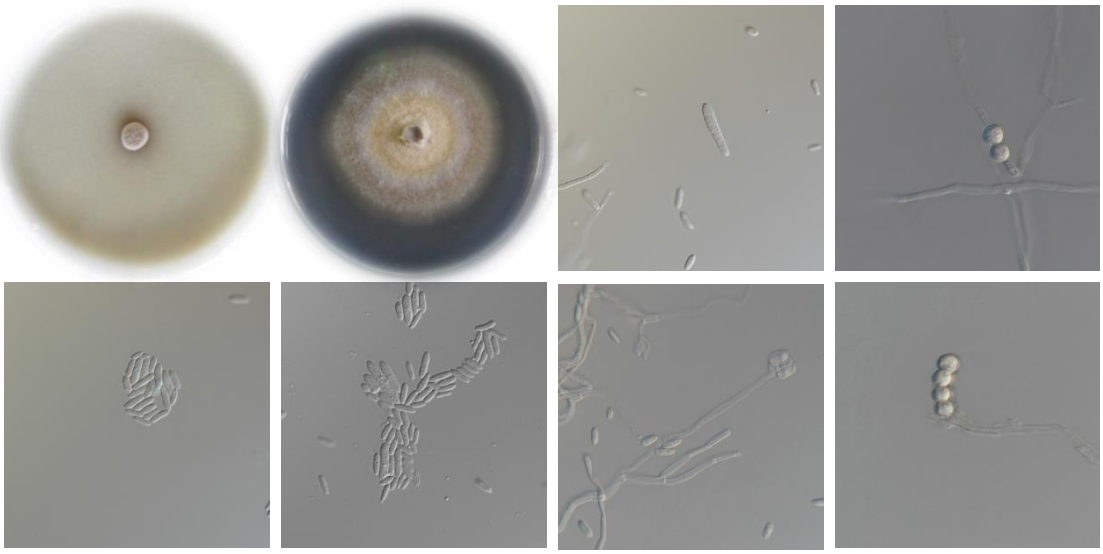
New insights into the biology, ecology and control of black-foot disease in grapevine



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2. Berlanas C, López-Manzanares B, Gramaje D (2017) Estimation of viable propagules of black-foot disease pathogens in grapevine cultivated soils and their relation to production systems and soil properties. Plant Soil 417:467-479.
3. Berlanas C,Berbegal M, Elena G, Laidani M, Cibriain JF, Sagüesa-Sara A, Gramaje D (2019) The fungal and bacteriar rhizosphere microbiome associated with grapevine rootstock genotypes in mature and young vineyards. Front Microbiol 10:1142.
4. Berlanas C, Andrés-Sodupe M, López-Manzanares B, Maldonado-González MM, Gramaje D (2018) Effect of white mustard cover crop residue, soil chemical fumigation and Trichoderma spp. root treatment on black foot disease control in grapevine. Pest Manag Sci 74:2864-2873.

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Summary

Grapevine trunk diseases (GTDs) are a growing concern in the wine sector worldwide. Among the, black-foot disease (BFD) has increased its incidence in grapevine nurseries and newly established vineyards. BFD is caused by numerous *Cylindrocarpon*-like asexual morphs species. This disease has been widely studied in Spain in recent years. However, the constant reclassification and taxonomic expansion of the species associated with BFD, the restriction in the application of fungicides, and the emergence in the use of high-throughput sequencing techniques has made necessary a review and update of the knowledge obtained so far. In this PhD thesis, the biology and ecology of the disease was studied in detail. The evaluation of different control strategies to improve disease management was also investigated.

Firstly, a wide collection of isolates associated to BFD, which were obtained from asymptomatic vines, were characterized. These isolates were studied with morphological and cultural characteristics as well as phylogenetic analyses of combined DNA sequences of the *his3*, *tef1* and *tub2* genes, and the ITS region. Two new species associated with the disease were described: *Dactylonectria riojana* and *Ilyonectria vivaria. I. pseudodestructans* and *Neonectria quercicola* were isolated for the first time from grapevine in Spain, raising the total number of fungal species associated to BFD in our country to 17. The development of a semi-selective medium based on previous research allowed the identification and quantification of viable propagules of fungi associated with BFD from soil samples. The presence of BFD inoculum in rotating nursery fields and in mature vineyards was confirmed. Moreover, a positive correlation was established between Colony Forming Units (CFU) of BFD pathogens and the $CaCO_3$ concentration in soil. On the other hand, the fungal and bacterial microbiome of the rhizosphere of 5 rootstocks in two vineyards located in La Rioja and Navarra has been characterized by high-throughput amplicon sequencing (HTAS). The results showed that grapevine rootstock genotype was the most important factor in shaping the microbiome in a mature vineyard (25-year-old), but not in a young vineyard (7-year-old). However, several bacterial and fungal species were found in both vineyards, demonstrating the existence of a “core” microbiome conserved in the vineyard, regardless of the geographic region. In addition, a positive correlation has been observed between the

relative abundance of pathogens of BFD obtained by HTAS and the one obtained by qPCR. Moreover, the rhizosphere compartment of the "140 R" and "161-49 C" rootstocks harboured lower number of these pathogens than the "1103 P", "110 R" and "41 B" rootstocks. Finally, regarding control measures, the efficacy of white mustard biofumigation was compared with the use of propamocarb + fosetyl-Al, as well as the effect of Tusal® application. Biofumigation with *Brassica* sp. is a promising alternative to the use of chemical fungicides for BFD control, while the application of commercial products based on *Trichoderma* sp. on the roots before planting resulted ineffective for disease control.

Resumen

Actualmente, las enfermedades fúngicas de la madera de la vid son muy graves y han sido señaladas en muchos foros como una de las principales preocupaciones actuales del sector vitivinícola, si no la mayor. Entre estas enfermedades destaca la enfermedad del pie negro, cuya incidencia es creciente en viveros de vid y en nuevas plantaciones. Esta enfermedad está causada por numerosas especies con formas asexuales del tipo "*Cylindrocarpon*". El pie negro ha sido extensamente estudiado en España durante los últimos años. Sin embargo, la constante reclasificación y ampliación taxonómica de las especies asociadas a la enfermedad, la restricción en la aplicación de fungicidas y la emergencia en el uso de técnicas de secuenciación de nueva generación ha hecho necesario una revisión y actualización de los conocimientos obtenidos hasta ahora. En esta tesis se ha estudiado en detalle la biología y ecología de la enfermedad, y se han evaluado diversas estrategias de control.

En primer lugar, se han caracterizado una amplia colección de aislados asociados al pie negro obtenidos de vides asintomáticas. Estos aislados fueron analizados mediante el estudio de sus caracteres fenotípicos y la secuenciación de los genes *his3*, *tef1* y *tub2* y la región ITS. Como resultado, se describieron dos nuevas especies asociadas al pie negro de la vid: *Dactylonectria riojana* e *Ilyonectria vivaria*, y por primera vez se han aislado de vid *I. pseudodestructans* y *Neonectria quercicola*, elevando a 17 el total de especies fúngicas asociadas con la enfermedad en España. Además, se ha desarrollado un medio semi-selectivo, basado en un trabajo ya publicado, para identificar y cuantificar propágulos viables de hongos asociados al pie negro en muestras de suelo. El uso de este medio ha permitido confirmar la presencia de inóculo en campos de vivero en rotación y en viñedos adultos. Además, se ha establecido una relación positiva entre Unidades Formadoras de Colonias (UFC) de los patógenos de la enfermedad del pie negro y la concentración de $CaCO_3$ en el suelo. A continuación, se ha caracterizado el microbioma fúngico y bacteriano de la rizosfera de 5 portainjertos en dos viñedos localizados en La Rioja y Navarra mediante secuenciación de amplicones. Los resultados mostraron que el genotipo es determinante en la selección del microbioma residente en la rizosfera en el viñedo adulto (25 años), mientras que este factor no influía en la selección del microbioma en el viñedo joven (7 años). Sin embargo, diversas especies

bacterianas y fúngicas se encontraron en ambos viñedos, lo que demuestra la existencia de un microbioma conservado en el viñedo, independientemente de la región geográfica. Además, se ha observado una correlación positiva entre la abundancia relativa de patógenos de la enfermedad del pie negro obtenida mediante secuenciación masiva de amplicones y la obtenida mediante qPCR. También se ha comprobado que la rizosfera de los portainjertos “140 R” y “161-49 C” contiene menor cantidad de estos patógenos que los portainjertos“1103 P”, “110 R” y “41 B”. Por último, en cuanto a medidas de control, se ha comparado la eficacia de la biofumigación con mostaza blanca con el empleo de propamocarb + fosetyl-Al, así como el efecto de la aplicación de Tusal®, un producto comercial basado en especies de *Trichoderma*. Se ha confirmado que la biofumigación con *Brassica* sp. es una alternativa prometedora al uso de fungicidas químicos para el control de la enfermedad del pie negro, mientras que la aplicación de *Trichoderma* spp. sobre las raíces antes de la plantación resultó ser inefectiva para el control de la enfermedad.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. Grapevine and viticulture

1.1.1 Origins of viticulture

Grapevine (*Vitis vinifera* L.) has been cultivated since the beginning of time. The first accounts of viticulture come from Armenia, Iran, Sumeria or Egypt. A vessel from 5,400 B.C. found in the Zagros Mountains is one of the most antiques evidence of wine elaboration and consumption. The most ancient winery has been discovered in Armenia, and dates from 6,000 B.C. One of the first big civilizations to practice viticulture was the Egyptian, with more than 4,000 years old mosaics showing viticulture practices (Figure 1.1). From this region, the Hebrews passed on viticulture tradition to the Phoenician and Greeks traders, arriving to Europe and Spain (Vicente-Elías 2000; Hidalgo and Hidalgo 2019).

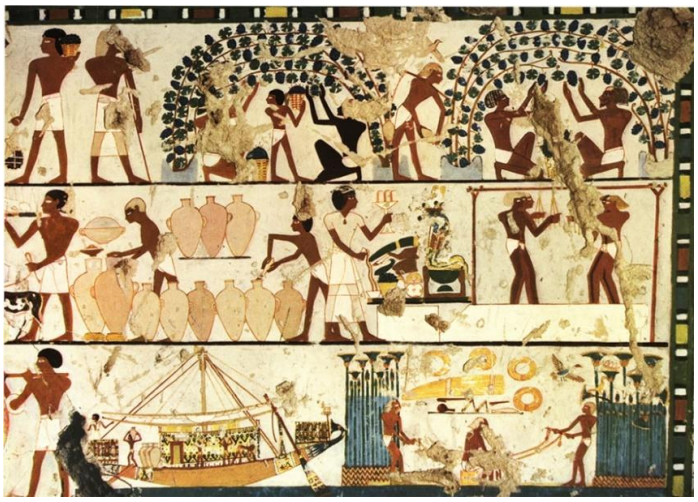

Figure 1.1. *Egyptian mosaic. Source:* Vicente-Elías (2000).

1.1.2. Viticulture in Europe

The Roman farmers inherited from the Greeks all the knowledge about the cultivation of grapevines and elaboration of wines, extending these practices throughout the Roman Empire. Towards the 3th century A.D., the Romans had already laid the foundations of today's great European vineyards. With the fall of the Roman Empire, the wine trade was interrupted and commercial vine plantations declined. During the Middle Ages, the monks were the guardians of the winemaking knowledge (Álvarez-Ramos and Villarías-Moradillo 2012).

In the 16th century, European viticulture expanded again. However, in 1863, with the arrival to Europe of the phylloxera disease from North America, European viticulture

underwent a serious reverse. For example, French producers were left on the verge of bankruptcy. The devastation could be controlled with grafting in imported resistant plants from California (Álvarez-Ramos and Villarías-Moradillo 2012). This resistance to phylloxera can be intrinsic, which depends in plant genetics, or extrinsic, depending on vigour, that is influenced by the environment. One resistant trait that American vines show is that their roots are more lignified and their tissues are more dense and narrow than the ones from *V. vinifera*, making more difficult the infection. *V. riparia*, *V. rupestris* and *V. berlandieri* are the three resistant species commonly use in breeding for the development of resistant rootstocks. These rootstocks can have pure linage, as 'Riparia Glorie de Montpellier' (*V. riparia*) or 'Rupestris du Lot' (*V. rupestris*), or be hybrids, for example '110 Richter' ('110 R') (*V. berlandieri x V. rupestris*) or 'Selection Oppenheim 4' ('SO4') (*V. berlandieri x V. riparia*) (Hidalgo and Hidalgo 2019).

1.1.3. Viticulture in Spain

Viticulture and winemaking have been present in the Iberian Peninsula from the beginning, even before the Phoenicians arrived. It is believed that this was possible thanks to grapevine varieties native to the Iberian Peninsula. During the Roman rule of Iberia, Iberian wine was sold throughout all the Roman Empire. With the Germanic invasions, part of the vineyard was destroyed, but some of the wine industry survived. At the beginning of the 8th century, the Arabs invaded the Iberian Peninsula, growing vineyards mainly for table use. However, although Muslim laws prohibit the consumption of wine, the Moorish rulers had an ambiguous stance towards wine (Álvarez-Ramos and Villarías-Moradillo 2012; Hidalgo and Hidalgo 2019).

With the Reconquest of the Iberian Peninsula by Christians, the possibility of growing vineyards and exporting Spanish wines was reopened, mainly towards the Anglo-Saxon market. The end of the Reconquest and the discovery of America in 1492 opened a new export market and new opportunities for wine production, taking Spanish missionaries and conquerors vines to the new world with them. During the 17th and 18th centuries the popularity of Spanish wines, especially Jerez, Malaga and Rioja wines, spread throughout Europe. This widespread acceptance was stopped by the delay with which the Industrial Revolution arrived to Spain, being surpassed by other countries, such as France. As mentioned before, the phylloxera crisis affected the neighbouring country

before than Spain, forcing French winemakers to look for new areas, such as La Rioja, Navarra or Catalonia, taking with them more modern techniques and new varieties. Finally, the phylloxera arrived in Spain in 1876. In 1903, the affected vineyard area reached 1,162,103 ha and only 862,516 ha of vineyard were left unaffected by phylloxera (Hidalgo and Hidalgo 2019) and, as in the rest of Europe, the solution was grafting (Álvarez-Ramos and Villarías-Moradillo 2012).

The first half of 20th century was difficult for the sector due to the Spanish Civil War and the Second World War paralyzing the markets. During the 50s, the Spanish economy began to stabilize, allowing the recovery of the wine sector. With the arrival of democracy, a new middle class emerged, demanding wine and favouring viticulture, which began to modernize and improve. Soon the volume of quality and production of wines increased and Spain got an important role in the world wine market, being one the most important producers of wine and grapes (Álvarez-Ramos and Villarías-Moradillo 2012; OIV 2014).

1.1.4. Viticulture in La Rioja

Viticulture has had an important role in La Rioja since the beginning, with Iberian and Breton farmers. However, grapevine cultivation was consolidate during the Roman occupation, although it could have begun before with Phoenician traders arriving through the Ebro River. Later, in spite of the fact that Goths and Muslims did not contribute to viticulture development, numerous cartulary documents from San Millán de la Cogolla, Nájera or Logroño confirm the existence of vineyards cultivated in the region during the Middle Ages. The passage of the Way of Saint James through La Rioja facilitated the transmission of knowledge about the cultivation of grapevine, the conservation and aging of wine and other cultural elements (Hidalgo and Hidalgo 2019).

Between the end of the 15th century and the beginning of the 16th, La Rioja vineyard reached its maximum area, occupying currently inappropriate lands thanks to the use of lost varieties that were able to adapt to those unsuitable climates and soils. During the 18th century, attempts to open new foreign markets and improve wine quality were made. In the 19th century, the arrival of French merchants after the phylloxera crisis in their country was a great opportunity for Rioja viticulture. However, the arrivals of the powdery mildew disease in 1855 and 1862, of downy mildew in 1885 and finally of the

phylloxera in 1900 were a very hard setback, disappearing more than 85% of the vineyard (Hidalgo and Hidalgo 2019). The reconstruction of the vineyard through the use of grafting and rootstocks was quite slow, beginning around 1910. Since then, the growth of the Rioja wine sector has been unstoppable until today, being supervised by the Corporation *Calificada* Designation of Origen (DOCa) Rioja (Fundación Caja Rioja 2001; Corporation DOCa Rioja 2019; Hidalgo and Hidalgo 2019). In fact, during the last 30 years, the production inside DOCa Rioja has multiplied by three and sales by 2.62. Moreover, there are more vineyard owners and the number of aging wineries has been multiplied by 6 (Barco-Royo 2018).

The 65,326 hectares of vineyards protected by the DOCa Rioja are distributed among Rioja Alta (27,347 ha), Rioja Oriental (24,590 ha) and Rioja Alavesa (13,389 ha) (Figure 1.2) (Corporation DOCa Rioja 2019). The DOCa Rioja settles its production area on different soils, each of which brings different qualities to Rioja wines. The soils that can be found are clay-calcareous, clay-ferrous and alluvial. Moreover, Rioja wine region is favourably affected by the confluence of two types of climate: Atlantic and Mediterranean. For this reason, there are mild temperatures throughout the year and average annual rainfall suitable for the cultivation of the vine, which due to its shortage in summer favours a good maturation (Fundación Caja Rioja 2001). Finally, the red varieties authorized by Corporation DOCa Rioja are 'Tempranillo', 'Garnacha tinta', 'Graciano', 'Mazuelo', and 'Maturana tinta'. On the other hand, the white varieties are 'Viura', 'Malvasía', 'Garnacha blanca', 'Tempranillo blanco', 'Maturana blanca', 'Turruntés', 'Chardonnay', 'Sauvignon blanc', and 'Verdejo' (Corporation DOCa Rioja 2019).

***Figure 1.2**. DOCa Rioja regions. Source: https://www.decantalo.com.*

1.2. Grapevine Trunk Diseases

1.2.1. Current situation

Dr. Luigi Chiarappa in collaboration with other scientists coined the term grapevine trunk disease (GTD) in the late 1990s. The term gather together several internal and external symptoms observed in grapevines, previously thought as caused by a group of fungi that first infect the pruning wounds, and then colonize the vascular tissues (Mugnai 2011).

The term GTD is moderately new, but the symptoms are old. In fact, the first official report of esca foliar symptoms is from the end of the 19th century in France (Ravaz 1898, 1909), although is believed that esca may be as old as viticulture (Mugnai et al. 1999). Despite the fact that the GTDs have been known for more than a century, their importance regarding plant health is relatively recent. In Spain, the incidence of GTDs in Castilla-León region increased 5.2% from 2001 to 2006 (Martin and Cobos 2007) and their incidence and severity have increased over time in La Rioja region (Pérez-Marín 2012). This could be linked to several reasons:

I) During the 1990s a planting boom occurred, increasing not only the movement of potentially infected plant material, but also the cultivated area allowing the grapevines to reach an age where the symptoms are expressed and can be assessed (Gramaje and Armengol 2011).

II) Changes in management practices that have led to a more favourable environment for fungal infection, such as converting traditional low-density head trained vines to more high density spur-pruned trellis vineyards (Gramaje et al. 2018).

III) The ban of some fungicides at the beginning of this century due to environmental and public health concerns, including sodium arsenite or methyl bromide (Decoin 2001; National Library of Medicine 2003), removed from use the most effective chemical products available against GTD pathogens.

Grapevine trunk diseases have an important economic impact, threatening the economic viability of viticulture worldwide (Kaplan et al. 2019). For example, in South Australia, AUD$2,800 per hectare are the estimated losses due to Eutypa dieback (Wicks and Davies 1999). In California, the difference in benefits between healthy and diseased vineyards is valued in around $33,000 per hectare over a 25-year vineyard lifespan (Hillis et al. 2017). In La Rioja region, €7.16 million per year are the estimated cost of the

replacement of 1% death cultivar 'Tempranillo' plants due to GTDs (Martínez-Diz et al. 2019b).

To date, 136 fungal species belonging to 35 genera have been associated with 6 different grapevine trunk diseases worldwide (Gramaje et al. 2018; Lawrence et al. 2019; Aigoun-Mouhous et al. 2019), and it is common that more than one GTD affect the same plant at the same time (Pérez-Marín 2012; Gramaje et al. 2018). However, the number of fungal species associated to GTDs is constantly increasing due to the continuous improvement of the molecular diagnosis techniques. Information about the current known GTDs is individually provided below, with the exception of black-foot disease, to which a detailed description in a separate section is provided due to be the major field of study of this thesis.

1.2.2. Esca disease

Early known as 'apoplexy' or 'folletage', esca was the first GTD reported in many European and Mediterranean countries (Ravaz 1909; Mugnai et al. 1999).

Traditionally, two forms of this disease have been described, chronic/mild and acute/apoplectic. In the mild form, also known as grapevine leaf stripe disease (Gubler et al. 2015), the symptoms recorded in the literature are highly erratic: yellowing, reddening, drying and dropping (Lecomte et al. 2012). However, there is a characteristic foliar symptom of this form, the 'tiger-stripe' pattern: leaves display multiple banding discolouration surrounding dry, light or red-brown necrotic tissue on the leaf blade. In red cultivars, it is often bordered by narrow red or yellow blotches, whereas on white cultivars the red colour is usually absent (Figure 1.3A), but black-measles can be eventually found on their berries (Surico 2009).

The apoplectic esca form is characterized by a sudden wilting of several shoots, one of the arms or the entire plant (Figure 1.3B). Leaf symptoms include scorching, dropping and shrivelling. Moreover, grape clusters can also dry on the vine (Mugnai et al. 1999).

In both cases, foliar symptoms appear in late spring or summer, and may be expressed one year and not the next, though it is common for vines to show symptoms in many consecutive years. Internal symptoms are diverse, including brown to black vascular streaking, black spot in the xylem surrounded by pink to brown wood

discolouration, or dry wood with a silver appearance (Figure 1.3C). In older vines, the wood may develop a yellow to white soft rot (Figure 1.3D) (Fischer 2002).

***Figure 1.3**. Esca sympytoms. **A**: 'tiger-stripe' pattern in red (left) and white (right) 'Tempranillo' cultivars. **B**: acute/apoplectic form. **C**: internal symptoms, black vascular streaking and black spot in the xylem surrounded by pink to brown wood discolouration. **D**: yellow to white soft rot developed in mature vines.*

The etiology of esca disease has been a matter of discussion among scientists over the last 20 years. The principal current hypothesis is that young vines infected with *Phaeomoniella chlamydospora* (W. Gams, Crous, Wingf. & Mugnai) Crous & W. Gams and/or species of *Phaeoacremonium* W. Gams, Crous & M.J. Wingf., can later develop esca symptoms following further colonization by several basidiomycetes species belonging to the genera *Inocutis* Fiasson & Niemelä, *Inonotus* P. Karst, *Fomitiporella* Murrill, *Fomitiporia* Murrill, *Phellinus* Quél, and *Stereum* Hill ex Pers. (Cloete 2015; Guerin-Dubrana et al. 2019). However, Koch's postulates are to be completed to support the role of basidiomycetes in the symptomatology of the diseases. Several species of the genus *Cadophora* Lagerb. & Melin have also been recently associated with esca-diseased vines (Travadon et al. 2015).

Perithecia (*Phaeoacremonium* spp.) or pycnidia (*P. chlamydospora*) begin developing during the hot, dry months of midsummer and mature by the end of the season. These

fruiting bodies are found in old, rotted vascular tissues, on pruning wounds, and on other injuries that expose rotted xylem (Gubler et al. 2015). Spore release occurs naturally during fall, winter, and spring rains (Gramaje et al. 2018), but it can also take place during irrigation events, especially via overhead sprinklers (Gubler et al. 2015). Spores are then spread from perithecia or pycnidia by rain droplets, 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). The potential for pruning shears to spread esca pathogens has been demonstrated recently under greenhouse conditions (Agustí-Brisach et al. 2015).

1.2.3. Petri disease

This disease affects young vines (<7-8 years old) and planting material in grapevine nurseries (Serra et al. 2000; Halleen et al. 2003).

Foliar symptoms of Petri disease include overall stunting, reduced foliage and wilting of leaves or entire shoots (Gramaje et al. 2018). These symptoms resemble those associated with abiotic disorders such as spring frost, winter damage, water stress and/or nutrient deficiency. The vine will struggle and show more severe symptoms each year, especially if under stress conditions. Severely affected vines may die within a few years.

Internal symptoms are more specific and allow recognizing the disease: black discolouration of the xylem vessels, which is a result of tyloses, gums, and phenolic compounds formed inside these vessels by the host in response to the fungus growing in and around the xylem vessels (Figure 1.4A and 1.4B) (Gramaje and Armengol 2011).

Petri disease is mainly caused by a combination of *Phaeomoniella chlamydospora* and 28 species of *Phaeoacremonium* (Gramaje and Armengol 2011; Gramaje et al. 2015; Araújo da Silva et al. 2017). *Phaeomoniella chlamydospora* has been more often associated with typical Petri disease symptoms than species of *Phaeoacremonium* (Mostert et al. 2006). Other species associated with this disease are *Cadophora luteo-olivacea* (J.F.H. Beyma) Harr. & McNew (Gramaje et al. 2011b) and *Pleurostoma richardsiae* (Nannfeldt) Réblová & Jaklitsch (Halleen et al. 2007b).

Infection occurs trough root wounds, rootstock base and grafting zone, affecting primary the basal end of the rootstock. Petri disease pathogens have been found during

different steps of the propagation process in nurseries, being grafting and callusing the most critical steps regarding disease infection (Gramaje and Armengol 2011; Gramaje et al. 2018).

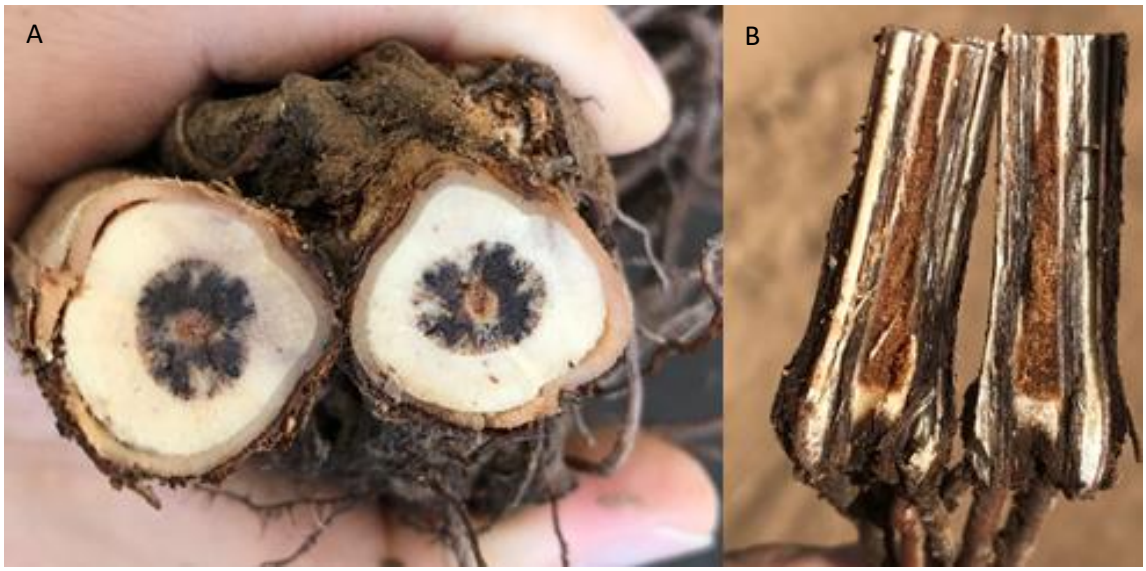

Figure 1.4. *Internal symptoms of Petri disease.* ***A****: Rootstock cross section showing a ring of necrotic xylem vessels surrounding the pith.* ***B****: rootstock longitudinal section showing dark brown to black streaking of the xylem tissues.*

1.2.4. Eutypa dieback

Eutypa dieback affects predominantly mature vineyards (>7-8 years old) (Gubler et al. 2013).

External symptoms include chlorotic leaves in stunted shoots with shorts internodes (Figure 1.5A). Leaves usually present necrotic margins and occasionally have dead interveinal tissues (Rolshausen et al. 2013b). These symptoms are caused by toxic metabolites produced by *Eutypa lata* (Pers.) Tul. & C. Tul. in the wood (Mahoney et al. 2005) and can vary year to year, similar to esca symptoms (Sosnowski et al. 2016).

Wood symptoms of Eutypa dieback include cordon dieback, with loss of spurs and internal, necrotic, wedge-shape staining in the cross-section of cordons and trunks (Figure 1.5B). External cankers appear as the disease advances, characterized by flattened areas of the wood with no bark surface, leading to eventual plant death (Gramaje et al. 2018).

Eutypa dieback is caused by 24 species in the Diatrypaceae family (Trouillas et al. 2010; Luque et al. 2012; Rolshausen et al. 2013b; Pitt et al. 2013b), *E. lata* being the most common and virulent fungus causing the symptoms (Trouillas and Gubler 2010). Other genera in the Diatrypaceae family have been isolated from symptomatic wood,

such as *Anthostoma* Nitschke, *Cryptosphaeria* Ces. & De Not., *Cryptovalsa* Ces. & De Not. Ex Fuckel, *Diatrype* Fr., *Diatrypella* (Ces. & De Not.) De Not., and *Eutypella* (Nitschke)

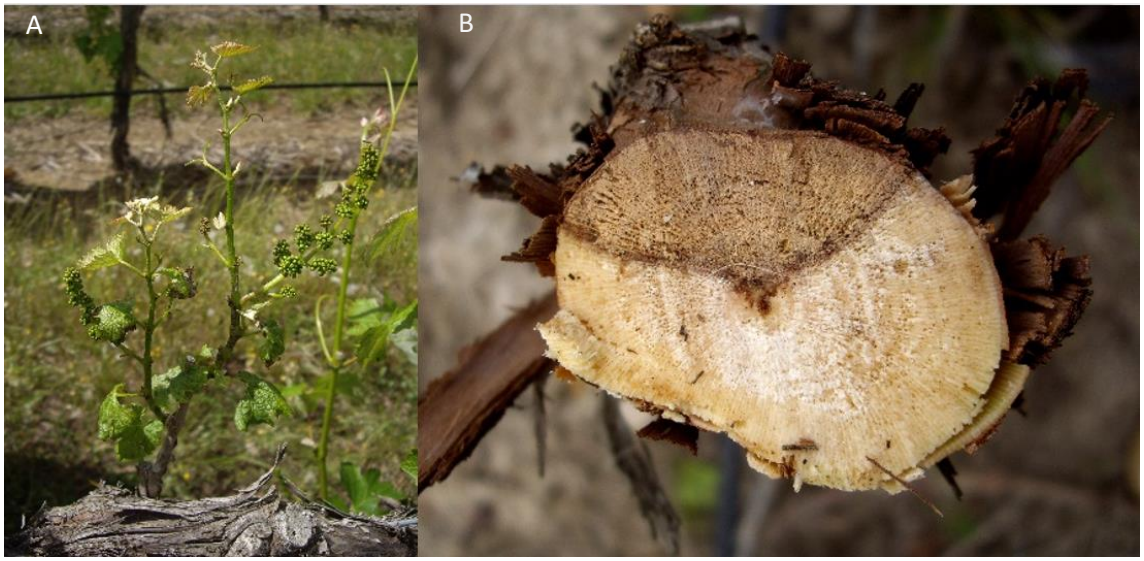

Figure 1.5. Eutypa dieback symptoms. ***A****: stunted shoots with chlorotic leaves often cupped and with necrotic margins, and short internodes.* ***B****: internal, necrotic, wedge-shaped internal necrosis of a cordon. Source: Gramaje et al. 2018.*

Sacc. (Trouillas et al. 2010; Luque et al. 2012).

As it usually happens in the GTDs affecting mature vineyards, the primary form of dispersal of Eutypa dieback pathogens is aerial (Legorburu et al. 2014). For instance, *E. lata* ascospores cause new infections where they land on susceptible open wounds (Moller et al. 1977). These spores are produced by perithecia formed in layers of stromatic tissue found on dead host tissue (Magarey and Carter 1986).

1.2.5. Botryosphaeria dieback

Previously known as black dead arm, this disease mainly affects mature vineyards, although it has been detected affecting 3 to 5 year old vines (Úrbez-Torres et al. 2008) and planting material in grapevine nurseries (Aroca et al. 2010).

The main wood symptom of Botyrosphaeria dieback is wedge-shape perennial cankers (Figure 1.6A) or circular to non-uniform central staining of the wood observed in cross-sections of affected wood (Úrbez-Torres 2011). These perennial cankers are indistinguishable from cankers caused by *E. lata*, and thus visual field diagnosis is difficult. However, grapevines affected by Botryosphaeria dieback fail to manifest the distinctive foliar symptoms caused by *E. lata* during the years following infection.

Grapevines infected with Botryosphaeria dieback, on the other hand, show either a total absence of spring growth or normal and healthy development of shoots (Figure 1.6B). These symptoms can appear only 1 or 2 years after the infections have occurred (Úrbez-Torres et al. 2006).

***Figure 1.6**. Botryosphaeria dieback symptoms. **A**: wedge-shape canker. **B**: cordon dieback along with a lack of spring growth. Source: Gramaje et al. 2018.*

At least 26 different species belonging to 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 Botryosphaeria dieback of grapevines (Úrbez-Torres and Gubler 2011; Pitt et al. 2013a, c, 2015; Rolshausen et al. 2013a; Yang et al. 2017). *Diplodia seriata* De Not, *Neofusicoccum parvum* (Pennycook & Samules) Crous, Slippers & A.J.L. Phillips and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not are the most frequently isolated fungus (Úrbez-Torres 2011). Several studies indicate that the fastest wood-colonizing fungi and therefore the most virulent belong to the genera *Lasiodiplodia* and *Neofusicoccum* (van Niekerk et al. 2004; Úrbez-Torres et al. 2008; Úrbez-Torres and Gubler 2009).

The sexual stage of the different Botryosphaeriaceae spp. causing cankers has rarely been found on grapevines; therefore, it is thought that conidia may be the main source of infection. Conidia are released from pycnidia, then colonize pruning wounds (Úrbez-Torres 2011). Botryosphaeriaceae spp. overwinters as pycnidia in diseased woody parts of the plant and in wood debris left in the vineyard after pruning (Elena and Luque 2016).

Botryosphaeria dieback pathogens can also be propagated through the use of infected cuttings (Gramaje and Armengol 2011).

1.2.6. Phomopsis dieback

Characteristic symptoms of Phomopsis dieback are similar to those resembling Botryosphaeria and Eutypa diebacks and include perennial cankers in the framework of the vine (Figure 1.7A), and budbreak failure from infected spurs (Úrbez-Torres et al. 2013; Guarnaccia et al. 2018).

Several species in the genus *Diaporthe* Nitschke have been associated with Diaporthe dieback (Baumgartner et al. 2013; Úrbez-Torres et al. 2013; Dissanayake et al. 2015; Guarnaccia et al. 2018). Among them, the disease is primarly caused by the most virulent *D. ampelina* (Berkeley & M.A. Curtis) R.R. Gomes, C. Glienke & Crous (formerly *Phomopsis viticola*) (Úrbez-Torres et al. 2013; Dissanayake et al. 2015), which has long been known as the causal agent of the grapevine disease named Phomopsis cane and leaf spot in the USA or excoriose in Europe (Figure 1.7B) (Phillips 2000; Úrbez-Torres et al. 2013).

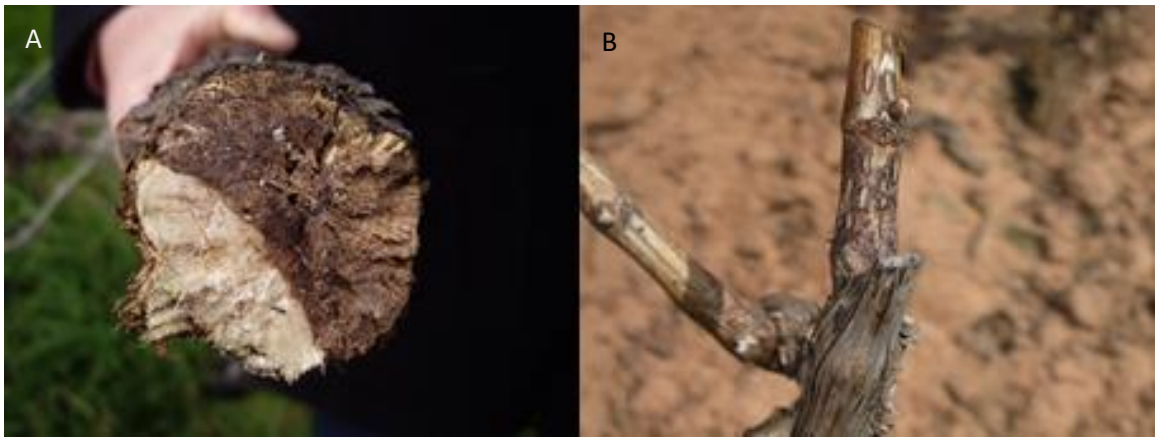

***Figure 1.7. A**: wedge-shape canker resembling Botryosphaeria and Eutypa diebacks. **B**: lesions of Phomopsis cane and leaf spot or excoriose on shoots.*

Spores released from overwintering pycnidia found on diseased spurs, canes and bark are splashed by rain onto newly developing shoots (Phomopsis cane and leaf spot or excoriose). Spores are thought to be also spread by wind or arthropods and colonize pruning wounds (Phomopsis dieback) (van Niekerk et al. 2010; Moyo et al. 2014).

