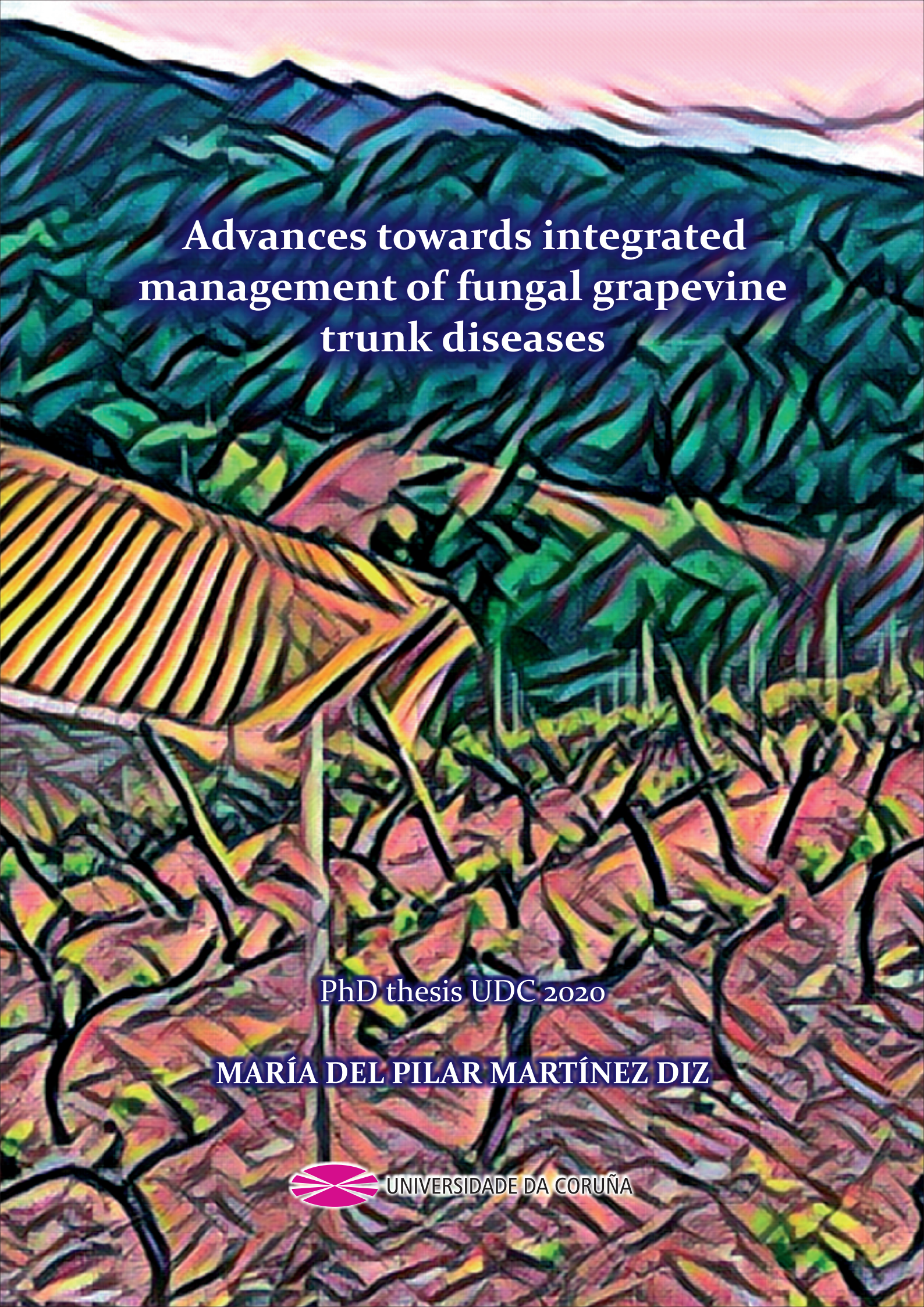

Advances towards integrated management of fungal grapevine trunk diseases

PhD thesis UDC 2020

MARÍA DEL PILAR MARTÍNEZ DIZ

UNIVERSIDADE DA CORUÑA

Cover picture: 'Armontes' vineyard, April 17th, 2019
Bodegas Godeval
O Barco de Valdeorras – Ourense
Spain

Foto portada: Viñedo de la finca "Armontes", 17 de abril del 2019
Bodegas Godeval
O Barco de Valdeorras – Ourense
España

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MARÍA DEL PILAR MARTÍNEZ DIZ

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PhD in Advanced Biotechnology

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Abstract

Grapevine trunk diseases (GTDs) are among the main biotic threats to the economic sustainability of the grapevine industry worldwide, reducing yields, productivity and longevity of vineyards. Several factors such as changes in vineyard cultural practices, low sanitary quality of the grapevine propagation material, the ban of most effective chemical active ingredients, and poor protection of pruning wounds have increased their incidence over last years. Moreover, these diseases are very complex and involve a broad spectrum of taxonomically unrelated fungi making their control very challenging. In this thesis, the development and implementation of novel molecular techniques that lead to the improvement of practical effective strategies to the GTD management were investigated. The assessment of sustainable alternatives to the use of fungicides to increase vineyards lifespan was also performed.

Initially, we comparatively assessed the accuracy, efficiency, and specificity of Droplet Digital PCR (ddPCR) and real-time PCR (qPCR) techniques for the detection and quantification of *Ilyonectria liriodendri* in bulk and rhizosphere soil, as well as, grapevine endorhizosphere obtained from healthy young vines in La Rioja region. The results revealed a strong correlation between both techniques with ddPCR being more sensitive to very low pathogen concentrations. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by black-foot disease pathogens.

Then, the spatial dynamics of the fungal communities of these three soil-plant compartments were also characterized by high-throughput amplicon sequencing (HTAS). Soil fungal assemblages had extensive local homogeneity across small distances, and soils with similar climatic and edaphic variables, thus suggesting that vineyard fungal communities are partially conserved in La Rioja region. The diversity and composition of the fungal communities were largely affected by the soil-plant compartment, with diversity decreasing in the endorhizosphere and suggesting that the root tissues entail a barrier for fungal colonization. The results of functional prediction suggested an increase in the relative abundances of potential plant pathogens, endophytes and arbuscular mycorrhiza, and a decrease in the relative abundance of potential wood and/or dung saprotrophs were predicted from bulk soil towards the endorhizosphere. Once more, it was found that the internal root tissues of asymptomatic vines are a microbial niche that is inhabited by GTD fungi.

The HTAS technique was also used to determine the effect of pruning time on the fungal microbiome diversity and composition that colonized pruning wounds naturally over two infection periods (November-February and February-May), in vineyards belonging to three Denominations of Origin (D.O.) in Galicia (Spain). The fungal communities colonizing grapevine pruning wounds were affected in their composition and diversity by the D.O., whereas the spatial variation within each region was low. The results showed the existence of a core community of fungal species conserved in grapevine pruned canes regardless of the infection period. Higher abundances for most GTD genera were detected after pruning in February (winter) than in November (mid-autumn), therefore suggesting a seasonal effect on pruning wounds infections. Additionally, a positive correlation was observed between the accumulated rainfall and the abundance of the total fungal microbiome and *Diaporthe* genus over eighth and eleventh weeks after pruning.

Planting tolerant grapevine cultivars is one of the alternatives to the use of fungicides in controlling GTDs. The tolerance to *Phaeomoniella chlamydospora* infection of commercial and minority grapevine cultivars from Spanish germplasm collections was evaluated in a rapid and effective detached cutting assay under greenhouse conditions. All cultivars developed internal wood lesions after artificial inoculation with the causal agent of Petri disease and esca, indicating that there is no evidence of qualitative resistance to this fungus. Nevertheless, the severity of internal wood symptoms varied considerably amongst cultivars allowing to classify them according to varying signs of partial tolerance to *Pa. chlamydospora*. The cultivars 'Estaldiña', 'Albillo Mayor' or 'Castañal' were classified as the most tolerant based on mean lengths of wood discolouration. No foliar symptoms were observed on artificially inoculated plants during the experiments.

Regarding disease management strategies, we assessed the effect of several biocontrol agents (BCAs) applied as root treatments at pre- and post-planting to control natural infections caused by black-foot and Petri diseases fungi, and their influence in plant growth parameters. Results showed that BCA effectiveness was dependent on the plant age and the plant part analyzed in reducing the incidence and severity of both diseases. Under specific scenarios, *Streptomyces* sp. E1 + R4 significantly reduced *Dactylonectria torresensis* and *D. macrodidyma* (black-foot) infections, while *Pythium oligandrum* P037 and *Trichoderma* spp. significantly reduced *Pa. chlamydospora* and *Phaeoacremonium minimum* (Petri disease) infections. BCAs treatments significantly reduced root weight with respect to the control while no effect was observed on the shoot weight.

Finally, we comparatively evaluated the efficacy of various fungicide and BCA formulations registered in Spain to act as pruning wound protectants against *Diplodia seriata* and *Pa. chlamydospora,* in the two vineyards of the D.O. Valdeorras (Galicia, Spain). Results showed that BCA-based treatments were less effective than fungicides. The commercial formulation containing pyraclostrobin + boscalid provided a high control of both GTD fungi while *Trichoderma*-based treatments showed a low efficacy. The poor performance and implantation of treatments based on *Trichoderma* spp. it is further discussed considering among other factors, the high spore load of GTD fungi used in the artificial inoculations, the short period elapsed between the BCAs treatments application and artificial inoculation with GTD fungi, the effect of unfavourable environmental conditions for the implantation of *Trichoderma* or the possibility of a low affinity of these fungi for the cultivar 'Godello'.

Resumen

Las enfermedades de la madera de la vid (EMV) se encuentran entre las principales amenazas bióticas para la sostenibilidad económica de la industria vitivinícola mundial, reduciendo el rendimiento, productividad y longevidad de los viñedos. Varios factores han sido atribuidos al aumento de su incidencia en los últimos años, como son los cambios en las prácticas culturales de los viñedos, la baja calidad sanitaria del material de propagación de vid, la prohibición de las materias activas químicas más efectivas y la deficiente protección de las heridas de poda. Además, estas enfermedades son muy complejas, implicando un amplio espectro de hongos no relacionados taxonómicamente, lo que hace que su control sea muy difícil. En esta tesis, se realizaron estudios para la puesta a punto de nuevas técnicas moleculares para la mejora en la detección y gestión de las EMV, así como, la evaluación de alternativas sostenibles al uso de fungicidas que permitan aumentar la vida útil de los viñedos.

En primer lugar, se evaluó comparativamente la precisión, la eficiencia y la especificidad de las técnicas de PCR digital (ddPCR) y PCR en tiempo real (qPCR) para la detección y cuantificación de *Ilyonectria liriodendri* a partir de muestras de suelo, rizosfera y endorizosfera de viñedos jóvenes y aparentemente sanos de La Rioja. Los resultados revelaron una fuerte correlación entre ambas técnicas, siendo la ddPCR más sensible a concentraciones muy bajas del patógeno. Se observó que las raíces de vides asintomáticas son un nicho microbiano que está habitado por patógenos asociados a la enfermedad del pie negro.

A continuación, se caracterizó la dinámica espacial de las comunidades fúngicas de estos tres compartimentos, mediante la técnica de secuenciación masiva de amplicones (HTAS), observándose que las comunidades de hongos fueron homogéneas entre viñedos. Teniendo en cuenta que los estudios se realizaron en viñedos con variables climáticas y edáficas similares, estos resultados indican que el microbioma del viñedo de La Rioja se conserva parcialmente en la región. Sin embargo, el compartimento suelo-planta constituyó la principal fuente de diversidad y composición de las comunidades fúngicas, con una disminución de la diversidad en la endorizosfera, sugiriendo que los tejidos de la raíz suponen una barrera para la colonización fúngica. El análisis predictivo de funcionalidad mostró un aumento en la abundancia relativa de posibles patógenos de plantas, endófitos y micorrizas arbusculares, y una disminución en la abundancia relativa de posibles saprótrofos de la madera y/o estiércol, del suelo hacia la endorizosfera. Se

corrobora así mismo que los tejidos internos de la raíz de las vides asintomáticas son un nicho microbiano que está habitado por hongos asociados a las EMV.

La HTAS también se empleó para determinar el efecto de la época de poda en la diversidad y composición del microbioma fúngico que coloniza las heridas de poda de forma natural durante dos períodos de infección (noviembre-febrero y febrero-mayo), en viñedos pertenecientes a tres Denominaciones de Origen (D.O.) de Galicia (España). Las comunidades fúngicas que colonizan las heridas de poda de la vid se vieron afectadas en su composición y diversidad por la D.O., mientras que la variación espacial dentro de cada región fue baja. Se constató la existencia de un microbioma fúngico estable en las heridas de poda, independientemente del período de infección, con abundancias más altas para la mayoría de los géneros de hongos asociados a EMV después de la poda de febrero (invierno) que en la de noviembre (mediados de otoño), lo que sugiere un efecto estacional en las infecciones de las heridas de poda. Además, se observó una correlación positiva entre la lluvia acumulada y la abundancia del microbioma fúngico total, y del género *Diaporthe* en particular, durante la octava y la undécima semana después de la poda.

Una de las alternativas al uso de fungicidas en el control de las EMV es la utilización de cultivares menos sensibles a estos patógenos. En este sentido se evaluó, en un ensayo bajo condiciones de invernadero, la tolerancia de cultivares de vid comerciales y minoritarios, presentes en colecciones de germoplasma españolas, ante la infección de *Phaeomoniella chlamydospora*, el principal agente causal de la enfermedad de Petri y la yesca. No se observaron síntomas foliares en las plantas, si bien todos los cultivares desarrollaron lesiones internas en la madera después de la inoculación artificial con el hongo, lo que indica que no hay evidencia de que exista resistencia cualitativa a *Pa. chlamydospora*. Sin embargo, la severidad de los síntomas internos de la madera varió considerablemente entre los cultivares, lo que permitió clasificarlos en base a diversos grados de tolerancia parcial a la infección fúngica. Los cultivares 'Estaldiña', 'Albillo Mayor' y 'Castañal' se clasificaron como los más tolerantes en función de las longitudes medias de necrosis de la madera.

En relación con las estrategias de manejo de estas enfermedades, se evaluó el efecto de varios agentes de control biológico (ACBs), aplicados como tratamientos radiculares en pre- y post-plantación, para controlar infecciones naturales causadas por hongos asociados con las enfermedades del pie negro y de Petri, así como, su influencia en los parámetros de crecimiento de la planta. La efectividad del ACB para reducir la incidencia y la severidad de ambas enfermedades varió con la edad y la parte de la planta analizada.

Bajo escenarios específicos, *Streptomyces* sp. E1 + R4 redujo significativamente las infecciones causadas por *Dactylonectria torresensis* y *D. macrodidyma* (pie negro), mientras que *Pythium oligandrum* P037 y *Trichoderma* spp. redujeron significativamente las infecciones causadas por *Pa. chlamydos*pora y *Phaeoacremonium minimum* (enfermedad de Petri). Los tratamientos con ACB redujeron significativamente el peso de la raíz con respecto al control, mientras que no se observó ningún efecto sobre el peso del brote.

Finalmente, se evaluó la eficacia de diversas formulaciones fungicidas y de ACB, registradas en España, como protectores de heridas de poda frente a infecciones artificiales de *Diplodia seriata* y *Pa. chlamydospora,* en dos viñedos de la D.O. Valdeorras (Galicia, España). Los resultados mostraron, en estas condiciones de estudio, que los tratamientos basados en ACBs fueron menos efectivos que los fungicidas. La formulación comercial basada en piraclostrobin + boscalida proporcionó un alto control de ambos hongos asociados a las EMV, mientras que los tratamientos basados en *Trichoderma* mostraron baja eficacia. El bajo rendimiento e implantación de los tratamientos basados en *Trichoderma* spp. es discutido más a fondo, valorando entre otros factores, la elevada carga de esporas utilizada en las inoculaciones artificiales de los hongos asociados con las EMV, el corto periodo de tiempo transcurrido entre la aplicación del ACB y la inoculación artificial de los mismos, el efecto de las condiciones ambientales adversas para la implantación de *Trichoderma* o la posibilidad de una baja afinidad de estos hongos por el cultivar 'Godello'.

Resumo

As enfermidades da madeira da vide (EMV) atópanse entre as principais ameazas bióticas para a sustentabilidade económica da industria vitivinícola mundial, reducindo o rendemento, produtividade e lonxevidade dos viñedos. Varios factores foron atribuídos ao aumento da súa incidencia nos últimos anos, como son os cambios nas prácticas culturais dos viñedos, a baixa calidade sanitaria do material de propagación de vide, a prohibición das materias activas químicas máis efectivas e a deficiente protección das feridas de poda. Ademais, estas enfermidades son moi complexas, implicando un amplo espectro de fungos non relacionados taxonómicamente, o que fai que o seu control sexa moi difícil. Nesta tese, realizáronse estudos para a posta a punto de novas técnicas moleculares para a mellora na detección e xestión das EMV, así como, a avaliación de alternativas sostibles ao emprego de funxicidas que permitan aumentar a vida útil dos viñedos.

En primeiro lugar, avaliouse comparativamente a precisión, a eficiencia e a especificidade das técnicas de PCR dixital (ddPCR) e PCR en tempo real (qPCR) para a detección e cuantificación de *Ilyonectria liriodendri* en mostras de solo, rizosfera e endorizosfera de viñedos novos e aparentemente sans de La Rioja. Os resultados revelaron unha forte correlación entre ambas as técnicas, sendo a ddPCR máis sensible a concentracións moi baixas do patóxeno. Observouse que as raíces de vides asintomáticas son un nicho microbiano que está habitado por patóxenos asociados á enfermidade do pé negro.

A continuación, caracterizouse a dinámica espacial das comunidades fúnxicas destes tres compartimentos mediante a técnica de secuenciación masiva de amplicóns (HTAS), observándose que as comunidades de fungos foron homoxéneas entre viñedos. Tendo en conta que os estudos se realizaron en viñedos con variables climáticas e edáficas similares, estes resultados indican que o microbioma do viñedo de La Rioja consérvase parcialmente na rexión. Porén, o compartimento solo-planta constituíu a principal fonte de diversidade e composición das comunidades fúnxicas, cunha diminución da diversidade na endorizosfera, suxerindo que os tecidos da raíz supoñen unha barreira para a colonización fúnxica. A análise preditiva de funcionalidade mostrou un aumento na abundancia relativa de posibles patóxenos de plantas, endófitos e micorrizas arbusculares, e unha diminución na abundancia relativa de posibles saprótrofos da madeira e/ou do esterco, do solo cara a

endorizosfera. Corrobórase así mesmo que os tecidos internos da raíz das vides asintomáticas son un nicho microbiano que está habitado por fungos asociados ás EMV.

A HTAS empregouse tamén para determinar o efecto da época de poda na diversidade e composición do microbioma fúnxico que coloniza as feridas de poda de forma natural durante dous períodos de infección (novembro-febreiro e febreiro-maio), en viñedos pertencentes a tres Denominacións de Orixe (D.O.) de Galicia (España). As comunidades fúnxicas que colonizan as feridas de poda da vide víronse afectadas na súa composición e diversidade pola D.O., mentres que a variación espacial dentro de cada rexión foi baixa. Constatouse a existencia dun microbioma fúnxico estable nas feridas de poda, independentemente do período de infección, con abundancias máis altas para a maioría dos xéneros de fungos asociados ás EMV despois da poda de febreiro (inverno) que na de novembro (mediados de outono), o que suxire un efecto estacional nas infeccións das feridas de poda. Ademais, observouse unha correlación positiva entre a chuvia acumulada e a abundancia do microbioma fúnxico total, e do xénero *Diaporthe* en particular, durante a oitava e a undécima semana despois da poda.

Unha das alternativas ao emprego de funxicidas no control das EMV é a utilización de cultivares menos sensibles a estes patóxenos. Neste sentido avaliouse, nun ensaio baixo condicións de invernadoiro, a tolerancia de cultivares de vide comerciais e minoritarios presentes en coleccións de xermoplasma españolas, ante a infección de *Phaeomoniella chlamydospora*, principal axente causal da enfermidade de Petri e da iesca. Non se observaron síntomas foliares nas plantas, se ben todos os cultivares desenvolveron lesións internas na madeira despois da inoculación artificial co fungo, o que indica que non hai evidencia de que exista resistencia cualitativa a *Pa. chlamydospora*. Porén, a severidade dos síntomas internos da madeira variou considerablemente entre os cultivares, o que permitiu clasificalos en base a diversos grados de tolerancia parcial á infección fúnxica. Os cultivares 'Estaldiña', 'Albillo Mayor' e 'Castañal' clasificáronse como os máis tolerantes en función das lonxitudes medias de necrose da madeira.

En relación coas estratexias de manexo destas enfermidades, avaliouse o efecto de varios axentes de control biolóxico (ACBs), aplicados como tratamentos radiculares en pre- e post-plantación para controlar infeccións naturais causadas por fungos asociados coas enfermidades do pé negro e de Petri, así como, a súa influencia nos parámetros de crecemento da planta. A efectividade do ACB para reducir a incidencia e a severidade de ambas enfermidades variou coa idade e a parte da planta analizada. Baixo escenarios específicos, *Streptomyces* sp. E1 + R4 reduciu significativamente as infeccións causadas por

Dactylonectria torresensis e *D. macrodidyma* (pé negro), mentres que *Pythium oligandrum* P037 e *Trichoderma* spp. reduciron significativamente as infeccións causadas por *Pa. chlamydos*pora e *Phaeoacremonium minimum* (enfermidade de Petri). Os tratamentos con ACB reduciron significativamente o peso da raíz con respecto ao control, mentres que non se observou ningún efecto sobre o peso do brote.

Finalmente, avaliouse a eficacia de diversas formulacións funxicidas e de ACB, rexistradas en España, como protectores das feridas de poda fronte a infeccións artificiais de *Diplodia seriata* e *Pa. chlamydospora*, en dous viñedos da D.O. Valdeorras (Galicia). Os resultados amosaron, nestas condicións de estudo, que os tratamentos baseados en ACBs foron menos efectivos que os funxicidas. A formulación comercial baseada en piraclostrobin + boscalida proporcionou un alto control de ambos os fungos asociados ás EMV, mentres que os tratamentos baseados en *Trichoderma* mostraron baixa eficacia. O baixo rendemento e implantación dos tratamentos baseados en *Trichoderma* spp. discútese máis a fondo, valorando entre outros factores, a elevada carga de esporas empregada nas inoculacións artificiais dos fungos asociados coas EMV, o corto período de tempo transcorrido entre a aplicación do ACB e a inoculación artificial dos mesmos, o efecto das condicións ambientais adversas para a implantación de *Trichoderma* ou a posibilidade dunha baixa afinidade destes fungos polo cultivar 'Godello'.

CHAPTER 1

General introduction

1.1. Introduction

Grapevine (*Vitis vinifera* L.) is known to host the broadest range of pathogens of any other woody agricultural crop (Martelli, 1997). Among them, grapevine is susceptible up to 29 fungal diseases (Wilcox et al., 2015) including grapevine trunk diseases (GTDs).

Fungal GTDs have been known since the late 19th century when esca foliar symptoms were officially described and named for the first time as 'folletage' and 'apoplexy' in France (Ravaz, 1898, 1909). Despite this fact, it is believed that esca may be as old as viticulture (Mugnai et al., 1999). Shortly after, the Italian scientist Lionel Petri accomplished Koch's postulates for the first time demonstrating that *Cephalosporium* and *Acremonium* spp. were responsible for the vascular necrosis detected in young grapevines (Petri, 1912). Similarly, in the early 1900s in North America the plant pathologist Donald Reddick demonstrated that *Fusicoccum viticolum*, now known as *Diaporthe ampelina* (syn. *Phomopsis viticola*), was linked with grapevine cankers and symptoms resembling what we know today as Phomopsis dieback (Úrbez-Torres et al., 2013), and Eutypa dieback, thus naming the syndrome dead-arm disease of grapevines (Reddick, 1914). Accordingly, the term dead-arm disease was commonly used for many years to describe similar symptoms including those shown for the first time to be caused by species in the Botryosphaeriaceae family (Chamberlain et al., 1964), now known as Botryopshaeria dieback (Úrbez-Torres, 2011). Eutypa dieback was first reported to occur in Australia on apricots and grapevines (Carter, 1957a, b) and later on grapevines in California (English et al., 1962; Moller et al., 1968) and Europe, among many others (Carter, 1991). Black-foot disease was first related to "*Cylindrocarpon*" species in Italy in 1975 (Grasso and Magnano Di San Lio, 1975) and its symptoms were firstly described in the early 1960s in France under the name of 'gangrene' (Maluta and Larignon, 1991).

Despite the fact that GTDs have been known for more than a century, the term GTD is comparatively fairly new being coined by Dr. Luigi Chiarappa together with other scientists from around the world in the late 1990s. This term combined some symptoms detected in both grapevines' foliage and vascular tissues, which were supposed to be caused by a group of fungi that mostly infect the vine via pruning wounds and subsequently colonize the vascular tissues (Mugnai, 2011). Nowadays, GTD fungi account for the largest group of pathogens known to infect grapevines (Gramaje et al., 2018; Lawrence et al., 2019; Berlanas et al., 2020) and are considered some of the most threatening and devasting diseases (Bertsch et al., 2013).

1.2. Importance and impact of Grapevine Trunk Diseases

The impact and significance of GTDs on plant health have not been recognized until recently, although they are long known. Decline symptoms in young and mature vineyards have dramatically increased worldwide over the years causing considerable economic losses and compromising the productivity and longevity of vineyards (Gramaje and Armengol, 2011; Gramaje et al., 2018). Currently, it is believed that this increase is due to several and complex aspects:

(i) the worldwide grapevine planting 'boom' in the 1990s, increased not only the movement of possibly infected propagation material (Gramaje and Armengol, 2011) but also the grapevine growing area around the world reaching a mature age where external GTD symptoms are expressed and thus becoming more visually prevalent.

(ii) changes in grapevine production systems with the increase of plant density in vineyards, more common use of double cordon and spur-pruned vines, thus presenting a significantly higher number of pruning wounds, and mechanization of vineyard practices, in particular pruning, have greatly favored and increased the chances of GTD pathogens infection on grapevines (Gramaje et al., 2018).

(iii) the banning of the most effective fungicides against GTDs in the early 21st century due to environmental and public health concerns (Decoin, 2001; Larignon et al., 2008; Spinosi et al., 2009; EPA, 1997), such as sodium arsenite, benzimidazoles and methyl bromide (Mondello et al., 2018).

Vines affected by GTDs reduce its productivity over time by death of the spurs, canes, and/or cordons. Yield losses between 30-50% and up to 94% have been reported in North America in severely Botryosphaeria dieback (Milholland, 1991) and Eutypa dieback (Johnson and Lunden, 1987) infected vineyards, respectively. In South Australia, when 47% of 'Shiraz' vines were affected by Eutypa dieback, yield losses were estimated to be of 1,500 kg/ha, leading to losses of AUD$2,800 per ha (Wicks and Davies, 1999). In California, Botryosphaeria and Eutypa dieback economic impact was estimated to be $USD260 million per year (Siebert, 2001). Studies conducted at the end of the 1990s in Italy, reported about 15% of the young vines in Sicily with symptoms of decline and high mortality after one year planted in the field (Sidoti et al., 2000). In southern Italy, many mature vineyards have reached up to 80% of esca incidence (Romanazzi et al., 2009). More recently, Yan et al. (2013) also reported a rising of GTD incidence and subsequent vine mortality

throughout Chinese vineyards. In the Canadian Province of British Columbia, 90% of the vineyards showed GTD symptoms, with some individual vineyards recording up to 54% incidence (Úrbez-Torres et al., 2014a, b). An annual loss of €1 billion has been estimated in France, with 12% of the vineyards being currently not economically viable, mainly due to Esca (Lorch, 2014). In Spain, GTD incidence increased from 1.8% in 2001 to 7% in 2006 in Castilla y León region (Martín and Cobos, 2007).

According to the afromentioned data, GTDs are currently considered one of the major threats to the sustainability of viticulture and wine industry, reducing yields and shortening life span of vineyards with the consequent significant economic losses that this entails (Bertsch et al., 2013; Kaplan et al., 2016). Until now, no curative measures are available to reduce the impact of GTDs once the vines are already infected making very difficult their management in the field. This is also influenced by the kind of disease and/or pathogens involved and by the fact that it is very common that one vine is affected by several GTDs at the same time (Gramaje et al., 2018). To date, up to 136 fungal species in 35 genera have been reported to cause six different GTDs over the world (Gramaje et al., 2018; Aigoun-Mouhous et al., 2019; Lawrence et al., 2019; Berlanas et al., 2020), and this number is continuously increasing due to the improvement of tools for their detection and identification. The loss of the most effective preventative chemical products increased even more the complexity of their control. Nowadays, information on control measures is very limited and sometimes varies among geographical regions, thus, control is being primarily focused on disease prevention and mitigation (Úrbez-Torres, 2011). Due to the banning of several effective chemical active ingredients, the only management strategy left for the grape growing industries to control GTDs was the remedial surgery of the vines (Creaser and Wicks, 2004; Sosnowski et al., 2011a), although this operation can be very expensive (Epstein et al., 2008). Therefore, the main priority during the last decade for researchers and wine industry have been looking for new active ingredients, as well as cultural practices that could successfully reduce GTD pathogens infection (Úrbez-Torres, 2011).

Adittionaly, the impact of GTD fungal pathogens transmitted in propagation material on the establishment and durability of vines is well-known (Gramaje and Armengol, 2011). Nurseries can be a source of infected planting material which results in cross-infection of entire batches of cuttings and the nursery young vines growing from them (Gramaje et al., 2018). An integrated pest management (IPM) strategy where several strategies are combined to reduce GTD fungal infections, such as the use of physical (e.g. hot-water

treatment), biological (e.g. antagonist microorganisms) and cultural practices (e.g. crop management, irrigation, soil preparation, etc.), throughout the nursery mother blocks and newly planted vineyards (Gramaje et al., 2018), has been suggested as the most effective approach to reduce infections caused by fungal trunk pathogens (Berstch et al., 2013).

1.3. Grapevine Trunk diseases: symptoms and fungi involved

Fungal GTD complex currently includes six main different pathologies affecting grapevine planting material in nurseries, and young and mature vineyards over the world, which are black-foot, Petri and esca diseases, and Eutypa, Botryosphaeria and Phomopsis diebacks (Bertsch et al., 2013; Gramaje et al., 2018; Mondello et al., 2018).

This pathosystem is very complex due to (i) the high number of related fungal species, (ii) GTDs affecting young and adult vines share common fungal pathogens, (iii) the individual vines can be affected by one or more fungi at the same time as a result of the numerous infection opportunities throughout the season and over the years, (iv) young vines infected by GTDs can show symptoms when they are mature, after a latency period, and (v) the different GTDs share similar external and internal symptomology that sometimes overlaps among them when several infections occur in a vine and/or resemble the associated with abiotic disorders, making difficult an accurate identification in the field (Gramaje et al., 2018; Mondello et al., 2018). Information about the current known GTDs is individually provided below.

1.3.1. Black-foot disease

Black-foot disease affects planting material and young vineyards (<5-years-old) contributing to the young vine decline (YVD) syndrome (Gramaje and Armengol, 2011). Field symptoms of black-foot disease include reduced vigour with small-sized trunks, shortened internodes, uneven wood maturity, sparse foliage, and small leaves with interveinal chlorosis and necrosis (Fig. 1.1.A) (Agustí-Brisach and Armengol, 2013). They usually resemble symptoms associated with abiotic disorders such as winter damage, spring frost, water stress and/or nutrient deficiency being frequently difficult to distinguish, and also some of them overlaps with Petri disease symptomatology (Gramaje et al., 2018). Characteristic internal symptoms of affected vines are black, sunken, necrotic lesions on roots with a reduction in root biomass and roots hairs (Rego et al., 2000; Hallen et al., 2006), being frequent the formation of a second crown of horizontally growing roots

close to the soil surface to compensate for the loss of functional roots, and a dark purplish or reddish-brown discolouration in the base of the trunk (Agustí-Brisach and Armengol, 2013). Bark removal reveals black discoloration, dark brown to black streaks and necrosis of wood vascular tissue that develops from the basal end of the rootstock (Fig. 1.1.B and 1.1.D) (Rego et al., 2000; Halleen et al., 2006). In many instances, black-foot disease can be found in association with the syndrome known as J-rooting (Fig. 1.1.C) (Gubler and Petit, 2013). This syndrome is the results of poor planting of the vines in which roots are oriented upward.

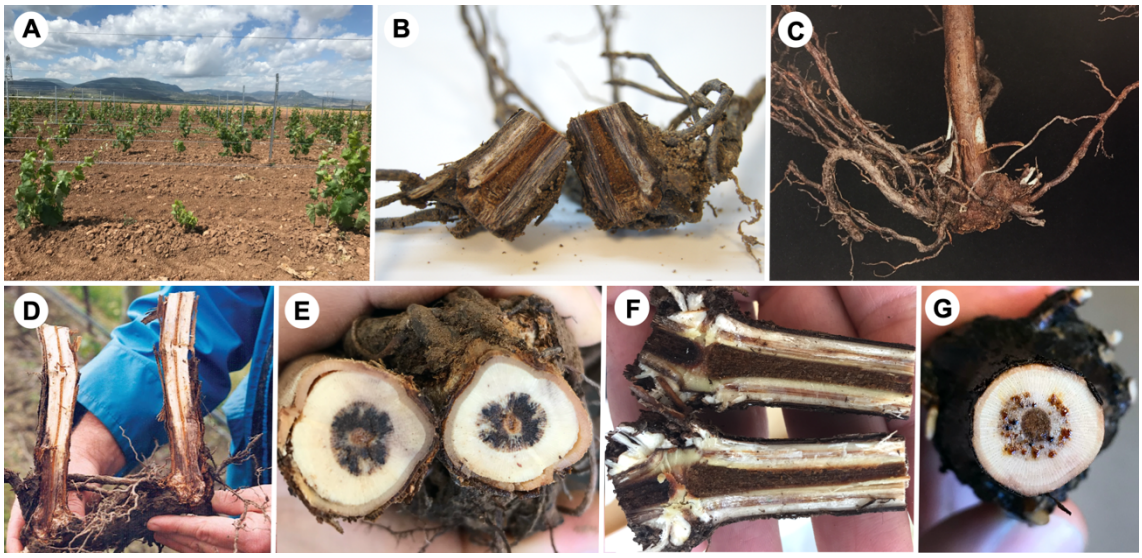

Figure 1.1. Petri disease and black-foot disease foliar and vascular symptoms. **A**, poor vigour vines affected by black-foot disease. **B** and **D**, Wood necrosis at the basal end of the rootstock in black-foot infected vines. **C**, "J-root" syndrome observed in declining young vines. Rootstock cross- **(E)** and longitudinal-section **(F)** showing dark xylem vessels and necrotic streaks infected by Petri disease fungi. **G**, Black goo caused by Petri disease pathogens seen in the cross-section of a 3-year-old rootstock.

To date, up to 32 species of the genera *Campylocarpon* Halleen, Schroers & Crous, *Cylindrocladiella* Boesew, *Dactylonectria* L. Lombard & Crous, *Ilyonectria* P. Chaverri & C. Salgado, *Neonectria* Wollenw., *Pleiocarpon* L. Lombard & D. Aiello and *Thelonectria* P. Chaverri & C. Salgado have been reported to cause black-foot disease (Gramaje et al., 2018; Aigoun-Mouhouse et al., 2019; Berlanas et al., 2019, 2020; Lawrence et al., 2019), *Dactylonectria torresensis* (A. Cabral, Rego & Crous) L. Lombard & Crous being the most prevalent species associated with diseased vines in Europe (Reis et al., 2013; Carlucci et al., 2017; Berlanas et al., 2017a).

1.3.2. Petri disease

Petri disease also contributes to YVD syndrome, affecting grapevine propagation material in nurseries and new plantations of up to 5-year-old (Gramaje and Armengol, 2011; Úrbez-Torres et al., 2015a; Gramaje et al., 2018). External symptoms include general stunting growth, delayed budbreak, retarded or absent sprouting, shortened internodes, chlorotic and sparse foliage with necrotic margins, leaves or entire shoots, wilting and dieback (Gramaje and Armengol, 2011). As mentioned in the previous section, they frequently resemble symptoms of abiotic disorders and are indistinguishable from those caused by black-foot disease. Internal symptoms of affected vines include the presence of dark-coloured phenolic compounds formed inside xylem vessels of the trunks in response to the fungus growing in and around them, which exude out when cut in cross-sections and dark streaks in longitudinal section (Fig. 1.1.E-G) (Rooney-Latham et al., 2005).

The main fungal species associated with Petri disease is *Phaeomoniella chlamydospora* (W. Gams, Crous, Wingf. & Mugnai) Crous & W. Gams (Mostert et al., 2006). However, other fungal species that have also been isolated in relatively high frequencies from Petri diseased vines are 29 species of the genus *Phaeoacremonium* W. Gams, Crous & M.J. Wingf., *Pleurostoma richardsiae* (Nannfeldt) Réblová & Jaklitsch, and 6 species of the genus *Cadophora* Lagerb. & Melin (Gramaje et al., 2018). Among those, *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous and *Cadophora luteo-olivacea* (J.F.H. Beyma) Harr. & McNew are the most prevalent worldwide (Mostert et al., 2006; Gramaje et al., 2011a).

1.3.3. Esca disease

Esca was the first reported GTD in France (Ravaz, 1909) and Italy (Mugnai et al., 1999) which predominantly affects mature grapevines with more than 8-year-old. It has been reported to occur in vineyards under two different forms which are chronic/mild, also known as grapevine leaf stripe disease, and acute/apoplectic, also known as esca disease (Gubler et al., 2015; Lecomte et al., 2012). The most characteristic foliar symptom of chronic form is the 'tiger-stripe' pattern (Fig. 1.2.A) (Surico, 2009; Gubler et al., 2015). Multiple banding discolorations are displaying on the leaves surrounding by dry, light or red-brown necrotic tissue on the leaf blade, often bordered by narrow red or yellow blotches, depending if the cultivar is red or white, respectively. Leaf symptoms of vines affected with the chronic forma can also be very variable such as drying, dropping, reddening and

yellowing (Lecomte et al., 2012). Small reddish and dark spots on the berry epidermis surface, known as 'black-measles', can also develop regardless of the cultivar colour (Surico, 2009). Apoplectic esca form is characterized externally by a sudden and unexpected wilting of the whole vine or one/several arms or shoots (Fig. 1.2.D). Leaf symptoms involve scorching, dropping and shriveling, and grape clusters can also dry on the vine (Mugnai et al., 1999). For both disease forms, foliar symptoms frequently appear in late spring or summer, and they can be very variable from year to year, being expressed erratically over the years. Internal wood symptoms usually reveal black spots in the xylem sometimes surrounded by pink to brown discolouration (Fig. 1.2.B), longitudinal brown to black vascular streaking, or silvery dry wood. A white to light yellow soft rot frequently developes in older vines wood (Fig. 1.2.C) (Fischer, 2002).

Esca disease etiology has been a matter of discussion among scientists over last years. A broad range of taxonomically unrelated fungal trunk pathogens and even endophytic bacteria have been isolated from wood tissues of esca affected vines (Hofstetter et al., 2012; Bruez et al., 2014, 2015, 2016), but it is still unknown how they interact with the primary fungi responsible for disease symptoms. The main hypothesis nowadays is that the pioneer fungi *Pa. chlamydospora* and/or species of *Phaeoacremonium*, *Pm. minimum* being the most virulent and prevalent, infect young vines. They could later develop disease symptoms after further colonization by several species of basidiomycetes belonging to genera *Inocutis* Fiasson & Niemelä, *Inonotus* P. Karst, *Fomitiporella* Murrill, *Fomitiporia* Murrill, *Phellinus* Quél, and *Stereum* Hill ex Pers. (Cloete et al., 2015; Guerin-Dubrana et al., 2019). Recent studies carried out by Brown et al. (2020) in California, suggested that basidiomycetes may not require a first infection step by *Pa. chlamydospora* in order to be able to colonize the wood, but further investigation is required. Several *Cadophora* spp. have also been lately related with esca affected vines (Travadon et al., 2015).

1.3.4. Eutypa dieback

Eutypa dieback mainly affects mature vineyards (>8-year-old). External symptoms include stunted shoots with chlorotic leaves, that are often cupped and with necrotic margins (Fig. 1.2.E and 1.2.F). Foliar symptoms are caused by toxic metabolites produced in the wood only by *Eutypa lata* (Pers.) Tul. & C. Tul. fungus (Moller and Kasimatis, 1981; Tey-Rulh et al., 1991; Molyneux et al., 2002; Mahoney et al., 2005; Trouillas and Gubler, 2010) and they can appear 3 to 8 years after infection occurred (Carter, 1978; Tey-Rulh et al., 1991) often varying from year to year (Sosnowski et al. 2007a). Clusters on stunted

shoots are small, ripen unevenly, and, in severe cases, berries shrivel and die (Gramaje et al., 2018). Internal wood symptoms include necrotic wedge-shaped staining in the cross-section of cordons and trunks, and cordon dieback with loss of spurs. As the dieback progresses, external cankers characterized by wood flatten areas with no bark appear, leading to eventual vine death. Perithecia of the fungus develop in the cankered wood and can be found embedded in the bark.

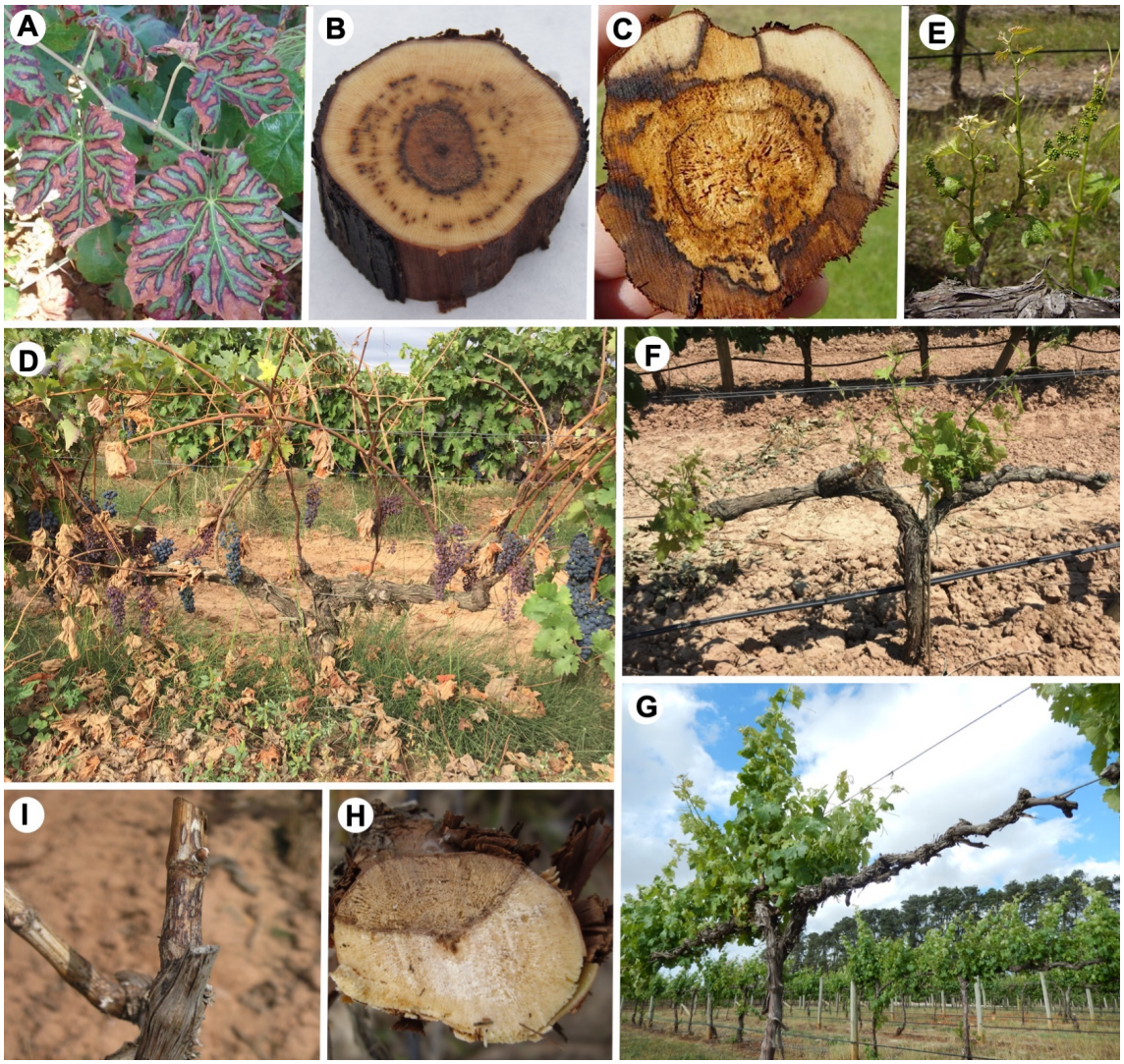

Figure 1.2. Symptoms of grapevine trunk diseases in mature plants. **A**, "Tiger-stripe" symptoms on leaves of a red cultivar characteristic of esca. Cross-section showing a central sectorial necrosis surrounded by black spots **(B)** or a central white rot surrounded by sectorial necrosis **(C)** of esca infected vines. **D**, Esca acute or apoplectic form is characterized by a sudden wilting of the entire plant. **E** and **F**, Foliar symptoms of Eutypa dieback include stunted shoots with chlorotic leaves often cupped and with necrotic margins. **G**, Cordon dieback along with lack of spring growth can be oversed in vines affected by Botryosphaeria dieback (courtesy J.R. Úrbez-Torres; source: Gramaje et al. 2018). **H**, Wedge-shaped canker in a Botryosphaeria dieback infected cordon similar to those observed in Eutypa and Phomopsis dieback affected vines. **I**, Lesions of Phomopsis cane and leaf spot on shoot (courtesy J.L. Ramos Sáez de Ojer; source: Guarnaccia et al., 2018).

To date, 24 species in the Diatrypaceae family belonging to the genera *Anthostoma* Nitschke, C*ryptosphaeria* Ces. & De Not., *Cryptovalsa* Ces. & De Not. Ex Fuckel, *Diatrype* Fr., *Diatrypella* (Ces. & De Not.) De Not., and *Eutypella* (Nitschke) Sacc. (Trouillas et al., 2010; Luque et al., 2012; Pitt et al., 2013a; Rolshausen et al., 2014) have been reported to cause internal wood symptoms of Eutypa dieback. The species *E. lata* is the most virulent and prevalent (Carter, 1991), and the only known to be responsible for the foliar symptoms (Trouillas and Gubler, 2010).

1.3.5. Botryosphaeria dieback

Botryosphaeria dieback is mainly observed in mature vineyards with over 8-year-old, although, cankers, dieback and plant death associated with this disease have been also found in planting material in grapevine nurseries (Gramaje and Armengol, 2011) and in 3- to 5-year-old vines (Úrbez-Torres et al., 2008). Typical external symptoms of this disease often present as lack of spring growth from affected spurs with shoot dieback (Fig. 1.2.G), bud mortality, leaf chlorosis and necrosis, inflorescences wilt, fruit rot and cane bleaching (Phillips, 2000; Larignon and Dubos, 2001; Larignon et al., 2001, 2009; Úrbez-Torres, 2011). In the field, Botryosphaeria dieback can be distinguished from Eutypa dieback by the lack of foliar symptomatology, i.e., chorotic leaves with necrotic margins (Leavitt, 1990, Úrbez-Torres et al., 2006a, 2008, 2015b). Symptom expression caused by Botryosphaeriaceae spp. can appear in the field only after 1 or 2 years after the infection occurred (Leavitt, 1990; Úrbez-Torres et al., 2006a) and it has been shown to differ from grape-growing areas and among grapevine cultivars (van Niekerk et al., 2004; Larignon et al., 2009). The main wood symptoms are characterized by wedge-shaped perennial cankers which are indistinguishable to those of Eutypa dieback (Fig. 1.2.H), and non-uniform to circular central staining of the wood observed in cross-sections of affected spurs, cordons and trunks vascular tissue that typically begins in pruning wounds (Úrbez-Torres et al., 2006a, 2008; Úrbez-Torres and Gubler, 2011; Gramaje et al., 2018). Botryosphaeria dieback has been also associated with orange/brown stripes located in the outer xylem appearing in the longitudinal direction just beneath the bark and extending from the base of the infected shoots to the graft unions, and even to the rootstock and annual stems (Larignon et al., 2001; Abou-Mansour et al., 2015).

To date, 26 botryosphaeriaceaous taxa in the genera *Botryosphaeria* Ces. & De Not., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Neofusicoccum* Crous, Slippers & A.J.L. Phillips, *Neoscytalidium* Crous & Slippers, *Phaeobotryosphaeria* Speg., and

Spencermartinsia A.J.L. Phillips, A. Alves & Crous have been associated with this disease (Úrbez-Torres, 2011; Pitt et al., 2013b, 2013c, 2015; Rolshausen et al., 2013; Yang et al., 2017), *Neofusicoccum parvum* (Pennycook & Samules) Crous, Slippers & A.J.L. Phillips, *Diplodia seriata* De Not and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not being the most frequently isolated fungi (Úrbez-Torres, 2011). Several pathogenicity studies have demonstrated that species within the botryosphaeriaceous genera *Lasiodiplodia* and *Neofusicoccum* are among the fastest wood-colonizing fungi and hence the most virulent GTD pathogens (van Niekerk et al., 2004; Úrbez-Torres et al., 2008; Úrbez-Torres and Gubler, 2009a).

1.3.6. Phomopsis cane and leaf spot / Excoriosis / Phomopsis dieback

Diaporthe ampelina (syn. *Phomopsis viticola*) is historically known to infect grapevines causing Phomopsis cane and leaf spot disease in USA and excoriosis in Europe (Hewitt and Pearson, 1988). Although *D. ampelina* occurs wherever grapes are grown (Farr and Rossman, 2012), Phomopsis cane and leaf spot, and excoriosis, are more severe in grape-growing regions characterized by a humid temperate climate through the growing season (Úrbez-Torres et al., 2013). *Diaporthe ampelina* can infect all green parts of the grapevine, and thus disease symptoms can be observed on leaves as small pale green to yellow spots with necrotic centers. Canes show brown to black necrotic irregular-shaped lesions (Fig. 1.2.I), and clusters show rachis necrosis and brown, shriveled berries close to harvest (Hewitt and Pearson, 1988). Both diseases are well-studied grapevine diseases, and much has been written about its etiology (Phillips, 2000; Mostert et al., 2001; Schilder et al., 2005), epidemiology (Hewitt and Pearson, 1988; Pscheidt and Pearson, 1989; Erincik et al., 2001; Nita et al., 2006), and management (Cucuzza and Sall, 1982; Pscheidt and Pearson, 1989; Gubler and Leavitt, 1992; Ellis et al., 2004; Nita et al., 2006).

However, *D. ampelina* has not always been associated exclusively with Phomopsis cane and leaf spot and excoriosis symptoms. Field surveys conducted throughout USA revealed *D. ampelina* associated with grapevine perennial cankers (Úrbez-Torres et al., 2006a, 2009, 2012, 2013; Baumgartner et al., 2013). Úrbez-Torres et al. (2013) re-evaluated the role of *D. ampelina* as a grapevine canker-causing agent, giving the disease name Phomopsis dieback to these internal wood symptoms resembling those of Botryosphaeria dieback and Eutypa dieback. In vineyards severely affected by Phomopsis cane and leaf spot, symptoms of Phomopsis dieback were shown to be particularly high (Baumgartner et al., 2013; Úrbez-Torres et al., 2013). Several *Diaporthe* spp. have been associated with diseased vines,

namely *D. ambigua*, *D. ampelina*, *D. amygdali*, *D. australafricana*, *D. baccae*, *D. celeris*, *D. eres*, *D. foeniculina*, *D. helianthi*, *D. hispaniae*, *D. hongkongensis*, *D. hungariae, D. kyushuensis*, *D. novem, D. perjuncta*, *D. phaseolorum*, *D. rudis* and *D. sojae* (van Niekerk et al., 2005; Kaliterna et al., 2012; Baumgartner et al., 2013; Úrbez-Torres et al., 2013; Dissanayake et al., 2015; Cinelli et al., 2016; Guarnaccia et al., 2018; Pintos et al., 2018).

1.4. Epidemiology of Grapevine Trunk Diseases

Grapevine trunk pathogens have the ability to be disseminated in many different ways. Most of the fungal species related with GTDs are mainly spread through the dispersion of airborne spores, such as the associated with esca disease, and Eutypa, Botryosphaeria and Phomopsis diebacks. Fungal species responsible for Petri disease can be dispersed by spores and also, they may become soil-borne from diseased pruning debris either on or in the soil (Gramaje et al., 2018). Furthermore, grapevine pathogens responsible for Botryosphaeria and Phomopsis diebacks, Petri and black-foot diseases and esca can be propagated by using infected planting material in nurseries (Gramaje et al., 2018). The use of pruning shears showed to have a certain potential to spread GTD pathogens under controlled conditions (Agustí-Brisach et al., 2015). Nevertheless, these authors found that high inoculum concentrations of fungal trunk pathogens are required to produce successful infection rates. It has been also reported that many of GTD fungi are able to cause cankers and dieback symptoms in other woody perennial crops (Carter, 1991; Gramaje et al., 2016; Moyo et al., 2019). These hosts can act as a source of inoculum mostly when near vineyards but also, in the case of *E. lata*, when located more than 50 km from grapevine-production areas (Ramos et al., 1975; Petzoldt et al., 1983a).

Depending on the fungal trunk pathogen species, spores (conidia or ascospores) are released from fruiting bodies (pycnidia or perithecia) embedded in the bark and/or on the surface of dead grapevine wood (Pearson, 1980; Trese et al., 1980; Eskalen and Gubler, 2001; Rooney-Latham et al., 2005; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010) under favourable environmental conditions, which have been mostly associated with rain events and/or high relative humidity (RH) along with temperatures above freezing, which also favour spore germination (Úrbez-Torres et al., 2010a, b; van Niekerk et al., 2010). An epidemiological equation model for *Pa. chlamydospora* was recently developed by González-Domínguez et al. (2020) in Spanish vineyards, who concluded that the dispersal dynamics of this fungus were best explained by hydro-thermal time which takes into

account the effect of both temperature and rain. Spores are disseminated from fruiting bodies by rain, wind or arthropods until they land on susceptible pruning wounds to germinate and start colonizing new xylem vessels and pith parenchyma cells (Mostert et al., 2006; Moyo et al., 2014). It has been shown that depending on the fungal trunk pathogen, geographical location and weather conditions spore release and therefore, high-risk infection periods may vary throughout the growing season and from year to year but mainly overlap with dormant seasons in both the Northern and Southern Hemispheres (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Amposah et al., 2009; Kuntzmann et al., 2009; Quaglia et al., 2009; Trouillas, 2009; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010; Cloete, 2015; Valencia et al., 2015; González-Domínguez et al., 2020).

Susceptibility of grapevine pruning wounds to GTD fungi primarily depends on the pruning month and the time elapsed between pruning and possible infection events. Studies using artificial spore inoculations suggest that pruning wound susceptibility is high when infections occur at pruning time but decreases as the interval between pruning and infection increases over the following weeks and months, with seasonal variation reported between regions, mainly due to climatic variances (Eskalen et al., 2007; Serra et al., 2008; Úrbez-Torres and Gubler, 2011; van Niekerk et al., 2011a; Ayres et al., 2016; Elena and Luque, 2016a). Eskalen et al. (2007) reported that pruning wounds can remain susceptible for up to 4 months. There are contradictory findings on the wood age effect on wound susceptibilty to *E. lata*. Moller and Kasimatis (1980) found a significantly less infection on wounds of 1-year-old compared with that of 2- to 4-year-old wood (*V. vinifera* 'Grenache'), whereas Trese et al. (1982) reported no difference in *E. lata* infection between 1- to 2- and 3-year-old wood (*V. labrusca* 'Concord'). More recently, Úrbez-Torres and Gubler (2011) showed that pruning wounds were equally susceptible to infection caused by *Lasiodiplodia theobromae* and *N. parvum*, in both 1- and 2-year-old wood in California. Susceptibility to GTD pathogens has been also linked with xylem vessel diameter and lignin content of grapevine (Rolshausen et al., 2008; Pouzoulet et al., 2014; Hamblin, 2015). Rainfall exposure has been linked with susceptibility to *E. lata* infection in apricot trees based on pruning wound microorganism activity (Carter and Moller, 1970; Price, 1973).

Black-foot disease pathogens are soil-borne (Agustí-Brisach et al., 2013a). They are known to persist as mycelium and conidia in rotten root fragments or as resting spores (chlamydospores) that can survive in the soil for extended periods of time after infected plants are removed (Petit et al., 2011; Agustí-Brisach and Armengol, 2013). These fungal species are commonly found in nursery fields and soils and thus, inoculum may already

exist in soils before planting (Agustí-Brisach et al., 2011, 2013a; Agustí-Brisach and Armengol, 2013; Berlanas et al., 2017a). Apparently healthy plants placed in infested nursery soil can become infected through small wounds made when roots break off during the planting process. The incomplete callusing of the lower trunk or wounds made in the grapevine propagation process, such as disbudding wounds, can also be a part of entry of GTD fungi (Halleen et al., 2006).

Some studies have shown evidence to support an endophytic phase of GTD fungal pathogens such as *Pa. chlamydospora*, *Pm. minimum* and several Botryosphaeriaceae spp. in grapevines (González and Tello, 2011), as they have been isolated from asymptomatic rootstock mother plants (Halleen et al., 2003, 2007; Edwards and Pascoe, 2004; Fourie and Halleen, 2004a; Aroca et al., 2010), graftlings (Berlanas et al., 2020) and mature plants (Hofstetter et al., 2012). It has been hypothesized that these fungi may become pathogenic to the grapevine following different biotic and/or abiotic stress factors and thus, they have been considered to play a role as latent pathogens in vines (Ferreira et al., 1999). Further investigation is required within the GTD complex to determine what triggers latent pathogens to transition from an endophyte to a pathogen, and to cause disease symptoms.

1.5. Tools for detection, identification and quantification of fungal trunk pathogens

Detection and identification of GTD fungal pathogens in grapevine have been traditionally performed by morphological approaches. This involves to culture small pieces of grapevine tissues onto nutrient-rich agar plates and incubate them during days or weeks until the existing fungi grow and can be phenotypically identified under the microscope. Culture-based methods are time-consuming, slow and also requires expert knowledge due to:

(i) not all fungi are able to grow out of the grapevine sample onto the culture media, so these will be never detected leading to false-negative results.

(ii) numerous fungal species and growing at different rates may be present in a sample, so faster wood-colonizing fungi could outgrow the target GTD pathogens.

(iii) once isolated, accurate identification of species can be complicated by the plasticity and paucity of morphological characters (Mostert et al., 2006; Cabral et al., 2012; Phillips et al., 2013). Within a given species, isolates can have variable phenotypes, such as colony

colours and textures, and the range of these phenotypic characters sometimes overlap between species (Cabral et al., 2012; Gramaje et al., 2015).

In recent years, molecular techniques have been a highly demanded and useful approach for the detection of fungal trunk pathogens being much less time-consuming, more sensitive and a faster alternative to traditional culture-based techniques. Detection and identification of main fungal pathogens associated with GTDs have been made from different kind of samples, such as grapevine wood, roots, water, soil and spore traps from nurseries and vineyards using different molecular-based techniques that we review below.

1.5.1. Conventional PCR

Several approaches have been developed for identification of black-foot disease pathogens. A method for fungal DNA extraction from soil was developed by Damm and Fourie (2005) to study the epidemiology of GTD pathogens in South African grapevine nurseries and vineyards. Then, the extracted DNA was tested to detect "*Cylindrocarpon"* spp. using specific primers (Hamelin et al., 1996) and these species were detected in 66% of the samples analysed (Damm and Fourie, 2005). A pair of genus-specific primers to detect "*Cylindrocarpon"* from infected grapevines in Californian nurseries was designed by Dubrovsky and Fabritius (2007). Mostert et al. (2010) developed species-specific primers from the β-tubulin nuclear gene area to identify "*C". liriodendri*, "*C". macrodidymum*, *Campyl. fasciculare*, and *Campyl. pseudofasciculare* from soil and grapevine root material.

Regarding fungal species associated with Petri disease and esca, the sequence of the ITS region was used for developing species-specific PCR primers to ease accurate identification of *Pa. chlamydospora* (Groenewald et al., 2000; Tegli et al., 2000). Moreover, both methods were not suitable for the detection of DNA from lignified wood because of the presence of PCR inhibitors. An extraction procedure and species-specific PCR assay using Pch1/Pch2 primers (Tegli et al., 2000) were therefore designed by Ridgway et al. (2002) to find *Pa. chlamydospora* in grapevine wood and was able to get less than 1 pg of fungal genomic DNA. Similarly, Retief et al. (2005) also developed a conventional PCR method for *Pa. chlamydospora* indentification in grapevine wood, being able to reach a similar limit of detection (up to 1 pg of fungal DNA). Tegli et al. (2000) also developed species-specific primers from the ITS1 and ITS2 regions of the rRNA gene to detect *Pm. minimum* (syn. *Pm. aleophilum*). Mostert et al. (2005, 2006) developed a fast identification method for 22 *Phaeoacremonium* spp. using 23 species-specific primers targeting the β-tubulin and the

actin genes and provided a polyphasic identification tool containing morphological and cultural characters as well as β-tubulin sequences. Species-specific primers based on the ITS region were developed for the detection of *C. luteo-olivacea* from pome fruits and kiwi (Spadaro et al., 2011) and from artificially (Navarrete et al., 2011) or naturally (Vicente et al., 2020) inoculated grapevine grafted plants. However, Maldonado-González et al. (2020) recently reported the ineffectiveness of the ITS region alone in resolving *Cadophora* species, and developed species-specific primers based on the β-tubulin to detected *C. luteo-olivacea* from grapevine plants and vineyard soil.

1.5.2. Nested PCR

For the detection of "*Cylindrocarpon*" spp. directly from infected grapevines, Nascimento et al. (2001) used a nested-PCR with the universal primer ITS4 and the fungus-specific primer ITS1F in a first-stage fungus specific amplification, and the primers described by Hamelin et al. (1996) in a second amplification stage, amplifying indistinctly "*Cylindrocarpon" destructans* (Zinssm.) Scholten and "*C". obtusisporum* (Cooke & Harkn.) Wollenw. Alaniz et al. (2009) designed three pairs of species-specific primers and developed a multiplex nested-PCR to detect "*C". liriodendri*, "*C". macrodidymum*, and "*C". *pauciseptatum* from artificially inoculated grapevine rootstock cuttings reaching a selective amplification of the target pathogens.

Concerning Petri disease and esca fungal pathogens, Whiteman et al. (2002, 2005) designed a sensitive nested-PCR using the primers developed by Tegli et al. (2000) to identify *Pa. chlamydospora* from artificially infested soil, detecting as little as 50 fg of genomic DNA. The development of a DNA extraction method from water and callusing media, along with other DNA extraction methods previously reported from soil (Damm and Fourie, 2005) and wood (Retief et al., 2005), led to the optimization of a one-tube nested-PCR technique to detect *Pa. chlamydospora* in all these grapevine nursery processes (Retief et al., 2006). A genus-specific primer pair based on the ITS region has been successfully used in a nested-PCR for the detection of any *Phaeoacremonium* spp. in plants (Aroca and Raposo, 2007). Diagnostic procedures based on nested-PCR for the identification of *Pa. chlamydospora* and *Phaeoacremonium* spp. at different grapevine nursery stages have been also commonly designed (Whiteman et al., 2004; Abbatecola et al., 2006; Borgo et al., 2009; Aroca et al., 2010).

Few nested-PCR studies have been reported for the detection and identification of Botryosphaeriaceae dieback fungi. Two ITS-rDNA-based nested-PCR assays were developed by Spagnolo et al. (2011), to detect *N. parvum* and closely associated species, and to identify 17 Botryosphaeriaceae spp. from naturally infected grapevine tissues. Ridgway et al. (2011) performed a nested-PCR protocol to assess the sensitivity of self-developed multi-species primers that were able to detect DNA of 6 Botryosphaeriaceae spp. commonly found in New Zealand vineyards.

1.5.3. Quantitative real-time PCR

Advances in DNA-based techniques over the years have offered new tools for accurately identification and relative quantification of fungal trunk pathogens DNA in grapevine tissue such as quantitative real-time PCR (qPCR). For the detection of black-foot disease fungi, Probst et al. (2010) developed a qPCR assay to identify "*C". liriodendri* and "*C". macrodidymum* from soil samples using species-specific primers from the β-tubulin nuclear gene being able to reach small quantities of the target fungal DNA. A qPCR assay using SYBR Green chemistry was developed by Tewoldemedhin et al. (2011) for simultaneous identification of four "*Cylindrocarpon"* spp. associated with apple tree roots in South Africa. These authors used a previously developed reverse primer (Dubrovsky and Fabitius, 2007) and designed a new forward one both based on the ITS region to detect 10 fg/µl for "*C". destructans* and "*C". pauciseptatum*, and 1 fg/µl for "*C". liriodendri* and "*C". macrodidymum*. In Spain, Agustí-Brisach et al. (2014) designed a qPCR assay using SYBR Green technology to quantify *Iyonectria* spp. in nursery soils, with concentrations ranging from 0.004 to 1,904.8 pg/µl.

Regarding Petri and esca diseases associated fungi, Overton et al. (2004, 2005) developed primer pairs for species- and genus-specific amplification of *Pa. chlamydospora* and *Phaeoacremonium* spp., respectively, using a qPCR assay with SYBR Green chemistry. Edwards et al. (2007a) compared several molecular analyses (single-PCR, nested-PCR, and qPCR with SYBR Green and TaqMan technology) to detect *Pa. chlamydospora* during grapevine nursery process, and results showed that nested-PCR and TaqMan qPCR were the most sensitive. Also, a comparison between a nested-PCR and qPCR using TaqMan technology for identification of *Phaeoacremonium* spp. in naturally infected grapevine cuttings was carried out by Aroca et al. (2008a) and concluded that qPCR assay was the most sensitive and reproducible. In Spain, Martín et al. (2012) designed a qPCR protocol using TaqMan technology with species-specific primers and probe set for *Pa.*

chlamydospora and *Pm. minimum*, based on the ITS2 region and β-tubulin gene, respectively, reaching a limit of detection around 100 fg for *Pa. chlamydospora* and 50 fg for *Pm. minimum*. Pouzoulet et al. (2013) developed a multiplex qPCR method with species-specific primers based on the β-tubulin gene to detect and quantify *Pa. chlamydospora* and *Pm. minimun* DNA in grapevine woody tissues and yielded a limit of detection of 250 fg of fungal DNA. More recently, González-Domínguez et al. (2020) developed a SYBR Green qPCR-based method the for the detection and quantification of *Pa. chlamydospora* airborne inoculum from spore traps being able to detect a minimum of 36 fg of this fungus genomic DNA.

Several studies have been also developed for the detection and quantification of Botryosphaeriaceae and Diatrypaceae species from grapevine samples. Luchi et al. (2009) effectively designed a TaqMan qPCR assay, developing taxon-specific primers for the detection of Botryosphaeriaceae spp. in grapevine propagation material. In California, Pouzoulet et al. (2017) designed a SYBR Green qPCR assay to identify and quantify *D. seriata*-complex and *E. lata* in naturally and artificially inoculated grapevine pruning wounds with a limit of detection of 250 fg of fungal DNA for both fungi. In Australia, Billones-Baaijens et al. (2018) developed multispecies primers and a TaqMan probe to anneal the β-tubulin gene region for accurate detection and quantification of 10 Botryosphaeriaceae spp. and reached up to 200 fg of genomic DNA. In France, Moisy et al. (2017) successfully designed a species-specific β-tubulin primer set of *E. lata* using a qPCR with SYBR Green chemistry to asses grapevine wood colonization by this fungus.

1.5.4. Droplet digital PCR

Nowadays, although qPCR is still one of the most powerful and sensitive gene analysis techniques and is still used for a broad range of applications, droplet digital PCR (ddPCR) has recently emerged as the next generation of PCR (Hindson et al., 2011). Droplet digital PCR is an endpoint and absolute quantitation approach of nucleic acids in a sample. A single PCR mixture is partitioned into approximately 20,000 water-in-oil droplets that support PCR amplification of single template molecules. The DNA binding dye included in the super-mix is used to distinguish positive droplets (amplification of target molecule) from negative droplets (no target molecule present) via automated droplet flow-cytometry. Then, the number of target molecules in the sample is calculated from numbers of positive and negative droplets using Poisson statistics (Hindson et al., 2011). Advantages over other quantitative technologies such as qPCR include: (i) the possibility for absolute

quantification without the need of standards construction, (ii) the insignificance of primer efficiencies, and (iii) the increased assay sensitivity due to dilution of PCR inhibitors in partitioning of reactions into thousands of nanodroplets (Hindson et al., 2011; Pinheiro et al., 2012; Cao et al., 2013; Rački et al., 2014; Pavšič et al., 2016; Hua et al., 2018).

Droplet digital PCR is the latest DNA quantification technology that can be broadly used in several scientific fields (Morisset et al., 2013; Kim et al., 2014; Yang et al., 2014, Cao et al., 2015; Cavé et al., 2016; Hussain et al., 2016; Palumbo et al., 2016; Porcelatto et al., 2016; Hua et al., 2018), including plant pathology (Dreo et al., 2014; Bahder et al., 2018, 2019; Voegel and Nelson, 2018). This thechnique also showed to increase the sensitivity over qPCR in several studies (Kim et al., 2014; Cavé et al., 2016; Porcellato et al., 2016; Bahder et al., 2016, 2018; Maldonado-González et al., 2020). Regarding GTD pathosystem, Holland et al. (2019) developed a ddPCR protocol and a specific primer pair and probe set targeting a portion of the β-tubulin region to quantify *Ilyonectria liriodendri* from grapevine roots. More recently, Maldonado-González et al. (2020) designed species-specific primers and a TaqMan probe based on the β-tubulin gene for the detection and quantification of *C. luteo-olivacea* from grapevine nursery stock and vineyard soil by qPCR and ddPCR, reaching a limit of detection of 25 fg/µl and 125 fg/µl, respectively.

1.5.5. Next generation sequencing

The new advances in next generation sequencing (NGS) technology have increased both the resolution and scope of fungal community analyses and have revealed a highly diverse and complex mycobiota of plant vascular systems (Studholme et al., 2011). NGS technology has transformed research in many fields of biology. Among its many applications, it provides and alternate approach for the detection of plant pathogens and it is currently being used for the discovery of novel bacterial, fungal and viral plant pathogens (Al Rwahnih et al., 2009; Barba et al., 2014; Rott et al., 2018).

In grapevine, NGS has been recently used to screen grapevine nuclear stock material in order to determine the health status in regard to different economically important and regulated viruses (Al Rwahnih et al., 2015; Padilla et al., 2019). However, there are few studies to expand the implementation of NGS technology for the detection of GTD pathogens. Eichmeier et al. (2018) used RNA-based high-throughput amplicon sequencing (HTAS) approach to investigate changes in the potentially active fungal communities associated with GTDs of hot-water treated planting material and concluded that HWT

reduced the infection caused by these fungi but was not completely effective in eliminating their growth. Berlanas et al. (2019) recently found lower abundances of *Cylindrocarpon*-like asexual morphs in 161-49 C rootstock among the five most common rootstocks grown in Spain by DNA HTAS.

1.5.6. Other molecular techniques

The use of other techniques has also been reported to detect and identify fungal trunk pathogens. Regarding black-foot disease fungi, Parkinson et al. (2019) recently developed three loop-mediated isothermal amplification (LAMP) diagnostic assays based on Histone H3 gene to fast identification of *Dactylonectria macrodidyma*, and species within the *Dactylonectria* genus in avocado roots. The authors found that the species-specific assays were sensitive and specific at DNA concentrations 0.01 ng/µl for *D. macrodidyma*, while the *Dactylonectria* genus-wide assay was sensitive to 0.1 ng/µl.

A DNAmacroarray based on reverse dot-blot hybridation containing 102 oligonucleotides complementary to portions of β-tubulin region was developed to detect 61 species of pathogens related with black-foot, Petri and esca diseases from planting material in Canadian nurseries (Úrbez-Torres et al., 2015a). Tegli et al. (2000) used restriction fragment length polymorphism (RFLP) patterns of the ITS region to distinguish *Pm. minimum* (syn. *Pm. aleophilum*), *Pm. inflatipes*, and *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf. Five *Phaeoacremonium* spp., namely *Pm. minimum* (syn. *Pm. aleophilum*), *Pm. inflatipes*, *Pm. parasiticum*, *Pm. rubrigenum*, and *Pm. viticola* J. Dupont, were differentiated using PCR-RFLP markers from the partial β-tubulin gene and the ITS regions (Dupont et al., 2002). Similarly, Aroca and Raposo (2007) developed a protocol based on enzymes digestion and RFLP patterns to specific detection and identification of several species of *Phaeoacremonium* from grapevine wood. Martos et al. (2011) adapted the co-operational PCR technique coupled with dot-blot hybridization for the detection of *Pa. chlamydospora* in infected grapevine wood designing a specific probe targeting the ITS2 region and getting a detection efficacy between 75 and 85%. Using the same technique, Martos et al. (2009) designed a sensitive tool for fast identification of Botryosphaeriaceae spp. affecting grapevines reaching a similar sensitive level to nested-PCR methods. In addition, using a single-stranded conformational polymorphism (SSCP) analysis, the single amplicon produced for all 6 species of Botryosphaeriaceae tested were resolved into 4 individual species and a duo of either *N. parvum* or *N. ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips (Ridgway et al., 2011).

1.6. Management of Grapevine Trunk Diseases

Research carried out over last years has let us reach a good understanding of the etiology, biology and epidemiology of GTD fungal pathogens, identifying high-risk infection and susceptibility periods throughout the year. This knowledge assisted in the development of effective management strategies in grapevine nurseries and newly established and mature vineyards (Gramaje et al., 2018).

Nowadays, it is well-accepted that a successful GTD management requires a holistic approach from the nursery to the vineyard. An IPM programme where several control strategies are combined has been suggested as the most successful approach to minimize GTD infections (Fourie and Halleen, 2006; Halleen et al., 2010; Berstch et al., 2013; Billones-Baaijens et al., 2015; Gramaje et al., 2018). These strategies are discussed below.

1.6.1. Cultural practices

1.6.1.1. Nursery processes management

A correct management throughout the different steps of propagation process in the nursery can have a great impact on GTD pathogens incidence, and hence in the quality of grapevine grafted material. Potential black-foot disease pathogen infection can be eliminated by trellising rootstock mother vines, but it is labour intensive and more expensive practice (Hunter et al., 2004). High relative humidity and temperature can increase the susceptibility of ground-sprawling shoots to be infected by soil-borne pathogens than vertical-positioned shoots (Whiteman et al., 2007). Overwatering benefits most soil-borne fungal pathogens and also reduces aeration in the root system, therefore maintaining an adequate soil moisture and aeration is very important (Toussoun et al., 1970). Recently, it has been showed that overhead sprinkler irrigation can elicit Botryosphaeriaceae conidia and ascospores release of the sexual morph of *Pm. minimum* (Úrbez-Torres et al., 2010a; Gubler et al., 2013), and Diatrypaceae spp. spores release (Úrbez-Torres et al., 2019) in Californian vineyards.

The hydration stage can promote GTD pathogens infections, so long periods of soaking cuttings in water could threaten the phytosanitary status of grapevine planting material (Pollastro et al., 2009; Aroca et al., 2010; Gramaje et al., 2011a; Agustí-Brisach et al., 2013b). Moreover, the large number of cuts and wounds resulted from disbudding, grafting, improperly matched or healed graft unions, or the rooting process make the propagation

material very susceptible to infection by fungal trunk pathogens (Gramaje and Armengol, 2011). Pruning shears, grafting machines and hydration tanks have been found to be source of viable propagules of black-foot and Petri diseases pathogens (Retief et al., 2006; Aroca et al., 2010; Gramaje et al., 2011a; Agustí-Brisach et al., 2013b, 2015; Cardoso et al., 2013; Waite et al., 2013), a frequent disinfection and cleaning is therefore recommended. High temperatures used in nurseries during callusing stage can create weakened callus unions increasing even more the susceptibility to trunk disease infection (Waite et al., 2015), and the humid, warm and dark environment of callusing rooms are also especially favourable for pathogens growth (Hartmann et al., 2001). Grapevine cuttings and young vines subjected to stressful conditions of increasing periods of cold storage before rooting and callusing exhibited an increased susceptibility to black-foot disease pathogens (Probst et al., 2012).

