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Plant health and food safety

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11th Special issue dedicated to grapevine trunk diseases

The international journal of the Mediterranean Phytopathological Union

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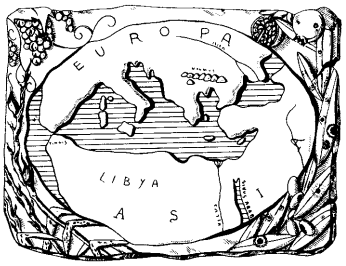

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

Phytopathologia Mediterranea is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.

The journal deals with all areas of plant pathology, including epidemiology, disease control, biochemical and physiological aspects, and utilization of molecular technologies. All types of plant pathogens are covered, including fungi, nematodes, protozoa, bacteria, phytoplasmas, viruses, and viroids. Papers on mycotoxins, biological and integrated management of plant diseases, and the use of natural substances in disease and weed control are also strongly encouraged. The journal focuses on pathology of Mediterranean crops grown throughout the world.

The journal includes three issues each year, publishing Reviews, Original research papers, Short notes, New or unusual disease reports, News and opinion, Current topics, Commentaries, and Letters to the Editor.

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Preface
11th Special Issue on Grapevine Trunk Diseases

Grapevine trunk diseases (GTDs) are a complex caused by many taxonomically unrelated fungi. Diseases in this complex are responsible for different vascular and foliar symptoms, which cause overall decline and eventual death of grapevines. Though GTDs have been known since the late 19th Century their significance and impacts on vine health have been fully recognized much more recently. Today, GTDs are considered major biotic factors reducing grapevine yields and vineyard lifespans, which cause substantial economic losses to grape and wine industries worldwide.

Emergence of these diseases in the early 1990s, and the urgent need for effective disease management strategies by growers and industry, focused scientific attention. As a result, a meeting in California took place in July of 1998, organized by the viticulturist Lucie Morton, who saw the potential threats posed by fungus infections of grapevine trunks. On that occasion, participants and founding members developed the structure and objectives of the International Council on Grapevine Trunk Diseases (ICGTD). Since then, the primary goal of the ICGTD has been to promote relevant research, and encourage collaboration and knowledge exchange among scientists and industry partners, on issues pertaining to GTDs.

Every two years, since 1999, the ICGTD has organized the International Workshop on Grapevine Trunk Diseases (IWGTD), where scientists and industry members from around the world present, share and discuss the latest research findings on different areas concerning this increasingly important disease complex.

This 11th Special Issue of Phytopathologia Mediterranea *on GTDs collects together presentations given at the 11th IWGTD, which was held on July 7-12, 2019, in Penticton, British Columbia, Canada. This conference marked the 20th Anniversary of the Workshop. The Workshop was attended by 151 participants from 21 countries. A total of 64 oral and 48 poster papers were presented in four sessions, covering Pathogen Detection and Identification, Epidemiology, Host-Pathogen Interactions, and Disease Management. In addition, a special session on GTD control provided the local grapevine industry with a practical summary and overview of the current GTD management options and the economic impacts they cause.*

A student competition, with awards for best oral and poster presentations, was held for the first time at IWGTDs, with 20 graduate students participating. For Best Poster presentations, first place was awarded to Pierluigi Reveglia (Charles Sturt University, Wagga, New South Wales, Australia), second to Edelweiss Rangel Montoya (Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California, Mexico), and third to Daina Grinbergs (Instituto Nacional de Investigaciones Agropecuarias, Chillán, Chile). For Best Oral Presentation, first place was awarded to Jinxz Pollard-Flamand (Agriculture and Agri-Food Canada Summerland RDC, British Columbia, Canada), second to Clément Labois (Université de Haute-Alsace, Colmar, France), and third to María del Pilar Martínez-Diz (Estación de Viticultura e Enoloxía de Galicia, Ourense, Spain).

The ICGTD Council Members have determined that these Workshops will now be convened at 3 year intervals. ***The 12th IWGTD will be held in the Czech Republic in 2022****.*

The editorial team of this Journal is indebted to all authors of papers presented in this Special Issue, for their support and contributions.

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Review

Grapevine trunk disease fungi: their roles as latent pathogens and stress factors that favour disease development and symptom expression

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Summary. Grapevine trunk diseases (GTDs) are major biotic factors reducing yields and limiting vineyard economic life spans. Fungi in the GTD complex cause a range of symptoms in host plants, although these pathogens are slow wood colonizers and potentially latent pathogens. Understanding has recently increased on the possible roles that GTD fungi may play as latent pathogens, and how this can be translated into disease management. This paper summarizes evidence for the latent nature of infections by these fungi in grapevines and other hosts. Abiotic and biotic stressors have been associated with symptom expression in many hosts, but limited information is available regarding their roles in symptom development in grapevines. Based on research conducted in other pathosystems, this review discusses how abiotic and/or biotic stress factors may influence the transition from the endophytic to the pathogenic phases for GTD fungi. Potential methods for stress mitigation are also outlined as alternative GTD control strategies to minimize the economic impacts that that these diseases have on grape production.

Keywords. Abiotic and biotic stresses, black foot disease, Botryosphaeria dieback, endophyte, Esca, Eutypa dieback, Petri disease, Phomopsis dieback.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are major threats to the economic sustainability of viticulture; they cause significant economic losses due to reduced yields, increased crop management costs and shortened life spans for vineyards (Wicks and Davies, 1999; Siebert, 2001; Bertsch *et al.*, 2013; Kaplan *et al.*, 2016; Gramaje *et al.*, 2018). The GTD complex includes Black foot disease, Botryosphaeria dieback, Esca, Eutypa dieback, Petri disease, and Phomopsis dieback (Gramaje and Armengol, 2011; Úrbez-Torres, 2011; Agustí-Brisach and Armengol, 2013; Bertsch *et al.*, 2013; Gramaje *et al.*, 2018; Mondello *et al.*, 2018). These diseases are primarily caused by taxonomically unrelated Ascomycete fungi, and to lesser extent by several Basidi-

omycetes which are primarily associated with the Esca complex (Gramaje *et al*., 2018). Black foot disease and Petri disease typically occur in grapevines aged 5 years or younger, while Botryosphaeria dieback, Esca, Eutypa dieback, and Phomopsis dieback are usually seen in older vineyards, although these diseases may also occur in young grapevines (Gramaje and Armengol, 2011; Úrbez-Torres *et al*., 2008; Agustí-Brisach and Armengol, 2013; Bertsch *et al*., 2013). Pruning wounds serve as major routes of infection by these pathogens in established vineyards. The discharge of spores from fruiting bodies and airborne transmission of these spores from plant to plant have primarily been associated with rainfall, with the exception of black foot pathogens, which are known to be soil-borne (Agustí-Brisach and Armengol, 2013; Agustí-Brisach *et al*., 2014; Eskalen and Gubler, 2001; Rooney-Latham *et al*., 2005; Trese, 1980; Úrbez-Torres *et al*., 2010a; Úrbez-Torres *et al*., 2010b).

Grapevine trunk diseases are caused by xylem-colonizing fungi, and are categorized as slow-progression diseases with symptoms sometimes taking several years to appear after infection. In addition, GTD symptoms are known to be expressed inconsistently from year to year on individual grapevines (Bertsch *et al*., 2013; Calzarano *et al*., 2018; Songy *et al*., 2019). General symptoms of GTDs include delayed bud-break, leaf chlorosis, reduced vigour, stunted growth, wood necroses, canker formation, dieback, and eventual death of affected grapevine plants (Gramaje and Armengol, 2011; Úrbez-Torres, 2011; Agustí-Brisach and Armengol, 2013; Bertsch *et al*., 2013; Gramaje *et al*., 2018; Mondello *et al*., 2018; Billones-Baaijens and Savocchia, 2019; Reis *et al*., 2019; Songy *et al*., 2019). Due to the inconsistent symptom expression from year to year for some GTDs, and the reported isolation of GTD fungi from asymptomatic tissues, it has been proposed that some of these fungi may act as latent pathogens (Úrbez-Torres, 2011; Gramaje *et al*., 2018). The presence of GTD fungi in asymptomatic tissues can be very problematic for growers and propagators, as infections can spread unnoticed in the field or in nurseries. Asymptomatic infections in rootstock and scion mother plants can provide possible sources of infection in nurseries (Fourie and Halleen, 2004; Mondello *et al*., 2018). In addition, abiotic and biotic stresses are likely to play important roles in GTD symptom development (Úrbez-Torres, 2011; Gramaje *et al*., 2018; Reis *et al*., 2019; Songy *et al*., 2019).

Endophytes and latent pathogens: from endophytic to pathogenic phases

Endophytes are organisms found in the internal tissues of plants, including in roots, xylem, phloem, and/or leaves, and these organisms may act as saprobes, mutualists, or latent pathogens (Wilson, 1995; Hyde and Soytong, 2008; Rodriguez *et al*., 2009). The definition of endophyte has changed several times; however, for this review, the definition of Petrini (1991) is followed, as *"all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to their host."* This definition includes latent pathogens during their asymptomatic phases (Stone *et al*., 2000; Wilson, 2000; Wilson, 1995; Sieber, 2007). The term "endophyte" is used here for the endophytic phase of an organism's life cycle, the life cycle of which may include several other phases that may be transitory and/or pathogenic (Wilson, 1995; Stone *et al*., 2000; Wilson, 2000).

Most fungal endophytes are in Ascomycota R.H. Whittaker, although some are in Basidiomycota Whittaker ex R.T. Moore, or Oomycota Arx (Hyde and Soytong, 2008; Petrini, 1986). Fungal endophytes can also be classified into two broad categories, as clavicipitalean endophytes, which live in the internal tissues of grasses and have been thoroughly researched, and non-clavicipatalean endophytes, which live in a wide range of hosts including woody perennials and non-vascular plants (Sieber, 2007; Hyde and Soytong, 2008; Rodriguez *et al*., 2009). Rodriguez *et al.* (2009) suggested an endophyte classification system with four classes. This included Class 1 as clavicipitaceous endophytes which are grass endophytes. Classes 2 to 4 are non-clavicipitaceous endophytes. Class 2 edophytes are distinguished from the rest by their colonization of roots, stems, and leaves, and their transmission via seed coats and rhizomes. Class 3 endophytes have diverse plant host ranges, including woody perennials, and these endopytes live in the inner bark, wood, fruit, or flowers of their hosts, and can reproduce via sporulation on dead plant tissues. Class 3 endophytes include xylem-colonizing endophytes. Class 4 endophytes are only found in host roots, where they form melanised structures.

The focus of the present review is on xylem-colonizing Class 3 endophytes, which make up a distinct guild of xylem-inhabiting endophytes (Stone *et al*., 2000). These include species in the order *Hypocreales* Lindau and in the genus *Hypoxylon* Bull, and to a lesser extent some Basidiomycetes. Survival strategies of many xylem-inhabiting endophytes consists of initial infection of host plants, followed by indeterminate periods of interrupted growth allowing for further invasion and exploitation of the substrates upon occurrence of favourable conditions, such as host stress (Stone *et al*., 2000). These xylem-colonizing endophytes typically disperse through horizontal transmission, with drop-splash from rainfall for spore

dispersal. Wind and/or other vectors such as animals or insects may also facilitate transmission of these fungi (Petrini, 1991; Wilson, 2000). Endophytes can either have wide host ranges, or as suggested by Petrini (1991), they may have coevolved with their host plants, allowing the endophytes to infect their hosts without activating host defense mechanisms.

There are three hypotheses for the causes of the transition from endophytic to pathogenic phases. The first is that some event occurs which increases host susceptibility, facilitating the transition. These events are most likely to be abiotic and/or biotic stressors, including drought, poor host nutrient supply, and/or host wounding (Petrini, 1991; Stone, *et al.*, 2000). The second hypothesis includes endophyte changes, including single point mutations, the transfer of virulence genes, and/or virus infections causing change to a pathogenic state (Sieber, 2007). The third hypothesis is the threshold model developed by Sieber (2007) for conifer needle endophytes, and this could be relevant to other types of endophytes. This model suggests that endophytes live in needle tissues, which senesce once a colonization threshold has been reached. This threshold is only reached under normal circumstances during the natural senescence of the needles; however, under stress conditions, the colonization threshold is reached sooner, leading to premature death (Sieber, 2007). All these scenarios could be applied to GTD fungi thought to have endophytic phases before becoming pathogenic. Most GTD fungi are slow wood colonizers causing slow-developing diseases (Bradshaw *et al.*, 2005; Agusti-Brisach *et al.*, 2019). In healthy grapevines, fungal growth is characteristically slow. When vines become stressed, however, fungal growth is accelerated and colonization thresholds are reached sooner than under non-stress conditions. Additionally, this threshold could be reached sooner when multiple GTD fungi are present within a grapevine (Figure 1).

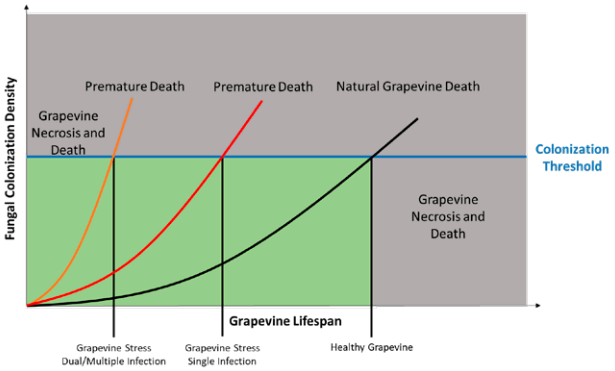

Figure 1. Illustration of the Threshold Model in grapevines, showing the relationship between the grapevine age and GTD fungal colonization density. Under normal conditions, fungal growth is slow, only reaching the colonization threshold in old grapevines during natural senescence. Under stress conditions, fungal growth is accelerated, and this threshold is reached rapidly in the grapevine lifespan, leading to disease progression and premature host death. This threshold is hypothesized to be reached sooner when multiple GTD fungi are present than when few fungi occur (adapted with permission from Sieber, 2007).

Endophytes in grapevines

Characterization of bacterial and fungal endophytes of grapevines has been a major focus of research, to identify potential biocontrol agents against pathogens (Bruisson *et al.*, 2019). Endophytes colonize above- and below-ground plant organs, including, in grapevines, flowers, berries, xylem, roots, and seeds (Compant *et al.*, 2011; Bruisson *et al.*, 2019). Bacterial endophyte richness is greater than fungal endophyte richness in grapevines, and *Proteobacteria* Garrity et al., and Ascomycota species, respectively are the most abundant bacteria and fungi in grapevines (Deyett and Rolshausen, 2020). In *Vitis vinifera* L., Ascomycota endophytes are primarily Class 3 xylem-colonizing endophytes in the *Hypocreales*, *Pleosporales* Luttrell ex M.E. Barr, and *Xylariales* Nannf., which is consistent with other woody perennial hosts (Petrini, 1986; Hyde and Soytong, 2008; González and Tello, 2011).

Among these Ascomycetes, species in the *Hypocreales* and *Pleosporales* were the most abundant endophytes found across multiple grapevine cultivars in a study in Spain (González and Tello, 2011). However, the cultivars were colonized by different endophyte populations, showing that grapevines cannot be viewed as a homogeneous group of hosts, but cultivars must be studied individually. For example, González and Tello (2011) found 'Tempranillo' and 'Merlot' cultivars harboured high levels of *Eurotiales* G.W. Martin ex Benny & Kimbr., while 'Cabernet Sauvignon' harboured the least variable endophyte diversity. This study also showed high prevalence of the GTD pathogen *Diaporthe ampelina* (Berkeley & M.A. Curtis) R.R. Gomes, C. Glienke & Crous, (syn. *Phomopsis viticola* Sacc.) in asymptomatic and symptomatic grapevines (González and Tello, 2011). Other GTD pathogens found less widely in asymptomatic vines include *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous, and *Phaeoacremonium inflatipes* W. Gams, Crous & M.J. Wingf. (involved in Petri disease

and Esca), *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous (associated with Black foot disease), and species in the *Botryosphaeriaceae* Theiss. & P. Syd. [causing Botryosphaeria dieback (Gonzalez and Tello, 2011)]. These results, along with other reports of GTD fungi isolated from asymptomatic grapevines (Halleen and Petrini, 2003; Zanzotto *et al*., 2007; Kaliterna *et al*., 2009; Aroca *et al*., 2010; Carlucci *et al*., 2017), support the hypothesis that some of these fungi may have roles as latent pathogens in grapevines (González and Tello, 2011; Gramaje and Armengol, 2011; Úrbez-Torres, 2011; Gramaje *et al*., 2018).

The objectives of this review are to provide up-to-date information indicating that GTD fungi may function as latent grapevine pathogens, to develop a synthesis of current knowledge available on the different abiotic and biotic stress factors that may influence the transition from endophytic to pathogenic phases, and to indicate future research directions for advancing understanding and management of these complex diseases.

GRAPEVINE TRUNK DISEASE FUNGI AS LATENT PATHOGENS

Most knowledge on the potential of GTD fungi to act as latent pathogens comes from studies in two main groups of Ascomycetes: *Botryosphaeriaceae* and *Ilyonectria* P. Chaverri & C. Salgado ("*Cylindrocarpon*" Wollenw.) spp., on grapevines as well as other woody perennials (Fourie and Halleen, 2004; Slippers and Wingfield, 2007; Úrbez-Torres, 2011). This paper also reviews the endophytic-pathogenic continuum of *Phaeomoniella chlamydospora*, *Diatrypaceae* and species of *Phaeoacremonium* and *Diaporthe* on grapevines.

Botryosphaeriaceae

Botryosphaeriaceae includes morphologically diverse fungi which have been found in gymnosperms and angiosperms in every climatic region except the polar regions (Phillips *et al*., 2013; Slippers and Wingfield, 2007). Not all fungi in the *Botryosphaeriaceae* produce disease symptoms, and some species may be true endophytes, which never produce disease symptoms (Slippers and Wingfield, 2007). Some *Botryosphaeriaceae* have been shown to be important pathogens and have become problems in economically important agricultural crops, with several different species found to be associated with decline symptoms in apple trees (Brown-Rytlewski and McManus, 2000; Úrbez-Torres *et al*., 2016), Eucalyptus (Burgess *et al*., 2005), dwarf cashew (Cardoso *et al*., 2006), hickory (Dai *et al*., 2017), macadamia (Jeff-Ego and Akinsanmi, 2019), bay laurel (Lawrence *et al*., 2017), almond, dried plum, pistachio, walnut (Luo *et al*., 2019), peach, pear, blueberry (Sessa *et al*., 2018), black currant (Singer and Cox, 2010), olive (Úrbez-Torres *et al*., 2013b), and grapevine (Úrbez-Torres, 2011). However, even in the most severe diseases caused by *Botryosphaeriaceae* in some hosts, virulence is probably favoured by abiotic and/or biotic stresses suffered by host plants (Slippers and Wingfield, 2007). Fungi in the *Botryosphaeriaceae* have been found in several hosts which were asymptomatic and symptomatic (Brown-Rytlewski and McManus, 2000; Tennakoon *et al*., 2018; Panahandeh *et al*., 2019; Scala *et al*., 2019). *Botryosphaeriaceae* isolated from asymptomatic hosts have been found to be pathogenic in pathogenicity trials conducted under controlled greenhouse conditions (Manzanos *et al*., 2017; Steinrucken *et al*., 2017). The *Botryosphaeriaceae* have gone through extensive taxonomic changes and revisions since first introduced in 1918, and have primarily been considered to be saprophytes (Phillips *et al*., 2013). Only in the last two decades have detailed studies on the *Botryosphaeriaceae,* supported by the use of molecular techniques, revealed the widespread presence of these fungi in asymptomatic hosts, which has led to further investigations of the hypothesis that some of *Botryosphaeriaceae* species may act as latent pathogens.

In grapevines, up to 26 species of *Botryosphaeriaceae* have been identified as pathogens responsible for symptoms associated with the GTD Botryosphaeria dieback. These symptoms include leaf chlorosis, bud and wood necrosis, weak spring growth, and vascular cankers primarily in the shape of wedges (Úrbez-Torres, 2011; Gramaje *et al*., 2018; Billones-Baaijens and Savocchia, 2019) (Figure 2). Among all species known to be associated with Botryosphaeria dieback, *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. has been described as a latent pathogen of global importance in woody perennials (Marsberg *et al*., 2017). Several other *Botryosphaeriaceae* species have been described as latent pathogens in other woody hosts, but have not been confirmed in grapevines (Slippers and Wingfield, 2007; Carlucci *et al*., 2015; Marsberg *et al*., 2017; Billones-Baaijens and Savocchia, 2019). Lecomte and Bailey (2011) found *Botryosphaeriaceae* species, primarily *Diplodia seriata* De Not., in wounded host tissues, which was attributed to presence of these fungi in the bark prior to wounding, suggesting that the fungi may act as latent pathogens in grapevines. This is important for grape production, as Botryosphaeria dieback is one of the most prevalent diseases of grapevines, and the causal pathogens may not be detected due to their latent nature (Marsberg *et al*.,

Figure 2. Botryosphaeria dieback, Eutypa, Phomopsis dieback, and Esca symptoms. Botryosphaeria dieback symptoms on wine (**A**) and table grapevines (**B**) are characterized by dead spurs and cordons with no spring growth. Characteristic foliar symptoms of Eutypa dieback are shoots with short internodes and chlorotic and cupped leaves (**C** and **D**). Phomopsis dieback symptoms resemble those of Botryosphaeria dieback, with dead spurs and lack of spring growth (**E**). Vines affected by Botryosphaeria, Eutypa and/or Phomopsis dieback have perennial cankers in spurs, cordons and trunks, often with wedge shapes (**F**). Tiger-striped leaves (**G**) and characteristic soft yellowish wood rot (**H**) caused by Basidiomycete fungi are commonly associated with Esca disease.

2017; Agusti-Brisach *et al*., 2019; Billones-Baaijens and Savocchia, 2019). *Neofusiccocum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips remained latent in 'Cabernet Sauvignon' grapevines, with limited pathogen spread or lesion growth for 1.5 months after inoculation; thereafter, lesion lengths increased (Czemmel *et al*., 2015). However, no cause for the transition from endophyte to pathogen was investigated, and more research should be conducted to determine whether this is the normal latency period of *N. parvum*, and/or if some external factors played roles in the transition, such as abiotic or biotic stress.

Some GTD fungi can be detected in asymptomatic host tissues far beyond necrotic or cankered wood. For example, *B. dothidea* colonizes asymptomatic tissues beyond canker margins in many different hosts, which supports the hypothesis of its endophytic nature (Wene, 1979; Schoeneweiss, 1981). Similarly, *Botryosphaeriaceae* known to be pathogenic in grapevines have been found in asymptomatic and symptomatic grapevines (Halleen and Petrini, 2003; Aroca *et al*., 2010; Abreo *et al*., 2013; Úrbez-Torres *et al*., 2015). Other *Botryosphaeriaceae* have also been shown to infect asymptomatic tissues beyond lesions in grapevines (Amponsah *et al*., 2011; Billones-Baaijens *et al*., 2013a). As in other hosts, the relationship between symptomatic tissue and/or hosts in grapevines has also become an area of interest, with particular emphasis on the role that drought stress may play on symptom expression and disease development.

Ilyonectria

Species of *Ilyonectria* are cosmopolitan soil-borne fungi, and the fungi are weak and/or opportunistic pathogens associated with root rot diseases in several economically important hosts, including apple (Manici *et al*., 2013), avocado (Parkinson *et al*., 2017), banana (Booth and Stover, 1974), Mexican blue palm, Guadalupe palm, Kentia palm (Aiello *et al*., 2014), clover (Barbetti *et al*., 2007), ginseng (Rahman and Punja, 2005), hazelnut (Guerrero *et al*., 2014), kiwifruit (Erper *et al*., 2013), loquat (Agustí-Brisach *et al*., 2016), olive (Úrbez-Torres *et al*., 2012), strawberry (Ceja-Torres *et al*., 2008), and other fruit trees and nut producing hosts (Lawrence et al. 2019). In addition, several *Ilyonectria* species and closely related *Campylocarpon* Halleen, Schroers & Crous, *Cylindrocladiella* Boesew., *Dactylonectria* L. Lombard & Crous, *Neonectria* Wollenw., and *Thelonectria* P. Chaverri & C. Salgado, are known to cause black foot of grapevines (Agusti-Brisach *et al.,* 2013). Black foot pathogens have been isolated from asymptomatic and symptomatic rootstock mother-plants, rootstock cuttings, and young grafted grapevines, in nurseries and young vineyards, as well as from other hosts, which has led to the hypothesis that Black foot pathogens act as latent and/or weak pathogens in grapevines (Agustí-Brisach and Armengol, 2013; Carlucci *et al*., 2017; Dubrovsky and Fabritius, 2007; Dumroese *et al*., 2002; Halleen and Petrini, 2003; Úrbez-Torres *et al*., 2015).

Phaeomoniella chlamydospora *and* Phaeoacremonium *species*

Petri disease is mainly caused by *P. chlamydospora* and *Phaeoacremonium* spp. in young grapevines, while Esca is thought to be caused by the same species of fungi along with colonization of various Basidiomycetes in older grapevines (Gramaje *et al*., 2018). While Petri disease and Esca are different, for the purpose of this review these two diseases are considered together (Figure 3). *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. have been isolated from asymptomatic and symptomatic wood from grapevines and other hosts (Halleen and Petrini, 2003; Edwards and Pascoe, 2004; Zanzotto *et al*., 2007; Abreo *et al*., 2011; Panahandeh *et al*., 2019). Some grapevines infected with Esca pathogens have displayed symptoms one year but not in the next, which has been proposed to correspond to differences in environmental conditions, primarily temperature and rainfall (Surico *et al*., 2000; Marchi *et al*., 2006; Péros *et al*., 2008; Calzarano *et al*., 2018). In addition, *P. chlamydospora* and *Phaeoacremonium* spp. have been found to widely occur in grapevines in several regions such as in Australia and British Columbia (Canada). However, Esca foliar symptoms are rarely observed in these regions in comparison with Europe, where these symptoms are common (M. Sosnowski, Personal Communication; Úrbez-Torres *et al*., 2014a). Though not yet studied, the lack of Esca symptom expression in Australia and/or Canada may result from the lack of Basidiomycete species associated with Esca symptoms in Europe, primarily those in *Fomitiporia* Murrill (Fischer, 2002; Fischer and Kassemeyer, 2003). On the other hand, other studies have tended to exclude a direct role of *Fomitiporia* in Esca leaf symptom expression (Calzarano and Di Marco, 2007). In a study in Australia, *P. chlamydospora* was widely found in south-eastern Australia, less commonly in western Australia, and absent from the northern region, indicating that host range may be mainly influenced by climatic factors (Edwards and Pascoe, 2004). Nevertheless, these observations suggest that Esca internal symptoms are not necessarily indicative of external symptoms, and that foliar symptom expression may rely also on external abiotic or biotic stress fac-

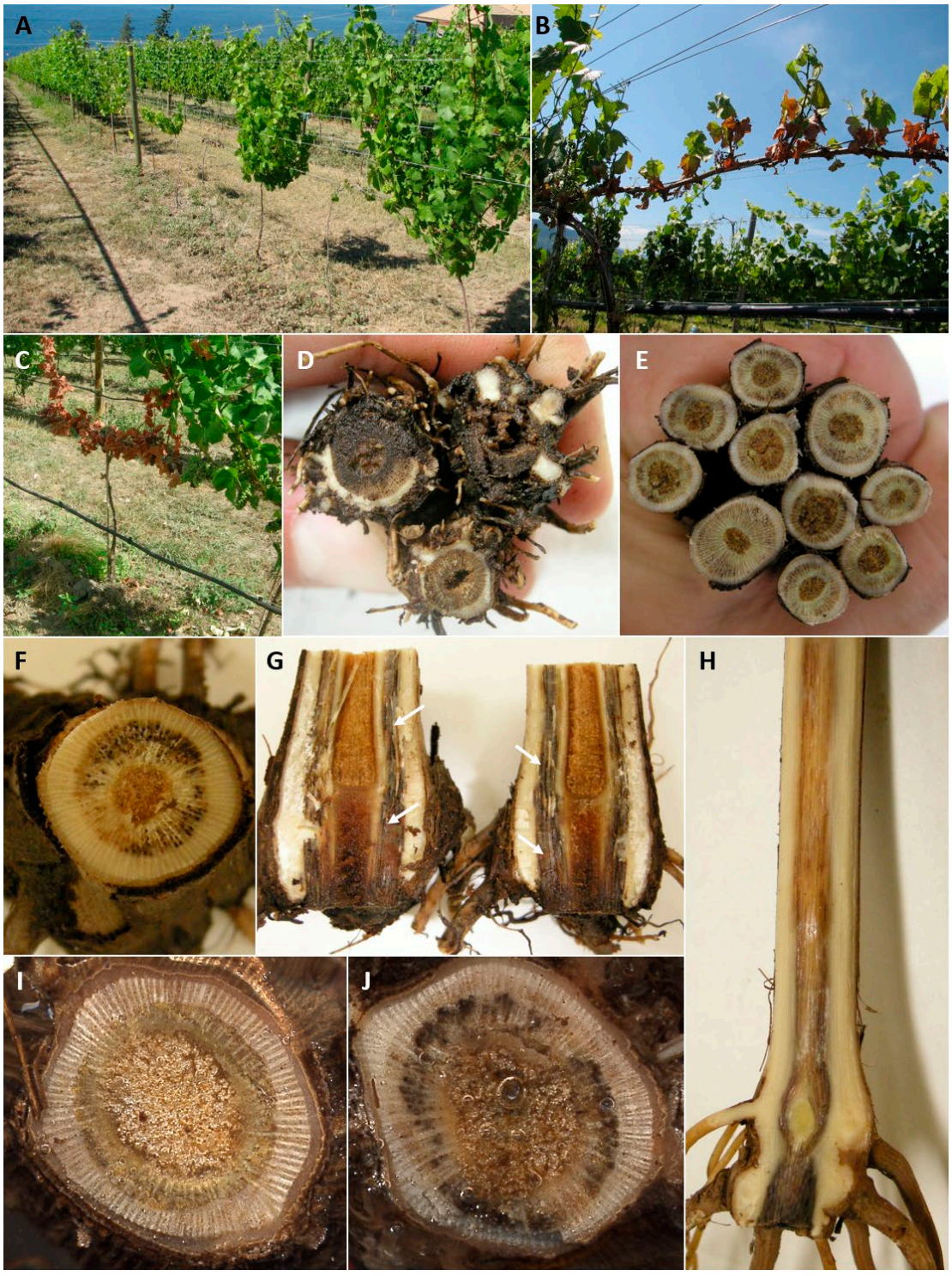

Figure 3. Decline of young vines caused by Petri disease and/or black foot. Overall symptoms in the field include poor vigour, leaf chlorosis, short internodes (**A** and **B**) and eventual or sudden collapse (**C**) during the growing season. Internal wood necrosis observed at the basal end of the rootstock (**D**) commonly associated with black foot. Tylosis plugged xylem vessels and necrosis observed at a graft union (**E**) and trunk base, usually associated with Petri disease in young vines (**F**). Longitudinal section of a ready-to-plant nursery vine showing vascular necrosis originating from the basal end of the rootstock (**G** and **H**). *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Ilyonectria* spp., and some *Botryosphaeriaceae* spp. can be isolated from asymptomatic (**I**) and symptomatic (**J**) wood tissues from nursery propagated material, which reveals their potential latent phases in grapevines.

tors (Surico *et al*., 2000; Zanzotto *et al*., 2007; Calzarano and Di Marco, 2018). Lack of correlation between extent of vascular alteration and foliar symptom expression in Esca may indicate a latent phase (Calzarano and Di Marco, 2007). In addition, rainfall has been suggested to be involved in foliar symptom expression regardless of the extent of fungal colonization (Calzarano *et al*., 2018).

Diatrypaceae

The causal organism for Eutypa dieback is the diatrypaceous fungus *Eutypa lata* (Pers.) Tul. & C. Tul. Although other 23 species in the Diatrypaceae Nitschke have been isolated from grapevine cankers, only *E. lata* has been proven to cause the foliar symptoms associated with Eutypa dieback (Trouillas and Gubler, 2010; Gramaje *et al*., 2018). These symptoms include internal wood wedge-shaped necroses, cordon dieback, leaf chlorosis, and stunted shoots (Rolshausen *et al*., 2015) (Figure 2). These symptoms mainly appear on grapevines that are 7 years or older, and foliar symptoms typically do not appear for 3 to 8 years post-infection (Rolshausen *et al*., 2015). Symptoms have also been observed to be inconsistent from year to year, with individual grapevines displaying symptoms one year, while appearing asymptomatic the next (Sosnowski *et al*., 2007b; Bertsch *et al*., 2013; Mundy and McLachlan, 2016). Several hypotheses have been proposed to explain why symptoms do not appear for extended periods, including the influence of environmental factors. Bruez *et al.* (2016) found *E. lata* among other GTD pathogens in asymptomatic grapevines aged 40 years or older and suggested that there was a balance between beneficial organisms and pathogenic fungi, which prevented the pathogens from developing further in grapevines. *Eutypa lata* and other *Diatrypaceae* have been isolated from asymptomatic or healthy tissues several centimeters ahead of disease margins, indicating pathogen latency (Pitt *et al*., 2013). Other hypotheses, including the threshold model (above), could also explain these observations.

Diaporthe

The main causal agent of Phomopsis dieback is *D. ampelina*, which has recently been added to the list of pathogens in the GTD complex (Úrbez-Torres *et al*., 2013a). Symptoms of Phomopsis dieback are similar to those observed in Botryosphaeria dieback, and include perennial cankers, lack of or delayed bud break and leaf chlorosis (Úrbez-Torres *et al*., 2013a) (Figure 2). Fungi in *Diaporthe* Nitschke are diverse, inhabiting many different hosts including grapevines, as pathogens, true endophytes, or saprobes (Gomes *et al*., 2013; Úrbez-Torres *et al*., 2013a). In grapevines, a diversity of *Diaporthe* species, including *D. ampelina*, have been found in asymptomatic and symptomatic grapevine tissues from mature vines and especially from nursery propagated plants, leading to the suggestion that some Phomopsis dieback pathogens may have endophytic phases. However, research into this is limited (Mostert *et al*., 2000; Kaliterna *et al*., 2009; Król, 2012; Guarnaccia *et al.,* 2018;).

In general, knowledge of the role that species in the *Diaporthaceae* Höhn. ex Wehm. may have as latent pathogens comes from hosts other than grapevines. For example, *Diaporthe toxica* P.M. Will., Highet, W. Gams & Sivasith., the cause of Phomopsis stem blight in *Lupinus albus* L., has been suggested as a latent pathogen, due to a prolonged latent period under which no visible symptoms occur (Cowley *et al*., 2012). Tongsri *et al.* (2016) also suggested an endophytic phase for *Diaporthe* pathogens in durian leaves and flowers, with symptoms typically occurring 32 d after infection. Similarly, lupins infected with *Phomopsis leptostromiformis* (J.G. Kühn) Bubák, exhibited a 20 d asymptomatic period after infection (Williamson *et al*., 1991). Furthermore, symptom expression from *D. toxica* and *P. leptostromiformis* infections in lupins has typically been associated with host senescence (Cowling *et al*., 1984; Williamson and Sivasithamparam, 1994). Mature durian leaves also contained a higher density of *Diaporthe* pathogens than immature leaves (Tongsri *et al*., 2016). This evidence indicates that these pathogens reach their thresholds for transition from endophytic to pathogenic phase during natural senescence in mature leaves and/or hosts, as suggested by Sieber (2007). However, further research is required to confirm these observations, particularly in grapevines, to determine if Phomopsis dieback pathogens act similarly across different host plants.

YOUNG VINE DECLINE AND GRAPEVINE NURSERY STOCK

As outlined above, some of the pathogens responsible for Botryosphaeria dieback, Black foot and Petri disease in grapevines have been isolated from ready-to-plant nursery material and from plants in young vineyards, either separately or together and either from symptomatic or asymptomatic tissues. Accordingly, some of these pathogens may often be latent or weak pathogens, remaining in asymptomatic grapevines for indeterminate periods, and being widespread in vine-

yards (Rumbos and Rumbou, 2001; Halleen and Petrini, 2003; Giménez-Jaime *et al.*, 2006; Dubrovsky and Fabritius, 2007; Halleen *et al.*, 2007a; Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013; Úrbez-Torres *et al.*, 2014a, 2014b; Carlucci *et al.*, 2017).

Black foot pathogens are soil-borne and can be present and ready to infect young grapevines when new vineyards are established (Halleen and Petrini, 2003; Carlucci *et al.*, 2017; Agusti-Brisach *et al.*, 2019;). However, serious infections and disease outbreaks by these pathogens have been found shortly after grapevines were planted (Úrbez-Torres *et al.*, 2014b). This rapid disease development has been suggested to result from: i) exposure to abiotic and/or biotic stress factors during planting in material that is already contaminated with *Ilyonectria* spp. (Halleen *et al.*, 2006); or ii) greater than normal infection thresholds that host plants cannot withstand (Úrbez-Torres *et al.*, 2014b; Gramaje *et al.*, 2018). In addition, Black foot pathogens have been found in soil in rootstock mother fields and young plant nurseries (Cardoso *et al.*, 2013; Whitelaw-Weckert *et al.*, 2013; Agustí-Brisach *et al.*, 2014).

These pathogens are prevalent in asymptomatic inner tissues of nursery stock, as shown in 15 nurseries in Northern Spain (Berlanas *et al.*, 2020). A total 1,427 isolates of Black foot pathogens were found, including species of *Dactylonectria, Ilyonectria, Neonectria,* and *Thelonectria* from 3,426 ready-to-plant grafted grapevines. Of these pathogens, *Dactylonectria torresensis* (A. Cabral, Rego & Crous) L. Lombard & Crous made up 75% of all isolates. Further research should be conducted in other regions on the incidence of Black foot and Petri disease pathogens in nurseries and young vineyards to better determine the scale of this problem. In particular, species of *Cadophora* Lagerb. & Melin have come to attention, since they have been found in grapevines in many regions, including Africa, Europe, North America, and South America (Halleen *et al.*, 2007b; Casieri *et al.*, 2009; Úrbez-Torres *et al.*, 2014a; Travadon *et al.*, 2015). *Cadophora* species have also been shown to be occur in nursery material in Northern Spain (Maldonado-Gonzalez *et al.*, 2020), with *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew found in 27% of 'Tempranillo'/'110R' dormant grapevines.

Recent studies have also identified species in *Fusarium* Link to occur in high numbers in nursery material and vineyards in Canada (Úrbez-Torres *et al.*, 2017). Pathogenicity studies have shown some *Fusarium* species to be capable of causing necrosis in grapevines to the same extent as *Ilyonectria* spp. under favourable conditions. This indicates that *Fusarium* species potentially act as weak or latent pathogens in grapevines, transitioning from endophytic to pathogenic phases under stress conditions, thus playing roles in young vine decline symptom development (Úrbez-Torres *et al.*, 2017). The suggestion that *Fusarium* is involved in grapevine decline is not new, and had been proposed by Highet and Nair (1995), when a clear association between *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen and vine decline was found in vineyards in New South Wales, Australia. However, the roles of *Cadophora* and *Fusarium* in nursery material and young vineyards, and any interactions of these fungi with Petri disease and Black foot pathogens is not well-understood, although the high incidence of these fungi in nursery material warrants further investigation of them as potentially relevant to young vine decline. In addition, the roles of different types of stress, and the effects of intermittent *versus* prolonged stress on young grapevines remains unknown. Research on the roles of *Cadophora* and *Fusarium* spp. in association with Black foot and Petri disease pathogens should be further investigated.

Infection during the grapevine propagation

Several Black foot pathogens have also been discovered in nurseries on grapevine scion cuttings, cutting tools, in water from hydration tanks, and in callusing medium, indicating that infections can occur at several steps in the propagation process, and in the field (Gramaje and Armengol, 2011; Cardoso *et al.*, 2013). The presence of pathogens during several steps of nursery processes has also been observed with several *Botryosphaeriaceae* species associated with Botryosphaeria dieback in New Zealand. In a study of three commercial nurseries, 33 to 100% of canes contained detectable amounts of *Botryosphaeriaceae* on the surfaces of the canes, and 15 to 68% of canes were internally infected with these fungi (Billones-Baaijens *et al.*, 2013b). In a separate study in New Zealand, three of ten apparently healthy '3309' mother grapevines and two out of ten '101-14' grapevines were internally infected with *Botryosphaeriaceae* in nursery mother blocks. External infection rates in plant bark were also high, with the bark in one out of three shoots infected in one grapevine. Therefore, it was suggested that these *Botryosphaeriaceae* remained latent in the bark of dormant cuttings, which may be a potential route of infection and spread during nursery propagation (Billones-Baaijens *et al.*, 2015). These results also indicate that disease control should begin in mother blocks from which scion and rootstock material is obtained, and precautions should also be taken during the propagation processes, in which there are several possible avenues of spread of these pathogens.

Current best practices for the prevention of transmission have been outlined (Gramaje and Armengol, 2011; Waite *et al.*, 2018). In addition to adopting these best practices, rootstocks are a promising avenue to reduce transmission. Berlanas *et al.*, (2019) found that '161-49C', '140 Ru', '1103P', and '110R'had lower abundance of "*Cylindrocarpon*"-like asexual morphs, compared to '41B' rootstocks.

Rootstock and scion resistance to GTD fungi

In grapevines infected with Botryosphaeria dieback pathogens, it has been shown that some scion varieties are more susceptible than others to these pathogens (Úrbez-Torres and Gubler, 2009). Studies in New Zealand vineyards showed 'Sauvignon Blanc', with 83% incidence of infection, was more susceptible to species of *Botryosphaeriaceae* compared to 'Pinot noir', which had 42% incidence (Baskarathevan *et al.*, 2012). Similarly, 'Shiraz' and 'Sauvignon Blanc' had greater susceptibility to *D. seriata* (pathogen recovery 12 to 21 mm from inoculation sites, while 'Green Veltliner' and 'Muscadelle' were less susceptible (pathogen recovery 8 to 17 mm from inoculation sites (Sosnowski *et al.*, 2017c). Pintos *et al.*, (2018) showed that 'Savagnin' grafted plants had greater Botryosphaeria dieback pathogen infections (66%) than other cultivars.

Rootstock susceptibility to Black foot has also been evaluated, and among the screened rootstocks, a majority were found to be susceptible, in particular '110R'. This rootstock was the most susceptible to infection by *Ilyonectria liriodendri* (Halleen, Rego & Crous) Chaverri & C. Salgado and *Ilyonectria macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado, which are considered the main pathogens of Black foot. These results were similar to those for susceptibility of '110R' to *P. chlamydospora* (Zanzotto *et al.*, 2008; Alaniz *et al.*, 2010; Nguyen, 2013). 'SO4', 'Freedom', and 'Riparia Glorie' were also susceptible to *I. liriodendri* and *I. macrodidyma*, while '44-53' and 'St. George' were more tolerant to these fungi (Nguyen, 2013). Susceptibility of different rootstocks to co-infection of Black foot pathogens and those causing either Petri disease or Botryosphaeria dieback should be studied further, along with the impacts of nematode damage on rootstocks infected by GTD pathogens.

Symptom expression of grapevines infected with Esca and/or Petri disease pathogens may be influenced by scion variety, with 'Merlot' found to be very tolerant to Esca disease (1.5 to 3.6% incidence), while 'Cabernet Sauvignon' was less resistant (20.2 to 27.8% incidence) (Christen *et al.*, 2007). Andreini *et al.*, (2009) showed that 'Trebbiano' and 'Sangiovese' grapevines were found to have lower disease incidence than 'Cabernet Sauvignon', which is probably one of the most affected cultivars. Several other studies have noted varying degrees of susceptibility between rootstocks and scions; however, the factors influencing scion and rootstock susceptibility remain unknown (Edwards and Pascoe, 2004; Edwards *et al.*, 2007a, 2007b). A detached cane assay study conducted by Martinez-Diz *et al.* (2019) found variations in colonization of xylem tissues among 20 cultivars, varying from 3 to 34 mm when infected with *P. chlamydospora* and 9 to 48 mm when infected with *P. minimum*. Gramaje *et al.* (2010) showed that 1-year-old rootstocks of '110R' and '140Ru' were very susceptible to *P. chlamydospora, Phaeoacremonium* spp., and *Cadophora* spp., while '161-49C' was the most tolerant rootstock to these pathogens. 'Fiano' and 'Sauvignon Blanc' scions grafted onto 'SO4' rootstock, which is intolerant to drought stress, displayed increased disease incidence compared to when grafted onto '1103P' rootstock, which is more drought tolerant (Murolo and Romanazzi, 2014). These results are similar to those of Cardoso *et al.* (2006), in that scion and/or rootstock resistance to abiotic or biotic stress factors may influence disease susceptibility. This requires further investigation, however.

Cultivar variety may affect symptom expression in grapevines infected with *E. lata*, with studies showing that different cultivars were more resistant or susceptible than others to Eutypa dieback (Baumgartner *et al.*, 2019; Cardot *et al.*, 2019). 'Merlot' was the most resistant to Eutypa dieback (38% plants asymptomatic), while 'Ugni Blanc' was very susceptible (87% of plants with symptoms) (Cardot *et al.*, 2019). Péros and Berger (1994) assessed different cultivars for foliar symptoms 5- and 10-weeks post-inoculation. 'Cabernet Sauvignon' was the most susceptible (90% of plants displaying foliar symptoms), followed by 'Ugni Blanc', while 'Sauvignon' was the least susceptible.

Research is being instigated on rootstock tolerance to GTD pathogens under abiotic or biotic stress conditions. However, further studies are required on the tolerance of different rootstock and cultivars to Esca disease while under stress (Sosnowski *et al.*, 2017a; Sosnowski *et al.*, 2017b). In a field grapevine study, trunk disease severity was greater in red than white cultivars when infected with *E. lata*. 'Petit Verdot' had the least trunk symptom severity while 'Odola' had the greatest for red cultivars, while 'Traminer', 'Petit Meslier', 'Muscadelle', and 'Green Veltliner' displayed no trunk symptoms. Among white cultivars infected with *E. lata* 'Sauvignon Blanc' had the greatest trunk symptom severity (Sosnowski *et al.*, 2017c). In a follow-up detached cane assay (Sosnowski *et*

al., 2017c), *E. lata* was recovered 20 to 23 mm from inoculation sites in 'Shiraz' and 'Sauvignon Blanc' compared to 4 to 14 mm in 'Petit Meslier', 'Petit Verdot', and 'Muscadelle' grapevines, confirming the field assessments. A later field inoculation experiment (Sosnowski *et al*., 2017c) also showed that 'Petit Verdot' and 'Muscadelle' had increased resistance to pathogen colonization. Clonal susceptibility for 'Shiraz' varied between clones aged 21 to 34 years, for 'Sauvignon Blanc' clones between 20 to 44 years, and 'Chardonnay' clones between 18 to 45 years, with less symptom severity in younger than older grapevines. Plant age and resistance to environmental stresses in relation to pathogen resistance should be further explored, to determine how resistance varies as grapevines age.

Rootstock and scion varieties present a promising avenue for tolerance to GTD infection. However, further research is required on this subject, including host tolerance to infection under abiotic and biotic stress conditions. Research has been conducted on rootstock performance in varying growing condition, but the roles that abiotic and biotic stress factors play for grapevine latent infections by GTD pathogens in rootstocks, particularly during the first few years after planting, have not been explored (Fort *et al*., 2017; Cuneo *et al*., 2020). In particular, rootstock and scion tolerance to stress and symptom expression should be assessed at the regional levels, to determine the best rootstock selections, and which ones are best suited for unique stress factors encountered in each region.

ABIOTIC STRESS FACTORS AND GTD SYMPTOM EXPRESSION

Botryosphaeriaceae

Many members of the *Botryosphaeriaceae* are not host specific, but rather the environment probably plays a major role in host affinity (Slippers and Wingfield, 2007; Ibrahim *et al*., 2017). Symptom expression in many different hosts has been found to coincide with periods of drought or other extreme weather events (Brown-Rytlewski and McManus, 2000; Ma *et al*., 2001; Slippers and Wingfield, 2007; Golzar and Burgess, 2011; Bendixsen *et al*., 2015; Crous *et al*., 2017; Acimovic *et al*., 2018; Jeff-Ego and Akinsanmi, 2019). Direct evidence for stress induced symptom expression includes the research of Old *et al.* (1990), which showed that *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, caused larger cankers in Eucalyptus when trees were defoliated or attacked by insects, than where insect attack was absent.

Drought stress is probably the most important factor in symptom expression, and has been the most extensively researched out of all potential stress factors (Brown-Rytlewski and McManus, 2000; Slippers and Wingfield, 2007; Scala *et al*., 2019). Similarly, water stress which reduced leaf water potentials increased the symptoms caused by *B. dothidea*. Plants remained resistant to *B. dothidea* infection until a water potential threshold of -12 to -13 MPa was reached (Wene, 1979; Schoeneweiss, 1981). However, it was necessary to sustain stem water potentials at less than -12 MPa for at least 3 d to allow for predisposition to *B. dothidea*, and that under field conditions, the effects were reversible if drought was not sustained. Similarly, Ma *et al*., (2001) in a greenhouse study showed that old pistachio trees could not recover from the combined effects of drought stress and *B. dothidea* infection, while young trees were more resilient. This may be explained by the ability of *B. dothidea* to grow rapidly when pistachio tree stem water potentials were decreased from 0 MPa to -2 MPa (Ma *et al*., 2001). Pusey (1989) also found that drought conditions resulting in leaf water potential of -3.0 MPa increased lesion length in peach trees. These results indicate that symptom expression is a combination of reduced host resistance due to stress and increased pathogen colonization due to optimal growth conditions. Similarly, Cardoso *et al.* (2006) hypothesized that resistance in a dwarf cashew clone to *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. was due to indirect mechanisms related to the drought tolerance of the clone. In grapevines, under drought conditions, 'Cabernet Sauvignon' grapevines infected with *N. parvum* had larger lesions than plants provided with adequate water; however, grapevines infected with *Neofusiccocum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips had larger lesions when grown at high soil moisture levels, indicating physiological conditions induced by low moisture content may affect wood symptom expression, but other factors may be involved that influence lesion length (Galarneau *et al*., 2019). Qiu *et al.* (2016) reported similar results, where 'Chardonnay' grapevines infected with *N. parvum*, *B. dothidea*, *L. theobromae*, and *D. seriata* produced larger necrotic wood lesions in water stressed grapevines than in unstressed vines. Van Niekerk *et al.* (2011a) also showed that 'Shiraz' grapevines infected with *Neofusiccocum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *N. parvum, L. theobromae*, and *D. seriata* developed larger lesions and reduced shoot mass when water stressed compared with unstressed plants.

Other host stress factors, including freezing damage, root damage from transplanting, pathogenic nema-

tode infestations, and suboptimal environmental conditions for growth (nutrient-poor or high-salt soils, excessive cold or heat) have also been associated with GTD symptom expression in multiple hosts. In addition to these associations, some research has been completed on freezing stress, showing similar effects to drought stress in *Cornus sericea* L. Localized cold exposure of this host to -30°C produced no visible signs of freeze damage, but colonization by *B. dothidea* occurred solely in the cold exposed areas and did not extend significantly beyond the margins. Similar results were also found in *Sorbus aucuparia* L. infected with *B. dothidea* (Wene, 1979; Schoeneweiss and Wene, 1980;). Manawasinghe *et al.* (2018) also suggested that *B. dothidea* outbreaks in China were associated with other stress factors, such as increased temperature and pest pressure.

Ilyonectria

Several factors have been shown to affect symptom expression in hosts infected with *Ilyonectria* spp. In apple and ginseng, root age affects lesion severity, with young roots developing more severe lesions compared to older roots, which displayed shallow lesions (Rahman and Punja, 2005; Manici *et al.*, 2018). Abiotic and biotic factors also probably affect symptom expression, including root damage, acidic soils, temperature, and drought. (Rahman and Punja, 2005; Barbetti *et al.*, 2007; Ruiz-Gómez *et al.*, 2019).

Black foot symptom expression on grapevines has been associated with three categories of stress factor: (1) nursery-related factors such as cold storage, grapevine defects, and low light and other conditions leading to low carbohydrate levels in the plants; (2) vineyard conditions and plant establishment stress factors, including nutrient deficient soils, poor soil drainage leading to low oxygen levels, drought, soil compaction, J-rooting, and other factors contributing to poor root development and temperature extremes; and (3) biotic factors, including fungal pathogens, pathogenic nematodes, and insect pests (Stamp, 2001; Rahman and Punja, 2005; Halleen *et al.*, 2006; Barbetti *et al.*, 2007; Gramaje and Armengol, 2011; Nguyen, 2013; Agusti-Brisach *et al.*, 2019; Ruiz-Gómez *et al.*, 2019) (Figure 4). Moreno-Sanz *et al.* (2013) suggested that symptom expression primarily occurs when grapevines are under stress, as defined by hindering of normal growth and development and photosynthesis, and causing physiological responses, e.g. increased stress hormones that lead to numerous responses that enhance plant survival at the expense of crop performance. As *Ilyonectria* spp. are likely weak or opportunistic pathogens, they probably only invade weakened or dead roots (Scheck *et al.*, 1998; Halleen *et al.*, 2006; Jankowiak *et al.*, 2016;). These conclusions were further supported by Probst *et al.* (2012), who found that increased cold storage time correlated with increased disease incidence and severity for several Black foot pathogens. Partial defoliation also decreased root dry weight and increased disease severity resulting in increased canker development in young grapevines infected with *I. destructans*, which was probably due to reduced carbohydrate levels (Brown *et al.,* 2012). This is similar to the results of Old *et al.* (1990) stated above.

Phaeomoniella chlamydospora *and* Phaeoacremonium species

Several stress factors have been associated with, or are thought to influence, symptom expression from Esca and Petri disease pathogens, including biological factors such as nematode or other fungal pathogens, and abiotic factors such as cold exposure, and those associated with poor soil quality including low water and nutrient supply, extreme pH, and excess salt (Stamp, 2001; Corino *et al.*, 2004; Oliveira *et al.*, 2009; Oliveira *et al.*, 2013; Fischer and Ashnaei, 2019). Drought has received the most attention. In young grapevines, wood symptom expression, plant growth, and likely survival of grapevines infected with Esca and/or Petri disease pathogens depends on sufficient water supply to avoid stress (Fischer and Kassemeyer, 2012). In two separate studies conducted in Australia, 'Cabernet Sauvignon' and 'Zinfandel' grapevines were infected with *P. chlamydospora* and subjected to either an irrigation regime that resulted in no measurable water stress, 50% irrigation, or 25% irrigation. While stomatal conductance and leaf water potentials were both progressively reduced in the 50% and 25% irrigation regimes, for both non-infected and *P. chlamydospora* infected grapevines, in each irrigation treatment the infected grapevines displayed greater stomatal conductance and less leaf water potential compared to non-infected grapevines. Furthermore, 'Chardonnay' grapevines displayed grater leaf water potentials than 'Cabernet Sauvignon' and 'Zinfandel', which may indicate 'Chardonnay' is more tolerant of *P. chlamydospora* infection (Edwards *et al.*, 2007a, 2007b). On potato dextrose agar (PDA) amended with either potassium chloride or sucrose to generate osmotic water potentials between -0.3 and -8.3 MPa, two out of three isolates of *P. chlamydospora* grew at down to -8.3 MPa (Whiting *et al.*, 2001), indicating that the the fungus could grow in severely water-stressed plant tissues. Results by Lima *et al.* (2017) indicated that a combination of water stress and Esca infection increased xylem sap nutrient concen-

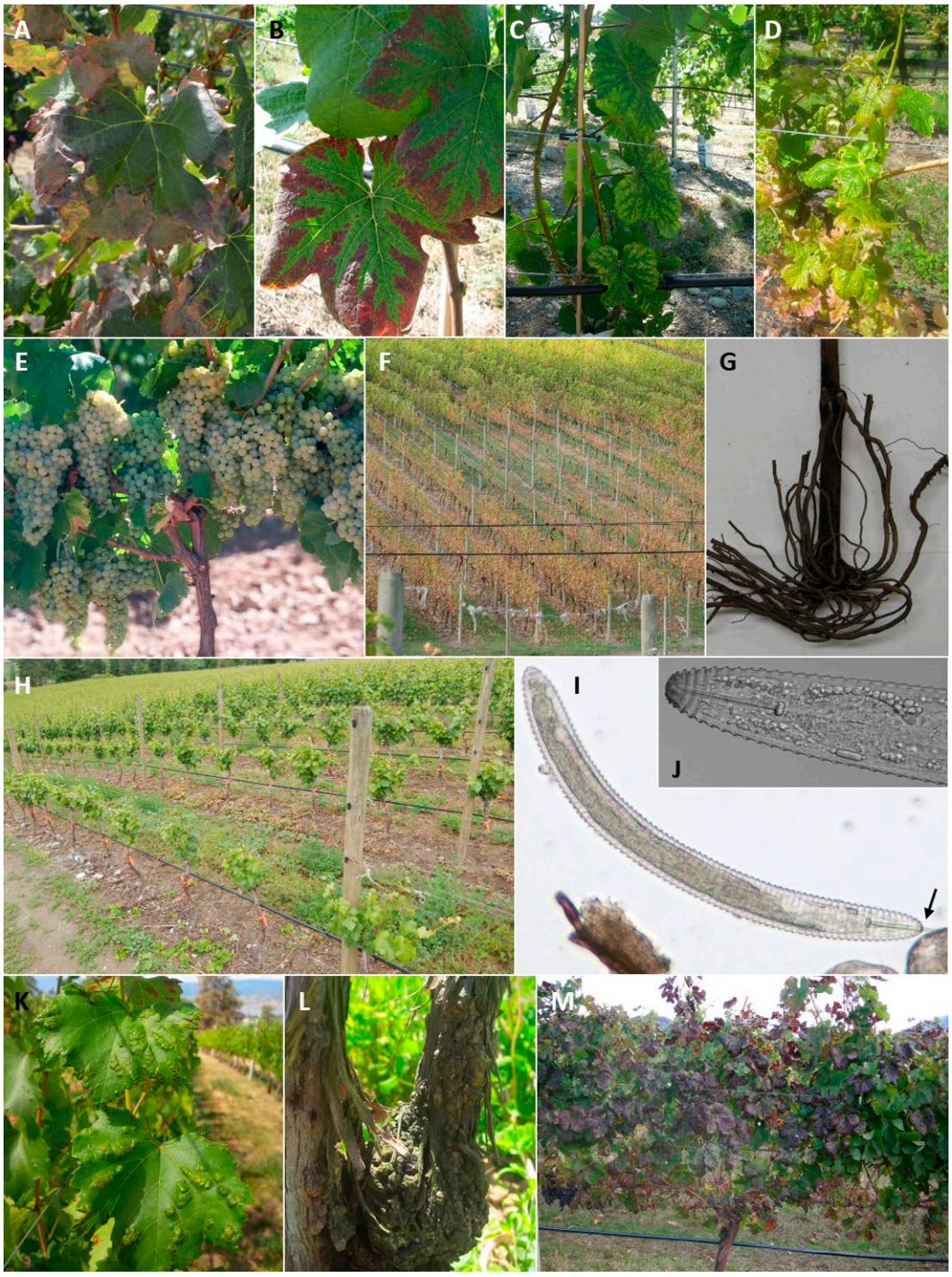

Figure 4. Abiotic and biotic stress factors hypothesized to contribute to grapevine trunk disease fungal development and symptom expression. **A.** Leaf scorching as a result of severe vine water stress. **B** and **C**. Leaves of young vines showing nutrient deficiency. **D.** Leaf deformities caused by herbicide damage. **E.** Severe over-cropping during the first years of vineyard establishment (courtesy of D. Gramaje, ICVV, Logroño, Spain). **F.** Winter damage and/or spring frost may favor disease development. **G.** J-rooting as a result of poor planting conditions. **H.** Low vigour vines caused by a nematode infestation. **I.** Ring nematode (*Mesocriconema xenoplax*) feeding on a root by introducing the stylet (black arrow). **J.** Close up of the ring nematode head. **K.** Leaf galls caused by erineum mite (*Colomerus vitis*). High levels of insect damage could increase vine stress. **L.** Grapevine crown gall caused by *Agrobacterium vitis*. **M.** Grapevine showing grapevine leaf roll symptoms caused by *Grapevine leafroll-associated virus 3*. Grapevines affected by biotic stress factors (fungal, bacterial or virus diseases) may favour GTD fungal infections, wood colonization and symptom expression.

trations, allowing for increased growth of the pathogens. In contrast, heavy rainfall from fruit set until veraison were associated with increasing Esca foliar symptoms, reducing grape quality (Calzarano and Di Marco, 2018). These results show that water stress may not be the only water status condition in grapevines that favour Esca development.

Diatrypaceae

It is well known that precipitation plays a role in *E. lata* distribution and frequency of occurrence. However, the effects of environmental conditions on symptom expression are not well understood. In a survey in France, the incidence and frequency of Eutypa dieback varied among different regions, indicating that climatic conditions, particularly temperature regimes which varied among regions, were major causes of differences in Eutypa occurrence (Guérin-Dubrana *et al.*, 2013). Magarey and Carter (1986) also found *E. lata* to be restricted to geographical locations where annual rainfall exceeded 350 mm, or in vineyards subject to overhead watering systems, indicating that precipitation affects *E. lata* distribution. Likewise, winter precipitation may be an additional requirement for the full development of *E. lata* in particular regions (Petzoldt *et al.*, 1983; van Niekerk *et al.*, 2011b; Úrbez-Torres *et al.*, 2019).

Guérin-Dubrana *et al.* (2013) and Sosnowski *et al.* (2007a) found that symptom expression and severity were influenced by climactic conditions in different regions. In Australia, *E. lata* isolates obtained from regions with intermediate climate had greater effects on symptom expression in a shadehouse experiment (Sosnowski *et al.*, 2007a). These isolates may have had greater virulence due to more favourable climactic conditions in the shade-house compared to the field. In a separate study in Australia, grapevines grown in extreme temperature and moisture conditions had increased foliar symptoms, but internal wood staining was not related to disease severity (Sosnowski *et al.*, 2007a; Sosnowski *et al.*, 2011). This is consistent with other reports on *Botryosphaeriaceae*, that indicate wood staining does not relate to colonization rates or correspond to foliar symptoms, but may be a consequence of wounding (Travadon *et al.*, 2013). Grapevines infected with *E. lata* also displayed decreased leaf water potential compared with non-infected grapevines when exposed to moisture deficits, suggesting that the pathogen increased sensitivity to water stress which may facilitate symptom development (Sosnowski *et al.*, 2011). Decreased leaf water potentials may be due to Eutypa dieback pathogens occluding the xylem vessels, but this needs to be confirmed (Sosnowski *et al.*, 2011).

Diaporthe

Host stress may also affect the transition from the endophytic to pathogenic phase. Hulke *et al.* (2019) found a correlation between increased disease incidence of Phomopsis stem blight of sunflowers and climate data over two decades. *Diaporthe citri* F.A. Wolf, J. Agric. also has been suggested to cause increased symptom expression in yuzu trees (*Citrus ichangensis* × *Citrus reticulata* var. *austera*) under adverse weather conditions (Kim *et al.*, 2015). However, investigations into the effects of stress on *Diaporthe* symptom expression are recent and limited, especially with grapevines.

ROLES OF BIOTIC STRESS FACTORS ON GTD SYMPTOM EXPRESSION

Though still limited, the number of studies investigating the roles that abiotic stress factors play in GTD symptom expression and disease progression is far greater than those investigating effects of biotic factors. Among the few studies available, two biotic factors have been identified: nematodes and other fungal pathogen interactions. In addition, few studies have primarily focused on the roles of biotic factors on GTD caused by *Ilyonectria* fungi.

Parasitic nematodes affecting Ilyonectria *symptom expression*

Plant parasitic nematodes have been shown to affect disease development and symptom expression across a range of pathosystems (Powelson and Rowe, 1993; LaMondia, 2003; Cao *et al.*, 2006), but data are scarce on their interactions with *Ilyonectria* spp. (Hastings and Bosher, 1938; Booth and Stover, 1974; Sutherland, 1977; Rahman and Punja, 2005).

While not involving *Ilyonectria*, bacterial canker of *Prunus* species fruit and nut trees provides an example of how nematodes can influence development and severity of a disease of woody perennials. Ring nematode infestation of roots has been shown to increase lesion length of bacterial canker in peach, caused by *Pseudomonas syringae* van Hall, (Cao *et al.*, 2005; Cao *et al.*, 2006). Ring nematode infestations lead to nitrogen deficiency in peach trees, which was suggested as the mechanism for nematodes increasing susceptibility to infection (Cao *et al.*, 2011). The effects of nitrogen deficiency on disease severity, measured by lesion length are mixed, however, with Cao *et al.* (2011) finding no association between host nitrogen levels and lesion length in peach,

while in a separate study, Cao *et al.* (2013) showed that nitrogen fertilization led to significantly decreased lesion lengths in almond trees. The mechanisms for nitrogen fertilization effects were not determined, but it was suggested that the nitrogen may decrease nematode populations in the soil or by indirect effects such as increased host vigour.

Hastings and Bosher (1938) showed that growth of seedlings of several hosts was inhibited by a combined treatment of root lesion nematodes (*Pratylenchus pratensis)* (de Man) Filipjev and *I. destructans* to a greater extent than either pathogen or nematode alone. Rahman and Punja (2005) also found that development of root rot in ginseng was contingent on minor wounds, which could occur from nematode damage. This was also shown for root rot in clovers, where increased stress symptoms occurred when root systems were damaged (Barbetti *et al.*, 2007). Sutherland (1977) inoculated Douglas fir seedlings with dagger nematodes (*Xiphinema bakeri* Williams) and *I. destructans*, separately or in combination, and showed that while the nematode caused corky root symptoms on its own, the combined inoculation did not give a synergistic interaction.

In vineyards, several genera of plant parasitic nematodes are ubiquitous, including root-knot nematodes (*Meloidogyne* Goeldi), ring nematodes (*Mesocriconema xenoplax* Andrassy), root lesion nematodes (*Pratylenchus* Filipjev), and dagger nematodes (*Xiphinema* Cobb) (Storey *et al.*, 2017) (Figure 4). Recently research has begun to focus on nematode/pathogen relationships. Rahman *et al.*, (2014) assessed population densities of the ring nematode (*Mesocriconema xenoplax* (Raski) Loof & De Grisse) and the citrus nematode (*Tylenchulus semipenetrans* Cobb) under symptomatic and non-symptomatic vines infected with *Ilyonectria* spp. Nematode population densities did not differ between the symptomatic and asymptomatic vines. However, this study was conducted during a drought period, which is not considered conductive to the spread of *Ilyonectria* spp. or to the buildup of nematode populations, and was carried out with 'Ramsey' rootstock, which is resistant to ring nematodes. Similarly, co-infections of Black foot pathogens with other pathogenic fungi have also been associated with an increase in disease incidence and severity (Probst *et al.*, 2012).

Fungal pathogen interactions and infection thresholds

The interactions between *Ilyonectria* spp. and other fungal pathogens has been documented in apples. For apple replant disease (ARD), a combination of *I. macrodidyma* and *Pythium irregulare* Buisman reduced seedling growth when compared to single inoculations of each pathogen (Tewoldemedhin *et al.*, 2011). Braun (1991) also measured reductions in plant weight and size when apple trees were co-inoculated with *P. irregulare* and *Thelonectria lucida* (Höhn.) P. Chaverri & C. Salgado, which had previously been shown to be pathogenic to apple seedlings (Jaffee, 1982).

Research on effects of co-infections on expression of GTDs has recently increased. Young 'Chardonnay' grapevines infected with pathogens causing Botryosphaeria dieback and Black foot gave more severe decline compared to single pathogen inoculation, while 'Sauvignon Blanc' grafted onto '101-14' infected with Petri disease and Black foot pathogens also had increased disease incidence and severity than with single pathogen infections (Probst *et al.*, 2012; Whitelaw-Weckert *et al.*, 2013). This is important, because co-infections with Black foot and Botryosphaeria dieback pathogens, and especially with those causing Black foot and Petri disease, are common in nurseries and young vineyards (Halleen and Petrini, 2003; Halleen *et al.*, 2007a; Úrbez-Torres *et al.*, 2014a; 2014b).

Fungal interactions in infected grapevines probably affect disease development. Several Basidiomycetes, particularly *Fomitiporia* species, have been associated with Esca symptoms in old grapevines, and are commonly associated with *P. chlamydospora* and *Phaeoacremonium* spp. (Fischer, 2002; Fischer and Kassemeyer, 2003; White *et al.*, 2011; Cloete *et al.*, 2014;). Fischer and Kassemeyer (2003) isolated *F. mediterranea* M. Fisch., along with *P. chlamydospora* and *Phaeocremonium* spp. from symptomatic tissues. In addition to *F. mediterranea*, other species of *Hymenchaetales* Oberw, have been associated with Esca symptoms, including species in *Fomitiporella* Murrill, *Inocutis* Fiasson & Niemela, *Inonotus* P. Karst., and *Phellinus* Quél. (Cloete *et al.*, 2015a; Cloete *et al.*, 2015b). This has led to the hypothesis that some Basidiomycetes are involved in the development of Esca symptoms, but has yet to be confirmed (Gramaje *et al.*, 2018). Similar to *Ilyonectria* spp. co-infections with other GTD pathogens discussed above, the presence of Basidiomycetes may act as inciting factors in symptom expression. However, further research is required to demonstrate specific roles that Basidiomycetes play in Esca disease of grapevines.

While environmental and biotic triggering factors are likely to be important, threshold fungal concentrations within grapevines may also affect the switch from endophyte to pathogen. Inoculation of up to 10^4 conidia mL^{-1} suspensions of *N. luteum* increased lesion lengths, but greater inoculum concentrations decreased lesion lengths (Amponsah *et al.*, 2014). This contrasts with *B.*

dothidea, where spore concentrations up to 10^6 conidia mL^{-1} gave increasing lesion lengths in non-wounded peach bark (Pusey, 1989). Reduced lesion length may be due to competition in small inoculation sites, which was 3 mm diam. In this case, further research into effects of spore concentration on disease severity and grapevine mortality has been suggested (Amponsah *et al*., 2014). Additionally, differences were observed in virulence among different isolates of individual species of *Botryosphaeriaceae* (Úrbez-Torres and Gubler, 2009). Secondary metabolites produced by different GTD fungi, such as Eutypines produced by *E. lata*, are known to be phytotoxic (Andolfi *et al*., 2011). Other species producing secondary metabolites include *P. chlamydospora* and several *Botryosphaeriaceae* involved in Botryosphaeria dieback (Andolfi *et al*., 2011). However, the roles these secondary metabolites play in symptom expression and differences in virulence, in particular among the other GTD fungi, are unclear. In addition, how secondary metabolites may respond in grapevines under either abiotic or biotic stress needs to be elucidated.

CLIMATE CHANGE AND VITICULTURE PRACTICES: IMPACTS ON ABIOTIC AND BIOTIC STRESS FACTORS AND GTD SYMPTOM EXPRESSION

The predicted effects of climate change vary from variations in average temperatures and precipitation, to extreme weather events such as flooding or droughts (Dixon, 2012; Ali, 2013). Several projections have predicted increasing global temperatures and variations in precipitation, as well as expressing concerns about these changes affecting global food security. Temperature increases associated with climate change are expected to increase occurrence of heat waves, agricultural droughts, and river floods, which will likely impact grape-growing regions (Arnell *et al*., 2019). Environmental stress factors such as drought, temperature increases, and salinity are of concern for plant health, particularly relating to climate change (Ahuja *et al*., 2010). Grapevines are sensitive to climate change, particularly temperature, which may put them at increased risk in major grape-growing regions such as the Mediterranean basin (Giorgi and Lionello, 2008; Biasi *et al*., 2019). In addition to likely crop stress due to climate change, it is expected that these climate changes will lead to increased pathogen incidence in crops, due to improved survivability in milder winters, altering the geographic distribution of pathogens, and possible modifications in host resistance to pathogens and plant-pathogen relationships (Dixon, 2012). In particular, climate change may affect grapevines grown in optimal grape-growing regions such as California, where increasingly mild winters may prevent latent bud hardening and pests and pathogens reduced by cold winters may increase (Dixon, 2012). Similar effects have been observed for *Fusarium* infections in maize, where mild winters resulted in increased biomass of *Fusarium* in maize debris while frost-thaw cycles reduced biomass and reduced risks of infection (Lukas *et al*., 2014). Extreme weather conditions such as drought may impact plant defenses, and increased temperatures may lead to plant stress in summers when temperatures peak. However, predictions on effects of climate change on plants are difficult to confirm, as experiments are typically carried out in controlled environments rather than in the field (Elad and Pertot, 2014).

Water deficit irrigation

Some viticulture practices employed to improve grape and wine quality may be stress factors with unintended consequences for GTD's. One example which requires further investigation is regulated deficit irrigation, which involves maintaining water deficits for particular seasonal development periods. For wine grapes, deficit irrigation is often employed from after fruit set until veraison, when normal irrigation recommences (Chaves *et al*., 2010; Intrigliolo and Castel, 2010; Santesteban *et al*., 2011). Deficit irrigation possibly increases grape and wine quality (Acevedo-Opazo *et al*., 2010; Santesteban *et al*., 2011), although there are conflicting reports on the effectiveness of regulated deficit irrigation for improved quality of grapes in all situations (Hepner *et al*., 1985; Acevedo-Opazo *et al*., 2010; Chaves *et al*., 2010; Santesteban *et al*., 2011; Lauer, 2012). Úrbez-Torres (personal communication) observed a difference in GTD incidence on mature 'Cabernet Sauvignon' vineyards grown for different wine quality in Chile. Vineyard blocks grown for premium quality wines at high elevation with poor soils and subjected to a severe water deficit irrigation to improve quality, showed a much greater incidence of Botryosphaeria dieback than other blocks cultivated for the production of bulk table wines grown in valleys on fertile soils and subjected to no water deficit irrigation. Although these observations require explanation, they are consistent with the hypothesis that water stress may enhance GTD development. In contrast, Sosnowski *et al*. (2016), studying 'Cabernet Sauvignon' in South Australia to assess effects of water stress on pruning wound susceptibility to *E. lata* and *D. seriata*, showed that water stress did not increased susceptibility to pruning wound infections. Their results indicated that drought and deficit irrigation practices

were not likely to contribute to increased prevalence of GTD in vineyards. Regulated deficit irrigation for grapevines involves moderate water stress, but the effects of this stress on GTD symptom expression and disease development are unclear. These should be further investigated as a potential grapevine health concern for plants that may contain asymptomatic infections (Santesteban *et al*., 2011; Tarara and Perez Peña, 2015).

Vine pruning and training practices

Travadon *et al.,* (2016), in a study in France assessed incidence of Esca on 'Syrah' 'Mourvèdre' vines grafted onto '140Ru' rootstocks, to assess effects of minimal pruning and spur pruning on a bilateral cordon system. Both cultivars displayed more symptomatic vines when spur pruned than those that had minimal pruning. Mean necrosis was likewise greater (35%) for spur pruned vines than those receiving minimal pruning (21%). Similar results were obtained in California, United States of America, in 'Cabernet Sauvignon' responses to Eutypa dieback symptoms, with minimal pruning in the summer producing less dieback than the pruning of dormant vines or using the Sylvoz system (Gu *et al*., 2015). Lecomte *et al*. (2012) observed that vineyards with the Lyra training system had foliar symptoms in 56% of grapevines, but also had a low proportion of trunks affected. This indicated that the long cordons of the Lyra training system may have been one of the causes. In a follow-up study in France, it was confirmed that different training practices affected Esca foliar symptom severity. Grapevines with short cordons had more severe symptoms than grapevines with long cordons. In addition, plants which were pruned less had less severe symptoms than those that were heavily pruned (Lecomte *et al*., 2018). These surveys only recorded foliar symptoms, but the results were similar to those of Travadon *et al*. (2016), who recorded decreased necrosis in minimally pruned grapevines. While these results are promising, further research is required for other GTD fungi, particularly for those causing Black foot, Botryosphaeria dieback, and Phomopsis. Furthermore, the effects of pruning and training practices require further investigation to determine if and/or how they influence wood necrosis. In addition, grapevines under different pruning regimes and training systems may respond differently to stress conditions, and thus may develop GTD symptoms differently. No studies have been reported on this subject, but these would likely provide greater understanding of these interactions, which would improve management of GTD under different pruning and training systems.

Endophytes as potential reducers of host stress

Endophytes may increase plant tolerance to abiotic and biotic stresses associated with climate change (Chakraborty and Newton, 2011). Endophytes possibly contribute several beneficial attributes to their hosts, including enhanced water use efficiency leading to improved survival during drought, increased nutrient uptake and recycling, reduced stress associated with temperature increases or decreases, and increased tolerance to soil salinity, alkalinity, and heavy metals (Lata *et al*., 2018). In two varieties of rice plants not well adapted to high soil salinity or drought, class 2 fungal endophytes enhanced host water use efficiency, and increased growth rates, reproductive yield, and biomass under drought and high salinity in greenhouse experiments. Class 2 endophytes conferred cold tolerance to rice plants subjected to temperatures ranging from 5°C to 20°C, in a plant growth chamber (Redman *et al*., 2011). Similarly, fungal endophytes increased grain yields and second-generation seed viability in wheat subject to drought and increased temperature (Hubbard *et al*., 2014).

The use of *Pythium oligandrum* Drechsler is another example of reduction of disease progression caused by *P. chlamydospora*. In a 4-month greenhouse assay with 'Cabernet Sauvignon' canes infected with *P. chlamydospora,* colonization of roots by *P. oligandrum* resulted in up to 50% reduction of necrosis, compared to grapevines infected with *P. chlamydospora* without *P. oligandrum* (Yacoub, *et al.,* 2016). In addition, in 'Cabernet Sauvignon', expression of several genes associated with *P. chlamydospora* infection was greater in vines with *P. oligandrum* colonization of the roots than in vines without *P. oligandrum*, suggesting that *P. oligandrum* induced resistance in grapevines. In addition, *P. oligandrum* may also reduce biotic stress by the way of fungus-produced tryptamine, an auxin compound involved in plant growth (Floch *et al.,* 2003; Benhamou *et al*., 2012). Further studies, in particular in other grapevine cultivars and during prolonged intervals are required to determine the efficacy of *P. oligandrum* as a potential biocontrol agent. Promotion of plant growth may aid reduction in the effects of abiotic and biotic plant stress, but this requires further confirmation to determine what, effects this endophyte may have on grapevines infected with GTDs.

In grapevines, *Pseudomonas fluorescens* Migula promoted high salinity tolerance via production of endogenous melatonin in host roots, and promoted plant growth (Ma *et al*., 2016). Bacterial and fungal endophytes have been associated with grapevine tolerance to drought, high salinity, heavy metals, and high and low tempera-

tures (Pacifico *et al*., 2019). These factors include associated biotic tolerance to some GTD pathogens such as *P. chlamydospora,* and *N. parvum*. The use of endophytes to reduce environmental stress should be further investigated in relation to GTD disease development, to determine the roles that bacterial and fungal endophytes may have.

Arbuscular mycorrhizal fungi as potential stress reducers

While not endophytes by definition, arbuscular mycorrhizal (AM) fungi (*Glomeromycota* Walker & A. Schüßler) colonize roots, and these organisms can reduce stress associated with high and low temperatures, as well as water stress, soil compaction, and salinity (Ellis *et al*., 1985; Charest *et al*., 1993; Harrier and Watson, 2003; Lu *et al*., 2007; Zhu *et al*., 2010). Suggested mechanisms for increased tolerance include improved plant nutrition, compensation for damaged roots, competition for infection sites, alterations in root anatomy or morphology, alterations in the mycorrhizosphere, and activation of plant defenses (Azcón-Aguilar and Barea, 1997).

In addition to increased environmental stress tolerance, some evidence suggests that AM fungi increase tolerance to pathogens, in particular root pathogens, although the mechanisms are as yet unclear (Whipps, 2004; Comby *et al*., 2017). AM fungi may also affect tolerance to airborne pathogens, and plants have been reported to have increased resistance to hemibiotrophic and necrotrophic fungal infection, although results have been varied and some have reported increased susceptibility to these pathogens (Comby *et al*., 2017). In grapevines, AM fungi were shown to increase root and shoot biomass in '110R' rootstocks infected with *Armillaria mellea* (Vahl) P. Kumm., compared to plants without AM fungi (Nogales *et al*., 2009).

In greenhouse studies, AM fungi have been shown to influence disease severity in hosts infected by GTD pathogens. Petit and Gubler (2006) inoculated *Vitis rupestris* Scheele with the AM fungus *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler and *I. macrodidyma*, a causal agent of Black foot, in a controlled greenhouse environment. While *I. macrodidyma* was recovered from both AM fungus-inoculated roots and the non-inoculated roots, grapevines inoculated with *R. irregularis* displayed decreased disease severity. Also, apple trees with roots inoculated with AM fungi had decreased symptom expression and increased survival when infected with *N. ribis* compared to experimental controls (Krishna *et al*., 2010).

However, research into the effects of AM fungi on disease severity has given mixed responses, and, in some cases, these fungi have been shown to be detrimental to the plant health. In 'Riparia gloire' rootstocks infected with *I. liriodendri*, colonization by *R. irregularis* increased abundance of the pathogen in the roots, and had no effect on plant growth (Holland *et al*., 2019). Likewise, in '101-14' rootstocks planted in soil inoculated with *I. liriodendri,* a positive linear relationship was demonstrated between AM fungus colonization of roots and proportions of necrosis was found (Vukicevich *et al*., 2018). These results indicate that the use of commercial AM fungi inoculants for *Vitis vinifera* should be further investigated to determine the impacts of these fungi on GTD development.

The use of AM fungi to reduce plant stress is a potential avenue of research, and should be considered for its potential to increase environmental stress and disease tolerances, and the mechanisms of these tolerances should be further investigated. This should particularly be in relation to the potential environmental stress and symptom expression relationships for many GTDs. Field studies are required to determine effects of AM fungi on health of GTD-infected grapevines in field conditions. Field inoculations with commercial AM fungi has also given inconsistent response, and inoculum may spread beyond the intended fields. Further research should therefore be conducted on the use of regionally-based AM fungi inoculants (Farmer *et al*., 2007; Kokkoris *et al*., 2019). This is of particular importance for *Vitis*, as AM fungal communities differ between grapevine rows and inter-row plants. This indicates that grapevines may select for particular AM fungal communities (Holland *et al*., 2014). Increasing the diversity of cover crops may increase the diversity of AM fungal communities, allowing greater chances of grapevine root colonization, and also reduce soil-borne pathogen populations (Holland *et al*., 2014; Vukicevich *et al*., 2016).

CONCLUSIONS AND FUTURE DIRECTIONS

Limited research has been conducted on endophytes in economically important crops, particularly on their roles as latent pathogens. This is an increasing area of interest particularly in grapevines, where many GTD fungi have been suggested to have endophytic phases. Many of the hypotheses addressing transition from the endophytic to the pathogenic phases remain to be tested, in particular, the threshold model developed by Sieber (2007).

GTD as latent pathogens

The roles of *Botryosphaeriaceae* as potential latent pathogens has been well-documented particularly in

forest trees. However, research has just begun in grapevines. Several *Botryosphaeriaceae* have been discovered in the asymptomatic and symptomatic grapevine wood. Effects of abiotic and biotic stress factors on disease development are still largely unknown, and research has only recently begun on the effect of pathogen quantities on symptom expression.

Likewise, the role of *Ilyonectria* spp. has been examined for a number of hosts, with several factors influencing symptom expression. Research on *Ilyonectria* spp. in grapevines is limited, however, with the most studies conducted in nurseries and young vineyards where these pathogens are mostly found. Biotic factors affecting nematode infection of the roots and co-infections of GTD fungi are likely to be important and productive areas of research. Petri disease pathogens in grapevine nurseries and young vineyards have been associated with stress factors in young grapevines, while Esca has been associated with biotic factors including Basidiomycete co-infections. Several abiotic factors have been associated with Petri disease and Esca. Eutypa dieback has received more research attention than other GTDs, but knowledge is limited and associations between symptom expression and climatic factors have only recently been assessed. Phomopsis dieback as a GTD is a new area of research, and most investigations of this disease have been in other hosts. Several species of *Diaporthe* have been suggested as latent pathogens, and their discovery in asymptomatic and symptomatic grapevines indicates that they are also latent pathogens in grapevines.

Latent pathogens in young vineyards

The roles of latent pathogens, particularly those causing Black foot and Petri disease in mother blocks, nurseries and young vineyards, are of particular importance. Many of these pathogens are widespread, and conditions during nursery propagation processes are optimal for the latent transmission of these fungi. Young grapevines may also be more susceptible to disease development and less able to recover, compared with mature grapevines which display symptoms erratically. Further study of the stress factors which may induce disease development is required to determine the nature of these pathogens, and indicate potential treatment options, including best planting conditions to avoid stress and the potential transition from endophytic to pathogenic phases by these fungi. Further study is required of abiotic stress, particularly in nurseries. Research on biotic stress, particularly from pathogenic nematodes and fungal interactions will also be worthwhile. Recently, work has begun on fungal interactions in GTDs, with grapevine initially infected with *D. seriata* followed by infection by one of *N. parvum,* or *P. chlamydospora*. Initial results have shown reduced lesion lengths and changes in host physiology (Wallis *et al.*, 2019). These indicate an urgent need for further research, particularly focusing on fungal interactions involving Black foot and Petri disease pathogens in young grapevines.

Methods for host stress reduction

Reducing stress on grapevines is an important first step, but not all stress in the field can be controlled. Therefore, bacterial and fungal endophytes and AM fungi known to enhance resistance of plants to some stress factors are likely to be productive research candidates. However, the effects of AM fungi on disease development are variable, and regional-based approaches to AM fungi are recommended for further study. Rootstock and scion variety is also likely to be a productive area of study, and research is required to show how resistance of rootstocks to abiotic and biotic stress factors affect GTD development in grapevines.

While GTD fungi potentially acting as latent pathogens has long been the subject of speculation, recent discoveries aided by molecular biology research tools have led to the resurrection of this hypothesis. Study on latent pathogens has recently commenced, and requires urgent focus given the growing impacts of GTDs in most grape production areas. Endophytic phases may lead to uncontrolled spread of potential pathogens throughout nurseries and vineyards, and may cause harm within young vineyards before grape growers can recoup capital investments in new plantings, or may shorten the economic lives of vineyards. Effects of climate change along with specific viticulture practices may further increase the incidence and severity of GTDs, causing increased economic losses. Understanding the effects of these stress factors and development of methods to minimize stress on grapevines in relation to GTD disease development, are crucial first steps to reducing the effects of future climate change, and increasing the economic lifespan of vineyards containing asymptomatically infected grapevines.

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Research Papers

Characterization of *Trichoderma* isolates from southern Italy, and their potential biocontrol activity against grapevine trunk disease fungi

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Summary. Grapevine trunk diseases (GTDs) are one of the most economically important diseases of grapevines, causing yield reductions and limiting vineyard lifespans. Fungal pathogens responsible for GTDs primarily infect grapevines through pruning wounds. The lack of systemic fungicides to stop the advance of fungi in vine vascular systems makes the use of pruning wound protection with either synthetic chemicals or biological control agents (BCAs) the main available GTD control strategy. Demands for sustainable grape production and increasing limitations on pesticide usage have raised interest in potential use of BCAs as pruning wound protectants against GTDs. The objectives of this study were to characterize 16 *Trichoderma* isolates from southern Italy using molecular methods, and to evaluate their potential activity as BCAs *in vitro* against the GTD canker-causing fungi *Diplodia seriata*, *Eutypa lata* and *Neofusicoccum parvum*. Molecular studies along with phylogenetic analyses identified eight species, including *Trichoderma atroviride*, *T. guizhouense*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and one unidentified *Trichoderma* sp. All these species had optimum mycelium growth between 25 and 35°C. *In vitro* dual culture experiments assessed antagonistic capabilities of all the *Trichoderma* isolates against *D. seriata*, *E. lata* and *N. parvum*, and showed that the isolates inhibited mycelial growth of the pathogens by up to 70%. The most inhibitory isolates were tested *in planta* for capability to protect pruning wounds against *D. seriata* and *N. parvum* in a detached cane assay. All the selected *Trichoderma* isolates gave >80% control of *D. seriata* and 60% control of *N. parvum*, and protected pruning wounds for up to 21 d after treatment.

Keywords. Biological control, *Botryosphaeriaceae*, canker, *Diatrypaceae*, *Vitis vinifera*.

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INTRODUCTION

Vitis vinifera L., with 7.15 million ha cultivated and 79.2 million tonnes of grapes produced in 2018, is one of the largest and most profitable perennial crops (FAO, 2020). However, grapes host the widest variety of pathogens of

any woody agricultural plant (Martelli, 1997), with fungi being the most prevalent (Wilcox *et al*., 2015). Among all fungal diseases affecting grapes, grapevine trunk diseases (GTDs) are considered the most destructive, causing significant economic losses to grape and wine industries (Bertsch *et al*., 2013; Gramaje *et al*., 2018). GTDs cause a wide range of foliar and vascular symptoms, and include black foot and Petri disease in young vineyards and Botryosphaeria dieback, esca, Eutypa dieback, and Phomopsis dieback causing overall decline and eventual vine death in mature vineyards (Gramaje *et al*., 2018).

With the exception of Black foot disease, caused by soil-borne fungi, most GTD fungal pathogens are air-borne, and these infect grapevines through pruning wounds (Gramaje *et al*., 2018). Under optimum environmental conditions, fruiting bodies (pycnidia or perithecia) embedded in the bark of cordons and/or trunks of infected grapevines, and/or from other woody hosts surrounding vineyards, discharge spores (conidia or ascospores). These are primarily spread short and/or long distances by rain droplets and wind (Kuntzmann *et al*., 2009; Petzoldt *et al*., 1983; Úrbez-Torres *et al*., 2010). Spores land on susceptible pruning wounds of nearby vines and infection commences. No systemic products are currently available to detain colonization by GTD fungi in vine vascular systems once infections move beyond points of entry. Preventive control measures such as pruning wound protection have been shown to be the most effective (Gramaje *et al*., 2018). Many different products, including synthetic, biological and organic fungicides are available and registered as pruning wound protectants against GTD fungi. Much has been published on this subject, and several reviews on specific products and their use for GTD control have been recently published (Gramaje *et al*., 2018; Mondello *et al*., 2018; Úrbez-Torres, 2011). Increasing consumer demands for more sustainable grapevine production and restrictions on synthetic pesticide usage have resulted in further interest by grape and wine industries to develop integrated pest management (IPM) programmes to control GTDs. These will include organic products, biological control agents (BCAs), cultural practices and responsible use of synthetic chemicals (Bertsch *et al*. 2013).

BCAs are organisms selected for their intrinsic antagonism towards a particular pathogen or pathogen groups, and that can function as effective alternatives to chemical pesticides (Wilson, 2003). BCAs are diverse and include either generalist species of *Bacillus*, *Pseudomonas*, *Streptomyces*, *Trichoderma*, *Clonostachys* or *Beauvaria*, or specialists in *Agrobacterium*, *Ampelomyces*, *Fusarium* or *Aspergillus* (Woo *et al*., 2014). Among them, *Trichoderma* species are the most studied as bio-pesticides, with over 60% of registered fungal BCAs products based on *Trichoderma* spp. (Verma *et al*., 2007). This is also true for grapevines, in which *Trichoderma*-based products are becoming more prevalent as alternatives for reducing pesticide use to control economically important diseases, including GTDs (Pertot *et al*., 2017; Mondello *et al*., 2018; Berbegal *et al*., 2020). Several *Trichoderma*-based commercial products using different *Trichoderma* species or strains are available as pruning wound protectants against GTDs, but there are conflicting reports on their effectiveness depending on the GTD fungal pathogen targeted and/or the grape cultivar and region (Mutawila *et al*., 2011; Gramaje *et al*., 2018; Mondello *et al*., 2018). The success of *Trichoderma* spp. as BCAs depends on their ability to survive under unfavourable conditions, their high reproductive capacity, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against plant pathogenic fungi, and efficiency in promoting plant growth and defense mechanisms (Verma *et al.,* 2007). Disease control efficacy by *Trichoderma* spp. may depend to their adaptability to host plants and regional environmental conditions.

The main objectives of the present study were; i) to characterize, using molecular methods, several *Trichoderma* isolates obtained from different substrates in southern Italy; and ii) to assess their potential biocontrol activity against commonly found canker-causing GTD fungi.

MATERIALS AND METHODS

Trichoderma *isolates and grapevine trunk disease fungal pathogens*

Sixteen hyphal-tipped *Trichoderma* isolates were selected for this study (Table 1). The isolates were stored in the fungal collection of the Department of Soil, Plant and Food Sciences at the University of Bari Aldo Moro, Bari, Italy. *Trichoderma* isolates were retrieved from the collection, revived on potato dextrose agar (PDA) and submitted to the Summerland Research and Development Centre (SuRDC), Summerland, British Columbia, Canada for characterization. The GTD pathogens *Diplodia seriata* (PARC91), *Eutypa lata* (PARC381) and *Neofusicoccum parvum* (PARC15) were selected for this study. Isolates of these pathogens were kept dormant as mycelium plugs in glass vials in autoclaved distilled water at 2°C in the Plant Pathology Fungal Collection at the SuRDC. The isolates were revived by placing three to four mycelium plugs on PDA and incubating at 22°C in the dark. When colonies were observed, they were transferred to fresh PDA and incubated until needed.