1.3. Black-foot disease

Black-foot disease (BFD) was first reported in France in 1961 (Maluta and Larignon 1991). Since then, the disease has been identified in all major viticulture regions throughout the world. It represents a major problem in nursery plants and young plantations, and the pathogens associated with BFD are commonly found in the soil, being able to infect the grapevines once they are planted (Agustí-Brisach and Armengol 2013).

Black-foot disease has been extensively studied in countries such as Italy (Carlucci et al. 2017), New Zealand (Jaspers 2013a, b), Portugal (Rego et al. 2001a, b; Reis et al. 2013), South Africa (Halleen et al. 2003, 2006b; Langenhoven et al. 2018) and USA (Petit and Gubler 2005; Dubrovsky and Fabritius 2007; Petit et al. 2011).

In Spain, the importance of BFD of grapevine has increased considerably over the last 15 years, leading to numerous research studies concerning the etiology, epidemiology and control of the disease (Aroca et al. 2006; Alaniz et al. 2007, 2009, 2010, 2011a, b; Alaniz 2008; Gramaje et al. 2010; Agustí-Brisach et al. 2011a, 2013a, b, 2016, 2019; Agustí-Brisach and Armengol 2012; Tolosa-Almendros et al. 2016; Martínez-Diz et al. 2018, Pintos et al. 2018).

1.3.1. Symptoms

External symptoms of BFD overlap with those of Petri disease and resemble several abiotic stresses as mentioned before. These symptoms include reduced vigour, short internodes, interveinal necrosis, sparse foliage and uneven wood maturity (Figure 1.8A) (Rego et al. 2000; Halleen et al. 2006a; Agustí-Brisach and Armengol 2013).

Internal symptoms of affected vines include black, sunken necrotic lesions on roots and reddish to brown discolouration in the base of the trunk (Halleen et al. 2006a) (Figure 1.8B and 8C). A reduction in root biomass and root hairs are also distinctive symptoms of BFD (Rego et al. 2000). Moreover, black discolouration and necrotic woody tissue that develops from the base of the rootstock can be found when the bark is removed and the pith of affected plants can be compacted and discoloured (Jaspers 2013a). In many instances, BFD can be found in association with the condition known as J-rooting (Figure 1.8D) (Gubler and Petit 2013). This condition is the results of poor planting of the vines in which roots are oriented upward.

Vines frequently have early autumn senescence and may die during the growing season or in winter when they fail to develop shoots (Jaspers 2013a). When young plants are attacked, they die very quickly, but as the vine ages, infection results in a more gradual decline and death might occur after more than a year (Gubler et al. 2004).

Figure 1.8. *Black-foot disease symptoms.* ***A****: reduced vigour and stunted growth in a young plantation.* ***B*** *and* ***C****: wood necrosis.* ***D:*** *J-rooting.*

1.3.2. Etiology and worldwide distribution

Up to 29 seven species belonging to 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 associated with BFD (Agustí-Brisach and Armengol 2013; Lombard et al. 2014; Carlucci et al. 2017; Lawrence et al. 2019; Aigoun-Mouhous et al. 2019). *Dactylonectria torresensis* (A. Cabral, Rego & Crous) L. Lombard & Crous is the most common species associated to BFD in Algeria (Aigoun-Mouhous et al. 2019), Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016).

Black-foot disease pathogens have been isolated from diseased vines in Italy (Grasso and Magnano di San Lio 1975; Grasso 1984; Carlucci et al. 2017), France (Maluta and Larignon 1991), United States (Scheck et al. 1998; Petit and Gubler 2005; Petit et al. 2011), Portugal (Rego et al. 2000), South Africa (Fourie et al. 2000), Argentina (Gatica et al. 2001), Greece (Rumbou and Rumbos 2001), Spain (Armengol et al. 2001a; Aroca et al. 2006; Giménez-Jaime et al. 2006) Germany (Fischer and Kassemeyer 2003), New Zealand (Halleen et al. 2004), Australia (Whitelaw-Weckert et al. 2007), Chile (Auger et al. 2007), Slovenia (Schroers et al. 2008), Iran (Mohammadi et al. 2009), Lebanon (Choueiri et al. 2009), Switzerland (Casieri et al. 2009), Uruguay (Abreo et al. 2010), Canada (O'Gorman and Haag 2011; Petit et al. 2011), Peru (Álvarez et al. 2012), Turkey (Özben et al. 2012), Brazil (dos Santos et al. 2014a, b), Bulgaria (Piperkova et al. 2017), Czech Republic (Pečenka et al. 2018), and more recently in Algeria (Aigoun-Mouhous et al. 2019).

1.3.3. Taxonomy

The generic term "*Cylindrocarpon" Wollenw.* was introduced by Wollenweber (1913). Following that, this author associated "*Cylindrocarpon"* with the sexual form *Neonectria* (Gordillo and Decock 2017). Later on, Booth (1966) split the genus in four groups based on the presence or absence of microconidia and chlamydospores:

I) *"Cylindrocarpon" magnusianum* (Sacc.) Wollenw., the asexual morph of the type species *Neonectria*.
II) *"C. cylindroides"* Wollenw., the type species of the genus.
III) *"C. destructans*" (Zinssm.), asexual morph of *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels.
IV) *"Cylindrocarpon"* species linked to sexual morphs of the *Nectria mammoidea* W. Phillips & Plowr. group.

Additionally, the genus *Neonectria* was also divided into five groups based on perithecial morphology, being reduced to three by Mantiri et al. (2001) after studying phylogenetic inference of mitochondrial small subunit rDNA sequences. Traditionally, BFD has been related to two "*Cylindrocarpon"* species: "*C." destructans* and "*C". obtusisporum* (Cooke & Harkn.) Wollenw. (Abreo et al. 2010). "*C." destructans* was firstly described affecting grapevine n France in 1961 (Maluta and Larignon 1991). On the other

hand, "*C". obtusisporum* has been found in California (Scheck et al. 1998) and Sicily (Grasso and Magnano di San Lio 1975). In 2004, Halleen et al. (2004) described "*C". macrodidymum* Schroers, Halleen & Crous associated to BFD in South Africa, Canada, Australia and New Zealand. Later on, a theory that "*C". obtusisporum* was misidentified in previous studies (Grasso and Magnano di San Lio 1975; Scheck et al. 1998) and it was in fact "*C". macrodidymum* was confirmed by Hallen et al. (2006a). Whereas, after DNA analysis of the ITS and β-tubulin regions of "*C". destructans* isolates from grapevine, the species was renamed as "*C". liriodendri* J.D. MacDon. & E.E. Butler, species first described associated to tulip poplar (*Liriodendron tulipifera* L.) (MacDonald and Butler 1981), and with identical sequences to *"C." destructans*. "*C*." *destructans* and "*C*."*liriodendri* were also indistinguishably by morphological characters (Halleen et al. 2006b). Through 2008, "*C." pauciseptatum* Schroers & Crous, a new species associated with BDF in grapevines in New Zealand and Slovenia was described (Schoers et al. 2008).

Several studies (Mantiri et al. 2001; Halleen et al. 2004, 2006b; Hirooka et al. 2005; Castlebury et al. 2006, Lombard et al. 2014) suggested that the genus *Neonectria* and its "*Cylindrocarpon"* asexual morphs could represent a generic complex. With the introduction of the asexual morph genus *Campylocarpon,* similar to *"Cylindrocarpon"* but lacking microconidia (Halleen et al. 2004) the first formal segregation from the genus "*Cylindrocarpon"* was done. The species *Campyl. fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous were described as causal agents of BFD in South Africa (Halleen et al. 2004).

Following this, new genera were identified within the *Neonectria*/"*Cylindrocarpon"* complex: *Ilyonectria*, *Rugonectria* P. Chaverri & Samuels and *Thelonectria* based on multigene phylogenetic analyses, morphological comparisons and ecological characters (Chaverri et al. 2011). *Ilyonectra* presented macroconidia, microconidia and chlamydospores, being found in roots and soils. *Rugonectria* did not produce chlamydospores and was isolated mainly from the bark of dying or recently dead trees. Some species of *Thelonectria* produced chlamydospores, whereas other did not, the microconidia were only found in natural substrate and the genus was found in the bark of dying trees. Some species of this genus were *T. coronato*, *T. discophora*, *T. olida* or *T. westlandica* (Chaverri et al. 2011). "*C." liriodendri* and "*C." macrodidymum* were included in the *Ilyonectria* genus, changing their names to *I. liriodendri* (Halleen, Rego &

Crous) Chaverri & C. Salgado and *I. macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado, respectively. Afterwards, in 2012, a multi-gene DNA analysis supported by morphological characters conducted by Cabral et al. (2012a) was able to define 12 new taxa in the *I. radicicola* (Gerlach & L. Nilsson) Chaverri & C. Salgado complex. After this, the existence of polymorphism into *I. macrodidyma* complex was also demonstrated (Cabral et al. 2012b).

As a consequence of the studies revealing that the genus *Ilyonectria* also was polyphyletic (Cabral et al. 2012a, b; Lombard et al. 2013), the genus *Dactylonectria* was introduced to accommodate a bunch of species after using multi-gene DNA data and morphological comparisons (Lombard et al. 2014). *Dactylonectria* presents perithicia ovoid to obpyriform, smooth to finely warted, dark-red papillate ostiolar region at the apex. Moreover, the asexual morph produces abundant macro and microconidia, but rarely chlamydospores in culture (Lombard et al. 2014). The genus *Dactylonectria* included species previously classified as *Ilyonectria*, such as *D. macrodidyma,* the type species of the genus*, D. alcacerensis* or *D. torresensis,* or as *"Cylindrocarpon", D. pauciseptata* (Lombard et al. 2014).

On the other hand*, Cylindrocladiella*, which is also *Cylindrocarpon*-like in morphology, has been associated with BDF on grapevines (van Coller et al. 2005; Agustí-Brisach et al. 2012; Jones et al. 2012). *Cyl. parva* (P.J. Anderson) Boesew. and *Cyl. peruviana* (Bat., J.L. Bezerra & M.P. Herrera) Boesew. are the main causal agents of black-foot within the genera in Spain (Agustí-Brisach et al. 2012, Agustí-Brisach and Armengol 2013). In New Zealand, *Cyl. parva* has been reported associated to BFD (Jones et al. 2012), whereas in South Africa two additional species of *Cylindrocladiella*, namely *Cyl. lageniformis* Crous, M.J. Wingf. & Alfenas and *Cyl. viticiola* Crous & Van Coller, have been associated to BFD (van Coller et al. 2005).

Finally, the genus *Pleiocarpon* has been recently added to the *Cylindrocarpon*-like complex (Aiello et al. 2017). In particular, the species *P. algeriense* W. Aigoun-Mouhous, A. Cabral & A. Berraf-Tebbal has been associated with BFD of grapevine in Algeria (Aigoun-Mouhous et al. 2019).

In conclusion, the causal agents of BFD are multiple and diverse, and are in continuous taxonomic reassessment. The development of molecular techniques allows identifying a considerable number of new species associated with this pathology, since

most of them are indistinguishable only by considering their morphological characteristics.

1.3.4. Biology and epidemiology

Cylindrocarpon-like asexual morphs are usually considered as pathogens and/or endophytes of a wide range of hosts and climates. Apart from grapevine, other economically important hosts where these fungi have been detected and identified include *Actinidia chinensis* Planch. (Erper et al. 2011), *Eriobotrya japonica* (Thunb) Lindl (Agustí-Brisach et al. 2016), *Liriodendron tulipifera* L. (MacDonald and Butler 1981), *Malus domestica* Borkh. (Cabral et al. 2012a), *Olea europaea L.* (Úrbez-Torres et al. 2012), *Panax quinquefolius* L. (Rahman and Punja 2005), *Persea americana* Mill. (Vitale et al. 2012), *Pinus radiata* D. Don (Agustí-Brisach et al. 2011b), *Pinus sylvestris* L. (Menkis and Burokienė 2012) or *Strelitzia reginae* Banks. (Aiello et al. 2017).

The disease cycle of black-foot pathogens on grapevines is not completely known, but the behaviour of *Cylindrocarpon*-like asexual morphs on other hosts has been studied in more detail (Booth 1966; Brayford 1993) and provided information about the likely disease cycle of associated genera on grapevines. By referring to other diseases that these fungal species cause on other hosts, it is assumed that they live in a dormant state in the soil and can infect susceptible plants under certain conditions (Figure 1.9).

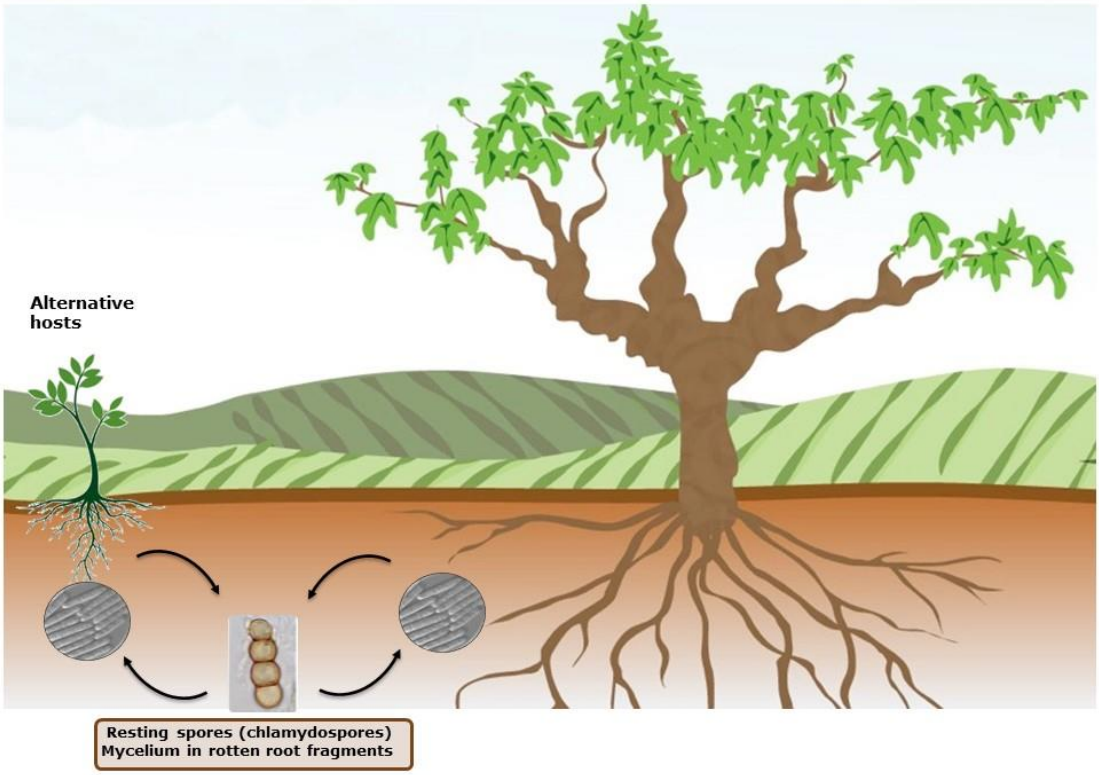

Figure 1.9. *Disease cycle of black-foot pathogens.*

At the moment of planting, small wounds appear when young callus roots break and the susceptible basal ends of the nursery cuttings are exposed, making the cutting susceptible to infection (Agustí-Brisach and Armengol 2013). Some species associated to BFD are able to produce chlamydospores, which allow them to survive in the soil for long time (Halleen et al. 2004). However, little is known about the survival of these pathogens and the role of chlamydospores during infections (Halleen et al. 2006a; Agustí-Brisach and Armengol 2013).

Agustí-Brisach and Armengol (2012) studied the effect of temperature, pH and water potential on the mycelial growth of "*Cylindrocarpon"* spp. They found that the isolates were able to growth from 5°C to 30°C, but not at 35°C. pH value were also little restrictive, allowing fungal growth from pH=4 to pH=8. The authors concluded that temperature, pH and water potential had limited impact in chlamydospores production. Finally, weeds also play a role in the propagation of BFD and can act as reservoir of inoculum, being present in established vineyards and field nurseries (Figure 1.9) (Agustí-Brisach et al. 2011a, 2013a, b; Langenhoven et al. 2018). The ability of BFD fungi of being endophytes on weeds or other hosts has important implications, such as symptomless plants inadvertently serving as sources of hidden diversity of black-foot species, or serving as inoculum reservoirs. Most research conducted on fungal trunk diseases has focused on pathogens infecting important agricultural commodities such as grapevine, almond or olive (Gramaje et al. 2016), leading to a lack of understanding of the true nature of the associations of these fungi with other plants and their environment. Moreover, some species are able to form a sexual form *in vitro*, for example *D. torresensis* (formerly *I. torresensis*) (Cabral et al. 2012b), *I. liriodendri* (formerly *N. liriodendri)* (Halleen et al. 2006b) and *I. novozelandica* (Cabral et al. 2012b). However, the role of the sexual stage in the epidemiology of the disease is still unknown.

There is evidence that BFD also has a connection with nursery production, and occurrence of the pathogens in the nurseries most likely plays an important role in the diseases (Aroca et al. 2010; Agustí-Brisach et al. 2011a, 2013a; Cardoso et al. 2013). BFD pathogens have been isolated from rootstock mother-plants, rooted rootstock cuttings, bench-grafts and young grafted vines from both symptomatic and asymptomatic plants (Rego et al. 2001a; Halleen et al. 2004; Oliveira et al. 2004; Petit and Gubler 2005; Langenhoven et al. 2018).

Finally, there are few reports about virulence diversity within the *Cylindrocarpon*-like asexual morphs to grapevine. Alaniz et al. (2009) studied differences in virulence inside the, for that time, *I. macrodidyma*-complex, concluding that isolates belonging to ISSR groups G6 and G7 were more virulent than the others. Cabral et al. (2012b) performed pathogenicity tests with 60 isolates belonging to 14 BFD species collected from grapevine and other hosts and concluded that *I. lusitanica*, *D. estremocensis*, and *I. europaea* were more virulent to grapevine than to the species previously accepted as the main causal agents of BFD, namely *D. macrodidyma* and *I. liriodendri*. Pathrose et al. (2014) found high degree of virulence diversity among 17 isolates collected in Australia, New Zealand, and South Africa. Probst et al. (2019) found that *I. liriodendri* was able to cause significantly higher disease incidence and severity in artificially inoculated grapevine rootstocks '101-14' and '5C' compared with *D. macrodidyma*, with high diversity in virulence between isolates within each species.

1.3.5. Control measures

Currently, no curative measures are known for control of GTDs, including BFD. The best approach is an integrated disease management combining the use of preventive measures and control options throughout the nursery mother blocks, the nursery process, nursery propagation beds, and newly planted vineyards (Gramaje et al. 2018).

Cultural practices

Several methods should be implanted in the nursery management programme because individual control methods are not totally efficient (Jaspers 2013b). For example, in nursery mother blocks, trellising can eliminate potential black-foot pathogens infection, but this practice is more expensive and labour intensive than cultivating mother plants along the ground (Hunter et al. 2004). Moreover, susceptibility to soilborne pathogens of shoots growing along the soil increases with high temperatures and humidity (Whiteman et al. 2007). Therefore, a correct soil moisture and aeration are needed (Gramaje et al. 2018). Drainage in heavy soil can be achieved by planting on raised beds and by moving away from the vine drip irrigation emitters (Gubler and Petit 2013).

In grapevine nurseries, wounds made during the grafting and callusing stages are vulnerable to fungal infection (Gramaje and Armengol 2011). Moreover, the conditions of the callusing rooms (i.e., darkness, humidity and warm temperature) are favourable for the development of fungal pathogens (Hartmann et al. 2001). Therefore, periodical disinfection treatments in the callusing rooms are essential for BFD control. Jaspers (2013b) recommends dipping in wax with fungicides the grafted join as soon as possible and the cleaning and daily disinfection of the grafting areas: tables and equipment. In addition, stress conditions, such as dehydration, soaking and anaerobic storage conditions, excessive wounding, or extreme temperatures, should be avoided during the propagation process (Waite et al. 2015). In this sense, Probst et al. (2012) found that black-foot disease incidence and severity increased with the length of time that the vines were cold stored, with greater incidence of *I. liriodendri* and *D. macrodidyma* (formerly "*C." destructans*, "*C." liriodendri* and "*C." macrodidymum*) in the inoculated treatments.

The practice of covering the graft unions with soil to prevent the callus for drying that is carry out in some countries could increase the infection threshold of black-foot pathogens (Fourie and Halleen 2006). It is also important that the grapevines develop an adequate root system preceding the early production years to avoid stress, reducing the probability of pathogenic infections (Gramaje et al. 2018).

Crop rotation plays a significant role in managing soilborne plant diseases (Peel 1998), but for many crops the outcome is inconsistent. This is especially true for pathogens with long-lived propagules like black-foot fungi (Cardoso et al. 2013; Gramaje et al. 2018; Langenhoven et al. 2018). Halleen et al. (2003) suggested that BFD inoculum may increase when planting grapevine cutting every second year in a nursery field, followed by a cover crop. In Portugal, Rego et al. (2009) found that *Cylindrocarpon*-like fungal inoculum was significantly influenced by the repeated use of land as grapevine nursery, even when followed by three years of rotation with other crops (e.g. potato, cabbage, carrot, garlic, and leek) and/or gramineous plants. Cardoso et al. (2013) also detected viable propagules of black-foot pathogens in soil after the rotation cycle, before or immediately after planting in nursery fields.

Finally, taking care before planting is crucial for maintaining the quality of the vines. The vineyard must be ready for planting, with weed control and irrigation infrastructure (Waite et al. 2015). Reducing the infection by black-foot pathogens can be achieved by

planting in well drained soils (Rego et al. 2000; Halleen et al. 2007a). In addition, site election should be made based on assessment of inoculum density and distribution in the soil. This can be done by using bait plants (Agustí-Brisach et al. 2013a), or by molecular methods (Probst et al. 2010; Tewoldemedhin et al. 2011; Agustí-Brisach et al. 2014; Martínez-Diz et al. 2019a).

Hot-water treatment

Hot-water treatment (HWT) of rootstock cuttings prior to grafting or HWT of dormant grafted vines after uprooting has been strongly recommended for their effectiveness in reducing BFD infection levels in nursery plants (Halleen et al. 2007a; Halleen and Fourie 2016). The standard HWT protocol is 50°C for 30 minutes (Crous et al. 2001; Halleen et al. 2007a; Gramaje et al. 2010; Halleen and Fourie 2016). Crous et al. (2001) found an important reduction in pathogen populations after HWT when the isolations were made immediately after the treatment. However, when the isolations where made after planting, this reduction was not significant. Halleen et al. (2007a) did not found any black-foot pathogens from roots of plants that were subjected to HWT (50°C for 30 min), whereas none of the other treatments evaluated (i.e., benomyl, carnauba wax, imazalil, or Trichoflow®) differed significantly from the control plants. Gramaje et al. (2010) observed *in vitro* that conidial germination for both *D. macrodidyma* (formerly "*C." macrodidymum*) and *I. liriodendri* ("*C." liriodendri*) isolates was inhibited at temperatures above 45°C, while temperatures above 48°C were necessary to inhibit the mycelial growth. These results indicate that the standard HWT protocols may be sufficient to control BFD pathogens. Another studied carried out by Halleen and Fourie (2016) found that HWT (50°C for 30 min) of dormant vines (after uprooting) was highly efficient in the eradication of black-foot pathogens.

In spite of its efficacy to reduce BFD infection, HWT is linked to some reports of unacceptably high losses when long duration is applied to commercial batches of cuttings and rootlings (Bazzi et al. 1991; Wample 1997). Waite and Morton (2007) suggested that tolerance of plants to HWT is affected by the climate in which the cuttings are grown. Crocker et al. (2002) indicated that grapevine cuttings taken from vines grown in warm climates are of better quality than cuttings taken from vines grown in cool climates and are better able to withstand HWT. In this sense, Graham (2007)

showed that cuttings grown in cool climates in New Zealand were susceptible to damage at 50°C for 30 min. Therefore, the recommendation in New Zealand is to apply HWT at 48°C (Bleach et al. 2013; Billones-Baaijens et al. 2015). On the contrary, in Spain, Gramaje et al. (2009c, 2010, 2014) determined that 53°C for 30 min is the most effective treatment to reduce conidial germination and mycelial growth of BFD pathogens without detrimental effects to grapevine cuttings.

Use of resistant rootstocks

Planting disease-resistant rootstocks is a time-tested and sustainable approach for BFD management. The existing infection assays for BFD pathogens have indicated that grapevine rootstocks might show varying levels of susceptibility to pathogen infection, but no evidence of qualitative resistance to black-foot fungi has been found (Gubler et al. 2004; Alaniz et al. 2010; Brown et al. 2013). Gubler et al. (2004) reported 'O39-16' and 'Freedom' rootstocks as moderate resistant to *I. liriodendri* (formerly "*C." destructans*). Bleach et al. (2007) examined the susceptibility of six rootstocks to "*Cylindrocarpon"* spp. infection under field conditions and found that there was a difference in susceptibility to BFD depending on the amount of inoculum in the soil. Rootstocks '5C' and '3309' were more resistant in soils with heavy disease pressure when compared to 'Schwarzmann', '420A', 'Riparia Gloire' and '101-14' which were the least resistant to infection (Bleach et al. 2007). Alaniz et al. (2010) evaluated the most common used rootstocks in Spain ('110 R', '1103 Paulsen', '140 Ruggeri', '161-49 Couderc', '196-17 Castel', 'Fercal' and 'SO4') against *I. liriodendri* ("*C.*" *liriodendri*) and *D. macrodidyma* ("*C.*" *macrodidymum*), and found that all of them were affected by BFD in some degree, '110 R' being the most susceptible. The susceptibility of 'Millardet et de Grasset 101-14', 'Schwarzmann', '5C' and 'Riparia Gloire' rootstocks was tested by inoculating 4-month-old callused rooted cuttings with *Cyl. parva* conidial suspension in New Zealand (Brown et al. 2013). The authors found that disease incidence and severity were significantly affected by rootstock variety, 'Riparia Gloire' being more susceptible to fungal infection than 'Millardet et de Grasset 101-14' (Brown et al. 2013).

Chemical control

Hydration tanks containing drench water (pre-storage, pre- and post-grafting) and callusing rooms are an important focal point for BFD management strategies. However, the use of fungicides in nurseries is difficult since common chemicals used for the control of surface pathogens do not penetrate dormant grapevine cuttings sufficiently to control fungi inhabiting the vascular tissues. In despite of this handicap, fungicides are used worldwide during the propagation process, but with varying effectiveness (Gramaje and di Marco 2015; Gramaje et al. 2018).

Rego et al. (2006) assessed the *in vitro* effectiveness of 12 fungicides and two mixtures against four different isolates of *I. liriodendri* (formerly "*C." destructans*). These authors found that the most effective fungicide in reducing mycelial growth was prochloraz, followed by carbendazim + flusilazole, benomyl and cypronidil + fludioxonil. However, only cyprodimil + fludioxonil was also able to inhibit conidial germination. Azoxystrobin, trifloxystrobin and tolylfluanid were only capable of inhibiting conidial germination of *I. liriodendri.* Regarding the *in vivo* assay, Rego et al. (2006) found that the plant response to the fungicide was dependent on the *I. liriodendri* isolate used for the plant inoculation. Nascimiento et al. (2007) evaluated the effectiveness of tebuconazole, cyprodinil + fludioxonil and carbendazim + flusilazole against *I. liriodendri* confirming the efficacy previously reported by Rego et al. (2006).

Halleen et al. (2007a) performed an *in vitro* evaluation of different fungicides against *Campyl. fasciculare*, *I. liriodendri* (formerly *"C". liriodendri)*, *Campyl. pseudofasciculare* and *D. macrodidyma* (*"C." macrodidymum*), selecting the most efficient ones for further semi-commercial nursery trials. Prochloraz and benomyl were the only ones able to reduce mycelial growth of all four species. In nurseries, benomyl was relatively effective in reducing BFD incidence compared to prochloraz, flusilazol and imazalil.

Rego et al. (2009) conducted a field trial soaking natural BFD infected grapevine '1103 P' rootstock and 'Aragonez' (synon. 'Tempranillo') scion cuttings in several fungicide suspensions for 50 min prior to grafting. Results showed that only cyprodinil + fludioxonil reduced significantly the incidence and severity of *Cylindrocarpon*-like asexual morphs in a soil without nursery history. On the other hand, in a field with nursery history the disease pressure was very high and all treatments, except for

cyprodinil, significantly reduced the incidence and severity of BFD fungi (Rego et al. 2009).

Alaniz et al. (2011a) tested the *in vitro* efficacy of 14 fungicides against two isolates of *I. liriodendri* (formerly *"C." liriodendri*) and two isolates of *D. macrodidyma* (*"C." madrodidymum*). Carbendazim, hydroxyquinoline sulphate, imazalil, and prochloraz were the most effective fungicides in reducing mycelial growth in both species, while captan, copper oxicloride, didecyldimethylammonium chloride and thiram were the most effective to inhibit conidial germination. A pot assay was also conducted to measure the effect of the previous efficient fungicides on the rooting phase by dipping plant roots during 1 hour in fungicides and planting them in an artificially inoculated substrate. The results showed that except for imazalil in *D. macrodidyma* all fungicides significantly decreased the root disease severity values in both species compared with control treatment. In the case of the percentage of reisolation, all values were lower than those obtained for the control treatment, but only captan, carbendazim and didecyldimethylammonium chloride were significantly different in the case of the cuttings inoculated with *I. liriodendri*, and prochloraz in the case of those inoculated with *D. macrodidyma* (Alaniz et al. 2011a).

In Spain, only mancozeb is registered against BFD. However, Halleen et al. (2007a) demonstrated the ineffectiveness of this active ingredient to reduce the *in vitro* mycelial growth of several BFD fungi.

Biological control

Species of the genus *Trichoderma*, particularly *Trichoderma atroviride* and *T. harzianum,* have been the focus of attention of the majority of studies regarding biological control for GTDs in nurseries. *Trichoderma* commercial products can be found in different formulations, such as dowels, pellets, or powder. As there are diverse formulations, there are also different methods of application: dipping the basal ends in dry formulation, dipping the basal ends in hydrated formulation or periodical drenching in the fields. The application method used can influence in the colonization success (van Jaarsveld et al. 2019). The growth and establishment in the wood of *Trichoderma* spp. is key in the efficacy of the control treatment.

In a study carried out by Fourie et al. (2001) the stimulating properties of commercial products of *Trichoderma* were confirmed, finding five weeks after planting that the vines treated with *Trichoderma* had longer shoots than the control ones. Total root mass also increased significantly in the treated plants eight months after treatments. These authors also found an effect on natural infection of *Cylindrocarpon*-like asexual morphs, reducing the percentage of isolation from 2.8 to 1.6 in the roots of *Trichoderma* treated plants. Halleen et al. (2007a) did not observe an increased root or shoot mass in naturally BFD infected material with *T. harzianum* application. In addition, treatments resulted inefficient in reducing disease incidence, maybe due to the insufficient colonization of the basal ends of rootstocks.

Later on, Halleen and Fourie (2016) evaluated the efficacy of an integrated management of GTDs during the propagation process in nursery. The application of selected strains of *T. harzianum* by drenching scions and grafting unions in biological agent suspensions for 30 minutes at different nursery stages was evaluated among other treatments. Results showed that the treatment with *T. harzianum* by itself increased root mass significantly, and also that the colonisation of the basal ends and graft unions by *Trichoderma* was better when applied alone, without other control strategies. However, *Trichoderma* performed better in the integrated strategies, such as the application of benomyl before cold storage, HWT and Sporekill® before grafting and *Trichoderma* after grafting and before planting.

Different species of *Trichoderma* have been tested *in vitro* and *in vivo* against several isolates of *D. macrodidyma*, including *T. harzianum*, *T. asperellum*, *T. atroviride*, and *T. viride* (dos Santos et al. 2016). The results obtained in the *in vitro* experiment showed that all treatments inhibited mycelial growth, *T. harzianum* being the most efficient biocontrol agent. However, there were differences in the reduction due to a variability in the *D. macrodidyma* isolate response to the *Trichoderma* spp. In the *in vivo* assay, dos Santos et al. (2016) found that the plants treated with *Trichoderma* spp. exhibited less disease symptoms in shoots and roots, such as reduced vigour, reduction in root biomass, root rots, sudden wilting of the foliage, and vascular necrosis, than the positive control plants.

Berbegal et al. (2019) evaluated the long-term protection from nursery to vineyard provided by *T. atroviride* SC1 against fungal grapevine trunk pathogens, including black-

foot pathogens. The plants were treated three times during the grapevine propagation process: prior to grafting, during stratification and before planting. In both scenarios, nursery and field conditions, *T. atroviride* SC1 treated plants showed lower levels of BFD incidence and severity when compared with the untreated plants. However, the infection levels were very variable and, in some cases, too low to be conclusive regarding the percentage of reductions obtained.

Arbuscular mycorrhizal (AM) fungi have also been evaluated against BFD pathogens. *Rhizophagus irregularis* (formerly *Glomus intraradices*) was able to reduce not only disease incidence, but also root lesions in *V. rupestris* 'St. George' rootstock watered and inoculated with *D. macrodidyma* (formerly *"C". macrodidymum*) conidial suspension (Petit and Gubler 2006). Nevertheless, Holland et al. (2019) found that the inoculation of *R. irregularis* increased the presence of *I. liriodendri* when the rootstock *V. rupestris* 'Riparia Gloire' was inoculated by pruning the roots and submerging them in the pathogen conidial suspension.

On the other hand, bacteria such as *Bacillus subtilis* has also been *in vitro* and *in vivo* evaluated as biocontrol agent against different isolates of *D. macrodidyma* (dos Santos et al. 2016). Results showed that the mycelial growth inhibition was dependent on the fungal isolate and plants treated with *B. subtilis* in the *in vivo* trial showed less disease symptoms than the control.

Natural compounds

Up to date, chitosan is the only natural compound tested against BFD (Nascimento et al. 2007). These researchers evaluated its efficacy against *I. liriodendri* (formerly *N. liriodendri*) and other pathogens associated with GTDs. *I. liriodendri* was the less sensitive fungal species to chitosan application in the *in vitro* assay. The effect of chitosan was further evaluated and compared with those achieved by three fungicides in 'Castelão' potted plants grown in soil mixture artificially infested with spore dilutions. Chitosan significantly promoted the plant growth and the total number of roots of *I. liriodendri* infected plants, and simultaneously reduced the disease incidence, in comparison with control plants. The efficacy of chitosan was equivalent to that achieved by conventional fungicides (Nascimento et al. 2007).

Biofumigation

Biofumigation with *Brassica* spp. has been widely studied in the last 50 years, confirming their long allelopathic effects (Brown and Morra 1997; Rosa and Rodrigues 1999). Research conducted in Australia and New Zealand has shown promising results both *in vitro* and *in vivo* to control BFD pathogens (Bleach 2013; Barbour et al. 2014; Weckert et al. 2014). Bleach (2013) studied the *in vitro* effect of biofumigation treatments against *Cylindrocarpon*-like asexual morphs, finding that mycelium was less sensitive to the isothiocyanates released from the crushed mustard plants and seeds than conidia. In an *in vivo* assay, Bleach (2013) reported that biofumigation with mustard plants and ground seeds had some potential for improving control of BFD in grapevines. Disease severity in plants that were planted in soil that had been treated with mustard meal was significantly reduced compared to control plants (Bleach 2013). Barbour et al. (2014) found that there were differences in the mustard efficacy as biofumigant agent depending on the cultivar, standard brown mustard or cv. 'Caliente 199'. However, amending soil with either mustard cultivar did not change the overall dynamics of propagule conversion over time, but it significantly affected the numbers of conidia and chlamydospores recovered from conidial inoculum after different incubation times. Moreover, Weckert et al. (2014) observed that mustard seed meal, with and without glucosinolates, significantly improved the growth and yield parameters.