1.6.1.2. Crop rotation in nursery fields

This approach may have a restricted effect with black-foot pathogens because they produce long-lasting spores and can survive as saprophytes in the soil for long periods of time (Petit and Gubler, 2005). A high proportion of plants was infected with "*Cylindrocarpon*" spp. in a Portuguese nursery field where grapevines had been planted consecutively for two years, followed by three years of rotation with several crops such as potato, cabbage, carrot, garlic, leek and cereals (Rego et al., 2009). Planting grapevine cuttings every second year followed by a cover crop in a nursery field in South Africa may have also led to raised black-foot disease inoculum (Halleen et al., 2003). Moreover, black-foot disease pathogens were found in soils during the rotation cycle with other crops (wheat and barley) in Portugal (Cardoso et al., 2013) and Spain (Berlanas et al., 2017a). Crop rotation with *Brassica* spp. was suggested to reduce black-foot (Jaspers and Billones-Baaijens, 2014; Berlanas et al., 2018) and Petri disease pathogens (Jaspers and Billones-Baaijens, 2014) infections in nursery fields. Similarly, a recent study performed by Richards et al. (2020) showed that using a monoculture of white mustard (*Sinapis alba*) as a cover crop significantly reduced the abundance of *I. liriodendri* in grapevine roots under controlled conditions.

1.6.1.3. Newly established vineyards

Pre-planting care is very important in maintaining vines quality and fitness. The vineyard must be ready for planting with soil cultivation, irrigation system, amendments and weed control among others, already done (Agustí-Brisach et al., 2011; Waite et al., 2015), and vines should be immediately planted upon their arrival. It is also recommended to do an evaluation of GTD fungal inoculum density and distribution in soil before site preparation and grapevine planting. The methods and techniques currently available to assess the soil phytosanitary status are: (i) the use of a semi-selective culture medium for black-foot disease pathogens (Berlanas et al., 2017a), (ii) traditional fungal isolation from grapevine seedlings roots or vascular tissues used as bait plants (Agusti-Brisach et al., 2013a), and (iii) using DNA-based molecular methods (Probst et al., 2010; Tewoldemedhin et al., 2011; Agustí-Brisach et al., 2014; Úrbez-Torres et al., 2015a; Maldonado-González et al., 2020).

Poorly drained and heavy soils should be avoided as they can favour black-foot pathogens infections (Rego et al., 2000; Halleen et al., 2007). Cover graft unions with soil to prevent callus tissue dehydration is usually performed in South Africa (Fourie and Halleen, 2006), but this practice could increase the incidence of black-foot disease pathogens in this plant zone. Suitable root and aerial part growth should be permitted in the first years of the vine development instead of placing a heavy fruit load in order to avoid stress situations (Gramaje et al., 2018).

1.6.1.4. Sanitation practices in vineyards

One of the best practices to reduce the number of new GTD fungal infections is removing and/or destroying all diseased wood from the vineyard. Fruiting bodies containing the fungal spores are mainly developed in dead or infected tissues of spurs, cordons and trunks. It would be advisable that these sanitation practices are implemented across production areas because nearby vineyards or orchards can also be a potential source of GTD pathogens inoculum (Gramaje et al., 2018; Moyo et al., 2019).

Pruning debris left in or close to the vineyard has also been shown as a long-lasting GTD fungal inoculum source. Pycnidia containing viable conidia of *D. seriata* have been found on pruning debris after three and a half years in a Spanish vineyard (Elena and Luque, 2016b). Infected wood and pruning debris can be destroyed by burning, burying, mulching, composting or incorporating them directly to vineyard soil (Gramaje et al.,

2018). The practice of burning has several environmental disadvantages, therefore it is recommended to preferably use eco-friendly options such as composting or mulching. Composting pruning debris along with garden residues and sheep manure for six months can reduce efficiently the inoculum of *D. seriata*, *Pa. chlamydospora*, *Pm. minimum* and *E. lata* from grapevine woody tissues (Lecomte et al., 2006).

1.6.1.5. Remedial surgery in mature vines

Remedial surgery has long been implemented in Australian vineyards to control Eutypa dieback (Carter, 1994; Creaser and Wicks, 2004; Sosnowski et al., 2011a) and it was also effective to control Botryosphaeria dieback in Californian vineyards (Leavitt, 1990). Vines showing foliar symptons and dieback in spring/summer should be identified and flagged and then infected wood can be removed at any time of the year (Sosnowski, 2016). The success of remedial surgery depends mainly on cutting any discoloured cordon and/or trunk wood, including disposal of an additional 10-20 cm of apparently healthy tissue beyond any evident necrosis to guarantee the removal of all infected wood. The missing part of the vine is then replaced in the following spring using new shoots or watershoots arising from the cordon or the trunk, respectively, returning to full production within 2–3 years (Sosnowski et al., 2007b, 2016a, b). Studies conducted in Australia showed that making cuts in the trunk 20-30 cm above the ground improved the likelihood of eradicating GTD pathogens from the vine (Sosnowski et al., 2011a). More recent studies also revealed the importance of low trunk cuts to ensure all affected wood is removed to control Botryosphaeria dieback (Savocchia et al., 2014).

Remedial surgery is difficult to accomplish in vines in which infection has reached graft union or the ground level. The complete removal of infected wood is particularly difficult in esca diseased plants where internal necrosis is usually observed in both rootstock and scion wood (Calzarano et al., 2004). The retraining is hindered when cuts were made 30-40 cm above graft union with very limited watershoot production in the scion part (Savocchia et al., 2014). Additionally, grafted vines cannot be retrained if infection has developed beneath the graft union. When infection has reached ground level in trunks of own-rooted vines, layering can be used to self-rejuvenate vines (Ahrens, 2010), or healthy canes can be taken from an adjacent vine to replace a diseased or dead vine (Nicholas et al., 2001). Layering involves taking a lignified cane and burying it at least 20 cm beneath the soil surface remaining the tip of the cane just above the soil surface (Sosnowski, 2016).

On the other hand, remedial surgery is a labour-intensive practice and an expensive operation with costs of up to USD$4.20/plant or USD$1,960/ha (Epstein et al., 2008). Highly skilled workers are also required. Despite this fact, this method still remains cost-effective compared with the cost of pulling out and replanting an entire vineyard (Sosnowski and McCarthy, 2017).

1.6.1.6. Pruning

Reduction of new GTD infections in the vineyard can be effectively achieved by managing pruning time based on the knowledge obtained from epidemiological studies conducted worldwide. Fungal trunk pathogens spore release has generally been shown to be linked with rain events and moderate temperatures. Consequently, it is recommended to prune grapevines during dry weather avoiding periods when inoculum is high in the environment and also favouring wound healing at the same time.

A late pruning (mid-February to early March) has been recommended to reduce infections caused by *E. lata* in California (Petzoldt et al., 1981, 1983b; Munkvold and Marois, 1995) and France (Chapuis et al., 1998), where weather conditions favour faster healing of pruning wounds than earlier in the season and also when inoculum is lower on the environment. Similarly, recent studies conducted in California also showed late pruning as an effective cultural practice to reduce Botryosphaeria dieback pathogen infections (Úrbez-Torres et al., 2010a; Úrbez-Torres and Gubler, 2011).

To follow these recommendations, finishing all pruning operations in late winter before budbreak may be difficult in large vineyards in California, so the double-pruning technique was suggested (Weber et al., 2007). It involves a first pre-pruning by trimming canes generally 30 to 45 cm above the spurs in November or December (early winter) and a second pruning is made in late February or March (late winter), at which time vines can be rapidly and selectively pruned to their final desired bud count. The main idea is that if canes get infected, the pathogen will not have enough time to reach the final two-bud spur left following the final prune in late winter (Weber et al., 2007). Inoculum levels of both Botryosphaeria and Eutypa dieback in California are much lower in late winter reducing the risk of infection in the pruned vines. Nowadays, double pruning is a usual cultural practice for control of Botryosphaeria dieback, esca and Eutypa dieback used by grape growers in California (Weber et al., 2007; Herche, 2009; Úrbez-Torres and Gubler, 2009b). Nevertheless, this technique resulted to be the costliest preventative practice (USD$247

per ha/year) in a survey of pest control advisers in California (Hillis et al., 2016). Double pruning costs can be minimised by using mechanical pruning systems which are best implemented in specific designed trellis systems. For instance, a mechanical pre-pruning practice in mid-atumn is common in Spanish vineyards.

Epidemiological studies conducted more recently in Cataluña region (Northeast Spain) showed that an early pruning (mid-November) can minimize natural infection rates caused by fungal trunk pathogens since much higher levels of these fungi were detected in pruning wounds made in late winter (February) than in late fall (November) (Luque et al., 2014). Additionally, watershoots removal can lead to sporadic infection in spring (Lecomte and Bailey, 2011; Makatini et al., 2014), so it is recommended that shoot thinning be made as possible during dry weather.

1.6.1.7. Training system

Training system have been demonstratared to have an influence in GTD fungal pathogens incidence. Three main key points related with the vine formation labour have been highlighted by several authors (Lafon, 1921; Dal et al., 2008, 2013; Simonit, 2016): (i) the number, location and size of pruning wounds on each vine, (ii) the interruption of the main sap routes selected by the pruning system, and (iii) a pruning close to the main trunk or cordon favouring a quick development of dry necrotizing wood under the wound surface from the tissues exposed to the open air (Grosclaude, 1993). In a 10-year field experiment in France, Dumot et al. (2004) found that Eutypa dieback foliar symptoms were more widespread in spur-pruned and cordon trained vines. When the same field experiment reached 20-year-old, Dumot et al. (2012) stated much higher mortality in cane-pruned and 'Guyot' trained vines. Then, external symptoms are usually seen earlier on training systems which cause a great number of small pruning wounds in the vine (spur-pruned) instead of fewer large wounds located on the crown (cane-pruned). Similarly, in California, head training system showed a lower Eutypa dieback incidence than bilateral cordons (Gu et al., 2005). In a 10-year field survey carried out in France by Lecomte et al. (2018), vine training forms with longs arms (cordons) showed to decline less rapidly due to esca disease than forms with no or short arms. 'Guyot mixte Poussard' system was reported to display less esca damage than 'Guyot simple' trained vines. The first training system avoids large wounds and minimizes interruptions of sap flow from one year to the next while the second usually have two very short or no arms with very close pruning wounds and many proximal 'dead wood cones' interfering with the sap routes (Lecomte et

al., 2018). The assessment of the effect of two pruning systems on wood mycobiota of 'Mourvedre' and 'Syrah' cultivars, determined that minimal pruning system, with fewer pruning wounds per vine, was related to a lower incidence of esca and a lesser wood necrosis than the spur-pruning system (Travadon et al., 2016).

1.6.1.8. Environmental factors and water stress

The environmental conditions have been shown to influence the symptom expression and/or the progress of GTDs. Foliar symptoms expression has been related to environmental factors reporting a seasonal variation of GTD external symptoms incidence in France (Dumot et al., 2004), USA (Butterworth et al., 2005) and Australia (Sosnowski et al., 2007a). Sosnowski et al. (2007a) observed a correlation between winter rainfall and Eutypa dieback foliar symptoms expression, thus suggesting that the transport of fungal toxins to the grapevine leaves could be facilitated by the greater water availability. These authors also found a correlation between higher temperatures and a lower disease incidence in spring, concluding that the greater biomass and vine growing as a consequence of warmer conditions reduced the fungal toxic metabolites concentration and hence foliar symptom expression (Sosnowski et al., 2011b). Significantly greater foliar symptoms were reported in potted vines inoculated with *E. lata* and subjected to extreme temperature and soil moisture conditions (high and low), although fungal mycelial growth did not correlate to wood tissue staining of stems (Sosnowski et al., 2011b). Vineyard experiments in two climatically different regions of South Australia (Barossa Valley and Riverland) showed that pruning wounds of grapevines under water stress in a dry and warm environment may be more susceptible to infection by *E. lata* (Sosnowski et al., 2011b). In contrast, Sosnowski et al., (2016a) observed that the distance of *E. lata* colonization was reduced within canes of water stressed vines compared with well watered vines.

Botryosphaeria dieback development has also been linked with water stress conditions. Lesion length caused by *Neofusicoccum australe*, *N. parvum*, *L. theobromae* and *D. seriata* was greater in water-stressed potted grapevines than in non-stressed vines (van Niekerk et al., 2011b). Amponsah et al. (2014) found that the stress imposed with high and low soil moisture levels on potted grapevines makes them more vulnerable to *Neofusicoccum luteum* infection. More recently, Lawrence et al. (2016) demonstrated that *N. parvum* caused more severe lesions on water-stressed potted 'Cabernet Sauvignon' vines than on non-stressed control vines. Conversely, Sosnowski et al. (2016a) reported no increase in

cane colonization by *D. seriata* of grapevines subjected to water-stress compared with non-stressed in a field assay.

Esca and Petri disease symptoms are aggravated in water-stressed vines, as was demonstrated with *Pa. chlamydospora* by Ferreira et al. (1999) and Edwards et al. (2007b, c). Apoplectic symptoms of esca are associated with water stress conditions during mid-summer, while chronic esca symptoms are linked with cool and rainy summers (Surico et al., 2000). The latter researchers also found a higher incidence of esca symptoms in level areas where water is usually accumulated. Lately, increased wood symptoms in grapevine cuttings inoculated with *Pa. chlamydospora* under water stress were also reported by Fischer and Kassemeyer (2012).

1.6.2. Physical control in grapevine nurseries: hot-water treatment

The application of hot-water treatment (HWT) to rootstock cuttings prior to grafting (Edwards et al., 2004; Fourie and Hallen, 2004b; Halleen and Fourie, 2016) or to young grafted vines prior to dispatch (Fourie and Halleen, 2004b; Halleen et al., 2007; Halleen and Fourie, 2016), has been reported as a method to reduce GTD levels of dormant grapevine propagation material. To date, the standard protocol used worldwide is 50°C for 30 minutes (Crous et al., 2001; Fourie and Halleen, 2004b; Waite and May, 2005; Halleen et al., 2007; Gramaje et al., 2009a, 2010a; Halleen and Fourie, 2016). In Spain, HWT at 53°C for 30 minutes significantly reduced *in vitro* conidial germination and mycelial growth of GTD pathogens without detrimental effects to cuttings (Gramaje et al., 2008a, 2010a, 2014). Recent studies using RNA HTAS demonstrated that HWT pre-planting treatments of nursery grafted grapevines at 50°C or 53°C for 30 min were not able to sterilize internal woody tissues, being only able to reduce fungal trunk pathogens infection but no completely effective in eliminating their growth (Eichmeier et al., 2018).

Unacceptably high losses have been reported when long-duration treatment is applied to commercial batches of grapevine propagation material (Ophel et al., 1990; Bazzi et al., 1991; Wample, 1993). In Italy, Habib et al. (2009) found negative side effects on shoot development and growth of scion graftlings, rootstocks and grafted plants ('Negroamaro' grafted onto 140 Ruggeri and 1103 Paulsen) treated at 50°C for 45 min after one growing season. HWT has also been shown to cause mortality of cuttings in cooler-climate grapevine regions such as New Zealand, leading to give a recommendation of a less effective protocol against GTD pathogens of 48°C for 30 minutes (Bleach et al., 2013;

Billones-Baaijens et al., 2015). The climate where the plant material is grown has been suggested to be related to their tolerance to HWT (Waite and Morton, 2007), thus vines grown in warm climates might be superior to cuttings taken from vines grown in cool climates and better able to withstand HWT (Billones-Baaijens et al., 2015; Gramaje et al., 2018). Other negative aspects of HWT include delayed or no rooting, retarded callusing and shoot development, and no bud burst or bud death in cuttings and/or grafted vines (Caudwell et al., 1997; Laukart et al., 2001; Waite and May, 2005; Gramaje et al., 2009a; Billones-Baaijens et al., 2015). Failed or incomplete healing of graft unions and fermentation during cold storage has been also reported (Waite and Morton, 2007).

1.6.3. Chemical control

The evaluation of fungicides to control GTD pathogens has been intensively studied in recent years. The loss of the most effective preventative chemical products, such as sodium arsenite, benzimidazole fungicides, and methyl bromide at the beginning of the 21st century eliminated the most effective chemical products available against fungal trunk pathogens. Nowadays, the registered active ingredients to control these diseases are very limited and generally do not provide wide control spectrum of the numerous taxonomically unrelated fungi or long-term protection to grapevines for the entire wound susceptibility period, being also their application expensive and very difficult (Rolshausen et al., 2010). Despite this fact, a recent survey reported that using fungicides during the propagation process is a very common practice to control GTDs in European nurseries (Gramaje and Di Marco, 2015). In Spain, only one commercial chemical product containing pyraclostrobin 0.5% and boscalid 1% (Tessior®) is currently authorized and specifically designed to protect grapevine pruning wounds against GTDs (MAPA, 2020).

Regarding the application method of fungicides, there has been extensive evaluation of several strategies with diverse levels of efficacy. In nurseries, traditional chemical dips and sprays used for external pathogens are not able to suitably penetrate grapevine cuttings to control fungal pathogens inhabiting the vascular tissues (Waite and May, 2005). In vineyards, wound protection is the most effective strategy for preventing GTD infection especially if adopted early in the life of the vineyard (Kaplan et al., 2016; Sosnowski and McCarthy, 2017).

Several studies have been carried out to assess the efficacy of fungicides in different scenarios. The *in vitro* screening of fungicides against GTD pathogens has been recently

reviewed by Mondello et al. (2018) and Gramaje et al. (2018). In this section, we review the research on chemical control performed in nursery, greenhouse and vineyard.

1.6.3.1. Nursery processes and greenhouse assays

Several trials perfomed during the nursery propagation process under controlled conditions demonstrated the ability of certain fungicides to control black- foot and Petri disease pathogens, as well as Botryosphaeriaceae fungi. Benomyl, tebuconazole, carbendazim + flusilazole and cyprodinil + fludioxonil applied as pre-planting roots dippings significantly decreased "*C*". *destructans* incidence in a potted greenhouse assay carried out by Rego et al. (2006). Similarly, *Ilyonectria liriodendri* infections were controlled by cyprodinil + fludioxonil as also by foliar spray applications of chitosan (Nascimento et al., 2007). Captan, carbendazim, and didecyldimethylammonium chloride applied at rooting stage significantly reduced the severity in grapevine roots of "*C*". *liriodendri,* and prochloraz of "*C*". *macrodidymum*, in a potted assay carried out by Alaniz et al. (2011a).

Concerning Petri disease fungi, spraying chitosan on grapevine leaves showed to be effective in controlling *Pa. chlamydospora* infections under greenhouse conditions (Nascimento et al., 2007). Grapevine cuttings soakings with didecyldimethylammonium chloride during the hydration stage showed a good performance in the prevention of Petri disease pathogens infection (Gramaje et al., 2009b). Fosetyl-Al applications limited wood necrosis length in young vines inoculated with *Pa. chlamydospora* (Laukart et al., 2001), and *Pa. chlamydospora* and *Pm. minimum* (Di Marco et al., 2011). Thiophanate methyl and thiram applied at different stages during nursery process reduced the infections of *Pa. chlamydospora* and *Pm. minimum*, respectively (Kun and Kocsis, 2014). Benomyl and carbendazim drenching at several propagation process stages and also subjected to HTW after vines uprooting, significantly reduced the incidence of *Pa. chlamydospora*, *Pleurostoma* (syn. *Pleurostomophora*) *richardsiae*, *Phaeoacremonium* spp. and Botryosphaeriaceae spp. (Halleen and Fourie, 2016).

Natural infection by Botryosphaeriaceae spp. were fully reduced by soaking grapevine canes in carbendazim prior to rooting and planting (Billones-Baaijens et al., 2015). These authors also observed that tebuconazole along with a polyether-modified trisiloxane adjuvant and carbendazim completely eliminated *N. luteum* bark infections.

Moreover, different assays were performend under controlled conditions to evaluate the efficacy of fungicides to act as a pruning wound protectant. Benomyl, pyraclostrobin, tebuconazole and thiophanate methyl were the most effective reducing pruning wound infections caused by *Inocutis* sp. and *Pa. chlamydospora* (Díaz and Latorre, 2013).

Benomyl, tebuconazole and prochloraz manganese chloride wound spraying limited lesion length produced by Botryosphaeriaceae spp. (Bester et al., 2007). Spraying freshly made pruning wounds with carbendazim, mancozeb, iprodione and flusilazole were effective to reduce artificial infections of *N. luteum* in an outdoor potted grapevine experiment (Amponsah et al., 2012). Fluazinam also provided pruning wound protection for control *N. luteum* infections in a greenhouse detached cane assay (Sosnowski and Mundi, 2019).

Regarding Diatrypaceae fungi, the active ingredients tebuconazole, fluazinam and pyraclostrobim showed a great potential to control *E. lata* pruning wounds infections in a greenhouse detached cane assay performed in Australia (Ayres et al., 2011, 2017). Likewise, in a similar assay performed in New Zealand, fluazinam also provided pruning wound protection against *E. lata* (Sosnowski and Mundi, 2019).

1.6.3.2. Field assays

In nursery field assays, benomyl, captan, and didecyldimethylammonium chloride treatments prior cold storage, grafting and planting reduced the natural incidence of GTD fungal pathogens in grapevine basal ends (Fourie and Halleen, 2006). Furthermore, applications of benomyl prior to grafting (Fourie and Halleen, 2004b) and carbendazim during the hydration stage (Gramaje et al., 2009b) were the most effective to control natural infections of Petri disease fungi in nursery fields. Soakings of rootstock and scion cuttings before grafting with cyprodinil + fludioxonil significantly reduced the incidence and severity of "*Cylindrocarpon*" spp. and incidence of Botryosphaeriaceae fungi of naturally infected nursery fields (Rego et al., 2009). Also, flusilazole protected trimming wounds against *N. luteum* and *N. parvum* infection in a field nursery trial (Billones-Baaijens et al., 2015).

Several assays have been carried out in the vineyard to assess the efficacy of fungicides as wounds protectans against fungal trunk pathogens. Fungicides belonging to the benzimidazole group have been widely applied in several grape growing regions worldwide. Thiophanate methyl was efficient in protecting vines against esca disease

pathogens in California (Rolshausen et al., 2010), Chile (Díaz and Latorre, 2013) and South Africa (Mutawila et al., 2015), Botryosphaeriaceae spp. in California (Rolshausen et al., 2010), Chile (Díaz and Latorre, 2013) and New Zealand (Amposah et al., 2012), and *E. lata* in California (Rolshausen et al., 2010). Benomyl showed high effectiveness in protecting pruning wounds against Botryosphaeriaceae spp. and esca disease pathogens in Chile (Díaz and Latorre, 2013), and *E. lata* in South Africa (Halleen et al., 2010). Carbendazim was also effective as wound protectant against esca disease pathogens in South Africa (Mutawila et al., 2015), Botryosphaeriaceae spp. in Australia (Pitt et al., 2012) and New Zealand (Amponsah et al., 2012; Sosnowski and Mundy, 2019), and *E. lata* in Australia (Sosnowski et al., 2008, 2013) and New Zealand (Sosnowski and Mundy, 2019).

Other fungicides such as tebuconazole showed good results against Botryosphaeriaceae spp. and *E. lata* infections in Australia (Pitt et al., 2012) and New Zealand (Amponsah et al., 2012; Sosnowski and Mundi, 2019). Wound protection control of esca disease pathogens, *Diaporthe* spp. and *E. lata* in South Africa (Halleen et al., 2010) and Botryosphaeriaceae spp. in New Zealand (Amposah et al., 2012) was achieved by applying flusilazole. Pyraclostrobin was also effective reducing Botryosphaeriacea spp. (Rolshausen et al., 2010) and *E. lata* (Ayres et al., 2017) infections in California and Australia, respectively. Fluazinam also showed high effectiveness against Botryosphaeriaecea spp. in Australia (Pitt et al., 2012) and New Zealand (Amponsah et al., 2012; Sosnowski and Mundy, 2019), and *E. lata* in New Zealand (Sosnowski and Mundy, 2019). The application of boric acid showed promising results against *Pa. chlamydospora* (Rolshausen et al., 2010) and *E. lata* (Rolshausen and Gubler, 2005) infections in California. Esca leaf symptom expression and vine mortality was limited by the application of a copper oxychloride formulation in standing vines (Di Marco et al., 2011).

The efficacy of several pastes and paints in protecting pruning wounds were also assessed against Botryosphariaceae, esca and Diatrypaceae fungi. A mixture of boric acid and a commercial latex paint had a good performance protecting pruning wounds of *E. lata* colonization (Rolshausen and Gubler, 2005). A self-priming latex paint was shown to be an effective barrier the protection of surgical wounds and new pruning wounds against Botryosphaeria-like fungi (Epstein et al., 2008). A commercial tree wound paste formulated with cyproconazole and iodocarb protected pruning wounds from the infection of *E. lata* (Sosnowski et al., 2008), *D. seriata* and *D. mutila* (Pitt et al., 2012). Similarly, ATCS tree wound dressing (acrylic paint) applied to freshly cut pruning wounds were effective reducing infection caused by *D. seriata* and *D. mutila*. The mixture of a vinyl

acrylic paint with thiophanate methyl provided good control of *D. seriata*, *Inocutis* sp. and *Pa. chlamydospora* in protecting pruning wounds in a Chilean vineyard (Díaz and Latorre, 2013).

1.6.4. Biological control

Investigation of different antagonist microorganisms that can act as biocontrol agents (BCAs) to prevent or at least reduce the development of GTDs has been intensively studied over the last years (Mondello et al., 2018). Nowadays, BCAs are considered a research priority not only because they can be used in organic viticulture but also, they are a key point into an IPM strategy (Gramaje et al., 2018). The integration of chemical and biological control approaches could benefit of the specific characteristics of both methods to control GTD pathogens, such as the immediate protective effect of chemical active ingredients, and the wide-spectrum, lasting efficacy and positive effects on plants development of BCAs. In this sense, Mutawila et al. (2015) recently developed benzimidazole-resistant mutant *Trichoderma* strains by gamma irradiation which were effective in protecting pruning wounds against fungal trunk pathogen infections. These authors also found that the wild type *Trichoderma atroviride* P. Karst. isolates were naturally resistant to thiophanate methyl.

The efficacy of fungi, bacteria and oomycetes to control GTD fungi have been assessed under different scenarios, mainly considering the prevention of fungal trunk pathogens infection (i) during plant propagation process in nurseries, and ii) protecting pruning wounds in the field. Most of the biological control assays using fungi have been carried out with *Trichoderma* spp. (Table 1.1.), while *Bacillus subtilis* and *Pythium oligrandum* Drechsler were the most prevalent bacterium and oomycete tested against GTDs, respectively (Table 1.2.).

Nowadays, the biological commercial products that are authorized to control GTDs in Spain are mainly based on fungal species belonging to the *Trichoderma* genus: (i) *T. atroviride* I-1237 (Esquive®), (ii) *T. atroviride* SC1 (Vintec®), and (iii) *T. asperellum* Lieckf. & Nirenberg ICC012 + *T. gamsii* Samuels & Druzhin. ICVV080 (Blindar®) (MAPA, 2020). In general, many of these studies provided promising findings, but the results have not been consistent, observing differences in efficacy depending on the BCA nature, the target pathogen, application method, time of exposure to the BCA and even the grapevine cultivars and rootstocks subjected to study.

Table 1.1. Fungal biocontrol agents (BCA) evaluated against fungal trunk pathogens.

Species and strain tested	Disease[a]	Pathogens[b]	Procedure[c]	Material treated[d]	Application method	Environment[e]	Reference
***Rhizophagus irregularis* INVAM CA501**	BFD	***"C." m.* (3)[f]**	A	Roots	Rooting in inoculated soil	*In vitro*	Petit and Gubler, 2006
***T. atroviride* STE-U 6514, STE-U 6515, STE-U 7733, STE-U 7734,** *T. harzianum* T77, *T. harzianum* MT77	ED, BD, PD, esca	***E. l.*, *D. s.*, *N. p.*, *P. c.*** (4)				*In vitro*	Mutawila et al., 2015
Fusarium proliferatum Fus	BD	*N. p.* (2), *D. s.* (2)	A	(i) Rooted CC (ii) Green stem	Mycelial plug	*In vitro*, (i) G, (ii) V	Mondello et al., 2019
T. harzianum (Trichodermil SC1306®), *T. asperellum* (TrichoderMax EC®), *T. asperellum* (Quality WG®), *T. harzianum* + *T. atroviride* + *T. viride* (Trichodel®)	BFD	*D. m.* (4)	A	Substrate	14-day drenching	G	Santos et al., 2016
***Epicoccum layuense* E24**	PD, esca	***P. c.* (3), *P.m.* (3)**	A	Rooted CC	Mycelial plug	G	Del Frari et al., 2019
R. irregularis (AGTIV®)	BFD	*I. l.* (1)	A	RC roots	Dipping, pre-planting	G	Holland et al., 2019
***T. harzianum* T39, *T. longibrachiatum* 6**	PD, esca	***P. c.* (3)**	A	(i) Basal end GP (ii) PW	(i) 30-min dipping, post-callusing and pre-planting (ii) spraying	(i) N (ii) G	Di Marco et al., 2004
***T. atroviride* SC1 (Vintec®)**	PD, esca	***P. c.* (3), *P.m.* (3)**	A	(i) RC + CC (ii) Sawdust (iii) Basal end GP	(i) 30-h soaking, pre-storage hydration (ii) 13-days, pre-callusing stratification (iii) 4-day dipping, pre-planting	N	Pertot et al., 2016
T. harzianum (Trichoflow-T™), *Trichoderma* + *Gliocladium*	PD, esca	*P. c.* (2), *P. m.* (2)	N	RC	1-h drenching, pre-grafting	NF	Fourie and Halleen, 2004b
T. harzianum (Trichoflow-T™)	PD, esca, BFD, BD, PhD	*P. c.* (2) *P. m.* (1), "*C.*" spp. (1), *Ca.* spp. (1), Bot. spp. (1), *Ph.* spp. (1)	N	RC + CC	(i) 1-h soaking, pre-cold storage (ii) 10-min soaking, pre-grafting (iii) 5-s dipping, pre-planting	NF	Fourie and Halleen, 2006
T. harzianum (Trichoflow-T™)	BFD, PD, esca	BFP (2), *P. c.* (2), *P. m.* (2)	N	Basal ends GP	1-min dipping, pre-planting	NF	Halleen et al., 2007
T. harzianum (Trichopel-R™, Trichogrow™)	BFD, PD, esca	BFP (1), *P. c.* (1), *P. m.* (1)	N	(i) Furrows (ii) Roots	(i) Trichopel-R™, pre-planting (ii) Trichogrow™, monthly drenching, post-planting	NF	
***T. atroviride* SC1 (Vintec®)**	BFD, PD, BD	BFP (1), PDP (2), **BDP (3)**	N	(i) RC + CC (ii) Sawdust (iii) basal end GP	(i) 1-day soaking, pre-grafting (ii) during stratification (iii) 1-h soaking, pre-planting	NF	Berbegal et al., 2020

(*Continued on next page*)

Table 1.1. (*Continued from previous page*)

Species and strain tested	Disease[a]	Pathogens[b]	Procedure[c]	Material treated[d]	Application method	Environment[e]	Reference
***Cladosporium herbarum* 10bf-021, *Fusarium lateritium* F1A**, *T. viride* Tr1, *Aureobasidium pullulans* 10bf-069, 5bf-012, *Rhodotorula rubra* 1f-077, *Altenaria alternata* 5f-020, *Candida famata* 1bf-053	ED	***E. l.*** (4)	A	PW	Paint brush	V	Munkvold and Marois, 1993
***T. harzianum* AG1, *T. harzianum* (7 selected strains, Trichoseal®), *Fusarium lateritium*, *T. harzianum* (AG2, AG11, AGSS28) + *T. atroviride* (AG3, AG5, AG8) (Vinevax®)**	ED	***E. l.* (3)**	A	PW	Paint brush	V	John et al., 2005
C. herbarum	ED	*E. l.* (2)	A	PW	Spraying	V	Rolshausen and Gubler, 2005
***T. harzianum* T77**, *T. harzianum* (Bio-Tricho®), ***T. harzianum* (Trichoseal-Spray®)**	ED	***E. l.*** (4)	A	PW	Spraying	V	Halleen et al., 2010
***T. harzianum* T77 (Eco-77®)**	BD, PD, ED, esca, PhD	Bot. spp. (1), *P. c.* (1), *E. l.* (1), *Ph.* spp. (1), ***Pha. spp.* (3)**	N	PW	Spraying	V	
***T. harzianum* (AG2, AG11, AGSS28) + *T. atroviride* (AG3, AG5, AG8) (Vinevax®)**	PD, esca, PhD, ED, and BD	Bot. spp. (1), *P. c.* (1), *Ph.* spp. (1), *E. l.* (2), ***Pha. spp.* (3)**	N	PW	Spraying	V	
***T. atroviride* STE-U 6514**	ED, PD, esca, PhD, BD	***E. l.* (3), *P. c.* (3), *Ph. v.* (3), *D. s.* (3), *L. t.* (3), *N. a.* (3), *N. p.* (3)**	A	PW	Spraying	V	Kotze et al., 2011
***T. atroviride* STE-U 6515, *T. harzianum* ECO 77 (ECO 77®), *T. harzianum* (AG2, AG11, AGSS28) + *T. atroviride* (AG3, AG5, AG8) (Vinevax®)**	ED, PD, esca, BD	***E. l.* (3), *P. c.* (3), *D. s.* (3), *L. t.* (3), *N. a.* (3)**	A	PW	Spraying	V	
T. atroviride* STE-U 6514, *T. atroviride* STE-U 7733	ED, BD, PD, esca, PhD	***N. spp.* (3), *Dip. spp.* (3), Dia. spp. (3), *Ph. spp.* (3), *P. c.* (3), *P. m.* (3)**	N	PW	Spraying	V	Mutawila et al., 2015
***T. atroviride* STE-U 6514, *T. harzianum* T77 (Eco-77®)**	PD, esca, PhD, ED, and BD	**Bot. spp. (3), Dia. spp. (3), *Ph. spp.* (3), *P. c.* (3), *P. m.* (3)**	N	PW	Pipette	V	Mutawila et al., 2016
T. atroviride SC1 (Vintec®)	BFD, PD, BD	BFP (1), PDP (2), BDP (2)	N	1st year: basal end 2nd year: PW	1st year: 1-h soaking, pre-planting 2nd year: spraying	V	Berbegal et al., 2020

[a] ED, Eutypa dieback; BD, Botryosphaeria dieback; BFD, Black-foot disease; PD, Petri disease; PhD, Phomopsis dieback.

[b] BFP, 'black-foot' pathogens; Bot. sp., Botryosphaeriaceae sp.; BDP, Botryosphaeria dieback pathogens; *Ca.* spp., *Campylocarpon* spp.; "*C.*" spp., "*Cylindrocarpon*" spp.; "*C.*" m., "*Cylindrocarpon*" *macrodidymum*; *D. m.*, *Dactylonectria macrodidyma*; *D.* spp., *Dactylonectria* spp.; Dia. spp., Diatrypaceae spp.; *D. s.*, *Diplodia seriata*; *Dip.* spp., *Diplodia* spp.; *E. l.*, *Eutypa lata*; *I. l.*, *Ilyonectria liriodendri*; *I.* spp., *Ilyonectria* species; *L. p.*, *Lasiodiplodia pseudotheobromae; N. a.*, *Neofusicoccum australe*; *N. p.*, *Neofusicoccum parvum*; *N.* spp., *Neofusicoccum* spp.; PDP, Petri disease pathogens; *P. m.*, *Phaeoacremonium minimum*; *Pha.* spp, *Phaeoacremonium* spp.; *P. c.*, *Phaeomoniella chlamydospora*; *Ph.* spp., *Phomopsis* spp.; *Ph. v.*, *Phomopsis viticola*.

[c] A, artificial inoculation; N, natural infection.

[d] PW, pruning wounds; RC, rootstock cuttings; CC, cultivar cuttings; GP, grafted plants.

[e] G, greenhouse; N, nursery; NF, nursery field; V, vineyard.

[f] In parentheses, BCA efficacy: 1, ineffective; 2, limited or reduced effectiveness; 3, effective, eliminating or significantly reducing fungal infection; 4, varying efficacy depending on the BCA-pathogen interaction. In bold letters, GTD pathogens and most effective BCA against them.

**T. atroviride* STE-U 6514 mutant, resistant to benzimidazoles.

Table 1.2. Bacterial and oomycetes biocontrol agents (BCA) tested against fungal trunk pathogens.

BACTERIA							
Species and strain tested	**Disease[a]**	**Pathogens[b]**	**Procedure[c]**	**Material treated[d]**	**Application method**	**Environment[e]**	**Reference**
***Bacillus subtilis* EE**	ED	***E. l.* (3)[f]**	A	PW	Spraying	*In vitro*, V	Ferreira et al., 1991
***B. subtilis* AG1**	PD, esca	***P. c.* (3), *P. m.* (3)**				*In vitro*	Alfonzo et al., 2009
19 bacterial strains The most effective: ***Bacillus* sp. B4, B6, B11, B15, B18, *Pseudomonas* sp. B16**	BD, esca	***L. p.* (3), *S. c.* (3), *N. p.* (3)**				*In vitro*	Rezgui et al., 2016
***B. subtilis* PTA-271**	BD	***N. p.* (3)**	A	CC roots	1-month pre-fungal inoculation	*In vitro*	Trotel-Aziz et al., 2019
***B. subtilis* F62**	BFD	***I. l.* (3), *D. m.* (3)**				*In vitro*	Russi et al., 2020
B. subtilis (Rizos®), *B. subtilis* (Rizolyptus®)	BFD	*D. m.* (4)	A	Substrate	14-day drenching, pre-fungal inoculation	G	Santos et al., 2016
46 bacterial strains The most effective: ***Pantoea agglomerans* S1, S2, S3, S4, *Enterobacter* sp. S24**	BD	***N. p.* (3)**	A	Rooted CC	Drop-inoculation	G	Haidar et al., 2016a
46 bacterial strains The most effective: ***Enterobacter* sp. S24, *Paenibacillus* sp. S18, S19, *B. pumilus* S32, *B. reuszeri* S28, *Pantoea agglomerans* S1, S3, *B. reuszeri* S27, *B. firmus* S41**	PD, esca	***P. c.* (3)**	A	(i), (ii) Rooted CC (iii) Soil	(i) Co-inoculation (ii) 4-day pre-inoculation (iii) 4-day pre-inoculation drenching	G	Haidar et al., 2016b
***B. subtilis* B6**	BD	***N. p.* (3)**	A	Rooted CC	Drop-inoculation	G	Rezgui et al., 2016
Pantoea agglomerans S1, *B. pumilus* S32	BD, PD, esca	*N. p.* (2), *P. c.* (2)	A	CC stem	Pipetting	G	Daraignes et al., 2018
B. subtilis F62	BFD	*D. m.* (2)	A	Substrate	Drenching	G	Russi et al., 2020
B. subtilis (1)	PD, esca	*P. c.* (1), *P. m.* (1)	N	RC	1-h drenching, pre-grafting	NF	Fourie and Halleen, 2004b
***Streptomyces* sp. VV/E1, VV/R1, VV/R4**	BFD, PD, esca	**D. spp. (3), I. spp. (3), P. c. (3), P. m. (3)**	N	Basal end GP	24-h immersion	NF	Álvarez-Pérez et al., 2017
***Streptomyces* sp. VV/R5**	BFD, PD, esca	**D. spp. (3), I. spp. (3), P. m. (3)**, P. c. (2)	N	Basal end GP	24-h immersion	NF	
Pseudomonas fluorescens 1b-023, *Penicillium* sp. 5bf-039, *Kokuria kristinae* ob-027, *B. megaterium* ob-001	ED	*E. l.* (1)	A	PW	Paint brush	V	Munkvold and Marois, 1993
B. subtilis EE	ED	*E. l.* (1)	A	PW	Spraying	V	Halleen et al., 2010
B. subtilis EE	BD, PD, esca, PhD	Bot. spp. (1), *P. c.* (1), *Ph.* spp. (1)	N	PW	Spraying	V	
***B. subtilis* EE1/10**	ED, PhD, BD, PD, esca	***E. l.* (3), *Ph. v.* (3), *D. s.* (3), *L. t.* (3), *N. a.* (3)**, *P. c.* (2), *N. p.* (2)	A	PW	Spraying	V	Kotze et al., 2011

(*Continued on next page*)

Table 1.2. (*Continued from previous page*)

OOMYCETES							
Species and strain tested	**Disease[a]**	**Pathogens[b]**	**Procedure[c]**	**Material treated[d]**	**Application method**	**Environment[e]**	**Reference**
Pythium oligandrum	PD, esca	***P. c.* (3)**	A	Collar rooted CC	Drenching	G	Yacoub et al., 2016
***P. oligandrum* St07**	PD, esca, BD	***P. c.* (3), *N. p.* (3)**	A	Soil	Drenching	G	Daraignes et al., 2018
P. oligandrum St07 + *Pa. agglomerans* S1, *P. oligandrum* St07 + *B. pumilus* S32	PD, esca, BD	*P. c.* (2), *N. p.* (2)	A	Soil (oomycete) CC stem (bacteria)	Drenching (oomycete) Pipetting (bacteria)	G	

[a] ED, Eutypa dieback; BD, Botryosphaeria dieback; BFD, 'black-foot' disease; PD, Petri disease; PhD, Phomopsis dieback.

[b] BFP, 'black-foot' pathogens; Bot. sp., Botryosphaeriaceae sp.; BDP, Botryosphaeria dieback pathogens; Ca. spp., *Campylocarpon* spp.; "C." spp., "*Cylindrocarpon*" spp.; "C". m., "*Cylindrocarpon*" *macrodidymum*; D. m., *Dactylonectria macrodidyma*; D. spp., *Dactylonectria* spp.; Dia. spp., Diatrypaceae spp.; D. s., *Diplodia seriata*; Dip. spp., *Diplodia* spp.; E. l., *Eutypa lata*; I. l., *Ilyonectria liriodendri*; I. spp., *Ilyonectria* species; L. p., *Lasiodiplodia pseudotheobromae;* N. a., *Neofusicoccum australe*; N. p., *Neofusicoccum parvum*; N. spp., *Neofusicoccum* spp.; PDP, Petri disease pathogens; P. m., *Phaeoacremonium minimum*; Pha. spp, *Phaeoacremonium* spp.; P. c., *Phaeomoniella chlamydospora*; Ph. spp., *Phomopsis* spp.; Ph. v., *Phomopsis viticola*; S. c., *Schizophyllum commune*.

[c] A, artificial inoculation; N, natural infection.

[d] PW, pruning wounds, RC, rootstock cuttings; CC, cultivar cuttings, GP, grafted plants.

[e] G, greenhouse; NF, nursery field; V, vineyard.

[f] In parentheses, BCA efficacy: 1, ineffective; 2, limited or reduced effectiveness; 3, effective, eliminating or significantly reducing fungal infection; 4, varying efficacy depending on the BCA-pathogen interaction. In bold letters, GTD pathogens and most effective BCA against them.

Regarding the latter, variations in the incidence of *Trichoderma* spp. among different grapevine cultivars were found after the field application of *Trichoderma*-based treatments on pruning wounds (Mutawila et al., 2011a). These authors concluded that the effect of the BCA on pruning wound protection was not only due to the suppressive effect on the fungal trunk pathogens but also dependent on the BCA-grapevine interaction.

1.6.5. Disease tolerance

A time-tested and sustainable strategy to manage GTDs is the use of disease-tolerant grapevine material, such as rootstocks, cultivars, and clones. This approach would be the least expensive, easiest, safest, and most effective means of controlling and reducing losses from these diseases. Cultivation of tolerant material is a key element in an IPM program, which could markedly decrease the need for chemical spray applications and curative control strategies reducing the level of toxic chemicals in the vineyard environment. Research to determine GTD tolerance of grapevine rootstocks has been mainly focused on fungal pathogens associated with black-foot and Petri diseases, and Botryosphaeria dieback (Table 1.3.), while studies with *Vitis vinifera* cultivars and other *Vitis* spp. determined the variation in susceptibility to pathogens related with the six main trunk diseases currently affecting grapevines (Table 1.4. and 1.5.). To date, no evidence of qualitative resistance to any of the fungal trunk pathogens has been found, but several infection assays have reported varying GTD tolerance of grapevine cultivars (Sosnowski et al., 2007b, 2016b; Travadon et al., 2013; Murolo and Romanazzi, 2014), clones (Murolo and Romanazzi, 2014; Berlanas et al., 2017b), and rootstocks (Eskalen et al., 2001; Gubler et al., 2004; Jaspers et al., 2007; Alaniz et al., 2010; Gramaje et al. 2010b).

Phenotyping assays mainly relies on mechanical artificial inoculations of the fungi on grapevine rootstocks and/or cultivar cuttings or canes under laboratory (Zanzotto et al., 2008; Landi et al. 2012; Guan et al., 2016), greenhouse (Eskalen et al., 2001; Sosnowski et al., 2007b, 2016b; Alaniz et al., 2010; Brown et al., 2013; Travadon et al., 2013; Billones-Baaijens et al., 2014; Markakis et al., 2017), and field conditions (Feliciano et al., 2004; Sosnowski et al., 2007b, 2016b; Gramaje et al., 2010b; Sofia et al., 2018).

Table 1.3. Disease tolerance studies of grapevine rootstocks to fungal trunk pathogens assessed by artificial inoculation.

Planting material	Disease[a]	Pathogens[b]	Environment[c]	Most tolerant material	Most susceptible material	Reference
1103P, 110R	PD, esca	*P. c.*, *P. i.*, *P. m.*	*In vitro*	1103P	110R	Zanzotto et al., 2008
Kober 5BB, SO4, 420A, 1103P, *Vitis rupestris*	PD, esca	*P. c.*-sGFP71 line*	*In vitro*	*V. rupestris*, SO4	420A, Kober 5BB, 1103P	Landi et al., 2012
41 B, 110 R	BD	*N. p.*, *D. s.*	*In vitro*	41B (*N. p.*)**	110R (*N. p.*) 110R, 41B (*D. s.*)	Guan et al., 2016
Freedom, 99R, 39-16, Riparia Gloire, 140 Ru, 16-16, 1103, Harmony, Dog Ridge, 44-53, SO4, 161-49, Salt Creek, St. George, Schwarzman, 5C, 110R, 420A, 3309, AXR	PD, esca	*P. c.*, *P. i.*, *P. m.*	G	Salt Creek (*P. c.*, *P. i.*, *P. m.*) 3309, 110R, 5C (*P. c.*, *P. i.*) 420A (*P. c.*, *P. m.*) AXR1 (*P. i.*, *P. m.*) Schwarzman, St. George (*P. c.*) 16-16, Freedom, 140Ru (*P. i.*) 1103, Harmony (*P. m.*)	39-16 (*P. c.*, *P. m.*) 161-49 (*P. i.*, *P. m.*) 99R, Freedom, Riparia Gloire, 140Ru, 16-16, 1103 (*P. c.*) 420A, St. George, Harmony (*P. i.*) 110R, SO4 (*P. m.*)	Eskalen et al., 2001
110R, 1103P, 140Ru, 161-49 C, 196-17, Fercal, SO4	BFD	"*C.*" *l.*, "*C.*" *m.*	G	140R ("*C.*" *l.*) Fercal ("*C.*" *m.*)	110R ("*C.*" *l.*, "*C.*" *m.*)	Alaniz et al., 2010
101-14, Schwarzman, 5C, Riparia Gloire	BFD	*C. p.*	G	101-14	Riparia Glorie	Brown et al., 2013
101-14, 5C, 3309, Riparia Gloire, Schwarzman, SO4	BD	*N. a.*, *N. l.*, *N. p.*	G	101-14, Schwarzman, 3309, Riparia Glorie	5C, SO4	Billones-Baaijens et al., 2014
41B Mgt, 140Ru, 161-49 C, 1103P, 110R	PD, esca	*C. lo.*, *P. m.*, *P. mo.*, *P. p.*, *P. s.*, *P. v.*, *P. c.*	F	161-49 C	110R, 140Ru	Gramaje et al., 2010b

[a] BD, Botryosphaeria dieback; BFD, Black-foot disease; PD, Petri disease.

[b] *C. lo.*, *Cadophora luteo-olivacea*; "*C.*" *l.*, "*Cylindrocarpon*" *liriodendri*; "*C.*" *m.*, "*C*". *macrodidymum*; *C. p.*, *Cylindrocladiella parva*; *D. s.*, *Diplodia seriata*; *N. a.*, *Neofusicoccum australe*; *N. l.*, *Neofusicoccum luteum*; *N. p.*, *Neofusicoccum parvum*; *P. i.*, *Phaeoacremonium inflatipes*; *P. m.*, *Phaeoacremonium minimum*; *P. mo.*, *Phaeoacremonium mortoniae* (fraxynop.); *P. p.*, *Phaeoacremonium parasiticum*; *P. s.*, *Phaeoacremonium scolyti*; *P. v.*, *Phaeoacremonium viticola*; *P. c.*, *Phaeomoniella chlamydospora.*

[c] G, greenhouse; F, field.

**Pa. chlamydospora* CBS 229.95 transformed using a pCT74 construct which contained the genetic markers for synthetic green fluorescent protein (sGFP) and hygromycin B phosphotransferase.

**In parentheses, GTD fungal pathogens which plant material tolerance information refers to. If plant material is not followed by parentheses it refers to all pathogens assessed in the study.

Table 1.4. Disease tolerance studies of *Vitis vinifera* L. cultivars and other *Vitis* spp. to fungal trunk pathogens assessed by artificial inoculation.

Planting material	Disease[a]	Pathogens[b]	Environment[c]	Most tolerant material	Most susceptible material	Reference
'Shiraz', 'Chardonnay'	BD	*B. l.*, *B. o.*	*In vitro*	'Shiraz'	'Chardonnay'	Savocchia et al., 2007
'Chardonnay', 'Aglianico'	PD, esca	*P. c.*, *P. i.*, *P. m.*	*In vitro*	'Aglianico'	'Chardonnay'	Zanzotto et al., 2008
'Montepulciano', 'Verdicchio', 'Sangiovese', 'Biancame', 'Cabernet Sauvignon'	PD, esca	*P. c.*-sGFP71 line*	*In vitro*	'Cabernet Sauvignon'	'Montepulciano', 'Verdicchio'	Landi et al., 2012
4 *Vitis* species and 16 *V. vinifera* cultivars	BD	*N. p.*, *D. s.*	*In vitro*	'Cabernet Sauvignon' (*N. p.*)** 'Fantasy seedless' (*D. s.*)	'Chasselas' (*N. p.*) 'Colombard' (*D. s.*)	Guan et al., 2016
'Chardonnay', 'Gewürztraminer', 8 *V. vinifera* subsp. *sylvestris* accesions	BD	*N. p.*, *D. s.*	*In vitro*	Ke33, Ke95 (*N. p.*) Hö29, Ke84, Ke83 (*D. s.*)	'Chardonnay' (*N. p.*, *D. s.*) 'Gewürztraminer' (*N. p.*)	
'Grenache', 'Cabernet Sauvignon', 'Merlot'	ED, PD, esca, PhD, BD	*E. l.*, *P. c.*, *Ph. p.*, *Ph.* sp., *B. r.*, *Lib.* sp., Bot. sp., *C. a.*, *P. m.*	G	'Cabernet Sauvignon', 'Merlot'	'Grenache'	Sosnowski et al., 2007b
'Cabernet Franc', 'Cabernet Sauvignon', 'Chardonnay', 'Merlot', 'Riesling', 'Petite Syrah', 'Thompson Seedless', *Vitis lambrusca* ('Concord')	BD, PD, esca, PhD, ED	*L. t.*, *N. p.*, *P. m.*, *P. c.*, *Di. a.*, *E.* sp., *E. l.*	G	'Cabernet Sauvignon' (*L. t.*, *P. m.*, *P. c.*) 'Concord' (*N. p.*) 'Petite Syrah' (*E.* sp.) 'Cabernet Franc' (*E. l.*)	'Thompson Seedless' (*L. t.*, *N. p.*, *P. m.*, *Di. a.*, *E.* sp., *E. l.*) 'Concord' (*P. c.*)	Travadon et al., 2013
2 *Vitis hybrid* and 1 *V. arizonica* disease-resistant breeding lines***	ED	*E.* sp., *E. l.*	G		*V. arizonica* 'b42-26'	
'Sauvignon Blanc', 'Chardonnay', 'Riesling', 'Pinot Noir', 'Merlot', 'Cabernet Sauvignon'	BD	*N. a.*, *N. l.*, *N. p.*	G	'Sauvignon blanc', 'Cabernet Sauvignon', 'Chardonnay'	Merlot, Pinot Noir	Billones-Baaijens et al., 2014
18 least symptomatic cultivars (field survey Sosnowski et al., 2016b), 'Shiraz', 'Sauvignon Blanc'	ED, BD	*E. l.*, *D. s.*	G	'Petit Meslier' (*E. l.*) 'Sangiovese' (*D. s.*)	'Sauvignonasse' (*E. l.*) 'Uguetta' (*D. s.*)	Sosnowski et al., 2016b
'Agiorgitiko', 'Asyrtiko', 'Roditis', 'Soultanina' (syn. 'Thompson Seedless'), 'Xinomavro'	PD, esca	*P. c.*	G	'Xinomavro'	'Agiorgitiko'	Markakis et al., 2017
'Thompson Seedless', 'Cabernet Sauvignon', 'Grenache'	PD, esca	*P. c.*, *P. m.*	F	'Cabernet Sauvignon'	'Thompson Seedless'	Feliciano et al., 2004
'Grenache', 'Merlot', 'Pinot Noir', 'Riesling', 'Semillon', 'Shiraz', 'Cabernet Sauvignon', 'Gamay'	ED	*E. l.*	F	'Merlot', 'Gamay', 'Grenache', 'Semillon'	'Cabernet Sauvignon', 'Shiraz'	Sosnowski et al., 2007b
18 least symptomatic cultivars (field survey Sosnowski et al., 2016b), 'Shiraz', 'Sauvignon Blanc'	ED, BD	*E. l.*, *D. s.*	F	'Petit Verdot' (*E. l.*) 'Petit Meslier' (*D. s.*)	'Sangiovese' (*E. l.*) 'Sauvignonasse' (*D. s.*)	Sosnowski et al., 2016b
'Alfrocheiro', 'Aragonez', 'Jaen', 'Touriga Nacional'	PD, esca	*P. c.*	F	'Jaen'	'Alfrocheiro'	Sofia et al., 2018

[a] ED, Eutypa dieback; BD, Botryosphaeria dieback; PD, Petri disease; PhD, Phomopsis dieback.
[b] Bot. sp., Botryosphaeriaceae sp.; *B. l.*, *Botryosphaeria lutea (*currently *N. l.*); *B. o.*, *Botryosphaeria obtusa (*currently *D. s.*); *B. r.*, *Botryosphaeria ribis (*currently *Neofusicoccum ribis*); *C. a.*, *Cryptovalsa ampelina*; *Di. a.*, *Diaporthe ampelina*; *D. s.*, *Diplodia seriata*; *E. l.*, *Eutypa lata*; *E.* sp., *Eutypa* sp.; *L. t.*, *Lasiodiplodia theobromae*; *Lib.* sp., *Libertella* sp.; *N. a.*, *Neofusicoccum australe*; *N. l.*, *Neofusicoccum luteum*; *N. p.*, *Neofusicoccum parvum*; *P. i.*, *Phaeoacremonium inflatipes*; *P. m.*, *Phaeoacremonium minimum*; *P. c.*, *Phaeomoniella chlamydospora*; *Ph.* sp., *Phellinus* sp.; *Ph. p.*, *Phellinus punctatus*.
[c] G, greenhouse; F, field.
**Pa. chlamydospora* CBS 229.95 transformed using a pCT74 construct which contained the genetic markers for synthetic green fluorescent protein (sGFP) and hygromycin B phosphotransferase.
**In parentheses, GTD fungal pathogens which plant material tolerance information refers to. If plant material is not followed by parentheses it refers to all pathogens assessed in the study.
*** *Vitis hybrid* 'Ill547-1' (powdery and downy mildew resistant), *V. hybrid* 'DVIT0166' (powdery mildew resistant), *V. arizonica* 'b42-26' (*Xylella fastidiosa* resistant).

Several inoculation methods have been reported for tolerance screening, such as soaking bases of grapevine cuttings or seedlings in spore suspensions (Eskalen et al., 2001), insertion of mycelial plugs (Sosnowski et al., 2007b) or conidial suspensions (Wallace et al., 2004; Feliciano et al., 2004; Zanzotto et al., 2008; Travadon et al., 2013; Santos et al., 2006; Pouzoulet et al., 2017) into side wounds or cut ends of the grapevine stems, and vacuum-inoculation of conidial suspensions throughout the vascular system of rootstock cuttings (Gramaje et al., 2010b). Most of these studies have examined the levels of grapevine tolerance to wood symptoms, such as discolouration and necrosis length formation caused by GTD related fungi.

Cultivar susceptibility has been also determined based on visual assessment of external symptoms in the field, mainly examining foliar symptomatology (Marchi, 2001; Fussler et al., 2008; Bruez et al., 2013; Murolo and Romanazzi, 2014; Borgo et al., 2016) (Table 1.5.). This kind of assessment have the limitation that the GTD pathogens often occur in mixed infections within the same vine (Gramaje et al., 2018) and thus there may be some uncertainty that the symptoms observed are due to the effects of a single GTD pathogen.

Table 1.5. Disease tolerance of *Vitis vinifera* L. cultivars to fungal trunk pathogens in field surveys assays assessed by natural infection.

Planting material	Disease[a]	Most tolerant	Most susceptible	Reference
17 white cultivars	Esca	'Roussane'	'Semillon'	Marchi, 2001
'Chardonnay', 'Semillon', 'Shiraz', 'Cabernet', 'Muscat'	ED, BD		'Semillon', 'Chardonnay'	Savocchia et al., 2007
12 white and 10 red cultivars	ED, esca	'Merlot'		Fussler et al., 2008
36 white and 31 red cultivars	Esca	'Pinot blanc', 'Chiapparù', 'Grechetto', 'Shiraz', 'Limberger', 'Brugentile', 'Lacrima'	'Passerina', 'Sauvignon blanc', 'Manzoni bianco', 'Riesling', 'Rebo', 'Traminer', 'Cabernet Sauvignon'	Murolo and Romanazzi, 2014
'Montepulciano', 'Trebbiano', 'Sangiovese', 'Maceratino', 'Verdicchio', 'Passerina'	Esca	'Montepulciano'	'Passerina'	
'Sauvignon blanc', 'Riesling', 'Aglianico', 'Chardonnay', 'Fiano', 'Pinot blanc', 'Falanghina'	Esca	'Chardonnay', 'Fiano', 'Pinot blanc'	'Sauvignon blanc', 'Riesling', 'Falanghina', 'Aglianico'	
40 white and 45 red cultivars	Esca	'Aglianico Taurasi', 'Merlot', 'Fiano', 'Trebbianno d'Abruzzo'	'Lambrusco Salamino', 'Croatina', 'Cabernet Sauvignon', 'Albana', 'Riesling'	Borgo et al., 2016
Germplasm collection: 83 red and 95 white cultivars	ED	'Sangiovese', 'Petit Verdot', 'Nebbiolo Fino', 'Traminer', 'Petit Meslier', 'Muscadelle'	'Odola', 'Dolcetto', 'Cascade', 'Tempranillo', 'Sauvignon Blanc', 'Verdelet', 'Himrod', 'Clairette Blanche'	Sosnowski et al., 2016b

[a] ED, Eutypa dieback; BD, Botryosphaeria dieback.

Biochemical and anatomical characters have been correlated with tolerance of planting material to GTD fungi. Relatively high lignin levels have been associated with wood and

cane tissue of grapevine cultivars having more tolerance to *E. lata* infection (Hamblin, 2015; Rolshausen et al., 2008). Furthermore, tolerance has also been correlated to xylem vessel diameter for esca pathogens (Pouzoulet et al., 2014, 2017) and *E. lata* (Hamblin, 2015).

1.6.6. Alternative methods

Several alternative treatments to reduce GTD incidence and severity have been also evaluated. In Italian nurseries, Di Marco and Osti (2009) found that the application of electrolyzed acid water to cuttings during the hydration stage reduced conidial germination of *Pa. chlamydospora* and *Pm. minimum* without affecting plant growth and development in the nursery field. More recently, ozonated water suppressed *Pm. minimum* spore germination *in vitro* and reduced fungal development by 50% *in planta* after 9 weeks of pruning wounds inoculation (Pierron et al., 2015). Several natural compounds including garlic extract, lactoferrin, tea tree oil, chitosan, oligosaccharide, lichen extract, lemon peel extract, and vanillin have also shown promising results for controlling GTD fungi (Nacimento et al., 2007; Sosnowski et al., 2013; Cobos et al., 2015; Ayres et al., 2017), but further research is necessary before recommendations can be made for widescale application.

Biofumigation with Indian mustard seed meal (*Brassica juncea*) was evaluated in nursery fields and vineyards in Australia as an alternative of chemical control of black-foot disease pathogens significantly improving the yield and growth parameters when buried under diseased grapevines (Whitelaw-Weckert et al., 2014). In New Zealand, *Brassica juncea* shown certain potential reducing black-foot disease incidence and severity when callused rootstock cuttings were planted into artificially infested soil with "*Cylindrocarpon"* spp. (Bleach, 2013). Barbour et al. (2014) also found that amended soils with standard brown mustard or 'Caliente 199' cultivar significantly reduced black-foot disease inoculum. Soil incorporation of white mustard (*Sinapis alba* 'Braco') biomass residues significantly reduced black-foot disease incidence and severity when grafted vines were planted into a naturally infested soil in Spain (Berlanas et al., 2018). More recently, Richards et al. (2020) also showed that planting 'Chardonnay' cuttings in a soil previously conditioned by a white mustard monoculture cover crop significantly reduced the necrosis on basal ends and abundance on roots of *I. liriodendri* under controlled conditions.

1.7. Current situation of Grapevine Trunk Diseases in Spain

Fungal trunk diseases have been known in Spain for a long time. Esca disease was first reported in the Levante region by Bellod (1947). In 1979, Eutypa dieback symptoms were observed in Badajoz region (Arias-Giralda, 1998). However, it was not until the early 21st century when Botryosphaeria dieback, and Petri and black-foot diseases were reported in our country (Armengol et al., 2001a, b).

Although GTD incidence has increased considerably over the last few years in Spanish vineyards (Martín and Cobos, 2007), few field surveys have been performed to know the real impact of these diseases. An average of 3% of plants have been estimated to be affected by GTDs in Spain, although there are newly established plantations in which their incidence could exceed 20%, generating very high annual economic losses (De la Fuente et al., 2016). Rubio and Garzón (2011) reported an increase of GTD incidence in Castilla-León vineyards from 1.8% in 2001 to 10.5% in 2007. A field survey made in 176 vineyards throughout the main grapevine-production areas of Bierzo, Cigales, Ribera del Duero, Rueda, and Toro in the Castilla y León region between 2000 and 2004 indicated that an 80% and 35% of vineyards surveyed showed symptoms resembling Eutypa dieback and esca, respectively (Úrbez-Torres et al., 2006b). Pérez-Marín (2012) highlighted that either incidence or severity of GTDs have increased over the years in La Rioja region. To date, 64 fungal species in 24 genera have been isolated from grapevines in Spain (Table 1.6.).

Table 1.6. Fungal trunk species isolated in Spain.

GTD	Fungal species	Reference
Petri disease/esca	*Cadophora luteo-olivacea*	Gramaje et al., 2011a
	Cadophora vitícola	Gramaje et al., 2011a
	Fomitiporia mediterranea	Armengol et al., 2001b
	Phaemoniella chlamydospora	Armengol et al., 2001b
	Phaeoacremonium cinereum	Gramaje et al., 2009c
	Phaeoacremonium fraxinopennsylvanicum	Gramaje et al., 2007
	Phaeoacremonium hispanicum	Gramaje et al., 2009c
	Phaeoacremonium inflatipes	Gramaje et al., 2009d
	Phaeoacremonium iranianum	Gramaje et al., 2009d
	Phaeoacremonium krajdenii	Gramaje et al., 2011b
	Phaeoacremonium minimum	Armengol et al., 2001b
	Phaeoacremonium parasiticum	Aroca et al., 2006
	Phaeoacremonium scolyti	Gramaje et al., 2008b
	Phaeoacremonium sicilianum	Gramaje et al., 2009d

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Table 1.6. (*Continued from previous page*)

GTD	Fungal species	Reference
Petri disease/esca	*Phaeoacremonium viticola*	Aroca et al., 2008b
	Pleurostoma richardsiae	Pintos et al., 2016
	Stereum hirsutum	Armengol et al., 2001b
Black-foot disease	*Campylocarpon fasciculare*	Alaniz et al., 2011b
	Cylindrocladiella parva	Agustí-Brisach et al., 2012
	Cylindrocladiella peruviana	Agustí-Brisach et al., 2012
	Dactylonectria alcacerensis	Agustí-Brisach et al., 2013a
	Dactylonectria hordeicola	Pintos et al., 2018
	Dactylonectria macrodidyma	Alaniz et al., 2007
	Dactylonectria novozelandica	Agustí-Brisach et al., 2013a
	Dactylonectria pauciseptata	de Francisco et al., 2009
	Dactylonectria riojana	Berlanas et al., 2020
	Dactylonectria torresensis	Agustí-Brisach et al., 2013a
	Ilyonectria liriodendri	Alaniz et al., 2007
	Ilyonectria pseudodestructans	Berlanas et al., 2020
	Ilyonectria robusta	Martínez-Diz et al., 2018
	Ilyonectria vivaria	Berlanas et al., 2020
	Neonectria quercicola	Berlanas et al., 2020
	Thelonectria olida	de Francisco et al., 2009
Eutypa dieback	*Anthostoma decipiens*	Luque et al., 2012
	Crytovalsa ampelina	Luque et al., 2006
	Eutypa lata	Martínez-Olarte et al., 1998
	Eutypa leptoplaca	Luque et al., 2009
	Eutypella citricola	Luque et al., 2012
	Eutypella microtheca	Luque et al., 2012
	Eutypella vitis	Luque et al., 2009
	Fomitiporia punctata	Armengol et al., 2001b
Botryosphaeria dieback	*Botryosphaeria dothidea*	Armengol et al., 2001b
	Diplodia corticola	Pintos et al., 2011
	Diplodia mutila	Martin and Cobos, 2007
	Diplodia seriata	Armengol et al., 2001b
	Dothiorella iberica	Martin and Cobos, 2007
	Dothiorella sarmentorum	Martin and Cobos, 2007
	Lasidiplodia theobromae	Aroca et al., 2008c
	Lasiodiplodia mediterranea	Redondo-Fernández, 2019
	Neofusicoccum australe	Aroca et al., 2010
	Neofusicoccum luteum	Martos and Luque, 2004
	Neofusicoccum mediterraneum	Aroca et al., 2010
	Neofusicoccum parvum	Martos and Luque, 2004
	Neofusicoccum vitifusiforme	Aroca et al., 2010
	Spencermartinsia viticola	Luque et al., 2005
Phomopsis dieback	*Diaporthe ambigua*	Guarnaccia et al., 2018
	Diaporthe ampelina	Martin and Cobos, 2007

(*Continued on next page*)

Table 1.6. (*Continued from previous page*)

GTD	Fungal species	Reference
Phomopsis dieback	*Diaporthe baccae*	Guarnaccia et al., 2018
	Diaporthe eres	Guarnaccia et al., 2018
	Diaporthe hispaniae	Guarnaccia et al., 2018
	Diaporthe hungariae	Guarnaccia et al., 2018
	Diaporthe novem	Pintos et al., 2018
	Diaporthe phaseolorum	Pintos et al., 2018
	Diaporthe rudis	Guarnaccia et al., 2018

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CHAPTER 2

Objectives and thesis outline

As highlighted in the General Introduction, grapevine trunk diseases (GTDs) are caused by several ascomycetous and basidiomycetous fungi that infect grapevines primarily through wounds, causing a slow or rapid decline of the vine as a result of vascular colonization and/or toxin production. Grapevine trunk diseases occur wherever grapes are grown and are one of the main factors limiting both vineyard longevity and productivity due to death of woody parts of the vine caused by the fungus.

Currently, it is well accepted that an integrated pest management (IPM) approach with a combination of several control options should be implemented to minimize GTD infections in nurseries and vineyards. The reduction in the availability of efficient chemical products provides a unique scenario for testing more sustainable management strategies against GTDs, such as the use of tolerant cultivars and biological control agents (BCAs). However, early, specific, accurate and sensitive molecular tools to detect GTD pathogens is a crucial step towards the application of these strategies. Novel high-throughput DNA technologies such as droplet digital PCR (ddPCR) or next-generation sequencing (NGS) can be deployed for absolute quantification of target fungi and for unravelling grapevine-GTD pathogens interactions.

The main aims of this thesis are to develop and implement novel molecular tools that lead to the development of practical effective strategies to manage GTDs, and to evaluate sustainable alternatives to the use of fungicides in order to improve vineyard productivity and longevity.

Chapter 3 comparatively evaluates the accuracy, efficiency, and specificity of a new developed ddPCR protocol and real-time PCR (qPCR) technique, for the detection and quantification of *Ilyonectria liriodendri* in bulk and rhizosphere soils, as well as grapevine endorhizosphere. The abundance of *I. liriodendri* in different habitats inside and outside of grapevine roots was also compared.

Chapter 4 deals with the application of novel high-throughput amplicon sequencing technology to study the GTDs pathosystem in different scenarios, soil and grapevine wood. Chapter 4.1. investigates how fungal communities are enriched in different habitats inside and outside of grapevine roots, with special emphasis on GTD pathogens, and analyzes the effects of plant compartment on the metabolic function of the fungal communities. Chapter 4.2. identifies the diversity and composition of the fungal microbiome, in particular GTD pathogens, infecting pruning wounds in mature vineyards at two pruning

periods. The relationship between the rate of fungal colonization and the main weather data recorded during the experimental period was also inspected.

Chapter 5 evaluates the susceptibility of minority and commercial grapevine cultivars retrieved from Spanish germplasm collections to the internal wood symptoms caused by *Phaeomoniella chlamydospora*.

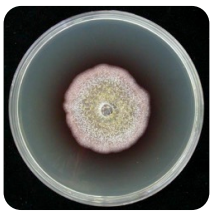
Chapter 6 covers the assessment of several treatments under field conditions to control infections caused by GTD fungal pathogens when applied at pre- and post-planting strategies as well as to act as grapevine pruning wound protectants. Chapter 6.1. evaluates the effectiveness of several BCA root treatments under field conditions in reducing natural infections of fungal pathogens associated with black-foot and Petri diseases over two growing seasons and also assesses the BCA root treatments influence in plant growth parameters. Chapter 6.2. analyses the efficacy of several fungicides and BCA formulations for their potential to prevent infection of grapevine pruning wounds by *Diplodia seriata* and *Pa. chlamydospora* in the vineyard over two growing seasons.

Chapter 7 contains a general and summarizing discussion of the results obtained in this thesis. These data are reviewed in light of what was known prior to this study, leading to several suggestions for future research initiatives.

Finally, **chapter 8** presents as concluding remarks, the most important achievements of this thesis.

CHAPTER 3

Molecular diagnostics



Chapter 3.1.

Droplet digital PCR technology for detection of *Ilyonectria liriodendri* from grapevine environmental samples

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Abstract

Black-foot disease is one of the most important soilborne diseases affecting planting material in grapevine nurseries and young vineyards. Accurate, early and specific detection and quantification of black-foot disease causing fungi are essential to alert growers and nurseries to the presence of the pathogens in soil, and to prevent the spread of these pathogens through grapevines using certified pathogen-free planting material and development of resistance. We comparatively assessed the accuracy, efficiency, and specificity of Droplet Digital PCR (ddPCR) and real-time PCR (qPCR) techniques for the detection and quantification of *Ilyonectria liriodendri* in bulk and rhizosphere soils, as well as grapevine endorhizosphere. Fungal abundance was not affected by soil-plant fractions. Both techniques showed a high degree of correlation across the samples assessed (R^2=0.95) with ddPCR being more sensitive to lower target concentrations. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by black-foot disease fungi.

Introduction

Soilborne pathogens can establish a parasitic relationship with their host plants in the rhizosphere. To infect roots, pathogens have to compete with other microbial organisms of the rhizosphere for available microsites and nutrients (Chapelle et al., 2016). To date, the complex grapevine-soilborne pathogen interactions in the rhizosphere are not fully understood. Among diseases caused by soilborne pathogens in grapevine, black-foot has received much attention in recent decades from plant pathologists as it has been implicated in contributing to young grapevine decline syndrome (Gramaje and Armengol, 2011). *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* have been associated with black-foot disease (Agustí-Brisach and Armengol, 2013; Lombard et al., 2014; Carlucci et al., 2017). The genus *Ilyonectria* represents one of several newly established genera of fungi with *Cylindrocarpon*-like anamorphs (Chaverri et al., 2011), with *Ilyonectria liriodendri* being one of the most prevalent causal agents of black-foot disease (Agustí-Brisach and Armengol, 2013).

Internal symptoms of black-foot diseased vines usually range from black, necrotic, sunken lesions on roots to reddish brown discoloration in the base of the rootstock

(Halleen et al., 2006). Foliar symptoms associated with black-foot disease are practically indistinguishable from those observed in Petri disease affected vines and include delayed bud break, chlorotic foliage with necrotic margins, overall stunting, and wilting of leaves or entire shoots (Agustí-Brisach and Armengol, 2013). These symptoms may also resemble those associated with abiotic disorders such as spring frost, winter damage, nutrient deficiency and/or water stress (Gramaje et al., 2018).

Black-foot disease is particularly important in grapevine nurseries and new plantations. *Cylindrocarpon*-like asexual morphs produce conidia and some species also produce chlamydospores in culture, which indicates that those propagules are likely to be produced on stem bases of infected vines and the diseased roots. The conidia are spread in soil water and the chlamydospores can allow these pathogens to survive in the soil for extended periods of time (Petit et al., 2011). Infection can occur through the small wounds made when roots break off during the planting process, through the incomplete callusing of the lower trunk or through wounds made in the grapevine propagation process, such as disbudding wounds, from which the infection progresses downward to the base of the trunk (Halleen et al., 2006).

Traditionally, detection and identification of black-foot disease fungi in grapevine has been performed by morphological approaches (Chaverri et al., 2011) or by multiplex PCR system (Alaniz et al., 2009). Although reliable for a preliminary identification and classification, these techniques are not practical to detect low levels of black-foot pathogens that anticipated during early stages of infection. Recently, real-time PCR (qPCR) has become a useful technique for increasing the sensitivity and specificity for detecting and quantifying *Cylindrocarpon*-like asexual morphs (Tewoldemehdin et al., 2011; Agustí-Brisach et al., 2014; Langenhoven et al., 2018). The Digital PCR (ddPCR) has only recently been adapted to detect plant pathogens in agricultural systems from biomedical disciplines where it showed to be more sensitive technology compared with qPCR (Dreo et al., 2014; Miotke et al., 2014; Racki et al., 2014; Bahder et al., 2016, 2018).

The objectives of this study were therefore: i) to design a ddPCR protocol that is capable to detect and quantify *I. liriodendri* in soil and roots, ii) to evaluate the overall sensitivity of ddPCR for detection of *I. liriodendri* compared with qPCR, and iii) to compare the abundance of *I. liriodendri* in different habitats inside and outside of grapevine roots.