DNA extraction, PCR amplification, sequencing and phylogeny of Trichoderma *isolates*

Trichoderma isolates were grown on PDA for up to 3 d at 22°C in the dark. Total genomic DNA was extracted from actively growing colonies of each isolate using the Power Soil DNA Isolation Kit (MO BIO Laboratories Inc.) following the manufacturer's instructions. Oligonucleotides primers ITS1/ITS4 (White *et al.*, 1990), tef71f /tef997R (Braithewaite *et al.*, 2017) and fRBP2-7cR/fRBP2-5F (Liu *et al.*, 1999) were used to amplify, respectively, the ITS1-5.8S-ITS2 gene (ITS), a portion of the translation elongation factor 1-α (*EF-1α*) gene, and the RNA polymerase II second largest subunit (*RPB2*). ITS PCR mixtures each contained 4 µL of 5× Phusion HF Buffer, 11.9 µL of SD-H_2O, 2 µL of 2.0 mM dNTPs, 0.4 µL of Blotto 10%, 0.25 µL of 20 mM of each primer, 0.2 µL of *Phusion*, 1 µL of DNA, and ultrapure water up to 20 µL. EF1-α and RPB2 polymerase chain reaction (PCR) mixtures each contained 2 µL of 10× PCR buffer, 13.85 µL of SD-H_2O, 2 µL of 2.0 mM dNTPs, 0.4 µL of Blotto 10%, 0.25 µL of 20 mM of each primer, 0.1 µL of *DreamTaq* DNA polymerase, 1 µL of DNA, and ultrapure water up to 20 µL. Amplifications were performed in a GeneAmp 2700 thermal cycler (Applied Biosystems) using the following PCR conditions. ITS PCR conditions were one cycle at 98°C for 30 s followed by 35 cycles at 98°C for 10 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. EF-1α PCR conditions were 95°C for 6 min followed by four cycles at 95°C for 60 sec, 70°C for 90 sec, 72°C for 90 sec; 26 cycles with the annealing temperature decreasing from 68°C to 55°C (reduction gradient 0.5°C per cycle), and 12 cycles with the annealing temperature at 55°C; with a final extension at 72°C for 7 min. RBP2 PCR conditions were of hot start at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 55°C for 2 min, an increase of 1°C per 5 s to 72°C, and 72°C for 2 min; with a final extension at 72°C for 10 min. All PCR products were purified using a QIAquick PCR purification Kit (QIAGEN Inc.), and both strands of the ITS, *EF1-α* and *RPB2* were sequenced using a eight-capillary AB 3500 genetic Analyzer Sanger Sequencer at the SuRDC.

Forward and reverse sequences from each *Trichoderma* isolate were edited and assembled using Lasergene SeqMan Pro version 9.1.1.4 (DNASTAR Inc.). Consensus sequences were then compared with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST), and closest related sequences were selected to conduct phylogenetic analyses (Table 1). *Trichoderma* sequences from southern Italy were aligned with published GenBank sequences, including ex-type specimens when available for comparison, using the computer software BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999) (Table 1). Alignments were corrected visually and manually edited. Single phylogenetic analyses for each ITS, *EF-1α* and *RPB2* dataset were first conducted using Maximum Parsimony (MP) with the bootstrap test (1,000 random additional sequence replicates) and the Tree-Bisection-Regrafting (TBR) algorithm in MEGA-X (Kumar *et al.*, 2018). In addition, each aligned locus was further analyzed by Neighbor-Joining (NJ) using the Maximum Composite Likelihood method with 1,000 replicates to assess the robustness and the Tamura-Nei model. Tree topologies from individual trees were compared. ITS, *EF-1α* and *RPB2* trees with the greatest log likelihood values were selected. *Trichoderma* sequences from this study were deposited into GenBank, and representative isolates are maintained in both University of Bari Aldo Moro and SuRDC fungal collections.

Optimum temperatures for mycelium growth of Trichoderma *isolates*

Mycelium plugs (4 mm diam.) from the edges of 3-d-old actively growing colonies on PDA from 12 *Trichoderma* isolates (Table 1), were used to inoculate 90 mm diam. Petri dishes each containing 20 mL of PDA. Three replicates of each isolate were incubated separately at eight different temperatures ranging from 5 to 40°C, at 5°C intervals, in the dark. The orthogonal diameters of developing colonies were measured after 3 d incubation. The experiment was conducted twice. Paired T-test analyses were conducted to test for statistical differences between the first and the second experiment, to determine if data from separate experiments could be pooled for analyses. The mean radial mycelial growth and standard error for each temperature/species combination was calculated. Statistical analyses were performed using R.

Dual culture antagonism assay

Antagonistic capability of the selected *Trichoderma* isolates was evaluated *in vitro* against the GTD pathogens *D. seriata* (PARC 91), *E. lata* (PARC 381) and *N. parvum* (PARC 15) using dual culture assays (Rahman *et al.*, 2009). Mycelium plugs (6 mm diam.) of each *Trichoderma* and pathogen were obtained from the edges of 3-d-old actively growing colonies on PDA, and were placed opposite to each other at the edge of 90 mm diam. Petri dishes containing PDA. Plates were incubated at 22°C in the dark for 5 d. *Diplodia seriata*, *E. lata* and *N. parvum* were individually grown without the

Table 1. *Trichoderma* isolates used in this study and their GenBank accession numbers.

Species	Isolate[a]	Host	Geographic origin	ITS[d]	EF-1α[e]	RPB2[f]
Trichoderma atroviride	CBS 142.95	*Quercus* sp.	Slovenia	MH862505	-	-
T. atroviride	CBS 693.94	Mushroom compost	Northern Ireland	MH862501	-	-
T. atroviride	CBS 119499	*Prunus padus*	Austria	FJ860726	-	FJ860518
T. atroviride	DAOM 222144	n/a	n/a	AF456916	-	FJ442754
T. atroviride	DAOM 230838	n/a	Japan	-	KJ871107	-
T. atroviride	DAOM 238037	n/a	Thailand	-	KJ871093	-
T. atroviride	DAOM 242935	n/a	n/a	-	KX463440	-
T. atroviride	DAOM 242940	n/a	n/a	-	KX463436	-
T. atroviride	**PARC1011[b] / FV146**	n/a	Italy	MT448957	MT454114	MT454130
T. atroviride	**PARC1014[b] / FV54**	n/a	Italy	MT448960	MT454117	MT454133
T. atroviride	**PARC1017[b] / FV178**	n/a	Italy	MT448963	MT454120	MT454136
T. atroviride	**PARC1018[c] / FV271**	n/a	Italy	MT448964	MT454121	MT454137
Trichoderma guizhouense	BG8	*Ceriops tagal*	China	MH712265	-	-
T. guizhouense	BG9	*Ceriops tagal*	China	MH712266	-	-
T. guizhouense	DAOM 231412	n/a	n/a	-	AY605764	-
T. guizhouense	DAOM 231435	n/a	n/a	-	EF191321	-
T. guizhouense	NJAU 4742	Plant tissue	China	-	KP292611	-
T. guizhouense	**PARC1022[b] / Tch_4**	*Prunus persica*	Italy	MT448968	MT454125	MT454141
T. guizhouense	**PARC1023[b] / 5B**	*Prunus persica*	Italy	MT448969	MT454126	MT454142
T. guizhouense	**PARC1025[b,c] / Tch_6**	*Prunus persica*	Italy	MT448971	MT454128	MT454144
T. guizhouense	**PARC1026[c] / Tch_7**	*Prunus persica*	Italy	MT448972	MT454129	MT454145
T. guizhouense	S628	n/a	Greece	-	-	KJ665273
Trichoderma harzianum	*CBS 226.95*	Soil	England	AF057606	-	AF545549
T. harzianum	CBS 354.33	Soil	U.S.A.	MH855457	-	-
T. harzianum	DAOM 233986	n/a	n/a	EF392757	EF392749	KJ842171
T. harzianum	DAOM 242937	n/a	n/a	-	KX463434	-
T. harzianum	PAN12-34	Soil	U.S.A.	MK32267	-	-
T. harzianum	**PARC1013[b] / FV185**	n/a	Italy	MT448959	MT454126	MT454132
T. harzianum	**PARC1019[b] / Tch_1**	*Prunus persica*	Italy	MT448965	MT454122	MT454138
Trichoderma koningiopsis	Arak-96	Soil	Iran	-	KP985652	-
T. koningiopsis	*GJS 93-20*	Wood	Cuba	DQ313140	-	-
T. koningiopsis	ITCC 7291	Soil	India	-	LN897322	-
T. koningiopsis	**PARC1024[c] / Tch_5**	*Prunus persica*	Italy	MT448970	MT454127	MT454143
T. koningiopsis	S359	n/a	France	-	-	KJ665285
Trichoderma longibrachiatum	*ATCC 18648*	Mud	U.S.A.	NR_120298	AY865640	HQ260615
T. longibrachiatum	CIB T13	n/a	Colombia	-	EU280033	-
T. longibrachiatum	DAOM 167674	n/a	n/a	EU280099	-	-
T. longibrachiatum	DAOM 234103	n/a	n/a	-	DQ125467	-
T. longibrachiatum	**PARC1015[b] / FV144**	n/a	Italy	MT448961	MT454118	MT454134
T. longibrachiatum	S328	n/a	n/a	JQ685875	-	KJ665291
Trichoderma nigricans	NBRC 31285	n/a	n/a	JN943368	-	-
Trichoderma paratroviride	*CBS 136489*	n/a	Spain	-	KJ665627	KJ665321
T. paratroviride	**PARC1012[b] / FV145**	n/a	Italy	MT448958	MT454115	MT454131
T. paratroviride	S489	n/a	Spain	-	KJ665628	KJ665322
T. paratroviride	TE11	n/a	n/a	MH549109	-	-
T. paratroviride	TES15	n/a	n/a	MH549108	-	-
Trichoderma paraviridescens	*Hypo 372*	n/a	Austria	MT187973	-	KC285763
T. paraviridescens	S122	n/a	Italy	-	-	KC285764

(Continued)

Table 1. (Continued).

Species	Isolate[a]	Host	Geographic origin	ITS[d]	EF-1α[e]	RPB2[f]
T. paraviridescens	ATCC 20898	Soil	U.S.A.	-	-	EU252009
T. paraviridescens	BMCC:LU786	n/a	New Zealand	-	KJ871271	-
T. paraviridescens	KX098484	n/a	New Zealand	-	KX098484	-
T. paraviridescens	**PARC1016[b] / FV154**	n/a	Italy	MT448962	MT454119	MT454135
Trichoderma simonsii	CBS 130431	Decaying bark	U.S.A.	NR_137297	-	-
T. simonsii	GJS 91-138	Wood	U.S.A.	-	-	FJ442757
T. simonsii	GJS 92-100	Wood	U.S.A.	-	-	FJ442710
T. simonsii	S7	n/a	Italy	-	-	KJ665337
Trichoderma sp.	**PARC1020[c] / Tch_2**	*Prunus persica*	Italy	MT448966	MT454123	MT454139
Trichoderma spirale	*DAOM 183974*	Soil	Thailand	DQ083014	-	AF545553
T. spirale	DIS 311D	*Irvingia gabonensis*	Cameroon	FJ442232	-	-
T. spirale	**PARC1021[b] / Tch_3**	*Prunus persica*	Italy	MT448967	MT454124	MT454140
T. spirale	UNISS 3b-11	n/a	n/a	-	EF596973	-
T. spirale	UNISS 23-9	n/a	n/a	-	EF596975	-

[a] Isolate numbers in bold represent *Trichoderma* isolates from Italy used in this study. Isolate numbers in italics and underlined are ex-type specimens. CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), DAOM (Department of Agriculture Ottawa Mycology, Ottawa, ON, Canada), PARC (Pacific Agri-Food Research Centre Fungal Collection, Summerland, BC, Canada). ATCC (American Type Culture Collection, Manassas, VA, USA).
[b] *Trichoderma* isolates used in the temperature study.
[c] *Trichoderma* isolates used in the detached cane assay experiment.
[d] ITS: Internal Transcribed Spacer.
[e] EF1-α: Translation elongation factor 1-α.
[f] RNA polymerase II second largest subunit.
n/a: Not available.

presence of *Trichoderma* under the same conditions as experimental controls. Each *Trichoderma*/pathogen combination was replicated four times, and the experiment was conducted twice. The percent of mycelium inhibition was calculated using the formula:

Percent Inhibition (%) = [(B – A) / B] * 100

where A is the radius of pathogen mycelium growth co-inoculated with *Trichoderma* and B is radius of the pathogen mycelium growth alone in the control plate.

Statistical data analyses were adapted from Haidar *et al.* (2016). The experimental data from the dual culture antagonism assays were subjected to analysis of variance (ANOVA) followed by Tukey-Kramer's Honest Significant Difference post-hoc comparison tests (Tukey HSD) ($P = 0.05$). The standard errors of the means were calculated for all mean values.

Detached grapevine cane assay

The antagonistic activity of most inhibitory *Trichoderma* isolates from the dual culture experiments was evaluated *in planta* against the GTD pathogens *D. seriata* (PARC 91) and *N. parvum* (PARC 15) using a detached cane assay (DCA) (Ayres *et al.*, 2014) (Table 1). *Trichoderma* inoculum was obtained by scraping the surfaces of 4-d-old *Trichoderma* colonies grown on PDA at 22°C with a UV light /darkness regime of 12 h:12 h, after flooding each plate with sterile water containing 0.05% Tween 20 and filtering through autoclaved 25 µm pore diam. Miracloth (Sigma-Aldrich) to remove mycelium fragments. Conidium suspensions of each *Trichoderma* isolate were adjusted to 10^6 conidia mL^{-1} with a haemocytometer. Pycnidiospores of *D. seriata* and *N. parvum* were obtained from pycindia formed on colonies growing on PDA in the dark at 25°C with a UV light/darkness regime of 12 h:12 h, with the UV light source(Phillips UVB TL 20W/12RS bulb) at 80 cm distance from the Petri plates. Pycnidia and pycniospores formed after 4-5 weeks were harvested by adding 1-2 mL of sterile distilled water previously amended with a drop of Tween 20, then gently scraping the upper layer of the PDA with a sterile thin spatula to expose the pycnidia. Suspensions containing pycnidia was transferred into a sterile pestle and ground with a mortar to release spores from pycnidia. Suspensions were filtered through Miracloth into test tubes, which were vortexed, and spore concentrations were adjusted

to 10^5 spores mL^{-1} for both *D. seriata* and *N. parvum* using a haemocytometer.

Dormant grapevine canes (cv. Chardonnay) were collected from an experimental vineyard block located at the SuRDC. The canes were cut into two node sections (≈ 20 cm length), and were placed vertically through holes in Styrofoam trays floating in water, in a contained plant growth chamber (CONVIRON) set at 25°C, 70% RH, and 12 h light:12 h dark regime. The canes were then each pruned to 4 cm above the upper bud to simulate a fresh pruning wound. Soon after pruning, the wounds were individually treated with 50 µL of 10^6 conidia mL^{-1} (50,000 conidia per wound) of each *Trichoderma* spp. Different wounds were then challenged 24 h, 7 d or 21 d after treatment with 50 µL of 10^5 conidia mL^{-1}(5,000 spores per wound) of *D. seriata* or *N. parvum* to determine how long any *Trichoderma* activity lasted on each pruning wound. Positive controls included non-treated but inoculated wounds with *D. seriata* or *N. parvum* at 24 h, 7 d, or 21 d after pruning. Negative controls included non-treated and non-inoculated canes to determine if natural infections occurred in the collected canes from the experimental vineyard. A total of 30 canes per treatment were used, and canes were placed in randomized blocks of ten canes each. The cane cuttings were maintained under controlled conditions in the plant growth chamber for 5 weeks. After this time, the canes were collected, roots and leaves were removed, and the canes were prepared for re-isolation of the inoculated pathogens. The bark around the pruning wounds was shaved off, then the surface of each cane was flame sterilized with 95% ethanol. A tissue piece (≈ 1 mm) from the surface of each cane was discarded and then ten pieces of tissue (≈ 0.5 cm^2) were plated onto PDA amended with 1 mg mL^{-1} tetracycline (Sigma-Aldrich) (PDA-tet). Plates were the incubated for up to 10 d at 22°C in the dark. If a plate yielded either *D. seriata* or *N. parvum*, the corresponding cane was rated as colonized by the respective pathogen. The mean percent infection (MPI) by each pathogen was determined from all positive controls and treatments. *Trichoderma* spp. inhibition effectiveness was calculated as the mean percent disease control (MPDC) using the formula:

MPDC = 100 × [1-(MPI treated canes / MPI control canes)]

Statistical data analyses were adapted from the method used by John *et al.* (2005). The binary (infected or not-infected) data produced from the detached cane assays were analyzed using a logistic regression to determine the significance of timing of inoculation, and were subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests to determine if there were statistically significant differences between the means (P = 0.05). All statistical analyses were performed using R (R Core Team, 2019).

RESULTS

Molecular characterization of Trichoderma *isolates from southern Italy*

PCR amplifications of ITS, *EF-1α* and *RPB2* regions gave, respectively, products of approx. 500, 700, and 800 bp. To identify the different isolates and study the phylogenetic relationships among *Trichoderma* from southern Italy, ITS, *EF-1α* and *RPB2* sequences were BLAST analysed to select closely related sequences for the phylogenetic analyses (Table 1). Thirty seven taxa were included in the phylogenetic analysis of the ITS dataset, 36 taxa were included in the *EF-1α* dataset, and 33 taxa were included in the *RPB2* dataset. There were a total of 608 (ITS), 819 (*EF-1α*) and 849 (*RPB2*) positions in each final dataset, including gaps. The ITS and *RPB2* MP evolutionary histories both generated ten equally parsimonious trees. The *EF-1α* MP evolutionary history generated nine equally parsimonious trees (trees not shown). Lengths, consistency indices (CI), retention indices (RI), and rescaled consistency indices (RC) were, respectively, 98, 0.956989, 0.995056, and 0.954441 for ITS; 659, 0.835385, 0.968994, and 0.811662 for EF-1α; and 423, 0.808290, 0.959496, and 0.791641 for RPB2. The NJ analyses of each ITS, *EF-1α* and *RPB2* dataset resulted in trees with similar topology to the MP tree. The ITS, *EF-1α* and *RPB2* trees with the greatest log likelihoods are shown, respectively, in Figures 1, 2 and 3.

Based on individual phylogenetic analyses, *Trichoderma* isolates from southern Italy were identified as *Trichoderma atroviride* (PARC1011, PARC1014, PARC1017 and PARC1018), *T. guizhouense* (PARC1022, PARC1023, PARC1025, and PARC1026), *T. harzianum* (PARC1013 and PARC1019), *T. koningiopsis* (PARC1014), *T. longibrachiatum* (PARC1015), *T. paratroviride* (PARC1012), *T. paraviridescens* (PARC1016), *T. spirale* (PARC1021) and *Trichodrma* sp. (PARC1020).

The ITS phylogenetic analyses resulted in eight clades, each corresponding to a well-supported species. However, ITS analyses were not informative enough to discriminate among *T. harzianum* and *T. guizhouense* isolates, and included isolates of both species in a single clade along with *T. nigricans*, another species within the *T. harzianum* species complex (Figure 1). In addition, a second well-supported clade (82% NJ) containing *T. har-*

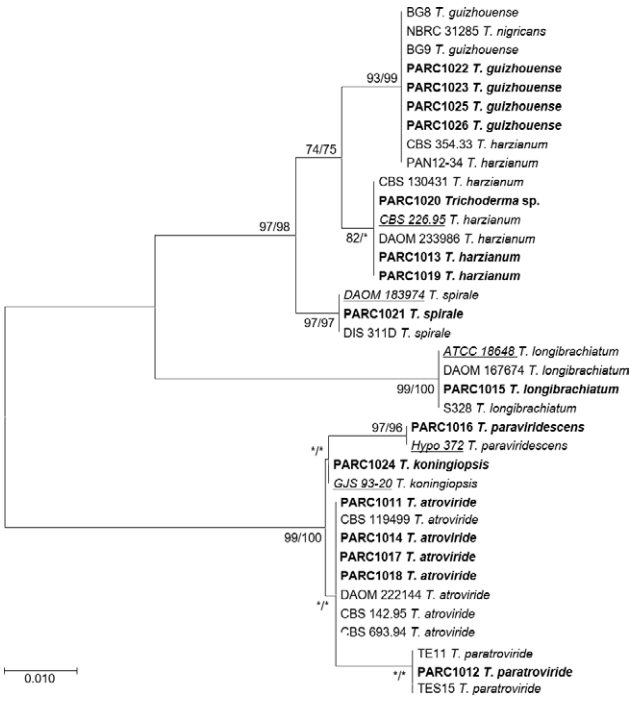

Figure 1. Greatest log likelihood ITS Neighbor-Join phylogenetic tree generated with 37 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values represented by asterisks were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

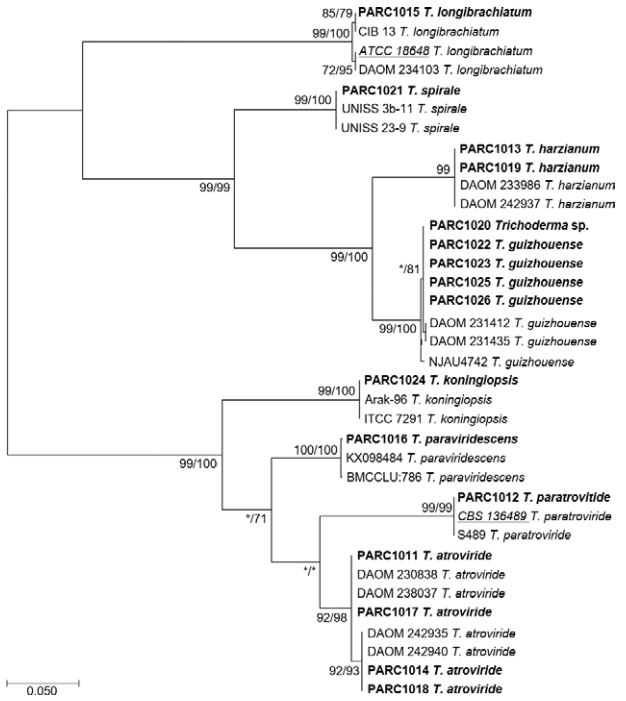

Figure 2. Greatest log likelihood EF-1α Neighbor-Join phylogenetic tree generated with 36 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values accompanied by asterisks were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

zianum isolates, including the ex-type CBS 226.95, was formed in the ITS analyses (Figure 1).

The *EF-1α* phylogenetic analyses placed *Trichoderma* isolates from southern Italy in eight different well-supported clades corresponding to each of the species identified, with the exception of isolate PARC1020, which grouped within one of the *T. harzianum* clades in the ITS analyses but fell within the *T. guizhouense* clade in the *EF-1α* analyses (Figure 2). Intraspecific variation was observed in three clades in the *EF1-α* analyses, including the *T. atroviride*, *T. guizhouense*, and *T. longibrachiatum* (Figure 2). Bootstrap values in the *EF-1α* analyses were greater than in the ITS analyses.

The *RPB2* phylogenetic analyses were the most informative, and these grouped *Trichoderma* isolates from Italy in eight well-supported clades, including the ex-types (Figure 3). Similar to ITS and *EF-1α* analyses, *RPB2* was did not group isolate PARC1020 with any identified clade, and it was closely related to *T. simonsii*, another species identified within the *T. harzianum* species complex. Therefore, isolate PARC1020 was identified as *Trichoderma* sp. (Figure 3). *RPB2* phylogenetic analyses resulted in the greatest bootstrap support for each clade.

Optimum temperatures for mycelium growth of Trichoderma *isolates*

Radial colony growth values for each *Trichoderma* isolate were plotted versus temperature (Figure. 4A). All isolates grew over the temperature range of 10 to 30°C, except for PARC1016, which did not grow at 30°C. Isolates PARC1013, PARC1015, PARC1019, PARC1022, PARC1023, PARC1025 grew at 35°C. None of the isolates used in the study grew at 5°C or 40°C (Figure 4A). Optimum temperatures for mycelium growth varied among isolates. The temperatures at which each *Trichoderma* isolate reached the maximum radial growth were 25°C for PARC1011, PARC1012, PARC1014, PARC1016, and PARC1017; 30°C for PARC13, PARC19, PARC1021, PARC1022, PARC1023, and PARC1025; and 35°C for PARC1015 (Figure 4A). Among all the isolates, PARC1017 had the least radial colony growth. Colony colours were cream for iso-

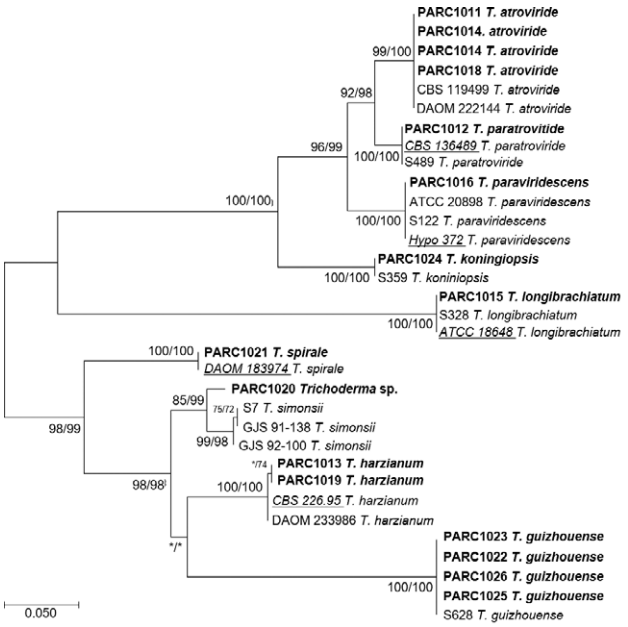

Figure 3. Greatest log likelihood RPB2 Neighbor-Join phylogenetic tree generated with 33 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values accompanied by an asterisk were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

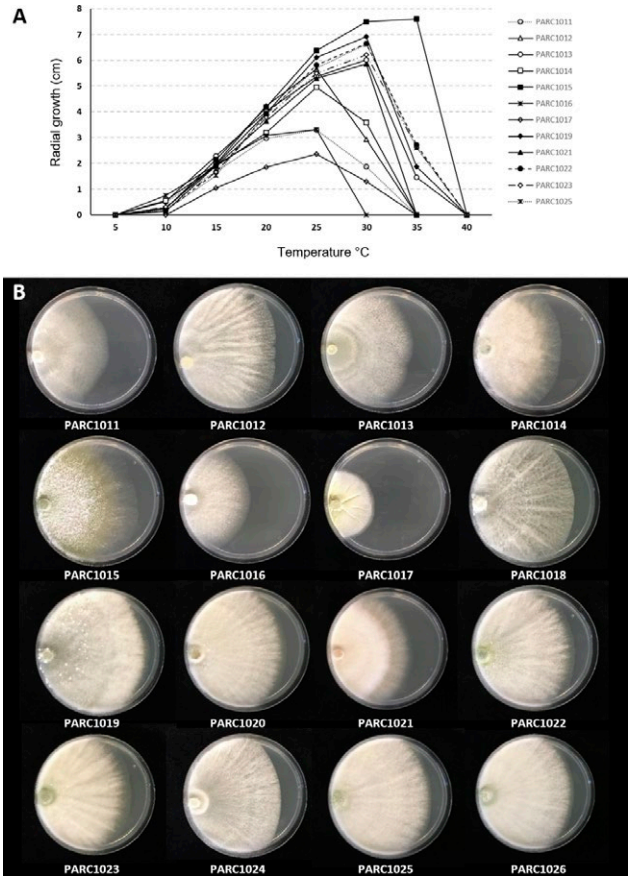

Figure 4. Phenotypical features of *Trichoderma* isolates from southern Italy. A. Mean radial mycelium growth at different temperatures after 72 h. B. Colony growth and colour after 72 h on potato dextrose agar for the different *Trichoderma* isolates from southern Italy used in this study.

lates PARC1014, PARC1018, and PARC1021, green for PARC1015, and white for the other *Trichoderma* isolates used in this study (Figure 4B).

Dual culture antagonism assays

The levels of antagonism of *Trichoderma* isolates used in this experiment against the GTD pathogens *D. seriata*, *N. parvum* and *E. lata* is shown in Figures 5 and 6. The mean percentage of inhibition of radial mycelial growth (PIRG) of *D. seriata*, *N. parvum* and *E. lata* by *Trichoderma* isolates used in this experiment ranged from 24.7% to 74.3% (Figure 6). Mean PIRG ranged from 51.4% to 69.6% for *D. seriata*, from 44.5% to 74.3% for *N. parvum* and from 24.7% to 68.2 for *E. lata* (Figure 6). The greatest PIRG for *D. seriata* (69.6%) and *E. lata* (68.2%) were recorded from *T. atroviride* PARC1018, and for *N. parvum* (74.3%) for *T. koningiopsis* PARC1024 (Figure 6). Overall, antagonism of *Trichoderma* isolates against *E. lata* mycelium growth was less than for both *D. seriata* and *N. parvum*, with seven out of 16 *Trichoderma* isolates giving mean PIRG less than 50% (Figure 6).

Detached grapevine cane assays

Results of the DCA trial are shown in Table 2. MPI of *D. seriata* and *N. parvum* from positive controls inoculated with 5,000 spores per wound were, respectively, 60% and 80% or greater, even when wounds were inoculated 21 d after pruning. *Diplodia seriata* and *N. parvum* were not isolated from non-treated and non-inoculated negative controls (natural infection, data not shown). All isolates tested in the DCA gave MPDC of 84 to 100% against *D. seriata* and 23 to 100% against *N. parvum* (Table 2). *Trichoderma atroviride* (PARC1018) and *T. harzianum* (PARC1020) gave the greatest MPDC (100%) against *D. seriata* from day one to day 21 after treatment. The other *Trichoderma* isolates gave high MPDCs (89 to 94%) when pruning wounds were challenged with *D. seriata* one day after treatment, and 100% MPDC when wounds were challenged 21 d after treatment

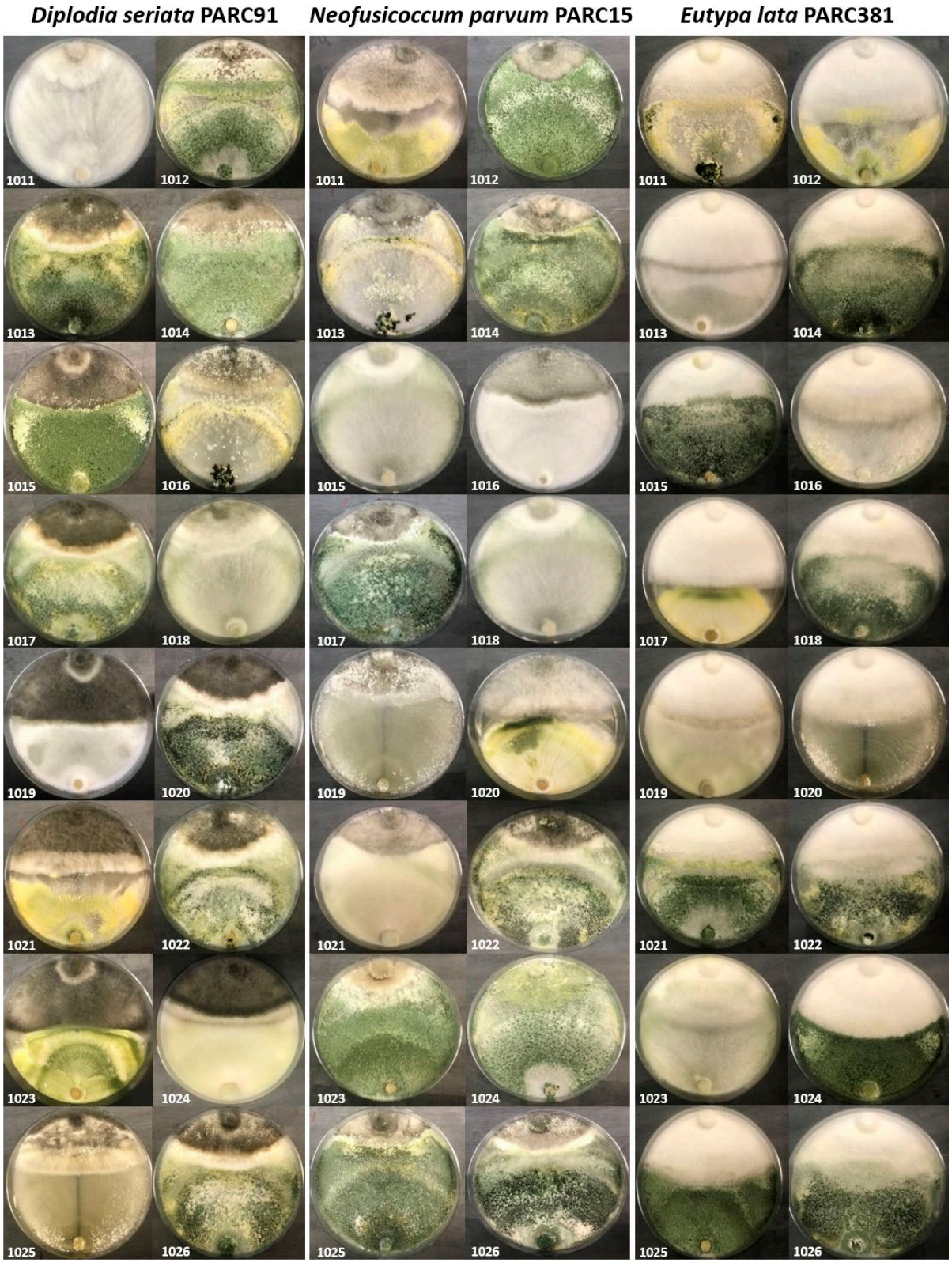

Figure 5. Dual culture antagonism experiment. Petri plates show antagonistic activity of each *Trichoderma* isolate used in this study (bottom side of Petri dish) against the GTD pathogens *Diplodia seriata* PARC91, *Neofusicoccum parvum* PARC15 and *Eutypa lata* PARC 381 (top of Petri plate) five days after culturing on PDA at 23°C.

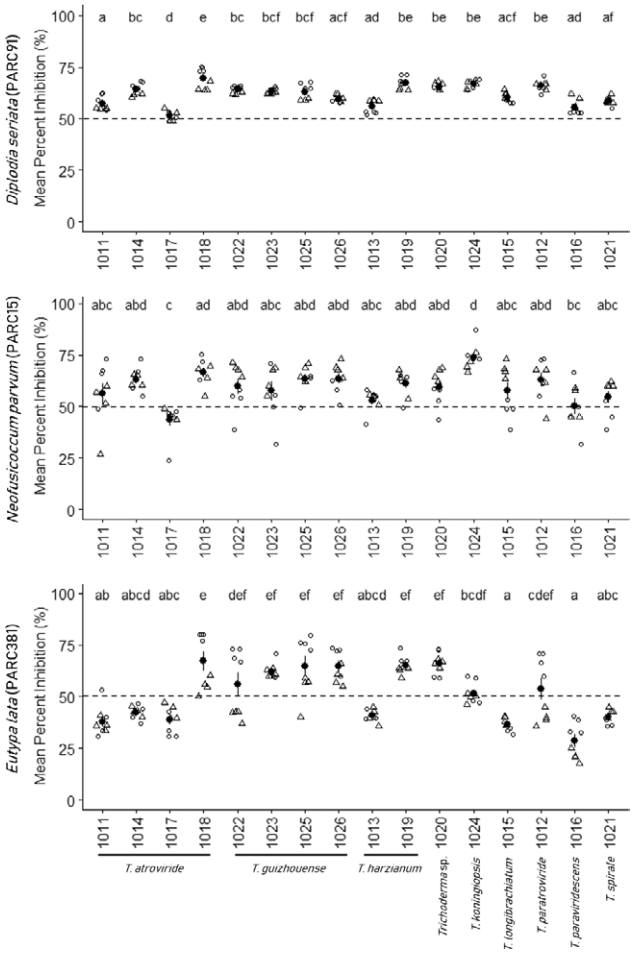

Figure 6. Mean percent inhibition of radial growths (PIRG) for *Trichoderma* isolates from southern Italy against GTD pathogens *Diplodia seriata* PARC91, *Neofusicoccum parvum* PARC15 and *Eutypa lata* PARC 381, measured after 5 d. White dots and triangles represent, respectively, data from experiment 1 and repeated experiment 2. Solid black dots represent the mean percent inhibition for each isolate calculated from eight replicates, and bars represent standard errors of the means. Dash line represents 50% inhibition threshold. Columns accompanied by the same letter were not statistically different ($P = 0.05$), Tukey-Kramer Honest Significant Difference post-hoc tests.

(Table 2). *Trichoderma atroviride* (PARC1018) and *T. paratroviride* (PARC1012) gave the greatest MPDC (88-100%) against *N. parvum* from day one to day 21 after treatment. The remaining isolates provided much lower MPDCs (23 to 70%) against *N. parvum*, except for *T. guizhouense* (PARC1026) that gave MPDCs of 69% at 1 d after treatment, 75% at 7 d, and 83% at 21 d after treatment. All the isolates tested in the DCA experiment gave MPDCs greater than 50% when pruning wounds were challenged with 5,000 *N. parvum* spores per wound 7 d after treatment (Table 2).

DISCUSSION

This study has identified nine different *Trichoderma* spp. from southern Italy and characterized their *in vitro* potential as biocontrol agents against the GTD fungal pathogens *D. seriata*, *N. parvum* and *E. lata*. *Trichoderma* isolates in this study were identified to species level based on molecular and phylogenetic analyses of the ITS, *EF1-α* and *RPB2* genes, and included *T. atroviride*, *T. guizhouense*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and an unidentified *Trichodrma* sp.

The genus *Trichoderma* was first described by Christiaan Hendrik Persoon in 1794 and later linked to the sexual state *Hypocrea*, which was described by Elias Fries in 1825. The type species of the *Hypocrea* is *Hypocrea rufa* (Pers. : Fr.) Fr. (anamorph *T. viride*) (Jaklitsch *et al.*, 2006). Species of *Trichoderma* and their *Hypocrea* teleomorphs can colonize different substrates; however, they are primarily isolated from soil. *Trichoderma* spp. have cosmopolitan distribution and have been isolated from many different ecosystems, including from the most extreme northern and southern latitudes (Chaverri and Samuels, 2003). Many studies made taxonomic reassessments of *Hypocrea* and *Trichoderma* anamorphs, and the current status and species delimitation of these fungi has been outlined by Braithwaite *et al.* (2017), Chaverri and Samuels (2003), Chaverri *et al.* (2015), Jaklitsch *et al.* (2006) and Jaklitsch and Voglmayr (2015). Most of the *Trichoderma* spp. identified in the present study have been previously described to commonly occur in many different regions, including Italy (Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015; Lorenzini *et al.*, 2016; Innocenti *et al.*, 2019). However, the present study describes for the first time the presence of *T. koningiopsis*, *T. paratroviride*, and *T. spirale* in Italy.

Trichoderma koningiopsis was recently described as a new species within the *T. koningii* species complex, based on multi-locus sequence analyses, and this species has been reported from Brazil, Canada, Cuba, Ecuador, France, Germany, New Zealand, Peru, Puerto Rico, and the United States of America (Samuels *et al.*, 2006; Braithwaite *et al.*, 2017). *Trichoderma koningii* has long been known to occur in Italy and was described from citrus in Sicily in the early 1990s (Greuter *et al.*, 1991). It is likely that *T. koningiopsis* could also have been long present in Italy as part of the *T. koningii* species complex, but only now has this species been confirmed using DNA analyses.

Trichoderma paratroviride is a recently described new species, and it has been only reported to occur in China (Zhang *et al.*, 2015), Hungary (Chen *et al.*, 2019),

Table 2. Detached cane assay results under controlled conditions. Values represent the mean percent recovery (MPR) of *Diplodia seriata* (PARC91) and *Neofusicoccum parvum* (PARC15) from pruning wounds treated with selected *Trichoderma* isolates from southern Italy and inoculated 1, 7 or 21 d after treatment. Values in each row followed by same letters are not statistically different (P = 0.05), Tukey-Kramer multiple comparison test.

Pathogen / Inoculation day[a]		I-Controlc	*T. paratroviride*	*T. atroviride*	*Trichoderma* sp.	*T. koningiopsis*	*T. guizhouense*	
			PARC1012	PARC1018	PARC1020	PARC1024	PARC1025	PARC1026
Diplodia seriata								
1 d	MPR	60a	0b	3b	0b	7b	3b	7b
	MPDCb		100	94	100	89	94	89
7 d	MPR	63a	0b	3b	0b	10b	0b	0b
	MPDC		100	95	100	84	100	100
21 d	MPR	60a	0b	0b	0b	3b	0b	0b
	MPDC		100	100	100	94	100	100
Neofusicoccum parvum								
1 d	MPR	87a	7b	10b	47c	37cd	67c	27bd
	MPDC		92	88	46	58	23	69
7 d	MPR	80a	3b	0b	40c	33cd	40c	20bd
	MPDC		96	100	50	58	50	75
21 d	MPR	80a	0b	0b	27c	23cd	33c	13bd
	MPDC		100	100	67	70	58	83

[a] Number of days after the *Trichoderma* treatment when pruning wounds were inoculated with *D. seriata* or *N. parvum*.
[b] MPDC: mean percent disease control was calculated as 100 x [1-(MPR treatment/MPR control)].
[c] Inoculated positive control.

and Spain (Jaklitsch and Voglmayr, 2015). The present study expands the geographical distribution of *T. paratroviride* into Italy. Whether the geographical distribution of this species is restricted to Europe, or more precisely, to southern European countries, cannot yet be confirmed. However, based on the cosmopolitan nature of *Trichoderma* spp., it is likely that future studies and surveys could expand the geographical distribution of *T. paratroviride* to more countries in Europe and/or other continents.

Trichoderma spirale was first described by Bissett (1991) from samples collected in Canada, Thailand, and the United States of America. This species has since been reported from several countries in Africa and Central America, from Iran and Turkey in the middle east and from New Zealand in Oceania (Kindermann *et al.*, 1998; Jaklitsch and Voglmayr, 2015; Braithwaite *et al.*, 2017). To date, *T. spirale* has only been found once in Europe, in the Canary Islands of Spain (Jaklitsch and Voglmayr, 2015). The present study also expands the distribution of *T. spirale* into another European country.

Hypocrea teleomorphs possess highly conserved structures, so they are of limited use for species identification (Chaverri and Samuels, 2003; Samuels *et al.*, 1998). Consequently, phenotypical and molecular characters of *Trichoderma* anamorphs have traditionally been used for species delineation (Chaverri and Samuels 2003). However, with over 250 *Trichoderma* spp. currently accepted (Bissett *et al.*, 2015) and many of them having overlapping morphological features, species differentiation based only on anamorph characters it is not definitive. Implementation of DNA sequencing and multi-locus sequence analyses has facilitated resolution of taxonomic uncertainty and species delineation in *Trichoderma* (Jaklitsch *et al.*, 2006; Chaverri and Samuels, 2003; Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015; Samuels *et al.*, 2006).

Different genetic markers, including a-actin (*ACT*), calmodulin (*CAL*), *EF-1α*, ITS, the large subunit of ATP citrate lyase (*acl1*), and *RPB2* have been commonly used in phylogenetic analyses of the genus (Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015). We used ITS, *EF-1α* and *RPB2* sequences in individual phylogenetic analyses to determine the phylogenetic relationships among *Trichoderma* isolates from southern Italy. All three markers supported species delineation, with *EF-1α* and *RPB2* giving the strongest bootstrap supports, as reported elsewhere (Chaverri and Samuels, 2003; Jaklitsch and Voglmayr, 2015). However, *Trichoderma* isolate PARC1020 could not be assigned to a specific species in the current study. ITS analyses grouped this isolate in the *T. harzianum* clade containing the Ex-type speci-

men and thus, it could have been named *T. harzianum*. However, the *EF-1α* analyses grouped PARC1020 in the *T. guizhouense* clade, while the *RPB2* analyses grouped it in a single clade closely related to *T. simonsii*. These species all belong to the *T. harzianum* species complex, in which a total of 14 cryptic species have been identified (Chaverri *et al*., 2015). We are confident, based on current information, that PARC1020 is not *T. harzianum* but belongs to the *T. harzianum* species complex. Further multi-locus sequence analyses, including other loci such as *ACT* and *CAL*, are required to identify this isolate to species, so we have named PARC1020 as *Trichoderma* sp. until further studies provide more precise nomenclature.

Trichoderma spp. are well-known for their antifungal properties, and they have long been used as BCAs against a wide range of pathogens in many different crops (Schuster and Schmoll, 2010). Species in *Trichoderma* are now the most studied organisms for use in products employed as BCAs, bio-pesticides, and/or bio-fertilizers. Over 60% of registered fungal BCA products are based on *Trichoderma* spp. (Verma *et al*., 2007). *Trichoderma* spp. are also being studied as potential BCAs against GTD fungi. Mondello *et al*. (2018) reviewed trials conducted and compounds used to control GTDs in the last 15 years, including BCAs and *Trichoderma* spp. To date, *T. asperellum*, *T. atroviride*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, and *T. polysporum* have been tested in different countries against GTD pathogens causing Botryosphaeria dieback, Esca and Eutypa dieback (Mondello *et al*., 2018). These *Trichoderma* spp. were shown to be highly efficient against GTD fungi in the different studies. However, most of these data were from experiments conducted *in vitro* and only very few studies completed trials *in planta* and/or under natural field conditions (Mondello *et al*., 2018). The present study adds six new taxa to the list of effective *Trichoderma* spp. against GTD fungi, including *T. guizhouense*, *T. koningiopsis*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and a yet unidentified *Trichoderma* sp. In addition, we report results from *in vitro* and *in planta* experiments.

The *in vitro* dual culture antagonism assays conducted showed at least one isolate from each *Trichoderma* spp. from southern Italy to be highly effective on reducing mycelium growth (60 to 75%) of *D. seriata* and *N. parvum*, two of the most common grapevine canker-causing fungi (Úrbez-Torres, 2011). These results agree with most *in vitro* experiments conducted against these two botryosphaeriaceous fungi (Mondello *et al*., 2018). However, only isolates of *T. guizhouense* and *Trichoderma* sp. and one isolate each of *T. atroviride* and *T. harzianum* gave similar reductions of mycelium growth of reduction against *E. lata* in the present study. This indicates less efficacy of *Trichoderma* spp. against this second canker-causing pathogen. *Trichoderma atroviride* and *T. harzianum* have routinely been shown to be effective against *E. lata*, although mostly used in trials as commercial products and not as pure isolates (Halleen *et al*., 2010; Kotze *et al*., 2011). These studies along with the present results indicate that *T. atroviride* and *T. harzianum* are the most promising species to be used as BCAs against *E. lata*. However, we also showed that isolates of *T. guizhouense* and one isolate of *Trichoderma* sp. were very effective against *E. lata*, but further research is required to expand the number of *Trichoderma* spp. that could be used as BCAs against *E. lata*.

No field trials to assess the efficacy of the different *Trichoderma* spp. under natural conditions were conducted in this study, due to the foreign origins of all the *Trichoderma* isolates. However, the selected isolates were screened against *D. seriata* and *N. parvum in planta* under controlled conditions in a growth chamber using a modified DCA similar to that described by Ayres *et al.* (2014). Due to the lack of a native source of *E. lata* ascospores at the time of conducting this study, we did not include this pathogen in the DCA. The DCA showed all species tested, including *T. atroviride*, *T. guizhouense*, *T. koningiopsis*, *T. paratroviride*, and *Trichoderma* sp., effectively protected pruning wounds against *D. seriata* for at least 21 d after treatment. In contrast, only *T. atroviride*, *T. paratroviride* and *T. guizhouense* isolate PARC1026 showed similar control against *N. parvum*. The greater control of *D. seriata* may correspond to the well-known lower virulence compare to *N. parvum* isolates (Úrbez-Torres, 2011). However, inconsistent efficacy has been shown for *Trichoderma* against GTD pathogens, including botryosphaeriaceous fungi (Halleen *et al*., 2010; Kotze *et al*., 2011; Mondello *et al*., 2018).

The antifungal activity of *Trichoderma* has been extensively documented, and is based on different factors, including antibiosis, mycoparasitism, and nutrient and/or space competition (Schuster and Schmoll, 2010). Though still not well-understood, it is hypothesized that nutrient and/or space competition in grapevine pruning wounds could be an important antifungal mode of action of *Trichoderma* spp. against GTD fungi. Therefore, effective disease control as pruning wound protectants requires time for *Trichoderma* to become established, so most commercial products report greatest antifungal activity several days after application. High levels of control activity were shown for *Trichoderma* spp. in the present study only 1 d after treatment. MPDCs greater than 90% 1 d after treatment were

recorded from four of six *Trichoderma* isolates against *D. seriata*, and from isolate PARC1012 against *N. parvum*. This could have resulted from conducting the DCA experiment under controlled conditions with optimum temperature and humidity for *Trichoderma* conidium germination and wound colonization, conditions that would not occur in field situations. However, grapevine pruning is conducted during the dormant plant period (late fall to late winter), in which temperatures are usually below optima for *Trichoderma* conidium germination and mycelium growth. Therefore, it is important to test the survival and effectiveness of these *Trichoderma* spp. under natural conditions during the time when pruning wound protection is normally carried out. Nevertheless, the results obtained in this study are promising and also showed the possibility of *Trichoderma* spp. to immediately protect pruning wounds if applied under optimum growing conditions.

The species identified in the present study showed optimum mycelium growth between 25 and 35°C. The differences in growth at different temperatures can be important in the selection of BCAs for aspects related to the "industrial production" of biomass, and for fitness in different environmental conditions in different habitats and for multiple pathogens with different biological requirements (Kredics *et al*., 2003). *Trichoderma* spp. from the present study are likely to be well-adapted for use in grape-growing regions with mild winters (main pruning season), as in southern Italy. Further studies are needed to determine the optimum application time in the field to give greatest pruning wound protection from GTD pathogens. In order to secure sufficient infection in the positive experimental controls, we used high inoculum pressure (5,000 spores per wound) of *D. seriata* and *N. parvum* to challenge the *Trichoderma* treatments tested. It is very unlikely that pruning wounds are exposed to such high levels of inoculum under natural field conditions. It is therefore possible that we have underestimated the efficacy of *Trichoderma* spp. against *N. parvum* or other GTD pathogens (Mondello *et al*., 2018). Field evaluations under natural conditions using lower inoculum pressures in southern Italy (origin of the isolates) should be carried out to better determine the capacity the *Trichoderma* spp. as BCAs against GTDs.

Trichoderma spp. have also been widely used to minimize the impacts of GTDs during grapevine nursery propagation processes, with some promising results primarily against fungi in the Esca complex (Gramaje *et al*., 2018; Mondello *et al*., 2018). Similar to studies conducted on the use of *Trichoderma* spp. for pruning wound protection, the number of *Trichoderma* spp. tested in propagation processes is limited to *T. atroviride*, *T. harzianum* and *T. longibrachiatum* (Gramaje *et al*., 2018; Mondello *et al*., 2018). The present study provides efficacy data for several different *Trichoderma* spp. against GTD fungi in the *Botryosphaeriaceae* and the *Diatrypaceae*. Further screening of *Trichoderma* spp. identified in this study should be conducted to assess their efficacy against other GTD fungi, including species of *Ilyonectria* and *Phaeoacremonium*, and *Cadophora luteo-olivacea* and *Phaeomoniella chlamydospora*, all of which are commonly found in the nursery propagated material, and are responsible for decline and death of young grapevines. These studies could provide more alternatives to the current BCAs available during nursery propagation processes.

Chemical pesticides have become increasingly relied upon for control of grapevine pathogens and those causing other crop diseases. The deleterious effects of many of these chemicals are due to overuse, toxic residues, applicator hazards, development of pathogen resistance, and adverse environmental impacts (Nicolopoulou-Stamati *et al*., 2016). Therefore, integrated pest management (IPM) approaches, which include the use of cultural practices, BCAs and responsible use of chemical pesticides, have been suggested as strategies for management of many crop diseases, including GTDs (Bertsch *et al*., 2013; Gramaje *et al*., 2018). The present study has demonstrated the potential of several different *Trichoderma* spp. as alternatives to chemicals to reduce the important economic impacts that GTD have for grape production. However, further research is required to determine: i) the antifungal modes of action these *Trichoderma* spp. have against GTD pathogens, ii) the adaptability of the potential BCAs under natural conditions, and iii) their potential to be used as effective commercial products.

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Research Papers

Precise nondestructive location of defective woody tissue in grapevines affected by wood diseases

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Summary. Grapevine trunk diseases are major threats to viticulture. A diverse array of Ascomycetes and Basidiomycetes can affect perennial and, indirectly, annual organs of grapevines. Early infections produce wood discolouration, brown wood streaking, black spots and wood necroses. However, all wood symptoms are internal, making nondestructive identification of infected plant material very difficult. To date, there are no nondestructive methods for detecting the presence of developing wood infections, neither in nursery nor field conditions. This means that infected propagation material is planted into new vineyards. Three technologies, magnetic resonance imaging (MRI), computed tomography scan (CT scan) and X-Ray microtomography (Micro-CT), were assessed for determining presence, location and extent of grapevine wood defects caused by fungal infections. Results indicated that MRI lacked resolution to differentiate between asymptomatic and defective wood. CT scan analyses revealed substantial differences in radiodensity when comparing asymptomatic wood to wood with black spots, necroses, and decay. Greatest resolution was achieved with micro-CT (6 µm). This technology precisely distinguished asymptomatic from defective wood, for wood symptoms including necrosis, decay, black spots and brown wood streaking affecting individual xylem vessels, in perennial wood and canes. Micro-CT was thus the best method for nondestructive identification of wood defects resulting from infections. Further work is required to make this technology feasible for the rapid screening of grapevine nursery stock, both in nurseries and at planting.

Keywords. CT scan, micro-CT, MRI, grapevine trunk diseases.

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INTRODUCTION

Nondestructive techniques have been increasingly used to observe and characterize the interior of trees, logs, fruit and vegetables, and other living organisms (Ruiz-Altisent *et al.*, 2010). These techniques are often applied in wood industries, in forestry and urban trees. Examples include the identifi-

cation of decayed woody tissues, the presence of which increases the risk of tree failure in urban areas, study of the health status of valuable tree specimens, and the commercial value of standing timber trees or industrial logs. The aim of these methods is to differentiate asymptomatic wood from defective necrotic or decayed wood (Bucur, 2003b; Nicolotti *et al*., 2003; Bucur, 2005; Baietto *et al*., 2010; Bieker and Rust, 2010). These methods also assist studies of anatomy and of some physiological processes in woody plants (Brodersen, 2013; Wang *et al*., 2013; Choat *et al*., 2016). The most popular nondestructive techniques include ultrasound-based analyses (Nicolotti *et al*., 2003; Bucur, 2005), magnetic resonance imaging (Pearce *et al*., 1997; Clearwater and Clark, 2003), and X-Ray-based techniques, such as computed tomography scan (CT scan) and X-Ray microtomography (micro-CT) (Lindgren, 1991; Bucur, 2003a; Milien *et al*., 2012; Earles *et al*., 2018).

Nondestructive techniques may assist detection and study of grapevine trunk diseases (GTDs). Grapevines (*Vitis vinifera* L.) affected by GTDs exhibit reduced vigour, productivity and overall life span, contributing to heavy economic losses (Hofstetter *et al*., 2012). Infected vines have internal symptoms for months to years before the appearance of visible external symptoms, on leaves, berries and shoots (Bertsch *et al*., 2013). Early internal wood symptoms are brown streaking, black spots, necrosis and decay. Several studies have attributed the former three symptoms to the action of different Ascomycetes, while wood decay, most often white rot, is the result of Basidiomycete infections (Surico *et al*., 2008; Bertsch *et al*., 2013; Fontaine *et al*., 2016; Gramaje *et al*., 2018). Within the esca complex, one of the most threatening GTDs, the Ascomycetes involved are *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., while *Neofusicoccum parvum*, *Lasiodiplodia theobromae*, and other *Botryosphaeriaceae* are associated with the syndrome Botryosphaeria dieback. *Phomopsis viticola* causes Phomopsis cane blight, and *Eutypa lata* and other *Diatrypaceae* cause Eutypa dieback. The pathogenicity of these fungi has been proven in controlled studies (Sparapano *et al*., 2000a; Savocchia *et al*., 2007; Úrbez-Torres *et al*., 2013). Under field conditions, however, several of these pathogens are often found simultaneously in symptomatic plants (Bruez *et al*., 2016; Del Frari *et al*., 2019). Among the Basidiomycetes, members of genera *Fomitiporella*, *Inonotus*, *Inocutis*, *Stereum* and *Fomitiporia* have been isolated in plants affected by wood decay (Gramaje *et al*., 2018), with *F. mediterranea* being a primary pathogen (Sparapano *et al*., 2000b).

Current GTDs control strategies are often unreliable, offering only partial protection of grapevines (Mondello *et al*., 2018). Therefore, non-destructive detection of wood defects in infected grapevines would benefit nurseries, by minimizing the production of contaminated nursery stock. Vines with defective wood could be treated (e.g. endotherapy; Del Frari *et al*., 2018) or discarded to prevent disease spread to newly established vineyards. The present research compared the nondestructive technologies magnetic resonance imaging, computed tomography scan, and X-Ray microtomography for assessing presence, location and extent of wood defects in grapevines.

MATERIALS AND METHODS

Sampling of vineyards for asymptomatic and symptomatic wood, and pathogen identification

One-year-old grapevine canes, young and adult grapevines (*Vitis vinifera* L.) of cultivars Cabernet-Sauvignon, Trincadeira, Touriga Nacional and Castelão, located in the experimental vineyards of the Instituto Superior de Agronomia, Lisbon, were collected in 2010, 2011 and 2017 for the different analyses. The two vineyards, Almotivo and Vinha Velha, had planting densities of 3,333 plants ha^{-1}, the soil was a vertisoil, and the vineyards were managed under conventional practices without irrigation. The Almotivo vineyard was planted in 1998 with cv. Trincadeira, Touriga Nacional and Cabernet-Sauvignon, grafted on 140 RU rootstocks. The vineyard had a history of esca, exclusively manifest in cv. Cabernet-Sauvignon, with approx. 1% of vines showing tiger stripe symptoms on leaves. The Vinha Velha vineyard, which hosted cv. Castelão, was uprooted during the study (2010) as it was heavily affected by GTDs. The prevalent symptoms observed during the two years prior to uprooting were apoplexy, black-dead arm, and greater than 20% plant incidence of tiger stripe foliar symptoms.

Sampling occurred as follows. For cv. Castelão, numerous plants were examined for the presence of GTD internal symptoms at the tops and bases of the trunks, during the vineyard uprooting, and ten symptomatic trunks were selected. For cv. Trincadeira and Touriga Nacional, six plants per cultivar were randomly selected and uprooted, and the trunks were stored for further examination. For cv. Cabernet-Sauvignon, six trunks and 20 1-year-old canes were randomly sampled and cut into 30 cm segments before storage. All sampled wood material was stored at 4°C until further processing.

Fungi were isolated from symptomatic wood, and its proximities, by cutting wood into small pieces (approx. 3 mm^3), then sterilizing them by flaming, followed by immersion in a NaClO solution (0.05% w/w

active chlorine) for 1 min, and double rinsing in sterile distilled water. Surface-sterilized wood was dried on sterile filter paper and placed on Petri dishes (90 mm diam.) each containing 15 mL of potato dextrose agar (BD-Difco Laboratories) supplemented with 250 mg L^{-1} chloramphenicol (BioChemica). The Petri dishes were then incubated at 25 ± 1°C, in the dark, for 21 d. Colonies emerging from the wood were identified based on morphological and cultural features (macro- and microscopic analyses of mycelium, conidia and conidiomata), revealing the presence of the ascomycete *Phaeomoniella chlamydospora* and other wood *Botryosphaeriaceae* pathogens. The Basidiomycete *Fomitiporia* sp. was consistently isolated from decayed wood, which was a white-rot type of decay. In a separate study, conducted on cv. Cabernet-Sauvignon plants, numerous wood pathogens, including *Phaeomoniella chlamydospora*, *Fomitiporia* sp., *Fomitiporella* sp., *Neofusicoccum* spp., *Diaporthe* sp. and *Eutypa* spp., were identified using DNA metabarcoding (Del Frari *et al*., 2019).

Nondestructive identification of wood defects

The nondestructive identification of brown wood streaking and black spots, wood defects associated with brown wood streaking of rooted cuttings and Petri disease (Surico 2009), esca, and other GTDs, was the prime objective of this study. However, symptoms of necrosis and white rot were frequently observed, so the following four categories were defined for symptoms identification:

(i) *Early stages of infection*. Presence of brown wood streaking and/or black spots (Figure 1A);
(ii) *Advanced stages of infection*. Presence of symptoms described in (i), and central or sectorial necroses of different size (Figure 1B and C);
(iii) *White rot stage*. Presence of white rot, defined as 'a soft, friable, spongy mass' (wood decay; Mugnai *et al*., 1999), which, in the present study, always occurred in plants exhibiting advanced stages of infection (Figure 1D);
(iv) *Asymptomatic wood*. Absence of all of the symptoms described in categories (i), (ii), and (iii) (Figure 4A).

Trunks and canes were scanned using the methods outlined below and, afterwards, they were manually sectioned to confirm the presence and locations of the symptoms.

Magnetic resonance imaging (MRI)

Measurements were performed in a medical laboratory (Laboratório de Radiologia, Alverca, Portugal), using

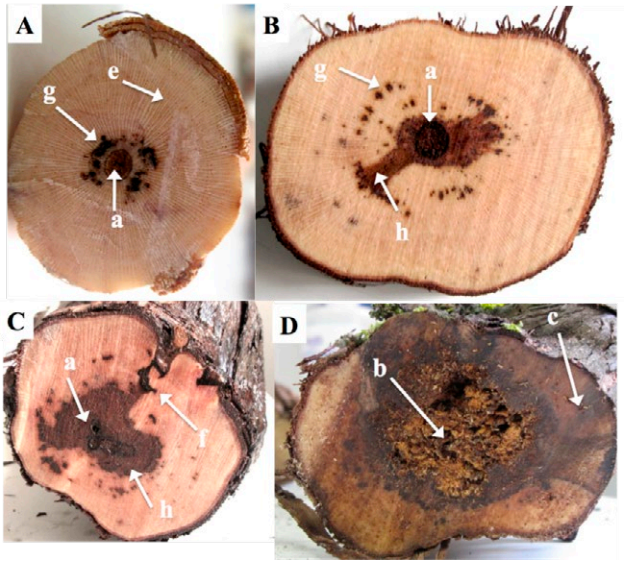

Figure 1. Cross-sections of grapevine trunks of different ages showing wood defects. (A) Early stages of wood infection, (B) and (C) advanced stages of infection, and (D) trunk affected by white rot. (a) Pith and holes, (b) decayed wood, (c) extended wood necrosis, (e) asymptomatic wood, (f) wood knots, (g) black spots, (h) central necrosis. Lower case letters represent some of the features identified in Table 1.

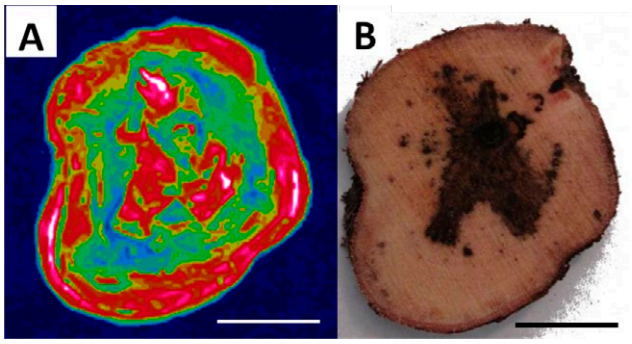

Figure 2. Magnetic resonance image (A) and the corresponding original photograph (B) of a grapevine trunk (cv. Castelão). Scale bars = 1 cm.

an Aris Elite, manufactured by Hitachi. This instrument employs 4-channel RF system with QD MER coil, and scalable Dual-Quad RF receiver system and support for RAPID parallel imaging. The instrument was equipped with self-shielded vertical permanent magnet of Tesla strength = 0.3 T. The gradient system characteristics were slew rate = 55 T m^{-1} sec^{-1} and amplitude = 21 mT m^{-1}. Three grapevine trunk samples (approx. length 20 cm, diam. = 3 cm) were scanned, and ten transversal slices per sample were recorded (slice thickness, SL =

3.0 mm). Spin echo imaging used repetition time (TR) = 4000 ms, and echo time (TE) = 80 ms. The image matrix was 256 × 256 pixels with a field of view (FOV) = 42 cm. Image processing was carried out with Hitachi dedicated software. Trunks of grapevine cv. Castelão were analysed using MRI (n = 3).

Computed tomography scan (CT scan)

CT scan is based on the attenuation of X-Rays by a sample, due to differences in its density, water content and chemical composition (Lindgren, 1991). A medical TOMOSCAN AV CT Philips scanner was used with the following CT scan settings: acceleration voltage = 120 kV, and exposure time + 2 s at 90 mA. Scans were made in radial cross-section, using optic slices of wood 270 mm × 5 mm. The reconstruction matrix of the resulting images was 512 × 512 pixels. The measured X-Ray intensity was converted into grey scale, in which darker regions represented low densities and bright regions high densities. The attenuation values calculated for each pixel were translated into grey scale or colour scale, and transformed into an image of the cross-sections of the samples. For medical scanners, the radiodensity, expressed in Hounsfield units (H), is -1000 H for air (0 kg m^{-3}), 0 H for water (1000 kg m^{-3}) and +1000 H for human bone. The scans were saved in DICOM format and viewed and analysed using Osirix v.3.7.1 32-bit software. Trunks of grapevine cv. Trincadeira and Touriga Nacional were analysed using CT-scan (n = 4).

X-Ray microtomography (micro-CT)

When compared to pre-existing X-Ray methods (including CT scan), the major difference of micro-CT is the scanning of objects from different radial positions, resulting in a series of different projections that are mathematically transformed and combined using appropriate algorithms. Each resulting tomogram is an image of known size and coordinates of each pixel. The combination of multiple tomograms along the axial vector of the object yields a full 3-dimensional representation. The different attenuations were quantified in Hounsfield units and, for visualization, they are expressed by grey values for each pixel. Grapevine trunks and 1-year-old canes were examined by this technique using a SkyScan 1174 compact micro-CT (courtesy of SkyScan, Kontich, Belgium, and Dias de Sousa SA, Lisbon, Portugal), and related SkyScan software to process the images. Trunks and canes of cv. Cabernet-Sauvignon vines were analysed using micro-CT (trunks, n = 4; canes, n = 10).

RESULTS

Magnetic resonance imaging

MRI failed to give enough resolution to accurately differentiate between asymptomatic and defective wood. The comparison between one MRI image of a sample with advanced stages of infection (Figure 3A) and the photograph of the corresponding section (Figure 3B) revealed no clear distinction between regions of defective and asymptomatic tissues, especially for brown streaking and black spots. Figure 3A shows that the high-intensity signal regions corresponded to affected areas as well as asymptomatic areas of woody tissue. The black spots visible in Figure 3B were not identified, in Figure 3A, as high-intensity signal areas.

Computed tomography scan (CT scan)

Visualization of the cross-sectional images obtained from the CT scanner and the analysis of the resulting tomographic reconstructions revealed clear differences between asymptomatic and defective wood areas, which were imputable to density alterations induced by fungal infections (Figure 3). Image contrast was defined as the signal intensity difference detected among different types of tissues.

Analysis of samples at different stages of wood infection revealed variations in radiodensity values, according to the various anatomical and symptom features (Table 1). Selected CT images were compared with the original photographed sections illustrating early (A1) and advanced (A2) stages of infection, as well as the white rot stage (A3) (Figure 3). When observing the grey-scale images (Figure 3B), dark shades indicated regions of low radiodensity, whereas bright areas indicated material of greater radiodensity. Comparisons between the original photographs and the corresponding tomography images showed that black spots, and to a lesser extent necrotic wood, presented brighter shades than asymptomatic wood, which was due to greater radiodensity (Hounsfield Units, H) of the diseased areas (Table 1; Figure 3). An alternative colour scale (UCLA colour scale, Osirix v. 3.7.1; Figure 3C) aided differentiation of black spots from necrotic wood tissue and other areas. In the section A3 of Figure 3, the cross-section shows (i) internal necrotic wood, exhibiting high radiodensity, (ii) sectorial necrosis, and (iii) wood decay. Sectorial necrosis and wood decay were regions of low radiodensity, due to absence of water and the transformation of woody tissue in response to the action of Basidiomycetes, and were visualized as dark-coloured areas in Figure 3 B3.

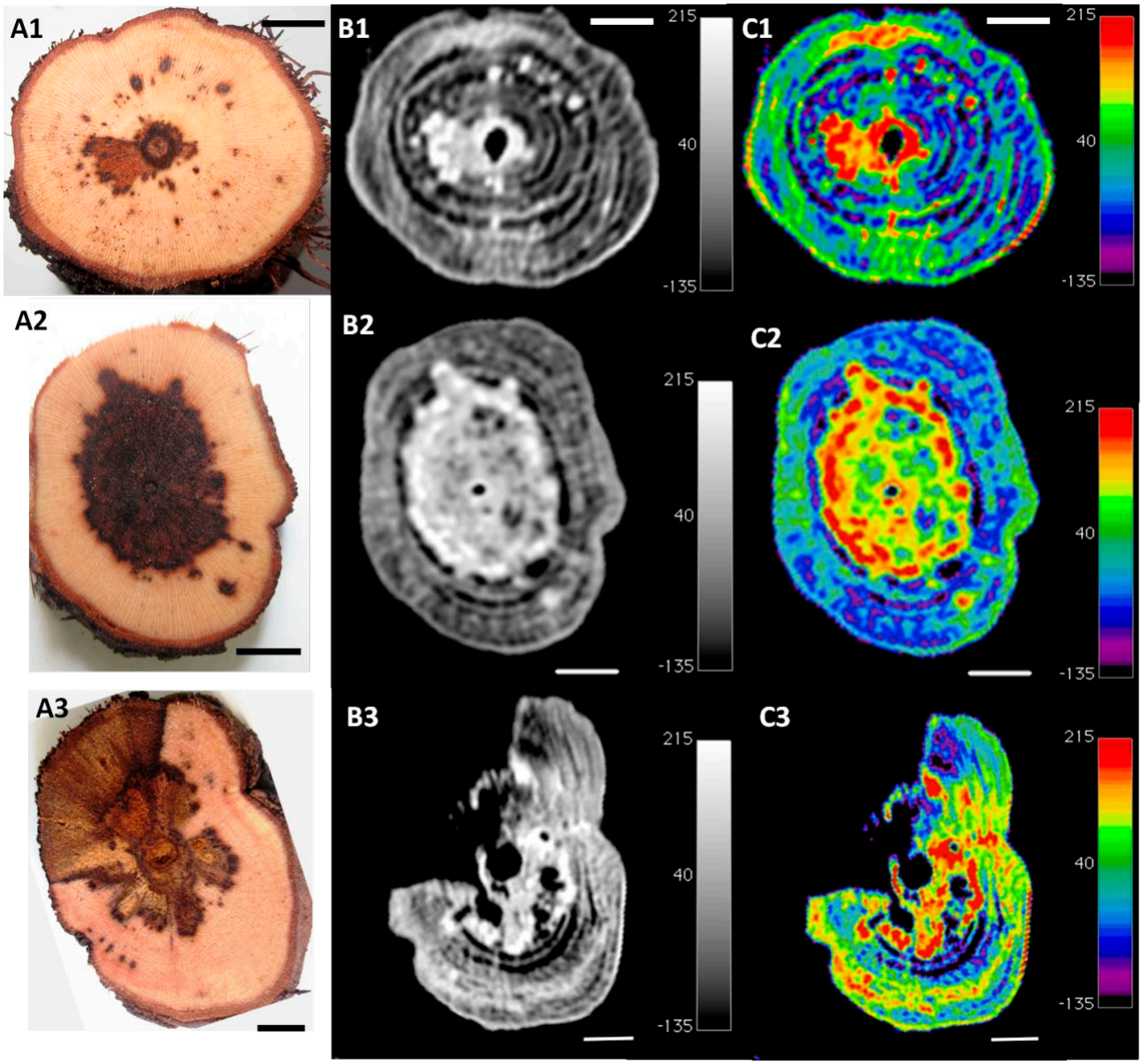

Figure 3. Original photographs (A) and computed tomography scan images (B and C) showing a symptomatic grapevine trunk (cv. Touriga Nacional) at (1) early, and (2) advanced stages of infection, and (3) white rot stage. (B) Tomographic analysis (grey colours; and (C) tomographic analysis (UCLA colours), according to the radiodensity scales presented on the right side of B and C. Scale bars = 1 cm. CT scan images extracted from Osirix v. 3.7.1.

X-Y plots extracted from Osirix v.1.3.7.v. (Figure 4) show three examples of variation (H) along cross-sections of asymptomatic wood (A), early stage of infection (B) and white rot stage (C). In asymptomatic vines, radiodensity values varied between -40 ± 47 H for empty xylem vessels and +76 ± 27 H for asymptomatic wood (Table 1; Figure 4A). During early stages of infection (Figure 4B), the radiodensities of the two black spots (g) in the section corresponded to +251 H and +264 H (from left to right). Overall, the pith and holes, as well as decayed wood and other necrotic tissue affected by Basidiomycetes, exhibited reduced radiodensity, ranging from approx. -900 H to -300 H. For example, during the white rot stage (Figure 4C), sectorial necrosis (c) gave radiodensity of -439 H and wood decay (b) of -760 H, revealing low density of these tissues. The wood transformed by the action of decay agents (e.g. *F. mediterranea*) had low water and high air contents, which

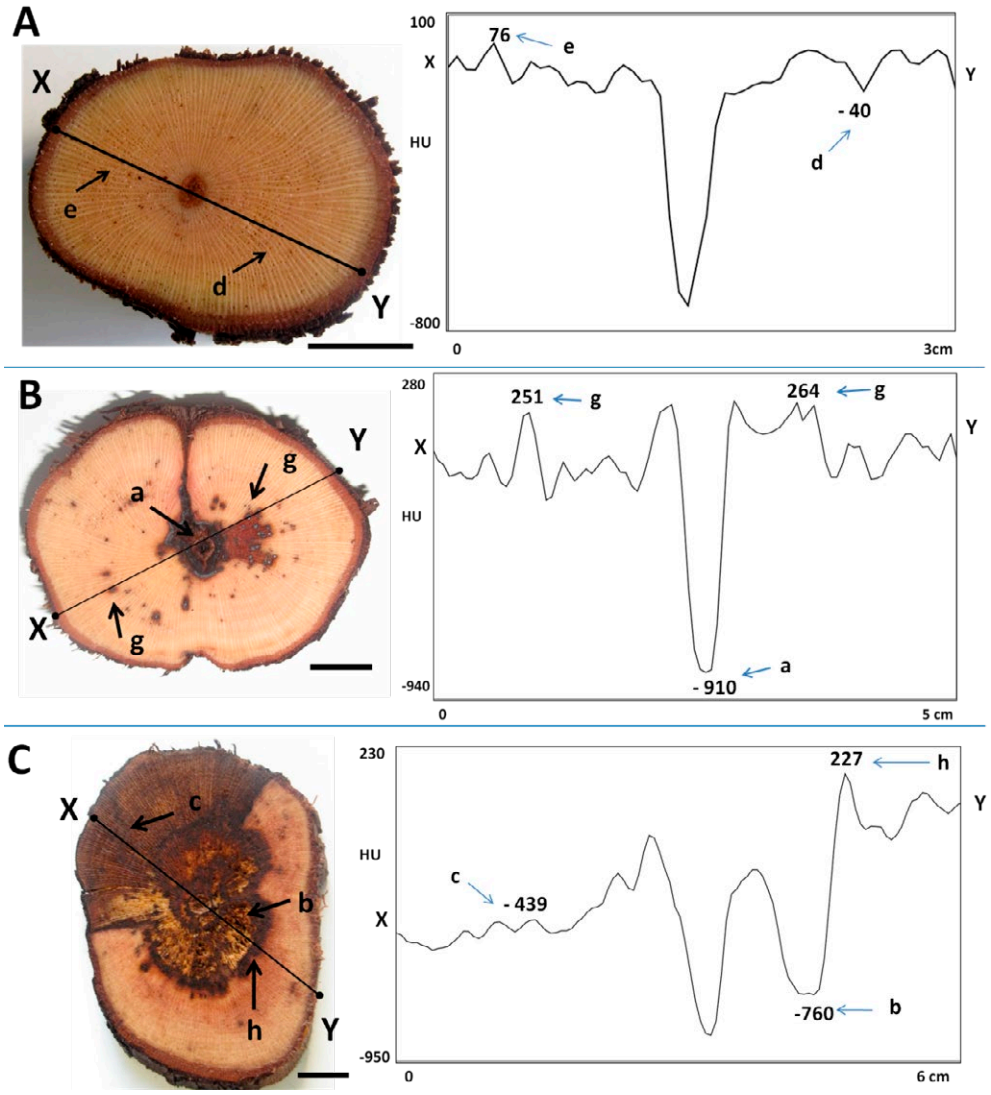

Figure 4. Grapevine trunk sections (cv. Trincadeira) and the corresponding X-Y plots, showing patterns of radiodensity variation across the lines indicated, in different areas of infection. (A) Asymptomatic grapevine, (B) early stage of infection and (C) white rot stage. Scale bars = 1 cm. X-Y plots extracted from OsiriX Viewer v.3.7.1. Lower case letters represent the features identified in Figure 1 and Table 1.

translated into low radiodensity CT images. The X-Y plot also shows an area of wood with high radiodensity, corresponding to necrotic wood (h), at +227 H (Figure 4C; Table 1), hypothetically not yet affected by decay.

X-Ray microtomography (micro-CT)

While CT scan gave promising results for black spot detection, as well as necrosis and wood decay, accurate identification of brown wood streaking was not attained. For this reason, micro-CT was assessed, as a technique capable of achieving greater resolution. Figure 5 shows micro-CT detection of high-density material (white colour) inside individual xylem vessels of a grapevine trunk (cv. Cabernet Sauvignon). On the cross-section (Figure 5A), the clear presence of xylem vessels partially or completely filled with gums was confirmed by visual inspection/photography after slicing the grapevine trunk (not shown). A longitudinal section of the same trunk is shown in Figure 5B, illustrating discontinuity of gums along the trunk xylem vessels.

One-year-old canes were also examined using this technique. In this case, high resolution images were acquired (6 µm), confirming the results presented above. To achieve rapid screening, fundamental for application of this technique in large-scale environments (e.g. nurs-

Table 1. Radiodensity values (H) obtained from computerized axial tomography for distinct parts of grapevine wood, either asymptomatic or with different wood defects. Lower case letters represent the features identified in Figures 1 and 4. Mean ± standard deviation (n = 40 wood sections, 4 grapevine trunks).

Feature	Radiodensity (Hounsfield Units, H) Mean +/- SD
Pith and hole (a)	-860 ± 55
Wood decay (b)	-700 ± 70
Extended central/sectorial necrosis (c)*	-430 ± 94
Empty vessels (d)	-40 ± 47
Asymptomatic wood (e)	+76 ± 27
Knots (f)	+204 ± 54
Black spots (g)	+234 ± 56
Early necrotic wood (h)	+264 ± 68

*in plants at white rot stage

eries), lower resolution (25 µm) images were acquired, such as that shown in Figure 6. This resolution achieved with micro-CT gave the possibility of identifying individual xylem vessels in the early stages of symptom expression, namely brown wood streaking.

Other non-invasive techniques tested by the authors of this study lacked the required levels of sensitivity to non-destructively detect symptoms of grapevine wood infection. Among them, there were techniques based on ultrasounds, Fourier transform infrared spectroscopy (FTIR), particle/proton-induced X-Ray emission (PIXE), electrical impedance spectroscopy (EIS) and radiography.

DISCUSSION

This study tested three nondestructive techniques for assessing the presence of fungus-induced wood

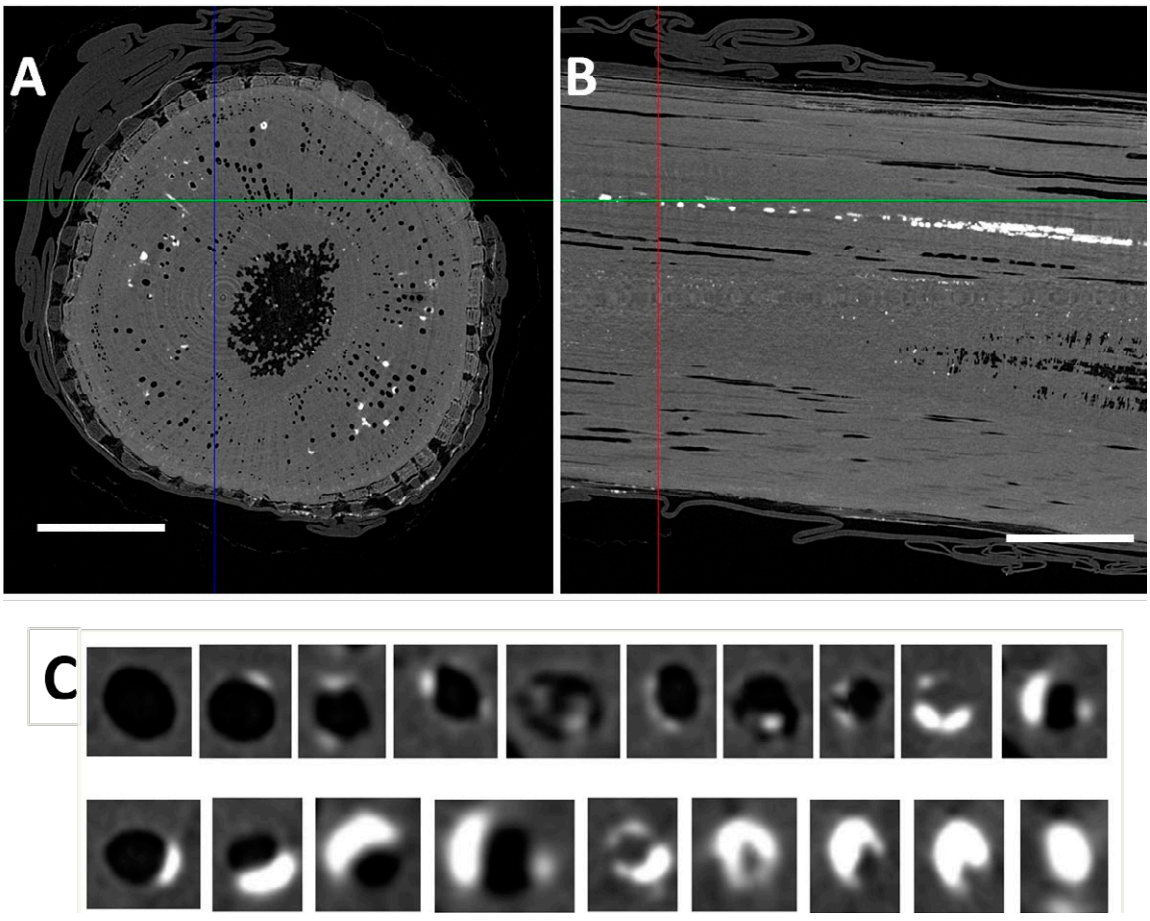

Figure 5. X-Ray microtomography (micro-CT) of a grapevine trunk (cv. Cabernet Sauvignon) infected by wood pathogens. (A) Cross-section showing some vessels partially or completely filled with gums. (B) Corresponding longitudinal section showing the accumulation of high-density material along the xylem vessels. (C) Individual xylem vessels with increasing gum accumulation in each from empty (top left) to filled (bottom right). Scale bars = 1 cm, resolution 6 µm.

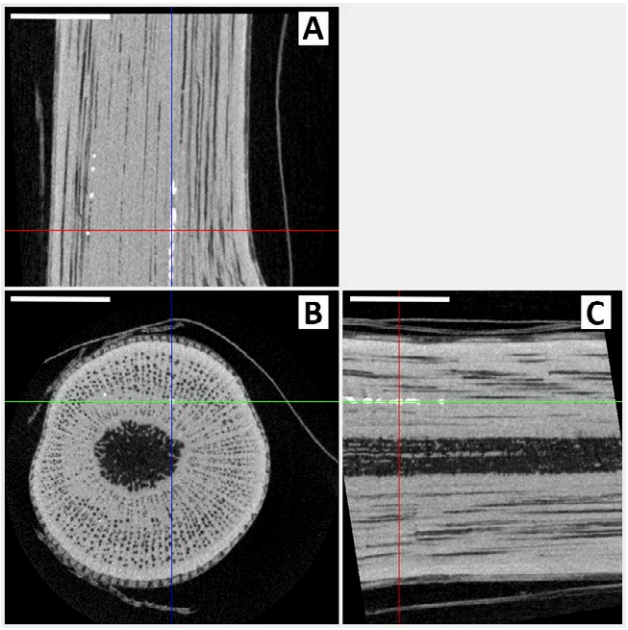

Figure 6. X-Ray microtomography (micro-CT) of a 1-year-old cane of cv. Cabernet Sauvignon. (A and C) Longitudinal sections, and (B) cross-section, with horizontal and vertical lines pinpointing individual xylem vessel(s) filled with gums. Scale bars = 5 mm, resolution 25 µm.

defects in grapevine trunk and stem tissues. CT scan and micro-CT were effective for detecting the presence of early and advanced stages of infection. Both methods detected black spots, wood necrosis and wood affected by the action of basidiomycetes (wood decay). In addition, micro-CT detected the earliest symptoms of wood infection, namely the appearance of brown wood streaking in individual xylem vessels. This highlights the potential of micro-CT for application in nursery environments. Despite these promising results, some wood defects (e.g. brown streaking) are not exclusively the results of a fungus infection, but may also be caused by other factors such as mechanical injuries. Furthermore, under some circumstances wood pathogens may occur in asymptomatic wood (Del Frari *et al*., 2019).

MRI has been frequently used to locate fungal decays or cavitation in trees (Müller *et al*., 2001; Bucur, 2003a), or to follow the development of core breakdown disorder in pear fruit (Lammertyn *et al*., 2003). Kuroda *et al*. (2006) showed that magnetic resonance micro-images can measure water distribution and areas affected by pathogenic microorganisms in living tree stems, but small vessels (20 to 30 µm diam.) filled with water could not be visualized. These authors found a theoretical resolution of 117 µm, in the best analyzed situation, which allowed the identification of xylem vessel aggregates, despite not being sensitive enough to pinpoint individual vessels (Kuroda *et al*., 2006). These observations are similar to those of the present study (Figure 2). Under our experimental conditions and with the instrument used, MRI did not reveal defective wood. However, other studies that employed high-resolution MRI (e.g. 7.05 Tesla, 1.0 T m^{-1}) in grapevines, were successful in detecting individual xylem vessels, for example in examination of water ascent in embolized xylem vessels (Wang *et al*., 2013) and water-stress induced cavitation (Choat *et al*., 2010). Therefore, MRI has potential to be exploited in the detection of defective wood. Further studies could also assess the contribution of secondary metabolites to MRI signals (Oven *et al*., 2008). From the practical viewpoint, a portable MRI was developed and tested in 2012 in living trees, but the low level of resolution only allowed analysis of water content in mature trees and wood (Jones *et al*., 2012). Nevertheless, a patent concerning a portable MRI system was recently granted (US9910115B2), increasing possibilities for the development of a prototype capable of increased resolution and applicability in vineyards.

Both X-Ray techniques used in the present study (CT scan and micro-CT) enabled clear imaging of wood defects in grapevines, at different stages of infection. When comparing these two techniques, micro-CT allowed more precise analysis of symptoms in grapevine wood, due to the increased resolution of the equipment, when compared to CT scan. A resolution of approx. 6 µm is achieved by using micro-CT, which, after translation into high-quality images, allowed rigorous identification of individual xylem vessels that were entirely or partially filled with gums. As far as the authors are aware, there is no other technology that allows non-invasive and nondestructive detection of these symptoms in vines affected by wood pathogens.

Successful applications of CT scanning in woody tissues include accurate and nondestructive measurements of wood density (Funt and Bryant, 1987; Lindgren, 1991) in plants affected by fungal diseases (Okochi *et al*., 2007), and examination of the water distribution in stems of living trees (Habermehl *et al*., 1990; Fromm *et al*., 2001). Our results support this understanding, showing that the action of wood pathogens alters wood composition, either by increasing (e.g. black spots) or decreasing wood density (e.g. wood decay; Figure 4), when compared to asymptomatic woody tissue, resulting in different radiodensity signals. The major limitation of CT scan is the typical size of the instruments, which prevents application under field conditions.

As postulated, micro-CT was the best technology for accurate identification of presence, extent and precise

location of wood defects in grapevines (Figure 5). Recent studies have shown similar results, in grapevines, where micro-CT was successfully applied to characterize wood anatomy (Milien *et al*., 2012; Brodersen, 2013), infection by the Botryosphaeria dieback pathogen *Neofusicoccum parvum* (Czemmel *et al*., 2015), and other physiological processes, such as xylem vessel dehydration and refilling (Choat *et al*., 2016; Brodersen *et al*., 2018), and estimation of starch content (Earles *et al*., 2018). Despite these promising results, to find a robust application of micro-CT in large-scale environments (e.g. nurseries), it is necessary to optimize scanning parameters (e.g. reducing resolution to increase scanning speed), and portability, always allowing for the potential hazards caused by radiation.

CONCLUSIONS

Early nondestructive detection of wood defects caused by grapevine wood pathogens represents an important step towards controlling these serious threats for grape production. No chemical treatment is capable of eradicating wood pathogens from infected wood. Successful *in vitro* efficacy of fungicides often did not translate to *in vivo* efficacy, so research has shifted to nurseries and pruning wound protection, as an integrated GTDs management strategy (Gramaje *et al*., 2018; Mondello *et al*., 2018). While investigating new chemicals and ways to deliver them to infected tissues, some of the technologies assessed in the present study could have short and medium term practical applications. In the short term, X-Ray-based scanning techniques could be implemented in nurseries, screening propagation material for internal GTDs symptoms and allowing removal of symptomatic individuals. This may reduce market inputs of infected plant material. In the medium term, when effective chemical treatments become available, this technology could be applied in the field, to find and treat young plants during early stages of infection. A patent application based on the results shown in this study was recently granted in the European Union, the United States and other countries (PCT/EP2011/068320). Future investigations will focus, therefore, on improving the portability, speed and efficiency of these techniques, as well as the search for new treatments to control *in vivo* wood pathogens.

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Research Papers

Fungal pathogens associated with grapevine trunk diseases in young vineyards in Sicily

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Summary. After the first report of grapevine decline caused by *Botryosphaeriaceae* in Sicily in 2007, epidemiological studies carried out in mature vineyards until 2011 confirmed the widespread occurrence of "Botryosphaeria dieback" and the "Esca complex" disease. Dieback symptoms were also recently observed in two young vineyards in Partanna and Castellammare del Golfo in western Sicily (Trapani province). Declining vines were inspected for grapevine trunk disease (GTD) symptoms, and were uprooted and submitted for analyses. Fungal isolates were collected and identified using culturing and molecular analyses. One isolate per identified species was inoculated to three grapevine shoots to evaluate pathogenicity and fulfil Koch's postulates. Several GTD *Botryosphaeriaceae* pathogens in the genera *Cadophora, Ilyonectria, Neonectria*, *Phaeoacremonium* and *Phaeomoniella* were isolated from the symptomatic young vines. Artificial inoculation confirmed the pathogenicity of these fungi. In addition, virulence variability was observed among the isolates, with *P. chlamydospora* causing the largest lesions. The different species were associated with specific symptoms and/or host vine parts, especially in the roots and around the grafting areas. Several fungi associated with Petri disease and black foot were shown to be responsible of young vine decline.

Keywords. Young vine decline, grapevine trunk diseases, Petri disease, black foot.

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INTRODUCTION

Within grapevine trunk diseases (GTDs), the term "young vine decline" (YVD) refers to a complex of diseases (Petri disease, black foot and young Esca) observed in vines less than 6 years old, and caused by different fungal pathogens (Agustı-Brisach and Armengol, 2013; Gramaje and Armengol, 2011a). The main external symptoms of YVD are progressive dieback characterized by reduced vigour, shortened internodes, leaf chlorosis and eventual plant death. Internally, infected vines show dark-brown streaking along the trunks and/or necrotic wood mainly at the bases of the rootstocks or scions in self-rooted vines. Decreased survival of young plants in nurseries and

newly established vineyards affected by YVD have been reported from many wine-producing areas (Halleen *et al.,* 2003; 2004; Gimenez-Jaime *et al.,* 2006; Gramaje *et al.*, 2009, 2018; Rego *et al.,* 2009; Agustì-Brisach *et al.,* 2011; Gramaje and Armengol 2011a; Cabral *et al.,* 2012; Pintos *et al.,* 2018). At least 51 GTD pathogens are now considered associated to the YVD (Úrbez-Torres *et al.,* 2015). Economic impacts of YVD are significant in nurseries and vineyards (due to required replacement of dead vines). In nurseries, the lack of effective prophylaxis techniques along the plant production process has increased GTD infections, causing grafting failures, poor plant vigour and, in general, the production of poor-quality vines with latent infections (Smart *et al.,* 2012; Gramaje and Di Marco, 2015; Waite *et al.,* 2015).

In Sicily, the first report of YVD was in 1912, when Lionello Petri found two isolates of *Cephalosporium* and one of *Acremonium* associated with symptomatic wood of young declining vines. Sixty years later, *Cylindrocarpon obtusisporum* (Grasso and Magnano di San Lio, 1975), *Phoma glomerata* (Granata and Refatti, 1981), and *Cylindrocarpon destructans* and *Fusarium solani* (Grasso, 1984) were identified as causal agents of YVD in different areas of Sicily. Sidoti *et al.* (2000) reported *Phaeomoniella chlamydospora* associated with YVD in a young vineyard of cv. Victoria vines in the southeast of Sicily.