1.4. Current situation of Grapevine Trunk Diseases in Spain

As previously stated, the incidence of GTDs has been increasing over the last years in Spain (Martin and Cobos 2007; Pérez-Marín 2012). Some of these GTDs have been known in our country for a long time. For instance, Bellod (1947) reported the incidence of esca in Levante region at the beginning of the 20th century. Eutypa dieback was also reported in Badajoz by 1979 (Arias-Giralda 1998). Botryosphaeria dieback, Petri disease and BFD were first reported at the beginning of this century (Armengol et al. 2001a, 2001b). Up until now, 60 species belonging to 24 genera have been isolated from grapevines in Spain (Table 1.1).

Table 1.1. Species of fungi associated to GTDs isolated in Spain.

Disease	Causal agent	Reference
Esca Petri Disease	*Cadophora luteo-olivacea*	Gramaje et al. 2011b
	Cadophora viticola	Gramaje et al. 2011b
	Fomitiporia mediterranea	Armengol et al. 2001b
	Phaemoniella chlamydospora	Armengol et al. 2001b
	Phaeoacremonium cinereum	Gramaje et al. 2009b
	Phaeoacremonium fraxinopennsylvanicum	Gramaje et al. 2007
	Phaeoacremonium hispanicum	Gramaje et al. 2009b
	Phaeoacremonium inflatipes	Gramaje et al. 2009a
	Phaeoacremonium iranianum	Gramaje et al. 2009a
	Phaeoacremonium krajdenii	Gramaje et al. 2011a
	Phaeoacremonium minimum	Armengol et al. 2001b
	Phaeoacremonium parasiticum	Aroca et al. 2006
	Phaeoacremonium scolyti	Gramaje et al. 2008
	Phaeoacremonium sicilianum	Gramaje et al. 2009a
	Phaeoacremonium viticola	Aroca et al. 2008a
	Pleurostoma richardsiae	Pintos et al. 2016
	Stereum hirsutum	Armengol et al. 2001b
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. 2008b
	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
Phomposis dieback	*Diaporthe ambigua*	Guarnaccia et al. 2018
	Diaporthe ampelina	Martin and Cobos 2007
	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
BFD	*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 torresensis	Agustí-Brisach et al. 2013a
	Ilyonectria liriodendri	Alaniz et al. 2007
	Ilyonectria robusta	Martínez-Diz et al. 2018
	Neonectria sp.	Not published
	Thelonectria olida	de Francisco et al. 2009

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CHAPTER 2.

OBJECTIVES AND OUTLINE OF THE THESIS

As highlighted in the General introduction, black-foot disease (BFD) caused by a broad range of *Cylindrocarpon*-like asexual morphs is particularly important in grapevine nurseries and new plantations in wine-producing countries around the world, including Spain.

Black-foot disease pathogens have a serious effect on the health and long-term productivity of vines and young vineyards, and there are no effective control measures once they are established in the plant. Although vines can eventually acquire this disease during the propagation process in grapevine nurseries, infected soil in nurseries and young vineyards are a major source of BFD infection and can result in the failure of new vineyards soon after planting.

The constant identification and description of new *Cylindrocarpon*-like asexual morphs associated with BFD, the emergence of novel high-throughput sequencing technologies and the restriction on the use of fungicides all over the word, required the implementation of new research approaches to study BFD. Therefore, the aim of this thesis has been to gain a better understanding about the biology, ecology and control of BFD of grapevine.

Chapter 3 describes the characterization of *Cylindrocarpon*-like asexual morphs fungi isolated from visually symptomless vines and asymptomatic internal wood tissue of grafted plants. A semi-selective culture medium adapted from the literature was also developed to estimate BFD pathogens populations in soils and to examine how shifts in the abundance and composition of black-foot pathogens corresponded to changes in specific soil properties. Finally, the rhizosphere bacterial and fungal microbiota across grapevine rootstock genotypes was characterized, with special emphasis on the comparison between the relative abundances of sequences reads and DNA amount of BFD pathogens.

Chapter 4 evaluates the effect of combined soil and root treatments to control BFD of grapevine under field conditions. In particular, chemical and biological control, and biofumigation, were evaluated in this thesis.

Chapter 5 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 6** presents as concluding remarks, the most important achievements of this thesis.

CHAPTER 3.

BIOLOGY AND ECOLOGY

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Occurrence and diversity of black-foot disease fungi in symptomless grapevine nursery stock in Spain

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Abstract

In this study, 3,426 grafted grapevines ready to be planted from 15 grapevine nursery fields in Northern Spain were inspected from 2016 to 2018 for black-foot causing pathogens. In all, 1,427 isolates of black-foot pathogens were collected from the asymptomatic inner tissues of surface sterilized secondary roots and characterized based on morphological features and DNA sequence data of the nuclear ribosomal DNA-internal transcribed spacer region, histone H3, translation elongation factor 1-alpha and β-tubulin genes. Eleven species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* were identified, including *Dactylonectria alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, *Ilyonectria liriodendri*, *I. pseudodestructans*, *I. robusta*, *Neonectria quercicola, Neonectria sp.* 1 and *Thelonectria olida*. In addition, two species are newly described, namely *D. riojana* and *I. vivaria*. Twenty-four isolates representing 13 black-foot species were inoculated onto grapevine seedlings cultivar 'Tempranillo'. The pathogenicity tests detected diversity in virulence among fungal species and between isolates within each species. The most virulent species was *D. novozelandica* isolate BV-0760, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. This study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic phase of these pathogens in grapevines.

Keywords: *Cylindrocarpon*-like fungi, endophyte, Nectriaceae, systematics, *Vitis vinifera*, tree fruits, etiology, pathogen diversity.

Introduction

Endophytes are defined as organisms living in the internal tissues of plants that exhibit no visible symptoms as a result of the colonization (Hallmann et al. 1997). This endophytic relationship is often unnoticed due to the lack of symptoms in the plant and is usually only discovered by examining internal tissues with a microscope, by aseptic isolations of endophytes from plants, or from polymerase chain reaction (PCR)-based methods of DNA extracted from surface-sterilized plant tissues (Stone et al. 2000). Many fungal endophytes have been sought and characterized for their ability to produce biologically active secondary metabolites with potential applications in agriculture, medicine, and other areas (Wang and Dai 2011).

In grapevine, numerous studies have been conducted to analyze the endophytic fungal communities in different plant organs (Casieri et al. 2009; Martini et al. 2009; González and Tello 2011; Hofstetter et al. 2012; Cosoveanu et al. 2014; Pinto et al. 2014; Bruez et al. 2014, 2016; Dissanayake et al. 2018; Kraus et al. 2019). However, special focus has been given on the fungal microbiome on its woody tissues, due to the problems arising from grapevine trunk diseases (GTDs) (Casieri et al. 2009; Hofstetter et al. 2012; Bruez et al. 2014, 2016; Kraus et al. 2019). These diseases, namely esca, eutypa, Phomopsis and Botryosphaeria diebacks, as well as black-foot and Petri diseases, are some of the most destructive fungal diseases of grapevine in grape growing areas of the world. Management of GTDs has been intensively studied for decades with some great advances made in the understanding of the causal pathogens, their epidemiology, impact, and control (Gramaje et al. 2018).

Black-foot disease is a soil-borne disease caused by a broad range of *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* (Gramaje et al. 2018). Among these, *Dactylonectria torresensis* is the most frequent species in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Berlanas et al. 2017). Internal symptoms of black-foot diseased vines usually range from black, sunken, necrotic lesions on roots to reddish brown discoloration in the base of the rootstock trunk (Fourie and Halleen 2001). Foliar symptoms associated with black-foot disease are practically indistinguishable from those observed in grapevines affected by Petri disease and include delayed bud-break, chlorotic foliage with necrotic margins, overall stunting, and

wilting of leaves or entire shoots (Agustí-Brisach and Armengol 2013). They also resemble symptomatology 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. 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. 2006a). Black-foot disease pathogens have also been detected, identified and quantified in soil samples by PCR-based methods (Damm and Fourie 2005; Probst et al. 2010; Cardoso et al. 2013; Agustí-Brisach et al. 2014; Úrbez-Torres et al., 2015; Langenhoven et al. 2018) or by dilution plating technique together with the use of a semi-selective medium (Berlanas et al. 2017).

Much of the current knowledge on black-foot disease pathogens of grapevine has been derived from research based on populations isolated from vines displaying foliar or internal wood disease symptoms. Recent research in South Africa has also suggested that black-foot causing fungi could be latent in visually healthy grapevine nursery stock (Langenhoven et al. 2018). In Spain, grapevine nursery surveys had been previously conducted for the detection of black-foot pathogens (Alaniz et al. 2007; Agustí-Brisach et al. 2013); however, this research focused on these organisms as plant pathogens causing necrotic symptoms in grapevine tissues. Therefore, the objectives of this study were to (i) conduct extensive surveys in grapevine nurseries and identify the fungal species associated with black-foot disease from asymptomatic endorhizosphere tissues of externally asymptomatic plants based on morphological and molecular methods, and (ii) determine the pathogenicity of these fungal endophytic species on grapevine.

Materials and Methods

Nursery survey and fungal isolation

Isolates used in this study were collected from externally symptomless grafted plants of '110 Richter' rootstock ready to be planted from 15 nursery fields in Northern Spain from 2016 to 2018 (Table 3.1). Fungal isolations were made from the asymptomatic endorhizosphere tissue of secondary roots. For this purpose, sections of externally symptomless roots (2 cm long and 1-3 mm diameter) 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. The bark was carefully peeled out and the endorhizosphere tissue was plated on malt extract agar (MEA) (Conda Laboratories, Madrid, Spain) supplemented with 0.35 g liter^{-1} streptomycin sulphate (MEAS) (Sigma-Aldrich Laboratories, St. Louise, MO), and incubated at 25°C in darkness for 12 days. Colonies resembling black-foot disease pathogens were subcultured on potato dextrose agar (PDA) (Conda Laboratories). All isolates were single-spored in order to obtain pure cultures and stored in filter paper at -20°C. The incidence was determined as the mean percentage of grafted plants that were infected by black-foot fungi. The disease severity in infected grafted plants was determined as the mean percentage of root segments (10 segments per plant) that were colonized by these fungi.

Morphological identification and characterization

Cultures were grown on PDA, oatmeal agar (OA) (Sigma-Aldrich laboratories), and synthetic nutrient agar (SNA) (Sifin Diagnostics, Berlin, Germany) with or without two 1 cm^2 pieces of sterile filter paper on the medium surface (Crous et al. 2009). Plates were incubated at 25°C under near UV light with a 12 h photoperiod. To induce perithecia of new species, heterothallic and homothallic crosses were performed according to Cabral et al. (2012a). The length and width of 40 conidia were measured at ×1,000 magnification with a compound Nikon Eclipse Ni-e microscope. Minimum, maximum, mean and standard deviation were calculated from the measurements. Conidial color, shape, and the presence of septa were also recorded. Colony morphological characters were observed and colony colors were determined with the color chart of Rayner (1970).

Table 3.1. Location of the field nurseries surveyed and the incidence and severity of black-foot disease and fungal species isolated.

Location	Year of sampling	Plants analyzed	Incidence (%)[a]	Severity (%)[b]	Fungal species (No. of isolates)[c]
Larraga, Navarra	2016	10	60.0	13.3	*D. torresensis* (8)
Larraga, Navarra	2016	10	90.0	11.1	*D. torresensis* (10)
Mendavia, Navarra	2016	5	80.0	10.0	*D. torresensis* (4)
Mendavia, Navarra	2017	598	23.4	15.8	*D. macrodidyma* (25) *D. novozelandica* (26) *D. torresensis* (168) *I. liriodendri* (1) *T. olida* (1)
Mendavia, Navarra	2017	614	23.0	13.7	*D. macrodidyma* (21) *D. novozelandica* (12) *D. torresensis* (154) *I. liriodendri* (5) *I. robusta* (1)
Larraga, Navarra	2017	40	12.5	10.0	*D. macrodidyma* (2) *D. torresensis* (3)
Larraga, Navarra	2017	120	60.8	13.3	*D. alcacerensis* (8) *D. torresensis* (89)
Larraga, Navarra	2017	220	14.1	11.9	*D. alcacerensis* (1) *D. macrodidyma* (2) *D. torresensis* (33) *I. liriodendri* (1)
O Barco de Valdeorras, Galicia	2018	50	26.0	14.6	*D. macrodidyma* (5) *D. pauciseptata* (3) *D. torresensis* (8) *I. liriodendri* (2) *I. robusta* (1)
O Barco de Valdeorras, Galicia	2018	243	4.9	16.7	*D. alcacerensis* (2) *D. torresensis* (6) *I. liriodendri* (12)
Larraga, Navarra	2018	136	8.8	10.8	*D. torresensis* (13)
Larraga, Navarra	2018	120	60.0	16.1	*D. alcacerensis* (25) *D. torresensis* (91)
Logroño, La Rioja	2018	260	19.2	14.0	*D. alcacerensis* (4) *D. novozelandica* (6) *D. torresensis* (54) *D. riojana* sp. nov. (2) *I. liriodendri* (3)
Larraga, Navarra	2018	960	51.8	12.3	*D. alcacerensis* (2) *D. torresensis* (425) *I. liriodendri* (132) *I. pseudodestructans* (11) *I. robusta* (27) *I. vivaria* sp. nov. (3) *N. quercicola* (2) *N.* sp. 1 (1) *T.* olida (3)
Logroño, La Rioja	2018	40	12.5	18.0	*D. novozelandica* (2) *D. torresensis* (5) *N. quercicola* (2)

[a] Disease incidence of black-foot pathogens was determined as the percentage of grafted plants that were infected by these fungi.
[b] Disease severity in infected grafted plants was determined as the percentage of root segments (10 segments per plant) that were colonized by black-foot fungi.
[c] Identification of black-foot pathogens was first confirmed by sequencing part of the *his3* gene. (*D.*): *Dactylonectria*; (*I.*): *Ilyonectria*; (*T.*): *Thelonectria*, (*N.*): *Neonectria*

Molecular characterization and phylogenetic analyses

Total genomic DNA was extracted from fresh mycelium after 10 days of incubation on PDA using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's instructions. The identification of black-foot pathogens was made by sequencing part of the histone H3 (*his3*) gene. In addition, the internal transcribed spacer and intervening 5.8S gene (ITS) region, partial regions of the translation elongation factor 1-alpha (*tef1*), and the β-tubulin (*tub2*) genes were also sequenced. Each 25 µL reaction volume contained 12.5 µl of FastGene Ready Mix (NIPPON Genetics Europe, Dueren, Germany), 1 µL of each primer, 1 µL of DNA template (10 ng/ µL) and 9.5 µL sterile distilled water and PCR was performed using a C1000 touch thermal cycler (Bio-Rad Laboratories, Hercules, CA). For *his3*, ITS and *tub2* genes amplification conditions included an initial denaturation step of 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 minute. A final extension was performed at 72°C for 1 min. For *tef1* gene, amplification conditions were as follow: initial denaturation step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. Primers used were CYLHEF and CYLH3R (Crous et al. 2004b) for *his3*, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS, CylEF-1 (5'-ATGGGTAAGGAVGAVAAGAC-3'; JZ Groenewald, unpublished) and CylEF-R2 (Crous et al. 2004b) for *tef1*, and T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for *tub2*. PCR products were visualized in 1% agarose gels (agarose D-1 Low EEO, Conda Laboratories). PCR products were sequenced in both directions by Eurofins GATC Biotech (Cologne, Germany). Sequences were edited and aligned using the program MEGA v. 6 (Tamura et al. 2013). The alignments for each locus were combined in a single file using GenTools (https://github.com/aberlanas/gen-tools).

Fungal sequences from grapevines from Spain were aligned with published GenBank sequences, including ex-type specimens from grapevines and other hosts for

comparison using MAFFT sequence alignment program v. 6 (Katoh and Toh 2010) (Supplementary Table 3.1). Alignments were corrected visually and manually edited in Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Firstly, phylogenetic analyses were conducted on the *his3*, ITS, *tef1*, and *tub2* single-locus alignments for representative isolates obtained in this study. In addition, a multi-locus phylogenetic analysis was performed on the combined *his3*, ITS, *tef1*, and *tub2* datasets. A partition homogeneity test was conducted in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003). The congruence among the *his3*, ITS, *tef1*, and *tub2* datasets were tested at 1,000 replicates.

Maximum Likelihood (ML) and Bayesian analyses were performed on the concatenated alignment, while individual gene alignments were only subjected to ML analyses. ML analyses were performed in MEGA v. 6 (Tamura et al. 2013) using the best fit model as estimated with the Bayesian information criterion in jModelTest 2.1.10 (Darriba et al. 2012). Branch support was calculated from 1,000 bootstrap replicates for the single and concatenated datasets. The Bayesian analysis of the combined four-loci was performed with MrBayes v. 3.2.1 (Ronquist et al. 2012) based on the results of the jModelTest. The Markov Chain Monte Carlo sampling (MCMC) analysis of four chains started in parallel from a random tree topology (Rodríguez et al. 1990). The number of generations was set at 10 M and the run was stopped automatically when the average standard deviation of split frequencies fell below 0.01. Trees were saved each 1,000 generations. Burn-in was set at 25%, after which the likelihood values were stationary and the remaining trees were used to calculate posterior probabilities. *Campylocarpon fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679) were used as outgroups in phylogenetic analyses.

Representative black-foot disease isolate sequences obtained in this study were submitted to GenBank (Supplementary Table 3.2), and the alignments in TreeBASE under the study number 24347 (http://treebase.org), and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous et al. 2004a).

Effect of temperature on mycelial growth

Twenty-four fungal isolates, belonging to 13 different species were randomly selected for temperature-growth assay (Table 3.2). Five-mm diameter mycelial plugs

Table 3.2. Temperature-growth relationship for black-foot disease associated fungal isolates[1].

Species	Isolate	Adjusted model[2]				Temperature (°C)			Growth (mm/day)[4]
		R^2	*a*	*b*	*c*	Minimum	Maximum	Optimal[3]	
Dactylonectria alcacerensis	BV-1240	0.71	-0.0157	0.6266	-3.2011	10	25	19.9 ab	3.0 bc
D. alcacerensis	BV-1469	0.82	-0.0124	0.4985	-2.4377	10	30	20.1 ab	2.6 bcd
D.macrodidyma	BV-1366	0.80	-0.0154	0.6179	-3.0275	10	30	20.1 ab	3.2 bc
D. macrodidyma	BV-0535	0.79	-0.0141	0.5684	-2.7668	10	30	20.2 ab	3.0 bc
D. novozelandica	BV-0760	0.78	-0.0148	0.5834	-2.6809	10	30	19.8 ab	3.1 bc
D. novozelandica	BV-1369	0.75	-0.0177	0.7107	-3.3549	10	30	20.1 ab	3.8 bc
D. pauciseptata	BV-1354	0.83	-0.0169	0.6898	-3.2981	5	30	20.4 ab	3.7 bc
D. pauciseptata	BV-1360	0.73	-0.0215	0.8871	-4.7891	10	30	20.6 a	4.4 ab
D. riojana sp. nov.	BV-1396	0.92	-0.0107	0.4272	-2.0464	10	30	20.0 ab	2.2 d
D. riojana sp. nov	BV-1397	0.93	-0.0109	0.4351	-2.068	10	30	20.0 ab	2.3 cd
D. torresensis	BV-0901	0.78	-0.0135	0.5373	-2.6696	10	30	19.9 ab	2.7 bcd
D. torresensis	BV-0666	0.76	-0.0181	0.7081	-3.2878	10	30	19.6 ab	3.6 bc
Ilyonectria liriodendri	BV-1591	0.81	-0.0138	0.5362	-2.1454	5	30	19.4 ab	3.1 bc
I. liriodendri	BV-1642	0.80	-0.0173	0.668	-2.5882	5	30	19.3 ab	3.9 bc
I. pseudodestructans	BV-2142	0.84	-0.0099	0.3956	-1.8645	10	30	20.0 ab	2.1 d
I. pseudodestructans	BV-2609	0.71	-0.0158	0.6301	-3.2205	10	30	19.9 ab	3.0 bc
I. robusta	BV-1593	0.80	-0.0191	0.7121	-2.4659	5	25	18.6 b	4.2 ab
I. robusta	BV-1654	0.80	-0.0245	0.9523	-4.1827	10	30	19.4 ab	5.1 a
I. vivaria sp. nov.	BV-1924	0.78	-0.0173	0.6657	-2.5358	5	35	19.4 ab	3.9 bc
I. vivaria sp. nov.	BV-2305	0.77	-0.0184	0.7116	-3.0192	5	30	19.3 ab	3.9 bc
Neonectria quercicola	BV-2137	0.82	-0.0191	0.7274	-2.6044	5	35	19.0 ab	4.3 ab
N. quercicola	BV-1661	0.85	-0.0194	0.7427	-2.6372	5	30	19.1 ab	4.5 ab
Neonectria sp. 1	BV-2682	0.78	-0.0149	0.5603	-1.7684	5	30	18.8 b	3.5 bc
Thelonectria olida	BV-0537	0.76	-0.0133	0.5287	-2.5958	5	30	19.9 ab	2.7 bcd

[1] Data are the mean of eight replicates for each isolate. For each column, means with the same letter are not significantly different ($P = 0.05$) according to the Kruskal-Wallis test and the Dunn's test for multiple comparisons of mean ranks.

[2] Mycelial growth on potato dextrose agar at 5 to 35°C was adjusted to a second-degree polynomial model: $Y = aT^2 + bT + c$, in which Y = mycelial growth (mm/day); a, b, and c are the regression coefficients; and R^2 = coefficient of determination.

[3] Optimal temperature estimated by the adjusted model.

[4] Maximum growth rate estimated by the adjusted model

were transferred to the center of PDA plates, and incubated in darkness from 5 to 35°C at 5°C intervals. Colony diameter was recorded after 10 days in two orthogonal directions. Four replicate plates per isolate were used and the experiment was conducted twice. Regression curves were fitted to the values of temperature versus radial growth in millimeters per day for each isolate. The optimum temperature for radial growth and the maximum daily radial growth were calculated in the fitted equation for each isolate. Data were analyzed using the Kruskal-Wallis test and mean ranks of isolates were compared at $P = 0.05$ using Dunn's test.

Pathogenicity tests

Two-month-old grapevine seedlings (cv. 'Tempranillo') were used for pathogenicity tests. Seedlings were planted individually in 250 cm^3 plastic pots containing sterilized peat moss and inoculated when one to two leaves had emerged by immersing the roots in a 10^5 mL^{-1} conidial suspension for 1 hour (Martínez-Diz et al. 2018). The viability of the conidia was tested by spreading 150 µL of each conidial suspension diluted to 100 conidia mL^{-1} onto PDA plates and counting the resulting colonies after 2-3 days incubation at 25°C in the dark. Twenty-four fungal isolates, belonging to 13 different species were used (Table 3.3). Six seedlings per isolate were inoculated and control plants were inoculated with sterile distilled water. The experiment was repeated twice. Seedlings were maintained in a growth chamber at 25°C in a completely randomized design. Plants were inspected every morning from day 1 to day 61 for the development of foliar symptoms. However, due to the lack of specificity of foliar symptoms in grapevine seedlings, as well as the short time interval from foliar symptom development to plant death, the following parameters were considered: the mean time from inoculation in which the plant stayed alive (MTA) and the percent of dead plants. Two months after inoculation, all plants were uprooted and washed free of soil. Roots, crown and stem were aseptically plated on MEAS to re-isolate the black-foot disease fungal species as described above.

Statistical analysis

Homogeneity of variance across treatments was evaluated using Levene's test prior to the analysis of variance (ANOVA) (Box et al. 1978). The percentage of dead plants and

Table 3.3. Pathogenicity of black-foot pathogens on seedlings of *Vitis vinifera* cv. 'Tempranillo'[a]

Genera	Fungal species	Isolate	% of dead plants	MTA (days)[b]	% of isolation (n=12)
Dactylonectria	*D. novozelandica*	BV-0760	100 a	12.9 f	100
	D. alcacerensis	BV-1240	75.0 b	41.2 e	100
	D. macrodidyma	BV-1366	66.7 bc	56.4 ab	100
	D. macrodidyma	BV-0535	41.7 cd	54.9 abc	83.3
	D. pauciseptata	BV-1360	41.7 cd	47.3 cde	100
	D. riojana sp. nov	BV-1397	41.7 cd	57.5 ab	100
	D. alcacerensis	BV-1469	33.3 cd	49.5 bcde	75
	D. riojana sp. nov.	BV-1396	33.3 cd	49.8 bcde	100
	D. torresensis	BV-0901	25.0 cde	58.2 ab	100
	D. novozelandica	BV-1369	16.7 cdef	57.4 ab	90.9
	D. pauciseptata	BV-1354	8.3 ef	55.4 abc	83.3
	D. torresensis	BV-0666	8.3 ef	56.3 ab	100
Ilyonectria	*I. vivaria* sp. nov.	BV-1924	50.0 c	53.3 abcd	100
	I. vivaria sp. nov.	BV-2305	50.0 c	49.8 bcde	41.7
	I. liriodendri	BV-1591	25.0 cde	60.3 a	100
	I. pseudodestructans	BV-2142	25.0 cde	56.2 ab	83.3
	I. liriodendri	BV-1642	16.7 cde	54.5 abc	91.7
	I. robusta	BV-1654	16.7 cde	59.3 a	83.3
	I. pseudodestructans	BV-2609	8.3 ef	59.0 a	75
	I. robusta	BV-1593	8.3 ef	60.5 a	66.7
Neonectria	*Neonectria* sp. 1	BV-2682	25.0 cde	55.3 abc	0
	N. quercicola	BV-2137	8.3 ef	60.4 a	50
	N. quercicola	BV-1661	0.0 f	61.0 a	100
Thelonectria	*T. olida*	BV-0537	58.3 c	45.4 de	100
	Control		10.4 ef	59.4 a	0

[a]For each column, means with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test

[b]MTA: Mean time from inoculation in which the plant stayed alive

the MTA data were subjected to ANOVA factorial design with the following factors: experiment (performed twice), pathogen and isolate nested in pathogen (two isolates for each pathogen species except for *Neonectria* sp. 1 and *Thelonectria olida*). Means were compared by Duncan's multiple range test at $P = 0.05$ using IBM SPSS Statistics 19.0.

Results

Incidence of black-foot disease pathogens

Based on colony morphology, conidial characteristics and phylogenetic analyses (see below), 1,427 isolates associated with 13 species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* were identified. *Dactylonectria torresensis* was isolated from grafted plants in all grapevine nursery fields, and accounted for 75.05% of all isolates (Table 3.1). The remaining isolates were identified as *D. macrodidyma* (3.85%), *D. novozelandica* (3.22%), *D. alcacerensis* (2.94%), *D. pauciseptata* (0.21%), *Dactylonectria* sp. (0.14%), *Ilyonectria liriodendri* (10.93%), *I. robusta* (2.03%), *I. pseudodestructans* (0.8%), *I.* sp. (0.21%), *Neonectria* sp. 1 (0.06%), *N. quercicola* (0.28%), *and T. olida* (0.28%). In all nursery fields surveyed, incidence ranged from 4.9 to 90%, while disease severity ranged from 10 to 18%.

Morphological characterization

The color of the colonies ranged from pale buff to chestnut or cinnamon to vinaceous on PDA, and from buff to chestnut or brown to cinnamon on OA. Based on the microscopic observations, almost all the isolates produced macroconidia, microconidia and chlamydospores which matched descriptions for *Cylindrocarpon*-like asexual morphs. Based on the morphological features, it was possible to separate the isolates into three different groups among black-foot disease isolates. The first consisted of isolates showing 5-septate, cylindrical, and generally straight macroconidia, and microconidia were ellipsoidal to oblong and no chlamydospores were observed. These characteristics matched those described earlier for the genus *Thelonectria* (Chaverri et al. 2011). The second group of isolates had 5-septate, fusiform and curved macroconidia. Microconidia and chlamydospores were uncommon. These matched descriptions for the genus *Neonectria* (Chaverri et al. 2011). The third consisted of

isolates showing 1 to 3-septate, cylindrical, and generally straight macroconidia. Microconidia were ellipsoidal to ovoid. These characteristics matched those described earlier for the genera *Dactylonectria* (Lombard et al. 2014) or *Ilyonectria* (Chaverri et al. 2011). According to Lombard et al. (2014), *Dactylonectria* can be distinguished from *Ilyonectria* by the characteristics of the sexual morph as well as the absence of chlamydospore production in culture.

Molecular characterization and phylogenetic analyses

The AIC best-fit nucleotide substitution model identified by jModelTest was Hasegawa-Kishino-Yano model (HKY) with gamma distributed with invariant sites rates (G+I) for *his*3 and ITS and HKY+G for *tef*1 and t*ub*2. In the *his*3, *tub2* and *tef1* single-locus alignments, three major clades belonging to the genera *Ilyonectria*, *Neonectria* and *Dactylonectria* were obtained (Figure 3.1, Supplementary Figures 3.1 and 3.2). Looking at individual gene trees obtained using the model proposed by jModelTest, the *his*3 tree enables the separation of all species with high bootstrap values (Figure 3.1). For *tub*2, all species could be resolved, except *Dactylonectria novozelandica* (Supplementary Figure 3.1). Individual *tef1* gene phylogeny revealed a lack of resolution for some species, including *I. lusitanica*/*I. europaea*, *I. rufa*/*I. panacis*, *I. crassa*/*I. pseudodestructans*/*I. protearum*, and *D. vitis*/*D. hispanica* (Supplementary Figure 3.2). The ITS tree does not resolve any species, as nucleotide sequences were indistinguishable for this region.

The partition homogeneity test (P = 0.112) led us to combine the *his3*, ITS, *tef1*, and *tub2* datasets (Figure 3.2). The ML tree and the Bayesian consensus tree had similar topology, and therefore only the ML tree is presented with bootstrap support values (BS) and posterior probability values (PP). The four loci alignment contained 91 taxa (including the two outgroups). The ML tree and the Bayesian consensus tree obtained with the four-loci alignment confirmed the existence of two novel taxa within our set of isolates.

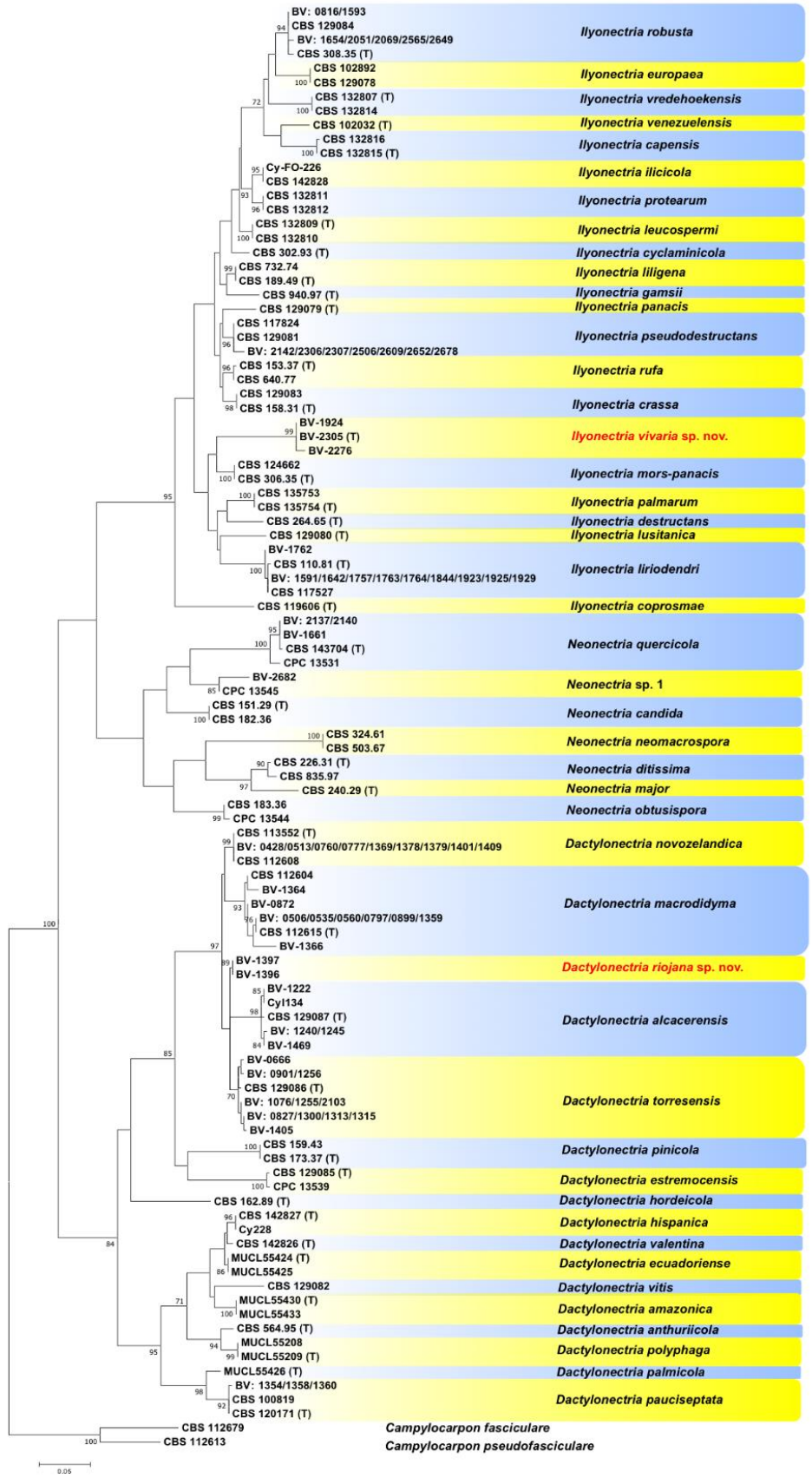

***Figure 3.1**. Maximum likelihood phylogeny of Cylindrocarpon-like asexual morphs as estimated from the alignment of the histone H3 gene sequences. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to Campylocarpon fasciculare (CBS 112613) and Ca. pseudofasciculare (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. Tentative new species are indicated in red.*

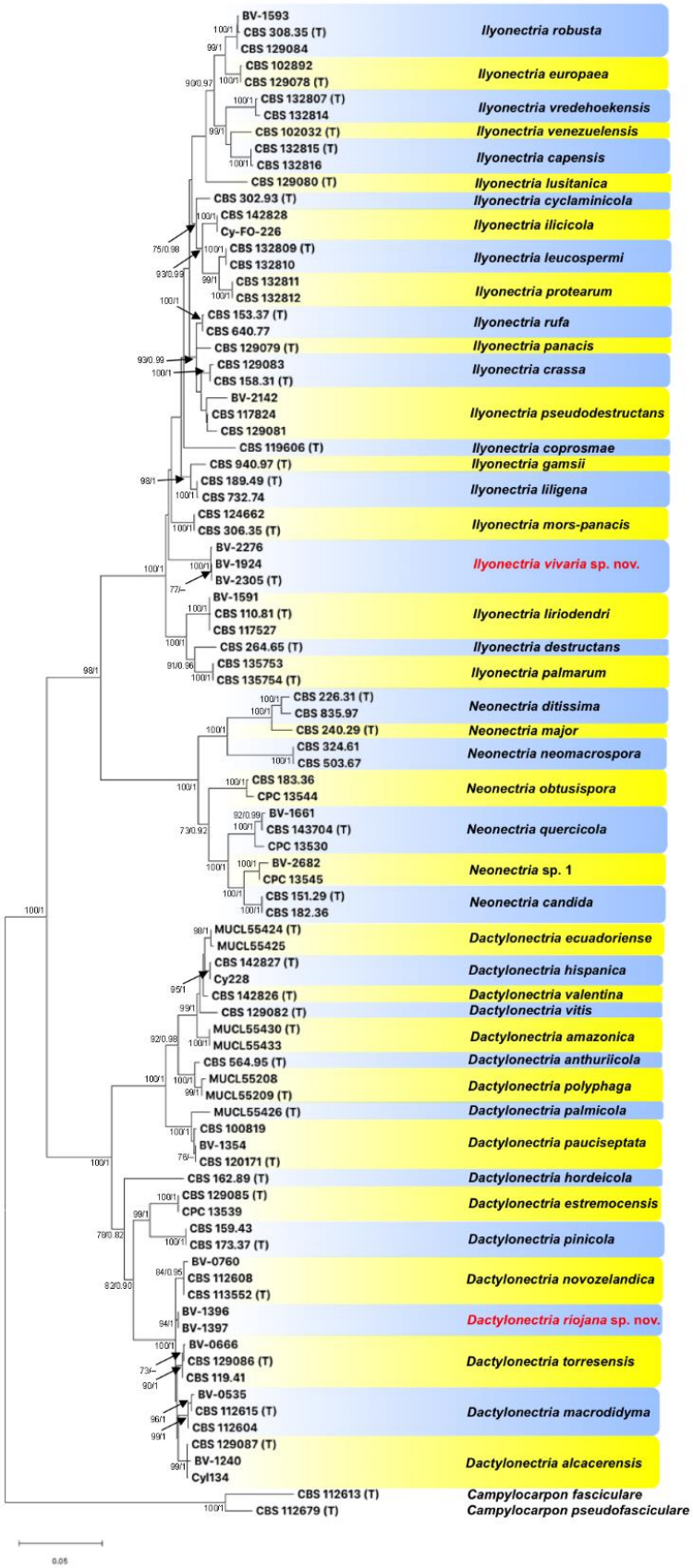

***Figure 3.2**. Maximum likelihood phylogeny of Cylindrocarpon-like asexual morphs as estimated from concatenated alignments of four gene dataset (ITS, tub2, his3, and tef1). Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes (ML/PP). Support values less than 70% bootstrap or 0.80 posterior probability are omitted or indicated with '–'. The tree was rooted to Campylocarpon fasciculare (CBS 112613) and Ca. pseudofasciculare (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. New species are indicated in red.*

Taxonomy

Based on the phenotypical characters previously recorded along with the phylogenetic analysis, one species each of *Ilyonectria* and *Dactylonectria* are described (Figures 3 and 4). No perithecia were observed in the heterothallic or homothallic crosses performed.