Materials and Methods

Fungal Isolate selection and DNA serial dilutions

Ilyonectria liriodendri isolate BV-0596 was obtained from the culture collection of the Instituto de Ciencias de la Vid y del Vino (ICVV) (Spain). Fungal mycelium and conidia from pure cultures grown on potato dextrose agar for 2 to 3 weeks at 25°C in the dark were scraped and homogenized in 2 ml tubes with 600 µl of P1 buffer of the kit E.Z.N.A. Plant Miniprep kit (Omega Bio-tek, Norcross, GA, USA) with 4 steel beads of 2.38 mm and 2 of 3 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, California, USA) at 5 m/s for 20 s twice. DNA integrity and quality were assessed by gel electrophoresis visualizing the samples previously stained with RedSafe (iNtRON Biotechnology, Lynnwood, WA, USA). DNA samples were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, MA, USA). Serial dilutions ranging from 10,000 to 1 fg μl^{-1} of the DNA were prepared for quantification purposes by ddPCR and qPCR. Three independent DNA standard curves were obtained using separate pathogen DNA sources that were treated as independent experiments.

TaqMan assay design and ddPCR parameters

Digital Droplet PCR (ddPCR) was performed on a Bio-Rad QX200 system using a TaqMan assay. A probe was designed using the PrimerQuest®Design Tool (Integrated DNA Technologies, Inc. Coralville, IA, USA) and labeled at the 5' end with Hetrachloro-6-carboxyfluorescin (HEX) and a double-quencher (internal ZEN with 3' Iowa Black FQ). The probe sequence is 5'-/HEX/TCCGAGCGT/ZEN/CATTTCAACCCTCAA/3IABkFQ/-3'. Primers YT2F (Tewoldemedhin et al., 2011) and Cyl-R (Dubrovsky and Fabritius, 2007) were used in the experiment. These primers amplify the main *Cylindrocarpon*-like asexual morphs associated with black-foot disease, in particular those belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria.* Each reaction contained 1x Supermix for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 20 µM of each forward and reverse primer solution (final concentration 750 nM for each primer), 10 µM of the probe and 2 µl of DNA template resulting in a final volume of 20 µl. The PCR reactions were mixed, centrifuged briefly, and 20 µl transferred into the sample well of a DG8TM cartridge (Bio-Rad). After adding 70 µl of QX200TM droplet generation oil (Bio-

Rad Laboratories) into the oil wells, the cartridge was covered using a DG8TM gasket, and droplets generated using the QX200TM droplet generator (Bio-Rad Laboratories). Droplets were carefully transferred into PCR plates using a multi-channel pipette and the plate was sealed using PCR plate heat seal foil and the PX1TM PCR plate sealer (Bio-Rad Laboratories). PCR was performed in a C1000 touch thermal cycler (Bio-Rad Laboratories) using the following thermal cycling conditions: initial denaturation stage of 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s and annealing temperature (59°C) for 60 s and a final extension of 10 min at 98°C. PCR plates were transferred into a QX200TM droplet reader (Bio-Rad Laboratories) and reads analyzed using QuantaSoftTM software (Bio-Rad Laboratories). A thermal-gradient PCR experiment was conducted to establish the optimal annealing temperature for the primers using DNA of *I. liriodendri* isolate BV-0596 as a template.

Real-time PCR assay parameters

Real-time PCR assays were performed on a CFX384 real time PCR system (Bio-Rad Laboratories) using the same primers and TaqMan probe as described above. Each reaction contained 2 µl of DNA template, 5 µl of 1x Supermix for Probes (Bio-Rad), containing 500 nM of probe and 750 nM of each primer. The reaction mix was adjusted to a final volume of 10 µl with sterile distilled water. Thermal cycling conditions were as follows: 10 min of initial denaturation at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and annealing at 62°C for 60 s. Both ddpCR and qPCR were performed at BIODONOSTIA Health Research Institute (San Sebastián, Spain).

Environmental sample collection

Grapevine samples were collected at five young vineyards of Tempranillo cultivar grafted onto 110 Richter rootstock maintained in La Rioja (Spain) located between 2.2 to 14.9 km distance from each other (Supplementary Table 3.1.1.). These vineyards were under similar soil, climatic and management conditions. In each vineyard, three different sample types were studied in June 2017 (flowering): bulk soil, soil surrounding roots (rhizosphere) and roots (endorhizosphere). Four plants per vineyard were chosen to represent the same aspect of the plant and position within the vineyard, and four samples were randomly collected from each soil-plant fraction (bulk, rhizosphere and endorhizosphere). Sampled vines did not show any symptom of disease or nutrient

deficiency and root tissue did not have any rot or necrosis that could be associated to black-foot or other diseases caused by soilborne pathogens. A total of 60 samples were collected.

Bulk soil samples were collected with a sterile spade 1 m from each stem at depths of 40 to 50 cm. Homogenized dry soil was then passed through a 1-mm-pore size sieve and divided into two subsamples, each one for *I. liriodendri* detection and quantification, and soil chemistry analyses, respectively. Roots and rhizosphere soil samples were collected with a sterile spade close to the stem at depths of 40 to 50 cm, where the root system was denser. All samples were stored on dry ice in sterile bags at the time of sampling, and brought to the laboratory for further processing within 24 h from the time of sampling. A total of 5 g of the sampled roots with rhizosphere soil particles attached were placed in sterile tubes containing 9 ml of physiological solution (9 g/l NaCl). They were vortexed for 5 min to detach the soil particles and immediately centrifuged at 1,503 g for 5 min. The supernatant was discarded and the remaining soil fraction was used to represent the rhizosphere fraction. The roots devoid of soil particles were placed in a new tube and surface sterilized according to Cherif et al. (2015).

DNA extraction

The bulk soil and rhizosphere DNA were extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). For endorhizosphere DNA, before DNA extraction, roots were sequentially washed in 70% ethanol and distilled water. Upon this treatment, bark was carefully peeled out and the DNA was extracted from 0.5 g tissue using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, South Korea).

Standard curves determination

A standard curve was constructed with DNA dilution series of *I. liriodendri* isolate BV-0596. Analyses were performed as previously described and the standard curve was generated following the MIQE guidelines (Bustin et al., 2009). The estimated number of target molecules per µl (ddPCR) and the quantification cycle (Cq) (qPCR) values obtained for each specific isolate DNA dilution were plotted against the logarithm of the concentration (fg μl^{-1}) of each isolate DNA dilution.

Sensitivity of ddPCR and qPCR was assessed estimating the limit of detection (LOD) of both techniques, using the resulting standards curves to determine the minimum DNA concentration that can be detected in three consecutive assays. The amplification efficiency (E) and the coefficient of determination (R^2) of the standard curve were obtained using the specific software of each system, QuantaSoftTM (Bio-Rad) for ddPCR and CFX Maestro (Bio-Rad) for qPCR. Signal threshold levels were set automatically by the instrument software and the LOD was identified by the last dilution in which successful amplification of all DNA replicates occurred.

Quantification of black-foot pathogens in environmental samples

To compare both quantification techniques in environmental samples, the 60 grapevine samples collected at 5 young vineyards in La Rioja were analyzed by both ddPCR and qPCR technologies. All samples, along with a non-template control (NTC) reaction (water), two positive controls containing DNA of a soil sample tested positive to *I. liriodendri* by ITS high-throughput amplicon sequencing, and DNA extracted from a pure culture of *I. liriodendri* isolate BV-0596, were analyzed by qPCR and ddPCR in triplicate. In addition, negative controls (serial dilutions of DNA from grapevine and soil lacking target fungi) were used alone or spiked with the same amount of target DNA in order to assess inhibition in the assays. The mean DNA concentration and the standard deviation were determined from five replicates per dilution. For the qPCR results, copy number was calculated with the following formula: (DNA amount (g) * 6.022 x 10^{23} (copy/mol) / (DNA length (bp) * 660 (g/mol/bp)) (Lee et al., 2006; Lee et al., 2016), where DNA amount was the concentration of DNA (g) and DNA length was the length of *I. liriodendri* BV-0596 genome, 60 Mbp (unpublished data). The efficiency of both ddPCR and qPCR technologies to quantify *I. liriodendri* from environmental samples was compared. Values from the *I. liriodendri* DNA concentration obtained with each technique were transformed by log (n/N ∗ 1000 + 1). Where n was the DNA concentration detected on each sample and N was the total DNA concentration detected. An analysis of correlation between both transformed datasets was performed in R version 3.5 (R Core Team, 2017) using the corrr package. DNA concentration values using both quantification methods were calculated for each fraction and vineyard. Significance levels for mean values were determined by the Kruskal-Wallis one-way analysis of variance on ranks and mean separation was conducted at $P < 0.05$. The analysis was performed using R package agricolae (Mendiburu, 2015).

Results

Detection and quantification limit of genomic DNA of cultured I. liriodendri by ddPCR and qPCR

The optimal annealing temperature for primers using pure culture *I. liriodendri* BV-0596 DNA in ddPCR was established at 59°C. Both methods showed good linearity within the quantification range with a high coefficient of determination (R^2) of 0.9917 and 0.9893 and a reaction efficiency of 0.83 and 0.97 for ddPCR and qPCR, respectively (Fig. 3.1.1). The minimum target concentration detectable was the 5 fg μl^{-1} dilution for ddPCR and the 10 fg μl^{-1} dilution for qPCR (Table 3.1.1), thus, the LOD was established at these concentrations for each technique. The NTC showed no positive amplification.

Quantification of black-foot pathogens from environmental samples

DNA of *I. liriodendri* was detected in all soil-plant fractions samples in the five vineyards assessed. Significant differences in the abundance of *I. liriodendri* were detected among vineyards with both techniques ($P < 0.01$). Concentrations ranged from 1.79 to 20.98 pg μl^{-1} in vineyard 1, 0.77 to 8.73 pg μl^{-1} in vineyard 2, 1.99 to 53.8 pg μl^{-1} in vineyard 3, 0.03 to 38.58 pg μl^{-1} in vineyard 4 and 0.34 to 29.43 pg μl^{-1} in vineyard 5 by qPCR (data not shown). In ddPCR, concentrations ranged from 96 to 2,350 copies μl^{-1} in vineyard 1, 75 to 860 copies μl^{-1} in vineyard 2, 190 to 8,680 copies μl^{-1} in vineyard 3, 4.7 to 9,470 copies μl^{-1} in vineyard 4 and 40 to 1,920 copies μl^{-1} in vineyard 5 (data not shown). Average number of copies in each vineyard per soil-plant fraction obtained by ddPCR are shown in Table 3.1.2. In each vineyard, no significant differences in the abundance of *I. liriodendri* were detected among soil-plant fractions with both techniques ($P > 0.05$). Overall, concentrations ranged from 0.5 to 38.68 pg μl^{-1} in bulk soil, 0.03 to 53.8 pg μl^{-1} in rhizosphere and 0.78 to 107.73 pg μl^{-1} in roots fraction by qPCR. Average concentrations in each soil-plant fraction were 10.07 pg μl^{-1} (bulk soil), 10.49 pg μl^{-1} (rhizosphere), and 11.53 fg μl^{-1} (endorhizosphere). In ddPCR, concentrations ranged from 40 to 9,470 copies μl^{-1} in bulk soil, 4.7 to 5,270 copies μl^{-1} in rhizosphere and 75 to 8,680 copies μl^{-1} in roots fraction. Average number of copies in each soil-plant fraction were 1,275 copies μl^{-1} (bulk soil), 1,028 copies μl^{-1} (rhizosphere), and 1,233 copies μl^{-1} (endorhizosphere). No PCR inhibition or positive droplets noticed using negative controls by qPCR or ddPCR (Fig. 3.1.2), respectively. The correlation analysis showed a high and positive significant

correlation between *I. liriodendri* DNA quantified using both the ddPCR and qPCR techniques (R^2= 0.95) (Fig. 3.1.3).

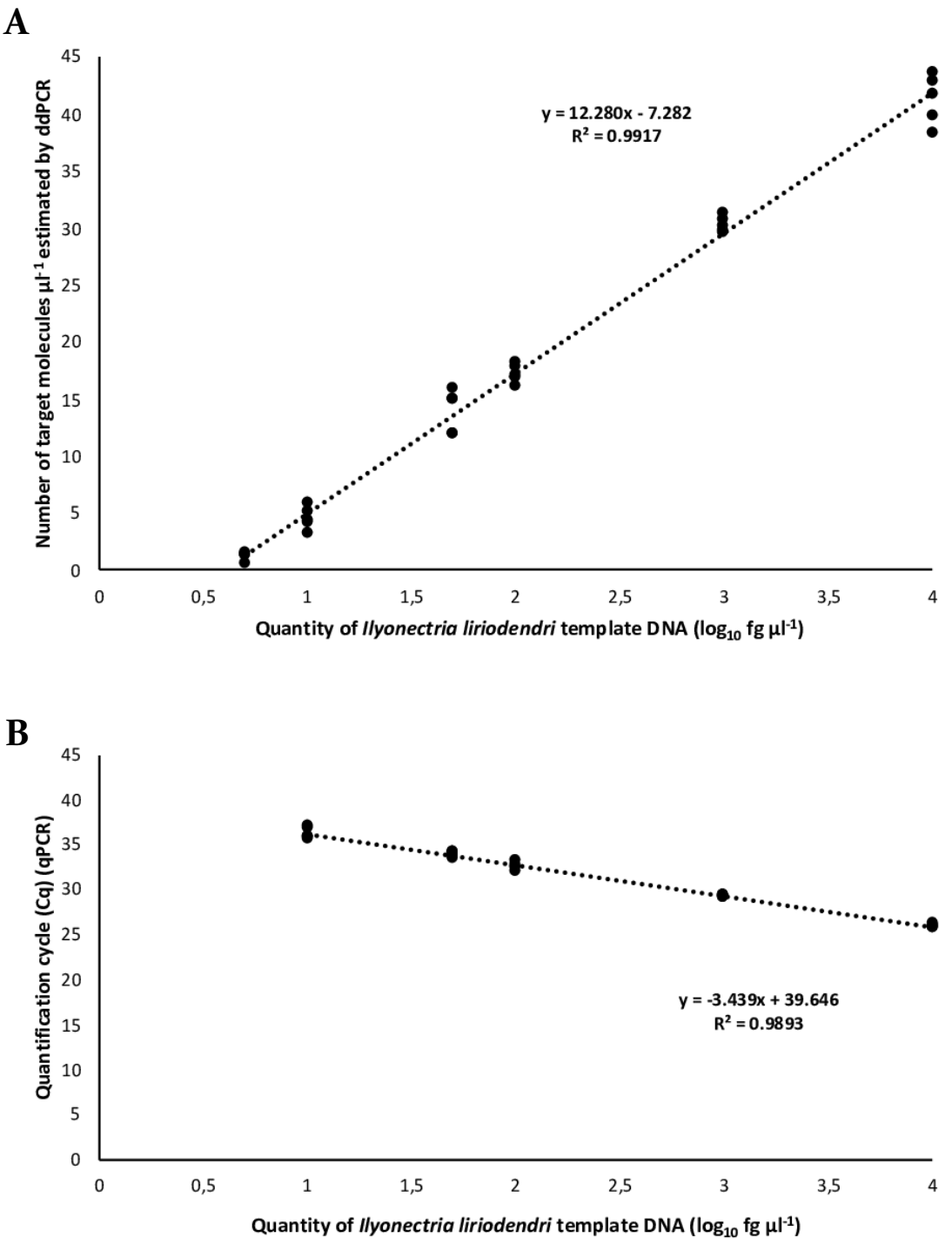

Figure 3.3.1. Standard curves obtained using *Ilyonectria liriodendri* isolate BV-0596 DNA dilutions ranging from 10,000 to 1 fg μl^{-1}. The logarithm of the concentration of each isolate DNA dilution was plotted against: **A**) Target DNA concentration (copy number μl^{-1}), in order to construct the ddPCR standard curve; **B**) Quantification cycle (Cq), in order to construct the qPCR standard curve. Data points represent amplification results of five replicates. The reaction efficiency was 0.83% and 0.97% for ddPCR and qPCR analysis, respectively.

Table 3.1.1. Droplet digital PCR and Quantification Cycle (Cq) obtained in real-time PCR average data for the serial dilutions of *Ilyonectria liriodendri* isolate BV-0596 DNA (n=5). Values represent the mean±SE.

DNA concentration (fg µl^{-1})	ddPCR (copies µl^{-1})	qPCR (Cq)
10,000	41.3 ± 0.98	26.0 ± 0.10
1,000	30.3 ± 0.32	29.3 ± 0.07
100	17.3 ± 0.36	32.6 ± 0.20
50	14.0 ± 0.84	33.9 ± 0.13
10	4.5 ± 0.45	36.3 ± 0.29
5	1.2 ± 0.16	N/A
1	N/A	N/A

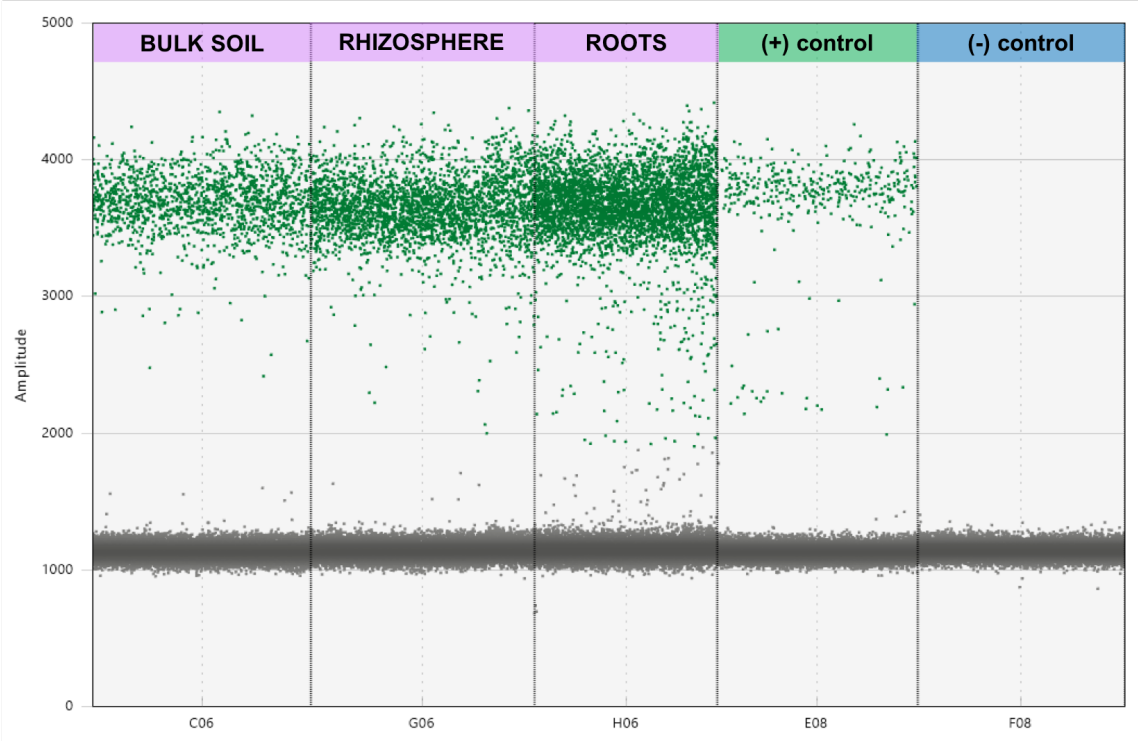

Figure 3.1.2. Droplet digital PCR amplitude plot showing all accepted droplets with a clear distinction between positives (green) and negatives (grey) in each soil-plant fraction (bulk soil, rhizosphere and roots), positive control (*I. liriodendri* isolate BV-0596 DNA) and non-template control (NTC), confirming the assay optimization.

Table 3.1.2. Droplet profile and digital PCR quantitation data from DNA extracts of the environmental samples from five vineyards (n=4). Values represent the mean±SE from four replicates.

	Bulk soil			Rhizosphere soil			Endorhizosphere		
	Accepted Droplets	**(+) Droplets**	**Copies µl^{-1}**	**Accepted Droplets**	**(+) Droplets**	**Copies µl^{-1}**	**Accepted Droplets**	**(+) Droplets**	**Copies µl^{-1}**
Vineyard 1	17,400 ± 930	417 ± 143	293 ± 103	16,400 ± 1,420	1,250 ± 723	889 ± 495	16,200 ± 971	482 ± 75	370 ± 79
Vineyard 2	12,400 ± 1,570	194± 44.0	183 ±28	11,800 ± 1,480	442 ± 147	441 ± 147	11,500 ± 1,230	285 ± 123	321 ± 166
Vineyard 3	18,100 ± 606	2,910 ± 784	2,090 ± 610	18,500 ± 751	2,270 ± 1,210	1,820 ± 1,150	18,500 ± 670	3,410 ± 2,360	2,700 ± 2,000
Vineyard 4	16,600 ± 653	3,320 ± 1,820	3,190 ± 2,100	18,200 ± 282	1,830 ± 649	1,260 ± 453	16,700 ± 604	2,770 ± 1,000	2,300 ± 894
Vineyard 5	14,900 ± 1,160	833 ± 445	624 ± 298	16,000 ± 756	925 ± 561	722 ± 428	16,400 ± 755	640 ± 126	468 ± 96
(+) control[1]	19,200 ± 0	20 ±0	12 ±0	19,200 ± 0	20 ± 0	12 ± 0	19,200 ± 0	20 ± 0	12 ± 0
(+) control[2]	16,400 ± 0	411 ±0	299 ±0	16,400 ± 0	411 ± 0	299 ± 0	16,400 ± 0	411 ± 0	299 ± 0
(-) control[3]	16,100 ± 436	0	N/A	16,100 ± 436	0	N/A	16,100 ± 436	0	N/A
(-) control[4]	18,600 ±1,660	0	N/A	18,600 ±1,660	0	N/A	18,600 ±1,660	0	N/A

[1]DNA of a pure culture of *I. liriodendri* isolate BV-0596 (n=1)
[2]DNA of soil sample tested positive to *I. liriodendri* (n=1)
[3]DNA from grapevine (n=2)
[4]Water (n=2)

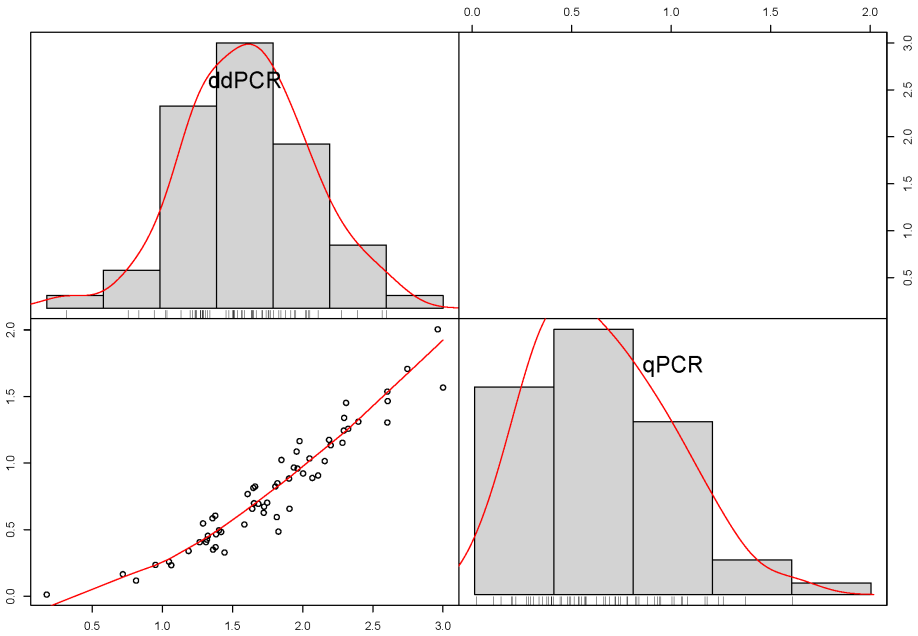

Figure 3.1.3. The distribution of DNA concentration of *I. liriodendri* values is shown on the diagonal. The bivariate scatter plot with a fitted line is displayed on the bottom of the diagonal and the Spearman correlation value ($P < 0.05$) is indicated on the top of the diagonal.

Discussion

Digital PCR is the latest DNA quantification technology that can be broadly used in several scientific fields (Morisset et al., 2013; Yang et al., 2014; Cao et al., 2015; Hussain et al., 2016; Palumbo et al., 2016; Porcellato et al., 2016), including plant pathology (Bahder et al., 2018, 2019; Voegel and Nelson, 2018). The present study represents the first approach to assess the ddPCR as a reliable tool to detect and quantify pathogenic fungi associated with grapevine trunk diseases. In particular, black-foot disease is one of the main soilborne diseases affecting planting material and young vineyards worldwide (Gramaje et al., 2018). DNA was not sheared prior to the experiments since our input DNA concentration was 10 ng/20 µl reaction. DNA shearing is recommended for input DNA concentrations >66 ng/20 µl reaction using the Bio-Rad QX100/200 system (Hindson et al., 2011). We found that both ddPCR and qPCR showed the potential of being efficient techniques to detect and measure *I. liriodendri* DNA associated with black-foot disease, with a strong correlation between them. These findings are in agreement with those obtained by Kim et al. (2014), who found a high quantitative agreement between DNA quantity measured with ddPCR and qPCR while examining population dynamics of bacteria in soil.

Quantification of copy number from complex samples containing multiple target species may be inaccurate. However, our attempt to design specific primers for black-foot disease genera (*Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria*) from available gene sequences in the GenBank database (internal transcribed spacer region, histone H3, translation elongation factor 1-alpha and β-tubulin genes) were unsuccessful, due to lack of highly conserved gene regions among these closely related phylogenetic genera. In this study, *I. liriodendri* BV-0596 genome size was used in the calculation of copy number across samples and the DNA of this isolate was also used for the standard curve determination and to establish the optimal annealing temperature for the primers. A limitation of this approach that needs to be stated is that bias in the calculation of the copy number may be introduced due to different genome sizes from the fungal species associated with black-foot disease: 58 Mbp in *D. macrodidyma* isolate JAC15-245 (Malapi-Wight et al., 2015) and 64 Mbp in *D. torresensis* isolate BV-0666 (Gramaje et al., 2019).

The ddPCR showed to be more sensitive as compared with qPCR in the detection and quantification of this fungal pathogen at very low concentrations. Increased sensitivity of digital PCR over qPCR has been reported in other studies (Kim et al., 2014; Cavé et al., 2016; Porcellato et al., 2016; Bahder et al., 2018) and similar sensitivity was highlighted by others (Dreo et al., 2014; Blaya et al., 2016) when comparing both techniques. Developing a robust ddPCR assay with increased sensitivity of ddPCR over qPCR would be beneficial to researchers and diagnostic laboratories by identifying early infections in grapevines and soil. Additional benefits were reported in several recent studies for ddPCR such as it obviates the preparation of reference DNA templates (Kim et al., 2014), the absolute quantitative target detection without the need of standard curves construction (Yang et al., 2014) and that the inhibitory substances had a little effect on DNA quantification using this technique (Hoshino and Inagaki, 2012). These features and the results obtained in the present study make ddPCR an attractive alternative for measuring environmental samples allowing a better understanding and monitoring of fungal pathogens associated with grapevine trunk diseases in the future.

The abundance of *I. liriodendri* was not affected by soil or plant as source of DNA. Recent studies have shown that black-foot inoculum pressure in vineyard soils is frequently high (Reis et al., 2013; Agustí-Brisach et al., 2014), even with the absence of grapevine (Cardoso et al., 2013; Berlanas et al., 2017). Black-foot disease fungi can survive in soil for multiple years in the absence of suitable host due to the production of

chlamydospores after infected vines have been removed (Petit et al., 2011). These pathogens have also been frequently associated with the roots of herbaceous plants (Langenhoven et al., 2018) and weeds (Agustí-Brisach et al., 2011). It is also important to note that *Cylindrocarpon*-like asexual morphs seem to be quite frequent in roots and the endorhizosphere of diverse plants, not just only in grapevines. Several studies show that they are indeed among the dominant fungi found in soils with strawberry (Xu et al., 2015) and forest trees (Bonito et al., 2014), and in roots of apple in South Africa (Tewoldemedhin et al., 2011) and USA (Manici et al., 2018). This frequent and high occurrence of *Cylindrocarpon*-like asexual morphs in different environments may explain the lack of specialization of these fungi to specific plant associated ecological niches (rhizosphere and endorhizosphere).

Both the qPCR and ddPCR methods revealed that healthy grapevine plants harbor *Cylindrocarpon*-like asexual morphs that are causal agents of black-foot disease, demonstrating that these fungi can act as endophytic and/or latent pathogenic microorganisms in grapevine. In the scientific literature, observations of black-foot disease fungi as endophytes colonizing asymptomatic vines (Langenhoven et al., 2018; Berlanas et al., 2019) or other plant species (Agustí-Brisach et al., 2011; Langenhoven et al., 2018) have been documented. Many of these asymptomatic plants are cereals and brassicaceous crops, used in crop rotations in grapevine nurseries (Langenhoven et al., 2018), and weeds, which may be present in field nurseries and established vineyards along with cultivated crops (Agustí-Brisach et al., 2011; Langenhoven et al., 2018). The occurrence of black-foot disease pathogens in asymptomatic vines highlights the urgent need to implement early, accurate and specific *in planta* detection and quantification of these fungi to prevent the spread of black-foot disease in grapevine propagation material.

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Chapter 3.1.

Supplementary material

Supplementary **Tables**

Supplementary Table 3.1.1. Soil physicochemical properties and management practices of the five vineyards examined in this study. Values represent the mean±SE.

	Vineyard-1	**Vineyard-2**	**Vineyard-3**	**Vineyard-4**	**Vineyard-5**
Coordinates	42,583560º, -2,853296º	42,588604º, -2,868726º	42,539882º, -2,766227º	42,499781º, -2,781054º	42,593068º, -2,851397º
Location	Haro	Haro	Briones	Briones	Haro
Year of plantation	2013	2013	2014	2012	2013
Extension (ha)	0.93	8.32	3.00	5.11	1.96
Altitude (m)	478	482	501	497	499
Physicochemical properties					
pH	8.1[a]±0.02	8.1±0.02	8.2	8.2	8.2±0.02
P mg/100g	3.39±0.25	2.6±0.2	2.4±0.3	3.1±0.3	3.2±0.2
K mg/100g	17.2±0.7	17.1±0.4	16.4±0.5	19.1±0.6	20.5±0.5
S mg/100g	4.3±0.5	3.9±0.4	4.1±0.3	4.1±0.4	4.2±0.4
Mg mg/100g	24.3±0.3	27.1±0.3	25.7±0.5	21.4±0.4	26.7±0.5
Mn mg/100g	3.5±0.8	2.9±0.7	2.8±0.7	2.8±0.7	2.8±0.8
Fe mg/100g	8.6±0.4	8.7±0.2	10.6±0.2	7.7±0.4	8.7±0.5
Ca mg/100g	3979.6±220.8	4346.9±120.4	4347.5±109.8	3731.3±176.4	3503.0±126.7
Na mg/100g	2.4±0.2	2.83±0.1	3.1±0.2	3.0±0.2	3.4±0.2
SOM%	0.95±0.03	1.05±0.2	1.05±0.03	0.95±0.05	1.07±0.08
Clay%	18.5±0.2	22.2±0.3	25.3±0.3	21.9±0.6	22.9±0.4
Sand%	39.9±0.3	33.8±0.4	34.3±0.5	35.1±0.6	34.3±0.9
Silt%	41.6±0.6	44.0±1.1	40.4±0.8	43.0±0.7	42.8±0.5
CO_3Ca	14.15±0.02	15.67±0.23	13.9±0.1	14.5±0.4	14.9±0.6
CEC mekv/100g	8.9±0.3	10.4±0.1	11.2±0.2	9.6±0.2	9.1±0.3
EC mS/cm	0.14	0.16	0.16	0.15	0.16
Assim. Ca mekv/100g	15.1±0.2	17.4±0.2	16.5±0.2	15.5±0.3	14.8±0.4
Assim. Mg mekv/100g	1.57±0.05	1.81±0.11	1.75±0.12	1.70±0.15	1.63±0.21
Soil temperature (ºC) (July)	20.9	21.0	21.6	21.3	20.9
Soil management practices					
Irrigation system	Drip irrigation				
Fertilization	2 applications per year				
Pest management practices	5 spray treatments against powdery and downy mildew per year				
Herbicide treatment	Yes				

CHAPTER 4

Microbial ecology

Chapter 4.1.

Soil-plant compartments affect fungal microbiome diversity and composition in grapevine

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Grapevine trunk disease
High-throughput amplicon sequencing
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Microbiome

Abstract

Plant compartments provide unique niches that lead to specific microbial associations. The microbiota colonizing the endophytic compartment (endorhizosphere) and the rhizosphere contribute to productivity, plant growth, phytoremediation and carbon sequestration. The main objective of this study was to investigate how fungal communities are enriched in different habitats outside and inside of grapevine roots. For this purpose, the spatial dynamics of the fungal communities associated with three soil-plant compartments (bulk soil, rhizosphere and endorhizosphere) were characterized by ITS high-throughput amplicon sequencing (HTAS). Fungal communities were largely affected in their diversity and composition by soil-plant compartments, whereas the spatial variation (i.e. across five vineyards) was low. The endorhizosphere compartment was the one that differed the most from the other two, suggesting that the root tissues entail a barrier for fungal colonization. The results of functional prediction via FUNGuild suggested an increase in the relative abundances of potential plant pathogens, endophytes and arbuscular mycorrhiza, and a decrease in wood, dung and undefined saprotrophs from bulk soil towards the endorhizosphere. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by soilborne fungi associated with grapevine trunk diseases, which opens up new perspectives in the study of the endophytic role of these pathogens on grapevines. Results obtained in this study provide helpful information to better know how the host shapes its microbiome and the implications for vineyard productivity and management.

Introduction

Grapevine belowground compartments (microbial habitats: rhizosphere, rhizoplane and endorhizosphere) provide unique niches and maintain microbes associated with stress protection, plant health, plant development and productivity (Zarraonaindia and Gilbert, 2015; Marasco et al., 2018; Yu and Hochholdinger, 2018; Berlanas et al., 2019). Some endophytes can originate from the soil and move towards the aerial compartments through the endorhizosphere, indicating that the roots act as the primary reservoir of microbial grapevine colonizers (Compant et al., 2008; Martins et al., 2013; Zarraonaindia et al., 2015). Zarraonaindia et al. (2015) suggested that the local biogeography of the soil

microbiota is actively involved in the formation of the regional wine terroir, thereby indirectly influencing wine characteristics.

Shifts in community structure from bulk soil towards roots are known to occur in commercial crops (Reinhold-Hurek et al., 2015). Such gradients in microbial diversity with lower diversity and a higher degree of specialization toward the inside of the root suggest that microbiome from adjacent soil might be modified by plant mutualistic associations (Reinhold-Hurek et al., 2015). Plant roots can influence rhizosphere microbial community composition through selective enrichment via root exudates (Berg and Smalla, 2009). A rhizosphere effect was recognized early in this research field by Starkey (1938), with bacterial community structure changing in rhizosphere in comparison to bulk soil. Several studies using cultivation-independent approached also confirmed community changes in bulk and rhizosphere soil (Smalla et al., 2001; Costa et al., 2006). Recent studies using high-throughput sequencing approaches allowed deeper insight into microbial community changes and their functions at the soil-root interface (Gottel et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012; Turner et al., 2013; Ofek et al., 2014; Wang et al., 2017). Significant dependencies of the biogeographic distribution of soil microbiome on environmental filters, such as environmental conditions (e.g. climate and topography), soil properties (e.g. pH, texture and carbon content), and even land cover have been previously demonstrated (Fierer and Jackson, 2006; Laubert et al., 2008; Rousk et al., 2010; Griffiths et al., 2011; Prévost-Bouré et al., 2014; Terrat et al., 2017). However, the reported evidence has not been conclusive in determining the main controlling factors. The spatial regulators of soil microbiome vary in different ecosystems and at different spatial scales (Xia et al., 2016). In grapevine, the soil physicochemical properties and moisture content (Fernández-Calviño et al., 2010; Corneo et al., 2014; Burns et al., 2015; Zarraonaindia et al., 2015; Holland et al., 2016), as well as the host genotype (Marasco et al., 2018; Berlanas et al., 2019), have been identified as major factors shaping the spatial scaling of the rhizosphere microbiome (Fernández-Calviño et al., 2010; Corneo et al., 2014; Burns et al., 2015; Zarraonaindia et al., 2015; Holland et al., 2016). Soil physicochemical properties can also influence the population structure of specific soilborne pathogens. For instance, Berlanas et al. (2017) observed that excessive calcium carbonate in soil may increase black-foot disease inoculum density.

To date, less attention has been paid to the fungal microbiota of grapevine. Differences among root, rhizosphere and bulk soil have been revealed by high-throughput amplicon sequencing (HTAS) analysis using 16S rRNA gene for bacteria

(Zarraonaindia et al., 2015; Marasco et al., 2018). Zarraonaindia et al. (2015) investigated the temporal and spatial dynamics of the bacterial microbiome associated with grapevine compartments of four Merlot cultivars in New York state and determined that root samples were not as diverse as the samples from bulk soil and root zone soil. Similar results were obtained by Marasco et al. (2018) when studying effects of the rootstock genotype on the selective recruitment of bacterial microbiome in the endorhizosphere and the rhizosphere compartments in a vineyard in Italy. The ecology of fungal mycobiome in grapevine is so far been obtained from the studies using pyrosequencing approach in bulk soil (Holland et al., 2016; Castañeda and Barbosa, 2017; Longa et al., 2017) or ARISA fingerprinting (Likar et al., 2017), PCR-DGGE (Manici et al., 2017) and ITS HTAS (Berlanas et al., 2019) approaches in rhizosphere soil. Previous studies showed that grapevine genotype influences the selection and recruitment of rhizosphere microbiome (Marasco et al., 2018; Berlanas et al., 2019) that may successively colonize roots and plant organs influencing the quality of the fruit (Zarraonaindia et al., 2015). In this context, knowledge of the biogeography patterns and spatio-temporal dynamics of the grapevine-associated microorganisms is fundamental to enhance crop properties. With the advent of metagenomics, vineyard productivity and management could be improved by manipulating the grapevine-associated microbiome, such as taxa related to nutrient uptake, pathogen defence and stress tolerance (Zarraonaindia and Gilbert, 2015). This manipulation of the plant microbiome has the potential to dramatically improve the yield of agronomically important crops (Turner et al., 2013).

The simultaneous study of plant microbiota, especially bacteria, across multiple compartments is common (Bulgari et al., 2012; Lundberg et al., 2012; Agler et al., 2016; Coleman-Derr et al., 2016; Dombrowski et al., 2017). However, to the best of our knowledge, there are no studies comparing fungal communities in different grapevine plant compartments. In this study, we tested the following hypotheses and questions: (1), there is a core grapevine fungal microbiome encompassing all fungal groups that are common across sites; (2) what are the changes in fungal communities and their functions at the soil-root interface?; (3) which fungi are in closer contact with grapevine roots?; and (4) fungi associated with grapevine trunk diseases frequently occur in healthy, asymptomatic endorhizosphere tissues. The objectives were therefore to (i) investigate how fungal communities are enriched in different habitats inside and outside of grapevine roots by ITS HTAS, and (ii) analyse the effects of plant compartment on the metabolic function of the fungal communities.

Materials and methods

Sample collection

Grapevine samples were collected at five young vineyards in La Rioja (Spain) which are between 2.2 and 14.9 km distant from each other (Supplementary Fig. 4.1.1; Supplementary Table 4.1.1). These vineyards were grown with Tempranillo cultivar grafted onto 110 Richter rootstock maintained under similar soil, climatic and management conditions (Supplementary Table 4.1.1). There were no significant differences in soil chemical analyses between soil types; therefore, the direct effect of soil type was not examined in the statistical analysis.

In each vineyard, three different sample types were studied in June 2017 (flowering): bulk soil, soil surrounding roots (rhizosphere soil) and roots (endorhizosphere). Four plants per vineyard were chosen to represent the same position and aspect, and four samples were randomly collected from each soil-plant compartment (bulk, rhizosphere and endorhizosphere). Sampled vines did not show any symptom of disease or nutrient deficiency. Root tissue did not show any type of rot or necrosis that could be associated with diseases caused by soilborne pathogens. A total of 60 samples were collected.

Bulk soil samples were collected with a sterile spade 1 m from each stem at depths of 40-50 cm. Homogenized soil was then passed through a 1-mm-pore size sieve and divided into two subsamples, each one for microbial community and soil chemistry analyses, respectively. Roots and rhizosphere soil samples were collected with a sterile spade close to the stem at depths of 40-50 cm, where the root system was denser. All samples were stored on dry ice in sterile bags at the time of sampling, and brought to the laboratory for immediate processing. Roots with rhizosphere soil particles attached were put in sterile tubes with 9 ml of NaCl (9 g/l). Samples were vortexed for 5 min to detach the soil particles and immediately centrifuged at 1,800g for 5 min. The supernatant was discarded and the remaining soil compartment was used to represent the rhizosphere compartment. Roots were then placed in a new tube and surface sterilized according to Cherif et al. (2015). Several washes with sterile distilled water were performed to remove any trace of contaminants. The last solution from the last rinse was cultured on malt extract agar medium supplemented with 0.35 g/l streptomycin sulphate (MEAS) to determine the efficiency of sterilization.

DNA extraction and sequencing

The bulk soil and rhizosphere DNA were extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Before DNA extraction, roots were sequentially washed in 70% ethanol and sterile distilled water. Bark was then carefully peeled out and the endorhizosphere DNA was extracted from 0.5 g using the i-genomic Plant DNA Extraction Mini Kit (Intron Biotechnology, South Korea). DNA yields from each sample were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, USA), and the extracts were adjusted to 10-15 ng/ml. The fungal ITS2 region was amplified using the universal primers ITS3/KYO2 and ITS4 (Toju et al., 2012). Primers for the second PCR were modified to include Illumina specific sequences allowing the ligation of adaptors. Genomic library was prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, USA). Purified PCR products were quantified using Agilent DNA 12,000 Kit in a Bioanalyzer 2100, the Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) in a Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, USA) and finally using MCNext™ SYBR® Fast qPCR Library Quantification Kit (MCLAB, San Francisco, USA) with the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Samples were sequenced on the MiSeq instrument (Illumina, San Diego, USA) at the Fundación FISABIO (Valencia, Spain) facility using a 2 x 300 nucleotide paired reads chemistry. Negative controls were included during the amplification and extraction steps, which were also prepared for sequencing to assess potential contaminations of the entire process.

Data analysis of the high-throughput amplification assay

Raw forward and reverse reads for each sample were assembled into paired-end reads using the fastq-join tool from the ea-tools suite (Aronesty, 2011). Sequences were then first demultiplexed and quality-filtered by retaining only those with 75% of their lengths with phred scores ≥ 30 (Glynou et al., 2018). Chimeric sequences were identified and filtered using the USEARCH tool (Edgar, 2010, 2018). The UCLUST algorithm (Edgar, 2013) in QIIME (Caporaso et al., 2010) was used to cluster sequences at a ≥97% sequence similarity against UNITE dynamic database (Abarenkov et al., 2010) for ITS reads. Operational taxonomic units (OTUs) matching mitochondrial, chloroplast or Viridiplantae sequences or with no kingdom-level classification were then removed from

the dataset. Unaligned sequences and singletons were excluded from the analysis. The dataset was optimized and each sample was rarefied to the same number of sequences per sample, that is, 30,137 fungal sequences. OTUs represented globally by less than five reads were removed (Glynou et al., 2018). The resulting quality-filtered dataset was used for the assessment of diversity and richness. The OTUs table, and corresponding taxonomic classifications, and metadata for all samples used in this study have been deposited in figshare (ID: 58,388). High-throughput amplicon sequencing data were deposited in GenBank/NCBI. Sequencing data are deposited under BioProject acc. no. PRJNA548498 and SRA study SRP201195. The group of 60 sequences (DG01-DG60) is represented by BioSamples acc. nos. SAMN12035086 - SAMN12035145.

Fungal diversity, taxonomy distribution and statistical analysis

Within sample type, alpha-diversity estimates were calculated by analyzing the Chao1 richness and Shannon diversity in Phyloseq package, as implemented in the tool MicrobiomeAnalyst (Dhariwal et al., 2017). Multiple mean comparisons using Tukey's test were performed to determine how fungal alpha-diversity differed among plant-soil compartments. *P* values were corrected for multiple comparisons using the sequential Bonferroni correction. Relationship in OTU composition among samples were investigated by calculating Bray Curtis metrics, and visualized by means of PCoA plots (Vázquez-Baeza et al., 2013) using MicrobiomeAnalyst. PERMANOVA was performed to investigate which OTUs significantly differed in abundance among experimental factors. Rarefaction curves and Good's coverage values were calculated using MicrobiomeAnalyst.

The Linear Discriminant Analysis Effect Size (LEfSe) algorithm was used to identify taxa (genus level or higher) that differed in relative abundance between soil-plant compartments (Segata et al., 2011). The online Galaxy Version 1.0 interface (http://huttenhower.sph.harvard.edu) was used; the threshold for the logarithmic Linear Discriminant Analysis (LDA) score was set at 1.0 and the Wilcoxon *p*-value at 0.05. The results are displayed in a cladogram and a bar graph. The dissimilarities between the compartments were explored by applying a Similarity Percentages (SIMPER) analysis with PRIMER 6 software. The phylogenetic groups that contribute to the dissimilarity were inferred using a table summarizing taxa at the genera and phylum levels. Unclassified OTUs amounting to less than 3% of the relative abundance were discarded from the analysis, according to Marasco et al. (2018). The fungal OTUs shared among

compartments were obtained by a Venn-diagram analysis using the software retrieved from http://bioinformatics.psb.ugent.be.

Functional prediction of fungal communities

FUNGuild v1.0 was used to investigate the function of fungal communities in the three soil-plant compartments (Nguyen et al., 2016). Twelve guilds were classified according to three trophic modes (pathotrophs, saprotrophs and symbiotrophs): arbuscular mycorrhizal fungi, ectomycorrhizal fungi, fungal endophytes, lichenized fungi, wood saprotrophs, soil saprotrophs, dung saprotrophs, litter saprotrophs, undefined saprotroph, animal pathogen, plant pathogen and lichen parasites. Three confidence ranks, namely, "possible", "probable", and "highly probable" were assigned according to the fungal database. Guilds considered "probable" and "highly probable" were selected for further analysis. All OTUs that not match taxa in the database were classified as "unassigned". ANOVA test was used with Statistix 10 software (Analytical Software) to assess the effect of soil-plant compartments on the relative abundance of OTUs inferred with FUNGuild. Transformed data means were compared using Tukey's honestly significant difference test at $P = 0.05$.

Results

High-throughput amplicon sequencing

After paired-end alignments, quality filtering and deletion of chimeric, singletons, and mitochondrial and chloroplast sequences, a total of 3,585,692 fungal internal transcribed spacer (ITS) sequences were generated from 58 samples (two samples were removed from the analysis due to the low number of sequences read) and assigned to 536 fungal OTUs (Supplementary Table 4.1.2). OTUs generated from the negative control used in the amplification step belonged only to the genus *Cladosporium* and the fungal endophyte acc. no. KY038596 GenBank/NCBI. The number of sequences of each OTU present in the negative control was subtracted from the sequence abundance of that OTU in the experimental samples according to Nguyen et al. (2015). No contamination was detected in the negative control used in the DNA extraction step. Good's coverage values in all samples ranged from 99.97 to 100%, capturing nearly all the diversity with an adequate sequencing depth (Supplementary Table 4.1.3; Supplementary Fig. 4.1.2).

Chao1 diversity estimator ranged from 63.1 ± 0.5 to 166.5 ± 3.9, while Shannon diversity estimator ranged from 1.4 ± 0.3 to 3.7 ± 0.6 (Supplementary Table 4.1.3).

Core grapevine mycobiota among vineyards

Alpha-diversity of fungal communities in soil and root samples did not differ significantly between vineyards (Table 4.1.1; Supplementary Fig. 4.1.3A and 4.1.3B). Comparing the fungal microbiota of the five vineyards, 41.4% of fungal OTUs were shared among them (Fig. 4.1.1). Specific OTUs associated with each vineyard ranged from 2.4 to 5.9% of their fungal communities (Fig. 4.1.1). The relative abundance of fungal phyla, order and family detected across all vineyards is shown in Supplementary Figure 4.1.4. The fungal phyla Ascomycota (from 50.2% to 60.6%) and Basidiomycota (from 15.0% to 20.9%) represented almost 70% of the total fungi detected (Supplementary Fig. 4.1.4A). The most abundant orders were Hypocreales (from 11.7% to 28.6%), Pleosporales (from 8.4% to 14.1%) and unidentified orders within the phylum Ascomycota (from 7.7% to 13.4%) (Supplementary Fig. 4.1.4B). The most abundant families were Nectriaceae (from 7.7% to 23.4%), Mortierellaceae (from 7.8% to 10.16%) and unidentified families within the phylum Ascomycota (from 7.7% to 13.4%) (Supplementary Fig. 4.1.4C).

Table 4.1.1. Experimental factors predicting α-diversity of bulk soil-, rhizosphere- and endorhizosphere- associated fungal communities.

	Indexes	
	Shannon	Chao1
Vineyard	$F = 2.14$	$F = 2.34$
	$P = 0.0882$	$P = 0.0760$
Plant-soil compartments	$F = 5.02$	$F = 27.79$
	$P = 0.0099$	**$P = 4.5e\text{-}09$**
Vineyard x plant-soil compartments	$F = 0.53$	$F = 1.65$
	$P = 0.0922$	$P = 0.2640$

ANOVA, analysis of variance
All P values were corrected for multiple comparisons using the sequential Bonferroni correction. Bold values indicate statistically significant results after correction for multiple comparisons. P = 0.05.

Fungal communities differed among soil-plant compartments

The alpha-diversity of fungal communities differed among soil-plant compartments (Table 4.1.1; Fig. 4.1.2), and principal coordinates analysis (PCoA) of Bray Curtis data

demonstrated that soil-plant compartment was the primary source of β-diversity (R^2 = 0.73, P < 0.001) (Fig. 4.1.3).

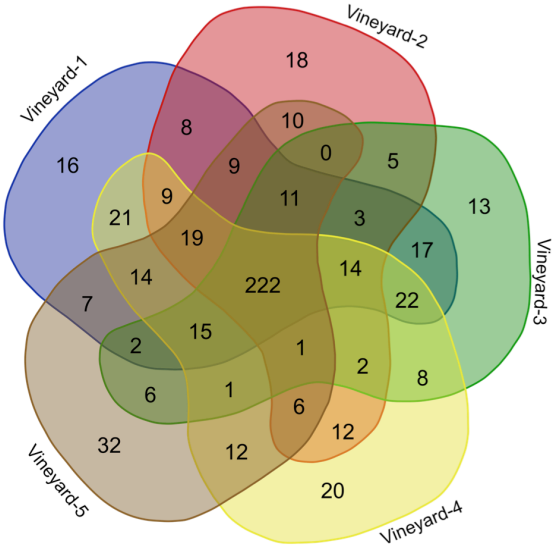

Figure 4.1.1. Venn diagram illustrating the overlap of the OTUs identified in the fungal microbiota among vineyards.

Richness and diversity of OTUs in the root compartment decreased to approximately half of the OTUs of the rhizosphere and bulk soil (Fig. 4.1.2A and 4.1.2B). The relative abundance of fungal phyla, order and family detected across all soil-plant compartments is shown in Supplementary Figure 4.1.5. Considering the three soil-plant compartments, the most abundant phyla were Ascomycota, followed by Basidiomycota and the former phylum Zygomycota (Supplementary Fig. 4.1.5A). The most abundant orders were Hypocreales, followed by Pleosporales and unidentified orders within the phylum Ascomycota (Supplementary Fig. 4.1.5B). The most abundant families were Nectriaceae, followed by unidentified families within the phylum Ascomycota, and the family Mortierellaceae (Supplementary Fig. 4.1.5C).

In bulk soil, most families were unidentified within the Ascomycota (13.9%), and the family Mortierellaceae (13.2%). The most abundant families in rhizosphere soil were unidentified families within the phylum Ascomycota (14.2%), followed by Mortierellaceae (10.7%) and Nectriaceae (10.1%). In the endorhizosphere, the most abundant families were Nectriaceae (30.7%), followed by Pleosporaceae (8.8%), unidentified families within the order Sebacinales (6.9%) and unidentified families within the phylum Ascomycota (6.0%).

Soil-plant compartments-specific and shared fungal assemblages

49.6% of fungal OTUs were shared among the three soil-plant compartments (Fig. 4.1.4). Specific OTUs associated with each soil-plant compartment ranged from 7.7 to 10.6% of their fungal communities (Fig. 4.1.4).

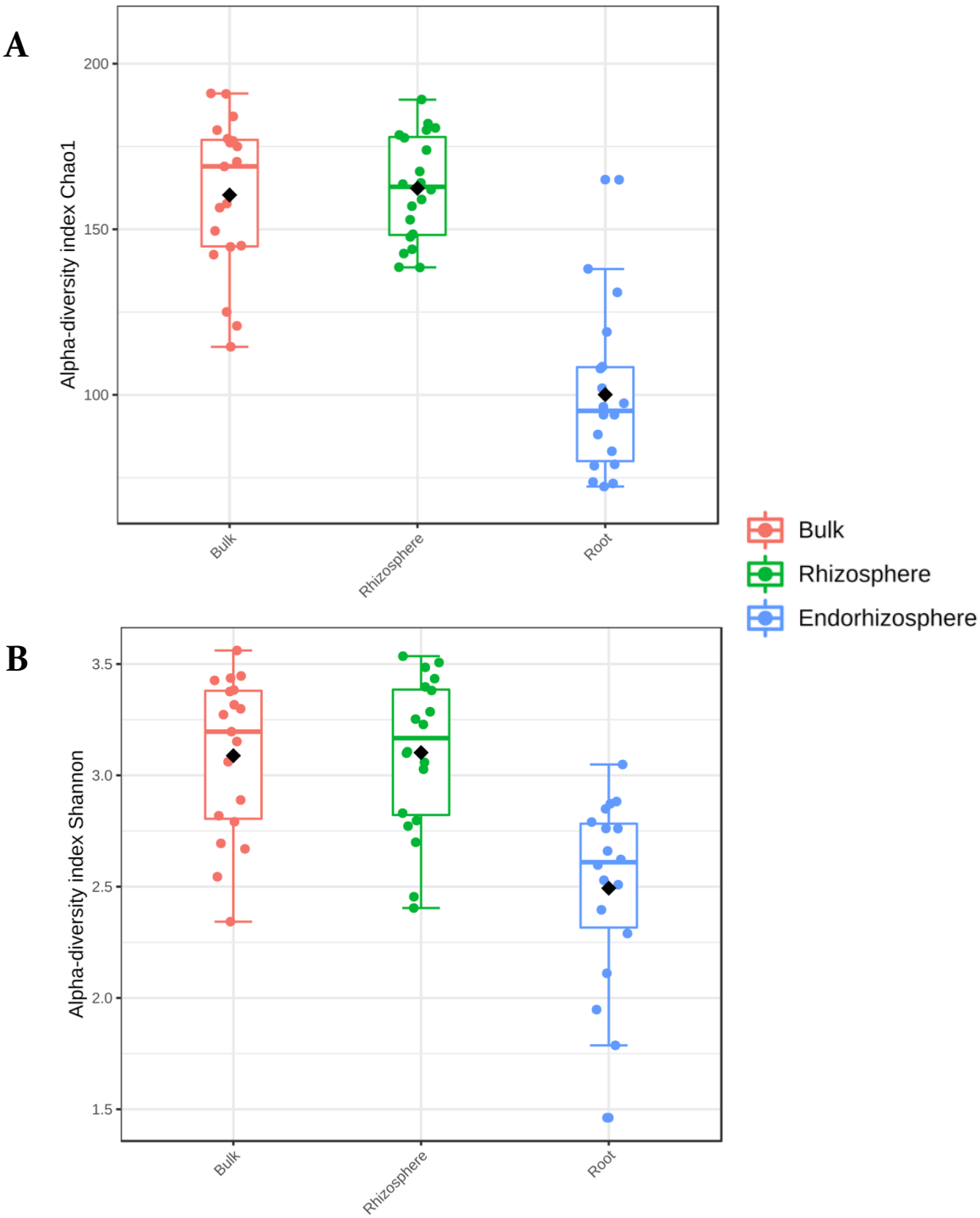

Figure 4.1.2. Boxplot illustrating the differences in Chao1 (**A**) and Shannon (**B**) diversity measures of the fungal communities in the soil-plant compartments.

The OTUs that were unique in each of the soil-plant compartments are shown in Supplementary Table 4.1.4. The linear discriminant analysis effect size (LEfSe) detected 25 fungal clades in the soil or root samples, which discriminated the microbial communities among the soil-plant compartments (Fig. 4.1.5). The rhizosphere compartment had a higher number of differentially abundant fungal clades (10) than the other compartments (8 in bulk soil and 7 in the endorhizosphere). The dominant fungal phyla were Ascomycota in bulk soil (75%) and rhizosphere soil (54.5%) compartments, and Basidiomycota in the endorhizosphere compartment (57.1%).

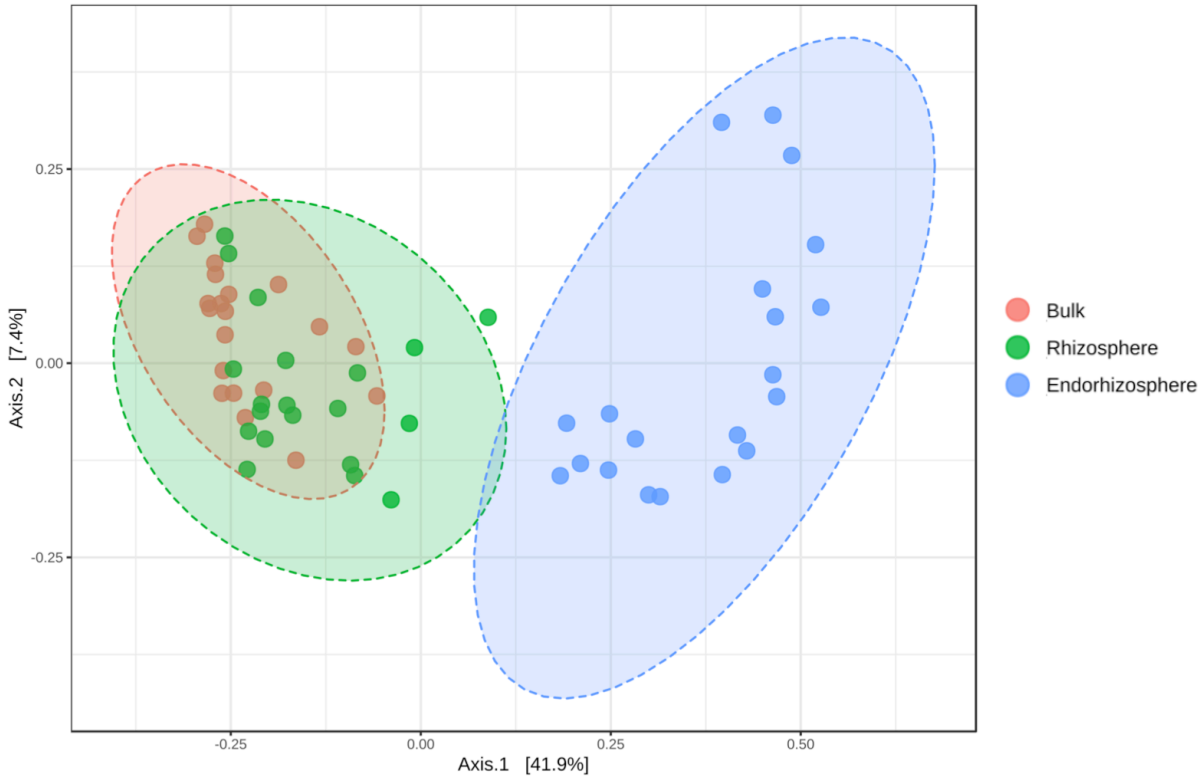

Figure 4.1.3. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the fungal communities among soil-plant compartments.

The soil-plant compartments-pairs dissimilarity, due to genera and phyla contribution in the samples, was calculated by SIMPER analysis (Supplementary Table 4.1.5). Higher microbial dissimilarity was observed between bulk soil-endorhizosphere and rhizosphere soil-endorhizosphere. No phyla contributed to differentiate the fungal communities between bulk and rhizosphere soils. Several genera were predominant and determined the dissimilarities among these soil compartments such as *Ramicandelaber*, *Clonostachys* and *Ilyonectria*. The former fungal phylum Zygomycota, and the phyla Basidiomycota and Chytridiomycota contributed to the dissimilarity between bulk soil and endorhizosphere. The fungal genera *Filobasidium*, *Pleospora* and *Psathyrella*

determined the dissimilarities among these soil-plant compartments. Unidentified Ascomycota, Zygomycota and Chytridiomycota were the major phyla that contribute to differentiate the fungal communities associated with rhizosphere soil and endorhizosphere. Several genera were predominant and determined the dissimilarities among these soil-plant compartments such as *Psathyrella*, *Malassezia* and *Filobasidium* (Supplementary Table 4.1.5).

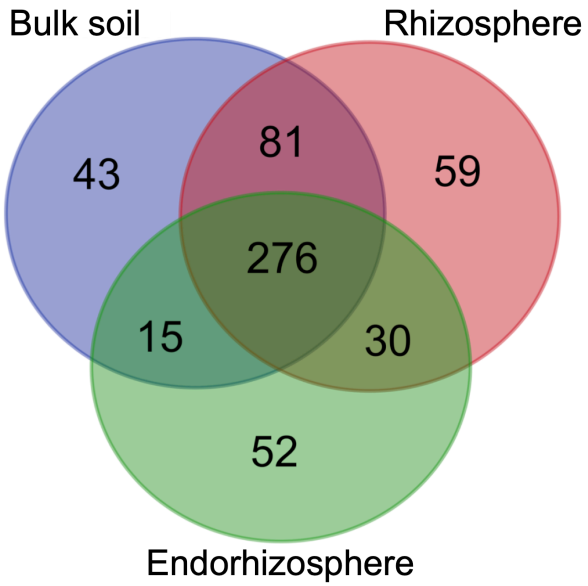

Figure 4.1.4. Venn diagrams showing the common and exclusive fungal OTUs of the soil-plant compartments.

Soil-plant compartments affect fungal functionality

Overall, a total of 74.7%, 78.3% and 78.1% of OTUs from bulk soil, rhizosphere and endorhizosphere, respectively, were identified as trophic modes with saprotrophs, symbiotrophs and pathotrophs while the remaining OTUs were unassigned (Fig. 4.1.6A; Supplementary Table 4.1.6). There were significant differences in the relative proportion of fungal functions within each soil-plant compartment ($P < 0.05$) (Supplementary Table 4.1.6). The trophic mode in bulk soil was dominated by saprotrophs which accounted for 38.7% of the total OTUs, but with no significant differences with unassigned modes (25.3%). In rhizosphere samples, there were no significant differences among trophic modes ($P > 0.05$), while saprotrophs (15.6%) were found in lower proportions than pathotrophs (29.8%), symbiotrophs (32.7%) and unassigned taxa (21.9%) in the endorhizosphere. Pathotrophs were found in the bulk soil (17.3%) in lower proportions compared with rhizosphere (25.7%) and endorhizosphere (29.8%).

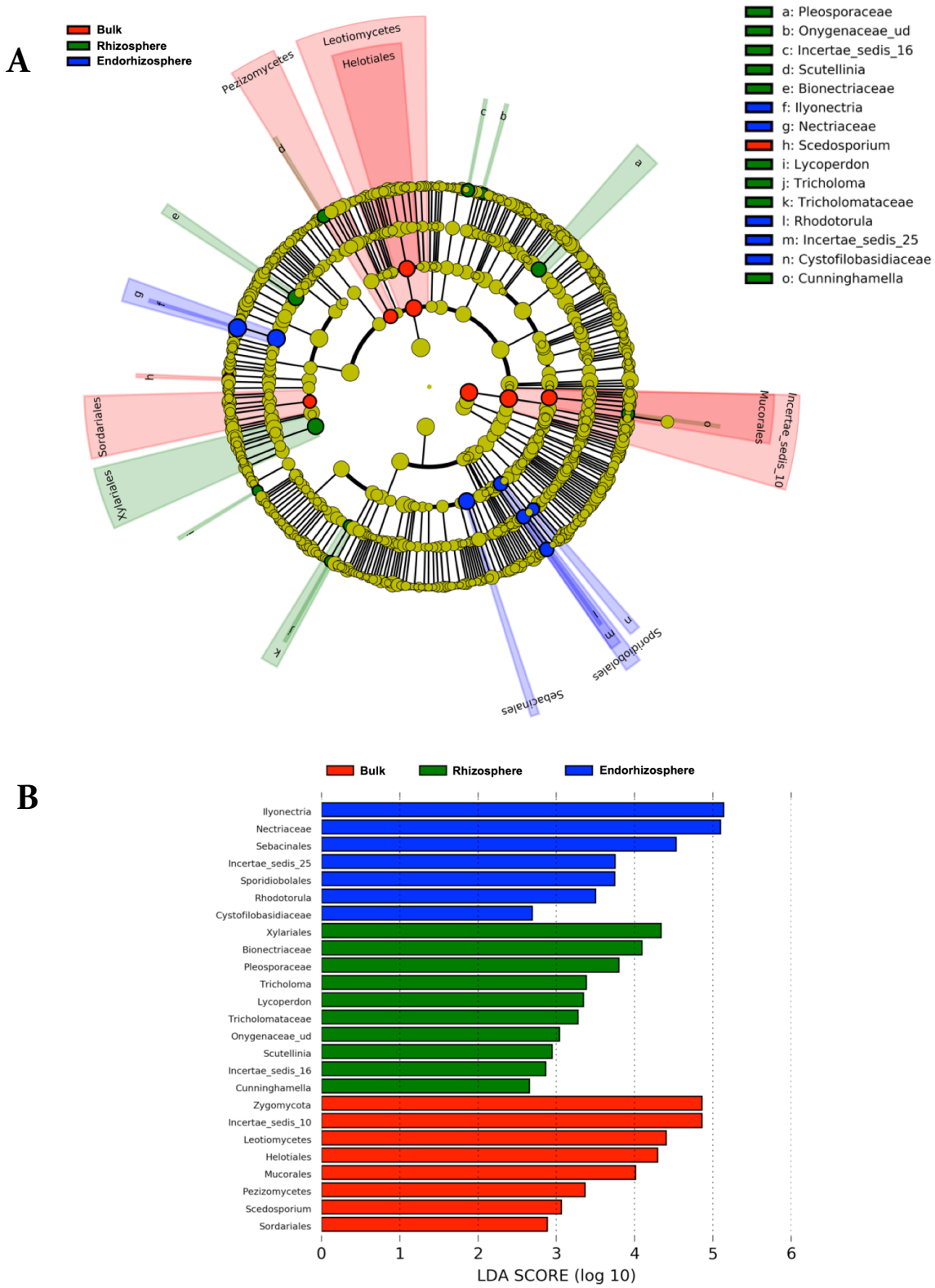

Figure 4.1.5. LEfSe was used to identify the most differentially abundant taxa among soil-plant compartments. (**A**) Cladogram generated by LEfSe indicating differences of fungi at phylum, class, family and genus levels between the three groups (relative abundance ≤ 0.5%). Each successive circle represents a phylogenetic level. Colour regions indicate taxa enriched in the different plant compartments. Differing taxa are listed on the right side of the cladogram. Bar graph showing LDA scores for fungi **(B)**. Only taxa meeting an LDA significant threshold >2 are shown.

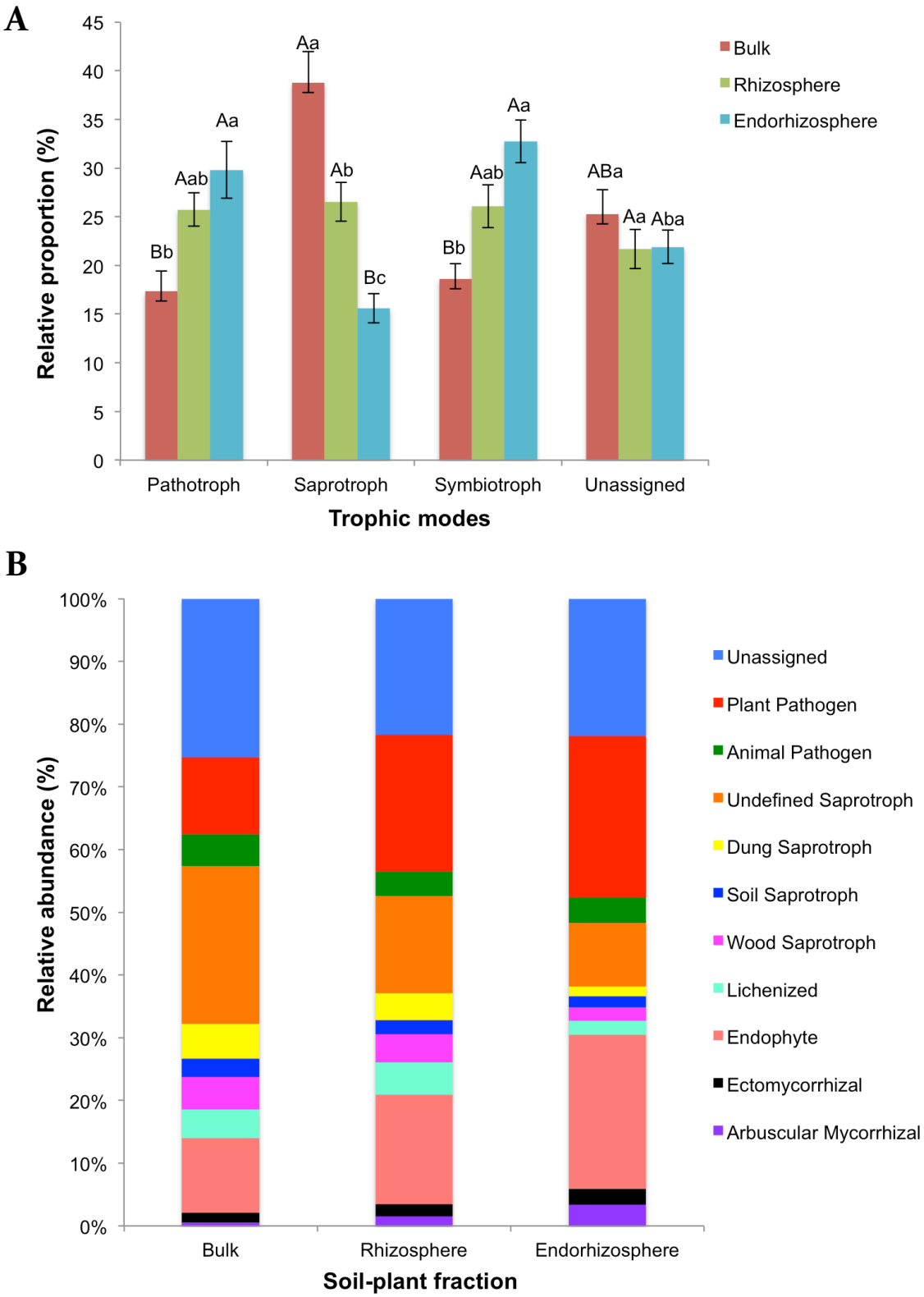

Figure 4.1.6. Variations in fungal function (**A**) and composition of fungal functional groups (guilds) inferred by FUNGuild (**B**). Tukey's test at $P < 0.05$ level. Means followed by the same letter do not differ significantly ($P < 0.05$). Capital letters are for comparison of means among functional groups within each compartment. Small letters are for comparison of means among plant-soil compartments within each functional group.

Regarding the saprotrophs, these organisms were found in bulk soil (38.7%) in higher proportions compared with rhizosphere (26.5%) and endorhizosphere (15.6%). Symbiotrophs were more frequently detected in the endorhizosphere (32.7%) compared to the bulk soil (18.6%) and the rhizosphere (26.1%) (Supplementary Table 4.1.6; Fig. 4.1.6A).

In the symbiotrophs group, the relative abundances of arbuscular mycorrhizal and ectomycorrhizal in the endorhizosphere compartment (3.40 and 2.48%, respectively) were significantly higher than in the other compartments ($P < 0.05$) (Fig. 4.1.6B; Supplementary Table 4.1.7). Endophytes were significantly more abundant in the rhizosphere (17.50%) and the endorhizosphere (24.61%) than in bulk soil (11.80%), while the relative abundances of lichenized fungi in the rhizosphere (5.1%) and bulk soil (4.64%) compartments were significantly higher than in the endorhizosphere (2.09%). In the pathotroph group, the relative abundances of plant pathogens in the endorhizosphere (25.70%) and rhizosphere (21.84%) compartments were significantly higher than in the bulk soil (12.22%) ($P < 0.05$) (Fig. 4.1.6B; Supplementary Table 4.1.7). There were no significant differences in the relative abundance in animal pathogens among soil-plant compartments ($P > 0.05$). In the saprotroph group, the relative abundances of wood and dung saprotrophs in the bulk and rhizosphere compartments were significantly higher than in the endorhizosphere ($P < 0.05$) (Fig. 4.1.6B; Supplementary Table 4.1.7). Undefined saprotrophs were detected in higher abundances in bulk (25.12%) compared to endorhizosphere (10.16%); but no significant differences were found with the rhizosphere compartment (15.5%). No significant differences were found in the relative abundance of soil saprotrophs among soil-plant compartments ($P > 0.05$) (Fig. 4.1.6B; Supplementary Table 4.1.7).

Discussion

This study is the first comprehensive effort to compare different microhabitats at the soil-grapevine root interface and evaluate the shifts in fungal community structure from bulk soil towards grapevine endorhizosphere, by ITS HTAS approach. We detected community changes in bulk and rhizosphere soils compared to grapevine endorhizosphere, with diversity decreasing in the endophyte compartment. Analysing ITS HTAS did not reveal major differences in diversity between the rhizosphere soil and bulk soil of grapevine. Our results are in accordance with previous research aimed to

unravel the bacterial microbiome of grapevine (Zarraonaindia et al., 2015) and other hosts (Bulgarelli et al., 2012; Lundberg et al., 2012; Ofek et al., 2014). This study represents the first approach to comparatively assess fungal communities in association with grapevine in different compartments by using a high-throughput sequencing approach. Although the ITS region, comprising the ITS1, 5.8S, and ITS2 segments, was selected by The Fungal Barcoding Consortium (Schoch et al., 2012) as the formal DNA barcode for the Fungal Kingdom using the same gene section proposed by White et al. (1990), some recent reports highlight its limitations for specific taxa. This region does not work well with taxa having narrow or no barcode gaps in their ITS regions, such as *Trichoderma* or *Fusarium* (Schoch et al., 2012). Moreover, the correct identification of morphologically similar cryptic species using the ITS regions is still problematic due to the lack of consensus in the lineage-specific cut-off value for species determination (Nilsson et al., 2008). There is also a lack of consensus regarding which ITS (ITS1 or ITS2) to use as a barcode (Blaalid et al., 2013), and still remains unclear which of the ITS primers sets has the best resolution for fungal diversity (Banos et al., 2018).

The major fungal phyla detected in our study were largely composed of Ascomycota and Basiodiomycota that accounted for almost 75% of the relative abundance in all vineyards. Previous studies conducted on grapevine soil fungal communities share this taxonomic pattern, indicating that the selective forces shaping fungal root microbiome composition at a high taxonomic rank are consistent against several environmental conditions (Castañeda and Barbosa, 2017; Longa et al., 2017; Manici et al., 2017; Berlanas et al., 2019). Soil fungal assemblages had extensive local homogeneity across small distances, and soils with similar climatic conditions and homogenous edaphic variables. These results suggest that vineyard fungal communities are partially conserved in La Rioja region.