More recently, vineyard surveys in the western part of Sicily (Agrigento, Caltanissetta, Palermo and Trapani provinces) have highlighted the presence and spread of Botryosphaeria dieback and Esca pathogens, but only in mature vines (Burruano *et al.*, 2008; 2010; Mondello *et al.,* 2013). In 2015 and 2018, contrary to previous studies, dieback symptoms were also observed in two young vineyards (2- and 1-year-old), located in the Trapani province. Different abiotic and biotic factors can be associated with the decline of young vineyards, including nutritional deficiencies, fungal, bacterial and viral diseases, and insect and nematode pests (Halleen *et al.,* 2003). Accordingly, the aim of this research was to investigate the observed decline of young vines, to identify the associated fungi and assess their virulence towards *Vitis vinifera*.

MATERIALS AND METHODS

Sampling and fungus isolation

Three surveys were carried out in a 2-year-old vineyard (cv. Grecanico) in Castellammare del Golfo in 2015. In addition, two surveys were carried out in a 1-year-old vineyard (cv. Muller-Thurgau) in Partanna in 2018. Both vineyards were in the province of Trapani, Sicily, Italy. Plants in both vineyards showed dieback and general reduced vigour, with foliar discolouration frequently observed in the Partanna vineyard. In both vineyards, vines with canes with stunted growth, including short internodes, uneven lignification, leaf zig-zag lesions and chlorosis, were collected in spring. In total, 20 samples were collected for analysis from the Castellammare del Golfo vineyard, and nine samples were from Partanna. After preliminary inspections, each vine trunk was superficially sterilized, the bark removed and the trunk divided into three different sections, including the base of the rootstock, the rootstock and the grafting point. Small wood portions (approx. 2 mm^2) from each trunk position were placed into Petri plates containing potato dextrose agar (PDA) supplemented with 500 mg L^{-1} streptomycin sulphate, and these were incubated at 24± 1°C in darkness. After 7 to 10 d incubation, fungal colonies similar to known GTD fungi were individually transferred to PDA in Petri plates, to obtain pure cultures. Isolation frequencies for each fungus per sample and per sample wood part were calculated. The obtained isolates are maintained in the fungal culture collection of the Dipartimento di Scienze Agrarie Alimentari e Forestali (University of Palermo).

Morphological characterization of fungus isolates

One isolate of each colony type was selected for identification to genus on the basis of macroscopic (colony colour, morphology, texture and type of growing margin), microscopic and biometric characteristics (Domsch *et al.,* 1980; Barnett and Hunter 1998; Pitt and Hocking 1999). Microscopic characteristics, including conidium length, width, shape and number of septa, were examined using a light microscope (Axioskop; Zeiss) coupled to an Axio-Cam MRc5 (Zeiss) digital camera, and images were captured using the AxioVision 4.6 software (Zeiss). Fifty conidia were measured per isolate, to calculate length and width means, standard deviations and 95% confidence intervals for the conidium dimension data.

Biometric analyses were used to evaluate effects of temperature on mycelium growth in the same isolates used for microscope measurements. Agar plugs (6 mm diam.) from 7-day-old colonies were inoculated onto PDA plates which were incubated at different temperatures of 5 to 40°C (5°C intervals) in the dark. Three replicates for each isolate and temperature were prepared, and the experiment was performed twice. Colony diameters were each measured along two perpendicular axes when the colony reached at least two-thirds of the plate diameter, and these data were converted to daily radial

growth (mm d^{-1}). For each isolate, average data of radial growth were adjusted to a regression curve using Statgraphics Plus 5.1 software (Manugistics Inc.), and the best polynomial model was chosen based on parameter significance ($P < 0.05$) and coefficient of determination (R^2) to estimate the optimum growth temperature for each isolate. Data were subjected to analysis of variance (one-way ANOVA) and significant differences between mean values were determined by Fisher's least significant difference (LSD) multiple range test at $P = 0.05$, using SAS version 9.0 (SAS Institute).

After 15 days, culture plates incubated at temperatures in which there was no growth were placed at 25°C to determine if these temperatures were fungistatic or fungicidal.

DNA extraction, PCR amplification and sequencing

Isolates grouped according their morphology were also processed for molecular identification. Total genomic DNA was extracted from each isolate using a standard cetyltrimethylammonium bromide (CTAB)-based protocol (O'Donnell *et al.,* 1998). The internal transcribed spacer region (ITS) of the ribosomal DNA operon, including the ITS1, 5.8S and ITS2, were amplified by PCR using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et *al.,* 1990). Each 40 µL reaction volume contained 50–100 ng of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM of each primer, 0.5 U of Taq DNA polymerase (Dream Taq, Fermentas) and 1 × Dream Taq buffer (Fermentas). PCR products were sequenced in both directions, and the nucleotide sequences were compared to those present in GenBank using the BLAST program (Altschul *et al.,* 1997). Sequences obtained in this study were deposited in GenBank (Table 1).

Pathogenicity tests

Following previous studies (Úrbez-Torres and O'Gorman, 2014; Carlucci *et al*, 2017; Aigoun-Mouhous *et al*., 2019), the identified isolates were tested for pathogenicity in 1-year-old dormant canes cut from 3-year-old grapevine plants (cv. Grecanico; five replicates per isolate). Before artificial inoculation, the canes were subjected a hot water treatment (53°C for 30 min: Carlucci *et al.,* 2017), to obtain shoots with low presence of pathogens.

A mycelium plug (6 mm diam.) from a 10-day-old PDA colony of each selected isolate was inserted into a wound made in the wood of each inoculated cane after removing the bark. Inoculated wounds were wrapped with wet sterile cotton wool for approx. 2 d, and the canes were then placed vertically in a plastic box sealed with cellophane film for another 13 d. Negative controls were inoculated with non-colonized PDA plugs. Each experiment included six replicates per isolate. After incubation at 23 ± 2°C for 15d, inoculated shoots were examined for necrosis by removing the bark from each shoot, and the length of brown streaking was measured. To fulfill Koch's postulates, all the inoculated canes were subjected to re-isolation of the inoculated pathogen. Data of lesion lengths were checked for normality using the Shapiro-Wilk test, and were subjected to analysis of variance (one-way ANOVA). Significant differences between mean values were determined by LSD tests at $P = 0.05$, using SAS version 9.0 (SAS Institute).

RESULTS

Sampling and fungal isolations

Sampled plants from the two different vineyards showed stunted/reduced vegetative vigour, interveinal leaf chlorosis, thin canes with shortened internodes and reduced root growth (Figure 1a, b, c and d). Longitudinal sections of symptomatic plants showed brown/black discolouration of the wood along almost the entire trunk lengths. In cross section, discolourations were often associated with dark-coloured exudates or with annular browning (Figure 1e and f). Most of the cv. Grecanico samples showed larger discolouration around the grafting-points than discolourations observed in basal ends and collars of the rootstocks (Figure 1g, h and i). In some of the observed rooting areas, severe blackening of the wood was also present. Muller-Thurgau vines all showed prominent vascular discolouration (Figure 1j).

Morphological and molecular identification of isolated fungi

A total of 570 fungal isolates were obtained from the symptomatic plants. Of these, 264 were classified as fungi belonging to the genera *Acremonium, Aspergillus, Cladosporium, Lophiostoma*, and *Penicillium*, which are known as endophytes and/or saprophytes in plants. These isolates were not further considered in the present study (Halleen *et al.,* 2007). The other 306 isolates, grouped according similarity for colony and conidium morphology, were classified as fungi associated with GTDs. Sixty-two isolates were associated with biometric features of Botryosphaeria dieback (BD) pathogens, 118 with features related to Black foot disease (BFD) patho-

Figure 1. Symptoms of YVD observed in western Sicilian vineyards. a-d, whole plants with stunted development in cv. Muller-Thurgau (a-b) and cv. Grecanico (c-d). Vines showed delayed growth, chlorosis, canes with short internodes and poor root system development. e-j, YVD internal symptoms: dark-coloured exudates in cv. Grecanico (e), and annular browning in cv. Muller-Thurgau (f). Declining vines in longitudinal section showed wood discolouration at the grafting points (g), along the trunk (h) and at the base of the rootstock (h, cv. Grecanico). j, longitudinal sections of vines cv. Muller-Thurgau.

gens, and 126 isolates had features of Petri disease (PD) pathogens.

Morphological features, biometric and molecular analyses of the selected isolates of GTD fungi identified two genera and four species (Table 1). Optimum temperatures for growth in culture varied among the tested isolates, ranging from 20.7°C of *Ilyonectria liriodendri* to 28.7°C of *Phaeoacremonium* sp. All optimum growth temperatures were statistically different among isolates, except between *Phaeomoniella chlamydospora* and *Cadophora lutea-olivacea*, which had the same optimum growth temperature (22.7°C). All tested isolates grew at 5°C except for *N. parvum* which did not grow at 5°C but did at 10°C. Different values were recorded for the upper temperature limits for growth, which were 25°C for *C. luteo-olivacea* and *I. liriodendri*, 30°C for *Neonectria* sp., and 35°C for *N. parvum*, *Phaeoacremonium* sp. and *P. chlamydospora*. When incubated at lower temperatures, mycelial plugs of *I. liriodendri* isolates maintained at 35°C did not show any growth, while 40°C killed all the other tested isolates, except *Phaeoacremonium* sp.

Comparisons between nucleotide sequences of the isolates and those present in Genbank confirmed the specific morphological identifications for *C. luteo-olivacea*, *I. liriodendri*, *N. parvum* and *P. chlamydospora*. For these species, the BLAST searches yielded 100% similarity with ex-type sequences in the GenBank database. For strains T2 and T21, the BLAST results did not allow their identification at species level.

Cadophora lutea-olivacea is reported to be associated with declining *V. vinifera* in Italy, confirming the report by Raimondo *et al.* (2019). *Cadophora lutea-olivacea* colonies on MEA were flat, felty, greenish-olivaceous in colour, with even edges (Figure 2a). All *C. luteo-olivacea* isolates grown on MEA had aerial mycelium consisting of branched and septate hyphae, singly or grouped up to three to five. Hyphal swellings were occasionally observed (Figure 2b). Conidiophores originating from hyphae were not usually branched, but were septate, mostly short, erect (Figure 2c) and sometimes flexuous (Figure 2d). Terminal or lateral phialides were mostly monophialidic (Figure 2e), smooth, hyaline, and with cylindrical collarettes, and were sometimes long with

Table 1. Morphological and molecular details of GTD fungi assessed in this study.

Isolate	Genus and species	*Vitis vinifera* cultivar	GenBank Access No. ITS	Conidium dimensions (µm)*	Mean ± SD (µm) **	L/W (µm) ***	Opt. growth T (°C)	GenBank Ex-type Access No. *(no ex-type)*	Coverage %	Similarity with ex-type *(no ex-type)*
T7	*Cadophora luteo-olivacea*	cv. Muller-Thurgau	MK589330	-	-	-	-	**NR_165945**	99.81	100
T23	*C. luteo-olivacea*	cv. Muller-Thurgau	MK589331	(4.6-)4.7 - 7.2(-7.9) x (1.6-)1.8 - 3.1(-3.4)	5.9 ± 0.8 x 2.5 ± 0.4	2.5 ± 0.5	22.7 b	**NR_165945**	**99.81**	**100**
T12	*Ilyonectria liriodendri*	cv. Muller-Thurgau	MK589332	microconidia (8.2-)8.6 - 19.0(-20.3) x (2.2-)2.5 - 6.5(-7.0) macroconidia (23.3-)23.6 - 38.5(-39.0) x (2.9-) 3.1 - 4.6(-5.7)	13.1 ± 3.4 x 4.0 ± 1.2	3.4 ± 0.6	20.7 a	**N_R119565.1**	**95**	**100**
T21	*Neonectria* sp.	cv. Muller-Thurgau	MK589335	microconidia (2.6-)5.2 - 7.3(-8.0) x (1.6-)2.0 x 3.7(-3.8) macroconidia (12.2-)12.3 - 35.5(-37.5) x (2.1-) 2.4 - 5.3(-6.0)	6.3 ± 0.9 x 2.8 ± 0.5	2.3 ± 0.4	24.7 c	***(HQ843921)***	**99**	***(100)***
T1	*Neofusicoccum parvum*	cv. Muller-Thurgau	MK589318	(11.7-)12.9 - 19.2(-21.1) x (5.2-)5.8 – 8.1(-9.0)	16.3 ± 1.9 x 7.1 ± 0.8	2.3 ±0.4	26.2 d	**FJ545228**	**99.63**	**100**
T9	*N. parvum*	cv. Muller-Thurgau	MK589319	-	-	-	-	**FJ545228**	**99.63**	**100**
T24	*N. parvum*	cv. Muller-Thurgau	MK589320	-	-	-	-	**FJ545228**	**99.63**	**100**
T2	*Phaeacremonium* sp.	cv. Muller-Thurgau	MK589321	(2.8-)3.6 – 4.8(-6.0) x (1.1-)1.2 - 2.1(-2.3)	4.2 ± 0.5 x 1.6 ± 0.3	2.6 ± 0.5	28.7 e	***(AF19783.1)***	**100**	***(99.73)***
PC1	*Phaeomoniella chlamydospora*	cv. Grecanico	MN597999	(2.7-)2.8 – 3.7(-3.8) x (0.7-)0.9 – 1.4(-1.5)	3.2 ± 0.3 x 1.1 ± 0.2	2.9 ± 0.5	22.7 b	**MH862510**	**99.82**	**100**
PC2	*P. chlamydospora*	cv. Grecanico	MN598000	-	-	-	-	**MH862510**	**99.82**	**100**
PC3	*P. chlamydospora*	cv. Grecanico	MN598001	-	-	-	-	**MH862510**	**99.82**	**100**

* Data presented as the lower and upper 95% confidence limits from 50 conidia, with minimum and maximum dimensions in parenthesis.
** Mean and S.D., standard deviation of 50 conidia.
*** L/W, length/width ratio, mean and standard deviation from 50 conidia.
Means accompanied by the same letters are not statistically different (Fisher's LSD tests at $P < 0.05$).
Data in bold are for ex-type specimens, in brackets to non-ex-type.

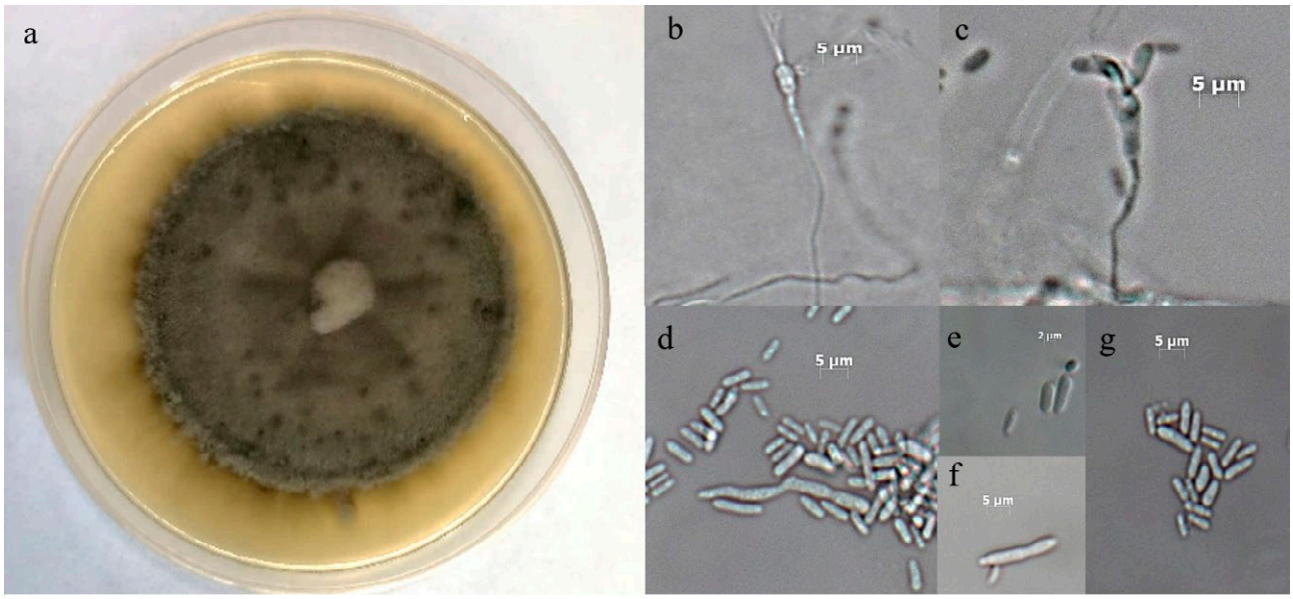

Figure 2. Macroscopic and microscopic features of *Cadophora luteo-olivacea* isolated from declining young grapevines cv. Muller-Thurgau in Sicily. a, colony growing on MEA. b, c and d, conidiophores of *C. luteo-olivacea*. d and e, short terminal or lateral phialides. g, hyaline conidia.

ampulliform bases (Figure 2f). Conidia were hyaline, with 2-3 guttules, ovoid or oblong ellipsoidal (Figure 2g). These microscopic features were similar to those reported for *C. luteo-olivacea* by Gramaje *et al.* (2011b).

Incidence of Botryosphaeria dieback, black foot and Petri disease fungi in sampled vines

Eighty-three percent of the analysed plants were infected by at least one GTD pathogen. Two GTD fungi were isolated from the same plant in 41% of the collected vines and 17% of these vines yielded three GTD fungi. Incidence of the different GTD pathogens, as indicated by the fungi isolated from young grapevines, is shown in Table 2. Overall, the proportion of vines infected by GTD pathogens was less for cv. Grecanico plants than for cv. Muller-Thurgau plants. BD associated fungi were the less isolated in both the cultivars. In cv. Grecanico vines, PD associated pathogens were dominant, while both BFD and PD associated pathogens prevailed in cv. Muller-Thurgau vines.

Distribution and relative presence of grapevine trunk disease pathogens in declining vines

The isolation frequencies (IFs) of fungi associated with GTDs are shown in Table 3. These were different, both in number and type, depending on grapevine cultivar and on trunk portion. Among the fungi identified, *Neonectria* sp. and *P. chlamydospora* were isolated from all cv. Grecanico trunk portions, and similarly for *I. liriodendri*, *C. luteo-olivacea* and *Phaeoacremonium* from cv. Muller-Thurgau trunk portions.

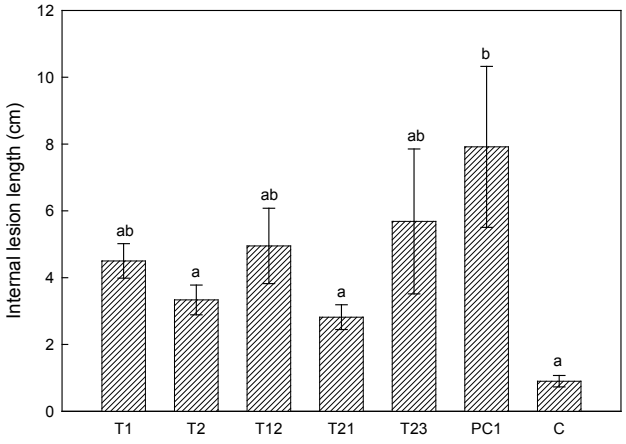

Figure 3. Mean internal lesion lengths (cm) on excised grapevine canes (cv. Grecanico) 15 d after inoculation with isolates of *Neofusicoccum parvum* (T1), *Phaeoacremonium* sp. (T2), *Ilyonectria liriodendri* (T12), *Neonectria* sp. (T21), *Cadophora luteo-olivacea* (T23), or *Phaeomoniella chlamydospora* (PC1), compared to non-inoculated controls (C). The vertical lines indicate standard errors. Bars accompanied by the same letter are not significantly different ($P < 0.05$, Fisher's LSD test).

Table 2. Incidence (%) of the different grapevine trunk diseases (GTDs) in whole grapevine plants and in plant parts of cvs Grecanico and Muller-Thurgau from two localities in Sicily. BD = Botryosphaeria dieback, BFD = black foot disease, PD = Petri disease. Percentages were calculated from the number of plants showing symptoms (in brackets) and the total number of samples per vineyard.

Locality (cultivar)	Number of plants	Disease	% of GTD-affected plants (N° of plants)	% of GTD affected plant parts (N° of plants)		
				apical	medium	basal
Castellammare (cv. Grecanico)	20	BD	25 (5)	25.0 (5)	5.0 (1)	0.0 (-)
		BFD	25 (5)	5.0 (1)	10.0 (2)	20.0 (4)
		PD	45 (9)	30.0 (6)	25.0 (5)	30.0 (6)
		Total GTD	***75 (15)***	***55.0 (11)***	***35.0 (7)***	***45.0 (9)***
Partanna (cv. Muller-Thurgau)	9	BD	66.7 (6)	66.7 (6)	0.0 (-)	0.0 (-)
		BFD	88.9 (8)	33.3 (3)	55.5 (5)	88.9 (8)
		PD	88.9 (8)	22.2 (2)	66.7 (6)	33.3 (3)
		Total GTD	*100.0 (9)*	*88.9 (8)*	*88.9 (8)*	*100.0 (9)*

Table 3. Isolation frequency (IF) of grapevine trunk disease (GTD) fungi from symptomatic cv. Grecanico and Muller-Thurgau grapevine samples from two localities in Sicily.

Locality (cultivar)	Disease	GTD fungi isolated	Total IF (%)	Isolation frequency of GTD fungi from trunk portions (%)		
				apical	medium	basal
Castellammare (cv. Grecanico)	BD	*Botryosphaeriaceae*	2.0	1.1	0.9	0.0
		Lasiodiplodia sp.	0.4	0.4	0.0	0.0
	BFD	*Neonectria* sp.	1.3	0.1	0.2	1.0
	PD	*P. chlamydospora*	4.7	1.0	1.7	1.9
Partanna (cv. Muller-Thurgau)	BD	*N. parvum*	4.1	4.1	0.0	0.0
	BFD	*I. liriodendri* sp.	10.4	1.7	3.2	5.4
		Neonectria sp.	2.2	0.0	2.0	0.2
	PD	*C. luteo-olivacea*	5.1	0.5	2.8	2.0
		Phaeoacremonium sp.	3.6	0.4	2.3	0.9

Pathogenicity tests

At 15 d after inoculation, control canes developed only slight subcortical discolouration upward and downward from the wound sites (mean total length = 0.9 cm). Canes inoculated with GTD pathogen isolates had bark lesions and subcortical longitudinal discolouration extending upward and downward from the inoculation points. Mean discolouration lengths varied among the fungal isolates. The greatest discoloration length (mean = 7.9 cm) resulted from inoculation with *P. chlamydospora*. The least discolouration length (2.8 cm) was caused by *Neonectria* sp., while *C. luteo-olivacea* (5.7 cm), *I. liriodendri* (5.0 cm) and *N. parvum* (4.5 cm) each gave similar amounts of discolouration. Only lesions from *P. chlamydospora* inoculations were significantly longer than the experimental controls (Figure 3). All the GTD fungi tested were re-isolated from inoculated plants, fulfilling Koch's postulates, and no fungal pathogens were isolated from the control plants.

DISCUSSION

This study has confirmed the presence of YVD in western Sicilian vineyards and has shown that different GTD pathogens were associated with the disease. All fungi identified in this study are known to be pathogens causing YVD in different wine growing regions (Rego *et al.,* 2006; Gramaje *et al.*, 2009; Agusti-Brisach and Armengol, 2013, Urbez-Torres and O'Gorman, 2014; Armengol, 2014, Carlucci *et al.*, 2015, 2017; Baranek *et al.*, 2018).

Morphological and molecular analyses identified six genera, including *Cadophora*, *Ilyonectria*, *Neonectria*, *Neofusicoccum*, *Phaeoacremonium* and *Phaeomoniella*

associated with YVD in cv. Grecanico and cv. Muller-Thurgau vines. According to their low IF and sporadic presence in the sub-samples, *Lasiodiplodia* and the other *Botryosphaeriaceae* were not considered to be associated with YVD in the two vineyards sampled in this study. Overall, a large proportion of vines was infected by at least one GTD fungus. This incidence was greater than previously recorded in Spain (minimum 9.6%, maximum 76.4%: Aroca *et al.,* 2010; Gramaje *et al.,* 2009; Gimenez–Jaime *et al.,* 2006), but similar to the values reported in 2018 by Pintos *et al.* (81-100%). Other recent studies carried out in Italy (Puglia and Molise regions) showed lower incidence of YVD fungi, both in nurseries and young plantations (4.4 to 24.4%), (Carlucci *et al.,* 2017).

In the present study, the incidence of the different GTDs (BD, BFD and PD) linked to the observed YVD was different for the two grapevine cultivars. In cv. Muller-Thurgau vines, all the samples were infected with GTD fungi with greater incidences of PD and BFD fungi than BD pathogens. For cv. Grecanico, lower incidence was recorded, with greater prevalence of PD than BD and BFD pathogens. Among fungi known to be associated with PD, *Phaeoacremonium* spp. have been the most frequently isolated (Raimondo *et al.,* 2014) along with *P. chlamydospora* (Mugnai *et al.,* 1999; Raimondo *et al.,* 2014), and *Pleurostoma richardsiae* (Carlucci *et al.,* 2013; Réblová *et al.,* in 2015). In the present study, the PD pathogens were *P. chlamydospora* from cv. Grecanico, and *P. chlamydospora*, *C. luteo-olivacea* and *Phaeoacremonium* sp. from cv. Muller-Thurgau vines.

The presence of BFD is now confirmed in many wine-growing areas, including Portugal, Spain, South Africa and the United States of America (Rego *et al.,* 2000; Halleen *et al.,* 2004; Petit and Gubler, 2005; Alaniz *et al.,* 2007; Gramaje *et al.,* 2010; Agusti-Brisach and Armengol, 2013). In the 1980s, BFD in Italy was associated with *C. obtusisporum*, *C. destructans* and *P. glomerata* (Grasso and Magnano di S. Lio, 1975; Granata e Refatti, 1981; Grasso, 1984), and more recently also with *Dactylonectria torresensis*, *I. liriodendri* and *Thelonectria* sp. (Carlucci *et al*., 2017). In the present study, the BFD-associated fungal genera were *Ilyonectria* and *Neonectria*, both present in cv. Muller-Thurgau vines, with *Neonectria* only found in cv. Grecanico samples.

Regarding the status of BD in Italy, the role of *Botryosphaeriaceae* damaging vineyards has been recognised since the late 1970s (Cristinzio, 1978; Rovesti and Montermini, 1987; Burruano *et al.,* 2008, Mondello *et al.,* 2013; Carlucci *et al.,* 2015). Results from the present study confirm the presence of *Botryosphaeriaceae* spp. in young vineyards, indicating a potential secondary role for these pathogens in YVD.

Among the four fungi identified to species level, the presence of *C. luteo-olivacea* isolated from symptomatic wood of cv. Muller-Thurgau is significant. This fungus is now considered a pathogen and part of the Petri disease causal agents in young vines (Halleen, *et al.,* 2007; Gramaje *et al.,* 2011b; Raimondo *et al*., 2019). The present study confirms *C. luteo-olivace*a to be associated with declining *V. vinifera* in Italy.

Despite most YVD-affected vines yielding multiple fungi associated with BD, BFD and PD, this study revealed different localization and abundance of specific GTD pathogens from different parts of the collected vines. In cv. Grecanico samples, *P. chlamydospora* was the most isolated fungus from along the rootstocks and grafting areas, and was always associated with brown wood streaking. In cv. Muller-Thurgau, the black foot pathogens *Ilyonectria* and *Neonectria* were the most abundant, and were specifically found at the bases of the rootstocks, associated with necrotic wood. These results indicate that PD could be the main GTD responsible for the vine decline observed in Castellammare vineyards, and BFD was important in Partanna vineyards.

High pathogen incidence in such young vines, and their abundance in the distal parts (grafting points and rootstock bases) indicate the pre-existence of the observed GTD infections before young vines are planted in the field. This has also been suggested by several authors, where *P. chlamydospora*, *Phaeoacremonium* spp., *Botryosphaeriaceae*, *Cylindrocarpon* spp., *Ilyonectria* and other pathogens have been commonly found during the vine propagation processes in nurseries, frequently resulting as mixed infections (Waite *et al.,* 2015). The frequency of mixed infections was also indicated in our assays, where the different genera associated with the observed YVD did not show significant differences in growth at low temperatures. This suggests high probability of multiple infections, in the field. Variability in the optimum and maximum growth temperature values would also influence the evolution of infections. Host colonization could vary, if environmental temperatures diverge from pathogen thermal needs, so affecting the incidence of the diseases they cause.

There is no single and simple control method for vine trunk diseases (Mondello *et al.,* 2018a), and the present results further confirm the urgency in adopting a "global strategy" for management of GTDs along all stages of grapevine vegetative and productive life (Armengol, 2014). Strategies using different methods can be suggested to nurserymen and winegrowers (Halleen and Fourie, 2016; Gramaje *et al.,* 2018, Mondello *et al*., 2018b) to limit the economic impacts of YVD and GTDs.

It is important to emphasize that the pathogenicity tests on detached canes did not show statistically significant differences among several isolates, except for *P. chlamydospora*. This result could be explained by the generally accepted complexity of interactions between *V. vinifera* and GTD pathogens. Disease symptoms are linked to the diversity of fungi, to the metabolites they produce (toxins and exopolysaccharides), the environmental conditions, and to the phenological stages of host grapevine plants (Spagnolo *et al*., 2017; Fisher and Peighami Ashnaei, 2019; Mondello *et al*., 2019; Reis *et al*., 2019). Models used to study GTD pathogen virulence are unlikely to represent all these variables.

Further studies are required to evaluate the spread of YVD in other young vineyards in Sicily, and to better characterize the roles of the different pathogens in YVD in this part of Italy.

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Research Papers

In vitro screening of *Trichoderma* isolates for biocontrol of black foot disease pathogens

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Summary. Management of black foot disease (BFD) of grapevines is difficult due to limited control options. Biological control fungi, in particular *Trichoderma* spp., hold potential as part of integrated management of BFD. *Trichoderma atroviride*, *T. fertile*, *T. harzianum* and *T. virens* were evaluated *in vitro* against four common BFD pathogens in South Africa, including *Campylocarpon fasciculare*, *C. pseudofasciculare*, *Dactylonectria macrodidyma* and *Ilyonectria liriodendri*. Effects of *Trichoderma* volatile organic and diffusible antifungal compounds (VOCs and DACs) and direct antagonistic effects were determined in Petri dish assays. Pathogen growth inhibition was determined in the VOC and DAC assays. Macro- and microscopic observations of fungus interaction zones were made in dual culture plate assays. Greater BFD pathogen growth inhibition occurred with the DACs than the VOCs. For both classes of compounds, *D. macrodidyma* was the most sensitive pathogen (100% inhibition by DACs and 65% by VOCs). In some cases, depending on the *Trichoderma* spp. isolate, growth stimulation occurred for *I. liriodendri*, *C. fasciculare* and *C. pseudofasciculare*. Macroscopically observed *Trichoderma* and BFD pathogen interactions included total or partial overgrowth, often associated with sporulation of the *Trichoderma* spp., and arrested growth or the formation of inhibition zones. Microscopic interactions included adhesion of the *Trichoderma* to pathogen hyphae, pathogen hyphae swelling, malformation and disintegration. In general, *in vitro* efficacy was isolate-dependent, both for *Trichoderma* spp. and the BFD pathogen.

Keywords. Volatile organic compounds, diffusible antifungal compounds, *Campylocarpon*, *Dactylonectria*, *Ilyonectria*.

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INTRODUCTION

Black foot disease (BFD) is a major fungus disease associated with young grapevine decline in nurseries and young vineyards (Halleen *et al*., 2004, 2006a, 2006b; Gramaje *et al*., 2010). Incidence and severity of BFD has increased and is now one of the major grapevine trunk diseases (Petit and Gubler, 2005; Rego *et al*., 2009; Compant *et al*., 2013; Úrbez-Torres *et al*.,

2014). The causal pathogens of this disease include species of *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew., *Dactylonectri*a L. Lombard and Crous, *Ilyonectria* P. Chaverri and C. Salgado, *Pleiocarpon* L. Lombard and D. Aiello 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, Aigoun-Mouhous *et al*., 2019). Fungi in these genera are common soil inhabitants, occurring as saprophytes, root colonizers or weak plant pathogens that can survive in soil for extended periods due to the formation of chlamydospores (Brayford, 1993; Halleen *et al*., 2004).

Apart from hot water treatments (HWT) of dormant nursery plants, no treatment is available to prevent nursery plants from becoming infected with BFD pathogens after planting. Hot water treatment (50°C for 30 min) is considered effective against BFD (Halleen *et al*., 2006a; Alaniz *et al*., 2011; Agustí-Brisach and Armengol, 2013), but may result in a short-term effect, and also vine failure if not correctly applied to low quality propagation material (Rego *et al*., 2009). The application of *Trichoderma* spp. as biological control agents (BCAs) offers sustainable and lasting protection against grapevine trunk diseases when applied as pruning wound protectants (Mondello *et al*., 2018), so BCAs offer potential as part of integrated disease management of BFD. However, *Trichoderma harzianum* was evaluated on nursery vines in South Africa, and although '*Cylindrocarpon*' re-isolation was reduced, this was not significantly less than untreated controls (Fourie *et al*., 2001). Other studies investigating effects of *Trichoderma* isolates on nursery vines and BFD did not consistently find lower BFD pathogen infections (Dos Santos *et al*., 2016; Berlanas *et al*., 2018; Berbegal *et al*., 2019).

Beneficial activity of *Trichoderma* that have been studied extensively *in vitro* includes production of secondary metabolites and antibiosis, and direct mycoparasitism of plant pathogens (Harman, 2006; Samuels and Hebbar, 2015). Secondary metabolite composition is specific to the *Trichoderma* strain (Vinale *et al*., 2008a, 2008b), and includes a variety of compounds that can be categorized as volatile organic compounds (VOCs) or diffusible antifungal compounds (DACs). However, factors contributing to overall plant health and productivity cannot be evaluated *in vitro*. For example, improved plant health can be attributed to the induction of host plant resistance (Harman *et al*., 2004; Harman 2006; Vinale *et al*., 2008a) and growth stimulation (Vinale *et al*., 2008a, 2009; Samuels and Hebbar, 2015).

Despite various commercial *Trichoderma*-based products being available in South Africa, none has yet been registered for control of BFD of grapevine. The aim of the present study was, therefore, to investigate the efficacy of *Trichoderma* spp. isolates, derived from commercial products or potential products, against BFD pathogens *in vitro*. The tests conducted included screening for VOCs and DACs and examination of competitive growth between *Trichoderma* isolates and BFD pathogens.

MATERIALS AND METHODS

Fungal isolates

Ten *Trichoderma* spp. isolates, of which eight were from commercial products (Table 1), were tested *in vitro* for abilities to inhibit mycelium growth of BFD pathogens, using the assay methods by Dennis and Webster (1971a; 1971b) with modifications. The BFD pathogens used were three isolates each of *C. fasciculare*, *C. pseudofasciculare*, *D. macrodidyma* and *I. liriodendri* (Supplementary Table 1). Pure *Trichoderma* cultures were made from the products TrichoPlus™ and Awegenic Tri-cure™, by making a spore suspension from each product, plating it onto potato dextrose agar (PDA) (Biolab), and hyphal tip subculturing of developing fungi. The other *Trichoderma* isolates were obtained as pure cultures from the respective companies or providers. Fungus cultures were grown on PDA plates at 25°C for approx. 10 d, cut into small blocks and stored in sterile 14 mL capacity McCartney bottles with 10 mL sterile distilled water at room temperature until required.

Screening Trichoderma *isolates for production of volatile organic compounds*

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 7 d. Mycelium plugs (5 mm diam.) cut from the growing margins of colonies were placed face down in the centres of 90 mm PDA Petri dishes. Petri dishes containing the pathogens were then inverted over Petri dishes containing *Trichoderma* isolates in all combinations, and were sealed with Parafilm® M (Bemis). The Petri dishes were then incubated in the dark at 25°C for 7 d. Control plates were set up in the same manner, except that BFD pathogen-inoculated dishes were combined with sterile PDA Petri dishes. Following incubation, two colony diameters (perpendicular and horizontal) of each BFD pathogen were measured.

Table 1. *Trichoderma* products and isolates used in this study.

Isolate code[a]	Species (commercial isolate)	Product name	Institution/Company	Recommended or registered use
T1	*Trichoderma atroviride* (USPP-MT1)		Stellenbosch University	Grapevine pruning wounds
T2	*Trichoderma atroviride* (USPP-T1)		Stellenbosch University	Grapevine pruning wounds
T3	*Trichoderma atroviride*	Eco 77®	Plant Health Products	Grapevine pruning wounds – *Eutypa*; Tomato and cucumber – *Botrytis cinerea*
T4 T5	*Trichoderma atroviride* (Vitic 2) *Trichoderma harzianum* (Sp)	Bio-Tricho	Agro-Organics	Multiple crops and diseases
T6	*Trichoderma fertile*	TrichoPlus™	BASF	Seed treatment of tobacco
T7 T8 T9	*Trichoderma harzianum* (K2) *Trichoderma atroviride* (K4) *Trichoderma virens* (K1)	Excalibur Gold™	Advanced Biological Marketing®	General seed treatment
T10	*Trichoderma harzianum* (MIT04)	Awegenic Tri-cure™ MBFi		Beans, maize – *Rhizoctonia* and *Fusarium*; Potatoes – stem canker, black scurf, *Rhizoctonia*; Wheat – Fusarium; Tomato, curcurbit, lettuce – *Fusarium*, *Pythium* spp.

[a] Isolate codes used in study.

Screening Trichoderma *isolates for production of diffusible antifungal compounds*

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 8 d. Mycelium plugs (5 mm diam.) cut from the growing margins of the *Trichoderma* colonies were placed face down on autoclaved 50 µm thick cellophane membranes (85 mm in diam.; Sigma) covering PDA in Petri dishes. The Petri dishes were then incubated in the dark at 25°C until colony diameters of 50 mm were reached (after 42 to 48 h incubation). Following the incubation period, the cellophane membranes were removed ensuring that the Petri dishes were completely free of *Trichoderma* conidia and mycelia. The Petri dishes were then re-inoculated with mycelium plugs (5 mm diam.) cut from the growing margins of BFD pathogen colonies, and were incubated in the dark at 25°C for 6 d. Control plates were set up in the same manner, with the exception that the cellophane membranes were not inoculated with the *Trichoderma* isolates. Following the incubation period, two colony diameters (perpendicular and horizontal) of each BFD pathogen were measured.

Competitive growth assessments

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 7 d. Mycelium plugs (5 mm diam.) of the pathogens were cut from the growing margins of the colonies and placed face down on one side of 90 mm diam. Petri dishes. The Petri dishes were then incubated in the dark at 25°C for 4 d. Mycelium plugs (5 mm diam.) cut from the growing margins of the *Trichoderma* colonies were placed face down opposing the BFD pathogen colonies and incubated for a further 6 d. These dual-inoculated plates were then used for macroscopic observations of the colony interaction zones. Hyphae interactions, in particular *Trichoderma* hyphal adhesion or coiling and pathogen hyphal swelling, malformation or disintegration, were observed by mounting agar blocks cut from the interaction zones between *Trichoderma* isolates and one isolate of each BFD pathogen on a microscope slide and viewing it with a Nikon Eclipse E600 compound microscope at different magnifications (×200, ×400 or ×1000 with oil immersion).

Statistical analyses

Six replicates were used for the VOC and DAC experiments. The percentage inhibition from each experimental treatment was calculated, 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 of the data was confirmed by the 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.). Fisher's least significant difference (LSD) was calculated at $P = 0.05$ to compare factor means (Ott and Longnecker, 2001), and this probability level was considered statistically significant for all tests.

RESULTS AND DISCUSSION

The *Trichoderma* spp. showed variable levels of growth inhibition towards the four BFD pathogen species tested, and, in a few cases, caused growth promotion of the pathogens. The biocontrol activity varied according to *Trichoderma* isolate, pathogen isolate, and according to the VOC or DAC tests.

Analyses of variance revealed significant pathogen × *Trichoderma* interactions ($P < 0.0001$), so the data are presented for each isolate (Tables 2 and 3). Results from the VOC assay differed to a large extent from those obtained in the DAC assay. When taking both classes of antifungal compounds into account, two isolates of *T. atroviride* (T1 and T8) gave the greatest overall mycelium growth inhibition, although growth inhibitioni-

Table 2. Mean percentage of mycelium growth inhibition of four species of black foot disease pathogens (three isolates each) from volatile organic compounds (VOCs) produced by different *Trichoderma* spp. Isolates (T1 to T10).

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	27.8ab[a]	33.8ab	23.2b-e	28.6a	15.8a	15.7a	55.3b	54.3a	66.9a	14.6a	15.8ab	21.9a
T2	23.9a-c	32.9ab	26.9bc	29.4a	11.8ab	10.4ab	37.7c	54.4a	58.0ab	-4.2b-d	10.6bc	14.5ab
T3	28.5a	33.9ab	30.8b	27.1a	11.3ab	12.1a	60.2ab	49.7a	41.0cd	5.7a-c	15.1ab	23.3a
T4	19.0b-d	23.5a-d	18.7c-e	21.3ab	3.6bc	2.6bc	60.7ab	53.6a	51.4bc	-11.0d	8.0bc	4.8bc
T5	24.7a-c	24.1a-c	22.1c-e	21.3ab	6.1bc	2.7bc	64.2a	58.3a	61.4ab	-10.7cd	7.9bc	3.6c
T6	6.3e	7.0e	9.0f	8.2c	-1.6c	-1.5c	8.3e	8.8c	21.8ef	-15.3d	4.4cd	-0.6c
T7	17.6cd	22.3b-d	24.7b-d	16.4bc	7.5a-c	9.7ab	33.1c	33.3b	34.2de	-7.3b-d	6.7c	17.3a
T8	28.6a	36.1a	39.6a	13.3bc	10.9ab	-2.5c	36.6c	37.6b	57.2ab	6.3ab	21.8a	23.9a
T9	12.6de	11.9c-e	18.6de	-2.6d	-1.2c	2.9bc	21.5d	14.1c	18.5f	-18.6d	-1.6d	-4.1c
T10	11.7de	10.6de	15.8ef	9.9c	6.4bc	3.9bc	18.4d	31.4b	30.5d-f	-5.2b-d	3.8cd	3.0c
LSD[b]	9.38	13.30	8.31	9.43	9.23	8.05	8.14	9.81	14.06	16.54	8.03	9.93

[a] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Least significant differences of each column.

Table 3. Mean percentage of mycelium growth inhibition of four species of black foot disease pathogens (three isolates each) from diffusible antifungal compounds (DACs) produced by *Trichoderma* spp. isolates (T1 to T10).

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	48.0a[a]	53.8a	52.8a	46.1c	30.7b	36.4a	100.0a	100.0a	100.0a	35.9a	70.7a	38.3b
T2	16.4c	22.3bc	19.6b	35.7d	17.4c	18.7bc	46.1b	43.9b	47.1c	19.1bc	25.6b	16.4de
T3	30.5b	20.6b-d	18.2bc	16.4f	17.4c	13.6cd	29.0cd	35.7bc	82.6b	9.5d	28.7b	16.4de
T4	20.0c	19.9cd	13.0bc	7.9fg	10.5d	3.7d	25.0c-e	12.4ef	26.8d	10.0cd	15.3bc	8.4ef
T5	20.6c	14.6c-e	18.6bc	26.1e	14.4cd	13.2cd	34.5bc	21.9de	23.7de	24.2b	24.9b	13.8d-f
T6	0.9e	-6.3f	-14.1e	2.6g	8.2d	3.6d	15.1e	13.0ef	16.8ef	2.5d	-1.1c	4.7f
T7	3.6de	5.0ef	-2.8de	2.7g	9.0d	4.0d	18.2de	9.9f	10.1f	6.6d	3.1c	8.2ef
T8	35.4b	48.4a	39.7a	100.0a	33.1b	29.9ab	96.4a	100.0a	94.5a	38.0a	57.5a	65.5a
T9	39.6ab	33.8b	52.0a	76.7b	97.8a	25.2ab	99.1a	29.9cd	53.2c	19.6b	23.0b	22.5cd
T10	11.5cd	8.7de	4.3cd	14.5f	13.7cd	13.9cd	21.1de	39.6bc	20.1de	19.2bc	14.7bc	26.0c
LSD[b]	9.37	13.33	14.69	8.68	6.34	11.30	11.67	11.27	9.74	9.50	16.81	9.08

[a] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Least significant differences of each column.

Table 4. Macroscopic interactions observed between *Trichoderma* isolates (T1 to T10), and the respective black foot disease pathogens (three isolates of each) in dual culture assays.

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	OGS[a]	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

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

was greatest in the DAC assay. In both assays, and for most of the pathogen isolates, isolates T1 and T8 gave the greatest growth inhibition. For example, with *C. fasciculare*, T1 DACs gave the greatest growth inhibition against all three pathogen isolates (mean = 48.0 to 53.8%), and T8 VOCs gave the greatest growth inhibition against all three pathogen isolates (mean = 28.6 to 39.6%).

Overall, *D. macrodidyma* was the most sensitive pathogen species, for which all three isolates were completely inhibited by T1 DACs, and inhibition from T8 not being significantly different from T1 (mean = 94.5 to 100.0%). Greater inhibition of *D. macrodidyma* isolates (maximum mean = 66.9%) was also measured from the VOCs in comparison to the other species (maximum mean = 39.6% for *C. fasciculare*, 29.4% for *C. pseudofasciculare* and 23.9% for *I. liriodendri*).

In some cases, *Trichoderma* metabolites stimulated growth of the BFD pathogens. This was more often observed for *I. liriodendri* (mean inhibition = -1.6 to -18.6%) and *C. pseudofasciculare* (mean = -1.2 to -2.6%) in the VOCs assay, and also occurred for *C. fasciculare* (mean = -2.8 to -14.2%) and once for *I. liriodendri* (mean = -1.1%) in the DACs assay.

The macroscopic interactions between *Trichoderma* and pathogen isolates are reported in Table 4. For 90% of the *Trichoderma* isolates, the interactions occurred simultaneously and are, therefore, shown for each isolate. The formation of inhibition zones and complete growth inhibition were probably due to the production of secondary metabolites by the BFD pathogens, and this was only observed in a few cases. *Trichoderma* spp. mostly overgrew the pathogens followed by profuse sporulation, which was not observed without direct contact to the pathogens. Numerous factors, including the metabolic rate of hypha cells and the production of antimicrobial metabolites, can cause conidiation (Steyaert *et al*., 2013). Such an example is cell wall degrading enzymes and secondary metabolites that are produced during this process, facilitating entry of *Trichoderma* hyphae into the lumina of the parasitized fungi (Zaidi and Singh, 2013).

These interactions often coincided with mycoparasitic actions at microscopic levels (Supplementary Table 2). Adhesion of the *Trichoderma* hyphae to pathogen hyphae, and disintegration of pathogen hyphae, were often observed, while coiling of the *Trichoderma* hyphae or swelling and malformation of pathogen hyphae were rarely seen. Similar hyphal interactions were observed in isolates of the same species.

This study found two isolates of *T. atroviride* (T1 and T8) gave general antagonistic efficacy, though with varying efficacy against different BFD pathogens. Combining these isolates could offer more effective management of BFD. A matter of concern are the low levels of inhibition observed for the *Campylocarpon* species and *I. liriodendri*. Overall efficacy towards BFD would, therefore, be dependent on the composition of the BFD species present in a particular vineyard, as well as the *Trichoderma* isolates utilized for BFD control. Combining existing knowledge of *Trichoderma* as BCAs with the knowledge obtained from the present study will aid in the screening and combination of *Trichoderma* isolates as BCAs against BFD before field evaluations commence.

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Research Papers

Synergistic effects of water deficit and wood-inhabiting bacteria on pathogenicity of the grapevine trunk pathogen *Neofusicoccum parvum*

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Summary. Grapevine trunk diseases (GTDs), including Esca and Botryosphaeria dieback, are major factors limiting grapevine productivity and longevity in France. The influence of combined biotic and abiotic stress factors on GTD development is not well understood. This study evaluated individual and combined effects of a biotic factor (bacterium occurrence) and abiotic stress (water deficit), on the pathogenicity to grapevine of the GTD pathogen *Neofusicoccum parvum*. Co-inoculation of 46 different bacterium strains with *N. parvum* into growing grapevine-cuttings showed synergistic relationships between several of the strains and the pathogen. *Bacillus pumilus* (strain S35) and *Xanthomonas* sp. (strain S45), which cause canker lesions, were selected for testing bacterial and fungal interactions in grapevine, under individual and combined stress conditions. *In vitro*, each of the bacterium strains neither inhibited *N. parvum* nor the co-inoculated bacterium strain. None of the three microorganisms degraded lignin, but all three degraded cellulose and hemicellulose. In a greenhouse experiment, 9 months after microbial inoculations in plants under normal and water-restricted conditions, effects on canker formation of water deficit combined with the bacteria and *N. parvum* interactions were assessed and on *N. parvum* DNA contents. Synergistic effects of biotic and abiotic stresses were demonstrated. The bacterial infection stress influenced the grapevine/*N. parvum* interaction by increasing canker lesions and *N. parvum* DNA contents in plants co-inoculated with *B. pumilus* and/or *Xanthomonas* sp. qPCR assays showed that high contents of *N. parvum* DNA occurred in water-restricted potted vines inoculated with *N. parvum*, especially in the inoculation zones. These results provide insights into the relative roles of biotic and abiotic stress factors in *Botryosphaeriaceae* symptom expression, which could assist development of future GTD management.

Keywords. Esca, Botryosphaeria dieback, *Bacillus pumilus, Xanthomonas* sp.

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INTRODUCTION

Grapevine is simultaneously exposed to multiple biotic and abiotic stresses, which can limit productivity. Grapevine trunk diseases (GTDs), including Eutypa dieback, Esca and Botryosphaeria dieback, have increased considerably in recent decades (De La Fuente *et al*., 2016; Gramaje *et al*., 2018). While most GTDs are likely to be multi-factorial diseases (Darrieutort *et al*., 2007; Lecomte *et al.,* 2011; Bertsch *et al*., 2013), research has mainly concentrated on plant responses to biotic stresses, especially to determine the infection processes associated with the complex of fungal GTD pathogens (Bertsch *et al*., 2013; Gramaje *et al*., 2018; Brown *et al.,* 2019). Several studies have dealt with the infection of grapevine by one or combinations of different fungi involved in GTDs (Laveau *et al*., 2009; Rezgui *et al*., 2018; Brown *et al*., 2019). However, the involvement of other biotic stresses, such as bacterial infections, has received little attention (Bruez *et al.,* 2015; Haidar, 2016). Beside the microbial aspects of GTDs, abiotic stress factors are reported to be involved in the development of these diseases (Bertsch *et al*., 2009; Lecomte *et al*., 2011; Fischer and Kassemeyer, 2012), but the particularly complex effects of multiple and combined biotic and abiotic stress factors are not well understood.

Infection of grapevines by pathogens causing canker and/or dieback, such as *Botryosphaeriaceae*, has been reported to be affected by stressful cultural practices and environmental conditions, including unfavourable climate and soil moisture deficit (Lecomte *et al*., 2011; Amponsah *et al*., 2014; Lawrence *et al*., 2016; Kovács *et al*., 2017; Billones-Baaijens and Savocchia, 2018). *Neofusicoccum parvum* and other *Botryosphaeriaceae* species, such as *N. australe*, *N. luteum*, *Lasiodiplodia theobromae*, *Diplodia mutila*, *D. seriata* and *Botryosphaeria dothidea*, are important pathogens associated with GTDs. These fungi cause dieback, cankers and central wood necroses, and eventually vine death (van Niekerk *et al*., 2006; Úrbez-Torres, 2011; Gramaje *et al*., 2018; Mondello *et al*., 2018).

Key environmental stress factors such as drought or water deficit, especially associated with climate change, could predispose vines to infection by GTD pathogens (Slippers and Wingfield, 2007; Bertsch *et al.,* 2013; Songy *et al*., 2019). Interactions between abiotic stresses and pathogens can result in enhanced reduced host susceptibility to infection, depending on the stress and pathogen (Ramegowda *et al*., 2013; Ramegowda and Senthil-Kumar, 2015; Songy *et al*., 2019). For *Botryosphaeriaceae*, different physiological and histochemical changes have been associated with infections, including formation of tyloses and production of gums blocking host xylem vessels (Bertsch *et al*., 2013). In Eucalyptus, as in grapevine, the impacts of infections by *Botryosphaeriaceae* in relation to climatic conditions and stress factors have been reported previously (Chen *et al*., 2011; Barradas *et al*., 2016, 2018; Marsberg *et al*., 2017).

In grapevine, previous studies of the effects of stress factors, such as drought or water deficit, on *Botryosphaeriaceae* disease expression, have produced variable results. Qiu *et al*. (2016) observed increased susceptibility to some species of *Botryosphaeriaceae,* including *B. dothidea*, *L. theobromae* and *N. parvum*, inoculated into water-restricted potted vines. Similarly, van Niekerk *et al*. (2011) reported that grapevine lesion lengths were greater in plants inoculated with some *Botryosphaeriaceae* species, when the vines were subjected to a low irrigation regime compared to well-irrigated vines. In contrast, Sosnowski *et al*. (2016) reported that water deficit did not increase grapevine susceptibility to *D. seriata*.

As GTD development may result from complex interactions *in natura*, there is a need to measure grapevine responses to different stress factors, tested singly or in combinations. Elucidation of the impacts of microorganisms and abiotic stress factors on grapevine responses is important for understanding the key factors that influence GTDs, and to develop effective management strategies for limiting GTD development.

The major objectives of the present study were as follows. Firstly, by inoculating grapevine plants with bacterial and fungal wood colonizing microorganisms, the combined effects were evaluated of two biotic stress factors on the expression of canker in cuttings. Two bacterium strains, *Xanthomonas* sp. (S45) and *B. pumilus* (S35) were used because previous research (Haidar *et al.,* 2016a) showed that wood necroses increased in young grapevines when they were co-inoculated with these two strains and *N. parvum*. Second, the consequences were assessed of grapevine exposure to inoculations with *N. parvum* and the two bacterium strains, combined with host abiotic stress (i.e. water deficiency). These biotic and abiotic stresses were tested individually and in combinations. Furthermore, by quantifying *N. parvum* DNA, the aim was to establish whether there was a link between symptom expression due to *N. parvum* infection and wood colonization by this pathogen. These results could give insights into the roles of biotic and abiotic stress factors in symptom expression, and provide new knowledge for developing management strategies for GTDs.

MATERIALS AND METHODS

Microorganisms and culture media

Neofusicoccum parvum *culture*

A *N. parvum* isolate (strain 'Cou 02') was selected from the INRAE-UMR 1065 SAVE collection, Bordeaux, France. This strain was originally obtained in 2008 from cv. Cabernet Sauvignon vines in an experimental INRAE vineyard, near Bordeaux, France. The isolate was characterized as very aggressive in previous studies (Laveau *et al*., 2009). The strain was subcultured on Malt Agar (MA) and incubated at 27°C (12 h light/12h dark) for 1 week, before being used in experiments.

Cultures of bacterium strains

Forty-six strains of bacteria were tested. These were from a previous study that included description of their origins and key features (Haidar *et al*., 2016a). For *in vitro* trials carried out with *B. pumilus* (S35) and *Xanthomonas* sp. (S45), the strains were grown for 24 h at 28°C on Trypto-Casein Soy Agar (TSA, Biokar Diagnostics). In two bioassays (see below), the bacterium preparations were made as described in Haidar *et al.* (2016a), with cell concentrations adjusted to 10^8 CFU mL^{-1}.

Stem disease bioassays

Plant material

Rooted cuttings of grapevine cv. Cabernet Sauvignon, originating from INRAE experimental vineyards near Bordeaux, were used in the bioassays. The cuttings were grown in an open greenhouse, and were processed and prepared as described by Laveau *et al.* (2009).

Bacterium and fungus inoculations

The bacterium and fungus inoculations were made as described by Haidar *et al.* (2016a). Briefly, for application of each bacterium strain, 40 µL of cell suspension was inoculated into a drilled hole in each stem cutting below the upper bud. Once the liquid inoculum had dried for 20 to 40 min at ambient temperature, the hole was inoculated with a mycelium plug of *N. parvum*. The treated wounds were covered with Parafilm® (Scellofrais film) to seal the inoculation zones.

Experimental design of the first bioassay (screening bioassay of 46 bacterium strains)

Forty-six bacterium strains were evaluated for their antagonistic activity against *N. parvum*. Two trials were conducted. The first included 26 bacterium strains (S1–S26), and the second included 20 strains (S27–S46). In the first trial, the experimental design was a completely randomized block, with two blocks, each of 224 plants. Twenty-eight treatments (with eight plants per treatment in each block) were applied, including the 26 bacterium strains co-inoculated with the *N. parvum* isolate and two experimental controls. In the second trial 252 plants were used. Eleven plants were tested for each bacterium strain co-inoculated with *N. parvum*. The experimental control treatments in both trials were; uninoculated uninfected control (UUC), where the plants were not inoculated with the fungus and not treated with bacteria; and an uninfected control (UC), where the plants were inoculated only with *N. parvum*.

Experimental design of the second bioassay (with the two selected bacterium strains)

The second bioassay was carried out between June 2017 and April 2018 with the two bacterium strains (S35 and S45) that gave the greatest synergistic effects with *N. parvum* in the first bioassay. The two strains were applied to grapevine cuttings by co-inoculation (as described above) to confirm their ability to enhance *N. parvum* symptoms in grapevine cuttings. To assess the impacts of water deficit on infection of the cuttings by *N. parvum*, the same experimentation was performed on well-watered (experimental control) or water-restricted grapevine cuttings. The experimental design was a randomized complete block with 30 cuttings per treatment and per watering condition. A total of 540 grapevine cuttings were used (270 plants for each watering condition). During the period of the experiment, the well-watered control grapevines received five times more water than the water-restricted grapevines (Lawrence *et al*., 2016). Each well-watered plant received water at 333.3 mL d^{-1}, while each water-restricted plant received 66.6 mL d^{-1}.

The experiment treatments consisted of cuttings that were: (i) co-inoculated with S35 and *N. parvum*; ii) co-inoculated with S45 and *N. parvum*; or (iii) co-inoculated with both the bacterium strains and *N. parvum*. The control treatments consisted of plants that were: i) inoculated with *N. parvum*; ii) treated with S35; iii) treated with S45; iv) treated with S45 and S35; v) not inoculated with *N. parvum* nor treated with bacteria, but treated

with sterile bacterium and fungus growth media (mock control); or vi) not inoculated with *N. parvum*, nor treated with bacterium strain.

Effects of water deficiency on the root mass of cuttings in the second bioassay

After measurement of stem cankers (see below), six plants from each treatment were destructively harvested to provide measurements of root mass. The plants were removed from the soil, remaining soil particles were washed from the roots, and the roots were then weighed (Kamiloglu *et al*., 2014).

Evaluation of stem cankers

At 4 months post-inoculation in the first bioassay or 9 months in the second, length (mm) of stem canker was measured on each cutting.

Detection and quantification of Neofusicoccum parvum *within the wood of grapevine cuttings using quantitative PCR (qPCR)*

In the second bioassay, qPCR assays were performed to quantify *N. parvum* DNA in inoculated and uninoculated grapevine cuttings. For each treatment, five plants were sampled at the end of the experiment. After symptom (canker) measurement, two wood sections each of 15 mm were sampled from two stem zones of each plant, corresponding to zone (A), around the site of inoculation, and zone (B) 15 mm above the site of inoculation. Thus, four wood chips (each of 5 × 2 × 2 mm) were sampled from the stem of each plant after symptom evaluation. Overall, 180 wood samples were collected and stored at -80°C for DNA extractions.

DNA extractions from the wood of grapevine cuttings

DNA extracts from plants samples were obtained using the methods of Pouzoulet *et al*. (2013). Briefly, wood samples were lyophilized and subsequently ground at room temperature, using a Tissue Lyser II (Qiagen). Approximately 100 mg of wood powder was used for each DNA extraction, using the commercial kit DNeasy Plant Mini Kit (Qiagen), and the adapted protocol for grapevine wood (Pouzoulet *et al*., 2013) was implemented. The quality and quantity of DNA obtained were measured using nanodrop (ND-1000, Thermoscientific, Labtech), and diluted to 10 ng μL^{-1}.

DNA extraction from pure cultures of Neofusicoccum parvum

A pure culture of the *N. parvum* isolate was grown on MA at 25°C for 4 d. Mycelium was harvested and then freeze-dried overnight. The dried mycelia were ground with a small glass ball in a TissueLyserII (Qiagen) before DNA extraction with a cetyltrimethylammonium bromide (CTAB) procedure (Bruez *et al*., 2015). The DNA concentration was measured by the nanodrop (ND-1000, Thermoscientific, Labtech), and diluted to 50 ng μL^{-1}.

Quantitative real-time PCR analyses on the wood of grapevine cuttings

Each reaction, conducted in duplicate, contained 2 µL of sample DNA, 0.6 µL of each primer at 10 µM and 12.5 µL of 2× SYBR Green Quantitect Master Mix (Qiagen), and H_2O to 25 µL total volume. Experiments were conducted with Stratagene Mx3005P qPCR system (Agilent Technologies). The thermal cycling conditions were: an initial denaturation step at 95°C for 15 min; 40 cycles each of 15 s at 95°C (for denaturation) and 45 s at 62°C (for both annealing and extension); and an additional melting analysis for 40 min from 60°C to 95°C. Pure DNA from *N. parvum* cultures was used as the standard. Standard solutions, ranging in concentration from 750 ng μL^{-1} to 7.5×10^{-5} ng μL^{-1}, were performed. The sequence primers used were as follows: BpvQF, 5'-GCGCGAATGGCAATGGCTGA-3', and BpvQR, 5'-TACGTGTTTGTGCAATTAGTGAGAGAG-3' (Pouzoulet, 2012), and the primers were each used at a final concentration of 0.5 mM. For each sample, the amount of fungus DNA was calculated as described by Reid *et al*. (2006). Supplementary Figure S1 shows the relationship between Ct sample data and quantity of *N. parvum* DNA.

In vitro *microbial confrontations*

To assess the potential interactions between the bacteria S35 and S45, each strain was grown in 100 mL tryptic soy broth (TSB) at 28°C for 48h. Cell-free supernatants were prepared by centrifuging at 10,000 rpm for 30 min at 4°C. Appropriate agar medium plates (TSA or potato dextrose agar, (PDA; Biokar)) were each inoculated 100 µL of each strain, and wells (8 mm diam.) were cut and filled with 100 µL of the supernatants of tester strain. Plates were incubated at 28°C, for 2–3 days, and the inhibition zones around the inoculum wells were

then measured. The interaction between the two bacterium strains was also studied by a cross streak method (Lertcanawanichakul and Sawangnop, 2008). Each tester strain (S35 or S45) was inoculated by a single streak in the centre of an agar plate (PDA or TSA). After incubation for 2 d at 28°C, the respective receiver strain (S35 or S45) was then applied as a perpendicularly streak to the tester stain, and the plates were then incubated for 3 d at 28°C. The bacterium interactions were analyzed by observing development of inhibition zones. To determine if the bacterium strains S35 and S45 inhibited *N. parvum*, their direct and indirect *in vitro* effects on mycelium growth of *N. parvum* were assessed using the methods of Haidar *et al.* (2016b).

Wood cell component decomposition by Neofusicoccum parvum, Bacillus pumilus *S35 and* Xanthomonas *sp. S45.*

Wood component decomposition capabilities of bacterium strains and *N. parvum* were tested using three selective media: i) minimum medium [1 g K_2HPO_4; 1 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4.H_2O$; 0.5 g NaCl; 5 g carboxymethyl-cellulose sodium salt (Sigma), and 20 g agar per liter), with 5 g xylan from beechwood (Appolo Scientific) or carboxymethylcellulose (CMC, Sigma) as the sole carbon sources (Hervé *et al*., 2016); ii) WYA medium (1 g NaCl, 0.1 g yeast extract (Difco), 1.95 g MES (Sigma) and 20 g per liter agar, adjusted to pH 5, and containing 0.05% Remazol Brilliant Blue R (Sigma) (Hervé *et al*., 2016); or iii) PDA plates containing 0.02% Guaiacol.

Pure cultures of each microorganism strain were spot inoculated at the centres of Petri dishes (90 mm diam., 15 mL medium per plate). The Petri dishes were then incubated for 7 d at 28°C in the dark. Lignolysis was indicated by the Remazol Brilliant Blue R (RBBR) medium changing from blue to pale pink. Cellulolytic activity, on CMC medium, and xylanolytic activity on xylan medium, were detected using 0.1% Congo red (Sigma) for staining during 40 min. Staining was then followed by washing with 1M NaCl to counterstain the plates revealing clear zones where enzymatic activity occurred. Diameters of clear zones surrounding microbe colonies were measured to indicate the enzymatic activity.

Statistical analyses

The experimental data were compared using analysis of variance (ANOVA) followed by Newman-Keuls' test. For qPCR analyses, the comparison of grapevine watering conditions, sampling position and the effects of treatments, data were subjected statistical analyses using the non-parametric Kruskal-Wallis test. These analyses were carried out with the software packages StatBox (Version 6.6, Grimmer© Logiciels, Paris) and the Rcmdr package of the R software (64 3.0.1).

RESULTS

Effects of 46 different bacterium strains on Neofusicoccum parvum*-induced cankers*

In the control cuttings inoculated only with *N. parvum* (controls uninfected with bacteria: UC), the mean lengths of the canker lesions were 9.8 mm (Figure 1). No cankers were observed in the uninoculated uninfected controls (UUC) in which the samples were not inoculated with the fungus, nor treated with bacteria. Except for three bacterium strains, *P. agglomerans* (S1 and S3) and *B. reuszeri* (S27), all the stem cuttings co-inoculated with one tested bacterium strain and *N. parvum* exhibited canker lesions. On the other hand, co-inoculation of the fungus with five bacterium strains including *B. licheniformis* (S33 and S44), *Xanthomonas* sp. (S45), *B. pumilus* (S35) and *Bacillus* sp. (S43) increased ($P \leq 0.05$) canker size compared to the control plants inoculated with *N. parvum* alone (Figure 1).

Assessments of grapevine growth under two water regimes (second bioassay)

Plant growth was assessed visually between June and September 2017. The development of plants subjected to the water deficit regime was reduced compared to that of the normally irrigated plants, and was associated with external symptoms, including wilting, foliar chlorosis and dead leaves. A total of 52 plants had died by the end of the bioassay (Table S1), 40 of which were under the water deficit condition, and all but two (including one under the normal water condition) had been artificially infected. Of the 39 inoculated plants that died under the reduced-water regime, 12 had been inoculated with *B. pumilus* alone. The remaining 27 dead plants were distributed as follows: seven Bacil/Xanth, five Xanth, four Mock control, four Bacil/Xanth/Np, three Xanth/Np, two Np and two Bacil/Np.

Measurements of root mass (second bioassay)

As shown in Figure 2, the watering conditions affected root mass. The water deficient plants showed reduced root mass, which was reduced by half compared to

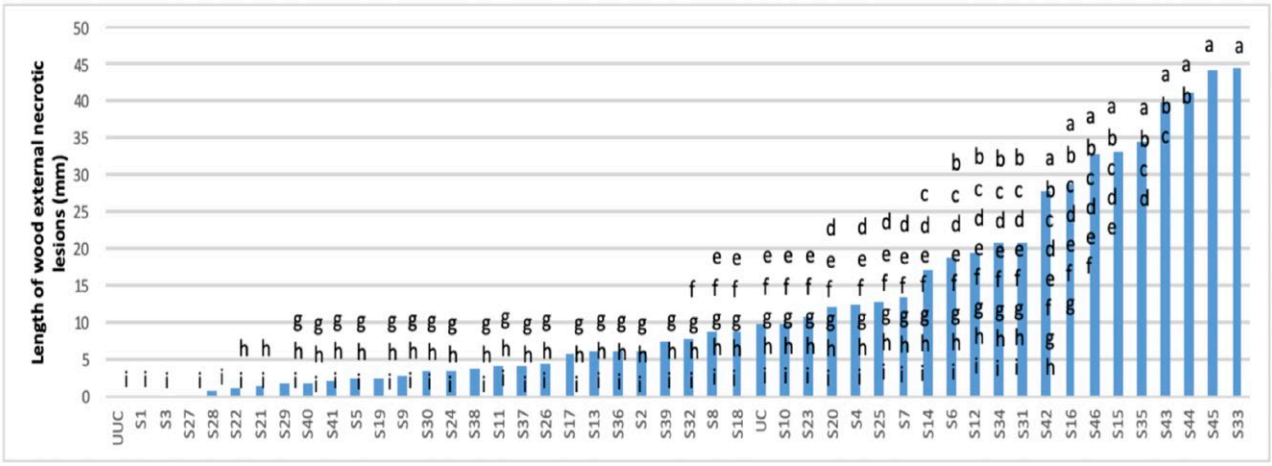

Figure 1. Mean wood canker lesion lengths (mm) in grapevine cuttings following inoculation with 46 different bacterium strains and co-inoculation with *Neofusicoccum parvum* in the first bioassay. Grapevine cutting stems (cv. Cabernet Sauvignon) were co-inoculated with bacteria and *N. parvum* before incubation in an open greenhouse for 4 months. The uninfected controls (UC) were inoculated only with *N. parvum*. The uninoculated, uninfected control (UUC) was not inoculated with the fungus and not treated with bacteria. Each value represents the mean of 16 or 11 cuttings, depending on the bioassay (Haidar *et al.,* 2016a). Means accompanied by the same letter are not significantly different ($P = 0.05$; Newman and Keuls' test after ANOVA).

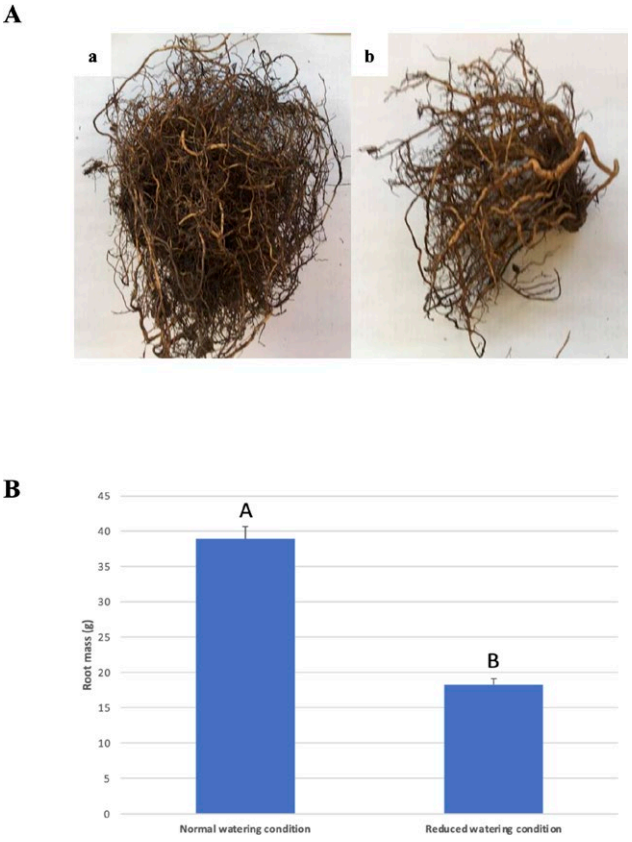

Figure 2. A. Mean (± SE; of 54 plants) root weights for potted grapevines in an open greenhouse. a) control plant roots. b) plant roots under water deficit. B. Mean root weights for potted grapevines in an open greenhouse. Different letters indicate differences ($P = 0.05$; Newman and Keuls' test after ANOVA)

that of normally irrigated plants. Under normal watering conditions, root mass reached a maximum mean of 46.1 g per plant, observed for the control plants, and a minimum of 31.9 g, observed in the plants co-inoculated with the three microorganisms.

Stem canker lesions in second bioassay

The results are presented in Figure 3. No cankers were observed when the fungus pathogen was not present (except for *B. pumilus* inoculation under the reduced water condition). All plants inoculated with *N. parvum* developed canker lesions regardless of the water regime applied. Furthermore, when the pathogen was inoculated alone or with one bacterium strain (*Xanthomonas* sp. (S45) or *B. pumilus* (S35)), there was no effect of water treatment on canker development. The data clearly showed that canker lesion length was affected by water regime in the plants co-inoculated with the three microorganisms. Under this multibiotic stress, mean lesion length was greater by a factor of two from the decreased irrigation treatment. On average, among the plants subjected to the three biotic stresses, mean lesion length was 12.3 mm from normal watering conditions and 26.2 mm from reduced watering. The enhancement of plant susceptibility to the pathogen due to these multiple stresses (especially reduced water supply) was probably responsible for the increased lesion lengths. Furthermore, within each watering condition (normal

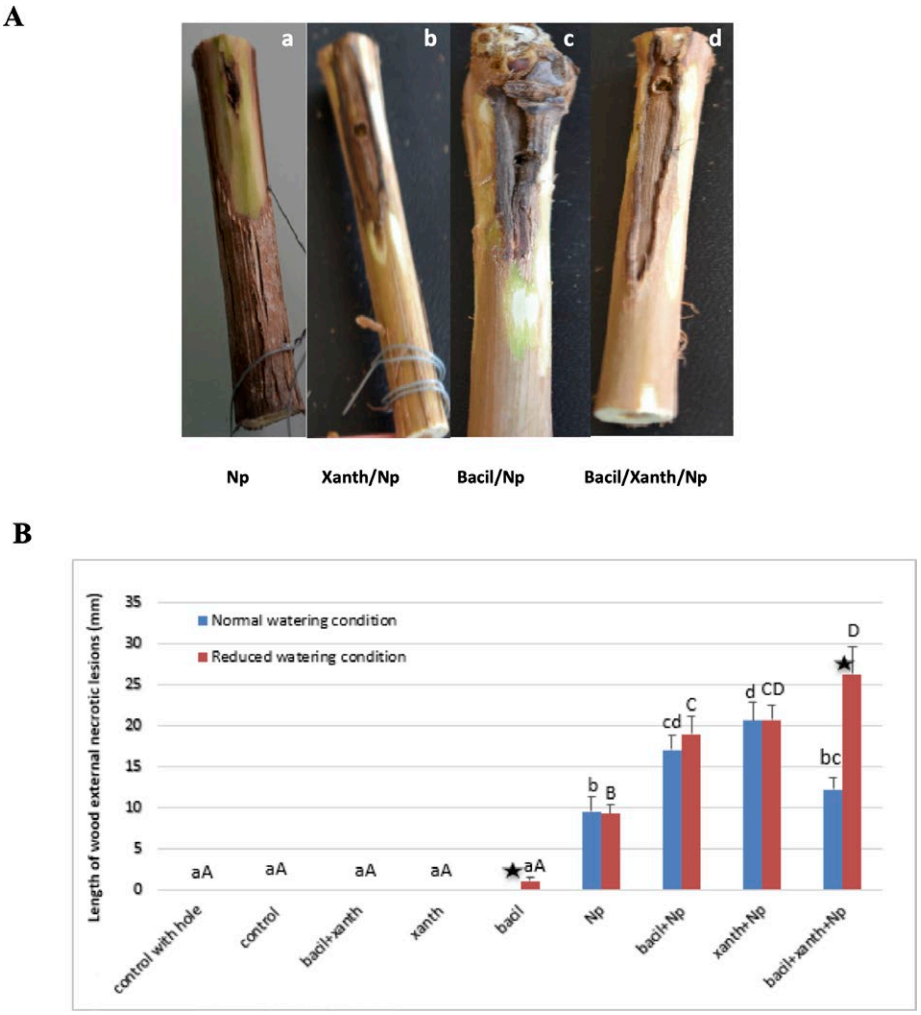

Figure 3. A. Mean wood canker lesion lengths on grapevine cuttings. (a) cuttings inoculated with *Neofusicoccum parvum* only; (b) cuttings co-inoculated with *N. parvum* and *Xanthomonas* sp. (S45); (c) cuttings co-inoculated with *N. parvum* and *B. pumilus* (S35); (d) cuttings co-inoculated with *N. parvum* and *Xanthomonas* sp. (S45) and *B. pumilus* (S35). B. Mean (±SE) wood canker lesion lengths from the different treatments in the second bioassay, under two water conditions, 9 months post-inoculation. Each value is the mean of 30 cuttings. Means accompanied by the same letter are not different ($P < 0.05$; Kruskal-Wallis test. indicates statistically significant interactions for the same modality under the two water conditions.

or reduced), a difference in canker length was detected between plants subjected to two or three stresses and those subjected to the stress caused by *N. parvum*. This difference was statistically significant in the reduced watering condition, with the plants co-inoculated with *N. parvum* and one or two bacterium strains exhibiting larger canker lesions than plants inoculated with *N. parvum* alone. This result clearly indicated potential synergetic interactions among the different microorganisms. Similarly, in the well-watered plants, the results also indicated a synergistic interaction, with plants subjected to two biotic stresses, i.e., *N. parvum* and either *B. pumilis* or *Xanthomonas* sp., developing larger lesions than those inoculated with *N. parvum* alone. However, the canker lesions were not significantly larger in the plants co-inoculated with the three microorganisms than in the control plants inoculated with *N. parvum* alone.

Quantification of Neofusicoccum parvum *DNA in the wood of grapevine cuttings*

The results of the quantification of *N. parvum* DNA are shown in Table 1. No amplification was detected in the treatments without *N. parvum* (data not shown). This confirmed that the primers used were specific to this pathogen and did not amplify nontarget DNA. In the

Table 1. Mean amounts (ng μL^{-1}; ±SE for five repetitions) of *Neofusicoccum parvum* DNA, measured in grapevine cuttings 9 months after the inoculation with different microorganisms, under two watering conditions. Means accompanied by different letters indicate that differences ($P < 0.05$) between modalities as determined by multiple comparison analyses (Kruskal-Wallis). Np: *Neofusicoccum parvum,* Bacill: *B. pumilus* (S35), Xanth: *Xanthomonas* sp. (S45).

Sampling position	Treatment	Normal watering condition	Reduced watering condition
Zone A (around the site of inoculation)	Np	$7.03 \times 10^{-3} \pm 2.03 \times 10^{-3}$ **b**	$1.43 \times 10^{-2} \pm 2.42 \times 10^{-3}$ **c**
	Bacil/Np	$8.54 \times 10^{-1} \pm 6.02 \times 10^{-1}$ **c**	$1.12 \pm 5.26 \times 10^{-1}$ **c**
	Xanth/Np	$3.48 \times 10^{-3} \pm 7.28 \times 10^{-4}$ **b**	$9.37 \times 10^{-3} \pm 3.98 \times 10^{-3}$ **b**
	Bacil/Xanth/Np	$4.53 \times 10^{-3} \pm 1.34 \times 10^{-3}$ **b**	$4.91 \times 10^{-3} \pm 1.33 \times 10^{-3}$ **b**
Zone B (above the site of inoculation)	Np	0 **a**	$4.91 \times 10^{-3} \pm 4.14 \times 10^{-4}$ **b**
	Bacil/Np	$9.93 \times 10^{-2} \pm 6.41 \times 10^{-2}$ **c**	$9.19 \times 10^{-2} \pm 3.99 \times 10^{-2}$ **c**
	Xanth/Np	$2.79 \times 10^{-3} \pm 1.58 \times 10^{-4}$ **a**	$1.11 \times 10^{-2} \pm 2.04 \times 10^{-3}$ **c**
	Bacil/Xanth/Np	0 **a**	$3.44 \times 10^{-3} \pm 7.18 \times 10^{-5}$ **b**

different treatments, depending on the sampling position and water supply condition, the amounts of fungus DNA were different within the same treatment. While *N. parvum* DNA was detected in all treatments with pathogen inoculation under the water deficit condition, no amplification of pathogen DNA occurred in samples from the zone above the site of inoculation in the plants inoculated with *N. parvum* alone or with the three microorganisms under the normal water condition. An increase in *N. parvum* DNA quantity was observed in the presence of *B. pumilus* (S35). However, plants co-inoculated with *N. parvum* and *B. pumilus* differed in quantity of *N. parvum* DNA from the control plants inoculated with *N. parvum* alone at both sampling zones under the normal watering condition. In contrast, under the reduced water condition, this difference was recorded only in the zone above the site of inoculation. This result indicates a favourable impact of reduced watering on *N. parvum* development.

In vitro *microbial interactions*

The susceptibility of each of the two selected bacterium strains to the antimicrobial substances produced by the other strain was tested using two *in vitro* methods (involving diffusible or volatile metabolites). These tests demonstrated that the two selected bacterium strains did not inhibit each other's growth. For the bacterium–*N. parvum* interactions, the results from the two *in vitro* experiments demonstrated that the two tested bacterium strains, S45 (*Xanthomonas* sp.) and S35 (*B. pumilus*), did not suppress growth of *N. parvum* (Figure S2).

Wood component decomposition ability

For ligninolytic activity, neither *N. parvum* nor the bacteria were able to degrade the Remazol Brilliant Blue R (RBBR) (data not shown). In contrast, all the tested microorganisms showed cellulase and xylanase activity halos, suggesting their potential capacities for grapevine wood degradation (Figure S3).

DISCUSSION

In vineyards, grapevines are exposed to multiple abiotic and biotic stresses. The impacts of combined stresses on plants may differ depending on the individual stresses involved. As combined stresses elicit complex plant responses, previous research on GTDs has mainly concentrated on grapevine responses to only individual biotic stresses, mostly those from fungus infections (Laveau *et al*., 2009; Spagnolo *et al*., 2017; Rezgui *et al*., 2018; Brown *et al*., 2019). In the present study, the aims were first assess grapevine responses to two combined biotic stresses (bacterium and fungus inoculation) using grapevine cuttings under open greenhouse conditions.

In a previous study, Haidar *et al*. (2016a) observed that eight bacterium strains, i.e., *B. pumilus* (S35), *Xanthomonas* sp. (S45), *B. licheniformis* (S33), *Paenibacillus polymyxa* (S15), *Curtobacterium* sp. (S42), *Bacillus* sp. (S43), *Bacillus* sp. (S46) and *Paenibacillus* sp. (S16), increased inner necrotic lesion lengths compared to that in control plants inoculated only with *N. parvum*. In the present study, four of these eight strains (*B. licheniformis* (S33), *Xanthomonas* sp. (S45), *Bacillus* sp. (S43) and *B. pumilus* (S35)) increased *N. parvum* canker lesion lengths (Figure 1). This result contributes to the growing

evidence that bacteria play roles in host wood decomposition and that the bacteria inhabiting wood could promote the ability of fungi to colonize and decompose wood structures (Hervé *et al*., 2014, 2016; Johnston *et al*., 2016, 2018; Válková *et al*., 2017; Probst *et al*., 2018). Detailed studies of bacterium/fungus interactions can increase understanding of the complex etiology of GTDs. Accordingly, in the present study, *B. pumilus* (S35) and *Xanthomonas* sp. (S45) were selected to test the effects of multiple stresses on grapevine plants. These bacteria were selected because *B. pumilus* and strains of *Xanthomonas* have been reported to be plant pathogenic bacteria (Galal *et al*., 2006; Bathily *et al*., 2010; Yan and Wang, 2011).

Combined stresses, including abiotic stresses, trigger responses in plants at physiological, biochemical, and molecular levels (Suzuki *et al*., 2014; Pandey *et al*., 2017). For *Botryosphaeriaceae* species, studies have shown that water deprivation increases the susceptibility of woody plants to these fungi (van Niekerk *et al*., 2011; Sherwood *et al*., 2015; Qiu *et al*., 2016). The present study is the first on effects of water deficit on the interactions between two xylanolytic and cellulolytic bacterium strains and *N. parvum*. Although multistress bioassays may include environmental conditions that do not perfectly simulate real vineyard conditions, in the present study the grapevines growing under normal water conditions exhibited developed greater root masses than vines growing under the reduced irrigation regime. These results are consistent with previous results from other plant hosts, showing that water-deficient plants were smaller to well-watered plants (Souza and Cardoso, 2003; Granda *et al*., 2011; Correia *et al*., 2014; McKiernan *et al*., 2014). In the present study, the large decrease in root mass (by a factor of two) under the reduced irrigation regime confirmed that this watering condition was stressful for the plant cuttings.

Under both watering conditions, the lengths of the canker lesions were greater in the plants subjected to two biotic stresses in combination, compared to plants subjected to *N. parvum* infection alone. Indirect effects of microbial community interactions, such as interactions of mutualistic or competing species with fungus pathogens, could be one of the reasons for this phenomenon (Wargo, 1996; Desprez-Loustau *et al*., 2006; Frey-Klett *et al*., 2011). A statistically significant effect of abiotic stress on the canker lesions was evident only when the plants were affected by three biotic stresses simultaneously (i.e., inoculated with three microorganisms). Our current hypothesis is that this phenomenon may have been caused by strong competition among the pathogenic microorganisms for water in the plants. Stated otherwise, this observation could depend on the amount of water available in the plants. However, watering limitation and water availability in the soil have quantitative effects on water available within plants. Further studies are needed to test this hypothesis. Furthermore, pathogens can produce primary and secondary compounds that affect plant growth and development (Berger *et al*., 2007). Therefore, the application of multiple types of stresses, as in the present study, as well as watering limitation, could increase the susceptibility of plants to pathogens. In addition, the present study showed the cellulolytic and xylanolytic activities of the different microorganisms tested. These activities could explain why more lesions were observed when the bacterium strains were present (co-inoculated) along with *N. parvum*.

In recent years, GTD fungus inoculum has been successfully quantified using quantitative real-time PCR (qPCR) for some major grapevine trunk pathogens, including *P. chlamydospora, P. aleophilum, D. seriata, E. lata* and several species of *Botryosphaeriaceae* (Pierron *et al*., 2016; Pouzoulet *et al*., 2017; Billones-Baaijens and Savocchia, 2018). This research was implemented with grapevine wood samples that had natural or inoculated infections from these pathogens. We detected *N. parvum* in all plants inoculated with this pathogen under both water conditions. In the plants inoculated only with the pathogen, in each watering condition, greater amounts of *N. parvum* were detected when sampling was conducted around the inoculation sites than from the zone just above the inoculation sites. This indicates a clear, effect of sampling location on the amount of *N. parvum* DNA measured. In the plants inoculated only with *N. parvum*, a significant effect of water condition was also observed, with *N. parvum* DNA detected more frequently under the reduced water condition than under the normal water condition. Similarly, *B. dothidea* (*Botryosphaeriaceae* family) was reported to preferentially colonize wounded and stressed tissues in *Eucalyptus* (Smith *et al*., 1996). McPartland and Schoeneweiss (1984) also found that *B. dothidea* hyphae in vessels of birch (*Betula alba*) stems were large and rectilinear in drought-stressed stems but were thin and contorted in unstressed stems. They also reported greater numbers of swollen (*vs.* intact) hyphal tips in unstressed stems than in stressed stems. The quantity of *N. parvum* DNA increased in the presence of the bacterium strains for plants tested under the two watering conditions.

In plants co-inoculated with *B. pumilus*, *N. parvum* DNA was detected in large amounts. Bacteria and fungi have been reported to influence the survival and colonization of their interacting partners (Frey-Klett *et al.,*

2011). Indirect effects of the modification of wood environments on bacterium-fungus interactions could also explain this result. For example, changes in pH were reported to influence the structure of microbial communities by either promoting or inhibiting the growth of particular organisms in different environments (Frey-Klett *et al*., 2011).

In summary, the present study, where up to four abiotic or biotic stresses were applied in combinations to grapevines, showed that strong synergistic effects occur between bacteria and fungus, and that positive interactions favour the *N. parvum* pathogenic process. Bacterium infection positively influenced the grapevine–*N. parvum* interaction as indicated by increased canker lengths. Further investigations are required to better understand bacterium–fungus interactions in GTDs, and the mechanisms associated with abiotic stress that may affect grapevine susceptibility to biotic infections. In addition, the present study has provided an example of the implementation of an experimental multifactorial model for study of combined stresses and their interactions in a grapevine pathosystem.

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Research Papers

Spore dispersal of *Eutypella* species under desert grape-growing conditions of southern California

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Summary. The seasonal abundance of *Diatrypaceae* spores was studied in southern California's desert table grape-growing region of Coachella Valley. Glass microscope slides covered with petroleum jelly were placed in a mature cv. Mid-Night Beauty® vineyard, and collected weekly from September 2006 to May 2009 (1400 samples for 140 consecutive weeks). Overall, *Diatrypaceae*-like colonies were recorded from Petri plates after processing spore traps in 93 (66%) of 140 weeks. Phylogenetic analyses showed *Eutypella citricola* and *Eutypella microtheca* to be the *Diatrypaceae* spp. captured from the spore traps. Though spores were captured throughout each year, their incidence varied among the different seasons. The greatest number of *Eutypella* spores were captured in autumn (38.7% of the total) followed by winter (30.6%), summer (19.7%), and spring (11%). The greatest numbers of spores were captured in October each year (15.7%) and least in June (1%). *Eutypella* spore release was correlated with rainfall only in 26 (28%) of the 93 weeks that spores were captured during the study. Analysis of diseased samples collected from the cv. Mid-Night Beauty® vineyard showed that *E. citricola*, *E. microtheca* and *E. scoparia* were the most prevalent fungi isolated from cankers. A pathogenicity study showed that *E. citricola* and *E. microtheca* isolates collected from spore traps caused larger vascular necrosis than the non-inoculated controls, indicating their role as pathogens on grapevines. This study has demonstrated *Eutypella* spp. to play an important role in grapevine health under desert growing conditions of southern California. In addition, and contrary to what it is largely accepted, results from this research suggest that *Diatrypaceae* spore release can occur in the absence of precipitation. This study expands current knowledge on epidemiology of *Diatrypaceae* spp. other than *Eutypa lata*, and provides important information for enhancing control strategies against grapevine trunk diseases under desert growing conditions.

Keywords. Canker, *Diatrypaceae*, epidemiology, *Eutypa*, *Eutypella*, grapevine trunk diseases, spore trap, *Vitis vinifera*.

INTRODUCTION

Eutypa dieback (ED) is an economically important grapevine trunk disease (GTD) with a cosmopolitan distribution, and is responsible for important yield losses and the shortening of vineyard lifespans (Kaplan *et al*., 2016; Munkvold *et al*., 1994; Siebert, 2001; Wicks and Davies, 1999). Grapevine symptoms associated with ED are characterized by stunted shoots, which often present chlorotic cupped leaves with necrotic margins. Symptomatic shoots may present clusters with poor fruit set early in the growing season, which develop into small bunches that ripen unevenly leading to berry shrivel before harvest. The main vascular symptoms associated with ED are cankers, usually in the form of a wedge-shape (Rolshausen *et al*., 2015). Infections start at pruning wounds and cankers develop through spurs, cordons and trunks. Eutypa dieback affects mature vineyards and symptoms of the disease can take up to 3 years to appear after infection (Gramaje *et al*., 2018). Once symptoms appear, infected vines start declining, and progressive death of spurs and cordons occurs in following years with eventual death of affected vines.

More than 20 species of *Diatrypaceae* have been associated with grapevine cankers and consequent dieback (Luque *et al*., 2006; 2012; Moyo *et al.* 2018a; Trouillas *et al*., 2010; 2011). However, *Eutypa lata* is considered the most virulent, and the only species proven to cause ED foliar symptoms (Pitt *et al*., 2013; Sosnowski *et al*., 2007; Trouillas and Gubler, 2010a). These symptoms probably result from secondary metabolites produced by the fungus and translocated to aerial parts of infected plants (Andolfi *et al*., 2011; Rolshausen *et al*., 2008; Rolshausen *et al*., 2015). *Diatrypaceae* species associated with grapevine dieback have also been reported to cause cankers and decline in a wide range of other woody perennial crops, and in native and/or introduced forest tree species (Carter 1991; Trouillas *et al*., 2011; Úrbez-Torres *et al*., 2013; Trouillas and Gubler, 2016; Moyo *et al*., 2018b).

Eutypa dieback has long been known to occur in grapevines in California (Moller and Kasimatis, 1978). This disease can be found in most grape-growing regions throughout California, with greatest incidence in vineyards in the Northern San Joaquin Valley, the Sacramento Valley and the North Coast (Úrbez-Torres *et al*., 2006, Trouillas and Gubler, 2010b). For many years, *E. lata* was thought to be the sole diatrypaceous fungus associated with cankers and dieback in California; however, studies conducted since mid-2000 have isolated and identified up to 11 *Diatrypaceae* species from grapevine cankers, including *Cryptosphaeria pullmanensis*, *Cryptovalsa ampelina*, *Diatrype oregonensis*, *D. stigma*, *D. whitmanensis*, *Diatrypella verrucaeformis*, *Eutypa leptoplaca*, *Eutypella citricola*, *E. scoparia*, *E. leprosa*, and *E. microtheca* (Trouillas and Gubler, 2004; Rolshausen *et al*., 2006; Trouillas *et al*., 2010; Trouillas *et al*., 2011). Pathogenicity studies showed some species, including *C. ampelina*, *D. stigma*, and *E. leptoplaca* capable of colonizing dormant canes and causing vascular necroses similar to those caused by *E. lata*. However, other species such as *D. oregonensis* and *D. verrucaeformis* did not produce significant lesions in shoots and canes, so they were suggested to be saprophytic rather than pathogenic to grapevine (Trouillas and Gubler, 2010a).

The epidemiology of *E. lata* in grapevines has been well-studied and there is good understanding of the pathogen life cycle (Rolshausen *et al*., 2015). Ascospores of *E. lata* are released from perithecia formed in old infected parts of vines such as spurs, cordons and/or trunks (Trouillas and Gubler, 2010b). Under favorable environmental conditions, ascospores are discharged and spread by rain droplets and/or wind to short or long distances. Ascospores land on susceptible exposed xylem tissues (pruning wounds and cuts) of vines, where they germinate and start new infections (Rolshausen *et al*., 2015; Gramaje *et al*., 2018). The presence of *E. lata* is restricted to geographical locations with at least 350 mm of annual rainfall, and release of ascospores occurs when temperatures are above freezing and with as little as 0.2 mm of rainfall (Trouillas and Gubler, 2010b; Rolshausen *et al*., 2015; Billones-Baaijens *et al*., 2017). Accordingly, high risk infection periods in vineyards may vary throughout the year and geographical regions, but primarily coincide with the dormant pruning season (Gramaje *et al*., 2018).