Dactylonectria riojana *C. Berlanas, M. Andrés-Sodupe, S. Ojeda & D. Gramaje, sp. nov. MycoBank MB 829945* (Figure 3.3). Etymology: name refers to the Spanish region of La Rioja, where the fungus was isolated.

Diagnosis: Morphologically *D. riojana*, can be distinguished by its shorter conidiophores when compared with *D. novozelandica*, *D. torresensis*, *D. alcacerensis* and *D. macrodidyma.* Nineteen polymorphisms can distinguish *D. riojana* from *D. alcacerensis*: two in *tub*2 locus, eleven in *his*3 locus, four in *tef*1 locus and two in ITS. Seventeen polymorphisms can distinguish *D. riojana* from *D. torresensis*: one in *tub*2 locus, four in *his*3 locus, ten in *tef*1 locus and two in ITS. Eleven polymorphisms can distinguish *D. riojana* from *D. novozelandica*: seven in *his*3 locus, two in *tef*1 locus and two in ITS. Twenty-three polymorphisms can distinguish *D. riojana* from *D. macrodidyma*: three in *tub*2 locus, thirteen in *his*3 locus and seven in *tef*1 locus.

Typus: Spain: La Rioja, Logroño, on roots of '110 Richter' grapevine rootstock (*Vitis berlandieri* x *Vitis rupestris*), 2018, C. Berlanas (CBS H-23883 – holotype; CBS 145413= BV-1396 – ex-type culture).

Conidiophores simple. Complex conidiophores not observed. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to three phialides, 1- to 2- septate, 35 to 65 µm long; phialides monophialidic, more or less cylindrical, tapering slightly towards the apex, 12 to 29 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.5 µm at the widest point, 1.5 to 3 µm near the aperture.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow towards the tip, more or less broadly rounded, and the base appearing acute due to the presence of the hilum; 1-septate (25–) 30 to 35 (–47) × (6.5–) 7 to 8 (–8.5) µm (av. 33 × 8 µm), L/W ratio (3–) 3.8 to 4.5 (–6) (av. 4.2), 2-septate (30–) 35 to 39 (–51) × (6–) 7 to 8.5 (–9.5) µm (av. 39 × 8 µm), L/W ratio (3–) 4 to 5 (–7) (av. 4.5), and 3-septate (36–) 44 to 48 (–55) × (7–) 7.5 to 8.2 (–9) µm (av. 45 × 8 µm), L/W ratio (4–) 4.5 to 6 (–7) (av. 5.5).

Microconidia 0-1 septate, aseptate microconidia subglobose to oval, (9–) 10 to 13.5 (–16.5) × (5–) 6 to 6.5 (–7) (av. 12 × 6 µm), L/W ratio (1.5–) 1.8 to 2.2 (–2.4) (av. 2); 1-septate, mostly subcylindrical, (12.5–) 18 to 21 (–23.5) × (6–) 7 to 8 (–8.5) (av. 20.3 × 7 µm), L/W ratio (2–) 2.5 to 2.8 (–3.2) (av. 2.7). Chlamydospores rarely observed.

Culture characteristics: Mycelium felty with average density (OA) or strong density (PDA). Surface on OA straw to buff; margin buff. Surface on PDA honey to buff; margin buff. Zonation absent, transparency homogenous and margin uneven (OA) and even (PDA). Reverse similar to surface, except in color, buff to sepia on OA and sepia to chestnut on PDA. Colonies reaching a radius of 26.8 mm after 7 d at 25°C. Minimum temperature for growth 10°C, optimum 20°C, maximum 30°C.

Host and distribution: *Vitis berlandieri* x *Vitis rupestris* (roots of '110 Richter' grapevine rootstock) (Spain, La Rioja).

Notes: *D. riojana* is closely related to *D. novozelandica*, *D. torresensis*, *D. alcacerensis* and *D. macrodidyma*. The morphology of these species is very similar, but *D. riojana* can be distinguished by its shorter conidiophores and the inability to grow at 5°C. No chlamydospores were observed in *D. riojana*, while these structures rarely occur in *D. novozelandica*, *D. torresensis* and *D. alcacerensis*, or sometimes occur in *D. macrodidyma*.

Ilyonectria vivaria *C. Berlanas, B. López-Manzanares, R. Bujanda & D. Gramaje, sp. nov. MycoBank MB 829951* (Figure 3.4). Etymology: Latin, from vivarium, meaning nursery. In reference to the environment it was frequently isolated from.

Diagnosis: Morphologically *I. vivaria*, can be distinguished by its slower growth at 25°C and the ability to grow at 35°C when compared with *I. mors-panacis.* Seventy six polymorphisms can distinguish *I. vivaria* from *I. mors-panacis*: seventeen in *tub*2 locus, thirty two in *his*3 locus, twenty five in *tef*1 locus and two in ITS.

Typus: Spain: Navarra, Larraga, on roots of '110 Richter' grapevine rootstock (*Vitis berlandieri* x *Vitis rupestris*), 2018, C. Berlanas (CBS H-23884 – holotype; CBS 145414 = BV-2305 – ex-type culture).

Conidiophores simple or complex. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to four phialides, 1- to 4- septate, 42 to 165 µm long; phialides monophialidic, more or less

cylindrical, tapering towards the apex, 21 to 47 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.0 µm at the widest point, 1.5 to 2.5 µm near the aperture.

Complex conidiophores aggregated in small sporodochia, repeatedly and irregularly branched; phialides cylindrical, but tapering towards the apex, 10 to 25 µm long, 1.5 to 2.5 µm wide at the base, 2.0 to 2.5 µm at the widest point, and 1.5 to 2.0 µm wide at the apex.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow towards the tip, more or less broadly rounded, and base sometimes with a visible, centrally hilum; 1-septate (22–) 27 to 34 (–41) × (6–) 6.5 to 8.0 (–9) µm (av. 31 × 7.5 µm), L/W ratio (3–) 3.5 to 4.2 (–6) (av. 4), 2-septate (26–) 33 to 38 (–47) × (6–) 7.0 to 8.0 (–9) µm (av. 35 × 7.5 µm), L/W ratio (3–) 4 to 5.5 (–7) (av. 4.5), and 3-septate (33–) 45 to 51 (–56) × (7–) 7.4 to 8 (–8.5) µm (av. 47 × 7.5 µm), L/W ratio (4–) 4.5 to 6.5 (–7) (av. 5).

Microconidia 0-1 septate, aseptate microconidia ellipsoidal to oval, (8–) 9 to 11.5 (–13.5) × (4.5–) 5.5 to 6.5 (–7) (av. 10.5 × 6 µm), L/W ratio (1.5–) 1.5 to 2 (–2.2) (av. 1.7); 1-septate, mostly ellipsoidal (12–) 15 to 21 (–18.5) × (5.5–) 7 to 8 (–8.5) (av. 18 × 6.5 µm), L/W ratio (1.5) 2.5 to 3 (–3.3) (av. 2.8). Chlamydospores globose to cylindrical, 10 to 17 × 10 to 20 µm diam., smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches, rarely intercalary, single, in chains or in clumps, hyaline, becoming medium brown.

Culture characteristics: Mycelium cottony to felty with average density in both OA and PDA. Surface on OA honey to buff; margin buff. Surface on PDA buff, to honey to isabelline towards the centre; margin buff. Zonation concentric (PDA) or absent (OA), transparency homogenous and margin uneven (OA) and even (PDA). Reverse similar to surface, except in color, buff to cinnamon on OA and chestnut to cinnamon on PDA. Colonies reaching a radius of 27.3 mm after 7 d at 25°C. Minimum temperature for growth 5°C, optimum 19.4°C, maximum 35°C.

Host and distribution: *Vitis berlandieri* x *Vitis rupestris* (roots of ‘110 Richter’ grapevine rootstock) (Spain, Navarra).

Notes: *I. vivaria* is closely related to *I. mors-panacis* based on the *his*3 sequence alignment. The morphology of both species is very similar, but *I. vivaria* can be distinguished by its slower growth after 7 d at 25°C and the ability to grow at 35°C.

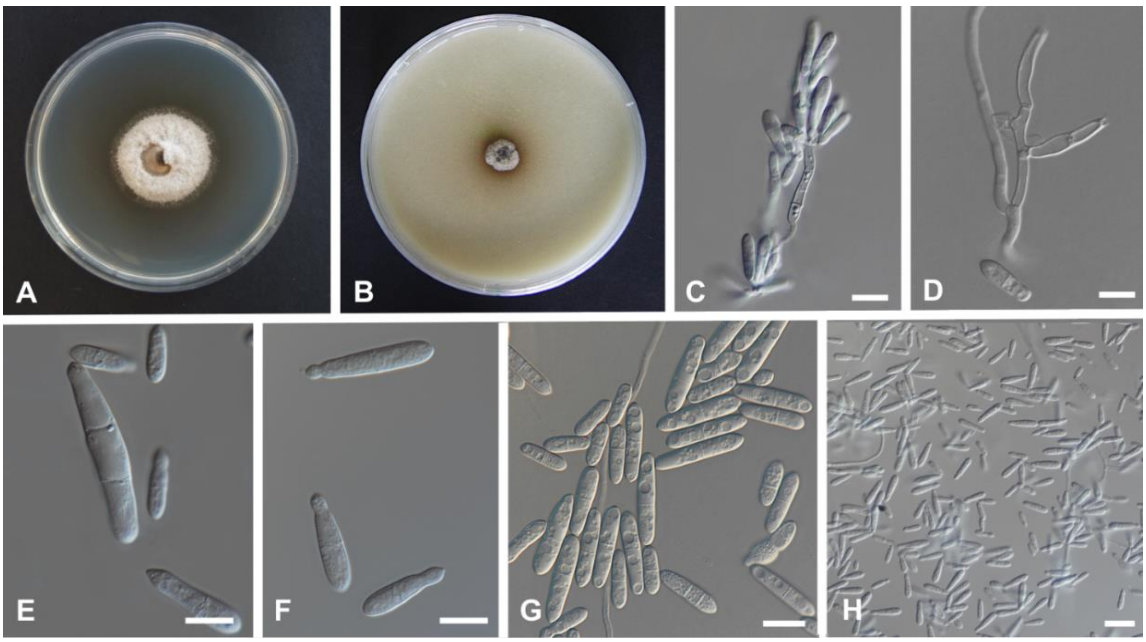

***Figure 3.3**. Dactylonectria riojana (ex-type culture BV-1396). Ten-day-old colonies grown at 20ºC in darkness on potato dextrose agar (PDA) (**A**) and oatmeal agar (OA) (**B**). **C-D**, Simple, sparsely branched conidiophore of the aerial mycelium. **E-H**, Micro- and macroconidia. Scale bars: C-F = 10 µm; G = 20 µm; H = 50 µm.*

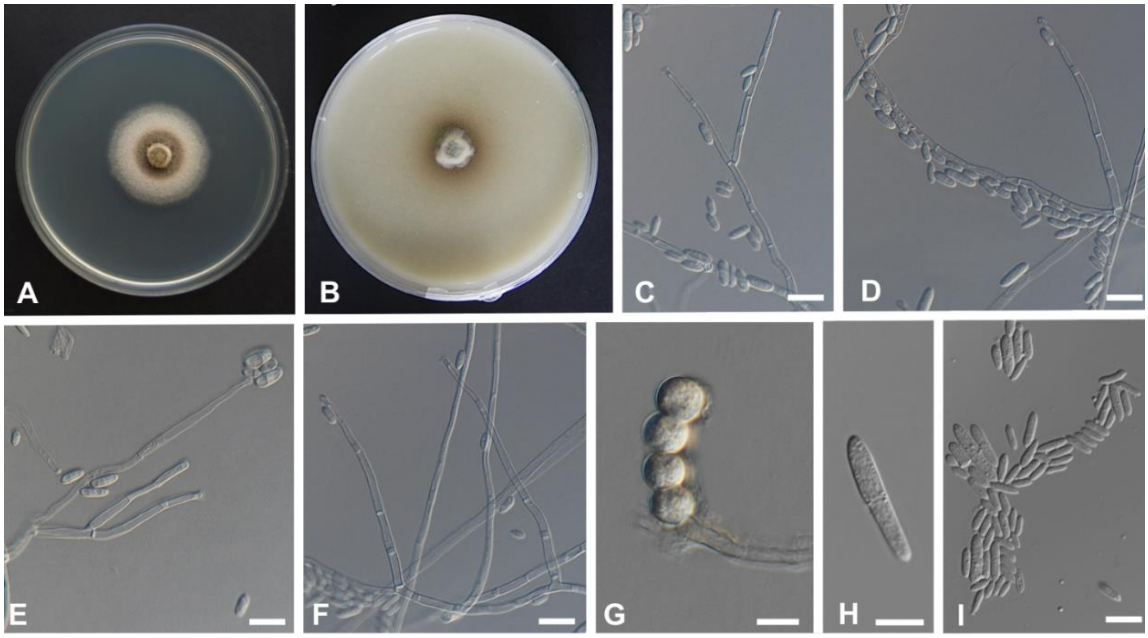

***Figure 3.4**. Ilyonectria vivaria (ex-type culture BV-2305). Ten-day-old colonies grown at 20ºC in darkness on potato dextrose agar (PDA) (**A**) and oatmeal agar (OA) (**B**). **C-F**, Simple, sparsely branched conidiophore of the aerial mycelium. **G**, Chlamydospores in mycelium. **H-I**, Micro- and macroconidia. Scale bars: C-I = 10 µm.*

Effect of temperature on mycelial growth

Analyses of variance indicated no significant differences of the mycelial growth between experiments ($P = 0.4115$), so data from both experiments were averaged. Most of the isolates studied failed to grow on PDA at 35°C, with the exception of *N. quercicola*

isolate BV-2137 and *I. vivaria* sp. nov isolate BV-1924, while some of them grew at 5°C (*D. pauciseptata* isolate BV-1354, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov isolates, *N.* sp. 1 isolate BV-2682, *N. quercicola* isolates, and *T. olida* isolate). Optimal temperatures for mycelial growth ranged from 18.6 to 20.6°C (Table 3.2). Significant differences were found in the optimal temperature between *D. pauciseptata* isolate BV-1360, and *Neonectria* sp. 1 isolate BV-2682 and *I. robusta* isolate BV-1593 ($P < 0.05$), but it was not possible to statistically differentiate the remaining isolates, because of the overlap in the optimal temperature measurements among them (Table 3.2). According to the Kruskal-Wallis test, maximum growth rates of isolates differed significantly ($P < 0.05$). The relationship between growth rate and temperature for all isolates was best described by a second-degree polynomial ($Y = aT^2 + bT + cT$). The three regression coefficients were highly significant in all cases ($P < 0.01$). The coefficient of determination (R^2) ranged from 0.71 to 0.93. Isolates with maximum growth rate $\leq$ 3 mm/day included *D. alcacerensis* isolates, *D. macrodidyma* isolate BV-0535, *D. riojana* sp. nov. isolates, *D. torresensis* isolate BV-0901, *I. pseudodestructans* isolates and *T. olida* isolate. Isolates with maximum growth rate between 3 and 5 mm/day included *D. macrodidyma* isolate BV-1366, *D. novozelandica* isolates, *D. pauciseptata* isolates, *D. torresensis* isolate BV-0666, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov. isolates, *N. quercicola* isolates, and *N.* sp. 1 isolate. The only species that grew more than 5 mm/day was *I. robusta* (isolate BV-1654).

Pathogenicity tests

In all fungi, the viability of conidia was at least 90%. For each species, there were differences between pathogen isolates when evaluating the percent of dead plants and the MTA ($P < 0.05$), so the data of isolates within species were not combined. Grapevine seedlings did not show any foliar symptoms during the experiment; however, the susceptibility to black-foot disease fungi measured as the percent of dead plants and the MTA varied among fungal isolates (Table 3.3). *Dactylonectria novozelandica* isolate BV-0760 was the most virulent species followed by *D alcacerensis* isolate BV-1240, *D. macrodidyma* isolate BV-1366 and *I. vivaria* sp. nov. isolates when measuring the percent of dead plants.

Grapevine seedlings inoculated with *D. novozelandica* isolate BV-0760 had significantly less MTA than those inoculated with other fungal species. *D. alcacerensis* isolates, *D. pauciseptata* isolate BV-1360, *D. riojana* sp. nov. isolate BV-1396, *I. vivaria* sp. nov. isolate BV-2305 and *T. olida* differed significantly with respect to the control when measuring the MTA. Percentage of re-isolation ranged from 41.7 to 100%. *Neonectria* sp. 1 was not successfully isolated from any inoculated seedling. No black-foot disease pathogens were isolated from the control treatment (Table 3.3).

Discussion

This study is the first comprehensive effort to characterize a group of fungi associated with GTDs isolated from visually symptomless vines and asymptomatic internal wood tissue. Our results demonstrate that black-foot disease fungi can live as latent pathogens within grapevine and might become pathogenic under specific conditions. The pathogenicity of black-foot disease fungi on grapevines have been so far demonstrated in tests performed under field (Sieberhagen 2016) and controlled conditions (Gubler et al. 2004; Jaspers et al. 2007; Alaniz et al. 2010; Cabral et al. 2012b; Brown et al. 2013; Probst et al. 2019). Recent studies reported the sporadic occurrence of other trunk disease fungi in grapevine as latent pathogens (Hofstetter et al. 2012, Eichmeier et al. 2018), without any disease symptoms ever becoming evident. Most of these studies employed molecular tools to analyze the grapevine fungal microbiome without the need to cultivate the fungal isolates. However, functions such as mutualism or pathogenicity in fungi can rarely be predicted by using these tools (Brader et al. 2017).

Several factors have been reported to be determinants in triggering pathogenicity in an endophyte that was previously asymptomatic, such as the host genotype, changes in plant gene expression, nutrient status, habitat or the locally occurring abiotic stress that might reduce host fitness, resulting in distortion of this delicate balance and thus influencing the symptomatology in plants (Johnson and Oelmüller 2009). Abiotic stress factors in grapevine nursery fields and new plantations include J-rooting, waterlogging, water stress, winter-kill, nutrition deficiency, soil compaction and/or overcropping (Gramaje et al. 2018).

In the scientific literature, observations of black-foot disease fungi as endophytes colonizing asymptomatic vines (Langenhoven et al. 2018) or other plant species (Agustí-

Brisach et al. 2011; Bonito et al. 2016; 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). We therefore suggest that these endophytic associations among black-foot disease fungi and symptomless hosts are not unusual relationships in nature. The fact that the black-foot disease fungi can be endophytes on weeds or other hosts has important implications, such as symptomless plants inadvertently serving as sources of hidden diversity of black-foot species, or serving as inoculum reservoirs. Because most research conducted on fungal trunk diseases has focused on pathogens infecting important agricultural commodities such as grapevine, almond or olive (Gramaje et al. 2016), we still lack a thorough understanding of the true nature of the associations of these fungi with other plants and their environment. In addition, the effects of weeds and/or other symptomless hosts on the genetic composition of black-foot disease pathogen populations have seldom been explored and thus remain poorly understood.

A wide diversity of black-foot disease pathogens were identified in this study, bringing the total number of fungal species associated with this disease isolated from grapevines in Spain to 17. These results confirm the richness of black-foot species in the Iberian Peninsula, with 14 species reported in Portugal (Cabral et al. 2012a, 2012c; Reis et al. 2013), compared to other countries in which black-foot disease is also prevalent, such as South Africa with 8 species (Halleen et al. 2004, 2006b; Langenhoven et al. 2018), New Zealand with 7 species (Bleach et al. 2006; Pathrose 2012), and Canada (Úrbez-Torres et al. 2014) and Italy (Carlucci et al. 2017) with 5 species each. Our findings also confirm *Dactylonectria* as a genus commonly associated with infections in grapevine as reported previously by Lombard et al. (2014), and *D. torresensis* being the most prevalent black-foot species in Spain. This study represents the first report of *I. pseudodestructans* and *N. quercicola* on grapevine in this country. Hosts and distribution of *I. pseudodestructans* include Kentucky bluegrass (Canada), *Quercus* sp. (Austria) and grapevine (Portugal). To our knowledge, *N. quercicola* has been reported only in holm oak root seedlings showing decline aerial symptoms in forest nurseries in Spain (Mora-Sala et al. 2018).

Two novel species are newly described, namely *D. riojana* and *I. vivaria*. Morphological characteristics have been reported to play a major role in the description of fungal species (Taylor et al. 2000). However, in our study, the use of such characters alone to delimit the new species was insufficient, thus highlighting the usefulness of DNA sequences for such purpose. The *his3* region has previously shown to be the most informative locus for the correct identification of black-foot disease fungi (Cabral et al. 2012a). In our study, the use of the *his3, tub*2 and *tef1* alone allowed us to describe these two novel species. However, the ITS tree could not resolve any species, as nucleotide sequences were indistinguishable for this region. These results are in agreement with previous studies aimed to characterize *Cylindrocarpon*-like fungi by using individual ITS dataset (Cabral et al. 2012c; Úrbez-Torres et al. 2014). Despite the ineffectiveness in resolving these fungal species, the ITS region has been widely used in multilocus sequence analyses of *Cylindrocarpon*-like fungi (Cabral et al. 2012a; Úrbez-Torres et al. 2014; Carlucci et al. 2017; Parkinson et al. 2017; Mora-Sala et al. 2018). The multilocus sequence analysis using *his3*, ITS, *tef1*, and *tub2* regions confirmed the level of polymorphism that enabled the fungal description.

Different inoculation methods, such as watering (Alaniz et al. 2009) or soaking (Martínez-Diz et al. 2018) the roots of grapevine seedlings in conidial suspensions, soaking bases of grapevine rootstock cuttings (Alaniz et al. 2010; Cabral et al. 2012b; Probst et al. 2019) or roots (Pathrose et al. 2014) in conidial suspensions, and vacuum-inoculation of conidial suspensions throughout the vascular system of rootstock cuttings (Sieberhagen 2016) have been used for virulence screening of black-foot disease fungi. Results of our study show that soaking roots of grapevine seedlings is a rapid and effective technique for evaluating virulence of *Cylindrocarpon*-like asexual morph isolates since most of the fungi collected from asymptomatic vines were capable of colonizing roots and causing disease under controlled conditions. Virulence varied among species and between isolates within each fungal species. High degree of virulence variability was also noticed in the 14 *D. macrodidyma* isolates (Alaniz et al. 2009) and 17 *I. liriodendri* isolates (Pathrose et al. 2014) collected in Spain and New Zealand, respectively. Subsequent taxonomic studies suggested that the *D. macrodidyma* isolates used by Alaniz et al. (2009) might represent separate species within the *D. macrodidyma*-species complex (Cabral et al. 2012b). In our study, *D.*

novozelandica isolate BV-0760 was considered the most virulent species, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. Cabral et al. (2012b) performed pathogenicity test with 60 isolates belonging to 14 black-foot species collected from grapevine and other hosts and concluded that *I. lusitanica*, *D. estremocensis* and *I. europaea* were more virulent to grapevine than to the species previously accepted as the main causal agents of black-foot disease, namely *D. macrodidyma* and *I. liriodendri*.

Pathogenicity in fungal trunk pathogens of grapevine is a complex phenomenon. The combination of many factors, such as pathogen and host genotypes and abiotic and other environmental stresses, as well as microbial interactions, seems to determine the outcome of the reaction of grapevine to the pathogen. The present study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic role of these pathogens on grapevines. The future research on black-foot disease needs to investigate (i) how these pathogens colonize the endorhizosphere and establish themselves inside, and (ii) what triggers latent black-foot disease fungi to transition from an endophyte to a pathogen, and cause disease symptoms in grapevine.

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Supplementary material

Supplementary Tables

Supplementary Table 3.1. Black-foot disease fungal isolates from GenBank included in the phylogenetic analyses.

Species	Strain number[a]	Host	Collector	Location	GenBank accession numbe			
					ITS[b]	*tub2*	*his3*	*tef1*
Campylocarpon fasciculare	**CBS 112613**	*Vitis vinifera*	F. Halleen	South Africa	AY677301	AY677221	JF735502	JF735691
C. pseudofasciculare	**CBS112679**	*V. vinifera*	F. Halleen	South Africa	AY677306	AY677214	JF735503	JF735692
Dactylonectria alcacerensis	**CBS 129087**	*V. vinifera*	A. Cabral & H. Oliveira	Portugal	JF735333	AM419111	JF735630	JF735819
	Cy134	*V. vinifera*	J. Armengol	Spain	JF735332	AM419104	JF735629	JF735818
D. amazonica	MUCL55433	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683707	MF683644	MF683686	MF683665
	MUCL55430	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683706	MF683643	MF683685	MF683664
D. anthuriicola	**CBS 564.95**	*Anthurium* sp.	R. Pieters	The Netherlands	JF735302	JF735430	JF735579	JF735768
D. ecuadoriensis	MUCL55425	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683705	MF683642	MF683684	MF683663
	MUCL55424	*Piper* sp.	A. Gordillo & C. Decock	Ecuador	MF683704	MF683641	MF683683	MF683662
D. estremocensis	**CBS 129085**	*V. vinifera*	C. Rego & T. Nascimento	Portugal	JF735320	JF735448	JF735617	JF735806
	CPC 13539	*Picea glauca*	R. C. Hamelin	Canada	JF735330	JF735458	JF735627	JF735816
D. hispanica	**CBS 142827**	*Pinus halepensis*	B. Mora-Sala	Spain	KY676882	KY676876	KY676864	KY676870
	Cy228	*Ficus* sp.	F. Caetano	Portugal	JF735301	JF735429	JF735578	JF735767
D. hordeicola	**CBS 162.89**	*Hordeum vulgare*	M. Barth	The Netherlands	AM419060	AM419084	JF735610	JF735799
D. macrodidyma	**CBS 112615**	*V. vinifera*	F. Halleen	South Africa	AY677290	AY677233	JF735647	JF735836
	CBS 112604	*V. vinifera*	F. Halleen	South Africa	AY677284	AY677229	JF735644	JF735833
D. novozelandica	CBS 112608	*V. vinifera*	F. Halleen	South Africa	AY677288	AY677235	JF735632	JF735821
	CBS 113552	*Vitis* sp.	R. Bonfiglioli	New Zealand	JF735334	AY677237	JF735633	JF735822
D. palmicola	**MUCL55426**	*Euterpe precatoria*	A. Gordillo & C. Decock	Ecuador	MF683708	MF683645	MF683687	MF683666
D. pauciseptata	CBS 100819	*Erica melanthera*	H. M. Dance	New Zealand	EF607090	EF607067	JF735582	JF735771
	CBS 120171	*Vitis* sp.	M. Zerjav	Slovenia	EF607089	EF607066	JF735587	JF735776
D. pinicola	**CBS 173.37**	*P. laricio*	T. R. Peace	UK	JF735319	JF735447	JF735614	JF735803
	CBS 159.43	-	H. W. Wollenweber	Germany	JF735318	JF735446	JF735613	JF735802
D. polyphaga	**MUCL55209**	*Costus* sp.	A. Gordillo & C. Decock	Ecuador	MF683689	MF683626	MF683668	MF683647
	MUCL55208	*Costus* sp.	A. Gordillo & C. Decock	Ecuador	MF683699	MF683636	MF683678	MF683657
D. torresensis	**CBS 129086**	*V. vinifera*	A. Cabral	Portugal	JF735362	JF735492	JF735681	JF735870
	CBS 119.41	*Fragaria* sp.	H. C. Koning	The Netherlands	JF735349	JF735478	JF735657	JF735846

D. vitis	**CBS 129082**	*V. vinifera*	C. Rego	Portugal	JF735303	JF735431	JF735580	JF735769
D. valentina	**CBS 142826**	*Ilex aquifolium*	B. Mora-Sala	Spain	KY676881	KY676875	KY676863	KY676869
Ilyonectria capensis	**CBS 132815**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231151	JX231103	JX231135	JX231119
	CBS 132816	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231160	JX231112	JX231144	JX231128
I. coprosmae	CBS 119606	*Metrosideros* sp.	G. J. Samuels	Canada	JF735260	JF735373	JF735505	JF735694
I. crassa	CBS 129083	*Panax quinquefolium*	S. Hong	Canada	AY295311	JF735395	JF735536	JF735725
	CBS 158.31	*Narcissus* sp.	W. F. van Hell	The Netherlands	JF735276	JF735394	JF735535	JF735724
I. cyclaminicola	**CBS 302.93**	*Cyclamen* sp.	M. Hooftman	The Netherlands	JF735304	JF735432	JF735581	JF735770
I. destructans	**CBS 264.65**	*Cyclamen persicum*	L. Nilsson	Sweden	AY677273	AY677256	JF735506	JF735695
I. europaea	CBS 102892	*Phragmites australis*	W. Leibinger	Germany	JF735295	JF735422	JF735569	JF735758
	CBS 129078	*V. vinifera*	C. Rego	Portugal	JF735294	JF735421	JF735567	JF735756
I. gamsii	**CBS 940.97**	Soil	J. T. Poll	The Netherlands	AM419065	AM419089	JF735577	JF735766
I. ilicicola	**CBS 142828**	*Ilex* sp.	B. Mora-Sala	Spain	KY676884	KY676878	KY676866	KY676872
	Cy-FO-226	*Ilex* sp.	B. Mora-Sala	Spain	KY676885	KY676879	KY676867	KY676873
I. leucospermi	**CBS 132809**	*Leucospermum* sp.	C. M. Bezuidenhout	South Africa	JX231161	JX231113	JX231145	JX231129
	CBS 132810	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231162	JX231114	JX231146	JX231130
I. liliigena	**CBS 189.49**	*Lilium regale*	M. A. A. Schippers	The Netherlands	JF735297	JF735425	JF735573	JF735762
	CBS 732.74	*Lilium* sp.	G. J. Bollen	The Netherlands	JF735298	JF735426	JF735574	JF735763
I. liriodendri	**CBS 110.81**	*Liriodendron tulipifera*	J. D. MacDonald & E.E	USA	DQ178163	DQ178170	JF735507	JF735696
	CBS 117527	*V. vinifera*	C. Rego	Portugal	DQ178165	DQ178172	JF735509	JF735698
I. lusitanica	**CBS 129080**	*V. vinifera*	N. Cruz	Portugal	JF735296	JF735423	JF735570	JF735759
I. mors-panacis	CBS 124662	*Pa. ginseng*	Y. Myazawa	Japan	JF735290	JF735416	JF735559	JF735748
	CBS 306.35	*Pa. quinquefolium*	A. A. Hildebrand	Canada	JF735288	JF735414	JF735557	JF735746
I. palmarum	CBS 135753	*Howea forsteriana*	G. Polizzi	Italy	HF937432	HF922609	HF922621	HF922615
	CBS 135754	*H. forsteriana*	G. Polizzi	Italy	HF937431	HF922608	HF922620	HF922614
I. panacis	**CBS 129079**	*Pa. quinquefolium*	K. F. Chang	Canada	AY295316	JF735424	JF735572	JF735761
I. protearum	**CBS 132811**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231157	JX231109	JX231141	JX231125
	CBS 132812	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231165	JX231117	JX231149	JX231133
I. pseudodestructans	CBS 117824	*Quercus* sp.	E. Halmschlager	Austria	JF735292	JF735419	JF735562	JF735751
	CBS 129081	*V.vinifera*	C. Rego	Portugal	AJ875330	AM419091	JF735563	JF735752
I. robusta	CBS 129084	*V. vinifera*	N. Cruz	Portugal	JF735273	JF735391	JF735532	JF735721
	CBS 308.35	*Pa. quinquefolium*	A. A. Hildebrand	Canada	JF735264	JF735377	JF735518	JF735707
I. rufa	**CBS 153.37**	*Dune sand*	F. Moreau	France	AY677271	AY677251	JF735540	JF735729
	CBS 640.77	*A. alba*	F. Gourbière	France	JF735277	JF735399	JF735542	JF735731
I. venezuelensis	**CBS 102032**	Bark	A. Rossman	Venezuela	AM419059	AY677255	JF735571	JF735760
I. vredehoekensis	**CBS 132807**	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231155	JX231107	JX231139	JX231123

	CBS 132814	*Protea* sp.	C. M. Bezuidenhout	South Africa	JX231158	JX231110	JX231142	JX231126
Neonectria candida	CBS 182.36	*Malus sylvestris*	H. W. Wollenweber	-	JF735314	JF735439	JF735603	JF735792
N. candida, authentic strain of *C. obtusiusculum*	**CBS 151.29**	*Ma. sylvestris*	H. W. Wollenweber	UK	JF735313	JF735438	JF735602	JF735791
N. ditissima, authentic strain of *C. wilkommii*	**CBS 226.31**	*Fagus sylvatica*	H. W. Wollenweber	Germany	JF735309	DQ789869	JF735594	JF735783
N. ditissima, representative strain of *N. galligena*	CBS 835.97	*Salix cinerea*	W. Gams	Belgium	JF735310	DQ789880	JF735595	JF735784
N. major	**CBS 240.29**	*Alnus incana*	H. W. Wollenweber	Norway	JF735308	DQ789872	JF735593	JF735782
N. neomacrospora	**CBS 324.61**	*A. concolor*	J. A. von Arx	The Netherlands	JF735312	DQ789875	JF735599	JF735788
	CBS 503.67	*A. alba*	F. Roll-Hansen	Norway	AY677261	JF735436	JF735600	JF735789
N. obtusispora	CBS 183.36	*Solanum tuberosum*	H. W. Wollenweber	Germany	AM419061	AM419085	JF735607	JF735796
	CPC 13544	*Prunus armenica*	J. A. Traquair	Canada	AY295306	JF735443	JF735608	JF735797
N. quercicola	**CBS 143704**	*Q. ilex*	P. Abad-Campos	Spain	KY676880	KY676874	KY676862	KY676868
	CPC 13530	*Pyrus* sp.	J. A. Traquair	Canada	AY295302	JF735441	JF735605	JF735794
Neonectria sp. 1	CPC 13545	*Pyrus* sp.	J. A. Traquair & B. Harrison	Canada	AY295303	JF735437	JF735601	JF735790

[a] Ex-type culture indicated in bold. **CBS**: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CPC**: culture collection of Pedro Crous, housed at CBS. **MUCL**: Mycothèque de l'Université catholique de Louvain.

[b] ITS = internal transcribed spacer, *tub2* = β-tubulin, *his3* = histone H3 and *tef1* = translation elongation factor 1- α.

Supplementary Table 3.2. Fungal species associated with black-foot disease recovered from grafted plants in Spain and nucleotide sequences used in the phylogenetic analyses.