The fungal community structure in the endorhizosphere was distinct to that found in the other compartments. Bulk and rhizosphere communities were composed mostly by Ascomycota, followed by Basidiomycota and the former phylum Zygomycota. However, Basidiomycota and the former phylum Zygomycota community members were consistently enriched and depleted, respectively, in the endorhizosphere compared to their surrounding soil biome. Some endophytes can be recruited from soil, but others are rarely found in the soil and may be transmitted with grapevine planting material. In fact, several taxa such as *Diplodia* or *Phaeomoniella*, were observed in the endorhizosphere but were not observed in bulk or rhizosphere soil. *Diplodia* and *Phaeomoniella* are two

fungal genera associated with grapevine trunk diseases. Species of these genera are primarily spread through the dispersion of airborne spores or through the use of infected cuttings (Gramaje et al., 2018). The presence of *Diplodia* in roots could have implications in the epidemiology of the disease since plant debris could become a potential inoculum source from which *Diplodia* spp. are rain splashed to aerial plant parts such as pruning wounds. The Ascomycete fungus *Phaeomoniella chlamydospora* can also be inhabitant of soil (Saccà et al., 2018) and penetrate into the plant through the root system or the base of the rootstock (Agustí-Brisach et al., 2013).

Models of root selection for soil microbiota have been proposed (Bulgarelli et al., 2013; Reinhold-Hurek et al., 2015). Roots provide a nutrient-rich niche for microbes through the secretion of vast amounts of photosynthetically fixed carbon as exudates that contain amino acids, carbohydrates, organic acid ions, and vitamins (Bais et al., 2006). In addition, roots can produce a great amount of organic compounds, contributing to nutrient depletion and gas exchange, thereby influencing the biological activity in the soil and on soil structure (Bertin et al., 2003). These factors may contribute to the gradients imposed by roots, leading to a refinement of the microbiome in the rhizosphere soil. The communities are strongly refined on the rhizoplane, with significant influence of the plant genotype. Research has just begun to uncover how grapevine shapes its microbiome. Different rootstocks are able to differentially select bacterial and fungal communities from the surrounding soil (Marasco et al., 2018; Berlanas et al., 2019), suggesting that rootstocks may affect further endophytic colonization, as it has been shown to play a key role in pathogen resistance (Keller, 2010). Finally, a least complex, highly specialized and taxonomically distinct microbiome is found in the endorhizosphere (Raaijmakers et al., 2008).

Prediction of fungal functional groups also varied among soil-plant compartments. Interestingly, an increase in the relative abundances of potential plant pathogens, endophytes and arbuscular mycorrhiza, and a decrease in the relative abundances of potential wood, dung and undefined saprotrophs were predicted from bulk soil towards the endorhizosphere. The rhizosphere compartment is the infection niche where soilborne pathogens can establish a parasitic relationship with the host plant. To proceed with root infection, pathogens have to compete with other members of the rhizosphere microbiome for available microsites and nutrients (Chapelle et al., 2016). The root immune system together with root exudates would finally select for those microbes that have adapted and produced mechanisms to colonize the endorhizosphere.

Potential grapevine pathogens found in the non-necrotic inner tissues of surface sterilized symptomless roots included fungal species belonging to genera associated with grapevine trunk diseases, namely *Botryosphaeria* and *Diplodia* (Botryosphaeria dieback), *Cadophora*, *Phaeoacremonium* and *Phaeomoniella* (Petri disease), *Diaporthe* (Diaporthe dieback) and *Ilyonectria* (black-foot disease) (Gramaje et al., 2018). This finding suggests that the root interior of asymptomatic vines, the endorhizosphere, is a microbial niche that is inhabited by fungi that act as endophytic and/or latent pathogens, and might become pathogenic under specific conditions. In particular, the enrichment of the genus *Ilyonectria* by HTAS from asymptomatic plants supports this statement. It is also important to note that *Ilyonectria* and related fungi seem to be frequent in roots and the endosphere of multiple plants, not only grapevine. Several studies show that they are indeed among the dominant fungi found in roots of several plants (Xu et al., 2015; Yang et al., 2018), and the same is true for dominant groups found in our study like the Nectriaceae and the Pleosporaceae (Glynou et al., 2016, 2018; Almario et al., 2017). This indicates that these fungi are not specific to grapes, but that they are common root-associated and endophytic fungi in plants. Other interesting taxa detected in the asymptomatic inner tissues of the roots included *Trichoderma atroviride* and *Aureobasidium pullulans*, two fungal species with high biocontrol potential against pathogens associated with Petri disease of grapevine (Pertot et al., 2016) and the pathogenic fungus *Diplodia seriata* (Pinto et al., 2018) respectively, and *Rhizophagus irregularis*, a mycorrhizal fungus tested with high efficacy against pathogens associated with black-foot disease of grapevine (Petit and Gubler, 2006).

In conclusion, our results show that fungal communities were largely affected in their diversity, composition and functionality by soil-plant compartment, whereas the spatial variation (i.e. across vineyards) was low. The endorhizosphere compartment was the one that differed the most from the other two, suggesting that the root tissues entail a barrier for fungal colonization. This study provides new advances in the knowledge on the fungal communities of grapevines and some the factors shaping it. In addition, it opens up new perspectives in the study of the endophytic role of several potential fungal pathogens on grapevines.

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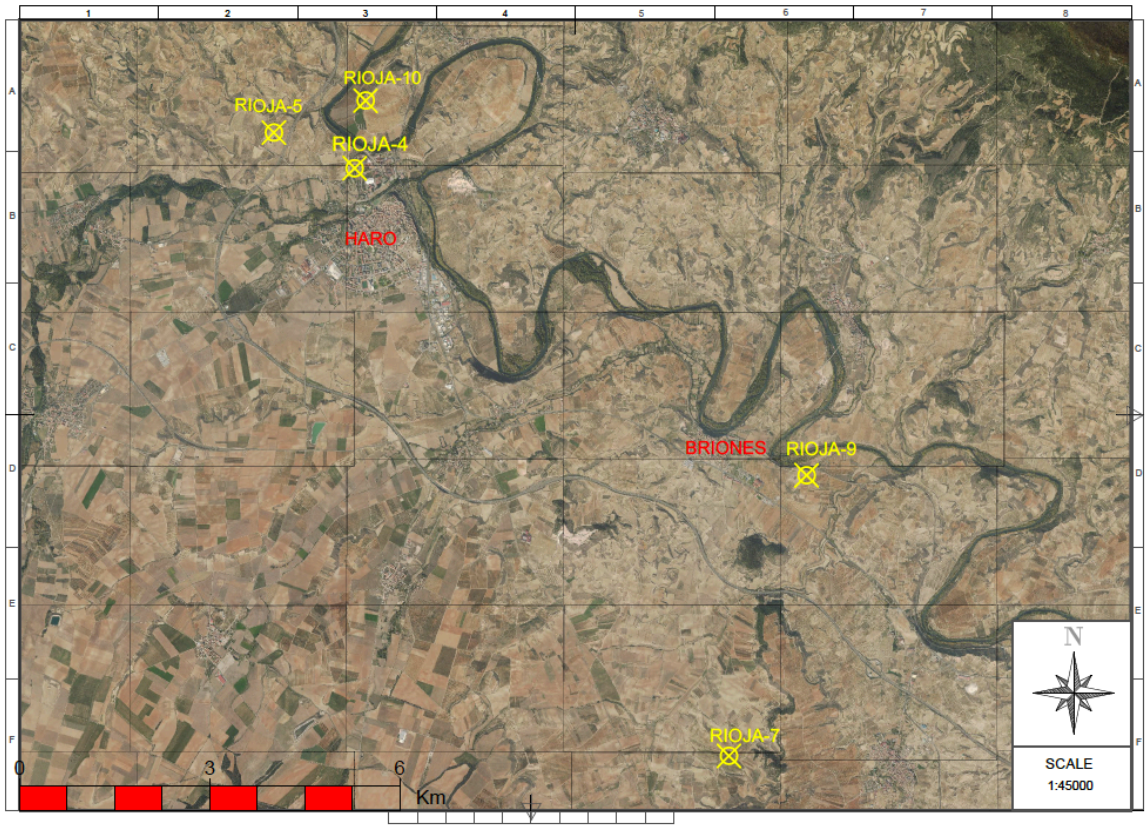
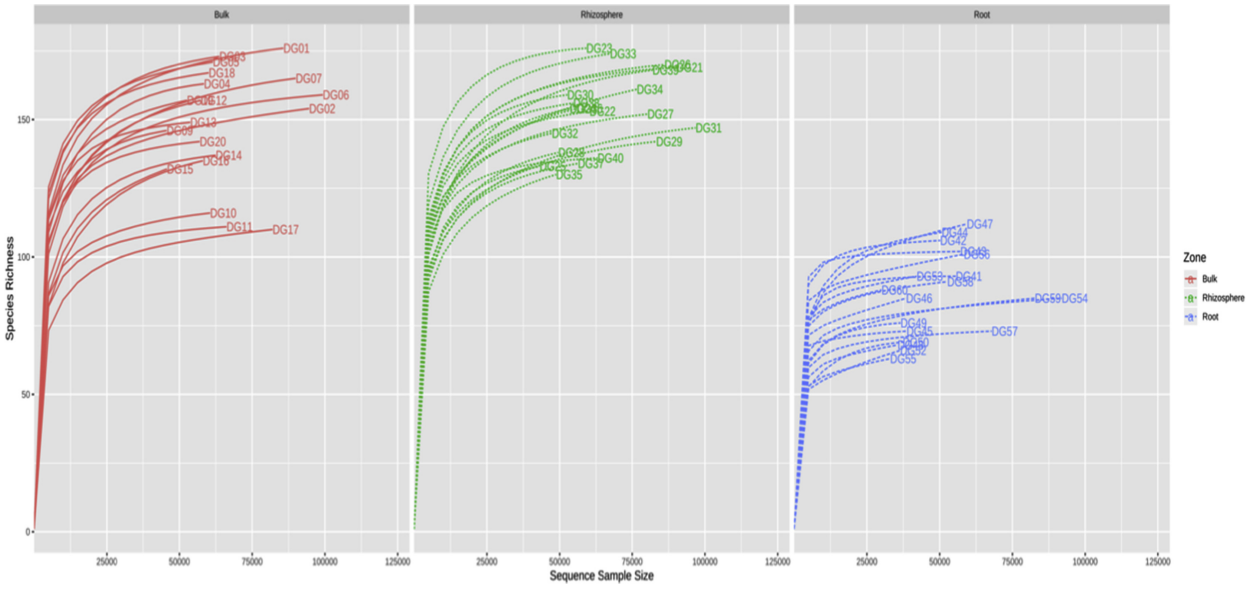
Chapter 4.1.

Supplementary material

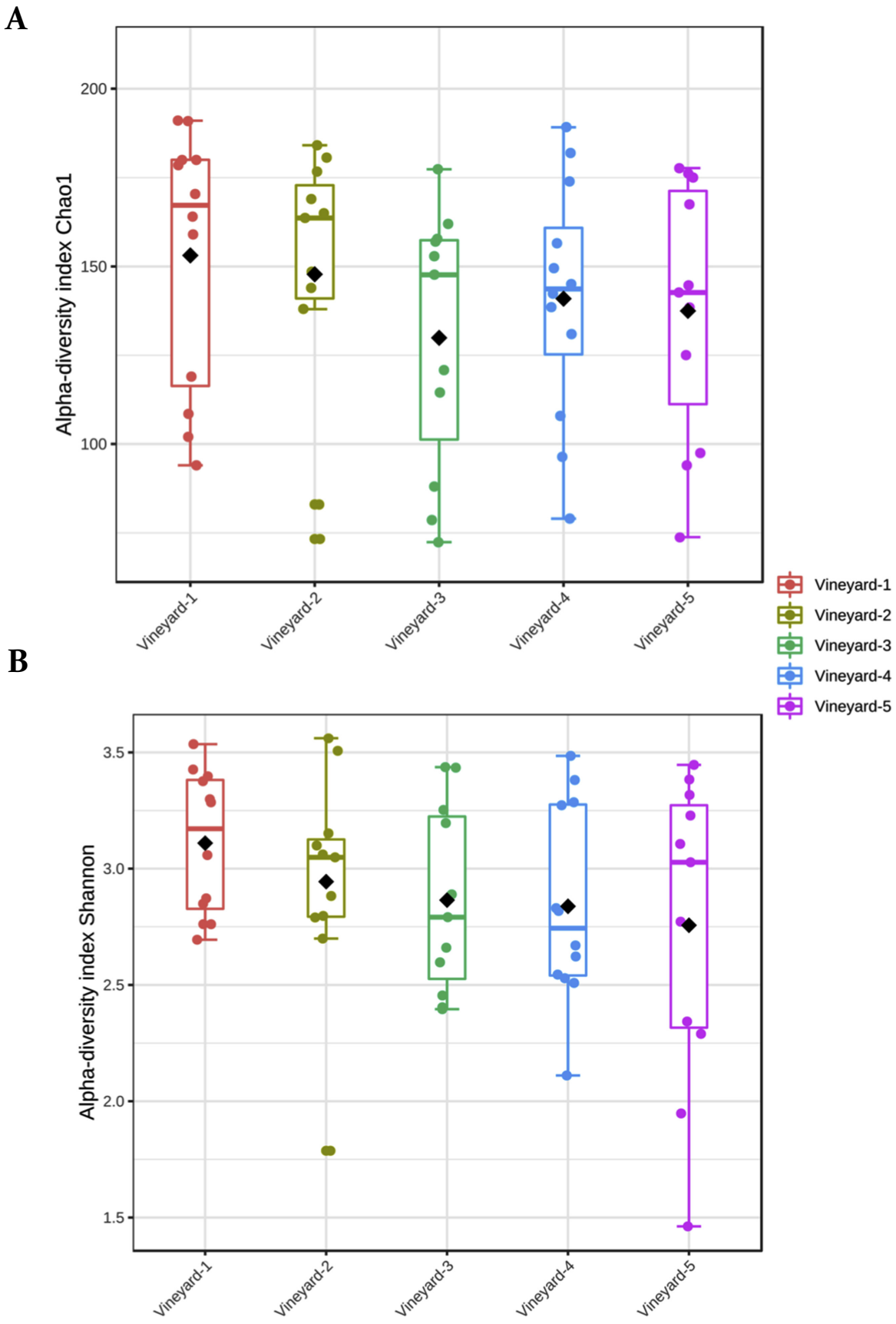
Supplementary **Figures**



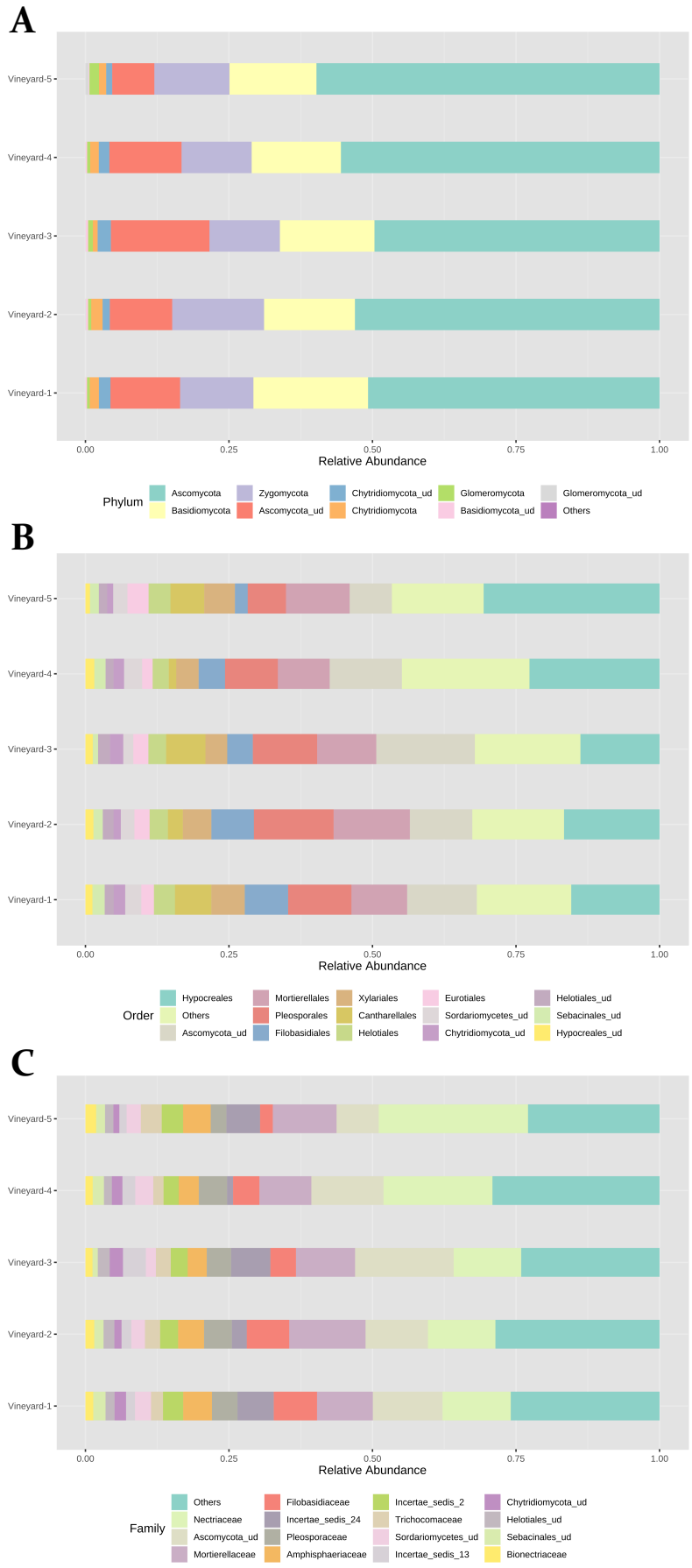
Supplementary Figure 4.1.1. Location of the five vineyards surveyed in this study.



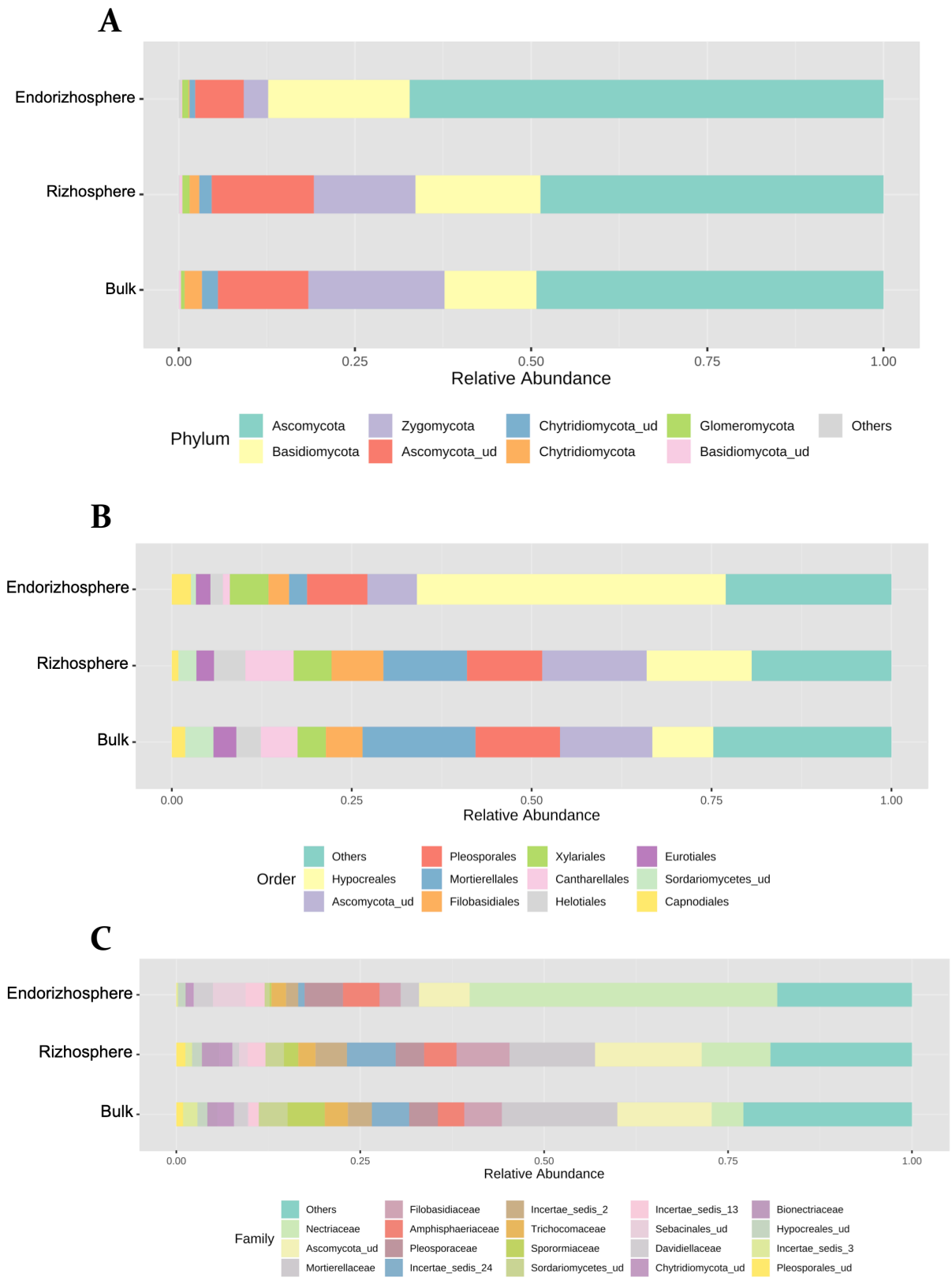
Supplementary Figure 4.1.2. Rarefaction curve values for each sample.



Supplementary Figure 4.1.3. Boxplot illustrating the differences in Chao1 (**A**) and Shannon (**B**) diversity measures of the fungal communities among vineyards.



Supplementary Figure 4.1.4. Relative abundance of different fungal phyla (**A**), orders (**B**) and families (**C**) in the five vineyards.



Supplementary Figure 4.1.5. Relative abundance of different fungal phyla (**A**), orders (**B**) and families (**C**) in the soil-plant compartments.

Supplementary **Tables**

Supplementary Table 4.1.1. Physicochemical properties and soil management practices of the five vineyard soils examined in this study. Values represent the mean±SE.

	Vineyard-1	**Vineyard-2**	**Vineyard-3**	**Vineyard-4**	**Vineyard-5**
Coordinates	42,583560º, -2,853296º	42,588604º, -2,868726º	42,539882º, -2,766227º	42,499781º, -2,781054º	42,593068º, -2,851397º
Location	Haro	Haro	Briones	Briones	Haro
Year of plantation	2013	2013	2014	2012	2013
Extension (ha)	0.93	8.32	3.00	5.11	1.96
Altitude (m)	478	482	501	497	499
Physicochemical properties					
pH	8.1[a]±0.02	8.1±0.02	8.2	8.2	8.2±0.02
P mg/100g	3.39±0.25	2.6±0.2	2.4±0.3	3.1±0.3	3.2±0.2
K mg/100g	17.2±0.7	17.1±0.4	16.4±0.5	19.1±0.6	20.5±0.5
S mg/100g	4.3±0.5	3.9±0.4	4.1±0.3	4.1±0.4	4.2±0.4
Mg mg/100g	24.3±0.3	27.1±0.3	25.7±0.5	21.4±0.4	26.7±0.5
Mn mg/100g	3.5±0.8	2.9±0.7	2.8±0.7	2.8±0.7	2.8±0.8
Fe mg/100g	8.6±0.4	8.7±0.2	10.6±0.2	7.7±0.4	8.7±0.5
Ca mg/100g	3979.6±220.8	4346.9±120.4	4347.5±109.8	3731.3±176.4	3503.0±126.7
Na mg/100g	2.4±0.2	2.83±0.1	3.1±0.2	3.0±0.2	3.4±0.2
SOM%	0.95±0.03	1.05±0.2	1.05±0.03	0.95±0.05	1.07±0.08
Clay%	18.5±0.2	22.2±0.3	25.3±0.3	21.9±0.6	22.9±0.4
Sand%	39.9±0.3	33.8±0.4	34.3±0.5	35.1±0.6	34.3±0.9
Silt%	41.6±0.6	44.0±1.1	40.4±0.8	43.0±0.7	42.8±0.5
CO_3Ca	14.15±0.02	15.67±0.23	13.9±0.1	14.5±0.4	14.9±0.6
CEC mekv/100g	8.9±0.3	10.4±0.1	11.2±0.2	9.6±0.2	9.1±0.3
EC mS/cm	0.14	0.16	0.16	0.15	0.16
Assim. Ca mekv/100g	15.1±0.2	17.4±0.2	16.5±0.2	15.5±0.3	14.8±0.4
Assim. Mg mekv/100g	1.57±0.05	1.81±0.11	1.75±0.12	1.70±0.15	1.63±0.21
Soil temperature (ºC) (July)	20.9	21.0	21.6	21.3	20.9
Soil management practices					
Irrigation system	Drip irrigation				
Fertilization	2 applications per year				
Pest management practices	5 spray treatments against powdery and downy mildew per year				
Herbicide treatment	Yes				

[a]Average of 4 replicates.

Supplementary Table 4.1.2. Number of reads, total OTUs, richness (Chao1 estimates of species richness) or diversity (Shannon's index of diversity) indices expressed as average and standard deviation.

Index	Bulk soil				
	Vineyard-1	Vineyard-2 [b]	Vineyard-3	Vineyard-4	Vineyard-5
Reads	73659.7±9308.4 [a]	82814.7±9937.4	55402.7±4388.0	54548.2±3592.9	61467.5±6853.3
OTUs	274	257	260	246	175
Chao1	158.9±4.4	157.2±3.6	127.7±4.1	142.4±6.1	141.6±4.9
Shannon	3.2±0.3	3.2±0.3	3.0±0.2	2.8±0.3	3.1±0.1

Index	Rhizosphere				
	Vineyard-1	Vineyard-2	Vineyard-3	Vineyard-4	Vineyard-5
Reads	65084.7±8273.7	64270.7±10635.6	68953.2±11698.8	61211.7±6375.6	63413.5±6303.2
OTUs	247	250	267	228	196
Chao1	154.7±2.1	144.3±3.2	137.9±2.4	151.3±5.2	143.9±3.2
Shannon	3.3±0.2	3.0±0.2	2.8±0.6	3.2±0.3	3.0±0.2

Index	Root				
	Vineyard-1	Vineyard-2	Vineyard-3 [b]	Vineyard-4	Vineyard-5
Reads	53186.7±1783.8	42370.0±5637.2	35891.7±460.3	55019.5±12922.2	57051.0±11481.3
OTUs	271	288	267	255	143
Chao1	100.6±2.4	98.4±14.1	73.3±6.8	96.1±12.2	84.3±4.3
Shannon	2.8±0.1	2.6±0.5	2.3±0.4	2.4±0.3	2.3±0.5

[a] Values are the mean of 4 replicates.

[b] Samples DG8 and DG51 were removed from the analysis due to the low number of sequences reads.

Supplementary Table 4.1.3. Estimates of number of reads, sample coverage and diversity indices at the genus level for fungal profiles.

Sample ID	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
DG01	85164	99.99	165.8±2.8	3.4±0.6
DG02	93812	99.99	155.0±8.4	2.7±0.5
DG03	58080	99.99	166.5±3.9	3.3±0.8
DG04	57580	99.99	152.1±2.2	3.3±0.5
DG05	60530	100	158.1±2.5	3.5±0.6
DG06	98706	99.99	155.5±4.1	3.0±0.5
DG07	89208	99.99	158.0±4.1	3.1±0.5
DG09	43949	99.99	138.1±2.3	3.4±0.6
DG10	58995	99.99	111.6±2.8	2.8±0.4
DG11	64628	99.99	106.0±2.8	2.8±0.4
DG12	54039	99.97	155.1±8.2	3.2±0.5
DG13	52597	99.99	150.0±3.4	3.2±0.6
DG14	62214	99.99	137.0±3.6	2.7±0.5
DG15	45526	99.98	148.4±13.1	2.8±0.5
DG16	57856	99.98	134.2±3.9	2.5±0.4
DG17	81494	99.99	111.3±8.8	2.3±0.3
DG18	58916	99.99	155.8±2.8	3.4±0.7
DG19	51435	99.99	161.3±5.7	3.4±0.5
DG20	54025	99.99	137.8±2.2	3.3±0.6
DG21	89506	99.99	163.5±0.9	3.2±0.6
DG22	59588	99.99	146.1±2.5	3.4±0.6
DG23	58430	99.99	163.3±0.7	3.5±0.9
DG24	52815	99.99	146.0±4.3	3.0±0.5
DG25	42921	99.99	138.5±4.5	2.7±0.8
DG26	85199	99.99	154.5±0.9	3.5±0.9
DG27	79705	99.99	147.1±2.5	3.0±0.5
DG28	49258	99.98	137.1±4.9	2.8±0.5
DG29	82824	99.99	134.0±2.8	2.4±0.4
DG30	51981	99.99	146.5±1.8	3.4±0.6
DG31	94557	99.99	133.0±2.8	2.4±0.5
DG32	46451	99.99	138.4±1.9	3.2±0.9
DG33	66910	99.99	160.4±1.9	3.5±0.6
DG34	76271	99.99	164.3±8.1	3.4±0.6
DG35	48274	99.99	121.2±2.9	2.8±0.4
DG36	53392	99.98	159.4±8.1	3.2±0.5
DG37	55920	99.98	129.4±5.4	3.0±0.6
DG38	53796	99.99	154.5±3.7	3.2±0.5
DG39	81501	99.99	157.0±2.1	3.7±0.6
DG40	62437	99.99	135.0±1.6	3.1±0.6
DG41	55483	100	92.0±0.4	2.7±0.6

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Supplementary Table 4.1.3. (*Continued from previous page*)

Sample ID	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
DG42	49680	99.99	100.5±1.3	2.7±0.5
DG43	56945	99.99	99.0±0.2	2.8±0.4
DG44	50639	99.98	111.0±8.0	2.8±0.4
DG45	38001	99.99	71.3±0.2	2.9±0.3
DG46	37171	99.97	124.0±13.4	3.0±0.5
DG47	59184	99.98	126.5±14.7	2.8±0.4
DG48	35124	99.98	72.0±10.4	1.8±0.5
DG49	36038	99.99	74.7±1.4	2.5±0.5
DG50	37135	99.99	63.1±0.5	2.6±0.6
DG52	35270	99.99	83.3±15.1	2.4±0.5
DG53	41130	99.99	118.0±21.7	2.5±0.5
DG54	91201	99.99	92.3±8.8	2.1±0.4
DG55	32636	99.99	65.0±5.5	2.5±0.5
DG56	55138	99.99	109.0±12.8	2.6±0.5
DG57	67426	99.99	71.7±1.4	1.9±0.4
DG58	50535	99.99	85.5±2.9	2.2±0.5
DG59	81809	99.99	91.3±8.8	1.4±0.3
DG60	28434	99.98	88.7±4.2	3.5±0.8

Supplementary Table 4.1.4. Fungal OTUs that were unique in each soil-plant compartment.

Bulk soil	Rhizosphere	Endorhizosphre
Ajellomycetaceae_ud	Albatrellaceae_ud	*Adisciso*
Archaeorhizomyces	Archaeosporales_ud	Ampelomyces
Asterostroma	*Aspergillus*	*Baeospora*
Athelia	Atheliaceae_ud	*Beauveria*
Aureobasidium	*Botryosphaeria*	Botryosphaeriaceae_ud
Ceratobasidium	*Cadophora*	*Bovista*
Chaetothyriales_ud	*Ceriporiopsis*	*Camarosporium*
Clavicipitaceae_ud	*Chaetomium*	Capnodiales_ud
Clavulina	*Chaetosphaeria*	*Catenulostroma*
Clonostachys	*Coprinellus*	Ceratobasidiaceae_ud
Coprinopsis	Cystofilobasidiales_ud	*Cladorrhinum*
Cytospora	*Cystofilobasidium*	*Coniella*
Dendryphion	*Eucasphaeria*	*Coniothyrium*
Dipodascaceae_ud	Erysiphaceae_ud	Diatrypaceae_ud
Entoloma	Filobasidiales_ud	*Diplodia*
Geomyces	*Filobasidium*	Eurotiomycetes_ud
Gigasporaceae_ud	Fomitopsidaceae_ud	Exobasidiomycetes_ud
Glomerellaceae_ud	Gaeumannomyces	*Exophiala*
Gymnopilus	*Ganoderma*	*Geminibasidium*
Hygrophoraceae_ud	Geastraceae_ud	*Golovinomyces*
Hyphodermella	Glomerales	Gymnoascaceae_ud
Hyphodontia	Glomeromycetes_ud	*Hypholoma*
Lachancea	Hymenochaetaceae_ud	Hypocreales_ud
Langermannia	*Hypomyces*	Incertae_sedis_25_ud
Lophiostoma	Hyponectriaceae_ud	*Lacrymaria*
Macrophomina	Incertae_sedis_11_ud	*Lecanicillium*
Melanoleuca	Incertae_sedis_13_ud	*Leohumicola*
Microbotryomycetes_ud	*Lecanora*	*Leptosphaeria*
Monacrosporium	*Lentinula*	*Letendraea*
Mucoraceae_ud	Malasseziales	*Leucoagaricus*
Ophiocordyceps	*Massarina*	*Magnaporthe*
Ophiocordycipitaceae_ud	*Minimedusa*	Marasmiaceae

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Supplementary Table S4. (*Continued from previous page*)

Bulk soil	Rhizosphere	Endorhizosphre
Phaeococcomyces	*Myrothecium*	Microascales_ud
Pochonia	*Neophaeosphaeria*	*Microdochium*
Rhizophydiales	*Penidiella*	*Myceliophthora*
Thelephoraceae_ud	*Peniophora*	*Ophiosphaerella*
Trichocomaceae_ud	Pezizaceae_ud	*Periconia*
Urocystis	*Pleurotus*	*Phaeomoniella*
Vibrisseaceae_ud	*Pluteus*	Polyporales_ud
Wallrothiella	Powellomyces	*Pyrenochaetopsis*
Xenasmatella	Pseudeurotiaceae_ud	*Rhinocladiella*
Xylomelasma	Pucciniaceae_ud	Rutstroemiaceae_ud
	Pyrenochaeta	*Scolecobasidium*
	Rhizopus	*Thermoascus*
	Saccharomycetaceae_ud	*Tomentella*
	Schwanniomyces	*Tomentellopsis*
	Scytalidium	*Tubulicrinis*
	Sporormiella	*Uromyces*
	Tremellales	*Wallemia*
	Trichocladium	Wallemiomycetes_ud
	Ustilaginales_ud	*Xylodon*
	Zygomycota_ud	

Supplementary Table 4.1.5. Similarity percentages (SIMPER) analysis determines the fungal phyla (**A**) and genera (**B**) contributions to the dissimilarity among soil-plant compartments. In the upper part of the table the soil-plant compartments pairwise comparison of average dissimilarity percentage has been reported. In the lower part, the overall top three phyla/genera contributing to the pairwise dissimilarity were listed, reporting in parenthesis their relative contribution to the observed dissimilarity expressed as percentage.

(A) SIMPER analysis determined the fungal phyla contributions in five vineyards

	Bulk soil	**Rhizosphere soil**	**Root**
Bulk soil		nd[†]	32.1[#]
Rhizosphere soil	nd		28.2[#]
Root	Zygomycota (23.5) Basidiomycota (12.7) Chytridiomycota (11.5)	Ud_Ascomycota (12.5) Zygomycota (10.6) Chytridiomycota (7.7)	

[#] Soil-plant compartments-pairs showing dissimilarity in phyla distribution higher than 10%.

[†] Soil-plant compartments-pair showing the lowest dissimilarity observed in phyla distribution.

(B) SIMPER analysis determined the fungal genera contributions in five vineyards

	Bulk soil	**Rhizosphere soil**	**Root**
Bulk soil		7.5[†]	34.8[#]
Rhizosphere soil	*Ramicandelaber* (21.3) *Clonostachys* (13.5) *Ilyonectria* (6.5)		29.9[#]
Root	*Filobasidium* (25.0) *Pleospora* (24.4) *Psathyrella* (21.0)	*Psathyrella* (17.5) *Malassezia* (9.1) *Filobasidium* (9.0)	

[#] Soil-plant compartments-pairs showing dissimilarity in genera distribution higher than 10%.

[†] Soil-plant compartments-pair showing the lowest dissimilarity observed in genera distribution.

nd: no dissimilarity.

Supplementary Table 4.1.6. Relative proportion (%) of fungal function from soil-plant compartments inferred by FunGuild.

	Pathotroph	**Saprotroph**	**Symbiotroph**	**Unassigned**
Bulk	17.3± 2.1 Bb	38.7±3.2 Aa	18.6±1.6 Bb	25.3±2.5 ABa
Rhizosphere	25.7±1.7 Aab	26.5±2.0 Ab	26.1±2.2 Aab	21.7±2.0 Aa
Endorhizosphere	29.8±2.9 Aa	15.6±1.5 Bc	32.7±2.2 Aa	21.9±1.7 ABa

Tukey's test at $P < 0.05$ level. Means followed by the same letter do not differ significantly ($P < 0.05$). Capital letters are for comparison of means in the same row. Small letters are for comparison of means in the same column.

Supplementary Table 4.1.7. Compositions and relative abundance (%) of fungal functional groups (guild) inferred by FunGuild.

	Symbiotroph				Saprotroph				Pathotroph		Unassigned
	Arbuscular Mycorrhizal	Ectomycorrhizal	Endophyte	Lichenized	Wood Saprotroph	Soil Saprotroph	Dung Saprotroph	Undefined Saprotroph	Animal Pathogen	Plant Pathogen	
Bulk	0.50 c	1.63 b	11.80 b	4.64 a	5.15 a	2.87 a	5.60 a	25.12 a	5.11 a	12.22 b	25.26 a
Rhizosphere	1.47 b	1.99 b	17.5 ab	5.1 a	4.54 a	2.22 a	4.27 a	15.5 ab	3.89 a	21.84 a	21.67 a
Endorhizosphere	3.40 a	2.48 a	24.61 a	2.25 b	2.09 b	1.75 a	1.59 b	10.16 b	4.14 a	25.70 a	21.90 a

Tukey's test at $P < 0.05$ level. Means followed by the same letter do not differ significantly ($P < 0.05$) among soil-plant compartments within each functional group.

Chapter 4.2.

Grapevine pruning time affects natural wound colonization by wood-invading fungi

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Abstract

Grapevine pruning wounds made during the dormant season are a port of entry of wood-invading fungi. Timing of pruning may affect the wound susceptibility to these fungi, such as those associated with grapevine trunk diseases (GTDs). This study aimed to determine the effect of pruning time on natural fungal infection in six vineyards in Galicia, Spain, belonging to three Denominations of Origin (D.O.) over two growing seasons. Pruning wounds were left unprotected physically and chemically during two periods of three months each, from November to February and from February to May. The diversity and composition of the fungal microbiome that colonized the pruning wounds were identified by ITS2 high-throughput amplicon sequencing (HTAS). A broad range of fungi was able to colonize grapevine pruning wounds at both infection periods. Fungal microbiome composition did not shift as year of sampling. Fungal communities were affected in their composition and diversity by the D.O., whereas the spatial variation (i.e. vineyard within each region) was low. Pruned canes harboured a core community of fungal species, which appeared to be independent of the infection period. Accumulated rainfall over 8 and 11 weeks after pruning positively correlated with the total fungal microbiome and in particular with the abundance of the GTD fungal genus *Diaporthe*. A seasonal effect on GTD fungal infection was detected for most genera, with higher percent detected after pruning in February (winter) as compared with that of pruning in November (mid-autumn). In light of the GTD colonization results and given the geographical location and the environmental conditions of this study, early pruning is recommended to reduce the infections caused by GTD fungi during the pruning season in Galicia.

Introduction

Pruning is the most important activity during the dormant season in vineyards. Grapevine pruning is recommended any time after leaf fall, which may occur late autumn or during the winter. The main objective of pruning is to allow adequate vegetative growth for the following season and to obtain maximum yields of high-quality grapes (Jackson, 2004). Timing of pruning may affect the grapevine phenology, and thus yield and fruit quality (Zheng et al., 2017). Early or late pruning can also affect the susceptibility of the plant to abiotic disorders, such as spring frost (Jackson, 2004), or the pruning wound susceptibility to infections caused by wood-invading fungi, such as those associated with grapevine trunk diseases (GTDs) (Luque et al., 2014).

Grapevine trunk diseases are caused by a wide range of taxonomically unrelated fungi that infect woody tissues. They reduce productivity and longevity of grapevines and thereby cause long-term economic losses to industry (Gramaje et al., 2018). To date, up to 135 fungal species belonging to 35 genera have been associated with GTDs worldwide (Gramaje et al., 2018; Aigon-Mouhous et al., 2019; Lawrence et al., 2019; Berlanas et al., 2020), thus accounting for the largest group of fungi known to infect grapevines (Gramaje et al., 2018). GTDs are mainly caused by fungal ascomycetes but some basideomiceteous fungi are also thought to play a relevant role in this pathosystem (Fischer, 2002; Cloete et al., 2015; Brown et al., 2020). GTD fungal spores can colonize any open and fresh wound, including those caused by de-suckering, re-training and trimming (Makatini et al., 2014). Nonetheless, pruning wounds, which are produced during pruning in the dormant season, are the primary point of infection providing many entry sites each growing season throughout the vineyards' lifespan (Gramaje et al., 2018).

The main GTDs in mature vines are Botryosphaeria dieback, Eutypa dieback, Phomopsis dieback and esca disease (Gramaje et al., 2018). In North America, several Cytospora spp. have also been recently reported causing dieback and wood cankers in grapevine (Lawrence et al., 2017). Grapevine pathogens responsible for these diseases are mainly spread through the dispersion of airborne spores. Previous studies showed that spore release and thus, high risk periods of infection vary during the growing season depending on the geographical location and fungal pathogen, but mainly overlay with dormant pruning seasons in both the Southern and Northern Hemispheres (Pearson, 1980; Petzoldt et al., 1983; Eskalen and Gubler, 2001; Amponsah et al., 2009; Trouillas, 2009; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010; Valencia et al., 2015). Pruning wounds susceptibility to GTD pathogens mainly depends on the pruning time and the period occurring between pruning and possible infection cases. Studies using artificial spore inoculations indicate that susceptibility of grapevine pruning wound significantly decreases as the length of time between pruning and inoculation increases, with seasonal variation noted between regions, due primarly to climatic differences (Moller and Kasimatis, 1980; Munkvold and Marois, 1995; Eskalen et al., 2007; Serra et al., 2008; Úrbez-Torres and Gubler, 2011; van Niekerk et al., 2011; Ayres et al., 2016).

The rate of natural fungal microbiome infections in pruned canes has been poorly studied so far, and data available is only referred in the context of GTD pathogens infections in France (Lecomte and Bailey, 2011) and northeast Spain (Luque et al., 2014). These studies employed standard culture-dependent microbial techniques; however,

these approaches tend to skew fungal activity and underestimate species richness, because fungi may be slow growing, hidden and highly selective. By contrast, molecular-based methods allow the detection and identification of a greater number of microorganisms in nature, including species that are unable to be isolated in culture (Amann et al., 1995). Recent advances of high-throughput sequencing (HTS) approaches have improved both the scope and resolution of fungal microbiome analyses and have reported a greatly complex and diverse fungal communities of vascular systems in plants (Studholme et al., 2011).

In recent years, grapevine has become a plant model system for microbiome research. HTS tools have been actively used to map the microbiome on grapevine organ epiphytes (i.e., root, berry and leaf) due of its importance with foliar and fruit diseases control, grape production along with the biological implication of indigenous microorganisms with the local signature of a wine (Perazzolli et al., 2014; Zarraonaindia et al., 2015). Culture-dependent microbial approaches have revealed the microbiome inhabiting the grapevine endosphere (West et al., 2010; Compant et al., 2011; Baldan et al., 2014; Kraus et al., 2019). However, culture-independent high-throughput amplicon sequencing (HTAS) techniques have recently been used to enhance the microbiome profile of grapevine woody organs such as cane and trunk (Faist et al., 2016; Deyett et al., 2017; Dissanayake et al., 2018; Eichmeier et al., 2018).

In this study, we tested the following hypotheses: (1) the diversity and composition of fungal microbiome that colonizes grapevine pruning wounds changes according to the pruning time and this shift is related to environmental conditions; (2) the susceptibility of pruning wounds to fungal infection and the ability of GTD pathogens to colonize them depend on the pruning time, therefore this would allow us to make pruning recommendations to growers in the short term in order to avoid high pathogen infection periods. The objective was therefore to identify the diversity and composition of the fungal microbiome, in particular GTD fungi, colonizing pruning wounds over two infection periods and seasons in six mature vineyards in Galicia, Spain, by HTAS: (i) from November to February, after an early pruning in mid-autumn; and (ii) from February to May, after a late pruning in winter. In addition, we investigated the relationship between the weather data registered throughout the experimental period and the rate of fungal colonization.

Materials and methods

Location and characteristics of the experimental vineyards

Experiments were conducted at six experimental plots located in three Denominations of Origin (D.O. Valdeorras, D.O. Ribeiro and D.O. Rías Baixas; two experimental plots per D.O.) in Galicia region, Spain, from November 2017 to May 2019 (Supplementary Table 4.2.1). Plots within each D.O. were < 10 km apart and had very similar climates. Standard cultural practices were applied in all experimental plots during the growing season. Powdery and downy mildews management was carried out using only wettable sulphur and copper compounds and applied at label dosages and following IPM guidelines, respectively, if required. Plots of 1,500 vines in these vineyards have been monitored biannually for the evolution of GTD symptoms since 2014 to the present. At the time this study was started (2017), approximately 12% of vines had shown GTDs symptoms in previous surveys. The main symptoms of GTDs observed during monitoring included chlorotic leaves, stunted shoots, and short internodes (Eutypa dieback), the arm and cordon death (Botryosphaeria, Eutypa and Phomopsis diebacks) and tiger-pattern foliar necrosis (esca). All vineyards were trained as bilateral cordons with spur pruning. Grapevine cultivars differed among D.O. (Supplementary Table 4.2.1), so data from each D.O. was analysed independently due to the previously reported variable degree of susceptibility of each grapevine cultivar to fungal trunk pathogen infections (Martínez-Diz et al., 2019a). The experimental plots were located < 6 km to automatic weather stations owned by MeteoGalicia (Weather Service of Galician Regional Government, Xunta de Galicia). Data obtained from the weather station in each D.O. were considered to be representative of the two experimental plots.

Pruning and sampling

A total of 200 vines were pruned in each experimental plot in mid-autumn (between November 13 and 14 for both years and experimental plots) leaving six buds. Then, 25 pruned canes in each vineyard were randomly selected and labelled for subsequent samplings. Wood of these 25 canes were taken to the laboratory for DNA extraction. Three months later in winter (between February 21 and 22 for both years and experimental plots), a 15-cm section was cut from the 25 labelled canes and taken to the laboratory for DNA extraction. On the same day of this sampling, vines were pruned to four buds, leaving a 5–

7 cm wood section above the top bud. Three months later in spring (between May 22 and 23 for both years and experimental plots), sampling for DNA extraction from approximately a 15-cm wood section was repeated following the same procedure earlier, and the labelled canes were definitely pruned to two buds. All canes were therefore exposed to natural infections for three months after each pruning (infection period 1: November-February; infection period 2: February-May). Pruning scissors were disinfested with 70% ethanol before and after every pruning cut. Pruning wounds were not protected chemically, nor physically during the trial.

DNA extraction and sequencing

Pruned canes were sequentially washed in 70% ethanol and sterile distilled water for 1 min each before DNA extraction. Bark was then carefully peeled out from the upper ends of canes with a flame-sterilised scalpel in order to expose the inner tissues starting from the pruning wound. A portion of approximately 3-mm from the end of the cane was cut and discarded to avoid bias by the colonization of saprophytic fungi. DNA was extracted from 0.5 g of xylem tissue collected between 3- to 8- mm from the pruning wound using the i-genomic Plant DNA Extraction Mini Kit (Intron Biotechnology, South Korea). DNA yields from each sample were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, USA), and the extracts were adjusted to 10-15 ng/µl. In total, DNA was extracted from 900 pruned canes (25 pruned canes x 6 experimental plots x 3 time-points x 2 seasons). After DNA quantification, samples of each pruning time and vineyard were pooled in groups of five, resulting in a total of five replicates for every batch of 25 canes. A total of 180 DNA samples was analysed. Complete fungal ITS2 region (around 300 bp) was amplified using the primers ITS86F (5' GTGAAT CATCGAATCTTTGAA 3'; Turenne et al., 1999) and ITS4 (5' TCCTCCGCTTATTGATATGC 3'; White et al., 1990), to which the Illumina sequencing primer sequences were attached to their 5' ends. PCRs were carried out in a final volume of 25 µl, containing 2.5 µl of template DNA, 0.5 µM of the primers, 12.5 µl of Supreme NZYTaq 2x Green Master Mix (NZYTech, Lisboa, Portugal), and ultrapure water up to 25 µl. The reaction mixture was incubated as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 49°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 minutes. The oligonucleotide indices, which are required for multiplexing different libraries in the same sequencing pool, were attached in a second PCR round with identical conditions but only five cycles and 60°C as the annealing temperature for a

schematic overview of the library preparation process. A negative control that contained no DNA was included in every PCR round to check for contamination during library preparation (BPCR). The libraries were run on 2% agarose gels stained with GreenSafe (NZYTech, Lisboa, Portugal) and imaged under UV light to verify the library size. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, Norcross, GA, USA), following the instructions provided by the manufacturer. They were then pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, USA). The pool was sequenced in a MiSeq PE300 run using MiSeq Reagent Kit v3 (Illumina, San Diego, USA). An additional negative control was included during the extraction step. A positive control containing DNA of a grapevine endorhizosphere sample previously evaluated by ITS HTAS was also included (Martínez-Diz et al., 2019b). All control samples were prepared for sequencing to evaluate potential contaminations of the entire process.

Data analysis of the high-throughput amplification assay

Quality of sequences was inspected using FastQC-0.10.1 (Andrews, 2010). Trimming and merging of the paired end reads was performed using CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark). The parameter Q30 was applied. Only reads longer than 100 nts were considered for further analysis. The distance of evaluated reads in the trimming and merging step was set from 200 to 400 nts. Primer and Illumina adapter sequences were trimmed out. The reads were exported to fasta format by CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark).

Exported fasta files were used for clustering in SCATA (https://scata.mykopat.slu.se/). Parameters for clustering were: Clustering distance 0.015; Minimum alignment to consider clustering 0.95; Missmatch penalty 0.1; Gap open penalty 0; Gap extension penalty 1; End gap weight 0; Collapse homopolymers 3; Downsample sample size 0; Remove low frequency genotypes 0; Tag-by-Cluster Max 10,000,000; Blast E-value cutoff 1e-60; Cluster engine USERACH; Number of repseqs to report 50. The CBS isolates were used as a reference sequences. Singleton operational taxonomic units (OTUs) were discarded. The sequences of non-singleton OTUs were used as the representative sequence and were identified using the blastn algorithm from the NCBI GenBank reference database (version 2.2.30+). OTUs matching mitochondrial, Viridiplantae or chloroplast sequences or with no kingdom-level classification were excluded from the dataset. In order to optimize the dataset, each sample was rarefied to the same sequence number per sample, that is, 21,287

fungal sequences. OTUs represented in global by less than five reads were discarded (Glynou et al., 2018). The resulting quality dataset was used for the estimation of richness and diversity. The metadata, OTUs table and associated taxonomic classifications deployed in this study have been deposited in figshare (ID: 79113; https://figshare.com/projects/Natural_fungal_infections_-_Galicia_Spain_/79113). HTAS data were deposited in NCBI GenBank under BioProject Acc. No. PRJNA625395.

Fungal diversity, taxonomy distribution and statistical analysis

Alpha-diversity estimates were calculated by analysing the Shannon diversity and Chao1 richness in Phyloseq package, as realized in the tool MicrobiomeAnalyst (Dhariwal et al., 2017). Differences in fungal alpha-diversity among year, D.O., vineyard within each D.O., and pruning time were inferred by multiple mean comparisons using Tukey's test in MicrobiomeAnalyst. PERMANOVA was performed to investigate which OTUs significantly differed in abundance among experimental factors after Bonferroni corrections. Bray Curtis metrics were calculated to investigate the relationship in OTUs composition among samples. PCoA plots were used to visualize these relationships using MicrobiomeAnalyst. Rarefaction curves and Good's coverage values were calculated using MicrobiomeAnalyst.

The Linear Discriminant Analysis Effect Size (LEfSe) algorithm was used to identify fungal taxa that differed in relative abundance between pruning times using MicrobiomeAnalyst (Segata et al., 2011). Wilcoxon *P*-value was set up at 0.05 and Linear Discriminant Analysis (LDA) threshold score at 1.0. The results are displayed in a bar graph designed with MicrobiomeAnalyst. The fungal OTUs shared between infection periods were visualized by a Venn-diagram analysis (http://bioinformatics.psb.ugent.be).

In order to compare the percentage of abundance of each fungal genus associated with GTDs between both infection periods, an ANOVA with log transforms was performed. Variance homogeneity was checked by Levene's test, and normality of residuals by Shapiro-Wilk's test. Tukey's Honestly Significant Difference range test ($P \leq 0.05$) was calculated to compare means.

Correlation with weather variables

The number of OTUs corresponding to the total fungal microbiome, and the fungal genera associated with GTDs was correlated with the main weather data (daily mean relative humidity, daily mean temperature and accumulated rainfall). Values from the number of OTUs were transformed by log (n/N * 1000 + 1), where n was the number of OTUs detected on each sample and N was the total number of OTUs detected. Temperature and humidity records were averaged over 1, 2, 4, 8, and 11 weeks post-pruning periods. Rainfall records were accumulated and log-transformed to make data conform to normality over the same periods. Spearman's correlation coefficients were calculated using the function *cor* of the 'stats' package of R v. 3.6.0 (R Core Team, 2019).

Results

High-throughput amplicon sequencing

After paired-end alignments, quality filtering and deletion of chimeras, singletons, a total of 10,740,761 fungal internal transcribed spacer (ITS2) sequences were generated from 180 samples, excluding controls, and assigned to 259 fungal OTUs.

The negative control included in the amplification step identified 30 fungal genera. OTU sequences identified in the negative control was subtracted from the sequence abundance of that OTU in the experimental samples (Nguyen et al., 2015). No contamination was detected in the negative control used in the DNA extraction step. Good's coverage values in all samples ranged from 99.25 to 100%, capturing nearly all the diversity with an adequate sequencing depth (Supplementary Fig. 4.2.1). Chao1 diversity estimator ranged from 5 to 26, while Shannon diversity estimator ranged from 0.31 to 2.27 (Supplementary Table 4.2.2).

Fungal communities differed among Denominations of Origin

The alpha-diversity of fungal communities differed among D.O. (Chao1: $P = 0.0047$, Shannon: $P < 0.001$; Fig. 4.2.1), and principal coordinates analysis (PCoA) of Bray Curtis data demonstrated that D.O. was the primary source of beta-diversity ($R^2 = 0.48$, $P < 0.001$) (Fig. 4.2.2). Therefore, data of each D.O. was analysed independently.

The relative abundance of fungal phyla, order and family detected across all D.O. is shown in Supplementary Figure 4.2.2. Considering the three D.O., the most abundant phyla were Ascomycota, followed by Basidiomycota (Supplementary Fig. 4.2.2A). The most abundant orders were Dothideales, followed by Capnodiales and Pleosporales (Supplementary Fig. 4.2.2B). The most abundant families were Dothioraceae, followed by Cladosporiaceae and Dermateaceae (Supplementary Fig. 4.2.2C). Comparing the fungal microbiota of the three D.O., 56.8% of fungal OTUs were shared among them (Fig. 4.2.3). Specific OTUs associated with each vineyard ranged from 12.1 to 18.4% of their fungal communities (Fig. 4.2.3).

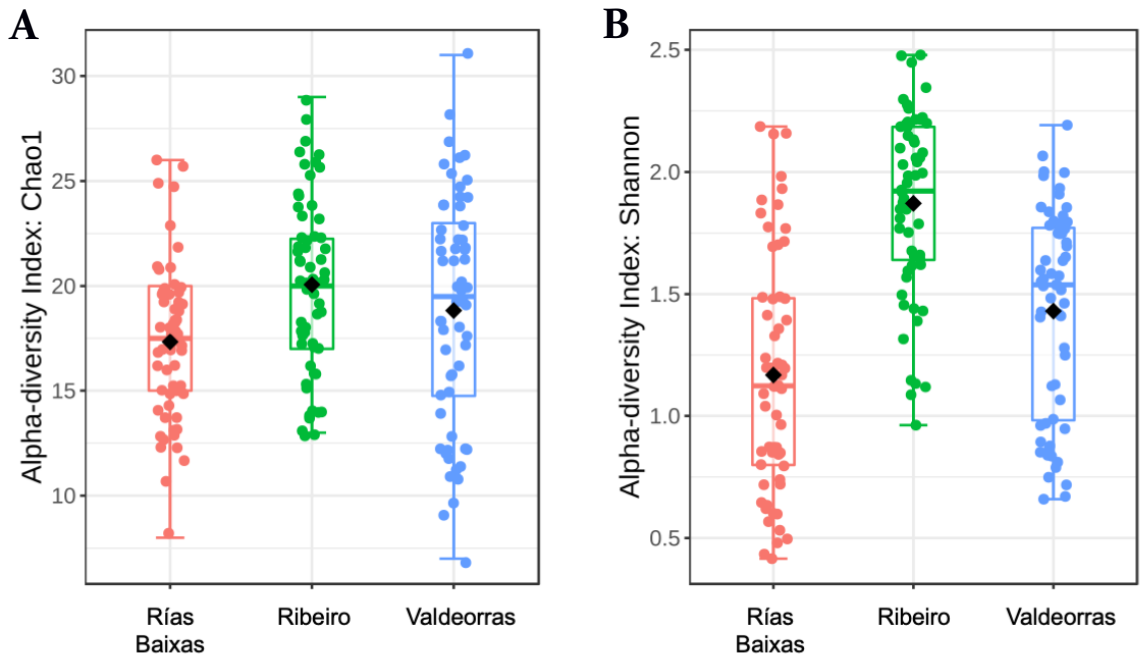

Figure 4.2.1. Boxplot illustrating the differences in Chao1 (**A**) and Shannon (**B**) diversity measures of the fungal communities in the three Denominations of Origin.

In D.O. Rías Baixas, the most abundant families were Dothioraceae (62.9%), followed by Cladosporiaceae (10.2%) and Pleosporaceae (9.9%) (Supplementary Fig. 4.2.2C). The most abundant families in D.O. Ribeiro were Cladosporiaceae (26.5%), followed by Dothioraceae (21.6%) and Dermateaceae (12.1%). In D.O. Valdeorras, the most abundant families were Dothioraceae (51.8%), followed by Cladosporiaceae (19.9%) and Tremellaceae (4.5%).

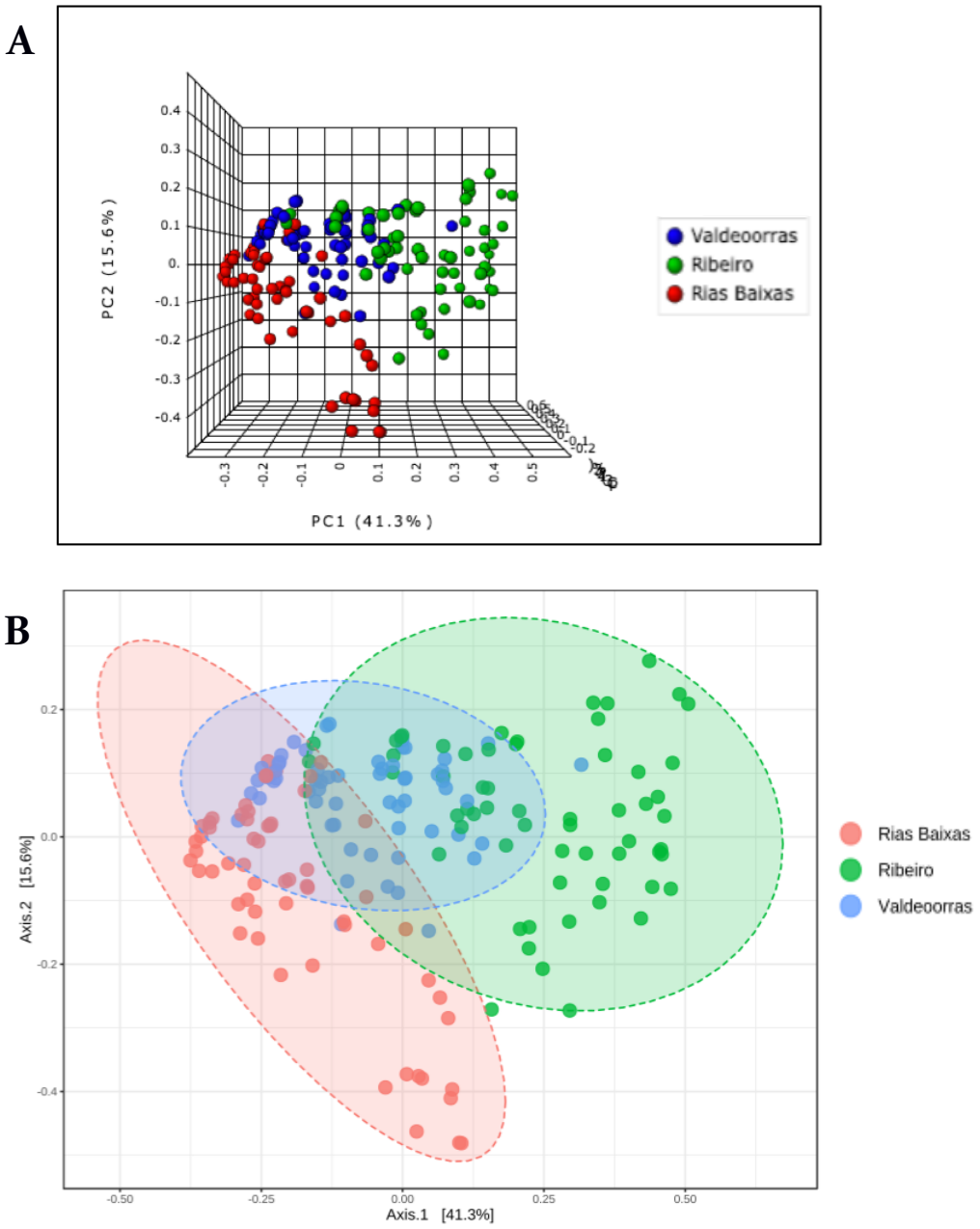

Figure 4.2.2. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics in 3D (**A**) and 2D (**B**), showing the distance in the fungal communities among Denominations of Origin.

Fungal diversity exhibits a temporal variation over the infection periods

Alpha-diversity of fungal communities in grapevine wood samples did not differ significantly between experimental plots (Supplementary Fig. 4.2.3) and year (Supplementary Fig. 4.2.4) within each D.O. (Table 4.2.1), thus the data of both years and experimental plots were combined for analyses.

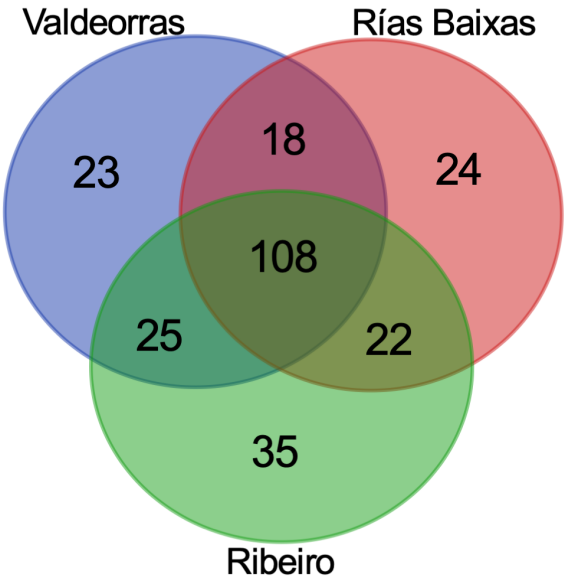

Figure 4.2.3. Venn diagram illustrating the overlap of the OTUs identified in the fungal microbiota among Denominations of Origin.

Comparing the microbiome in the grapevine inner tissue at the three sampling times (1: November, 2: February and 3: May), higher fungal diversity was mostly observed towards the sampling time 3 ($P < 0.05$) (Fig. 4.2.4). Excluding the initial fungal microbiome estimated in November, and considering the two infection periods, fungal community diversity was significantly different between both periods in D.O. Rías Baixas (Table 4.2.1; Fig. 4.2.5B). A PCoA further demonstrated that variation in the D.O. Rías Baixas dataset could be attributed to infection periods (R^2 = 0.60; Supplementary Fig. 4.2.5B). In D.O. Ribeiro, the infection periods did not predict Shannon diversity (Table 4.2.1; Fig. 4.2.5A), and any summary metrics of alpha-diversities in D.O. Valdeorras (Table 4.2.1; Fig. 4.2.5C). Infection periods did not affect the Bray Curtis metric of beta-diversity in D.O. Ribeiro and D.O. Valdeorras ($R^2 < 0.40$; Supplementary Fig. 4.2.5A and 4.2.5C).

The relative abundance of fungal families detected across sampling times is shown in Figure 4.2.6. In D.O. Ribeiro, the most abundant families were Cladosporiaceae (34.2%), Dothioraceae (33.1%) and Sporidiobolaceae (6.3%) (initial microbiome); Cladosporiaceae (22.9%), Dothioraceae (18.4%) and Dermateaceae (14.6%) (infection period 1); and Cladosporiaceae (22.2%), Dothioraceae (15.1%) and Dermateaceae (14.9%) (infection period 2). In D.O. Rías Baixas, the most abundant families were Dothioraceae (81.3%), Cladosporiaceae (9.9%) and Pleosporaceae (4.4%) (initial microbiome); Dothioraceae (66.5%), Cladosporiaceae (9.4%) and Pleosporaceae (7.1%) (infection period 1); and Dothioraceae (26.9%), Pleosporaceae (20.4%) and Dermateaceae (8.3%) (infection period 2).

Table 4.2.1. Experimental factors predicting alpha-diversity of pruning wounds associated fungal communities in three Denomination of Origin (D.O.) in Galicia.

	D.O. Ribeiro		**D.O. Rías Baixas**		**D.O. Valdeorras**	
	Shannon	Chao1	Shannon	Chao1	Shannon	Chao1
Year	$F = 3.01$ $P = 0.9129$	$F = 2.99$ $P = 0.6998$	$F = 2.99$ $P = 0.4553$	$F = 2.79$ $P = 0.211$	$F = 4.55$ $P = 0.1552$	$F = 4.22$ $P = 0.4761$
Experimental plot	$F = 2.13$ $P = 0.1455$	$F = 1.26$ $P = 0.0647$	$F = 3.05$ $P = 0.3550$	$F = 3.77$ $P = 0.1295$	$F = 3.12$ $P = 0.6772$	$F = 3.79$ $P = 0.8890$
Year x experimental plot	$F = 0.85$ $P = 0.8773$	$F = 0.95$ $P = 0.4103$	$F = 1.14$ $P = 0.1445$	$F = 1.92$ $P = 0.2301$	$F = 2.99$ $P = 0.9778$	$F = 3.76$ $P = 0.9110$
Infection period*	$F = 4.21$ $P = 0.7468$	$F = 3.72$ $\mathbf{P = 0.0416}$	$F = 3.75$ $\mathbf{P < 0.001}$	$F = 5.06$ $\mathbf{P < 0.001}$	$F = 4.25$ $P = 0.9079$	$F = 4.20$ $P = 0.1221$
Year x infection period	$F = 1.83$ $P = 0.2340$	$F = 0.90$ $P = 0.0981$	$F = 2.19$ $P = 0.1221$	$F = 4.26$ $P = 0.2543$	$F = 2.35$ $P = 0.4551$	$F = 1.88$ $P = 0.2989$
Experimental plot x infection period	$F = 3.24$ $P = 0.1134$	$F = 4.01$ $P = 0.0944$	$F = 3.11$ $P = 0.0987$	$F = 5.79$ $P = 0.1556$	$F = 5.67$ $P = 0.2375$	$F = 4.15$ $P = 0.6780$

*Infection period: 1 (from November to February) and 2 (from February to May).
All *P* values were corrected for multiple comparisons using the sequential Bonferroni correction. Bold values indicate statistically significant results after correction for multiple comparisons. $P < 0.05$.

In D.O. Valdeorras, the most abundant families were Dothioraceae (70.9%), Cladosporiaceae (23.1%) and Filobasidiaceae (1.2%) (initial microbiome); Dothioraceae (45.2%), Cladosporiaceae (17.8%) and Tremellaceae (8.5%) (infection period 1); and Dothioraceae (40.2%), Cladosporiaceae (20.9%) and Dermateaceae (12.8%) (infection period 2).

Infection periods-specific and shared fungal assemblages

The percentage of shared fungal OTUs among the three sampling times were similar in all D.O.: 31.5% (D.O. Ribeiro), 31.4% (D.O. Rías Baixas), and 28.7% (D.O. Valdeorras) (Fig. 4.2.7). Specific OTUs associated with each sampling time ranged from 15.8 to 21.4% (D.O. Ribeiro), from 6.9 to 23.8% (D.O. Rías Baixas), and from 9.7 to 17.2% (D.O. Valdeorras). Excluding the initial fungal microbiome and comparing the two infection periods, shared fungal OTUs among infection periods were also similar: 54.1% (D.O. Ribeiro), 56.3% (D.O. Rías Baixas), and 56.0% (D.O. Valdeorras).

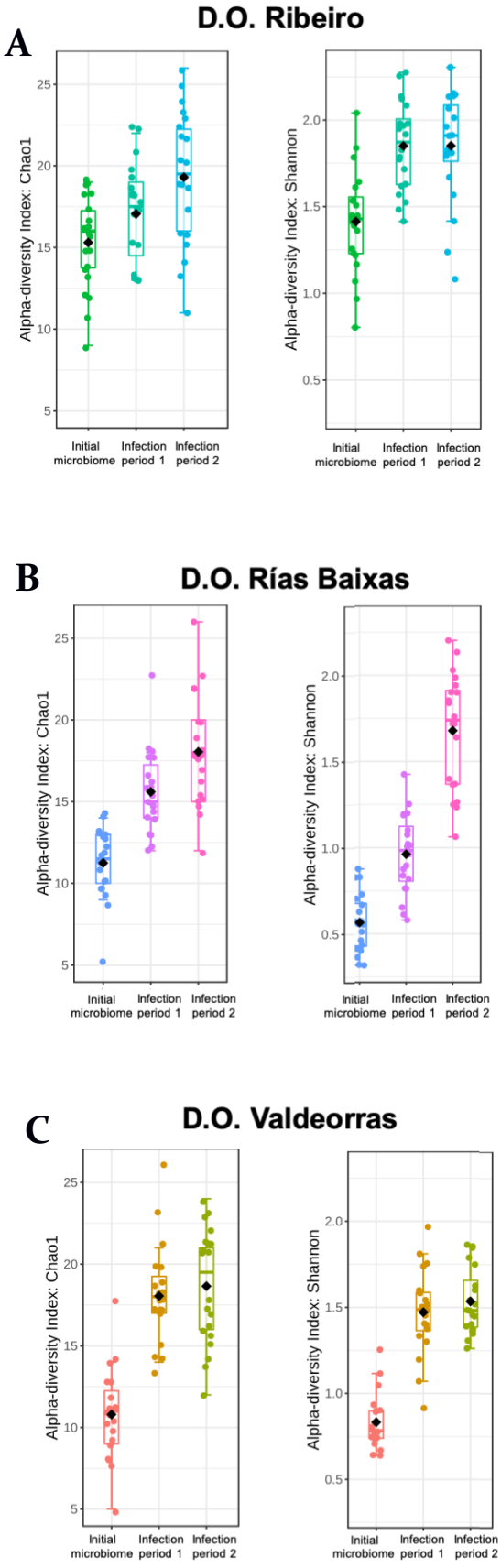

Figure 4.2.4. Boxplot illustrating the differences in Chao1 and Shannon diversity measures of the fungal communities among sampling times in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).

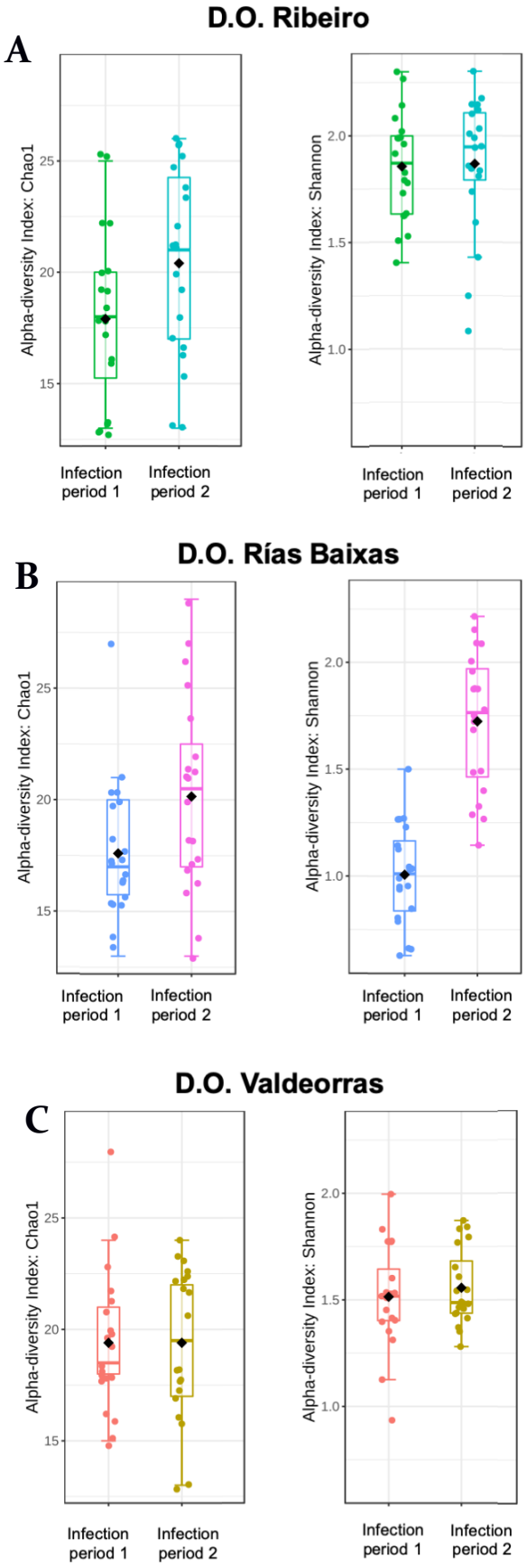

Figure 4.2.5. Boxplot illustrating the differences in Chao1 and Shannon diversity measures of the fungal communities between both infection periods in D.O. Ribeiro **(A)**, D.O. Rías Baixas **(B)**, and D.O. Valdeorras **(C)**.