Eutypa dieback spore trapping studies have primarily been conducted in grape-growing regions with temperate climates, where most viticulture takes place in the Northern and Southern Hemispheres (Gramaje *et al*., 2018). Discharge of *E. lata* ascospores from perithecia is primarily correlated with rainfall events. In regions with moderate winters, such as Australia, California and South Africa, ascospores are primarily released from late autumn to late winter (Ramos *et al*., 1975; Petzoldt *et al*., 1983a; 1983b; Trouillas *et al*., 2009; van Niekerk *et al*., 2010; Billones-Baaijens *et al*., 2017). In regions where temperatures below 0°C are common throughout the winter, as in New York and Michigan in the United States of America or British Columbia in Canada, ascospores discharge primarily occurs from late winter to late spring when temperatures above freezing are reached (Pearson, 1980; Trese *et al*., 1980; Úrbez-Torres *et al*., 2017). However, grapevine production, primarily table grapes, also occurs under tropical, sub-tropical

and desert climates (Winkler *et al*., 1974). In California, the third largest table grape production area is located in the Coachella Valley (Riverside County) in southern California. This region has a desert climate, classified as BWh under the Köppen climate classification and characterized by warm winters (14.7°C average temperature), hot summers (31.5°C), and annual precipitation of only 2.7 mm. Though this area is a desert, abundant underground water and aqueducts built from the Colorado River make production possible of a broad range of economically important agriculture commodities, including table grapes (USDA-NASS, 2018).

Field surveys conducted in California between 2004 and 2007 to determine the main fungal pathogens associated with grapevine cankers and dieback throughout California did not find *E. lata*, but other diatrypaceous taxa were associated with grapevine dieback in southern California, particularly in table grapes grown in the Coachella Valley (Trouillas *et al*., 2010; Úrbez-Torres *et al*., 2006). In addition, spore trapping studies conducted in different grape growing regions throughout California to determine the epidemiology of *Botryosphaeriaceae* spp. also yielded *Diatrypaceae* colonies from spore traps from different regions, including the Coachella Valley (Úrbez-Torres *et al*., 2010). Some of these colonies were later identified as *E. citricola* and *E. microtheca*, highlighting the important role that diatrypaceous fungi other than *E. lata* may play in grapevine dieback in this desert region (Trouillas *et al*., 2010; 2011).

The main objectives of the present study were to; (i) further investigate the epidemiology of *Diatrypaceae* spp. under desert climate conditions using spore trapping, (ii) identify the main *Diatrypaceae* spp. present in spore traps by morphological and molecular assessments, and (iii) complete pathogenicity assays to determine the roles that these diatrypaceous fungi play in grapevine health.

MATERIAL AND METHODS

Study site

The study was conducted in a *Vitis vinifera* cv. Mid-Night Beauty® commercial block located near Indio, Coachella Valley, Riverside County, in California. At the start of the study, the vineyard was 16 years old, with vines spaced 1.5 m apart and rows oriented north-south. Vines were trained on an open gable trellis system with up to four spur-pruned cordons in each vine (Figure 1). Vines were drip irrigated and fertilized as required, based on standard practices for the cultivar and the region. To increase the chilling hours, overhead sprinkler irrigation was applied for hydro-cooling the vineyard for 12 h each day from mid October to early December. The vineyard was selected based on previous studies indicating high incidence of vines showing dieback symptoms (Úrbez-Torres *et al*., 2006).

Glass microscope slide spore trapping

Glass microscope slides were used as spore traps to capture *Diatrypaceae* spores in the selected vineyard, as previously described (Eskalen and Gubler, 2001; Úrbez-Torres *et al*., 2006). Glass microscope slides (25 × 76 mm) each coated on both sides with a thin layer of white petroleum jelly were placed on grapevine cordons at approx. 80 cm above the soil surface (Figure 1). One microscope glass slide was placed on each of the cordons of ten different vines. Vines containing a spore trap were separated from each other by four vines, and all vines were selected in the centre of the block. Spore traps were replaced each week and individually collected into sterile 50 mL capacity screw-cap tubes (Sarstedt, Inc.) from 6 September, 2006 to 27 May, 2009. The tubes containing microscope slides were sent each week to the Plant Pathology laboratory at the University of California, Davis for processing. Screw-caps of the tubes were open inside a laminar flow cabinet, and 10 mL of autoclaved distilled water previously warmed to 20°C were added into each tube containing a microscope slide. The tubes were closed with the screw-caps and shaken by hand for approx. 60 sec. The microscope slides were then removed and two aliquots of 200 µL per tube were collected and plated onto two different 85 mm diam. Petri dishes (20 Petri dishes per week) containing potato dextrose agar (PDA: Difco Laboratories) amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich) (PDA-tet). The 200 uL aliquot was spread over the agar medium in each Petri plate using a curved glass rod, which was sterilized between plates. The inoculated Petri plates were air-dried for 10 min. inside the laminar flow hood, and were then all placed on a laboratory bench and incubated in room light and temperature (25°C ± 1°C) conditions. *Diatrypaceae*-like fungal colonies were recorded after 7 d (Figure 1) based on described taxonomic characters (Trouillas and Gubler, 2004), and were subcultured onto fresh PDA for morphological and molecular identifications.

Individual *Diatrypaceae* colonies growing from the isolation plates were counted as single spores captured from the spore trap, and were recorded each week during the study period. Total numbers of spores per week were calculated as the sum of all colonies observed from the 20 isolation plates, and multiplying the total spore

Figure 1. Spore trap study. **A** and **B**. Cv. Mid-Night Beauty® vineyard where the study was conducted in the Coachella Valley in southern California. **C.** Microscope glass spore trap coated with petroleum jelly and placed on the cordon of a grapevine. **D.** Petri dishes showing fungal colonies obtained from spore traps. Black arrows indicate characteristic *Diatrypella*-like colonies. **E** and **F.** Wedge-shape cankers observed and collected from symptomatic grapevines from the studied vineyard.

count by 25 to account for the subsampling factor. Total number of spores per week were superimposed with weekly environmental data, including average temperature and total precipitation accumulated per week. Meteorological data were collected from a California Irrigation Management Information System (CIMIS, Oasis, Imperial, Station 136) weather station located in the proximity of the vineyard.

Diatrypaceae *species identification*

Diatrypaceae isolates collected from spore traps in different weeks during the study were selected for molecular identification (Table 1). In addition, seven *Diatrypaceae* isolates identified in a previous spore trapping study conducted in California by Úrbez-Torres *et al.* (2010) were included in phylogenetic analyses for comparison and identification purposes (Table 1). A field survey was also conducted at the vineyard site on 15 July 2007 to determine *Diatrypaceae* spp. associated with wood cankers. In total, 20 symptomatic samples from 20 different vines in the vineyard block were collected. Cordons showing dieback symptoms, including dead spurs and wedge-shaped cankers in cordon cross sections were collected (Figure 1). Fungal pathogen isolation from cankers was conducted as described by Úrbez-Torres *et al.*

Table 1. *Diatrypaceae* isolates from spore traps and cankers identified in this study, and isolates retrieved from GenBank included in phylogenetic analyses.

Species	Isolate	Source - Week Collected	ITS[b]	*TUB*[c]
Cryptovalsa ampelina	A001	*Vitis vinifera*	GQ293901	GQ293972
C. ampelina	UCD2Mo	Spore trap	**MT845608**	**MT857226**
C. ampelina	UCD1Na	Spore trap	**MT845609**	**MT857227**
C. ampelina	UCD2Na	Spore trap	**MT845610**	**MT857228**
C. ampelina	UCD3Na	Spore trap	**MT845611**	**MT857229**
C. ampelina	UCD1SLO	Spore trap	**MT845612**	**MT857230**
Cryptovalsa rabenhorstii	WA08CB	*V. vinifera*	HQ692619	HQ692523
Eutypa lata	DCA900	*V. vinifera*	GQ293948	GQ294007
Eutypella australiensis	CNP03	*Acacia longifolia*	HM581945	HQ692479
Eutypella citricola	T3R2S2	*V. vinifera*	HQ692576	HQ692519
E. citricola	HVIT08	*V. vinifera*	HQ692583	HQ692513
E. citricola	UCRDC83	*Citrus limon*	KF620372	KF620408
E. citricola	STEU 8103	*V. vinifera*	KY111639	KY111592
E. citricola	UCD5Co [a]	Spore trap - Jan/30/07	**MT845613**	**MT857231**
E. citricola	UCD6Co [a]	Spore trap - Jan/30/07	*GQ293959*	*GQ294023*
E. citricola	UCD7Co [a]	Spore trap - Jan/30/07	*GQ293961*	*GQ294024*
E. citricola	UCD8Co [a]	Spore trap - May/15/07	*GQ293960*	*GQ294025*
E. citricola	UCD9Co	Spore trap - May/15/07	**MT845614**	**MT857232**
E. citricola	UCD11Co	Spore trap - Jul/24/07	**MT845615**	**MT857233**
E. citricola	UCD12Co	Spore trap - Sep/18/07	**MT845616**	**MT857234**
E. citricola	UCD18Co	Spore trap - Jul/14/08	**MT845617**	**MT857235**
E. citricola	UCD19Co	Spore trap - Dec/22/08	**MT845618**	**MT857236**
E. citricola	UCD20Co	Spore trap - Dec/22/08	**MT845619**	**MT857237**
E. citricola	UCD21Co	Spore trap - Dec/22/08	**MT845620**	**MT857238**
E. citricola	UCD22Co	Spore trap - Jan/13/09	**MT845621**	**MT857239**
E. citricola	UCD25Co	Spore trap - Apr/15/09	**MT845622**	**MT857240**
E. citricola	UCD1SJ	Spore trap	**MT845623**	**MT857241**
E. citricola	UCD2339Co	Canker in cordon - Jul/15/07	**MT845624**	**MT857242**
E. citricola	UCD2342Co	Canker in cordon - Jul/15/07	*GQ293968*	*GQ294022*
E. citricola	UCD2343Co	Canker in cordon - Jul/15/07	**MT845625**	**MT857243**
E. citricola	UCD2348Co	Canker in cordon - Jul/15/07	**MT845626**	**MT857244**
E. citricola	UCD2349Co	Canker in cordon - Jul/15/07	*GQ293969*	*GQ294021*
E. citricola	UCD2350Co	Canker in cordon - Jul/15/07	*GQ293970*	**MT857245**

(Continued)

Table 1. (Continued).

Species	Isolate	Source - Week Collected	ITS[b]	*TUB*[c]
E. citricola	UCD2353Co	Canker in cordon - Jul/15/07	*GQ293971*	*GQ294020*
Eutypella cryptovalsoidea	HVFIG05	*Ficus carica*	HQ692574	HQ692525
Eutypella leprosa	UCD713SJ	*V. vinifera*	GQ293955	GQ294016
Eutypella microtheca	UCRDC103	*Citrus paradisi*	KF620387	KF620423
E. microtheca	BCMX01	*V. vinifera*	KC405563	KC405560
E. microtheca	BCMX02	*V. vinifera*	KC405562	KC405561
E. microtheca	YC16	*V. vinifera*	HQ692561	HQ692529
E. microtheca	STEU-8107	*V. vinifera*	KY111629	KY111608
E. microtheca	UCD1Co [a]	Spore trap - Sep/20/06	**MT845627**	**MT857246**
E. microtheca	UCD2Co [a]	Spore trap - Sep/20/06	*GQ293958*	*GQ294018*
E. microtheca	UCD3Co [a]	Spore trap - Nov/18/06	*GQ293957*	*GQ294019*
E. microtheca	UCD4Co [a]	Spore trap - Jan/11/07	**MT845628**	**MT857247**
E. microtheca	UCD10Co	Spore trap - Jul/24/07	**MT845629**	**MT857248**
E. microtheca	UCD13Co	Spore trap - Jan/21/08	**MT845630**	**MT857249**
E. microtheca	UCD14Co	Spore trap - Feb/19/08	**MT845631**	**MT857250**
E. microtheca	UCD15Co	Spore trap - Feb/19/08	**MT845632**	**MT857251**
E. microtheca	UCD16Co	Spore trap - Feb/19/08	**MT845633**	**MT857252**
E. microtheca	UCD17Co	Spore trap - Jul/14/08	**MT845634**	**MT857253**
E. microtheca	UCD23Co	Spore trap - Jan/13/09	**MT845635**	**MT857254**
E. microtheca	UCD24Co	Spore trap - Apr/15/09	**MT845636**	**MT857255**
E. microtheca	UCD2345Co	Canker in cordon - Jul/15/07	**MT845637**	**MT862687**
E. microtheca	UCD2346Co	Canker in cordon - Jul/15/07	**MT845638**	**MT862688**
E. microtheca	UCD2352Co	Canker in cordon - Jul/15/07	**MT845639**	**MT857256**
E. microtheca	UCD2354Co	Canker in cordon - Jul/15/07	**MT845640**	**MT857257**
E. microtheca	UCD2355Co	Canker in cordon - Jul/15/07	**MT845641**	**MT857258**
E. microtheca	UCD2SJ	Spore trap	**MT845642**	**MT857259**
Eutypella scoparia	DFMAL100	*Robinia pseudoacacia*	GQ293962	GQ294029
E. scoparia	UCRDC142	*C. limon*	KF620391	KF620427
E. scoparia	UCRDC210	*C. paradisi*	KF620393	KF620429
E. scoparia	UCD2331Co	Canker in cordon - Jul/15/07	**MT845643**	**MT857260**
E. scoparia	UCD2332Co	Canker in cordon - Jul/15/07	**MT845644**	**MT857261**
E. scoparia	UCD2333Co	Canker in cordon - Jul/15/07	**MT845645**	**MT857262**
E. scoparia	UCD2334Co	Canker in cordon - Jul/15/07	*GQ293963*	*GQ294027*
E. scoparia	UCD2335Co	Canker in cordon - Jul/15/07	*GQ293964*	*GQ294028*
E. scoparia	UCD2336Co	Canker in cordon - Jul/15/07	*GQ293965*	*GQ294027*
E. scoparia	UCD2344Co	Canker in cordon - Jul/15/07	**MT845646**	**MT857263**
E. scoparia	UCD2347Co	Canker in cordon - Jul/15/07	**MT845647**	**MT857264**
Eutypella vitis	MSUELM13	*V. vinifera*	DQ006943	DQ006999
E. vitis	UCD2428TX	*V. vinifera*	FJ790851	GU294726
Lasiodiplodia theobromae	UCD2337aCo	Canker in cordon - Jul/15/07	**MT845648**	n/a
L. theobromae	UCD2337bCo	Canker in cordon - Jul/15/07	**MT845649**	n/a
L. theobromae	UCD2341aCo	Canker in cordon - Jul/15/07	**MT845650**	n/a
L. theobromae	UCD2341bCo	Canker in cordon - Jul/15/07	**MT845651**	n/a
Phaeoacremonium parasiticum	UCD2351Co	Canker in cordon - Jul/15/07	**MT845652**	n/a

[a] *Eutypella* isolates used in the pathogenicity study.
[b] ITS: Internal Transcribed Spacer GenBank Accession Numbers.
[c] *TUB:* beta-tubulin GenBank Accession Numbers.
GenBank Accession Numbers in bold were generated in this study.
GenBank Accession Numbers in italics were reported by Trouillas *et al.* (2010).
GenBank Accession Numbers in underlined italics were reported by Trouillas *et al.* (2011).
n/a: Not available.

(2006). Pure isolates of *Diatrypaceae*-like colonies selected from spore traps and from grapevine cordon cankers were obtained by hyphal tip isolations from PDA cultures, and were incubated in Parafilm-sealed Petri plates at room light and temperature conditions for 7 to 10 d. Total genomic DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN Inc). Oligonucleotide primers ITS1 and ITS4 (White *et al*., 1990) and T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) were used to amplify the nuclear rDNA ITS1-5.8S-ITS2 region (ITS) and part of the beta-tubulin (*TUB2*), in a thermal cycler (PTC-100TM, MJ Research), following the PCR temperature profiles described by Trouillas *et al*. (2010). Amplified products were purified using the QIAquick PCR purification Kit (QIAGEN Inc), and forward and reverse ITS and *TUB2* sequences were obtained using a ABI Prism 377 DNA Sequencer (Perkin-Elmer), at the Division of Biological Sciences sequencing facility at the University of California, Davis.

Sequences were edited and assembled using Sequencher™ version 4.1 (Gene Codes). Consensus sequences were aligned using the ClustalW multiple alignment program (Thompson *et al*., 1994) in BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999), and manually adjusted. *Diatrypaceae* spp. sequences from GenBank were selected based on their high similarity with the query sequences using MegaBLAST. Combined ITS and *TUB2* phylogenetic analyses were conducted first using the Neighbor-Joining (NJ) with the Maximum Composite Likelihood method and 1000 replicates to assess branch robustness and the Tamura-Nei model. The tree with the greatest log likelihood value was selected. In addition, the combined dataset was further analyzed using Maximum Parsimony (MP) with the bootstrap test (1000 random additional sequence replicates) and the Tree-Bisection-Regrafting (TBR) algorithm in MEGA-X (Kumar *et al*., 2018). *Diatrypaceae* sequences from this study were deposited into GenBank, and isolates were stored in the Plant Pathology Department fungal collection at the University of California, Davis.

Pathogenicity study

Four different isolates from the two species identified from the spore traps were used in the pathogenicity test (Table 1). The trial was conducted in a 7-year-old double cordon and spur pruned and trained cv. Red Globe vineyard at the University of California Field Station in Davis, California. Green shoots of the new vegetative growth were inoculated with mycelium plugs obtained from 7-d-old active growing colonies of fungus isolates, by wounding between the 4th and 5th internodes of each shoot, in June 2007 as described by Úrbez-Torres and Gubler (2009). One shoot per grapevine, on each of five grapevines per fungus treatment, was inoculated. The same number of negative controls were inoculated using non-colonized agar plugs. Shoots were collected 5 months after inoculation and brought to the laboratory for processing. The shoots were surface disinfected by submerging them on 4% sodium hypochlorite for 5 min. Samples were then air-dried and each sectioned in half longitudinally through the point of inoculation. Upward and downward vascular discolouration was measured from the point of inoculation, and results are presented as the mean of both measurements from each sample. In order to fulfill Koch's postulates, inoculated fungi were re-isolated as described by Úrbez-Torres and Gubler (2009). Data from the pathogenicity test were analyzed using SAS (Version 9.1.3; SAS Institute). Differences in length of vascular discolouration caused by each *Diatrypaceae* isolate were determined by one-way analyses of variance. Treatment means were compared using Fisher's least significant difference (LSD) test at the 5% significance level.

RESULTS

Spore trapping

A total of 1400 spore traps, corresponding to 140 consecutive weeks, were collected and processed from 6 September 2006 to 27 May, 2009 (Figure 2). *Diatrypaceae* fungi were recorded from 93 (66%) of the 140 weeks monitored. *Diatrypaceae* spores were captured throughout each year, but their incidence varied among the different seasons. The greatest number of *Diatrypaceae* spores were captured in autumn (38.7% of the total), followed by winter (30.6%), summer (19.7%), and spring (11%). The greatest numbers of *Diatrypaceae* spores were captured each year in October (15.7% of the total), followed by December (14.1%) and January (13.9%) (Figure 2). The month with fourth greatest numbers of spores captured was August (11.6%), which with July were the hottest and driest months of each year. The least numbers of spores were detected in June (1% of the total), followed by May (5.5%) and April and November (all 5.5%).

No rainfall was recorded from the start of the study on 6 September 2006 to 2 January 2008, and again from 2 February 2008 to 18 November 2008 (Figure 2). *Diatrypaceae* spore release was correlated with rainfall only in 26 (28%) of the 93 weeks when spores were captured (Figure 3). Although rain was not the main factor contributing to spore discharge, results showed that the

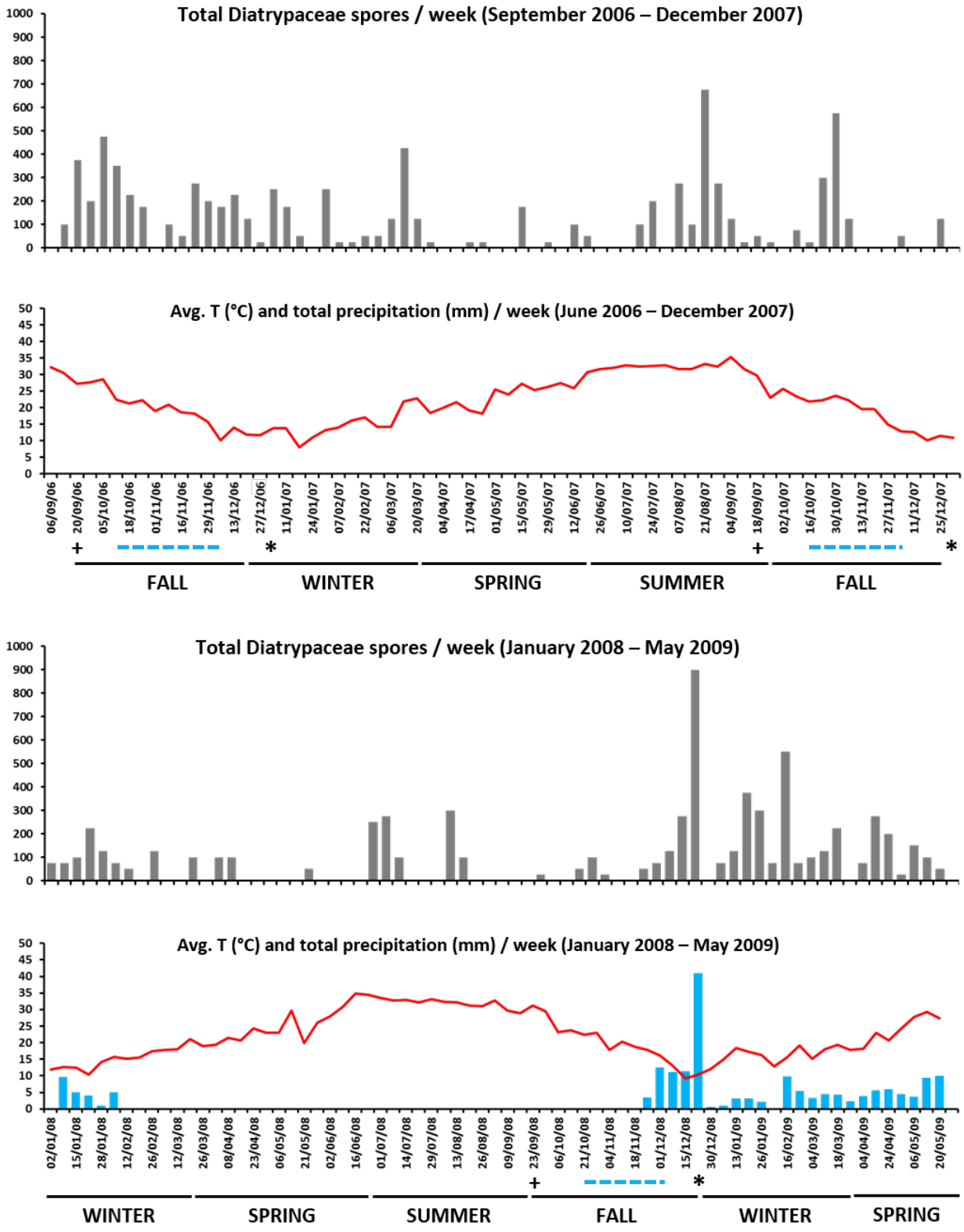

Figure 2. Total numbers of *Eutypella* spores trapped per week using glass microscope slides in the cv. Mid-Night Beauty® vineyard, average temperature and accumulated rainfall per week. Dashed blue line under the x axis shows overhead sprinkler irrigation period at the vineyard. + indicates week that overhead sprinklers were tested; * indicates pruning dates.

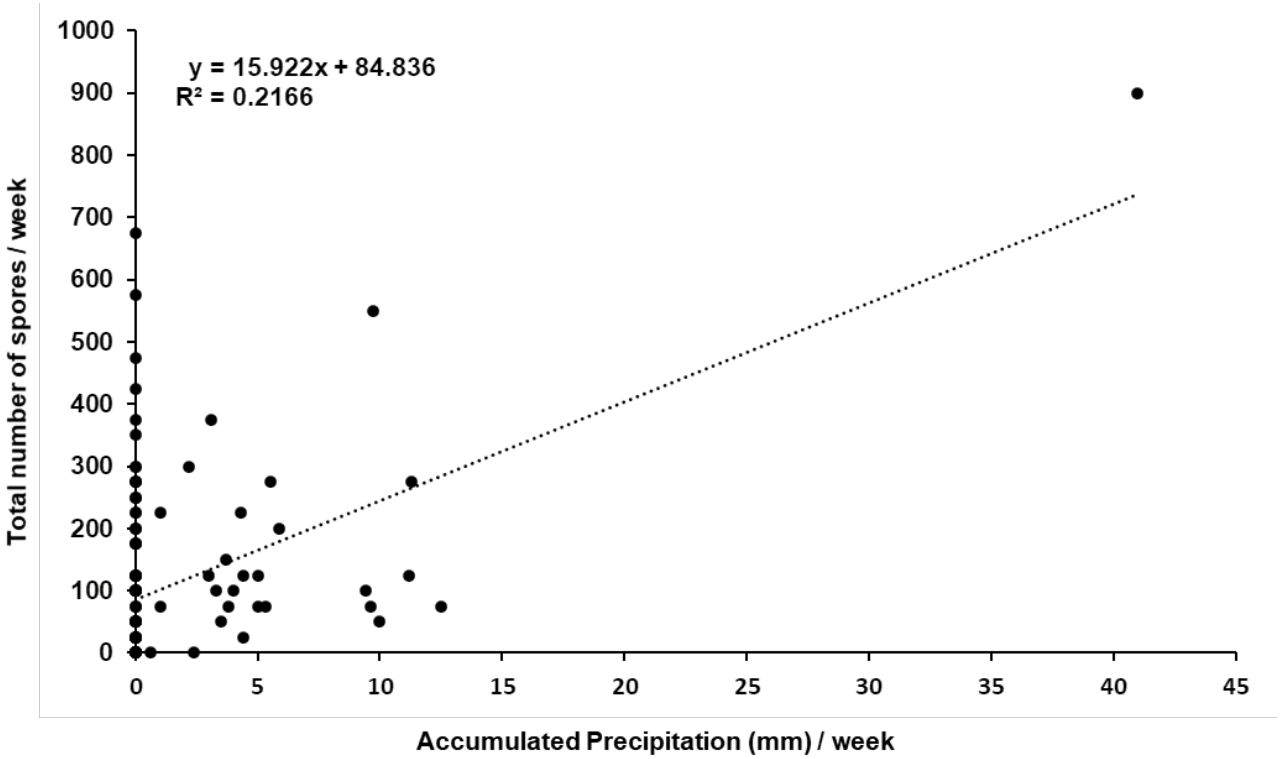

Figure 3. Scatter plot of the relationship between total spores trapped per week using glass microscope slides and precipitation. Linear regression line and equation are also shown.

largest numbers of spores were released during the week of 12 December 2008, when the greatest amount of precipitation was recorded (41 mm) during the course of this study (Figure 3). Overhead sprinkler irrigation was turned on in the studied vineyard from 18 October to 29 November in 2006, from 23 October to 27 November in 2007, and from 21 October to 25 November in 2008. *Diatrypaceae* spores were captured in six of seven weeks of overhead irrigation in 2006, three of six weeks in 2007, and four of six weeks in 2008. However, the total number of spores recorded during the weeks irrigation was not significantly greater when compared to the numbers of spores captured in weeks without irrigation (Figure 2). *Diatrypaceae* spore release showed no obvious correlation in this study with temperature, and spore release occurred under average temperatures ranging from 8°C to 35°C (Figure 4).

Diatrypaceae *species identifications*

PCR amplifications of ITS and *TUB2* regions gave respective products of approx. 500 and 700 bp. To study the phylogenetic relationships among *Diatrypaceae* isolates obtained in this study, ITS and *TUB2* sequences were BLASTed to select closely related sequences for the phylogenetic analysis (Table 1). In total, 72 isolates were included in the combined phylogenetic analysis with 1391 positions in the final dataset, including gaps. The greatest log likelihood Neighbor-Join phylogenetic tree of the combined ITS and *TUB2* sequences is shown in Figure 4. The MP evolutionary history generated seven most parsimonious trees (length = 1088; consistency index = 0.781855; retention index = 0.970641; composite index = 0.779724). The MP analysis resulted in trees with similar topology to the NJ tree. Based on this phylogenetic study, *Diatrypaceae* isolates obtained from spore traps placed in the Mid-Night Beauty® vineyard in Coachella Valley grouped in two well-supported clades with previously identified isolates of *E. citricola* (99% bootstrap for both NJ and MP) and *E. microtheca* (99% bootstrap for both NJ and MP), from different hosts from California and other countries (Figure 5). Additional *Diatrypaceae* isolates obtained from spore traps placed in vineyards in Monterrey, Napa, and San Luis Obispo, California, from a previous study (Úrbez-Torres *et al.*, 2010), grouped with *C. ampelina* isolate A001 from *V. vinifera* from Australia in a well-supported separate clade (99% bootstrap for both NJ and MP). In addition,

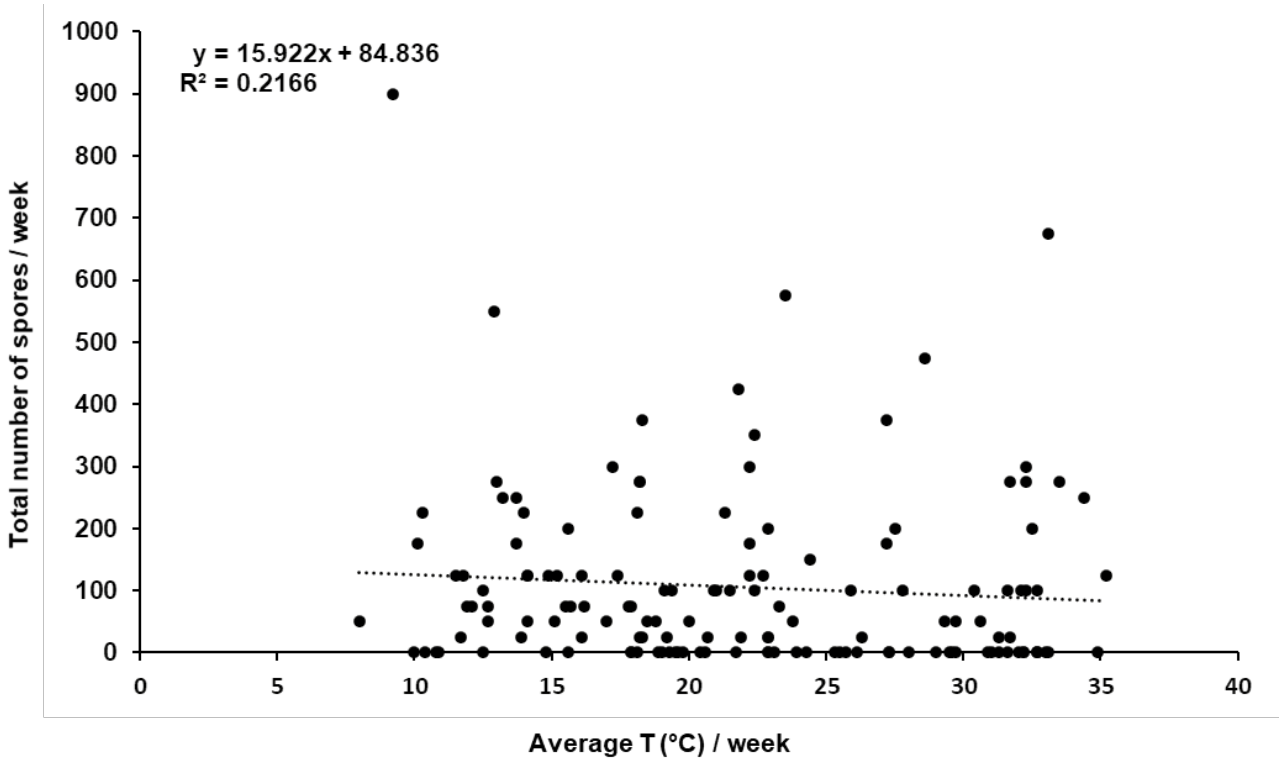

Figure 4. Scatter plot of the relationship between total spores trapped per week using glass microscope slides and average temperature. Linear regression line and equation are also shown.

two isolates from spore traps collected from San Joaquin County, California, (Úrbez-Torres *et al.*, 2010) grouped with *E. citricola* isolates.

A total of 25 fungal colonies were isolated and identified from 20 symptomatic cordon samples collected in a field survey at the spore trap block on 15 July 2007. BLAST results along with the combined ITS and *TUB2* phylogenetic analyses identified eight isolates as *E. scoparia* (including three isolates previously identified by Trouillas *et al.*, 2010; 2011), seven isolates as *E. citricola* (including three isolates previously identified by Trouillas *et al.*, 2010; 2011), five isolates as *E. microtheca*, four isolates as *Lasiodiplodia theobromae*, and one isolate as *Phaeoacremonium parasiticum* (Table 1; Figure 5).

Pathogenicity test

Results of the pathogenicity tests are summarized in Figure 6. Mean lesion lengths varied from 15.5 to 21.2 mm for *E. citricola* isolates, and from 13.5 to 16.9 for *E. microtheca* isolates (Figure 6). *Eutypella citricola* isolate UCD6Co caused the greatest mean lesion length, and while this was not significantly different from the mean lesion length from *E. citricola* isolate UCD8Co, it was significantly different from the mean lesion lengths from the other isolates tested (Figure 6). Mean lesion lengths obtained from inoculated canes were all longer than the negative controls ($P < 0.05$). Five months after inoculation, vascular necroses upwards and downwards from the inoculation sites were observed in longitudinal sections made from fungus inoculated canes (Figure 6B). Negative controls had significantly less discolouration extending from the inoculation sites (mean = 3.5 mm). *Eutypella citricola* and *E. microtheca* were re-isolated from lesions from all the inoculated canes, confirming that these fungi were the same as the inoculated isolates based, on morphology of the colonies. No fungal pathogens were isolated from the negative controls.

DISCUSSION

Eutypa lata has been known as the main causal agent of Eutypa dieback (ED) of grapevines since the early 1970s. However, few epidemiological studies have been conducted to understand spore dispersal patterns of this pathogen in vineyards (Pearson, 1980; Trese *et al.*, 1980; Trouillas and Gubler, 2010; van Niekerk *et al.*, 2010;

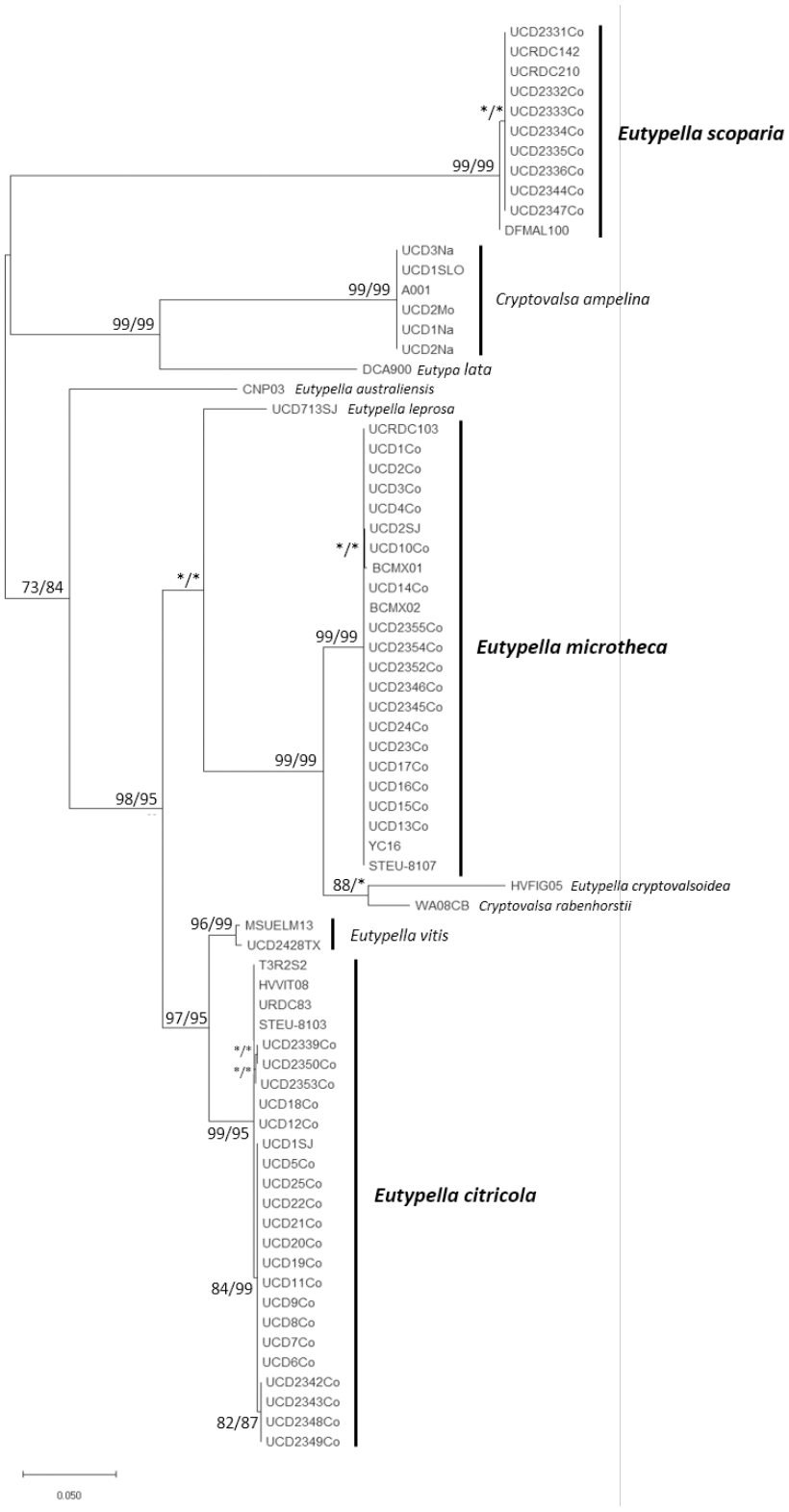

Figure 5. Greatest log likelihood Neighbor-Joining phylogenetic tree of the combined ITS and *TUB2* tree generated with 72 *Diatrypaceae* nucleotide sequences. Numbers in front and after the slashes represent, respectively, likelihood and parsimony bootstrap values from 1000 replicates. Values accompanied by an asterisk were less than 70% bootstrap. *Eutypella* isolates from spore traps from this study are indicated in bold font.

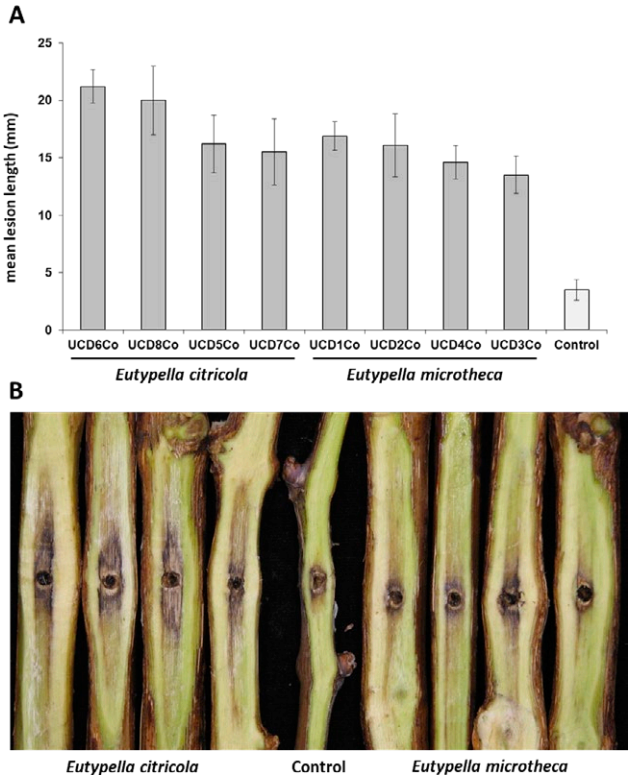

Figure 6. Pathogenicity test on cv. Red Globe grapevine plants inoculated with *Eutypella citricola* and *E. microtheca* isolates from vineyard spore traps. **A.** Mean lesion lengths caused by *E. citricola* and *E. microtheca* 5 months after inoculation. **B.** Vascular necrosis caused by *E. citricola* and *E. microtheca* in cv. Red Globe dormant canes compared to non-inoculated control.

Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017). Only recently, the use of DNA molecular identification has allowed the discovery of other diatrypaceous fungi associated with grapevine dieback, though their biology and epidemiology are still not fully understood (Trouillas and Gubler, 2004; Luque *et al.*, 2006; 2012; Trouillas *et al.*, 2010; 2011; Moyo *et al.*, 2018a). Only recent studies completed in South Australia (Billones-Baaijens *et al.*, 2017) and British Columbia, Canada (Úrbez-Torres *et al.*, 2017) have investigated spore dispersal patterns of *E. lata* and *Diatrypaceae* spp., using multi-species primers in quantitative PCR (qPCR) or droplet digital™ PCR (ddPCR™) assays. The present study adds to these latter studies investigating the role that diatrypaceous fungi may play in vineyards, and reports for the first time the seasonal spore release patterns of *Diatrypaceae* spp. other than *E. lata* under the desert conditions of California.

Based on previous spore trapping studies conducted in vineyards, it is well-accepted that spores of *E. lata* are released from perithecia within a few hours after the onset of rainfall, and release continues throughout each rain event and can last for up to 36 h after rain ends and until each stroma dries (Pearson, 1980; Trese *et al.*, 1980; Petzoldt *et al.*, 1983b; Trouillas and Gubler, 2010b). Similar conclusions can be reached from *E. lata* spore trapping studies conducted on apricots (Moller and Carter, 1965; Petzoldt *et al.*, 1983a; 1983b; Ramos *et al.*, 1975). It has also been suggested that a minimum of 2 mm of rainfall is required for stroma to start discharging *E. lata* ascospores (Trese *et al.*, 1980). However, a study conducted in South Africa showed a minimum of 1 mm of rainfall was enough for spores of *E. lata* to be discharged from perithecia in the Stellenbosch grape-growing region (van Niekerk *et al.*, 2010). More recent studies conducted in Australia and Canada have shown that spores of *Diatrypaceae* spp. were captured after as little as 0.5 mm of rain (Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017). Therefore, a strong correlation between rain events and *E. lata* spore release has been suggested, primarily during the grapevine dormant period (late autumn to early spring).

Most of the above-mentioned studies only investigated *E. lata* and did not consider other diatrypaceous fungi, although these have been commonly recognized as grapevine pathogens. However, based on morphological and taxonomic similarities between *E. lata* and other *Diatrypaceae* spp. in the genera *Cryptosphaeria*, *Cryptovalsa*, *Diatrype*, *Diatrypella*, and *Eutypella*, it is likely that these pathogens require similar environmental conditions for spore release, infection and perithecium formation to those for *E. lata* (Trouillas *et al.*, 2010; 2011). Nevertheless, the present study conducted under the desert conditions of the Coachella Valley in southern California showed rainfall was only responsible for 28% of the spore release events in the study vineyard, while overhead sprinkler irrigation contributed to another 22%. The greatest amount of rain recorded during this study occurred between the last week of November 2008 and the last week of May 2009 (89.4 mm), and this corresponded also with the greatest number of spores captured (4325). During this period, 900 spores were also captured in a single week, which also correlated with the greatest rainfall within a single week (41 mm). These results indicate a strong correlation between rainfall and *Eutypella* spp. spore release in the Coachella Valley. However, if we compare the same period from the last week of November of 2006 to the last week of May of 2007, from which no rainfall was recorded, the total number of spores captured was only slightly less (3000). Accordingly, if rainfall is suggested as the main factor responsible for spore release of *E. lata*, over 70% of *Eutypella* spp. spores captured in this study occurred without the

occurrence of rainfall events. These results are similar to those reported by Úrbez-Torres *et al.* (2017) from spore trapping studies conducted in the Okanagan Valley, a semi-arid grape-growing region located in the southern interior of British Columbia. In this study, Úrbez-Torres *et al.* (2017) showed that a significant number of *Diatrypaceae* spp. spore release events did not always correlate with rainfall. In addition, recent spore trapping studies conducted in Australia have also reported diatrypaceous spores to be captured during dry periods (Billones-Baaijens *et al.*, 2017). Based on these results, and as suggested by Billones-Baaijens et al. (2017), we hypothesize that environmental conditions other than rainfall (e.g. temperature, relative humidity, dew and/or wind) may be involved with spore release of *Diatrypaceae* spp., particularly under desert conditions where rainfall is scarce or limited to few days throughout each year.

Previous studies have correlated spore release of *E. lata* with high RH values (Trese *et al.*, 1980; Pearson, 1980; van Niekerk *et al.*, 2010). In the present study, relative humidity for most of the duration of the study were below 30% (data not shown), and only during rain events reached greater than 70%. Therefore, relative humidity probably did not play a role on *Eutypella* spp. spore release. Studies conducted in Australia (Moller and Carter, 1965) and California (Ramos *et al.*, 1975) suggested that wind currents can disseminate spores of *E. lata* long distances (50 and 160 km) from inoculum sources, but this always follows rain events. It is possible that some of the *Eutypella* spores captured in the present study did not originate in the monitored vineyard, but originated from the large number of citrus orchards planted in the region, where high incidence of *Eutypella* spp. infections has been reported (Mayorquin *et al.*, 2016). However, the desert climate under which the study was conducted occupies a large area of southern California, so even if spores came from surrounding vineyards, orchards and/or other native or introduced hosts, similar environmental conditions would have occurred in those locations, including the lack of precipitation. It is also possible that spores can be disseminated from distances greater than those previously reported, but further research is required to support this hypothesis. It is difficult to explain the high inoculum of *E. citricola* and *E. microtheca* spores present in this desert region during long periods without rain. Therefore, further research is required determine which other environmental conditions play roles in spore release and perithecium formation of *Eutypella* spp. under desert climatic conditions.

Eutypella spore captures were most common in winter months, followed by autumn. However, greatest spore quantities were captured during autumn (38.7% of the total), with almost half of spores captured in October. A large proportion (30.7%) of spores were also captured in winter with 50% of these captured in January. These results agree with previous studies conducted in California on *E. lata*. Ramos et al. (1975) and Petzoldt et al. (1983b) reported *E. lata* spore release from perithecia on apricots in the Salinas and Sacramento valleys to be high in mid- and late-autumn, medium in winter, high again in mid- and late-spring and low or nil in summer. Similar results were reported by Trouillas and Gubler (2009) for data from volumetric spore traps placed in vineyards in Sonoma, Napa and San Joaquin counties. Different results were reported by Moller and Carter (1965) based on *E. lata* spore trapping studies conducted on apricot in South Australia, where high spore release was detected from mid-autumn to early-winter, low release from mid-winter to early-spring, and high to medium release from mid-spring to mid-summer. The periods of high, medium and low discharge are partly explained by the more than 12 d required for released asci to be replenished with new mature ascospores in the perithecia under optimum environmental conditions (Petzoldt *et al.,* 1983b; Rolshausen *et al.*, 2015). The present study recorded similar total numbers of spore capture events occurring in spring and summer in the Coachella Valley. However, much greater numbers of spores were captured in summer (19.7% of the total) compare to spring (11%). In addition, August was the month with the greatest number of *Eutypella* spores captured after October, December and January. Though Moller and Carter (1965) reported some of the greatest numbers of *E. lata* spores captured during summer months, these releases were always correlated with rainfall. In contrast, July and August are among the hottest and driest months in the Coachella Valley, with average temperatures reaching up to 39°C. Úrbez-Torres *et al.* (2017) also reported large numbers of *Diatrypaceae* spores captured during July and August in British Columbia during dry periods. This is further evidence that environmental factors other than moisture favour spore release of *Eutypella* spp., and more specifically, release of *Eutypella* spores under desert climatic conditions.

Only *E. citricola* and *E. microtheca* were identified from spore traps in the present study, and both fungi were also isolated from symptomatic samples in the studied vineyard, indicating their roles as pathogens causing grapevine cankers. *Eutypella scoparia*, the third *Diatrypaceae* spp. isolated from cankers, was not identified from spore trap samples. Considering the difficulty to discriminate among *Diatrypaceae* spp. using colony morphology (Trouillas *et al.*, 2010; 2011), along with the

small number of samples selected each week to complete molecular identifications, it is possible that *E. scoparia* was missed from all of the spore samples collected. A much larger number of colony samples observed per week would need to be selected for molecular identification to confirm the presence of *E. scoparia* from spore traps. In addition, this would also allow more precise spore dispersal patterns for each *Eutypella* spp. found in this study. Trouillas *et al.* (2011) showed common occurrence of the perithecia of *E. citricola* and *E. microtheca* on diseased grapevine wood while perithecia of *E. scoparia* have not yet been found on this host.

To date, *E. lata* is the sole diatrypaceous fungus known to cause the characteristic ED foliar symptoms. In this study, the absence of *E. lata* from spore traps and cankers correlates with the lack of ED symptoms observed in the vineyard. This result is similar to those from surveys conducted in mid-2000s, in which ED symptoms along with *E. lata* isolated from cankers were mostly recorded from grape-growing regions in the Northern San Joaquin Valley, Sacramento Valley and North California (Úrbez-Torres *et al.*, 2006), with much greater precipitation than the present study area. *Eutypa lata* is restricted to geographical locations with at least 350 mm of annual rainfall, and perithecia are rarely found in areas with less than 250 mm (Ramos *et al.*, 1975; Trouillas and Gubler, 2010b; Rolshausen *et al.*, 2015). The historical average annual precipitation accumulated in the region of study (Indio, Coachella Valley) is about 200 mm, which may explain the absence of *E. lata*. These results agree with previous studies in which no *E. lata* but other *Diatrypaceae* spp. were found associated with dieback of perennial woody crops grown under desert conditions. Paolinelli-Alfonso *et al.* (2015) found *E. microtheca* but not *E. lata* as the cause of grapevine cankers in the grape-growing region of Baja California, Mexico. Similarly, Mayorquin *et al.* (2016) identified three *Diatrypaceae* spp., including *E. citricola*, *E. microtheca* and a *Eutypella* sp., to be involved in branch dieback of citrus in the southern California desert regions of Riverside (including Coachella Valley), Imperial and San Diego counties. The phylogenetic analyses conducted in the present study showed the unidentified *Eutypella* sp. from citrus in southern California to share 99% similarity with *E. scoparia* isolates from grapevine cankers, confirming that *E. scoparia* also occurs in citrus in California. Similarly, a much greater prevalence of *Diatrypaceae* spp. other than *E. lata* was found associated with grapevine cankers in the Okanagan Valley semi-arid region of British Columbia (Úrbez-Torres and O'Gorman, 2016). In addition, Trouillas *et al.* (2011) found *Eutypella* spp. to be the only diatrypaceous taxa occurring in vineyards in Western Australia. Based on these results *Diatrypaceae* spp., primarily in the genus *Eutypella*, are probably well-adapted to regions with desert or semi-arid climates. This information is important and deserves further investigation to determine the most prevalent and important fungal species involved on grapevine dieback under desert conditions, so that appropriate control strategies can be developed. Furthermore, molecular identification conducted in this study from spore trap samples previously collected from different regions in California (Úrbez-Torres et al., 2010) and thought to be *E. lata* resulted in colonies being identified as *C. ampelina*, *E. citricola* and *E. microtheca*. This highlights the difficulty to discriminate among *Diatrypaceae* spp. based solely on spore and colony morphology. To avoid fungal species misidentification in spore trapping studies, it is imperative to include molecular identification and quantification assays in future studies investigating fungal spore dispersal patterns (Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017; González-Domínguez *et al.*, 2020).

This study has confirmed the pathogenicity of *E. citricola* and *E. microtheca* isolates obtained from spore traps when artificially inoculated onto healthy grapevine shoots. Average necroses lengths recorded from both species after 5 months were small (18.2 mm for *E. citricola* and 15.2 mm for *E. microtheca*), but were greater than necroses from non-inoculated controls. These results agree with those published by Pitt *et al.* (2013), where *E. citricola* and *E. microtheca* isolates from grapevines in Australia resulted in average lesion lengths slightly greater than 20 mm after 9 months incubation on cv. Cabernet Sauvignon potted vines. In contrast, Paolinelli-Alfonso *et al.* (2015) showed that *E. microtheca* caused grapevine necrotic lesions of lengths 5 to 7 mm, which can be explained by the 3 month incubation period used. Necrotic lesions measured in the present study from both *E. citricola* and *E. microtheca* on grapevines were similar to those measured by Mayorquin *et al.* (2016) on inoculated lemon tree branches. In contrast, Moyo *et al.* (2018b) showed *E. citricola* and *E. microtheca* isolates from dieback on apricot and plum trees in South Africa caused larger lesions when inoculated onto these hosts under similar experimental conditions. Based on these results, fruit trees are probably more susceptible to these species than grapevines, but further studies are required to confirm this suggestion.

Mayorquin *et al.* (2016) showed a *Eutypella* sp., renamed in the present study as *E. scoparia*, to be highly virulent when inoculated onto branches of lemon trees, causing necrosis lengths greater than 80 mm after 8 months. Although *E. scoparia* was isolated from grape-

vine cankers in the present study, this fungus was not included in the pathogenicity assay. To the best of our knowledge, the pathogenicity of *E. scoparia* has not been evaluated on grapevines, although the results obtained by Mayorquin *et al.* (2016) on citrus indicate that this species could also be pathogenic to grapevine, and possibly more virulent than *E. citricola* and *E. microtheca*. Studies to determine the role that *E. scoparia* has in grapevine health are required, since this species is probably involved in dieback of economically important woody perennial crops in desert regions of southern California. Virulence of *E. citricola* and *E. microtheca* on grapevines is comparable to other *Diatrypaceae* spp., such as *C. ampelina*, *D. stigma*, *D. whitmanensis*, *Diatrypella* sp., and *E. leptoplaca* but less overall than *E. lata* (Luque *et al.*, 2006; Trouillas and Gubler, 2010; Pitt *et al.*, 2013). In addition, no foliar symptoms similar to those caused by *E. lata* were observed from any of the vines inoculated with *E. citricola* and *E. microtheca* in the present study.

Eutypa lata has long been known to be the most important diatrypaceous fungus causing dieback of a wide range of woody perennial hosts, including grapevines (Carter 1991). In general, *Diatrypaceae* spp. have been considered as saprophytes on decaying wood of angiosperms (de Almeida *et al.*, 2016), and the importance of some of these species as grapevine pathogens has only recently been demonstrated. The present study has shown that pruning dates in the studied block (29 December 2006; 27 December 2007; 22 December 2008) coincided with some of the greatest amounts of *Eutypella* spores in the vineyard. Since pruning wound infection has been suggested to occur after spores are discharge from perithecia during rain events, it is possible that the lack of precipitation in this region persuaded growers to not treat and protect pruning wounds to prevent pathogen infections. The present study demonstrated the importance of protecting pruning wounds soon after pruning under desert conditions, even if no rainfall occurs. In addition, further studies are required to better understand length of pruning wound susceptibility to *Eutypella* spp. under desert conditions. This information will assist development of appropriate fungicide spray programmes to ensure that pruning wounds are protected while susceptible. Results from this study are relevant, since they add to knowledge of the aetiology and epidemiology of GTD, especially under desert grape-growing conditions, which have not been extensively investigated. In other regions, large areas of table grapes are produced in desert climates. For instance, Coachella Valley represents about 7% of the total table grapes produced in California, with a farm gate value of $US118 million (USDA-NASS, 2018). Understanding the epidemiology of these pathogens in vineyards of southern California provides valuable information for improving disease control strategies under desert growing conditions.

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Research Papers

Distribution of three grapevine trunk pathogens in Chilean vineyards, determined using molecular detection from asymptomatic woody pruning material

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Summary. Grapevine Trunk Diseases (GTDs) cause important economic losses in vineyards. Diagnosis of pathogens causing GTDs is usually achieved using culture methods from symptomatic plants, but these methods may not be definitive. This study aimed to detect three fungi associated with GTDs in Chile (*Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata*) using qPCR methods, after extracting DNA from asymptomatic woody pruning material taken from 912 grapevine plants and 346 rootstocks. Pathogen incidence and distribution across different regions, vineyards, grape and wine cultivars, plant ages and rootstocks were determined. Forty percent of assessed grapevine plants and 42% of rootstocks were positive for the assayed GTD pathogens. The fungus with the greatest incidence was *D. seriata* (36% of plants and 30% of rootstocks), followed by *E. lata* (6% of plants and 20% of rootstocks) and *P. chlamydospora* (4% of plants and 5% of rootstocks). Positive relationships were detected between the presence of *D. seriata* and *P. chlamydospora* and grapevine age. However, the three fungi were randomly distributed across graprevine plants and rootstocks since none of the assessed factors had statistically significant influence in their distribution. This study is the first to assess incidence and distribution of *E. lata* in Chile. The qPCR-based method described could be used for detecting fungi in asymptomatic nursery plants, and be used to avoid distribution of infected plants into new vineyard plantings.

Keywords. qPCR, early detection, *Vitis vinifera*, trunk pathogens, wood fungi.

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INTRODUCTION

Grapevine Trunk Diseases (GTDs) causes major economic problems in vineyards, decreasing their longevity, yields and quality of wine produced. International cost for the replacement of dead grapevine plants resulting from GTDs is estimated to be over 1.5 billion dollars each year (Hofstetter *et al.,* 2012). Accordingly, much research has been carried out to understand these important diseases in many countries (Mondello *et al.,* 2018; Grozić *et al.,* 2019; Songy *et al.,* 2019). The currently accepted GTDs are Esca, Botryosphaeria dieback and canker, Eutypa dieback and Phomopsis dieback (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016; Gramaje *et al.,* 2018). The status of these diseases in Chilean wine production is scarce but they are likely to be detrimental to the wine industry (Besoain, 2018).

Chile is the world's sixth most important wine producer and the fourth most important wine exporter. Grapevines are grown in several valleys between the northern Coquimbo region (29°54'S, 71°15'W), and the southern Biobío region (36°46'22"S, 73°03'47"W). A total of 137,000 ha of cultivated wine grapes are grown, with the Maule (53,687 ha) and O´Higgins (45,782 ha) regions being the main wine producers (SAG, 2018).

Several fungi have been internationally associated with GTDs, and have been identified and described in Chile during the last decade. These include, the Ascomycete species of the *Botryosphaeriaceae*, *Phaeoacremonium* spp., *Diatrypaceae* spp., *Phaeomoniella chlamydospora*, and some Basidiomycetes such as *Inocutis* spp. (Auger *et al.,* 2004; Díaz *et al.,* 2009; 2011). In addition, Díaz *et al.* (2013) in a survey including 67 vineyards from the Antofagasta region (27°18'S) to the Biobío region (37°42'S), and using traditional culture methods, as well as molecular identification techniques, found a total of 12 fungal species. These fungi were present at varying frequencies, all were pathogenic, coexisting in plants in most cases, and inducing dark brown streaking in grapevine wood tissues of different ages. The most frequently isolated species were *P. chlamydospora* (85% incidence), *Diplodia seriata* (56%), and *Inocutis* sp. (47%). These results were partially confirmed in a survey from 2016 to 2018, where vineyards from the Valparaiso (33°02'S) to Maule (35°25'S) region were assessed. This focused on GTD symptomatic plants from vineyards of different ages. The pathogens most frequently isolated were *Inocutis* sp. (49% of isolates), *Botryosphaeriaceae* (*i.e. D. seriata* and *N. parvum,* 23%) and *P. chlamydospora* (18%) (Unpublished data). *Eutypa lata* was also reported from Chile, using molecular and morphological analyses (Lolas *et al.,* 2020). This species is one of the most internationally common GTD pathogen (Siebert, 2001; Travadon *et al.,* 2012). However, and due to recent findings, distribution of *E. lata* is not known within the Chilean wine production regions.

Diagnosis of GTD pathogens from grapevine plants has traditionally been carried out using culture isolation methods, morphological analyses, and molecular markers based on the Internal Transcribed Spacer (ITS) region and/or the beta-tubulin gene (Rolshausen *et al.,* 2013; Luque *et al.,* 2014; Úrbez-Torres *et al.,* 2015; Gramaje *et al.,* 2018). However, these methods can lead to inconsistent results, and identification of pathogen species can be complicated due the plasticity of morphological characters (Rolshausen *et al.,* 2004; Pavlic *et al.,* 2009). Based on the increasing availability of fungal genomic sequences from public repositories (Blanco-Ulate *et al.,* 2013a,b; Morales-Cruz *et al.,* 2015), and on the PCR specificity and sensibility, strategies using quantitative real-time PCR (qPCR) have been shown to be more suitable for detecting and quantifying the DNA of most of the main fungi associated to GTDs (Pouzoulet *et al.,* 2013, 2017; Reis *et al.,* 2019).

Most of the GTD diagnoses described above have been conducted on symptomatic plants. In nursery propagation processes, the sensitivity of the PCR-based diagnostic tool becomes relevant (compared with traditional culture methods) where mother plants and the propagation material (mostly asymptomatic) are important sources of inoculum for fungal trunk pathogens (Aroca and Raposo, 2007; Aroca *et al.,* 2010).

Factors affecting the diversity of fungi associated with GTDs are poorly understood (Del Frari *et al.,* 2019). Grape cultivar has been related to the fungi causing these diseases (Travadon *et al.,* 2016), but this has not been studied in Chilean vineyards. Molecular tools can provide accurate identification of GTD pathogens.

The aim of the present study was to determine the incidence of two of the most important fungi associated with GTDs in Chile (*D. seriata* and *P. chlamydospora*), and the recently reported *E. lata*, using qPCR-based methods and extracting DNA directly from asymptomatic woody pruning material. This was to develop understanding of the distribution patterns of the fungi associated with GTDs in this country.

MATERIALS AND METHODS

Sampling

Eight vineyards (hereafter designated V0 to V7), in the main wine grapevine production valleys of Chile, were surveyed from 2016 to 2019. The vineyards were

between 5 to 23 years old and were in several regions of Chile, including Coquimbo (29°54'S), Valparaíso (33°02'S), O´Higgins (34°10'S) and Maule (35°25'S), with mean annual rainfall ranging from 1 to 1,000 mm, mostly during winter months. Collected samples consisted of at least 5–8 pruned 1-year-old canes per plant, taken from asymptomatic woody pruning material (no visually evident wood symptoms). Sixteen *V. vinifera* cultivars were sampled, including: 'Cabernet franc' (CF), 'Cabernet Sauvignon' (CS), 'Carménère' (CA), 'Chardonnay' (CH), 'Gewürztraminer' (GT), 'Malbec' (MA), 'Marselan' (Ma), 'Merlot' (ME), 'Pedro Jimenez' (PJ), 'Petit Syrah' (PS), 'Petit Verdot' (PV), 'Pinot noir' (PN), 'Riesling' (RI), 'Sauvignon blanc' (SB), 'Syrah' (SY), and 'Viognier' (VI). Five rootstocks located in V6 from Maule region were also sampled, including: 101-14 MGt (*V. riparia* Michx × *V. rupestris* Scheele), 110 Richter (*V. berlandieri* Planch × *V. rupestris*), 1103 Paulsen (*V. berlandieri* × *V. rupestris*), 3309 Couderc (*V. riparia* × *V. rupestris*), and SO4 (*V. berlandieri* × *V. riparia*). The samples were transported at 4°C to a laboratory within 24 hours of collection and were subsequently stored at -80°C until processed.

Incidence of the three main GTD pathogens considered for this study (*D. seriata*, *E. lata* and *P. chlamydospora*) was determined as the percentage of the positively infected samples in relation to the total number of plants samples per region, vineyard, grape cultivar or wine cultivar (red or white), depending on the particular case. For the rootstock samples, pathogen incidence was determined as the percentage of positively infected plants in relation to the total number of plants sampled per rootstock type.

Sequence alignment and primer design

Partial sequences of the *P. chlamydospora* beta-tubulin gene were retrieved from the GenBank database at the NCBI website (https://www.ncbi.nlm.nih.gov/genbank/). To evaluate sequence variability between international and Chilean isolates, *P. chlamydospora* beta-tubulin sequences were aligned using Clustal Omega from the European Molecular Biology Laboratory, EMBL-EBI. The international sequences used were *P. chlamydospora* strain CBS 229.95 (accession No. AF253968), STE-U 3066 (AF253969), Y83-8-1 (EU078329), Y121-20-1 (EU078333), Pch-2 (GQ903724), Pch-3 (GQ903725), UCD2548MO (HQ288313), UCD-2586MO (HQ288316), PARC143 (KF764664), PARC326 (KF764667), and IBVD01 (KP213108). Chilean sequences used were *P. chlamydospora* isolate Pach-525 (JX679869), Pach-394 (JX679870), Pach-304 (JX679871), Pach-300 (JX679873), Pach-297 (JX679874), Pach-132 (JX679877), Pach-128 (JX679878), Pach-102 (JX679880), Pach-59 (JX679882), Pach-36 (JX679885), Pach-7 (JX679888), and Pach-6 (JX679889). Primers and TaqMan probe designs were carried out using the GenScript Real-time PCR (TaqMan) Primer Design tool (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool). To detect *D. seriata* and *E. lata*, the SYBR Green primer sets described by Pouzoulet *et al.* (2017) were used.

All primer conditions have been standardized for efficiency between 90 to 110%, R^2 ≥0.995. Specificity of primers has been validated by sequencing amplicons obtained and using BLASTN (NCBI), and in all cases the sequenced amplicons matched the expected amplicons. For *D. seriata*, the sequencing results coincided with *D. seriata* beta-tubulin annotated sequences from New Zealand (accession nº MN245025), United States of America (MN318125), Spain (MK522087) and Chile (MG952722), among other beta-tubulin sequences from *D. seriata*. For *E. lata*, amplicon sequences aligned with Australian sequences (MN433695), and others annotated in the United States of America (MN329778, HM164739, HM164762). In the case of *P. chlamydospora*, sequencing results aligned with beta tubulin sequences from Brazil (MN747132, MK903808), Spain (MG7458109), and Canada (KF764664), among other sequences from *P. chlamydospora*. In addition, the sequence from this fungus had 100% similarity with the Chilean *P. chlamydospora* beta-tubulin sequences annotated in GenBank used for primer design (JX679869, JX679870, JX679871, JX679873, JX679874, JX679877, JX679878, JX679880, JX679882, JX679885, JX679888, JX679889).

Total DNA purification

Frozen wood samples from all the pruned 1-year-old canes sampled per plant were each ground transversally at the center of the cane using a drill and liquid nitrogen, without grinding bark. Approx. 100 mg of wood chip representative of the sample was used to isolate total DNA using the DNeasy Plant Mini Kit (Qiagen, Germany). DNA quality and quantity were determined using an Infinite M200Pro NanoQuant spectrophotometer (Tecan) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific Inc.).

Quantitative real-time PCR (qPCR) analyses

To assess DNA quality and absence of inhibitors, all samples were first amplified using Actin primers with the TaqMan probe (Bruisson *et al.*, 2017). The TaqMan system was also used to detect *P. chlamydospora*. In both

cases, the reactions were each prepared in a final volume of 10 µL, containing 5 µL of TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific), 2 µL of DNA sample, and primers were each used at a final concentration of 600 nM and probe at 200 nM. The amplification profile used was 50ºC for 2 min, 95ºC for 20 sec, followed by 40 cycles each of 95ºC for 1 sec, 56ºC for 5 sec and 60ºC for 15 sec.

To detect *D. seriata* and *E. lata,* the SYBR green system was used. Reactions were performed in a final volume of 10 µL, containing 5 µL of PowerUp™ SYBR© Green Master Mix (Thermo Fisher Scientific), 2 µL of DNA sample, and primers were each used at a final concentration of 500 nM. The amplification profile used was 50°C for 2 min, 95°C for 2 min, followed by 40 cycles each of 95°C for 1 sec and 60°C for 30 sec. The melting curve was at 95°C for 15 sec and then from 60°C to 95°C (increasing 1.5°C sec^{-1}). All reactions were run in the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Primers and probe sequences are outlined in Table 1.

Statistical analyses

In order to understand the distribution patterns of the fungi associated with GTDs and the factors that might be explaining these patterns, the presence/absence data matrix of *P. chlamydospora*, *D. seriata* and *E. lata* was used. Samples were grouped by region, vineyard, grape cultivar, wine cultivar or age, constituting 25 different samples. Incidence was calculated for each sample. With both data sets (presence/absence and incidence), several analyses were performed using R 3.5.1 (R Development Core Team, 2020).

Firstly, whether age of the grape cultivar affected GTDs presence/absence was analyzed. For this the ggpubr package (Kassambara, 2020) was used, using the *stat_compare_means* function, in order to perform mean comparisons with a Kruskal-Wallis test.

Secondly, and to understand the distribution of the GTD pathogens across samples, a heatmap was built using the function *heatmap.2* of the gplots package (Warnes *et al.,* 2020). This made it possible to visualize how similar the samples were in terms of the fungal species of interest. Possible correlations between the incidence of GTD pathogens and the age of plants was then assessed. For the regressions the ggpubr package (Kassambara, 2020) was used for these regressions, using the *stat_regline_equation* function. To understand which factors affected the GTD pathogen composition in the samples and considering the unbalanced design, ANOSIM tests were performed to determine whether the region, vineyard, grape cultivar, wine cultivar (red or white) or vine age had effects on pathogen composition. Analyses were carried out with the incidence table, using the *anosim* function of the *vegan* package (Oksanen *et al.,* 2019), considering a Jaccard distance matrix with 999 permutations. The samples with absence of all the three species of fungi were removed in order to conduct the analysis.

For rootstocks, the presence/absence data matrix was used to calculate the incidence of the GTD pathogens. Since five rootstocks were sampled, the analysis considered five different samples, and a heatmap was constructed as described above. The matrix of pathogen incidence was used to test whether the type of rootstock had an influence of pathogen composition, through an ANOSIM.

RESULTS

Samples from a total of 912 plants, from four regions of Chile (Table 2), and from eight vineyards (Table 3) containing a total of 16 grape cultivars (Table 4), were

Table 1. Oligonucleotides used on this study.

Target	Detection system	Primer sequence (5'->3')	Reference
Phaeomoniella chlamydospora	TaqMan	PchF: GCTGACGACGTCCAGGGTAA PchR: TCAAGGCCGTGTTCACCAGA PchProbe: CCTCGACGACTACAGACGCGCCA	This study
Diplodia seriata	SYBR Green	DseCQF: CTCTGCAATCGCTGACCCTTG DseCQR: ACGTGTTTGTCTAACTAGTAGAGAGTACC	Pouzoulet *et al.,* 2017
Eutypa lata	SYBR Green	ElQF:GCCAGCTAATAAAACAATTGCTTACCT ElQR: AGATAACCTCGTGTGATTGTGTGATT	Pouzoulet *et al.,* 2017
Actin	TaqMan	ActF: GTATTGTGCTGGATTCTGGTGAT ActR: GCAAGGTCAAGACGAAGGATAG ActProbe: CACTGTGCCAATTTATGAAGGTTATGCACTTC	Bruisson *et al.,* 2017

Table 2. Incidence of GTD pathogens by region in Chile. The regions are ordered from north to south of the country. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s = Diplodia seriata*, *E_l = Eutypa lata* and *P_c = Phaeomoniella chlamydospora*. The number below each GTD fungus corresponds to the number of positive plants infected by that fungus.

Region	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Coquimbo	7	7	100	0	0	0	0	0
Valparaíso	50	37	74.0	13	26.0	13	3	0
O`Higgins	316	121	38.0	195	62.0	175	20	18
Maule	539	382	71.0	157	29.0	137	32	18
No. Total	912	547		370		325	55	36
%			60.0		40.6	35.6	6.0	3.9

Table 3. Incidence of GTDs and GTD pathogens in seven Chilean vineyards. The vineyards are ordered from north to south of Chile. V = Vineyard, C = Coquimbo, V = Valparaíso, O = O´Higgins and M = Maule, n = number, GTD- = plants with no GTD fungi, GTD+ = plants with presence of GTD fungi. *D_s = Diplodia seriata*, *E_l = Eutypa lata* and *P_c = Phaeomoniella chlamydospora*. The numbers below each GTD fungus are the number of positive plants for that fungus.

Vineyard (Region)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
V0 (C)	7	7	100	0	0	0	0	0
V1 (V)	50	37	74.0	13	26.0	13	3	0
V2 (O)	59	54	91.5	5	8.5	2	1	2
V3 (O)	257	67	26.1	190	73.9	173	19	16
V4 (O)	93	78	83.9	15	16.1	13	1	1
V5 (M)	24	21	87.5	3	12.5	2	1	0
V6 (M)	334	203	60.8	131	39.2	116	29	15
V7 (M)	88	80	90.9	8	9.1	6	1	2
No. Total	912	547		365		325	55	36
%			60.0		40.0	35.6	6.0	3.9

analyzed in this study. The samples included white and red cultivars (Table 5), and the sampled plants were of age between 5 and 23 years.

The data analyzed for GTD pathogen incidence in the different regions indicated that in the Valparaíso and Maule regions, the proportions of plants that were negative for these fungi were greater than the proportions of those infected. The samples from the O`Higgins region, on the other hand, had greater incidence of GTD pathogens in relation to plants with no pathogenic fungal species. The most frequently detected fungus was *D. seriata*, and samples from the O'Higgins and Maule regions were infected by *D. seriata*, *P. chlamydospora* and *E. lata* (Table 2). None of these pathogens were found in Coquimbo region, and *P. chlamydospora* was not detected in samples from Valparaíso.

Samples from two vineyards (V3 and V6) had high numbers of GTD pathogens. Vineyard V3 was in a coastal area, while vineyard V6 was located towards the Central Valley of Chile.

Incidence of GTD pathogens per grape cultivar showed the presence of at least one GTD fungus in most of the cultivars examined (Table 4). High incidence was detected in several cultivars, and *D. seriata* was always the dominant pathogen in these cultivars.

White wine cultivars had higher incidence of GTD pathogens than red wine cultivars (Table 5). However, the proportion of healthy plants was higher for both cases.

The age of the vines had a significant effect on the presence/absence of *D. seriata* (Figure 1a) and *P. chlamydospora* (Figure 1c). For both pathogens, that the older plants had more infections with these fungi than young plants.

The regressions between age of plants and the incidence of GTD pathogens showed positive but not statistically significant relationships for the three fungal species: *D. seriata* (R = 0.3, *P* = 0.19), *E. lata* (R = 0.12, *P* = 0.71) and *P. chlamydospora* (R = 0.5, *P* = 0.21).

Diplodia seriata had the greatest incidence across most samples, followed by *E. lata* (Figure 2). *Phaeomo-*

Table 4. Incidence of GTDs by grape cultivar. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The numbers below each fungus are the number of positive plants for that fungus.

Grape cultivar (vine age: years)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Cabernet franc (8 –11)	29	25	86.2	4	13.8	4	0	0
Cabernet Sauvignon (10–13)	157	99	63.1	58	36.9	51	20	9
Carménère (7–3)	84	69	82.1	15	17.9	11	1	4
Chardonnay (11–15)	90	48	53.3	42	46.7	40	5	1
Gewürztraminer (11)	10	9	90.0	1	10.0	1	0	0
Malbec (11)	10	10	100	0	0	0	0	0
Marselan (11)	9	8	88.9	1	11.1	1	0	0
Merlot (5–11)	141	120	85.1	21	14.9	18	2	1
Pedro Jimenez (7–11)	7	7	100	0	0	0	0	0
Petit Syrah (11)	2	2	100	0	0	0	0	0
Petit Verdot (11)	18	1	5.6	17	94.4	17	0	0
Pinot noir (14)	98	6	6.1	92	93.9	91	7	0
Riesling (11)	5	4	80.0	1	20.0	1	0	0
Sauvignon blanc (11–14)	225	117	52.0	108	48.0	85	20	21
Syrah (13)	6	3	50.0	3	50.0	3	0	0
Viognier (6)	21	19	90.5	2	9.5	2	0	0
No. Total	912	547		365		325	55	36
%			60.0		40.0	35.6	6.0	3.9

niella chlamydospora had the least incidence across the 25 samples studied. For the Coquimbo region, none of the seven analyzed plants from the cultivar 'Pedro Jimenez' were positive for GTD pathogens, in contrast with the other three regions. In addition, in the O`Higgins region and specifically in V2, the 20 plants of 'Cabernet franc' analyzed were also negative for all three GTD pathogens investigated. In the Maule region and specifically in V6, 'Malbec' (ten plants) and 'Petit Syrah' (two plants) cultivars were negative for all three GTD pathogens (Figure 2).

The analysis of GTD pathogen composition in relation to region, vineyard, grape cultivar, wine cultivar and vine age factors showed that none of these factors had statistically significant effects upon the composition of GTD pathogens detected in the sampled grapevine plants. However, region had an almost significant effect ($P = 0.06$) on pathogen composition, explaining 20% of the variance between regions.

Assessments of GTD pathogen incidence in rootstock plants, showed that *D. seriata* and *E. lata* were the most frequently detected species in all five sampled rootstocks (Table 5). None of the rootstocks were free of GTD fungi and almost half of the rootstocks were infected with *D. seriata* and *E. lata* (Table 6).

The heatmap (Figure 3) showed that rootstocks 101-14 and 3309 had similar incidences of GTD patho-

Table 5. Incidence of GTD pathogens by wine cultivar type. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The numbers below each fungus are the number of positive plants for that fungus.

Wine cultivar type	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Red	554	343	62.0	211	38.0	196	30	14
White	358	204	57.0	154	43.0	129	25	22
No. Total	912	547		365		325	55	36
%			60		40.0	35.6	6.0	3.9

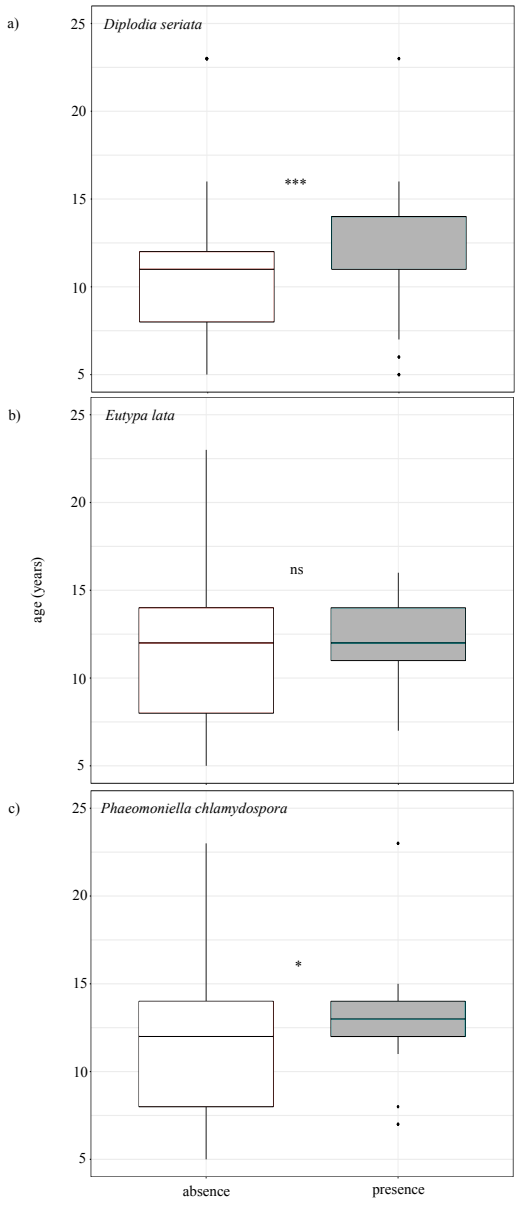

Figure 1. Effect of age of plants on the presence and absence of a) *Diplodia seriata* (*** $P < 0.001$) b) *Eutypa lata* (not significantly different) and c) *Phaeomoniella chlamydospora* (*$P < 0.05$).

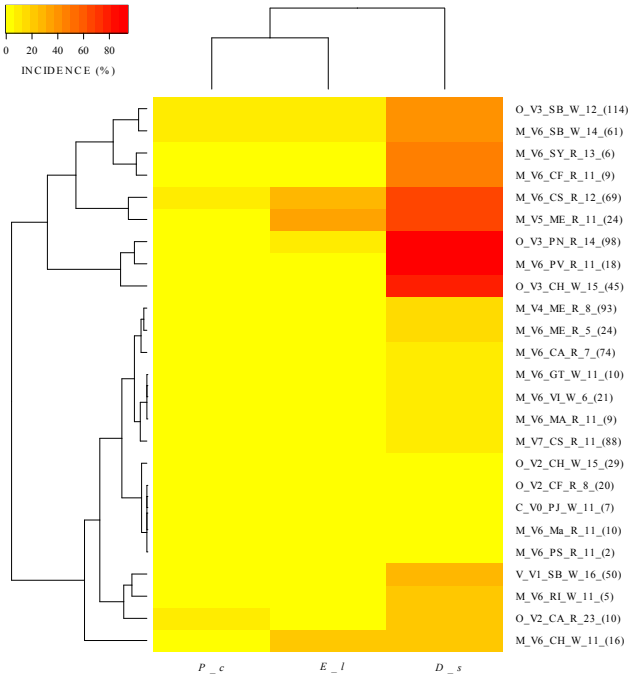

Figure 2. Heatmap with GTDs fungal distribution in the 25 samples of this study. M: Maule region, O: O`Higgins region, V: Valparaíso region, C: Coquimbo region, V0-V7: vineyards 0 to 7, CF: 'Cabernet franc', CS: 'Cabernet Sauvignon', CA: 'Carménère', CH: 'Chardonnay', GT: 'Gewürztraminer', MA: 'Malbec', Ma: 'Marselan', ME: 'Merlot', PJ: 'Pedro Jimenez', PS: 'Petit Syrah', PV: 'Petit Verdot', PN: 'Pinot noir', RI: 'Riesling', SB: 'Sauvignon blanc', SY: 'Syrah', VI: 'Viognier', W: white wine cultivar, R: red wine cultivar, numbers in the fifth position of each sample name indicates age in years. Next to each sample in brackets the number of plants per sample is depicted. *P_c*: *Phaeomoniella chlamydospora*, *E_l*: *Eutypa lata* and *D_s*: *Diplodia seriata*.

gens, with the pathogens occurring simultaneously and *D. seriata* and *E. lata* occurring more frequently. In the other three rootstocks, only *E. lata* and *D. seriata* were detected (Figure 3). The pathogen composition analysis revealed that the type of rootstock had a statistically significant effect ($P < 0.01$)on GTDs fungal composition.

Table 6. Incidence of GTD pathogens in grapevine rootstock plants. NO. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The number below each GTD fungus corresponds to the number of positive plants infected by that fungus.

Rootstock (age in years)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
101-14 (20)	80	39	49.0	41	51.3	27	27	10
1103P (20)	77	53	69.0	24	31.0	15	13	2
110-R (20)	46	21	46.0	25	54.0	21	9	0
3309 (20)	49	24	49.0	25	51.0	16	15	5
SO4 (20)	94	63	67.0	31	33.0	25	6	2
No. Total	346	200		146		104	70	19
%			58.0		42.0	30.0	20.0	5.0

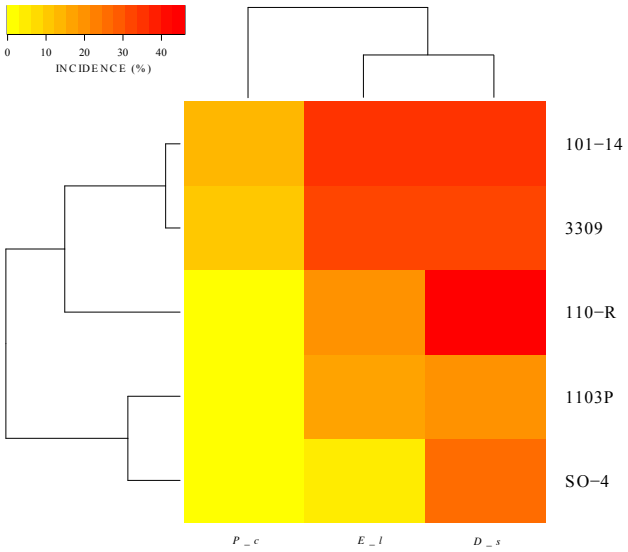

Figure 3. Heatmap with GTDs fungal distribution in the five rootstocks considered on this study. *P_c: Phaeomoniella chlamydospora, E_l: Eutypa lata* and *D_s: Diplodia seriata.*

DISCUSSION

Part of the aim of this study was to detect two of the most frequently occurring fungi (*P. chlamydospora* and *D. seriata*) associated with GTDs in Chile, and the globally important pathogen *E. lata* recently reported for Chile. In this research, the three pathogens were detected using qPCR-based methods and obtaining DNA material directly from asymptomatic grapevine woody pruning material. Using these methods is an advance compared with traditional methods of pathogen detection (Diaz *et al.,* 2013). Traditional methods have involved isolation and morphological identification of pathogens, and have been widely used due to simplicity and low cost. However, they require considerable time investment and have several disadvantages. They lack specificity, sometimes giving inaccurate results (false negatives) (Pouzoulet *et al.,* 2017), and are particularly complex since the fungi involved do not form key reproductive structures that allow identification from *in vitro* cultures (*e.g. E. lata*) (Rolshausen *et al.,* 2004; Pavlic *et al.,* 2009). Mycological experience is also essential to achieve accurate pathogen identification (Fischer and Dott, 2002). Use of qPCR for the detection of fungal pathogen has recently been demonstrated for grapevine pruning wounds (Pouzoulet *et al.,* 2017) and for apple trees (Gringbers *et al.,* 2020). Pouzoulet *et al.* (2017) demonstrated this for *E. lata* and *D. seriata,* and the present study has confirmed their results with Chilean material, and has added the possibility of detection of *P. chlamydospora* in grapevines through the design of a specific probe and primers based on international and Chilean isolates.

The present study has demonstrated of high sensitivity and specificity through direct diagnosis using qPCR, which efficiently differentiated several GTD-causing fungi from complex samples of woody pruning material. This study has confirmed that asymptomatic grapevine mother plants and propagation material can also be sources of fungal trunk pathogens as has been previously suggested (Aroca and Raposo, 2007; Aroca *et al.,* 2010). Consequently, sensitive PCR-based diagnostic tools become relevant in early grapevine propagation stages to assist prevention of pathogen dissemination. Another advantage of this approach is that it can be rapidly applied, and the results are quickly obtained relative to classical *in vitro* culturing methods. These methods are useful for rapid and accurate decisions in vineyards, and for early detection of pathogens in nursery plants, to assist high quality and production standards of for young plants that are free of GTD pathogens.

The present study also determined the relative incidence of the three fungal pathogens in the main grape-

vine growing regions of Chile. In the samples examined, incidence of *D. seriata* was 36%, *E. lata* was 6% and *P. chlamydospora* was 4%. This was found in analyses of samples from 912 plants, from four regions of Chile, eight vineyards, 16 grapes cultivars of white and red grape varieties, with plants from 5 to 23 years old. These results contrast with those of Diaz *et al.* (2013), who reported incidence of *D. seriata* at 56% and *P. chlamydospora* at 85%. However, Diaz *et al.* (2013) examined only GTD symptomatic plants. This difference suggests the presence of biotic or abiotic filtering in the environment, that could favour one pathogen species over another once symptoms appear (Crowther *et al.,* 2014). It is widely acknowledged that once the fungi sporulate, airborne spores are transported after rain (Úrbez-Torres *et al.,* 2010), and natural or artificial wounds are sites of host infection, followed by develop and reproduction (Bertsch *et al.,* 2013). However, even when different fungal species spores attach to the same wound, other factors, such as intrinsic species traits, may determine infection success of one species over another (Crowther *et al.,* 2014).

The incidence of *E. lata* infections was only 6% in asymptomatic plants. Future research should also consider symptomatic plants, to establish accurate incidence of this GTD pathogen. However, GTD pathogens were only detected only in some grape cultivars ('Sauvignon blanc', 'Pinot noir', 'Merlot', 'Chardonnay', 'Carménerè' and 'Cabernet Sauvignon'), and it has been reported that their occurrence can be related to warm temperatures and high humidity (Ramírez and Pineda, 2003).

In contrast for rootstocks, incidence for *D. seriata* was 30%, *E. lata* 20%, and *P. chlamydospora*, 5%. The only rootstock for which particular GTD pathogens were not detected was 110-R, where *P. chlamydospora* was not found. This suggest that rootstocks are important reservoirs for GTD pathogens. This raises the possibility that during grafting processes, nursery treatments (disinfection), biotic approaches (cultivar choice) or abiotic filtering (climatic conditions) could inhibit pathogen at least for *E. lata* (Crowther *et al.,* 2014).

The current study also considered to provide understanding of distribution patterns of the fungi associated with GTDs, and the factors that explain those patterns. A relationship was detected between grapevine age and the presence of GTD pathogens. This occurred for *D. seriata* and *P. chlamydospora*, but not for *E. lata*. Older plants had greater presence of the two GTD fungi than young plants, which was probably related to greater exposure to pruning and more exposed wounds susceptible to infections from pathogen inoculum.

In contrast with previous studies (Travadon *et al.,* 2016), the present research did not indicate effects of region, vineyard, grape and wine cultivar or plant age on GTD pathogen composition, factors which have not been previously considered in Chilean viticulture. Similarly, different rootstocks did not influence fungal composition. In most cases, random distributions of the GTD fungi occurred for all the plants investigated. This strongly suggests that these fungi were opportunists, and the first to arrive at susceptible host sites will be the successful colonizer. This study has demonstrated that GTD pathogens can be detected in asymptomatic plants, and in in grapevine nurseries. Avoiding transmission of these pathogens into productive vineyards is likely to be a prominent option for managing GTDs.

CONCLUSIONS

This study reports a rapid, accurate and reliable method for detecting three GTD fungi (*D. seriata, E. lata* and *P. chlamydospora*) in asymptomatic grapevine plants, and random distribution of the GTD pathogens in relation to environmental conditions. This knowledge supports adoption of appropriate disease management practices for detecting the fungi in asymptomatic nursery plants, which will reduce transmission of these pathogens into vineyard through young planting material.

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Research Papers

Aerial inoculum patterns of Petri disease pathogens in South African vineyards and rootstock mother blocks

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Summary. Petri disease is caused by the xylem inhabiting fungi *Phaeomoniella* (*Pa.*) *chlamydospora* and several *Phaeoacremonium* (*Pm.)* species. Pruning wounds are known host ports of entry for aerial spores of these pathogens. However, knowledge is lacking on occurrence of these pathogens as aerial inoculum within South African vineyards. This study determined when spores of Petri disease pathogens are released in Western Cape Province vineyards and how these spore release events coincided with pruning activities when infections could occur. The research was conducted for two seasons from mid-May to early December 2012 and from mid-March to early December 2013. Microscope slide spore traps were affixed to arms of infected vines in six vineyards and mother vines in two rootstock mother vine nurseries. The slides were replaced weekly and fungal spores were retrieved from them, cultured, counted and identified. Colonies resembling those of *Pa. chlamydospora* and *Phaeoacremonium* spp. were subcultured for further molecular identification. Species of *Phaeoacremonium* were identified by amplification of the partial beta-tubulin gene. Taqman probes and primers were developed to facilitate fast detection of the most frequently occurring species (*Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum*), using real-time PCR. Petri disease pathogens occurred throughout the periods investigated. *Phaeomoniella chlamydospora* and *Pm. minimum* were trapped in all vineyards. A total of 14 *Phaeoacremonium* species were identified, with the greatest diversity ever recorded in vineyards, including *Pm. australiense, Pm. griseo-olivaceum, Pm. griseorubrum, Pm. inflatipes*, *Pm. iranianum, Pm. italicum, Pm. minimum, Pm. parasiticum, Pm. prunicola, Pm. scolyti, Pm. sicilianum, Pm. subulatum, Pm. venezuelense* and *Pm. viticola*. Of these, only *Pm. minimum* and *Pm. inflatipes* have been reported as aerial inoculum within vineyards. Spore release coincided with winter and spring pruning activities. The occurrence of six *Phaeoacremonium* species in rootstock mother vine nurseries highlights the high risk of pathogen spread through infected nursery material. This is the greatest *Phaeoacremonium* species diversity ever recorded in vineyards and the first detection of *Phaeoacremonium* species aerial inoculum in grapevine rootstock mother vine nurseries. The high species diversity and frequency of spore release in vineyards and rootstock mother vine nurseries coinciding with traditional pruning practices emphasizes the need to develop effective wound protection strategies to avoid infection of unprotected grapevine pruning wounds.

Keywords. *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Vitis* spp., epidemiology, spore release.