Species	Isolate	Location	GenBank accession number[a]			
			ITS	*tub2*	*his3*	*tef1*
Dactylonectria alcacerensis	BV-1240	Larraga, Navarra	MK602783	MK602798	MK579234	MK602813
D. alcacerensis[b]	BV-1222	Larraga, Navarra	-	-	MK579235	-
D. alcacerensis	BV-1245	Larraga, Navarra	-	-	MK579236	-
D. alcacerensis[b]	BV-1469	Larraga, Navarra	-	-	MK579237	-
Dactylonectria macrodidyma	BV-0535	Mendavia, Navarra	MK602784	MK602799	MK579238	MK602814
D. macrodidyma[b]	BV-0506	Mendavia, Navarra	-	-	MK579239	-
D. macrodidyma	BV-0560	Mendavia, Navarra	-	-	MK579240	-
D. macrodidyma	BV-0797	Mendavia, Navarra	-	-	MK579241	-
D. macrodidyma	BV-0872	Mendavia, Navarra	-	-	MK579242	-
D. macrodidyma	BV-0899	Mendavia, Navarra	-	-	MK579243	-
D. macrodidyma	BV-1359	O Barco de Valdeorras, Galicia	-	-	MK579244	-
D. macrodidyma	BV-1364	O Barco de Valdeorras, Galicia	-	-	MK579245	-
D. macrodidyma[b]	BV-1366	O Barco de Valdeorras, Galicia	-	-	MK579246	-
Dactylonectria novozelandica	BV-0760	Mendavia, Navarra	MK602785	MK602800	MK579247	MK602815
D. novozelandica	BV-0513	Mendavia, Navarra	-	-	MK579248	-
D. novozelandica[b]	BV-0428	Mendavia, Navarra	-	-	MK579249	-
D. novozelandica	BV-0777	Mendavia, Navarra	-	-	MK579250	-
D. novozelandica[b]	BV-1369	Mendavia, Navarra	-	-	MK579251	-
D. novozelandica	BV-1378	Mendavia, Navarra	-	-	MK579252	-
D. novozelandica	BV-1379	Mendavia, Navarra	-	-	MK579253	-
D. novozelandica	BV-1401	Mendavia, Navarra	-	-	MK579254	-
D. novozelandica	BV-1409	Mendavia, Navarra	-	-	MK579255	-
Dactylonectria pauciseptata[b]	BV-1354	O Barco de Valdeorras, Galicia	MK602786	MK602801	MK579256	MK602816
D. pauciseptata	BV-1358	O Barco de Valdeorras, Galicia	-	-	MK579257	-
D. pauciseptata[b]	BV-1360	O Barco de Valdeorras, Galicia	-	-	MK579258	-
Dactylonectria torresensis[b]	BV-0666	Mendavia, Navarra	MK602787	MK602802	MK579259	MK602817

D. torresensis	BV-0827	Mendavia, Navarra	-	-	MK579260	-
D. torresensis[b]	BV-0901	Mendavia, Navarra	-	-	MK579261	-
D. torresensis	BV-1076	Larraga, Navarra	-	-	MK579262	-
D. torresensis	BV-1255	Larraga, Navarra	-	-	MK579263	-
D. torresensis	BV-1256	Larraga, Navarra	-	-	MK579264	-
D. torresensis	BV-1300	Larraga, Navarra	-	-	MK579265	-
D. torresensis	BV-1313	Larraga, Navarra	-	-	MK579266	-
D. torresensis	BV-1315	Larraga, Navarra	-	-	MK579267	-
D. torresensis	BV-2103	Larraga, Navarra	-	-	MK579268	-
Dactylonectria riojana sp. nov.[b]	BV-1396	Mendavia, Navarra	MK602796	MK602811	MK602831	MK602826
D. riojana sp. nov.[b]	BV-1397	Mendavia, Navarra	MK602797	MK602812	MK602832	MK602827
Ilyonectria liriodendri[b]	BV-1591	O Barco de Valdeorras, Galicia	MK602788	MK602803	MK579269	MK602818
I. liriodendri[b]	BV-1642	Larraga, Navarra	-	-	MK579270	-
I. liriodendri	BV-1757	Larraga, Navarra	-	-	MK579271	-
I. liriodendri	BV-1762	Larraga, Navarra	-	-	MK579272	-
I. liriodendri	BV-1763	Larraga, Navarra	-	-	MK579273	-
I. liriodendri	BV-1764	Larraga, Navarra	-	-	MK579274	-
I. liriodendri	BV-1844	Larraga, Navarra	-	-	MK579275	-
I. liriodendri	BV-1923	Larraga, Navarra	-	-	MK579276	-
I. liriodendri	BV-1925	Larraga, Navarra	-	-	MK579277	-
I. liriodendri	BV-1929	Larraga, Navarra	-	-	MK579278	-
Ilyonectria pseudodestructans[b]	BV-2142	Larraga, Navarra	MK602789	MK602804	MK579279	MK602819
I. pseudodestructans	BV-2306	Larraga, Navarra	-	-	MK579280	-
I. pseudodestructans	BV-2307	Larraga, Navarra	-	-	MK579281	-
I. pseudodestructans	BV-2506	Larraga, Navarra	-	-	MK579282	-
I. pseudodestructans[b]	BV-2609	Larraga, Navarra	-	-	MK579283	-
I. pseudodestructans	BV-2652	Larraga, Navarra	-	-	MK579284	-
I. pseudodestructans	BV-2678	Larraga, Navarra	-	-	MK579285	-
Ilyonectria robusta	BV-1593	Mendavia, Navarra	MK602790	MK602805	MK579286	MK602820
I. robusta[b]	BV-0816	O Barco de Valdeorras, Galicia	-	-	MK579287	-

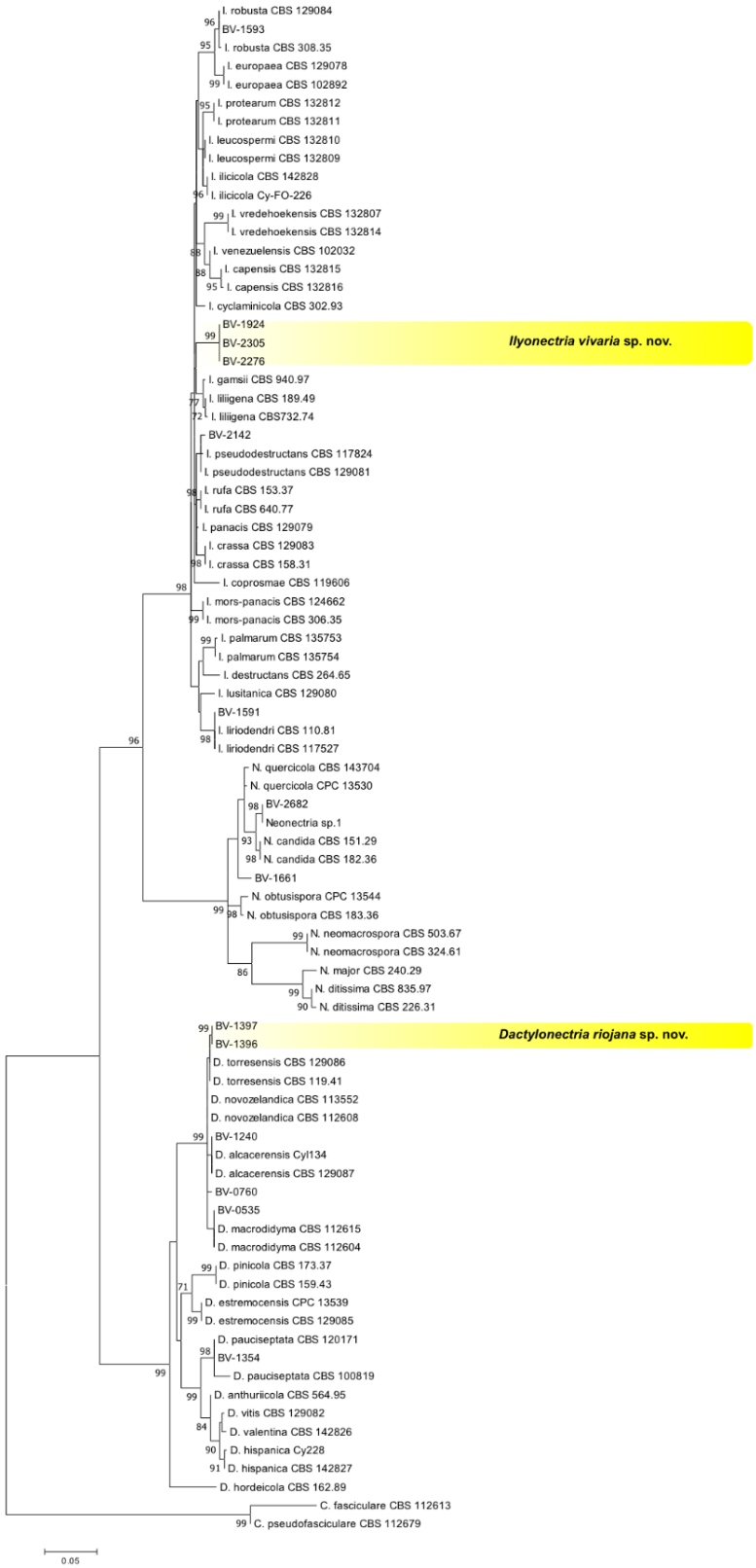
I. robusta[b]	BV-1654	Larraga, Navarra	-	-	MK579288	-
I. robusta	BV-2051	Larraga, Navarra	-	-	MK579289	-
I. robusta	BV-2069	Larraga, Navarra	-	-	MK579290	-
I. robusta	BV-2565	Larraga, Navarra	-	-	MK579291	-
I. robusta	BV-2649	Larraga, Navarra	-	-	MK579292	-
Ilyonectria vivaria sp. nov.[b]	BV-1924	Larraga, Navarra	MK602793	MK602808	MK602828	MK602823
I. vivaria sp. nov.[b]	BV-2276	Larraga, Navarra	MK602794	MK602809	MK602829	MK602824
I. vivaria sp. nov.	BV-2305	Larraga, Navarra	MK602795	MK602810	MK602830	MK602825
Neonectria quercicola[b]	BV-1661	Larraga, Navarra	MK602791	MK602806	MK579293	MK602821
N. quercicola[b]	BV-2137	Larraga, Navarra	-	-	MK579294	-
N. quercicola	BV-2140	Larraga, Navarra	-	-	MK579295	-
Neonectria sp. 1[b]	BV-2682	Larraga, Navarra	MK602792	MK602807	MK579296	MK602822
Thelonectria olida[b,c]	BV-0537	Mendavia, Navarra	MK602793	-	MK579297	-

[a] ITS = internal transcribed spacer, *tub2* = β-tubulin, *his3* = histone H3 and *tef1* = translation elongation factor 1- α.

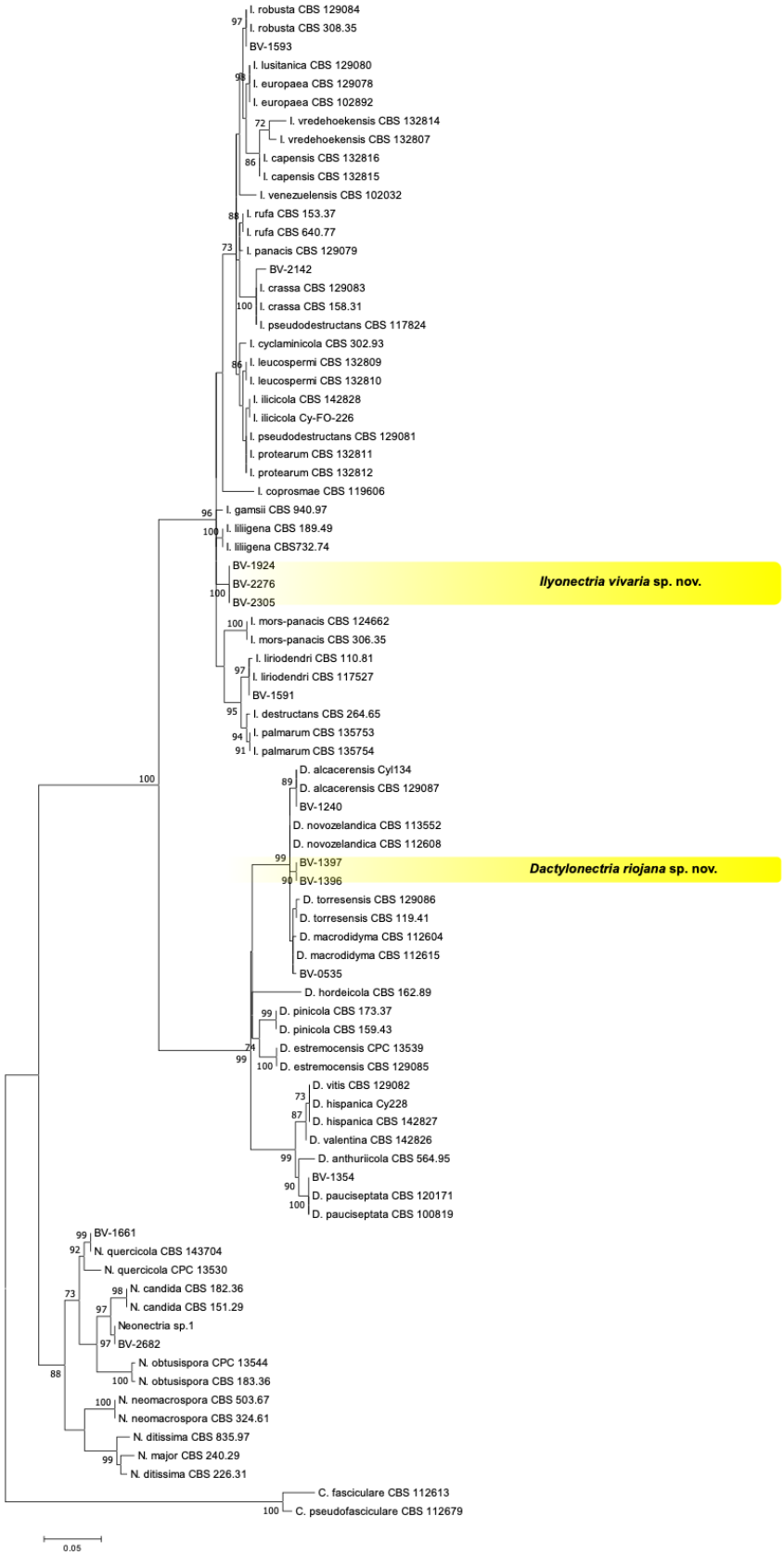
[b] Isolates used for colony and conidial morphology, in the temperature growth assay and for pathogenicity tests

[c] *Thelonectria olida* was not included in the phylogenetic analyses

Supplementary Figures



Supplementary Figure 3.1. *Maximum likelihood phylogeny of Cylindrocarpon-like asexual morphs as estimated from the alignment of the β-tubulin (tub2) gene sequences. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to Campylocarpon fasciculare (CBS 112613) and Ca. pseudofasciculare (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. Tentative new species are highlighted in yellow.*



***Supplementary Figure 3.2**. Maximum likelihood phylogeny of Cylindrocarpon-like asexual morphs as estimated from the alignment of the translation elongation factor 1-alpha (tef1) gene sequences. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to Campylocarpon fasciculare (CBS 112613) and Ca. pseudofasciculare (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. Tentative new species are highlighted in yellow.*

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Estimation of viable propagules of black-foot disease pathogens in grapevine cultivated soils and their relation to production systems and soil properties

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Abstract

Aims The study aimed to assess comparatively the accuracy and efficiency of three culture media protocols for estimating black-foot disease pathogens populations in soils and to examine how shifts in the abundance and composition of black-foot pathogens correspond to changes in specific soil properties.

Methods Firstly, culture media were compared by evaluating the mycelial growth of selected black-foot pathogens and by estimating the population of *Dactylonectria torresensis* from artificially infested soils. Secondly, the most efficient culture medium was selected for estimating the viable propagules of black-foot disease pathogens in eight naturally infested soils. An analysis of the soil physicochemical properties was conducted. Data were statistically analyzed in order to explore possible relationships between the studied variables.

Results Glucose-Faba Bean Rose Bengal Agar (GFBRBA) was selected as the most efficient culture medium. All naturally infested soils tested positive for the presence of black-foot pathogens. *D. torresensis* was the most frequently isolated species, followed by *Dactylonectria alcacerensis* and *Ilyonectria liriodendri.* A positive relationship between calcium carbonate and the Colony-Forming Units (CFUs) level of black-foot pathogens in soil was obtained.

Conclusions In this study, we provide an early, specific, and accurate detection of viable propagules of black-foot pathogens in soil, which is critical to understand the ecology of these fungi and to design effective management strategies.

Keywords: *Dactylonectria torresensis*, fungal ecology, grapevine trunk disease, soilborne inoculum.

Abbreviations

CEC	Cation Exchange Capacity
CFU	Colony-Forming Unit
EC	Electric conductivity
GFBA	Glucose-Faba Bean Agar
GFBRGA	Glucose-Faba Bean Rose Bengal Agar
ITS	Internal Transcribed Spacer
MRBA	Modified Rose Bengal Agar
NMDS	Non-Metric Multidimensional Scaling Analysis
LSD	Least Significant Difference
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDAC	Potato Dextrose Agar supplemented with 250 mg l^{-1} of chloramphenicol
SNA	Spezieller Nährstoffarmer Agar
SOM	Soil Organic Matter

Introduction

Black-foot disease is one of the main soilborne fungal diseases affecting grapevine production worldwide, being particularly important in grapevine nurseries and new plantations, where it causes the young vine decline syndrome (Halleen et al. 2006; Gramaje and Armengol 2011; Agustí-Brisach and Armengol 2013). Young vines affected by black-foot disease generally appear normal at planting, but differences in vigour become marked: reduced trunk caliper, shortened internodes, reduced foliage/canopy fill, and reduced leaf area. During the first 3 to 5 years after planting, foliar symptoms may appear as small leaves with interveinal chlorosis, followed by necrosis and early defoliation (Agustí-Brisach and Armengol 2013). Removal of rootstock bark of declining plants reveals black discolouration and necrosis of wood tissues which develop from the base of the rootstock. Below ground, symptoms include reduced total root biomass, low numbers of feeder roots, and black, sunken, necrotic root lesions (Agustí-Brisach and Armengol 2013).

Cylindrocarpon-like anamorphs 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), with *Dactylonectria torresensis* the most common species associated with diseased vines in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016). The disease cycle of black-foot pathogens on grapevines is not completely known, but the behaviour of *Cylindrocarpon*-like anamorphs on other hosts has been studied in more detail (Booth 1966; Brayford 1993), and provided information about the likely disease cycle of associated genera on grapevines. *Cylindrocarpon*-like anamorphs readily produce conidia and some species also chlamydospores in culture, which indicates that these propagules are likely to be produced on the diseased roots and stem bases of infected vines. The conidia are dispersed in soil water and the chlamydospores can allow these fungi to survive in the soil for extended periods of time (Petit et al. 2011). They infect grapevines through natural openings or wounds, such as the non-callused part of the lower trunk. Infection can also occur through wounds in canes, such as disbudding wounds, from which the infection progresses downward to the base of the trunk (Halleen et al. 2006). The occurrence of black-foot disease pathogens during the nursery propagation process

(Halleen et al. 2003, 2007; Agustí-Brisach et al. 2013a; Cardoso et al. 2013; Reis et al. 2013) and in the vineyard (Agustí-Brisach et al. 2013b, 2014), has resulted in a number of hypotheses on the sources of primary inoculum and their dissemination.

The detection, identification and quantification of black-foot pathogens from soil samples have been mainly focused on the use of DNA based molecular techniques (Damm and Fourie 2005; Probst et al. 2010; Cardoso et al. 2013; Agustí-Brisach et al. 2014). Although these methods are very useful, it is important to consider that the mere presence of DNA does not indicate whether viable pathogen propagules are present and active (England et al. 1997; Demanèche et al. 2001).

The use of traditional methods to identify fungal pathogens in soil environments is challenging. These methods rely mainly on the dilution plating technique together with the use of selective media, and microscopy to identify sporulating fungal structures. The advantages in the use of these methods reside in their simplicity and low cost. They have provided quite a sensitive detection of soil fungi and have been widely used in diversity studies in different habitats (Elmholt et al. 1999; Cho et al. 2001; Cabello and Arambarri 2002). By contrast, one of the major constraints with cultural studies is that fungal diversity may be quite high in soil, processing of cultures can be time consuming and laborious when a large number of isolates has to be handled, and the risk of culture contamination is always high and in most cases fast growing fungi will overgrow others and fill the plate (Jeewon and Hyde 2007). In addition, there can be many species that behave similarly under cultural conditions and exhibit similar morphology but are in fact different species.

The alternative to isolate black-foot disease pathogens from soil is to bait for them using susceptible hosts (Agustí-Brisach et al. 2013b), which is an efficient method, but usually takes time, since this method involves growing grapevine seedlings and planting them in target soils till recovery and isolation after a long time (Agustí-Brisach et al. 2013b). In addition, baiting introduces a potential bias because of host adaptation, so these isolates might not be valid for use in further studies aimed to determine and compare the genetic composition of black-foot pathogen populations strictly living in soil. The effect of resident plants on fungal soil populations has been previously reported for *Fusarium oxysporum* (Edel et al. 1997).

The development of an improved medium for detecting viable propagules of black-foot pathogens would allow us (i) to improve our knowledge on the ecology of these fungi in soil, (ii) to alert nurseries and growers to the presence of the pathogens in soil, (iii) to use disease risk as a factor in making management decisions, (iv) to characterize the diversity, genetic structure and pathogenicity of black-foot pathogen populations in soil and compare them with those collected from grapevine roots and rootstock and from asymptomatic secondary hosts (rotational crops in nursery fields and weeds), and (v) to compare the results of the soil plating assays using different types of field soil with existing quantitative molecular protocols (Probst et al. 2010; Agustí-Brisach et al. 2014).

With this aim, the goal of this study was (i) to compare modified culture media already published in the literature for the detection of *Cylindrocarpon*-like anamorphs or other soilborne pathogens phylogenetically related to black-foot genera, (ii) to assess the usefulness of the most efficient culture medium for estimating black-foot disease pathogens populations in naturally infested soils, and (iii) to examine how shifts in the abundance and composition of black-foot pathogens correspond to changes in specific soil properties.

Material and Methods

Culture media preparation

Three media were prepared in order to compare their efficiency for growing selected black-foot pathogens and the recovery of *Dactylonectria torresensis* from sterile and non-sterile soil: (i) a modification of Rose Bengal Agar (MRBA; Reedler et al. 2003) medium consisted of 32 g of Rose Bengal Agar (Difco, Detroit, MI, USA) (5 g Bacto peptone, 10 g dextrose, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 15 g Bacto agar), 30 mg streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA), 250 mg ampicillin (Sigma-Aldrich, St. Louis, MO, USA), 10 mg rifampicin (Sigma-Aldrich, St. Louis, MO, USA) (dissolved in 1 mL ethanol), 500 mg pentachloronitrobenzene, 500 mg dicloran, and 1 L distilled water; (ii) the Glucose-Faba Bean Rose Bengal Agar (GFBRBA), modified from Hunter et al. (1980), consisted of a faba bean infusion (boiling 62.5 g of fresh faba beans in 1 L distilled water for 5 min, keeping them in water for 12 h and filtering through cheesecloth, saving 0.75 L effluent), 32 g of Rose Bengal Agar (Difco) (5 g

Bacto peptone, 10 g dextrose, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 15 g Bacto agar), 90 g of glucose (Scharlab S.L., Barcelona, Spain), 50 mg of chloramphenicol (Sigma-Aldrich), 30 mg streptomycin sulphate (Sigma-Aldrich), 250 mg ampicillin (Sigma-Aldrich), 10 mg rifampicin (Sigma-Aldrich) (dissolved in 1 mL ethanol), and 0.25 L distilled water; and (iii) the Glucose-Faba Bean Agar (GFBA), modified from Singleton et al. (1993), consisted of a faba bean infusion as described above, 15 g of Bacteriological agar (Panreac AppliChem, Barcelona, Spain), 100 g of glucose (Scharlab S.L.), 50 mg of chloramphenicol (Sigma-Aldrich), 30 mg streptomycin sulphate (Sigma-Aldrich), 250 mg ampicillin (Sigma-Aldrich), 10 mg rifampicin (Sigma-Aldrich) (dissolved in 1 mL ethanol), and 0.25 L distilled water.

In all media, antibiotics and fungicides were added and thoroughly mixed after autoclaving at 121°C for 15 min and cooling to 55°C. The pH of all media was measured using a pH meter (Fisher Scientific, Santa Clara, CA) when the media temperature ranged between 39 and 45°C after each batch was prepared.

Evaluation of mycelial growth

Seven single spore isolates (one isolate of the species *Campylocarpon fasciculare*, *Dactylonectria alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis* and *Ilyonectria liriodendri*) obtained from different geographic locations and grapevine rootstock–scion combinations in Spain, and one isolate each of the species *Fusarium oxysporum* and *Rhizoctonia solani* were evaluated for mycelia growth on the media described above (Table 3.4). Isolates were obtained from the culture collection of the Instituto Agroforestal Mediterráneo at the Universitat Politècnica de València (Spain). Three plates of each medium were inoculated centrally with a 5-mm diameter agar disk from the advancing margins of 2-week-old cultures of each isolate on PDA. All plates were incubated at 25°C. Colony diameters were measured after 10 days of incubation. The experiment was conducted twice.

Preparation of artificially infested soil and recovery of Dactylonectria torresensis

Bulk soil was collected from a 10-year old vineyard in La Grajera (Logroño, Spain), with a shovel at 10 to 30 cm depth (field code C) (Table 3.5). Four sub-samples were randomly taken from the interrow space of asymptomatic grapevine plants (about 0.5

kg of soil/sample) in May 2016. Samples were mixed well, air-dried for one week and sieved (2-mm to 5-mm mesh size) prior to soil physicochemical analyses and culture media test. One half-soil sample was sterilized twice by autoclaving at 121°C for 1 hr at one-day interval.

Mycelium of one isolate of *D. torresensis* (BV-048) was produced on sterilised wheat grains. The wheat grains (200 g) were placed in a 1 L conical flask containing 500 mL of tap water and heated to boiling. The grains were left to settle for 10 min then washed three times with tap water and the excess drained off. The grains were autoclaved at 121°C for 15 min and left for 24 h, after which the process was repeated. Each flask of wheat grains was inoculated with ten 8 mm mycelium plugs of the BV-048 isolate taken from the edge of the *D. torresensis* cultures grown on PDA plates and incubated at 25°C for three weeks. The flasks were incubated at 20°C in the dark for one month, during which they were shaken daily by hand (5 sec) to assist colonisation by mycelium, which was confirmed visually.

Sterile and non-sterile soil (10 g, fresh weight) was mixed in a 1:1 proportion with 10 g of *D. torresensis* inoculum produced in wheat grains. Each mixture was incubated for three weeks at 25°C in the darkness. After this period, mixtures (20 g) were diluted in 100 mL of 0.1% water agar (dilution 1). The mixture was homogenised by shaking the bottle in a reciprocating shaker for 10 min at 250 rpm. One mL aliquots were serially diluted 100 times in 100 mL of 0.1% water agar (dilution 2) and then 10 times in 9 mL falcon tubes with sterile water (dilution 3). Sterile pipettes were used to transfer 150 µl each of the last two dilutions onto the surface of an agar plate. The soil suspension was spread evenly across the surface of the plate by the use of sterile glass spreading rods. Nine plates were prepared for each of the four soil samples, dilution and culture media (MRBA, GFBRBA and GFBA). The experiment was repeated three times. Plates were incubated at 25°C in the dark for 10 days, and examined daily for development of *D. torresensis* colonies using a stereomicroscope at 7.5 x magnification. Colony counts were converted to colony-forming units (CFU) per gram of dry soil.

Table 3.4. Fungal isolates used in this study and radial growth (in mm after 10 days at 25°C) on different culture media.

Species	Isolate	Year	Location	Host	GenBank accessions[a]	Radial growth on MRBA[b]	Radial growth on GFBRBA[c]	Radial growth on GFBA[d]
Campylocarpon fasciculare	GIHF-102	2015	Aielo de Malferit (Valencia)	Grapevine rootstock '110 Richter'	KY887653	23.2[b] b B[c]	26.7 b A	45.0 a BC
Dactylonectria alcacerensis	DA-1	2016	Aielo de Malferit (Valencia)	Grapevine rootstock '110 Richter'	KY887654	17.7 c D	24.2 b B	46.2 a B
D. macrodidyma	057	2016	Aielo de Malferit (Valencia)	Grapevine rootstock '110 Richter'	KY887655	15.2 c E	26.5 b A	41.2 a D
D. novozelandica	058	2016	Aielo de Malferit (Valencia)	Grapevine rootstock '110 Richter'	KY887656	23.7 b B	24.5 b B	38.7 a E
D. pauciseptata	GIHF-100	2016	Madrid	Common box	KY887657	19.5 b C	21.7 b C	41.2 a D
D. torresensis	BV-149	2015	Mendavia (Navarra)	Grapevine rootstock '110 Richter'	KY887658	16.7 c D	20.7 b C	38.5 a E
Ilyonectria liriodendri	IL-1	2016	Aielo de Malferit (Valencia)	Grapevine rootstock '110 Richter'	KY887659	24.0 c B	26.5 b A	43.7 a C
Fusarium oxysporum	GIHF-125	2016	El Perelló (Valencia)	Tomato	KY887660	26.2 c A	28.2 b A	72.0 a A
Rhizoctonia solani	GIHF-140	2016	Calasparra (Murcia)	Spinach	KY887661	25.5 c A	28.0 b A	72.0 a A

[a]GenBank accessions correspond to histone (black-foot pathogens), elongation factor (*F. oxysporum*) and internal transcribed spacer (*R. solani*) sequences.

[b]MRBA: Modification of Rose Bengal Agar; GFRBA: Glucose-Faba Bean Rose Bengal Agar; GFBA: Glucose-Faba Bean Agar

[c]Average of six replicates.

[d]Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$). Capital letters are for comparison of means in the same column. Small letters are for comparison of means in the same row.

Detection of black-foot pathogens in naturally infested soils

Eight fields located in different regions in Spain were surveyed in June 2016: four established vineyards (A, B, C and D), two nursery fields (E and F), and two nursery fields in rotation (G and H) (Table 3.5). Each field had a sampling area marked by a "V" pattern that included four interrow vineyard spaces, except for fields G and H, for soil sampling. Four bulk soil samples were taken with a shovel at 10 to 30 cm depth in each field (about 0.5 kg of soil/sample), and then processed and analysed for physicochemical properties in the laboratory. Samples were diluted as described before and soil suspensions were spread at dilution 2 onto GFBRBA medium, which was selected as the most efficient dilution/medium combination in the previous experiment. Four plates were prepared for each sub-sample. The experiment was repeated three times (48 plates per soil sample). Plates were incubated at 25°C in the dark for 10 days, and examined daily for development of fungal colonies using a stereomicroscope at 7.5 x magnification. Colonies emerging from the agar were transferred to PDA for further morphological and molecular identification, and those identified as black-foot pathogens were converted to CFU per gram of dry soil.

Table 3.5. Field sites used for soil sampling.

Field code	Geographical origin		Field type
	Location	Province	
A	Aldeanueva del Ebro	La Rioja	27-year-old vineyard ('Tempranillo' / '110 Richter')
B	Olite	Navarra	7-year-old vineyard (different scion/rootstock combinations)
C	Logroño	La Rioja	10-year-old vineyard ('Tempranillo' / '110 Richter')
D	Logroño	La Rioja	2-year-old vineyard ('Tempranillo' / '110 Richter')
E	Canals	Valencia	Field nursery ('Tempranillo' / '110 Richter')
F	Canals	Valencia	Field nursery ('Tempranillo' / '110 Richter')
G	Mendavia	Navarra	Field nursery during third year of rotation (bare fallow land)
H	Canals	Valencia	Field nursery during first year of rotation (fallow land with weeds)

Fungal identification

Fungal isolates resembling black-foot pathogens were identified morphologically by macroscopic characters, including colony texture, colour, and the shape of the growing

margin on PDA. Conidia were observed and measured from colonies growing on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Alaniz et al. 2007; Chaverri et al. 2011).

For DNA extraction, 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 mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. 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 stain and was stored at −20°C.

All fungal species were identified by analysis of the Internal Transcribed Spacer (ITS) region of DNA amplified using the fungal universal primers ITS1F (Gardes and Bruns 1993) and ITS4. The identification of black-foot pathogens was confirmed by sequencing part of the histone H3 gene using CYLH3F and CYLH3R primers (Crous et al. 2004; Cabral et al. 2012a, 2012b). Polymerase chain reaction (PCR) products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). The sequences obtained were then blasted in GenBank.

Soil physicochemical properties analysis

Soils sampled were tested for pH and electric conductivity (EC) in water with a soil solution ratio of 1:5, soil organic matter (SOM) by dichromate oxidation (Nelson and Sommers 1982), soil texture by laser diffraction particle size (Diffractometer LS 13 320, Beckman Coulter Inc., Brea, Calif.), total carbonate by infrared (Equilab CO-202; Equilab, Jakarta, Indonesia), cation exchange capacity (CEC) by the cobaltihexamine method (Orsini and Remy 1976), assimilable calcium and magnesium by the cobaltihexamine method and by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA) 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).

Data analyses

For the mycelial growth and the culture media assays, data were analysed using one-way ANOVA. Student's t test least significant difference (LSD) was calculated at the 5% significance level to compare mean mycelial growth between the different media and fungal species, and to compare mean CFUs between the different media, respectively.

Due to a non-uniformity of variance, black-foot populations CFU counts from naturally infested soils were transformed to $\log_{10}$ (x+1), where x is the number of CFUs per g of soil per petri dish. This transformation was better at achieving homogeneity of variance than several others tried. Data were analysed using one-way ANOVA. LSD test was used to determine the differences between black-foot total counts in different fields. Data from all experiments were analysed using the Statistix 10 software (Analytical Software).

Soil physicochemical variables were subjected to principal component analysis (PCA) in order to group the different fields tested and to reduce the observed variables into a smaller number of principal components (artificial variables) that will account for most of the variance in the observed variables. PCA analysis was performed using the R version 3.3.2 (The R Foundation for Statistical Computing) (R Core Team 2016) packages stats (R Core Team 2016) and devtools (Wickham and Chang 2016). Non-metric multidimensional scaling analyses (NMDS) based on dissimilarities calculated using the Bray–Curtis index after Hellinger standardization were run with MetaMDS functions within the vegan package (Oksanen et al. 2008) using R and soil physicochemical variables vectors were fitted using the envfit routine. Correlations with CFU (R^2) were obtained by fitting linear trends to the NMDS ordination obtained for each variable and significance (P) was determined by permutation (nperm = 1000).

Results

Effect of media on mycelial growth

The pH of GFBA medium was the lowest (pH = 5.9 ± 0.04), MRBA medium was the highest (pH = 7.0 ± 0.06) and GFBRBA medium was intermediate (pH = 6.3 ± 0.09). Colony diameters of black-foot isolates as well as *F. oxysporum* and *R. solani* isolates in GFBA were significantly greater than on the other two tested media ($P < 0.05$) (Table

3.4). In the MRBA and GFBA media, colony diameters of *F. oxysporum* and *R. solani* isolates were significantly greater than black-foot pathogens ($P < 0.05$). In the GFBRBA medium, colony diameters of *C. fasciculare*, *D. macrodidyma* and *I. liriodendri* did not show significant differences with mycelial growth of *F. oxysporum* and *R. solani* isolates ($P > 0.05$). Most of the species of black-foot produced a brown or yellow pigment on the agar of the media MRBA and GFBRBA.

Detection of Dactylonectria torresensis in artificially infested soils

Recovery of *D. torresensis* from sterile soil samples was affected by the type of media used (Table 3.6). In dilution 2, the CFUs/g soil in GFBRBA and GFBA media were significantly greater than in MRBA medium. In dilution 3, recovery of *D. torresensis* on GFBRBA medium was increased over MRBA and GFBA media. Regarding the non-sterile soil samples, no significant differences were found among media when measuring the CFUs/g soils in dilution 2 (Table 3.6). In dilution 3, the CFUs/g soil in GFBRBA and GFBA media were significantly greater than in MRBA medium.

Table 3.6. Comparison of media for recovery efficiency of *Dactylonectria torresensis* from artificially infested soils.

	Sterile soil		Non-sterile soil	
	Dilution 2	Dilution 3	Dilution 2	Dilution 3
Media[a]	Mean estimated population density (CFU / g soil)	Mean estimated population density (CFU / g soil)	Mean estimated population density (CFU / g soil)	Mean estimated population density (CFU / g soil)
MRBA	7407.4[b] ± 79.1 b[c]	1538.5 ± 148.1 b	8592.6 ± 1002.3 a	1481.5 ± 148.1 b
GFBRBA	9037.0 ± 120.4 a	4444.4 ± 326.2 a	8740.7 ± 1328.2 a	1538.5 ± 148.1 a
GFBA	9185.2 ± 135.1 a	1481.5 ± 148.1 b	8888.9 ± 1405.4 a	1538.5 ± 148.1 a

[a]MRBA: Modification of Rose Bengal Agar; GFRBA: Glucose-Faba Bean Rose Bengal Agar; GFBA: Glucose-Faba Bean Agar

[b]Average of 3 replicates of 9 plates each.

[c]Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$).