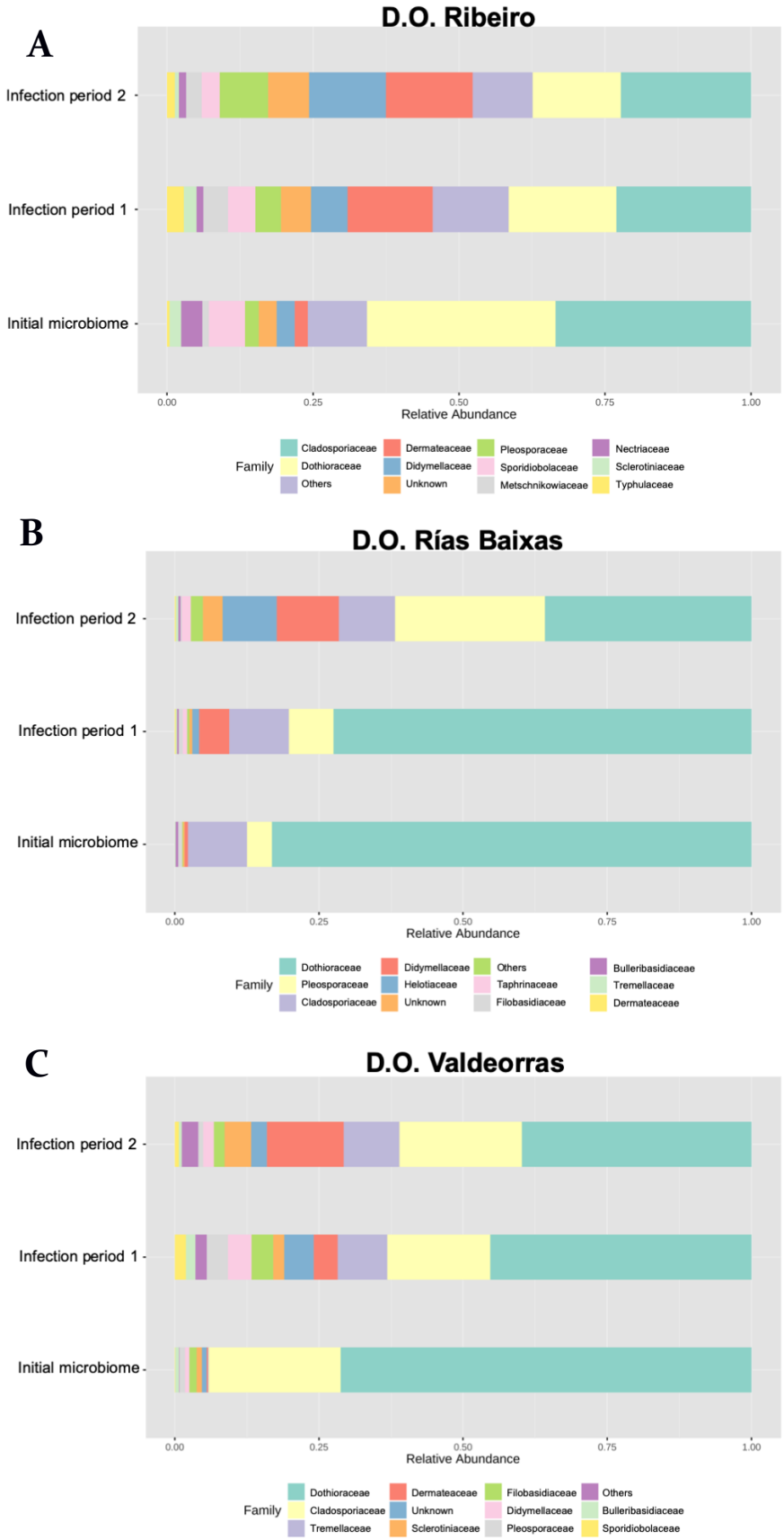

Figure 4.2.6. Relative abundance of different fungal families detected across sampling times (initial microbiome, infection period 1 and infection period 2) in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).

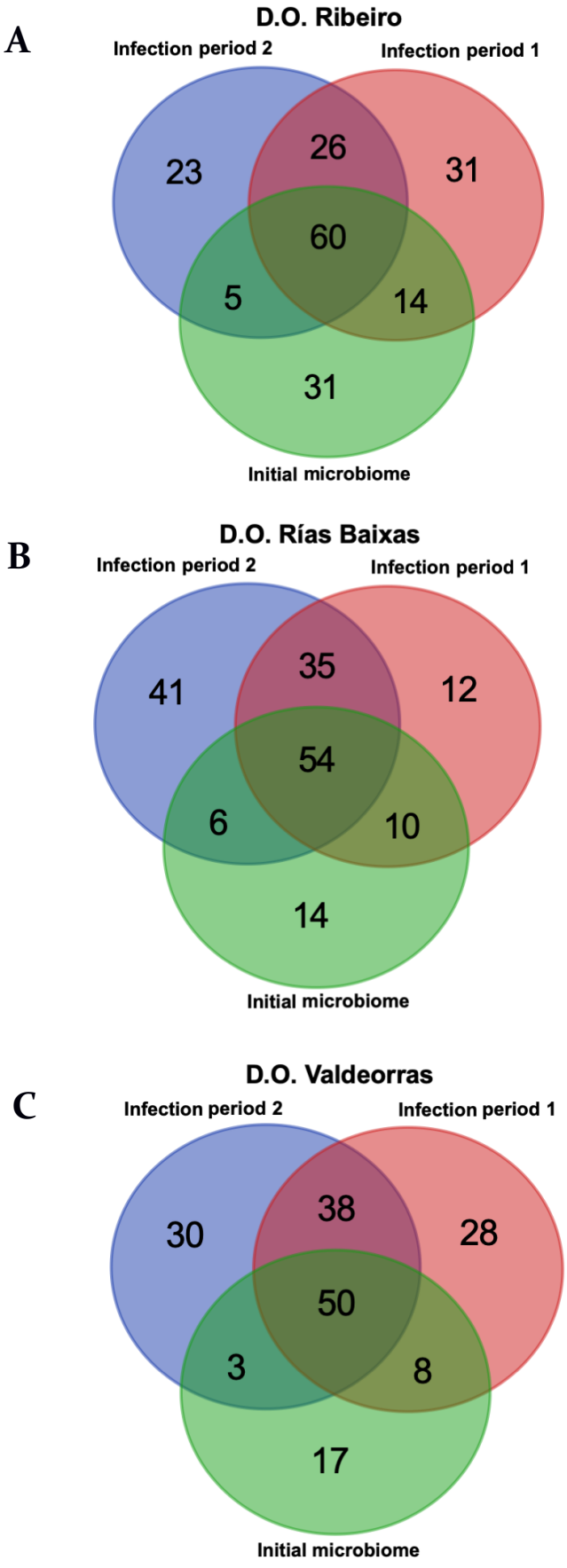

Figure 4.2.7. Venn diagram illustrating the overlap of the OTUs identified in the fungal microbiota among sampling times in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).

The OTUs that were unique in both infection periods for each D.O. are shown in Supplementary Table 4.2.3. Genera *Eucasphaeria* and *Penicillium* were unique to the infection period November-February, while *Cryptodiaporthe* genus was unique to the infection period February-May in the three D.O.

The LEfSe detected 3, 9 and 4 fungal clades in the grapevine inner tissues, which discriminated the fungal communities between infection periods in D.O. Ribeiro, D.O. Rías Baixas and D.O. Valdeorras, respectively (Fig. 4.2.8). The infection period 2 showed higher number of differentially abundant fungal clades (2, 8, and 3 in D.O. Ribeiro, D.O. Rías Baixas and D.O. Valdeorras, respectively). In the infection period 1, the dominant fungal genus in all D.O. was *Aureobasidium*. In the infection period 2, the dominant fungal genera were *Epicoccum* (D.O. Ribeiro), an unknown genus within the Pleospareaceae family (D.O. Rías Baixas), and *Cyanodermella* (D.O. Valdeorras).

The natural infection rates caused by fungal trunk pathogens differ between pruning times

Among the identified fungal taxa, 10 genera are associated with GTDs: *Botryosphaeria*, *Cadophora*, *Cryptovalsa*, *Cytospora*, *Diaporthe*, *Diplodia*, *Eutypa*, *Neofusicoccum*, *Phaeoacremonium* and *Phaeomoniella*. Alpha-diversity of fungal communities associated with GTDs in grapevine wood samples did not differ significantly among D.O. (Chao1: P = 0.1328, Shannon: P = 0.7608; Supplementary Fig. 4.2.6). The alpha-diversities of fungal communities differed between infection periods in D.O. Ribeiro (Chao1: P = 0.041, Shannon: $P < 0.001$) and D.O. Rías Baixas (Chao1: $P < 0.001$, Shannon: $P < 0.001$), richness and diversity being higher in the infection period 2 (Fig. 4.2.9A and 4.2.9B). The alpha-diversity of fungal GTD communities did not differ between infection periods in D.O. Valdeorras ($P > 0.05$; Fig. 4.2.9C).

In the annual shoots (November: initial fungal microbiome), the average number of GTD fungal OTUs ranged from 1.1 to 735 OTUs (Supplementary Fig. 4.2.7). Regarding the infection periods, the average number of GTD fungal OTUs ranged from 2.9 to 2,223.1 OTUs (infection period 1) and from 3.1 to 2,900 OTUs (infection period 2) (Fig. 4.2.10). The percentages of fungal GTD abundances with respect to the total fungal microbiome ranged from 0.2 to 1.2% (infection period 1) and from 0.3 to 1.9% (infection period 2). The abundances of several fungal GTD genera increased significantly in the infection period 2 compared to the infection period 1 ($P < 0.05$; Fig. 4.2.10): *Cadophora* and *Diplodia* in D.O.

Ribeiro, *Cadophora*, *Cytospora*, *Diaporthe*, *Diplodia*, *Eutypa* and *Neofusicoccum* in D.O. Rías Baixas, and *Diaporthe* and *Phaeomoniella* in D.O. Valdeorras. The abundance of *Cadophora* increased significantly in the infection period 1 compared to the infection period 2 in D.O. Valdeorras ($P < 0.05$; Fig. 4.2.10C).

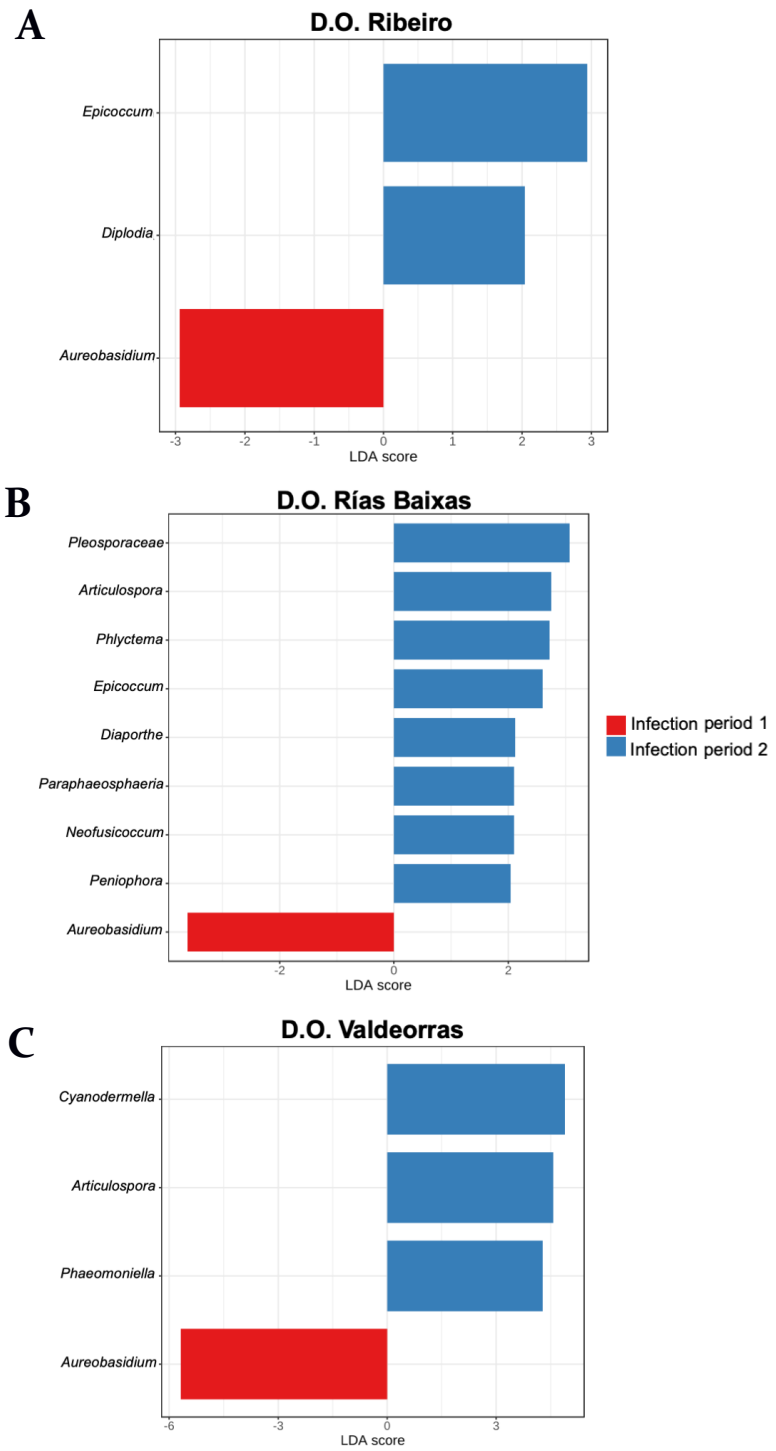

Figure 4.2.8. LEfSe was used to identify the most differentially abundant taxa between infection periods (infection period 1: November-February; infection period 2: February-May). Bar graph showing LDA scores for fungal genera in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**). Only taxa meeting an LDA significant threshold > 2 are shown.

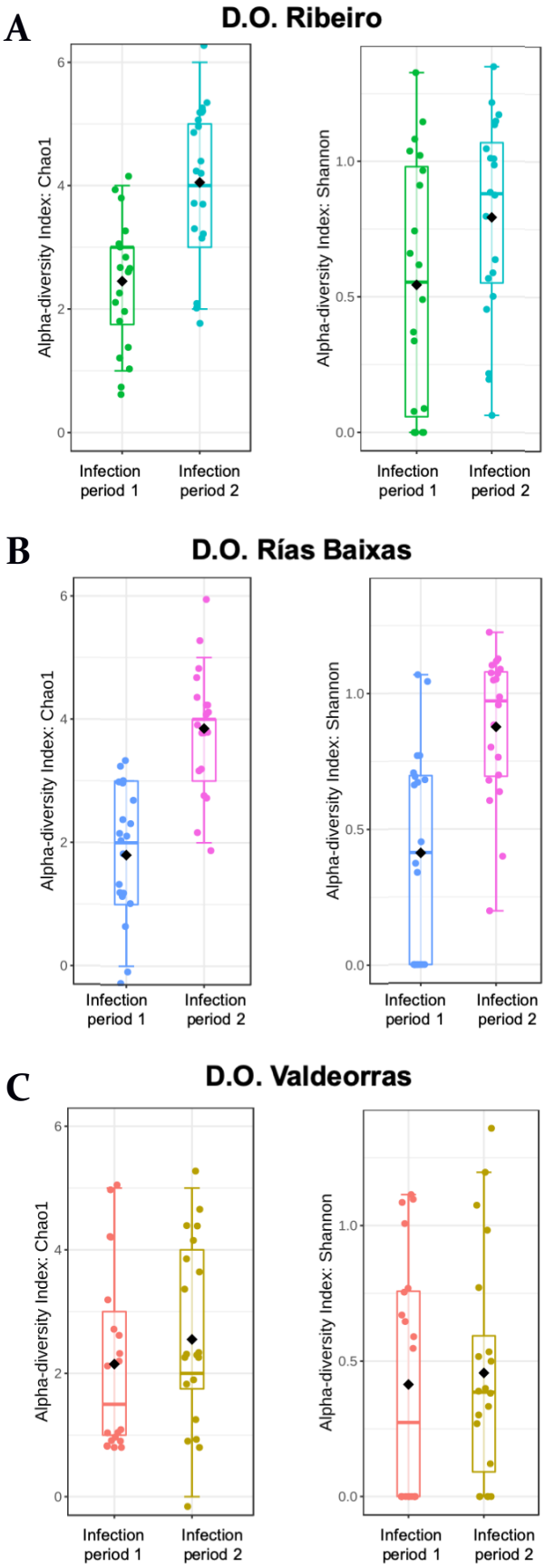

Figure 4.2.9. Boxplot illustrating the differences in Chao1 and Shannon diversity measures of the grapevine trunk disease pathogens between both infection periods in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).

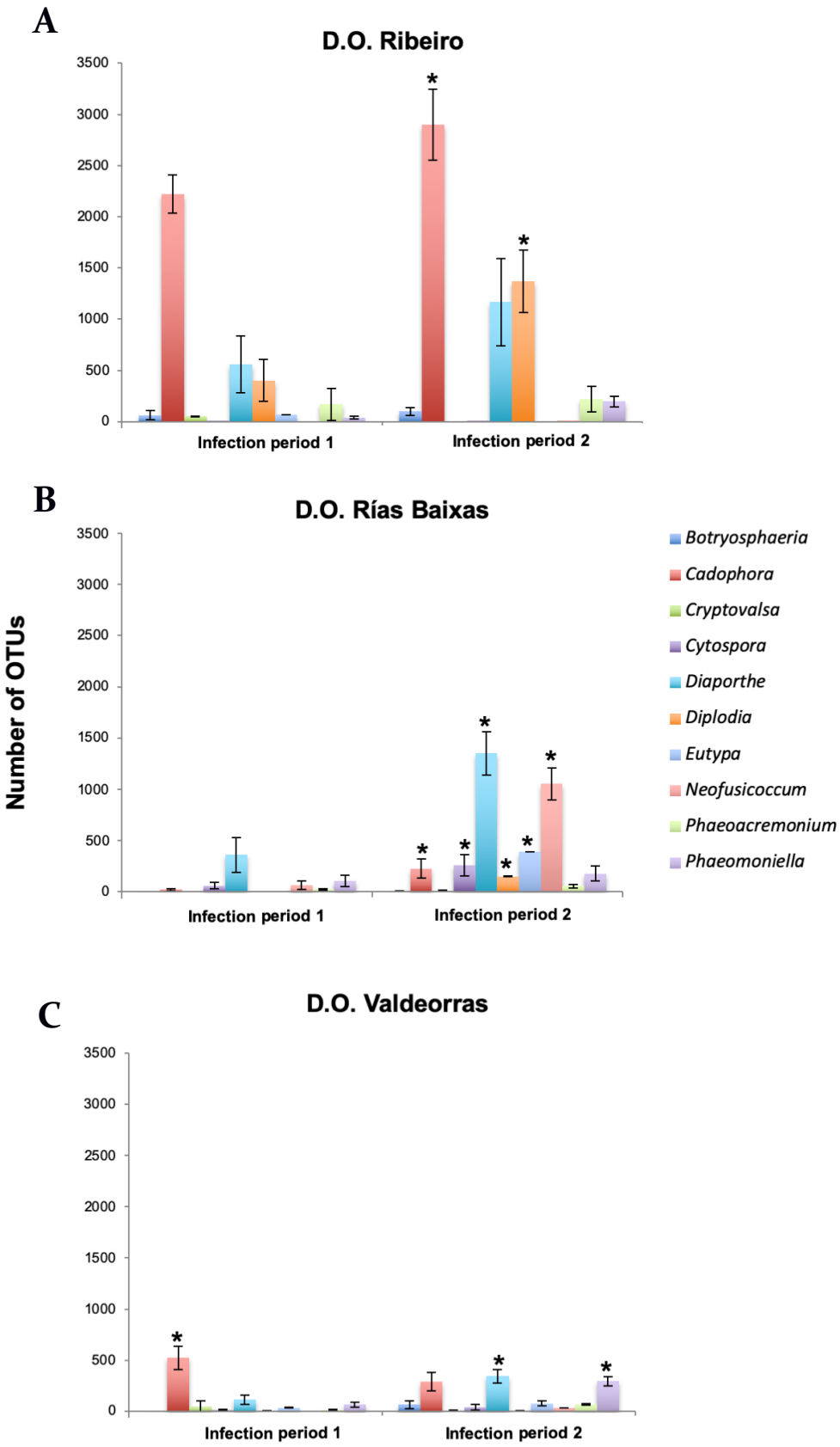

Figure 4.2.10. Distribution of the relative abundance of fungal trunk diseases genera obtained by high-throughput amplicon sequencing in both infection periods in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**). Asterisks (*) indicate significant differences in fungal abundances between infection periods according to the Tukey's Honestly Significant Difference range test ($P = 0.05$).

Correlation with weather variables

Climate conditions in each D.O., experimental season and infection period is shown in Supplementary Table 4.2.4. Climate variables varied between pruning seasons and locations. The mean values of temperature were similar during the winter season in D.O. Valdeorras (2017/2018: 6.52°C; 2018/2019: 7.04°C) and D.O. Ribeiro (2017/2018: 6.79°C; 2018/2019: 7.44°C), while they were around 3 degrees on average higher in D.O. Rías Baixas (2017/2018: 9.42°C; 2018/2019: 10.22°C). In general, temperature declined after November pruning reaching its yearly minimum during the winter season (Supplementary Table 4.2.4). Temperature increased steadily from February pruning until May pruning. Accumulated rainfall was very stable after November pruning (winter season) at both D.O. Valdeorras (2017/2018: 298.40; 2018/2019: 309.60) and D.O. Ribeiro (2017/2018: 322.20 mm; 2018/2019: 294.40 mm), but it was around 100 mm on average higher in D.O. Rías Baixas (2017/2018: 393.30 mm; 2018/2019: 439.10 mm). After February pruning (spring season), this parameter increased in 2017/2018 but decreased in 2018/2019, at the three D.O. studied. In general, D.O. Rías Baixas averaged the highest rainfall among the three D.O. The relative humidity was highly stable at all three locations and seasons, and as expected higher rates were recorded during winter.

A significant correlation between the weather variables and the OTU abundances of the total fungal microbiome, *Diaporthe* and *Phaeomoniella* was detected (Table 4.2.2). Average daily temperature for the 8-week period after pruning was negatively correlated ($P < 0.05$) with the OTU abundances of the total fungal microbiome. Accumulated rainfall over 8 and 11 weeks positively correlated with the fungal microbiome abundances ($P < 0.05$). Regarding GTD fungal genera, a negative correlation with temperature ($P < 0.05$) was observed for *Diaporthe* and *Phaeomoniella* in the first week after pruning. Accumulated rainfall over 8 and 11 weeks positively correlated with *Diaporthe* abundances ($P < 0.05$) (Table 4.2.2).

Table 4.2.2. Spearman's correlation coefficients of the relationships between weather data and OTUs number of the total fungal microbiome, *Cadophora*, *Diaporthe*, *Diplodia*, *Phaeoacremonium* and *Phaeomoniella*. All OTU data are log transformed.

Correlation coefficient and significance	Fungal microbiome			***Cadophora***			***Diaporthe***			***Diplodia***			***Phaeoacremonium***			***Phaeomoniella***		
	T1*	**RH1**	**LCR1**	**T1**	**RH1**	**LCR1**	**T1**	**RH1**	**LCR1**	**T1**	**RH1**	**LCR1**	**T1**	**RH1**	**LCR1**	**T1**	**RH1**	**LCR1**
r	**-0.938**	0.143	0.306	-0.146	0.262	-0.067	**-0.599**	0.303	0.161	-0.380	0.303	0.161	-0.068	0.102	-0.298	**-0.731**	0.102	-0.298
P	**<0.01**	0.658	0.333	0.649	0.409	0.833	**0.039**	0.337	0.676	0.229	0.337	0.676	0.831	0.750	0.346	**<0.01**	0.750	0.346
	T2	**RH2**	**LCR2**	**T2**	**RH2**	**LCR2**	**T2**	**RH2**	**LCR2**	**T2**	**RH2**	**LCR2**	**T2**	**RH2**	**LCR2**	**T2**	**RH2**	**LCR2**
r	**-0.754**	0.103	-0.188	-0.148	0.251	-0.224	-0.372	0.265	-0.017	-0.212	0.254	-0.430	0.066	0.161	0.328	-0.382	-0.074	0.006
P	**0.004**	0.751	0.558	0.646	0.414	0.492	0.231	0.404	0.955	0.507	0.424	0.162	0.838	0.617	0.282	0.219	0.818	0.984
	T4	**RH4**	**LCR4**	**T4**	**RH4**	**LCR4**	**T4**	**RH4**	**LCR4**	**T4**	**RH4**	**LCR4**	**T4**	**RH4**	**LCR4**	**T4**	**RH4**	**LCR4**
r	**-0.819**	-0.048	0.494	-0.213	0.190	0.170	-0.436	0.022	0.522	-0.341	0.129	-0.303	0.071	0.027	0.014	-0.487	-0.242	0.268
P	**<0.01**	0.882	0.102	0.505	0.552	0.596	0.156	0.944	0.081	0.277	0.689	0.338	0.824	0.933	0.966	0.107	0.447	0.398
	T8	**RH8**	**LCR8**	**T8**	**RH8**	**LCR8**	**T8**	**RH8**	**LCR8**	**T8**	**RH8**	**LCR8**	**T8**	**RH8**	**LCR8**	**T8**	**RH8**	**LCR8**
r	**-0.634**	0.777	**0.733**	-0.092	0.070	0.166	-0.244	0.165	**0.708**	-0.197	0.127	0.447	0.137	-0.188	-0.002	-0.327	-0.160	0.481
P	**0.027**	0.580	**0.006**	0.775	0.827	0.605	0.443	0.608	**<0.01**	0.588	0.692	0.145	0.670	0.557	0.847	0.299	0.619	0.113
	T11	**RH11**	**LCR11**	**T11**	**RH11**	**LCR11**	**T11**	**RH11**	**LCR11**	**T11**	**RH11**	**LCR11**	**T11**	**RH11**	**LCR11**	**T11**	**RH11**	**LCR11**
r	-0.280	-0.242	**0.685**	-0.073	0.070	0.066	0.005	0.344	**0.822**	-0.019	0.018	0.441	0.481	-0.028	-0.071	-0.310	0.473	0.493
P	0.337	0.940	**0.013**	0.820	0.828	0.838	0.986	0.915	**<0.01**	0.953	0.955	0.518	0.113	0.929	0.8253	0.326	0.120	0.103

T, mean daily temperature; RH, mean daily relative humidity; LCR, logarithm of accumulated rainfall.
* Numbers following abbreviations of T, RH and LCR refer to the weather data summarized at 1, 2, 4, 8 and 11 weeks of the experimental periods in all seasons.
Significant values ($P \leq 0.05$) are shown in bold.

Discussion

In this study, we characterized the fungal community composition that colonizes grapevine pruning wounds at two pruning times in six vineyards belonging to three D.O. in Spain. The fungal microbiome across the three D.O. was largely composed by Ascomycota, followed by Basidiomycota. The predominant fungal phylum found in this work is consistent with the results obtained in other studies that explored the grapevine vascular tissue by culture dependent (González and Tello, 2011; Hofstetter et al., 2012; Pancher et al., 2012; Bruez et al., 2014, 2016, 2017; Dissanayake et al., 2018; Eichmeier et al., 2018; Kraus et al., 2019) or by HTAS (Dissanayake et al., 2018; Eichmeier et al., 2018; Deyett and Rolshausen, 2019, 2020; Martínez-Diz et al., 2019b) approaches. The core microbiome included the ubiquitous, fast-growing fungi *Aureobasidium* (Dothioraceae), *Cladosporium* (Cladosporiaceae), *Neofabraea* (Dermateaceae) and *Epicoccum* (Didymellaceae). This result is in line with recent studies aiming to decipher the fungal microbiome that resides in the xylem vessels of healthy grapevine branches in Germany (Kraus et al., 2019), and in the grapevine sap under high Pierce's disease pressure in California (Deyett and Rolshauen, 2019).

The results obtained in D.O. Rías Baixas showed a significant fraction of variation in fungal diversity (both the alpha and beta-diversity) that could be attributed to the infection period. It is interesting to note that fungal richness and diversity obtained in the infection period November-February was high relative to the period February-May in all D.O. In Mediterranean climates, drier and colder conditions usually occur after early pruning in mid-autumn, while warmer and wetter conditions favourable for fungal growth and infection occur progressively after pruning in late winter (Luque et al., 2014). The lack of significant trend in fungal microbiome abundances in both infection periods for all D.O. can be attributed to the Oceanic climate conditions in Galicia region, with temperate and rainy periods from autumn to spring, which may have favoured fungal spread and infection. In addition, two factors could also contribute to the high abundance of microbial infection during November-February, namely the wound healing and the bleeding processes. The wound healing involves the drying of the cane tissues below the pruning wounds (Bostock and Stermer, 1989), which results in a dead wood area called the drying cone (Lafon, 1921). In late winter and early spring, environmental conditions are favourable for a rapid wound healing. When the weather is cold, pruning wounds heal slowly leaving them open to fungal infection. In addition, bleeding of sap from the cut

ends of canes or spurs is the first sign of renewed activity. Bleeding alone might provide some wound protection by flushing away fungal spores in early spring.

Spores are usually spread from sexual or asexual structures by wind, arthropods or rain droplets, until they land on freshly and susceptible pruning wounds and with conditions of optimal air temperature and moisture begin to germinate (Bettiga, 2013). In this study, the correlation coefficients inferred between fungal microbiome infections and the accumulated rainfall or the mean daily temperature showed negative values for temperature until eight week after pruning, and positive and statistically significant correlations for rainfall at 8 and 11 weeks after pruning. An explanatory hypothesis for the negative correlations with temperature variable might be related with a combination of favourable climatic conditions promoting a faster and suitable pruning wound healing, which physically impeded the entrance of fungal spores into the grapevine vascular tissue. Pruning grapevines in dry and warm weather is known to enhance the mechanisms which reduce pruning wounds susceptibility (Munkvold and Marois, 1995; Rolshausen et al., 2010). However, further research is required to confirm this hypothesis. Positive correlations with accumulated rainfall could indicate that rain events have an effect in increasing fungal microbiome abundance, and hence, pruning wounds infections. Several studies found that spore release and airborne inoculum spread of fungal trunk pathogens in vineyards coincided with the beginning and/or after periods of rain or irrigation events (Pearson, 1980; Carter, 1991; Michailides and Morgan, 1993; Eskalen and Gubler, 2001; Gubler et al., 2005; Amponsah et al., 2009; Kuntzmann et al., 2009; Trouillas and Gubler, 2009; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010; Baskarathevan et al., 2013; Gubler et al., 2013; Úrbez-Torres et al., 2019). It has also been reported that rain can likely contribute to pycnidia and conidia masses development (Anco et al., 2013; Onesti et al., 2017), and to the splash-dispersal of conidia from pycnidia (González-Domínguez et al., 2020).

The linear discriminant analysis effect size detected several fungal clades, which discriminated the fungal communities between infection periods. The fungal genus *Aureobasidium* was predominant during the period November-February. Species of this genus, in particular *A. pullulans*, is known to prevail in the core microbiome of grapevine (Sabate et al., 2002; Martini et al., 2009; González and Tello, 2011; Barata et al., 2012; Pinto et al., 2014; Dissanayake et al., 2018; Deyett and Rolshausen, 2019; Martínez-Diz et al., 2019b). *A. pullulans* has evidenced great capacity to colonize grapevine pruning wounds (Munkvold and Marois, 1993) and to act as a biocontrol agent of several grapevine post-

harvest diseases (Schena et al., 2002; Martini et al., 2009). This yeast-like fungus also showed antagonistic abilities against *Eutypa lata*, the main causal agent of Eutypa dieback of grapevine, reducing of up to 50% fungal infection in pruning wounds (Munkvold and Marois, 1993). In a recent study, *A. pullulans* reduced the *in vitro* mycelial growth of *Diplodia seriata*, one of the causal agents of Botryosphaeria dieback of grapevine, but no significant reduction of necrotic lesions were found in grapevine cuttings (Pinto et al., 2018).

Several fungal genera associated with GTDs, such as *Cadophora*, *Cytospora*, *Diaporthe*, *Diplodia* and *Phaeomoniella*, were mostly identified during the infection period February-May and explained the differences observed between periods. Cross-infection throughout both periods was unlikely to occur given the long wood section of approximately 15-cm left between sampling periods. Using artificial inoculations with extreme disease pressure, the farthest downward growth for a fast-growing fungus such as *E. lata* was estimated to be 4 cm at 5 months after inoculation (Weber et al., 2007), and the overall mean of the GTD pathogens *Phaeomoniella chlamydospora* and *D. seriata* recovery five months after inoculation were 54.2% and 46.9%, respectively, at 4.5 cm below the pruning wound (Elena and Luque, 2016). Noticeably, low GTD fungal abundance were detected in annual shoots. The data support the evidence that these fungi prefer perennial woody stems, which is where wood symptoms associated with GTDs are commonly found (Gramaje et al., 2018).

Trunk disease fungi are mainly spread through aerially dispersed spores infecting grapevines via pruning and/or natural wounds (Rolshausen et al., 2010; van Niekerk et al., 2011; Gramaje et al., 2018). Spore release varies throughout the growing season depending on the fungal pathogen, geographical location and environmental conditions (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Quaglia et al., 2009; Úrbez-Torres et al. 2010a, 2010b; van Niekerk et al., 2010; Billones-Baaijens et al., 2018; González-Domínguez et al., 2020); therefore, information related to the dispersal patterns of GTD pathogens are indispensable to identify high-risk infection periods and to guide growers in timing management practices such as pruning time. In this sense, González-Dominguez et al. (2020) recently developed a model to predict disease risk caused by *Pa. chlamydospora* in vineyards and estimated that the pathogen dynamics were best explained when time was expressed as hydro-thermal time accounting for the effects of both temperature and rain. In the present study, evolution patterns of the correlation coefficients between weather data and OTUs abundance of GTD pathogens have been irregular with negatively and

positively values being rarely statistically significant. In the first week after pruning, temperature was negatively correlated with *Diaporthe* and *Phaeomoniella* genus abundances and as previously discussed for the fungal microbiome, this fact could be associated with a mixture of proper climatic conditions favouring the pruning wound healing process. Negative correlations values between mean daily temperature and *D. seriata* and *Pa. chlamydospora* natural infections were also found in the first weeks after pruning by Luque et al. (2014). Accumulated rainfall was found to have a positive significant correlation with *Diaporthe* from eight weeks highlighting again the role of rain events in the infection and development of GTDs fungal pathogens, as earlier considered for the fungal microbiome. This same trend was also observed by Luque et al. (2014) for natural infections caused by *D. seriata*, *Pa. chlamydospora* and species of Diatrypaceae in Catalonian vineyards.

Susceptibility of grapevine pruning wounds to GTD pathogens have been evaluated by artificial fungal inoculations in many grape-growing regions such as Australia (Ayres et al., 2016), California (Moller and Kasimatis, 1978; Munkvold and Marois, 1995; Eskalen et al., 2007; Úrbez-Torres and Gubler, 2011), France (Chapuis et al., 1998; Larignon and Dubois, 2000; Lecomte and Bailey, 2011), Italy (Serra et al., 2008), Michigan (Trese et al., 1982), South Africa (van Niekerk et al., 2011) and Spain (Elena and Luque, 2016). Globally, these reports indicated that susceptibility of pruning wounds decreased as the period between pruning and wound inoculation increased, and it could be extended up to four to seven weeks for most GTD fungi under favourable conditions. Little is known about the rate of natural infections in pruned canes. Research available has been only inferred from spontaneous fungal infections of vines included as non-inoculated controls in artificial inoculations trials.

Results obtained in our study on the natural infections of pruning wounds in three D.O. in Galicia showed that higher fungal GTD infection abundances occurred more frequently in spring than in winter, thus suggesting that pruning wounds could be more susceptible to GTD fungi overall after a late pruning in winter. Similar results were obtained by Luque et al. (2014), who observed higher isolation percentages of several GTD fungi in culture medium following late pruning (February-May) compared with that following early pruning (November-February). In contrast, mean percentage values of natural infections caused by *Eutypa lata* were approximately 2% after pruning in spring and 13% after the winter pruning in France (Lecomte and Bailey, 2011). Studies based on artificial inoculations also recommended late pruning to reduce GTD pathogens infections

(Petzoldt et al., 1981; Munkvold and Marois, 1995; Chapuis et al., 1998; Larignon and Dubos, 2000; Eskalen et al., 2007; Serra et al., 2008, Úrbez-Torres and Gubler, 2011). However, these trials did not consider the presence of natural GTD fungal inoculum during the experimental period, thus underestimating the real potential risk of fungal infections.

In conclusion, a broad range of fungi was able to colonize grapevine pruning wounds at both infection periods. Pruned canes harbour a core community of fungal species, which appear to be independent of the infection period. In light of the GTD colonization results and given the environmental conditions and the geographical location of this study, early pruning is recommended to reduce the infections caused by GTD fungi during the pruning season in Galicia. It is important to note that read counts in HTAS approach are considered as semi-quantitative (Amend et al., 2010). This means that there is no real quantitative relationship between spore count and read count, although a significant correlation between sequencing reads and the relative abundance of DNA of GTD fungi have been recently observed in soil samples (Berlanas et al., 2019). If specific assessment of aerial spore amount for a fungal species is needed, real-time quantitative PCR would be the method of choice. In this sense, high-throughput droplet digital PCR protocols have been recently developed for absolute quantification of GTD fungi from environmental samples (Holland et al., 2019; Maldonado-González et al., 2020; Martínez-Diz et al., 2020).

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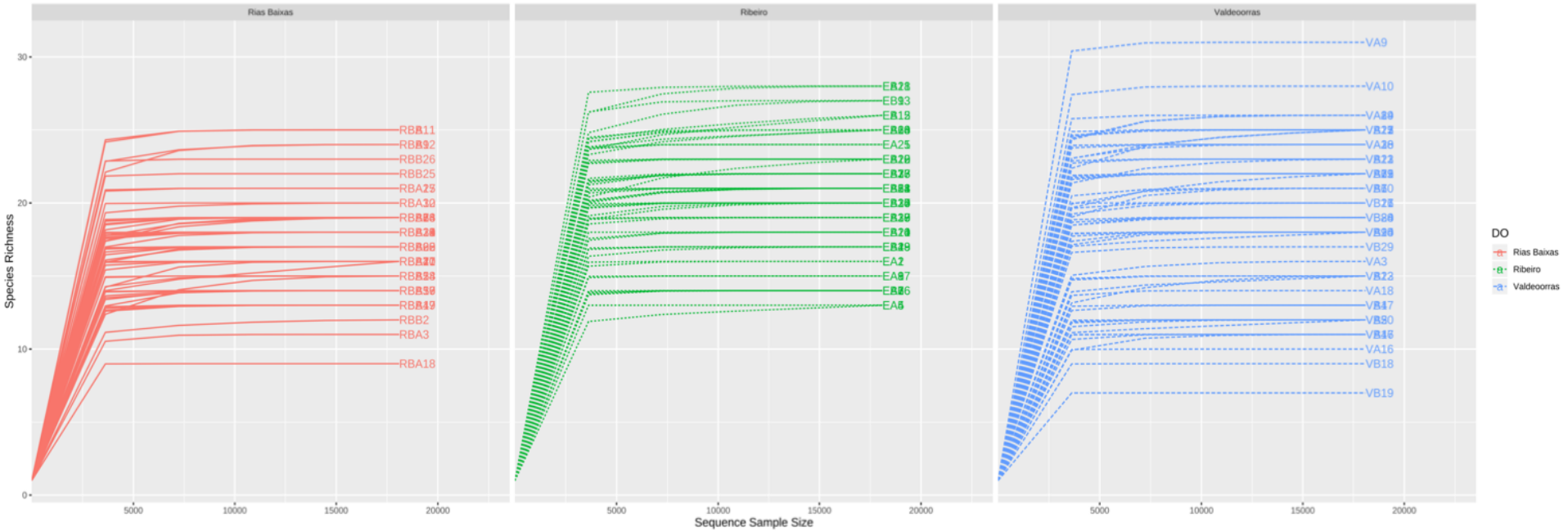
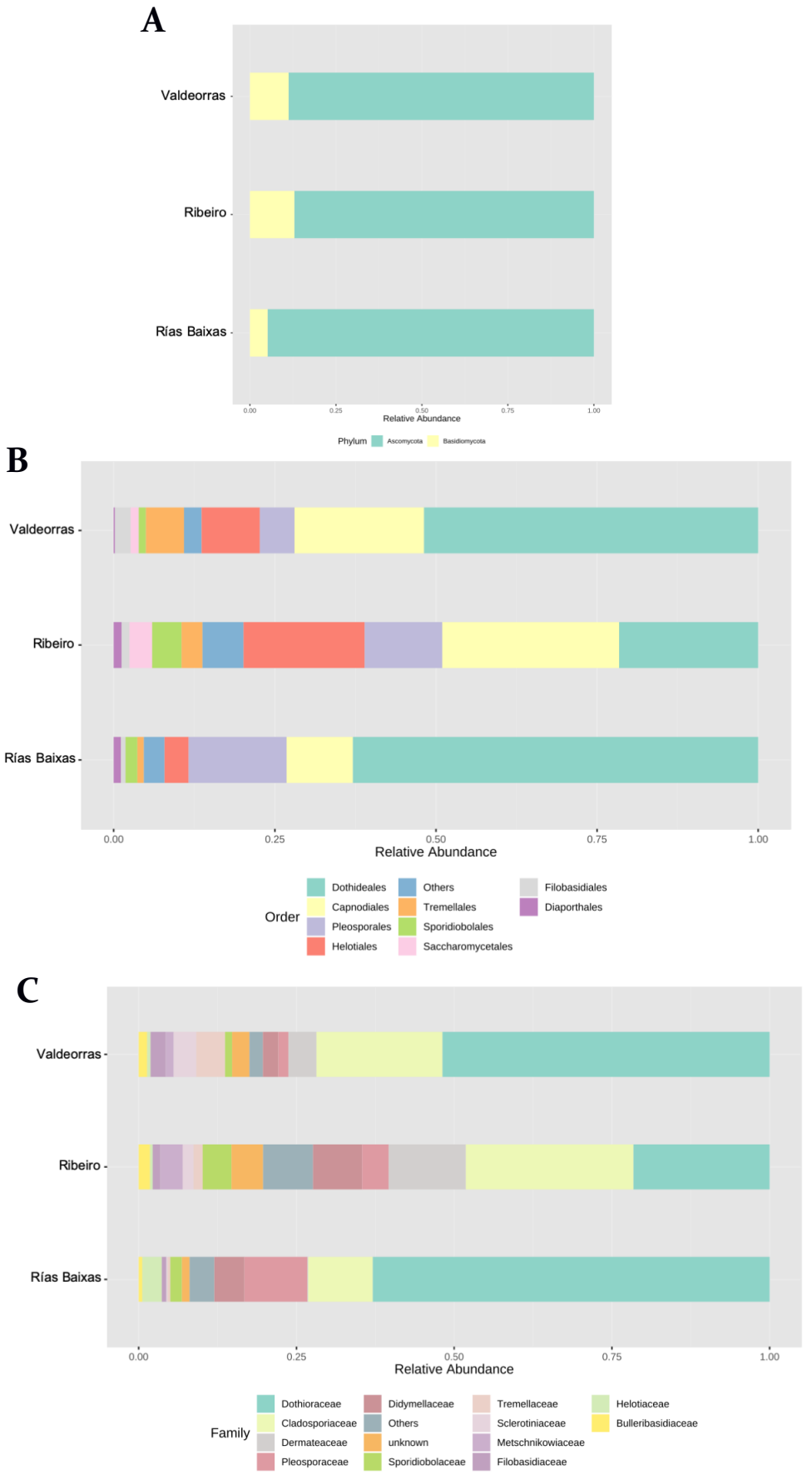
Chapter 4.2.

Supplementary material

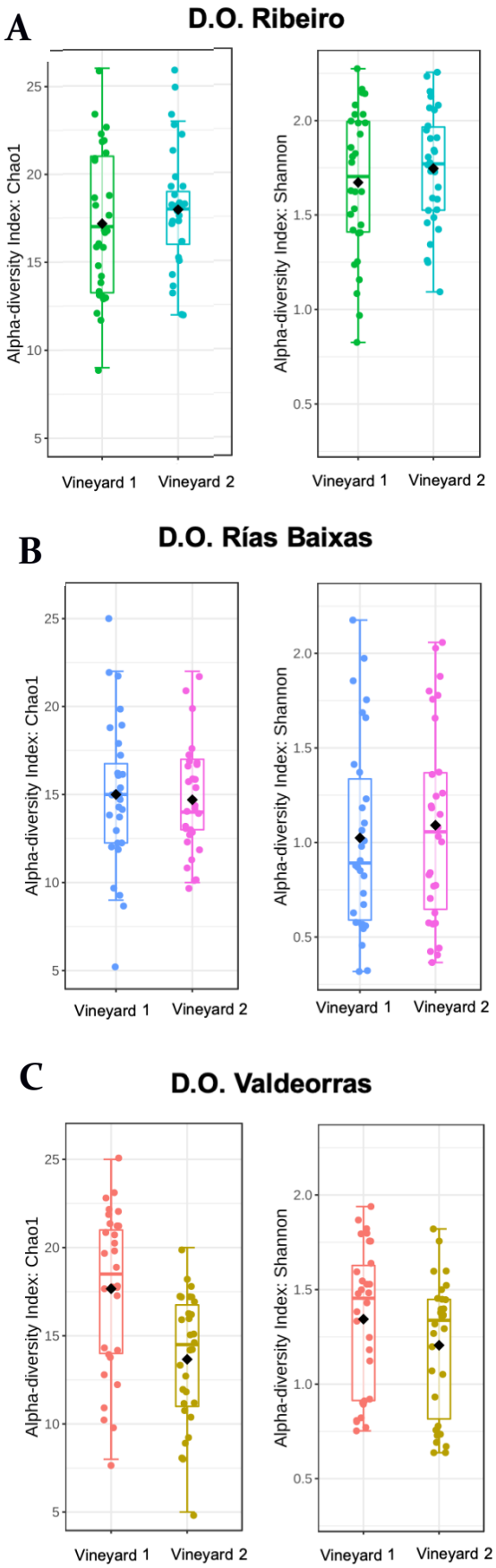
Supplementary **Figures**



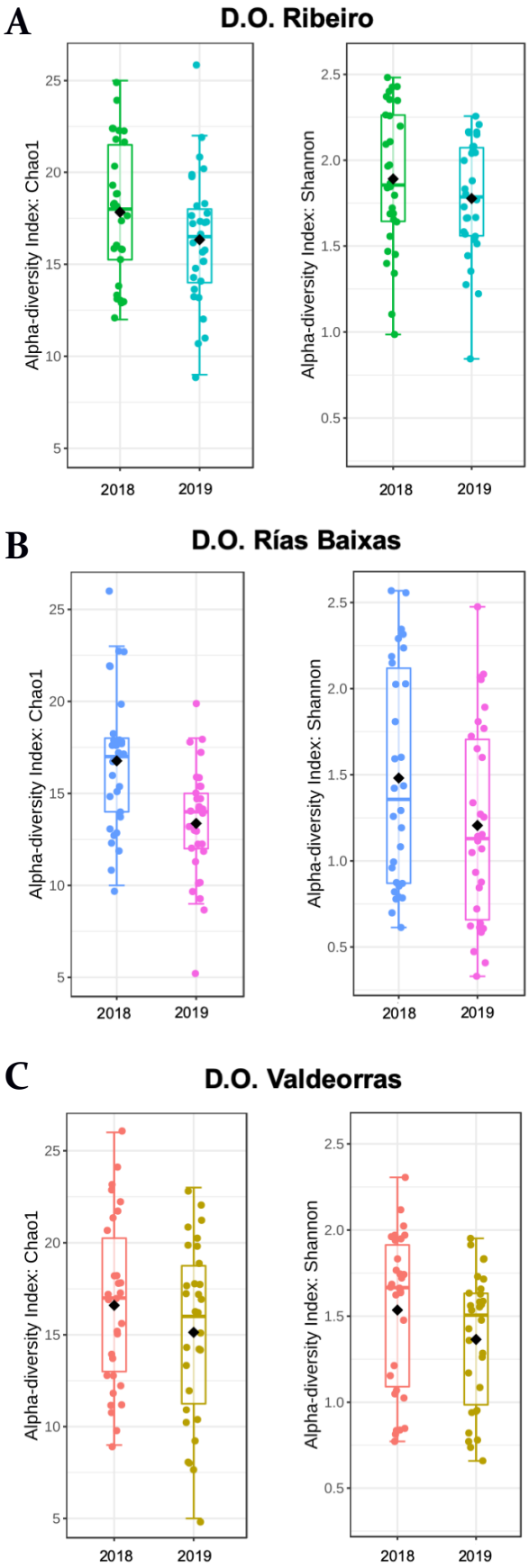
Supplementary Figure 4.2.1. Rarefaction curve values for each sample in each Denomination of Origin.



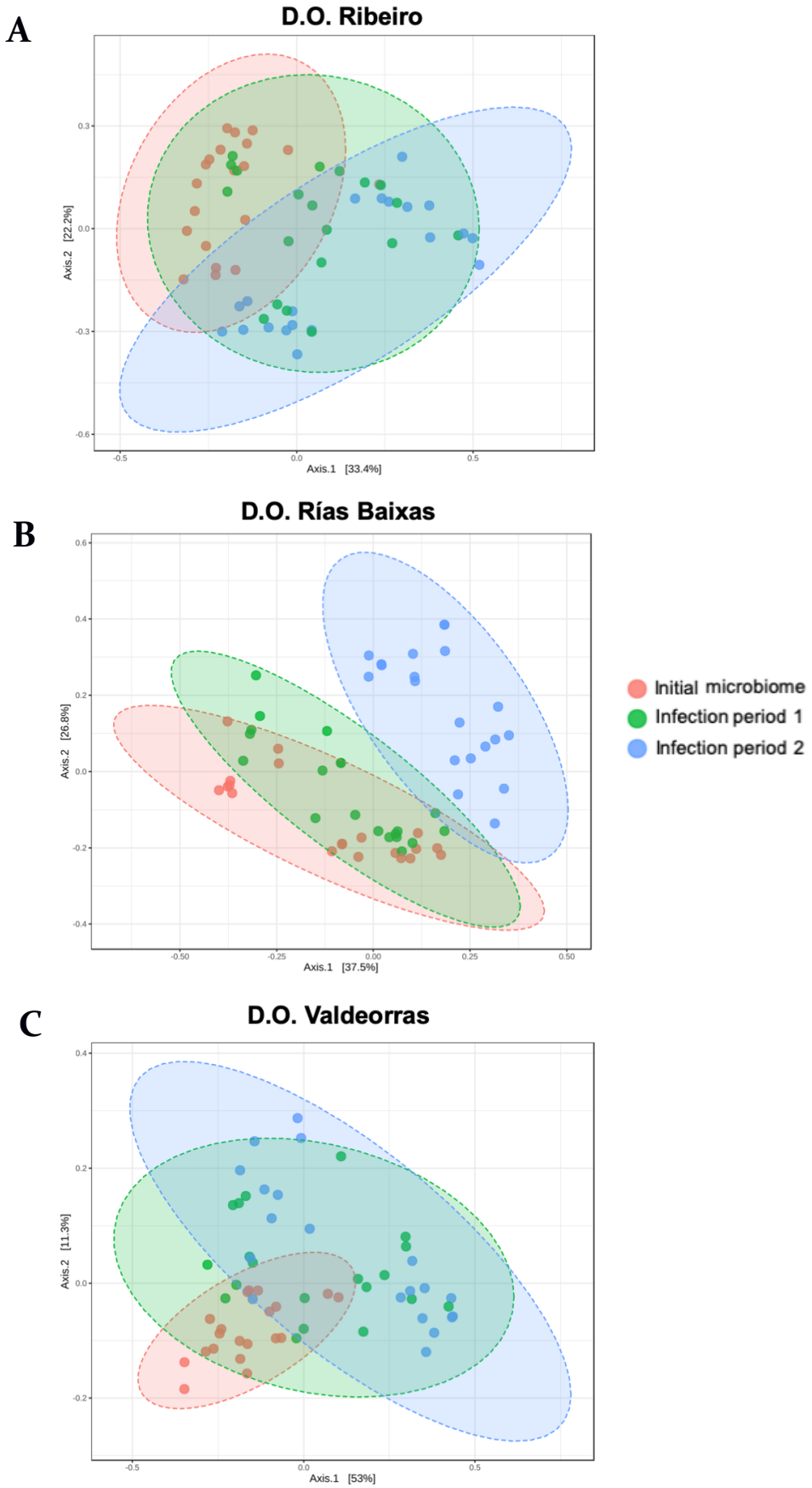
Supplementary Figure 4.2.2. Relative abundance of different fungal phyla (**A**), orders (**B**) and families (**C**) detected across Denominations of Origin.



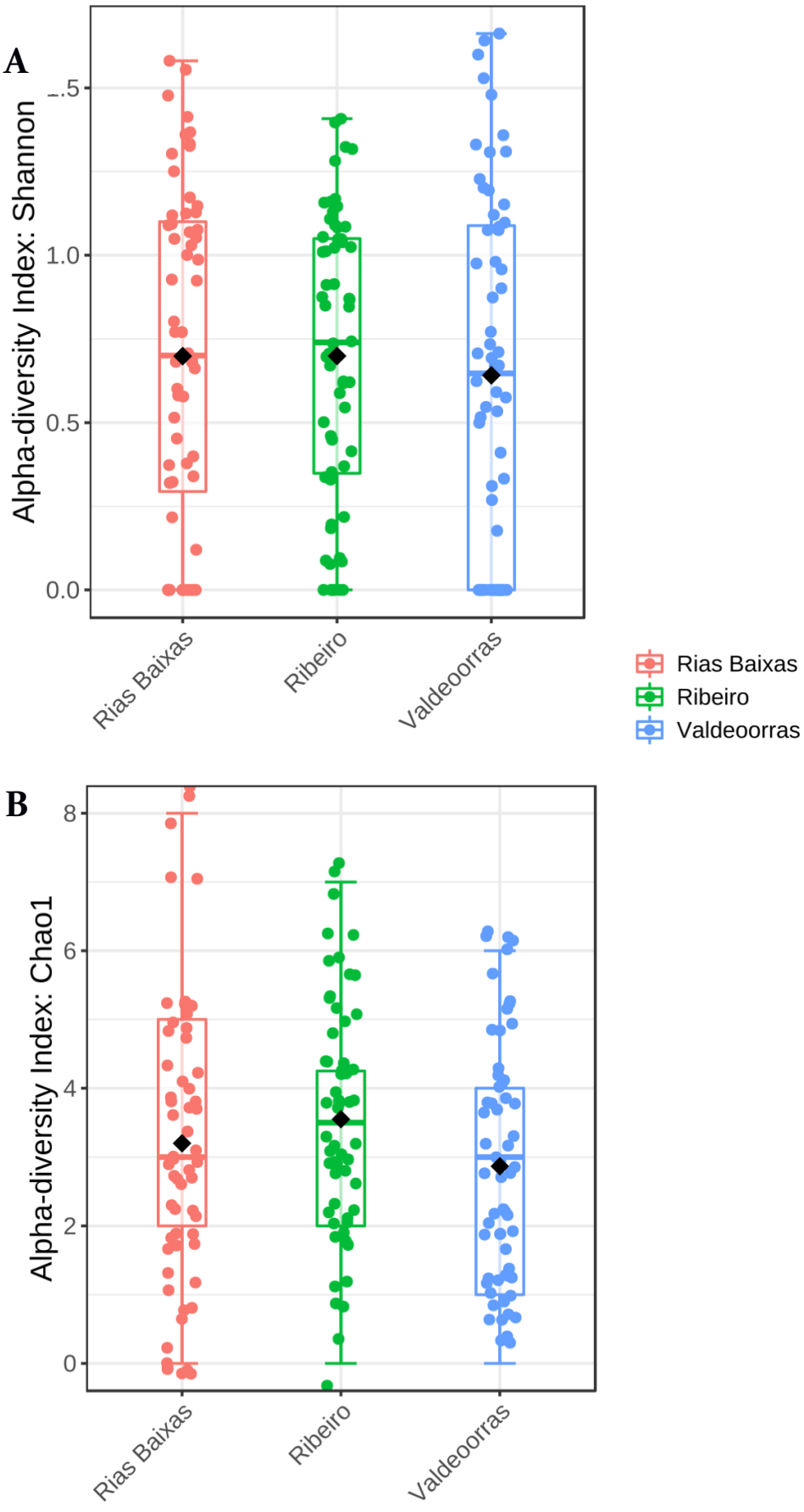
Supplementary Figure 4.2.3. Boxplot illustrating the differences in Chao1 and Shannon diversity measures of the fungal communities between vineyards in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).



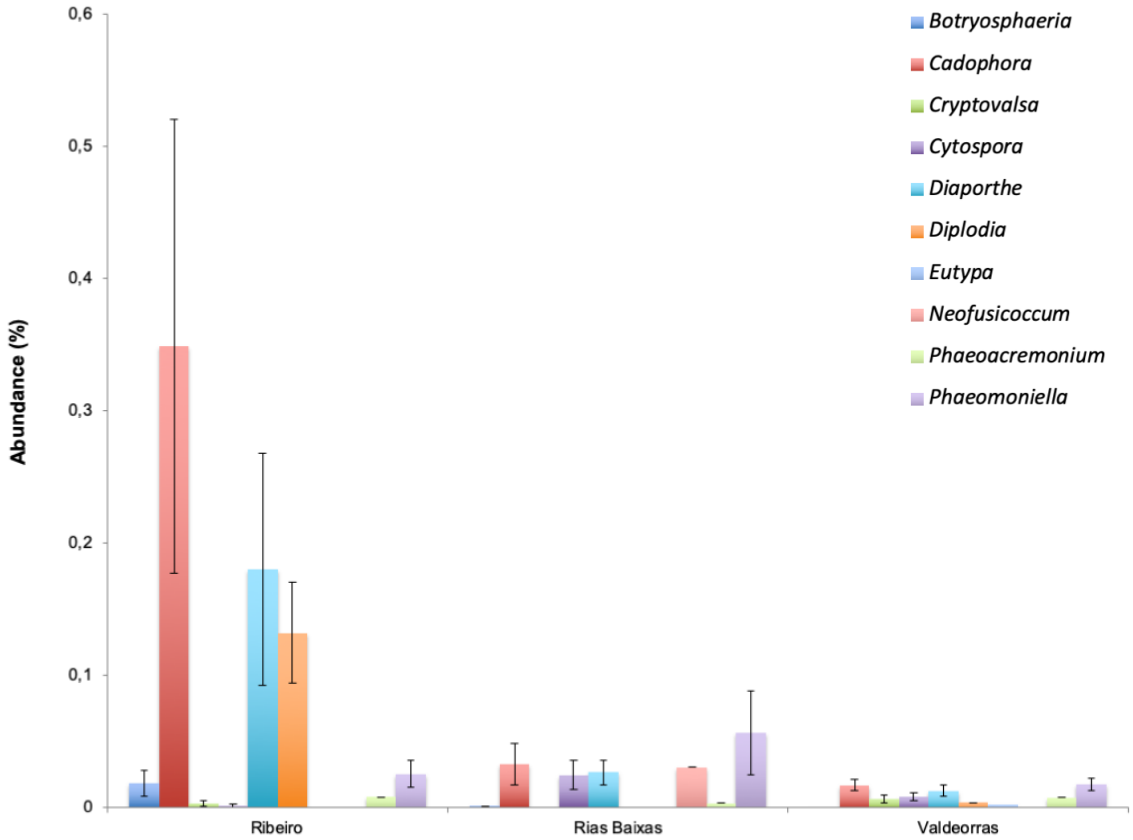
Supplementary Figure 4.2.4. Boxplot illustrating the differences in Chao1 and Shannon diversity measures of the fungal communities between years in D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).



Supplementary Figure 4.2.5. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics showing the distance in the fungal communities among sampling times D.O. Ribeiro (**A**), D.O. Rías Baixas (**B**), and D.O. Valdeorras (**C**).



Supplementary Figure 4.2.6. Boxplot illustrating the differences in Chao1 (**A**) and Shannon (**B**) diversity measures of the grapevine trunk disease pathogens among Denominations of Origin.



Supplementary Figure 4.2.7. Distribution of the relative abundance of fungal trunk diseases genera obtained by high-throughput amplicon sequencing in the annual shoot (sampling in November: initial microbiome) in the three Denominations of Origin.

Supplementary **Tables**

Supplementary Table 4.2.1. Main characteristics of the six vineyards used in this study.

	Vineyard-1	Vineyard-2	Vineyard-3	Vineyard-4	Vineyard-5	Vineyard-6
Coordinates	42°26'06.8"N 7°00'19.3"W	42°25'37.5"N 7°00'18.3"W	42°21'44.2"N 8°06'56.5"W	42°21'34.4"N 8°07'09.5"W	42°31'22.1"N 8°44'33.7"W	42°31'22.8"N 8°44'31.9"W
Location	O Barco de Valdeorras	O Barco de Valdeorras	Leiro	Leiro	Ribadumia	Ribadumia
Province	Ourense	Ourense	Ourense	Ourense	Pontevedra	Pontevedra
Denomination of Origin	Valdeorras	Valdeorras	Ribeiro	Ribeiro	Rías Baixas	Rías Baixas
Age	37	29	13	25	13	19
Rootstock	110 Richter	110 Richter	196-17 Castel	196-17 Castel	196-17 Castel	196-17 Castel
Cultivar	'Godello'	'Godello'	'Mencía'	'Mencía'	'Albariño'	'Albariño'

Supplementary Table 4.2.2. Estimates of number of reads, sample coverage and diversity indices at the genus level for fungal profiles.

Sample ID*	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
VA1	30997	99.99	14	0.82
VA2	37265	99.91	13	0.80
VA3	32082	99.25	13	0.89
VA4	55980	99.90	10	0.77
VA5	57752	100	12	0.92
VA6	44741	99.99	18	1.52
VA7	75471	99.26	17	1.18
VA8	102463	99.99	21	1.75
VA9	85422	99.99	26	1.95
VA10	110137	99.99	22	1.49
VA11	157989	99.97	21	1.39
VA12	151246	99.99	22	1.54
VA13	144063	99.29	21	1.79
VA14	112761	99.98	22	1.79
VA15	126625	99.98	23	1.82
VB1	21438	99.99	9	0.72
VB2	42497	99.99	11	0.75
VB3	29866	99.99	11	0.67
VB4	52414	99.99	12	0.77
VB5	38232	99.37	11	0.69
VB6	81483	99.99	17	1.82
VB7	85432	99.42	17	1.49
VB8	100993	99.99	17	1.40
VB9	73418	99.99	15	1.44
VB10	142701	99.99	19	1.76

(*Continued on next page*)

Supplementary Table 4.2.2. (*Continued from previous page*)

Sample ID*	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
VB11	153446	99.88	18	1.36
VB12	102367	99.98	15	1.35
VB13	126372	99.99	13	1.45
VB14	98737	99.97	16	1.26
VB15	143041	99.96	15	1.59
EA1	34336	99.97	16	1.39
EA2	47911	99.91	16	1.15
EA3	60592	99.99	14	1.08
EA4	39445	99.98	13	0.96
EA5	30599	99.95	12	1.41
EA6	30950	99.98	13	1.40
EA7	91545	99.43	13	1.62
EA8	35994	99.55	13	1.62
EA9	53704	99.90	13	1.82
EA10	77680	100	16	2.03
EA11	78617	99.99	16	1.80
EA12	110987	99.71	18	2.14
EA13	110881	99.98	22	2.27
EA14	113505	99.77	22	1.95
EA15	120807	99.97	22	2.13
EB1	30916	99.98	18	1.34
EB2	44533	99.98	18	1.25
EB3	65869	99.29	19	1.77
EB4	57978	99.53	15	1.48
EB5	62357	99.99	18	1.58
EB6	49423	99.92	19	1.94
EB7	62882	99.45	17	1.71
EB8	39264	99.61	18	1.99
EB9	123476	99.99	22	2.23
EB10	95639	99.99	21	2.24
EB11	99738	99.97	25	1.87
EB12	109848	99.83	23	2.04
EB13	139663	99.98	25	2.13
EB14	91304	99.99	19	1.67
EB15	87918	99.99	20	1.78
RBA1	78947	99.99	12	0.45
RBA2	107572	99.99	14	0.53
RBA3	74950	99.98	10	0.86
RBA4	122474	99.98	15	0.85
RBA5	95505	99.99	14	0.73
RBA6	92223	100	17	0.98

(*Continued on next page*)

Supplementary Table 4.2.2. (*Continued from previous page*)

Sample ID*	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
RBA7	50186	100	16	0.62
RBA8	72830	99.93	17	1.23
RBA9	107543	99.91	23	1.08
RBA10	92547	99.78	15	0.63
RBA11	91007	99.34	26	2.19
RBA12	101431	99.72	22	1.92
RBA13	122762	99.98	17	1.67
RBA14	95197	99.88	16	1.75
RBA15	76690	99.99	20	2.03
RBB1	73988	99.99	13	0.82
RBB2	107288	99.98	12	0.42
RBB3	37573	99.98	14	0.58
RBB4	32966	99.98	11	0.70
RBB5	100935	99.98	13	0.57
RBB6	71106	99.99	17	1.14
RBB7	102172	99.99	18	1.20
RBB8	72704	99.99	17	1.23
RBB9	60729	99.99	14	1.08
RBB10	133564	100	18	1.39
RBB11	74115	99.99	23	2.06
RBB12	71541	99.98	22	1.80
RBB13	120200	100	17	1.65
RBB14	95079	99.90	17	1.75
RBB15	108127	99.99	18	1.88
VA16	22135	99.98	8	0.89
VA17	25774	99.98	10	0.75
VA18	32272	99.99	11	0.80
VA19	31106	99.99	18	1.24
VA20	42558	99.99	14	1.12
VA21	36801	99.99	20	1.52
VA22	46534	99.98	18	0.91
VA23	26136	99.98	19	1.48
VA24	25613	99.98	14	1.33
VA25	37975	99.83	18	1.59
VA26	55837	99.72	21	1.47
VA27	45041	99.85	22	1.75
VA28	36321	99.79	20	1.43
VA29	34329	99.56	23	1.86
VA30	73180	99.97	21	1.63
VB16	20896	99.96	9	0.73
VB17	33556	99.98	10	1.05

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Supplementary Table 4.2.2. (*Continued from previous page*)

Sample ID*	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
VB18	29260	99.98	8	0.63
VB19	23805	99.98	5	0.63
VB20	33390	100	8	0.93
VB21	39692	99.99	17	1.39
VB22	49296	99.99	17	1.07
VB23	32440	99.98	21	1.63
VB24	29700	99.98	14	1.29
VB25	34674	99.98	14	1.20
VB26	48616	99.98	16	1.36
VB27	43326	99.98	17	1.52
VB28	41102	99.99	16	1.37
VB29	32632	99.95	12	1.31
VB30	65962	99.90	15	1.44
EA16	30996	99.99	9	1.23
EA17	26365	99.99	11	0.81
EA18	30523	99.99	15	1.53
EA19	24200	99.98	13	1.22
EA20	32822	99.81	16	1.44
EA21	34981	99.78	20	1.58
EA22	37116	100	18	1.96
EA23	34795	99.99	18	1.48
EA24	41678	99.98	17	1.76
EA25	42096	99.99	21	1.97
EA26	42101	99.90	21	1.88
EA27	43259	99.93	20	1.81
EA28	48662	99.93	25	2.06
EA29	46391	99.93	20	2.14
EA30	35451	99.96	16	2.01
EB16	25525	99.98	16	1.44
EB17	38998	99.98	17	2.04
EB18	38530	99.98	17	1.84
EB19	22581	100	14	1.52
EB20	36746	99.99	19	1.64
EB21	46211	99.89	17	2.11
EB22	44363	99.91	17	2.07
EB23	46907	99.99	12	1.90
EB24	62412	99.99	14	1.76
EB25	45877	99.99	15	1.52
EB26	29742	99.97	11	1.40
EB27	39639	99.90	16	1.57
EB28	23434	99.93	14	1.81

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Supplementary Table 4.2.2. (*Continued from previous page*)

Sample ID*	Number of reads	Good's coverage (%)	Chao1 richness	Shannon diversity
EB29	43869	100	13	1.24
EB30	45514	100	17	1.07
RBA16	31241	99.56	14	0.56
RBA17	40820	99.91	9	0.32
RBA18	21700	99.99	5	0.31
RBA19	30113	99.66	9	0.67
RBA20	42209	99.61	12	0.55
RBA21	32981	99.45	13	0.88
RBA22	36942	99.78	15	0.82
RBA23	30676	99.61	14	0.92
RBA24	32895	99.67	12	0.56
RBA25	42133	99.45	16	1.01
RBA26	23946	99.48	18	1.12
RBA27	30540	99.40	19	1.42
RBA28	33873	99.39	14	1.23
RBA29	35837	99.54	15	1.37
RBA30	45960	99.32	18	1.64
RBB16	35758	99.81	10	0.36
RBB17	27059	99.34	10	0.44
RBB18	25019	99.48	11	0.62
RBB19	28702	99.88	10	0.40
RBB20	42310	99.90	13	0.57
RBB21	38842	99.28	15	1.02
RBB22	40254	99.32	14	0.78
RBB23	36802	99.56	14	0.84
RBB24	38137	99.98	16	1.03
RBB25	33501	99.98	16	0.77
RBB26	34645	100	21	2.06
RBB27	21287	99.99	14	1.77
RBB28	32235	99.81	12	1.26
RBB29	35528	99.94	16	1.47
RBB30	27240	99.98	16	1.25

*VA/VB: D.O. Valdeorras; EA/EB: D.O. Ribeiro; RBA/RBB: D.O. Rías Baixas.

Supplementary Table 4.2.3. OTUs that were unique in both infection periods for each D.O.

D.O. Ribeiro		D.O. Rías Baixas		D.O. Valdeorras	
Nov-Feb	**Feb-May**	**Nov-Feb**	**Feb-May**	**Nov-Feb**	**Feb-May**
U* *Pleosporaceae*	*Arthrinium*	*Aleurobotrys*	*Bannozyma*	*Acremonium*	*Apiognomonia*
Athelia	*Aspergillus*	*Bensingtonia*	*Botryosphaeria*	*Apiognomonia*	*Bloxamia*
Bensingtonia	U *Heliotaceae*	*Boeremia*	*Bulleromyces*	*Apiotrichum*	*Botryosphaeria*
Colletotrichum	*Ceratobasidium*	*Camptophora*	*Coniosporium*	*Athelia*	U *Heliotales*
Conlarium	*Coniosporium*	*Clavaria*	*Coniothyrium*	*Boeremia*	*Candida*
Craterellus	*Cryptodiaporthe*	*Colacogloea*	*Constantinomyces*	*Buckleyzyma*	*Coniozyma*
Cyanodermella	*Cyclothyrium*	*Eucasphaeria*	*Cryptodiaporthe*	*Ceratobasidium*	*Constantinomyces*
Devriesia	*Dendrothyrium*	*Glomus*	*Cryptosporiopsis*	*Curvularia*	*Cryptodiaporthe*
Dioszegia	*Devriesia*	*Krasilnikovozyma*	*Cyanodermella*	*Eucasphaeria*	*Cryptovalsa*
Derxomyces	U *Gnomoniaceae*	*Malassezia*	U *Orbiliaceae*	*Exobasidium*	*Dendrophoma*
Erythrobasidium	*Fellozyma*	*Metschnikowia*	U *Gnomoniaceae*	*Exophiala*	*Devriesia*
Eucasphaeria	U *Tremellales*	*Penicillium*	*Derxomyces*	*Flagelloscypha*	*Unknown*
Exobasidium	*Keissleriella*	*Phaeotremella*	*Eutypa*	*Heterocephalacria*	*Gnomoniaceae*
Flagelloscypha	*Kwoniella*	*Phialophora*	*Exophiala*	*Krasilnikovozyma*	U *Dothideomycetes*
Kondoa	*Lachnella*	*Piskurozyma*	*Flagelloscypha*	U *Nectriaceae*	*Endoconidioma*
Lachancea	U *Pleosporaceae*	*Reddellomyces*	U *Physalacriaceae*	*Microdochium*	*Unknown*
Lecanicilium	*Lopthiotrema*	*Sampaiozyma*	*Fonsecazyma*	*Microstroma*	*Physalacriaceae*
U *Nectriaceae*	*Mulderomyces*	*Septoriella*	*Herpotrichia*	*Murilentithecium*	*Gnomoniopsis*
Neocucurbitaria	*Neofusicoccum*	*Sporidiobolales*	*Hyalotiella*	*Neophaeocryptopus*	*Hyalotiella*
Neophaeocryptopus	*Papiliotrema*	*Strigula*	*Hypocreales*	*Niesslia*	*Italica*
Penicillium	*Plagiostoma*		*Hypsotheca*	*Penicillium*	*Kalmusia*
Phaeococcomyces	*Raffaelea*		*Lachancea*	*Periconia*	*Lanzia*
Phialophora	U *Phanerochaetaceae*		*Lanzia*	*Phlyctema*	*Unknown*
Pleospora			*Lasionectria*	*Piskurozyma*	*Cystobasidiomycetes*
Pseudohyphozyma			U *Pleosporaceae*	*Pithomyces*	*Neofusicoccum*
Sarocladium			*Leucosporidium*	*Praetumpfia*	*Neosetophoma*
Seimatosporium			*Lewia*	*Rhizopus*	*Unknown*
Stagonospora			U *Niaceae*	*Septoriella*	*Pleosporales*
Sterigmatomyces			*Microdochium*	*Sporobolomyces*	*Papiliotrema*
Tygervalleyomyces			*Mucor*	*Sugiyamaella*	*Pleospora*
Xenoramularia			U *Filobasidiaceae*	*Tetracladium*	*Reddellomyces*
			Neoacrodontiella	*Tremellomycetes*	*Rhexocercosporidium*
			Neocucurbitaria	*Triposporium*	*Rhizoscyphus*
			Niesslia	*Vestigium*	*Sphaceloma*
			Periconia	*Xenoramularia*	*Sterigmatomyces*
			Phaeococcomyces		*Syncephalis*
			Powellomyces		
			Pyrenophora		
			Rachicladosporium		
			Rhizopus		
			Sclerostagnospora		
			Seiridium		
			Setophaeosphaeria		
			Sydowia		
			Syncephalis		
			Xylopsora		

*Unknown.

Supplementary Table 4.2.4. Mean values of temperature and relative humidity, and accumulated rainfall values at 1, 2 and 3 periods in each experimental season (winter: infection period Nov-Feb or spring: infection period Feb-May) for the two years of assay (2017/2018 and 2018/2019), in the three locations studied: (**A**) D.O. Ribeiro (Ourense), (**B**) D.O. Rías Baixas (Pontevedra), and (**C**) D.O. Valdeorras (Ourense).

A **D.O. RIBEIRO**

Season	Period	Days	Mean temperature (ºC)	Mean relative humidity (%)	Accumulated rainfall (mm)
Winter 2017/18	1	30	5.90	89.93	171.60
	2	30	7.77	89.80	125.60
	3	18	6.64	86.67	25.00
Winter 2017/18 total		78	6.79	89.13	322.20
Winter 2018/19	1	30	10.15	89.03	107.40
	2	30	5.51	90.23	81.60
	3	40	6.87	86.20	105.40
Winter 2018/19 total		104	7.44	88.26	294.40
Winter total (all years)			**7.12**	**88.69**	**308.30**
Spring 2018	1	30	6.96	83.23	180.60
	2	30	9.45	81.43	284.00
	3	39	13.95	71.08	62.60
Spring 2018 total		99	10.47	77.90	527.20
Spring 2019	1	30	10.24	76.60	88.80
	2	30	11.80	73.47	119.60
	3	31	14.75	70.10	59.20
Spring 2019 total		91	12.29	73.35	267.60
Spring total (all years)			**11.38**	**75.63**	**397.40**

B **D.O. RÍAS BAIXAS**

Season	Period	Days	Mean temperature (ºC)	Mean relative humidity (%)	Accumulated rainfall (mm)
Winter 2017/18	1	30	8.66	84.40	173.60
	2	30	10.57	88.93	206.20
	3	10	8.25	81.90	13.50
Winter 2017/18 total		70	9.42	85.99	393.30
Winter 2018/19	1	30	11.60	88.00	243.40
	2	30	9.38	79.67	34.40
	3	35	9.74	83.03	161.30
Winter 2018/19 total		95	10.22	83.54	439.10
Winter total (all years)			**9.82**	**84.76**	**416.20**
Spring 2018	1	30	8.85	77.97	200.20
	2	30	10.38	80.17	218.10
	3	39	13.20	77.44	143.20
Spring 2018 total		99	11.03	78.42	561.50
Spring 2019	1	30	11.71	76.37	83.60
	2	30	13.14	70.77	173.70
	3	31	14.61	73.87	87.10
Spring 2019 total		91	13.17	73.67	344.40
Spring total (all years)			**12.10**	**76.05**	**452.95**

C D.O. VALDEORRAS

Season	Period	Days	Mean temperature (ºC)	Mean relative humidity (%)	Accumulated rainfall (mm)
Winter 2017/18	1	30	6.08	84.17	133.60
	2	30	7.08	86.93	149.00
	3	16	6.30	81.94	15.80
Winter 2017/18 total		76	6.52	84.79	298.40
Winter 2018/19	1	30	9.06	86.73	81.40
	2	30	4.76	86.10	46.40
	3	44	7.20	80.14	181.80
Winter 2018/19 total		104	7.04	83.76	309.60
Winter total (all years)			**6.78**	**84.27**	**304.00**
Spring 2018	1	30	6.22	76.93	158.60
	2	30	8.66	76.20	214.40
	3	39	13.49	67.85	55.20
Spring 2018 total		99	9.83	73.13	428.20
Spring 2019	1	30	11.13	63.63	35.60
	2	30	11.15	71.63	148.40
	3	24	15.72	63.17	21.80
Spring 2019 total		84	12.45	66.36	205.80
Spring total (all years)			**11.14**	**69.74**	**317.00**

CHAPTER 5

Grapevine-pathogen interaction

Chapter 5.1.