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INTRODUCTION

Petri disease is a serious problem in grapevines affecting grape production worldwide including South Africa, where it has been a major problem since the early 1990's (Ferreira *et al*., 1994). Petri disease is caused by *Phaeomoniella* (*Pa*.) *chlamydospora* and *Phaeoacremonium* (*Pm*.) species (Scheck *et al*., 1998a; Mugnai *et al*., 1999; Mostert *et al*., 2006a), and is usually associated with 1- to 5-year-old vines in newly established vineyards (Mugnai *et al*., 1999; Halleen and Groenewald, 2005). Seventeen species of *Phaeoacremonium* have been isolated from symptomatic vines in South Africa, including *Pm. australiense, Pm. austroafricanum*, *Pm. fraxinopennsylvanicum, Pm. griseo-olivaceum, Pm. griseorubrum, Pm. inflatipes*, *Pm. iranianum*, *Pm. italicum* (reported as *Pm. alvesii*), *Pm. krajdenii*, *Pm. minimum*, *Pm. parasiticum*, *Pm. prunicola*, *Pm. scolyti*, *Pm. sicilianum*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* (Crous *et al*., 1996; Groenewald *et al*., 2001; Mostert *et al*., 2006b; White *et al*., 2011a; Spies *et al*., 2018). Infected vines show stunted growth, shortened internodes and dieback. Internal symptoms include black to brown spots and brown streaking in the xylem tissues (Ferreira, 1994; Scheck *et al*., 1998a). The black discolouration is a result of host responses to infection, which include the formation of tyloses and phenolic compounds in the xylem tissues (Mugnai *et al*., 1999; Lorena *et al*., 2001; Del Río *et al*., 2004). These responses prohibit host water uptake (Edwards *et al*., 2007), and decreases the lifespan of infected vineyards. Replanting of young vineyards is subsequently required (Scheck *et al*., 1998b; Ferreira *et al*., 1999). The pathogens associated with Petri disease are well-adapted endophytes that cause disease during stress conditions (Ferreira *et al*., 1999).

Petri disease pathogens spread within vineyards as aerial inoculum from reproductive structures (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). The asexual stage of *Pa. chlamydospora* (pycnidia) has been reported as the source of aerial inoculum, but a sexual stage has not been reported (Crous and Gams, 2000; Edwards *et al*., 2001; Eskalen *et al*., 2002; Edwards and Pascoe, 2004; Baloyi *et al*., 2016). Since Mostert *et al.* (2003) confirmed the sexual stage of *Phaeoacremonium* spp. by *in vitro* pairings, five *Phaeoacremonium* sexual morph connections from isolates originating from grapevines were made, including *Pm. austroafricanum, Pm. krajdenii, Pm. viticola, Pm. parasiticum* and *Pm. minimum*. However, only three *Phaeoacremonium* sexual morphs have been found on grapevines in nature, including *Pm. minimum* (Rooney-Latham *et al*., 2005a; Baloyi *et al*., 2013), *Pm. viticola* and *Pm. fraxinopennsylvanicum* (Eskalen *et al*., 2005a; b). *Phaeoacremonium viticola* and *Pm. fraxinopennsylvanicum* perithecia have also been found on ash trees near vineyards in California, emphasizing the potential role of alternative hosts as inoculum sources (Eskalen *et al*., 2005a; b). Furthermore, the known host range of *Phaeoacremonium* species has greatly increased to include a broad range of fruit trees, ornamental plants and natural vegetation, most of which can be found in close proximity to vineyards (Spies *et al*., 2018).

Spore trapping studies have shown the presence of aerial inoculum of Petri disease pathogens in vineyards of France, California and Italy, where large spore counts coincided with rainfall events, although spores were also detected in periods of no rainfall (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Roony-Latham *et al*., 2005b; Quaglia *et al*., 2009). The spore release mechanisms of *Phaeoacremonium* perithecia might explain this phenomenon. In the absence of rainfall, but sufficient relative humidity, perithecia ooze ascospores which result in small release events, but under sufficient wetting periods, i.e. during rainfall, ascospore release takes place through forcible discharge (Rooney-Latham *et al.* 2005b).

Petri disease pathogens infect grapevines through susceptible pruning wounds (Larignon and Dubos, 2000; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011a). Wound susceptibility is influenced by several factors including cultivar susceptibility, wound age, pruning date, and climate, but pruning wounds remain susceptible for at least 3 to 4 weeks (Van Niekerk *et al*., 2011a), although they could remain susceptible for as long as 3 to 4 months (Larignon and Dubos, 2000; Eskalen *et al*., 2007; Elena and Luque, 2016). Due to the long period of wound susceptibility, it is crucial that winter and spring pruning practices do not coincide with prolific spore release, as this will lead to increased probability of new wound infections.

For South African vineyards, the frequency and patterns of spore release of Petri disease pathogens are unknown. A previous attempt to quantify and correlate spore release of Petri disease causing-pathogens with rainfall using a volumetric spore trap was unsuccessful (Van Niekerk *et al*., 2010). The aim of the present study was, therefore, to determine if spores of Petri disease pathogens were present in South African vineyards, when these spores were released, and the risks these pose as inoculum during traditional pruning periods. Whiteman (2004) developed a species-specific PCR-based detection method to detect *Pa. chlamydospora* aerial inoculum in samples collected from a grapevine rootstock block. To our knowledge, the present study is the first to investigate *Phaeoacremonium* spp. aerial inoculum in grapevine rootstock mother vine nurseries.

MATERIALS AND METHODS

Site selection

Six vineyards within the Western Cape Province, South Africa, known to be infected with Petri disease pathogens, were selected for this study. These were two vineyards each in Paarl and Stellenbosch, and one vineyard in Durbanville and one in Rawsonville (Van Niekerk *et al*., 2011b; White *et al*., 2011b; Baloyi *et al*., 2013; 2016; Moyo *et al*., 2014). Two rootstocks mother vine nurseries were also included, one in Slanghoek and the other in Wellington (Table 1). Within each vineyard, five vines or five rootstock mother plants were selected and marked. The Western Cape has a Mediterranean climate with rainy winters and dry, warm to hot summers.

Spore trapping

The protocol was adopted from Eskalen and Gubler (2001). Spore traps consisted of microscopic slides coated with Vaseline® petroleum jelly on both sides, and were affixed with wire and binder clips to the vine cordon on each of the five plants. The slides were arranged directly above old pruning wounds or cracks.

The study was conducted over two seasons, from late May to the first week of December in 2012 and from mid-March to the first week of December in 2013. The spore trap slides were replaced weekly. The slides were placed individually in sterile Falcon tubes, and immediately taken to a laboratory for processing. Each slide was washed in 5 mL of sterile distilled water (dH_2O) to suspend the spores, and the resulting spore suspension was then filtered through 5 µm and 0.45 µm microfilters. This separated large from small spores. The filters were then backwashed with 1 mL dH_20 of which 200 µL was then plated onto each of three PDA-chloramphenicol (PDA-C) agar plates. The plates were sealed with Parafilm® and incubated at 25°C for 4–8 weeks. The number of *Pa. chlamydospora* and *Phaeoacremonium* spp. colonies were monitored and recorded as a total from the three plates. *Phaeomoniella chlamydospora* colonies were morphologically identified according to Crous and Gams (2000). *Phaeoacremonium* species were subcultured for further identification. In cases where more than one colony of the same species occurred on the three dishes from one grapevine, a representative sample was subcultured.

DNA extraction, PCR and sequencing

DNA was extracted from mycelia harvested from 2-week-old *Phaeoacremonium*cultures using a CTAB protocol as described by Damm *et al*. (2008). The partial beta-tubulin gene were amplified with PCR using primers T1 and Bt2b (Glass and Donaldson, 1995; O'Donnel and Cigelink, 1997). The reactions were each performed using 0.65 units of Biotaq polymerase, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.25 pmol of each primers, 1 mg mL^{-1} of bovine serum albumin (BSA) and 5 µL of DNA solution. The amplifications were performed on a GeneAmp PCR System 2700 (Applied system Biosystems, Foster City California). The cycling conditions were 5 min at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, 1 min and 30 s at 72°C and a 7 min extension step at 72°C to complete the reaction. PCR products were visualised on a 1% agarose gel. PCR products were purified with Nucleospin MSB PCRapace kit (Invitek) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems). Sequencing products were analysed on an ABI PRISM 3130XL DNA sequencer (Perkin-Elmer) at the Central Analytical Facility of Stellenbosch University. Sequences were trimmed in Geneious 3.5.6 (Kearse *et al*. 2012). Species identification was done using the

Table 1. Vineyards and rootstock mother vine nurseries in which spore trapping studies were conducted in 2012 and 2013.

Vineyard	Cultivar	Age of vineyard	Pruning dates	
			2012	2013
Paarl A	Red Muscadel	30	26/06/2012	27/08/2013
Paarl Z	Hanepoot	40	17/07/2012	08/07/2013
Stellenbosch B3	Mixed table grape cultivar	27	24/07/2012	27/07/2013
Stellenbosch P2	Pinotage	35	10/07/2012	27/07/2013
Durbanville	Sauvignon blanc	29	31/07/2012	18/06/2013
Rawsonville	Chenin blanc	24	24/07/2012	27/05/2013
Slanghoek	Ramsey	19	29/05/2012	06/05/2013
Wellington	Ramsey	17	04/06/2012	04/06/2013

Table 2. Primers and hydrolysis probes used in this study. Oligonucleotide modifications and template specificity are indicated.

Name	Sequence	Modifications	Specificity	Reference
(Primers)				
F_ibt_Paleo	GCTTCGACGTCCTCGA	none	*Pm. minimum*	This study
F_ibt_Ppara	GCTTCGACGACCTCGA	none	*Pm. parasiticum*	This study
F_ibt_Psicil	AGCTTCGAACCATCTCGA	none	*Pm. sicilianum*	This study
R_ibt_uni	GCATTGGCCGGTCTG	none	universal	Martin *et al.* (2012)
(Hydrolysis probes)				
Paleo	CAGAATCTACCCCAGATCATCGACCAGC	5'-FAM™, 3'-QSY®	*Pm. minimum*	This study
Ppara	CGACTCTGACCCCAAAAGCATCGAC	5'-VIC®, 3'-QSY®	*Pm. parasiticum*	This study
Psicil	CCTCGATATCGTCCTCAAAATGTCTCTCAGAC	5'-JUN®, 3'-QSY®	*Pm. sicilianum*	This study

megablast function of the NCBI's GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Primer design and qPCR analysis of Phaeoacremonium minimum*,* Pm. parasiticum *and* Pm. sicilianum isolates

Primers (IDT) and hydrolysis probes (Life Technologies) were designed to bind within the beta-tubulin gene region and are listed in Table 2. Three different species-specific primer/probe sets were designed for the respective detection of *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum*, following a similar design strategy to that used for *Pm. minimum* detection by Martin *et al.* (2012). Although this meant that the *Pm. minimum* detection system for the present study was very similar to that of Martin *et al.* (2012), there were significant sequence differences in the assay primers and probe, to provide sufficient discrimination between these *Phaeoacremonium* species. Species-specific forward primers were designed, including F_ibt_Paleo for *Pm. minimum*, F_ibt_Ppara for *Pm. parasiticum* and P_ibt_Psicil for *Pm. sicilianum*, intended for use with the universal reverse primer R_IBT_uni that recognises all *Phaeoacremonium* spp. templates. Species-specific hydrolysis probes (Paleo for *Pm. minimum*, Ppara *Pm. parasiticum* and Psicil for *Pm. sicilianum*) were 5' labelled with unique fluorophores (FAM, VIC and JUN) allowing for a multiplexed experimental design. Furthermore, all hydrolysis probes carried a 3' QSY quencher (Table 2). *Phaeoacremonium minimum* detection assays were run individually, and those for *Pm. parasiticum* and *Pm. sicilianum* were in in duplex. Reactions for *Pm. minimum* detection were each run in a 20 µL final volume containing 2× KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems), 0.2 µM each of F_ibt_Paleo, R_ibt_uni and Paleo, and 0.5 µL of template (genomic DNA 10× diluted in PCR grade water). Reactions were run in a CFX96 Touch™ cycler (Bio-Rad Laboratories Inc.) using the following programme: 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 67°C. Reactions for *Pm. parasiticum* and *Pm. sicilianum* detection were each run in duplex, in a 20 µL final volume containing 2× KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems), 0.1 µM each of F_ibt_Ppara and F_ibt_Psicil, 0.2 µM each of R_ibt_uni, Ppara and Psicil, and 0.5 µL of template (genomic DNA 10× diluted in PCR grade water). Alternatively, reactions were run using the following programme: 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 65°C also using the CFX96 Touch™ cycler. All assays included non-template controls as well as controls for *Pm. minimum*, *Pm. parasiticum* and *Pm. sicilianum*. All isolates not positively identified as either *Pm. minimum*, *Pm. parasiticum* or *Pm. sicilianum* using the above assays were identified through Sanger sequencing of the beta-tubulin gene region (Central Analytical Facility, Stellenbosch University).

RESULTS

Phaeoacremonium species identification

A total of 1532 representative *Phaeoacremonium* spp. isolates were recorded from the 2012 and 2013 spore trapping seasons. Of these, 919 isolates were amplified with the *Pm. minimum* Taqman probe, 157 with the *Pm. sicilianum* probe and 24 with the *Pm. parasiticum* probe. A total of 432 isolates which did not amplify with the Taqman probes were sequenced as described above. The results for the representative sequence results are presented in Table 3.

Species diversity in vineyards

Petri disease pathogens, *Pa. chlamydospora* and a number of *Phaeoacremonium* species were found in both

Table 3. *Phaeoacremonium* species identification with the partial beta-tubulin gene using the megablast search of GenBank.

Species	GenBank accession	Identities	Gaps
Pm. italicum	EU883990.1	609/612 (99%)	0/612 (0%)
Pm. australiense	EU128073.1	683/683 (100%)	0/683 (0%)
Pm. griseorubrum	EU128075.1	747/750 (99%)	0/750(0%)
Pm. griseo-olivaceum	EU128097.1	671/676 (99%)	0/676 (0%)
Pm. inflatipes	GQ903719.1	575/582 (99%)	0/582 (0%)
Pm. iranianum	KJ200941.1	504/506 (99%)	0/506 (0%)
Pm. minimum	HQ605018.1	618/620 (99%)	0/620 (0%)
Pm. parasiticum	KF870482.1	694/695 (99%)	0/695 (0%)
Pm. prunicola	EU128095.1	551/556 (99%)	0/556 (0%)
Pm. sicilianum	KM016927.1	600/603 (99%)	0/603 (0%)
Pm. subulatum	EU128092.1	616/618 (99%)	0/618 (0%)
Pm. scolyti	EU128090.1	744/751 (99%)	0/751(0%)
Pm. venezuelense	AY579318.1	441/442 (99%)	0/442 (0%)
Pm. viticola	EU128093.1	587/590 (99%)	0/590 (0%)

Table 4. Diversity of Petri disease pathogens collected from spore traps placed in six vineyards and two rootstock mother vine nurseries in the Western Cape Province during 2012 and 2013. (X) Denotes during which year and in which vineyard a particular species was trapped.

Pathogen	Vineyard												Nursery			
	PaarlA		PaarlZ		Stellenbosch B3		StellenboschP2		Durbanville		Rawsonville		Slanghoek		Wellington	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013[a]	2012	2013
Pa. chlamydospora	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pm. minimum	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pm. alvesii								X								
Pm. australiense												X	X			
Pm. griseorubrum								X								
Pm. griseo-olivaceum									X							
Pm. parasiticum	X	X	X	X		X	X	X		X	X	X				
Pm. prunicola					X			X								
Pm. inflatipes				X				X		X						
Pm. iranianum		X		X				X								
Pm. sicilianum											X	X	X			
Pm. scolyti											X	X	X			
Pm. subulatum											X	X	X			
Pm. venezuelense												X				
Pm. viticola		X			X	X										
Total	3	5	3	5	4	4	3	8	3	4	6	8	6	2	2	2

[a] Rootstock mother block was removed 13/05/2013.

seasons of spore trapping, nine *Phaeoacremonium* species were trapped in 2012 and 14 were trapped in 2013 (Table 4). Species diversity differed between vineyards, ranging from two to six Petri disease pathogens within a vineyard in 2012, and two to eight species in 2013. The least number of species within a vineyard were found in the Wellington rootstock mother block, with only two species trapped in both seasons. The greatest diversity during 2012 was recorded in Slanghoek and Rawsonville, with six species in each vineyard, whereas Stellenbosch P2 and Rawsonville vineyards recorded the greatest diversity (eight species in each vineyard) during 2013. *Phaeomoniella chlamydospora* and *Pm. minimum* were the only species trapped in all vineyards in both seasons. *Phaeoacremonium parasiticum* was the second most frequently found *Phaeoacremonium* species in all vine-

yards at least in one of the sesons, except in Slanghoek and Wellington. Pathogens found in both seasons, but not necessarly in the same vineyard, were *Pa. chlamydospora*, *Pm. minimum*, *Pm. australiense*, *Pm. parasiticum*, *Pm. prunicola*, *Pm. sicilianum*, *Pm. scolyti*, *Pm. subulatum* and *Pm. viticola*. Pathogens only reported in 2013 were *Pm. italicum*, *Pm. griseorubrum*, *Pm. inflatipes*, *Pm. iranianum* and *Pm. venezuelense*. All the species found in the 2012 trapping period except *Pm. griseo-olivaceum* were trapped again in the 2013 season. *Phaeoacremonium sicilianum*, *Pm. scolyti* and *Pm. subulatum* were only found in Rawsonville and Slanghoek. *Phaeoacremonium italicum* and *Pm. griseorubrum* were only found in Stellenbosch P2, and *Pm. griseo-olivaceum* was only found in Durbanville. *Phaeoacremonium prunicola* was found in Stellenbosch B3 during 2012 and Stellenbosch P2 during 2013.

Spore release events

Spore trapping was carried out in the six vineyards for 29–30 weeks in 2012 and 38 weeks in 2013 (Table 5). Spore release events were observed throughout the trapping periods in all the vineyards, with only a few weeks in each where no spore events were recorded. The number of weeks with spore release events varied between vineyards, ranging from 63–100% of the total number of weeks in 2012, and 87–100% in 2013. The number of weeks with spore release events during winter (73-100 % of this 11 week period in 2012, and 82–100% in 2013) and spring pruning periods (50-100 % in 2012, and 86–100% in 2013) was of particular interest, because these are the known periods for trunk pathogen infections.

Spore trapping in the two rootstock mother vine nurseries were carried out for 27–29 weeks during 2012, and 8-38 weeks during 2013 (Table 5). The Slanghoek mother block was unexpectedly removed in 2013 so only 8 weeks of data could be collected for this nursery. In the Western Cape Province it is recommended that Ramsey rootstock canes are harvested between April and July (Hunter *et al*., 2004). Within this period 78-89% of the weeks recorded spore trapping events.

Spore release in individual vineyards and rootstock mother blocks

Paarl A

Three pathogen species were trapped in 2012, including *Pa. chlamydospora*, *Pm. minimum* and *Pm. parasiticum* (Figure 1). Spore release occurred throughout the trapping period from early May to the end of November. The greatest number of colony forming units (cfu) were detected for *Pm. minimum* (525 cfu), with release detected during 45% of the trapping weeks. However, the majority (95%) of the cfu were detected between mid July and the end of November. The greatest release event occurred at the end of November (168 cfu). *Phaeomoniella chlamydospora* spores were trapped during 66% of the weeks, but these were at very low numbers of cfu (1 to 84 cfu per week), with most of these events occurring between mid August (also the highest peak) and late November. This accounted for 91% of all cfu detected. *Phaeoacremonium parasiticum* was only recorded once, late in November (21 cfu in total). The same pathogens were trapped in 2013, together with *Pm. iranianum* and *Pm. viticola* (Figure 2). *Phaeomoniella chlamydospora* was the dominant species, with 1922 cfu detected, and was recorded during 71% of the weeks. Most of the release events and cfu counts occurred between early

Table 5. Number of weeks with spore release events in the 2012 and 2013 trapping seasons.

Year	Pruning	Vineyard						Nursery	
		Paarl A	Paarl Z	Stellenbosch B3	Stellenbosch P2	Durbanville	Rawsonville	Slanghoek[c]	Wellington[c]
2012	Winter pruning[a]	8 of 11	9 of 11	10 of 11	11 of 11	11 of 11	9 of 11	8 of 11	8 of 9
	Spring pruning[b]	10 of 13	7 of 14	12 of 14	14 of 14	13 of 14	13 of 14	N/A	N/A
	Total[e]	23 of 30	19 of 30	26 of 29	29 of 29	28 of 30	25 of 29	25 of 29	25 of 27
2013	Winter pruning[a]	11 of 11	9 of 11	11 of 11	10 of 11	11 of 11	11 of 11	6 of 6[d]	10 of 18
	Spring pruning[b]	12 of 14	14 of 14	13 of 14	14 of 14	13 of 14	13 of 14	N/A	N/A
	Total[e]	35 of 38	38 of 38	33 of 38	37 of 38	37 of 38	37 of 38	7 of 8	37 of 38

[a] Winter pruning refers to pruning from mid-June to end-August.
[b] Spring pruning refers to pruning occurring from beginning of September to end-November.
[c] The optimum time for collecting Ramsy rootststock cuttings in the Western Cape is between April and June (Hunter *et al*., 2004).
[d] Rootstock mother block was pulled out during the week of 13/05/2013 and therefore data for only 6 weeks of the optimum collection time are available.
[e] Total number of spore trapping weeks for each vineyard and the total number of weeks with spore release events.

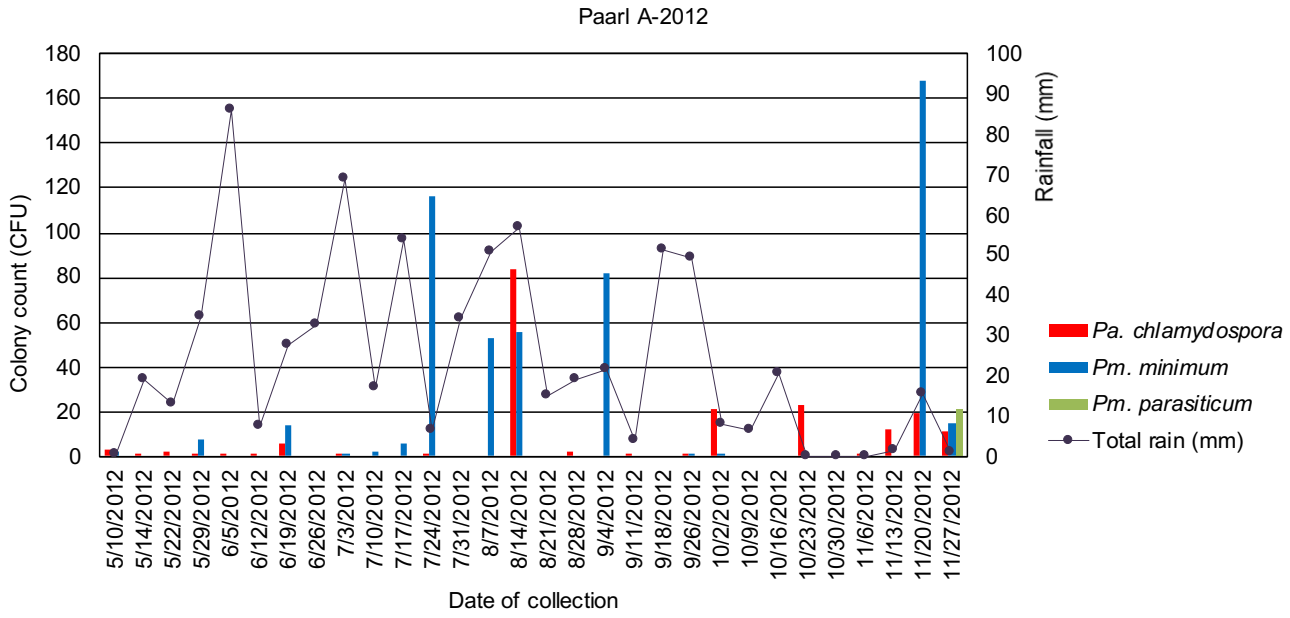

Figure 1. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Paarl A, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

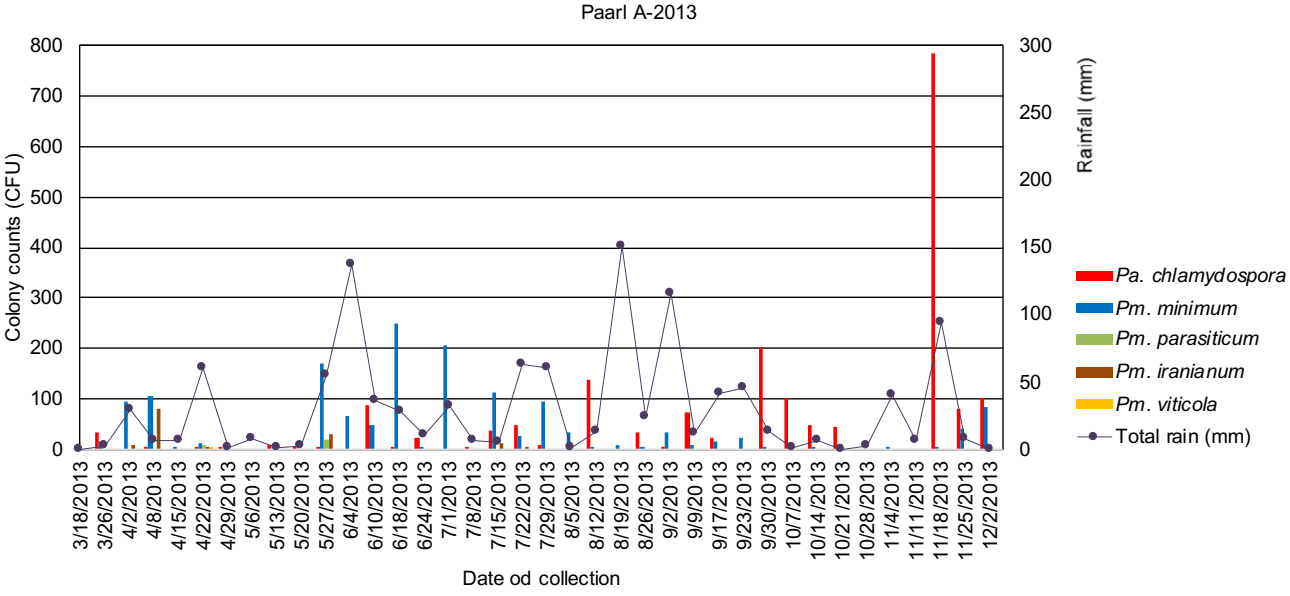

Figure 2. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Paarl A, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, *Pm. viticola* detection date is 22/04.

June and early December, accounting for 91% of all cfu detected, with the highest spore release peak in mid November (785 cfu). *Phaeoacremonium minimum* spores were released throughout the trapping period during 76% of the weeks (1472 cfu in total). The most cfu were detected between early April and late July, account-

ing for 81% of all cfu detected, with the highest peak of 250 cfu in mid June. *Phaeoacremonium iranianum* cfu were only detected during 16% of the weeks, and at low counts (140 cfu in total), between early April and mid July, with the highest peak of 80 cfu in early April. Only two small *Pm. parasiticum* release events occurred (27 cfu in total), one in mid April and one in early May, and there was only one release for *Pm. viticola* in mid April (1 cfu).

Paarl Z

Three pathogens, *Pa. chlamydospora*, *Pm. minimum* and *Pm. parasiticum*, were recorded in 2012 (Figure 3). *Phaeoacremonium minimum* was the predominant pathogen, based on the total number of cfu detected (1820) and the number of spore release events. CFU were detected in 47% of the weeks between mid May and mid November, but the most cfu were detected between early June and mid August, accounting for 95% of all the cfu detected of which 73% were in June. *Phaeomoniella chlamydospora* was recorded in 40% of the weeks between mid May and early November (453 cfu in total), with the most cfu detected in August, accounting for 69% of all cfu. *Phaeoacremonium parasiticum* was recorded only during 3 weeks (18 cfu in total), in mid May, late July and late November. The same three pathogens together with *Pm. inflatipes* and *Pm. iranianum* were trapped in 2013 (Figure 4). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of cfu detected (6613), and the number of spore release events. Spores of *Pm. minimum* were released in 84% of the weeks between mid March and late November. The highest peaks of cfu occurred between early April and early September, accounting for 97% of all cfu, with the highest peak (1340 cfu) in early April. *Phaeomoniella chlamydospora* spore release was recorded in 53% of the weeks between mid March and early December (422 cfu in total), but at low numbers of 1 to 99 cfu per week, of which 62% were detected between September and early December. The highest peak of 87 cfu was recorded in mid October. *Phaeoacremonium parasiticum* was recorded in 18% of the weeks between mid March and early November (691 cfu in total), although 95% of the total cfu were detected in April and May, with the highest peak was in early April (600 cfu). *Phaeoacremonium iranianum* was detected, mostly at low counts (217 cfu in total), during only 13% of the weeks between early April and mid July, with April accounting for 84% of the total cfu. Only one spore release event of 110 cfu was recorded for *Phaeoacremonium inflatipes* in mid June.

Stellenbosch B3

Four Petri disease pathogens were trapped during 2012, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. prunicola* and *Pm. viticola* (Figure 5). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of cfu detected (1208) and the number of large spore release events. Spore release occurred in 59% of the weeks between mid May and late November, although 91% of the total number of cfu were detected between late May and early September, with the highest cfu peak (615) in mid July. *Phaeomoniella chlamydospora* spore release occurred, at low counts (320 cfu in total), in 76% of the weeks between mid May to late November, with 85% of the cfu detected between June and early October. The highest peak occurred in early August (74 cfu). Only two low spore release events of *Phaeoacremonium prunicola* were recorded, one in late May (3 cfu) and one in early July (2 cfu), and one release event was detected for *Pm. viticola* in late May (1 cfu). Four pathogens were recorded in 2013, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum* and *Pm. viticola* (Figure 6). *Phaeoacremonium minimum* was the predominant pathogen based on the total numbers of cfu (2564) and spore release events. Spore release was recorded in 74% of the weeks from late March to late November. The majority of cfu (92%) were detected between early April and early September, with the highest peak in late May (998 cfu). *Phaeomoniella chlamydospora* was recorded, at low numbers (404 cfu in total), in 63% of the weeks between late March to late November, with 81% of the total number of cfu detected between early May and late August. The highest spore release peak (129 cfu) occurred in mid August. The only *Pm. parasiticum* spore release event was recorded in early March (4 cfu). *Phaeoacremonium viticola* was detected in early March (3 cfu) and mid October (1 cfu).

Stellenbosch P2

During 2012, *Pa. chlamydospora*, *Pm. minimum* and *Pm. parasiticum* twere trapped (Figure 7). *Phaeomoniella chlamydospora* was the predominant pathogen based on the total number of cfu (1798), with cfu detected during 90% of the weeks between mid May and late November. Consistent release events occurred between mid June and late October during which 94% of the total number of cfu were detected, with the highest peak observed in mid June (751 cfu). *Phaeoacremonium minimum* spore releases were recorded during 90% of the weeks between mid May and late November (1145 cfu in total), with three cfu peak periods, in late

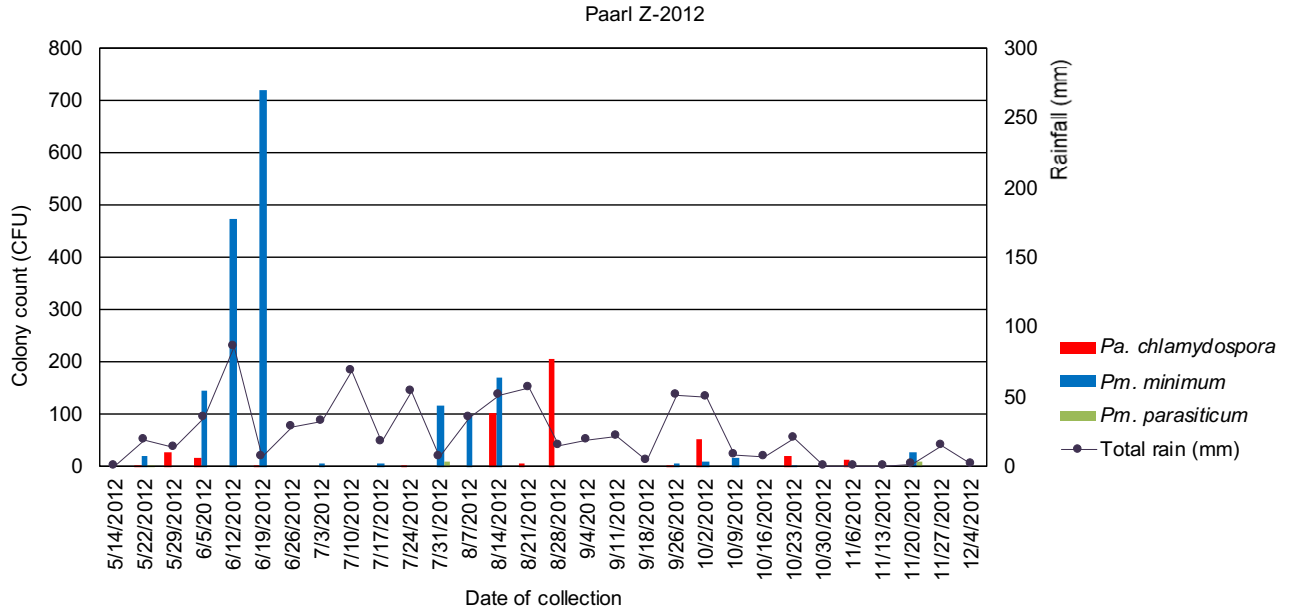

Figure 3. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Paarl Z, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, *Pm. parasiticum* detection dates are 22/05, 31/07 and 20/11.

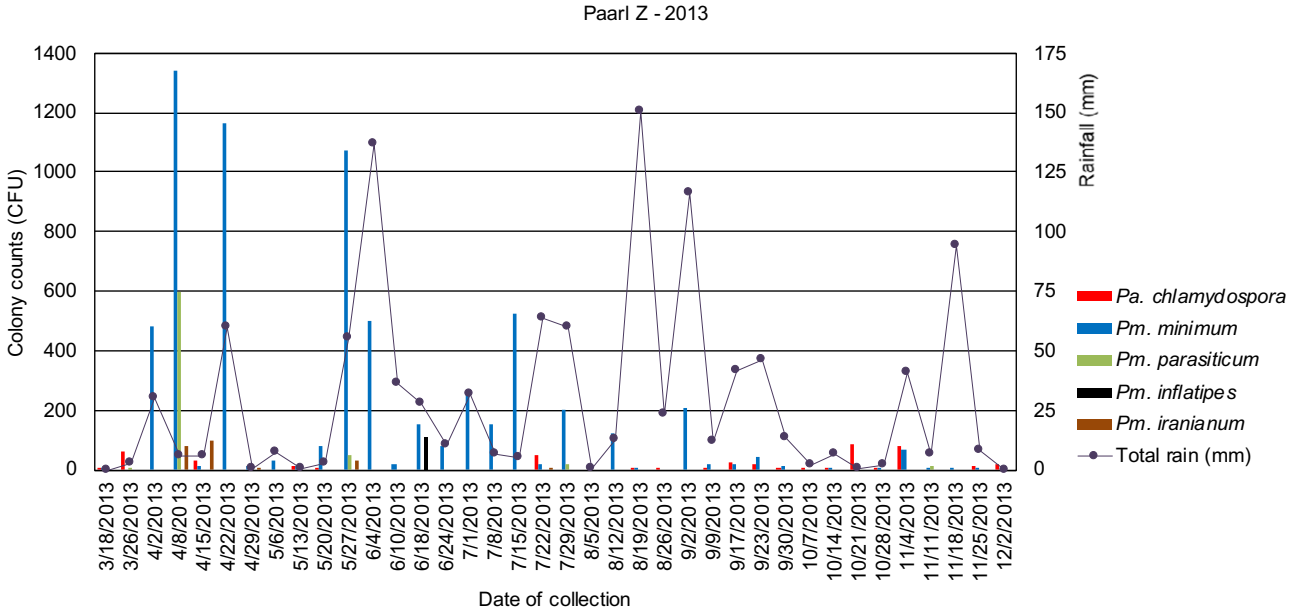

Figure 4. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Paarl Z, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

June/early July (highest peak of 279 cfu), early October (111 cfu) and mid/late November (294 cfu). These accounte for, respectively, 39, 10 and 26% of the total number of cfu detected. *Phaeoacremonium parasiticum* spore release was recorded in 34% of the weeks between mid May and early December, at very low numbers (47

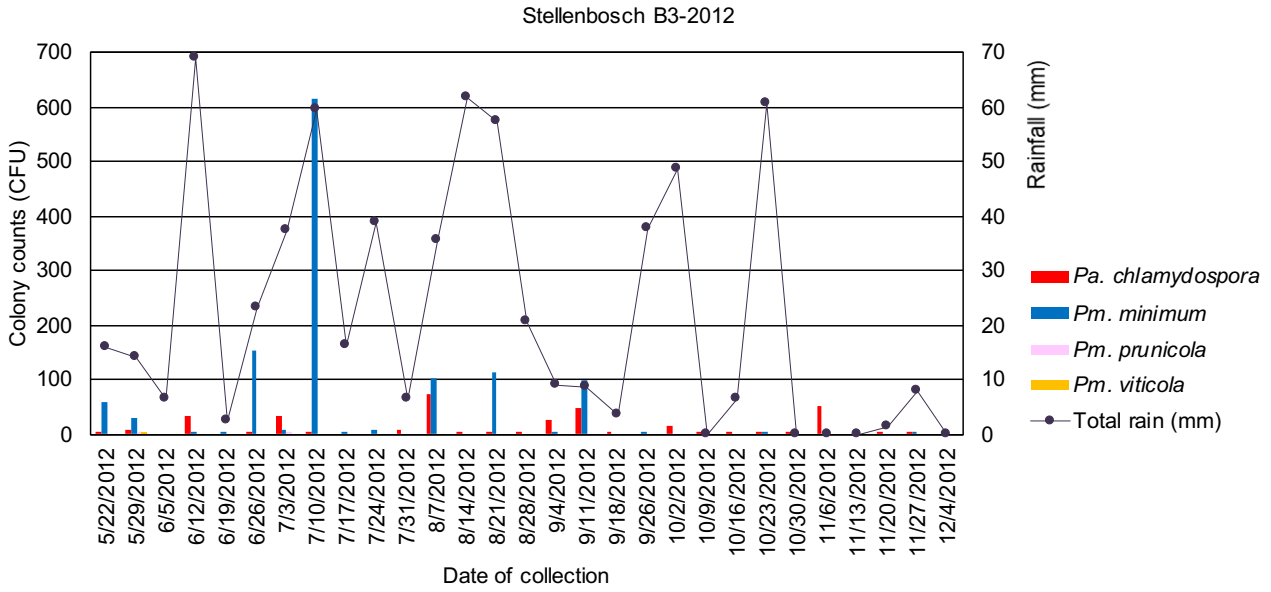

Figure 5. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Stellenbosch B3, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, *Pm. prunicola* detection dates are 29/05 and 03/07 and *Pm. viticola* is 29/05.

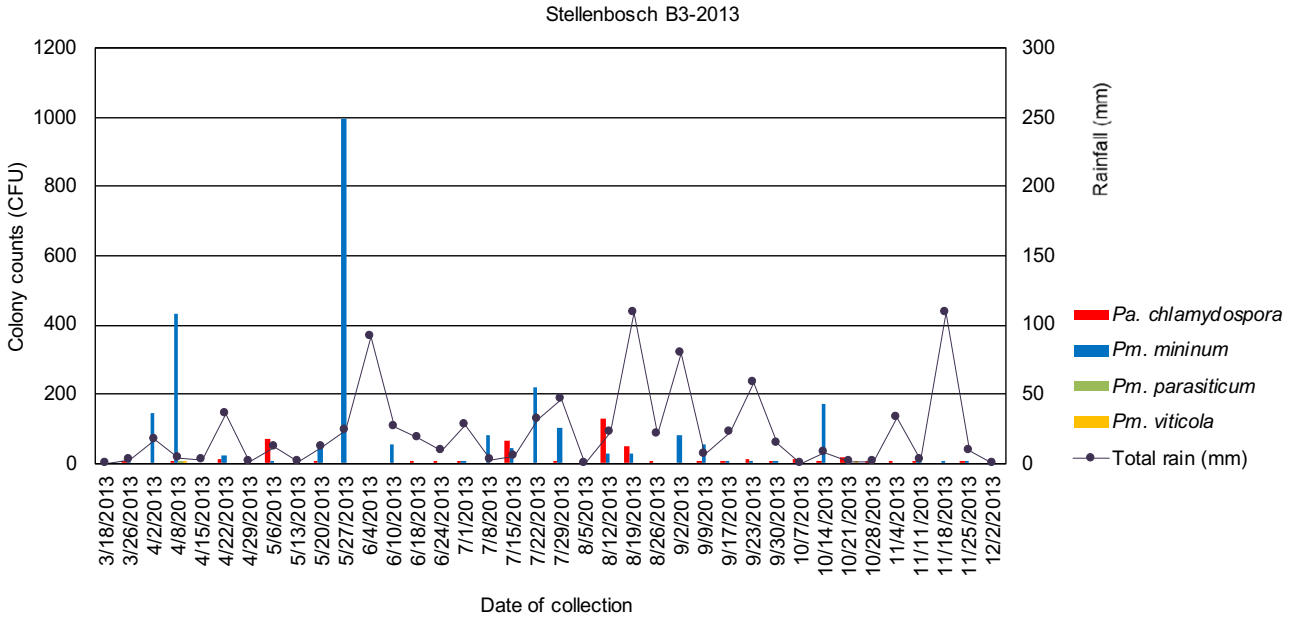

Figure 6. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Stellenbosch B3, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, detection dates for *Pm. viticola* are 08/04 and 21/10 and for *Pm. parasiticum* is 08/04.

cfu in total), with the greatest count (11 cfu) in late May. During 2013, eight Petri disease pathogens were recorded, including *Pa. chlamydospora, Pm. minimum*, *Pm. parasiticum*, *Pm. italicum*, *Pm. prunicola*, *Pm. iranianum*, *Pm. inflatipes* and *Pm. griseorubrum* (Figure 8). *Phaeomoniella chlamydospora* was the predominant pathogen based on the total number of cfu detected (2677) and number of large spore release events. Cfu of

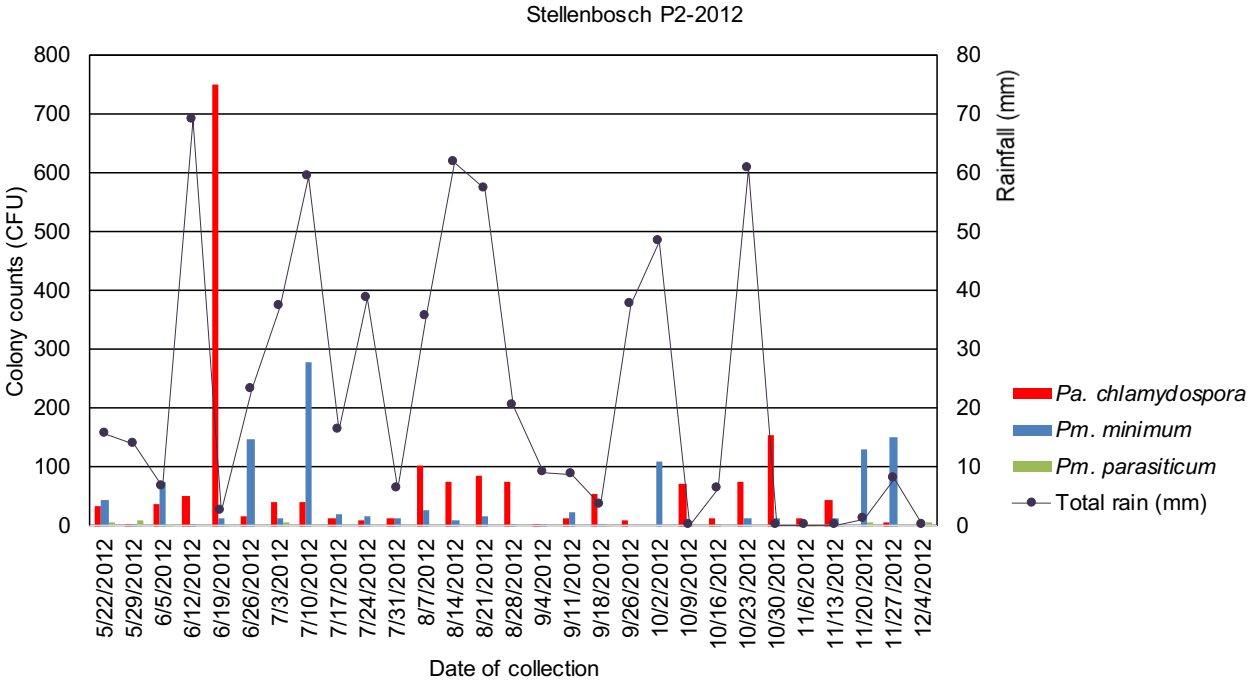

Figure 7. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Stellenbosch P2, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

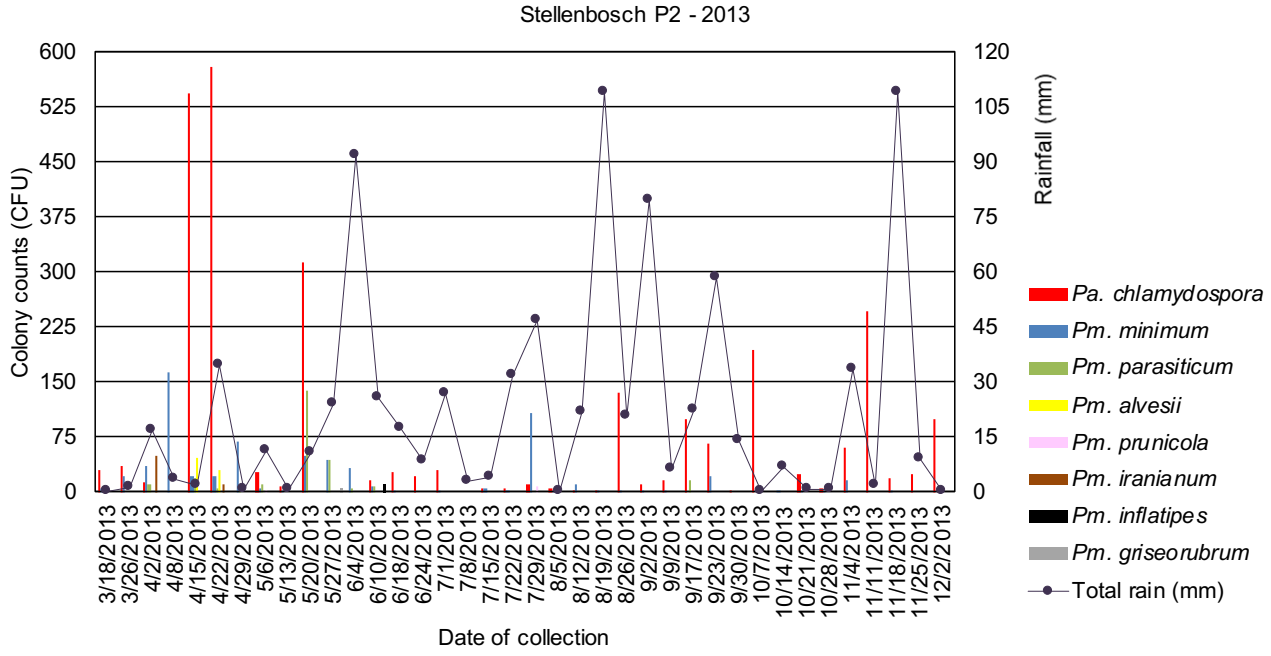

Figure 8. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in vineyard Stellenbosch P2, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, detection dates for *Pm. prunicola* are 06/05 and 29/07 and for *Pm. griseorubrum* is 27/05.

Pa. chlamydospora were recorded in 84% of the weeks between mid March to early December, with the highest cfu detection peaks observed in mid April (greatest number 580 cfu) to mid May, accounting for 55% of the total number of cfu detected, and then again during a period between late August and early December, representing 38% of the total cfu. *Phaeoacremonium minimum* cfu were detected during 79% of the weeks between mid March to late November (666 cfu in total). However, the greatest proportion (87%) of the cfu were detected between early April and late July, with the highest cfu peak recorded in early April (164 cfu). Spore release of *Pm. parasiticum* was recorded in 32% of the weeks between mid March and early December (267 cfu in total). The majority of cfu (94%) were detected between mid March and early June, with the highest cfu detection peak (139 cfu) in mid May. *Phaeoacremonium griseorubrum* and *Pm. inflatipes* were detected once in late May (5 cfu) and early June (10 cfu). *Phaeoacremonium italicum* was detected in mid March (total cfu 79), *Pm. iranianum* in early and mid March (respectively, 50 and 10 cfu), and *Pm. prunicol*a in early May (1 cfu) and late July (7 cfu).

Durbanville

Three Petri disease pathogens, *Pa. chlamydospora*, *Pm. minimum* and *Pm. griseo-olivaceum*, were trapped in 2012 (Figure 9). *Phaeomoniella chlamydospora* (626 cfu) and *Pm. minimum* (601 cfu) had very similar total cfu, and similar numbers of spore release events. *Phaeomoniella chlamydospora* spore release was recorded in 83% of the weeks between mid May to early December, although at low counts, with the highest peak (123 cfu) in late August. Spore release of *Pm. minimum* was recorded during 80% of the weeks, also at low numbers, from late May to early December, with the highest peak (122 cfu) recorded during mid August. *Phaeoacremonium griseo-olivaceum* was recorded only once (37 cfu) in early December. During 2013, four Petri disease pathogens were recorded, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum* and *Pm. inflatipes* (Figure 10). *Phaeoacremonium minimum* was the predominant pathogen, with the greatest total number of cfu detected (4016), as well as the most spore release events. Spore release was recorded during 82% of the weeks between mid March to late November, with the majority (95%) of the cfu detected between early April and late July. Although the highest cfu peak occurred at the end of June (750 cfu), the greatest total number of cfu detected per month was in April (1877 cfu). *Phaeomoniella chlamydospora* was recorded during 74% of the weeks between mid March and early December, although at low numbers (484 cfu in total). Two distinct spore release periods occurred, the first was between mid April to mid June, with the highest peak (90 cfu) occurring in late March, accounting for 59% of all the cfu. The second peak occurred between early August and early December, accounting for 38% of the toal number of cfu. *Phaeoacremonium parasiticum* was recorded during 11% of the weeks, at low numbers (26 cfu in total) between mid March and late May. *Phaeoacremonium inflatipes* was recorded in early June (50 cfu) and early July (4 cfu).

Rawsonville

During 2012, six pathogens were recorded, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. sicilianum* and *Pm. subulatum* (Figure 11). *Phaeoacremonium sicilianum* was the predominant pathogen, based on the total number of cfu detected (5605) and number of spore release events. *Phaeoacremonium sicilianum* was released during 62% of the weeks between mid May and early December, although the majority of cfu (90%) were detected between mid September and early December, with the highest cfu detection peak (1838 cfu) in mid November. *Phaeoacremonium scolyti* was released during 24% of the weeks between mid May and mid October (2194 cfu in total), with the highest cfu peaks in mid July (700 cfu) and mid October (1100 cfu). *Phaeoacremonium minimum* was recorded during 62% of the weeks, but at low counts (740 cfu in total), between early June and late December, with the highest cfu peak in mid October (313 cfu). *Phaeomoniella chlamydospora* spores were released during 55% of the weeks, also at low counts (560 cfu in total), between mid May and mid November, with the highest cfu peak (210) in mid September. However, the majority (89%) of these cfu were detected between mid July and mid November. *Phaeoacremonium parasiticum* was recorded at low counts during 10% of the weeks (46 cfu in total) in early June, early November and early December, and early December was when the highest cfu peak of 25 cfu was recorded. *Phaeoacremonium subulatum* was only trapped once in late June (16 cfu).

During 2013, eight pathogen species were trapped, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. sicilianum*, *Pm. subulatum*, *Pm. australiense*, *Pm. scolyti* and *Pm. venezuelense* (Figure 12). *Phaeoacremonium sicilianum* was the predominant pathogen, with the greatest total number of cfu detected (5748) and the greatest number of spore release events, with cfu detected during 74% of the weeks between mid March and early December. How-

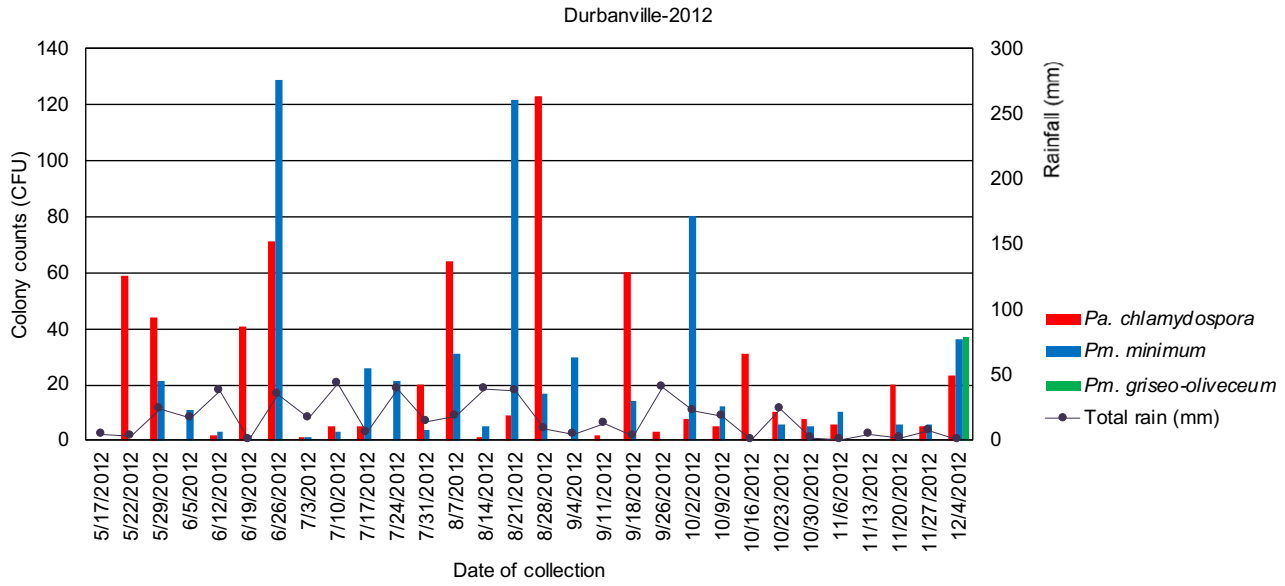

Figure 9. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in a vineyard in Durbanville, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

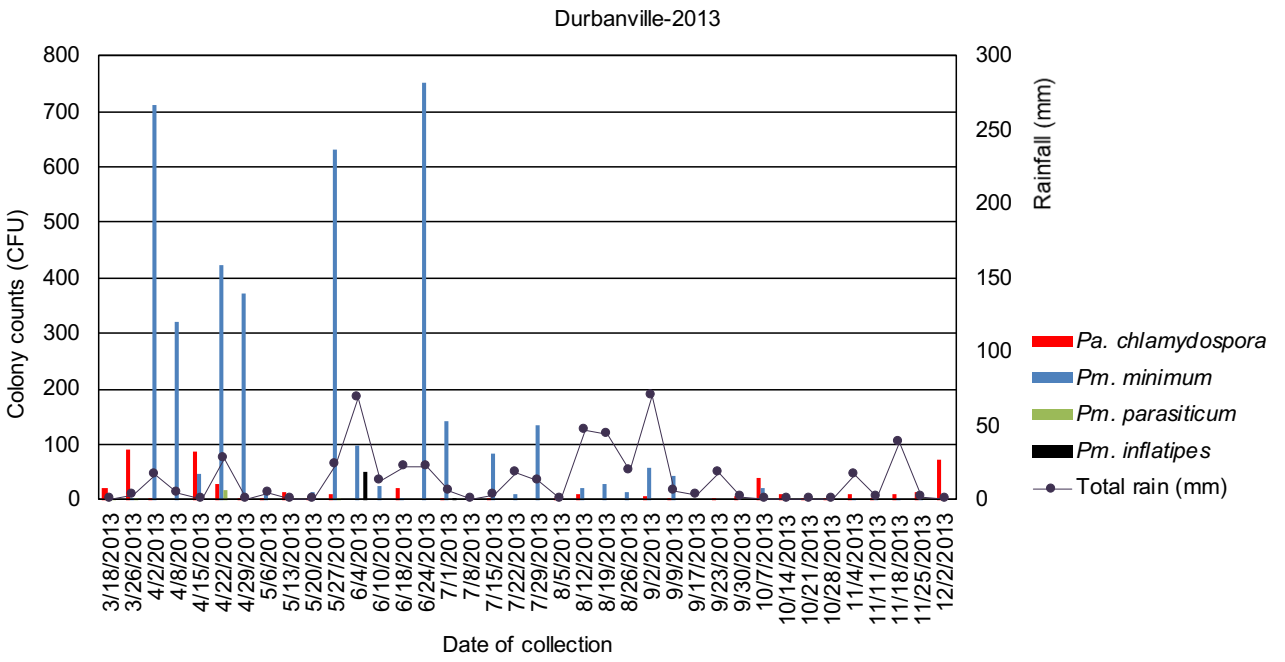

Figure 10. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in a vineyard in Durbanville, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

ever, the majority of the cfu were detected between mid March and late July, accounting for 74% of the total cfu detected, with the highest cfu detection peaks in mid April (665 cfu) and mid May (608 cfu). A high peak also occurred in early December (663 cfu), accounting for 12% of the total cfu. *Phaeoacremonium minimum* was trapped during 55% of the weeks between late March and late November (1506 cfu in total). The

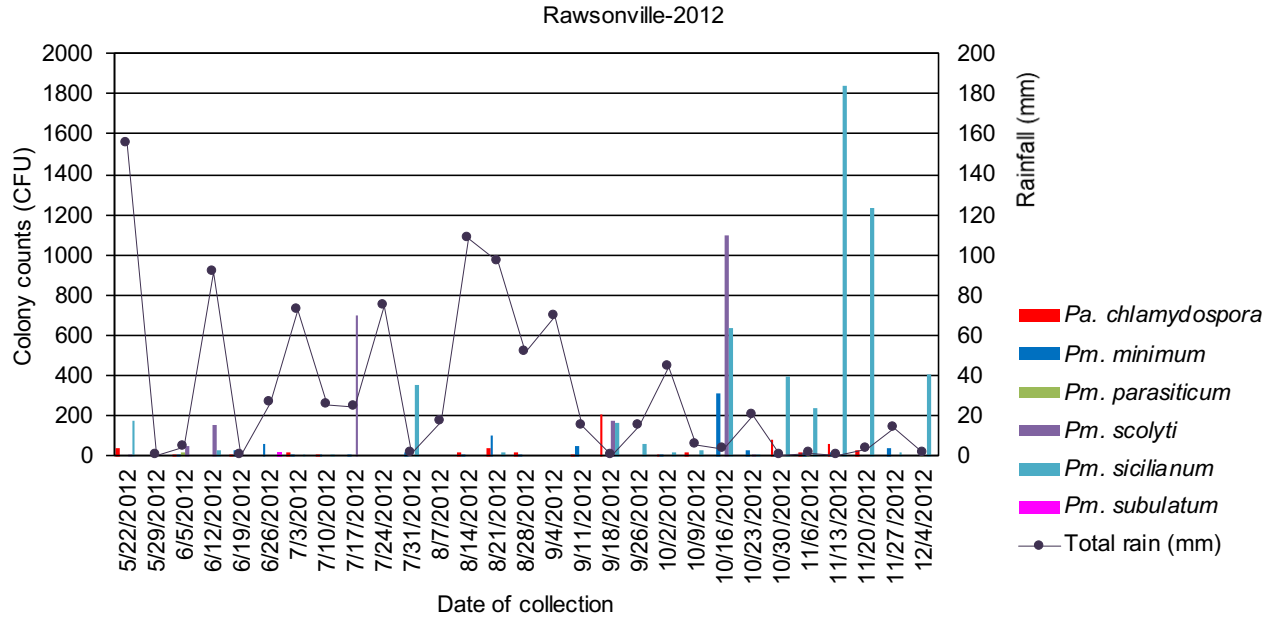

Figure 11. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in a vineyard in Rawsonville, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, detection dates for *Pm. parasiticum* are 05/06, 06/11 and 04/12 and for *Pm. subulatum* is 26/06.

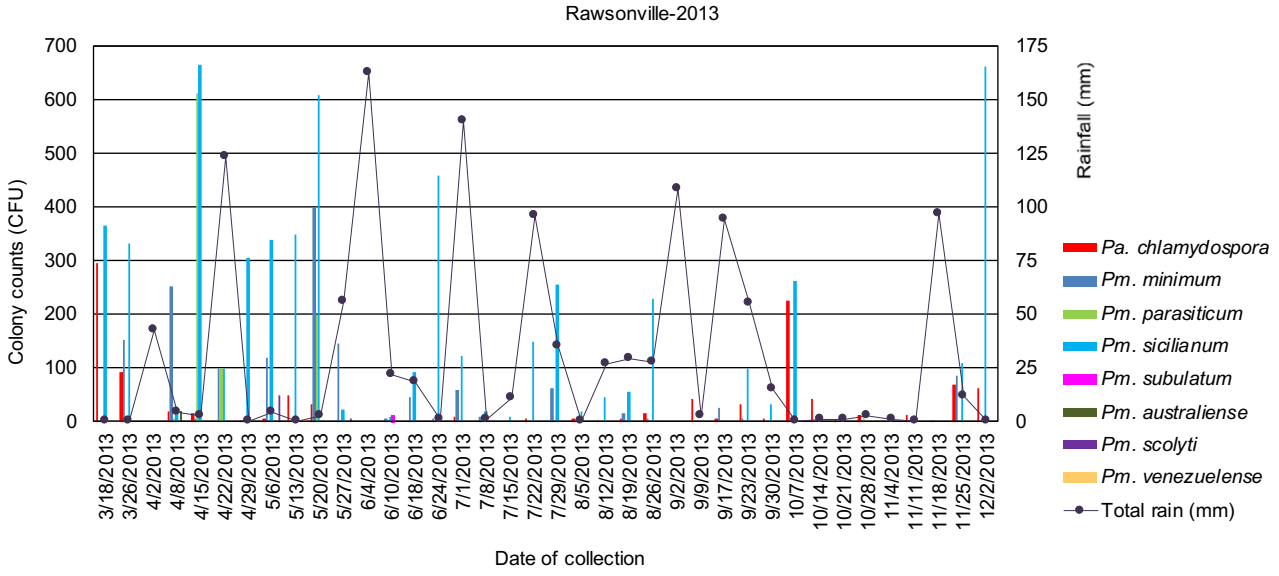

Figure 12. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to the cordons on each of five vines in a vineyard in Rawsonville, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, the detection date for *Pm. australiense* is 08/04 and for *Pm. venezuelense* is 13/05.

majority of these cfu were detected between early April and late July, accounting for 80% of all cfu detected, with the highest peak of 400 cfu recorded in mid May. *Phaeoacremonium parasiticum* was trapped during 13% of the weeks of spore trapping (924 cfu in total). The majority of these cfu (99%) were detected between early April and late May with, the highest peak in mid April (614 cfu). *Phaeomoniella chlamydospora* spores were

released during 66% of the weeks between mid March to early December (1059 cfu). The greatest number of cfu were detected during two distinct periods. The first was between mid March and late May (49% of all cfu detected), with the highest peak (295 cfu) in mid March. The second period was between early September and early December, representing 47% of the total number of cfu, with the highest peak (225 cfu) in early October. *Phaeoacremonium scolyti* spores were trapped in early May (50 cfu) and in late May (5 cfu). *Phaeoacremonium australiense* was trapped in early April (20 cfu), *Pm. venezuelense* in early May (1 cfu) and *Pm. subulatum* in early June (13 cfu).

Slanghoek

Six Petri disease pathogens were reported in 2012, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. australiense*, *Pm. scolyti*, *Pm. subulatum* and *Pm. sicilianum* (Figure 13). *Phaeomoniella chlamydospora* had the greatest total cfu count (947), and spores were released during 66% of the weeks between early June and late November. The greatest cfu numbers were detected between early July and late August, accounting for 85% of all cfu, with the highest peak in early July (587 cfu). *Phaeoacremonium minimum* was trapped during 76% of the weeks between mid June and early December (827 cfu in total), although the majority (76%) of the cfu were detected between mid June and late August, with the highest peak in late June (239 cfu). *Phaeoacremonium scolyti* was recorded during 34% of the weeks between mid June and early November (595 cfu in total), with the greatest number of cfu detected between mid June and mid August, and the highest peak (195 cfu) occurred in late June. *Phaeoacremonium australiense* was only recorded between early August, with the highest peak of 100 cfu, and early October (108 cfu in total). *Phaeoacremonium sicilianum* was only trapped once in early December (7 cfu). *Phaeoacremonium subulatum* was trapped twice, in early September (1 cfu) and mid October (3 cfu). Due to the unforeseen removal of grapevine plants, only 8 weeks of spore trapping were completed in 2013, between mid March and early May. During this period, *Pa. chlamydospora* and *Pm. minimum* were recorded (Figure 14). *Phaeoacremonium minimum* was the predominant pathogen trapped within this period, with 2693 cfu in total. Six high spore release events of 301 to 810 cfu per week were recorded, with the greatest release occurring in early April. *Phaeomoniella chlamydospora* was only detected during mid March (15 cfu in total).

Wellington

Only two pathogens, *Pa. chlamydospora* and *Pm. minimum*, were trapped during 2012 (Figure 15). *Phaeomoniella chlamydospora* was the predominant pathogen (total of 1281 cfu detected) and the number of spore release events, with spores released during 85% of the weeks between early June and early December, although the majority (82%) of the cfu were detected between early June and end of August. The highest peak of 578 cfu occurred during early June with a second peak of 188 cfu in late August. *Phaeoacremonium minimum* spore release was recorded during 74% of the weeks between early June and early December (575 cfu in total), although the majority (91%) of cfu were detected between early June and early September, with the highest peak (103 cfu) in early July. During 2013, only *Pa. chlamydospora* and *Pm. minimum* were trapped (Figure 16). *Phaeoacremonium minimum* was the predominant pathogen based on the total number of cfu (4061) and number of high spore release events, with spores trapped during 68% of the weeks between late March and early December. The majority (81%) of the spores were trapped between mid April and mid September, with two high peaks in late May (800 cfu) and mid July (800 cfu). *Phaeomoniella chlamydospora* was recorded during 42% of the weeks between mid April and early December. The majority (82%) of the cfu were detected between the early July and mid October, with the highest peak (465 cfu) detected in late July.

Spore release events during and after pruning and rootstock cane harvesting

The pruning and rootstock harvesting dates are listed in Table 1. A conservative arbitrary period of 4 weeks has been selected for illustration purposes, as the period when grapevines are likely to be most susceptible to infections after pruning, although it is known that pruning wounds remain susceptible to infection for 4 to 16 weeks (Eskalen *et al*., 2007; Elena and Luque, 2016). *Phaeomoniella. chlamydospora* and/or *Pm. minimum* spore release events occurred during the week of pruning or within 4 weeks after pruning, and rootstock cane harvesting in all the vineyards and rootstock mother blocks, although only a few *Pa. chlamydospora* and *Pm. minimum* cfu were detected during this period in the Paarl A vineyard in 2012. Cfu of *Pm. sicilianum* were also detected after pruning in Rawsonville in 2012 and 2013, while *Pm. subulatum* and *Pm. scolyti* were also trapped in 2013. *Phaeoacremonium inflatipes* spores were detected, although at very low numbers, 3 weeks after

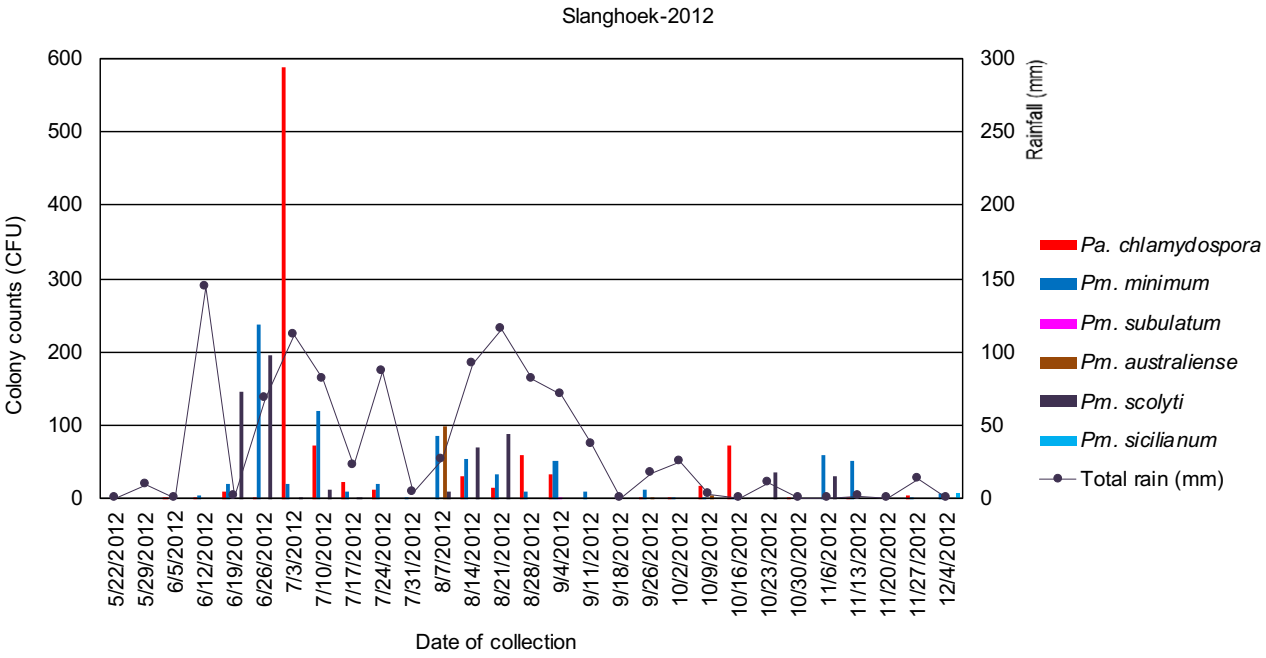

Figure 13. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to each of five rootstock mother vines in a rootstock mother block in Slanghoek, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, detection dates for *Pm. subulatum* are 04/09 and 16/10.

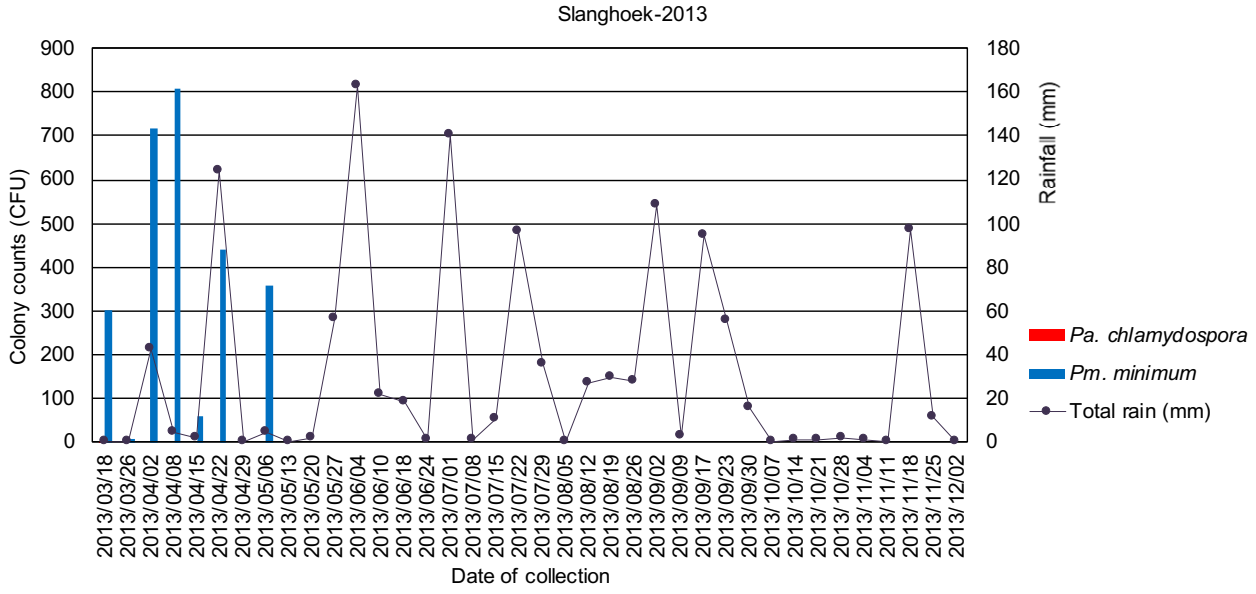

Figure 14. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to each of five rootstock mother vines in a rootstock mother block in Slanghoek, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes. Not clear on the graph, the detection date for Pa. chlamydospora is 18/03. The rootstock mother block was removed 13/05/2013 and therefore no sampling occurred beyond this date.

pruning in Durbanville in 2013, and *Pm. prunicola* in Stellenbosch P2 in 2013. *Phaeoacremonium iranianum* and *Pm. parasiticum* were trapped within 3 and 4 four weeks of pruning in Paarl Z in 2013, while *Pm. parasiticum* was trapped within 3 weeks in 2012. No cfu were detected during the first 2 weeks of harvesting rootstock

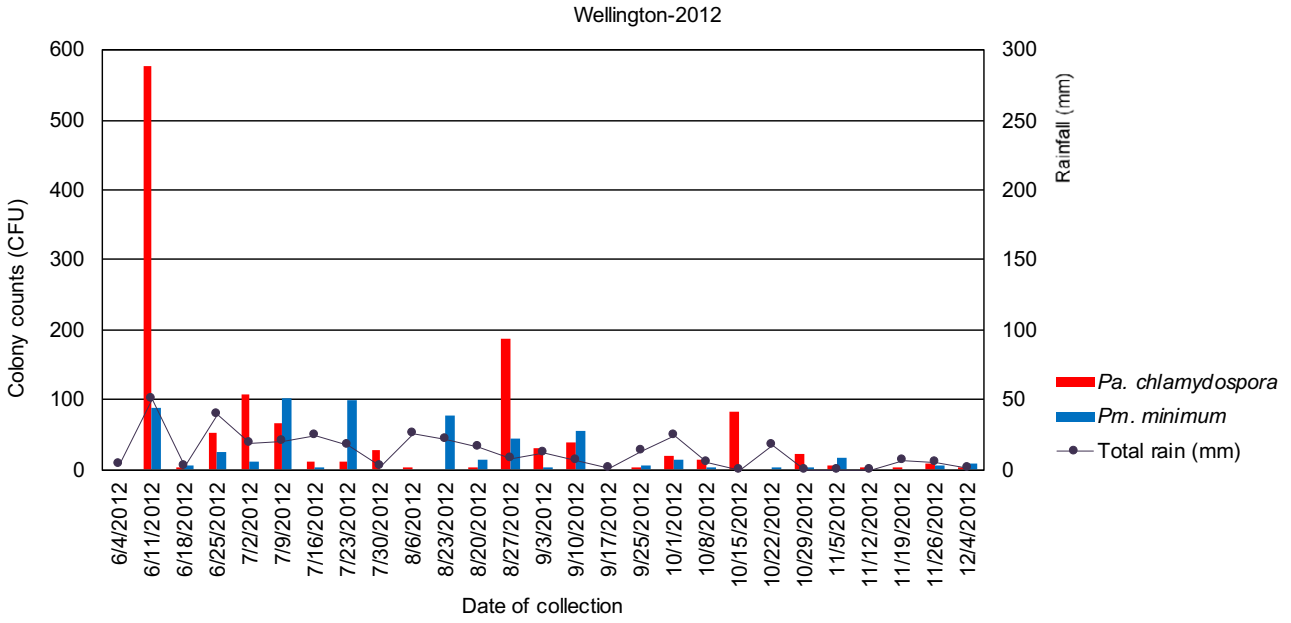

Figure 15. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to each of five rootstock mother vines in a rootstock mother block in Wellington, Western Cape province in 2012. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

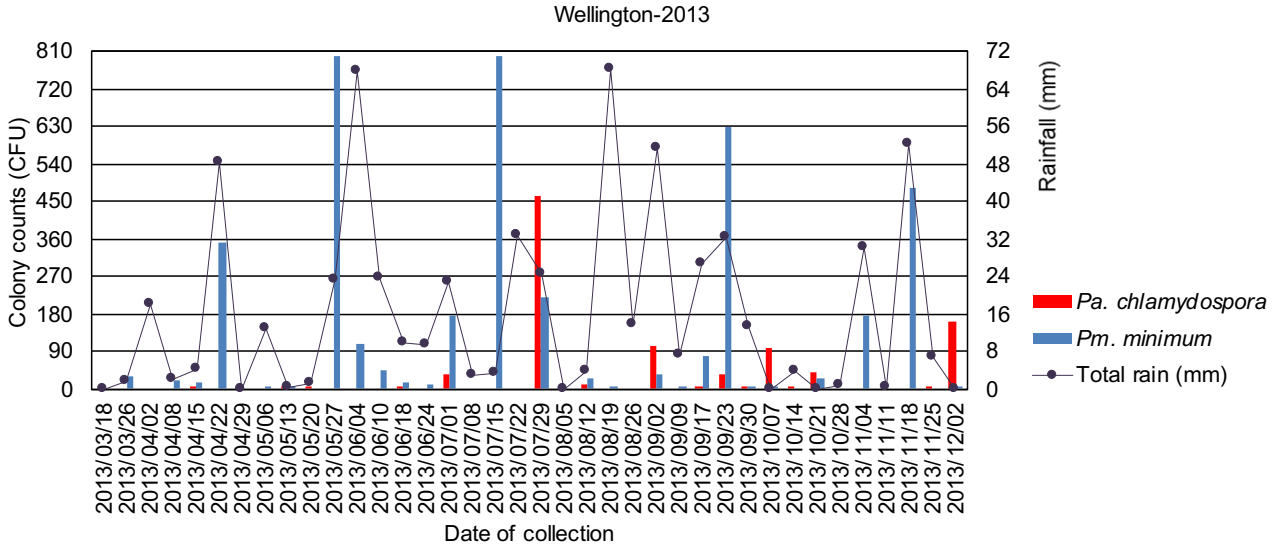

Figure 16. *Phaeomoniella* and *Phaeoacremonium* spp. cfu cultured from five vaseline coated microscope slides affixed to each of five rootstock mother vines in a rootstock mother block in Wellington, Western Cape province in 2013. The slides were replaced weekly. Each bar represents the total number of cfu cultured on three Petri dishes.

canes in Slanghoek, although a few *Pa. chlamydospora* and *Pm. minimum* cfu were detected 3 and 4 weeks after harvesting, with significant cfu counts of *Pm. scolyti* during weeks 4 and 5.

DISCUSSION

The present study conducted during the 2012 and 2013 seasons, trapped aerial spores of *Pa. chlamydospo-*

ra and *Phaeoacremonium* species from six vineyards and two rootstock mother vine nurseries located in six grape-growing areas of the Western Cape Province. A total of 14 *Phaeoacremonium* species were detected, including *Pm. australiense*, *Pm. griseo-olivaceum*, *Pm. griseorubrum, Pm. inflatipes, Pm. iranianum, Pm. italicum, Pm. minimum*, *Pm. parasiticum*, *Pm. prunicola, Pm. scolyti*, *Pm. sicilianum*, *Pm. subulatum, Pm. venezuelense* and *Pm. viticola*. Compared to similar spore trapping studies conducted in other countries, this is the greatest species diversity recorded in vineyards. Only two Petri disease pathogens were detected in vineyards in France (*Pa. chlamydospora* and *Pm. minimum*) (Larignon and Dubos, 2000) and three pathogens in California (*Pa. chlamydospora*, *Pm. minimum* and *Pm. inflatipes*) (Eskalen and Gubler, 2001). Of the 17 *Phaeoacremonium* species previously isolated from diseased grapevines in South African vineyards (Crous *et al*., 1996; Mostert *et al*., 2005; Mostert *et al*., 2006a; White *et al*., 2011a: Spies *et al*., 2018), only *Pm. krajdenii*, *Pm. austroafricanum* and *Pm. fraxinopennsylvanicum* could not be cultured from spore traps during the present study. This study is the first to report aerial inoculum of *Pm. australiense, Pm. griseo-olivaceum, Pm. griseorubrum, Pm. iranianum, Pm. italicum, Pm. parasiticum, Pm. prunicola, Pm. scolyti, Pm. sicilianum, Pm. subulatum, Pm. venezuelense and Pm. viticola* in vineyards.

Two pathogen species, *Pa. chlamydospora* and *Pm. minimum*, were trapped in all the vineyards and rootstock mother vine nurseries during both seasons of the study. The reason for their abundance as aerial inoculum was probably linked to the occurrence of their fruiting bodies in Western Cape vineyards (Baloyi *et al*., 2013; 2016), and possibly for *Pm. minimum,* the presence on *Proteaceae* twig litter (Marincowitz *et al*., 2008). One of these two pathogens was the predominant species detected in all vineyards, except in Rawsonville where *Pm sicilianum* was the predominant species. *Phaeomoniella chlamydospora* and *Pm. minimum* are widely distributed throughout most grape growing regions of the world (Mostert *et al*., 2006a; Gramaje *et al*., 2015), which is testimony to their adaptive characters and abilities to grow in a wide range of biotic conditions (Whiting *et al*., 2001; Mostert *et al*., 2006a). *Phaeoacremonium sicilianum* was previously isolated from vineyards in Calitzdorp (White *et al*., 2011a) and was also found in Italian (Essakhi *et al*., 2008) and Spanish vineyards (Gramaje *et al*., 2009). However, fruiting structures of this species have not been found in nature or through *in vitro* mating studies, and the origin of this inoculum is still unclear. Similar to previous isolation studies, after *Pm minimum*, *Pm. parasiticum* appears to be the most common *Phaeoacremonium* species associated with grapevine (Mostert *et al*., 2006a), as this species was detected in four and six vineyards, respectively, during 2012 and 2013, with significant numbers of spores released in at least two vineyards during 2013. Perithecia of *Pm. parasiticum* have been found on *Proteaceae* twig litter in the Western Cape (Marincowitz *et al*., 2008), but not from grapevines. Altough *Pm. scolyti* only occurred in Rawsonville (in 2012 and 2013) and Slanghoek (only 2012), significant spore releases occurred especially during the 2012 season in Rawsonville. Together with *Pm minimum*, *Pm. scolyti* was also the most dominant species isolated from *Prunus* spp. (Damm *et al*., 2008).

The total number and composition of *Phaeoacremonium* species varied between vineyards and seasons. Vineyards of Stellenbosch P2 and Rawsonville had the greatest species diversity (eight species each), compared to the two species found in the Wellington rootstock mother vine nursery. The reason for this is unknown, but the age of the vineyards, proximity to other older vineyards, proximity to other woody hosts and sanitation practices may play roles. The Wellington rootstock mother vine nursery was more isolated from other fruit orhards and woody hosts, and although there are other rootstocks and vineyards within the immediate vicinity, the sampled nursery was surounded by wheat fields. Some of the *Phaeoacremonium* species that were detected less frequently occurred within the same or nearby geographical areas. For example *Pm. prunicola* only occurred in the two Stellenbosch vineyards, *Pm. australiense*, *Pm. subulatum*, *Pm. scolyti* and *Pm. sicilianum* were trapped in the Rawsonville and Slanghoek areas, while *Pm. iranianum* was trapped in the two Paarl vineyards. These results suggests that aerial spores were dispersed between vineyards that were in close proximity to each other. This can also be the case for vineyards established in close proximity to fruit orchards, ornamental trees or numerous other woody hosts. All the vineyards were in close proximity to other vineyards, fruit orchards or other woody hosts. Stellenbosch P2 was located directly next to a plum orchard and various other *Prunus* spp. were just a few metres further away. The extent of the host ranges associated with *Phaeoacremonium* species in South Africa was recently confirmed by Spies *et al.* (2018), who linked *Pm. scolyti* with 20 woody hosts, *Pm. minimum* with 19, *Pm. parasiticum* with 17, *Pm. inflatipes* with 11, *Pm. italicum* with ten, *Pm. subulatum* with ten, *Pm. prunicola* with ten, *Pm. viticola* with eight, *Pm. iranianum* with seven, and *Pm. sicilianum* with four woody hosts.

Eight *Pm.* species associated with *Prunus* spp. in South Africa (Damm *et al*., 2008) were trapped as aeri-

al inoculum in the present study, including *Pm. scolyti, Pm. minimum, Pm. australiense, Pm. griseo-olivaceum, Pm. griseorubrum, Pm. iranianum, Pm. prunicola* and *Pm. parasiticum*. The possibility that *Phaeoacremonium* sexual morphs may form on alternative hosts in close proximity to vineyards further emphasizes the importance of these hosts as inoculum sources. Eskalen *et al.* (2005a; b) found *Pm. fraxinopennsylvanicum* and *Pm. viticola* perithecia on ash trees growing in close proximity to vineyards in California.

Petri disease pathogens were detected throughout the trapping periods in all the vineyards, with only occasional weeks of no spore release. However, cfu numbers differed between the vineyards. The reasons for this are unknown, but these could be related to differences in inoculum type (sexual or asexual), inoculum abundance and/or favourable microclimatic conditions. Spore release of the two most predominant species, *Pa. chlamydospora* and *Pm. minimum,* occurred in a large proportion of the vineyards during most of the sampling months, although sometimes in very low numbers. However, if the total numbers of cfu detected in all the vineyards are taken into consideration, the greatest spore release events for *Pa. chlamydospora* differed between the two seasons. During 2012, the peak spore release months occurred in winter declining towards spring, while during the 2013 season spore release increased in spring and early summer with another high peak in autumn. The greatest spore release events for *Pm. minimum* were in winter 2012 and in autumn/winter 2013. *Phaeoacremonium sicilianum* possibly favoured the warm humid months of spring/early summer (2012) and autumn (2013), while *Pm. parasiticum* spore numbers peaked in autumn and those of *Pm. scolyti* peaked in winter/spring. For the other species, it is difficult to determine specific patterns, or to link them to specific seasons, because of the low detection numbers, species distribution amongst the vineyards and seasonal differences. In Californian vineyards, *Pa. chlamydospora*, *Pm. minimum* and *Pm. inflatipes* were also trapped throughout the year, but *Pa. chlamydospora* and *Pm. inflatipes* spore release coincided with rainfall events mostly in late winter and early spring. Trapping of *Pm. minimum* coincided less with rainfall events and even during periods of no rain during early to mid summer (Eskalen and Gubler, 2001). However, Rooney-Latham *et al.* (2005b) contradicted this when they reported large numbers of *Pm. minimum* spores that were trapped following rain in Californian vineyards, a phenomenon linked to the ability of *Pm. minimum* asci to forcibly discharge ascospores after thorough wetting. Trapping of *Pm. minimum* spores in dry summer months was attributed to overhead irrigation. In French vineyards *Pa. chlamydospora* was also trapped throughout the year, although pruning wound infections correlated with winter rainfall events, whereas *Pm. minimum* were mostly trapped during the grapevine vegetative period (Larignon and Dubos, 2000).

Spore release occurring during winter (June-August) and spring (September-November) coincides with traditional winter and spring grapevine pruning periods in the Western Cape Province. Winter and spring wounds remain susceptible for long periods (Eskalen *et al*., 2007; Van Niekerk *et al*., 2011a; Makatini, 2014). In the present study, spore release events were observed during the 4-week period after winter pruning when wounds are at their most susceptible to infections. During this period, aerial inoculum of *Pa. chlamydospora*, *Pm. minimum*, *Pm. parasiticum*, *Pm. inflatipes*, *Pm. prunicola*, *Pm. iranianum*, *Pm. sicilianum*, *Pm. scolyti* and *Pm. subulatum* were recorded. The capability of *Pa. chlamydospora* to infect spring wounds under field conditions has recently been shown (Makatini, 2014). Spring wounds also stayed susceptible to infection for 4 weeks, although susceptibility declined after the first week. The large number of spore release events, large aerial inoculum numbers and large diversity of Petri disease pathogens emphasize that pruning activities in South African vineyards occur during periods of high risk of infections. This shows that wound protectants that provide prolonged protection against Petri disease pathogens and those that cause other grapevine trunk diseases are very important.

The occurrence of aerial spore inoculum of *Pa. chlamydospora* and *Phaeoacremonium* species in rootstock mother vine nurseries is of concern, especially since these pathogens were trapped within the week in which cuttings were harvested as well as subsequent weeks when the wounds were still susceptible. The high *Phaeoacremonium* species diversity (six) within the Slanghoek mother vine nursery should be of major concern for South African vine improvement efforts. There are currently no guidelines on the productive lifespans of rootstock mother vines. From the present results it is clear that inoculum may build up in particular blocks, most likely increasing as the vines age. There are also no guidelines, or any regulations, regarding pruning wound protection or management of grapevine trunk diseases in general. The risks of mother vines becoming infected and supplying propagation material with compromised phytosanitary status could seriously impact spread of trunk diseases, as infected asymptomatic shoots may be used for grafting or planting into newly established vineyards with severe disease transmission results (Bertelli *et*

al., 1998; Fourie and Halleen, 2002; Halleen *et al*., 2003; Fourie and Halleen, 2004).

Establishing new vineyards in close proximity to older vineyards is a common practice in South Africa. In this study, spore traps were also placed in newly established vineyards adjacent to two of the spore trapping vineyards, one in Rawsonville and one in Paarl (data not shown). Pathogens trapped in the older vineyards were also trapped in the young vineyards, including *Pa. chlamydospora*, *Pm. minimum*, *Pm. sicilianum* and other trunk disease pathogens. Petri disease pathogens can also be dispersed from one vineyard to another by arthropods which act as vectors (Moyo *et al*., 2014), and on pruning shears (Augustí-Brisach *et al*., 2015). This emphasizes the need to adopt integrated disease approaches throughout vineyards, from as early as the establishment phase by sanitation practices to reduce inoculum sources and wound protection strategies to prevent infections. Gramaje and Armengol (2011) and Gramaje *et al*. (2018) have provided a comprehensive international overview of grapevine trunk disease management during the propagation process as well as in established vineyards.

The aim of this study was to provide new insight into the inoculum ecology of Petri disease pathogens in South African vineyards and rootstock mother vine nurseries. In total, 15 Petri disease pathogens, including *Pa. chlamydospora* and 14 *Phaeoacremonium* species were trapped as aerial spore inoculum. The spore release events coincided with periods of pruning and rootstock harvesting. Spores were available even during spring pruning and the late summer periods, which some viticulturist believe to be free of spores and consequently correct times to conduct clean pruning practices where large wounds are created. Wound protectants should be applied whenever vine wounding occurs, irrespective of the time of the year. New wound protectant formulations should consider the period of wound susceptibility, as well as the constant availability of aerial spore inoculum of many pathogens. However, to determine the best timing for such preventive management options, further research into the development of epidemiological models accounting for the specific environmental conditions required for spore dispersal and infection should be undertaken. A recent first step was achieved with a model to evaluate disease risk for *Pa. chlamydospora* in Spain, where hydro-thermal time was shown to be the best descriptor for predicting dispersal of this pathogen (González-Domínguez *et al*., 2020). Further studies on sources of inoculum within vineyards and mother vine nurseries are also highly recommended in order to reduce aerial pathogen inoculum.

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Research Papers

Yield loss estimation and pathogen identification from Botryosphaeria dieback in vineyards of Central Chile over two growing seasons

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Summary. Dieback symptoms have been increasingly reported in Chilean vineyards over recent years. Although there have been studies on *Botryosphaeriaceae* species and associated trunk disease incidence and severity in table grape-producing vineyards, their impacts on 'Cabernet Sauvignon', the most planted red wine grape in Chile, is unknown. This study determines the fungus species, incidence, disease severity, and yield losses associated with Botryosphaeria dieback in Chilean 'Cabernet Sauvignon' vineyards. Nine vineyards were surveyed during two growing seasons (2010 and 2018), and symptomatic wood samples were taken. Total potential production and yield losses were estimated from spur counts (2010) from harvested vines (2018) with different degrees of infection. Overall disease incidence was 87% in 2010 and 84% in 2018. Severity was 49% in 2010 and 47% in 2018. Yield losses were 39% in 2010 and 46% in 2018. *Diplodia seriata* was the most prevalent fungus isolated from symptomatic plants in both growing seasons. This study highlights the impacts of grapevine trunk diseases in vineyards in Central Chile, and indicates the need for improved disease management strategies.

Keywords. *Vitis vinifera*, 'Cabernet Sauvignon' disease incidence, disease severity, yield loss.

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is the most widely cultivated fruit species in Chile with an area of approx. 190,000 ha for the production of table grapes, spirits and wine. As the leading table grape exporter and the fourth largest exporter of wine worldwide (ODEPA, 2019a, 2019b), Chile exported

846 million liters of wine with a value of US $ 2 billion (ODEPA, 2019c) in 2018. 'Cabernet Sauvignon', which represents 19% of total Chilean wine exports, is the most widely planted nationwide, principally concentrated in Central Chile and particularly in the O'Higgins and Maule regions (SAG, 2019). Reports of generalized grapevine dieback (i.e., vine decay, perennial cankers, wood necroses, progressive sprouting failure, death of cordons and plants) have significantly increased in Chilean vineyards in recent years (Morales *et al*., 2012; Díaz *et al*., 2013; Besoain, 2018). While dieback symptoms in table grape cultivars are commonly associated with *Botryosphaeriaceae* spp. (Latorre *et al*., 1986; Auger *et al*., 2004; Díaz *et al*., 2011; Morales *et al*., 2012) with incidence of up to 69% in adult plants (Morales *et al*., 2012), symptoms have also been described in other Chilean winegrape cultivars (Auger *et al*., 2004; Besoain *et al*., 2013; Díaz *et al*., 2011, 2013). Although 'Cabernet Sauvignon' has been shown to be susceptible to grapevine trunk diseases (GTDs) (Larignon *et al*., 2009; Travadon *et al*., 2013), incidence, severity and impacts caused by *Botryosphaeriaceae* in Chilean 'Cabernet Sauvignon' remains unknown.