Detection of black-foot pathogens in naturally infested soils

All fields were positive for the presence of black-foot pathogens. *D. torresensis* was isolated from all soil samples evaluated with an inoculum density ranging from 2.793 to 0.631 log_{10} CFU/g soil (Figure 3.5). Significant differences were found in the CFU of *D. torresensis* among soils ($P < 0.01$) (Figure 3.5). This species was isolated most frequently from field B (2.824 ± 0.207 log_{10} CFU/g soil), with no significant differences with field A (2.699 ± 0.250 log_{10} CFU/g soil). In general, established vineyards (A, B, C: 2.523 ± 0.201 log_{10} CFU/g soil, D: 2.224 ± 0.220 log_{10} CFU/g soil) and nursery fields (E: 2.399 ± 0.097 log_{10} CFU/g soil, F: 2.399 ± 0.028 log_{10} CFU/g soil) had higher inoculum density of *D. torresensis* than nursery fields in rotation (G: 1.925 ± 0.035 log_{10} CFU/g soil, H: 2.224 ± 0.211 log_{10} CFU/g soil). The inoculum density of *D. torresensis* in nursery fields (E and F) was not significantly different from established vineyards (A and C). *Dactylonectria alcacerensis* was also isolated from fields B (1.262 ± 0.21 log_{10} CFU / g soil) and F (2.621 ± 0.02 log_{10} / g soil), while *I. liriodendri* was only detected in field F (0.631 ± 0.18 log_{10} / g soil).

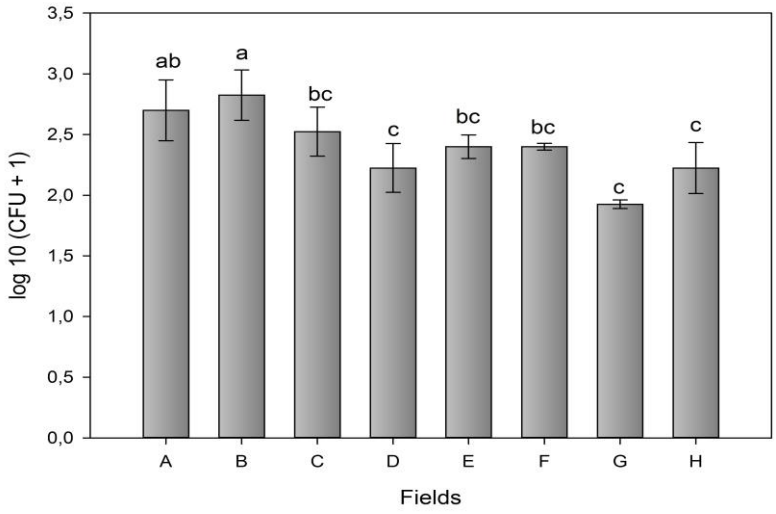

***Figure 3.5**. Mean log_{10} (Colony Forming Units (CFU) + 1) of Dactylonectria torresensis isolated from eight field soils. Results are reported as mean values of 48 plates from four soil samples per field. Means followed by the same letter are not significantly different (P < 0.05). Bars represent standard error of the mean.*

Physicochemical properties of the soil

The soil physicochemical properties varied between the experimental fields (Table 3.7). The PCA analysis showed the ordination of experimental fields according to soil physicochemical properties as well as the relationships among studied variables (Figure 3.6). The first coordinate explained 44.7% of the variation, the second

coordinate explained 36.7% of the variation, and the third coordinate explained 11.4% of the variation. In the plot of the first and second coordinates, axis 1 was clearly associated with soil texture (percentages of clay, sand and silt), as well as with $CaCO_3$, P, S and Fe concentrations. The percentage of clay, silt and $CaCO_3$ increased to the positive side of the axis, whereas the percentage of sand, P, S and Fe increased to the negative side, thus separating field B (high $CaCO_3$ concentration and percentage of silt) from field E (high K concentration and percentage of sand). The second PCA axis was associated mainly with K concentration and moderately with CEC (increasing to the positive side of the axis), and moderately with pH and Ca (increasing to the negative side of the axis). This separated the fields in two groups: soils with medium-high Ca concentration (fields A, C and G), and those with high percentages of K concentration (fields F and H).

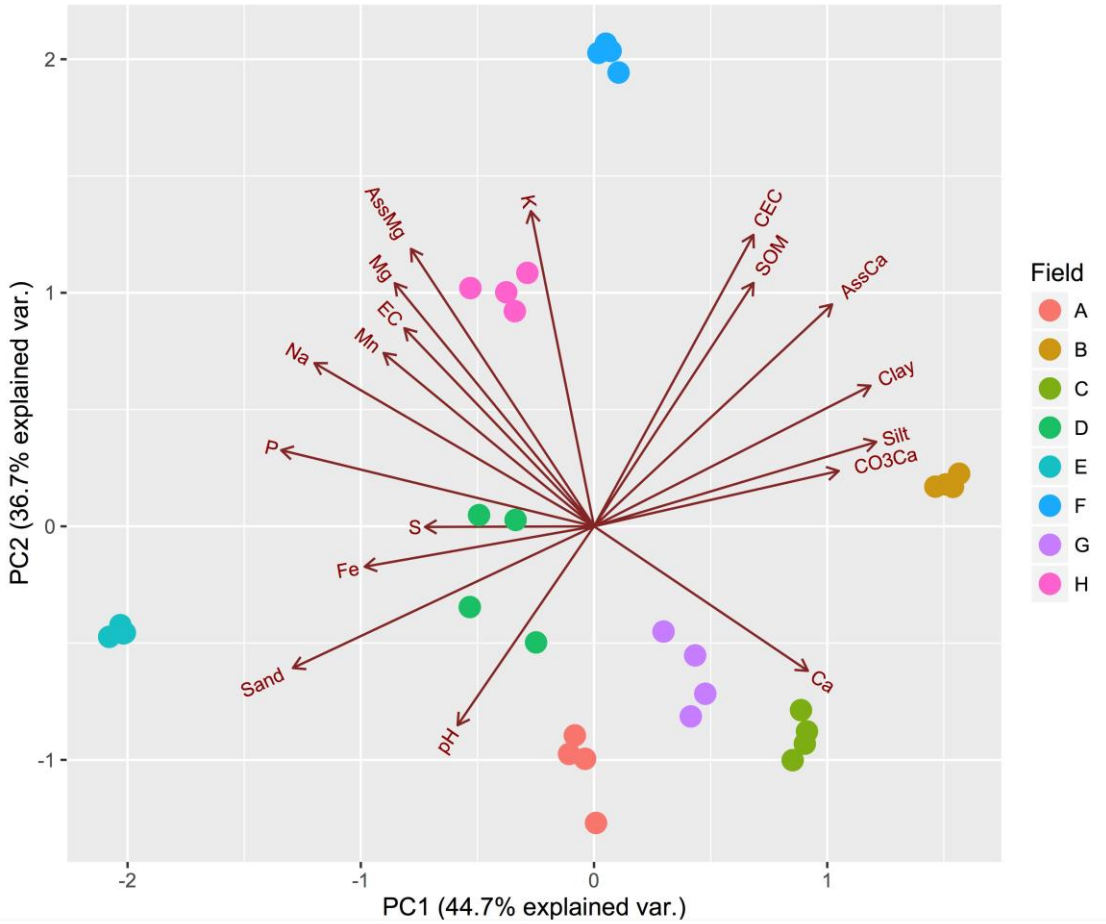

Figure 3.6. *Principal component analysis (PCA) of the eight studied fields in terms of the physicochemical properties of the soil. The percentage value given within parentheses corresponds to the variance explained by each principal component.*

Table 3.7. Physicochemical properties of the eight soils examined in this study. Values represent the mean±SE.

Soil	pH	P mg/100g	K mg/100g	S mg/100g	Mg mg/100g	Mn mg/100g	Fe mg/100g	Ca mg/100g	Na mg/100g	SOM%	Clay%
A	8.42[a]±0.02	3.47±0.27	15.52±0.59	4.37±0.38	25.57±0.29	9.31±0.87	7.5±0.44	1570.92±220.8	6.08±0.19	0.74±0.02	21.6±0.25
B	8.1	1.8±0.11	17.3±0.44	0.9±0.27	15.0±0.26	9.23±0.11	3.27±0.07	1862.15±12.6	1.46±0.05	1.75±0.01	29.42±0.28
C	8.12±0.05	1.91±0.20	19.97±0.42	7.95±0.11	23.3±0.45	2.44±0.12	3.18±062	3437.9±79.6	1.64±0.02	1.15	24.97±0.27
D	8.12±0.02	8.07±1.88	33.05±2.57	15.65±1.26	49.37±3.52	9.62±0.66	9.6±0.38	2512.05±231.1	3.31±0.36	1.61±0.13	15.6±0.26
E	8.3	23.92±0.73	23.17±0.16	14.02±0.33	44.57±0.83	15.6±0.07	7.71±0.46	534.3±15.1	12.57±0.16	0.67±0.01	14.37±0.07
F	8.0	10.42±0.08	61.55±0.66	13.6±0.36	60.65±0.69	11.1±0.04	5.91±0.15	1359.02±12.2	9.95±0.15	2.26±0.04	27.47±0.20
G	8.27±0.02	2.5±0.20	26.55±1.83	13.87±0.29	32.75±1.11	6.37±1.49	4.95±0.49	2173.97±282.7	2.22±0.17	1.12±0.02	26.45±0.43
H	8.27±0.02	9.01±0.10	50.92±0.63	2.42±0.05	66.2±0.51	20.37±0.22	4.42±0.10	993.9±10.7	10.17±0.19	0.99±0.01	26.3±0.28

Sand%	Silt%	CO_3Ca	CO_3Ca	CEC mekv/100g	EC mS/cm	Assim. Ca mekv/100g	Assim. Mg mekv/100g
37.25±0.29	41.12±0.05	15.05±0.03	15.05±0.03	9.7±0.23	0.15	10.2±0.19	1.47±0.03
21.62±0.66	49.0±0.41	18.67±0.23	18.67±0.23	21.25±0.10	0.16	20.4±0.15	0.8±0.04
32.75±0.62	42.27±0.33	14.97±0.06	14.97±0.06	11.3±0.18	0.15	11.92±0.14	0.87±0.01
38.27±0.45	46.1±0.37	11.02±0.56	11.02±0.56	10.45±0.51	0.22±0.01	8.92±0.37	2.33±0.21
61.47±0.08	24.12±0.12	5.77±0.18	5.77±0.18	8.75±0.43	0.28	7.92±0.08	2.32±0.02
26.67±0.67	44.85±0.53	16.32±0.42	16.32±0.42	21.55±0.22	0.31	19.05±0.15	3.71±0.04
29.6±1.09	43.95±0.71	7.0±0.71	7.0±0.71	10.67±1.03	0.12	14.35±0.13	1.42±0.01
27.72±0.25	42.17±3.86	7.35±1.01	7.35±1.01	18.45±0.18	0.16	14.3±0.33	3.47±0.17

[a]Average of 4 replicates.

Relationships between CFU of black-foot pathogens and physicochemical properties of the soil

Non-metric multidimensional scaling analysis (NMDS) grouped the variable CFU of black-foot pathogens per g of soil with $CaCO_3$ concentration (Figure 3.7). Moreover, CFU of black-foot pathogens per g of soil correlated positively with $CaCO_3$ concentration (r=0.61; $P < 0.05$).

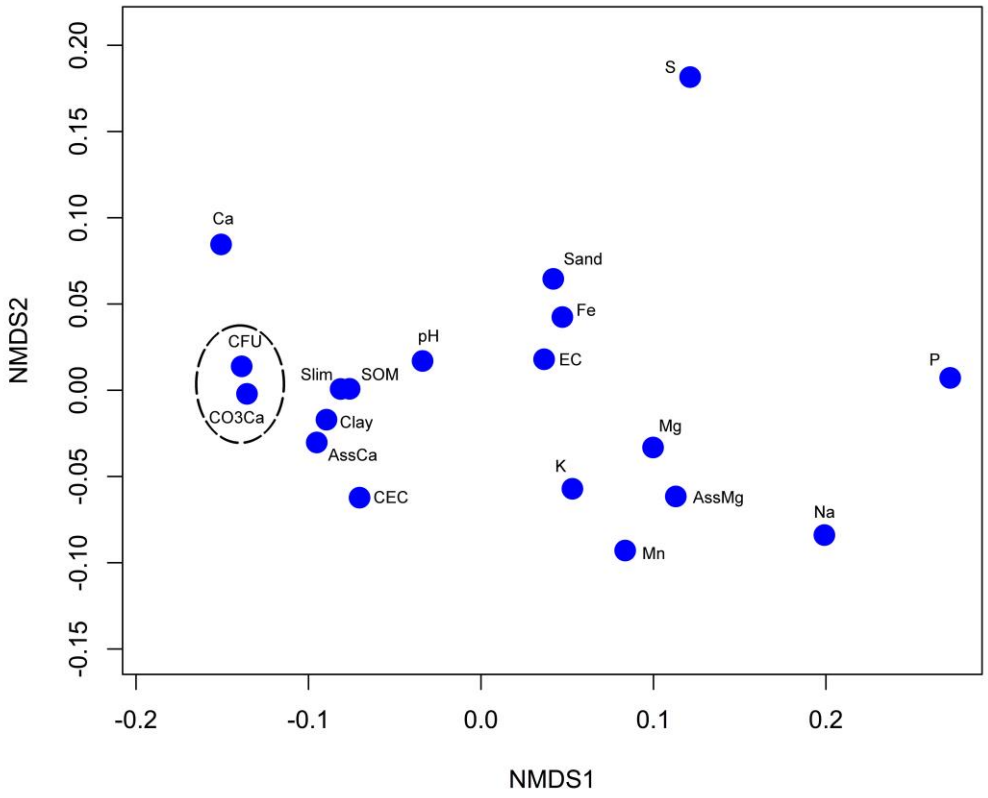

***Figure 3.7**. Non-Metric Multidimensional Scaling Analysis (NMDS) based on similarity matrices among soil physicochemical properties and Colony Forming Units (CFU) of black-foot pathogens in soil.*

Discussion

Early, specific, and accurate detection of black-foot pathogens in soil is essential to prevent the infection of grapevine planting material by these pathogens in field nurseries and also during the first years after planting. In this study, the accuracy and efficiency of three modified culture media protocols published in the literature for the detection of *Cylindrocarpon*-like anamorphs or other soilborne pathogens phylogenetically related to black-foot genera based on plating dry soil samples was

assessed comparatively (Hunter et al. 1980; Singleton et al. 1993; Reedler et al. 2003). We selected a robust protocol, modified from Hunter et al. (1980), that was efficient in detecting black-foot pathogens in naturally infested soils. The culture medium was selected based on the ability of most of the black-foot species to produce a brown or yellow pigment on the agar and the good performance obtained in the recovery of *D. torresensis* from both sterile and non-sterile soil samples. In addition, the mycelial growth of other relevant soilborne fungi (*F. oxysporum* and *R. solani*) was slowed down on GFBRBA and no significant differences were observed with the mycelial growth of some black-foot species. The use of this culture medium would avoid the overgrowing of black-foot species by fast-growing fungi such as *F. oxysporum* and *R. solani* in soil plating assays. Although the emphasis of this study was put on viticulture, other agricultural systems could equally benefit from our optimised protocol.

Detection of *D. torresensis* using the selected protocol confirmed this species as the most frequent black-foot pathogen isolated from the studied soils. Previous research aimed to determine the incidence of black-foot pathogens in grapevine plants in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016) also identified *D. torresensis* as the most frequent black-foot pathogen species. *D. torresensis* was the species with the widest occurrence in the study carried out by Cabral et al. (2012a), being present on four continents, and associated with *Vitis vinifera*, *Abies nordmanniana*, *Fragaria* sp., and *Quercus* sp. This species is also able to affect other woody hosts such as *Protea* sp. in South Africa (Lombard et al. 2013), *Viburnum tinus* in Italy (Aiello et al. 2015) and *Actinidia chinensis* in Turkey (Erper et al. 2013), and has been reported as the most frequently isolated species from necrotic lesions of loquat roots (Agustí-Brisach et al. 2016). Our findings are in agreement with the results obtained by Cardoso et al. (2013), who reported *D. torresensis* as the predominant species in rhizosphere soil samples collected from rootstock mother fields and nursery fields in Portugal.

Recent studies in rhizosphere research on grapevine (Zarraonaindia et al. 2015) and other crops (Turner et al. 2013) suggested that plant species has a clear, significant influence on the microbiota composition in rhizosphere soil. In addition, these studies recognized changes in the microbiota composition between rhizosphere and bulk soil. Active selection of the root microbiota, and in particular plant pathogens, as a

consequence of the use of specific plant genotypes has been studied extensively in other pathosystems (Lannou and Mundt 1997; Gandon and Michalakis 2002; Brown and Tellier 2011). Thus, more research is needed to explore the effects of rootstock selection on black-foot pathogen populations in rhizosphere soil and the changes of these populations between bulk soil and rhizosphere samples.

The inoculum density of *D. torresensis* (CFU per gram of soil) varied among field soils, with this species most often found from bulk soil of 7-year-old and 27-year-old vineyards versus a 2-year-old vineyard and nursery fields during third year of rotation. The richness of black-foot species found in a field nursery was greater than in the other field samples tested. This confirms nursery fields are an important source of soilborne inoculum of black-foot pathogens, which agrees with previous research (Halleen et al. 2003, 2007; Agustí-Brisach et al. 2013a, 2013b, 2014; Cardoso et al. 2013; Reis et al. 2013). Black-foot disease pathogens cause necrosis in the basal end of the rootstock, leading to the early decline and the death of vines exclusively in nurseries and young vineyards (<8 years old) (Agustí-Brisach and Armengol 2013). In this study, we found that *D. torresensis* inoculum is also present in a 27-year-old mature vineyard. However, at this stage, plants are usually less susceptible to black-foot infection than those planted in grapevine nurseries or recently established vineyards. This decrease in susceptibility to black-foot pathogens with host age may be linked to the development of defence mechanisms by older plants that inhibit disease development, such as changes in root exudates, or changes in the composition and structure of the root system that make it less vulnerable to soilborne pathogen infections.

It is thought that most of the species associated with black-foot disease survive in soil for extended periods due to the production of chlamydospores (Schroers et al. 2008; Chaverri et al. 2011), which can survive for multiple years in absence of suitable hosts. In our study, we demonstrated that viable inoculum of *D. torresensis* is still present during the rotation cycle as conidia or chlamydospores, which agrees with the results obtained by Cardoso et al. (2013) in Portugal. In Spain, according to the current nursery legislation, nursery fields used for the rooting phase cannot be planted for more than two consecutive growing seasons, and must have not been used for grapevine cultivation at least for the previous 12 years. These periods could be reduced to one and 6 years respectively if the nursery field is previously disinfested

against nematodes. Planting material is usually planted the first year, followed by three years of rotation with other crops such as wheat and/or barley, and land in a bare fallow strategy. Periodic bare fallow treatments (every third year) have proven effective in reducing pathogen populations in bareroot conifer nurseries (Jones and Benson 2003). This strategy works by removing the host and by decreasing the amount of organic matter in the soil, thereby eliminating or decreasing the food base for soilborne pathogens. However, bare fallow is less likely to work as well for certain soilborne pathogens, such as black-foot pathogens, which produce resistant structures that survive for long periods in soil (Schroers et al. 2008; Chaverri et al. 2011).

Despite the impressive success of crop rotation for managing numerous plant diseases, it has had mixed results for some soilborne pathogens with broad host ranges and long-lived inoculum (Easton et al. 1992; Davis et al. 1999). The unsuccessful results of rotation in some cases raise the possibility that asymptomatic or “hidden” hosts used in rotational programs in grapevine nurseries may be maintaining the inoculum bank. We therefore hypothesize that asymptomatic secondary hosts, specifically rotational crops and weeds, maintain populations of black-foot pathogens in grapevine nurseries and young vineyards. Some species of black-foot pathogens are able to colonize weeds, even though these hosts do not show symptoms of decline (Agustí-Brisach et al. 2011). In South Africa, Halleen et al. (2003) already suggested that the standard nursery practice of a 2-year rotation system, alternated with a cover crop, might have led to a build-up of black-foot pathogens in these soils. Pathogen diversity maintained by asymptomatic hosts may have a detrimental long-term consequence for disease management.

Our results showed a relationship between calcium carbonate and the CFUs of black-foot pathogens in soil. Calcium carbonate is a widely used amendment to neutralize soil acidity and to supply calcium for plant nutrition. In general, previous research has showed that calcium carbonate amendments have reduced infection by soilborne diseases, such as Fusarium wilt (Woltz and Jones 1981) and those caused by *Phytophthora* spp. (Boughton et al. 1978), but excessive calcium may increase disease severity (Schmitthenner and Canady 1983; Spiegel et al. 1987). Soil is essential for the production of healthy vines and nursery stock. It provides physical support for roots and supplies mineral nutrients and water necessary for growth. The soil is also the

environment in which plant roots interact with soilborne pathogens. Therefore, an understanding of how soil properties affect both plant and pathogen health is critical for making effective pest management decisions. The physiochemical characteristics of the soil may favour certain microbial groups over others, thereby leading to shifts in the composition of microbial communities (Brant et al. 2006; Kaiser et al. 2010). More research is needed to elucidate interactions of primary and secondary macronutrients, micronutrients and other physicochemical properties, with disease in enhancing or minimizing black-foot disease incidence in grapevine.

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The fungal and bacterial rhizosphere microbiome associated with grapevine rootstock genotypes in mature and young vineyards

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Abstract

The microbiota colonizing the rhizosphere and the endorhizosphere contribute to plant growth, productivity, carbon sequestration and phytoremediation. Several studies suggested that different plants types and even genotypes of the same plant species harbor partially different microbiomes. Here, we characterize the rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over two years by 16S rRNA gene and ITS high-throughput amplicon sequencing. In addition, we use quantitative PCR (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease. The objectives were to (1) unravel the effects of rootstock genotype on microbial communities in the rhizosphere of grapevine and (2) to compare the relative abundances of sequence reads and DNA amount of black-foot disease pathogens. Host genetic control of the microbiome was evident in the rhizosphere of the mature vineyard. Microbiome composition also shifted as year of sampling, and fungal diversity varied with sampling moments. Linear discriminant analysis identified specific bacterial (i.e., *Bacillus*) and fungal (i.e., *Glomus*) taxa associated with grapevine rootstocks. Host genotype did not predict any summary metrics of rhizosphere α- and β- diversity in the young vineyard. Regarding black-foot associated pathogens, a significant correlation between sequencing reads and qPCR was observed. In conclusion, grapevine rootstock genotypes in the mature vineyard were associated with different rhizosphere microbiomes. The latter could also have

been affected by age of the vineyard, soil properties or field management practices. A more comprehensive study is needed to decipher the cause of the rootstock microbiome selection and the mechanisms by which grapevines are able to shape their associated microbial community. Understanding the vast diversity of bacteria and fungi in the rhizosphere and the interactions between microbiota and grapevine will facilitate the development of future strategies for grapevine protection.

Keywords: bacterial and fungal recruitment, black-foot disease, microbial ecology, microbiome, rhizosphere, rootstock selection.

Abbreviations

OTU	Operational taxonomic unit
ITS	Internal transcribed spacer
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA
SIMPER	Similarity percentages

Introduction

Plants have evolved to cope with biotic and abiotic stresses in association with soil microorganisms (Lemanceau et al. 2017). These microorganisms are known as plant microbiota and, together with the plant, they form an holobiont (Liu et al. 2018). Plant-soil microbiome interactions are complex and, until recent times, the study of these relationships has been mainly focused in the pathogenicity of some microbial agents and how they use and compete for the resources (Philippot et al. 2013; Zancarini et al. 2013; Gilbert et al. 2014; Sapkota et al. 2015). Recent investigations have shown that soil microbiota can directly and indirectly interact with the plants improving their fitness and health (Sapkota et al. 2015). For example, these interactions help plants to deal with abiotic stress and diseases, improving the exchange of substances such as nitrogen or phosphate, or by acting as biocontrol agents through competition with pathogens (Reinhold-Hurek et al. 2015; Vega-Avila et al. 2015; Gallart et al. 2018).

Roots are surrounded by a narrow zone of soil known as rhizosphere. This area, which is influenced by the roots, has a high microbial diversity and its community structure is expected to be different than the one found in the bulk soil (Reinhold-Hurek et al. 2015). The rhizosphere microbiome community composition is affected by different factors, such as ambient conditions, soil properties and background microbial composition (Qiao et al. 2017). In addition, plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host specific microbial communities when grown on the same soil (Aira et al. 2010; Berendsen et al. 2012; Bazghaleh et al. 2015).

As reviewed by Philippot et al. (2013), plant roots release a huge variety of carbon-containing compounds known as rhizodeposits (nutrients, exudates, border cells and mucilage) which make the rhizosphere more nutritive than the bulk soil, which is mostly mesotrophic/oligotrophic, inducing therefore changes on soil microbial communities. It has been reported that the biodiversity in the rhizosphere is lower than in the corresponding bulk soil (Reinhold-Hurek et al. 2015; Lemanceau et al. 2017) since carbon availability often limits microbial growth (Dennis et al. 2010). Rhizodeposits released by the plants considerably vary according to the age and development of plants, among species and even among different genotypes of the same species (Inceoğlu et al. 2010; Philippot et al. 2013; Gilbert et al. 2014; Bazghaleh

et al. 2015; Hacquard 2016; Wagner et al. 2016; Lemanceau et al. 2017; Qiao et al. 2017).

The rhizosphere is also the infection court where soil-borne pathogens establish a parasitic relationship with the plant. To infect root tissue, pathogens have to compete with members of the rhizosphere microbiome for available nutrients and microsites (Chapelle et al. 2016). Exploiting genetic variation in host plant species and understanding interactions between microbiota and their hosts plants will allow the rhizosphere microbiota to be incorporated into plant breeding programs to promote beneficial associations between plants and microorganisms.

Common grapevine (*Vitis vinifera* L.) is one of the most extensively grown and economically important woody perennial fruit crop worldwide with an annual production in 2014 exceeding 74 million tons of grapes and 30 million tons of wine (FAO 2018). Since the late 19th century, *V. vinifera* cultivars have been grafted onto resistant rootstock of other *Vitis* species and hybrids to combat the devastating root phylloxera pest. Several major criteria have been outlined for choosing rootstocks: resistance to phylloxera and nematodes, and adaptability to drought, salinity, limestone content and poor mineral nutrition (Reynolds and Wardle 2001). In addition, the rootstock influence may affect scion vigour, yields, and fruit and wine qualities (Warschefsky et al. 2016).

Plant genetic control over microbial communities in the rhizosphere has been reported for different genotypes of the same species (Aira et al. 2010; Bouffaud et al. 2012; Peiffer et al. 2013; Marques et al. 2014; Jiang et al. 2017; Gallart et al. 2018). However, within grapevine species, the impact of genetic variation on the composition of the bacterial and fungal microbiota is poorly understood. In a recent study, Marasco et al. (2018) observed that five grapevine genotypes influenced the bacterial microbiome from both the root tissues and the rhizosphere fractions at a single vineyard, sampling date and year.

To better understand the players and processes that operate in the rhizosphere, a variety of molecular techniques, such as metagenomics have been applied over the past decade. Here, we characterize the rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over two years by 16S rRNA gene and ITS high-throughput

amplicon sequencing (HTAS). This design allowed us to evaluate the effect of the growing region, year, sampling date, grapevine genotype, and their interactions on the bacterial and fungal community diversity. In addition, we used quantitative Polymerase Chain Reaction (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease, one of the main soil-borne fungal diseases affecting grapevine production worldwide.

Materials and methods

Sample collection

Grapevine rhizosphere samples of five rootstocks ('110 R', '140 Ru', '1103 P', '41 B' and '161-49 C') were collected at two vineyards located in Aldeanueva de Ebro (abbreviated as 'Aldea') (La Rioja, Spain) and Olite (Navarra, Spain). Features of the selected rootstocks are reported in Supplementary Table 3.3. All the selected rootstocks were cultivated in the same vineyard and had been grafted onto 'Tempranillo' cultivar. Soil physicochemical properties showed significant differences between soil types. Climate and soil management practices for fertilization, irrigation and disease control also varied between vineyards (Supplementary Table 3.4). Aldea vineyard was 25-year-old vines at the moment of sampling and contained four randomized blocks of 48 vines per rootstock and block. Olite vineyard was 7-year-old vines at the moment of sampling and contained three randomized blocks of 15 vines per rootstock and block. In each vineyard, three rhizosphere samples were randomly collected per rootstock at two sampling dates (June and November) over two years (2016 and 2017). Sampled vines did not show any symptom of disease or nutrient deficiency. A total of 60 samples were collected per vineyard.

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 in sterile bags on dry ice at the time of sampling, and brought to the laboratory for further processing within 24 h from the time of sampling. The sampled roots with rhizosphere soil particles attached were placed in sterile tubes containing 9 mL of physiological solution (9 g/L NaCl). The tubes were vortexed for 5 min to detach the soil particles and then centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the remaining soil fraction was used for DNA extraction.

DNA extraction and sequencing

The rhizosphere DNA was extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) and DNA samples were randomized across plates. The bacterial V4 region of the 16S rRNA gene was amplified using the protocol described by Lundberg et al. (2013). The universal primer pair 515F and 806R was used to generate bacterial-derived 16S rRNA amplicons. PNA PCR clamps were used to reduce host organelle contamination. The fungal ITS2 region was amplified using the universal primers ITS3/KYO2 and ITS4 (Toju et al. 2012). All primers were modified to include Illumina adaptors (www.illumina.com). Each 25 µL reaction contained 12.5 µL of HiFi HotStart Ready Mix (KAPA Biosystems, Woburn, MA, USA), 1.0 µL of each primer (10 µM), 2.5 µL of DNA template (5 ng/µL), and 8.0 µL PCR-grade water. PCR amplifications (performed in triplicate for each sample) consisted of a 3 min denaturation at 95°C; 25 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; and 5 min at 72°C. Samples were cleaned using the AMPure beads XP purification system (Beckman Coulter, UK) and sequenced on the Illumina MiSeq platform at the Fundación FISABIO (Valencia, Spain) facility using a 2 x 300 nucleotide paired reads protocol.

Data analysis

Raw forward and reverse reads for each sample were assembled into paired-end reads considering the minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using the fastq-join tool from the ea-tools suite (Aronesty 2011). The paired reads were then quality trimmed with a minimum of Q20. Sequences without either primer were discarded. 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 aginast UNITE dynamic database (Abarenkov et al. 2010) for ITS reads and Greengenes database (DeSantis et al. 2006) using the QIIME implementation of the RDP classifier for 16S rRNA reads (Caporaso et al. 2010). A tree was constructed from a gap-filtered alignment using FastTree (Price et al. 2009). A final OTU table was created excluding unaligned sequences and singletons. OTUs with no kingdom-level classification or matching chloroplast, mitochondrial or Viridiplantae sequences were then removed from the data set. Good's coverage values were calculated using the

Mothur computer software (Schloss et al. 2009). The rarefied OTU table and the phylogenetic tree were used as inputs for the subsequent analyses of α- and β-diversity. The OTU table was log transformed for statistical analysis (McMurdie and Holmes 2014). As a final filter, taxa whose total abundances were less than 1% of the mean abundance were excluded, and only the OTUs present in at least two-thirds of the replicates of each sample were selected.

Bacterial and fungal diversity, taxonomy distribution and statistical analysis

Biodiversity indexes and principle statistics analyses on taxonomic profiles were analyzed in R version 3.5 using the vegan (Oksanen et al. 2018) and Phyloseq packages (McMurdie and Holmes 2014). Data in each vineyard was analyzed separately due to the differences in soil chemistry and climate (Supplementary Table 3.4). Technical noise (variation attributable to sequencing depth or batch effects) was controlled by including MiSeq run as a random effect.

Within sample type, α-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). The normalized OTU table was analyzed using Bray Curtis metrics (Bray and Curtis 1957) and utilized to evaluate the β- diversity and to construct PCoA plots (Vázquez-Baeza et al. 2013) using MicrobiomeAnalyst. In order to compare bacterial and fungal communities composition and to partition of variance in different categories, Bray-Curtis distance matrices were subjected to PERMANOVA (Anderson 2001) using the adonis function with a permutation number of 999 available in the vegan package of R. PERMANOVA was performed to investigate which OTUs significantly differed in abundance among experimental factors.

The variance-partitioning model tests for effects of year, sampling date and genotype on microbiome communities, while year-by-genotype and date-by-genotype interaction terms describe how the distinct fungal and bacterial communities at different common rootstocks respond differently to each of these factors. The linear mixed models were fit using the lme4 package (Bates et al. 2015). Statistical significance of fixed predictors (Year + Sampling Date + Genotype + Year x Genotype + Date x Genotype) was assessed using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom in the package InnerTest

(Kuznetsova et al. 2016), and of random effects (MiSeq run) using likelihood ratio tests. This model was used to predict community descriptors that were continuous and approximately normally distributed in α-diversity metrics (Shannon entropy and Chao1 estimated richness) as described above.

The Linear Discriminant Analysis Effect Size (LEfSe) algorithm was used to identify taxa (genus level or higher) that differed in relative abundance between the rootstocks (Segata et al. 2011). The online Galaxy Version 1.0 interface (The Huttenhower Lab) was used, the threshold for the logarithmic 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. A Similarity Percentages (SIMPER) analysis was performed with PRIMER 6 software to explore the dissimilarities between the rootstock factor. Summarized taxa tables at the phylum and genera levels were used to investigate the phylogenetic groups that contribute to the dissimilarity. Unclassified OTUs amounting to less than 3% of the relative abundance in the rhizosphere were discarded from the analysis, according to Marasco et al. (2018). The bacterial and fungal OTUs shared among vineyards and rootstocks were defined by a Venn-diagram analysis using the software available at (van de Peer et al. 2018).

Quantitative PCR amplification and quantification of black-foot disease pathogens

Quantitative PCR analyses were performed with the DNA extracted from the soil samples, as Agustí-Brisach et al. (2014) developed in previous research, using the primers YT2F and Cyl-R (Dubrovsky and Fabritius 2007; Tewoldemedhin et al. 2011). 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.* Rotor-Gene 6000 real-time rotary analyser (Qiagen, Hilden, Germany) was used to perform the qPCR amplifications. Each reaction contained 2 µL of DNA, 1x of SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Bio Inc., Shiga, Japan) and 0.4 µM of each primer. The reaction mix was adjusted to a final volume of 20 µL with sterile distilled water. The thermocycling profile consisted of 30 s at 95°C and 50 cycles of 10 s at 95°C, 10 s at 60°C, and 30 s at 72°C. To evaluate amplification specificity, melting curve analysis was performed at the end of the qPCR runs according to the manufacturer's recommendations. Each analysis included three

replicates of each sample, a non-template control reaction (water) and a positive control containing DNA extracted from a pure culture of the *D. torresensis* isolate GTMF DT097, obtained from the collection of the Instituto Agroforestal Mediterráneo, Universitat Politècnica de Valencia, Spain. *D. torresensis* is the most common fungal species associated with black-foot diseased vines in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Berlanas et al. 2017). For DNA extraction, fungal mycelium of this isolate grown on potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France) for 2 weeks at 25°C in darkness, was scraped from the surface of the plate with a sterile scalpel. Total DNA was extracted using the E.Z.N.A. Plant Miniprep kit (Omega Bio-Tek, Doraville, USA) following the manufacturer's instructions and mycelia was previously homogenized 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 5m/s for 20 s twice. DNA extracted was quantified with Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, USA).

DNA of the *Cylindrocarpon*-like asexual morphs species was quantified using a standard curve constructed with the isolate GTMF DT097, consisting of a dilution series from 275 µg/µL to 0.275 fg/µL. Quantitative PCR analysis were perform as previously explained and the standard curve was generated following the MIQE guidelines (Bustin et al. 2009), by plotting quantification cycle (C_q) values obtained for each specific DNA concentration, versus the logarithm of the initial concentration of isolate DNA. The mean DNA concentration and the standard deviation were determined from three replicates per dilution. Sensitivity of the qPCR assay was assessed using the standard curve to determine the minimum DNA concentration that can be detected. The amplification efficiency (E) and the coefficient of determination (R^2) of the standard curve were obtained using the Rotor-Gene 6000 Series software v. 1.7 (Qiagen, Hilden, Germany). Signal threshold levels were set automatically by the instrument software and the limit of detection (LOD) was identified by the last dilution when successful qPCR amplification of DNA occurred, accompanied by a melting curve peak temperature specific to *D. torresensis*.

Values from the *Cylindrocarpon*-like asexual morphs number of OTUs and DNA concentration were transformed by log ($n/N * 1000 + 1$). Where n was the number of OTUs or the DNA concentration detected on each sample and N was the total number

of OTUs and the total DNA concentration detected. An analysis of correlation between both transformed datasets was performed in R version 3.5 using the corrr package.