Screening of Spanish *Vitis vinifera* germplasm for resistance to *Phaeomoniella chlamydospora*

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Highlights

Inoculation with fungal mycelial plugs was an efficient method to evaluate resistance to *Pa. chlamydospora* infection.

A broad range of susceptibility to *Pa. chlamydospora* infection was observed among Spanish minority and commercial cultivars.

This study allowed classifying grapevine cultivars according to varying signs of partial resistance to *Pa. chlamydospora*.

Keywords

Disease susceptibility
Esca
Germplasm screening
Grapevine
Leaf stripe disease

Abstract

Esca and leaf stripe diseases are two of the most destructive grapevine trunk diseases which are caused by a complex of fungal species, being the Ascomycete *Phaeomoniella chlamydospora* the most frequently isolated species from affected vines. To date, no curative measures are known for control these diseases; therefore, planting disease-resistant cultivars is a time-tested and sustainable approach for disease management. The aim of this study was to identify sources of resistance to *Pa. chlamydospora* among minority and commercial grapevine germplasm collections in Spain. For this purpose, 15 and 38 cultivars from the ITACyL and the EVEGA collections, respectively, were selected based on the visual assessment of symptom expression in standing vines over the last 3 years, and inoculated with *Pa. chlamydospora* in a detached cutting assay under greenhouse conditions over two consecutive years. Seven months after inoculation, cuttings were collected and inspected for lesion length. The severity of internal wood symptoms caused by *Pa. chlamydospora* varied considerably amongst the cultivars. No foliar symptoms were observed during the experiment. All cultivars were susceptible to fungal necrotic infection, indicating that there is no evidence of qualitative resistance to this Ascomycete fungus.

Introduction

Esca and grapevine leaf stripe diseases are two of the most destructive grapevine trunk diseases (GTDs) which are caused by a complex of fungal species, being the Ascomycete *Phaeomoniella chlamydospora* the most frequently isolated species from affected vines (Gubler et al., 2015). *Pa. chlamydospora* is endemic to all viticulture regions worldwide and is responsible for significant economic losses to the grape industry (Bertsch et al., 2013). Two forms of the symptom expression, chronic/mild and acute/apoplectic, have been traditionally reported to occur in vineyards. In the chronic or mild form, grapevine leaf stripe disease, leaf symptoms of affected vines are highly variable according to the literature: drying, dropping, reddening, and yellowing (Lecomte et al., 2012). The most characteristic foliar symptom of this form corresponds to the 'tiger-stripe' pattern, which can vary from year to year (Surico, 2009; Gubler et al., 2015). The acute or apoplectic form, esca, is characterized by a sudden wilting of the entire plant or of one arm or several shoots. Leaf symptoms include scorching, dropping, and shrivelling. The drying of grape clusters is also frequently observed (Mugnai et al.,

1999). Foliar symptoms of both forms appear in late spring or summer. Cross-sections of affected trunks reveal a variety of internal wood symptoms, such as black spots in the xylem eventually surrounded by pink to brown wood discoloration, brown to black vascular streaking, or dry wood with a silver appearance. In older vines, the wood may develop a white to yellow soft rot, also called as white rot syndrome. As it is very common to have wood rot and leaf stripe disease on the same vine, especially on vines greater than 8-10 years old, the term esca proper has been suggested to indicate the syndrome of vascular disease and wood rot and therefore to include foliar symptoms, i.e., a complex of two different diseases (Gubler et al., 2015).

Phaeomoniella chlamydospora is primarily spread through the dispersion of airborne spores and infect grapevine through any type of open wound (Gubler et al., 2015). In addition, this fungus can also be propagated through the use of infected cuttings (Gramaje and Armengol, 2011). Complete eradication is not possible, so effective control of esca and grapevine leaf stripe diseases relies mainly on disease prevention and mitigation (Gramaje et al., 2018). Once a mature vine is infected by other GTDs such as Botryosphaeria or Eutypa diebacks, the only options are to either remove diseases tissues and retrain new cordons or canes from the healthy part of the trunk or replant (Creaser and Wicks, 2004; Sosnowski et al., 2011). However for esca and grapevine leaf stripe diseased vines, where internal necrosis is often observed in both scion and rootstock wood of affected plants, the success of remedial surgery is very limited (Calzarano et al., 2004).

Disease resistance is a key element in an Integrated Pest Management program. The use of tolerant cultivars, clones, and rootstocks would be the least expensive, easiest, safest, and most effective means of controlling esca and leaf stripe diseases. Cultivation of tolerant material would not only reduce losses from the diseases, but also would markedly decrease the need for spray treatments and curative control strategies, and reduce the level of toxic chemicals in the vineyard environment. The existing infection assays for *Pa. chlamydospora* have indicated that grapevine cultivars and rootstocks might show varying levels of susceptibility to pathogen infection, but no evidence of qualitative resistance to *Pa. chlamydospora* has been found. These phenotyping assays on grapevine relies on mechanical inoculation of detached shoots under laboratory conditions (Zanzotto et al., 2008; Santos et al., 2015), attached dormant rootstock or cultivar cuttings under greenhouse conditions (Eskalen et al., 2001; Sosnowski et al., 2007b; Travadon et al., 2013; Markakis et al., 2017; Pouzoulet et al., 2017), and rootstock

cuttings or cultivars under field conditions (Feliciano et al., 2004; Gramaje et al., 2010; Sofia et al., 2018). Most of these studies are focused so far on examinating the levels of grapevine resistance to wood symptoms, such as discolouration and canker formation, caused by the fungus. This is because foliar symptoms of esca and grapevine leaf stripe diseases have very rarely been reproduced consistently under controlled conditions (Lecomte et al., 2012). The present study was therefore undertaken to identify sources of resistance to *Pa. chlamydospora* among minority and commercial grapevine germplasm collections in Spain based on the severity of internal wood symptoms.

Materials and methods

Plant material

Inoculation experiments were conducted on 1-year-old grapevine cuttings of 15 (9 red and 6 white cultivars) and 38 (20 red and 18 white cultivars) cultivars collected from the Instituto Tecnológico Agrario de Castilla y León (ITACyL) and the Estación de Viticultura y Enología de Galicia (AGACAL-EVEGA) germplasm collections, respectively. Plant material was selected based on the visual assessment of grapevine trunk diseases symptom expression from consistent asymptomatic vines over the last 3 years. In total, 2,544 dormant cuttings were cut into uniform lengths containing four buds during winter time. Grapevine cuttings were kept in cold storage for one month. After this period, each dormant cutting was individually buried into sterilized peat moss in 20 cm diameter polyurethane pots, and placed in a callusing room at 25°C and 100% humidity for two months. After callusing and rooting, cuttings were placed in the greenhouses of the ITACyL and the EVEGA at 25°C with a relative humidity of 40% until inoculation.

Fungal isolate and inoculation experiments

The *Pa. chlamydospora* strain BV-130, which was obtained from the culture collection of the Instituto de Ciencias de la Vid y del Vino (ICVV) and selected among eight *Pa. chlamydospora* isolates in a previous virulence assay carried out at the ICVV, was used. This strain was isolated from internal wood symptoms such as black spots and brown streaking in a 43-year vineyard cultivar 'Tempranillo' grafted onto '41 Berlandieri' rootstock with apparent foliar symptoms of grapevine leaf stripe disease in 2015.

Cuttings were wounded between the two upper internodes with a 5-mm cork borer (Péros and Berger, 1994). Mycelium plugs were inserted as inoculum into the wounds. After inoculation, each wound was covered with moist cotton wool, sealed with a strip of Parafilm and wrapped with foil paper to prevent drying. Mycelium plugs were obtained from the periphery of actively growing fungal colonies cultivated on potato dextrose agar (PDA, 2% malt extract, Oxoid Ltd., England; 1.5% agar, Difco, USA). PDA plugs were used for control inoculations. Twelve plants were inoculated with the fungus. Twelve plants were used for control inoculations. The experiment was repeated over two consecutive years (2016 and 2017). Pots were arranged in a completely randomised design and watered twice weekly. Plants were collected after 7 months and inspected for lesion development. Extent of vascular discolouration was measured upward and downward from the inoculation point by the ImageJ software. In 2016 and 2017, three inoculated cuttings of each cultivar were selected and small pieces (0.5 to 1 cm) of necrotic tissue from the edge of each lesion were cut and placed on malt extract agar (MEA, 2% malt extract, Oxoid Ltd., England; 1.5% agar, Difco, USA) supplemented with 0.5 g/l of streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA) in an attempt to recover the inoculated fungi and complete Koch's postulates. In 2017, fungal isolation was also performed from 1 cm intervals above the inoculation point. This was done to a maximum of 8 cm beyond staining on three inoculated cuttings and tissues were transferred to MEAS plates. Recovery rates were calculated as the percentage of plants from which a pathogen was recovered out of the total number of inoculated plants. Plates were incubated at 25°C in darkness and fungi subcultured onto PDA after 7-10 days. Two weeks later, cultures were identified as *Pa. chlamydospora* based on colony morphology.

Statistical analysis

Prior to analysis of variance (ANOVA), homogeneity of variance across treatments was evaluated using Levene's test (Box et al., 1978). To satisfy the assumption of homogeneity of variance, a square-root transformation to total lesion length was applied. Lesion length over 2 years was analysed using ANOVA. Data from both greenhouse trials were analysed using the Statistix 10 software (Analytical Software). Transformed data means were compared using Tukey's honestly significant difference test at $P = 0.05$.

Results

In both greenhouse trials, there were no differences in lesion length data between the two years (P = 0.3265 for the ITACyL trial and P = 0.1138 for the EVEGA trial), so data from both years were combined. The trunk inoculations result of the greenhouse trials showed that the fungal species *Pa. chlamydospora* caused vascular discolouration on all grapevine cultivars wood, significantly longer than control plants. An example of the vascular discolouration caused by the fungus in several cultivars in the EVEGA trial is shown in Figure 5.1.1.

Mean lengths of the extent of vascular discolouration caused by *Pa. chlamydospora* on inoculated one-year-old grapevine wood of 9 red and 6 white cultivars in the ITACyL trial are shown in Figures 5.1.2A and 5.1.2B, respectively. In the red cultivar evaluation, mean lesion lengths caused by the fungus ranged from 27.0 ('Estaladiña' cultivar) to 58.6 mm ('Merenzao' cultivar) (Fig. 5.1.2A), while this parameter ranged from 31.9 ('Albillo Mayor' cultivar) to 70.8 mm ('Doña Blanca' cultivar) in white cultivars (Fig. 5.1.2B).

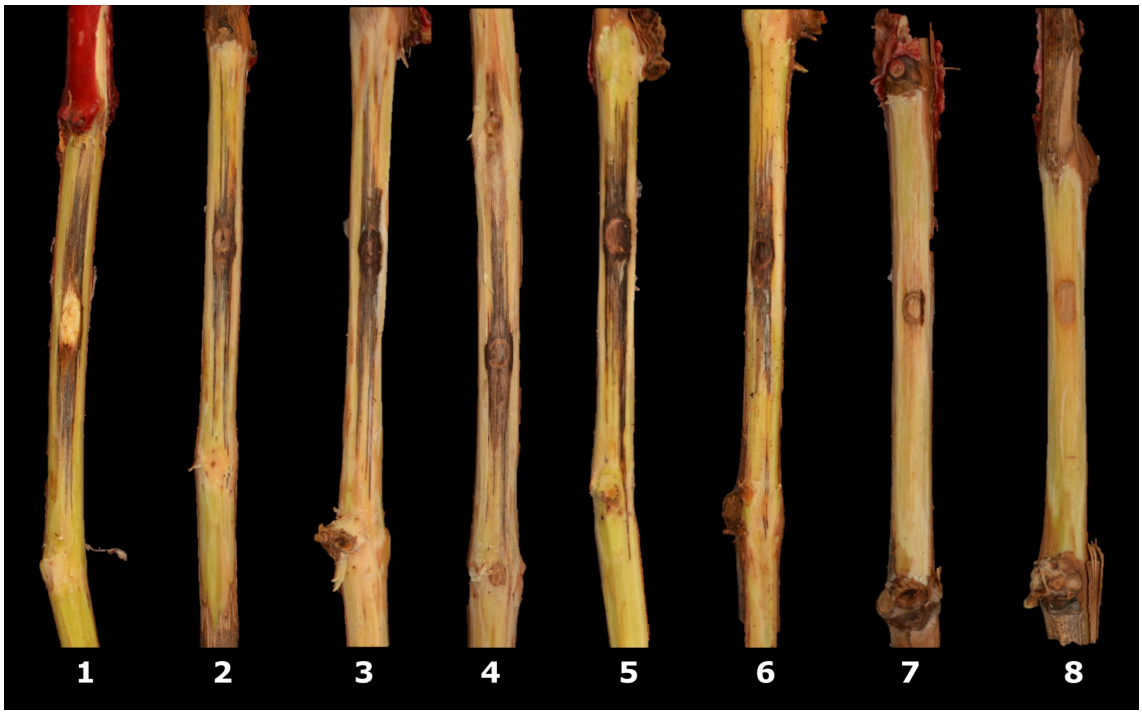

Figure 5.1.1. Vascular necrosis and dark streaking of the wood caused on grapevine cuttings cultivars 'Godello' (**1**), 'Albarín Blanco' (**2**), 'Doña Blanca' (**3**), 'Pan y Carne' (**4**), 'Palomino' (**5**), 'Zamarrica' (**6**), 'Mencía' (**7**) and 'Brancellao' (**8**), 7 months after inoculation by *Phaeomoniella chlamydospora* (**1–6**) and control (**7–8**).

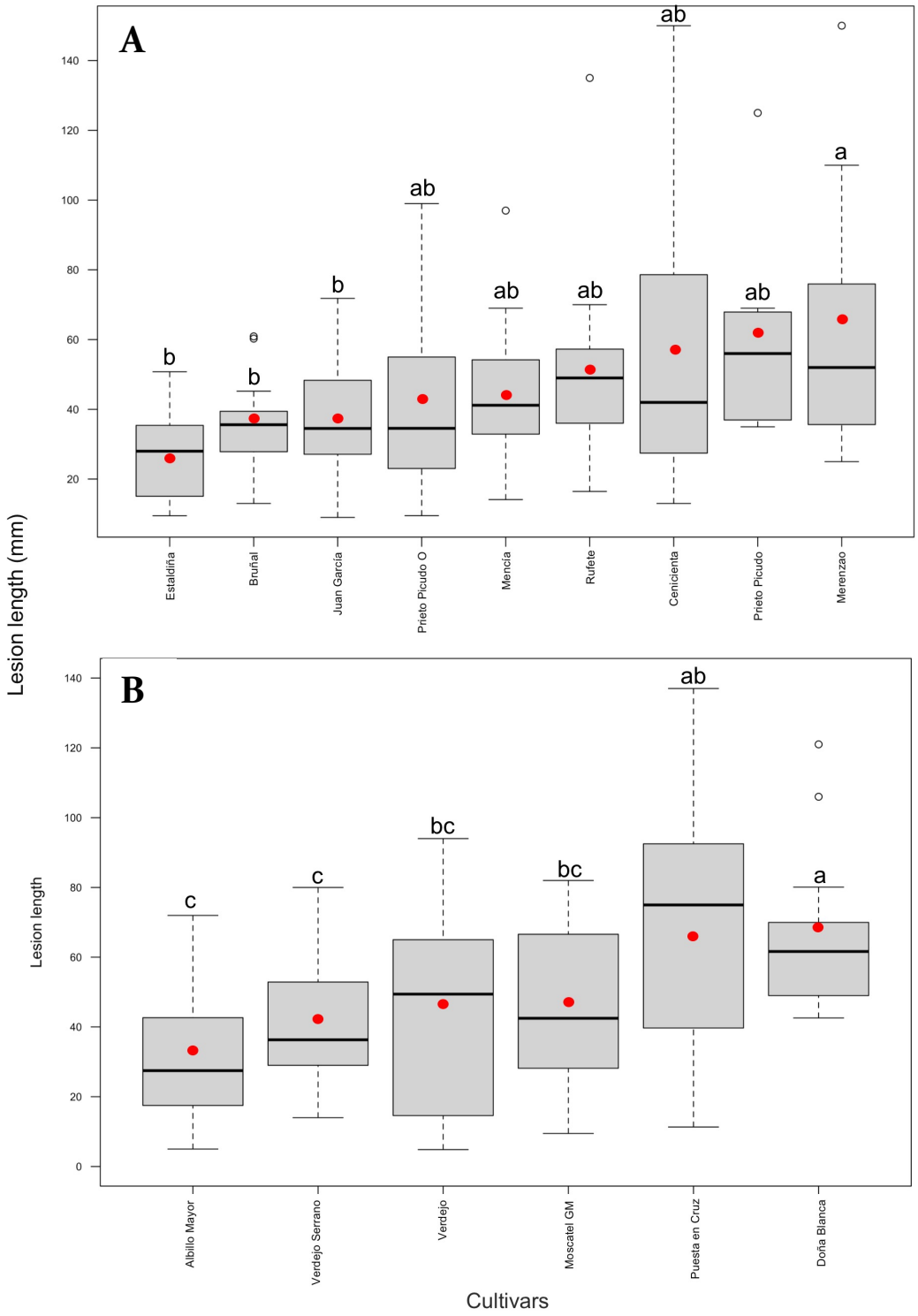

Figure 5.1.2. Grapevine susceptibility to *Phaeomoniella chlamydospora* in the ITACyL trial. Box plots illustrating the distribution of length of wood discolouration measured in 9 red (**A**) and 6 white (**B**) at 7 months after inoculations. Results are ordered according to the mean. Solid lines and red circles within the box correspond to the median and the mean, respectively. Top and bottom lines of the box correspond to the 25th and 75th percentiles of the data, respectively. Error bars represent the 10th and 90th percentiles, and circles represent the 5th and 95th percentiles. Mean lengths of wood discolouration of cultivars with different letters are significantly different at $P < 0.05$, Tukey's test. Cultivar abbreviations: 'Prieto Picudo O': 'Prieto Picudo Oval'; 'Moscatel GM': 'Moscatel Grano Menudo'.

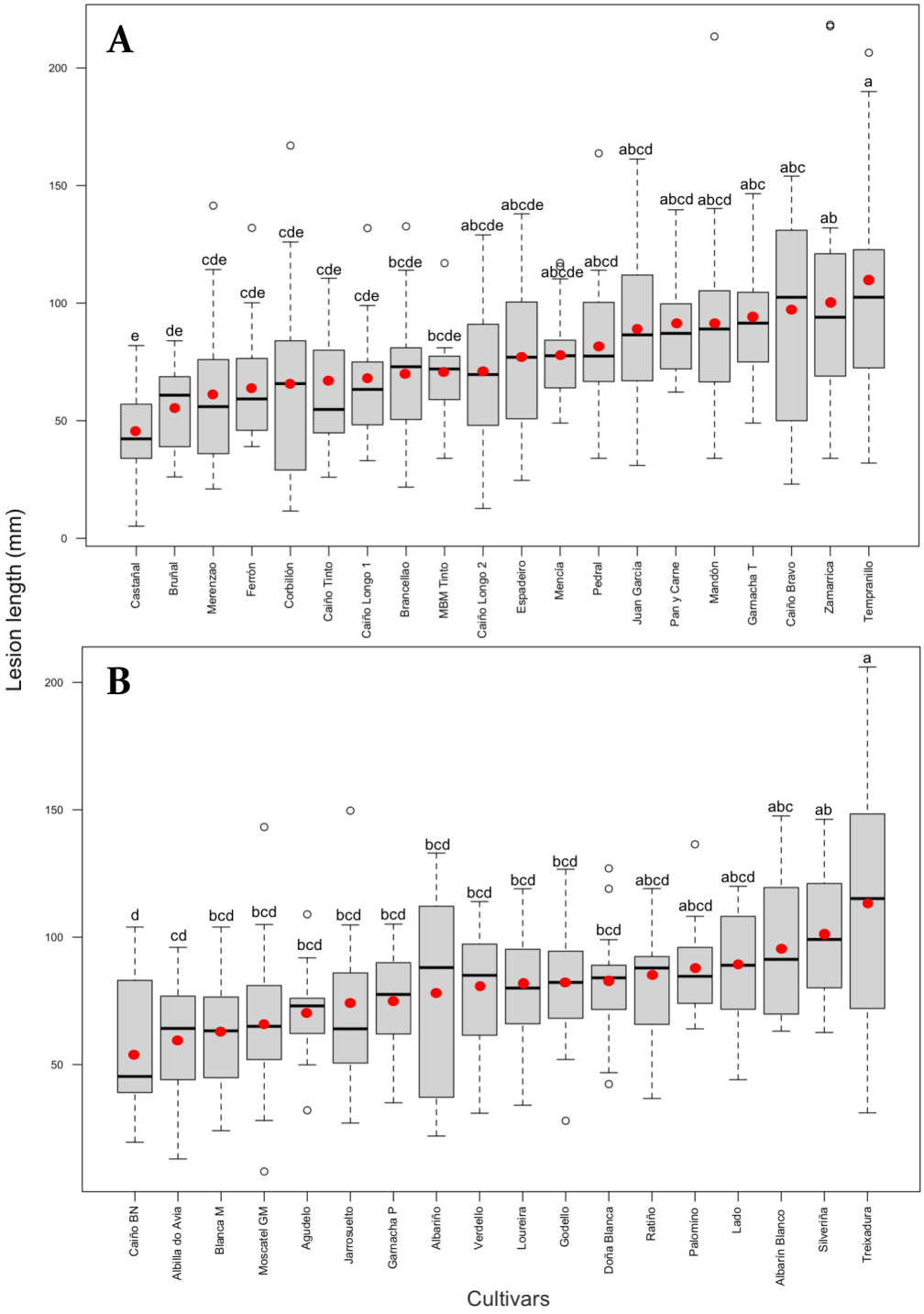

Figure 5.1.3. Grapevine susceptibility to *Phaeomoniella chlamydospora* in the EVEGA trial. Box plots illustrating the distribution of length of wood discolouration measured in 20 red (**A**) and 18 white (**B**) at 7 months after inoculations. Results are ordered according to the mean. Solid lines and red circles within the box correspond to the median and the mean, respectively. Top and bottom lines of the box correspond to the 25th and 75th percentiles of the data, respectively. Error bars represent the 10th and 90th percentiles, and circles represent the 5th and 95th percentiles. Mean lengths of wood discolouration of cultivars with different letters are significantly different at $P < 0.05$, Tukey's test. Cultivar abbreviations: 'Garnacha T': 'Garnacha Tintorera'; 'Caiño BN': 'Caiño Blanco'; 'Blanca M': 'Blanca de Monterrei'; 'Moscatel GM': 'Moscatel Grano Menudo'; 'Garnacha P': 'Garnacha Peluda'.

Mean lengths of the extent of vascular discolouration caused by *Pa. chlamydospora* on inoculated one-year-old grapevine wood of 20 red and 18 white cultivars in the EVEGA trial are shown in Figures 5.1.3A and 5.1.3B, respectively. In the red cultivar evaluation, mean lesion lengths caused by the fungus ranged from 40.7 ('Castañal' cultivar) to 94.0 mm ('Tempranillo' cultivar) (Fig. 5.1.3A), while this parameter ranged from 58.8 mm ('Caiño Blanco' cultivar) to 91.2 mm ('Treixadura' cultivar) in white cultivars (Fig. 5.1.3B).

In the ITACyL trial, *Pa. chlamydospora* was reisolated from the edge of each lesion and beyond the staining in asymptomatic tissue in 86.6% and 33.3% of the cultivars, respectively. In the EVEGA trial, *Pa. chlamydospora* was reisolated from the edge of each lesion and beyond the staining in asymptomatic tissue in 100% and 36.8% of the cultivars, respectively. No *Pa. chlamydospora* recovery was observed in the non-inoculated plants.

Discussion

This study represents the first comprehensive phenotyping assay to identify tolerant grapevine cultivars to *Phaeomoniella chlamydospora* infection in germplasm collections. Assessment of the Spanish minority and commercial cultivars in our controlled inoculation conducted in duplicate greenhouse experiments indicated that the severity of internal wood symptoms caused by *Pa. chlamydospora* varied amongst the cultivars. Nevertheless, the statistical analyses did not allow us to clearly distinguish tolerant and susceptible cultivars within the grapevine germplasm collections. *Pa. chlamydospora* caused vascular discolouration on all grapevine cultivars wood, significantly longer than control plants, but no foliar symptoms were observed on inoculated plants during the experiments. Results were generally consistent with those obtained by Travadon et al. (2013), who reported similar degree of susceptibility of seven commercial *Vitis vinifera* cultivars ('Cabernet Franc', 'Cabernet Sauvignon', 'Chardonnay', 'Merlot', 'Riesling', 'Petite Syrah' and 'Thompson Seedless') and one *Vitis* hybrid cultivar ('Concord') to *Pa. chlamydospora* infection 11 months after inoculating potted vines under greenhouse conditions. There is no known qualitative resistance in grapevine that prevents infections by fungal trunk pathogens. Rather, all cultivars, clones and rootstocks can potentially be infected, but symptom expression and severity vary among them.

Quantitative resistance, also called partial, race nonspecific, general, polygenic, field or horizontal resistance, is usually controlled by several genes. These genes seem to exert

their influence by controlling the numerous steps of the physiological processes in the plant that provide the structures and materials that create the defence mechanisms of the plant (Niks et al., 2015). Quantitative resistance does not prevent infection, but nonetheless limits the development of individual infection loci on a plant, thereby reducing the spread of the disease and the development of epidemics in the field over time (Stuthman et al., 2007). Such resistance is affected by and may vary considerably when environmental or plant tissue conditions are favourable to disease (Lindhout, 2002). This is well illustrated by the annual variation in symptoms of another trunk disease of mature vines, Eutypa dieback (Sosnowski et al., 2007a).

Several critical factors, namely stem age, isolate virulence, inoculation environment, inoculum type and amount, and evaluation timing, could have an influence in determining disease resistance. Different inoculation methods, such as soaking bases of grapevine cuttings or seedlings in spore suspensions (Eskalen et al., 2001), insertion of mycelial plugs (Sosnowski et al., 2007b) or conidial suspensions (Feliciano et al., 2004; Wallace et al., 2004; Zanzotto et al., 2008; Travadon et al., 2013; Santos et al., 2015; Pouzoulet et al., 2017) into side wounds or cut ends of the grapevine stems, and vacuum-inoculation of conidial suspensions throughout the vascular system of rootstock cuttings (Gramaje et al., 2010) have been used for resistance screening of grapevine against *Pa. chlamydospora*. In general, these phenotyping assays have also indicated that grapevine cultivars and rootstocks might show varying levels of susceptibility to *Pa. chlamydospora* infection in terms of foliar symptoms and wood canker formation. However, we were not able to compare the results of our phenotyping assays with those obtained by other researchers due to the different range of cultivars evaluated.

Under field conditions, the screening procedure mainly relies on visual assessment for esca and leaf stripe disease severity foliar symptoms (Marchi et al., 2001; Fussler et al., 2008; Bruez et al., 2013; Murolo and Romanazzi, 2014; Borgo et al., 2016). The problem with some field evaluations of cultivar susceptibility is that the GTD pathogens often occur in mixed infections within a vine (Úrbez-Torres et al., 2006) and so there may be some uncertainty that the symptoms are due to the effects of a single trunk pathogen. For esca and leaf stripe diseases in particular, their assessment of prevalence on the basis of foliar symptoms might not be a reliable variable due to the uncertain correlation between foliar symptoms and wood deterioration, the erratic nature of the diseases and the time required for foliar symptoms to appear (Lecomte et al., 2012, 2017).

In our study, *Pa. chlamydospora* was reisolated from asymptomatic tissue beyond necrotic staining in 33.3% and 36.8% of the cultivars in the ITACyL and the EVEGA trials, respectively. Reisolations of GTD pathogens are frequently made from the edge of the necrosis, thus completing Koch's postulates. However, in several studies published by Sosnowski et al. (2007b, 2016a, b), the distance recovery of the fungal trunk pathogens *Diplodia seriata* and *Eutypa lata* and lesion length were not correlated. These authors hypothesized that lesion length might not be a reliable measure of susceptibility to pathogen colonization. Plant genotypes may display resistance, susceptibility or tolerance to vascular pathogens (Beckman and Roberts, 1995; Fradin and Thomma, 2006). Resistance is characterized by the ability of the plant to successfully compartmentalize the pathogen, whereas in susceptible and tolerant genotypes the plant is not able to restrict pathogens movement often leading to systemic infection. Nevertheless, tolerant plant genotype exhibits little disease symptoms despite pathogen colonization as oppose to susceptible plant genotypes suggesting that tolerant plants have the ability to counteract the effect of virulence factors produced by the vascular pathogen (Beckman and Roberts, 1995). According to these concepts, the detection of the vascular pathogen ahead of the stained wood in asymptomatic tissue would not imply that the cultivar is more susceptible to fungal infection than in those in which the pathogen is restricted to the necrotic staining. Future research is needed to clarify the relevance of the *Pa. chlamydospora* detection in asymptomatic tissue beyond the necrotic staining.

'Tempranillo' cultivar was shown to be one of the most susceptible red cultivars to *Pa. chlamydospora* infection. This is consistent with findings by Sosnowski et al. (2013, 2016b), who visually assessed Australian germplasm collections in search of disease resistance to grapevine trunk diseases and found 'Tempranillo' cultivar to be very susceptible to overall trunk disease symptoms (Sosnowski et al., 2013) and cordon dieback caused by Eutypa dieback (Sosnowski et al., 2016b). 'Tempranillo' is the most important cultivar of Spain's two most respected fine red wine regions, Rioja and Ribera del Duero in the north of the country. This cultivar is the third most cultivated red wine cultivar in the world, covering an area of 231,000 ha in 2015 (OIV, 2018). 'Tempranillo' is not widely grown outside of Spain; it may be present in 17 countries but 88% of its cultivated area is in Spain (OIV, 2018). The high susceptibility of 'Tempranillo' cultivar to esca and leaf stripe diseases, and other GTDs, is causing substantial economic losses and compromising the stable production in viticulture and the Spanish wine industry. For

instance, according to an estimation model of total cost of vine replacement (Wineseq, 2018), the annual financial cost of the replacement of death plants cv Tempranillo in Rioja due to GTDs is estimated to be 7.16 million €/year (an estimation based on the following parameters: plant cost: 1.5 €; plant maintenance: 0.3 €/year; cost of plantation: 2.8 € per plant; plant density: 3,000 vines/ha; death vines due to GTDs: 1 %; extension of vineyards cv. Tempranillo in Rioja: 51,896 ha). There is evidence of variation amongst 'Tempranillo' clones in susceptibility to trunk diseases (Berlanas et al., 2017), which could open new areas of future research in order to guarantee the sustainability of the Spanish viticulture.

Presently, no curative measures are known for control of esca and leaf stripe diseases. These diseases would be best managed by an integrated disease management strategy that combines the use of less susceptible rootstocks and cultivars with appropriate culture practices, remedial control strategies, and application of preventative fungicides and/or biological agents to wounds.

Conclusions

The greenhouse technique reported in this article provides a rapid and effective technique for evaluating disease resistance to *Pa. chlamydospora*. All cultivars developed wood lesions after inoculation with the causal agent of esca and leaf stripe diseases, indicating that there is no evidence of qualitative resistance to this Ascomycete fungus. However, this study allowed classifying different grapevine cultivars according to varying signs of partial resistance to *Pa. chlamydospora*. Certain cultivars (i.e., 'Estaladiña', 'Albillo Mayor' or 'Castañal' cultivars) may be promising candidates for commercially relevant host resistance in grape-production systems where the dominant cultivars (i.e., 'Tempranillo' cultivar) are very susceptible.

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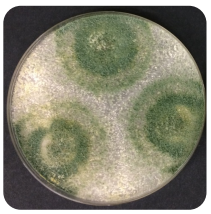
CHAPTER 6

Disease management



Chapter 6.1.

Field evaluation of biocontrol agents against black-foot and Petri diseases of grapevine

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Vitis vinifera L.

Abstract

Black-foot and Petri diseases are the main fungal diseases associated with young grapevine decline. Two field experiments were established to evaluate the preventive effect of two potential biocontrol agents (BCAs), i.e. *Streptomyces* sp. E1 + R4 and *Pythium oligandrum* P037, and three BCA-commercial products containing *Trichoderma atroviride* SC1, *Trichoderma koningii* TK7 and *Pseudomonas fluorescens+Bacillus atrophaeus* on fungal infection in grafted plants and plant growth parameters. The effectiveness of some BCA in reducing the incidence and severity of both diseases was dependent on the plant part analyzed and the plant age. No single BCA application was able to control both diseases. *Streptomyces* sp. E1+R4 were able to reduce significantly the infection of the most prevalent black-foot disease fungi while *P. oligandrum* P037 and *Trichoderma* spp. were able to reduce significantly *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Petri disease) infection. BCA treatments had no effect on the shoot weight, and root weight was significantly lower in all BCA treatments with respect to the control. The combination of the disease-suppressive activity of two or more beneficial microbes in a biocontrol preparation is required to prevent infection by black-foot and Petri disease fungi in vineyards.

Introduction

Grapevine trunk diseases (GTDs) are one of the most damaging diseases affecting the grapevine industry in all grape-growing regions worldwide, being responsible for yield and productivity loss, and one of the main causes of an early vines death (Gramaje et al., 2018). Among them, black-foot and Petri diseases are the two most common GTDs affecting planting material at nurseries, newly planted vines and young vineyards (<5 years old) (Halleen et al., 2006; Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013). In La Rioja (northern Spain), the annual financial cost of the replacement of death plants cv Tempranillo in due to black-foot and Petri diseases is estimated to be 7.16 million €/year (Martínez-Diz et al., 2019a). Field symptoms of black-foot and Petri diseases affected vines include overall stunting growth, delayed budbreak, retarded or absent sprouting, shortened internodes, chlorotic and sparse foliage with necrotic margins, leaves or entire shoots wilting, and dieback (Gramaje and Armengol, 2011). However, these symptoms also resemble those associated with abiotic disorders such as spring frost, winter damage and/or nutrient deficiency (Gramaje et al., 2018). Characteristic symptoms of black-foot

disease include dark brown and soft areas in roots and black discolouration and necrosis in the basal end of the rootstock (Halleen et al., 2006). Regarding Petri disease, dissected affected vines display brown and black vascular streaking, mainly in the rootstock, and gumming that turns dark when exposed to air (Gramaje and Armengol, 2011).

Up to 32 species of the genera *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria, Neonectria, Pleiocarpon* and *Thelonectria* have been reported to cause black-foot disease (Gramaje et al., 2018; Aigoun-Mouhous et al., 2019; Lawrence et al., 2019; Berlanas et al., 2020), *Dactylonectria torresensis* being the most prevalent species associated with diseased vines in Europe (Reis et al., 2013; Berlanas et al., 2017; Carlucci et al., 2017). These fungal species are known to be soilborne and persist as mycelium and conidia in rotten root fragments or as resting spores (chlamydospores) that can survive in the soil for extended periods of time after infected plants are removed (Petit et al., 2011; Agustí-Brisach and Armengol, 2013). Apparently healthy plants placed in infested nursery soil can become infected through trunk wounds or roots, such as the incomplete callused rootstock end (Halleen et al., 2006).

The main fungal species associated with Petri disease is *Phaeomoniella* (*Pa.*) *chlamydospora* (Mostert et al., 2006). However, other fungal species that have also been isolated in relatively high frequencies from Petri diseased vines are 29 species of the genus *Phaeoacremonium, Pleurostoma richardsiae*, and 6 *Cadophora* spp. (Gramaje et al., 2018). Among those, *Phaeoacremonium* (*Pm.*) *minimum* and *Cadophora luteo-olivacea* are the most prevalent (Mostert et al., 2006; Gramaje et al., 2011). *Pa. chlamydospora* and *Phaeoacremonium* spp. can spend part of their disease cycle in soil as mycelium and conidia in infected rootstock wood, roots or pruning debris (Gubler et al., 2013; Gramaje et al., 2015), or chlamydospores in the case of *Pa. chlamydospora* (Gubler et al., 2013). The presence of *Cadophora* spp. in soils has been recently confirmed using ITS high-throughput amplicon sequencing (HTAS) approach (Martínez-Diz et al., 2019b). Therefore, the main hypothesis is that these fungi could gain entry into the xylem of young plants at the nursery or newly established vineyards through root and/or basal end of the rootstock infections. In addition, they are also disseminated through the dispersion of airborne spores (conidia and/or ascospores) by rain, wind or arthropods until they land on susceptible and fresh pruning wounds (Gubler et al., 2013).

Presently, no curative measures are available to reduce the impact of these diseases once the vines are infected, making their management in the field difficult. Furthermore, the loss of the most effective preventative chemical products such as the banning in the

early 2000s of sodium arsenite or benzimidazoles (Mondello et al., 2018), and the high current restrictions and difficulties that chemicals are facing in most countries around the world because of the risks for human health and the environment (Larignon et al., 2008; Spinosi et al., 2009), increase even more the complexity of their control. Nowadays, the best way to handle these diseases is by using an Integrated Pest Management (IPM) strategy (Halleen and Fourie, 2016) where several strategies are combined to reduce GTDs infections, such as the use of physical (e.g. hot-water treatment), biological (e.g. antagonist microorganisms) and cultural practices (e.g. crop management, irrigation, soil preparation, etc.), throughout the nursery mother blocks and newly planted vineyards (Gramaje et al., 2018).

Investigation of BCAs able to prevent or at least reduce the development of GTDs are considered a research priority (Gramaje et al., 2018). In fact, over the last 10 years there has been a frantic search by the GTDs research community for microbial antagonists, including fungi (Fourie et al., 2001; Di Marco et al., 2004; Fourie and Halleen, 2004, 2006; Petit and Gubler, 2006; Mutawila et al., 2011; Halleen and Fourie, 2016; Pertot et al., 2016; Santos et al., 2016; Berlanas et al., 2018; Del Frari et al., 2019; Mondello et al., 2019; Berbegal et al., 2020), bacteria (Alfonzo et al., 2009; Haidar et al., 2016a,b; Rezgui et al., 2016; Santos et al., 2016; Álvarez-Pérez et al., 2017; Andreolli et al., 2019; Trotel-Aziz et al., 2019), and oomycetes (Yacoub et al., 2016; Daraignes et al., 2018). Although some of these studies provided promising findings, the results have not been consistent, observing differences in efficacy depending on the nature of the BCA, the target pathogen, application method, time of exposure to the BCA and even the grapevine cultivars and rootstocks subjected to study. In addition, most of these studies have been performed so far under *in vitro* laboratory (Petit and Gubler, 2006; Alfonzo et al., 2009; Haidar et al., 2016a, b; Rezgui et al., 2016; Santos et al., 2016; Álvarez-Pérez et al., 2017; Andreolli et al., 2019; Del Frari et al., 2019; Mondello et al., 2019; Trotel-Aziz et al., 2019), greenhouse (Haidar et al., 2016a,b; Rezgui et al., 2016; Santos et al., 2016; Yacoub et al., 2016; Daraignes et al., 2018; Del Frari et al., 2019; Mondello et al., 2019) or nursery (Fourie et al., 2001; Di Marco et al., 2004; Fourie and Halleen, 2004, 2006; Halleen and Fourie, 2016; Pertot et al., 2016; Álvarez-Pérez et al., 2017; Berbegal et al., 2020) controlled conditions by using rootstock or scion cuttings.

Three *Trichoderma*-based biological products are currently registered in Spain for the preventive protection of pruning wounds against GTD fungi, namely *Trichoderma atroviride* I-1237, *Trichoderma asperellum* ICC012 + *Trichoderma gamsii*, and *T. atroviride*

SC1 (MAPA, 2020). Only *T. atroviride* SC1 has been additionally registered to control Petri disease pathogens in grapevine grafted nursery stock (MAPA, 2020). Therefore, we propose to apply registered BCA products in Spain for control of GTD fungi both on grapevine and/or other hosts, and other potential BCAs as a preventive strategy in pre- and post-planting. The main objectives of this study were: (i) to evaluate the effectiveness of several BCA root treatments under field conditions in reducing natural infections of fungal pathogens associated with black-foot and Petri diseases over two growing seasons, and (ii) to assess the BCA root treatments influence in plant growth parameters.

Materials and methods

Planting material

One-year old grapevine grafted plants of 'Tempranillo'/110 Richter combination with uniform root distribution were obtained from a commercial nursery in Spain and used in this experiment. Roots were trimmed to 10 cm length and dormant plants were hot-water treated at 53°C for 30 min to reduce any existing infections by black-foot and Petri disease pathogens (Gramaje et al., 2010; Eichmeier et al., 2018) and then acclimatized for 24 h at 20°C before biological control agents (BCA) inoculation.

Grafted plants inoculation and experimental design

Hot-water treated plants were inoculated by dipping the roots and the basal part of the plants for 24 h at room temperature with 25 l water suspensions of the following treatments: (T1) *Streptomyces* sp. E1 + R4 (1.35×10^9 CFU ml^{-1}) at 7.5 ml l^{-1}, (T2) *Trichoderma koningii* TK7 (Condor Shield®, ATENS; 1×10^9 CFU g^{-1} formulated product) at 2 g l^{-1}, (T3) *T. atroviride* SC1 (Vintec®, Belchim Crop Protection; 2×10^{10} CFU g^{-1} formulated product) at 2 g l^{-1}, (T4) *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Stilo Cruzial®, SIPCAM Iberia; $1x\ 10^8$ CFU g^{-1} formulated product) at 2 g l^{-1}, (T5), *Pythium oligandrum* P037 (Biovitis, France; 1.28×10^6 CFU g^{-1}) at 2 g l^{-1}, and (C) water as untreated control. We selected T1 and T5 due to the previously demonstrated efficacy against GTD fungi in young vines (Yacoub et al., 2016; Álvarez-Pérez et al., 2017). T2 and T4 are not registered as a phytosanitary product in Spain yet. The viability of the *Trichoderma* conidia in the products T2 and T3 was checked to be at a minimum of 85% before the trial, as described by Pertot et al. (2016).

Inoculated grafted plants were immediately planted in May 2017 in two field sites located in Logroño (La Rioja, Spain). Both fields were under grapevine nursery planting material rotation, which is very common in the area of study. Standard cultural practices were used in both sites during the grapevine growing season. The plant groups (40 plants) were spaced 100 cm from other groups, plants being 30 cm apart from center to center. Each field plot was 12 m long and included 24 rows, each with a plant group of 40 plants (960 plants per field). In both sites, the experimental design consisted of four randomized blocks, each containing a plant group (40 plants) of each treatment (160 plants per treatment), with 200 cm between each block. Plots were less than 1 km apart and had very similar climates. Soil samples were taken for physicochemical properties analysis as described below. A drip irrigation system was laid on the soil of each row. An additional stock of 50 grafted plants was used to check for their phytosanitary status immediately after hot-water treatment (HWT).

GTD fungal isolation and identification

In February 2018, once grafted plants had completed their cycle of vegetative growth and were in a dormant state, 50% of the 2-year-old plants in each field were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing. In order to isolate black-foot and Petri disease pathogens, two plant parts were evaluated, roots and the basal ends of the rootstocks. Root necrotic sections from 2-3 cm near the basal end of the rootstock and wood sections of 3 cm length of the basal end of the rootstock were cut, washed under running tap water, surface sterilized in 33% sodium hypochlorite (commercial 40 g Cl/l) for 1 min and rinsed twice with sterile distilled water. Five small root or xylem pieces were plated on Malt Extract Agar (MEA) supplemented with 0.35 g l^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS). Four MEAS plates were used per plant (two per plant part). Plates were incubated for 10-15 days at 25°C in the dark and all colonies were transferred to Potato Dextrose Agar (PDA). Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair, 1995).

In May 2018, the remaining 50% of the plants in each field were drip inoculated with all treatments (0.5 l per plant using the same inoculum concentration as described above). In February 2019, these 3-year-old plants were carefully dug out and processed for fungal isolation as described above. All planting material was washed and also assessed for undried shoot and total root weight. The disease incidence (DI) of black-foot and Petri

disease pathogens was determined as the mean percentage of grafted plants that were infected by these fungi. The disease severity (DS) in infected grafted plants was determined as the mean percentage of root or wood segments (ten segments per plant each) that was colonized by these fungi. The presence of *Trichoderma* spp. was also recorded to provide an indication of the extent of colonization following treatment with the *Trichoderma* formulations (T2 and T3). The stock of 50 plants was also analysed after HWT as described before.

Fungal isolates resembling black-foot and Petri disease pathogens were identified by molecular techniques. For DNA extraction, 300 mg of fungal mycelium and conidia from single spore isolates grown on PDA for 2 to 3 weeks at 25°C in the dark were scraped and homogenised twice in a Fastprep®-24 tissue homogenizer (MP Biomedicals, USA). Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer's instructions. DNA was visualized on 1% agarose gels stained with RedSafe (iNtRON Biotechnology, Lynnwood, WA, USA). DNA was stored at -20°C. Black-foot species were identified by sequencing part of the histone gene (*his3*) using CYLH3F and CYLH3R primers (Crous et al., 2004; Cabral et al., 2012a, b). The identification of *Pa. chlamydospora* isolates was performed by analysis of the ITS region of DNA amplified using the fungal primers Pch1/Pch2 (Tegli et al., 2000). *Pm. minimum* and *C. luteo-olivacea* were identified by sequence analysis of the β-tubulin (*tub2)* using the primer pairs T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) for *Phaeoacremonium*, and BTCadF/BTCadR (Travadon et al., 2015) for *Cadophora*. *Trichoderma* spp. were isolated on MEAS and identified at species level by sequencing the ITS region using the universal primers ITS1F/ITS4 (Gardes and Bruns, 1993). *P. oligandrum* was isolated on Corn Meal Agar added with Pimaricin, Ampicilliun, Rifampicin and Pentachloronitrobenzene (CMA-PARP) and identified by morphological features (Yacoub et al., 2016). Polymerase chain reaction (PCR) products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). The sequences obtained were then blasted in GenBank.

Soil physicochemical properties analysis

Four soil cores were collected to a depth of 20 cm from each field and bulked into a single soil sample per field. Samples were mixed well, air-dried for one week and sieved

(2-mm to 5-mm mesh size) prior to soil physicochemical analyses. Soil samples were tested for electric conductivity (EC) in water and pH with a soil solution ratio of 1:5, soil texture by laser diffraction particle size (Diffractometer LS 13 320, Beckman Coulter Inc., Brea, Calif.), soil organic matter (SOM) by dichromate oxidation (Nelson and Sommers, 1982), cation exchange capacity (CEC) by the cobaltihexamine method (Orsini and Remy, 1976), carbonate total by infrared (Equilab CO-202; Equilab, Jakarta, Indonesia), assimilable magnesium and calcium by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA) and the cobaltihexamine method and P, K, S, Mg, Mn, Fe, Ca and Na by ICP and Mehlich method (Mehlich, 1984). Analyses were conducted in the official Regional Laboratory of La Grajera (Logroño, Spain) in April 2017, before the beginning of the experiment.

Data analysis

Prior to statistical analyses, data were checked for normality and homogeneity of variances, and transformed when needed. Percentage data were transformed into arcsin $(DI \text{ or } DS/100)^{1/2}$. Each treatment means (DI, DS, root and shoot weights) was calculated from the corresponding values in each sampling moment. The statistical analysis of the experimental results was carried out in a two-way ANOVA with blocks and treatments as independent variables, and the following dependent variables: DI (%), DS (%), root weight (g) and shoot weight (g). In the 3-year-old plants, the percentage of reduction (PR) of the fungal pathogen detection at each isolation plant part and for each fungal GTD species was calculated as $PR = 100(PC - PT)/PC$, where PC is the mean pathogen incidence or severity in the control and PT is the mean pathogen incidence or severity in the BCA treatment. Means were compared by the Student's *t* least significant difference (LSD) at $P < 0.05$. Soil physicochemical variables were subjected to analyses of variance. LSD test was calculated to compare variable means. Data from all experiments were analysed using the Statistix 10 software (Analytical Software).

Results

Plant viability and fungal identification

None of the treatments had a negative influence on callus or initial shoot growth. The viability of planting material was estimated to be of 94% and 92% for the 2-year-old and

3-year-old plants at the end of growing season, respectively. After HWT, six and four out of the 50 grafted plants stock tested positive for *Diplodia seriata* and *Neofusicoccum parvum*, respectively, other fungi associated with GTDs. No black-foot and Petri disease pathogens were isolated from hot-water treated plants. In the 2-year-old plants, a total of 1,650 Petri disease (83.6% from the basal end of the rootstock and 16.4% from roots) and 896 black-foot disease pathogens (15.8% from the basal end of the rootstock and 84.2% from roots) isolates were collected. Petri disease pathogens were identified as *C. luteo-olivacea* (57.8%), followed by *Pa. chlamydospora* (27.3%) and *Pm. minimum* (14.9%).

Black-foot pathogens were identified as *Dactylonectria torresensis* (66.4%), followed by *Dactylonectria macrodidyma* (22.6%), *Ilyonectria liriodendri* (6.2%) and *Dactylonectria alcacerensis* (4.8%). In the 3-year-old plants, a total of 1,825 Petri disease (89.4% from the basal end of the rootstock and 10.6% from roots) and 1,632 black-foot pathogens (26.9% from the basal end of the rootstock and 73.1% from roots) isolates were collected. Petri disease pathogens were identified as *C. luteo-olivacea* (54.6%), followed by *Pa. chlamydospora* (31.1%) and *Pm. minimum* (14.3%). Black-foot pathogens were identified as *D. torresensis* (66.0%), followed by *I. liriodendri* (16.0%), *D. macrodidyma* (9.2%), *Ilyonectria robusta* (4.4%), *D. alcacerensis* (2.5%) and *Ilyonectria pseudodestructans* (1.8%). Representative black-foot and Petri diseases isolate sequences obtained in this study were deposited to GenBank (Supplementary Table 6.1.1).

Trichoderma atroviride was isolated from 30 and 22% of the 2-year-old and 3-year-old plants, respectively. *Trichoderma koningii* was isolated from 12 and 18% of the 2-year-old and 3-year-old plants, respectively. Our attempts to isolate *P. oligandrum* were unsuccessful.

Disease incidence and disease severity in grafted plants

Neither field site, nor block, nor its interaction significantly affected the DI and DS ($P > 0.05$, ANOVA not shown). Therefore, data from both field sites were combined and analysed together. There was a significant effect of treatment on mean Petri disease incidence values in the roots and the basal ends for both 2-year-old and 3-year-old plants (Supplementary Table 6.1.2). In the 3-year-old plants, percentage of infected plants (DI) in the basal ends were significantly lower in treatments with *T. atroviride* SC1 (T3) (40.2% ± 8.3) than in the control treatment (61.5% ± 5.6) (Fig. 6.1.1A). In both 2-year-old and 3-year-old plants, percentage of infected plants (DI) in the roots were significantly lower in

treatments with *P. oligandrum* P037 (T5) (2-year-old plants: 7.5% ± 1.4, and 3-year-old plants: 4.8% ± 1.3) than in the control treatment (2-year-old plants: 23.1% ± 2.8, and 3-year-old plants: 18.3% ± 3.9) (Fig. 6.1.1B). Biocontrol treatments had a significant effect on mean Petri disease severity in basal ends of 2-year-old plants, and in roots and basal ends for 3-year-old plants (Supplementary Table 6.1.2). *T. atroviride* SC1 (T3) in the 2-year-old plants (19.4% ± 1.4) and both *Trichoderma* spp. treatments (T2: 25.5% ± 2.5, and T3: 25.8% ± 2.3) in the 3-year-old plants significantly reduced the percentage of DS in the basal ends compared to the control treatment (2-year-old plants: 36.1% ± 4.3, and 3-year-old plants: 39.5% ± 4.9) (Fig. 6.1.1A). *Trichoderma* spp. treatments (T2: 9.1% ± 1.3, and T3: 10.8% ± 1.8) resulted in significant lower DS in roots of the 3-year-old plants than the control treatment (16.8% ± 3.8) (Fig. 6.1.1B).

Analysis of variance showed no significant effect of biocontrol treatments on black-foot disease incidence and severity in roots of both 2-year-old and 3-year-old plants (Supplementary Table 6.1.2). There was a significant effect of treatment on mean black-foot disease incidence values in the basal ends for both 2-year-old and 3-year-old plants (Supplementary Table 6.1.2). In the 2-year-old plants, all treatments resulted in significant lower DI in the basal ends than the control treatment (Fig. 6.1.2A). In the 3-year-old plants, percentage of infected plants (DI) in the basal ends were significantly lower in treatments with *Streptomyces* sp. E1 + R4 (T1) (4.8% ± 2.1) than in the control treatment (13.2% ± 3.2) (Fig. 6.1.2A). There was a significant effect of treatment on mean black-foot disease severity values in the basal ends of 2-year-old plants (Supplementary Table 6.1.2). *Streptomyces* sp. E1 + R4 (T1) (10.0% ± 2.3) significantly reduced the percentage of DS in the basal ends compared to the control treatment (19.1 ± 0.8) (Fig. 6.1.2A).

Fungal species incidence and severity in grafted plants

Considering the fungal species within each disease individually, *P. oligandrum* P037 (T5) and *T. atroviride* SC1 (T3) significantly reduced the DI of *C. luteo-olivacea* in the roots and the basal ends, respectively, of 2-year-old plants compared to the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1). Percentage of DI in the roots of both 2-year-old and 3-year-old plants, and DS in the roots of 2-year-old plants caused by *Pa. chlamydospora* were significantly lower in treatments with *P. oligandrum* P037 (T5) than in the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1).

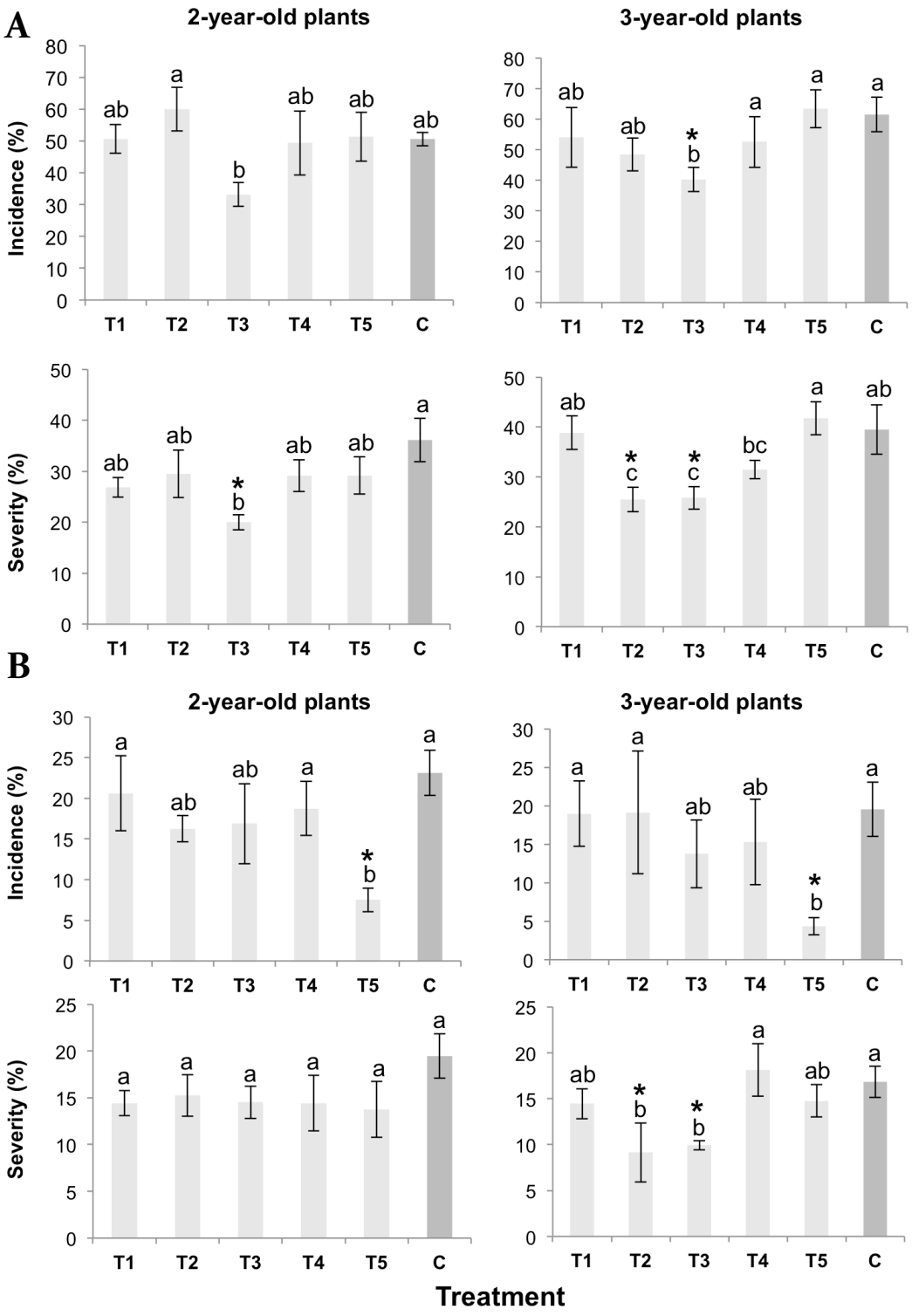

Figure 6.1.1. Petri disease incidence (DI) (%) and severity (DS) (%) of 2- and 3-year-old grafted plants in basal ends of the rootstock (**A**) and in roots (**B**). Values are the mean of four replicates and vertical bars are the standard errors of the mean. Bars followed by the same letter do not differ significantly (P = 0.05). Asterisks (*) indicate significant differences between the BCA treatment and untreated control (C) (P = 0.05). Treatments: (T1) *Streptomyces* sp. E1 + R4; (T2) *Trichoderma koningii* TK7 (Condor Shield®, ATENS); (T3) *Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection); (T4) *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Cruzial®, SIPCAM Iberia); (T5), *Pythium oligandrum* P037; (C) untreated control.

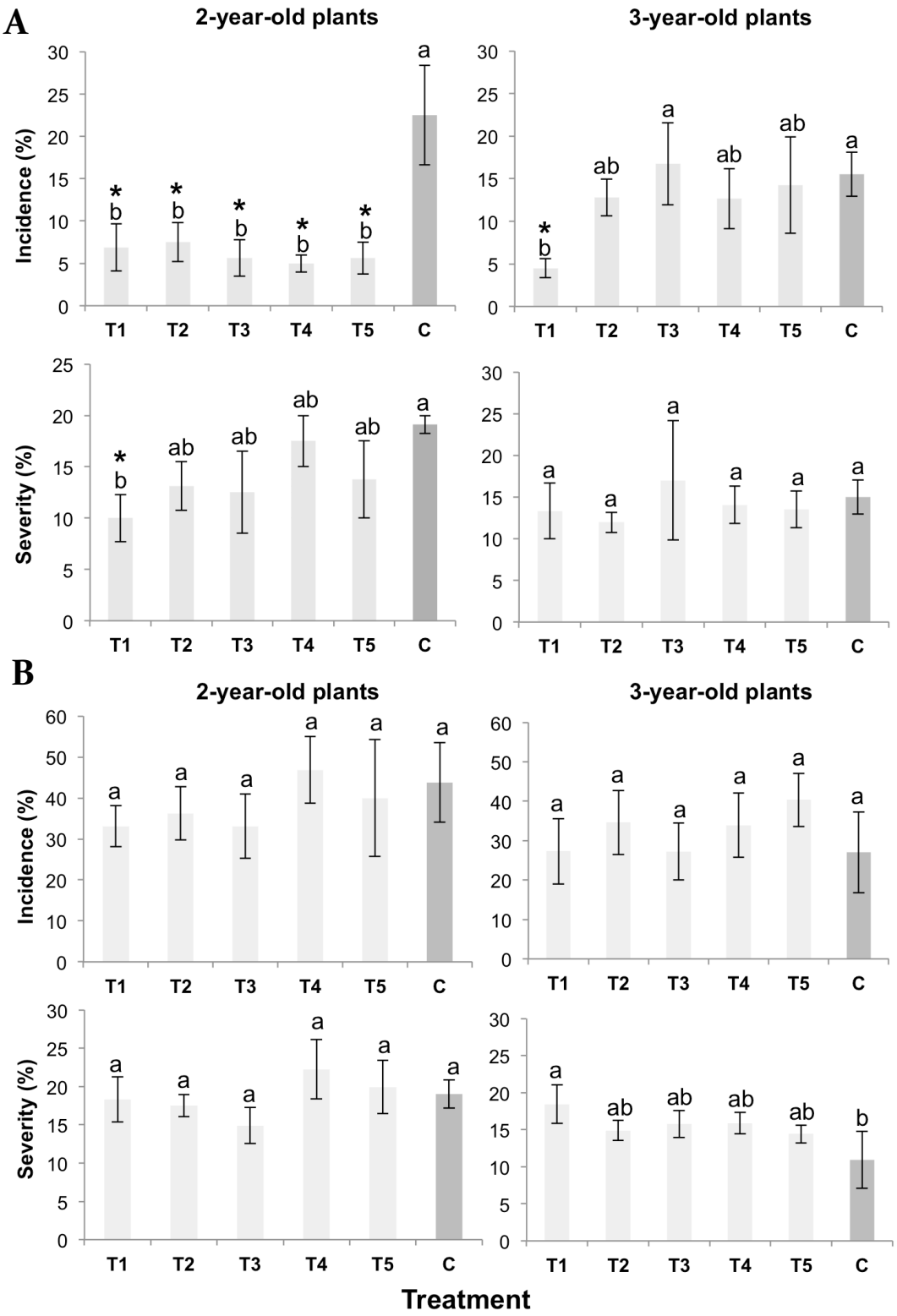

Figure 6.1.2. Black-foot disease incidence (DI) (%) and severity (DS) (%) of 2- and 3-year-old grafted plants in basal ends of the rootstock (**A**) and in roots (**B**). Values are the mean of four replicates and vertical bars are the standard errors of the mean. Bars followed by the same letter do not differ significantly (P = 0.05). Asterisks (*) indicate significant differences between the BCA treatment and untreated control (C) (P = 0.05). Treatments: (T1) *Streptomyces* sp. E1 + R4; (T2) *Trichoderma koningii* TK7 (Condor Shield®, ATENS); (T3) *Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection); (T4) *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Cruzial®, SIPCAM Iberia); (T5), *Pythium oligandrum* P037; (C) untreated control.

In the 3-year-old plants, *T. atroviride* SC1 (T3) significantly reduced both DI and DS caused by *Pa. chlamydospora* in the basal ends compared to the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1). Both *T. koningii* TK7 (T2) and *P. fluorescens* + *B. atrophaeus* (T4) treatments resulted in significant lower DI caused by *Pm. minimum* in the roots of 2-year-old plants than the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1). Furthermore, *T. koningii* TK7 (T2) treatment resulted in significant lower DS caused by *Pm. minimum* in the roots of 3-year-old plants than the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1). *T. atroviride* SC1 (T3) significantly reduced the DS of *Pm. minimum* in the roots of both 2-year-old and 3-year-old plants compared to the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.1).

Regarding black-foot pathogens, all treatments significantly reduced the DI of *D. torresensis* and *D. macrodidyma* in the basal ends of 2-year-old plants compared to the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.2). *Streptomyces* sp. E1 + R4 (T1) significantly reduced *D. torresensis* DS in the basal ends of 2-year-old plants and DI in the basal ends of 3-year-old plants compared to the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.2). In both the 2-year-old and 3-year-old plants, percentages of DI in the roots and DS in the basal ends caused by *D. macrodidyma* were significantly lower in treatments with *Streptomyces* sp. E1 + R4 (T1) than in the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.2). *T. atroviride* SC1 (T3) also resulted in significant lower DS in the basal ends of 3-year-old plants than the control treatment ($P < 0.05$, ANOVA not shown) (Table 6.1.2). Low levels of *Trichoderma* spp. (< 30%) were isolated from roots and basal ends of 2-year-old and 3-year-old plants subjected to T2 and T3 treatments in both fields.

The percentage of reduction (PR) was calculated for treatments statistically different from the control in the 3-years-old plants (Table 6.1.3). In roots, *P. oligandrum* P037 (T5) provided 93.6% disease incidence reduction of *Pa. chlamydopora*. On *Trichoderma* spp. treated plants, there was a reduction in *Pm. minimum* severity when compared with untreated controls, which ranged from 80% for *T. koningii* TK7 (T2) and 69.6% for *T. atroviride* SC1 (T3). In the basal ends, *T. atroviride* SC1 (T3) provided 69.4% disease incidence and 56.6% disease severity reduction of *Pa. chlamydopora*, while *T. koningii* TK7 (T2) provided 52.3% disease severity reduction of *Pm. minimum*. None of the BCA treatments statistically reduced the disease incidence and severity of black-foot disease fungi in roots (Tables 6.1.2 and 6.1.3).

Table 6.1.1. Mean disease incidence (DI) and severity (DS) of Petri disease pathogens isolated from the roots and basal ends of the rootstock of 2-year-old and 3-year-old grafted plants subjected to various treatments prior to planting in two fields in Logroño (La Rioja).

	2-year-old plants				3-year-old plants			
Cadophora luteo-olivacea	DI (%)[a]		DS (%)[a]		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	8.1 a	24.4 ab	20.4 a	26.7 a	6.9 a	25.6 a	19.4 a	31.3 a
T2. *Trichoderma koningii* TK7	5.6 ab	34.4 a	20.4 a	24.5 a	5.6 a	34.4 a	19.6 a	28.4 a
T3. *Trichoderma atroviride* SC1	6.9 ab	20.6 b	21.0 a	30.3 a	5.0 a	28.1 a	19.2 a	27.6 a
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	6.3 ab	31.3 ab	22.3 a	30.7 a	6.3 a	31.3 a	20.4 a	35.0 a
T5. *Pythium oligandrum* P037	3.8 b	31.9 ab	21.3 a	30.3 a	5.0 a	31.9 a	17.5 a	31.0 a
Control (C)	7.5 a	38.1 a	25.2 a	33.8 a	7.5 a	36.9 a	22.3 a	33.4 a
LSD (P = 0.05)	2.7	8.2	7.5	7.7	2.5	7.8	7.0	7.5
Phaeomoniella chlamydospora	2-year-old plants				3-year-old plants			
	DI (%)		DS (%)		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	6.3 a	11.3 a	10.0 ab	27.6 a	7.5 a	15.6 a	9.2 a	28.2 ab
T2. *Trichoderma koningii* TK7	6.3 a	18.8 a	10.6 ab	25.0 a	8.1 a	18.8 a	14.2 a	35.0 a
T3. *Trichoderma atroviride* SC1	7.5 a	14.4 a	14.8 ab	29.8 a	8.1 a	6.3 b	15.8 a	15.0 b
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	6.3 a	17.5 a	13.3 ab	28.5 a	7.5 a	17.5 a	11.7 a	38.7 a
T5. *Pythium oligandrum* P037	1.9 b	22.5 a	7.5 b	20.9 a	0.6 b	22.5 a	12.5 a	31.0 a
Control (C)	9.4 a	20.6 a	15.4 a	28.3 a	9.4 a	20.6 a	10.6 a	34.6 a
LSD (P = 0.05)	3.1	5.5	3.6	7.4	3.3	3.8	3.8	7.1
Phaeoacremonium minimum	2-year-old plants				3-year-old plants			
	DI (%)		DS (%)		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	4.4 ab	11.3 a	16.3 a	15.1 a	4.0 a	15.0 a	13.8 a	21.0 a
T2. *Trichoderma koningii* TK7	3.1 b	16.9 a	18.8 a	13.6 a	4.1 a	16.5 a	2.5 b	9.5 b
T3. *Trichoderma atroviride* SC1	5.0 ab	11.3 a	7.1 b	11.8 a	3.9 a	14.7 a	3.8 b	22.4 a
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	3.1 b	17.5 a	23.8 a	12.8 a	4.3 a	15.1 a	15.0 a	17.7 ab
T5. *Pythium oligandrum* P037	3.8 ab	16.9 a	16.3 a	10.3 a	4.0 a	15.8 a	12.5 a	18.3 ab
Control (C)	5.6 a	16.9 a	21.7 a	17.0 a	4.0 a	17.2 a	12.5 a	19.9 ab
LSD (P = 0.05)	2.3	3.6	3.5	3.3	2.7	3.1	3.6	3.5

[a] At each plant part, percentages of disease incidence (DI) and disease severity (DS) are the mean of 160 plants analyzed (40 plants per replicate). Values in the same column followed by the same letter do not differ significantly (P = 0.05).

Table 6.1.2. Mean disease incidence (DI) and severity (DS) of the most prevalent black-foot disease pathogens isolated from the roots and basal ends of rootstock of 2-year-old and 3-year-old grafted plants subjected to various treatments prior to planting in two fields in Logroño (La Rioja).

Dactylonectria torresensis								
	2-year-old plants				3-year-old plants			
	DI (%)[a]		DS (%)[a]		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	31.3 a	6.3 b	20.5 a	3.8 b	21.3 a	1.3 b	12.3 a	20.0 a
T2. *Trichoderma koningii* TK7	30.0 a	6.3 b	15.3 a	22.1 a	26.9 a	10.0 a	13.5 a	19.7 a
T3. *Trichoderma atroviride* SC1	25.0 a	5.0 b	23.0 a	17.5 a	21.9 a	14.4 a	13.8 a	16.7 a
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	31.3 a	4.4 b	18.5 a	22.5 a	26.9 a	10.0 a	10.8 a	18.3 a
T5. *Pythium oligandrum* P037	25.0 a	3.8 b	17.6 a	28.3 a	30.6 a	12.5 a	10.0 a	20.5 a
Control (C)	25.0 a	14.4 a	20.6 a	18.1 a	20.6 a	11.9 a	11.5 a	19.9 a
LSD (P = 0.05)	6.6	6.2	6.0	6.5	5.8	3.8	3.2	3.5
Dactylonectria macrodidyma								
	2-year-old plants				3-year-old plants			
	DI (%)		DS (%)		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	8.8 b	2.5 b	16.3 a	0 b	3.6 b	3.8 a	5.8 a	0 b
T2. *Trichoderma koningii* TK7	9.4 ab	3.1 b	17.8 a	3.8 ab	10.6 a	3.1 a	4.8 a	3.3 ab
T3. *Trichoderma atroviride* SC1	11.3 ab	1.3 b	18.8 a	2.5 ab	7.5 a	3.8 a	4.1 a	0 b
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	13.8 ab	1.3 b	18.5 a	2.5 ab	10.0 a	4.4 a	5.1 a	2.5 ab
T5. *Pythium oligandrum* P037	16.9 ab	3.8 b	11.8 a	1.7 ab	9.4 a	3.1 a	4.2 a	5.0 ab
Control (C)	16.3 a	10.0 a	14.8 a	6.1 a	8.8 a	3.8 a	4.0 a	6.3 a
LSD (P = 0.05)	3.6	3.3	3.7	2.3	3.0	2.5	2.6	2.3
Ilyonectria liriodendri								
	2-year-old plants				3-year-old plants			
	DI (%)		DS (%)		DI (%)		DS (%)	
	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends	Roots	Basal ends
T1. *Streptomyces* sp. E1 + R4	3.8 a	0.6 a	11.7 a	0 b	3.8 a	0.6 a	30.8 a	5.0 a
T2. *Trichoderma koningii* TK7	2.5 a	1.9 a	12.5 a	6.3 ab	3.1 a	0.6 a	32.5 a	2.5 a
T3. *Trichoderma atroviride* SC1	4.4 a	1.9 a	12.5 a	7.5 a	3.8 a	0.6 a	30.0 a	5.0 a
T4. *Pseudomonas fluorescens* + *Bacillus atrophaeus*	2.5 a	0.6 a	16.3 a	2.5 ab	3.1 a	1.3 a	36.3 a	5.0 a
T5. *Pythium oligandrum* P037	2.5 a	1.3 a	12.5 a	7.5 a	3.1 a	0.6 a	28.8 a	5.0 a
Control (C)	3.8 a	0.6 a	10.0 a	2.5 ab	2.5 a	0.6 a	30.0 a	5.0 a
LSD (P = 0.05)	2.5	2.4	3.1	2.9	2.5	2.2	7.1	2.5

[a] At each plant part, percentages of disease incidence (DI) and disease severity (DS) are the mean of 160 plants analyzed (40 plants per replicate). Values in the same column followed by the same letter do not differ significantly (P = 0.05).

In the basal ends, *Streptomyces* sp. E1 + R4 (T1) reduced the incidence of *D. torresensis* and the severity of *D. macrodidyma* by 89.1 and 100%, respectively. *T. atroviride* SC1 (T3) provided 100% disease severity reduction of *D. macrodidyma*.

Table 6.1.3. Pathogen reduction achieved by BCA treatments in the 3-year-old plants, associated with Petri and black-foot disease.

Petri disease			
Plant part	**Biocontrol agent**	**Pathogen**	**Reduction**[a]
Roots	*Pythium oligandrum* P037	*Pa. chlamydospora*	93.6% (DI[b])
	Trichoderma koningii TK7	*Pm. minimum*	80% (DS[c])
	Trichoderma atroviride SC1	*Pm. minimum*	69.6% (DS)
Basal ends	*Trichoderma atroviride* SC1	*Pa. chlamydospora*	69.4% (DI) 56.6% (DS)
	Trichoderma koningii TK7	*Pm. minimum*	52.3% (DS)
Black-foot disease			
Basal ends	*Streptomyces* sp. E1 + R4	*D. torresensis*	89.1% (DI)
	Streptomyces sp. E1 + R4	*D. macrodidyma*	100% (DS)
	Trichoderma atroviride SC1	*D. macrodidyma*	100% (DS)

[a] The percentage of reduction (PR) of the pathogen detection at each plant part was calculated as PR = 100(PC – PT)/PC, where PC is the mean pathogen incidence or severity in the control and PT is the mean pathogen incidence or severity in the biocontrol agent treatment.
[b] Disease incidence.
[c] Disease severity.

Root and shoot weights in grafted plants, and physicochemical properties of the soil

Analysis of variance showed no significant effect of biocontrol treatments on the shoot weight of 3-year-old plants ($P > 0.05$, ANOVA not shown) (Fig. 6.1.3). Mean shoot weight ranged from 55.3 g ± 5.7 (T3) to 64.9 g ± 8.2 (T2). Biological control treatments had a significant effect on the root weight of 3-year-old plants ($P < 0.05$, ANOVA not shown) (Fig. 6.1.3). Mean root weight ranged from 41.9 g ± 3.7 (T3) to 52.9 g ± 2.9 (C). All treatments resulted in significant lower root weight than the control treatment (Fig. 6.1.3). Analyses of variance indicated no significant differences for the soil physicochemical properties between fields ($P > 0.05$, ANOVA not shown).