Studies of pathogens associated with Botryosphaeria dieback have identified 26 species (Gramaje *et al*., 2018), with prominent representation from *Botryosphaeria, Diplodia, Lasiodiplodia* and *Neofusicoccum* (Úrbez-Torres, 2011; Gramaje *et al*., 2018; Billones-Baaijens and Savocchia, 2019). Botryosphaeria dieback of grapevines is mainly associated with cankers and dead spurs and cordons with no general foliar symptoms and wood damage, with brown streaks, discolouration, and characteristic wedge-shaped perennial cankers (Larignon *et al*., 2001; Van Niekerk *et al*., 2004; Úrbez-Torres, 2011; Spagnolo *et al*., 2014; Fontaine *et al*., 2015; Besoain, 2018; Gramaje *et al*., 2018).

Botryosphaeria dieback symptoms are ubiquitous (Duthie *et al*., 1991; Úrbez-Torres, 2011; Morales *et al*., 2012; Baskarathevan *et al*., 2012; Bertsch *et al*., 2013; Gramaje *et al*., 2018), and the disease causes significant production losses and disease management costs. For example, Hillis *et al*. (2016) reported that Californian vineyards spent an average of $US 477 ha^{-1} per year to prevent and manage GTDs. In Napa, California, cumulative losses due GTDs (including Botryosphaeria dieback) in 'Cabernet Sauvignon' vineyards was estimated at $US 160,000 per acre ($US 400,000 ha^{-1}) over a 25 -year vineyard lifespan (Kaplan *et al*., 2016).

Given the importance of 'Cabernet Sauvignon' to the Chilean wine industry, the present study aimed to: (i) determine the incidence, severity, and yield losses associated with Botryosphaeria dieback in 'Cabernet Sauvignon' vineyards in two wine-producing regions of Chile; and (ii) identify the fungi causing this disease.

MATERIALS AND METHODS

Vineyard sampling

Samples were taken from fourteen blocks in nine vineyards distributed across the O'Higgins and Maule regions of Chile, during the growing seasons of 2010-2011 and 2018-2019. The exception was for vineyard number 9, block 14, which was only sampled in the 2010-2011 growing season (Table 1). All the contained

Table 1. Vineyards and blocks 'Cabernet Sauvignon' analyzed, located in Central Chile.

Region/Locality	Vineyard	Block	Planting Year	Sampling season/plant age	Sampling season/plant age
O'Higgins/Chépica	1	1	1996	2010-11/15	2018-19/23
O'Higgins/Palmilla	2	2	1991	2010-11/20	2018-19/28
O'Higgins/Palmilla	2	3	2005	2010-11/06	2018-19/14
O'Higgins/Palmilla	3	4	1995	2010-11/16	2018-19/24
O'Higgins/Peralillo	4	5	1991	2010-11/20	2018-19/28
O'Higgins/Peralillo	5	6	1996	2010-11/15	2018-19/23
O'Higgins/Peralillo	5	7	1996	2010-11/15	2018-19/23
O'Higgins/Peralillo	5	8	1995	2010-11/16	2018-19/24
Maule/Batuco	6	9	1997	2010-11/14	2018-19/22
Maule/Pencahue	7	10	1997	2010-11/14	2018-19/22
Maule/Pencahue	7	11	2002	2010-11/09	2018-19/17
Maule/Pencahue	8	12	1997	2010-11/14	2018-19/22
Maule/Pencahue	8	13	1997	2010-11/14	2018-19/22
Maule/Talca	9	14	2003	2010-11/08	

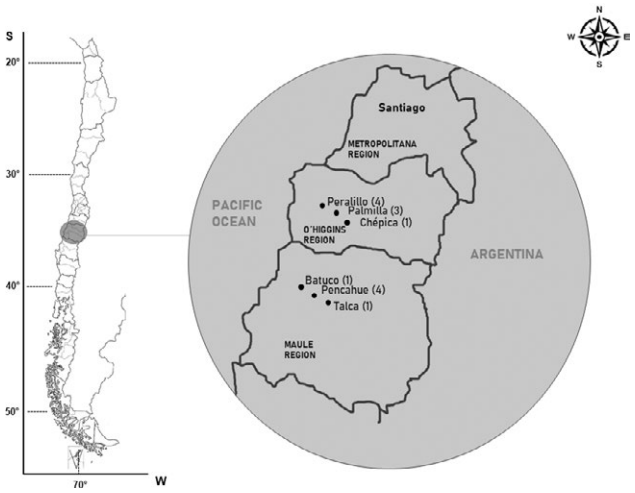

Figure 1. Sampling localities (black dots) in the Maule and O'Higgins Regions, Central Chile. The number of blocks analyzed in each locality is indicated in parentheses.

'Cabernet Sauvignon' non-grafted vines trained using the bilateral cordon system, which were spur pruned to an average of 12 spurs per plant. The majority of the vineyards also used Winter pruning (June-August). The vineyards were located in temperate climate regions in the Colchagua (34° S; O'Higgins Region) and Maule Valleys (35° S; Maule Region) (Figure 1), which generally receive rain in Autumn and Winter months. All nine vineyards were on flat or slightly sloping land. Vineyards in the Colchagua area were mainly on loamy soils with some variations from sandy loam to clay loam soils (Covarrubias *et al*., 2004), and were at altitudes between 133 and 201 m.a.s.l. In the Maule region, the vineyard soils were loamy to clay loamy (Gallardo *et al*., 1994), and between 107 and 131 m.a.s.l. In this part of Chile, altitude is the main factor determining climatic conditions (4 months rainfall season; Tmax = 28.1ºC; Tmin = 2.9ºC; rainfall = 582 mm; pan evaporation = 1,230 mm: Novoa and Villaseca, 1989).

Assessments of Botryosphaeria dieback incidence and severity

Incidence and severity of symptoms were determined during spring season (October-November), in 14 vineyard blocks in 2010, and 13 blocks 2018 respectively (Table 1). Each block included a randomly selected quadrant of 100 plants for determining incidence and severity of decay symptoms. Researchers recorded data on dead cordons of each plants as: wood canker, spur necrosis, dead spurs with grayish colour (non-sprouting spurs), or spurs taken from the plant in the winter pruning period. Leaf symptoms were also recorded. All diseased plants had cankers, mostly wedge-shaped (Figure 2A). Disease incidence was calculated as the percentage of symptomatic plants within the 100 plant quadrant. Severity of the damage was recorded using a rubric severity score of 0 to 4, where: 0 = healthy plant (both cordons complete and asymptomatic); 1 = one asymptomatic cordon and one cordon with up to 50% of symptoms (1-3 dead or missing spurs); 2 = one asymptomatic cordon and an entire symptomatic cordon (4-6 dead or missing spurs), 3 = one symptomatic cordon and the second with up to 50% of symptoms (7-9 dead or missing spurs); and 4 = dead plant (both cordons dead or totally missing) (Figure 2). Disease Index (DI) were calculated using a formula modified from Mc Kinney (1923):

$$DI\ (\%) = \frac{\sum nv}{VN}\ 100 \quad (1)$$

where n = number of plants per score degree; v = score; N = total number of plants evaluated; and V = maximum score.

Estimation of production loss per plant

Total production losses for the 2010-2011 harvest season were estimated using the procedure of Munkvold *et al*. (1994). The number of diseased spurs from all plants (n = 100) from each quadrant were compared to healthy plants, which were shown to have an average of 12 spurs per plant (data obtained in the field). At the end of the summer, when fruit ripened, average production per plant was calculated by harvesting ten healthy plants from each block. These provided potential yield (PY) and estimated yield loss (EYL), as follows:

$$PY_1 = \bar{P}_x\, NP_{ha} \quad (2)$$

$$EYL_1 = \left(\frac{S_d S_p N}{100}\right) NP_{ha} \quad (3)$$

where: PY_1 = potential yield (kg ha^{-1}); $\bar{P}_x$ = the mean of fruit weight (kg) from ten score 0 samples (healthy plants); NP_{ha} = number of plants ha^{-1}; EYL_1 = estimated yield loss (kg ha^{-1}); S_d = total number of dead spurs per quadrant; and S_p = mean fruit weight (kg) per spur.

Total production losses for the 2018-2019 harvest season were estimated using the following procedure. Grapes from three plants per disease severity score (1–4) were harvested in each quadrant (the same quadrants used to determine disease incidence and severity). For score 0, bunches from ten healthy plants were randomly

Figure 2. Damage and degrees of disease severity associated with dead arms in 'Cabernet Sauvignon' grapevines: A) Cross-section and old cordon, exhibiting double V-shaped dark brown necrosis; B) Severity score 0, healthy plant (both cordons complete and asymptomatic); C) Score 1, one asymptomatic cordon and one cordon with up to 50% of symptoms (one to three dead or missing spurs); D) Score 2, one asymptomatic cordon and an entire symptomatic cordon (four to six dead or missing spurs); E) Score 3, one symptomatic cordon and the second with up to 50% of symptoms and seven to nine dead or missing spurs; F) Score 4, dead plant (both cordons dead or totally missing).

selected and harvested from each quadrant, or at least in the same block. A calculation was made of average weight (kg) of fruit per severity score, multiplied by the number of plants with the same score in the quadrant (determined in spring severity assessments). Potential yields and yield losses were calculated as:

$$EFW = \frac{\sum \bar{P}_{xGi}\, n_{Gi}}{100}\, NP_{ha} \quad (4)$$

$$PY_2 = \bar{P}_x\, NP_{ha} \quad (5)$$

$$EYL_2 = PY_2 - EFW_2 \quad (6)$$

where: EFW = estimated fruit yield (kg ha^{-1}); $\bar{P}_{xGi}$ = mean fruit weight (kg) from three plants per severity score (based on *DI*) in the quadrant (for score 0, i.e., healthy plants, average fruit weight per plant was determined from ten plants); n_{Gi} = number of plants per severity score in each quadrant; PY_2 = potential yield (kg ha^{-1}); and EYL_2 = estimated yield loss (kg ha^{-1}).

Linear regression analyses were performed for estimated production loss for each year *versus* GTD severity measured in the corresponding Spring.

Fungus isolates

Samples were randomly chosen from diseased cordons from each evaluated block in Autumn and Spring of 2010 and 2018. A total of 108 samples were analyzed, 56 samples obtained in 2010 and 52 in 2018. Wood samples (<1 cm^2) were taken from the margins of canker lesions, disinfected in 70% ethanol for 30 s, rinsed in sterile distilled water, dried and then added to two different media in Petri plates: potato dextrose agar acidified with 0.5 ml of 96% lactic acid (APDA) and malt extract agar (Difco Laboratories). The plates were incubated for 3–7 d at 24°C until fungal colonies developed. Hyphal tip subcultures were the incubated on APDA for 5 d at 24°C. *Botryosphaeriaceae*-like colonies were subcultured and left in an incubation chamber with near ultraviolet light (λ = 320 nm) at room temperature (19–21°C) to stimulate pycnidium production and conidium development. From conidia of each isolate, monospore subcultures (on water agar and APDA subcultures) were made for morphological and molecular identifications.

Morphological and molecular identification of isolates

Lengths and widths of 30 conidia were measured for each *Botryosphaeria*-like isolate. For molecular characterizations, total DNA was extracted from each monospore culture using the DNeasy Plant Mini Kit (Qiagen). For all isolates obtained in 2010 and 2018, the ITS1-5.8S-ITS2 region was amplified using primer ITS4/ITS5 (White *et al.,* 1990), and the β-tubulin (BT) region using primer Bt2a/ Bt2b (Glass and Donaldson, 1995). Part of the translation elongation factor gene (*EF1-α*) was amplified for isolates obtained in 2018, using EF1-728F/ EF1-986R primers (Carbone and Kohn, 1999). Amplification reactions were each prepared in a final volume of 22 µL following manufacturer instructions (SapphireAmp Fast PCR Master Mix). A 1% electrophoresis gel was prepared and PCR products in TAE buffer were visualized (Winkler) by staining with GelRed (Biotium) and viewing with UV transilluminator (Vilber Lourmat). Amplified products were purified and sequenced in both directions by Macrogen (South Korea), and were assembled and edited using Geneious 10.0.6 software. The products were compared against reference sequences from the National Center of Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), using the BLAST tool. All sequences were deposited in the GenBank Database.

A multi-locus phylogenetic analysis was performed using Maximum Parsimony (MP) in MEGAX (Kumar *et al*., 2018). Bootstrap values were calculated using 1,000 replicates yielding the MP tree using Tree Bisection and Reconnection algorithms. The tree was rooted with *Lasiodiplodia gonubiensis* strain CBS 115812 and other reference isolates of different *Botryosphaeriaceae* were used from GenBank (Table 2). Tree length, consistency index, retention index, rescaled consistency index, and homoplasy index were calculated using MEGA X.

RESULTS

Estimation of incidence and severity of Botryosphaeria dieback in 'Cabernet Sauvignon' grapevines

Incidence and severity were measured in Spring 2010 and 2018. In 2010, the mean incidence of Botryosphaeria dieback was 77.6% for blocks in the O'Higgins Region and 98.7% in the Maule Region. In 2018, mean incidence in the O'Higgins Region increased to 82.4%, and decreased to 87.2% in the Maule Region. The average severity in 2010 was 40.1% in the blocks sampled from the O'Higgins Region and 60.1% in the Maule Region. In 2018, mean severity was 43.6% in the blocks sampled in the O'Higgins Region, and 51.4% in the Maule Region (Table 3). Furthermore, incidence and severity of the disease in the O'Higgins Region were related to the age of the vineyards, increasing with the increasing age of the plants (Figure 3).

Table 2. Isolates of *Diplodia seriata*, *D. mutila*, *Neofusicoccum parvum*, *N. australe*, and *Spencermartinsia viticola* obtained from the GenBank Database included in this study for phylogenetic analyses.

Species	Strain	Host	Reference	GenBank accession number[a]	
				ITS	BT
Diplodia seriata	CBS112555	*Vitis vinifera*	Alves *et al*., 2004	AY259094	DQ458856
	CBS119049	*Vitis* sp.	Alves *et al*., 2006	DQ458889	DQ458857
	CMW7774	*Ribes* sp.	Slippers *et al*., 2004	AY236953	AY236931
	CMW7775	*Ribes* sp.	Slippers *et al*., 2004	AY236954	AY236932
	CMW8230	*Picea glauca*	De Wet *et al*., 2003	AY972104	AY972119
	UCD244Ma	*Vitis vinifera*	Úrbez-Torres and Gubler, 2009	DQ008314	DQ008337
	UCD352Mo	*Vitis vinifera*	Úrbez-Torres and Gubler, 2009	DQ008315	DQ008338
	UCD614Tu	*Vitis vinifera*	Úrbez-Torres and Gubler, 2009	DQ008318	DQ008341
	UCD710SJ	*Vitis vinifera*	Úrbez-Torres and Gubler 2009	DQ008321	DQ008344
	USD770St	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	DQ008322	DQ008345
	UCD1010BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012377	EU012429
	UCD1015BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012378	EU012430
	UCD1035BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012379	EU012431
	UCD1038BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012380	EU012432
	UCD1052BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012381	EU012433
	UCD1061BC	*Vitis vinifera*	Úrbez-Torres *et al*., 2008	EU012382	EU012434
Diplodia mutila	CMW7060	*Fraxinus excelsior*	Slippers *et al*., 2004	AY236955	AY236933
	CBS230.30	*Phoenix dactylifera*	Alves *et al*., 2006	DQ458886	DQ458849
	CBS112553	*Vitis vinifera*	Alves *et al*., 2006	AY259093	DQ458850
	CBS112554	*Pyrus communis*	Alves *et al*., 2006	AY259095	DQ458851
	JL375	*Fraxinus excelsior*	Alves *et al*., 2006	DQ458887	DQ458852
	UCD288Ma	*Vitis vinifera*	Urbez-Torres *et al*., 2006	DQ008313	DQ008336
	UCD1953SB	*Vitis vinifera*	Urbez-Torres *et al*., 2006	DQ233598	DQ233619
	UCD1965SB	*Vitis vinifera*	Urbez-Torres *et al*., 2006	DQ233599	DQ233620
Neofusicoccum parvum	CBS110301	*Vitis vinifera*	Alves *et al*., 2004	AY259098	EU673095
	CCA189	*Ferula communis*	Lopes *et al*., 2016	KX871879	KX871766
	CMW9081	*Populus nigra*	Lopes *et al*., 2016	AY236943	AY236917
Neofusicoccum australe	CAA723	*Tilia platyphyllos*	Lopes *et al*., 2016	KX871862	KX871747
	CAA741	*Acacia longifolia*	Lopes *et al*., 2016	KX871863	KX871748
Spencermartinsia viticola	PD285	*Vitis* sp.	Inderbitzin *et al*., 2010	GU251166	GU251826
	UCP105	*Citrus* (cv. *Parent Washington)*	Adesemoye and Eskalen, 2011	JF271748	JF271766
Lasiodiplodia gonubiensis	CBS 115812	*Syzygium cordatum*	Alves *et al*., 2006	DQ458892	DQ458860

[a] ITS = internal transcribed spacer region; BT = β-tubulin gene.

Production loss estimations

In the 2010-2011 harvest season, average fruit production losses were 4.5 t ha^{-1} in the O'Higgins Region and 6.2 t ha^{-1} in Maule (Table 4). Production loss estimates represented 30.7% of total production potential in the O'Higgins Region and 52.9% in the Maule Region. For the 2018-2019 harvest season, average fruit production loss of 10.7 t ha^{-1} was estimated for the O'Higgins Region, and 11.9 t ha^{-1} for the Maule Region (Table 4). Production loss estimates represented 42% of total production potential in the O'Higgins Region and 52.3% in Maule Region.

Linear regressions had the best model fit for the relationship of disease severity (x) and production losses (y) (Figures 4A and 4B).

Table 3. Incidence and severity of Botryosphaeria dieback in Chilean vineyards of 'Cabernet Sauvignon' in O'Higgins and Maule Regions.

Region/Locality/Vineyard-Block	Planting year	Spring 2010		Mean number of dead spurs	Spring 2018	
		Mean Incidence (%)	Mean Severity (%)		Mean Incidence (%)	Mean Severity (%)
O'Higgins/Chépica 1-1	1996	64	27.3	2.5	73	29.0
O'Higgins/Palmilla 2-2	1991	71	36	3.6	94	66.0
O'Higgins/Pamilla 2-3	2005	22	7.8	0.6	63	18.5
O'Higgins/Palmilla 3-4	1995	99	62.3	6.5	84	52.0
O'Higgins/Peralillo 4-5	1991	100	70.5	7.7	83	47.0
O'Higgins/Peralillo 5-6	1996	83	31.3	2.8	94	49.3
O'Higgins/Peralillo 5-7	1996	84	30.3	2.8	88	48.3
O'Higgins/Peralillo 5-8	1995	98	55.0	5.6	80	38.5
O'Higgins average		77.6	40.1	4.0	82.4	43.6
Maule/Batuco 6-9	1997	100	66.8	6.9	82	43.0
Maule/Pencahue 7-10	1997	100	60.3	6.2	88	46.5
Maule/Pencahue 7-11	2002	99	61.3	6.5	92	59.0
Maule/Pencahue 8-12	1997	99	54.0	5.7	86	54.5
Maule/Pencahue 8-13	1997	94	47.0	4.6	88	54.0
Maule/Talca 9-14	2003	100	71.3	7.7		
Maule average		98.7	60.1	6.3	87.2	51.4

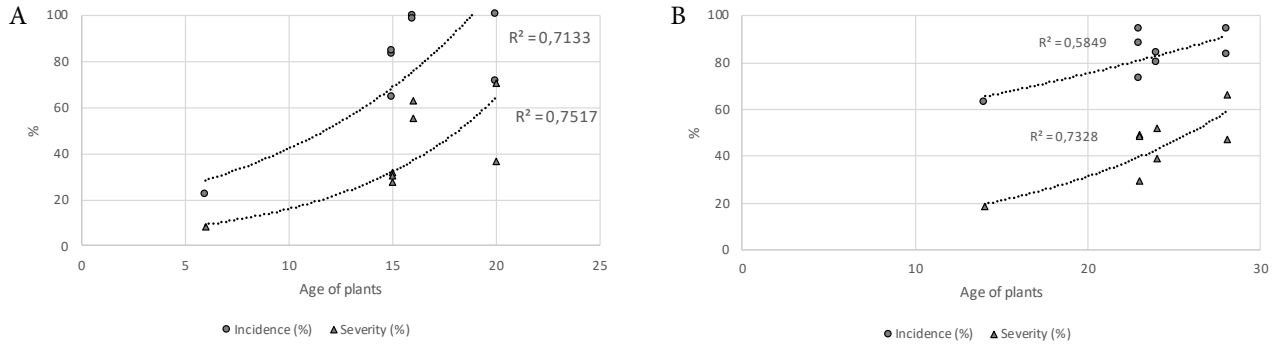

Figure 3. Relationships between vineyard age ('Cabernet Sauvignon') in the O'Higgins Region and the incidence and severity of Botryosphaeria dieback in A) spring 2010; and B) spring 2018.

Morphological and molecular identification of fungus isolates

Seventy *Botryosphaeriaceae* isolates were obtained from 108 diseased vine samples collected from nine vineyards during two growing regions. Isolates were obtained in autumn and spring of 2010 and 2018. Thirty-seven *Botryosphaeriaceae* isolates were obtained in 2010 and 33 in 2018. The study found that colonies of *Diplodia mutila*, *D. seriata*, *Neofusicoccum australe*, *N. parvum* and *Spencermartinsia viticola* developed pycnidia after 20-30 d growth on APDA. The conidia identified as *D. seriata* were unicellular, initially hyaline, and then brown, each with a rounded apex and truncated base, measuring 22.9 ± 1.3 × 11.9 ± 0.4 µm. Conidia of *D. mutila* were aseptate, hyaline, ellipsoid, each with rounded apex and base, measuring 24.7 ± 1.1 × 12.9 ± 0.1 µm. Conidia of *Neofusicoccum* were unicellular, hyaline, ellipsoidal, and thin-walled, measuring 16.1 ± 0.9 × 5.9 ± 0.6 µm. Conidia of *S. viticola* were initially hyaline but still attached to the conidiogenic cells, turning brown, and each developing a central septum; they were ellipsoid, with thick walls, rounded at both ends, or in some cases with truncated bases, and measuring 22.2 ± 0.2 × 9.2 ± 0.1 µm. These conidium characteristics have been respectively described for these species.

Table 4. Estimated yield losses in 'Cabernet Sauvignon' vineyards, sampled in two years (2010 and 2018) in O'Higgins and Maule Regions, Central Chile.

Vineyard block	Planting year	Potential yield (kg ha^{-1}), 2010	Estimated yield loss (kg ha^{-1}), 2010[a]	Potential yield (kg ha^{-1}), 2018	Estimated yield loss (kg ha^{-1}), 2018[b]
O´Higgins Region					
1-1	1996	23,011	4,756	34,257	4,185
2-2	1991	18,264	5,433	18,785	13,529
2-3	2005	14,337	753	9,198	1,441
3-4	1995	12,402	6,666	16,118	8,446
4-5	1991	8,087	5,156	24,769	14,765
5-6	1996	11,822	2,788	31,811	15,554
5-7	1996	15,058	3,564	33,325	15,493
5-8	1995	15,058	7,077	35,688	12,303
Average		14,755	4,524	25,494	10,715
Maule Region					
6-9	1997	11,208	6,445	15,571	6,458
7-10	1997	14,184	7,376	35,947	16,800
7-11	2002	14,188	7,626	26,346	19,617
8-12	1997	10,163	4,794	17,278	7,170
8-13	1997	8,916	3,447	18,681	9,488
9-14	2003	11,666	7,525	-	-
Average		11,721	6,202	22,765	11,907

[a] Estimated yield losses based on dead spurs per evaluated quadrant.
[b] Estimated yield losses based on the harvest of plants with different degrees of GTD damage per evaluated quadrant.

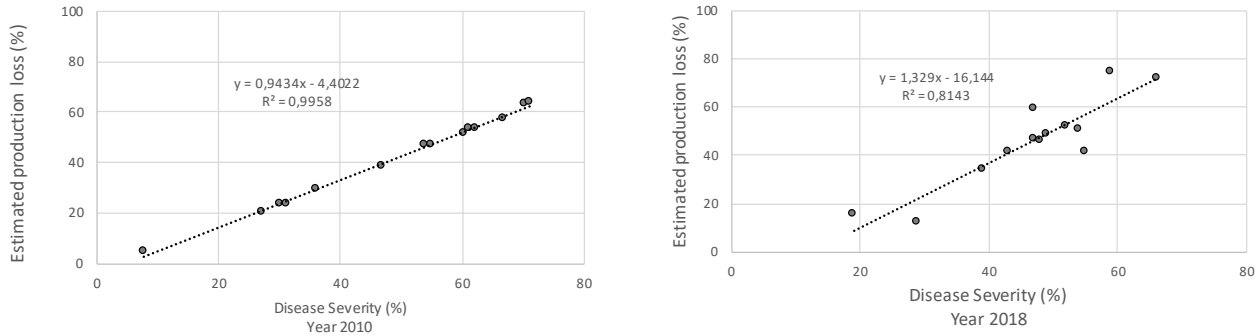

Figure 4. Regression analyses between severity of Botryosphaeria dieback (measured in spring) in 'Cabernet Sauvignon' vineyards located in the O'Higgins and Maule Regions, Central Chile. A) Production losses (%), estimated in 2010 by number of dead spurs; and B) Production losses (%), estimated in 2018 by harvesting plants with different degrees of disease severity.

Comparison of DNA sequences identified in 2010 using BLASTn (NCBI) showed *D. seriata* to be the most prevalent species (68%), followed by *D. mutila* (13%), *N. parvum* (11%), *N. australe* (5%), and *S. viticola* (3%) (Supplementary Table 1). DNA sequences showed ≥98% similarity matches with previously deposited sequences for these species in GenBank. All the species were isolated in autumn and spring, except for *S. viticola*, which was obtained only in autumn. Isolates collected in 2018 were identified as *D. seriata* (91%), *D. mutila* (6%) and *N. parvum* (3%) (Supplementary Table 2). Sequence similarities for these species were also ≥98% with those in GenBank database. *Diplodia seriata* was obtained in samples taken in autumn and spring, while *D. mutila* and *N. parvum* were detected only in spring. In both sampling years (2010 and 2018), isolates obtained from

vines showed dieback in cordons with canker lesions (Figure 2A).

The concatenated ITS and BT phylogenetic analyses included 68 *Botryosphaeriaceae* sequences. The analyses contained 799 nucleotides of which 621 were constant and 111 were parsimony-informative. The analyses yielded the ten most parsimonious trees (one is shown in Figure S1), with tree length (TL) = 236, consistency index (CI) = 0.7965; retention index (RI) = 0.9721, recalled consistency index (RC) = 0.7743, and homoplasy index (HI) = 0.2035. MP analyses showed two main branches, with 68 isolates from Chile grouping into five different clades, including *D. seriata*, *D. mutila*, *N. parvum*, *N. australe*, and *S. viticola* (Figure S1).

DISCUSSION

This study reports estimates of Botryosphaeria dieback incidence and severity, as well as production losses, in 'Cabernet Sauvignon' vineyards in Central Chile over two different growing season sampled 8 years apart. In both growing seasons, the incidence of Botryosphaeria dieback (average by region) varied between 78% and 99%. Morales *et al*. (2012) previously reported incidence of *Botryosphaeriaceae*-associated dieback between 22% and 69% for Chilean table grape vineyards. Although this was less than in the present study, the vineyards assessed by Morales *et al*. (2012) were located in warmer, drier regions (Valparaíso and Metropolitan Regions) than those assessed here. The previous study used the same methodology for determining disease incidence as the present study.

Between 2010 and 2018, average incidence of Botryosphaeria dieback was similar (respectively, 87% and 84%). However, between regions, average incidence in the O'Higgins Region increased by 5% from 2010 to 2018, but decreased 12% in the Maule Region, although average incidence in the Maule Region (93%) was greater than in the O´Higgins Region (80%) (Table 3). This could be due to differences in rainfall. During 2015 to 2018, the O'Higgins Region (San Vicente Meteorological Station, https://agrometeorologia.cl) averaged 384 mm p.a., while the Maule Region (San Clemente Meteorological Station, https://agrometeorologia.cl) averaged 669 mm p.a., 74% more rainfall than in the O'Higgins Region.

Some vineyards in both regions have used of potassium phosphite in attempts to decrease the incidence and severity of Botryosphaeria dieback. Between 2010 and 2018, the use of pruning paste with DMI fungicide additives also increased. Pruning wound treatments have been effective under laboratories conditions for Chilean *Botryosphaeriaceae* isolates (Torres *et al*., 2013), or under field conditions (Díaz and Latorre, 2013). The use of benzimidazole-, QoL- or and DMI-containing pastes was suggested to provide better disease control than spray applications (Díaz and Latorre, 2013).

The overall average severity of Botryosphaeria dieback was 42% in O'Higgins and 56% in Maule. Morales *et al*. (2012) reported average disease severity 13% across seven table grape vineyards, which are analogous because they used similar analyses to those used in the present study. In 2010 and 2018, overall average severity was similar, at 49% in 2010 and 47% in 2018, although there were differences in incidence between regions and vineyards. Average severity in O'Higgins increased by 4% from 2010 to 2018, but decreased in the Maule by 8.5%. In individual vineyard blocks, average severity increased or decreased between both in O'Higgins and in Maule. Considering differences between vineyards in the same region could be due to effective disease management than to the impacts of climatic conditions.

Gubler *et al*. (2005) showed that the impacts of GTDs were proportional to vineyard age, where the most severe symptoms occurred in older vineyards. Morales *et al*. (2012) also found that Botryosphaeria dieback affecting table grape vineyards increased with the age of the plants. This was also true in the present study (Figure 3), where disease incidence and severity increased with increasing vineyard age, as shown with regression analysis for the O'Higgins Region data. In the Maule region, the surveyed vineyards were of similar age, and were fewer vineyards were sampled, so the regression analysis did not show any correlation between disease and vineyard age.

Grape yield losses for the O'Higgins Region averaged 30.7% in 2010 and 42% in 2018; and were similar in the Maule Region, at 52.9% in 2010 and 52.3% in 2018. A similar study in France estimated yield losses due to Botryosphaeria dieback of 25–30% for 'Cabernet Sauvignon', 'Cabernet Franc', and 'Sauvignon Blanc' (*Úrbez*-Torres *et al*., 2015). Munkvold *et al*. (1994) estimated that production losses caused by Eutypa dieback in Californian vineyards were between 30.1% and 61.9%. Siebert (2001) estimated 14% yield losses due to Eutypa and Botryosphaeria dieback (Bot canker). The estimated yield losses for these GTDs in other vineyards could also be significant, and similar to those determined in the present study.

The analysis of Kaplan *et al*. (2016) (see Munkvold *et al*. (1994), indicated that 'Cabernet Sauvignon' yields in a symptomless San Joaquin Valley vineyard in California was estimated at 25 t ha^{-1}, while in an untreated, infect-

ed vineyard, the estimated potential yield loss was 90% for plants at least 20 years old. These effects are similar to those determined in the present study. In 2018, estimated potential yield was 25.5 t ha^{-1} in O'Higgins Region and 22.8 t ha^{-1} in the Maule Region (Table 4). Average potential loss of both years was 36.5% in O'Higgins and 52.5% in Maule. These losses were potentially of considerable importance, but if no management was carried out, these losses would be greater and up to 90% (Munkvold *et al*., 1994; Kaplan *et al*., 2016).

Disease severity scales were evaluated in this study for Botryosphaeria dieback, and were expressed as severity and linearly associated with losses of fruit production in the vineyards. For every 10% increase in dieback severity, approx. 9% (2010) and 13% (2018) of fruit production was lost, either for estimation of production using dead spurs or from the harvest of plants with varying degrees of disease. The novel severity scale used here is an easier method for detecting losses compared to the spur count method, and is similarly robust. The severity scale also allows damage to be measured before harvest, and to estimate harvest losses (Figure 4). This new method estimates damage and losses by measuring the intensity or severity of losses caused by specific pathogens Chester (1950).

Diplodia seriata has also been reported as the most prevalent *Botryosphaeriaceae* species in vineyards in other parts of the world, including New South Wales (Castillo-Pando *et al*., 2001; Pitt *et al*., 2010; Qiu *et al*., 2011), Western Australia (Taylor *et al*., 2005), United States of America (California) (Úrbez-Torres *et al*., 2006), and South Australia (Pitt *et al*., 2010).

Gubler *et al*. (2005) reported that 36% of cankers in 'Cabernet Sauvignon' vineyards in California were due to *Botryosphaeriaceae* species. Qiu *et al*. (2011) showed that 36% of diseased grapevines in grape growing regions of Australia, among them 'Cabernet Sauvignon', were infected with *Botryosphaeriaceae* species. Proportions of *Botryosphaeriaceae* recovered from diseased plants in a broad California survey found values similar to Díaz *et al*. (2013), at 47%. In New South Wales and South Australia, 56% of diseased plants were infected by *Botryosphaeriaceae*, 68% in New Zealand and 83% in Queensland, Australia (*Úrbez*-Torres *et al*., 2006; Pitt *et al*., 2010; Baskarathevan *et al*., 2012; Sosnowski *et al*., 2013).

The present study contributes to knowledge highlighting the importance of Botryosphaeria dieback for grape production, particularly for the principal wine grape cultivar in Chile. This disease has had severe impacts on yields in Chile during the last 10 years, so these results should encourage implementation of control measures based on estimated potential production losses in each vineyard. The results also indicate the need to implement control measures, assess damage for translation into yield losses, and estimate whether newly implemented management strategies have reasonable cost/benefit outcomes.

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Research Papers

The role of melanin in the grapevine trunk disease pathogen *Lasiodiplodia gilanensis*

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Summary. *Lasiodiplodia* (*Botryosphaeriaceae*) includes fungi that are considered among the most aggressive to grapevine, capable of causing cankers and necrotic lesions which eventually lead to death of host plants. A common characteristic of this genus is the presence of melanin in conidia and mycelium. Melanin is produced by the oxidation of phenolic and/or indolic compounds. For some fungi, this pigment is an essential factor for pathogenicity. This study characterized the types and the roles of melanin produced by *Lasiodiplodia gilanensis*. Using specific melanin inhibitors, *L. gilanensis* was shown to synthesize DOPA-melanin, DHN-melanin, and pyomelanin. DOPA-melanin was shown to be involved in production of aerial mycelium and protection against enzymatic lysis and oxidative stress; DHN-melanin to be involved in ramification of mycelium when exposed to nutrient deficiency; and pyomelanin to be related with hyphae development. The fungus used tyrosine as a precursor of DOPA-melanin and as carbon and nitrogen sources, and produced melanin inside the piths of infected plants. Genes involved in melanin synthesis were conserved among the *Botryosphaeriaceae*, highlighting the importance of melanin in this family.

Keywords. Grapevine trunk diseases (GTDs), Botryosphaeria dieback, *Botryosphaeriaceae*, fungal melanin, tyrosine catabolism.

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INTRODUCTION

The *Botryosphaeriaceae* contains several fungal plant pathogens of a wide range of woody plants (Slippers and Wingfield, 2007), with cosmopolitan distribution (Damm *et al*., 2007; Phillips *et al*., 2013). The ability of these fungi to infect multiple hosts increases their economic impacts and ecological risks in many regions (Mehl *et al*., 2017). More than 30 *Botryosphaeriaceae* species from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium, Neofusicoccum*, *Phaeobotryosphaeria*, and

Spencermartinsia, have been associated with grapevine trunk diseases (GTDs) (Úrbez-Torres, 2011; Gramaje *et al*., 2018). Interactions of these fungi with their hosts have been studied, as well the virulence factors that trigger disease development. Infections of healthy plants occur mainly through pruning wounds, and some of the symptoms caused are leaf spot, fruit rot, dieback, shoot necrosis, vascular discolouration, and perennial cankers (Úrbez-Torres, 2011). In the states of Baja California and Sonora, Mexico, grapevine is one of the most economically important crops, and in these areas, several species of fungi causing GTDs have been reported, including *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl (Úrbez-Torres *et al*., 2008; Candolfi-Arballo *et al*., 2010).

Lasidiplodia theobromae is the type species of *Lasiodiplodia*. This fungus has widespread distribution, but is most common in tropical and subtropical regions, and has been found in more than 500 hosts (Punithalingam, 1976). In grapevine, *L. theobromae* was reported as one of the most aggressive pathogens causing Botryosphaeria dieback (Úrbez-Torres *et al*., 2008). To date, more than eighteen species of *Lasiodiplodia* have been reported, including *L. exigua, L. parva, L. crassispora, L. gilanensis, L. brasiliense, L. laeliocattleyae* (formerly *L. egyptiacae*)*, L. euphorbicola* and, *L. hormozganensis* (Yan *et al*., 2013; Linaldeddu *et al*., 2015; Correia *et al*., 2016; Rodríguez-Gálvez *et al*., 2017). The main differences in these species are the morphology and dimensions of conidia and the morphology of paraphyses (Phillips *et al*., 2013). Conidia are initially hyaline, and later, a single median septum is formed in each conidium. Then, the cell walls become dark brown, and melanin granules are deposited longitudinally on the inner surface, giving a striated appearance (Phillips *et al*., 2013). Since not all conidia become melanized at the same time, it is possible to find hyaline and pigmented conidia in individual pycnidia.

Melanins are macromolecules of high molecular weight, derived from oxidative polymerization of phenolic and/or indolic compounds (Jacobson, 2000). They are usually dark pigments distributed in all biological kingdoms (Butler and Day, 1998; Eisenman and Casadevall, 2012). Melanin is considered a stable, insoluble, and resistant biopolymer due to its complex structure and physicochemical properties (Solano, 2014; Cordero and Casadevall, 2017). Several types of fungal melanin have been described, determined by their precursors; among them, DHN-melanin, DOPA-melanin and pyomelanin are related to pathogenicity or antifungal drug protection (Toledo *et al*., 2017). DHN-melanin is synthesized through a series of dehydration and reduction reactions from 1,3,6,8-tetrahydronaphthalene (1,3,6,8-THN), in which the final product is 1,8-dihydroxynaphthalene (1,8-DHN) that polymerizes to form melanin (Langfelder *et al*., 2003; Eisenman and Casadevall, 2012). DOPA-melanin is synthesized from the oxidation of tyrosine to L-DOPA or directly from L-DOPA to dopaquinone (Langfelder *et al*., 2003; Eisenman and Casadevall, 2012). Pyomelanin is related to tyrosine catabolism and is synthesized from the oxidation of homogentisic acid and its subsequent polymerization (Schmaler-Ripcke *et al*., 2009).

Melanization is not an essential factor for fungus growth, but is involved in a broad spectrum of biological functions (Wu *et al*., 2008). The primary functions of melanin is to protect cells from UV radiation, and from oxidizing agents; melanin interacts with free radicals and other reactive species because of the presence of unpaired electrons (Butler and Day, 1998; Shcherba *et al*., 2000), contributing to the survival of cells under environmental stress conditions (Wu *et al*., 2008; Eisenman *et al*., 2020). Melanin is a virulence factor in some phytopathogenic fungi acting as a non-specific "body-armour" during infection, protecting the fungus against host defense mechanisms and promoting its survival to cause the disease (Hamilton and Gomez, 2002; Nosanchuk and Casadevall, 2003; Eisenamn *et al*., 2020). DHN-melanin has been the most studied melanin in fungi. In the rice pathogen *Magnaporthe oryzae*, production of DHN-melanin is vital in appressorium formation and for the stationary phase of mycelial growth (Howard and Valent, 1996; Nosanchuk and Casadevall, 2003; Eisenman and Casadevall, 2012). In *Colletotrichum lagenarium* (Kubo *et al*., 1982), *C. lindemuthianum* (Wolkow *et al*., 1983), *C. kahawae* (Chen *et al*., 2004), and *Setosphaeria turcica* (Shuangxin *et al*., 2017), DHN-melanin is associated to the appressorium formation. The grapevine trunk disease fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* produce naphthoquinones (scytalone and isosclerone), intermediate metabolites of melanin biosynthesis. These metabolites have been associated with oxidative properties and production of esca disease symptoms in grapevines (Evidente *et al*., 2000; Andolfi *et al*., 2011).

Melanin inhibitors have been used to study the synthesis pathways of melanin in some fungi (Woloshuk and Sisler, 1982; Wheeler and Klich, 1995; Butler *et al*., 2009; Kumar *et al*., 2015). Gonçalves *et al*. (2012) reported that tropolone (DOPA-melanin inhibitor) added to growth media, inhibited the synthesis of melanin in *Aspergillus nidulans*, unlike the use of DHN-melanin inhibitors. Pal *et al*. (2014) used tricyclazole, phthalide, tropolone, and kojic acid in *Aspergillus* spp., and found that in *Aspergillus niger*, *Aspergillus tamarii,* and *Aspergillus flavus*, synthesis of melanin was inhibited

by kojic acid and tropolone, while in *Aspergillus terreus* and *Aspergillus tubingensis* melanin was inhibited in the presence of tricyclazole and phthalide. In the hypersaline yeast *Hortaea werneckii*, which produces DHN-melanin, tricyclazole was used to study the effect of melanin in NaCl tolerance (Kejžar *et al*., 2013). Nitisinone has been primarily used in bacteria, for example, in *Pseudomonas aeruginosa,* in which this compound inhibited the synthesis of pyomelanin (Ketelboeter *et al*., 2014).

In a transcriptomic analysis of *L. gilanensis* UCD-256Ma (formerly *L. theobromae*), genes related to DHN-melanin and pyomelanin pathways were differentially expressed under heat shock and in the presence of grapevine wood. In contrast, genes related to the DOPA-melanin pathway were expressed only in the presence of grapevine wood without heat shock (Paolinelli-Alfonso *et al*., 2016). The present study aimed to identify the types and roles of melanin produced by *L. gilanensis* to increase understanding of this fungus-host interaction.

MATERIALS AND METHODS

Fungus strain and growth conditions

Lasiodiplodia gilanensis UCD256Ma (formerly *L. theobromae*) was isolated from grapevine plants showing Botryosphaeria dieback symptoms in Madera County, California, United States of America (Úrbez-Torres *et al*., 2006), and this isolate was provided by Dr. Douglas Gubler (University of California, Davis) to the laboratory of Phytopathology of CICESE. The stock culture was recovered on potato dextrose agar (PDA) and incubated at 30°C. For the assays described in the following sections, mycelial disks (5 mm diam.) were individually inoculated at one border of Petri dishes containing relevant media, and fungus growth was measured every 24 h. In each experiment, vegetative growth, pigmentation, and formation of aerial mycelium were evaluated, and changes in pigmentation of the fungal colonies were recorded. All experiments were carried out in triplicate and repeated once.

Evaluation of the behaviour of Lasiodiplodia gilanensis *in the presence of melanin inhibitors*

Tropolone and kojic acid, inhibitors of DOPA-melanin; phthalide and tricyclazole, inhibitors of DHN-melanin; and nitisinone, an inhibitor of pyomelanin, were used to perform the following assays. First, the effect of each inhibitor on growth of *L. gilanensis* was evaluated in PDA media supplemented with either 5, 10, 15, 20, 30, 100, or 200 μg mL^{-1} of each inhibitor, as described above. All treatments were incubated at 30°C for up to 4 d. Growth of the fungus was measured in each plate every 24 h. The inhibitors and concentrations in which the fungus showed a decrease in the pigmented dark colour compared to the non-inhibitor experimental controls, were selected for subsequent analyses.

Melanin production in response to environmental stress

Melanin production in response to enzymatic lysis, oxidative stress, and UV radiation was evaluated by combining the environmental stress conditions with the melanin inhibitors that showed effects on melanin production.

Enzymatic lysis stress was assessed using a lyophilized powder of lysing enzymes from *Trichoderma harzianum* (L1412; Sigma) at 10 mg mL^{-1}, and oxidative stress was assessed using 5% v/v hydrogen peroxide (H_2O_2), both added to PDA media. In each plate, a *L. gilanensis* mycelium disk was inoculated at one border, incubated at 30°C, and evaluated as described above. Vegetative growth of the fungus was measured every 24 h up to 4 d.

Effects of UV radiation were assessed using pigmented and hyaline conidia, exposed to UV light using a transilluminator (UVP). The conidia were obtained by inducing the production of pycnidia in Vogel's minimal medium (VMM) supplemented with ground grapevine wood at 5% (w/v). The wood was obtained from 'Cabernet Sauvignon' canes, frozen in liquid nitrogen, ground with a blender (Waring), and then autoclaved. Cultures were incubated under a near-UV electromagnetic radiation lamp for 15 to 20 d. Conidia were then collected under a stereoscopic microscope (Zeigen) using a dissection needle. Hyaline and pigmented conidia were taken individually and suspended in 0.05% Tween 20 (P9416; Sigma). Fifty hyaline or pigmented conidia were placed in PDA, exposed to high-intensity UV radiation of 365 nm for 30 or 60 min, and incubated in darkness at 30°C. The percentage of conidia germination was evaluated after 24 or 48 h, under a stereoscopic microscope (Zeigen).

Evaluation of L-tyrosine metabolism in Lasiodiplodia gilanensis

The ability of *L. gilanensis* to metabolize tyrosine was evaluated as follows. The fungus was grown in Minimal Medium 9 (MM9) (10.0 g L^{-1} glucose, 5.8 g L^{-1} Na_2HPO_4, 3.8 g L^{-1} K_2HPO_4, 0.5 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl) supplemented with 10 mM tyrosine. The use of tyrosine as

carbon, nitrogen, or carbon and nitrogen source was evaluated by preparing the medium without glucose (MM9 -C), without NH_4Cl (MM9 -N) or without both elements (MM9 -CN). After inoculation with the fungus, plates were incubated at 30°C for 7 d. The formation of a halo from decrease in medium opacity indicated catabolic use of tyrosine. Vegetative growth of the fungus was measured every 24 h for each treatment. Experiments were repeated in the presence of previously selected melanin inhibitors.

Evaluation of biomass production in the presence of melanin inhibitors and stress conditions

For each assay, biomass was evaluated as follows. A sterilized cellophane membrane was placed on the top of the medium, inoculated with a mycelium disk of *L. gilanensis*, and plates were incubated at 30°C. In the experiments with melanin inhibitors and stress conditions, membranes were carefully removed after 4 d and weighed. For tyrosine and melanin inhibitors, the membranes were recovered after 7 d. Biomass data were obtained by subtracting the weight of the membrane and the mycelium disk used for the inoculation.

Microscopic observations of Lasiodiplodia gilanensis *colonizing grapevine tissue plants*

For the evaluation of melanin production during colonization by the fungus, one-year-old cuttings of *V. vinifera* 'Cabernet Sauvignon' were used. Plants were drilled in the woody stems and then each inoculated with a 3 mm diam. mycelium plug of a 3-d-old culture of *L. gilanensis*, and the drill wound was covered with parafilm. Control plants were each inoculated with a 3 mm diam. plug of PDA. The plants were left in a growth chamber (PGR15; CONVIRON) for 28 d, with a day/night 30°C/10°C temperature cycle at 50% humidity. Samples (each approx. 2 cm) were then taken from the infection zones (1 cm above and 1 cm below). Lesions were observed under a stereo microscope, and the tissues were fixed in FAA solution (formaldehyde, ethanol, acetic acid; 10%:50%:5%). The samples were then dehydrated in ethanol solutions (25, 50, 80%) and preserved in 80% ethanol at 4°C until analysis. Samples were cut into cross- and longitudinal sections 70 µm thick with a microtome (EMS 5000 Oscillating Tissue Slicer). The sections were stained using the Masson-Fontana technique (modified from Lillie, 1965) to assess for the presence of melanin in the hyphae of *L. gilanensis*. This staining procedure is based on the ability of melanin to reduce ammoniacal silver nitrate solution to metallic silver without using an external reducing agent. Stained samples were analyzed using light microscopy (DM4000; Leica Microsystems).

Comparison of Lasiodiplodia gilanensis *genes involved in melanin synthesis with other* Botryosphaeriaceae *species*

In order to determine if previously reported melanin genes of *L. gilanensis* UCD256Ma (Paolinelli-Alfonso *et al*., 2016) were present in other *Botryosphaeriaceae*, seven genes from three different melanin pathways were compared against the sequenced genomes from the family available in the GenBank database. These were: for the DHN-melanin pathway, short-chain dehydrogenase reductase (*sdr*) and thioesterase (*thr*) genes; for the DOPA-melanin pathway, tyrosinase (*tyr*), and multicopper oxidase (*mco*), and for the pyomelanin pathway, laccase (*lcc*), hydroxyphenylpyruvate dioxygenase (*hppD*) and homogentisate dioxygenase (*hmgD*). Putative gene sequences involved in melanin synthesis were recovered from the transcriptome data of *L. gilanensis* (Paolinelli-Alfonso *et al*., 2016) and a tBLASTx (percent query coverage per hsp: ≥70%, and minimum percent similarity: ≥70%) analysis was performed against *Botryosphaeriaceae* genomes available in the GenBank database to search for sequence similarities (Table S1). In addition, the genome sequenced from *Phaeoacremonium minimum*; another grapevine trunk fungus, was used for comparison.

Data analyses

Statistical analyses were carried out using one-way ANOVA followed by a *post hoc* Fisher analysis, with an $\alpha < 0.05$ for the determination of statistical significance, using STATISTICA 8.0. All the experiments were carried out in triplicate. Graphs of presented data were made with SigmaPlot 11.0 software.

RESULTS

Different melanin inhibitors produce contrasting effects in Lasiodiplodia gilanensis

Kojic acid at concentrations up to 500 µg mL^{-1} did not affect colony pigmentation or mycelial growth. For this reason, Kojic acid was discarded as a DOPA-melanin inhibitor of *L. gilanensis*. Tropolone, another DOPA-melanin inhibitor, diminished the growth and pigmentation at 10 µg mL^{-1}, and colonies had less aerial

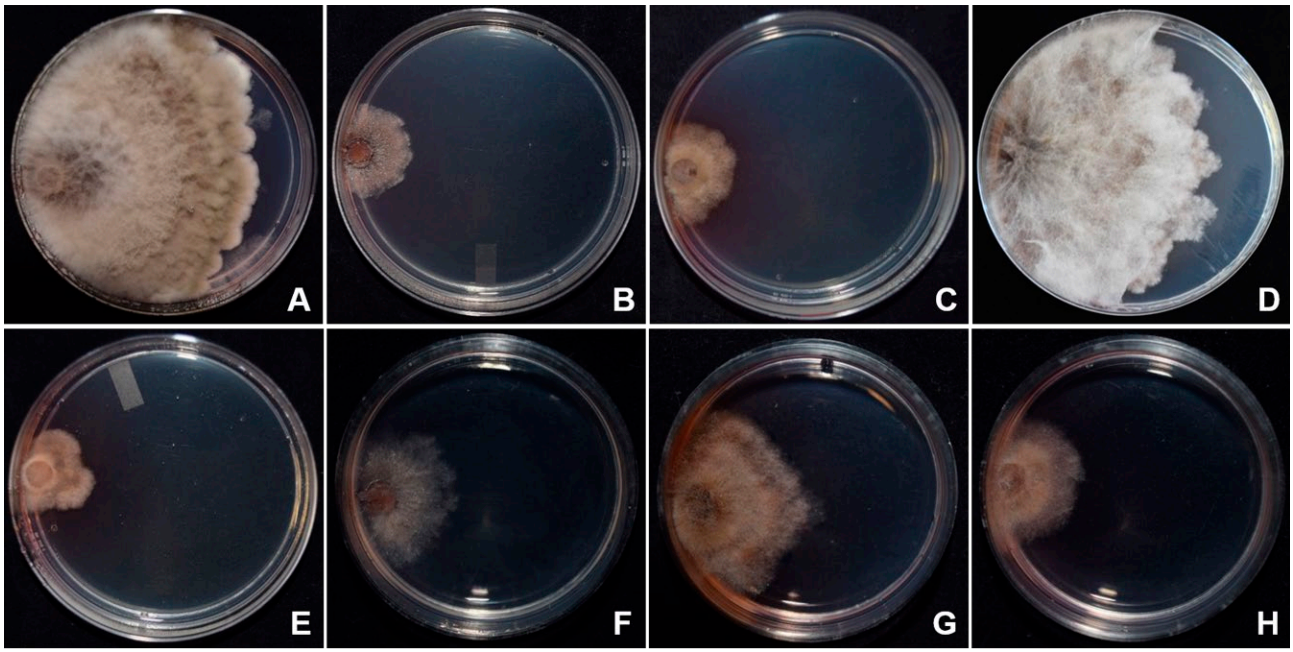

Figure 1. Growth of *Lasiodiplodia gilanensis* on PDA medium with different melanin inhibitors: A) Control. B) PDA + 15 µg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. C) PDA + 500 µg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. D) PDA + 100 µg mL^{-1} nitisinone (NTBC), pyomelanin inhibitor. E) PDA + Tp + Ph. F) PDA + Tp + NTBC. G) Ph + NTBC. H) Tp + Ph + NTBC.

mycelium in comparison to controls. At 15 µg mL^{-1} flat colonies and the formation of guttation droplets were observed (Figure 1B), and at 20 µg mL^{-1}, the growth of the fungus was inhibited. The concentration of 15 µg mL^{-1} of tropolone was therefore used for subsequent experiments.

For the DHN-melanin inhibitors, the fungus growing in phthalide (100 µg mL^{-1}) developed light-coloured mycelium. At 500 µg mL^{-1}, a noticeable change in morphology, growth inhibition and a slightly brown-orange pigmentation in the colony was observed (Figure 1C). With tricyclazole, at 10 µg mL^{-1}, the colour changed, but the morphology of the fungus was not affected. At 15 and 30 µg mL^{-}, growth inhibition and an orange-brown pigmentation were observed, similar to that observed with phthalide. Since both compounds inhibit the reductase reactions of two hydroxynaphthalene compounds to scytalone and vermelone in the DHN-melanin pathway (Wheeler and Klich, 1995; Suwannarach *et al*., 2019), but phthalide is cheaper, phthalide was selected for subsequent tests.

Nitisinone, a pyomelanin inhibitor, affected the fungus less, since only at 100 µg mL^{-1} was the colony colour lightened and growth slightly diminished (Figure 1D). This concentration was used in subsequent tests.

When phthalide (500 µg mL^{-1}) and tropolone (15 µg mL^{-1}) were used together, less aerial mycelium, slightly orange pigmentation, guttation droplets and growth inhibition were observed (Figure 1E). When nitisinone 100 µg mL^{-1} and tropolone 15 µg mL^{-1} were used together, growth, pigmentation, and formation of aerial mycelium were affected (Figure 1F). The greatest growth inhibition, least formation of aerial mycelium and less colony pigmentation were observed from the combination of phthalide 500 µg mL^{-1}, tropolone 15 µg mL^{-1}, and nitisinone 100 µg mL^{-1} (Figure 1H, 4; Table S2).

Responses of Lasiodiplodia gilanensis *to environmental stress*

Responses to enzymatic lysis

The use of lysing enzymes from *T. harzianum* reduced growth of the fungus, but the formation of aerial mycelium and biomass were similar to the experimental control (Figures 2A and B; 4, and S1; Table S2). The greatest inhibition was observed from the lysing enzymes, with tropolone at 15 µg mL^{-1} (Figures 2D and S1). From phthalide at 500 µg mL^{-1}, mycelium growth was inhibited and a brown-orange colour was observed (Figure 2F). When combining lysing enzymes with tropolone or with phthalide, biomass was similar (Figure 4; Table S2). Nitisinone at 100 µg mL^{-1} reduced growth of *L. gilanensis* compared with the lysing enzymes alone, but not as much as for tropolone (Figures 2H, 2D, and S1).

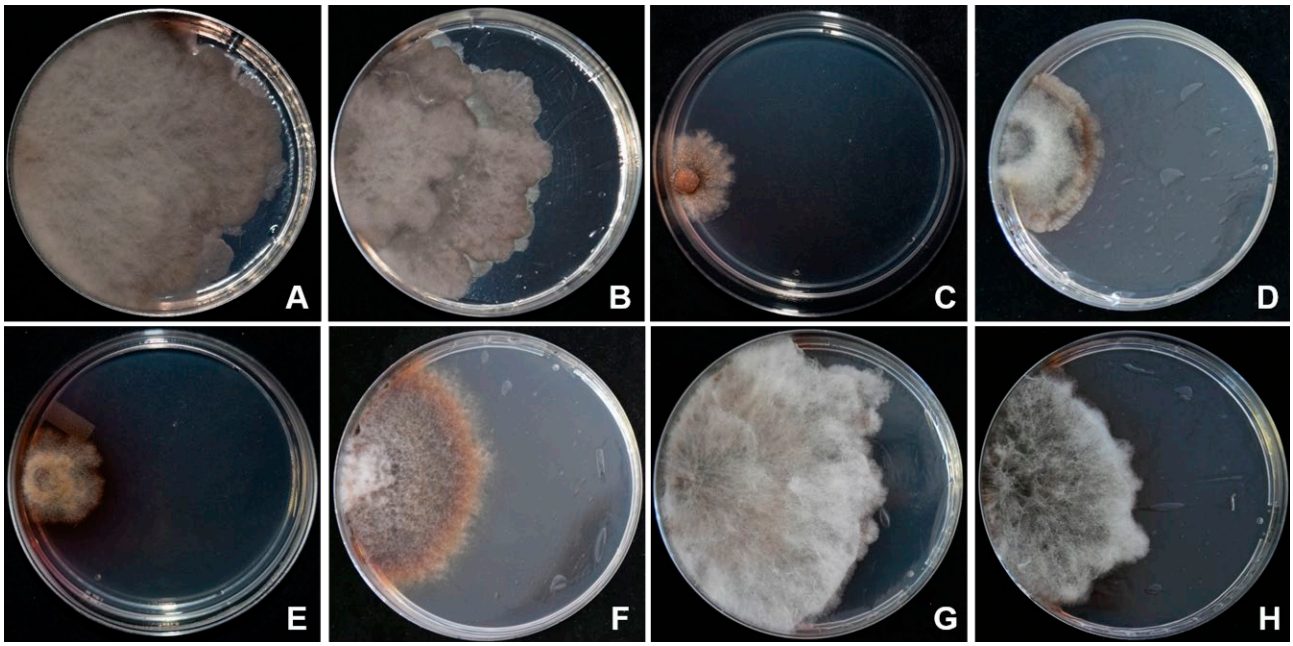

Figure 2. Growth of *Lasiodiplodia gilanensis* under enzymatic stress conditions. A) Control on PDA. B) PDA + 10 mg mL^{-1} lysing enzymes of *Trichoderma harzianum* (LE). C) PDA + 15 µg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. D) PDA + LE + Tp. E) PDA + 500 µg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. F) PDA + LE + Ph. G) PDA + 100 µg mL^{-1} nitisinone, pyomelanin inhibitor (NTBC). H) PDA + LE + NTBC.

Responses to oxidative stress

Exposure to hydrogen peroxide at 0.5 to 2.5% did not affect vegetative growth of *L. gilanensis*, but at 5%, colony growth was reduced (Figures 3A and B, and S1). When H_2O_2, tropolone and phthalide were combined, decreased tolerance to oxidative stress was observed, since the fungus grew less compared with the experimental control (Figure 3A), with H_2O_2 alone (Figure 3B), or with each inhibitor (Figure 3C-3D). In the combination treatment with nitisinone and H_2O_2, less colony aerial mycelium was observed (Figure 3H), compared with nitisinone alone (Figure 3G). Biomass production was only affected in the presence of H_2O_2 with tropolone (Figure 4; Table S2). The assays of the lysing enzymes and H_2O_2 indicated that DOPA-melanin was the main melanin that protected the fungus from oxidative stress.

Responses to UV radiation

Hyaline and pigmented conidia without exposure to UV radiation both had 100% germination before 24h of incubation. Exposure to UV radiation generally delayed germination and growth of conidia. Hyaline conidia exposed to UV radiation for 30 or 60 min did not germinate after 24h, and at 48h had, respectively, 33% and 20% germination. Melanized conidia exposed to UV for 30 min or 60 min germinated before 24h, reaching, respectively 80% and 40% germination. After 48h, melanized conidia exposed to 30 min of UV radiation reached 83% germination, and 73% of those exposed for 60 min germinated. The mycelia originating from hyaline conidia exposed for 30 or 60 min remained white for longer time than mycelia from melanized conidia (Figure S3). These results indicated that melanin in conidia cell walls enabled the conidia to maintain viability under UV radiation conditions.

Use of tyrosine as carbon and nitrogen sources

The presence of tyrosine 10 mM in MM9 increased growth and biomass of *L. gilanensis* (Figures 5A, 5E, 6 and S2). The fungus without a carbon source (Figure 5B), a nitrogen source (Figure 5C), or without both (Figure 5D) had shallow growth. When tyrosine was added to these treatments (Figure 5F to H), aerial mycelium and colony pigmentation formed, and a pink pigment was secreted and degradation halos developed. This showed that the fungus catabolized tyrosine and used it as carbon and nitrogen sources. When adding the melanin inhibitors in the MM9-CN treatment (Figure 5I to

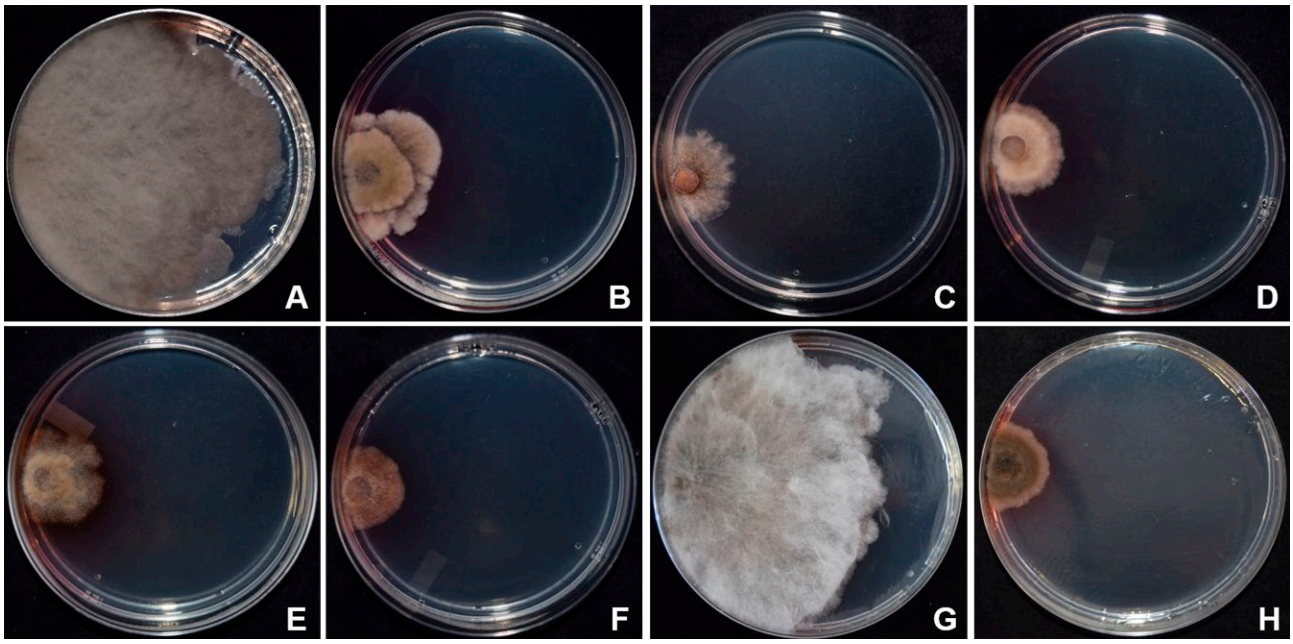

Figure 3. Growth of *Lasiodiplodia gilanensis* under oxidative stress. A) Control. B) PDA + 5% hydrogen peroxide (H_2O_2). C) PDA + 15 μg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. D) PDA + H_2O_2 + Tp. E) PDA + 500 μg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. F) PDA + H_2O_2 + Ph. G) PDA + 100 μg mL^{-1} nitisinone, pyomelanin inhibitor (NTBC). H) PDA + H_2O_2 + NTBC.

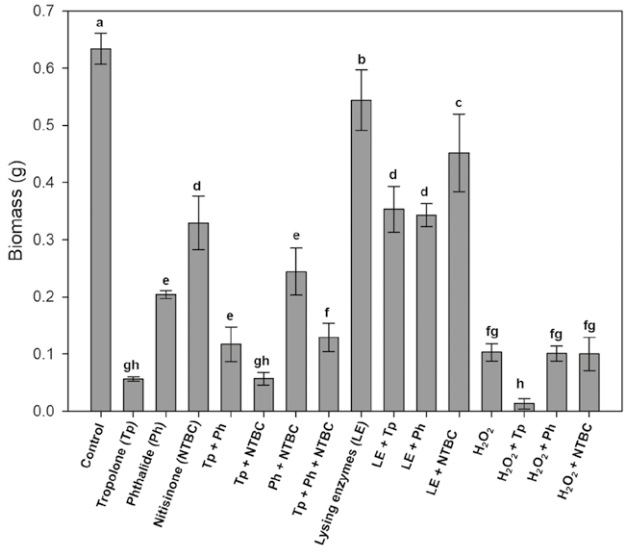

Figure 4. Mean weights of *Lasiodiplodia gilanensis* biomass in the presence of melanin inhibitors and under stress conditions. Bars indicate standard deviations. Means accompanied by the same letters are not significantly different ($\alpha < 0.05$)

L), only in the presence of tropolone was the fungus unable to use tyrosine, since growth was reduced (Figure 5I) and biomass production was greatly reduced (Figure 6; Table S3). The fungus did not grow in the MM9-CN treatment with the three inhibitors of melanin and 10mM tyrosine (Figure 5L).

Melanized mycelium of Lasiodiplodia gilanensis *growing inside grapevine plants*

Lasiodiplodia gilanensis melanized mycelia growing mainly in the pith and vascular bundles of the plants was observed with the use of Masson-Fontana stain (Figure 7). Xylem vessel occlusions were observed in both infected and control plants, probably due to the response of the plant to wounding.

Comparison of Lasiodiplodia gilanensis *genes involved in melanin synthesis with other* Botryosphaeriaceae

The similarity analysis showed that most of the genes were conserved among the *Botryosphaeriaceae*, with similarity percentages greater than 71%. All genes analyzed, previously identified through *L. gilanensis* transcriptome, had over 90% similarity with the genomes of *L. theobromae* CSS-01s and LA-SOL3. Most of the genes involved in the three different melanin pathways (including *sdr*, *thr*, *tyr*, *mco*, *lcc* and, *hmgD*), showed similarity to sequences in *D. seriata* DS831 and F98.1, and *D. corticola* CBS 112549. *Neofusicoccum parvum*

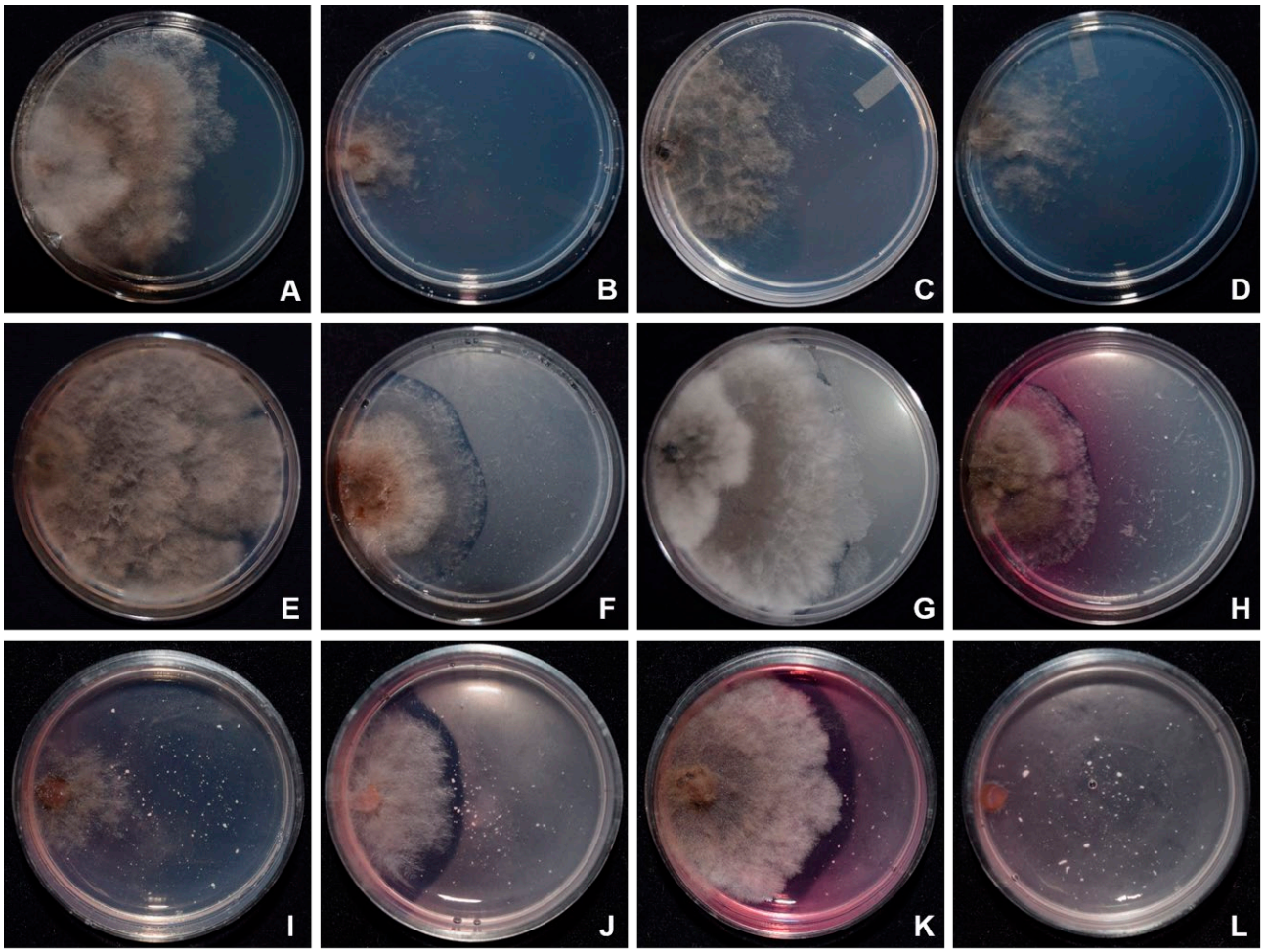

Figure 5. Assessment of effects of tyrosine on growth in *Lasiodiplodia gilanensis*. A) Minimal Medium 9 (MM9). B) MM9 without carbon source (MM9-C). C) MM9 without nitrogen source (MM9-N). D) MM9 without carbon and nitrogen sources (MM9-CN). E) MM9 + 10 mM tyrosine (Tyr). F) MM9-C + Tyr. G) MM9-N + Tyr. H) MM9-CN + Tyr. I) MM9-CN + Tyr + 15 μg·mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. J) MM9-CN + Tyr + 500 μg·mL^{-1} phthalide (Ph), DHN-melanin inhibitor. K) MM9-CN + Tyr + 100 μg·mL^{-1} nitisinone (NTBC), pyomelanin inhibitor. L) MM9-CN + Tyr + Tp + Ph + NTBC.

UCRNP2 had similarity in four genes (*mco*, *lcc*, *hppD* and, *hmgD*), and *Botryosphaeria dothidea* LW030101 only in three genes (*mco*, *lcc* and, *hmg*) (Table 1). The genes *mco*, *lcc* and, *hmgD* were present in all *Botryosphaeriaceae* genomes. Only *hmgD*, which is involved in the tyrosine catabolism and is a precursor in the pyomelanin pathway, was also found in the genome of *P. minimum* with 75% similarity.

DISCUSSION

Melanin inhibitors of three different pathways affected pigmentation, morphology, and the growth of *L. gilanensis*, indicating that this fungus produces DOPA-melanin, DHN-melanin, and pyomelanin. Biosynthesis of three types of melanin has been reported in *S. schenckii* (Romero-Martinez *et al*., 2000; Almeida-Paes *et al*., 2012), *Aspergillus fumigatus* (Schmaler-Ripcke *et al*., 2009; Pal *et al*., 2014), and *Penicillium marneffei* (Liu *et al*., 2014; Boyce *et al*., 2015; Sapmak *et al*., 2015). Ability to synthesize melanin using different pathways may be an adaptation of the fungus to cope with unfavourable conditions, increasing the protection against environmental stress and during host infection (Almeida-Paes *et al*., 2009; Sapmak *et al*., 2015).

Similar gene sequences involved in melanin synthesis are present in the *Botryosphaeriaceae*, and are highly conserved within *Lasiodiplodia*. The genomes from *Diplodia* had high percentages of similarity, for six of

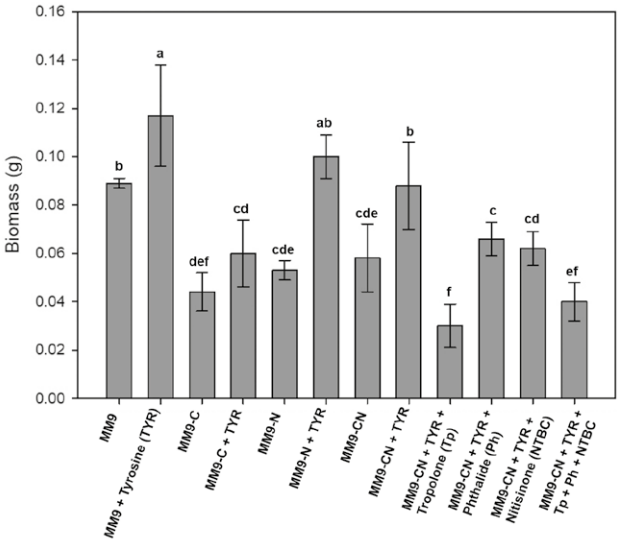

Figure 6. Mean weights of *Lasiodiplodia gilanensis* biomass in the presence of tyrosine and melanin inhibitors. Bars indicate standard deviations. Means accompanied by the same letters are not significantly different ($\alpha < 0.05$).

the seven genes compared, with the *hppD* gene being the only one not found. This is because *Diplodia* is phylogenetically closer to *Lasiodiplodia* than *Neofusicoccum* and *Botryosphaeria*.

A cluster of three genes coding for putative short-chain dehydrogenase/reductases (SDRs) was reported in *N. parvum* (Massonnet *et al*., 2018). The similarity standard was set above 70% in the present study, and these sequences were not so similar with the sequence of the gene *sdr* of *L. gilanensis* (63%). However, this highlights the importance of melanin in the pathogenicity of *Botryosphaeriaceae*.

The homogentisate dioxygenase gene (*hmgD*), which is involved in tyrosine catabolism and is a precursor in the pyomelanin pathway, was detected in all *Botryosphaeriaceae* genomes and in *P. minimum*. This indicates that *hmgD* is essential in the metabolism of fungi causing grapevine trunk diseases.

Aerial mycelium is essential in fungi for the formation and dispersal of reproductive structures (Braun, 2007). For example, a mutant of the *mve1* gene in *Mycosphaerella graminicola* with an albino phenotype produced less aerial mycelium than the wildtype, which indicated a relationship between the lack of melanin and absence of aerial mycelium (Choi and Goodwin, 2011). In *L. gilanensis,* DOPA-melanin is related to the production of aerial mycelium, since the presence of tropolone produced flat colonies. This effect was not due to the interference of any essential pathway. When *Fusarium oxysporum* FFOCR-SQ, and *Trichoderma asperellum* TTORO, isolates with hyaline mycelium from our collection, were grown in the presence of tropolone, no significant effects were observed (Figure S4). Therefore, DOPA-melanin is likely to provide structural strength to the mycelia of *L. gilanensis*, which is necessary for vegetative growth, and DOPA-melanin biosynthesis is likely to be the primary pathway for melanin synthesis in this fungus under *in vitro* conditions. Further genetic studies should confirm this.

The protective role of melanin in enzymatic lysis has been little studied, but melanin-producing mutants of *A. nidulans* (Kuo and Alexander, 1967) and *Aspergillus phoenicis* (Bloomfield and Alexander, 1967) were more susceptible to enzymatic lysis than their respective wild types. In the present study, the presence of cell wall degrading enzymes in the growth media partially inhibited the growth of *L. gilanensis*, but these enzymes did not inhibit formation of aerial mycelium. Instead, when the DOPA-melanin pathway was blocked, growth of the fungus was strongly inhibited. This indicates that the presence of DOPA-melanin helps to maintain cell wall integrity. Since melanin is associated with chitin in fungus cell walls (Nosanchuk *et al*., 2015), as reported for *A. nidulans* (Bull, 1970) and *Cryptococcus neoformans* (Banks *et al*., 2005), this pigment could help conceal target molecules from cell wall degrading enzymes, preventing cell walls from damage and weakening.

DOPA-melanin has been associated with protection against oxidative stress damage in *C. neoformans* (Jacobson and Tinnell, 1993), *Sporothrix* spp., (Almeida-Paes *et al*., 2012), and *A. fumigatus* (Heinekamp *et al*., 2013). In *Inonotus obliquus*, production of DOPA-melanin is one of the primary responses to oxidative stress in the presence of H_2O_2, together with an increment in production of mycelia (Zheng *et al*., 2009). In contrast, in the hypersaline yeast *H. werneckii,* inhibition of DHN-melanin by tricyclazole did not affect survival under H_2O_2 oxidative stress (Kejžar *et al*., 2013), as further evidence that DOPA-melanin is the main melanin involved in protection from oxidative stress. In the present study, the presence of H_2O_2 inhibited growth of *L. gilanensis* but did not affect morphology, pigmentation, or formation of aerial mycelium. However, when the DOPA-melanin pathway was blocked, the fungus did not grow, produced less aerial mycelium, and had different colour, indicating that DOPA-melanin protected the fungal cells against oxidative stress.

Another function of melanin in fungi is the protection against UV radiation (Bell and Wheeler, 1986; Jacobson, 2000; Cordero and Casadevall, 2017). Al-Laaeiby *et al*. (2016) reported differences in the germi-

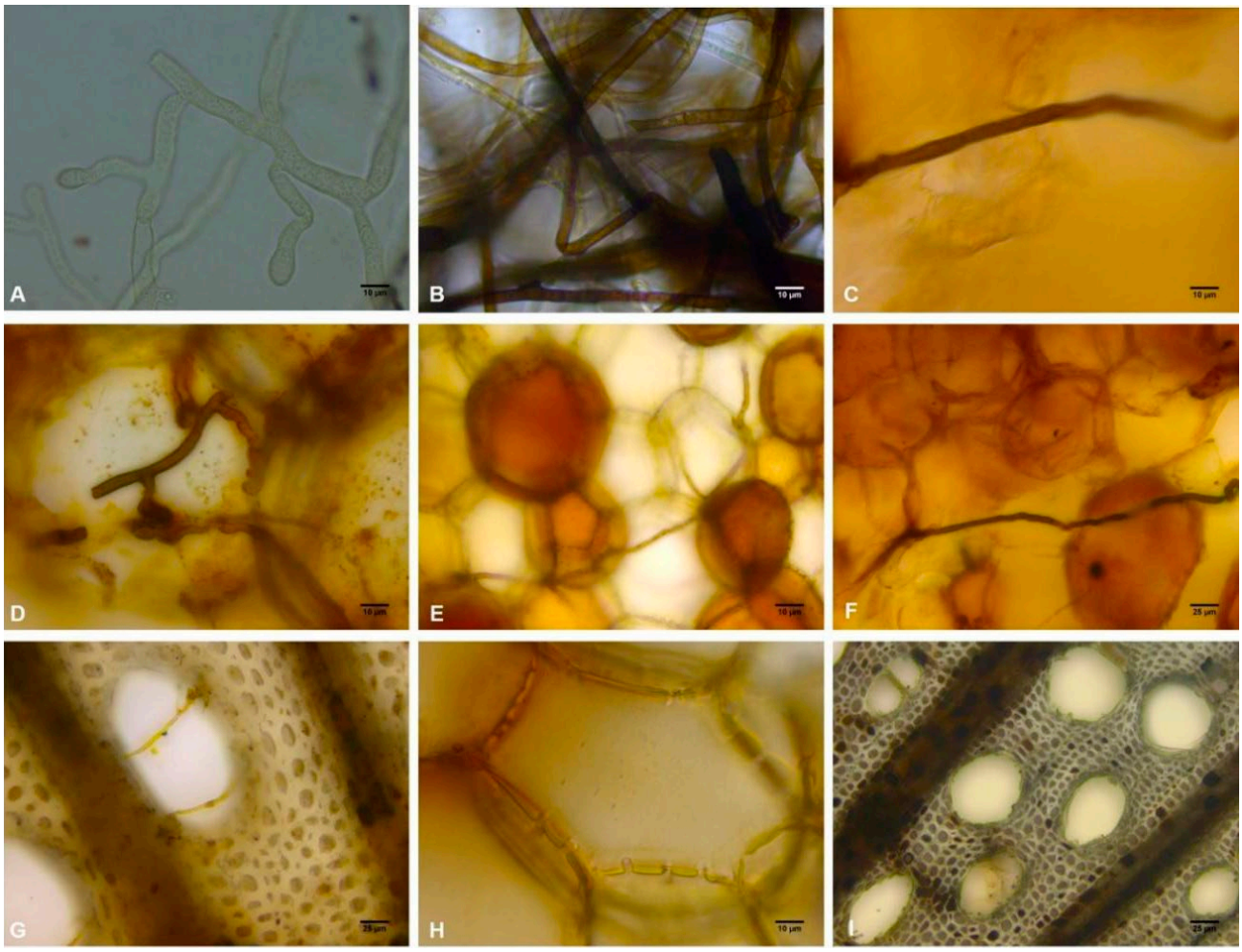

Figure 7. *In planta* melanin staining of *Lasiodiplodia gilanensis* using the Masson-Fontana method. A) Non-melanized fungus on PDA). B) Melanized fungus on PDA). C), D), E), and F) Melanized fungus colonizing the host plant piths. G) Fungus colonizing vascular bundles. H) plant pith in an uninfected plant. I) Vascular bundles in an uninfected plant.

nation of melanized conidia of *Lomentospora prolificans* exposed to 200 mJ cm^{-2} of UV radiation (51% germination) in comparison to conidia deficient in the PKS1 enzyme (28% germination). Hyaline conidia of. *L. gilaniensis* had lower and delayed germination compared with pigmented conidia. Melanin has been reported to absorb UV light, thereby acting as a shield of photons of high energy and protecting cells from damage (Almeida-Paes *et al*., 2012). In the present study, presence of melanin in conidia of *L. gilanensis* gave protection from UV radiation, most probably allowing the conidia to survive under harsh environmental conditions once released from the pycnidia.

Data from the present study also showed that *L. gilanensis* used tyrosine as carbon and nitrogen sources for growth. Use of this amino acid was affected in the presence of tropolone. It was unexpected that nitisinone did not affect growth of the fungus in the presence of tyrosine, since pyomelanin results from tyrosine catabolism. Under *in vitro* conditions, the fungus may use tyrosine mostly for the synthesis of DOPA-melanin, so tyrosine could be the precursor of synthesis of DOPA-melanin, and be essential for growth. As additional evidence, Paolinelli-Alfonso *et al*. (2016) reported induction of *L. gilanensis* genes associated with production of DOPA-melanin, tyrosinase (TYR) and multicopper oxidase (MCO), in the presence of grapevine wood. Phenylalanine is another precursor of DOPA-melanin related to pyomelanin, derived from tyrosine catabolism (Plonka and Grabacka, 2006; Boyce *et al*., 2015). When phenylalanine was evaluated in MM9, growth, aerial mycelium formation and colony pigmentation of the fungus were similar to that observed in the presence of tyrosine. However, the fungus did not use phenylalanine as a car-

Table 1. Comparison of genes involved in the synthesis of melanin among genomes of the *Botryosphaeriaceae*, using tBLASTx analysis of similarity percentages.

Pathway	Gene	Organism	Isolate	Query cover	Similarity Percentage	Accession Number
DHN-melanin	*sdr*	*Diplodia seriata*	DS831	88%	91%	LAQI01000021.1
		Diplodia seriata	F98.1	87%	91%	MSZU01000111.1
		Diplodia corticola	CBS	83%	89%	NW_017971532.1
		Lasiodiplodia theobromae	CSS-01s	100%	94%	KZ107829.1
		Lasiodiplodia theobromae	LA-SOL3	100%	93%	VCHE01000036.1
		Macrophomina phaseolina	MP2_2471002	89%	84%	WHMB01000016.1
	thr	*Diplodia seriata*	DS831	82%	89%	LAQI01000195.1
		Diplodia seriata	F98.1	79%	87%	MSZU01000075.1
		Diplodia corticola	CBS 112549	83%	89%	NW_017971483.1
		Lasiodiplodia theobromae	CSS-01s	96%	98%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	96%	98%	VCHE01000163.1
DOPA-melanin	*tyr*	*Diplodia seriata*	DS831	90%	71%	LAQI01000197.1
		Diplodia seriata	F98.1	90%	71%	MSZU01000087.1
		Diplodia corticola	CBS	89%	73%	NW_017971497.1
		Lasiodiplodia theobromae	CSS-01s	99%	94%	KZ107831.1
		Lasiodiplodia theobromae	LA-SOL3	99%	94%	VCHE01000030.1
	mco	*Neofusicoccum parvum*	UCRNP2	83%	74%	KB915882.1
		Diplodia seriata	DS831	89%	92%	LAQI01000063.1
		Diplodia seriata	F98.1	92%	92%	MSZU01000075.1
		Botryosphaeria dothidea	LW030101	83%	87%	MDSR01000123.1
		Diplodia corticola	CBS 112549	94%	88%	NW_017971485.1
		Lasiodiplodia theobromae	CSS-01s	100%	99%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	100%	99%	VCHE01000017.1
		Macrophomina phaseolina	MP2_2471002	86%	79%	WHMB01000018.1
	lcc	*Neofusicoccum parvum*	UCRNP2	84%	81%	KB916.432.1
		Diplodia seriata	DS831	88%	85%	LAQI01000030.1
		Diplodia seriata	F98.1	88%	85%	MSZU01000080.1
		Botryosphaeria dothidea	LW030101	78%	85%	MDSR01000049.1
		Diplodia corticola	CBS 112549	91%	85%	NW_017971480.1
		Lasiodiplodia theobromae	CSS-01s	98%	97%	KZ107828.1
		Lasiodiplodia theobromae	LA-SOL3	98%	97%	VCHE01000007.1
		Macrophomina phaseolina	MP2_2471002	84%	81%	WHMB01000012.1
Pyomelanin	*hppD*	*Neofusicoccum parvum*	UCRNP2	90%	74%	KB916303.1
		Lasiodiplodia theobromae	CSS-01s	93%	92%	KZ107826.1
		Lasiodiplodia theobromae	LA-SOL3	94%	92%	VCHE01000035.1
	hmgD	*Neofusicoccum parvum*	UCRNP2	82%	93%	KB915800.1
		Diplodia seriata	DS831	95%	95%	LAQI01000171.1
		Diplodia seriata	F98.1	95%	95%	MSZU01000075.1
		Botryosphaeria dothidea	LW030101	85%	94%	MDSR01000002.1
		Diplodia corticola	CBS 112549	93%	95%	NW_017971483.1
		Lasiodiplodia theobromae	CSS-01s	100%	99%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	100%	99%	VCHE01000037.1
		Macrophomina phaseolina	MP2_2471002	87%	95%	WHMB01000018.1
		Phaeoacremonium minimum	UCRPA7	72%	75%	NW_006920969.1

bon or nitrogen source (Figure S5). Thus, phenylalanine did not have an essential role in the melanin pathway of *L. gilanensis*.

For some pathogenic fungi, amino acids are essential sources of carbon and nitrogen (Boyce *et al*., 2015). In plants, tyrosine and phenylalanine give rise to the synthesis of compounds involved in structure and defense, including lignin, suberin, phenylpropanoids, anthocyanins, plastoquinones, isoquinoline alkaloids and flavonoids (Tzin and Galili, 2010; Nelson *et al*., 2013). Some of these compounds are produced near the sites of pathogen infections, and they accumulate in the necrotic tissues, acting in host resistance and defense (Ahuja *et al*., 2012; Nelson *et al*., 2013). Although further *in planta* experiments are required, results of the present study indicate that the ability of *L. gilanensis* to degrade tyrosine confers an advantage during the processes of infection and colonization of grapevine.

It is important to establish how *Lasiodiplodia* infects plants, and the mechanisms these fungi use to counteract host responses to infection. The present study demonstrated that the hyphae of *L. gilanensis* were melanized, mainly among the parenchymal cells in host pith tissues, but not in the vascular bundles. This indicates that the fungus became melanized as host colonization progresses. Although additional study is required using fungus mutants for key melanin genes to fully understand the role of melanin in *L. gilanensis,* results from the present study indicate that melanin protected *L. gilanensis* from adverse environmental conditions, such as the reactive oxygen species, for example, superoxide (O_2^-), or its dismutation product hydrogen peroxide (H_2O_2), which is generated within plants as a primary defense mechanism (Torres *et al*., 2006).

In conclusion, this study demonstrated that *L. gilanensis* synthesized three types of melanin: DOPA-melanin, DHN-melanin, and pyomelanin. DOPA-melanin is the essential melanin, involved in vegetative growth and formation of aerial mycelium. This pathogen utilized tyrosine as a nutrient source, and as a precursor of DOPA-melanin. Melanin protected the fungus against enzymatic lysis and oxidative stress, and conidia from UV radiation. This pathogen also produced melanin within its host as part of the colonization processes.

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Research Papers

Pythium oligandrum induces grapevine defence mechanisms against the trunk pathogen *Neofusicoccum parvum*

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Summary. Grapevine trunk diseases (GTDs) are increasing in vineyards in many grape production regions. Among the pathogens causing these diseases, *Neofusicoccum parvum*, is one of the most frequent and virulent. To control GTDs, biocontrol is being developed using plant beneficial microorganisms. Strains of the oomycete *Pythium oligandrum* have been shown to naturally colonize grapevine roots in vineyards in several countries in Europe. This study examined the ability of the root-coloniser *P. oligandrum* to induce grapevine resistance against *N. parvum*, by deciphering the gene expression changes in a set of 62 genes involved in different grapevine defence pathways. Two greenhouse assays showed that the wood necrosis of vine cuttings caused by *N. parvum* was reduced by 65% when *P. oligandrum* colonized root systems of the plants. The relative expression levels of selected genes in the host trunks were studied by real-time PCR. Plant responses were assessed after inoculation by *P. oligandrum* and/or *N. parvum*, at three different sampling time points (0, 14 and 150 d after *N. parvum* inoculation). Sampling time influenced gene expressions for the different inoculation treatments. At each sampling time, specific host responses to the different treatments were also detected, for controls, and for inoculations with *P. oligandrum*, *N. parvum* or *P. oligandrum* + *N. parvum*. When *P. oligandrum* colonized grapevine root systems, inoculation with the pathogen was associated with increased up-regulation and over-expression of particular genes, including those regulating Pathogen-Related proteins, cell wall reinforcement proteins and hormone signalling pathways. A priming effect of the grapevine defence system was induced in roots colonized by *P. oligandrum*.

Keywords. Biocontrol, induced resistance, gene expression.

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INTRODUCTION

Grapevine Trunk Diseases (GTDs), mainly Esca, have become major concerns for the wine industry. GTDs have deleterious effects on vineyards,

associated with decreased harvest quality and quantity (Lorrain *et al.,* 2012; Bertsch *et al*., 2013; Gramaje *et al*., 2018; Mondello *et al.,* 2018). GTDs affect the wood of grapevines, in trunks, cordons and rootstocks. They complex pathosystems for research, mainly due to the long periods before wood necroses develop and leaf symptoms appear (Maher *et al.,* 2012). The three main GTDs are Esca, Botryosphaeria dieback and Eutypa dieback. In France, from 2012 to 2017, the proportion of unproductive vineyard area was approx. 12% due to these diseases (Doublet and Grosman, 2018). The resulting production losses in France were estimated to be worth approx. €1 billion (Lorch, 2014).

Botryosphaeria dothidea, *Diplodia seriata*, *Neofusicoccum parvum, N. australe*, *N. luteum* and *Lasiodiplodia theobromae* are among the most important pathogens associated with *Botryosphaeria* dieback (Úrbez-Torres and Gubler, 2011; Billones-Baaijens and Savocchia, 2019). These fungi are cosmopolitan and polyphagous. They have been isolated from different plant species and can cause large amounts of decay in host plants (Slippers and Wingfield, 2007). On grapevine, wood symptoms of the disease consist of sectoral and longitudinal brown wood streaking, and perennial cankers (Lecomte *et al.,* 2012). *Botryosphaeriaceae* species are known for differences in pathogenicity. *Neofusicoccum parvum* is one of the most virulent species, as shown by the extent of necroses it causes (Laveau *et al.,* 2009; Pitt *et al.,* 2013; Chen *et al.,* 2014; Bellée *et al.,* 2017).

Given the absence of chemical treatments against GTDs, some assessments of potential naturally-occurring biocontrol microorganisms have been carried out in nurseries and vineyards. Some studies have shown the ability of microorganisms to reduce infections caused by *Botryosphaeraceae* species. Bacteria, including *Bacillus subtilis* strains, *Pantoea agglomerans*, and *Enterobacter* sp., reduced necroses caused by *N. parvum* or *L. theobromae* (Kotze *et al.,* 2011; Haidar *et al.,* 2016a; Rezgui *et al.,* 2016). Additionally, the potential of *B. subtilis*, *B. pumilus*, *Paenibacillus sp*. and some actinobacteria to inhibit *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* infections has also been demonstrated *in vitro* and *in planta* (Alfonzo *et al.,* 2009; Kotze *et al.,* 2011; Compant *et al*., 2013; Haidar *et al*., 2016b; Alvarez-Pérez *et al.,* 2017).