Results

High-throughput amplicon sequencing

After paired-end alignments, quality filtering and deletion of chimeric, singletons, and mitochondrial and chloroplast sequences, a total of 4,337,395 bacterial 16S rRNA sequences and 6,216,366 fungal internal transcribed spacer (ITS) sequences were generated from 117 (three samples were removed from the analysis due to the low number of sequence reads) and 120 samples, respectively, and assigned to 975 bacterial and 567 fungal operational taxonomic units (OTUs) (Supplementary Table 3.5). Good's coverage values indicated that on average 94.5% and 90.1% of the total species richness were accounted for in bacteria and fungal communities, respectively (Supplementary Table 3.6). Chao1 diversity estimator ranged from 143.6 to 549.5 in the bacterial microbiome, and from 90.5 to 254.9 in the fungal microbiome. Shannon diversity estimator ranged from 1.80 to 4.68 in the bacterial microbiome, and from 1.80 to 3.84 in the fungal microbiome (Supplementary Table 3.6).

Core grapevine phylogeny between vineyards

The two habitats used as vineyard sites (Aldeanueva del Ebro, abbreviated 'Aldea' in the figures and tables; and Olite) were separated by 45 km, and varied in most of soil physico-chemical properties (Supplementary Table 3.4). Bacterial communities of rhizosphere soil samples did not differ significantly between vineyards (Supplementary Table 3.7). However, α-diversity differed among sites when studying the fungal microbiota, and principal coordinates analysis (PCoA) of Bray Curtis data demonstrated that vineyard was the primary source of β-diversity (Supplementary Figure 3.3). Comparing the fungal and bacterial microbiota of the two vineyards, 82.9 and 58.7% of bacterial and fungal OTUs, respectively, were shared between vineyards, demonstrating the existence of a "core" grape phylogeny that is independent of the growing region (Figure 3.8).

The relative abundance of bacterial and fungal phyla detected across all samples is shown in Figure 3.8. In both vineyards, the bacterial phyla Proteobacteria (26.1% and

28.1% in Aldea and Olite, respectively) and Actinobacteria (24.1% and 18.5%) represented almost 50% of the total bacteria detected. These phyla were followed by Acidobacteria (13.7 and 16.4%), unidentified bacteria (11.4 and 11.7%) and Bacteroidetes (5.2 and 6.1%) (Figure 3.8).

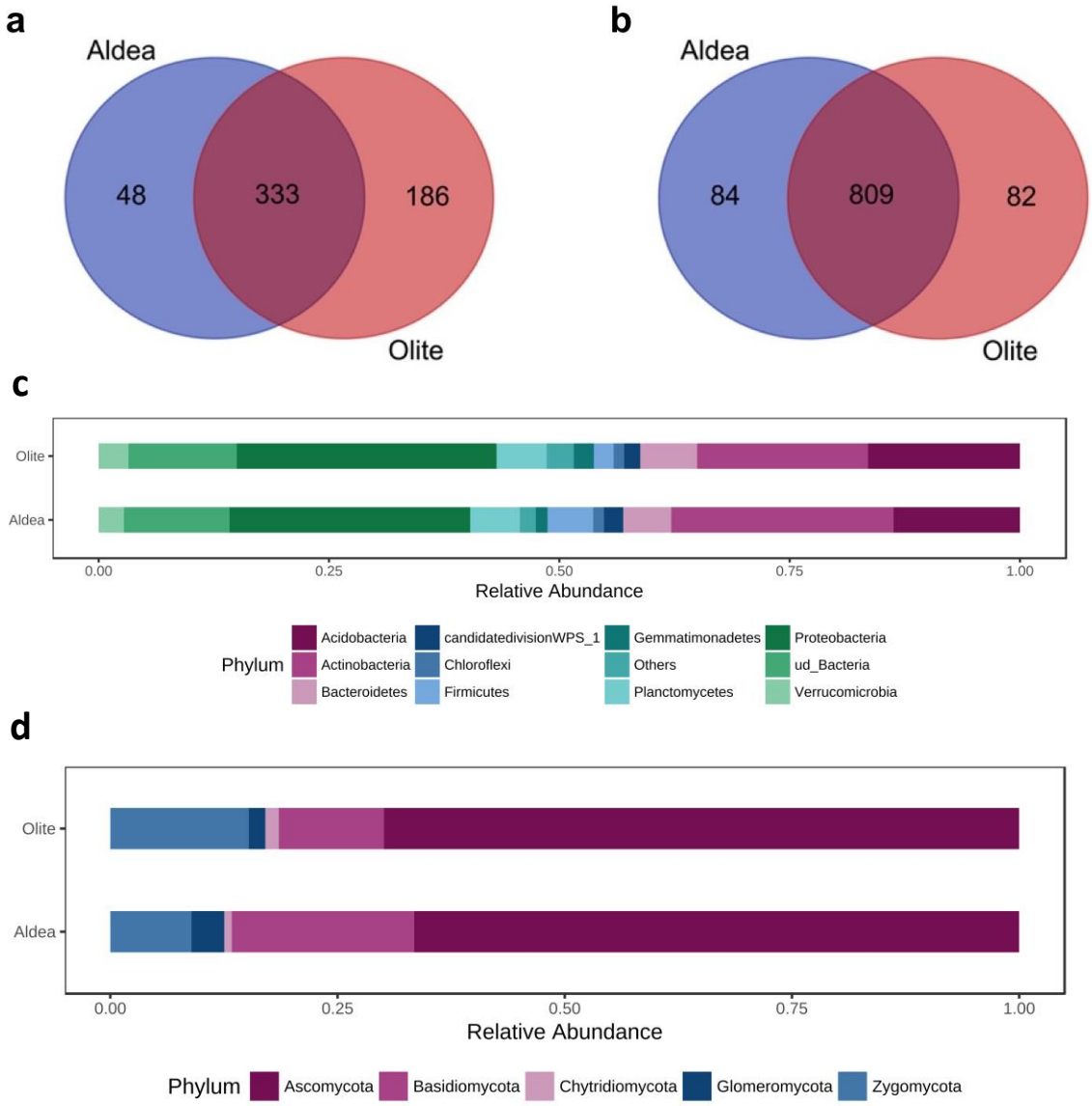

Figure 3.8. *Venn diagram illustrating the overlap of the OTUs identified in the fungal (a) and bacterial (b) microbiota between vineyards. Relative abundance of different bacterial (c) and fungal (d) phyla in the rootstock rhizospheres in both vineyards representing OTUs showing more than 1% relative abundance of all reads and present in at least 2/3 of replicates. Phyla representing less than 1% of the total reads are grouped in 'Others'.*

The most abundant families within the Proteobacteria phylum were unidentified families from the order Rhizobiales (13.0% and 10.4% in Aldea and Olite, respectively), unidentified families from the class Betaproteobacteria (9.8% and 13.0%) and Sphingomonadaceae (7.6% and 10.7%). The most abundant families within the

Actinobacteria phylum were unidentified Actinobacteria (29.1% and 22.5% in Aldea and Olite, respectively), Gaiellaceae (16.0% and 15.2%) and Streptomycetaceae (6.2% and 6.7%) (Supplementary Figure 3.4). Regarding the fungal taxa, the most abundant fungal phylum was Ascomycota (66.6% and 69.9% in Aldea and Olite, respectively), followed by Basidiomycota (20.1% and 11.5%) and Zygomycota (8.9 and 15.2%) (Figure 3.8). The most abundant families within the Ascomycota phylum were Nectriaceae (15.4%), unidentified Ascomycota (8.8%) and Bionectriaceae (9.1%) in Aldea vineyard, and Nectriaceae (17.7%), unidentified Ascomycota (11.1%), Pyronemataceae (9.6%) and Trichocomaceae (8.4%) in Olite vineyard (Supplementary Figure 3.4).

Host genetic influence on the rhizosphere microbiota

Bacterial and fungal diversity in rhizosphere soil samples differed significantly among rootstocks in Aldea vineyard. However, plant genotype did not predict Chao1 diversity (Table 3.7). Host genotype was the most important factor in structuring bacterial (R^2 = 0.65, P < 0.001) and fungal (R^2 = 0.86, P < 0.001) communities in the entire dataset, and also when the data were split by year and date (Table 3.8). A PCoA further demonstrated the variation in the total dataset could be attributed to host genotype in Aldea vineyard (Figure 3.9). In Olite vineyard, plant genotype had a much weaker influence on rhizosphere-associated bacterial and fungal communities. Host genotype did not predict any summary metrics of rhizosphere α and β-diversities (Tables 3.8 and 3.9).

The linear discriminant analysis effect size (LEfSe) detected 27 bacterial and 36 fungal clades in the rhizospheres, which discriminated the microbial communities between the different rootstock genotypes in Aldea vineyard (Figures 3.10 and 3.11). Both rootstocks ‘1103 P’ and ‘41 B’ showed higher number of differentially abundant bacterial clades (8 each) than the other rootstocks (5, 4 and 2 in ‘161-49 C’, ‘110 R’ and ‘140 Ru’, respectively). The dominant bacterial phyla were Firmicutes (37%) in rootstock ’41 B’, Actinobacteria and Planctomycetes (50% each) in rootstock ‘140 Ru’, and Actinobacteria in rootstocks ‘161-49 C’, ‘110 R’ and ‘1103 P’ (60%, 75% and 75%, respectively) (Figure 3.10). The dominant fungal phyla were Basidiomycota (73%) in rootstock ‘140 Ru’, and Ascomycota in rootstocks ‘41 B’, ‘161-49 C’, ‘110 R’ and ‘1103 P’ (75%, 100%, 36% and 71%, respectively) (Figure 3.11).

Table 3.8. Experimental factors predicting α-diversity of rhizosphere associated fungal and bacterial communities in Aldea and Olite vineyards.

Bacteria	Aldea		Olite	
	Shannon	Chao1	Shannon	Chao1
Genotype	$F_{4,54}$ = 3.47	$F_{4,54}$ =0.34	$F_{4,54}$ = 0.90	$F_{4,54}$ = 0.32
	***P* = 0.0134**	*P* = 0.8480	*P* = 0.4693	*P* = 0.8648
Year	$F_{1,57}$ = 6.83	$F_{1,57}$ = 17.39	$F_{1,57}$ = 4.66	$F_{1,57}$ = 7.55
	***P* = 7.3^{-09}**	***P* = 1.5^{-20}**	***P* = 1.6^{-04}**	***P* = 4.7^{-10}**
Year x Genotype	$F_{4,49}$ = 0.73	$F_{4,49}$ = 1.48	$F_{4,49}$ = 2.33	$F_{4,49}$ = 6.08
	***P* = 0.0122**	*P* = 0.3661	*P* = 0.0623	*P* = 0.2143
Date	$F_{1,57}$ = 0.05	$F_{1,57}$ = 0.18	$F_{1,57}$ = 0.68	$F_{1,57}$ = 0.13
	P = 0.9555	*P* = 0.8502	*P* = 0.4989	*P* = 0.8941
Date x Genotype	$F_{4,49}$ = 1.55	$F_{4,49}$ = 0.74	$F_{4,49}$ = 0.19	$F_{4,49}$ = 1.67
	P = 0.1812	*P* = 0.7702	*P* = 0.1802	*P* = 0.2561
MiSeq r	χ^2_1 = 0.55	χ^2_1 = 0.74	χ^2_1 = 0.28	χ^2_1 = 1.59
	P = 0.3623	*P* = 0.4565	*P* = 0.7712	*P* = 0.3421
Fungi				
Genotype	$F_{4,55}$ = 2.80	$F_{4,55}$ = 1.12	$F_{4,55}$ = 0.82	$F_{4,55}$ = 2.27
	***P* = 0.0232**	*P* = 0.3529	*P* = 0.5130	*P* = 0.0929
Year	$F_{1,58}$ = 0.95	$F_{1,58}$ = 10.62	$F_{1,58}$ = 0.37	$F_{1,58}$ = 5.25
	P = 0.3415	***P* = 3.2^{-15}**	*P* = 0.7112	***P* = 3.5^{-06}**
Year x Genotype	$F_{4,50}$ = 2.85	$F_{4,50}$ = 1.15	$F_{4,50}$ = 0.35	$F_{4,50}$ = 3.85
	P = 0.1126	*P* = 0.3601	*P* = 0.1831	*P* = 0.3126
Date	$F_{1,58}$ = 8.52	$F_{1,58}$ = 2.17	$F_{1,58}$ = 0.44	$F_{1,58}$ = 1.31
	***P* = 1.08^{-11}**	*P* = 0.0640	*P* = 0.6597	*P* = 0.1937
Date x Genotype	$F_{4,50}$ = 0.71	$F_{4,50}$ = 0.91	$F_{4,50}$ = 1.91	$F_{4,50}$ = 6.81
	***P* = 0.0112**	*P* = 0.2903	*P* = 0.6351	*P* = 0.7443
MiSeq run	χ^2_1 = 0.74	χ^2_1 = 2.92	χ^2_1 = 1.77	χ^2_1 = 0.12
	P = 0.4912	*P* = 0.2551	*P* = 0.8135	*P* = 0.7331

ANOVA, analysis of variance

Statistics describe linear random-intercept models of Shannon diversity and Chao1 richness in the rhizosphere. All *P* values were corrected for multiple comparisons using the sequential Bonferroni correction. Significance was assessed using Type III ANOVA with F tests for fixed effects and likelihood ratio tests for the random effect. Bold values indicate statistically significant results after correction for multiple comparisons $P < 0.05$.

The rootstock-pairs dissimilarity, due to phyla and genera contribution in the rhizosphere was calculated by SIMPER (similarity percentages) analysis (Supplementary Table 3.8). Higher microbiome dissimilarity among rootstocks was revealed in Aldea vineyard compared to Olite vineyard, considering bacterial (Supplementary Table 3.8A) and fungal phyla (Supplementary Table 3.8C), and bacterial (Supplementary Table 3.8B) and fungal genera (Supplementary Table 3.8D) distribution. Firmicutes and Acidobacteria were the major phyla that contribute to differentiate the bacterial communities associated with the different rootstock types in Aldea and Olite vineyards, respectively (Supplementary Table 3.8A). Several genera were predominant and determined the dissimilarities among rootstocks such as *Bacillus* in Aldea vineyard or *Aridibacer* in Olite vineyard. The genus *Bacillus* appeared

to be rhizosphere genotype biomarker of '140 Ru' and '161-49 C' rootstocks (Supplementary Table 3.8B).

The fungal phyla Ascomycota and Basidiomycota contributed to the dissimilarity among rootstocks in Aldea vineyard, while only the phylum Basidiomycota contributed to differentiate fungal communities among rootstocks (Supplementary Table 3.8C). The fungal genera *Geopyxis*, *Clonostachys* and *Lecanicillium* determined the dissimilarities among rootstocks in Aldea vineyard, being *Geopyxis* a rhizosphere genotype biomarker of '110 R' rootstock and *Clonostachys* of '1103 P' and '140 Ru' rootstocks (Supplementary Table 3.8D). In Aldea vineyard, '161-49 C' rootstock showed the highest dissimilarity with the other rootstocks in bacterial and fungal microbiome distribution.

Year strongly influenced microbiomes

Our results demonstrate that bacterial microbiome varied profoundly between years. This pattern was consistent to community-level measure of α- diversity in both Aldea and Olite vineyards (Table 3.8) Richness increased between 2016 and 2017 in both vineyards (Supplementary Figure 3.5). However, year of sampling affected the Bray Curtis metric of β-diversity in only Olite vineyard (R^2 = 0.494) (Supplementary Figure 3.5). Regarding the fungal microbiome, richness also varied between vineyards and increased between 2016 and 2017 in both vineyards (Table 3.8; Supplementary Figure 3.6). However, year of sampling did not predict Shannon diversity and affected the Bray Curtis metric of β-diversity in only Olite vineyard (Table 3.9; Supplementary Figure 3.6). Sampling date also contributed to α-diversity variation indicating temporal changes in relative abundance of fungal OTUs in Aldea vineyard. Fungal composition decreased between June and November (Table 3.8; Supplementary Figure 3.7). Fungal community structure varied individually in each rootstock with date (R^2 ranging from 0.42 to 0.61), but not in the total dataset ($R^2 < 0.1$) (Table 3.9).

Rootstock-specific and shared bacterial and fungal assemblages

The rhizosphere compartments of grapevine rootstocks showed specific fungal and bacterial OTUs for each rootstocks and a cluster of shared OTUs. In Aldea, specific OTUs associated with most of the rootstocks ranged from 4.3 to 5.8% of their bacterial

communities (Figure 3.12). Specific OTUs associated with the rootstocks '140 Ru', '1103 P', '41 B' and '110 R' represented less than 9% of their fungal communities, where the '161-49 C'-specific OTUs enriched only 4.5% of the relative abundance (Figure 3.12). In Olite, specific OTUs associated with most of the rootstocks represented less than 9% of their bacterial and fungal communities, with the exception of bacterial communities associated with 140 Ru rootstock that represented 21.3% of its total (Figure 3.13). The OTUs that were unique in each of the grapevine rootstock are shown in Supplementary Tables 3.9 and 3.10.

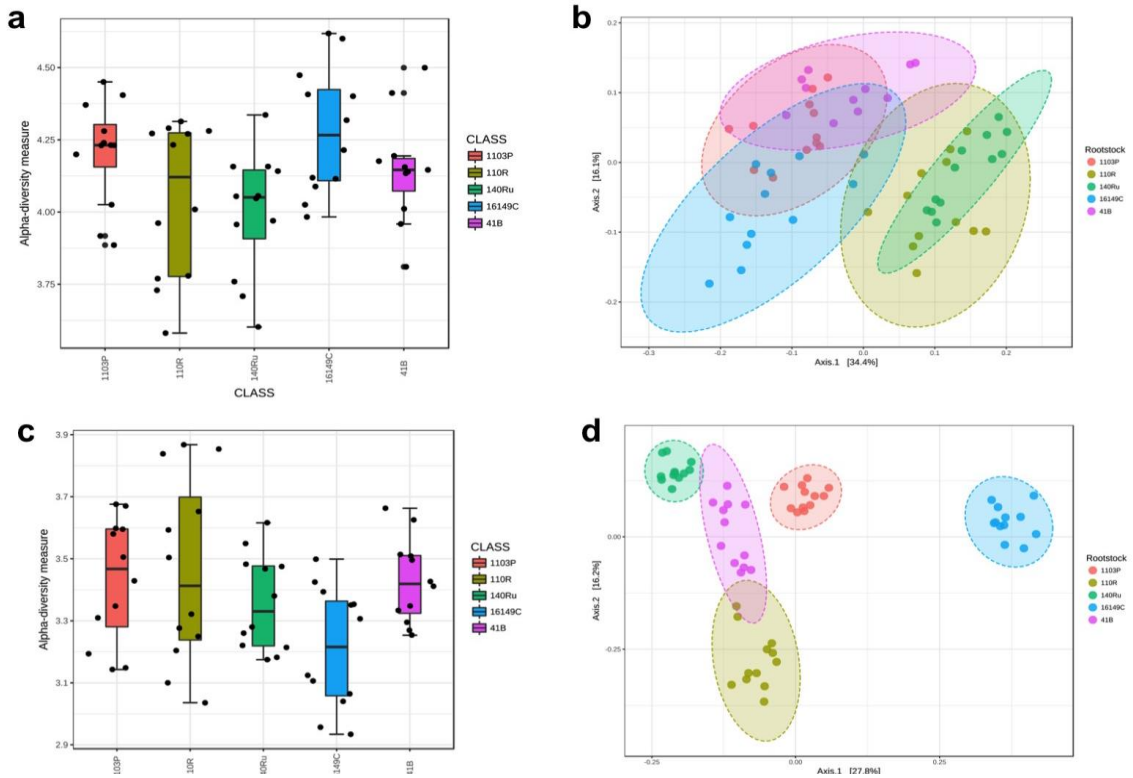

Figure 3.9. *Boxplot illustrating the differences in Shannon diversity measures of the bacterial* ***(a)*** *and fungal* ***(c)*** *communities in the grapevine rootstocks in Aldea vineyard. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the bacterial* ***(b)*** *and fungal* ***(d)*** *communities among grapevine rootstocks.*

Quantification of black-foot disease pathogens using quantitative PCR

The standard curve, constructed with serial dilutions of the DNA of *Dactylonectria torresensis* isolate GTMF DT097, revealed high correlations between C_q and DNA, with R^2 value of 0.99 and reaction efficiency of 0.90. The minimum DNA concentration detectable of *D. torresensis* was at C_q value of the dilution D7 thus, the limit of detection (LOD) was established at 2.75 fg/µL.

DNA of *Cylindrocarpon*-like asexual morphs was detected in all rootstock rhizosphere samples, in both vineyards and years, with concentrations ranging from 0.39 pg/µL to 4.06 pg/µL in Aldea 2016, from 3.52 pg/µL to 14.14 pg/µL in Aldea 2017, from 0.88 pg/µL to 8.45 pg/µL in Olite 2016 and from 2.65 pg/µL to 59 pg/µL in Olite 2017. The year and vineyard factors had a significant effect on *Cylindrocarpon*-like asexual morphs DNA concentration detected ($P < 0.01$).

Table 3.9. Adonis test of category effect on bacterial and fungal Bray-Curtis distance matrix.

Bacteria		Aldea			Olite	
Dataset	Factor	R^2	*P* value	Factor	R^2	*P*-value
Total	Genotype	0.658	0.001	Genotype	0.058	0.015
	Year	0.163	0.001	Year	0.494	0.001
	Date	0.109	0.002	Date	0.059	0.004
'110 R'	Year	0.564	0.002	Year	0.438	0.005
	Date	0.028	0.116	Date	0.204	0.066
'140 Ru'	Year	0.235	0.006	Year	0.458	0.005
	Date	0.355	0.002	Date	0.092	0.333
'1103 P'	Year	0.220	0.011	Year	0.379	0.005
	Date	0.461	0.002	Date	0.174	0.036
'41 B'	Year	0.087	0.071	Year	0.453	0.005
	Date	0.670	0.002	Date	0.129	0.092
'161 49 C'	Year	0.228	0.003	Year	0.471	0.005
	Date	0.228	0.005	Date	0.221	0.040
2016	Genotype	0.868	0.001	Genotype	0.206	0.031
	Date	0.067	0.035	Date	0.165	0.001
2017	Genotype	0.768	0.001	Genotype	0.240	0.001
	Date	0.135	0.004	Date	0.138	0.002
June	Genotype	0.634	0.001	Genotype	0.145	0.365
	Year	0.110	0.005	Year	0.331	0.001
November	Genotype	0.831	0.001	Genotype	0.240	0.020
	Year	0.123	0.004	Year	0.354	0.001
Fungi						
Total	Genotype	0.864	0.001	Genotype	0.096	0.027
	Year	0.052	0.004	Year	0.564	0.001
	Date	0.084	0.001	Date	0.042	0.005
'110 R'	Year	0.183	0.122	Year	0.438	0.005
	Date	0.501	0.002	Date	0.204	0.066
'140 Ru'	Year	0.142	0.137	Year	0.458	0.005
	Date	0.615	0.002	Date	0.092	0.333
'1103 P'	Year	0.266	0.031	Year	0.379	0.005
	Date	0.496	0.002	Date	0.174	0.036
'41 B'	Year	0.241	0.033	Year	0.453	0.005
	Date	0.425	0.002	Date	0.129	0.092
'161 49 C'	Year	0.191	0.066	Year	0.471	0.005
	Date	0.472	0.002	Date	0.221	0.040
2016	Genotype	0.841	0.001	Genotype	0.144	0.305
	Date	0.110	0.002	Date	0.070	0.002
2017	Genotype	0.928	0.001	Genotype	0.274	0.001
	Date	0.130	0.002	Date	0.127	0.002
June	Genotype	0.808	0.001	Genotype	0.220	0.012
	Year	0.066	0.080	Year	0.289	0.001
November	Genotype	0.753	0.001	Genotype	0.200	0.003
	Year	0.105	0.004	Year	0.208	0.001

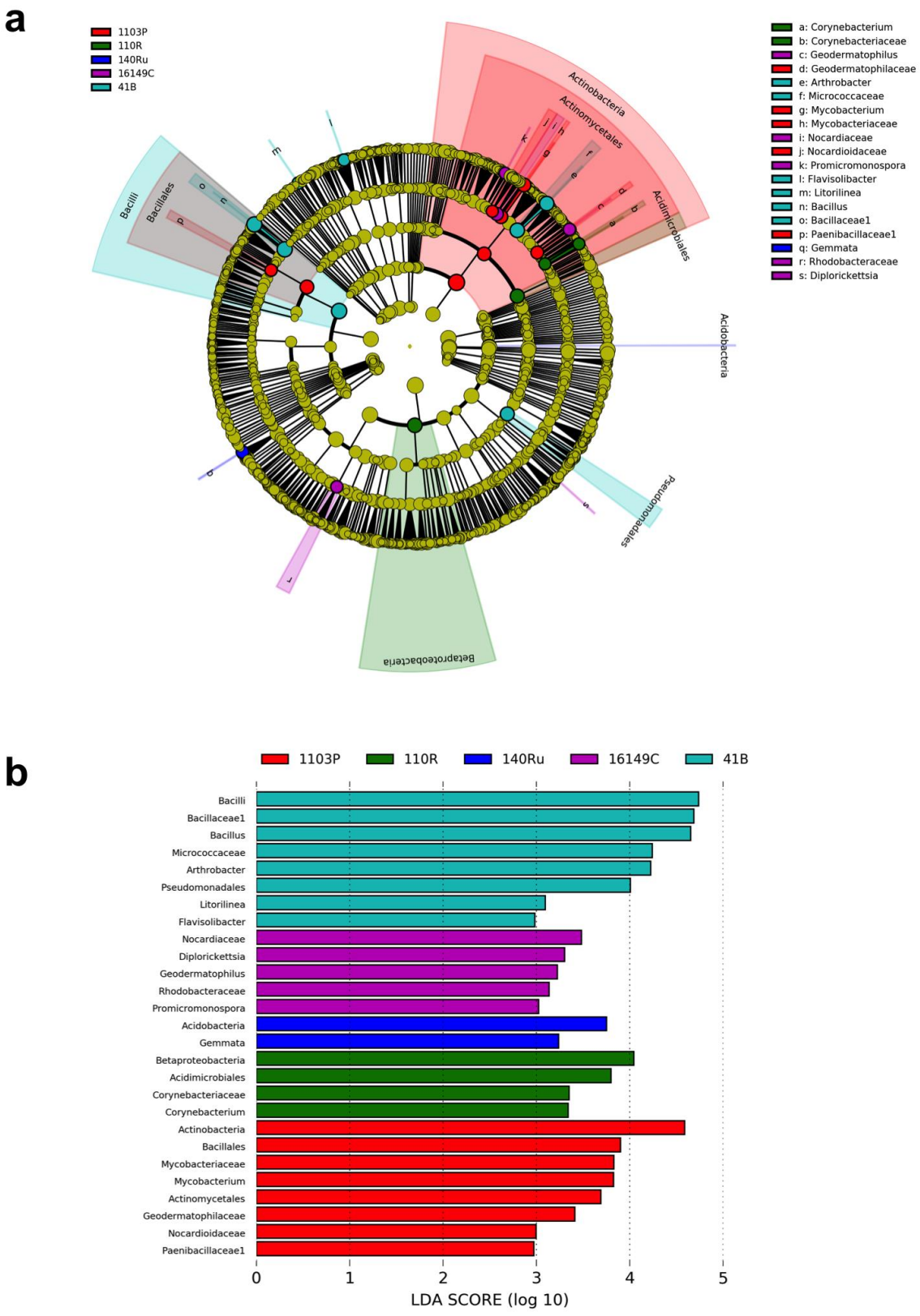

Figure 3.10. *LEfSe was used to identify the most differentially abundant taxa among grapevine rootstocks in Aldea vineyard. Cladogram generated by LEfSe indicating differences of bacteria* ***(a)*** *at phylum, class, family and genus levels between the five groups (relative abundance* $\leq$ *0.5%). Each successive circle represents a phylogenetic level. Color regions indicate taxa enriched in the different rootstocks. Differing taxa are listed on the right side of the cladogram. Bar graph showing LDA scores for bacteria* ***(b)****. Only taxa meeting an LDA significant threshold < 2 are shown.*

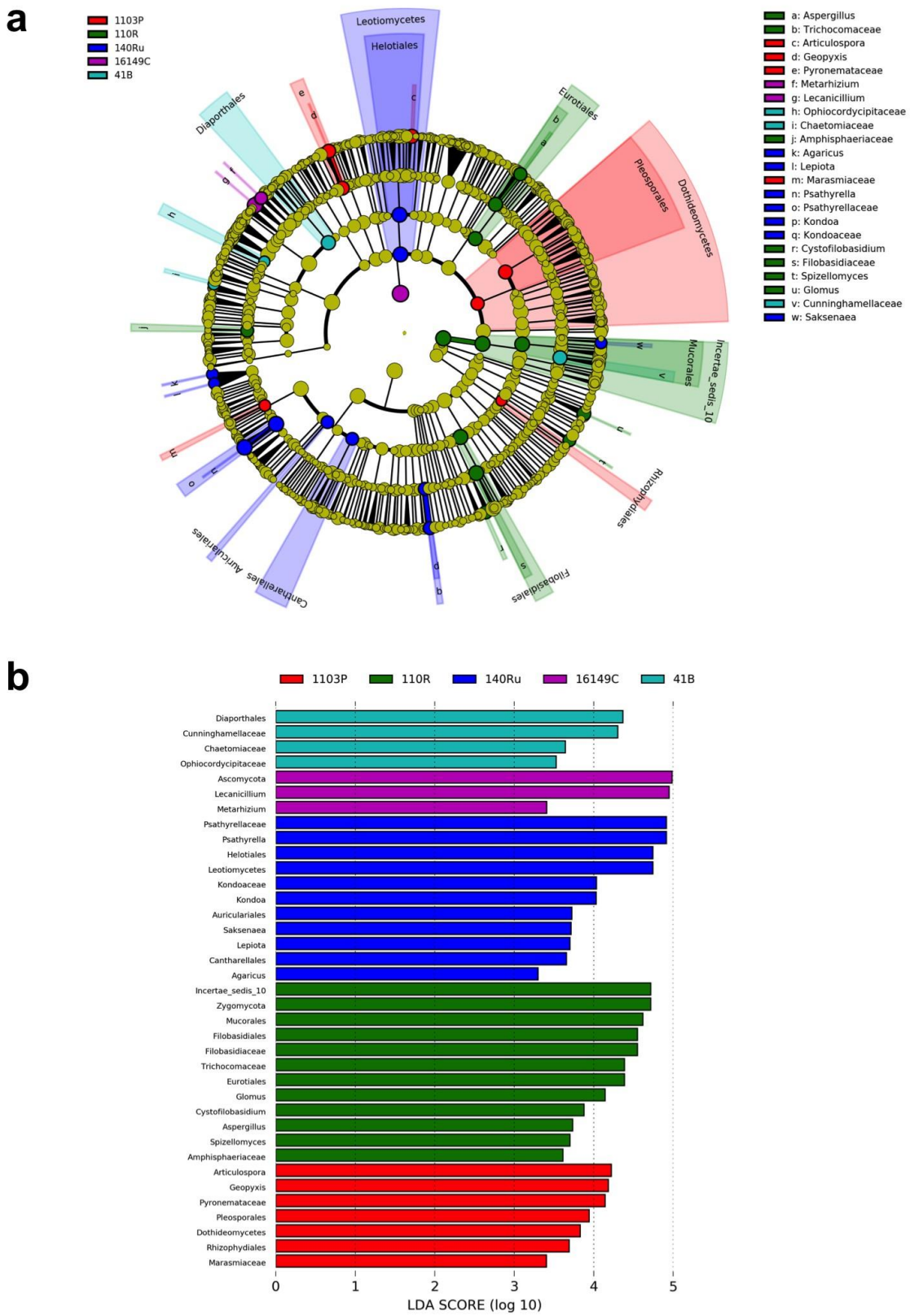

Figure 3.11. *LEfSe was used to identify the most differentially abundant taxa among grapevine rootstocks in Aldea vineyard. Cladogram generated by LEfSe indicating differences of fungi* ***(a)*** *at phylum, class, family and genus levels between the five groups (relative abundance ≤ 0.5%). Each successive circle represents a phylogenetic level. Color regions indicate taxa enriched in the different rootstocks. 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.*

The concentration of DNA detected was significantly higher in Olite vineyard compared with Aldea vineyard, especially in year 2017. The rootstock factor had a significant effect on the DNA concentration detected in Aldea vineyard for 2017 samples (P = 0.0156). Rootstocks '161-49 C', '140 Ru', '1103 P' and '110 R' showed similar DNA concentrations values that were significantly lower when compared with '41 B' rootstocks (Figure 3.14). The analysis showed a positive significant correlation between the number of OTUs and the *Cylindrocarpon*-like asexual morphs DNA quantified using the real-time approach (P < 0.01, Spearman correlation coefficient = 0.72) (Figure 3.15).