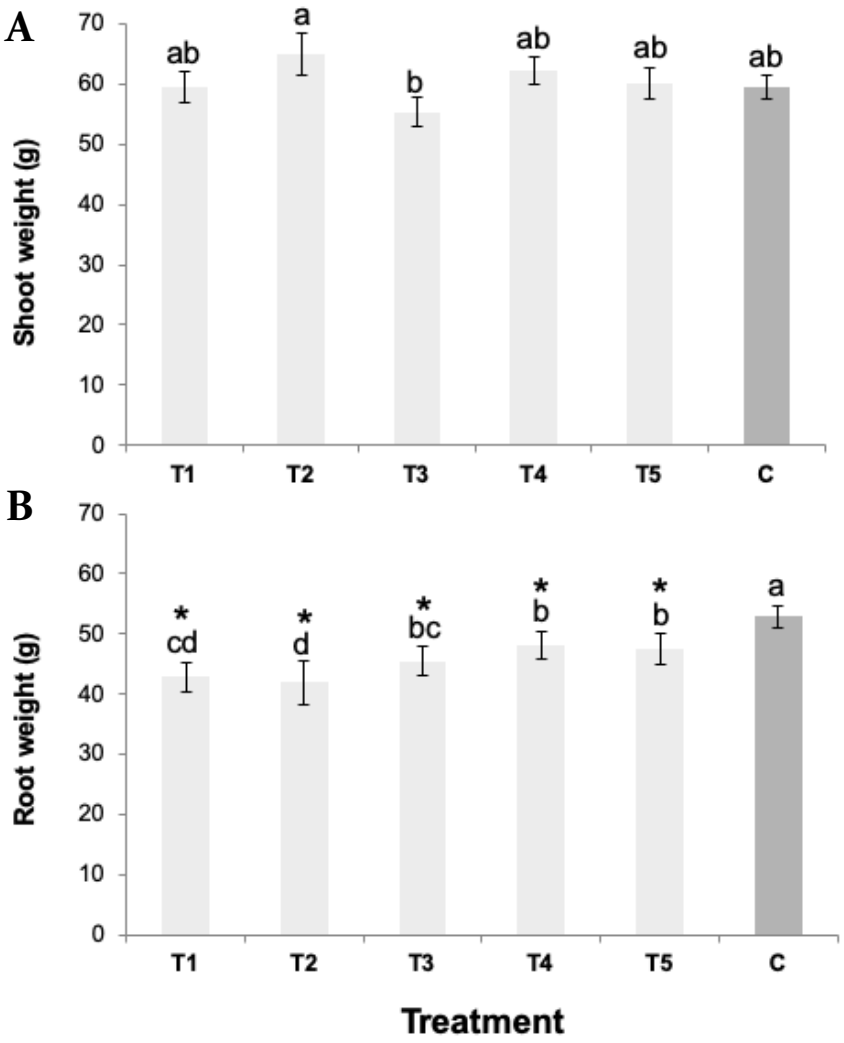

Figure 6.1.3. Fresh root mass (g per plant) (**A**) and shoot weight (g per plant) (**B**) in 3-year-old vines. Values are the mean of four replicates and vertical bars are the standard errors of the mean. Bars followed by the same letter do not differ significantly (P = 0.05). Asterisks (*) indicate significant differences between the BCA treatment and untreated control (C) (P = 0.05). Treatments: (T1) *Streptomyces* sp. E1 + R4; (T2) *Trichoderma koningii* TK7 (Condor Shield®, ATENS); (T3) *Trichoderma atroviride* SC1 (Vintec®, Belchim Crop Protection); (T4) *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Cruzial®, SIPCAM Iberia); (T5), *Pythium oligandrum* P037; (C) untreated control.

Discussion

This study represents the first approach to evaluate the effectiveness of different antagonistic microorganisms (bacteria, fungi and an oomycete) applied preventively to control black-foot and Petri diseases under field conditions. The use of BCA against soilborne pathogens are on the forefront of research; however, most experiences are on a laboratory scale, thus avoiding the problems related to the production of large quantities of antagonists and their formulations, and disease control trials are performed in

simplified environment such as growth chambers or experimental greenhouses, thus avoiding the risk of large-scale experiments in the field.

In this study, Petri disease infection was mainly detected in the basal ends of the rootstock, while fungi associated with black-foot disease were most frequently isolated from roots. *D. torresensis* was the most frequent isolated species (>60%) associated with black-foot disease at both plant ages. This agrees with previous research carried out on black-foot in Europe (Reis et al., 2013; Berlanas et al., 2017; Carlucci et al., 2017). Regarding Petri disease, more than 80% of the fungi were identified as *C. luteo-olivacea* and *Pa. chlamydospora* at both plant ages. Both fungal species were frequently isolated from nursery stock and young vines worldwide (Gramaje and Armengol, 2011).

In our specific pathosystems, the effectiveness of some BCA in reducing the incidence and severity of both diseases under field conditions were dependent on the plant part analysed and the plant age. *Streptomyces* sp. E1 + R4 had a biocontrol effect against the most prevalent black-foot disease pathogens obtained in this study, namely *D. torresensis* and *D. macrodidyma*. This BCA treatment was highly effective in reducing black-foot disease incidence of both fungi at both plants ages and the severity of 2-year-old plants in the basal ends. However, the effect of these actinobacteria against Petri disease pathogens after 2 years in the field was very low. In contrast, Álvarez-Pérez et al. (2017) evaluated the effectiveness of these bacterial strains individually, previously isolated from the endo- (strain E1) and rhizosphere (strain R4) of the grapevine root system and found significant reductions of the infection rates at the lower end of the rootstock of the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *Pm. minimum* and *Pa. chlamydospora* (Álvarez-Pérez et al., 2017). These differences in the effectiveness of the bacteria against Petri disease between experiments could be due to the commonly unpredictably behaviour of BCA when tested in different environments (Vannacci and Gullino, 2000).

Other bacterial treatment tested in our study was a commercial product containing *Pseudomonas fluorescens* and *Bacillus atrophaeus*. No biocontrol effect of this treatments was observed on fungal pathogens associated with black-foot and Petri diseases. Despite this fact, some strains of these bacterial species have been previously reported as plant growth-promoting bacteria (PGPB) and have been found to be potential BCA of plant diseases in several crops (Compant et al., 2005; Zhang et al., 2013; Guardado-Valdivia et al., 2018; Ma et al., 2018). In grapevine, different *P. fluorescens* strains were identified as prospective new BCA against *Botrytis cinerea* (Trotel-Aziz et al., 2008) and to induce

systemic resistance against *Plasmopara viticola* and *B. cinerea* by priming common and distinct defensive pathways (Lakkis et al., 2019).

Most studies on biological control of GTDs have examined the application of *Trichoderma* spp. in grapevine nurseries and young vineyards (Fourie et al., 2001; Di Marco et al., 2004; Fourie and Halleen, 2004, 2006; Halleen and Fourie, 2016; Santos et al., 2016). In our study, we individually evaluated two *Trichoderma*-based products containing *T. koningii* strain TK7 and *T. atroviride* strain SC1. A certain effect was observed in reducing *Pm. minimum* disease incidence for 2-year-old plants and disease severity for 3-year-old plants at the root level by *T. koningii* TK7 treatment. Little information is still available related to the biocontrol effect of TK7 strain to combat plants' fungal pathogens. Howell et al. (2000) showed that the application of *T. koningii* TK7 to cotton seeds before planting was ineffective to control cotton seedlings damping-off in artificially *Rhizoctonia solani*-infested cotton field soil flats.

Trichoderma atroviride SC1 was effective in reducing *Pa. chlamydospora* disease incidence and severity in the basal ends of 3-year-old plants. In accordance with our results, a study carried out in Spain by Berbegal et al. (2020) also found reductions in the incidence and severity of *Pa. chlamydospora* and *Pm. minimum* when analysed the rootstock basal end and root system of 1-day *T. atroviride* SC1 inoculated grafted plants in nurseries. Under field conditions, Berbegal et al. (2020) observed no BCA effect on incidence and severity of black-foot disease associated pathogens and significant reductions on pathogens associated with Petri disease after the first growing season. In Italian grapevine nurseries, the application of *T. atroviride* strain SC1 at several stages of the nursery processprotected plants from infection by *Pm. minimum* and *Pa. chlamydospora* after a single artificial inoculation with both pathogens following the grafting stage (Pertot et al., 2016).

Regarding *P. oligandrum* P037 treatment, a significant reduction of Petri disease incidence and severity was observed in 2-year-old plants and disease incidence in 3-year-old plants, at roots level. Yacoub et al. (2016) reported a significant reduction in necrosis length caused by *Pa. chlamydospora* when the roots of 'Cabernet Sauvignon' cuttings were colonized by different *P. oligandrum* strains. The ability of *P. oligandrum* strain P037 to act as an inducer of plant systemic resistance against pathogens is thought to be due to the presence of three elicitin-like proteins in its genome (Berger et al., 2016).

Diverse formulations (dry or water suspensions), application methods and times of exposure of plants to BCA have been tested in the different studies carried out to assess the biocontrol potential of antagonist microorganisms (Pertot et al., 2016; Yacoub et al., 2016; Álvarez-González et al., 2017; Berlanas et al., 2018; Daraignes et al., 2018; Yacoub et al., 2018; González-García et al., 2019; Van Jaarsveld et al., 2019; Berbegal et al., 2020). In our assay, a 24-h soaking of the trimmed root systems and the basal end of the plants in BCA water suspensions was carried out before planting, but the percentage of *Trichoderma* spp. recovery was low in all cases (<30%). In this sense, Halleen et al. (2007) were also able to only isolate a 2.3% of *Trichoderma* spp. from the basal ends of the rootstock and none from roots of grafted plants subjected to *Trichoderma* treatments, applied by dipping the basal ends of the rootstock for 1 min before planting, after 7 months in a nursery field. In a recent study, González-García et al. (2019) evaluated the colonization efficiency of *Streptomyces* sp. in the root system by comparing two inoculation methods, plant immersion in a bacterial suspension or direct injection of the bacterial suspension into the vegetal tissues and concluded that both methods allowed effective BCA colonization. This is also in accordance with Berbegal et al. (2020) who used 24-h soak in *T. atroviride* SC1 water suspension to inoculate 110R rootstock cuttings before grafting, with percentages of recovery over 80% at both nursery and vineyard experiments. Van Jaarsveld et al. (2019) evaluated different methods of application of *T. atroviride* on commercially planted nursery vines and concluded that dipping of basal ends in the *Trichoderma* dry formulation consistently gave higher colonization percentages than the 1-h soak of bases of vines before planting or *Trichoderma* field drenching. Further research is needed to evaluate the effectiveness of soaking vines in *T. koningii* TK7 or *T. atroviride* SC1 dry formulations compared to soaking vines for 24-h in BCA water suspensions before planting.

Biological control agent treatments did not affect the shoot weight, and root weight was significantly lower for all BCA treatments with respect to the untreated control at the end of the second growing season (3-year-old plants). The impact of BCA treatments on grapevine development was very variable on previous research (Halleen et al., 2007; Santos et al., 2016; Van Jaarsveld et al., 2019). *Trichoderma* spp. and *B. subtilis*-based treatments resulted in lower mean root and shoot dry weight values when compared with the negative controls (Santos et al., 2016). Nevertheless, Halleen et al. (2007) found that none of the *Trichoderma* formulations tested yielded plants with roots or shoots mass significantly different than the water treated controls. Berbegal et al. (2020) observed a significantly

higher undried shoot weight for *T. atroviride* SC1 treated plants at the end of the first growing season, but this effect was not observed in the second growing season. Likewise, the application of actinobacteria to grafted grapevine plants did not show a significant effect, either positive or negative, on plants growth (Álvarez-Pérez et al., 2017). In contrast, Fourie et al. (2001) observed that *T. harzianum* treatments significantly improved root development but not shoot mass in comparison with the control vines in nurseries. Several studies indicate that BCA treatments can enhance the growth of other crops, such as tomato (Kaur et al., 2019) or rice (Suárez-Moreno et al., 2019). All this variability could be related to the lack of proper long-standing implantation by these antagonist microorganisms in grapevine roots or the vigour level of the rootstock cultivar tested. BCA are living organisms whose activities depend mainly on the different physicochemical environmental conditions to which they are subjected (Benitez et al., 2004), and the greatest long-term effects probably occur with rhizosphere-competent strains with the ability to colonize and grow in association with plant roots (Harman et al., 2004).

Conclusions

This study highlighted the potential of some BCA applied preventively to reduce the infection caused by the most prevalent black-foot disease fungi and the majority of Petri disease pathogens isolated in this study under field conditions. No single BCA application was able to control both diseases. Further studies should evaluate the combination of the disease-suppressive activity of two or more beneficial microbiomes in a biocontrol preparation against black-foot and Petri diseases. Our results also open up the possibility to combine the application of BCA as a pre- planting strategy with other measures in an Integrated Pest Management (IPM) programme against GTDs. For example, BCA can be applied after hot-water treatment (HWT) of dormant grafted plants or after soil biofumigation. In this regard, recent research highlighted the effectiveness of HWT at 53ºC for 30 min (Eichmeier et al., 2018) and white mustard biofumigation (Berlanas et al., 2018) to reduce GTD incidence in planting material and grapevine nursery soil, respectively.

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Chapter 6.1.

Supplementary material

Supplementary **Tables**

Supplementary Table 6.1.1. Fungal species associated with black-foot and Petri diseases recovered from grafted plants in Spain and nucleotide sequences used for species identification.

Species	**Isolate**	Accession number		
		ITS	*tub2*	*his3*
Cadophora luteo-olivacea	BV-3125	-	MN233210	-
C. luteo-olivacea	BV-3126	-	MN233211	-
C. luteo-olivacea	BV-3127	-	MN233212	-
C. luteo-olivacea	BV-3128	-	MN233213	-
C. luteo-olivacea	BV-3129	-	MN233214	-
Dactylonectria alcacerensis	BV-1240	-	-	MK579234
D. alcacerensis	BV-1222	-	-	MK579235
D. alcacerensis	BV-1245	-	-	MK579236
D. alcacerensis	BV-1469	-	-	MK579237
Dactylonectria macrodidyma	BV-0535	-	-	MK579238
D. macrodidyma	BV-0506	-	-	MK579239
D. macrodidyma	BV-0560	-	-	MK579240
D. macrodidyma	BV-0797	-	-	MK579241
D. macrodidyma	BV-0872	-	-	MK579242
D. macrodidyma	BV-0899	-	-	MK579243
Dactylonectria torresensis	BV-1076	-	-	MK579262
D. torresensis	BV-1255	-	-	MK579263
D. torresensis	BV-1256	-	-	MK579264
D. torresensis	BV-1300	-	-	MK579265
D. torresensis	BV-1313	-	-	MK579266
D. torresensis	BV-1315	-	-	MK579267
D. torresensis	BV-2103	-	-	MK579268
Ilyonectria liriodendri	BV-1642	-	-	MK579270
I. liriodendri	BV-1757	-	-	MK579271
I. liriodendri	BV-1762	-	-	MK579272
I. liriodendri	BV-1763	-	-	MK579273
I. liriodendri	BV-1764	-	-	MK579274
I. liriodendri	BV-1844	-	-	MK579275
I. liriodendri	BV-1923	-	-	MK579276
I. liriodendri	BV-1925	-	-	MK579277
I. liriodendri	BV-1929	-	-	MK579278
Ilyonectria pseudodestructans	BV-2306	-	-	MK579280

(*Continued on next page*)

Supplementary Table 6.1.1. (*Continued from previous page*)

Species	Isolate	Accession number		
		ITS	*tub2*	*his3*
I. pseudodestructans	BV-2307	-	-	MK579281
I. pseudodestructans	BV-2506	-	-	MK579282
I. pseudodestructans	BV-2609	-	-	MK579283
I. pseudodestructans	BV-2652	-	-	MK579284
I. pseudodestructans	BV-2678	-	-	MK579285
Ilyonectria robusta	BV-1654	-	-	MK579288
I. robusta	BV-2051	-	-	MK579289
I. robusta	BV-2069	-	-	MK579290
I. robusta	BV-2565	-	-	MK579291
I. robusta	BV-2649	-	-	MK579292
Phaeoacremonium minimum	BV-2780	-	MN233215	-
Pm. minimum	BV-2781	-	MN233216	-
Pm. minimum	BV-2782	-	MN233217	-
Pm. minimum	BV-2783	-	MN233218	-
Pm. minimum	BV-2784	-	MN233219	-
Phaeomoniella chlamydospora	BV-1105	MN233220	-	-
Pa. chlamydospora	BV-1106	MN233221	-	-
Pa. chlamydospora	BV-1107	MN233222	-	-
Pa. chlamydospora	BV-1108	MN233223	-	-
Pa. chlamydospora	BV-1148	MN233224	-	-

[a] ITS = internal transcribed spacer, *tub2* = β-tubulin and *his3* = histone H3.

Supplementary Table 6.1.2. Effects of variables on disease incidence (DI) and disease severity (DS) in roots and basal ends of rootstock of grapevine grafted plants.

Petri disease	**2-year-old plants**					**3-year-old plants**				
	DI (%)			DS (%)		DI (%)			DS (%)	
Variables	df[a]	MS[b]	*P* value[c]	MS	*P* value	df	MS	*P* value	MS	*P* value
Roots										
Block	3	0.0034	0.9920	0.0129	0.8115	3	0.0036	0.5770	0.0332	0.9112
Treatment	5	0.0162	0.0003	0.0196	0.2107	5	0.0202	0.0010	0.0708	0.0418
Error	63	0.0029		0.0133		63	0.0042		2.1901	
Basal ends										
Block	3	0.03102	0.7304	0.01033	0.9031	3	0.03008	0.4951	0.02974	0.9954
Treatment	5	0.03810	0.0411	0.00806	0.0397	5	0.03400	0.0433	0.011761	0.0462
Error	63	0.01686		0.01890		63	0.01770		0.02023	
Black-foot disease	**2-year-old plants**					**3-year-old plants**				
	DI (%)			DS (%)		DI (%)			DS (%)	
Variables	df	MS	*P* value	MS	*P* value	df	MS	*P* value	MS	*P* value
Roots										
Block	3	0.0141	0.9945	0.0071	0.9110	3	0.1212	0.9932	0.0448	0.8234
Treatment	5	0.0024	0.9973	0.0121	0.7817	5	0.0082	0.9162	0.0044	0.0526
Error	63	0.0393		0.0247		63	0.0282		0.0467	
Basal ends										
Block	3	0.0076	0.8712	0.0081	0.7883	3	0.0135	0.9003	0.0328	0.9991
Treatment	5	0.0282	0.0413	0.0328	0.0417	5	0.0167	0.0389	0.0151	0.9404
Error	63	0.0136		0.0501		63	0.0233		0.0613	

[a] df = degrees of freedom.

[b] MS = mean square.

[c] Significance level $P < 0.05$.

Chapter 6.2.

Protection of grapevine pruning wounds against *Phaeomoniella chlamydospora* and *Diplodia seriata* by biological and chemical methods

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Vitis vinifera L.

Abstract

The grapevine trunk diseases (GTDs) Botryosphaeria dieback and esca threaten the sustainability of the grapevine industry worldwide. This study aimed to evaluate and compare the efficacy of various liquid (pyraclostrobin + boscalid and thiophanate methyl) and paste (paste + tebuconazole) formulation fungicide treatments, and biological control agents (*Trichoderma atroviride* SC1 and *T. atroviride* I-1237), for their potential to prevent infection of grapevine pruning wounds by *Diplodia seriata* and *Phaeomoniella chlamydospora* in two field trials over two growing seasons. Treatments were applied to freshly pruned wounds following their label dosages recommendations. After 24 hours, wounds were artificially inoculated with 400 spores of *D. seriata* or 800 spores of *Pa. chlamydospora*. Isolations were made from the treated pruning wounds after 12 months to evaluate the efficacy of the treatments. Fungicide formulations were superior to *Trichoderma*-based treatments for the control of both pathogens during both growing seasons, with mean percent disease control of 44 to 95% for *D. seriata* and 46 to 67% for *Pa. chlamydospora*. Pyraclostrobin + boscalid was the most effective treatment. *Trichoderma atroviride*-based treatments did not reduce infection by *D. seriata* or *Pa. chlamydospora* compared to the untreated inoculated control in both vineyards and seasons. This study represents the first vineyard assessment of several chemical and biological treatments to protect pruning wounds against GTDs fungi in Europe and provides growers with tangible preventative control practices to minimize yield losses due to GTDs.

Introduction

Botryosphaeria dieback and esca are two of the most harmful grapevine trunk diseases (GTDs) affecting vineyards in all major grape-producing areas worldwide. They currently are among the main biotic threats to the economic sustainability of viticulture reducing yields, productivity and longevity of vines and vineyards (Gramaje et al., 2018). Yield losses of 30-50% have been reported by Botryosphaeria dieback in highly infected vineyards of North America (Milholland, 1991). The economic impact of Botryosphaeria dieback along with another GTD such as Eutypa dieback in California was estimated to be $USD260 million per year (Siebert, 2001). Esca incidence has reached up to 80% in several vineyards of Southern Italy (Romanazzi et al., 2009), and 12% of vineyards in France are currently no

longer economically viable, due mainly to esca, with an annual estimated loss of €1 billion (Lorch, 2014).

Botryosphaeria dieback is currently associated with 26 botryosphaeriaceaous taxa in the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria*, and *Spencermartinsia* (Úrbez-Torres, 2011; Pitt et al., 2013a, b, 2015; Rolshausen et al., 2013; Yang et al., 2017) with the species *Diplodia seriata* being one of the most frequently isolated fungi from diseased vines in several grape growing regions such as Australia (Savocchia et al., 2007), California (Úrbez-Torres et al., 2010), Chile (Auger et al., 2004), China (Yan et al., 2013), France (Larignon et al., 2001), Mexico (Úrbez-Torres et al., 2008), Portugal (Phillips, 2002), South Africa (van Niekerk et al., 2004) and Spain (Luque et al., 2014). Botryosphaeria dieback frequently shows as complete absence of spring growth from affected spurs due to necrosis formation in wood vascular tissues with bud-break failure, and shoot and trunk dieback (Úrbez-Torres, 2011). Wood symptoms are characterized by wedge-shaped perennial cankers and dark streaking in spurs, cordons and trunks vascular tissues usually beginning in pruning wounds (Úrbez-Torres et al., 2010).

Esca is mainly caused by the fungus *Phaeomoniella chlamydospora* along with *Phaeoacremonium minimum* and other *Phaeoacremonium* spp. (Gramaje et al., 2015), some *Cadophora* spp. (Travadon et al., 2015), and several basidiomycetous taxa belonging to genera *Inocutis*, *Inonotus*, *Fomitiporella*, *Fomitiporia*, *Phellinus*, and *Stereum* (Cloete et al., 2015). The most characteristic external symptoms of the chronic esca comprise multiple banding discolourations on leaves known as 'tiger-stripe' pattern (Surico, 2009; Gubler et al., 2015). Internal wood symptoms involve black spots in the xylem vessels, longitudinal brown to black vascular streaking, and white to light yellow soft rot that frequently develops in wood of older vines (Fischer, 2002; Lecomte et al., 2012). Apoplectic esca form is characterized by a sudden and unexpected wilting of the whole vine or one/several arms or shoots (Lecomte et al., 2012).

Infection of grapevines by GTD fungal pathogens primarily occurs through annual pruning wounds made during the dormant season (Gramaje et al., 2018). Pycnidia of Botryosphaeriaceae spp. and *Pa. chlamydospora* develop from dead/cankered wood, old pruning wounds, grapevine canes, crevices, cracks and on the bark of infected grapevines (Úrbez-Torres and Gubler, 2011; Baloyi et al., 2016), and in the case of *Pa. chlamydospora*, mycelium on infected wood can also be a source of conidia (Edwards and Pascoe, 2001; Edwards et al., 2001; Baloyi et al., 2016). Fruiting bodies of these fungi can also be found in

pruning debris left in the vineyard, thus becoming a potential inoculum source for new infections (van Niekerk et al. 2010; Úrbez-Torres, 2011; Elena and Luque, 2016b).

Conidia release of Botryosphaeriaceae spp. and *Pa. chlamydospora* has been shown to be primarily correlated with rain events (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Kuntzmann et al., 2009; van Niekerk et al., 2010; Úrbez-Torres et al., 2010; Valencia et al., 2015). The dynamics of *Pa. chlamydospora* dispersal in Spain were recently described by an epidemiological equation that integrated the effects of both rain and temperature (González-Domínguez et al., 2020). Conidia of Botryosphaeriaceae spp. has been shown to be primarily dispersed by rain splash (Úrbez-Torres et al., 2010), while inoculum of *Pa. chlamydospora* is predominantly aerially dispersed (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Gubler et al., 2015; Quaglia et al., 2009). Infection occurs when conidia land on exposed and susceptible pruning wounds, germinate in xylem vessels and colonize the vine spur, cordon and trunk (Mostert et al., 2006; Epstein et al., 2008; Gubler et al., 2013; Moyo et al., 2014).

Susceptibility of pruning wounds to GTD pathogens is mainly dependent on the time of pruning, and the period between pruning and possible infection case. Several studies using artificial spore inoculations showed that susceptibility of grapevine pruning wounds is high when fungal infection occurs at the moment of pruning but decreases as the period between pruning and infection increases up to several weeks or months (Petzold et al., 1981; Munkvold and Marois, 1995; Eskalen et al., 2007; Serra et al., 2008; Úrbez-Torres and Gubler, 2011), with seasonal variation reported between grape regions caused primarily by climatic differences (Gramaje et al., 2018).

Protection of pruning wounds is essential for the management of Botryosphaeria dieback and esca in grapevine, especially if adopted early in the vineyard lifespan (Kaplan et al., 2016; Sosnowski and McCarthy, 2017). The efficacy of fungicide wound treatments against Botryosphaeriaceae spp. and *Pa. chlamydospora* has been demonstrated in Australia (Pitt et al., 2012), California (Rolshausen et al., 2010), Chile (Díaz and Latorre, 2013), New Zealand (Amponsah et al., 2012; Sosnowski and Mundi, 2019) and South Africa (Mutawila et al., 2015). The use of physical barriers such as paints and pastes formulated with or without fungicides have also shown to be effective to control infections caused by Botryosphariaceae fungi and *Pa. chlamydospora* (Epstein et al., 2008; Rolshausen et al., 2010; Pitt et al., 2012; Díaz and Latorre, 2013).

The high restrictions that most effective chemical active ingredients are currently facing in Europe because of environmental and human health risks (Larignon et al., 2008; Spinosi et al., 2009), make indispensable address new alternatives for controlling GTDs. Over the last years, research on biological control of GTD fungi with antagonistic microorganisms has shown promising results primarily under controlled conditions (Alfonzo et al., 2009; Mutawila et al., 2011a; Haidar et al., 2016; Rezgui et al., 2016; Álvarez-Pérez et al., 2017; Daraignes et al., 2018; Mondello et al., 2018; Andreolli et al., 2019; Del Frari et al., 2019; Mondello et al., 2019; Trotel-Aziz et al., 2019; Niem et al., 2020). Field trials with biological control agents (BCAs) have shown variable results for preventing infection by Botryosphaeriaceae and esca fungi (Kotze et al., 2011; Mutawila et al., 2011b, 2015, 2016; Mounier et al., 2014; Reis et al., 2017; Martínez-Diz et al., 2020a).

To our knowledge, no comparative studies to evaluate the efficacy of chemical and BCA products as pruning wound protectants against GTD fungi have been performed in Europe so far. Four pruning wound treatments are currently registered in Spain for the control of GTD fungi: three *Trichoderma*-based biological products, namely Esquive, Blindar and Vintec, and Tessior, a liquid polymer containing boscalid and pyraclostrobin (MAPA, 2020). In addition, thiophanate methyl is registered in Spain against fungal trunk pathogens in almond (MAPA, 2020). The aim of this study was to evaluate and compare the efficacy of various liquid and paste formulation fungicide treatments, and BCAs, for their potential to prevent infection of grapevine pruning wounds by *D. seriata* and *Pa. chlamydospora* in field trials. The products assessed were those registered in Spain for control of fungal trunk pathogens or other diseases on grapevine and/or other hosts.

Materials and methods

Location and characteristics of the experimental vineyards

The assays were carried out at two commercial vineyards located in O Barco de Valdeorras, Galicia region (Spain), in 2018 and 2019. The vineyards were planted on 1981 (37-years-old) and 1989 (29-years-old) with 'Godello' cultivar grafted onto 110 Richter rootstock. Vines were spaced 120 cm from center to center, and with an interrow spacing of 225 cm, trained as bilateral cordons in a trellis system with a spur-pruning (Royat).

Vineyards were less than 500 m apart and had very similar climates. Standard cultural practices were used in both vineyards during the growing season, and the management of

powdery and downy mildews was performed using only wettable sulphur and copper compounds applied at label dosages and following Integrated Pest Management (IPM) guidelines, respectively, when required. At the beginning of the study (2018), about 8% and 12% of vines had shown GTDs symptoms in each vineyard, respectively. The presence and evolution of GTDs symptoms have been inspected biannually from 2014 to present in plots of 1,500 vines at both vineyards. GTDs symptoms detected during inspection were associated mainly with esca such as tiger-pattern foliar necrosis, and shoots, arm and/or cordon death.

Both vineyards were located less than 4 km to an automatic weather station owned by MeteoGalicia (Weather Service of Galician Regional Government, Xunta de Galicia) and its climatic data was considered to be representative.

Fungal isolates and inoculum preparation

Diplodia seriata isolate CJL-398 and *Phaeomoniella chlamydospora* isolate BV-130 were used. *Pa. chlamydospora* BV-130 was selected due to its high virulence on grapevine in previous assays (Martínez-Diz et al., 2019). This strain was isolated from a 43-year-old esca diseased vine cultivar 'Tempranillo' grafted onto '41 Berlandieri' rootstock in 2015. *D. seriata* JL-398 was the most virulent isolate among 14 in a detached grapevine cane assay (Elena et al., 2015a). This strain was isolated from cankers and wood necrosis of grapevine.

Conidial suspensions of each pathogen were used for artificial inoculations in the field and inoculum was obtained using methods similar to those described by Elena and Luque (2016a). In the case of *D. seriata*, a mycelial plug previously plated on Potato Dextrose Agar (PDA, Conda Laboratories, Spain) at 25°C for 7 days was cultured upside down over the center of a water agar (WA, Conda Laboratories, Spain) plate. Maritime pine (*Pinus pinaster* L.) needles were cut to 1 cm long fragments and then sterilized in an autoclave following the standard protocol of 121°C for 20 min. Then, approximately 20 sterile needles fragments were placed on the WA media surface surrounding the *D. seriata* mycelial plug at about 1 to 1.5 cm and plates were incubated under warm white fluorescent and near ultraviolet light for a 12-h photoperiod regime at 25°C for 4 weeks until pycnidia formation. The day before inoculation, pine needles fragments (about n=40) with *D. seriata* pycnidia were placed along with 30 ml of sterile distilled water (SDW) in a beaker. The solution was kept overnight (about 16 h) at 4°C in permanent agitation with the aid of a magnetic stirrer to induce conidia release from the pycnidia and prevent conidia germination. The

inoculation day, the resulting solution was vacuum-filtered through a 60-mm Steriflip filter (Millipore Corporation, Billerica, MA) to get a cleaner suspension. Then, conidial suspension was adjusted to 2 x 10^4 conidia ml^{-1} using a hemocytometer (Brand™ Blaubrand™ Neubauer Counting Chamber, Thermo Fisher Scientific Inc., MA, USA).

Phaeomoniella chlamydospora strain was grown on PDA plates at 25°C for 3 weeks. Same day of inoculation, conidia were released from cultures by adding 10 ml of SDW and gently scraping with a sterile stick and the collected suspension adjusted to a concentration of 4 x 10^4 conidia ml^{-1} based on counts from the hemocytometer. Both conidial suspensions were stored at 4°C until inoculation time to avoid early conidia germination.

Spore germination was assessed for both fungal trunk pathogens by placing four drops of the spore suspension on a PDA plate, which was then incubated at 25°C under fluorescent light for a 12-h photoperiod. After approximately 24 h, a glass cover slip was placed over each drop area on the PDA. The number of non-germinated spores over a total of 100 in each drop was counted using an optical microscope (Nikon Eclipse E400) at 100x magnification. The mean percentage of germinated spores was determined.

Pruning wound protection treatments

Wound protection treatments tested in the present assay are listed in Table 6.2.1. We evaluated the efficacy of two chemical and two BCA formulated products, and also a paste mixed with a fungicide. In general, the chemical and biological products assessed were those commercially formulated and currently registered and available in Spain for control of fungal trunk pathogens, except tebuconazole (Song), which is registered to control botrytis bunch rot (*Botrytis cinerea*) and powdery mildew (*Erysiphe necator*) in grapevine. Applications rates were selected based on the registered label dosages recommendations. Liquid formulations were prepared by the suspension of the products in tap water, which is the procedure normally used for spraying vineyard treatments in Galicia region. Pyraclostrobin + boscalid (Tessior) treatment contains a liquid polymer and it is already formulated to be directly sprayed to pruning wounds without any previous mixing. Paste treatment was prepared by mixing a liter of the paste formulation (Master) with 80 ml of tebuconazole (Song).

Table 6.2.1. Pruning wound treatments evaluated for control of *Diplodia seriata* and *Phaeomoniella chlamydospora* under field conditions.

Trade name	Nature	Chemical/Biological group[a]	Active ingredient	Application rate	Supplier
Enovit Metil	Chemical	MBC[b] / Thiophanates	Thiophanate methyl 70%	1 g l^{-1}	Sipcam Inagra S.L.
Tessior	Chemical	QoI[c] / methoxy-carbamates + SDHI[d] / pyridine-carboxamides	Pyraclostrobin 0.5% + boscalid 1%	n/a[*]	BASF Española S.L.U.
Master + Song	Paste + chemical	DMI[e] / Triazoles	Paste (resin 55% + vegetal oil and healing substances 45%) + tebuconazole 25%	n/a	Sipcam Jardin S.L.+ Sipcam Iberia S.L.
Vintec	BCA[f]	Microbial (fungi)	*Trichoderma atroviride* SC1 (2×10^{10} CFU g^{-1})	2 g l^{-1}	Belchim Crop Protection España S.A.
Esquive	BCA	Microbial (fungi)	*T. atroviride* I-1237 (1×10^{8} CFU g^{-1})	100 g l^{-1}	Idai Nature S.L.

[a] According to Fungicide Resistance Action Committee (FRAC) Code List© (2020): Fungal control agents sorted by cross resistance pattern and mode of action (https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2020-final.pdf?sfvrsn=83014a99_2)
[b] MBC, Methyl Benzimidazole Carbamates
[c] QoI, Quinone outside Inhibitors
[d] SDHI, Succinate-dehydrogenase Inhibitors
[e] DMI, Demethylation Inhibitors
[f] BCA, Biological Control Agent
[*] n/a, not applicable

Regarding BCA treatments, the conidia viability of both *Trichoderma atroviride* strains (SC1 and I-1237) in the commercial products was tested to be at a minimum of 85% before the assay was set up (Pertot et al., 2016). A serial dilution of the conidia suspension was plated on PDA and the colony-forming units were counted after 24-48 h incubation at room temperature.

Field assay and experimental design

On 19 February 2018, 1-year-old canes of all vines to be treated were spur-pruned to three buds using secateurs in both vineyards, coinciding with the common pruning time in this region of Spain. Wounds treatments were applied by hand until runoff within 2 h after pruning to three wounds per vine. Liquid formulations were applied using a 500 ml hand-held spray bottle with a plastic shield on the nozzle to minimise spray drift and the paste formulation were applied with the aid of a paintbrush. Untreated controls, positive (artificially inoculated, IC) and negative (non-artificially inoculated, NC) were mock treated with sterile distilled water (SDW).

On the following day, wounds were moistened by spraying with SDW immediately prior to inoculation with the fungal trunk pathogens and a drop of Tween 20 (Sigma-Aldrich, San Luis, MO, USA) was added to each conidial suspension as a surfactant to assist spreading the spores over the pruning wound surface (Sosnowski and Mundi, 2019). Approximately 400 and 800 conidia of *D. seriata* and *Pa. chlamydospora*, respectively, suspended in a drop of 20 µl of SDW were then applied per wound using a micropipette. All pruning wounds were inoculated with the pathogen inoculum except NC controls, which were mock inoculated with a drop of 20 µl of SDW alone instead and being exposed to natural infection. Inoculum drops placed onto the pruning wounds were left to air dry (from some minutes to 1 h) before being wrapped with Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA) to avoid fast dehydration and favour fungal spores' penetration into xylem vessels. Due care was taken to avoid the rain for the entire duration of the trials set up, namely pruning, wound treatments application and artificial fungal inoculation (2 days).

The experiment was set up as a randomized block design with three replicates of ten plants (thirty canes) per wound protectant treatment and pathogen in each vineyard. Three replicates of ten plants per pathogen were also used for IC in each vineyard. Additionally, three replicates of ten plants were used as NC in each vineyard. The

experiment was repeated the following season (2019-20), with pruning and wound treatments applied on 12 February 2019, and artificial fungal inoculations on 13 February 2019.

Fungal recovery and identification

Canes were harvested from vines above the second bud (about 10 cm long pieces) approximately 12 months after artificial inoculation and stored in a 4°C cool room prior to laboratory assessment. Bark was first removed using a sharp knife from each cane. Then, canes were surface sterilised for 1 min in 33% sodium hypochlorite (commercial 40 g Cl/l) and rinsed twice for 1 min each in SDW. After air drying on sterile filter paper to remove moisture excess, each cane was cut into small pieces (about 12 mm^2) taken from the margin between discoloured or dead and live or apparently healthy wood tissue using sterilised secateurs. Five wood fragments were plated onto each of two plates of Malt Extract Agar (MEA) amended with 0.35 g l^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS) giving a total of ten wood pieces per cane. Cultures were incubated at 25°C under warm fluorescent light for a 12-h photoperiod and inspected daily for 15 days. All growing fungal colonies were transferred to PDA plates and then assessed for the presence or absence fungal mycelial growth resembling *D. seriata*, *Pa. chlamydospora* or *Trichoderma* spp.

Identification of GTD fungal cultures was then assessed under a stereoscopic (Olympus SZX9, Olympus Corporation, Tokyo, Japan) and optical microscopes (Nikon Eclipse E400, Nikon Corporation, Tokyo, Japan) based on cultural and morphological features previously described including colony growth pattern, colour, mycelial and other characteristics such as conidial shape, size and colour (Crous and Gams, 2000; Phillips et al., 2007). Identity of GTD fungal isolates and *Trichoderma* spp. was confirmed by molecular methods. Fungal DNA was extracted from fresh mycelium after 3 weeks of incubation in PDA using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA) following manufacturer's instructions. *D. seriata* was confirmed by sequencing part of the translation elongation factor 1- using the primer pairs EF1F-EF2R (Jacobs et al., 2004). *Pa. chlamydospora* was detected by PCR using the primers Pch1-Pch2 (Tegli et al., 2000). Identity of *Trichoderma* spp. was confirmed at species level by sequencing the ITS region using the universal primers ITS1F/ITS4 (Gardes and Bruns, 1993). All PCR products were visualized in 1% agarose gels (agarose D-1 Low EEO, Conda Laboratories) and sequenced in both direction by Eurofins GATC Biotech (Cologne, Germany).

Data analysis

Efficacy of each wound treatment was calculated as mean percentage recovery (MPR) of *D. seriata* and *Pa. chlamydospora* from each cane per treatment (Sosnowski et al., 2008, 2013). Data were checked for normality and homogeneity of variances prior to statistical analyses and transformed when required into the arcsine of the square root of the proportion $(MPR/100)^{1/2}$. The statistical analysis of the experimental results was carried out in a two-way ANOVA with blocks and treatments as independent variables, and MPR (%) as dependent variable. Mean percentage disease control (MPDC) was also determined as the reduction in MPR (%) as a proportion of the artificially inoculated control (IC) (MPDC=100 × [1 – (MPR treatment/MPR IC)]) (Sosnowski et al., 2008, 2013). Means were compared with ICs by the Student's *t* least significant difference (LSD) at $P < 0.05$. Data from all experiments were analysed using the Statistix 10 software (Analytical Software).

Results

Wound treatment evaluation against Diplodia seriata

During 2018-19 and 2019-20 seasons, *D. seriata* spore germination on PDA was 94% and 98.5%, respectively, and it was recovered from 58 and 68% of IC wounds, respectively (Table 6.2.2). *D. seriata* was recovered from 1% of NCs wounds at both seasons. Analysis of variance showed that there were significant differences in the relative recovery data from the different treatments between seasons ($P < 0.05$). No significant differences were found in the recovery data between vineyards in each season (2018-19, $P = 0.904$; 2019-20, $P = 0.593$), so data from each vineyard were combined and the analysis was performed separately for each season (Table 6.2.2).

Treatment with pyraclostrobin + boscalid, thiophanate methyl, and the paste + tebuconaloze significantly reduced the MPR of *D. seriata* from pruning wounds with respect to the IC at both seasons ($P < 0.05$) (Table 6.2.2). During 2018-19 season, pyraclostrobin + boscalid, thiophanate methyl, and the paste + tebuconaloze provided MPDC of 95, 90 and 76%, respectively, whereas these products provided MPDC of 69, 54 and 44%, respectively, during 2019-20 season. During both seasons, there was not a significant treatment effect with *Trichoderma*-based wound protectants ($P > 0.05$). During 2018-19 season, *T. atroviride* SC1 and *T. atroviride* I-1237 provided MPDC of 10, and 26%,

respectively, while these products provided MPDC of 22 and 32%, respectively, during 2019-20 season.

Table 6.2.2. Efficacy of wound treatments when applied 24 h before inoculation with *Diplodia seriata* in two growing seasons.

Trade name	Active ingredient	Growing season			
		2018/2019		2019/2020	
		MPR[a]	MPDC[b]	MPR	MPDC
	Inoculated control (IC)	58 a	-	68 a	-
Vintec	*T. atroviride* SC1	52 a	10	53 ab	22
Esquive	*T. atroviride* I-1237	43 a	26	46 ab	32
Master + Song	Paste (resin 55% + vegetal oil and healing substances 45%) + tebuconazole 25%	14 b	76	38 bc	44
Enovit Metil	Thiophanate methyl 70 %	6 b	90	31 bc	54
Tessior	Pyraclostrobin 0.5% + boscalid 1%	3 b	95	21 c	69

[a] Efficacy was based on the mean percent recovery (MPR) of *Diplodia seriata* from the treated canes by traditional isolation. Values in the same column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Mean percent disease control (MPDC) of treatments was calculated as the reduction in MPR as a proportion of the inoculated control.

Wound treatment evaluation against Phaeomoniella chlamydospora

During 2018-19 and 2019-20 seasons, *Pa. chlamydospora* spore germination on PDA was 89% and 93%, respectively, and it was recovered from 27 and 42% of IC wounds, respectively (Table 6.2.3). *Pa. chlamydospora* was recovered from 0% and 3% of NCs wounds during 2018-19 and 2019-20 seasons, respectively. There were no significant differences in the relative recovery data from the different treatments between vineyards ($P = 0.500$) and seasons ($P = 0.080$), so data were combined for analysis.

There was a significant treatment effect ($P < 0.05$) with the paste + tebuconaloze and pyraclostrobin + boscalid treatments reducing MPR of *Pa. chlamydospora* to 12 and 18% compared with 36% from the IC wounds (MPDC of 67 and 51%; Table 6.2.3). There was not a significant treatment effect with thiophanate methyl and *Trichoderma*-based wound protectants ($P > 0.05$). Thiophanate methyl provided MPDC of 46%, whereas *T. atroviride* SC1 and *T. atroviride* I-1237 provided MPDC of 0 and 17%, respectively.

Table 6.2.3. Efficacy of wound treatments when applied 24 h before inoculation with *Phaeomoniella chlamydospora*.

Trade name	Active ingredient	MPR[a]	MPDC[b]
	Inoculated control (IC)	36 ab	-
Vintec	*T. atroviride* SC1	45 a	0
Esquive	*T. atroviride* I-1237	30 ab	17
Enovit Metil	Thiophanate methyl 70%	19 bc	46
Tessior	Pyraclostrobin 0.5% + boscalid 1%	18 c	51
Master + Song	Paste (resin 55% + vegetal oil and healing substances 45%) + tebuconazole 25%	12 c	67

[a] Efficacy was based on the mean percent recovery (MPR) of *Phaeomoniella chlamydospora* from the treated canes by traditional isolation. Values in the same column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Mean percent disease control (MPDC) of treatments was calculated as the reduction in MPR as a proportion of the inoculated control.

Trichoderma-based treatments colonization

The conidia viability was on average of 97% and 95% for *T. atroviride* SC1 during 2018-19 and 2019-20 seasons, respectively. Regarding *T. atroviride* I-1237, the conidia viability was 94% during 2018-19 season and 96% during 2019-20 season. *Trichoderma* spp. were exclusively recovered from pruning wounds treated with *Trichoderma*-based formulations at varying levels. There were no significant differences in the relative recovery data between vineyards ($P = 0.180$) and seasons ($P = 0.075$). During 2018-19, recovery percentages were 5 and 10% for *T. atroviride* SC1 and *T. atroviride* I-1237, respectively. During 2019-20, recovery percentages were 9% and 14% for *T. atroviride* SC1 and *T. atroviride* I-1237, respectively.

Weather data

During 2018-19 season, the average of the daily mean temperature and relative humidity in the week from the day of pruning and wound treatments application (from 19 to 25 February 2018) was 6.5°C and 67.5%, respectively, with no rain events in that period. The average of daily mean temperature, daily mean relative humidity and accumulated rainfall for the whole month of February 2018 was 5.8°C, 76.6% and 84.2 mm, respectively, with nine rain events (of > 1 mm) in total.

During 2019-20 season, the week from the day of pruning and application of wound treatments (from 12 to 18 February 2019) registered an average of the daily mean temperature of 8.2°C and a 71.1% on average of daily relative humidity. For the same period, there was only one rain event (18 February 2019) with a total rainfall of 10.6 mm. Regarding the whole February 2019 month, the average of the daily temperature was 8.2°C and of the daily relative humidity 73.3%. The total rainfall in the same month was 37 mm received in a total of four rain events.

Discussion

The present study represents the first vineyard comparison of the efficacy of paste and liquid fungicides, and BCA treatments to protect pruning wounds against GTDs fungi in Europe. Considering the high incidence of GTDs, particularly esca, and the restrictions on the use of chemicals in Europe (Mondello et al., 2018), this study provides growers with tangible preventative control practices to minimize yield losses due to GTDs. By focussing on products already registered for control of trunk diseases in almond or foliar diseases of grapevines in Spain, the lower cost of label extension compared to new product registration will increase the likelihood and success of registration for GTDs. *D. seriata* was chosen to represent Botryosphaeria dieback, because it is one the most common cited Botryosphaeriaceae species occurring on grapevines worldwide and is reported to be a virulent species in Spain (Luque et al., 2009; Elena et al., 2015b). *Pa. chlamydospora* was chosen to represent esca, because it is the most frequently isolated species from affected vines in most grape growing regions worldwide (Berstch et al., 2013; Gubler et al., 2015).

Our results demonstrate that paste and liquid fungicide formulations were superior to *Trichoderma*-based treatments for the control of *D. seriata* and *Pa. chlamydospora.* All paste and liquid fungicide treatments tested reduced recovery of both pathogens from inoculated wounds compared with the untreated inoculated control, with the exception of thiophanate methyl for *Pa. chlamydospora*. Similar results were observed in other studies where several fungicides and BCA treatments were compared as pruning wound protectants in the same field trial. In Australian vineyards, liquid and paste fungicide formulations were more effective than *Trichoderma*- and *Bacillus subtilis*-based formulations against *D. seriata* and *Diplodia mutila* (Pitt et al., 2012), and *Eutypa lata* (Ayres et al., 2017) infections, respectively. Halleen et al. (2010) also reported that fungicides were more effective than *Trichoderma* spp. against *E. lata* infection in field trials

carried out in South Africa, in spite of the efficacy of *Trichoderma* treatments in reducing GTD fungal infection.

Application of pyraclostrobin + boscalid to pruning wounds provided high mean percentage of disease control (MPDC) for both pathogens. To date, only preliminary studies have been carried out in field trials in Germany (Kühn et al., 2017; Lengyel et al., 2019), Greece (Kühn et al., 2017; Samaras et al., 2019) and Spain (Kühn et al., 2017), where pyraclostrobin and boscalid (Tessior) was effective as pruning wound protectant reducing the grapevine wood infection caused by *Diplodia* spp. and *Pa. chlamydospora*. The application of a similar commercial product based on pyraclostrobin and boscalid without the liquid polymer (BASF516, BASF Australia Ltd, Sidney, New South Wales, Australia) showed a low efficacy against *E. lata* artificial pruning wound inoculations in Australian vineyards (Sosnowski et al., 2008). Wound applications of pyraclostrobin alone were effective for the control of *D. seriata* and *Pa. chlamydospora* in Chile (Díaz and Latorre, 2013) and California (Rolshausen et al., 2010) vineyards. Moreover, this active ingredient significantly reduced infections caused by fungi associated with Botryosphaeria dieback (Rolshausen et al., 2010), Eutypa dieback (Sosnowski et al., 2008, 2013; Rolshausen et al., 2010; Ayres et al., 2017), and esca (Rolshausen et al., 2010), under field conditions.

The only treatment to provide a similar level of control than pyraclostrobin + boscalid for both pathogens was the paste with tebuconazole. Accordingly, applications of paste and liquid formulations containing tebuconazole on pruning wounds of 'Cabernet Sauvignon' vines significantly reduced the mean vascular discolouration length and the reisolation percentage of *D. seriata* and *Pa. chlamydospora* in Chilean vineyards (Díaz and Latorre, 2013). In Australia, a gel and a paint with tebuconazole applied by paintbrush to freshly pruned canes reduced *E. lata* infections to 100% and 94%, respectively (Sosnowski et al., 2013). Pitt et al. (2012) also demonstrated that a tebuconazole paste formulation provided a 38% control of *D. mutila* in a trial performed in Australia. Other physical barriers containing a paste with fungicides have resulted effective at reducing pruning wound infections by other GTD fungi (Rolshausen and Gubler, 2005; Sosnowski et al., 2008; Rolshausen et al., 2010; Pitt et al., 2012). Liquid spray applications of tebuconazole were also significantly effective reducing the recovery of *D. seriata* in Australia (Pitt et al., 2012).

Thiophanate methyl was effective in reducing infection by *D. seriata*, while no significant effect was observed against *Pa. chlamydospora*. Similar findings were reported by Rolshausen et al. (2010) in California, where pruning wounds applications of

thiophanate methyl reached a disease control of 80% for *D. seriata* infections but did not perform as well against *Pa. chlamydospora* with only a 52% of disease control. In Chile, Díaz and Latorre (2013) reported the efficacy of both liquid and paste formulations of thiophanate methyl to control *D. seriata* and *Pa. chlamydospora* infections in pruning wounds. This chemical compound was also effective in reducing the pruning wound infections caused by *Pa. chlamydospora* and *Neofusicoccum luteum* in field trials carried out in South Africa (Mutawila et al., 2015) and New Zealand (Amponsah et al., 2012), respectively.

Pastes and paints are considered the most reliable protectants of pruning wounds against GTD fungi, especially when they are mixed with fungicides (Moller et al., 1977; Rolshausen and Gubler, 2005; Rolshausen et al., 2010; Sosnowski et al., 2008, 2013; Díaz and Latorre, 2013). They provide a physical barrier to protect pruning wounds from GTD fungal infection while the fungicide can also act on the pathogens if the physical barrier is compromised by rain, sap flow, or cracking when drying (Sosnowski et al., 2008). However, some other studies reported no differences in effectiveness between application of acrylic paint with or without fungicides (Sosnowski et al., 2008; Mayet and Lecomte, 2014). Pastes and paints are usually applied by hand with a paint brush, unless the product contains a liquid polymer to act as a physical barrier, which is the case of Tessior commercial product. It should be noted that application by hand is more time-consuming and can be at least two to four times the application cost with a tractor mounted sprayer (Sosnowski and McCarthy, 2017). Further research is therefore required to determine the protective mechanisms of each component and if their efficacy is also influenced by other factors such as wound size, application time, and weather variables.

Species of the fungal genus *Trichoderma* have been the most investigated BCA to act as pruning wound protectant against GTDs pathogens (John et al., 2005; Halleen et al., 2010; Kotze et al., 2011; Mutawila et al., 2015, 2016; Reis et al., 2017). Our results shown that *Trichoderma atroviride*-based treatments did not reduce infection by *D. seriata* or *Pa. chlamydospora* compared to the untreated inoculated control in both vineyards and seasons. This is the first report to assess the efficacy of *T. atroviride* SC1 in protecting grapevine pruning wounds from infection by GTD fungi in mature vineyards. In recent research, Berbegal et al. (2020) applied *T. atroviride* SC1 to pruning wounds of 3-year-old vines but its efficacy as wound protectant against GTD pathogens was not tested in this specific plant part. In nurseries, *Trichoderma atroviride* SC1 showed high efficacy to reduce artificial (Pertot et al., 2016) or natural (Berbegal et al., 2020) *Pa. chlamydospora* infection

when applied at different propagation stages. Preliminary results showed the efficacy of *T. atroviride* I-1237 to reduce the disease incidence and severity of *Pa. chlamydospora* and *N. parvum* on pruning wounds in Portuguese vineyards (Reis et al., 2017). Similarly, Mounier et al. (2014) demonstrated that spraying pruning wounds with *T. atroviride* I-1237 over two years significantly reduced the esca, and Botryosphaeria and Eutypa diebacks foliar symptoms expression, and the plant mortality rate due to GTDs in French vineyards. Dipping young grapevine plants in *T. atroviride* I-1237 during the nursery propagation process decreased *D. seriata* and *Pa. chlamydospora* DNA and necrotic lesion length compared to the untreated plants (Mounier et al., 2014). Other *Trichoderma* strains or *Trichoderma*-based commercial products have shown high efficacy in reducing the recovery of GTDs fungal pathogens from artificially inoculated pruning wounds under field conditions (John et al., 2005; Halleen et al., 2010; Kotze et al., 2011; Pitt et al., 2012; Mutawila et al., 2015).

These inconsistences found in the *Trichoderma* products performance among our study and previous reports could be due to different reasons. Although *Trichoderma* spp. have the ability to provide long-term protection to pruning wounds and thus preventing fungal trunk pathogens infections, they firstly need to establish itself, grow and colonize wounds instead of a simply temporal establishment (Munkvold and Marois, 1993; Mutawila et al., 2011a, b). In this study, pruning wound colonization by both strains of *T. atroviride* was very low at both vineyards and seasons ranging from 5 to 14%. Environmental conditions such as the temperature at the time of application might have a negative influence in the persistence and implementation of *Trichoderma* spp. (Elmer and Reglinski, 2006; Pertot et al., 2017). According to the label recommendations of each product, *T. atroviride* SC1 formulation should be applied when environmental temperature is equal or higher than 10°C for a minimum of five hours on the day of application in the field, while *T. atroviride* I-1237 formulation is supposed to be biologically active at temperatures above 5°C. The average of the daily mean temperature experienced in the week of the trials set up was 6.5°C and 8.2°C during 2018-19 and 2019-20 seasons, respectively, a fact that could explain the low colonization performed by *Trichoderma*-based formulations. In addition, a slightly higher *Trichoderma* recovery was registered during 2019-20 season (9.4 to 13.5%) than 2018-19 (4.8 to 9.8%) probably also explained by the warmer temperatures registered in this season.

Timing of pruning within the dormant season should be adjusted to periods with mild and favourable temperature values that might lead to a better implantation and

development of the BCA on pruning wounds and thus increasing its effectiveness against GTD pathogens. *Trichoderma* spp. application after pruning in late winter or early spring would likely provide higher disease control than normal pruning in winter. However, late pruning is not feasible in all vineyards. In those vineyards with limited labour force, growers need to begin pruning early in the winter to ensure completion of the activity before bud break. An alternative would be to prune in late autumn or early winter. Recent research carried out in the same grape-growing region of the present study reported low abundances of GTDs pathogens infecting naturally pruning wounds after an early pruning made in November (Martínez-Diz et al., 2020b). The age and physiological state of the vine as well as the dose and product formulation have also been suggested as factors that could have an influence on effective colonization by *Trichoderma* spp. (Schubert et al., 2008; Halleen et al., 2010; Mutawila et al., 2016). However, further research is required to confirm these hypotheses.

Label instructions of most fungicide and BCA commercial formulations to protect pruning wounds recommend their application shortly after pruning to minimize the chances of GTDs infection. In our study, we followed the official method of European countries to evaluate pruning wounds protection products against *E. lata,* which suggests carrying out fungal inoculations 24 hours after the application of preventive treatments (EPPO, 2017). Accordingly, in most of the previous pruning wound protection trials, the time elapsed between pruning wound protection and GTD fungal inoculation was 24 hours (John et al., 2005; Sosnowski et al., 2008, 2013; Halleen et al., 2010; Rolshausen et al., 2010; Kotze et al., 2011; Amponsah et al., 2012; Pitt et al., 2012; Díaz and Latorre, 2013; Ayres et al., 2017; Sosnowski and Mundi, 2019). This short time between BCA treatments application and artificial fungal inoculations could also have explained the poor performance exhibited by *Trichoderma*-based commercial formulations in our study. Previous research reported a greater biocontrol efficacy when artificial GTD pathogen infection was delayed 7 (Kotze et al., 2011; Mutawila et al., 2015) and 14 (Munkvold and Marois, 1993; John et al., 2005) days after application of *Trichoderma* spp. on pruning wounds. These findings suggest BCAs might need a period to colonise the pruning wound surface and grapevine wood to be effective, as reported by John et al. (2005).

The use of pathogen artificial inoculations is very common in the assessment of the efficacy of pruning wound protectants against GTDs to guarantee a substantial establishment of infection in untreated inoculated controls for statistical analysis (Halleen et al., 2010; Rolshausen et al., 2010; Sosnowski et al., 2008, 2013; Amponsah et al., 2012; Pitt

et al., 2012; Ayres et al., 2017; Sosnowski and Mundi, 2019). In the present study, artificial inoculations with 400 (*D. seriata*) and 800 (*Pa. chlamydospora*) conidia were applied per wound to obtain optimal recovery percentages for robust evaluation of treatments according to doses recommendations made by Elena et al. (2015b). This fact represents a significantly higher 'disease pressure' than that which might be expected to occur under natural conditions. Wounds were infected naturally up to 1% by *D. seriata* and 3% by *Pa. chlamydospora*, in contrast with the artificially inoculated controls recovery with up to 68% and 42%, respectively. This indicates that wound protectants that showed lower efficacy rates in this study, such as BCA formulations, will most likely provide better control of both *D. seriata* and *Pa. chlamydospora* under 'natural disease pressure' in the vineyard. The efficacy of pruning wound protectants under lower artificial GTD inoculum levels or natural infections in the vineyard should be tested in future studies. Different affinities of *T. atroviride* strains for specific grapevine cultivars has been previously reported in South Africa (Mutawila et al., 2011b), and this should not be discarded as a possible cause of the low *Trichoderma* colonization rates obtained in this study.

To conclude, this study highlighted the efficacy of several fungicides with or without a physical barrier to protect grapevine pruning wounds against *D. seriata* and *Pa. chlamydospora* infections under field conditions. In particular pyraclostrobin + boscalid (Tessior), a registered product against GTD fungi in several countries in Europe, is recommended as pruning wound protectant to prevent infection by the most prevalent pathogens associated with Botryosphaeria dieback and esca. *Trichoderma*-based treatments showed lower efficacy against GTD fungi than that provided by fungicides and their performance seems to be related to environmental conditions and wound colonisation prior to infection by the pathogens. Good pruning practices along with strict sanitation procedures and pruning wound protection by the application of authorized products can significantly reduce the impact of GTD pathogens infections and thus increasing the lifespan of vineyards.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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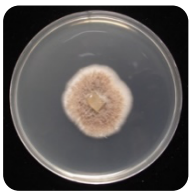
CHAPTER 7

General discussion



Grapevine trunk diseases (GTDs) have become a major concern worldwide, causing significant economic impact by reduced production and vineyard longevity. These diseases are caused by a wide range of fungal pathogens producing a range of symptoms including leaf and shoot distortion and discolouration, external wood cankers and dieback, internal wood necrosis and staining, poor growth, mortality of roots and sudden vine collapse. The prevalence of GTDs has significantly increased with changes in production practices, loss of effective chemicals, predominance of susceptible cultivars and ageing of vineyards. Pruning wounds are the main infection portal for these pathogens and inoculum sources include a wide range of alternative hosts such as fruit crops and many introduced and/or native tree species.

Due to the rapid expansion of wine regions, young vine decline (YVD) is escalating, as pathogens can be introduced during propagation, and diseases are often associated with poor planting practices and stress. Control is limited to hot-water treatment (HWT) and fungicide or biocontrol agents (BCAs) dips, with mixed results. The rapid development of molecular diagnostic capabilities is essential to prevent pathogen-infected vines from being planted and, thus, to facilitate the management of GTDs. Among them, culture-independent amplicon sequencing has recently emerged as a powerful tool to test hypotheses related to grapevine-microbiome interactions.

Different aspects of the GTD pathosystem have been studied in this thesis. But the overall objective was to develop and implement high-throughput DNA technologies that lead to the development of practical effective strategies to manage GTDs, and to evaluate sustainable alternatives to the use of fungicides in order to improve vineyard productivity and longevity.

Molecular diagnostics

In recent years, molecular techniques have been reported to be useful for detecting pathogens from different environmental samples because they are rapid and more sensitive than conventional techniques. For example, the quantitative real-time PCR (qPCR) that utilizes fluorescence chemistry allows the detection and quantification of very low amounts of nucleic acids in a wide range of samples (Bustin et al., 2009). The qPCR has been widely employed for sensitive and specific quantification of different fungal pathogens associated with GTDs (Tewoldemedhin et al., 2011; Martín et al., 2012; Pouzoulet

et al., 2013; Agustí-Brisach et al., 2014; Moisy et al., 2017; Pouzoulet et al., 2017; Billones-Baaijens et al., 2018; Langenhoven et al., 2018).

Recently, droplet digital PCR (ddPCR) has emerged as the third generation of PCR technology for absolute quantification of target nucleic acids based on sample nanodroplet partitioning and using binomial Poisson statistics (Hindson et al., 2011; Pinheiro et al., 2012). This novel technique is a direct end-point measurement that allows standards-free quantification of the target concentration reducing bias from amplification efficiency, susceptibility to PCR inhibitors and the difficulty in multiplexing (Kim et al., 2014; Yang et al., 2014a; Cao et al., 2015; Pavšič et al., 2016). The development of ddPCR protocols to quantify plant pathogens is still very limited (Dreo et al., 2014; Bahder et al., 2018; Voegel and Nelson, 2018). In particular, the application of ddPCR for GTD fungal quantification has only been recently realized for some pathogens (Úrbez-Torres et al., 2017; O´Gorman et al., 2019; Maldonado-González et al., 2020; Holland et al., 2019).

In chapter 3.1, we set up the innovative ddPCR technique for the absolute detection and quantification of *Ilyonectria liriodendri* in bulk and rhizosphere soil, as well as grapevine endorhizosphere and we compared it with the qPCR approach. We used previously developed primers (YT2Y/Cyl-R) (Tewoldemedhin et al., 2011; Dubrovsky and Fabritius, 2007) that amplify the main *Cylindrocarpon*-like asexual morphs associated with black-foot disease, in particular those belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria.* These primers proved a good performance in the simultaneous detection of "*Cylindrocarpon" macrodidymum*, "*C". destructans*, "*C". liriodendri,* and "*C". pauciseptatum* associated with apple tree roots in South Africa (Tewoldemedhin et al., 2011). A specific probe was also designed for the experiment. The use of probe-based assays leads to an accurate, specific and sensitive target quantification (Wong et al., 2015). Recently, the detection and quantification of Botryosphaeria dieback fungi spores from environmental samples was shown to be more specific and sensitive using a probe-based ddPCR assay (O'Gorman et al., 2019).

Both ddPCR and qPCR showed the potential of being efficient techniques to detect and quantify *I. liriodendri* DNA from grapevine environmental samples, with a strong correlation between them. This high quantitative agreement between techniques was also previously reported (Kim et al., 2014; Yang et al., 2014a; Cao et al., 2015; Porcellato et al., 2016). Increased sensitivity of ddPCR over qPCR was found in the detection of *I. liriodendri* at very low concentrations. A higher sensitivity of ddPCR as compared with qPCR was previously highlighted (Kim et al., 2014; Porcellato et al., 2016; Cavé et al., 2016; Bahder et

al., 2018), and comparable with qPCR technique by others (Dreo et al., 2014; Blaya et al., 2016). We also found that the abundance of *I. lirodendri* was not affected by the soil-plant fraction. *Cylindrocarpon*-like asexual morphs are commonly found in the endorhizosphere and roots of different plant hosts including grapevines (Tewoldemedhin et al., 2011; Bonito et al., 2014; Xu et al., 2015; Manici et al., 2018; Berlanas et al., 2020). This presence in dissimilar environments could explain the lack of specialization of these fungi to specific ecological compartments. Asymptomatic roots showed to harbour *Cylindrocarpon*-like asexual morphs by both quantification techniques. This is consistent with findings obtained by Berlanas et al. (2020), who reported the occurrence of fungal species associated with black-foot disease as pathogenic or non-pathogenic endophytes in visually symptomless vines and asymptomatic root vascular tissue. This fact was also documented in scientific literature for other plant species, such as weeds (Agustí-Brisach et al., 2011) or cereals (Langenhoven et al., 2018). We therefore hypothesize that some black-foot disease fungi may become pathogenic to the grapevine following different biotic and/or abiotic stress factors and thus, they can be considered as latent pathogens in vines. In USA, black-foot disease fungi are considered weak pathogens that are more damaging in soils with high moisture or compaction (Gubler and Petit, 2013). Further investigation is required within black-foot species complex to determine what triggers latent pathogens to transition from non-pathogenic endophyte to pathogenic endophyte, and cause disease symptoms in grapevine.

The ddPCR technology developed in this thesis to detect and quantify black-foot pathogens from soil and plant tissue can be adapted to other GTD pathogens, in order to implement early, accurate, specific and sensitive detection methods of these fungi. This would alert nurseries and growers to the presence of these pathogens in soil, and to prevent the spread of GTDs in grapevine propagation material.

Microbial ecology

Fungal communities in grapevine have been largely studied based on culture-dependent morphological methods (Casieri et al., 2009; Martini et al., 2009; González and Tello, 2010; Hofstetter et al., 2012; Pancher et al., 2012; Bruez et al., 2014, 2016, 2017). Culturing usually mislead fungal activity and underrate species richness, because the proportion of culturable microbial species are very low (Amman et al., 1995). Molecular-based technologies such as high-throughput amplicon sequencing (HTAS) has increased both the range and resolution of fungal communities' analyses and have discovered a

highly complex and wide-ranging microbiome of grapevine compartments (Zarraonaindia et al., 2015; Holland et al., 2016; Eichmeier et al., 2018; Berlanas et al., 2019; Deyett and Rolshausen, 2019, 2020).

Grapevine has become as an exceptional plant model system for woody perennial crops microbiome research (Bokulich et al., 2014; Perazzolli et al., 2014; Zarraonaindia et al., 2015; Deyett and Rolshausen, 2020). In this thesis, we studied the fungal microbiome diversity in two different scenarios by using HTAS technology: below- (bulk soil, rhizosphere and root endosphere) (chapter 4.1.) and aboveground (canes) (chapter 4.2) grapevine compartments.

To date, the diversity of fungal communities in soil has been mostly inferred by pyrosequencing approach (Holland et al., 2016; Castañeda and Barbosa, 2017; Longa et al., 2017), ARISA fingerprinting (Likar et al., 2017) and PCR-DGGE (Manici et al., 2017). Recently, the fungal and bacterial microbiome has been characterized in the rhizosphere (Berlanas et al., 2019), or throughout the vine (Deyett and Rolshausen, 2020) by HTAS. Regarding bacterial diversity, differences among grapevine root, rhizosphere and bulk soil have been reported by HTAS (Zarraonaindia et al., 2015; Marasco et al., 2018).

In chapter 4.1., we characterized the spatial dynamics of the fungal communities in three soil-plant compartments (bulk soil, rhizosphere and endorhizosphere) of young vines located in La Rioja (Northern Spain) by the nuclear ribosomal DNA-internal transcribed spacer region (ITS) HTAS. Results showed an increase of the relative abundance of potential plant pathogens, endophytes and arbuscular mycorrhiza from bulk soil towards endorhizosphere. These results agree with those obtained recently by Deyett and Rolshausen (2020), who analysed the bacterial and fungal communities across six grapevine compartments (bulk soil, rhizosphere, root, cordon, cane and sap) to determine the origin of the microbial endophytes inhabiting grapevine vascular system. Symptomless grapevine roots were found to be a microbial niche colonized by GTD pathogens, such as *Ilyonectria*, *Cadophora*, *Phaeomoniella*, *Phaeoacremonium*, *Diaporthe*, *Botryosphaeria* and *Diplodia*, suggesting an endophytic phase of these fungi until some of them become pathogenic triggered by specific conditions. This agrees with the results obtained in chapter 3.1, and by Berlanas et al. (2020) who found 13 fungal species associated with black-foot disease colonizing root vascular tissue without causing any kind of internal or external symptom in grapevine nursery plants ready to dispatch.

Several studies showed the significance of grapevine below-ground compartments in providing and maintaining exclusive niches and microorganisms linked with stress protection and health, development and productivity of the plant (Zarraonaindia and Gilbert, 2015; Marasco et al., 2018; Yu and Hochholdinger, 2018; Berlanas et al., 2019). Microbial endophytes can originate from the soil and move towards the aboveground compartments via the endorhizosphere, demonstrating that soil is the main reservoir of microbial grapevine colonizers (Compant et al., 2008; Martins et al., 2013; Zarraonaindia et al., 2015). Soil microbiota was suggested to be involved in the creation of the local wine terroir and thus ultimately affecting wine characteristics (Zarraonaindia et al., 2015). Unravelling the dynamics of fungal communities in different soil-plant compartments would allow researchers to improve the knowledge on the biology and ecology of GTD fungi in order to develop effective management strategies.

In chapter 4.2., we developed an ITS HTAS assay to ascertain the effect of pruning time in composition and diversity of fungal communities naturally colonizing pruning wounds, in particular, those responsible for GTDs. Pruned canes were taken from six vineyards located in three Denominations of Origin (D.O. Ribeiro, D.O. Rías Baixas and D.O. Valdeorras,) in Galicia region in mid-autumn (November), winter (February) and spring (May), over two growing seasons. Our results showed that fungal microbiome richness and diversity differed among the three D.O., being usually higher in D.O. Ribeiro. Environmental variables did not predict alpha-diversity variation. Although in general fungal microbiome richness and diversity was higher during the period February-May, there were not significant differences for these indexes between both infection periods in specific scenarios. Theoretically, fungal growth and infection usually occurs during wet and warm conditions. The linear discriminant analysis effect size detected several fungal clades which discriminated the fungal communities between infection periods. Several fungal genera such as *Vishniacozyma*, *Filobasidium*, *Rhodotorula*, *Aureobasidium*, and *Udeniomyces* were predominant during the period November-February. Some species belonging to these genera are known to be psychrophilic yeast-like fungi which means that they can thrive at low temperatures (Han et al., 2012; Buzzini et al., 2018). The species *Aureobasidium pullulans* was shown to prevail in the grapevine core microbiome (Sabate et al., 2002; Martini et al., 2009; González and Tello, 2011; Barata et al., 2012; Pinto et al., 2014; Dissanayake et al., 2018; Deyett and Rolshausen, 2019) as well as to have a great capacity to colonize grapevine pruning wounds (Munkvold and Marois, 1993). This yeast-like fungus also showed antagonistic abilities against GTD fungi such as *Diplodia seriata*

and *Eutypa lata*, reducing the *in vitro* mycelial growth (Pinto et al., 2018) and pruning wounds infections (Munkvold and Marois, 1993), respectively.

Several fungal genera associated with GTDs, such as *Cadophora*, *Cytospora*, *Diaporthe*, *Diplodia* and *Phaeomoniella*, were mostly identified during the infection period February-May and explained the differences observed between periods. These fungi are mainly spread through aerially dispersed spores infecting grapevines via pruning and/or natural wounds (Rolshausen et al., 2010; van Niekerk et al., 2011; Gramaje et al., 2018). Spore release varies throughout the growing season depending on the fungal pathogen, geographical location and environmental conditions (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Quaglia et al., 2009; van Niekerk et al., 2010; Billones-Baaijens et al., 2018; Úrbez-Torres et al. 2010a, b; González-Domínguez et al., 2020). Information related with the dispersal patterns of GTD pathogens are therefore indispensable to identify high-risk infection periods and to guide growers in timing management practices such as pruning time.

Susceptibility of grapevine pruning wounds to trunk pathogens have been studied through artificial fungal inoculations in several grape-growing regions such as Australia (Ayres et al., 2016), California (Moller and Kasimatis, 1978; Munkvold and Marois, 1995; Eskalen et al., 2007; Úrbez-Torres and Gubler, 2011), France (Chapuis et al., 1998; Larignon and Dubos, 2000; Lecomte and Bailey, 2011), Italy (Serra et al., 2008), Michigan (Trese et al., 1982), South Africa (van Niekerk et al., 2011) and Spain (Elena and Luque, 2016). In general, these studies showed that wound susceptibility decreased as the period between pruning and inoculation of wounds increased, and it can be extended up to 4 to 7 weeks for most pathogens under favourable conditions. The rate of natural infections in pruned canes (i.e., those not obtained through artificial inoculations), however, has not been extensively studied to date, and they can be estimated only through the spontaneous infections of the vines included as non-inoculated controls in artificial inoculations.

Results obtained in our study on the natural infections of pruning wounds in three D.O. in Galicia showed that higher fungal GTD infection abundances occurred in spring than in winter, thus suggesting that pruning wounds could be more susceptible to pathogens overall after a late pruning in winter. Similar results were obtained by Luque et al., (2014), who observed higher isolation percentages of several GTD fungi in culture medium following late pruning (February-May) compared with that following early pruning (November-February). In contrast, mean percentage values of natural infections caused by *Eutypa lata* were about 2% after the spring-pruning (mid-May to late June) and 13%

after the winter pruning (January to February) in France (Lecomte and Bailey, 2011). Studies based on artificial inoculations also recommended late pruning to reduce GTD pathogens infections (Petzold et al., 1981; Munkvold and Marois, 1995; Chapuis et al., 1998; Larignon and Dubos, 2000; Eskalen et al., 2007; Serra et al., 2008, Úrbez-Torres and Gubler, 2011), although the real potential risk of infections may have been biased since these trials did not consider the presence of natural pathogenic inoculum along the experimental period.

The correlation patterns between weather data and OTUs abundance of GTD fungi obtained in this study have been irregular with negatively and positively values being hardly statistically significant. The abundances of genera *Diaporthe* and *Phaeomoniella* were negatively correlated with temperature in the first week after pruning. This fact could be associated with climatic conditions favouring the pruning wound healing process. Similarly, negative correlations values between mean daily temperature and *D. seriata* and *Phaeomoniella chlamydospora* natural infections were found by Luque et al. (2014) in the first weeks after pruning. Accumulated rainfall was found to have a positive correlation with *Diaporthe* genus from eight weeks after pruning highlighting the role of rain events in the infection and development of GTD fungi as previously reported (Amponsah et al., 2009; Kuntzmann et al., 2009; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010; Baskarathevan et al., 2013). This same trend was also observed by Luque et al. (2014) for natural infections caused by *D. seriata*, *Pa. chlamydospora* and species of Diatrypaceae in Catalonian vineyards.

In the light of the information presented in chapter 4.2, further research is needed to determine the effects of the pruning season and wound age on the susceptibility of pruning wounds to fungal trunk pathogens in Galicia. This information can be complemented with spore-trapping studies throughout the pruning season in order to develop models to predict the conditions under which spores infect pruning wounds of grapevine. In this sense, González-Dominguez et al. (2020) recently developed a model to predict disease risk caused by *Pa. chlamydospora* in Spanish vineyards concluding that the dynamics of this pathogen were best explained when time was expressed as hydro-thermal time accounting for the effects of both temperature and rain.

Grapevine-pathogen interaction

The grape and wine industry are currently looking for easy, safe, inexpensive and environmentally friendly alternatives to combat GTDs due to difficulties in their control. The use of disease-tolerant cultivars, clones, and rootstocks is a time-tested and sustainable method that can be included in IPM strategies against fungal trunk pathogens. This approach would not only diminish losses from the disease, but also, would notably reduce the necessity for chemical treatments and curative control strategies, and the level of toxic compounds in the vineyard environment.

The Ascomycete fungus *Pa. chlamydospora* is the most prevalent species associated with two GTDs affecting both young and mature vineyards, namely Petri and esca diseases, respectively (Larignon and Dubos, 1997; Mugnai et al., 1999; Crous and Gams, 2000; Bertsch et al., 2013; Gubler et al., 2015). This pathogen is endemic to all grapevine growing regions worldwide causing significant economic losses to grape and wine industry (Bertsch et al., 2013; Gramaje et al., 2018).