Trichoderma spp. have also been assessed as potential biocontrol agents against GTD pathogens (Fourie *et al*., 2001; Di Marco *et al.,* 2005; Kotze *et al.,* 2011; Mutawila *et al.,* 2011, 2015, 2016; Pertot *et al.,* 2016). Many formulations based on *Trichoderma* spp. strains, including Esquive WP®, Remedier®, Trichoflow-T®, Trichodex® and Vintec®, have been assessed for protection of grapevines against Esca. In France, two biofungicides based on different strains of *T. atroviride*, Esquive® WP (I-1237 strain) and Vintec® (TASCA strain), have been registered to control this disease. Mounier *et al*. (2016) applied Esquive® WP during 2 years on pruning wounds of mature grapevines, and these treatments reduced the expression of foliar symptoms of Esca by 50% and reduced plant mortality. The ability of certain other fungi such as: *Aureobasidium pullulans, Epicoccum layuense, Fusarium lateritium*, or *Cladosporium herbarum* to protect grapevine pruning wounds (John *et al.,* 2005; Rolshausen and Gubler, 2005), and to reduce necrosis in GTD pathogen-infected cuttings, has been shown (Gramaje *et al.,* 2018; Pinto *et al.,* 2018; Del Frari *et al.,* 2019). Most of these studies were made by directly applying fungi, bacteria or their metabolites onto pruning wounds. However, recent studies have shown that particular microorganisms, applied to host roots, can induce grapevine defences against GTD pathogens (Haidar et *al.,* 2016b; Yacoub *et al*., 2016; Daraignes *et al*., 2018; Trotel-Aziz et *al.,* 2019).

One of these microorganisms is the oomycete *Pythium oligandrum,* which is known as a biocontrol agent against many plant pathogens (Rey *et al*., 2008; Benhamou *et al*., 2012; Gerbore *et al*., 2014a). *Pythium oligandrum* strains produce different types of elicitor molecules (oligandrin and cell wall proteins) which induce plant resistance (Gerbore *et al*., 2014a). This oomycete naturally colonizes grapevine roots, and Gerbore *et al.* (2014b) showed that the isolated strains produced high amounts of oligandrin. A previous study showed that grapevine root treatments with *P. oligandrum* reduced by half the size of necroses caused by *P. chlamydospora* on young vines (Yacoub *et al.,* 2016). Moreover, Daraignes *et al.* (2018) observed that necroses caused by *N. parvum* and *P. chlamydospora* on young grafted grapevines were reduced, following plant treatment with *P. oligandrum*, either alone or in combination with beneficial bacteria.

The aim of the present study was to understand the mechanisms contributing to the biocontrol effects of the root biocontrol agent *P. oligandrum* against the grapevine trunk pathogen *N. parvum*. The ability of *P. oligandrum* to enhance grapevine resistance against *N. parvum* infection and induce the expression of genes involved in different pathways of plant defences, were investigated. High-throughput gene expression quantification was measured by microfluidic dynamic array (Fluidigm) technology as defined by Dufour *et al.* (2016). The genes analyzed were associated with Pathogen-Related (PR) proteins (18); secondary metabolite biosynthesis (13); cell wall reinforcement (11); indole pathway (5); Redox status regulation (3); oxylipin pathway (3); and hormone signalling pathway (9).

MATERIALS AND METHODS

Plant material

Two similar independent experiments were carried out in 2017 and 2018. Each experiment was conducted with rooted 'Cabernet Sauvignon' clone 191 (*Vitis vinifera* L.) cuttings, provided from the INRAE experimental vineyards near Bordeaux, France. The cuttings were grown for 2 months before inoculation, and grown under controlled conditions, as described by Laveau *et al.* (2009).

Oomycete and fungus inoculations

Inoculum of *P. oligandrum* strain Po37 was prepared by Biovitis SA Company (Saint Etienne Chomeil, France). At the five to six leaf stage, 50 mL of *P. oligandrum* inoculum was applied at the collar level of each plant. The inoculum concentration was adjusted to 3×10^4 mL^{-1}. Seven days after root inoculation with *P. oligandrum*, selected plants were inoculated with *N. parvum* strain COU02 (GenBank accession number is KT306957; INRAE-UMR SAVE collection, Bordeaux, France). This strain was originally obtained in 2008 from a 'Cabernet Sauvignon' cultivar Bordeaux, and was characterized as highly aggressive (Laveau *et al.,* 2009). The strain was subcultured on malt agar (MA) (15 g L^{-1} of malt (Biokar Diagnostics), 20 g L^{-1} agar (Setaxam®), and was incubated at 22°C (12 h light/12 h dark) for 1 week before plant inoculation.

The pathogen inoculations were carried out as described by Laveau *et al.* (2009). Briefly, each grapevine cutting was drilled at stem level, and then inoculated with 4 mm diam. agar plug from a *N. parvum* culture. The inoculation sites were then each covered with a protective film (Cellofrais®) to prevent external contamination.

Experimental layout

For each experiment, 250 plants were used and distributed between five treatments. The experimental design a randomized complete block with 50 plants per treatment. The different treatments were: (i) plants inoculated at root level with *P. oligandrum*; (ii) plants inoculated only with *N. parvum* at trunk level; (iii) plants inoculated on roots with *P. oligandrum* and then, 1 week later, with *N. parvum* at trunk level; (iv) plants not inoculated with microorganisms (experimental control); and (v) plants inoculated with sterile agar plugs (mock control).

Plant tissue samplings and evaluation of wood necrosis

Wood samples were collected at the end of each experiment (five months after the pathogen inoculations). For each treatment, 30 plants were collected, the stem of each plant was cut longitudinally and the length of wood necrosis was measured. The extent of necrosis was determined by calculating the ratio between the length of necrosis and the total length of the stem. Analysis of Variance (ANOVA, at $P \leq 0.05$) followed by pairwise comparisons of means using Tukey's *post hoc* test was performed to assess differences between treatments, using software R.3.1.2.

For the gene expression investigation, samples from the first experiment (2017) were analysed. Wood samples (1 cm above and 1 cm below the inoculation site on each plant) were collected at three time intervals after treatments were applied: 0 (five h), 14 or 150 d after pathogen inoculation (dpi). All samples were immediately frozen in liquid nitrogen, and stored at -80°C for gene expression analyses.

RNA extraction and cDNA preparation

For each sampling interval, the wood parts of six plants per treatment were sampled. The six samples were randomly grouped to obtain three biological repetitions. After crushing in liquid nitrogen, 100 mg of powder per sample was weighed into a 1.5 mL capacity tube pre-chilled with liquid nitrogen. Then, 1 mL of an extraction buffer (300 mM Tris HCl, pH 8.0; 25 mM EDTA; 2 mM NaCl; 2% CTAB; 2% polyvinylpolypyrrolidone; 0.05% spermidine trihydrochloride; and 2% β-mercaptoethanol, added extemporaneously), preheated to 56°C, was added to the wood powder. The mixture was stirred vigorously and incubated in a water bath at 56°C for 10 min under regular stirring. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added, and the solution was centrifuged at 3500g for 15 min. The following steps were conducted, with RNA extracted with a MagMAX™-96 Total RNA Isolation Kit (ThermoFisher Scientific) following the manufacturer's instructions. RNA concentrations were determined with a Qubit3 fluorimeter (Invitrogen). The total amount of RNA obtained from each sample was reverse-transcribed using 2 µM oligo-$d(T)_{15}$, ribonuclease inhibitor and M-MLV reverse transcriptase (Invitrogen), following the manufacturer's instructions.

High-throughput gene expression quantification was carried out using microfluidic dynamic array (Fluidigm Corporation, California, USA) technology. The relative gene expression of 67 of the "NeoViGen96" chip was

quantified. The primers used were designed by Dufour *et al.* (2016). Among these genes, 62 are involved in different grapevine pathway defences (Figure 1 and Table S1), and five (*EF1γ*, *GAPDH*, *TIP41*, *TUA*, and *THIORYLS8*) are used as housekeeping genes.

Before analyses using qPCR Fluidogm technology, cDNA were preamplified as described by Dufour *et al.* (2016). Briefly, a reaction mixture containing all the pairs of primers (primers pool, 50 mM) and the TaqMan PreAmp Master Mix (1:2, Applied Biosystems) was added to cDNAs. The preamplification programme was as follows: 14 cycles of 95°C for 15 s followed by 60°C for 4 min. The cDNAs were then used for qPCR analyses in a reaction mixture containing TaqMan Gene Expression Master Mix (Applied Biosystems), DNA Binding Dye Sample Loading Reagent (Fluidigm Corporation, California, USA), and EvaGreen (Interchim). The preamplified cDNAs were stored at -20°C before being sent, in dry ice, to the GeT platform (Toulouse, France) for subsequent qPCR analyses. Real-time qPCR was carried out using a BioMark HD system (Fluidigm Corporation, California, USA). The 96.96 dynamic array was used for qPCR, following the manufacturer's protocol (http://www.fluidigm.com/ user-documents).

Data analyses

The fold changes (FCs) of gene expression were calculated using the 2 -ΔΔCT method (Vandesompele *et al.*, 2002), based on multiple gene normalization. The geometric mean of the five reference genes was used as a normalization factor. For each treatment, ER Relative expression gene level was calculated according to the corresponding control (control for *P. oligandrum* treatment, and the mock control for *N. parvum* and *P. oligandrum* + *N. parvum* treatments). FC values between 0 and 1 indicated a low gene expression level in treated samples compared to control samples. In this case, FC were considered biologically significant when 0.5 × < FC or FC > 2 ×, as in Spagnolo *et al.* (2012). The FCs obtained were studied using Principal Component Analysis (PCA) to assess differ-

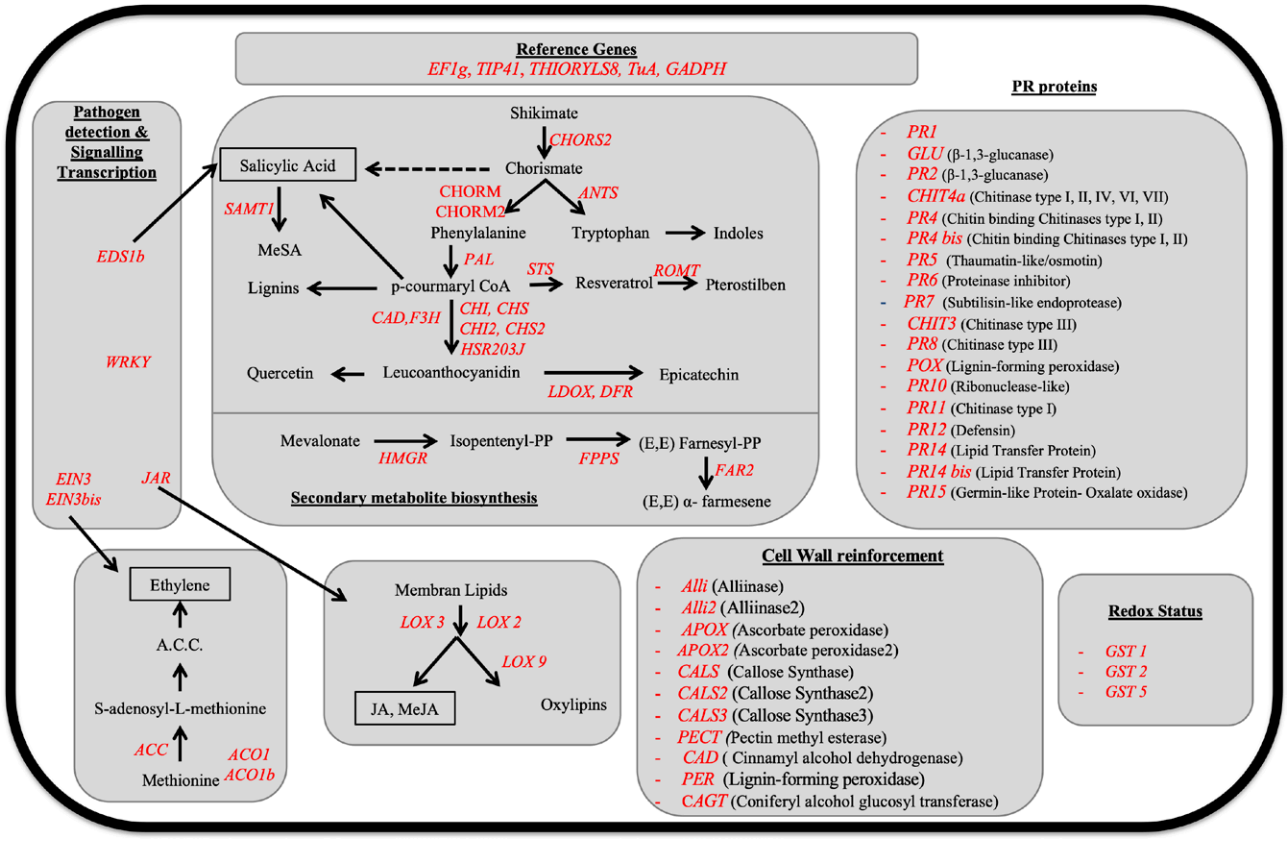

Figure 1. Sixty-two selected genes of "the NeoVigen96" chip are involved in different pathways of the grapevine defence system (Dufour *et al.*, 2016). Housekeeping (five genes), PR proteins (18), secondary metabolite biosynthesis (13), cell wall reinforcement (11), indole pathway (five), redox status regulation (three), oxylipin pathway (three) and hormone signalling pathway (nine genes).

ences between treatments. PCA was carried out using the RCMD package (version 2.6-2) and the plug-in FactoMiner (version 1.7) of R statistical software (version R 3.1.2). For each gene, differential gene expression was then subjected to statistical analysis, using nonparametric Kruskal Wallis tests (at $P \leq 0.05$), and statistically significant differences were determined compared to untreated controls.

To assess the evolution of gene expression, the FC ratios for each treatment were determined by calculating relative gene expressions, first between 0 and 14 dpi, then between 0 and 150 dpi (data not shown).

RESULTS

Assessment of wood necrosis caused by Neofusicoccum parvum *with or without* Pythium oligandrum

The ratios of necroses were measured in the grapevine wood at the end of the two experiments (150 dpi), to evaluate effects of *P. oligandrum* on the necrosis size caused by *N. parvum*. It should be noted that numerous previous studies showed that *N. parvum* strain 'COU02' is able to induce internal necroses in grapevine wood (Laveau *et al.,* 2009; Haidar *et al.,* 2016a; Daraignes *et al.,* 2018). Furthermore, *N. parvum* 'COU02' strain was re-isolated from different tissues sampled from infected plants, Laveau *et al.* (2009). Wood necroses were not observed in control or *P. oligandrum* inoculated plants.

Our results showed that plants inoculated by *N. parvum* at trunk level showed about 65% and 82% of necrosis ratios, in 2017 and 2018, respectively. However, for plants treated at root level with *P. oligandrum* before *N. parvum* inoculation, the ratios of necroses were significantly reduced; by 25% in 2017 and by 36% in 2018 (Figures 2A and B). Overall, the amounts of necrosis reduction were estimated to be 62% in 2017 and 56% in 2018, when the roots were inoculated with *P. oligandrum*. The results also showed that the inoculation method (drilling a hole at trunk level) induced necroses which were smaller than those induced by inoculations with *N. parvum*.

Grapevine trunk-specific responses detected from qPCR analyses

Effects of *Pythium oligandrum* and inoculation method on grapevine responses

In order to explore the effects of *P. oligandrum* root inoculation and the inoculation method on grapevine defences, expression levels of 62 grapevine defence genes were assessed (Figure 3). The gene expression levels in control plants were used as references. PCA was performed to evaluate effects of *P. oligandrum* and inoculation method (mock control) on transcriptomic grapevine responses, at three different sampling time points (0, 14 and 150 dpi) (Figures 3A and B). PCA eigenvalues indicated that the first two principal components explained 47.04% of total data variance. For each treatment, confidence ellipses revealed three statistically significant clusters corresponding to sampling time points, which were separated on Dim 1 (30.25% of the variability) for

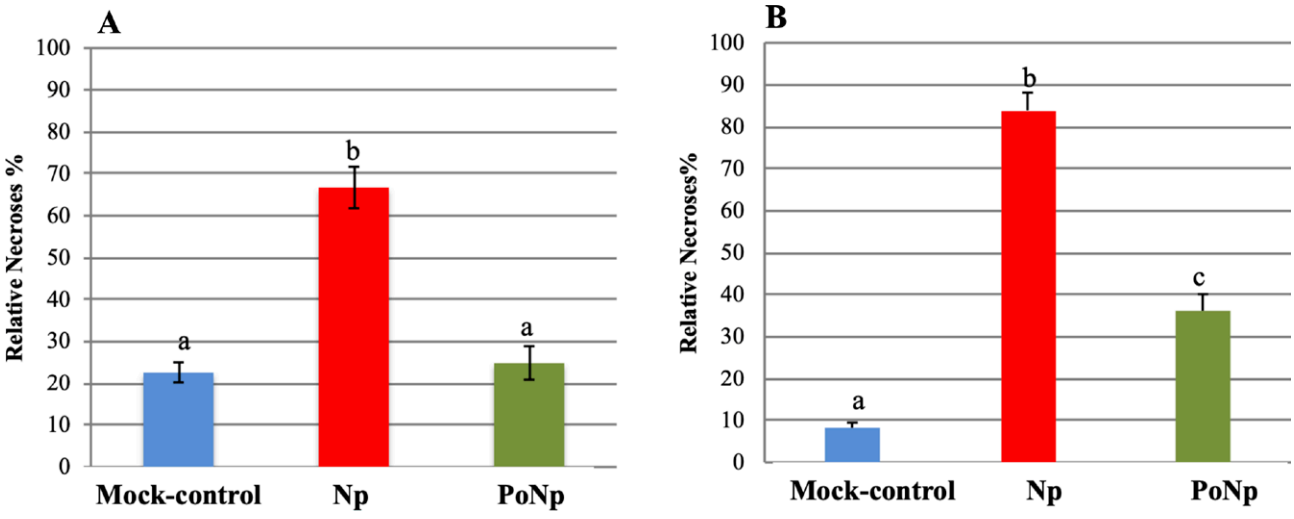

Figure 2. Wood necrosis resulting from inoculations with *Neofusicoccum parvum* in trunk cuttings with or without inoculation with *P. oligandrum* (Po) at root level, 150 days post-inoculation. The values reported are means (± SE) of 30 samples collected from each treatment. PoNp = Po + *N. parvum*, and Np = *N. parvum*. Different letters indicate differences ($P > 0.05$) between treatments.

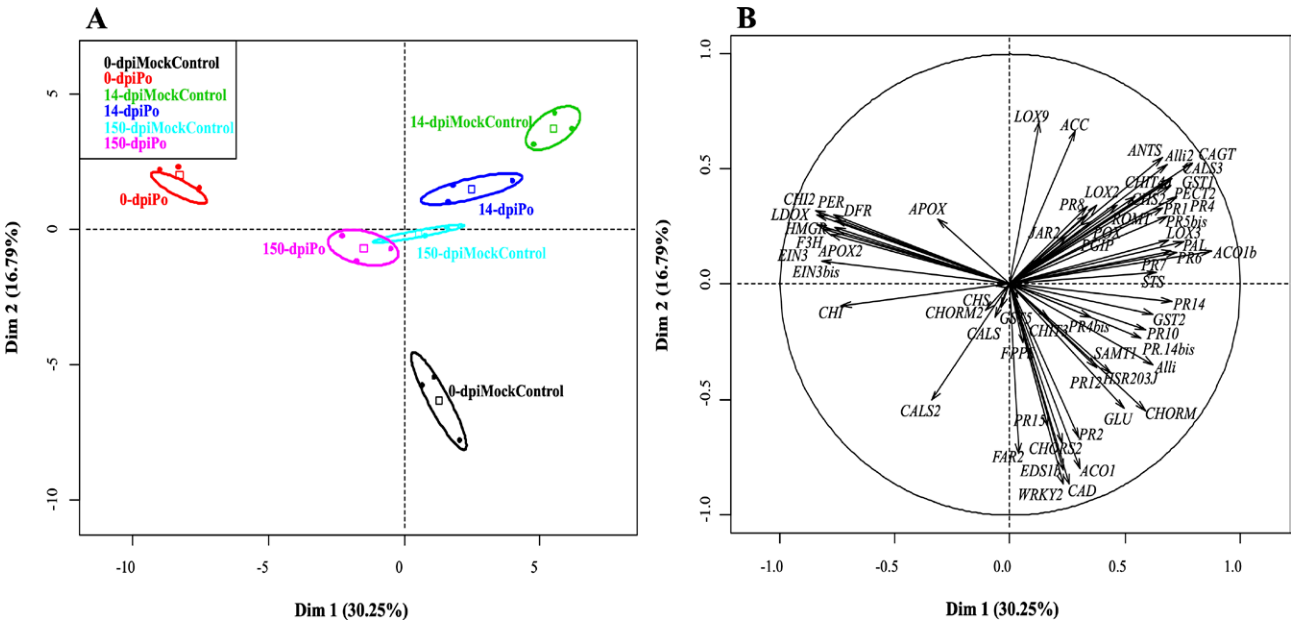

Figure 3. A. Principal component analysis of specific grapevine responses to *Pythium oligandrum* and mock-control treatments (relative expression levels of the 62 genes involved in plant defences) at 0, 14 or 150 d after treatments applied at trunk level. Gene expression of control plants was used as the reference to calculate relative expression. Ellipsoids represent the centres of factors with 95% confidence. The different groups are indicated by different colours. Po = *P. oligandrum*. B. Distribution into the correlation circles of the relative expression levels of the 62 genes studied.

P. oligandrum and Dim 2 (16.79% of the variability) for the mock-controls. Moreover, for each sampling time point, expression levels of the assessed genes were different according to the treatment, except at 150 dpi. Plant responses to *P. oligandrum* and mock-control treatments were greater at 0 dpi (5 hours after pathogen inoculation) than those assessed at 14 or 150 dpi.

In order to characterize the effects of *P. oligandrum* and mock-control treatments on grapevine defence responses, the corresponding correlation circles were examined (Figure 3B). Only well-represented genes are presented, and most of these genes correlated with grapevine responses to the mock-control treatment at 0 dpi. Eighteen genes involved in all the examined grapevine defence families (except the oxylipin family) were up-regulated following drilling of inoculation holes. Seven of these genes (*GLU*, *PR2*, *PR10*, *PR12*, *PR14*, *PR14bis* and *PR15*) belong to the PR protein family, , three genes (*HSR203J*, *CHORM* and *CHORS2*) are involved in the indole signalling pathway, and four genes (*EDS1*, *ACO1, SAMT1* and *WRKY2*) are involved in the hormone signalling pathway. Additionally, two (*CAD*, *Alli*) of the 18 genes induced after the hole drilling are in the cell wall reinforcement family, one (*FAR2*) is involved in phenylpropanoid synthesis and another (*GST2*) is involved in redox status regulation.. From the *P. oligandrum* treatment, grapevine responses were mostly associated with expression of six genes (*CHI2*, *CHI*, *LDOX*, *DFR*, *HMGR* and *F3H*) involved in secondary metabolite biosynthesis and three (*PER*, *APOX2* and *CHI*) involved in cell wall reinforcement, 7 days after the inoculation of *P. oligandrum* (0 dpi). The treatment with *P. oligandrum* is also correlated with up regulation of two genes (*EIN3* and *EIN3bis*) coding for a transcription factor acting as a positive regulator in the ethylene response pathway. At 14 and 150 dpi, grapevine responses to *P. oligandrum* and the mock-control were correlated with the same group of genes which are involved in different families of grapevine defences (Figure 3B).

Effects of *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* on grapevine responses

The biological system studied here included wounding in the pathogen infection process, and this induced strong plant responses. In order to subtract responses due to the inoculation method, and to evaluate effects of microorganism inoculations on grapevine responses, gene relative expression levels in *N. parvum* and *P. oligandrum* + *N. parvum* inoculated plants were calculated in relation to those measured in mock-control plants. The effects of *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments on grapevine responses were

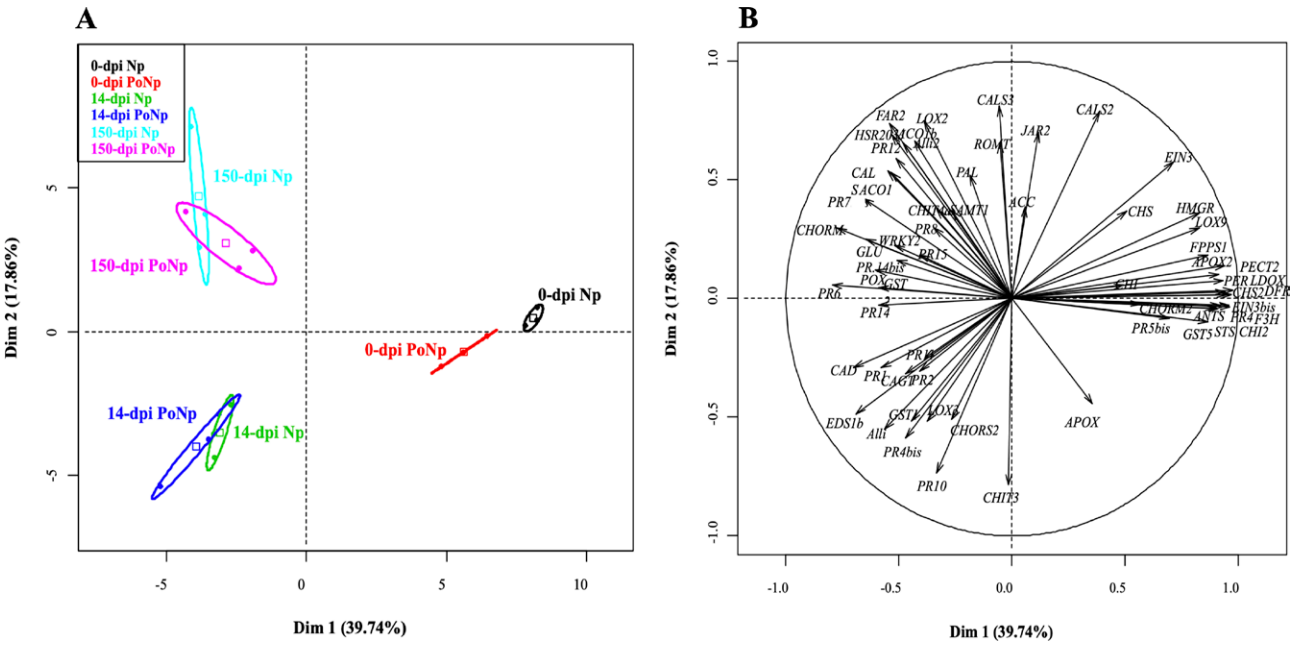

Figure 4. A. Principal component analysis of specific plant responses to *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* inoculation treatments (relative expression levels of the 62 genes involved in plant defences), at 0, 14 or 150 days after inoculations at trunk level.. Ellipsoids represent the centre of factors with 95% confidence. The different groups are indicated by different colours. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*. B. Distribution into the correlation circles of the relative expression levels of the 62 genes studied.

then analysed with PCA, at 0,14 and 150 dpi (Figure 4A). PCA eigenvalues indicated that the first two principal components explained 57.6% of total data variance. Results showed that relative gene expression levels of the assessed genes involved in grapevine defences were more differentiated at the sampling time points than to the inoculation treatment factor. PCA showed that, at each sampling time point, the same genes were modulated following the *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments. However, the amplitude of gene expressions differed according to the treatment.

The correlation circle corresponding to grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments showed that three different groups of genes were distinguished (Figure 4B). Each group of genes was associated with grapevine responses to the two inoculation treatments, at each sampling time point. The first group, correlated with grapevine responses at 0 dpi, included genes mostly involved in secondary metabolite biosynthesis (*CHS*, *HMGR*, *FPPS1*, *LDOX*, *CHS2*, *DFR*, *F3H*, *STS* and *CHI2*). However, grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments, at 14 or 150 dpi were more associated with the up-regulation of PR proteins.

Relative gene expression levels were separately compared between *N. parvum* and *P. oligandrum* + *N. parvum* treatments at each sampling time point, to focus on the effect of *P. oligandrum* on host responses to *N. parvum* inoculation (Figure 5). PCA eigenvalues indicated that the first principal components Dim1 and Dim2 explained 59.16% of the total data variance at 0 dpi, 63.11% at 14 dpi, and 51.35% at 150 dpi. Furthermore, at 0 dpi, Dim1, which represented 35.8% of total data variance, separated grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments (Figure 5A). At 14 dpi, host responses to the two inoculation treatments were separated by Dim 2, which represented 23.26 % of total data variance (Figure 5B). No statistically significant differences, were observed at 150 dpi, however, for responses between the two treatments (Figure 5C). Overall, at each sampling time point, *P. oligandrum* significantly modulated grapevine responses to *N. parvum* inoculation, except at the end of the experiment (150 dpi).

The distribution into a correlation circle of FC of the 62 genes involved in plant defences at 0 dpi, showed that two groups of genes were differentiated by Dim1 (Figure 5D). Most of the genes studied (approx. 70%) were associated with grapevine responses to infection by *N. parvum*. Only nine genes (*PR6*, *PR7*, *PR11*, *PR14bis*, *CHORS2*, *HSR203J*, *GST*, *CAGT* and *POX*) were associated with grapevine responses to the *P. oligandrum* + *N. parvum* inoculation treatment. However, at 14 dpi, after

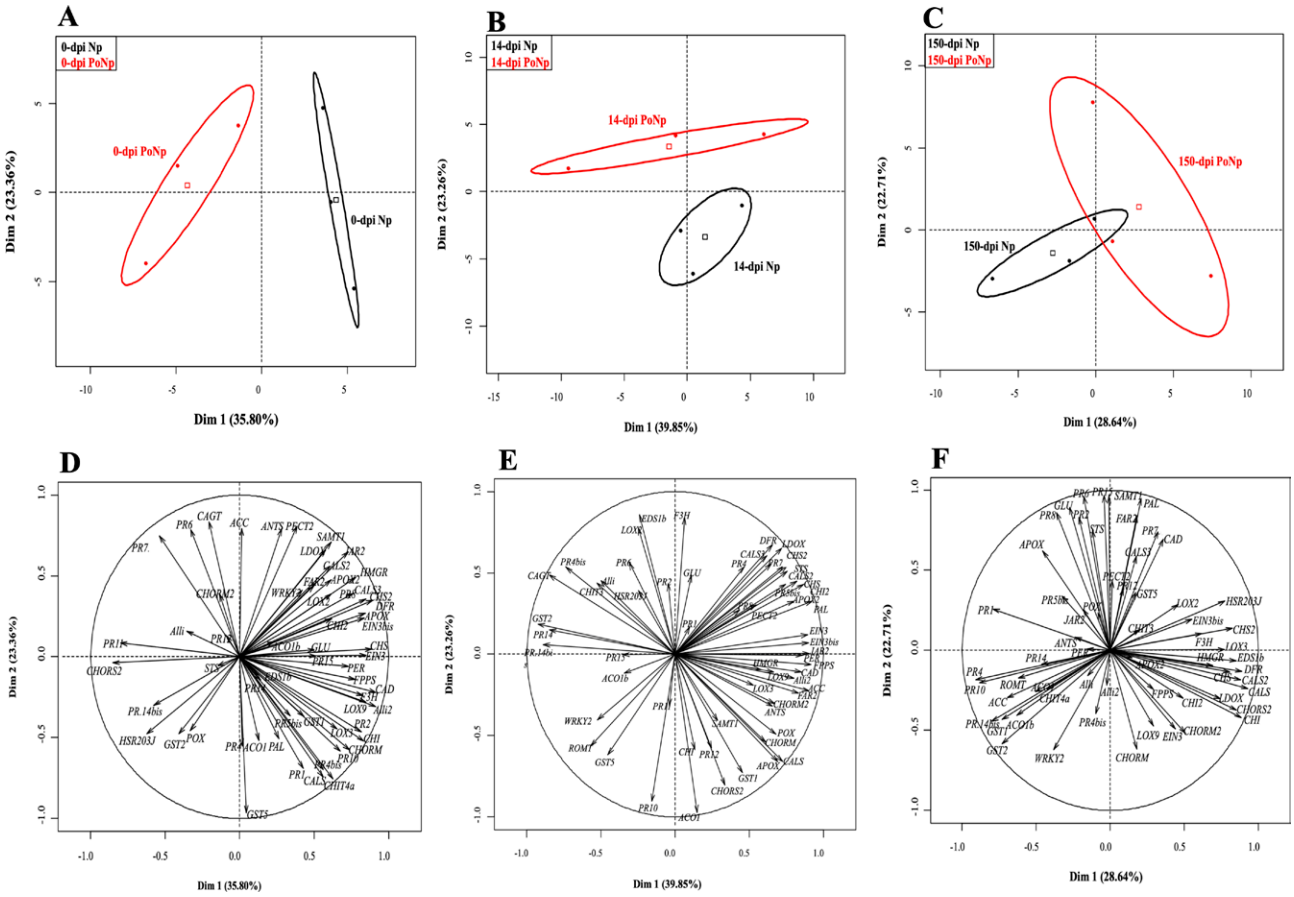

Figure 5. A, B and C. Principal component analyses of specific plant responses to *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* treatments (relative expression levels of the 62 genes involved in plant defences) at each trunk level sampling time (A for 0 days after pathogen inoculation (dpi), B for 14 dpi, and C for 150 dpi). Ellipsoids represent the centre of factors, with 95% confidence. The different groups are indicated by different colours. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*. D, E and F. Distributions into correlation circles of the relative expression levels of the 62 genes studied (D for 0 dpi, E for 14 dpi and F 150 days dpi).

P. oligandrum inoculations to roots, more host genes (30) were associated with response to *N. parvum* inoculations than those in plants inoculated only with the pathogen (25 genes) (Figure 5E). At 150 dpi, the numbers of genes correlated with each treatment decreased (Figure 5F). Despite this decrease of over-expressed genes (32 genes), the results were similar to those observed at 14 dpi, with more correlated genes with responses to *P. oligandrum* + *N. parvum* inoculation (21 genes) than to *N. parvum* inoculation (11 genes).

In order to obtain more details about gene expression changes induced by *P. oligandrum* on host responses to *N. parvum* inoculation, relative expression levels of all the studied genes associated with *N. parvum* and *P. oligandrum* + *N. parvum* treatments was examined at the three different sampling time points (Figure 6). Overall, the host responses to *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments were similar, at 0-dpi. For both treatments, all the assessed genes involved in phytoalexin biosynthesis were strongly up-regulated, except *FAR2* which was repressed by both treatments at 0 dpi. All the genes involved in the other assessed defence gene families were strongly repressed at 0 dpi, except for a few genes from each family. Overall, ten genes were significantly up-regulated by both treatments at 0 dpi. One of these genes, *PR4*, encoded PR proteins; four genes (*APOX2, PECT2, PER* and *CALS2*) are in the cell wall reinforcement family; two (*EIN3* and *EIN3bis*) are involved in the hormone signalling pathway; and *GST5*, *ANTS* and *LOX9* are involved, respectively, in redox status regulation, the indole pathway, and the oxylipin pathway. For all the over-expressed genes, grapevine responses to *N. parvum* inoculation were slightly reduced after *P. oligandrum* inoculation of the root system.

Fourteen days after *N. parvum* inoculation, particular genes, especially those encoding PR proteins includ-

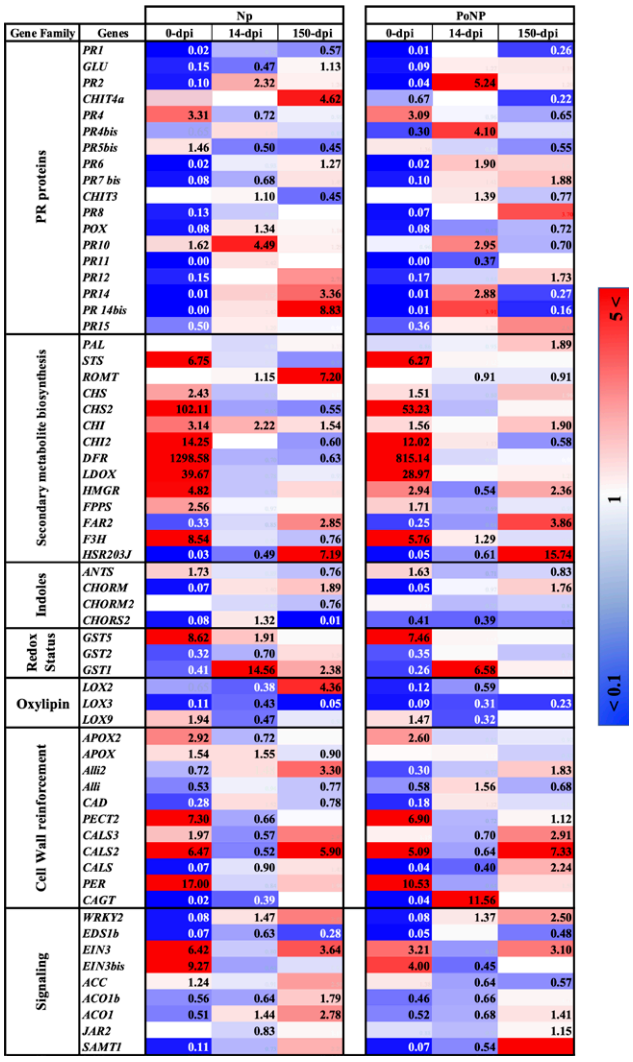

Figure 6. Relative expression levels of 62 defence genes in grapevine wood 0 ,14 or 150 days after inoculations. Gene expression of mock-control plants was used as reference to calculate the relative expression. Each column represents the time point after inoculation treatments (Np = *Neofusicoccum parvum* and PoNp = *Pythium oligandrum* + *N. parvum*), and each line corresponds to one gene, represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent samples. Up-regulated genes are shown in shades of red, with relative expression levels greater than 5 in bright red. Down-regulated genes are shown in shades of blue, with relative expression less than 0.1 in dark blue. Numbers in boxes represent the significant changes ($P < 0.05$; Kurskall-Wallis test) in gene expression compared to the mock-control. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*.

ing *PR2*, *PR4bis, PR6*, *CHIT3*, *PR10* and *PR14*, were more highly expressed in plants inoculated with *P. oligandrum* + *N. parvum* than in those inoculated with *N. parvum* alone. One gene involved in the cell wall reinforcement pathway, i.e. *CAGT*, was strongly over-expressed after *P. oligandrum* inoculation of the roots.

At the end of the experiment, relative expression levels of most of the studied genes were similar for the two inoculation treatments, but some differences were detected. Three genes, *CALS2* involved in cell wall reinforcement, *FAR2* in phytoalexin synthesis and *HSR203J* in the indole pathway, were more up-regulated in plants inoculated with the two microorganisms than in those only inoculated with the pathogen. In contrast, eight genes, associated with PR proteins (*CHIT4*a, *PR14* and *PR14bis*), secondary metabolite synthesis (*ROMT*), Redox status (*GST1*), the oxylipin pathway (*LOX2*), cell wall reinforcement (*Alli2*) and hormone signalling (*ACO1*), were over-expressed in plants inoculated only with *N. parvum*.

To highlight effects of *P. oligandrum* on the evolution of grapevine relative gene expression levels, the ratios of relative gene expression were calculated, first between 0 and 14 dpi (Figure 7), and then between 0 and 150 dpi (data not shown). Eleven grapevine genes encoding PR proteins were considerably more over-expressed in plants inoculated with *P. oligandrum* and *N. parvum*, than in those only inoculated with *N. parvum*. In plants inoculated with *P. oligandrum*, the ratios of three PR protein genes (*GLU*, *PR2* and *PR4*) were approx. five times more expressed than in plants inoculated with *N. parvum*. The same trend occurred for three other genes, *LOX2* (oxylipin pathway), *CAGT* (cell wall reinforcement) and *EDS1b* (hormone signalling). However, after *P. oligandrum* inoculation of host roots, the *CAGT* gene was the most up-regulated between 0 dpi and 14 dpi after *N. parvum* inoculation. Expression of this gene was 19 times greater in plants pre-treated with *P. oligandrum* and then inoculated with *N. parvum* (ratio = 323.3) than in those only inoculated with *N. parvum* (ratio = 17.01).

DISCUSSION

Microorganisms have been used as Biocontrol Agents (BCAs) for control of GTDs. *Trichoderma* spp. and *B. subtilis* strains are known to have direct effects on GTD pathogens, through competition for nutriments, antibiosis and mycoparasitism. These organisms are frequently applied onto pruning wounds, or by dipping grapevine cuttings in the BCA solutions (Bertsch *et al.* 2013; Gramaje *et al.* 2018; Mondello *et al.*, 2018). In the present study, a root BCA, *P. oligandrum*, which naturally colonises grapevine roots (Gerbore *et al.*, 2014b), was studied to evaluate inoculation with this oomycete to induce grapevine responses against *N. parvum*. It is important

to underline that *P. oligandrum* is able to colonize grapevine plant roots during, at least, 4 months following its inoculation (Yacoub *et al.,* 2016; 2018).

The study has provided evidence that grapevine root treatment with *P. oligandrum* reduced wood necrosis (about 60%) resulting from *N. parvum* inoculation. This confirms the results obtained by Daraignes *et al.* (2018), who observed that wood necroses caused by *N. parvum* and *P. chlamydospora* were reduced following the application of *P. oligandrum*, either alone or in combination with particular beneficial bacteria, in young grafted grapevines. That study there was no contact between the root BCA and the trunk pathogen, so it was assumed that protection by *P. oligandrum* was due to the induction of the grapevine defence system, but this point was not investigated. Some previous studies have shown the capacity of root BCAs to induce systemic resistance against GTD pathogens. Yacoub *et al.* (2016) demonstrated that three different inocula of *P. oligandrum* applied to grapevine roots reduced wood necroses caused by *P. chlamydospora* in host trunks. Expression of 22 grapevine defence genes was differentiated for each combination in this tripartite interaction (i.e. control, *P. oligandrum*, *P. chlamydospora* and *P. oligandrum* + *P. chlamydospora* treatments). Trotel-Aziz *et al.* (2019) showed that grapevine root treatment with *B. subtilis* strain PTA-271 reduced, by 63–75%, cankers and stem lesions, caused by *N. parvum*, compared to non-bacteria pre-treated plants.

In the present study, following observation of reductions of wood necroses, and to decipher the grapevine responses in the plant/*P. oligandrum*/*N. parvum* interaction, high throughput gene expression quantification was carried out using microfluidic dynamic array (Fluidigm) technology. The relative expression levels of the 62 genes involved in grapevine defence mechanisms (Dufour *et al.,* 2016) were studied at 0,14 and 150 dpi, and effects of the inoculation method used (drilling holes in the plant stems) on host responses over time were evaluated.

Compared to plants inoculated only with *P. oligandrum* (not wounded), the inoculation method induced strong modulation of gene expression, especially a few hours (0 dpi) after plant wounding. The most modulated genes were mainly those affecting PR proteins (*PR2*, *PR10*, *PR12*, *PR14*, *PR14bis* and *PR15*) and those involved in the indole (*HSR203J*, *CHORM* and *CHORS2*) and hormone signalling pathways (*EDS1*, *ACO1, SAMT1* and *WRKY2*). PCA analyses indicated that grapevine molecular responses to mock-control and *P. oligandrum* inoculation were differentiated at 0 and 14 dpi, but not at 150 dpi. This indicated that the effects of the inoculation method on grapevine defences was transient. This conclusion is partly supported by the results of Pierron *et al.* (2016), who showed that plant internodes responded intensely to injuries 10 to 120 h following wounding. In the present study, six of the 11 selected genes (*PAL*, *PR10.3*, *TL*, *TLb*, *Vv17.3* and *STS*) were up-regulated, but expression of other genes, including *PIN*, was unaffected.

For responses to *P. oligandrum* inoculations 7 d after the oomycete inoculation (0 dpi), several genes involved in secondary metabolite biosynthesis, cell wall reinforcement and the ethylene response pathway were up-regulated. This result is similar to those obtained by Miotto-Vilanova *et al.* (2019), who showed that *Paraburkholderia phytofirmans* PsJN systemically induced overexpression of all genes implied in phenylpropanoid and flavonoid pathways. Activation of ethylene pathway genes after BCAs inoculation has also been demonstrated in previous studies (reviewed by Pieterse *et al.,* 2014).

As wounding caused significant host stress, a separate investigation of the grapevine trunk responses was required to evaluate BCA and pathogen effects on plant defences, so grapevine relative gene expression levels were calculated following microorganism inoculations. Mock-inoculated plants were used as references. Overall, sampling time point after inoculations had a major effect on relative gene expression levels, whatever the treatment. This result is similar to those in previous studies, showing that effects of GTD pathogens on the grapevine defence system, applied individually or in combinations with BCAs, differ according to periods post inoculation (Haidar *et al.,* 2016b; Pierron *et al.* 2016; Massonnet *et al.,* 2017; Mutawila *et al.,* 2017; Trotel-Aziz *et al.,* 2019; Zhang *et al.,* 2019). For effects of *N. parvum* and *P. oligandrum* + *N. parvum* treatments on grapevine gene expression, at each sampling time point, PCAs indicated that the relative expression levels of the 62 studied genes were different at 0 and 14 dpi. However, no significant differences were observed at the end of experiment (150 dpi). This indicates that *P. oligandrum* modulated grapevine responses only at early stages post inoculation.

After an initial analysis of grapevine responses to *N. parvum* and/or *P. oligandrum* inoculations, a specific analysis was carried out to evaluate the modulation, over time, of each gene expression after each treatment. Firstly, the heatmap analyses of relative gene expression levels indicated that a similar tendency was observed in plant responses to *N. parvum* and to *P. oligandrum* + *N. parvum* treatments, with strong repression of genes affecting PR proteins, and strong up-regulation of most of the genes involved in phenylpropanoid biosynthesis, 5 h after pathogen inoculation (0 dpi). These results are sim-

ilar to those of Yacoub *et al.* (2016), who reported that wood infections by *P. chlamydospora* induced repression of *PR10* and *GLU* genes affecting PR proteins, and over-expression of *PAL*, which embodies the phenylpropanoid pathway. Pierron *et al.* (2016) and Massonnet *et al.* (2017) also showed that *PAL* and *STS*, involved in Stilbene synthase, were up-regulated in grapevine trunks a few hours after GTD pathogen inoculations.

Fourteen d after pathogen inoculations, the trends observed at 0 dpi were reversed for *N. parvum* and *P. oligandrum* + *N. parvum* treatments. Most of the studied PR Protein genes were up-regulated, as were genes involved in secondary metabolite biosynthesis. This was similar to the results of Haidar *et al.* (2016b), who showed that *P. chlamydospora* infections in grapevine trunks gave over-expression of PR protein genes *PR10* and *CHIT3*, and repression of the gene encoding *PAL*, 2 weeks after pathogen inoculation.

To focus more on effects of *P. oligandrum* on grapevine responses to *N. parvum* infection, the evolution of the FC gene levels were calculated between 0 and 14 dpi (Figure 7). This showed that, after inoculations with *P. oligandrum* to plant roots, stronger over-expression of genes encoding PR proteins was detected than in plants inoculated with *N. parvum* alone. This confirmed the ability of *P. oligandrum* to induce amplification of the PR proteins genes *PR1*, *GLU*, *PR2*, *PR4bis*, *PR6*, *PR7*, *PR8*, *PR14* and *PR14bis*, which has also been observed in tomato leaves after infection with *Botrytis cinerea* (Le Floch *et al.,* 2003, 2009). In addition, plants pretreated with *P. oligandrum* and inoculated with *N. parvum* showed more rapid up-regulation of *LOX2* (oxylipin pathway) and *GST2* (glutathione-S-transferase genes) relative gene expression levels, than was measured in plants inoculated only *N. parvum*. This result is similar to those of Yacoub *et al.* (2016), who showed, in three experiments, that grapevine root treatment with *P. oligandrum* induced over-expression of *GST2* and *LOX2* in response to *P. chlamydospora* inoculation. Oxylipin pathway and glutathione-S-transferase genes are known to be induced in plants after elicitor application (Dufour *et al.,* 2013; Harel *et al.,* 2014; Bellée *et al.,* 2018).

For genes involved in cell wall reinforcement, *CAD* and *CAGT* were more induced in *P. oligandrum* + *N. parvum* inoculated plants than in those inoculated only with *N. parvum*. As the *CAD* and *CAGT* genes are involved in the lignin pathway, this result suggests that over-expression of these genes following *P. oligandrum* root colonisation enhanced the ability of the grapevine plants to reinforce cell walls via lignin accumulation.

Among the genes involved in pathogen detection signalling transcription, *P. oligandrum* induced

		FC 14-dpi/FC 0-dpi	
Gene Family	**Genes**	**Np**	**PoNp**
PR proteins	*PR1*	35.38	68.92
	GLU	3.22	14.10
	PR2	22.37	147.95
	CHIT4a	NA	NA
	PR4	0.22	0.31
	PR4bis	2.28	13.79
	PR5	0.34	0.62
	PR6	57.11	88.99
	PR7	8.01	14.01
	CHIT3	NA	NA
	PR8	6.30	15.33
	POX	16.51	7.06
	PR10	2.77	3.08
	PR11	1167.32	137.97
	PR12	6.52	4.81
	PR14	120.72	208.29
	PR 14bis	306.34	675.69
	PR15	2.56	3.52
Secondary metabolite biosynthesis	*PAL*	0.84	1.11
	STS	0.13	0.18
	ROMT	NA	NA
	CHS	0.31	0.53
	CHS2	0.01	0.01
	CHI	0.71	0.67
	CHI2	0.08	0.11
	DFR	0.00	0.00
	LDOX	0.02	0.04
	HMGR	0.16	0.18
	FPPS	0.38	0.40
	FAR2	2.61	2.40
	F3H	0.11	0.22
	HSR203J	16.89	11.67
Indoles	*ANTS*	0.51	0.43
	CHORM	19.99	19.16
	CHORM2	0.84	0.65
	CHORS2	16.43	0.97
Redox Status	*GST5*	0.22	0.15
	GST2	2.20	3.14
	GST1	35.95	24.87
Oxylipin	*LOX2*	0.59	4.71
	LOX3	3.91	3.30
	LOX9	0.24	0.22
Cell Wall reinforcement	*APOX2*	0.25	0.33
	APOX	1.01	1.10
	Alli2	2.05	2.61
	Alli	1.78	2.69
	CAD	5.41	7.36
	PECT2	0.09	0.10
	CALS3	0.29	0.58
	CALS2	0.08	0.13
	CALS	13.72	9.62
	PER	0.05	0.06
	CAGT	17.01	323.30
Signalling	*WRKY2*	17.74	16.94
	EDS1b	8.83	20.18
	EIN3	0.12	0.19
	EIN3bis	0.07	0.11
	ACC	0.73	0.46
	ACO1b	1.14	1.44
	ACO1	2.85	1.31
	JAR2	0.79	0.85
	SAMT1	6.80	7.52

Figure 7. Ratios of relative expression levels of the 62 defence genes in grapevine wood between 0 d post inoculation (dpi: 5 h after inoculations) and 14 dpi. Gene expression of mock-controls was used as the reference to calculate relative gene expression. Each column represents the time point after treatment (Np = *Neofusicoccum parvum* and PoNp = *Pythium oligandrum* + *N. parvum*), and each line corresponds to one gene represented by a single row of boxes. Color gradient from blank (low values) to dark green (high values) was used to indicate the magnitude of FC values.

genes involved in the Salicylic Acid (SA) pathway, with the SA-dependent (Enhanced Disease Suceptibility, *EDS1b*) gene, and that controlling SA-methyl transferase (*SAMT1).* This result indicates the involvement of the SA pathway in induction of systemic resistance. This result differs from those in previous studies, which have demonstrated the implication of Jasmonic Acid and Ethylene signalling pathways in other *P. oligandrum*/pathogen/plant interactions (Hase et *al.,* 2006, 2008; Wang *et al.,* 2011; Ouyang *et al.,* 2015). Other studies have also showed an over-expression of *EDS1* induced systemic host resistance against pathogens (Gao *et al.,* 2010; Tahir *et al.,* 2017; Fan *et al.,* 2018).

By comparing the evolution of relative gene expression levels between 0 and 14 dpi, the present study has shown that *P. oligandrum* induced strong over-expression of particular grapevine defence-related genes and activation of priming after pathogen inoculation. The oomycete root colonization induced enhanced expression of genes involved in three different categories. These were:

(i) PR proteins genes, including *PR1*, a marker of the SA pathway and antifungal activity, *GLU* and *PR2* encoding β-1,3-glucanase, *PR4bis* encoding chitinase and *PR14* involved in the defense signalling pathway;
(ii) cell wall reinforcement genes (*CAD* and *CAGT*); and
(iii) genes affecting the SA pathway (*SAMT1* and *EDS1*)

These genes were expressed in grapevine trunks, after *N. parvum* inoculations, indicating that priming was not restricted to JA signalling. A similar result was obtained by Song *et al.* (2015) in leaves of tomato plants colonized with *Funneliformis mosseae* upon *Alternaria solani* infections. The ability of BCAs to induce the 'priming state' has also been demonstrated in many host plants, and with different microorganisms (Perazzolli *et al.,* 2011; Spagnolo *et al.,* 2012, 2014; Gruau *et al.,* 2015; Magnin-Robert et *al.,* 2016). The present study has demonstrated that the root oomycete *P. oligandrum* also induced the priming state in the grapevine/*N. parvum* interaction.

The results of the present study provide evidence that grapevine root inoculation with the oomycete *P. oligandrum* reduced grapevine trunk necrosis caused by *N. parvum*. *Pythium oligandrum* induced plant systemic resistance as indicated by the strong priming of genes involved in the grapevine defence system. After the pathogen inoculations, the FC of genes involved PR proteins, redox status, oxylipin and SA signalling pathways were induced when plants were pre-treated with *P. oligandrum*. These genes could be used as markers of plant resistance, induced by *P. oligandrum* against *N. parvum* in future studies, involving experiments performed in the vineyards.

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Research Papers

Cross pathogenicity of *Neofusicoccum australe* and *Neofusicoccum stellenboschiana* on grapevine and selected fruit and ornamental trees

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Summary. *Neofusicoccum australe* is one of the most important *Botryosphaeriaceae* pathogens occurring on fruit and vine crops. This fungus was recently taxonomically reassessed, identifying *N. stellenboschiana* as a separate species. Previous pathogenicity studies used *N. stellenboschiana* and *N. australe* isolates as *N. australe*, so assessment of the pathogenicity of these two species on grapevine and other hosts was required. A pathogenicity trial was conducted on detached shoots of grapevine, plum, apple, olive and Peruvian pepper tree. Shoots were individually inoculated with 11 *N. australe* and eight *N. stellenboschiana* isolates originally isolated from grapevine, plum, apple, olive, Peruvian pepper and fig. Both species formed lesions on all five hosts and were re-isolated 5 weeks post-inoculation. In general, the largest lesions were formed on plum and smallest on Peruvian pepper. Isolate host origin did not influence ability to cause lesions on other hosts. Isolates of *N. australe* and *N. stellenboschiana* differed in virulence on the various hosts, ranging from those that caused the largest lesions, a group causing intermediate lesions, and another causing lesions similar to uninoculated controls. The study demonstrates that *N. australe* and *N. stellenboschiana* isolates originating from various fruit hosts can infect alternative hosts including grapevine and other major fruit crops.

Keywords. *Botryosphaeriaceae*, pome fruit, stone fruit, olive, virulence.

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INTRODUCTION

Grapevine and fruit industries play major roles in the South African economy, in particular in the Western Cape Province. Approximately

125,800 ha are planted with grapevine, 36,421 ha with pome fruit and 17,680 ha with stone fruit (Hortgro, 2017). In recent years, several other fruit crops, including olive, have also become of major importance. Olive plantings increased by 135% to 3190 ha during the last 11 years, of which 92% are planted in the Western Cape (Hortgro, 2020). Fungi in the *Botryosphaeriaceae* are well-recognized trunk pathogens of grapevine, pome and stone fruit trees, as well as olives (Slippers *et al*., 2007; Úrbez-Torres and Gubler, 2009; Cloete *et al*., 2011).

Twenty-six species from *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinisa* have been reported as pathogens of grapevine (Billones-Baaijens and Savocchia, 2019). Although pathogenicity and virulence vary between species, numerous studies have shown that these fungi are capable of colonising grapevine wood causing disease, and are recognised as important grapevine trunk pathogens. Some of the most virulent grapevine trunk pathogens include *L. theobromae*, *N. australe*, *N. luteum, N. parvum, B. dothidea, D. mutila* and *D. seriata* (Van Niekerk *et al*., 2004; Úrbez-Torres and Gubler, 2009; Qiu *et al*., 2016; Billones-Baaijens and Savocchia, 2019). *Botryosphaeriaceae* reported from or associated with trunk diseases on apple include *B. dothidea*, *D. bulgarica*, *D. intermedia*, *D. malorum*, *D. mutila*, *D. seriata*, *N. australe*, *N. parvum*, *N. stellenboschiana, N. algeriense*, *N. vitifusiforme, N. viticlavatum*, and *N. ribis* (Slippers *et al*., 2007; Cloete *et al.,* 2011; Phillips *et al*., 2012; Lopes *et al*., 2016; Zafari and Soleiman, 2017; Havenga *et al*., 2019). In South Africa, several *Botryosphaeriaceae* have been associated with Botryosphaeria dieback in stone fruit including plum. These pathogens include *D. africana*, *D. mutila*, *D. pinea*, *D. seriata*, *D. viticola*, *L. plurivora*, *N. australe*, and *N. vitifusiforme* (Damm *et al.,* 2007, Slippers *et al.,* 2007). Some of the species associated with olive trunk diseases worldwide include *B. dothidea*, *D. corticola*, *D. mutila, D. seriata*, *Dothiorella iberica*, *L. theobromae*, *N. luteum, N. mediterraneum*, *N. parvum*, *N. vitifusiforme, N. australe*, and *N. ribis* (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al.,* 2010; 2017; 2019; Kaliterna *et al*., 2012; Carlucci *et al*., 2013; Úrbez-Torres *et al.,* 2013; Triki *et al*., 2014; Lopes *et al*., 2016; Spies *et al*., 2020).

Botryosphaeriaceae can infect grapevines and fruit trees through wounds, especially through those caused by pruning (Van Niekerk *et al*., 2010; Kotze *et al*., 2011; Havenga *et al*., 2019). Symptoms associated with Botryosphaeria dieback in grapevine include shoot dieback, wood necroses and perennial cankers (*Úrbez-Torres,* 2011). These symptoms are usually observed in the field 1-2 years after infection, and mainly occur in mature vineyards that are 8 years and older (Úrbez-Torres *et al.,* 2008). In cross sections through affected plant parts, symptoms are wedge- and arch-shaped lesions, watery discolouration of the wood, and sometimes also necrotic spots. Other symptoms include moderate chlorosis of the leaves caused by some species of *Botryosphaeriaceae* (Van Niekerk *et al.,* 2006; White *et al*., 2011). As trunk pathogens of pome and stone fruit trees, *Botryosphaeriaceae* are associated with stem and branch cankers, dieback, twig blights, wood rots, bark discolouration, gummoses, scaling-off of the host bark, and tree death in severe cases (Slippers *et al.,* 2007; Cloete *et al.,* 2011; Zafari and Soleimani, 2017). In olive trees, *Botryosphaeriaceae* cause cankers, twig dieback and branch dieback (Romero *et al.,* 2005; Moral *et al.,* 2017). Cankers are usually observed in stem, branch or trunk cross sections, and these reduce water and nutrient movement through host vascular tissues (Úrbez-Torres *et al.,* 2013).

In the Western Cape, vineyards are usually located in close proximity to fruit orchards and other woody plants used as ornamentals and windbreaks. In recent years, many unproductive vineyards have been removed and replanted with fruit orchards, and *vice versa*.

Within the South African grapevine and fruit industries, *N. australe* is recognized as one of the most important *Botryosphaeriaceae* pathogens. In pathogenicity studies conducted with detached green and mature grapevine shoots, as well as mature field grown vines, *N. australe* consistently produced the largest lesions or lesions within the largest lesion group (Van Niekerk *et al*., 2004). Pathogenicity studies conducted on detached green nectarine and plum shoots, also showed that *N. australe* caused lesions within the largest lesion group (Damm *et al*., 2007). Furthermore, on detached woody apple and pear shoots, the pathogen caused lesions within the largest group (Cloete *et al*., 2011).

Neofusicoccum australe recently underwent taxonomic reassessment (Yang *et al*., 2017) and South African isolates from fruit trees and grapevine previously identified as *N. australe* now also include *N. stellenboschiana*. Some of the previous pathogenicity studies (e.g. Van Niekerk *et al*., 2004) used *N. stellenboschiana* and *N. australe* isolates identified as "*N. australe*". Therefore, it is important to conduct pathogenicity tests with these two species on grapevine, as well as multiple hosts representative of the woody host diversity found close to vineyards, to determine whether these plants can be alternative hosts. In the present study, *N. australe* and *N. stellenboschiana* cultures previously isolated from grapevine and other woody hosts were subjected to cross pathogenicity studies on grapevine, apple, plum, olive and Peruvian pepper, to determine if these hosts could be alternative hosts to each other.

MATERIALS AND METHODS

Fungal inoculum and inoculations

The pathogenicity trials were conducted on 1-year-old detached shoots of *Vitis vinifera* cv. Cabernet Sauvignon, *Olea europaea* subsp. *europaea* cv. Frantoio, *Prunus salicina* cv. Sun Kiss, *Malus domestica* cv. Granny Smith and *Schinus molle* (Peruvian pepper tree). For each of the five inoculated hosts, 200 shoots were independently cut from different plants. Eleven *N. australe* and eight *N. stellenboschiana* isolates (Table 1), and uncolonized agar plugs for negative controls, were used for the inoculations, resulting in a total of 20 treatments. These isolates were originally isolated from grapevine (four isolates), olive (four), plum (two), apple (three), Peruvian pepper (four) and fig (two isolates), all from plants showing characteristic dieback symptoms and internal wood necroses associated with trunk diseases. The *Neofusicoccum* isolates are maintained in the fungal culture collection of the Department of Plant Pathology, University of Stellenbosch (STE-U) as well as the Agricultural Research Council (ARC) Infruitec - Nietvoorbij, Stellenbosch, South Africa. Before inoculations, isolates were subcultured in Petri dishes onto Potato Dextrose Agar (PDA, Biolab) amended with 250 mg L^{-1} chloromycetin (PDA-C). The cultures were grown for 3-7 d at 23-24°C, with daily cycles of approx. 12 h daylight and 12 h of darkness. Fungal plugs (4 mm diam.) were cut from the margins of these colonies with a cork-borer for use as inoculum. The shoots were cut into uniform size (25 cm), and were surface sterilized by immersing in 70% ethanol for 30 sec, followed by sodium hypochlorite (3.5%) for 1 min, and ethanol for 30 sec. The shoots were then air dried in a laminar flow cabinet. Each shoot was then wounded with an electric drill with a 4 mm drill bit. The wound was made deep enough to reach into the xylem tissue but not deep enough to reach the pith. The 4 mm agar plugs were then inserted into the wounds, mycelium side first, immediately after wounding, and the inoculated wounds were wrapped with parafilm to prevent drying. The inoculated shoots were put on moist sterile paper towels inside plastic boxes measuring 29 × 23.5 × 5.5 cm, were covered with a plastic lid, and left on a lab bench for 5 weeks at 22-24°C. The boxes were opened daily to check for fungal contamination on shoots or paper towels and to ensure that the paper towels remained moist. The moist paper towels were replaced weekly. After 5 weeks the shoots were removed from the moist chambers. The shoots were each split longitudinally with a meat saw through the point of inoculation, and the vascular discolouration on both sides of the point of inoculation (lesion lengths) were measured using digital callipers.

Not all inoculations of the different test hosts could be completed simultaneously. Therefore, the different inoculated hosts were assessed as separate trials. For each host, the experimental design was a randomised complete block with the 20 treatments replicated at random in five plastic boxes. Each plastic box contained 20 shoots of a specific host, each inoculated with one of the 20 fungal isolates. The trial for each inoculated host was repeated on a second occasion using new inoculum for each isolate.

Table 1. *Neofusicoccum stellenboschiana* and *Neofusicoccum australe* isolates used in cross pathogenicity experiments in this study.

Isolate identification number (STEU)	Original host	Area obtained	Species
9129	Fig	Durbanville	*N. stellenboschiana*
9130	Peruvian pepper	Durbanville	*N. stellenboschiana*
9131	Fig	Durbanville	*N. australe*
9132	Peruvian pepper	Durbanville	*N. australe*
9133	Peruvian pepper	Durbanville	*N. stellenboschiana*
9134	Olive	Durbanville	*N. stellenboschiana*
9135	Olive	Durbanville	*N. australe*
9136	Olive	Hermanus	*N. stellenboschiana*
9137	Olive	Hermanus	*N. australe*
9138	Peruvian pepper	Hermanus	*N. australe*
9139	Grapevine	Constantia	*N. australe*
9140	Grapevine	Constantia	*N. australe*
9141	Grapevine	Constantia	*N. stellenboschiana*
9142	Grapevine	Constantia	*N. stellenboschiana*
8302	Apple	Riviersonderend	*N. australe*
8307	Apple	Riviersonderend	*N. stellenboschiana*
8852	Plum	Clanwilliam	*N. australe*
8851	Plum	Clanwilliam	*N. australe*
8301	Apple	Riviersonderend	*N. australe*

Fungus re-isolation and identification

After taking lesion measurements, two pieces of each shoot were surface sterilized as described above. For each plant sample, 12 pieces of tissue (each ≈ 1 × 1 x 2 mm) were cut, with the first piece from the margin of healthy and necrotic tissue and the other 11 pieces from the entire length of each lesion. The tissue pieces were then placed onto PDA-C in three Petri dishes. The Petri dishes were incubated at 23-24°C for 4 weeks. The dishes were monitored daily, and fungal growth resembling *Neofusicoccum* was hyphal tip sub-cultured onto PDA-C

Petri plates. Morphological characteristics were initially used to compare re-isolated cultures with those of the originally inoculated isolates. Representative subsamples, one culture from each of the 19 isolates were also subjected to DNA extraction and molecular identification, to satisfy Koch's postulates.

DNA extraction, PCR amplification and DNA sequencing

Mycelia of the different isolates were scraped directly from the Petri dishes and transferred into Eppendorf tubes. DNA was isolated using a CTAB extraction method (Damm *et al.*, 2008). To confirm the species identity, the β-tubulin region was amplified using primers Bt2a and Bt2b (Glass and Donaldson, 1995). PCR amplifications were performed in 20 µL reactions containing 1× KAPA Taq Ready Mix (KAPA Biosystems), 0.08 µM of each primer, 2 µL DNA and the remaining volume was filled with double-distilled H_2O. PCR conditions consisted of initial denaturation step at 94°C for 5 mins, followed 36 cycles each of 45s at 94°C, 45s at 55°C and 90s at 72°C, with final extension step at 72°C for 6 min. An applied Biosystems 2700 PCR machine (Carlsbad) was used to carry out PCR reactions. Gel electrophoresis was performed on a 1% (w/v) agarose gel in TAE running buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.5) after staining with ethidium bromide. To visualize the gel under the ultraviolet (UV) light, the GeneGenius Gel Documentation and Analysis System (Syngene) alongside a 100-bp DNA ladder (GeneRuler, Thermo Fischer Scientific) were used. PCR products were then purified using the MSB Spin PCRapase kit (Invitek), and were prepared for reverse and forward sequencing. Thermocycler conditions were set for 1 min at 95°C, 30 cycles each of 10s at 95°C, 5s at 50°C and 4 min at 60°C, with a final extension of 30s at 60°C. The nucleotide samples were then sent to the DNA Sequencing Unit at the Central Analytical Facility of Stellenbosch University for sequencing.

Data analyses

For each inoculated host, analysis of variance (ANOVA) was performed per trial on the lesion length data obtained, according to the randomized block experimental design, using General Linear Models (GLM) procedure of SAS statistical software (Version 9.4; SAS Institute Inc.). Trial results were also combined in one analysis of variance after testing for trial homogeneity of variance using Levene's test (Levene, 1960). For the inoculated hosts apple, olive, and Peruvian pepper trial, variances were not equal; so weighted analyses of variance were performed as described by John and Quenouille (1977). The Shapiro-Wilk test (Shapiro and Wilk, 1965) was performed to test for deviation from normality. Fisher's least significance was calculated at the 5% level to compare isolate means (Ott, 1998). A probability level of 5% was considered statistically significant for all tests.

RESULTS

According to the ANOVA's (Supplementary Tables S1 to S5) there were no Isolate × Experiment interactions between the two repeat trials for all five hosts ($P > 0.05$), so combined analyses could be performed for each host. There were statistically significant ($P < 0.05$) differences between *Neofusicoccum* isolates for all the hosts (Supplementary Tables S1 to S5). Although the hosts data were analysed separately, the largest lesions developed on plum (mean lesion length = 154.4 mm) followed by grapevine (128.3 mm), apple (113.2 mm), olive (94.0 mm) and Peruvian pepper (43.2 mm) (Tables 2 to 6). The lesion lengths could be grouped into three categories: those that did not differ significantly from the experimental controls, those that grouped together with the largest lesions, and an intermediate group.

Lesion formation by Neofusicoccum australe *and* Neofusicoccum stellenboschiana *isolates within hosts*

Apple

Neofusicoccum australe (isolate STEU 9131) was the most virulent isolate to apple shoots (mean lesion length = 113.2 mm: Table 2). *Neofusicoccum stellenboschiana* isolate STEU 9141 caused the largest lesions among the *N. stellenboschiana* isolates (mean lesion length = 107.1 mm). These two isolates and isolates STEU 9140, STEU 8852, STEU 8301, STEU 9134, STEU 9138, STEU 9139, STEU 9129, and STEU 8307 caused lesions which were significantly larger than those from the controls, and were the group causing the largest lesions. This group included six *N. australe* and four *N. stellenboschiana* isolates, which were originally isolated from fig, grapevine, plum, apple, olive or Peruvian pepper. *Neofusicoccum australe* STEU 8851 caused the smallest lesions (mean = 21.5 mm). Isolates STEU 8851, STEU 9136, STEU 9132, STEU 8302, STEU 9135, STEU 9137, STEU 9130, STEU 9133, and STEU 9142 caused lesions not significantly different from the non-inoculated controls (mean = 11.9 mm). This group included five *N. australe* and four *N. stellenboschiana* isolates, originally from grapevine,

Table 2. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old apple shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9131	*N. australe*	Fig	113.2a	1.15	84
9141	*N. stellenboschiana*	Grapevine	107.1ab	1.25	97
9140	*N. australe*	Grapevine	92.9abc	0.75	83
8852	*N. australe*	Plum	87.5abc	1.12	91
8301	*N. australe*	Apple	83.8abcd	1.10	85
9134	*N. stellenboschiana*	Olive	76.1abcde	1.44	96
9138	*N. australe*	Peruvian pepper	74.7abcdef	0.96	67
9139	*N. australe*	Grape	73.1abcdef	0.93	80
9129	*N. stellenboschiana*	Fig	72.9abcdef	0.94	75
8307	*N. stellenboschiana*	Apple	68.2abcdef	1.00	79
9142	*N. stellenboschiana*	Grape	57.3bcdefg	1.25	70
9133	*N. stellenboschiana*	Peruvian pepper	55.0bcdefg	1.15	73
9130	*N. stellenboschiana*	Peruvian pepper	49.9cdefg	0.75	88
9137	*N. australe*	Olive	48.9cdefg	0.87	80
9135	*N. australe*	Olive	42.7cdefg	0.95	73
8302	*N. australe*	Apple	30.8defg	0.54	62
9132	*N. australe*	Peruvian pepper	28.2efg	0.81	68
9136	*N. stellenboschiana*	Olive	24.9efg	0.59	76
8851	*N. australe*	Plum	21.5fg	0.60	36
Control	Non-inoculated		11.9g	0.50	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 53.45.

plum, apple, olive or Peruvian pepper. Two of the three isolates originally from apple (STEU 8301 and STEU 8307) caused lesions in the largest lesion group, whereas the third (STEU 8302) caused lesions not significantly different from the non-inoculated controls. All the *Neofusicoccum* isolates were re-isolated from inoculated tissues, with re-isolation percentages ranging from 67 to 97% for the group causing large lesions, and 35 to 88% for the group for which lesion lengths did not differ from the non-inoculated controls.

Grapevine

All the isolates caused lesions in grapevine that were significantly larger than for the non-inoculated controls (mean = 0.0 mm: Table 3). *Neofusicoccum stellenboschiana* isolate STEU 9134 caused the largest lesions (mean = 128.3 mm) and together with *N. australe* STEU 9131 and STEU 8852 were the group causing the largest lesions. These isolates were originally from, respectively, olive, fig or plum. The rest of the *Neofusicoccum* isolates caused lesions in the intermediate size group (mean = 40.3 to 95.1 mm), although there was an upper intermediate lesion size group (STEU 9133, STEU 9132, STEU 8307, STEU 9141, STEU 9135, STEU 9138, and STEU 9130) and lower intermediate size group. The upper intermediate group included four *N. stellenboschiana* and three *N. australe* isolates, whereas the lower intermediate group included three *N. stellenboschiana* and six *N. australe* isolates. One of the four isolates originally from grapevine (STEU 9141) caused lesions in the upper intermediate lesion size group, whereas the other three were in the lower intermediate size group. The re-isolation percentages ranged from 68 to 77% for the large lesion group, 61 to 94% for the upper intermediate group, and 28 to 79% for the lower intermediate lesion size group.

Olive

Neofusicoccum stellenboschiana (STEU 9133) caused the largest lesions on olive (mean = 94.0 mm: Table 4). However, *N. stellenboschiana* isolates STEU 9142, STEU 9130, STEU 9141 and STEU 9129 caused lesions that

Table 3. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old grapevine shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9134	*N. stellenboschiana*	Olive	128.3a	1.42	77
9131	*N. australe*	Fig	110.7ab	1.04	70
8852	*N. australe*	Plum	103.8abc	1.00	68
9133	*N. stellenboschiana*	Peruvian pepper	95.1bcd	0.59	61
9132	*N. australe*	Peruvian pepper	92.0bcde	1.04	78
8307	*N. stellenboschiana*	Apple	79.6bcdef	1.06	80
9141	*N. stellenboschiana*	Grapevine	79.3cdef	0.95	61
9135	*N. australe*	Olive	78.7cdef	0.88	94
9138	*N. australe*	Peruvian pepper	66.2defg	0.94	80
9130	*N. stellenboschiana*	Peruvian pepper	65.4defg	0.45	65
8301	*N. australe*	Apple	63.3efg	0.62	69
9139	*N. australe*	Grapevine	62.9efg	1.08	69
9140	*N. australe*	Grapevine	62.1efg	0.91	73
9136	*N. stellenboschiana*	Olive	59.2fg	0.45	70
8302	*N. australe*	Apple	55.5fg	1.05	28
8851	*N. australe*	Plum	52.2fg	0.31	79
9129	*N. stellenboschiana*	Fig	49.8fg	0.23	77
9137	*N. australe*	Olive	43.6g	0.39	78
9142	*N. stellenboschiana*	Grapevine	40.3g	0.72	77
Control	Non-inoculated		0.0h	0.00	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD= 31.20.

were not significantly different from isolate STEU 9133, hence in the group causing the largest lesions. These isolates were originally from Peruvian pepper, fig or grapevine. *Neofusicoccum australe* STEU 9132, STEU 9137, STEU 9140, STEU 9139, STEU 8851, STEU 8301, and STEU 8302 formed lesions (mean = 23.5 to 44.2 mm) that were not significantly different from the controls (23.8 mm). *Neofusicoccum stellenboschiana* isolates STEU 8307, STEU 9136, and STEU 9134, and *N. australe* STEU 9131, STEU 9138, STEU 9135, STEU 8852, and STEU 9132 produced lesions that were significantly smaller than those from isolate STEU 9133, and were in the intermediate lesion size group. All the isolates in the large lesion group were *N. stellenboschiana*. Isolates of this pathogen were only in the large or intermediate lesion group, whereas all the lesions that did not differ from the controls were from *N. australe* inoculations. Three of the isolates originally from olive caused lesions in the intermediate group, and one was in the group where lesions were not different from the controls. The re-isolation percentages were from 27 to 100% for all the isolates. In the two cases where 100% re-isolations were obtained, both the isolates originated from olive.

Peruvian pepper

All the isolates caused lesion lengths significantly greater than those for the non-inoculated controls (mean = 9.9 mm: Table 5). *Neofusicoccum stellenboschiana* isolate STEU 9142 caused the largest lesions (mean = 43.2 mm). However, this was not significantly different from the lesions caused by 14 other isolates, six of *N. stellenboschiana* and eight of *N. australe*. These isolates were originally from olive, grapevine, Peruvian pepper, apple, fig or plum. *Neofusicoccum australe* isolates STEU 9135, STEU 8851 and STEU 9138 and *N. stellenboschiana* STEU 9136 caused lesions that were significantly shorter (mean lengths = 24.8 to 28.3 mm) than STEU 9142, and were in the intermediate lesion size group. Three of the four isolates originally from Peruvian pepper caused lesions in the large lesion group. The re-isolation percentages from Peruvian pepper shoots ranged from 59 to 93% in the large lesion group and 76 to 91% in the intermediate group.

Table 4. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old olive shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9133	*N. stellenboschiana*	Peruvian pepper	93.8 a	0.69	73
9142	*N. stellenboschiana*	Grapevine	91.8 ab	0.96	90
9130	*N. stellenboschiana*	Peruvian pepper	87.5 abc	0.50	89
9141	*N. stellenboschiana*	Grape	78.0 abcd	0.52	90
9129	*N. stellenboschiana*	Fig	76.2 abcde	0.76	80
8307	*N. stellenboschiana*	Apple	70.8 bcdef	0.62	73
9136	*N. stellenboschiana*	Olive	69.3 cdef	0.74	100
9131	*N. australe*	Fig	63.5 defg	0.81	93
9134	*N. stellenboschiana*	Olive	61.1 defg	0.84	90
9138	*N. australe*	Peruvian pepper	56.2 efg	0.55	90
9135	*N. australe*	Olive	51.7 fgh	0.84	100
8852	*N. australe*	Plum	45.8ghi	0.96	83
9132	*N. australe*	Peruvian pepper	44.2 ghij	0.69	83
9137	*N. australe*	Olive	43.3 ghijk	0.69	97
9140	*N. australe*	Grapevine	34.6 hijk	0.78	80
9139	*N. australe*	Grapevine	32.1 hijk	0.49	83
8851	*N. australe*	Plum	29.4 ijk	0.87	43
Control	Non-inoculated		23.8 jk	0.99	0
8301	*N. australe*	Apple	23.5 jk	0.75	36
8302	*N. australe*	Apple	17.5 k	0.50	28

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 7.05.

Plum

All the isolates caused lesion lengths that were significantly greater than the controls (mean = 17.1 mm: Table 6). *Neofusicoccum australe* isolate STEU 9138 caused the largest lesions (mean = 154.4 mm). However, mean lesion size was not significantly different from the lesions caused by 15 other isolates, seven of *N. stellenboschiana* and eight of *N. australe*. These isolates were originally from Peruvian pepper, apple, grapevine, olive, fig or plum. *Neofusicoccum stellenboschiana* isolate STEU 9130 and *N. australe* STEU 8302 and STEU 8851 caused lesions which were significantly smaller (mean lengths = 70.1 to 105.6 mm) than those caused by STEU 9138, and were in the intermediate lesion size group. One of the isolates originally from plum (STEU 8852) caused lesions in the large lesion group, whereas the second isolate from plum caused lesions in the intermediate size group. The re-isolation percentages from plum shoots ranged from 60 to 100%, and were 70 to 100% in the large lesion group and 60 to 90% in the intermediate group.

Lesion formation from Neofusicoccum australe *and* Neofusicoccum stellenboschiana *isolates across different hosts*

Five isolates, *N. australe* STEU 8852 and STEU 9131, *and N. stellenboschiana* STEU 9134, STEU 9129, and STEU 9141, caused lesions within the large lesion size group on four hosts, and lesions on one host in the intermediate size group. None of these isolates caused lesions similar to the non-inoculated controls on any of the tested hosts. Isolates STEU 8852, STEU 418 and STEU 9134 formed lesions within the large size group on apple, grape, plum and Peruvian pepper, whereas intermediate size lesions were formed on olive. Isolates STEU 9129 and STEU 9141 formed lesions within the large lesion group on apple, plum, Peruvian pepper and olive, whereas intermediate-sized lesions were formed on grape. These five isolates were therefore virulent across all the hosts tested.

Isolate STEU 8307 of *N. stellenboschiana* formed lesions within the large lesion group on three hosts, apple, plum, and Peruvian pepper, intermediate lesions on grape and olive, and no lesions similar to the non-inoculated controls on any host. This isolate was, there-

Table 5. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old Peruvian pepper shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9142	*N. stellenboschiana*	Grapevine	43.2 a	1.23	90
9130	*N. stellenboschiana*	Peruvian pepper	40.2 ab	1.28	89
8307	*N. stellenboschiana*	Apple	39.9 ab	1.04	63
8302	*N. australe*	Apple	39.1 abc	0.91	91
9129	*N. stellenboschiana*	Fig	39.0 abc	0.85	91
9131	*N. australe*	Fig	37.8 abc	0.73	93
9137	*N. australe*	Olive	36.8 abc	1.59	66
9141	*N. stellenboschiana*	Grapevine	36.1 abc	0.98	90
9134	*N. stellenboschiana*	Olive	34.7 abc	1.02	76
8852	*N. australe*	Plum	34.7 abc	0.99	90
9133	*N. stellenboschiana*	Peruvian pepper	33.5 abc	1.16	73
9140	*N. australe*	Grapevine	33.0 abc	0.81	59
8301	*N. australe*	Apple	30.5 abc	0.78	90
9139	*N. australe*	Grapevine	30.2 abc	1.28	84
9132	*N. australe*	Peruvian pepper	29.1 abc	0.83	93
9135	*N. australe*	Olive	28.3 bc	0.79	91
9136	*N. stellenboschiana*	Olive	26.7 bc	0.90	87
8851	*N. australe*	Plum	26.5 bc	0.47	76
9138	*N. australe*	Peruvian pepper	24.8c	0.27	89
Control	Non-inoculated		9.9d	0.63	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 14.30.

fore, virulent across all the hosts tested. Five isolates, including STEU 9133 and STEU 9142 of *N. stellenboschiana*, and STEU 9140, STEU 8301, and STEU 9139 of *N. australe*, also formed lesions within the large lesion size group on three hosts each, plum, Peruvian pepper and olive for STEU 9133 and STEU 9142, and plum, Peruvian pepper and apple for STEU 9140, STEU 8301 and STEU 9139. These five isolates also produced intermediate sized lesions on grape, and lesions that did not differ from the non-inoculated controls in apple (STEU 9133 and STEU 9142) and olive (STEU 9140, STEU 8301 and STEU 9139). These isolates were, therefore, virulent on at least four of the hosts tested.

Four isolates, three of *N. australe* (STEU 9138, STEU 9132, STEU 9137) and one of *N. stellenboschiana* (STEU 9129), formed large lesions on two hosts each, but then differed in the number of hosts where they caused intermediate-sized lesions or lesions similar to the non-inoculated controls. Isolate STEU 9138 produced large lesions on plum and apple and intermediate-sized lesions on grape, olive and Peruvian pepper. This isolate was, therefore, virulent across all the tested hosts. Isolate STEU 421 formed large lesions on olive and pepper, intermediate-sized lesions on grape and plum, and lesions similar to the non-inoculated controls on apple. Isolates STEU 9132 and STEU 9137 formed large lesions on plum and Peruvian pepper, intermediate-sized on grapevine, and lesions similar to the non-inoculated controls on apple and olive. Although isolates STEU 9129, STEU 9132 and STEU 9137 were virulent on three to four hosts, these isolates showed more variation in virulence across the different hosts than other isolates.

Isolates STEU 9136 of *N. stellenboschiana* and STEU 9135 and STEU 8302 of *N. australe* produced large lesions only on one host each, plum in the case of STEU 9136 and STEU 9135 and Peruvian pepper for STEU 8302. Isolates STEU 9136 and STEU 9135 produced intermediate-sized lesions on grape, olive and Peruvian pepper, with lesions similar to the non-inoculated controls on apple. Isolate STEU 8302 produced intermediate-sized lesions on plum and grape, so displayed high virulence variation. Isolate STEU 8851 was by far the weakest pathogen, not being able to form large lesions on any of the tested hosts, intermediate-sized lesions on grapevine, plum and Peruvian pepper, and lesions not

Table 6. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old plum shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion Length (mm)[a]	Std dev	Re-isolation (%)
9138	*N. australe*	Peruvian pepper	154.4a	0.73	100
8301	*N. australe*	Apple	151.7a	0.85	97
9136	*N. stellenboschiana*	Olive	150.0a	1.18	96
9141	*N. stellenboschiana*	Grapevine	148.4a	0.61	98
9134	*N. stellenboschiana*	Olive	146.8ab	0.71	85
8307	*N. stellenboschiana*	Apple	146.5ab	0.74	90
9135	*N. australe*	Olive	145.0abc	0.89	73
9129	*N. stellenboschiana*	Fig	144.9abc	0.85	77
9133	*N. stellenboschiana*	Peruvian pepper	143.0abc	0.88	76
9139	*N. australe*	Grapevine	141.5abc	1.03	92
9137	*N. australe*	Olive	140.7abc	1.13	89
9132	*N. australe*	Peruvian pepper	128.0abc	1.09	70
8852	*N. australe*	Plum	128.0abc	0.70	87
9142	*N. stellenboschiana*	Grapevine	124.4abc	1.25	77
9131	*N. australe*	Fig	121.8abc	0.71	100
9140	*N. australe*	Grapevine	112.3abcd	0.96	87
8851	*N. australe*	Plum	105.6bcd	0.60	7
8302	*N. australe*	Apple	103.7cd	0.65	90
9130	*N. stellenboschiana*	Peruvian pepper	70.1d	0.91	60
Control	Non-inoculated		17.1e	0.35	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments.LSD= 42.67.

significantly different from the non-inoculated controls in apple and olive.

DISCUSSION

All the *Neofusicoccum australe* and *N. stellenboschiana* isolates evaluated in this pathogenicity study were able to infect, colonise and cause necrosis on apple, grapevine, olive, Peruvian pepper and plum. Lesions extended upward and downward from the points of inoculation, with variable lengths depending on the pathogen-host combination. The inoculated fungi were re-isolated from each of these hosts and together with the characteristic lesions produced, *N. australe* and *N. stellenboschiana* can therefore be regarded as pathogens of these hosts. This is the first report of *N. australe* and *N. stellenboschiana* as pathogens of Peruvian pepper. *Neofusicoccum ribis* is the only *Botryosphaeriaceae* species that had previously been associated with Peruvian pepper, in Hawaii (Stevens and Shear, 1929). Although *N. stellenboschiana* was associated with trunk disease symptoms on nursery apple trees, no pathogenicity studies were conducted (Havenga *et al*., 2019). Furthermore, only two previous studies have linked *N australe* with olive plants showing canker and dieback symptoms. However, the study by Lopes *et al*. (2016), conducted in Portugal, did not perform pathogenicity studies, whereas the study by Triki *et al*. (2014) in Tunisia conducted a pathogenicity study showing formation of a brown colour on stems of 2-year-old plug-inoculated potted olive trees. Triki *et al*. (2014) did not disclose whether the symptom was external or internal, nor was the extent of the lesion formation provided. Pathogenicity studies were conducted by Lazzizera *et al*. (2008) with *N. australe*, however, on olive drupes in Italy where the pathogen was regarded as highly virulent.

Although the data for inoculation of different hosts could not be compared statistically, the present study has shown that the largest lesions resulting from inoculations were formed on plum, followed by grapevine, apple, olive and Peruvian pepper. This indicates that plum could be more susceptible to infection by *N. australe* and *N. stellenboschiana* than the other four hosts. The fact that all 19 isolates evaluated caused lesions on plum shoots that were larger than those from

the non-inoculated controls indicates that plum was highly susceptible to these pathogens. This was also the case in Peruvian pepper, for which none of the evaluated isolates caused lesions similar to those from the non-inoculated controls, confirming that all 19 isolates were pathogenic to this host. The high re-isolation percentages obtained from Peruvian pepper is further confirmation of the susceptibility of this host. Peruvian pepper trees often grow in close proximity to vineyards and fruit orchards in the Western Cape of South Africa, confirming that they are probably alternative hosts of *Neofusicoccum* spp., and are sources of inoculum to closely associated vineyards and fruit orchards.

Considering the virulence of *N. australe* and *N. stellenboschiana* isolates within the different hosts evaluated, no clear patterns were identified in most of the hosts. The exception was olive, where all the isolates producing large lesions were *N. stellenboschiana*, and all the isolates where lesions were not different to the non-inoculated controls were *N. australe*. Significant virulence differences within the same species was observed for all the tested hosts, except for Peruvian pepper inoculated with *N. australe*, where the lesion lengths did not differ significantly. For example, *N. australe* isolate STEU 9131 produced lesions that were five times longer than those from *N. australe* STEU 8851 in apple shoots. Also in apple, *N. stellenboschiana* isolate STEU 9141 caused lesions that were four times longer than those from *N. stellenboschiana* STEU 9136. High virulence diversity within *N. australe* has been documented on several fruit trees and other woody hosts (Cloete *et al*., 2011; Jami *et al*., 2015; Baskarathevan *et al*., 2017; Aćimović *et al*., 2018; Arjona-Girona *et al*., 2019).

Lesion formation from *N. australe* and *N. stellenboschiana* isolates across the tested hosts showed that within both fungi there were isolates that were more virulent than others. Some isolates caused large lesions on four hosts and lesions different from the non-inoculated controls in a fifth (isolates STEU 8852, STEU 9131, STEU 9129, STEU 9141 and STEU 9134). Some of the isolates of *N. australe* (i.e. STEU 8302) and *N. stellenboschiana* (i.e. STEU 9136) had variable virulence across hosts, forming lesions in all three size categories. Only *N. australe* isolate STEU 8851 was unable to cause large lesions in on any of the hosts. This isolate was therefore a weak pathogen, although it could infect, colonise and cause lesions significantly different from the non-inoculated controls in three of the five test hosts.

The isolates of *N. australe* and *N. stellenboschiana* used in the present study were originally obtained from different woody hosts, but this did not affect their abilities to cause lesions on different hosts. None of the isolates originally from a specific host caused large lesions on that host. The two isolates originally from fig (STEU 9131 and STEU 9129) produced large lesions on apple, Peruvian pepper and plum, but on grape and olive, one isolate caused large lesions and the other caused intermediate-sized lesions. These results are similar to those of Amponsah *et al*. (2011), where isolates of *N. luteum, N. parvum* and *N. australe* from grapevine and non-grapevine hosts were able to infect grapevine shoots and produce lesions. Also, Sessa *et al*. (2016) showed in Uruguay, that *N. australe* isolates from apple branches showing papyraceous cankers caused large lesions when 1-year-old apple, pear or peach shoots were inoculated with six Botryosphaeriaceous species, in a field trial. *Neofusicoccum australe* is known from at least 46 hosts from 18 plant families (Sakalidis *et al*., 2011). Based on microsatellite data, native populations *N. australe* in Australia were highly diverse, with no discernible host or habitat restrictions, but rather geographic distribution and life-strategies controlled by environmental factors (Sakalidis *et al*., 2011). Even in a different pathosystem, *N. australe* originally isolated from avocado and blueberry caused leaf lesions on macadamia and blueberry leaves when the petioles of detached leaves were inoculated with the pathogen (Liddle *et al*., 2019). *Neofusicoccum australe* isolates isolated from healthy leaves and branchlets of *Acacia karroo*, a native tree with wide distribution in South Africa, were shown to be highly virulent when 1-year-old *A. karroo* seedling stems were inoculated with the fungus (Jami *et al*., 2015).

In blueberries in Portugal, Hilário *et al*. (2020) showed that *N. australe* occurred alone in branches with dieback, but also from dead branches in association with other *Botryosphaeriaceae* and from the same asymptomatic branch, as endophytes or latent pathogens co-existing with *N. parvum*, which was the more pathogenic of the two species on blueberry. Moyo *et al*. (2016) surveyed trunk pathogens and associated symptom types on *Diospyros kaki* (persimmon) in South Africa. *Neofusicoccum australe* co-existed in 11 trunk pathogen combinations in various symptom types. Co-inoculation of *Seimatosporium vitifusiforme* with *D. seriata*, previously isolated from one canker in a vineyard, caused an increase in lesion length (Lawrence *et al*., 2018). Co-infection by *N. australe* and other trunk pathogens has not been investigated previously, and, due to its very wide host range and virulence on many hosts, this is likely to be a fruitful study. Detached shoot assays would be good starting points, but field trials are also highly recommended.

The present study has showed that *N. australe* and *N. stellenboschiana* isolates originating from different

fruit tree hosts can cross-infect alternative hosts, causing lesions. These infections are likely to be inoculum sources for infestation of grapevine and other major fruit crops. In the South African fruit and vine industries, only one previous study (Van Niekerk *et al*., 2010) investigated spore release patterns associated with *Botryosphaeriaceae* species in vineyards, but spore release in fruit orchards has not been investigated. Little is known about the inoculum sources and plant-to-plant dispersal of *Botryosphaeriaceae* species within vineyards and fruit orchards. Moyo *et al*. (2014) reported *Botryosphaeriaceae* species (including *N. australe*) on the exoskeletons of arthropods in close proximity to grapevine pruning wounds. To effectively manage Botryosphaeria canker and dieback of grapevine and fruit trees, growers will have to be cognizant of the importance of appropriate sanitation practices for vineyards and orchards, but also for other closely associated woody hosts. Results of the present study have shown that in addition to *N. australe*, *N. stellenboschiana* is likely to be an important trunk pathogen of grapevine and fruit trees. Future research should investigate the biology of *N. stellenboschiana* to support appropriate disease management strategies. Most importantly, testing of pruning wound protectants against *N. stellenboschiana* infections is urgently required.