In chapter 5.1., we set up a rapid and effective detached cutting assay for evaluating disease tolerance to *Pa. chlamydospora* among minority and commercial grapevine germplasm collections in Spain. The cultivars 'Estaldiña', 'Albillo Mayor' or 'Castañal' were categorized as the most tolerant based on mean lengths of wood discolouration. However, all cultivars developed wood lesions after inoculation with the fungus, indicating that there is no evidence of qualitative resistance to *Pa. chlamydospora*. This agrees with previous research where grapevine cultivars, clones, and rootstocks showed different levels of tolerance to *Pa. chlamydospora* (Eskalen et al., 2001; Feliciano et al., 2004; Zanzotto et al., 2008; Gramaje et al., 2010; Landi et al., 2012; Travadon et al., 2013; Markakis et al., 2017; Sofia et al., 2018). Partial or quantitative resistance to *Pa. chlamydospora* on grapevine would limit the infection development, thus reducing the dispersion and epidemics in the vineyard over the years (Stuthman et al., 2007). This type of resistance is frequently controlled by several genes related to the physiological processes which help to build the plant defense mechanisms (Niks et al., 2015). To date, little is known regarding the mechanisms of woody tissues to counteract GTD infections. A study carried out by Pouzoulet et al. (2017) showed that xylem vessel diameter of grapevine cultivars could be an important trait to explain their tolerance to *Pa. chlamydospora* infection. High tolerance of grapevine woody tissues to *E. lata* infection have been associated to high lignin levels (Rolshausen et al., 2008; Hamblin, 2015).

Planting tolerant grapevine material is of utmost importance for the future management of fungal trunk diseases and to ensure the longevity of vineyards. Research focused on the screening of different grapevine genotypes, i.e. cultivars, clones and/or rootstocks within existing germplasm collections is required to identify tolerant grapevine plant material to GTDs. For instance, 161-49 Couderc showed to be the least susceptible among five grapevine rootstocks vacuum inoculated with several esca and Petri disease pathogens (Gramaje et al., 2010). A wide screening of grapevine cultivars in Australian germplasm collections provided evidence for Eutypa and Botryosphaeria dieback tolerance among cultivars (Sosnowski et al., 2016). Future studies should aim to determine if there is a significant correlation between the anatomical and biochemical data and the extent of wood-lesions measure in screening assays.

Disease management

The banning of the most effective fungicides available against GTD fungi in the early 21st century along with the increasing restrictions that chemicals are currently facing worldwide both due to environmental and human health concerns, make indispensable address new alternatives for controlling GTDs in nurseries and vineyards. An IPM program which includes the use of HWT in combination with the application of BCA treatments have been reported as the best alternative strategy to reduce GTD incidence in grapevine nurseries (Fourie and Halleen, 2004; Halleen and Fourie, 2016; Martínez-Diz et al., 2019). In particular, over the last 10 years there has been a frantic search by the GTD research community for microbial antagonists. Most of these experiments have been performed under *in vitro* (Alfonzo et al., 2009; Haidar et al., 2016a, b; Santos et al., 2016; Álvarez-Pérez et al., 2017; Andreolli et al., 2019; Del Frari et al., 2019; Mondello et al., 2019), greenhouse (Rezgui et al., 2016; Santos et al., 2016; Yacoub et al., 2016; Daraignes et al., 2018), and grapevine nursery (Fourie and Halleen, 2004, 2006; Halleen and Fourie, 2016; Pertot et al., 2016; Álvarez-Pérez et al., 2017; Berbegal et al., 2020) controlled conditions showing preliminary promising results. To date, few field trials have been performed with BCAs applied as a pre-planting strategy (Fourie and Halleen, 2006; Halleen et al., 2007; Halleen and Fourie, 2016; Berlanas et al., 2018; Berbegal et al., 2020) or acting as pruning wound protectants (John et al., 2005; Rolshausen and Gubler, 2005; Halleen et al., 2010; Kotze et al., 2011; Mutawila et al., 2011, 2015, 2016; Pitt et al., 2012; Mounier et al., 2014; Ayres et al., 2017; Reis et al., 2017) to prevent infection by GTD fungi, with variable results.

In chapter 6.1., we assessed the effect of pre- and post-planting root applications of two potential BCAs and three BCA-commercial products to control natural infections caused by black-foot and Petri diseases fungi. Results showed that BCA effectiveness in reducing the incidence and severity of both diseases was dependent on the plant age and the plant part analyzed. We found that *Streptomyces* sp. E1 + R4 in basal ends and *Pythium oligandrum* in roots were effective to reduce most black-foot and Petri disease fungal infections, respectively. Similarly, significant reductions of infections rates caused by black-foot fungi were observed after the root application of several *Streptomyces* sp. strains (Álvarez-Pérez et al., 2017), and *P. oligandrum* was also effective in reducing the necrosis length caused by *Pa. chlamydospora* (Yacoub et al., 2016; Daraignes et al., 2018). In our study, *Trichoderma atroviride* SC1 was able to reduce *Pa. chlamydospora* incidence and severity at basal ends, while *T. koningii* TK7 reduced *Phaeoacremonium minimum* infection in roots. In nurseries, the application of *T. atroviride* SC1 at different stages of the propagation process was also capable to reduce the infection caused by *Pa. chlamydospora* and *Pm. minimum* (Pertot et al., 2016; Berbegal et al., 2020). Combining two or more BCAs could improve not only the biocontrol efficacy against a disease (Yang et al., 2013, 2014b), but also cover a broader spectrum of target pathogens (Deketelaere et al., 2017). This approach needs to be extensively studied to better understand the ecological basis of the interactions among BCAs, as the predicted individual performance of each BCA does not have to be preserved or synergic when they are in a mixture (Meyer and Roberts, 2002).

In chapter 6.2., we set up vineyard trials aimed to evaluate and compare the efficacy of several liquid and paste fungicides, and BCA formulations currently registered in Spain, for their potential as preventive treatment to protect pruning wounds against *D. seriata* and *Pa. chlamydospora* infections. Our results showed that fungicide formulations were superior to BCA-based treatments for the control of both GTD pathogens. Similar results were observed in studies carried out in Australian (Pitt et al., 2012; Ayres et al., 2017) and South African (Halleen et al., 2010) vineyards where fungicides showed a higher effectivity than BCAs to protect pruning wounds against Botryosphaeria and Eutypa diebacks fungi when compared at the same trial. The treatment with pyraclostrobin + boscalid provided a high control of both GTD fungi. This formulation was also effective controlling *Diplodia* spp. and *Pa. chlamydospora* in preliminary studies carried out in field trials in Germany (Kühn et al., 2017; Lengyel et al., 2019), Greece (Kühn et al., 2017; Samaras et al., 2019) and Spain (Kühn et al., 2017). On the contrary, the application of a similar commercial product based on the same chemical active ingredients showed a low efficacy against *E. lata* in

Australian vineyards (Sosnowski et al., 2008). We also found that *T. atroviride*-based formulations did not reduce infection caused neither by *D. seriata* nor *Pa. chlamydospora*. Conversely, *T. atroviride* SC1 previously shown to be effective reducing *Pa. chlamydospora* infection when applied at different stages of the nursery process (Pertot et al., 2016; Berbegal et al., 2020). Regarding *T. atroviride* I-1237, preliminary studies demonstrated its ability to control *Pa. chlamydospora* and Botryosphaeria dieback fungi on pruning wounds in French (Mounier et al., 2014), and Portuguese vineyards (Reis et al., 2017). These inconsistencies found among studies could be due to diverse aspects. The different affinity of *Trichoderma* spp. for specific grapevine cultivars (Mutawila et al., 2011), the time elapsed between *Trichoderma* treatments application and GTD fungal infections (Munkvold and Marois, 1993; John et al., 2005), the environmental conditions at the time of *Trichoderma* treatments application (Elmer and Reglinski, 2006; Pertot et al., 2017), the age and physiological state of the vine (Mutawila et al., 2011, 2016), the pruning time (Mutawila et al., 2016), and the dose and formulation of the product (Schubert et al., 2008), have been suggested as factors that could influence the effectiveness of *Trichoderma*-based formulations.

Long-term protection can be provided by *Trichoderma* spp. and thus preventing GTD fungi infections, but they first need to establish itself, grow and colonize grapevine tissues (Munkvold and Marois, 1993; Mutawila et al., 2011). The complex interactions that occur among the antagonist, the pathogen, the host plant and its related microbial community, and also the physical environmental conditions make the control of GTD pathogens difficult (Elmer and Reglinski, 2006; Ojiambo and Scherm, 2006). The effectiveness and survival of antagonistic microorganisms to act as BCA depends on many key factors such as the kind of microorganism, the strain, application method, time and dose, soil type and moisture, humidity, temperature, UV light and nutrient level or availability (Elmer and Reglinski, 2006; Ojiambo and Scherm, 2006; Abd-Elgawad and Askary, 2020). The employment of plant microbiome and endophyte microorganisms in disease management has been postulated (Gimenez et al., 2007; Pinto et al., 2014; Lamichhane and Venturi, 2015) as they could take part in plant resilience and be a source of bioactive metabolites (Campisano et al., 2014, 2015).

Future research should focus on the development of effective BCA treatments, and searching for existing or new microbial strains with the potential to degrade phytotoxic disease factors of GTD pathogens through the use of in-depth microbial ecology studies. In this regard, culture-independent amplicon metagenomic approaches have been

recently developed to better-known grapevine microbiome which can establish a framework for discovering new microbial antagonists of pathogens or new advances to improve the efficacy of the biocontrol microorganisms (Massart et al., 2015; Deyett and Rolshausen, 2019, 2020). Future research should also benefit from the availability of sequenced and annotated genomes of BCAs to determine the genes associated with the mechanisms of action of the most effective BCA strains.

Current recommendations on GTD control are not pathogen-specific due to gaps in the knowledge of pathogen biology, ecology, epidemiology and host resistance. Since the indistinct symptoms of GTDs in dormant propagation materials and young vines has made it difficult for nurseries to detect this problem before selling plants to the growers, the application of sensitive detection techniques such ddPCR, as demonstrated in this research, should be incorporated into any program that develops an IPM strategy specific for nurseries. This method could also be used for quality testing of the cuttings and grafted vines to prevent the latent infections of GTD pathogens being carried over to new vineyards. Decreasing costs and increased accessibility have enabled researchers to develop a rich catalog of HTAS applications in Plant Pathology. Our research applied the latest HTAS technology in the GTD pathosystem in order to make pruning time recommendations to growers in the short term. The identification of disease tolerant rootstocks and cultivars, along with the use of effective BCA are key elements in the IPM program due to the restrictions on the use of chemicals in nurseries and established vineyards. The information generated in this thesis is already available for nurseries and specially for grapegrowers, who plan to establish or replant vineyards and want to reduce both their reliance on fungicides and their costs for controlling trunk diseases.

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CHAPTER 8

Conclusions

1. Droplet digital (ddPCR) and real-time PCR (qPCR) showed the potential of being efficient techniques to detect and measure *Ilyonectria liriodendri* DNA associated with black-foot disease, with a strong correlation between them.

2. The ddPCR was more sensitive compared with qPCR in the detection and quantification of *I. liriodendri* at very low concentrations, making this approach very suitable for identifying early fungal infections in grapevines and soil.

3. The abundance of *I. liriodendri* was not affected by soil or plant as source of DNA, which may explain the lack of specialization of *Cylindrocapon*-like asexual morphs to specific plant-associated ecological niches (rhizosphere and endorhizosphere).

4. Both the ddPCR and qPCR methods revealed that healthy grapevine plants harbor *Cylindrocarpon*-like asexual morphs, demonstrating that these fungi can act as pathogenic or non-pathogenic endophytes in grapevine.

5. Soil fungal assemblages had extensive local homogeneity across small distances, and soils with similar climatic conditions and homogeneous edaphic variables, thus suggesting that vineyard fungal communities are partially conserved in La Rioja region.

6. Fungal community changes in bulk and rhizosphere soils compared to grapevine endorhizosphere were detected by high-throughput amplicon sequencing (HTAS), with diversity decreasing in the endorhizosphere compartment.

7. An increase in the relative abundances of potential plant pathogens, endophytes and arbuscular mycorrhiza, and a decrease in the relative abundance of potential wood, dung and undefined saprotrophs were predicted from bulk soil towards the endorhizosphere.

8. The root interior of asymptomatic vines is a microbial niche that is inhabited by fungi associated with grapevine trunk diseases (GTDs) and might become pathogenic under specific conditions.

9. Grapevine pruned canes harbour a core community of fungal species, which appear to be independent of the infection period.

10. The fungal communities colonizing grapevine pruning wounds were affected in their composition and diversity by the Denomination of Origin (D.O.), whereas the spatial variation within each region was low.

11. A seasonal effect on GTD fungal infection of pruning wounds was detected for most genera, with higher percentages of abundance detected after pruning in February (winter) as compared with that of pruning in November (mid-autumn).

12. Weather variables were significantly correlated with the abundance of the total fungal microbiome and two GTD fungal genera. Temperature negatively correlated with the fungal microbiome abundance until the eighth week after pruning and with *Diaporthe* and *Phaeomoniella* genera abundance in the first week after pruning. Accumulated rainfall positively correlated with both the fungal microbiome and *Diaporthe* genus abundance over eighth and eleventh weeks after pruning.

13. *Phaeomoniella chlamydospora* caused vascular discolouration on all minority and commercial grapevine cultivars wood, significantly longer than control plants, but no foliar symptoms were observed on inoculated plants during the experiments.

14. 'Estaldiña', 'Albillo Mayor' or 'Castañal' were categorized as the most tolerant cultivars based on mean lengths of wood discolouration and may be promising candidates for commercially relevant host resistance in grape production systems.

15. The effectiveness of some BCA in reducing the incidence and severity of black-foot and Petri diseases under field conditions were dependent on the plant part analysed and the plant age.

16. *Streptomyces* sp. E1 + R4 were able to reduce significantly the infection caused by the most prevalent black-foot disease fungi, while *Pythium oligandrum* P037 and *Trichoderma* spp. were able to reduce significantly *Pa. chlamydospora* and *Phaeoacremonium minimum* infections in specific scenarios.

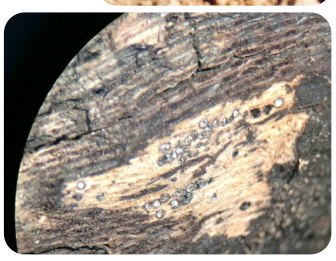
17. Treatments with BCAs had no effect on the shoot weight, and root weight was significantly lower in all BCA treatments with respect to the control.

18. BCA-based treatments showed lower efficacy as pruning wound protectants against *Diplodia seriata* and *Pa. chlamydospora* than that provided by fungicides.

19. The low efficacy showed by *Trichoderma*-based treatments might be attributed to their ability to colonise grapevine pruning wounds as well as the environmental conditions prior to infection by GTD pathogens.

20. The commercial formulation containing pyraclostrobin + boscalid was the most effective pruning wound protectant to prevent infection by the most prevalent fungal pathogens associated with Botryosphaeria dieback and esca.

Appendix



Appendix A

Resumen extenso

Introducción

Las enfermedades de la madera de la vid (EMV) se encuentran entre las principales amenazas bióticas para la sostenibilidad económica de la industria vitivinícola mundial, reduciendo el rendimiento, productividad y longevidad de los viñedos. El complejo de las EMV incluye principalmente seis patologías diferentes que afectan al material de propagación de vid en viveros y a viñedos jóvenes y adultos en todo el mundo, que son el pie negro de la vid, la enfermedad de Petri, la yesca, la eutipiosis y los decaimientos por Botryosphaeria y por Diaporthe. Varios factores han sido atribuidos al aumento de su incidencia en los últimos años, como son los cambios en las prácticas culturales de los viñedos, la baja calidad sanitaria del material de propagación de vid, la prohibición de las materias activas químicas más efectivas y la deficiente protección de las heridas de poda. Estas enfermedades poseen una complejidad añadida, ya que al menos 136 especies fúngicas no relacionadas taxonómicamente pertenecientes a 35 géneros distintos están asociadas a las distintas patologías en todo el mundo, lo que hace que su control sea muy difícil. La biología y epidemiología de estos patógenos es también muy compleja, ya que algunos hongos se caracterizan por ser habitantes comunes del suelo (pie negro), otros se dispersan principalmente mediante esporas a través del viento, del agua de lluvia y de los artrópodos (enfermedad de Petri, yesca, eutipiosis y decaimiento por Botryosphaeria y por Diaporthe) y algunos tienen la capacidad de dispersarse mediante esporas y además, de llegar a ser habitantes del suelo, a través de restos de poda enfermos (enfermedad de Petri). Asimismo, algunos de estos hongos pueden dispersarse a través del material de propagación infectado en el vivero (decaimiento por Botryosphaeria y por Diaporthe, pie negro, enfermedad de Petri y yesca).

Tradicionalmente, la detección e identificación de los hongos asociados as estas patologías se ha realizado mediante evaluaciones morfológicas dependientes del cultivo tradicional en placa. Estas técnicas, aunque son fiables para una identificación y clasificación preliminar, no son capaces de detectar bajos niveles del patógeno, ni tampoco a la gran mayoría de los microorganismos presentes. En los últimos años se han impuesto las técnicas moleculares de la PCR, PCR anidada, PCR en tiempo real (qPCR), PCR digital (ddPCR) y las técnicas de secuenciación de nueva generación (NGS) como una alternativa a las técnicas de cultivo tradicional, ya que permiten obtener resultados en un menor

tiempo y son más específicas en la identificación y/o cuantificación de los patógenos. En este sentido, las NGS, y dentro de estas fundamentalmente la secuenciación masiva de amplicones (HTAS), han supuesto un avance en el conocimiento de las comunidades microbianas, y la ddPCR ha emergido como una técnica novedosa y más sensible que la qPCR, capaz de cuantificar de forma absoluta el organismo diana.

Una gestión exitosa de las EMV requiere estrategias de control que implican a todo el sistema de cultivo, desde el material de propagación hasta el viñedo adulto. La prohibición, a principios de la última década, de productos químicos como los benzimidazoles carbendazima y benomilo, altamente eficaces para el control de las EMV, junto con las altas restricciones a las que se enfrentan actualmente los fungicidas químicos en la mayoría de los países del mundo por sus efectos nocivos para la salud humana y el medio ambiente, hacen muy complejo el manejo de estas enfermedades.

Los tratamientos de termoterapia con agua caliente (TAC) de hasta 53°C durante 30 minutos del material de propagación, la utilización de organismos antagonistas, sobre todo hongos del género *Trichoderma* y la biofumigación de campos de vivero con mostaza blanca, reducen significativamente la presencia de los agentes patógenos causantes de las EMV sin que se vea afectada la viabilidad de la planta, aunque su eficacia no es total.

Para el control de las EMV en plantaciones adultas se suelen recomendar medidas de lucha de carácter general, como utilizar plantones de buena calidad, evitar el cultivo intensivo durante los primeros años tras la plantación, retirar o quemar los restos de poda y proteger las heridas de poda con productos autorizados. La retirada de restos de poda requiere un importante coste económico y la práctica de la quema es cada vez más restrictiva. El compostaje o la incorporación al suelo se han propuesto como alternativas eficaces en la eliminación de los hongos asociados a las EMV.

En relación con la protección de las heridas de poda, la utilización de agentes de control biológico (ACBs), aunque han mostrado resultados prometedores, éstos no han sido consistentes, observándose diferencias en la eficacia dependiendo, entre otros factores, del ACB y su cepa específica, del patógeno objetivo, del método de aplicación, del tiempo de exposición al ACB e incluso de los cultivares de vid y portainjertos objeto de estudio. Por otra parte, la mayoría de los estudios realizados hasta el momento se han llevado a cabo bajo condiciones controladas, por lo que se hace necesaria la realización de más ensayos en campo para conocer la efectividad real de estos productos.

La sostenibilidad ambiental de la producción vitícola también ha aumentado el interés por el uso de cultivares, clones y portainjertos más tolerantes a las enfermedades, siendo un elemento clave para la gestión integrada de las EMV, a las cuales son muy susceptibles los cultivares y portainjertos de mejor adaptación pedoclimática e interés comercial.

Justificación y objetivos

El manejo integrado de las EMV implica la implementación de diferentes medidas de control, encaminadas a minimizar el efecto de los hongos causantes de estas enfermedades. Las restricciones en la utilización de fungicidas químicos altamente efectivos determinan la búsqueda de estrategias alternativas más sostenibles para su control, como la utilización de ACBs o el uso de cultivares más tolerantes.

Sin embargo, la aplicación de nuevas estrategias requiere de la utilización de técnicas moleculares altamente específicas, precisas y sensibles para la detección y cuantificación de estos patógenos. Tecnologías de alto rendimiento basadas en el ADN, como la ddPCR o la HTAS, deben de ser implementadas para la cuantificación absoluta de estos patógenos y para desentrañar las interacciones entre estos y la vid.

Distintos aspectos relacionados con el patosistema de las EMV han sido evaluados en esta tesis con el objetivo de implementar nuevas técnicas moleculares para la mejora en la detección y gestión de estas, y de desarrollar alternativas sostenibles al uso de fungicidas que permitan aumentar la vida útil de los viñedos.

Los estudios realizados para alcanzar este objetivo general se engloban en cuatro grandes bloques: diagnóstico mediante técnicas moleculares, ecología de comunidades microbianas, interacción planta-patógeno y estrategias de control de las EMV.

Diagnóstico mediante técnicas moleculares

Recientemente, la ddPCR se ha convertido en la tercera generación de PCR haciendo posible la cuantificación absoluta de ácidos nucleicos sin la utilización de una curva estándar, proporcionando mayor precisión, mayor robustez en presencia de inhibidores de la PCR y permitiendo el diseño de ensayos multiplex sin comprometer la sensibilidad del sistema de cuantificación. Hasta el momento los protocolos para cuantificar patógenos en plantas mediante esta técnica aún son muy limitados.

Por tanto, en este capítulo se ha diseñado un protocolo de ddPCR para la detección y cuantificación de *Ilyonectria liriodendri*, uno de los principales agentes causales del pie negro de la vid, evaluando la precisión, la eficiencia y la especificidad de esta técnica frente a la qPCR.

Para ello, se evaluaron muestras de suelo, rizosfera (suelo que rodea a las raíces) y raíz (endorizosfera), procedentes de cinco viñedos jóvenes de La Rioja, con condiciones similares de suelo, clima y manejo del cultivo, y vides aparentemente sanas del cultivar 'Tempranillo' injertado sobre el portainjerto 110 Richter. Se seleccionaron cuatro plantas por viñedo y en cada planta se recogieron muestras al azar de cada compartimento suelo-planta. Los ensayos de ddPCR y qPCR se realizaron utilizando los cebadores YT2F y Cyl-R, y una sonda TaqMan. Se construyeron curvas estándar a partir de diluciones seriadas de 10.000 a 1 fg μl^{-1} con ADN de *I. liriodendri*, con la finalidad de determinar el límite de detección de cada una de las técnicas. Se cuantificó la concentración de ADN de hongos asociados al pie negro mediante la ddPCR y qPCR, y se evaluó la correlación entre ellas, así como, la existencia de diferencias significativas entre compartimentos.

Los resultados mostraron la validez de la técnica de la ddPCR como una herramienta para detectar y cuantificar hongos patógenos asociados a la enfermedad del pie negro en muestras de suelo y planta. A pesar de que se utilizó exclusivamente el genoma de *Ilyonectria liriodendri* para el cálculo del número de copias de cada muestra, lo que puede introducir un sesgo en las especies de hongos asociados a la enfermedad del pie negro, tanto la ddPCR como la qPCR mostraron ser técnicas eficientes para detectar y cuantificar ADN de formas asexuales similares a "*Cylindrocarpon*" asociadas a esta enfermedad. Ambos métodos mostraron una buena linealidad dentro del rango de cuantificación con un alto coeficiente de correlación de las curvas estándar (R^2 = 0,9917 y 0,9893), y eficiencias de 0,83 y 0,97 para la ddPCR y la qPCR, respectivamente. Además, los resultados de la cuantificación con ambas técnicas mostraron una alta correlación (R^2 = 0,95). La ddPCR resultó ser la técnica más sensible ante concentraciones del patógeno bajas, presentando un límite de detección de 5 fg μl^{-1} frente a los 10 fg μl^{-1} detectados mediante qPCR, lo que indica que esta técnica es más adecuada para la detección de especies fúngicas asociadas a las EMV en las primeras fases de infección.

La ausencia de diferencias significativas en la concentración de hongos asociados al pie negro entre el suelo, la rizosfera y la endorizosfera, obtenidas tanto por ddPCR como por qPCR, indican que los compartimentos suelo-planta no afectaron a la abundancia de estos hongos. Las raíces de vides asintomáticas son un nicho microbiano que está habitado por

patógenos asociados a la enfermedad del pie negro, lo que demuestra que estos hongos pueden actuar como endófitos y/o patógenos latentes de la vid.

La metodología desarrollada en esta tesis en base a la utilización de la técnica ddPCR para detectar y cuantificar hongos asociados al pie negro en suelo y tejido vegetal, puede ser adaptada para la evaluación de otros patógenos asociados con las EMV, constituyendo así un método de detección temprana, precisa, específica y sensible, que permitiría alertar a viveristas y viticultores de la presencia de estos patógenos en el suelo o en el material de propagación, evitando así su dispersión a través de la venta y la plantación de material vegetal contaminado.

Ecología de comunidades microbianas

Numerosos estudios han demostrado el importante efecto que tiene el microbioma presente en el suelo sobre el desarrollo vegetativo, la productividad y la sanidad de los cultivos. Se sabe que los organismos endófitos residentes en el suelo son transmitidos hacia la parte aérea de las plantas a través de la endorizosfera, por lo que la raíz actúa como el reservorio primario de los colonizadores microbianos de la vid. Las novedosas técnicas de secuenciación masiva han sido utilizadas fundamentalmente, en el caso de la vid, para valorar la diversidad del microbioma presente en los suelos de viñedos y su variación en función de las condiciones ambientales. Desentrañar la dinámica de las comunidades fúngicas entre el ambiente y la planta podrá permitir mejorar el conocimiento sobre la biología y la ecología de los hongos asociados a las EMV y, por tanto, el desarrollo de estrategias de manejo más efectivas.

Por ello, en esta tesis se ha estudiado la diversidad microbiana fúngica en dos escenarios diferentes utilizando la tecnología HTAS: en la interfaz suelo-planta (suelo, rizosfera y endorizosfera) y en el entorno aéreo-planta (heridas de poda de los sarmientos).

En primer lugar, a partir de las muestras de suelo, rizosfera y raíz de los cinco viñedos utilizadas en el ensayo precedente, se realizó una caracterización de la dinámica espacial de las comunidades fúngicas de estos tres compartimentos suelo-planta. Para ello, se amplificó la región fúngica ITS2 de las muestras utilizando los cebadores universales ITS3/KYO2 y ITS4. La genoteca se preparó de acuerdo con el protocolo de secuenciación metagenómica 16S de Illumina y las muestras se secuenciaron con el sistema MiSeq utilizando una química de lecturas pareadas de 2 × 300 nucleótidos. Las estimaciones de la diversidad alfa se calcularon mediante el análisis de los índices de Chao1, que estima el

número de especies en una comunidad basado en el número de especies menos frecuentes en la muestra, y de Shannon, que contempla la riqueza y abundancia de las especies. La relación en la composición de unidades taxonómicas operativas (OTUs) entre muestras se estableció mediante el cálculo de métricas de Bray Curtis y se visualizó mediante un gráfico de componentes principales (PCoA). El análisis discriminante lineal (LEfSe) se utilizó para identificar los taxones (nivel de género o superior) que diferían en la abundancia relativa entre los compartimentos. Se estudió también la función de las comunidades fúngicas en los tres compartimentos suelo-planta.

La evaluación de los cambios espaciales del microbioma mostró que tanto la diversidad como la riqueza de las comunidades de hongos (diversidad alfa), no difirió significativamente entre viñedos y el 41,4% de las OTUs fueron comunes a los cinco viñedos. Los filos fúngicos estaban compuestos principalmente por Ascomycota y Basiodiomycota, representando casi el 70% del total de hongos detectados. El complejo de hongos en el suelo fue muy homogéneo a nivel local y en viñedos con condiciones climáticas similares y variables edáficas homogéneas, lo que sugiere que el microbioma fúngico se conserva parcialmente en los viñedos de La Rioja. La interfaz suelo-planta constituyó la principal fuente de heterogeneidad de las comunidades fúngicas y la estructura de estas fue distinta entre compartimentos. En la endorizosfera, se observó una disminución de la riqueza y la diversidad de OTUs de aproximadamente la mitad en relación con la rizosfera y una comunidad fúngica distinta a la de los otros dos compartimentos.

Se detectaron 25 clados de hongos que permitieron discriminar las comunidades microbianas entre los compartimentos suelo-planta. Ningún filo permitió diferenciar las comunidades de hongos entre suelo y rizosfera. Hongos de los filos Zygomicota, Basidiomycota y Chtridiomycota contribuyeron a la diferenciación de las comunidades entre suelo y endorizosfera, siendo los géneros *Filobasidium*, *Pleospora* y *Psathyrella* los más implicados en la misma. Los filos Zygomicota y Chytridiomycota, así como, hongos no identificados del filo Ascomycota, contribuyeron a la diferenciación de la interfaz rizosfera y endorizosfera, siendo los géneros *Psathyrella*, *Malassezia* y *Filobasidium* los más implicados en esta.

Estos resultados indican que existe una selección del microbioma del suelo por parte de la planta y en la endorizosfera se encuentra finalmente un microbioma más especializado y taxonómicamente distinto, que ha sido capaz de adaptarse y de desarrollar mecanismos que le permitan la colonización de la raíz.

En nuestro estudio, la familia Nectriaceae, en la que se incluyen los principales hongos asociados a la enfermedad del pie negro, ha sido detectada en los compartimentos de la rizosfera (10,1%) y endorizosfera (30,7%), y concretamente el género *Ilyonectria* representó un papel importante en la discriminación entre ambas, lo que corrobora el papel selectivo de la raíz y la presencia endófita de estos patógenos en las raíces de vides asintomáticas.

El análisis predictivo de funcionalidad mostró un aumento en la abundancia relativa de posibles patógenos de plantas, endófitos y micorrizas arbusculares, y una disminución en la abundancia relativa de posibles saprótrofos de la madera y/o del estiércol, del suelo hacia la endorizosfera.

En segundo lugar, la HTAS también se empleó para determinar el efecto de la época de poda en la diversidad y composición del microbioma fúngico que coloniza las heridas de poda de forma natural, durante dos períodos de infección (noviembre-febrero y febrero-mayo), en viñedos de Galicia (España) durante dos años de ensayo. Los experimentos se llevaron a cabo en un total de 6 viñedos ubicados en tres Denominaciones de Origen (D.O. Valdeorras, D.O. Ribeiro y D.O. Rías Baixas; 2 viñedos por D.O.) distantes entre sí aproximadamente 10 km dentro de cada D.O. y con condiciones ambientales muy similares. De un total de 200 vides se seleccionaron y marcaron al azar 25 sarmientos en cada viñedo y año, y se realizaron podas en tres épocas distintas, mediados de otoño (noviembre), invierno (febrero) y primavera (mayo), y se reservaron fragmentos de 15 cm de la madera podada para la extracción de ADN. Todos los sarmientos estuvieron expuestos a la infección natural durante tres meses tras la realización de la poda. La identificación del microbioma fúngico presente en las muestras se realizó mediante la amplificación de la región ITS2 usando los cebadores ITS86F e ITS4. Las muestras se secuenciaron siguiendo la metodología descrita anteriormente y las estimaciones de la diversidad alfa se calcularon nuevamente mediante el análisis del índice de diversidad de Chao1 y el de riqueza de Shannon. Las diferencias en la diversidad alfa fúngica entre año, D.O., viñedo dentro de cada D.O. y época de poda, se infirieron mediante comparaciones de medias múltiples usando la prueba estadística de Tukey. El análisis LEfSe se usó para identificar los taxones fúngicos cuya abundancia relativa difería entre las épocas de poda. También se realizaron correlaciones entre las principales variables climáticas (humedad relativa media diaria, temperatura media diaria y lluvia acumulada) y el número de OTUs correspondiente al microbioma fúngico total y a los géneros de hongos más prevalentes asociados con las EMV.

El microbioma fúngico que coloniza las heridas de poda de la vid se vio afectado en su composición y diversidad por la D.O. El 56,8% de las OTUs se compartieron entre D.O. mientras que las OTUs específicas asociadas a cada viñedo variaron entre el 12,1 y el 18,4% de sus comunidades fúngicas totales. Los filos más abundantes en las tres D.O., fueron Ascomycota seguido de Basidiomycota.

La diversidad alfa de las comunidades fúngicas no fue significativamente diferente entre viñedos y años dentro de cada D.O., pero si lo fue entre periodos de infección, con una tendencia a una mayor diversidad de hongos hacia la época de primavera.

En relación con el microbioma fúngico total, se han identificado 10 géneros asociados a las EMV: *Botryosphaeria*, *Cadophora*, *Cryptovalsa*, *Cytospora*, *Diaporthe*, *Diplodia*, *Eutypa*, *Neofusicoccum*, *Phaeoacremonium* y *Phaeomoniella*. La diversidad alfa de las comunidades fúngicas asociadas a las EMV no difirió significativamente entre D.O.

Se detectaron abundancias significativamente más altas para la mayoría de los géneros de hongos asociados a las EMV después de la poda de febrero (invierno) que en la de noviembre (mediados de otoño). Este hecho sugiere un efecto estacional en las infecciones de las heridas de poda, con una tendencia a una mayor susceptibilidad a estos patógenos después de la poda de invierno.

Además, se observó una correlación positiva entre la lluvia acumulada y la abundancia del microbioma fúngico total y del género *Diaporthe* en particular, durante la octava y la undécima semana después de la poda.

Estudios epidemiológicos basados en la captura de esporas de hongos asociados con las EMV y el desarrollo de modelos para predecir los periodos de mayor riesgo de infección, constituyen investigaciones complementarias a la información aquí obtenida, que podrían determinar los efectos que la época de poda puede tener sobre la susceptibilidad de las heridas de poda a hongos asociados a las EMV en Galicia.

Interacción planta-patógeno

Estudios recientes han mostrado diferentes grados de susceptibilidad de cultivares, clones y portainjertos de vid ante infecciones causadas por hongos asociados a las EMV. La utilización de material vegetal tolerante a estas patologías se postula como una de las principales estrategias para su manejo, siendo una alternativa económicamente eficiente y a su vez ambientalmente respetuosa.

En la presente tesis, se ha evaluado la tolerancia de cultivares de vid comerciales y minoritarios presentes en colecciones de germoplasma españolas ante la infección por el hongo Ascomiceto *Phaeomoniella chlamydospora*, principal agente causal de la enfermedad de Petri y la yesca. Este patógeno, frecuente en viñedos tanto jóvenes como adultos, es endémico de todas las regiones vitícolas a nivel mundial causando importantes pérdidas económicas para la industria vitivinícola.

El ensayo se realizó bajo condiciones de invernadero, utilizando, para ello, estaquillas enraizadas de 1 año de 15 cultivares (9 tintos y 6 blancos) procedentes del banco de germoplasma del Instituto Tecnológico Agrario de Castilla y León (ITACyL), y 38 cultivares (20 tintos y 18 blancos) procedentes del banco de germoplasma de la Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA). Las inoculaciones se llevaron a cabo haciendo una herida entre los dos nudos superiores de las estaquillas e inmediatamente se insertaron discos de agar con micelio de colonias fúngicas en crecimiento activo. Las estaquillas control se inocularon con discos de agar estéril. Se utilizaron 12 plantas por cultivar y tratamiento dispuestas en un diseño completamente aleatorizado. Pasados 7 meses, se cosecharon y se evaluó la longitud de la necrosis vascular producida por el hongo mediante el software ImageJ. También se determinó el porcentaje de reaislamiento de *Pa. chlamydospora* teniendo en cuenta el número de plantas de las que se recuperó el patógeno frente al total de plantas inoculadas.

Todos los cultivares desarrollaron lesiones internas en la madera después de la inoculación artificial con *Pa. chlamydospora*, lo que indica que no hay evidencia de que exista resistencia cualitativa a este hongo. Sin embargo, la severidad de los síntomas internos de la madera varió considerablemente entre los distintos cultivares evaluados, lo que permitió clasificarlos en base a los diversos grados de tolerancia parcial a *Pa. chlamydospora*. Los cultivares 'Estaldiña', 'Albillo Mayor' y 'Castañal' se clasificaron como los más tolerantes en función de las longitudes medias de necrosis vascular de la madera.

Es importante por tanto fomentar el cultivo de este material genético tolerante como medida para preservar la longevidad de los viñedos. Las estrategias de futuro deben dirigirse a identificar material vegetal tolerante a las EMV mediante bioensayos bajo condiciones que aseguren el desarrollo de la enfermedad. En concreto, se debe investigar en bancos de germoplasma de variedades comerciales y minoritarias, clones y portainjertos de vid. Además, el estudio de los componentes físicos, químicos, morfológicos y genéticos que puedan explicar la tolerancia a la infección por hongos asociados a las EMV, se postula como un elemento clave en esta investigación.

Estrategias de control de las EMV

Evaluar estrategias de control alternativas al uso de fungicidas químicos ha sido otro de los retos de esta tesis doctoral. En los últimos 10 años, la investigación se ha centrado en la búsqueda de microorganismos antagonistas a los hongos asociados a las EMV, observándose resultados prometedores, si bien los estudios de efectividad se realizaron mayoritariamente en condiciones *in vitro*, de invernadero o de vivero.

En este sentido, en el presente trabajo se han planteado dos ensayos de campo para valorar la efectividad de ACBs para el control de las EMV.

Se ha evaluado el efecto de diversos ACBs, aplicados como tratamientos radiculares en pre- y post-plantación, para controlar las infecciones naturales causadas por hongos asociados con el pie negro y la enfermedad de Petri, así como, su influencia en los parámetros de crecimiento de la planta. Se utilizaron plantas de vid de 1 año del cultivar 'Tempranillo' injertado sobre el portainjerto 110 Richter. Las raíces de estas se recortaron a una longitud uniforme de 10 cm y las plantas se trataron por TAC a 53°C durante 30 minutos, para reducir cualquier infección existente por hongos asociados al pie negro y la enfermedad de Petri. Antes de la inoculación con los ACBs, las plantas se aclimataron durante 24 h a 20°C. Las inoculaciones se llevaron a cabo sumergiendo las raíces y la parte basal de las plantas durante 24 h, a temperatura ambiente, en suspensiones acuosas de los siguientes ACBs: *Streptomyces* sp. E1 + R4, *Trichoderma koningii* TK7 (Condor Shield®, ATENS), *T. atroviride* SC1 (Vintec®, Belchim Crop Protection), *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Stilo Cruzial®, SIPCAM Iberia) y *Pythium oligandrum* P037. Se estableció un control en el que las plantas se sumergieron solamente en agua. Una vez inoculadas, se plantaron inmediatamente (mes de mayo), en dos parcelas de La Rioja (España) distantes entre sí aproximadamente 1 km. Las plantas se dispusieron en un diseño experimental de cuatro bloques aleatorios, cada uno con 40 plantas por tratamiento (160 plantas por tratamiento). En febrero del año siguiente, el 50% de las plantas (plantas de 2 años) de cada campo se arrancaron para su análisis, manteniendo intacto el sistema radicular. En mayo, la mitad restante de las plantas que permanecieron en campo, se inocularon nuevamente a través del riego por goteo con todos los tratamientos de ACB (0,5 l por planta) y se arrancaron en febrero del siguiente año (plantas de 3 años). Se realizaron aislamientos fúngicos para identificar los patógenos asociados con el pie negro y la enfermedad de Petri presentes en dos partes diferentes de la planta: las raíces y la base del portainjerto. La identificación de los hongos que se asemejaban morfológicamente a

los patógenos asociados con ambas enfermedades se confirmó mediante técnicas moleculares. Se determinó la incidencia (DI) (porcentaje de plantas infectadas) y la severidad (DE) (porcentaje de fragmentos de madera positivos a la presencia del patógeno) de hongos asociados con el pie negro y la enfermedad de Petri, así como, el porcentaje de control de la enfermedad (PR). Para las plantas de 3 años se evaluó, además, el desarrollo vegetativo mediante el peso del brote y de la raíz. Los resultados mostraron que la efectividad del ACB para reducir la incidencia y la severidad de ambas enfermedades dependía de la edad y de la parte analizada de la planta. Bajo escenarios específicos, *Streptomyces* sp. E1 + R4 redujo significativamente las infecciones causadas por *Dactylonectria torresensis* y *D. macrodidyma* (pie negro), mientras que *Pythium oligandrum* P037 y *Trichoderma* spp. redujeron significativamente las infecciones causadas por *Pa. chlamydos*pora y *Phaeoacremonium minimum* (enfermedad de Petri). En cuanto a los parámetros de desarrollo vegetativo, los tratamientos con ACB redujeron significativamente el peso de la raíz con respecto al control, mientras que no se observó ningún efecto sobre el peso del brote.

La combinación de dos o más ACBs podría suponer una mayor eficacia en el control de estas enfermedades, además de actuar sobre un mayor espectro de patógenos. Las estrategias de futuro deben dirigirse a evaluar posibles efectos sinérgicos de mezclas de varios ACBs para controlar las EMV.

Finalmente, se evaluó la eficacia de diversas formulaciones fungicidas y de ACBs registradas actualmente en España, como protectores de las heridas de poda contra la infección causada por *Diplodia seriata*, uno de los principales agentes causales del decaimiento por Botryosphaeria, y *Pa. chlamydospora*. El estudio se llevó a cabo en dos viñedos adultos de 37 y 29 años, del cultivar 'Godello' injertado sobre el portainjerto 110 Richter, localizados en O Barco de Valdeorras (D.O. Valdeorras, Galicia, España). Los productos utilizados en el ensayo fueron: dos fungicidas, metil tiofanato (Enovit Metil®, Sipcam Inagra) y piraclostrobin + boscalida (Tessior®, BASF); una pasta mezclada con un fungicida, pasta + tebuconazol (Máster® + Song®, Sipcam jardín y Sipcam Ibérica); y dos productos formulados con ACBs, *T. atroviride* SC1 (Vintec®, Belchim Crop Protection) y *T. atroviride* I-1237 (Esquive®, Idai Nature). Los aislados de *D. seriata* y *Pa. chlamydospora* utilizados en las inoculaciones artificiales fueron seleccionados en ensayos previos por su alta virulencia en vid. Se estableció un diseño experimental en bloques al azar con un total de 150 plantas por viñedo y hongo. En el mes de febrero, coincidiendo con la época habitual de poda de la zona, se podaron, por cepa, tres sarmientos del año a tres yemas.

Inmediatamente tras la poda, se aplicaron los tratamientos sobre las heridas siguiendo las dosis de aplicación recomendadas por las casas comerciales y transcurridas 24 horas se realizaron las inoculaciones artificiales con suspensiones de esporas a razón de 400 y 800 esporas para *D. seriata* y *Pa. chlamydospora,* respectivamente. Además, se estableció un control positivo (inoculación sin tratamiento protector) y un control negativo (infección natural, sin tratamiento ni inoculación), con 30 plantas cada uno, por viñedo y hongo. Al cabo de 12 meses, se cortaron fragmentos de unos 10 cm de los sarmientos tratados, y se procedió al reaislamiento e identificación de los hongos inoculados artificialmente. Se calculó la eficacia de cada tratamiento como el porcentaje de reaislamiento promedio (MPR), así como, el porcentaje medio de control de la enfermedad (MPDC) con respecto al control positivo.

Los resultados mostraron que los tratamientos basados en ACBs fueron menos efectivos que los tratamientos fungicidas. La aplicación de piraclostrobin + boscalida y de la pasta + tebuconazol redujo significativamente el porcentaje de reaislamiento de *Pa. chlamydospora*, con un MPDC del 51 y 67 %, respectivamente. La aplicación de metil tiofanato redujo la enfermedad en un 46%, a pesar de que no mostró diferencias significativas con el control positivo en relación al porcentaje de reaislamiento del hongo ($P > 0,05$). En el caso de la infección causada por *D. seriata*, la aplicación de piraclostrobin + boscalida, metil tiofanato y de la pasta + tebuconazol redujo significativamente el porcentaje de reaislamiento del hongo en las heridas de poda en ambas campañas ($P < 0,05$), con un porcentaje de control que en la primera campaña varió entre el 90-95% para piraclostrobin + boscalida y metil tiofanato, y del 76% para la pasta + tebuconazol, y en la segunda campaña varió entre el 54-69% para piraclostrobin + boscalida y metil tiofanato, y del 44% para la pasta + tebuconazol.

En nuestro estudio, los dos productos evaluados de ACBs han mostrado un MPDC bajo con valores inferiores al 32%. *T. atroviride* SC1 no ha mostrado efecto protector en las heridas de poda frente a *Pa. chlamydospora* y *T. atroviride* I-1237, si bien presentó un MPDC del 17%, el MPR del hongo no mostró diferencias significativas frente al control positivo.

El bajo rendimiento e implantación de los tratamientos basados en *Trichoderma* spp. es discutido más a fondo, valorando entre otros factores, la elevada carga de esporas utilizada en las inoculaciones artificiales de los hongos asociados con las EMV, el corto periodo de tiempo transcurrido entre la aplicación del ACB y la inoculación artificial de

los mismos, el efecto de las condiciones ambientales adversas para la implantación de *Trichoderma* o la posibilidad de una baja afinidad de estos hongos por el cultivar 'Godello'.

Appendix **B**

Resumo extenso

Introdución

As enfermidades da madeira da vide (EMV) encóntranse entre as principais ameazas bióticas para a sustentabilidade económica da industria vitivinícola mundial, reducindo o rendemento, produtividade e lonxevidade dos viñedos. O complexo das EMV inclúe principalmente seis patoloxías diferentes que afectan ao material de propagación de vide en viveiros e a viñedos novos e adultos en todo o mundo, que son o pé negro da vide, a enfermidade de Petri, a iesca, a eutipiose e os decaementos por Botryosphaeria e por Diaporthe. Varios factores foron atribuídos ao aumento da súa incidencia nos últimos anos, como son os cambios nas prácticas culturais dos viñedos, a baixa calidade sanitaria do material de propagación de vide, a prohibición das materias activas químicas máis efectivas e a deficiente protección das feridas de poda. Estas enfermidades posúen unha complexidade engadida, xa que polo menos 136 especies fúnxicas non relacionadas taxonómicamente pertencentes a 35 xéneros distintos están asociadas ás distintas patoloxías en todo o mundo, o que fai que o seu control sexa moi difícil. A bioloxía e epidemioloxía destes patóxenos é tamén moi complexa, xa que algúns fungos caracterízanse por ser habitantes comúns do solo (pé negro), outros dispérsanse principalmente mediante esporas a través do vento, da auga de chuvia e dos artrópodos (enfermidade de Petri, iesca, eutipiose e decaemento por Botryosphaeria e por Diaporthe) e algúns teñen a capacidade de dispersarse mediante esporas e ademais, de chegar a ser habitantes do solo, a través de restos de poda enfermos (enfermidade de Petri). Así mesmo, algúns destes fungos poden dispersarse a través do material de propagación infectado no viveiro (decaemento por Botryosphaeria e por Diaporthe, pé negro, enfermidade de Petri e iesca).

Tradicionalmente, a detección e identificación dos fungos asociados a estas patoloxías realizouse mediante avaliacións morfolóxicas dependentes do cultivo tradicional en placa. Estas técnicas, aínda que son fiables para unha identificación e clasificación preliminar, non son capaces de detectar baixos niveis do patóxeno, nin tampouco á gran maioría dos microorganismos presentes. Nos últimos anos impuxéronse as técnicas moleculares da PCR, PCR aniñada, PCR en tempo real (qPCR), PCR dixital (ddPCR) e as técnicas de secuenciación de nova xeración (NGS) como unha alternativa ás técnicas de cultivo tradicional, xa que permiten obter resultados nun menor tempo e son máis específicas na

identificación e/ou cuantificación dos patóxenos. Neste sentido as NGS, e dentro destas fundamentalmente a secuenciación masiva de amplicóns (HTAS), supuxeron un avance no coñecemento das comunidades microbianas, e a ddPCR emerxeu como unha técnica novidosa e máis sensible que a qPCR, capaz de cuantificar de forma absoluta o organismo diana.

Unha xestión exitosa das EMV require estratexias de control que implican todo o sistema de cultivo dende o material de propagación ata o viñedo adulto. A prohibición, a principios da última década, de produtos químicos como os benzimidazoles carbendazima e benomilo, altamente eficaces para o control das EMV xunto coas altas restricións ás que se enfrontan actualmente os funxicidas químicos na maioría dos países do mundo, polos seus efectos nocivos para a saúde humana e o medio ambiente, fan moi complexo o manexo destas enfermidades.

Os tratamentos de termoterapia con auga quente (TAC) de ata 53°C durante 30 minutos do material de propagación, o emprego de organismos antagonistas, sobre todo fungos do xénero *Trichoderma*, e a biofumigación de campos de viveiro con mostaza branca, reducen significativamente a presenza dos axentes patóxenos causantes das EMV sen que se vexa afectada a viabilidade da planta, aínda que a súa eficacia non é total.

Para o control das EMV en plantacións adultas adóitanse recomendar medidas de loita de carácter xeral, coma empregar plantóns de boa calidade, evitar o cultivo intensivo durante os primeiros anos tras a plantación, retirar ou queimar os restos de poda e protexer as feridas de poda con produtos autorizados. A retirada de restos de poda require un importante custo económico e a práctica da queima é cada vez máis restritiva. A compostaxe ou a incorporación ao solo propuxéronse como alternativas eficaces na eliminación dos fungos asociados ás EMV.

En relación coa protección das feridas de poda, o emprego de axentes de control biolóxico (ACBs), aínda que amosaron resultados prometedores, estes non foron consistentes, observándose diferenzas na eficacia dependendo, entre outros factores, do ACB e a súa cepa específica, do patóxeno obxectivo, do método de aplicación, do tempo de exposición ao ACB e incluso dos cultivares de vide e portaenxertos obxecto de estudo. Por outra banda, a maioría dos estudos realizados ata o momento leváronse a cabo baixo condicións controladas, polo que se fai necesaria a realización de máis ensaios en campo para coñecer a efectividade real destes produtos.

A sustentabilidade ambiental da produción vitícola tamén aumentou o interese polo emprego de cultivares, clons e portaenxertos máis tolerantes ás enfermidades, sendo un elemento chave para a xestión integrada das EMV, ás cales son moi susceptibles aos cultivares e portaenxertos de mellor adaptación pedoclimática e interese comercial.

Xustificación e obxectivos

O manexo integrado das EMV implica a implementación de diferentes medidas de control, encamiñadas a minimizar o efecto dos fungos causantes destas enfermidades. As restricións no emprego de funxicidas químicos altamente efectivos determinan a procura de estratexias alternativas máis sostibles para o seu control, como o emprego de ACBs ou o uso de cultivares máis tolerantes.

Sen embargo, a aplicación de novas estratexias require do emprego de técnicas moleculares altamente específicas, precisas e sensibles para a detección e cuantificación destes patóxenos. Tecnoloxías de alto rendemento baseadas no ADN, como a ddPCR ou a HTAS, deben de ser implementadas para a cuantificación absoluta destes patóxenos e para desentrañar as interaccións entre estes e a vide.

Distintos aspectos relacionados co patosistema das EMV foron avaliados nesta tese co obxectivo de implementar novas técnicas moleculares para a mellora na detección e xestión destas, e de desenvolver alternativas sostibles ao uso de funxicidas que permitan aumentar a vida útil dos viñedos.

Os estudos realizados para alcanzar este obxectivo xeral englóbanse en catro grandes bloques: diagnóstico mediante técnicas moleculares, ecoloxía de comunidades microbianas, interacción planta-patóxeno e estratexias de control das EMV.

Diagnóstico mediante técnicas moleculares

Recentemente, a ddPCR converteuse na terceira xeración de PCR facendo posible a cuantificación absoluta de ácidos nucleicos sen o emprego dunha curva estándar, proporcionando maior precisión, maior robustez en presencia de inhibidores da PCR e permitindo o deseño de ensaios múltiplex sen comprometer a sensibilidade do sistema de cuantificación. Polo momento os protocolos para cuantificar patóxenos en plantas mediante esta técnica aínda son moi limitados.

Polo tanto, neste capítulo deseñouse un protocolo de ddPCR para a detección e cuantificación de *Ilyonectria liriodendri*, un dos principais axentes causais do pé negro da vide, avaliando a precisión, a eficiencia e a especificidade desta técnica fronte á qPCR.

Para elo, avaliáronse mostras de solo, rizosfera (solo que rodea ás raíces) e raíz (endorizosfera), procedentes de cinco viñedos novos de La Rioja, con condicións similares de solo, clima e manexo do cultivo, e vides aparentemente sans do cultivar 'Tempranillo' enxertado sobre o portaenxertos 110 Richter. Seleccionáronse catro plantas por viñedo e en cada planta recolléronse mostras ao azar de cada compartimento solo-planta. Os ensaios de ddPCR e qPCR realizáronse empregando os cebadores YT2F e Cyl-R, e unha sonda TaqMan. Construíronse curvas estándar a partir de dilucións seriadas de 10.000 a 1 fg μl^{-1} con ADN de *I. liriodendri*, coa finalidade de determinar o límite de detección de cada unha das técnicas. Cuantificouse a concentración de ADN de fungos asociados ao pé negro mediante a ddPCR e qPCR e avaliouse a correlación entre elas, así como a existencia de diferenzas significativas entre compartimentos.

Os resultados mostraron a validez da técnica da ddPCR como unha ferramenta para detectar e cuantificar fungos patóxenos asociados á enfermidade do pé negro en mostras de solo e planta. A pesar de que se empregou exclusivamente o xenoma de *Ilyonectria liriodendri* para o cálculo do número de copias de cada mostra, o que pode introducir un sesgo nas especies de fungos asociados á enfermidade do pé negro, tanto a ddPCR como a qPCR mostraron ser técnicas eficientes para detectar e cuantificar ADN de formas asexuais similares a "*Cylindrocarpon*" asociadas a esta enfermidade. Ambos os métodos mostraron unha boa linealidade dentro do rango de cuantificación cun alto coeficiente de correlación das curvas estándar (R^2 = 0,9917 e 0,9893), e eficiencias de 0,83 e 0,97 para ddPCR e qPCR, respectivamente. Ademais, os resultados da cuantificación con ambas as técnicas mostraron unha alta correlación (R^2 = 0,95). A ddPCR resultou ser a técnica máis sensible ante concentracións de patóxeno baixas, presentando un límite de detección de 5 fg μl^{-1} fronte aos 10 fg μl^{-1} detectados mediante qPCR, o que indica que esta técnica é máis adecuada para a detección de especies fúnxicas asociadas ás EMV nas primeiras fases de infección.

A ausencia de diferenzas significativas na concentración de fungos asociados ao pé negro entre o solo, a rizosfera e a endorizosfera, obtidas tanto por ddPCR como por qPCR, indican que os compartimentos solo-planta non afectaron á abundancia destes fungos. As raíces de vides asintomáticas son un nicho microbiano que está habitado por patóxenos

asociados á enfermidade do pé negro, o que demostra que estes fungos poden actuar como endófitos e/ou patóxenos latentes da vide.

A metodoloxía desenvolvida nesta tese en base ao emprego da técnica ddPCR para detectar e cuantificar fungos asociados ao pé negro no solo e tecido vexetal, pode ser adaptada para a avaliación de outros patóxenos asociados coas EMV, constituíndo así un método de detección temperá, precisa, específica e sensible, que permitiría alertar aos viveiristas e viticultores da presenza destes patóxenos no solo ou no material de propagación, evitando así a súa dispersión a través da venta e da plantación de material vexetal contaminado.

Ecoloxía de comunidades microbianas

Numerosos estudos demostraron o importante efecto que ten o microbioma presente no solo sobre o desenvolvemento vexetativo, a produtividade e a sanidade dos cultivos. Sábese que os organismos endófitos residentes no solo son transmitidos cara a parte aérea das plantas a través da endorizosfera, polo que a raíz actúa coma o reservorio primario dos colonizadores microbianos da vide. As novidosas técnicas de secuenciación masiva foron empregadas fundamentalmente, no caso da vide, para valorar a diversidade do microbioma presente nos solos de viñedos e a súa variación en función das condicións ambientais. Desentrañar a dinámica das comunidades fúnxicas entre o ambiente e a planta poderá permitir mellorar o coñecemento sobre a bioloxía e a ecoloxía dos fungos asociados ás EMV e, polo tanto, o desenvolvemento de estratexias de manexo máis efectivas.

Polo tanto, nesta tese estudouse a diversidade microbiana fúnxica en dous escenarios diferentes empregando a tecnoloxía HTAS: na interface solo-planta (solo, rizosfera y endorizosfera) e no entorno aéreo-planta (feridas de poda das varas).

En primeiro lugar, a partir das mostras de solo, rizosfera e raíz dos cinco viñedos empregadas no ensaio precedente, realizouse unha caracterización da dinámica espacial das comunidades fúnxicas destes tres compartimentos solo-planta. Para iso, amplificouse a rexión fúnxica ITS2 das mostras empregando os cebadores universais ITS3/KYO2 e ITS4. A xenoteca preparouse de acordo co protocolo de secuenciación metaxenómica 16S de Illumina, e as mostras secuenciáronse co sistema MiSeq empregando unha química de lecturas pares de 2 × 300 nucleótidos. As estimacións da diversidade alfa calculáronse mediante a análise dos índices de Chao1, que estima o número de especies nunha comunidade baseado no número de especies menos frecuentes na mostra, e de Shannon,

que contempla a riqueza e abundancia das especies. A relación na composición de unidades taxonómicas operativas (OTUs) entre mostras estableceuse mediante o cálculo de métricas de Bray Curtis e visualizouse mediante un gráfico de compoñentes principais (PCoA). A análise discriminante lineal (LEfSe) empregouse para identificar os taxons (nivel de xénero ou superior) que diferían na abundancia relativa entre os compartimentos. Estudouse tamén a función das comunidades fúnxicas nos tres compartimentos solo-planta.

A avaliación dos cambios espaciais do microbioma mostrou que tanto a diversidade como a riqueza das comunidades de fungos (diversidade alfa) non diferiu significativamente entre viñedos e o 41,4% das OTUs foron comúns aos cinco viñedos. Os filos fúnxicos estaban compostos principalmente por Ascomycota e Basiodiomycota, representando case o 70% do total de fungos detectados. O complexo de fungos no solo foi moi homoxéneo a nivel local e en viñedos con condicións climáticas similares e variables edáficas homoxéneas, o que suxire que o microbioma fúnxico consérvase parcialmente nos viñedos de La Rioja. A interface solo-planta constituíu a principal fonte de heteroxeneidade das comunidades fúnxicas e a estrutura destas foi distinta entre compartimentos. Na endorizosfera, observouse unha diminución da riqueza e a diversidade de OTUs de aproximadamente a metade en relación coa rizosfera e unha comunidade fúnxica distinta á dos outros dous compartimentos.

Detectáronse 25 clados de fungos que permitiron discriminar as comunidades microbianas entre os compartimentos solo-planta. Ningún filo permitiu diferenciar as comunidades de fungos entre solo e rizosfera. Fungos dos filos Zygomicota, Basidiomycota e Chtridiomycota contribuíron á diferenciación das comunidades entre o solo e endorizosfera, sendo os xéneros *Filobasidium*, *Pleospora* e *Psathyrella* os máis implicados na mesma. Os filos Zygomicota e Chytridiomycota, así como, fungos non identificados do filo Ascomycota, contribuíron á diferenciación da interface rizosfera e endorizosfera, sendo os xéneros *Psathyrella*, *Malassezia* e *Filobasidium* os máis implicados nesta.

Estes resultados indican que existe unha selección do microbioma do solo por parte da planta, e na endorizosfera atópase finalmente un microbioma máis especializado e taxonómicamente distinto, que foi capaz de adaptarse e de desenvolver mecanismos que lle permitan a colonización da raíz.

No noso estudo, a familia Nectriaceae, na que se inclúen os principais fungos asociados á enfermidade do pé negro, foi detectada nos compartimentos da rizosfera (10,1%) e

endorizosfera (30,7%), e concretamente o xénero *Ilyonectria* representou un papel importante na discriminación entre ambas, o que corrobora o papel selectivo da raíz e a presencia endófita destes patóxenos nas raíces de vides asintomáticas.

A análise preditiva de funcionalidade mostrou un aumento na abundancia relativa de posibles patóxenos de plantas, endófitos e micorrizas arbusculares, e unha diminución na abundancia relativa de posibles saprótrofos da madeira e/ou do esterco, do solo cara a endorizosfera.

En segundo lugar, a HTAS tamén se empregou para determinar o efecto da época de poda na diversidade e composición do microbioma fúnxico que coloniza as feridas de poda de forma natural, durante dous períodos de infección (novembro-febreiro e febreiro-maio), en viñedos de Galicia (España) durante dous anos de ensaio. Os experimentos leváronse a cabo nun total de 6 viñedos situados en tres Denominacións de Orixe (D.O. Valdeorras, D.O. Ribeiro e D.O. Rías Baixas; 2 viñedos por D.O.) distantes entre si aproximadamente 10 km dentro de cada D.O. e con condicións ambientais moi similares. Dun total de 200 vides seleccionáronse e marcáronse ao azar 25 varas en cada viñedo e ano, e realizáronse podas en tres épocas distintas, mediados de outono (novembro), inverno (febreiro) e primavera (maio), e reserváronse fragmentos de 15 cm da madeira podada para a extracción do ADN. Todos as varas estiveron expostas á infección natural durante tres meses tras a realización da poda. A identificación do microbioma fúnxico presente nas mostras realizouse mediante a amplificación da rexión ITS2 usando os cebadores ITS86F e ITS4. As mostras secuenciáronse seguindo a metodoloxía descrita anteriormente e as estimacións da diversidade alfa calculáronse novamente mediante a análise do índice de diversidade de Chao1 e o de riqueza de Shannon. As diferenzas na diversidade alfa fúnxica entre ano, D.O., viñedo dentro de cada D.O. e época de poda, inferíronse mediante comparacións de medias múltiples usando a proba estatística de Tukey. A análise LEfSe empregouse para identificar os taxons fúnxicos cuxa abundancia relativa difería entre as épocas de poda. Tamén se realizaron correlacións entre as principais variables climáticas (humidade relativa media diaria, temperatura media diaria e chuvia acumulada) e o número de OTUs correspondente ao microbioma fúnxico total e aos xéneros de fungos máis prevalecentes asociados coas EMV.

O microbioma fúnxico que coloniza as feridas de poda da vide viuse afectado na súa composición e diversidade pola D.O. O 56,8% das OTUs compartíronse entre D.O. mentres que as OTUs específicas asociadas a cada viñedo variaron entre o 12,1 e o 18,4%

das súas comunidades fúnxicas totais. Os filos máis abundantes nas tres D.O., foron Ascomycota seguido de Basidiomycota.

A diversidade alfa das comunidades fúnxicas non foi significativamente diferente entre viñedos e anos dentro de cada D.O., pero si o foi entre períodos de infección, cunha tendencia a unha maior diversidade de fungos cara a época de primavera.

En relación co microbioma fúnxico total, identificáronse 10 xéneros asociados ás EMV: *Botryosphaeria*, *Cadophora*, *Cryptovalsa*, *Cytospora*, *Diaporthe*, *Diplodia*, *Eutypa*, *Neofusicoccum*, *Phaeoacremonium* e *Phaeomoniella*. A diversidade alfa das comunidades fúnxicas asociadas ás EMV non diferiu significativamente entre D.O.

Detectáronse abundancias significativamente máis altas para a maioría dos xéneros de fungos asociados ás EMV despois da poda de febreiro (inverno) que na de novembro (mediados de outono). Este feito suxire un efecto estacional nas infeccións das feridas de poda, cunha tendencia a unha maior susceptibilidade a estes patóxenos despois da poda de inverno.

Ademais, observouse unha correlación positiva entre a chuvia acumulada e a abundancia do microbioma fúnxico total e do xénero *Diaporthe* en particular, durante a oitava e a undécima semana despois da poda.

Estudos epidemiolóxicos baseados na captura de esporas de fungos asociados coas EMV e o desenvolvemento de modelos para predicir os períodos de maior risco de infección, constitúen investigacións complementarias á información aquí obtida, que poderían determinar os efectos que a época de poda pode ter sobre a susceptibilidade das feridas de poda a fungos asociados ás EMV en Galicia.

Interacción planta-patóxeno

Estudos recentes mostraron diferentes grados de susceptibilidade de cultivares, clons e portaenxertos de vide ante infeccións causadas por fungos asociados ás EMV. O emprego de material vexetal tolerante a estas patoloxías postúlase como unha das principais estratexias para o seu manexo, sendo unha alternativa economicamente eficiente e á súa vez ambientalmente respectuosa.

Na presente tese, avaliouse a tolerancia de cultivares de vide comerciais e minoritarios presentes en coleccións de xermoplasma españolas ante a infección polo fungo Ascomiceto *Phaeomoniella chlamydospora*, principal axente causal da enfermidade de

Petri e a iesca. Este patóxeno, frecuente en viñedos tanto novos como adultos, é endémico de todas as rexións vitícolas a nivel mundial causando importantes perdas económicas para a industria vitivinícola.

O ensaio realizouse baixo condicións de invernadoiro, empregando, para elo estacas enraizadas de 1 ano de 15 cultivares (9 tintos e 6 brancos) procedentes do banco de xermoplasma do Instituto Tecnológico Agrario de Castilla y León (ITACyL), e 38 cultivares (20 tintos e 18 brancos) procedentes do banco de xermoplasma da Estación de Viticultura e Enoloxía de Galicia (AGACAL-EVEGA). As inoculacións leváronse a cabo facendo unha ferida entre os dous nós superiores das estacas e inmediatamente inseríronse discos de agar con micelio de colonias fúnxicas en crecemento activo. As estacas control inoculáronse con discos de agar estéril. Empregáronse 12 plantas por cultivar e tratamento dispostas nun deseño completamente aleatorizado. Pasados 7 meses, colleitáronse e avaliouse a lonxitude da necrose vascular producida polo fungo mediante o software ImageJ. Tamén se determinou a porcentaxe de reillamento de *Pa. chlamydospora* tendo en conta o número de plantas das que se recuperou o patóxeno fronte ao total de plantas inoculadas.

Todos os cultivares desenvolveron lesións internas na madeira despois da inoculación artificial con *Pa. chlamydospora*, o que indica que non hai evidencia de que exista resistencia cualitativa a este fungo. Sen embargo, a severidade dos síntomas internos da madeira variou considerablemente entre os distintos cultivares avaliados, o que permitiu clasificalos en base aos diversos grados de tolerancia parcial a *Pa. chlamydospora*. Os cultivares 'Estaldiña', 'Albillo Mayor' e 'Castañal' clasificáronse coma os máis tolerantes en función das lonxitudes medias de necrose vascular da madeira.

É importante polo tanto fomentar o cultivo deste material xenético tolerante como medida para preservar a lonxevidade dos viñedos. As estratexias de futuro deben dirixirse a identificar material vexetal tolerante ás EMV mediante bioensaios baixo condicións que aseguren o desenvolvemento da enfermidade. En concreto, débese investigar en bancos de xermoplasma de cultivares comerciais e minoritarias, clons e portaenxertos de vide. Ademais, o estudo dos compoñentes físicos, químicos, morfolóxicos e xenéticos que poidan explicar a tolerancia á infección por fungos asociados ás EMV, postúlase como un elemento chave nesta investigación.

Estratexias de control das EMV

A avaliación de estratexias de control alternativas ao uso de funxicidas químicos foi outro dos retos desta tese doutoral. Nos últimos 10 anos, a investigación centrouse na procura de microorganismos antagonistas aos fungos asociados ás EMV, observándose resultados prometedores, se ben os estudos de efectividade realizáronse maioritariamente en condicións *in vitro*, de invernadoiro ou de viveiro.

Neste sentido, no presente traballo desenvolvéronse dous ensaios de campo para valorar a efectividade de ACBs para o control das EMV.

Avaliouse o efecto de diversos ACBs, aplicados como tratamentos radiculares en pre- e post-plantación, para controlar as infeccións naturais causadas por fungos asociados co pé negro e a enfermidade de Petri, así como, a súa influencia nos parámetros de crecemento da planta. Empregáronse plantas de vide de 1 ano do cultivar 'Tempranillo' enxertado sobre o portaenxertos 110 Richter. As raíces destas recortáronse a unha lonxitude uniforme de 10 cm e as plantas trataronse por TAC a 53°C durante 30 minutos, para reducir calquera infección existente por fungos asociados ao pé negro e á enfermidade de Petri. Antes da inoculación cos ACBs, as plantas aclimatáronse durante 24 h a 20°C. As inoculacións leváronse a cabo mergullando as raíces e a parte basal das plantas durante 24 h, a temperatura ambiente, en suspensións acuosas dos seguintes ACBs: *Streptomyces* sp. E1 + R4, *Trichoderma koningii* TK7 (Condor Shield®, ATENS), *T. atroviride* SC1 (Vintec®, Belchim Crop Protection), *Pseudomonas fluorescens* + *Bacillus atrophaeus* (Stilo Cruzial®, SIPCAM Iberia) e *Pythium oligandrum* P037. Estableceuse un control no que as plantas se mergullaron soamente en auga. Unha vez inoculadas, plantáronse inmediatamente (mes de maio), en dúas parcelas de La Rioja (España) distantes entre si aproximadamente 1 km. As plantas dispuxéronse nun deseño experimental de catro bloques aleatorios, cada un con 40 plantas por tratamento (160 plantas por tratamento). En febreiro do ano seguinte, o 50% das plantas (plantas de 2 anos) de cada campo arrincáronse para a súa análise, mantendo intacto o sistema radicular. En maio, a metade restante das plantas que permaneceron no campo, inoculáronse novamente a través da rega por goteo con todos os tratamentos de ACB (0,5 l por planta) e arrincáronse en febreiro do seguinte ano (plantas de 3 anos). Realizáronse illamentos fúnxicos para identificar os patóxenos asociados co pé negro e a enfermidade de Petri presentes en dúas partes diferentes da planta: as raíces e a base do portaenxertos. A identificación dos fungos que se asemellaban morfoloxicamente aos patóxenos asociados con ambas enfermidades confirmouse

mediante técnicas moleculares. Determinouse a incidencia (DI) (porcentaxe de plantas infectadas) e a severidade (DE) (porcentaxe de fragmentos de madeira positivos á presenza do patóxeno) de fungos asociados co pé negro e a enfermidade de Petri, así como, a porcentaxe de control da enfermidade (PR). Para as plantas de 3 anos avaliouse, ademais, o desenvolvemento vexetativo mediante o peso do brote e da raíz. Os resultados amosaron que a efectividade do ACB para reducir a incidencia e a severidade de ambas enfermidades dependía da idade e da parte analizada da planta. Baixo escenarios específicos, *Streptomyces* sp. E1 + R4 reduciu significativamente as infeccións causadas por *Dactylonectria torresensis* e *D. macrodidyma* (pé negro), mentres que *Pythium oligandrum* P037 e *Trichoderma* spp. reduciron significativamente as infeccións causadas por *Pa. chlamydos*pora e *Phaeoacremonium minimum* (enfermidade de Petri). En canto aos parámetros de desenvolvemento vexetativo, os tratamentos con ACB reduciron significativamente o peso da raíz con respecto ao control, mentres que non se observou ningún efecto sobre o peso do brote.

A combinación de dous ou máis ACBs podería supoñer unha maior eficacia no control destas enfermidades, ademais de actuar sobre un maior espectro de patóxenos. As estratexias de futuro deben dirixirse a avaliar posibles efectos sinérxicos de mesturas de varios ACBs para controlar as EMV.

Finalmente, avaliouse a eficacia de diversas formulacións funxicidas e de ACB rexistradas actualmente en España, como protectores das feridas de poda contra a infección causada por *Diplodia seriata*, un dos principais axentes causais do decaemento por Botryosphaeria, e *Pa. chlamydospora*. O estudo levouse a cabo en dous viñedos adultos de 37 e 29 anos, do cultivar 'Godello' enxertado sobre o portaenxertos 110 Richter, localizados en O Barco de Valdeorras (D.O. Valdeorras, Galicia, España). Os produtos empregados no ensaio foron: dous funxicidas, metil tiofanato (Enovit Metil®, Sipcam Inagra) e piraclostrobin + boscalida (Tessior®, BASF); unha pasta mesturada cun fungicida, pasta + tebuconazol (Máster® + Song®, Sipcam jardín y Sipcam Ibérica); e dous produtos formulados con ACBs, *T. atroviride* SC1 (Vintec®, Belchim Crop Protection) e *T. atroviride* I-1237 (Esquive®, Idai Nature). Os illados de *D. seriata* e *Pa. chlamydospora* empregados nas inoculacións artificiais, foron seleccionados en ensaios previos pola súa alta virulencia en vide. Estableceuse un deseño experimental en bloques ao azar cun total de 150 plantas por viñedo e fungo. No mes de febreiro, coincidindo coa época habitual de poda da zona, podáronse, por cepa, tres varas do ano a tres xemas. Inmediatamente tras a poda, aplicáronse os tratamentos sobre as feridas seguindo as doses de aplicación recomendadas

polas casas comerciais, e transcorridas 24 horas realizáronse as inoculacións artificiais con suspensións de esporas a razón de 400 e 800 esporas para *D. seriata* e *Pa. chlamydospora,* respectivamente. Ademais, estableceuse un control positivo (inoculación sen tratamento protector) e un negativo (infección natural, sen tratamento nin inoculación), con 30 plantas cada un, por viñedo e fungo. Ao cabo de 12 meses, cortáronse fragmentos duns 10 cm das varas tratadas, e procedeuse ao reillamento e identificación dos fungos inoculados artificialmente. Calculouse a eficacia de cada tratamento como a porcentaxe de reillamento media (MPR), así como a porcentaxe media de control da enfermidade (MPDC) con respecto ao control positivo.

Os resultados amosaron que os tratamentos baseados en ACBs foron menos efectivos que os tratamentos funxicidas. A aplicación de piraclostrobin + boscalida e da pasta + tebuconazol reduciu significativamente a porcentaxe de reillamento de *Pa. chlamydospora*, con MPDC do 51 e 67 %, respectivamente. A aplicación de metil tiofanato reduciu a enfermidade nun 46%, a pesar de que non amosou diferenzas significativas co control positivo en relación á porcentaxe de reillamento do fungo ($P > 0,05$). No caso da infección causada por *D. seriata*, a aplicación de piraclostrobin + boscalida, metil tiofanato e da pasta + tebuconazol reduciu significativamente a porcentaxe de reillamento do fungo nas feridas de poda en ambas campañas ($P < 0,05$), cun porcentaxe de control que na primeira campaña variou entre o 90-95% para piraclostrobin + boscalida e metil tiofanato, e do 76% para a pasta + tebuconazol, e na segunda campaña variou entre o 54-69% para piraclostrobin + boscalida e metil tiofanato, e do 44% para a pasta + tebuconazol.

No noso estudo, os dous produtos avaliados de ACBs amosaron un MPDC baixo con valores inferiores ao 32%. *T. atroviride* SC1 non amosou efecto protector nas feridas de poda fronte a *Pa. chlamydospora,* e *T. atroviride* I-1237, se ben presentou un MPDC do 17%, o MPR do fungo non amosou diferenzas significativas fronte ao control positivo.

O baixo rendemento e implantación dos tratamentos baseados en *Trichoderma* spp. discútese máis a fondo, valorando entre outros factores, a elevada carga de esporas empregada nas inoculacións artificiais dos fungos asociados coas EMV, o corto período de tempo transcorrido entre a aplicación do ACB e a inoculación artificial dos mesmos, o efecto das condicións ambientais adversas para a implantación de *Trichoderma* ou a posibilidade dunha baixa afinidade destes fungos polo cultivar 'Godello'.

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