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Short Notes

Metabolite fingerprints of Chardonnay grapevine leaves affected by esca is both clone- and year-dependent

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Summary. Esca is one of the most widespread grapevine trunk diseases affecting vineyards. This complex disease leads to leaf alterations, wood necrosis and eventually to plant death. Esca symptoms are caused by several fungi inhabiting the xylem of host plants and degrading the wood structure. The main pathogens causing the disease are *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Fomitiporia mediterranea* and other wood-rotting basidiomycetes. Grapevine susceptibility to esca can be predisposed by several factors, especially climate, vine age, and cultivar. An experiment was carried out (in 2015) to assess if esca expression on leaves could also be clone-dependent. Chardonnay clones 76 and 95 grown in the same plot were compared according to their developmental and physiological traits, metabolome, and foliar symptom expression. Leaves were sampled during summer on visually healthy vines as controls (C), and from asymptomatic (D-) and symptomatic (D+) shoots of esca-affected vines. Analysis of their metabolomes highlighted a clone-dependent metabolite fingerprint associated to esca expression. Opposite variations of specific metabolites were found between C and D+ leaves of both clones. The experiment was repeated (in 2018). Leaf samples could be discriminated, especially the C and D+ samples for each clone, but the differences were less marked than in the first experiment. Discriminant compounds were all different between the two experiments, and showed no opposite variations between C and D+ samples of both clones, which indicated variable metabolite responses from year to year for both clones. These results confirm that the leaf metabolite fingerprint associated to esca expression is clone-dependent, and is year-dependent in intensity and nature.

Keywords. *Vitis vinifera*, grapevine trunk diseases, metabolomics, clone.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are widespread and progressively cause vine death in all wine producing countries (De la Fuente *et al.,* 2016). They affect and weaken vines, eventually leading to their death, and adversely affect vineyard yields and wine quality causing economic losses for wine industries. Gramaje and Armengol (2011) observed that increase of GTD incidence may be partly due to the worldwide grapevine planting "boom" during the 1990s, as it contributed to the propagation of potentially contaminated planting material and increased the area of vineyards reaching the age of esca symptom expression. Other factors also contributed to GTD spread, such as changes of production methods that favoured fungal infection, and the banning in 2003 in some countries of sodium arsenite and other chemicals that were widely used to control GTDs (Gramaje and Di Marco, 2015; Gramaje *et al.,* 2018; Mondello *et al.,* 2018). Since these bans, no efficient products have been available for effective GTD control, leading to important economic losses in all major vine-growing countries, including Spain, France, Italy, and the United States of America (De la Fuente *et al.,* 2016; Guerin-Dubrana *et al.,* 2019). In 2012, it was estimated that if only one percent of vines within vineyards were replaced each year because of GTDs, the worldwide annual financial cost would exceed one and a half billion $US (Hofstetter *et al.,* 2012).

Esca is one of the main GTDs, and is caused by several fungus genera and species, including the Ascomycetes *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and Basidiomycetes such as *Fomitiporia mediterranea* (Surico *et al.,* 2006). These pathogens induce wood symptoms, including black spots, vascular streaking, discolouration and necrosis. This complex disease is also associated with foliar and berry symptoms (Mugnai *et al.,* 1999; Surico, 2009; Mondello *et al.,* 2018). Foliar symptoms, known as Grapevine Leaf Stripe Disease (GLSD), consist of tiger-stripe patterns leaves, and this is considered as a chronic form. Apoplectic symptoms can also occur, and these include leaf wilting rapidly followed by partial or total death of plants (Mugnai *et al.,* 1999). The mechanisms leading to the appearance of esca foliar symptoms are unclear, since on individual vines they can appear in one growing season, but not the next. It is also difficult to reproduce external symptoms in artificial conditions (Mondello *et al.,* 2018; Reis *et al.,* 2019).

Several factors can affect the symptom appearance and the level of expression, including region, soil, vine training system, cultivar, rootstock, vine age, and climatic conditions, which vary from one vintage to another (Mugnai *et al.,* 1999; Van Niekerk *et al.,* 2011; Lecomte *et al.,* 2012; Andreini *et al.,* 2014; Murolo and Romanazzi, 2014). In addition, the grapevine clone is important, as symptom expression is modulated by rootstock and clone combination (Murolo and Romanazzi, 2014). We showed (in an experiment in 2015) that different metabolite fingerprints are associated with expression of esca symptoms between leaves of vines of Chardonnay clones 76 and 95, with opposite variation of specific secondary metabolites between control and diseased leaves of both clones (Moret *et al.,* 2019). Due to the erratic occurrence of the esca foliar symptoms we repeated the experiment to validate the previous observations in another vintage. The present study thus assessed the metabolite fingerprint of leaves from symptomatic and asymptomatic vines of Chardonnay clones 76 and 95 in a second growing season (in 2018), and results were compared to those of the 2015 study (Moret *et al.,* 2019).

MATERIALS AND METHODS

Experimental plot

The experimental plot was located in Chablis vineyard, Burgundy, France (126 m above sea level, GPS coordinates 47_47046.9600N, 3_47026.1700E), and the vines were planted in 2002, half with *Vitis vinifera* cv. Chardonnay clone 76 and the other half with clone 95, both grafted on Fercal rootstocks (Moret *et al.,* 2019). The plot was on a clay soil, and the vines were trained in a double Guyot system. The plot was of total area of 0.7 ha, and contained a total of 4361 vines growing at spacing of 1 × 1.5 m.

Climate data

Climatic parameters were recorded by a meteorological station located at Saint-Bris le Vineux (Burgundy, France) <8 km from the experiment plot. The station is representative of plot climate conditions, and is used as a reference by the Chambre d'Agriculture. Rainfall and daily temperatures were recorded. Available data were from year 2005 to year 2018.

Incidence of Esca symptoms

The occurrence of diseased vines within the plot was established by visual observations of esca symptom expression. Diseased (D) and healthy (as controls, C) vines were counted at the end of August every growing season since 2005. On diseased vines, asymptomatic (D-)

and symptomatic (D+) shoots were distinguished. Control vines were considered healthy when they did not display foliar symptoms during the year of sampling or during any other year.

Agronomic and physiological parameters

Two series each of ten successive visually healthy (asymptomatic) vines were selected in the plot to be representative of the whole plot, and were used to follow agronomic parameters and for $\Delta^{13}C$ analyses.

Agronomic parameters

Bud burst, fertility and vine vigour were determined as described in Moret *et al.,* (2019), only on visually healthy vines (C). Bud burst and fertility were calculated as follows:

Bud burst percentage = (number of shoots / number of buds remaining after Chablis pruning) × 100

Fertility = number of clusters/number of shoots

Vine vigour was determined by measurements of cane diameter using calipers. Measurements were performed between the first and second node of the third shoot starting from the old part of the trunk (Chablis pruning).

Water status

The water stress of control and diseased vines, was assessed using $\Delta^{13}C$ measurements, which were made at the end of summer on must obtained from 150 fruit bunches collected from the four grapevine modalities (Farquhard *et al.,* 1989). The GISMO Platform (University of Burgundy, France) performed these analyses.

METABOLIC ANALYSIS

Sampling

Asymptomatic green leaves were sampled from randomly selected vines, from five healthy (C) and five esca-affected (D) vines showing partial apoplexy symptoms, for each clone, on July 24, 2018, during symptom expression at the early veraison vine growth stage. For each clone, five control leaves were collected, and for diseased samples, five leaves were sampled on asymptomatic canes (D-), plus three leaves on symptomatic canes (D+). Leaves were taken from the same foliar rank (approximately the same age), and were conserved in liquid nitrogen during sampling then ground and stored at -80°C.

GC-MS analyses

GC-MS analyses were carried out as described by Krzyzaniak *et al*., (2018) and Moret *et al.,* (2019). Briefly, 50 mg of ground leaves were resuspended in 1 mL of frozen water:acetonitrile:isopropanol (2:3:3) containing ribitol (4 µg mL^{-1}), and were then extracted for 10 min at 4°C with shaking (1400 rpm). Insoluble material was removed by centrifugation (20,000×g for 5 min). Three blank tubes underwent the same steps as the samples and a quality control was made by pooling an equal volume of each sample type. Thirty µL were collected and dried overnight at 35°C in a Speed-Vac vacuum centrifuge. For derivatization, 10 µL of 20 mg mL^{-1} methoxyamine in pyridine were added to each sample, and the reaction was performed for 90 min at 28°C under continuous shaking. Ninety µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (Aldrich 394866–10 × 1 mL) were then added and the reaction continued for 30 min at 38°C. After cooling, 45 µL were transferred to an Agilent vial for injection. Four h after the end of derivatization, the whole sample series was first injected (1 µL) in splitless mode, and then in split mode (1/30), in an Agilent 7890A gas chromatograph coupled to an Agilent 5977B mass spectrometer, as previously detailed (Krzyzaniak *et al*., 2018).

Data processing and statistical analyses

Data processing and statistical analyses were carried out using R-3.6.1 and RStudio software. For climate data assessments, mean temperature and the sum of rainfall were calculated for the 2008-2017 period, and for the year 2018. The differences of foliar disease expression count data were analyzed with Chi-square tests of proportions (at $P < 0.05$).

For processing of GC-MS data, the data files in NetCDF format were analyzed with AMDIS software. A home retention indices/mass spectra library built from the NIST, Golm2, and Fiehn databases and standard compounds were used for the identification of metabolites. Chromatogram peak areas were also determined with the Targetlynx software (Waters) after conversion of the NetCDF file in Masslynx format. AMDIS, Target Lynx in splitless and split 30 mode data were compiled into a single Excel file for comparison. After blank mean subtraction, peak areas were normalized to ribitol and fresh weight (µg mg^{-1} fresh weight). These data were fil-

tered by removing entries with 50% or more missing values across samples, except for those that were significant at $P < 0.01$ for Fisher's Exact Test, which compared the number of missing and non-missing values between the biological groups of interest. After filtering, missing values were replaced by an estimate of the limit detection (half the lowest value). Multivariate descriptive analyses were carried out for these processed data, such as PLS and Volcano plots (Goodacre *et al.,* 2007; Vinaixa *et al.,* 2012; Worley and Powers 2013; Schiffman *et al.,* 2019). $\Delta^{13}C$ and agronomic data were checked for homogeneity of variance (Levene test) and for normality (Shapiro Wilk test). $\Delta^{13}C$ data were then tested using Kruskall Wallis tests followed by a Dunn's *post hoc* test. For agronomic parameters, bud burst percentage was evaluated with Chi-square tests, and vigour and fertility parameters were evaluated using Mann Whitney two-sample tests.

RESULTS

Climate data

Recorded climate data are presented in Figure 1. Mean temperatures during summer were slightly higher in 2018 compared to the past 10 years, especially in July and August (respectively, 22.7 and 21.2°C in 2018, compared to 10 year means of 19.9 and 19.3°C). Precipitation was less in 2018 compared to the past 10 years, particularly in June, July and August.

Esca foliar symptom expression

Esca symptom expression is described in Table 1. Symptom expression was moderate in 2018, with 4.42% of clone 76 vines and 3.16% of clone 95 vines affected. In 2016, 7.69% of clone 76 vines and 6.97% of clone 95 were affected, and in 2017, 9.08% of clone 76 and 8.02% of clone 95 vines showed esca symptoms. There were no statistically significant differences between the two clones within each year.

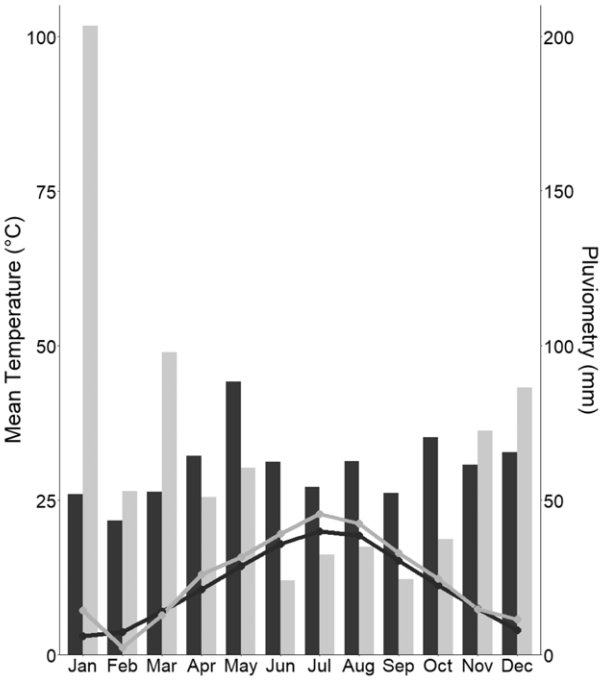

Figure 1. Ombrothermic diagram of monthly data relevant the study site showing rainfall (mm; histograms) and mean temperatures (°C, symbols connecting lines). Mean data for the 10-year period 2008-2017 are displayed in black, and 2018 data are displayed in grey.

Agronomic parameters

No statistically significant differences were found between clones 76 and 95 for the mean bud burst, fertility or vigour parameters (Table 2).

Water status

For both clones, $\Delta^{13}C$ values ranged from -22.82 to -23.75, which correspond to weak to moderate water stress (Van Leeuwen *et al.,* 2009). No significant differences were detected between the two clones, nor between the control or diseased vines of both clones (Table 2).

Table 1. Proportion (%) of grapevines displaying esca symptoms during August of eight years within the study plot, for Chardonnay clones 76 and 95. Statistically significant differences of expression ($P < 0.05$) between clones within each year are indicated by different letters.

Clone	Year							
	2009	2010	2011	2012	2015	2016	2017	2018
76	0.20a	0.40a	1.38a	2.39a	0.41a	7.69a	9.08a	4.42a
95	0.00a	0.79a	1.60a	3.82a	0.62a	6.97a	8.02a	3.16a

Table 2. Means and ranges of $\Delta^{13}C$, vigour, fertility and bud burst parameters obtained for the Chardonnay grapevine clones 76 and 95 from C (control) and D (esca-affected) leaf samples. Bud burst percentage = (number of shoots / number of buds remaining after Chablis pruning) × 100. Fertility = number of clusters/number of shoots. Vine vigour was determined by measurements of cane diameter between the first and second node of the third cane starting from the old part of the trunk (Chablis pruning). Statistically significant differences ($P < 0.05$) between clones are indicated by different letters.

Clone			$\Delta^{13}C$	Vigour	Fertility	Bud burst
Clone 76	C	Range	-23.03 to -22.92	0.48 to 1.19	1.11 to 2.42	87.50 to 100
		Mean	-22.97a	0.84a	1.85a	97.2a
	D	Range	-23.75 to -22.82	-	-	-
		Mean	-23.26ab	-	-	-
Clone 95	C	Range	-23.28 to -23	0.46 to 1.96	1.30 to 3.37	55.60 to 100
		Mean	-23.12ab	0.86a	2.06a	91.36a
	D	Range	-23.75 to -23.30	-	-	-
		Mean	-23.48b	-	-	-

Metabolite analyses

GC-MS analyses of leaf samples revealed 227 compounds, of 157 were identified (data not shown). Principal component analysis (PCA) showed overlapping groups for C, D- and D+ leaf samples from clones 76 and 95 (data not shown). PLS allowed separation of clone 76 D+ group compounds from those in clone 76 C and clone 95 C samples, whereas the compounds from the clone 76 D- group overlapped with both 76 C and D+. For clone 95, groups C, D- and D+ overlapped (Figure 2). Further analyses focused on C *versus* D+ comparisons. Volcano plots were produced to compare metabolite accumulation in C and D+ samples for each clone (Figure 3). Metabolites and their respective values are displayed in Table 3. For metabolites that were detected in several samples, after data processing with the different software packages, only the most relevant ones were selected and listed. Compounds with aberrant concentrations across samples (*i.e.* not constant during analyses and high variability in sample groups C, D or D+) were also not included in Table 3. For clone 76, only glycerate was more accumulated in C samples, whereas serine, and three other non-identified compounds were more accumulated in D+ samples. For clone 95, D-erythronic acid, myo-inositol-1-P, threonatelactone, and one unknown compound were more accumulated in C samples. In contrast, asparagine, galactosylglycerol, resveratrol, and four unknown compounds were more accumulated in the D+ samples.

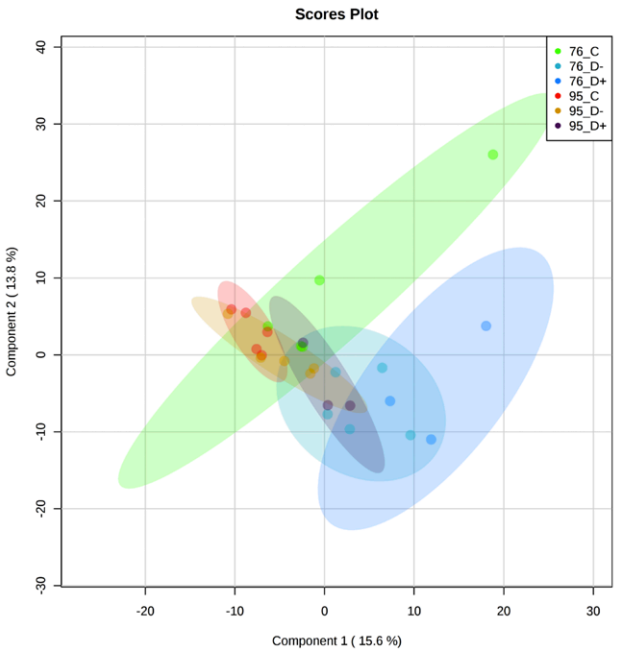

Figure 2. Partial Least Square (PLS) analysis of metabolite data obtained from GC-MS analyses of grapevine leaf extracts from different grapevine leaf samples of Chardonnay clones 76 and 95. For each group, individual samples are displayed as symbols and concentration ellipses are included. (C): green leaves sampled on heathy vines, as controls; (D-): green leaves sampled on asymptomatic canes of esca-affected vines showing partial apoplexy symptoms; (D+): green leaves sampled on symptomatic canes of esca-affected vines showing partial apoplexy symptoms.

DISCUSSION

In 2015, we studied a field plot planted with the two Chardonnay grapevine clones 76 and 95, to determine if esca foliar expression was clone dependent. The results highlighted that metabolites differentially accumulated between C and D+ leaves for both clones, and that there were differences in the metabolite profiles of C and D- leaves only for clone 95. Accumulation of some metab-

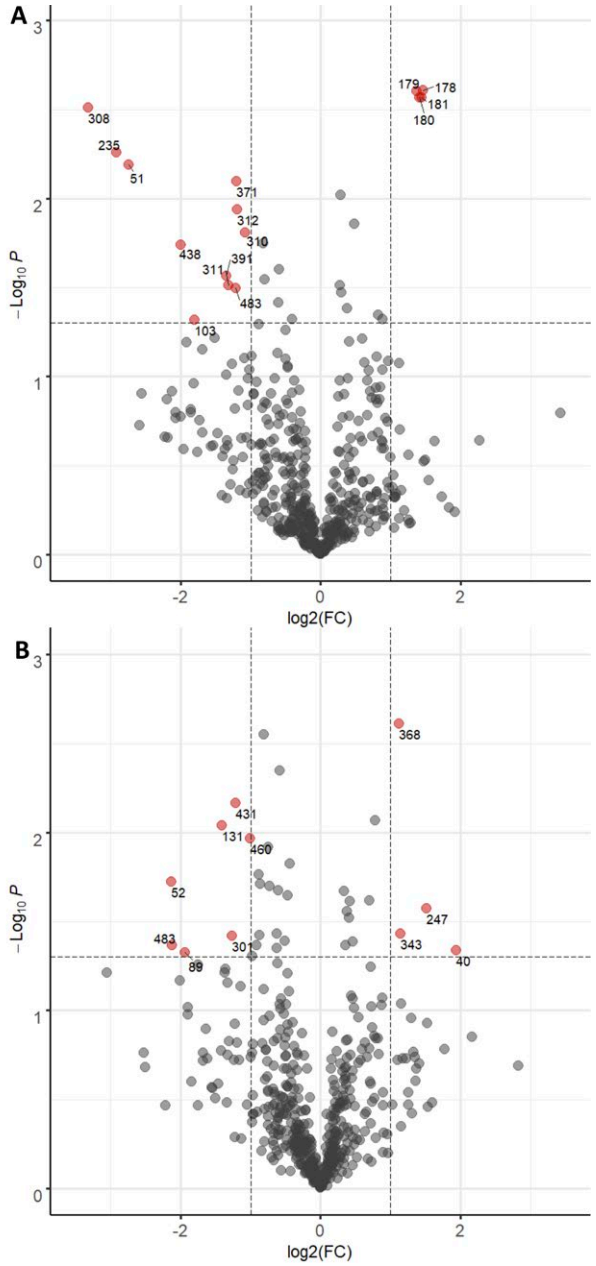

Figure 3. For each metabolite, fold change (C/D) and Student's T test p-value were calculated between C (control) and D (diseased) samples. The results are displayed on a Volcano plot, with $P < 0.05$ and FC > 2 thresholds. Each metabolite is displayed by a symbol, in red for $P < 0.05$, FC > 2, or in grey for $P > 0.05$, FC > 2. Only metabolites that had fold differences < 12 are displayed. Metabolites that were significant and validated the fold change threshold (red symbols) are annotated with their ID numbers. The panels are organized as A) clone 76 C *versus* D+, B) clone 95 C *versus* D+.

olites in opposite ways was also shown in D+ leaves of both clones, with lower amounts than in the control leaves of clone 76, and greater amounts for clone 95. The present study aimed to confirm these results for a second year (2018), where different environmental conditions applied to vines that were 3 years older.

Table 3. Metabolite analyses. Significant metabolite ID numbers, names, values and respective accumulations of significant metabolites ($P < 0.05$, fold change (FC) > 2) in grapevine leaf samples from Chardonnay grapevine clones 95 and 76. (C = greater accumulation in control leaf samples, D = greater accumulation in esca-affected leaf samples).

ID	Metabolite	*P*	FC	Accumulation	Clone
69	D-Erythronic acid	0.0099	1.05 10^2	C	95
245	Myo Inositol-1-P	0.0266	2.85	C	95
339	Threonatelactone	0.0371	2.20	C	95
364	U1579/306	0.0024	2.17	C	95
27	Asparagine	0.0134	0.02	D	95
129	Galactosylglycerol	0.0091	0.38	D	95
298	Resveratrol	0.0379	0.42	D	95
426	U2472.4/204	0.0068	0.43	D	95
436	U2565.5/179	2.84E-05	0.13	D	95
454	U2802.8/355	0.0108	0.50	D	95
476	U3142.4/202 Leu-Trp	0.0431	0.23	D	95
179	Glycerate	0.0027	2.70	C	76
308	Serine	0.0308	0.40	D	76
367	U1601.5/292	0.0080	0.43	D	76
432	U2540.8/204	0.0182	0.25	D	76
476	U3142.4/202 Leu-Trp	0.0319	0.43	D	76

In 2018, incidence of esca symptoms was low (4.42% for clone 76 and 3.16% for clone 95). This weak symptom expression can be explained by the relatively young age of vines (16 years). However, incidence was less than in 2016 (7.69% for clone 76 and 6.97% for clone 95) and 2017 (9.08% for clone 76 and 8.02% for clone 95). This can be partly explained by the particular climatic conditions that applied, especially during July, which is one of the most determinant months for expression of esca symptoms as affected by climatic conditions (Calzarano *et al.,* 2019). Temperatures were higher and precipitation was less in July 2018 compared to the average conditions of the previous 10 years. These elevated temperature and rainfall conditions during the growing season have been previously associated with high numbers of asymptomatic plants, whereas high rainfall and low temperatures may be correlated with increased symptom expression (Calzarano *et al.,* 2019; Serra *et al.,* 2019). Also, in July and August, rainfall was recorded on few days, with no rainfall precipitation for the rest of the months. In July, rainfall was recorded on 2 d, followed by 7 d of high temperatures 2 weeks later, and followed by 1 d of very high precipitation (20.5 mm). These climatic conditions led to moderate esca symptom expression and weak-to-

moderate water stress. Since the precise effects of precipitation on symptoms expression are not fully understood (Andreini *et al.,* 2014), and because expression and climatic data are variable, we could not directly correlate the two. Over the previous 8 years, esca has progressed with vine age, despite year-dependent expression. However, no statistically significant difference in expression between clones 76 and 95 was detected, indicating that these two clones do not have different susceptibilities to esca disease in the local French conditions.

Agronomic parameters were analyzed for control samples, to compare both clones in relation to the environmental conditions, independently of the disease. No statistically significant differences in vigour, bud burst or fertility parameters between clones 76 and 95 were detected. The differences observed for metabolite comparisons were therefore not due to clone-dependent physiological or agronomic factors.

PCA analyses of metabolite data showed overlapping groups, indicating that the analyses did not discriminate the different leaf sample groups analyzed, so there were no statistically significant differences when all the metabolites were considered. The deeper PLS analysis allowed discrimination of clone 76 D+ from C groups from both clones. For clone 95, the leaf sample groups C, D- and D+ overlapped. The overlapping area between C and D+ was small, indicating that, while the metabolites detected did not differ between these groups, some specific compounds were differentially accumulated. Volcano plots were therefore used to compare C and D+ samples from clones 76 and 95, and this highlighted different metabolite fingerprints for C and D+ for each clone. For clone 76, only glycerate was more accumulated in C samples, whereas serine was one of the compounds more accumulated in D+ samples. Beside photorespiration, serine can be synthesized in cell cytosol from 3-phosphoglycerate *via* glycerate as an intermediate (Igamberdiev and Kleczkowski, 2018). Despite being a minor pathway, this may play a role in highly specific stress conditions by regulating redox balance. For clone 95, resveratrol accumulated in D+ samples. This compound is a known grapevine phytoalexin and its accumulation in diseased organs is well documented (Chong *et al*., 2009;Adrian *et al.,* 2012). In our previous study, we observed accumulation of glycosylated resveratrol in D+ samples (Moret *et al.,* 2019). No other similarities in the metabolite profiles between years 2015 and 2018 were detected.

The results obtained in 2018 were very different from those obtained in 2015, for esca incidence, climate conditions and discrimination between samples and clones. The discriminating metabolite compounds were also different. Metabolomics still allowed detection of a clone-dependent leaf response to esca. However, this response was year-dependent and differed qualitatively and quantitatively when discriminating compounds were compared. Several factors are likely to explain these differences. Environmental conditions can affect leaf metabolism, and also esca foliar symptom expression (Fischer and Ashnaei 2019; Songy *et al.,* 2019). In addition, metabolite differences could be influenced by environmental conditions, as suggested by Bettenfeld *et al.,* (2020). They could also be due to changes in pathogen metabolism from year to year, indirectly influenced by the climatic conditions. Since esca pathogens produce toxins (Masi *et al.,* 2018), it is possible that their release varies qualitatively and quantitatively over time, leading to subsequent differences in the leaf metabolism.

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Short Notes

Greeneria uvicola associated with dieback in vineyards of Sonora, Mexico

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Summary. The state of Sonora is the main grape production area in Mexico. Grapevine trunk diseases (GTD) are serious disease in this region. During the springs of 2017 and 2018, symptoms of yellow and wilted leaves, and necrotic buds, were observed in several vineyards in Sonora. Affected plants had numerous small, black and bright acervuli. This study aimed to identify the causative agent of this disease. Isolates were obtained from small pieces of damaged plant parts, and were cultured on potato dextrose agar. The developing mycelium was white but turned greyish white after 3 d. Multi-shaped black mucilaginous droplets appeared in the cultures after 2 d, and the agar in Petri dishes was completely covered with mycelium after 7 d, and the colonies had five or six rings with large numbers of conidiomata. Conidia were hyaline or light cream, fusiform, oval or ellipsoid, with truncated bases and narrow almost pointed apices, and were 6–10 mm long and 2–3 mm wide. Phylogenetically analysed concatenated sequences of the DNA from two representative isolates, from the internal transcribed spacer region, and large ribosomal subunit, showed they were in a separate clade which aligned with several strains of *Greeneria uvicola*, confirming the presumptive morphological identity of the isolates. This cosmopolitan ascomycete is responsible for bitter rot of grapes, but the role of this fungus as a cause of grapevine trunk diseases is little known. Pathogenicity tests of the isolates were performed on 1-year-old 'Passion Fire' grapevines plants, one of the new cultivars planted in Sonora. All of strains *G. uvicola* were pathogenic, and the fungus was recovered from the lesions, fulfilling Koch's postulates. This is the first report on the pathogenicity of *G. uvicola* in wood tissues of *Vitis vinifera* in Mexico.

Keywords. *Diaporthales*, *Melanconiellaceae*, multilocus typing, grapevine trunk diseases.

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INTRODUCTION

The state of Sonora is the major grape production region in Mexico. The total production of fresh table, distillate, and raisin grapes was 415,889 tonnes in 2017, with an 18.4% increase compared with that in 2016. Sonora contributed 334,355 tonnes to the total domestic production of grapes (more than 80%), generating 350 to 400 million USD. Eighteen grape varieties recognized in the international market are grown in Sonora for four different markets: table grapes (92.8%), wine grapes (3.7%), raisins (3.3%), and table grapes grown under shade cloth (0.2%). Hermosillo, Caborca, and Guaymas-Empalme are the three main grape-producing regions (SAGARPA, 2018).

Grapevine trunk diseases (GTDs) caused by fungi are considered the most destructive diseases in vineyards (Larignon and Dubos, 1997; Agustí-Brisach and Armengol, 2013; Gramaje *et al*., 2018). Among the main GTD agents are *Phaeomoniella chlamydospora* and *Phaeoacre-monium* sp., which cause for Petri disease (Mostert *et al*., 2006); *Botryosphaeriaceae* species (Úrbez-Torres, 2011), *Phomopsis viticola* (*Diaporthe ampelina*; Úrbez-Torres *et al*., 2013) and *Eutypa lata* (Rudelle *et al.,* 2005), which cause regressive death of branches, and *Cylindrocarpon* sp. which cause blackfoot disease (BFD) (Agustí-Brisach and Armengol, 2013). Some of these pathogens can act independently or synergistically, and have been isolated from old grapevines and from symptomatic and asymptomatic root-stock mother-plants, rootstock cuttings, and young grafted vines (Carlucci *et al.,* 2017). Infected plants may be stunted with reduced loss of vigour, retarded or absent sprouting, biomass reduction, chlorotic and necrotic foliage, shoot mortality, fruit rot, wilting, and death. Necrotic lesions in the roots and reductions in root biomass may also occur (Gramaje and Armengol, 2011).

Castillo-Pando *et al.* (2001) determined that *Botryosphaeria obtusa* (syn. *Diplodia seriata*) was responsible for the regressive death of several vineyards in the Hunter Valley region of Australia. The presence of *Greeneria uvicola* and *Pestalotiopsis* sp. was also observed in the majority of the cases, but the role that these fungi remains uncertain. Subsequently, Abreo *et al.* (2008) found that *G. uvicola*, in addition to *Eutypella vitis* and species of Botryosphaeriaceae, was mainly responsible for GTDs in Uruguayan vineyards.

Although *G. uvicola* can infect grapevine leaves, tendrils, and stems, this fungus primarily attacks the fruit, especially if wet weather conditions persist during harvest (Longland and Sutton, 2008; Wilcox *et al*., 2015; Steel and Greer, 2008).

Greeneria uvicola (syn. *Melanconium fuligineum*; *Diaporthales*) is a cosmopolitan ascomycete responsible for bitter rot of grapes. This fungus reproduces asexually, and the teleomorph is not known. The fungus survives during the winter in stem lesions and mummified berries, and has been shown to attack the fruits of several species of *Vitis*, including *V. aestivalis*, *V. labrusca*, *V. rotundifolia* and *V. vinifera* (Farr *et al*., 2001; Longland and Sutton, 2008).

During April and May of 2017 and 2018, some gravevines in different vineyards in Sonora, located in Hermosillo (Costa de Hermosillo and Pesqueira) and Guaymas-Empalme, showed damage and symptoms of GTDs. Initially, the symptoms were dry leaves, buds, and woody shoots. Cross-sections of affected wood showed triangular black spots in some samples, so these symptoms were associated with the presence of *Lasiodiplodia theobromae*, a fungus responsible for GTD in these Sonora regions. However, microbiological tests revealed the presence of pycnidia similar to those produced by *Botryosphaeriaceae* sp., and conidia like those of *Neofusicoccum* sp. were observed. Nevertheless, an initial molecular analysis amplifying internal transcribed spacer region using ITS1/ITS4 primers indicated a high probability that *G. uvicola* was the causative agent of this disease.

Because *G. uvicola* is considered a pathogen of the grape berries and that it only occurs as a secondary opportunist in GTDs, the present study aimed to confirm presence and identification the fungus using molecular phylogeny, and pathogenicity tests were carried out to determine whether this fungus can be a primary agent capable of infecting vineyards in Sonora, Mexico.

MATERIALS AND METHODS

Sampling and isolation of the causative agent

Fifteen *Vitis vinifera* vineyards were sampled (seven on the Hermosillo Coast, four in the Pesqueira Valley, and four in the Guaymas-Empalme Valleys). The varieties planted in these vineyards were 'Summer Royal', 'Early Sweet', 'Flame Seedless', 'Sweet Celebration', and 'Passion Fire'. Wood was collected from plants with dieback symptoms and the presence of black subepidermal structures in the woody shoots. The samples were separately wrapped in wet paper, placed in plastic bags, labelled and placed on ice for transfer to a laboratory for processing. Each sample was directly examined under a stereoscopic microscope for characteristics and relevant symptoms.

Tissue sections (>5 mm^2) were cut from the margins of necrotic wood and were disinfected in 75% ethanol for 1 min, 1% sodium hypochlorite for 5 min and then

rinsed three times with sterile distilled water. The wood tissue pieces were then placed in Petri dishes containing potato dextrose agar (PDA), which were incubated at 25°C for 7 d. Pure cultures were obtained by transferring a hyphal tip to individual PDA dishes. The resulting grown cultures were stored at 0°C for no longer than 1 month, until further processing.

Morphological analysis

The isolated fungi were characterised for growth rate, and colony shape, consistency, and colour. Preparations were made on microscope slides and examined using a light microscope to determine characteristics of reproductive structures. A microscope stage micrometer (Carl Zeiss Canada Ltd) was used for calibrating the optical systems to determine dimensions of acervuli and conidia. Twenty acervuli and 100 conidia were measured for each isolate.

DNA extraction

Two representative isolates were subjected to molecular analysis. DNA was extracted from mycelia grown in pure cultures on PDA, using the Power Soil DNA Isolation Kit (MoBIO Laboratories). Cell lysis was conducted with a Precellys Evolution homogeniser (Bertin Technologies). DNA integrity was verified on 2% agarose gel. The extracted DNA was quantified using a NanoDrop 1000 (ThermoScientific). Only samples with a 260/280 absorbance ratio between 1.8 and 2 were amplified. The DNA was stored at -20°C until used.

Molecular identification and phylogenetic analysis

DNA amplification reactions were performed for each of the two isolates in a Biorad C1000 Thermal Cycler. Sets of ITS1 and ITS4 primers were used to amplify and sequence the ITS1-5.8-ITS2 region (White *et al*.,1990), and LROR and LR5 primers were used to amplify and sequence the fragment corresponding to the large ribosomal subunit (LSU) gene (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). The amplification products were purified with ExoSap-IT PCR Product Cleanup (Affymetrix). The purified products were separately sequenced in both directions, using the same pair of initiators. The sequencing equipment used was an ABI 3100 Genetic-Analyser (Applied Biosystems). Each sequence was manually checked, and nucleotides in ambiguous positions were corrected using the complementary sequences obtained with both primers, using ChromasPro v2.1.5. Several of the sequences of interest were aligned with existing sequences of different *Melanconiellaceae* family, obtained from the National Center for Biotechnology Information (NCBI) gene database. Evolutionary history was inferred using the neighbour-joining method. *Gaeumannomyces graminis* var. *avenae* CBS 187.65 was used as an external group. In total, 1447 positions were analysed. Evolutionary analyses were performed using MEGA X (Kumar *et al*., 2018).

Pathogenicity tests

Two representative isolates from affected grapevines in the sampling areas were used. Five plants of 'Passion Fire' were grown in polyethylene pots, and were inoculated 1 year after transplanting. Each inoculation was into a 4-month-old shoot or a 1-year-old trunk. A 5 × 2 mm longitudinal incision (1 mm deep) was made iat each inoculation point, and an 8 mm PDA disc with fungal mycelium was then placed in the incision and covered with parafilm to avoid dehydration and contamination (Figures 4A and 4B). This procedure was also performed for five control plants of the same variety, but the inoculations were with mycelia-free culture media.

RESULTS AND DISCUSSION

Field observations

Between 5 and 10% of the plants in each sampled vineyard showed typical GTD symptoms in all varieties. Figure 1A shows symptoms at the beginning of disease development (yellow and dry leaves and black spots in stems). Deformed stems and acervuli were also observed (Figure 1B). Other plants showed more severe symptoms, including dead twisted and deformed branch stems with pronounced cankers, and with acervuli (Figure 1C) and gummosis (Figure 1D).

Morphology of fungal isolates

The initial mycelium of the isolates on PDA was white and then turned greyish-white 3 d later. On the second day, small black droplets with mucilaginous consistency were observed on the mycelium, and these contained bright and shiny black conidiomata of varying shapes (Figure 2A). Initially, the acervuli were aqueous, but they became solid, opaque, and less shiny, with variable shapes as they matured (Figure 2B). Within 1 week,

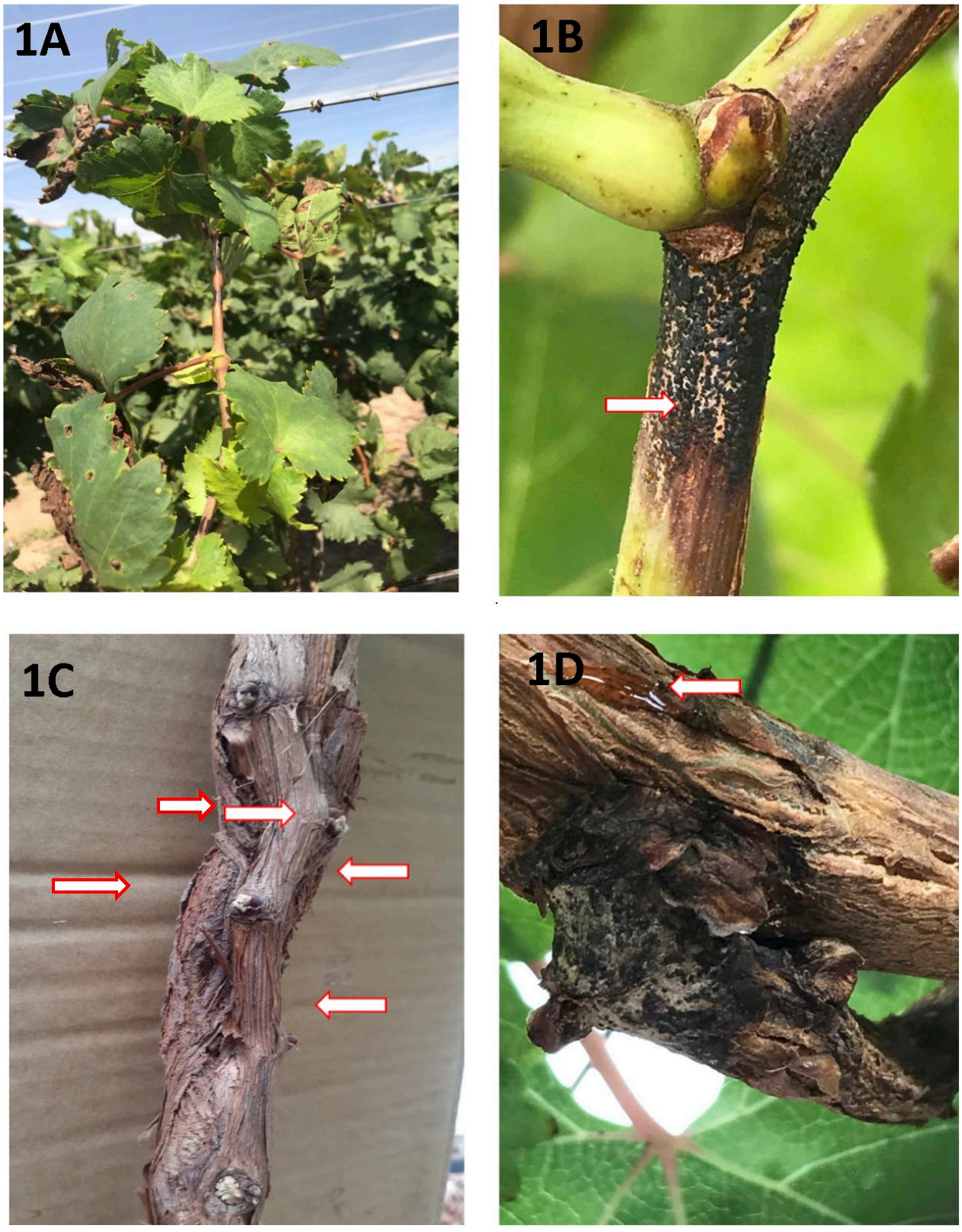

Figure 1. Grapevine trunk diseases caused by *Greeneria uvicola*. The arrows indicate details of the symptoms and damage. 1A: Beginning of the disease in young branches (leaves and stems). 1B; *Greeneria uvicola* acervuli around one shoot. 1C; Dead branch with pronounced canker, twist deformed stem, and presence of acervuli. 1D; Gummosis in dead tissues.

the medium in each Petri dish was completely covered with mycelia, with five to six rings of multiple acervulus-type conidiomata (Figure 2C). These were of dimensions from 400 to 1040 µm (average = 690 µm). Conidia were single-celled, fusiform or ovoid and smooth. The conidiogenic structures inside the acervuli were simple phialides with variable shapes and conidia at their ends. The conidia were hyaline or cream, and fusiform, oval or

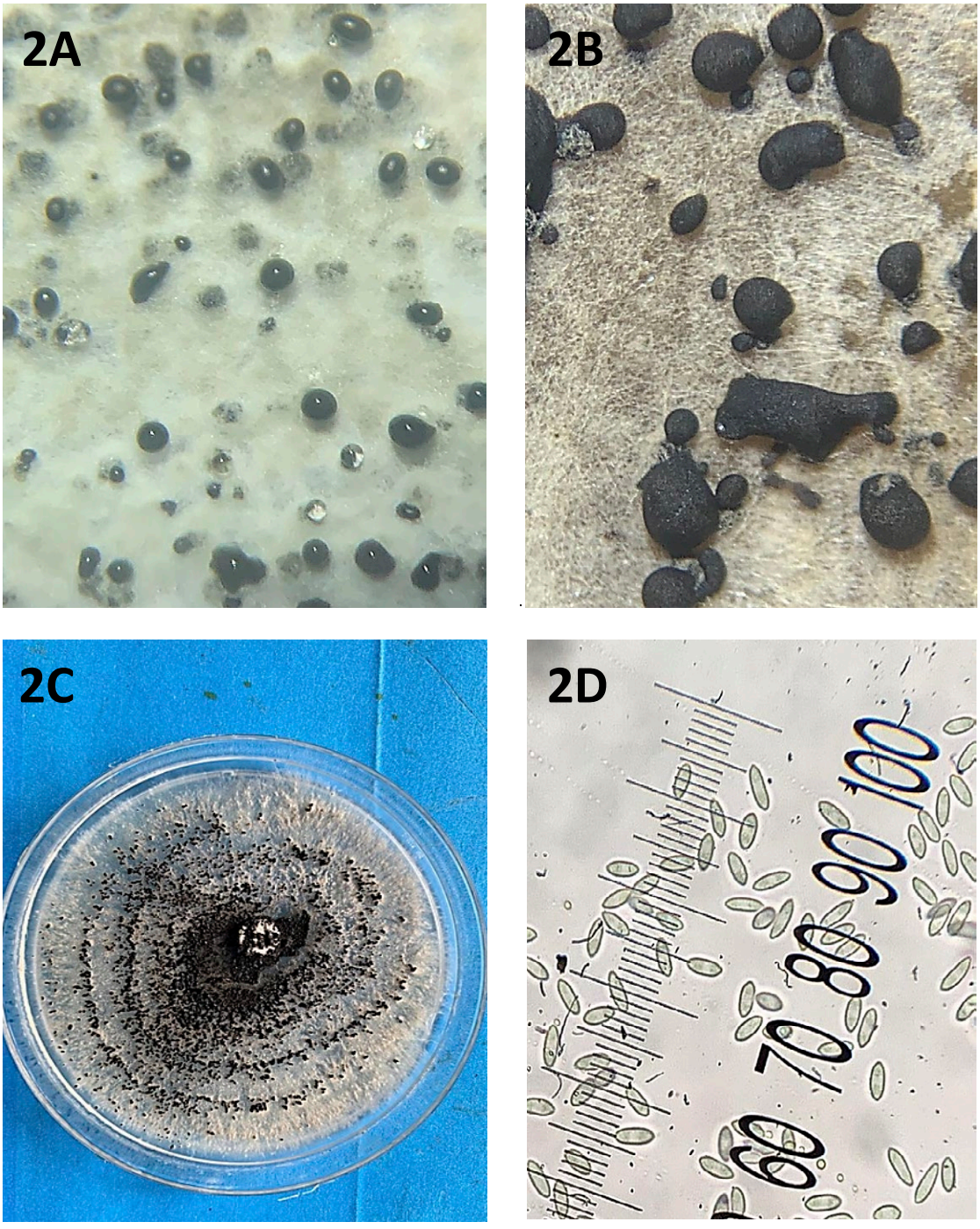

Figure 2. Morphological characteristics of *Greeneria uvicola* cultivated on PDA. 2A: Conidiomata growing over mycelium 2 d after inoculation. 2B: Mature, opaque and deformed acervuli. 2C; Petri dish covered with mycelia, showing concentric rings of multiple conidiomata at 7 d after inoculation. 2D. Morphology of conidia. Scale: each division = 2.5 µm.

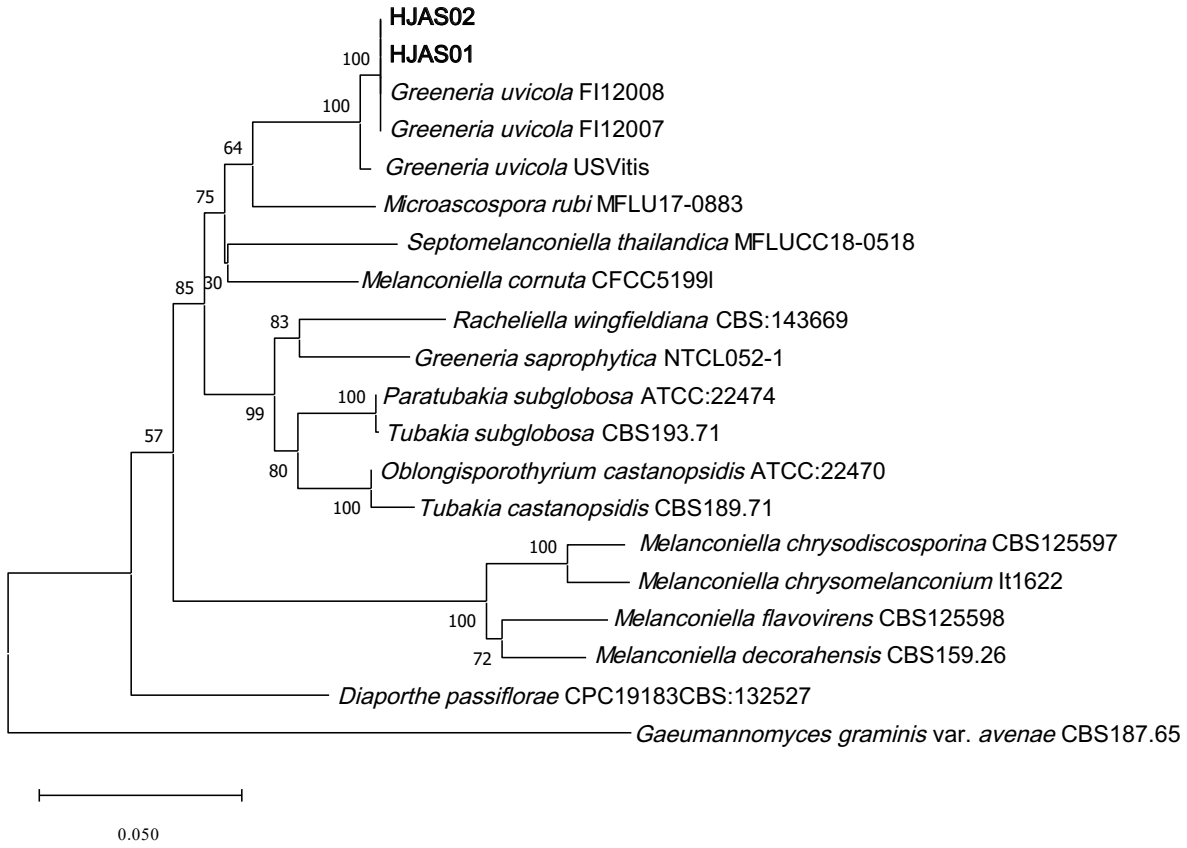

Figure 3. Evolutionary analyses conducted in MEGA X using nucleotide sequences of the *ITS-LSU* genes. Representative isolates of different *Melanconiellaceae* family were included, along with two representative isolates (HJAS01 and HJAS02) from grapevines in the Sonora state of Mexico. This analysis involved 20 nucleotide sequences. The evolutionary history was inferred using the Neighbour-Joining method. A total of 1447 positions were in the final dataset. The scale bar represents a genetic distance of 0.05.

ellipsoid with truncated bases and narrow, almost pointed apices, measuring 6–10 µm (mean = 7.7 ± 2.8 µm) by 2–3 µm (mean = 2.8 ± 0.4) (Figure 2D). These characteristics were consistent with those of *Greeneria*, described by Scribner and Viala (1887).

Molecular identification and phylogenetic analysis

The nucleotide sequences of the ITS and LSU regions of two representative isolates (HJAS01, HJAS02) were deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/), with accession numbers MN611374, MN611375, MN628296, MN628297. Sequences were edited, aligned, and compared with those of fungal species within *Melanconiellaceae*, and were analysed phylogenetically using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980), and are in the units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Phylogenetic analysis showed that isolates HJAS01 and HJAS02, which caused downward death of grapevines in vineyards, belonged to *G. uvicola*, because a well-defined clade (100%) was formed with several isolates of this species from GenBank (Figure 3).

Pathogenicity tests

Three weeks after inoculation, the plants began to show symptoms including general wilting of shoots at the inoculation points, and with chlorosis and leaf necrosis. All shoots and leaves were dried by the 4th week after inoculation (Figure 4D). The non-inoculated plants, which remained healthy (Figure 4C). Longitudinally cut shoots had necrotic interiors and portions

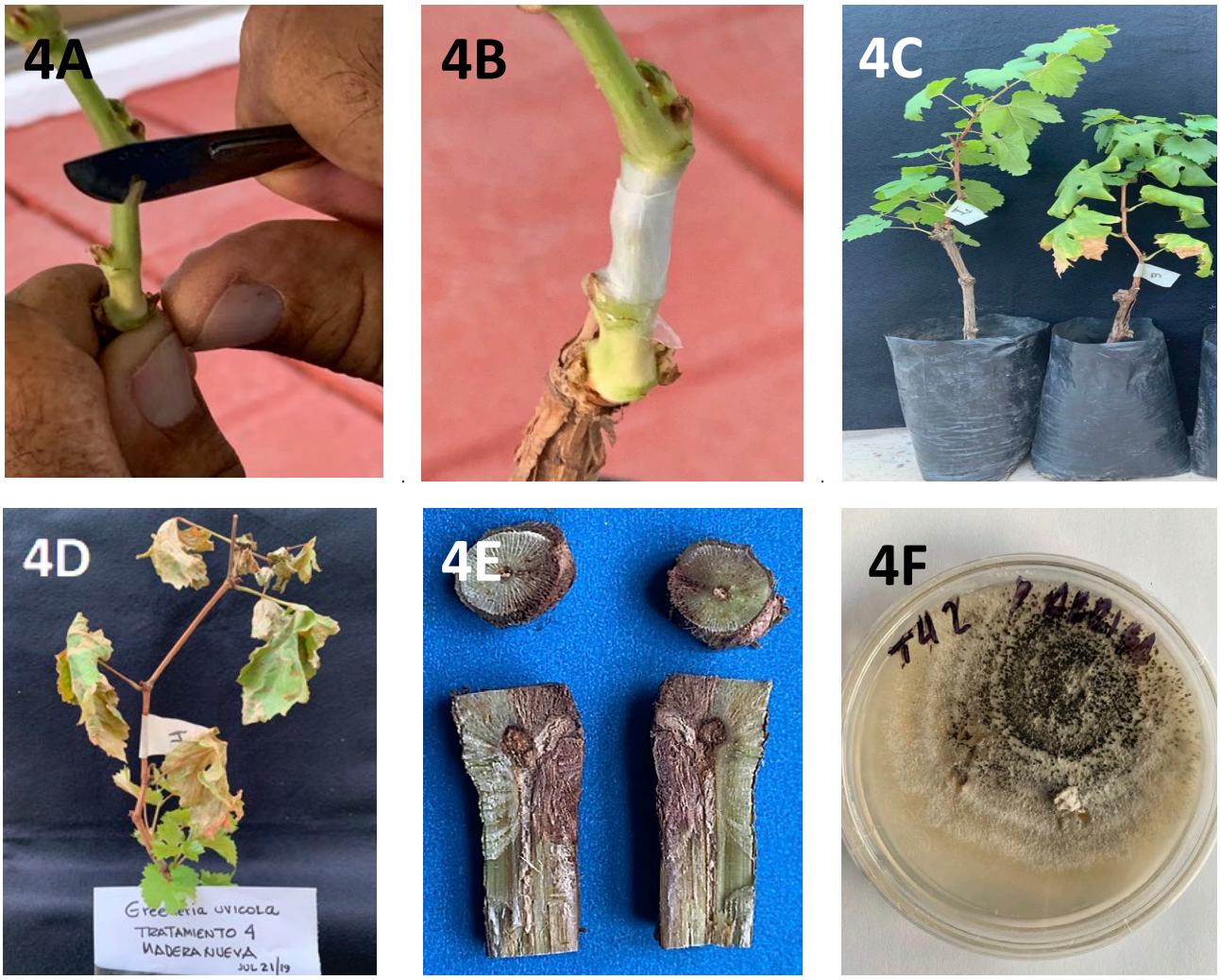

Figure 4. Pathogenicity tests. 4A and 4B; Inoculation. 4C: Healthy plants 30 d after treatment with PDA without *Greeneria uvicola*. 4D: Diseased plants after 30 d of inoculation with *G. uvicola* on PDA. 4E: Transverse and longitudinal cuts through stems of inoculated plants. 4F: *G. uvicola* re-isolated from segments of diseased plant stems.

of dead tissue (Figure 4E). Many acervuli were also observed on the opposite sides and above the inoculation sites. Tissues from these shoots were obtained and cultured in Petri dishes with PDA. *Greeneria uvicola* was isolated and confirmed in all inoculated tissues, fulfilling the Koch's postulates (Figure 4F).

Although *G. uvicola* is typically considered as an ascomycete responsible for the bitter rot of *Vitis vinifera* fruit bunches, some investigations have demonstrated the ability of this fungus to infect host arms and cause their death in vineyards. This species was detected as causal agent of arm dieback in grapevines in Australia (Castillo-Pando *et al.*, 2001) and Uruguay (Abreo *et al.*, 2008).

Because this fungus could be detected from both symptomatic and non-symptomatic plants, Navarrete *et al.* (2011), suggested that it was latent pathogen, capable of producing symptoms when plants are under stress. In the present study, during the field sampling, asymptomatic plants containing *G. uvicola* were detected. After a few weeks, wilt and yellowing were observed on the tips and lateral leaves of the infected shoots, followed by formation of dark rings and general death of leaves on the branches (Figure 1A). The symptoms of *G. uvicola* in Sonora grapevines could be confused with those caused by *Lasiodiplodia theobromae* (Úrbez-Torres *et al.*, 2008). Both fungi produced wedge-shaped staining in cross-section of cordons. However, the presence of numerous visual conidiomata in the affected areas differentiates these two pathogens.

The symptoms of dead arm caused by *G. uvicola* can be confused with those caused by other pathogens, such

as *Eutypa lata* and species of Botryosphaeriaceae (Navarrete *et al*., 2009). Accurate and timely diagnoses are essential for good prevention and control of the disease, and molecular techniques for pathogen detection and identification are very reliable.

In conclusion, the symptoms observed in the field, the morphological characteristics, phylogenetic analysis, and pathogenicity tests carried out for isolates obtained from disease grapevine plants, allowed identification of *G. uvicola* as the causative agent of dieback of arms, bud necrosis, and disease symptoms in shoots and branches in vineyards of Sonora state. This is the first report of the infectious capacity of *G. uvicola* in wood tissues of *Vitis vinifera* plants in Mexico.

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Short Notes

Host defence activation and root colonization of grapevine rootstocks by the biological control fungus *Trichoderma atroviride*

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Summary. Several *Trichoderma* species can act as biocontrol agents and hold the potential to control soilborne diseases through different modes of action. Little is known about the colonization pattern of *Trichoderma atroviride* in grapevine roots and activation of induced systemic resistance *in planta*. A laboratory model was developed to assess root colonization and its impact on grapevine defence activation. Rootstock cuttings from 1-year-old dormant canes were inoculated with conidium suspensions of *T. atroviride* T-77 or *T. atroviride* USPP T1, and host and inoculum colonisation were assessed after 21 d. The two strains of *T. atroviride* were re-isolated from the treated plants (from 70% of the roots and 20% of crowns). Colonization rates did not depend on the *Trichoderma* strain or rootstock cultivar. However, up-regulation of targeted defence genes was dependent on the inoculated *Trichoderma* strain and rootstock cultivar. Furthermore, in leaves of rootstock cultivars 'US 8-7' and 'Paulsen 1103', genes were up-regulated which encode for PR proteins involved in plant defence or production of stilbenic phytoalexins. *Trichoderma atroviride* T-77 was transformed with tdTomato fluorescent protein to allow visualization by confocal laser scanning microscopy. These results give new insights into the mechanisms of grapevine-*Trichoderma* interactions, and allow detection of establishment of potential biocontrol agents within host tissues.

Keywords. *Vitis* spp., *Trichoderma* spp., defence response.

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INTRODUCTION

The worldwide drive for environmentally sustainable practices has influenced methods used to control plant diseases. Disease management needs to decrease reliance on synthetic chemicals and increase use of biological control

agents (BCAs) (Edreva, 2004; Woo *et al.,* 2014). *Trichoderma* species are widely used and well-known BCAs. Sixty percent of fungal available biocontrol products contain *Trichoderma* species (Verma *et al.,* 2007), as individual species or species mixtures (Harman *et al.,* 2004).

Trichoderma species are saprophytic fungi commonly found in soil, while some can also be endophytes in plants. Endophytes can colonize healthy plant tissues without causing host symptoms or losses, and have minimal environmental impacts. The mechanisms of action of *Trichoderma* spp. as BCAs include mycoparasitism (Harman *et al.,* 2004), secretion of mycolytic enzymes (Reino *et al.,* 2007), competition for limiting resources (Harman *et al*., 1993; Haran *et al*., 1995; Howell, 2006) and/or production of antibiotic metabolites (Harman and Kubicek, 2002; Vinale *et al.,* 2006; Mutawila *et al.,* 2016b). *Trichoderma* species also have positive effects on their hosts, including growth enhancement and resistance activation (Harman, 2006; Vinale *et al.,* 2008a; Gallou *et al.,* 2009; Parrilli *et al*., 2019).

Activation of host defence genes by *Trichoderma* is an important component of biological control. As *Trichoderma* colonizes plants, endogenous compounds are released that lead to the recognition by the hosts and triggering of host defence reactions (Lorito *et al.,* 1994; Viterbo and Chet, 2006; Woo *et al.,* 2006; Brotman *et al.,* 2008; Vinale *et al.,* 2008b; Morán-Diez *et al.,* 2009; Hermosa *et al.,* 2013; Salas-Marina *et al.,* 2015; Contreras-Cornejo *et al.,* 2016; Guzmán-Guzmán *et al.,* 2017; Mendoza-Mendoza *et al.,* 2018; Nogueira-Lopez *et al.,* 2018). The different *Trichoderma* antagonisms, mechanisms and soil habitats make these fungi ideal candidates in controlling soilborne diseases.

Blackfoot disease (BFD) of grapevines is soilborne, and affects nursery grapevine plants and vines in newly established vineyards, causing economic losses in most grapevine industries (Halleen *et al*., 2004; 2006a; Gramaje and Armengol, 2011; Probst *et al.,* 2012; Úrbez-Torres *et al.,* 2014). Management of BFD relies on integrated programmes that include the use of hot water treatments (HWT), cultural practices and BCAs (Gramaje *et al.,* 2010; Úrbez-Torres *et al.,* 2014; Halleen and Fourie, 2016). Application of *Trichoderma* for control of BFD has given variable results (Fourie *et al*., 2001; Fourie and Halleen, 2006; Halleen *et al*., 2007; dos Santos *et al*., 2016; Halleen and Fourie, 2016; Berlanas *et al*., 2018; Gramaje *et al*., 2018). Improved understanding of host colonization and resistance activation by *Trichoderma* would aid utilisation of this biocontrol fungus for BFD control.

Grapevine rootstocks usually have American *Vitis* spp. origins, and include *V. riparia*, *V. berlandieri*, *V. champinii*, *V. aestivalis*, *V. riparia* or (sometimes) *Muscadinia rotundifolia*. This makes study of rootstock disease resistance difficult, because plant genomes could differ dependent on the rootstock cultivar. To date, molecular protocols developed for the study of *Vitis* resistance are rarely optimized for rootstocks.

Trichoderma species can activate either systemic acquired resistance (SAR) and/or induced systemic resistance (ISR) in host plants (Segarra *et al.,* 2007; Shoresh *et al.,* 2010; Rubio *et al.,* 2014; Martínez-Medina *et al.,* 2017; Manganiello *et al.,* 2018). ISR activated by *Trichoderma* species induces a state of priming by increasing the plant immune activation, leading to rapid and effective defence responses against pathogens in distal plant parts (Segarra *et al.,* 2009; Lorito *et al.,* 2010; Pieterse *et al.,* 2014; Conrath *et al.,* 2015; Martínez-Medina *et al.,* 2017). Mutawila *et al.* (2016a) showed that *Trichoderma* species triggered the activation of defence genes in an artificial system of grapevine cells. Di Marco and Osti (2007) showed that systemic activation of host resistance by *Trichoderma* root treatments of nursery vines reduced necrotic areas of *Botrytis cinerea*-inoculated grapevine leaves. Nevertheless, plant reactions when colonized by *Trichoderma*, at molecular and biochemical levels, are still not well understood (Contreras-Cornejo *et al.,* 2016; Guzmán-Guzmán *et al.,* 2017).

Endophytic growth and activation of grapevine rootstock defence genes by *Trichoderma* is not known, and these would aid understanding of the efficacy of this biocontrol fungus. Knowledge of root colonization and defence activation is an essential first step in evaluating potential use of *Trichoderma* spp. as BCAs for BFD or other diseases in nurseries and new vineyards. The aim of the present study was to increase understanding of the internal establishment of *T. atroviride* and activation of grapevine defence genes in different rootstock cultivars. The objectives were: i) to investigate the colonization by *T. atroviride* of different rootstock cultivars; and ii) to assess activation of host defence genes during colonization. This knowledge will help to ensure potential BCAs are well-established within host plants to prevent pathogen infections.

MATERIALS AND METHODS

Evaluation of Trichoderma atroviride *colonization of rooted rootstock plants by re-isolation*

Fungal isolates

Trichoderma atroviride isolate T-77 and *T. atroviride* isolate USPP T1 (Department of Plant Pathology, Stellenbosch University) were grown on Potato Dextrose Agar (PDA, 39 g L^{-1}) and subcultured every 21 d. *Tricho-*

derma atroviride T-77 is a commercial inoculum that was originally isolated from grapevine roots and is the component of Eco77® (Plant Health Products). Isolate USPP T1 originated from grapevine shoots.

Plant material

One-year-old dormant canes of the grapevine rootstock cultivars 'Richter 110' (*V. berlandieri* × *V. rupestris*), 'US 8-7' (Jacquez: *V. aesativalis* × *V. cinerea* × *V. vinifera* and 'Richter 99') and 'Paulsen 1103' (*V. berlandieri* × *V. rupestris*) were collected from a nursery mother block near Wellington, South Africa. The cuttings were disinfected by soaking a didecyldimethylammonium chloride compound (Sporekill®) for 1 h followed by hot water treatment for 45 min at 50°C. The basal end of each two-bud cutting was dipped in 4-indole-3-butyric acid powder and rooted in a perlite-filled mist bed maintained at 26°C under daylight conditions. The cuttings were watered for 10 s every 15 min for the first 5 weeks, then reduced to 2 min twice each day for 1 week. The rooted cuttings were then transplanted into perlite-containing cups after 6 weeks. Plants were then maintained for 1 week, receiving water *ad libitum* to avoid potting stress, before application of experimental treatments.

Colonization by Trichoderma atroviride

Rootstocks inoculation

Plants of 'Richter 110', 'US 8-7' and 'Paulsen 1103' were inoculated by drenching with conidium suspensions of either *T. atroviride* T-77 or *T. atroviride* USPP T1 (final concentration of 1×10^6 conidia mL^{-1}) into the perlite. Inoculum was freshly prepared on the day of inoculation. *Trichoderma atroviride* cultures were grown on PDA plates for 2 weeks before covering each plate with 5 mL of tap water and scraping the culture to collect conidia. The resulting conidium suspension was filtered through a double layer of sterile cheesecloth and adjusted to the required concentration after counting with haemocytometer. Seventy-five plants were used per treatment, and a total of 225 plants were inoculated. For each trial, each plant was inoculated with 100 mL of conidium suspension or 100 mL of sterile water for the control plants.

Re-isolation of *Trichoderma atroviride*

Roots were harvested for re-isolations 21 d after inoculation. The roots were then rinsed with water and surface sterilized (30 s in 70% ethanol, 1 min in 3.5% sodium hypochlorite and 30 s in 70% ethanol), to ensure that re-isolated fungi originated from the inner root tissues of the plant and not from rhizospheres. Four pieces of roots were plated onto each Petri plate containing PDA amended with streptomycin (40 $mg.L^{-1}$; PDA+) and two plates were used per plant. Four small sections from the internal tissue of the crown were plated onto one PDA+ plate per plant. The plates were then incubated at room temperature in day light conditions. Fungal growth from roots of each rootstock was determined as proportion (%) of the isolated segments colonized. Infection by *Trichoderma* isolates was scored based on the number of root pieces or crowns colonized on the plates.

Evaluation of Trichoderma atroviride *colonization of rooted rootstock plants using an Agrobacterium-transformed* T. atroviride *isolate*

Agrobacterium transformation of *Trichoderma atroviride* with tdTomato fluorescent protein

Agrobacterium tumefaciens AGL-1 was used as host for plasmid pBHt2-tdTom (Caasi et *al.*, 2010). This strain contains a gene coding for a tdTomato (orange-red) fluorescent protein, under control of the *Pyrenophora tritici-repentis* toxA promoter, the hygromycin phosphotransferase hph for selection of fungal transformants, aminoglycoside phosphotransferase, and the kanamycin resistance marker for selection of bacterial transformants. Transformation was based on an optimized protocol from Gorfer *et al.* (2007). Briefly, the *Agrobacterium* vector was induced in a minimal medium (AtIND) containing acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone 200 µM; AS) and kanamycin (50 mg mL^{-1}). *Trichoderma atroviride* T-77 conidia were inoculated into the AtIND medium, after induction of the *Agrobacterium*. After 2 d , 200 µL of medium containing *Agrobacterium* and *T. atroviride* T-77 were plated on a thin layer of MoserIND medium. Once the fungal thalli developed enough (2–3 d), they were overlayed with a layer of Moser medium supplemented with 200 mg L^{-1} of hygromycin B. Transformants that crossed the selection layer were plated onto selective PDA and checked for fluorescence. Single conidium isolations were then repeated three times onto PDA plates supplemented with hygromycin B (200 µg L^{-1}) to avoid chimeras. The transformation of *T. atroviride* T-77 was confirmed by confocal laser microscopy.

Confocal laser microscopy

Confocal laser microscopy was performed at the Confocal & Light Microscope Imaging Facility, UCT (University of Cape Town), using a LSM880 Airyscan (Carl Zeiss) and a Fast Airyscan module confocal. The EC "Plan-Neofluar"20×/0.5 M27 objective was used for low magnification at a zoom factor of 1. The objective used for high magnification was LCI "Plan-Apochromat"63×/1.4 Oil DIC M27 with a zoom factor of 1. A 561 nm solid-state red laser was used with emission detection from 566 nm to 609 nm, with 2% laser power and a pinhole size of 74 µm. The maximum intensity projections were obtained by capturing and processing Z-stacks. The imaging software ZEN 2.3 SP1 (Carl Zeiss) was used to process the images.

Experimental design and re-isolation from rootstock plants

Plants of 'Richter 110', 'US 8-7' and 'Paulsen 1103' each with six fully developed leaves, were inoculated with conidium suspensions of *T. atroviride* strain T-77::*tdTomato* (finale concentration of 1×10^6 conidia mL^{-1}) for confocal laser microscopy and re-isolation. Each plant was inoculated with 100 mL of the conidium suspension, or 100 mL of water for the control plants. In total, 96 plants were inoculated, 36 plants for the control and 60 for the treatment with the conidium suspension. After 3, 7, 10 and 21 d post inoculation (dpi), re-isolations were carried out from the roots and crowns of the plants (as described above). *Trichoderma atroviride* T-77::*tdTomato* was confirmed by making slides of the cultures and visualisation with an epifluorescence Zeiss Axioscope microscope (Carl Zeiss). Colonization by *T. atroviride* T-77::*tdTomato* was scored based on the frequency of occurrence in plates, not number of plant pieces.

Induced systemic responses in leaves by Trichoderma *atroviride root colonization*

Experimental design

The plants of 'Richter 110', 'Paulsen 1103' and 'US 8-7' used to determine host defence activation in leaves were the same as those used for the colonization experiment (above). The top fully developed leaf from each of five plants were harvested at 21 dpi and then combined as one repeat of one treatment, resulting in a total of three biological repetitions. The leaves were then stored at -80°C for later analysis.

RNA extraction, cDNA synthesis and qPCR from leaves

Frozen leaves (-80°C) were ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from 150 mg of ground leaf material using the RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's instructions, and was quantified at 260 nm wavelength using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). Residual genomic DNA was removed by DNase1 digestion on an extraction column with the RNase-free DNase set (QIAGEN) at 25°C for 15 min during the RNA extraction. cDNAs were synthesized from 0.5 µg of DNase-treated RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Real-time PCRs were carried out on a CFX96 Real Time System C1000 Touch™ Thermal Cycler (Bio-Rad). qPCR reactions were each carried out in a reaction buffer containing 2× iQ SYBR® Green Supermix, 0.2 mM of forward and reverse primers, and 10 ng of reverse transcribed RNA, in a final volume of 20 µL. Thermal cycling conditions were as follows: 30 s at 95°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The defence genes and primer pairs associated with defence in *Vitis vinifera* (*Vv*) used for quantitative real-time PCR are listed in Table 1. The study included genes encoding for a wide range of defence reactions including *VvCAM* that encodes for cell signalling and calcium fluxes and *VvSOD* for reactive oxygen species (ROS) metabolism. *VvPR2*, *VvCHIT4C* and *VvPR6* are related to the production of different pathogenesis-related proteins (PR proteins), and *VvLTP* to the production of lipid transfer proteins and the PR14 proteins. *VvPAL* is associated with the phenylpropanoid pathway, *VvACO1* with the ethylene pathway, *VvLOX9* with the jasmonic pathway and *VvSTS* with the stilbene pathway. The data obtained were analyzed using CFX Manager Software (Bio-Rad). The results obtained for each gene of interest were normalized to the expression of two reference genes, *VvEF1-y*, an elongation factor 1 gene, and *VvActin* encoding actin proteins. Induction ratios compared to the controls were calculated according to Hellemans *et al.* (2007), as:

$$\text{Induction ratio} = [(1+E)\Delta CT(REF1)\times(1+E)\Delta CT(REF2)]^{0,5}$$

Gene expression was considered significant at an induction ratio of 4. The calculation takes the PCR efficiency (E) as well as the ΔCT, representing the difference between the Ct of the negative control and the Ct of the sample. For each sample, tests were carried out with two technical repetitions (the Ct results from two data values) and three biological repetitions (five leaves from five plants

Table 1. Sequences of the primer pairs derivative of *Vitis vinifera* and used for RT-qPCR.

Gene (Target)	Primer sequences[a]	Reference	Gene ID/ Gene bank accession
VvEF1-y (Elongation factor 1)	F : 5'-CAAGAGAAACAATCCCTAGCTG-3' R : 5'-TCAATCTGTCTAGGAAAGGAAG-3'	Rossdeutch *et al.,* 2016	VIT_12s0035g01130
VvActin (Actin)	F : 5'-CTTGCATCCCTCAGCACCTT-3' R : 5'-TCCTGTGGACAATGGATGGA-3'	Rossdeutch *et al.,* 2016	VIT_04s0044g00580
VvCAM (Calmodulin)	F : 5'- TATTCCAGTAGTTTGGGTTGGTAGTG-3' R : 5'-AAGAAGCACCAAACAAGAAAGGAG-3'	Perazzolli *et al.*, 2010	GR911644.1
VvSOD (Superoxide dismutase)	F : 5'-TGCCAGTGGTAAGGCTAAGTTCA-3' R : 5'-GTGGACCTAATGCAGTGATTGA-3'	Stempien *et al.*, 2018	AF056622
VvPR2 (PR protein 2)	F : 5'-GGGGAGATGTGAGGGGTTAT-3' R : 5'-TGCAGTGAACAAAGCGTAGG-3'	Bellee *et al.,* 2017	AF239617
VvCHIT4C (Acidic class IV chitinase)	F : 5'-GTGTGTCCGGGAAGGATTACT-3' R : 5'-TCAAGCCATCAAACCCAATGC-3'	Mutawila *et al.,* 2016a	XM002275480
VvPR6 (PR protein 6)	F : 5'-AACCATTAAGAGGGAGAATCCTCA-3' R : 5'-CACGGACCCTAGTGCAGTAAA-3'	Mutawila *et al.,* 2016a	XM002284411
VvLTP (PR protein 14)	F : 5'-CTGGCATCAATTTCGGTCTT-3' R : 5'-AAGGCTGAGTGGTCCAAGTG-3'	Bruisson, 2015	NM_001281191.1
VvACO1 (Aconitase 1)	F : 5'-GCCGGTTTGAAGTTCCAGGCCA-3' R : 5'-ACTCAAACTGTGGCAATGGGACCC-3'	Bellee *et al.,* 2018	XM_002273394.1
VvLOX9 (Lipoxygenase)	F : 5'-CCCTTCTTGGCATCTCCCTTA-3' R : 5'-TGTTGTGTCCAGGGTCCATTC-3'	Perazzolli *et al.*, 2010	AY159556
VvSTS (Stilbene synthase)	F : 5'-AAGGGTCCGGCCACCATCCT-3' R : 5'-ACGCAGTCATGTGCTCGCTCT-3'	Mutawila *et al.,* 2016a	XM002268806
VvPAL (Phenylalanine ammonia-lyase-like)	F : 5'-GGTGAGCTTCACCCCTCCAGGT-3' R : 5'-GGAGCTGCAGGGGTCATCAATGT-3'	Mutawila *et al.,* 2016a	XM002281763

[a] Primer efficiency of 1.8 used for all the sets of primers.

were used for each repetition). PCR amplification tests were each conducted in 20 µL final volume (10 µL of Taq DNA Polymerase Master Mix 2x [AMPLIQON], 1 µL of forward and reverse *VvActin* primers, 2 µL of cDNA and 6 µL of sterile water), to verify the quality of the cDNA.

Statistical analyses

For *Trichoderma* re-isolation and RT-qPCR gene expression analyses, data evaluation was performed with R 3.6.1. Software (R Development Core Team, 2016) through a multifactorial ANOVA, and multiple comparison of the means with the Tukey test ($P \leq 0.05$).

RESULTS

Evaluation of Trichoderma *colonization of rooted rootstock plants by re-isolation*

The inner roots of all three tested rootstock cultivars were efficiently colonized at 21 dpi by both *Trichoderma* strains. The re-isolation percentages showed that the strains T-77 and USPP T1 colonized the plant parts to a similar extent, at an average of 70% in roots and 20% in the crowns (Figure 1). The roots were significantly more colonized than the crowns. The average colonization percentages for both strains for the roots were 78% for 'Richter 110' and 'Paulsen', and 74% for 'US 8-7'. The colonization percentages for the crowns were 25% for 'Richter 110', 30% for 'Paulsen' and 19% for 'US 8-7'. However, there were no statistically significant differences in root and crown colonization by the *T. atroviride* strains between the three rootstocks.

Evaluation of Trichoderma *colonization of rooted rootstock plants using an Agrobacterium-transformed* T. atroviride

Trichoderma atroviride T-77 was successfully transformed. The transformant *T. atroviride* T-77::*tdTomato* expressed the expected intensity of fluorescence. Mycelium and conidia were observed from pure cultures (Figure 2), allowing to test the ability of this strain to colonize rootstock roots. Observations *in planta* were hampered by the hardening of roots and required specialized

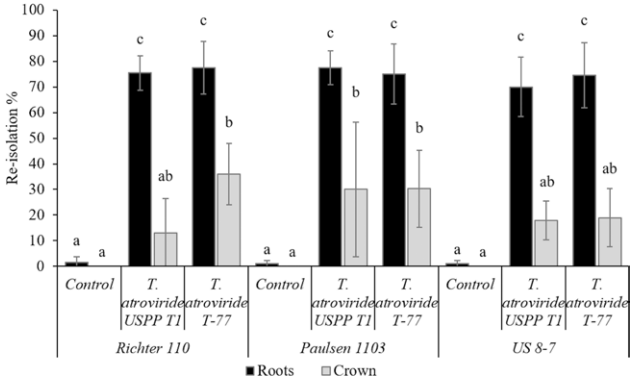

Figure 1. Mean proportions (%) of re-isolations of *Trichoderma atroviride* T-77 and *T. atroviride* USPP T1 from the grapevine rootstock cultivars 'Richter 110', 'Paulsen 1103' and 'US 8-7'. Re-isolations were made from plant roots and crowns 21 d post-inoculation. Each of the data points shows the average of 25 replicates (five biological replicates and five technical replicates). Error bars indicate standard deviations of the means. Different letters accompanying the means indicate significant differences ($P \leq 0.05$; Tukey Contrasts).

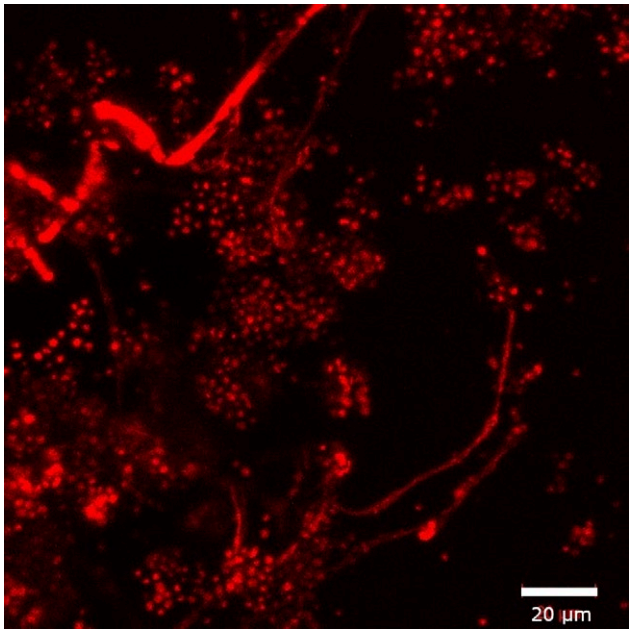

Figure 2. Confocal microscope image of *Trichoderma atroviride* T-77::*tdTomato* mycelia and conidia.

sectioning of plant material, so assessments of colonization were made based on re-isolation of the fluorescent strain.

Re-isolations of *T. atroviride* T-77::*tdTomato* were carried out (described above) from roots and crowns at the different time points: 3, 7, 11 and 21 dpi. Whereas *T. atroviride* T-77::*tdTomato* was absent from the control plants, it was present in all three cultivars after 3 dpi in the roots and crowns (Table 2). Colonization of roots was from 80% to 100%, and of crowns from 20% to 60%. After 21 dpi, colonization in the roots was 80% to 100% and 60% in the crown. However, in the roots and the crowns, the colonization was not significantly different ($P \leq 0.05$, Tukey Contrasts) between the three cultivars or between the four periods post-inoculation.

Systemic response in leaves induced by Trichoderma atroviride *root colonization*

Firstly, primers designed for *Vitis vinifera* were evaluated to assess gene expression of rootstocks. Expression of defence related genes was assessed at 21 dpi in parallel with re-isolations for the two *T. atroviride* strains (Figure 3). In 'Paulsen 1103', only the defence genes encoding for PR proteins (*VvPR6*) and stilbene synthase (*VvSTS*) were significantly induced by both *Trichoderma* strains, although the induction was greater with *T. atroviride* T-77 than with *T. atroviride* USPP T1 (Figure 3 B). In 'US 8-7' leaves, *VvChit4c*, *VvLTP*, *VvACO1* and *VvSTS* were significantly up-regulated, but only by *T. atroviride* USPP T1 (Figure 3 C). For 'Richter 110', no modulation of defence gene expression was detected after 21 dpi (Figure 3A).

DISCUSSION

Several *Trichoderma* species are well known BCAs, and these could have potential for use against grapevine trunk diseases via root applications. To use a BCA to prevent pathogen infection, the mechanisms of grapevine-BCA interactions and establishment of the potential BCA inside host plants must be well understood. However, how *Trichoderma* species colonize grapevine, and how they react on molecular or biochemical levels, are not well-understood. In the present study, a protocol to inoculate *Trichoderma* on grapevine was develop under laboratory conditions, to investigate colonization of roots and bases of grapevine rootstock plants by *Trichoderma*, and the abilities of these fungi to activate host defence in leaf tissues.

Strains of *Trichoderma* have been found to inhabit root systems of many plant species (Shoresh *et al.,* 2010; Hermosa *et al.,* 2012). However, to our knowledge there has been no laboratory study confirming early colonization of grapevine roots by these fungi. *Trichoderma* spp. readily colonize roots in field applications, as reported by Fourie *et al.* (2001), where less BFD pathogens were isolated from grapevine roots after treatment

Table 2. Numbers of plants from which *Trichoderma atroviride* T-77::*tdTomato* were re-isolated from roots and crowns after 3, 7, 10 or 21 d post-inoculation (dpi).

Cultivar	Incidence in roots (per plant)[a]				Incidence in crowns (per plant)[a]			
	3 dpi	7 dpi	10 dpi	21 dpi	3 dpi	7 dpi	10 dpi	21 dpi
'Richter 110'	4	5	5	5	1	5	3	3
'Paulsen 1103'	5	5	5	5	2	4	3	3
'US 8-7'	5	4	5	5	3	5	4	3

[a] Five plants for each treatment and time point.

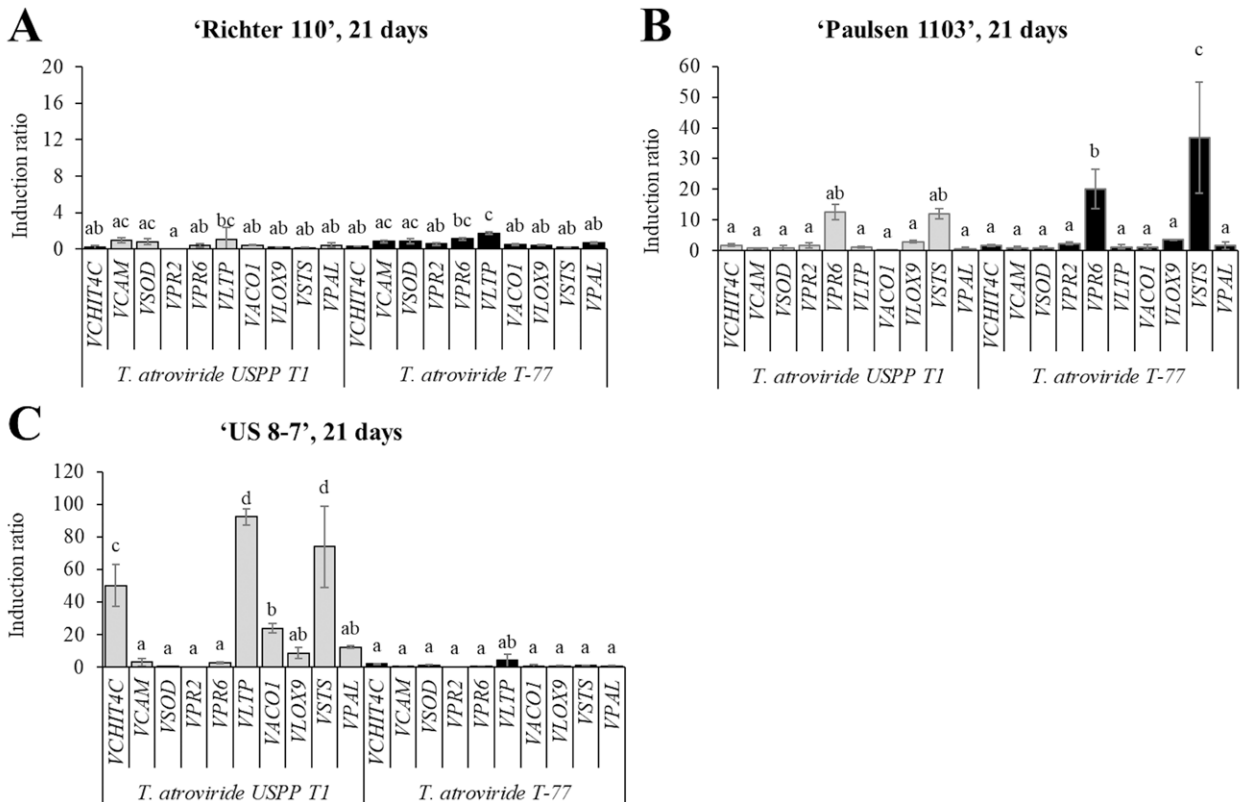

Figure 3. Mean gene induction ratios for different gene expressions (as indicated from RT-qPCR) in the leaves of grapevine rootstock cultivars 'Richter 110' (A), 'Paulsen 1103' (B) and 'US 8-7' (C) after 21 d after inoculations with *Trichoderma atroviride* T-77 (grey bars) or *T. atroviride* USPP T1 (black bars). Individual data points are means of two technical and three biological replicates. Error bars indicate standard deviations of the means. Different letters accompanying the means indicate significant differences ($P \leq 0.05$; Tukey Contrasts).

with *Trichoderma* species. In the present study, the ability of *Trichoderma* to colonized grapevine root tissues was evaluated with two *T. atroviride T. atroviride* strains inoculated onto three grapevine rootstock cultivars. After 21 dpi, approx. 70% of roots and 20% of crowns of inoculated plants were colonized by both strains. The lower colonization in crowns than in roots could be explained by the growth of *T. atroviride* from the roots to the crowns, but the infections could also have occurred directly into the crowns since these host tissues were not completely callused. The amount of colonization in the roots and crowns of the rootstock plants was neither dependent on the cultivar nor the *T. atroviride* strain. The *T. atroviride* transformed with tdTomato allowed assessment of colonization using re-isolations and confocal laser microscopy. Protocols for visual observation *in planta* need to be optimized. The results showed that *T. atroviride* successfully colonized the roots and crowns of grapevine plants after 3 dpi, and colonization was similar in the three studied cultivars.

This study has demonstrated rapid and high frequencies of colonization of grapevine roots by *T. atroviride*. However, several studies have showed low levels of field colonization of grapevine rootstocks by *Trichoderma* species. Ferrigo *et al.* (2017) reported low amounts of colonization of 'Richter 110' roots after a *T. atroviride* soil inoculation. However, these treatments were effective in controlling disease caused by *Agrobacterium vitis*. Berlanas *et al.* (2018) applied *Trichoderma* root dips to 'Richter 110' plants for 24 h, and colonization rate was less than 1% 2 months after inoculation.

After determining that the potential BCA was established in its host, the interactions with the host were assessed. The mechanism by which plants perceive *T. atroviride* is not well understood. Beneficial microbes only induce limited levels of host immune systems after recognition during local colonization of roots. Defence responses are activated in plant hosts due to Microbe-Associated Molecular Patterns (MAMPs) and Damage-Associated Molecular Patterns (DAMPs) perceived as danger indicators (Boller and Felix, 2009). *Trichoderma* spp. secrete structural proteins and secondary compounds that act as MAMPs, and their secreted enzymes that act against plant host cell walls operate as DAMPs (Hermosa *et al.,* 2013). In recent studies of the *Trichoderma* genome, it was demonstrated that the genome could encode for potential effector proteins that assist plant colonization (Mendoza-Mendoza *et al.,* 2018; Nogueira-Lopez *et al.,* 2018). Furthermore, *Trichoderma* spp. have been shown to form attachment structures that are similar to appressoria that penetrate through production of cellulolytic and proteolytic enzymes (Brotman *et al.,* 2008; Contreras-Cornejo *et al.,* 2016). These attachment structures were probably the results of the germinating conidia identified in the present study using confocal laser microscopy. Once recognized by hosts, *Trichoderma* spp. prime the plant defence systems (Guzmán-Guzmán *et al.,* 2018), and induce expression of defence genes, which confer local resistance (site inhabiting resistance) in roots and ISR against pathogens in distal plant parts (Pieterse *et al.,* 2014).

Development of a system to assess activation of grapevine defence genes after *Trichoderma* conidium drenching required considerable optimization. The extraction of mRNA from roots was abandoned after several failed attempts. Obtaining mRNA from roots is known to be difficult, most probably due to inhibitors present within the extraction. Extraction of mRNA from leaves is more reliable and this allowed demonstration of ISR by induction of defence genes in the leaves after only the roots were inoculated with *T. atroviride* T-77 or *T. atroviride* USPP T1 conidium suspensions. Induction of defence genes was studied after 21 dpi. Differences between rootstock cultivars were demonstrated. In leaves of 'Richter 110', no modulation of expression of the targeted genes was observed in response to *T. atroviride* inoculation with either of the fungus strains. This was confirmed by a separate assay (unpublished data), in which no impact of *T. atroviride* inoculation was detected for expression of defence genes at 10 dpi. Absence of induction in 'Richter 110' by *T. atroviride* could be explained by low sensitivity of the cultivar. In leaves of 'US 8-7', up-regulation was detected for the defence genes *VvChit4c* and *VvLTP* encoding two PR proteins, *VvACO1* encoding a protein involved in ethylene pathway synthesis, and *VvSTS* encoding a stilbene synthase. However, the expression of these genes was only induced in leaves of grapevine plants inoculated with strain USPP T1, suggesting that the modulation of defence gene expression is dependent on the *Trichoderma* strain. In leaf tissues of 'Paulsen 1103', two genes (*VvPR6* and *VvSTS*) were up-regulated by both strains of *Trichoderma*. However, the gene expression was slightly greater when induced by strain T-77 than strain USPP T1. Furthermore, these results can be correlated with information on rootstock susceptibility to pathogens. Sieberhagen (2017) inoculated rootstock cuttings with conidium suspensions of different pathogens, including those causing BFD, to determine the resistance or susceptibility of grapevine rootstocks used in South Africa. 'US 8-7' and 'Paulsen' presented the least disease severity against all the pathogens tested including BFD pathogens, whereas 'Richter 110' developed the greatest disease severity. The high susceptibility of 'Richter 110' to BFD pathogens could explain inefficient priming by *Trichoderma* due to a lack of host defence activation, in comparison to 'Paulsen 1103' and 'US 8-7'.

The two experiments carried out in the present study have demonstrated that the intensity of induction of defence genes was dependent on the *Trichoderma* strain and the grapevine rootstock cultivar. In both experiments, the *Trichoderma* inoculations lead to up-regulation of gene encoding PR proteins or proteins involved in stilbene synthesis. These compounds may represent an effective defense response to protect grapevine plants from BFD. High expression of the stilbene synthase gene (*VvSTS*) results in increased resistance responses to pathogens (Adrian and Jeandet, 2012). It is not certain that priming of host defence will be sufficient for protection against, for example, infection by black foot pathogens. Field experiments have shown that the use of *Trichoderma* did not consistently prevent black foot pathogen infections of nursery vines (Berlanas *et al.,* 2018; van Jaarsveld *et al.*, 2020).

In conclusion, the present study has increased understanding of the mechanisms of grapevine-*Trichoderma* interactions. Knowledge of these interactions is important to assist screening for potential biocontrol agents that can be used against BFD and other grapevine trunk diseases. Although *T. atroviride* similarly colonized the three rootstock cultivars tested, activation of host defence was cultivar dependent and therefore needs to be evaluated for more rootstock cultivars. *Trichoderma* treatments need to be evaluated in combination with a pathogen with measurable symptoms, in controlled environment experiments and in the field. This will aid optimization of the use of *Trichoderma* spp. for grapevine root application.

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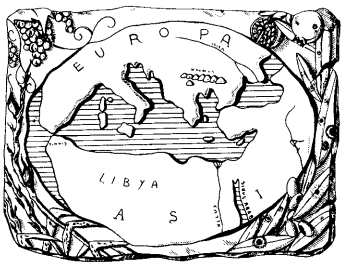

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