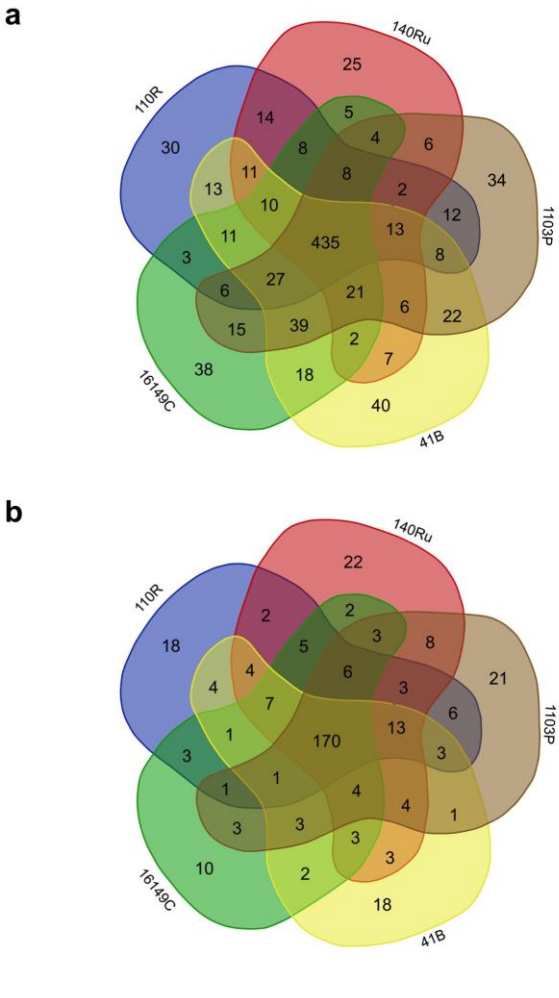

Figure 3.12. *Venn diagrams showing the common and exclusive bacterial* ***(a)*** *and fungal* ***(b)*** *OTUs of the rhizosphere of the grapevine rootstocks in Aldea vineyard.*

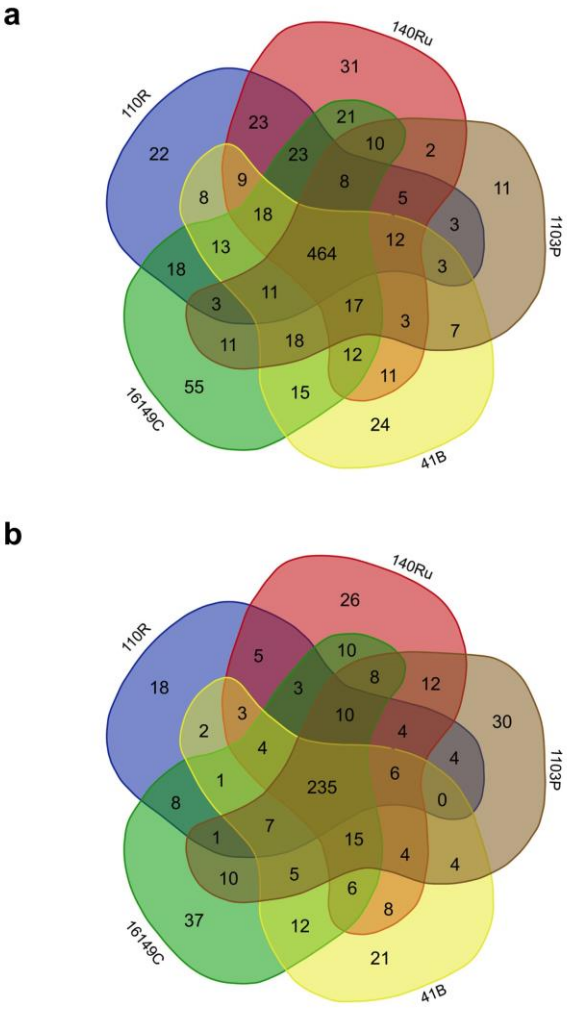

Figure 3.13. *Venn diagrams showing the common and exclusive bacterial* ***(a)*** *and fungal* ***(b)*** *OTUs of the rhizosphere of the grapevine rootstocks in Olite vineyard.*

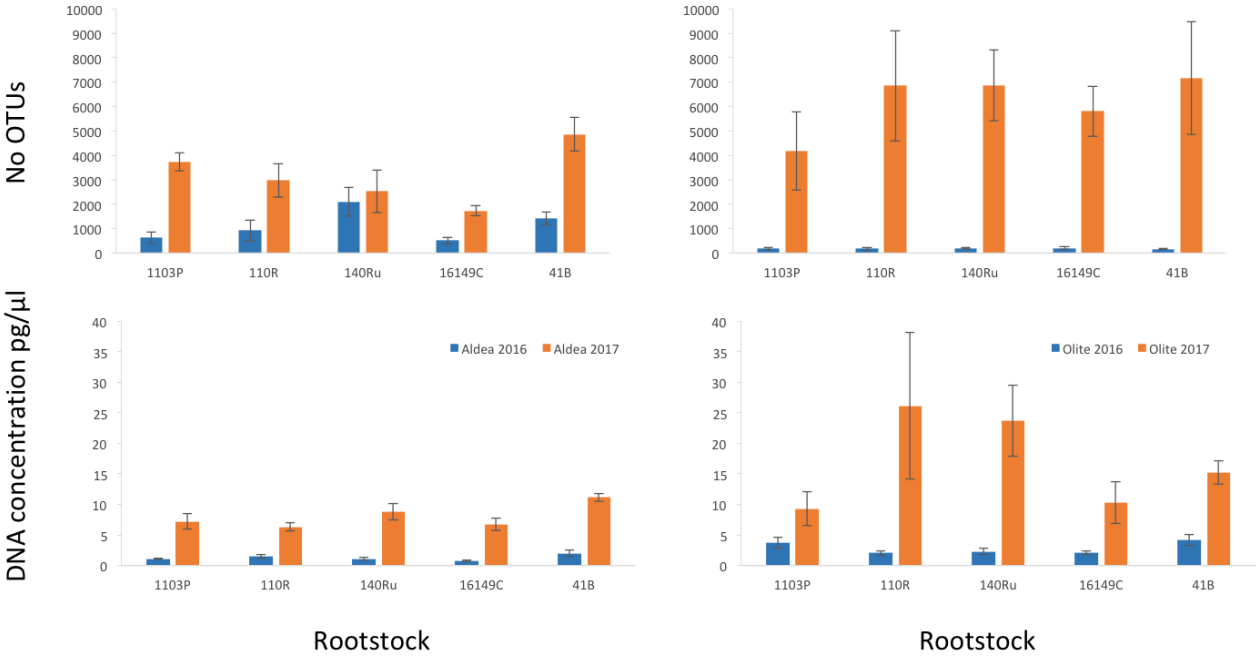

Figure 3.14. *Number of OTUs identified and DNA concentration of Cylindrocarpon-like asexual morphs for the five rootstocks analyzed in Aldea and Olite geographic regions in both years studies. Values are the mean of six replicates (3 samples per sampling time) and twelve replicates (3 samples per sampling time and 2 runs for each one) for qPCR and high-throughput amplicon sequencing analysis, respectively. Vertical bars represent the standard errors.*

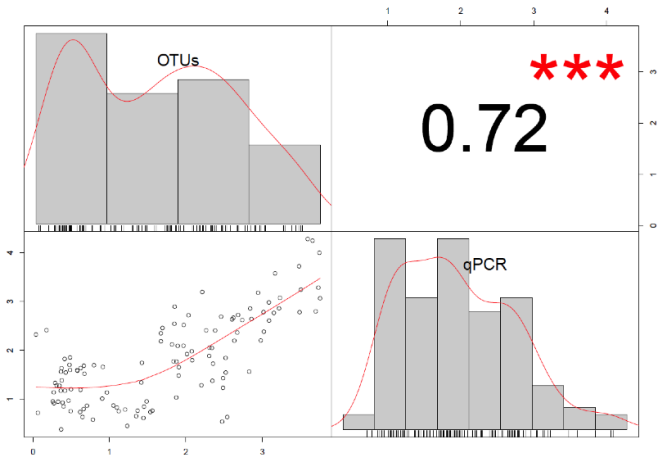

Figure 3.15. *The distribution of the number of OTUs and DNA concentration of Cylindrocarpon-like asexual morphs values are shown on the diagonal. The bivariate scatter plot with a fitted line is displayed on the bottom of the diagonal and the Spearman correlation value ($P < 0.05$) in indicated on the top of the diagonal.*

Discussion

In this study, we characterized the rhizosphere microbial community composition across five commercial grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over two years. The analysis of bacterial and fungal populations in the grapevine rhizosphere targeting 16S rRNA and ITS region, respectively, have been proved effective in previous studies (Corneo et al. 2014; Holland et al. 2016; Longa et al. 2017; Manici et al. 2017; Stefanini and Cavalieri 2018). Especially for bacterial barcoding, the choice of partial sequence regions is pivotal and can significantly affect the results because the 16S rRNA gene regions have different divergence (Youssef et al. 2009). In our study, we used the V4 region because according to recent *in silico* studies (Youssef et al. 2009), V4 along with V5-V6, and V6-V7 regions were considered as the most suitable regions for metagenomic purposes because they provided estimates comparable to those obtained with the complete 16S rRNA gene sequence (Youssef et al. 2009).

Our study represents the first approach to investigate the rhizosphere fungal microbiome of grapevine by HTAS. In grapevine, the ecology of fungal communities is so far largely derived 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) and PCR-DGGE (Manici et al. 2017) approaches in rhizosphere soil. Even though the ITS region was ratified by The Fungal Barcoding Consortium (Schoch et al. 2012) as the universal DNA barcode for the fungal kingdom using the same gene section proposed by White et al. (1990), some recent reports point out 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 *Fusarium* or *Trichoderma* (Schoch et al. 2012). In addition, 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).

The bacterial microbiomes of the different rootstocks were largely composed of Proteobacteria and Actinobateria that accounted for almost 50% of the relative abundance in both vineyards. The predominant bacterial phyla found in this work is consistent with the results obtained in other studies in vineyard soil (Opsi et al. 2014;

Vega-Avila et al. 2015; Castañeda and Barbosa 2017; Longa et al. 2017; Marasco et al. 2018). Proteobacteria and Actinobacteria are known for their role in the carbon biochemical cycle and their production of second metabolites (Jenkins et al. 2009). 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 both vineyards. Previous studies also agree on the most common fungal phyla detected in grapevines fields (Castañeda and Barbosa 2017; Longa et al. 2017; Manici et al. 2017). These results suggest that vineyard microbiome in Navarra and La Rioja regions is partially conserved.

The results obtained in the Aldea vineyard showed a significant fraction of variation in fungal and bacterial diversity (both the α- and β-diversity) that could be attributed to host genetics. Recent research indicated that rootstock genotypes could have a notable influence in shaping the bacteria taxa distribution in the root and rhizosphere systems of grapevine (Marasco et al. 2018). This effect of the host genotype in the rhizosphere microbiome has been reported in other woody crops, such as apple (Liu et al. 2018) and pines (Gallart et al. 2018), as well as in several annual crops, such as maize (Peiffer et al. 2013), potato (Inceoğlu et al. 2010) and chickpea (Bazghaleh et al. 2015). This could be due to the influence of the genotype in the root metabolism, including immune response and exudate composition, which impact in the rhizosphere microbiome (Wagner et al. 2016). Rootstocks show different level of tolerance to distinct diseases; and this could be decisive in their effect in the microbiome (Sapkota et al. 2015). Moreover, as reviewed by Liu et al. (2018), several studies hint to a possible co-evolution of the holobiont. However, further research is needed to validate this hypothesis. On the other hand, the Olite vineyard showed a lower microbiome dissimilarity among rootstocks, suggesting that the effect of genotype in shaping the microbiome might be influenced by other factors.

The differences between Olite and Aldea vineyards could lie in the soil physicochemical properties, in the soil and cultivar management practices, or in the age of the plants, being vines cultivated in Olite vineyard younger than in Aldea vineyard. Environmental heterogeneity, such as the soil physicochemical properties and moisture content have been identified as major factors shaping the spatial scaling of the rhizosphere microbiome in many previous studies (Costa et al. 2006; Tan et al.

2013; Schreiter et al. 2014;), including grapevine (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 soil-borne pathogens. For instance, Berlanas et al. (2017) observed that excessive calcium carbonate in soil may increase black-foot disease inoculum density.

Field management practices have been also reported as an important driver of the microbiome diversity (Hacquard 2016; Santhanam et al. 2015; Sapkota et al. 2015; Gallart et al. 2018), including the grapevine soil microbiome (Vega-Avila et al. 2015; Likar et al. 2017; Longa et al. 2017;). Nevertheless, other studies showed a long-term effect of cultivation rather than field management on soil microbial diversity (Buckley and Schmidt 2001; Peiffer et al. 2013). Microbiome studies should consider the high degree of temporal variability in the sample design, because sampling the same point in different times can give different results due the variability of the own microbial community through time (Redford and Fierer 2009). The year to year variation found in our study could be explained by the different root response to distinct environmental factors, such as temperature or precipitation (Wagner et al. 2016). Further research is needed to determine if environment plays a much greater role than host genetics in determining the composition of the rhizosphere microbiome of grapevine.

Several studies have remarked the effect of the growth stage of the plant in its associated rhizosphere microbiome (Baudoin et al. 2002; Inceoğlu et al. 2010; Li et al. 2014; Okubo et al. 2014; Yuan et al. 2015; Wagner et al. 2016; Qiao et al. 2017). Changes in the quantity and quality of root exudates as plants develop have been proposed as the main source of variation of the rhizosphere microbiome composition present during different developmental stages of maize cultivars (Baudoin et al. 2002). However, most of the published studies are focused in annual plant systems. In grapevine, Manici et al. (2017) recently investigated shifts in bacterial and fungal communities between mature and young replaced vines in Italy. At a single sampling moment, these researchers concluded that long-term growth legacy overcame plant age in shaping rhizosphere microbiome (Manici et al. 2017). Further research is therefore needed to determine the long-term effect of the grapevine age on the associated microbiome as plants develop. This could be accomplished by comparing

the rhizosphere microbiome (i) in a single vineyard over time, or (ii) in two vineyards in close proximity with identical environmental conditions and soils, but with vines on different aging process.

Our results showed that the root system type is able to select specific bacterial and fungal OTUs as biomarkers for the different genotypes. Members of the bacterial genus *Bacillus*, which was only found in '140 Ru' and '161-49 C' rootstocks in Aldea vineyard, has wide diversity of physiological ability with respect to heat, pH and salinity. Therefore, *Bacillus* species can be found in a wide range of habitats, being a few of them pathogenic to vertebrates or invertebrates (Holt et al. 1994). *Bacillus subtilis* and *B. amyloliquefaciens* have been described as potential biocontrol agents against *Aspergillus parasiticus* and stem rot disease (Le et al. 2018; Siahmoshteh et al. 2018). *In vitro* assays of the heat stable metabolites of *B. subtilis* showed promising results in reducing the growth of the fungal trunk pathogens *Lasiodiplodia theobromae*, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Alfonzo et al. 2009). Rezgui et al. (2016) recently identified several *B. subtilis* strains inhabiting the wood tissues of mature grapevines in Tunisia with antagonistic traits against fungal trunk pathogens. On the other hand, some species of the arbuscular mycorrhizal (AM) fungal genus *Glomus*, one of the most differentially abundant taxa for '110 R' rootstock in Aldea vineyard, are catalogued as biocontrol agents (Tahat et al. 2010). For instance, inoculation of grapevine roots with *Rhizophagus irregularis* (syn. *Glomus intraradices*) reduced both the disease severity and the number of root lesions caused by black-foot disease pathogens (Petit and Gubler2006). AM fungi form one of the most interesting beneficial plant–micro-organism associations (Smith and Read 2008) and are known to colonize the roots of the majority of land plants, including grapevines (Schreiner and Mihara 2009; Trouvelot et al. 2015). Several genera within the Glomeromycota phylum have been identified from the rhizosphere samples obtainted in this study, namely *Claroideoglomus*, *Diversispora*, *Entrophosphora* and *Rhizophagus*. Trouvelot et al. (2015) reported that soil management can greatly impact the diversity of AM fungi. In fact, AM fungal communities are highly influenced by the soil characteristics but also to a smaller extent by the host plant development stage (Schreiner and Mihara 2009; Balestrini et al. 2010).

High-throughput amplicon sequencing is a powerful method for the analysis of microbial populations. It is accomplished by sequencing specific marker genes amplified directly from environmental DNA without prior enrichment or cultivation of the target population (Franzosa et al. 2015). The advantages of this approach is the detection of rare taxa at the genus level given the availability of large and comprehensive reference databases as well as several pipelines for bioinformatics analysis (Stefanini and Cavalieri 2018). Drawbacks of HTAS include the biased relative quantification of bacterial communities since bacterial species bear various number of copies of 16S rRNA genes, the sequencing of matrix (e.g., grape ITS, chloroplast 16S) and the low confidence for taxonomic assignment at the species level (Stefanini and Cavalieri 2018). A step forward consists of the understanding of how changes in the composition of microbial communities impact the population's biological functions (Ravin et al. 2015). Unfortunately, HTAS only allows inference of functional annotation while in whole-genome sequencing, functional annotation can be carried out by gene enrichment (Stefanini and Cavalieri 2018). A further drawback of using DNA-based metagenomic data to infer the biological functions potentially exploited by microbial populations is that the detected DNA may belong to dead organisms. However, an approach based on RNA sequencing would give a direct report of the functions achievable by the viable microbial populations. In grapevine, the study of the active fungal communities of internal grapevine wood by HTAS in extracted total RNA has been recently accomplished by Eichmeier et al. (2018).

The quantitative significance of next-generation sequencing data for microorganisms is often debated (Amend et al. 2010). Fortunately, we were able to compare the relative abundance of reads with the relative abundance of DNA of black-foot disease pathogens, and we observed significant positive correlation. From the fungal soilborne pathogens affecting grapevine, *Cylindrocarpon*-like asexual morphs associated with black-foot disease are among the most important limiting factor of the production worldwide (Halleen et al. 2006; Agustí-Brisach and Armengol 2013). Therefore, *Cylindrocarpon*-like asexual morphs can be considered model pathogens to monitor the healthy status of the grapevine planting material when analyzing the fungal microbial composition of soil/rhizosphere samples.

Grapevine rootstocks have different susceptibilities towards pathogens, including trunk disease pathogens (Alaniz et al. 2010; Eskalen et al. 2001; Gramaje et al. 2010; Brown et al. 2013; Billones-Baaijens et al. 2014), which may be an important factor in shaping not only pathogens abundance but also entire communities. Nevertheless, we did not observe a clear correlation between known disease resistances in individual genotypes and the fungal communities, although *Cylindrocarpon*-like asexual morphs were found in lower abundance in '161-49 C' rootstock by both high-throughput amplicon sequencing and qPCR approaches. The use of '161-49 C' rootstock was previously recommended within an integrated management program for other grapevine trunk diseases, such as Petri disease and esca (Gramaje et al. 2010).

Conclusion

We have studied the effects of genotype, year, sampling date and location on bacterial and fungal communities in the grapevine rhizosphere. We found that grapevine genotype was the most important factor in shaping the microbiome in the mature vineyard. Many bacterial and fungal species were found in all rootstocks and in both locations in our study, demonstrating the existence of a "core" grape phylogeny that is independent of the growing region. Interestingly, the rhizosphere compartments of '140 Ru' and '161-49 C' rootstocks, the latter showing high tolerance to esca and Petri disease pathogens in previous research (Gramaje et al. 2010), harboured lower number of black-foot pathogens than the other grapevine rootstocks. Also of interest was the presence of high relative abundance of the genus *Bacillus* in both grapevine rootstocks, a bacterial genus recognized as biocontrol agents. A more comprehensive study is needed to decipher the cause of the rootstock microbiome selection and the mechanisms by which grapevines are able to shape their associated microbial community. Understanding the vast diversity of bacteria and fungi in the rhizosphere and the interactions between microbiota and grapevine will facilitate the development of future strategies for grapevine protection.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The study was conceived by CB, MB and DG. All authors contributed to the data collection. Data interpretation and manuscript preparation were performed by CB, MB, GE and DG. CB, MB, DG, GE and ML performed the experiments. CB, MB, GE and DG contributed to bioinformatics data analysis. All authors critically reviewed and edited the manuscript, and approved its publication.

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Data Availability Statement

The unrarefied OTU tables, and corresponding taxonomic classifications, sequence files and metadata for all samples used in this study have been deposited in Figshare (https://figshare.com/projects/Grapevine_rhizosphere_microbiome/36155).

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Supplementary material

Supplementary Tables

Supplementary Table 3.3. Information about rootstock selected in this study. The information is based in published studies (Martínez-Cutillas et al. 1990, Hidalgo 2002; Keller 2010).

Rootstock Type	Rootstock germplasm	Phylloxera resistance*	Nematode resistance	Grafted Scion Vigour	Tolerance			Ease of rooting
					Drought	Wet soil	Salt	
'110 R'	*V. berlandieri* x *V. rupestris*	High	Poor	Medium	High	Low	Poor	Low
'140 Ru'	*V. berlandieri* x *V. rupestris*	High	Low	High	High	Low	High	Poor
'1103 P'	*V. berlandieri* x *V. rupestris*	High	Medium	Medium	Medium	Low	High	Medium
'41 B'	*V. vinifera* x *V. berlandieri*	High	Poor	n.d.	High	Poor	Low	Medium
'161-49 C'	*V. berlandieri* x *V. riparia*	Excellent	Poor	Medium	Low	Low	Low	Low

* Excellent > High > Medium > Poor > Low

Supplementary Table 3.4. Physicochemical properties, soil management practices and climate of the two vineyard soils examined in this study. Values represent the mean±SE.

	Aldea vineyard	Olite vineyard
Coordinates	42,234961º, -1,899365º	42,252659º, 1,394441º
Altitude (m)	347	396
Physicochemical properties		
pH	8.4[a]±0.02	8.1
P mg/100g*	3.47±0.27	1.8±0.11
K mg/100g	15.52±0.59	17.3±0.44
S mg/100g*	4.37±0.38	0.9±0.27
Mg mg/100g*	25.57±0.29	15.0±0.26
Mn mg/100g	9.31±0.87	9.23±0.11
Fe mg/100g*	7.5±0.44	3.27±0.07
Ca mg/100g*	1570.92±220.81	1862.15±12.65
Na mg/100g*	6.08±0.19	1.46±0.05
SOM%*	0.74±0.02	1.75±0.01
Clay%	21.6±0.25	29.42±0.28
Sand%*	37.25±0.29	21.62±0.66
Silt%	41.12±0.05	49.0±0.41
CO_3Ca	15.05±0.03	18.67±0.23
CEC mekv/100g*	9.7±0.23	21.25±0.10
EC mS/cm	0.15	0.16
Assim. Ca mekv/100g*	10.2±0.19	20.4±0.15
Assim. Mg mekv/100g*	1.47±0.03	0.8±0.04
Soil temperature (ºC) (June)	25.7	22
Soil temperature (ºC) (November)	10.5	10.6
Soil management practices		
Irrigation system	Drip irrigation	Drip irrigation
Fertilization	1 application per year	6 applications per year
Pest management practices	5 spray treatments against powdery and downy mildew per year	6 spray treatments against powdery and downy mildew per year
Herbicide treatment	Yes	No
Climate		
Precipitation (mm)	529	462
Mean temperature (ºC)	13.5	12.8

[a]Average of 4 replicates. Asterisk indicates statistically significant results ($P < 0.05$)

Supplementary Table 3.5. 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 in the rootstock studied, for both bacteria and fungi analysis.

Aldea vineyard

Index	Bacteria				
	'110 R'	'140 Ru'	'1103 P'	'41 B'*	'161-49 C'
Reads	29769.8±13217.1	36838.8±20139.9	30159.1±10864.9	32881.7±11279.7	28512.2±10966.7
OTUs	611	577	658	683	650
Chao1	370.6±163.5	332.7±185.2	385.2±171.4	373.7±158.8	339.5±188.3
Shannon	4.0±0.7	4.1±0.5	4.2±0.2	4.2±0.3	4.3±0.2
Index	Fungi				
	'110 R'	'140 Ru'	'1103 P'	'41 B'	'161-49 C'
Reads	51139.7±29265.5	45952.4±28535.3	51436.2±28284.0	49659.7±16547.8	45420.4±29794.6
OTUs	246	259	250	241	224
Chao1	168.9±31.2	183.4±43.1	176.5±31.9	180.3±52.0	176.2±47.0
Shannon	3.4±0.1	3.3±0.1	3.4±0.2	3.3±0.3	3.1±0.4

Richness and diversity indices calculated at an even sequencing depth of 1,000 sequences/sample for both bacteria and fungi
*Sample DG126 was removed from the analysis due to the low number of sequence reads.

Olite vineyard

Index	Bacteria				
	'110 R'*	'140 Ru'	'1103 P'	'41 B'	'161-49 C'
Reads	37315.3±7632.7	4117.25±15790.9	39529±16251.5	34539.9±14862.6	58237.9±16430.6
OTUs	643	669	588	645	717
Chao1	400.5±66.0	413.5±103.8	427.6±102.9	373.6±123.9	400.5±99.8
Shannon	4.2±0.4	4.3±0.2	4.3±0.07	4.1±0.3	4.2±0.2
Index	Fungi				
	'110 R'	'140 Ru'	'1103 P'	'41 B'	'161-49 C'
Reads	53623.8±23695.8	57160.5±27945.5	49059.2±21623.0	40862.6±23264.0	61027.7±33281.9
OTUs	311	359	355	333	372
Chao1	189.2±64.2	223.7±33.6	213.7±51.3	202.1±33.1	224.4±43.9
Shannon	2.6±0.9	2.9±0.4	2.9±0.9	2.5±0.5	2.9±0.4

Richness and diversity indices calculated at an even sequencing depth of 1,000 sequences/sample for both bacteria and fungi
*Samples DG47 and DG48 were removed from the analysis due to the low number of sequence reads

Supplementary Table 3.6. Estimates of sample coverage and diversity indices at the genus level for bacteria and fungal profiles.

BACTERIA			
Sample ID	Good's coverage	Chao1 richness	Shannon diversity
DG01	1.000	275.8	3.85
DG02	0.995	265.9	3.73
DG03	0.991	315.4	3.75
DG04	0.999	220.3	3.70
DG05	0.994	175.0	4.33
DG06	0.988	155.1	4.21
DG07	1.000	180.2	4.03
DG08	0.981	210.1	3.85
DG09	0.933	161.1	4.04
DG10	0.921	250.4	4.10
DG11	0.995	321.7	4.25
DG12	0.973	335.0	4.11
DG13	0.933	245.0	3.99
DG14	0.933	245.1	3.88
DG15	0.999	219.9	3.95
DG16	0.987	240.9	3.80
DG17	0.931	276.8	4.68
DG18	0.910	299.9	3.75
DG19	0.923	310.0	3.85
DG20	0.962	325.4	4.15
DG21	0.987	260.5	4.30
DG22	0.987	305.4	4.25
DG23	0.892	216.6	4.23
DG24	0.900	351.8	4.11
DG25	0.903	391.8	4.04
DG26	0.987	255.0	3.95
DG27	0.911	200.8	3.89
DG28	0.987	428.9	3.99
DG29	0.927	335.6	4.01
DG30	0.987	350.4	3.97
DG31	0.994	402.5	3.97
DG32	0.991	265.7	3.99
DG33	0.991	270.2	4.23
DG34	1.000	221.4	4.26
DG35	1.000	170.6	4.29
DG36	0.995	170.7	3.95
DG37	0.981	145.7	4.33
DG38	0.988	143.6	3.98
DG39	0.981	158.8	3.91
DG40	0.991	230.4	3.80
DG41	0.909	399.1	4.08
DG42	1.000	225.7	4.09

DG43	1.000	403.6	4.09
DG44	0.909	175.9	4.12
DG45	0.999	224.5	3.58
DG46	0.987	214.4	3.42
DG49	0.994	221.1	3.65
DG50	0.895	215.9	3.66
DG51	0.958	380.9	3.77
DG52	0.910	235.9	4.10
DG53	0.895	270.6	3.41
DG54	0.899	390.7	3.64
DG55	0.895	399.8	3.76
DG56	0.920	448.1	3.80
DG57	0.962	461.6	2.49
DG58	0.958	365.7	3.67
DG59	0.900	375.7	4.35
DG60	0.910	378.6	4.15
DG61	0.999	410.1	4.33
DG62	0.855	419.3	4.21
DG63	0.995	448.4	4.15
DG66	0.981	465.6	4.21
DG67	0.988	465.2	4.09
DG68	0.981	410.4	4.13
DG71	0.995	503.0	3.96
DG72	0.995	499.8	3.95
DG73	0.960	470.4	4.01
DG76	0.995	518.5	4.10
DG77	0.855	475.6	3.94
DG78	1.000	460.7	4.12
DG81	0.960	475.6	4.21
DG82	0.994	500.1	4.15
DG83	1.000	501.2	4.17
DG86	0.884	426.6	4.68
DG87	0.910	446.7	3.76
DG88	0.899	455.8	3.85
DG91	0.910	478.0	3.86
DG92	0.899	480.9	4.02
DG93	0.884	449.9	4.05
DG96	0.905	515.6	4.25
DG97	0.967	535.9	4.30
DG98	0.899	462.1	4.29
DG101	0.905	501.0	4.14
DG102	0.884	508.0	4.21
DG103	0.905	445.5	4.20
DG106	0.904	456.8	4.19
DG107	0.884	425.9	4.15
DG108	0.899	485.9	4.23

DG111	0.994	470.2	3.54
DG112	0.981	465.7	4.24
DG113	0.889	480.8	4.51
DG116	0.995	501.8	4.52
DG117	0.949	525.0	4.35
DG118	1.000	485.1	4.35
DG121	0.949	519.4	4.12
DG122	0.973	490.5	4.27
DG123	0.973	485.7	4.28
DG127	0.995	405.2	4.35
DG128	0.991	406.3	3.98
DG131	0.889	410.0	4.11
DG132	0.889	445.0	4.13
DG133	0.995	443.7	4.01
DG136	0.911	448.9	4.11
DG137	0.857	540.1	4.24
DG138	0.908	525.3	4.31
DG141	0.912	250.2	4.15
DG142	0.900	251.2	4.13
DG143	0.900	445.6	4.11
DG146	0.851	510.5	4.10
DG147	0.896	509.8	4.21
DG148	0.884	505.0	3.76
DG151	0.911	475.0	4.21
DG152	0.904	549.5	4.20
DG153	0.893	234.4	4.11
DG156	0.884	421.3	4.19
DG157	0.896	446.7	4.19
DG158	0.911	447.5	4.13
Average	0.945	370.0	4.00

	FUNGI		
Sample ID	Good's coverage	Chao1 richness	Shannon diversity
DG01	0.966	103.0	3.41
DG02	0.991	130.5	3.43
DG03	0.991	130.6	3.50
DG04	0.872	155.7	3.52
DG05	0.952	156.0	3.30
DG06	0.930	175.1	3.54
DG07	0.891	140.1	3.57
DG08	0.988	138.3	3.55
DG09	0.875	139.0	3.55
DG10	0.991	170.3	3.83
DG11	0.873	140.5	3.33
DG12	0.872	110.4	3.34
DG13	0.829	119.4	3.51
DG14	0.973	172.0	3.50
DG15	0.853	175.1	3.54
DG16	0.875	165.2	2.55
DG17	0.855	180.3	1.80
DG18	0.971	196.2	2.68
DG19	0.891	163.7	2.76
DG20	0.992	90.8	1.80
DG21	0.829	241.0	3.60
DG22	0.856	185.6	3.25
DG23	0.905	190.7	3.33
DG24	0.875	200.3	3.34
DG25	0.971	204.1	3.50
DG26	0.849	170.1	3.41
DG27	0.849	175.8	3.34
DG28	0.853	175.6	3.32
DG29	0.875	147.5	3.30
DG30	0.905	223.6	3.29
DG31	0.904	215.8	3.04
DG32	0.890	216.0	3.10
DG33	0.930	165.3	3.12
DG34	0.991	112.4	3.43
DG35	0.973	128.5	3.11
DG36	0.944	141.1	3.13
DG37	0.930	225.5	3.18
DG38	0.952	165.5	3.15
DG39	0.872	225.8	3.04
DG40	0.855	148.0	3.02
DG41	0.930	173.0	3.14
DG42	0.973	164.3	3.21
DG43	0.905	160.2	3.22
DG44	0.966	135.1	3.24

DG45	0.872	138.5	3.14
DG46	0.837	90.5	2.75
DG47	0.973	155.8	2.87
DG48	0.857	161.0	3.10
DG49	0.952	162.5	3.05
DG50	0.857	165.6	3.03
DG51	0.835	225.5	3.09
DG52	0.849	205.4	2.87
DG53	0.880	185.9	2.91
DG54	0.991	224.0	3.36
DG55	0.890	203.6	3.34
DG56	0.830	140.7	2.99
DG57	0.829	148.6	3.13
DG58	0.900	181.5	3.12
DG59	0.831	182.6	3.50
DG60	0.904	159.1	3.84
DG61	0.836	208.3	3.34
DG62	0.844	206.4	3.59
DG63	0.849	223.5	3.56
DG66	0.904	244.9	3.54
DG67	0.896	189.0	3.51
DG68	0.966	190.7	3.44
DG71	0.896	250.7	3.44
DG72	0.857	211.8	3.82
DG73	0.880	188.7	3.58
DG76	0.844	177.6	3.59
DG77	0.893	207.3	3.33
DG78	0.971	185.4	3.44
DG81	0.833	250.5	3.58
DG82	0.930	220.0	2.65
DG83	0.845	205.0	2.51
DG86	0.952	225.7	2.65
DG87	0.911	210.1	2.23
DG88	0.893	208.4	2.51
DG91	0.860	203.4	3.65
DG92	0.860	192.1	3.62
DG93	0.930	191.9	3.10
DG96	0.829	235.0	3.12
DG97	0.893	240.4	3.70
DG98	0.911	202.5	2.90
DG101	0.899	191.5	3.55
DG102	0.896	226.0	3.43
DG103	0.880	212.2	3.13
DG106	0.902	203.4	3.15
DG107	0.956	221.3	3.12
DG108	0.938	175.1	3.26

DG111	0.944	183.6	3.31
DG112	0.934	240.9	2.92
DG113	0.845	215.3	3.34
DG116	0.893	186.6	3.33
DG117	0.881	210.0	2.93
DG118	0.872	245.5	3.42
DG121	0.833	220.4	3.32
DG122	0.911	231.5	3.24
DG123	0.905	227.6	3.25
DG126	0.846	214.2	3.21
DG127	0.890	223.3	3.24
DG128	0.841	201.1	3.26
DG131	0.841	223.1	2.24
DG132	0.842	220.6	3.35
DG133	0.833	218.7	3.41
DG136	0.896	180.6	3.45
DG137	0.966	220.5	3.23
DG138	0.967	221.9	3.24
DG141	0.923	254.9	3.25
DG142	0.994	230.7	3.25
DG143	0.900	215.0	3.03
DG146	0.847	240.1	2.99
DG147	0.848	222.0	3.11
DG148	0.988	191.2	3.23
DG151	0.841	223.3	3.14
DG152	0.993	248.9	3.15
DG153	0.960	190.3	3.25
DG156	0.950	222.8	3.23
DG157	0.860	234.8	3.11
DG158	0.983	241.9	3.24
Average	0.901	190.6	3.21

Supplementary Table 3.7. Experimental factors predicting α- and β-diversity of rhizosphere associated bacterial and fungal communities between vineyards.

	α-diversity		β-diversity
	Shannon	Chao1	Bray Curtis
Bacteria	$F_{1,115}$ = 0.27 *P* = 0.7840	$F_{1,115}$ = 1.42 *P* = 0.1567	R^2 = 0.19 *P* = 0.1134
Fungi	$F_{1,117}$ = 2.15 ***P* = 0.033**	$F_{1,117}$ = 1.37 *P* = 0.1724	R^2 = 0.69 ***P* < 0.001**

ANOVA, analysis of variance

All *P* values were corrected for multiple comparisons using the sequential Bonferroni correction. Significance was assessed using Type III ANOVA. Bold values indicate statistically significant results after correction for multiple comparisons, $P < 0.05$.

Supplementary Table 3.8. Similarity percentages (SIMPER) analysis determines the bacterial phyla **(A)** and genera **(B)**, and fungal phyla **(C)** and genera **(D)** contributions to the dissimilarity among rootstocks in the rhizosphere. In the upper part of the table the rootstock pairwise comparison of average dissimilarity percentage has been reported. In the lower part, the overall top one, two or 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 bacterial phyla contributions in Aldea and Olite vineyards.

ALDEA	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		9.2	16.1[#]	7.2	5.5[†]
'140 Ru'	Latescibacteria (21.3) Firmicutes (19.3) Planctomycetes (11.2)		8.2	10.1[#]	10.7[#]
'1103 P'	Acidobacteria (24.5) Firmicutes (15.6) Gemmatimonadetes (11.3)	Acidobacteria (31.5) Firmicutes (19.5) Planctomycetes (9.3)		11.0[#]	18.1[#]
'41 B'	Firmicutes (23.1) Nitrospirae (21.6) Actinobacteria (8.5)	Firmicutes (24.4) Acidobacteria (15.6) Latescibacteria (11.1)	Actinobacteria (19.4) Bacteroidetes (13.4) Planctomycetes (12.2)		8.4
'161-49 C'	Firmicutes (29.5) Acidobacteria (21.5) Nitrospirae (13.8)	Acidobacteria (32.4) Firmicutes (18.6) candidatedivisionWPS_1 (13.4)	Firmicutes (30.6) candidatedivisionWPS_1 (25.6) Verrucomicrobia (16.7)	Firmicutes (29.5) Actinobacteria (17.8) Acidobacteria (8.9)	
OLITE	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		8.1	9.5	7.6	10.3[#]
'140 Ru'	No significant phyla		3.7	2.5	5.0
'1103 P'	Nitrospirae (14.4)	Nitrospirae (22.3) Acidobacteria (17.8)		5.1	1.8[†]
'41 B'	Parcubacteria (16.5) Acidobacteria (13.2)	Acidobacteria (25.0)	Acidobacteria (27.6)		4.8
'161-49 C'	Acidobacteria (21.6)	Acidobacteria (24.3) Nitrospirae (14.4) Ud_Bacteria (11.0)[¥]	Acidobacteria (25.4) Chlamydiae (10.1)	No significant phyla	

[#] Rootstock-pairs showing dissimilarity in phyla distribution higher than 10%.

[†] Rootstock-pair showing the lowest dissimilarity observed in phyla distribution.

¥ Ud: unidentified.

(B) SIMPER analysis determined the bacterial genera contributions in Aldea and Olite vineyards.

ALDEA	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		5.5	14.8[#]	9.7	12.8[#]
'140 Ru'	*Corynebacterium* (24.3) Ud_Microbacteriaceae (23.5)[¥] Nocardioides (11.7)		9.9	6.3	6.1
'1103 P'	*Gp6* (26.0) Ud_Betaproteobacteria (25.6) *Gemmatimonas* (21.1)	*Bacillus* (26.6) *Gp4* (23.4) *Gp6* (14.2)		4.9	4.7[†]
'41 B'	*Ilumatobacter* (23.6) *Propionibacterium* (22.5) *Rubrobacter* (21.3)	*Bacillus* (25.7) *Nocardioides* (14.5) Ud_Bacillales (11.0)	*Mycobacterium* (25.1) Ud_Betaproteobacteria (22.0) *Ilumatobacter* (9.8)		5.1
'161-49 C'	Ud_Bacillales (25.6) *Bacillus* (12.3) Ud_Rhodocyclaceae (12.1)	*Bacillus* (24.5) *Gaiella* (15.5) *GP4* (8.8)	*Bacillus* (27.8) *Serratia* (18.4) *Pesudomonas* (14.0)	*Bacillus* (29.8) *Ilumatobacter* (22.6) *Propionibacterium* (20.5)	
OLITE	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		3.6	3.8	2.5	2.1[†]
'140 Ru'	*Aquicella* (15.4) *Flavobacterium* (9.8) Ud_Bradyrhizobiaceae (9.7)[¥]		3.0	4.1	3.1
'1103 P'	*Aridibacter* (21.0) *Aquicella* (17.6) *Chitinophaga* (15.0)	*Aridibacter* (17.7) Ud_Cytophagales (12.3) *Vasilyevaea* (6.7)		6.8	3.5
'41 B'	No significant genera	*Gp6* (14.4) *Povalibacter* (14.1) Ud_Proteobacteria (10.9)	*Gp10* (17.0) *Gemmata* (11.4) *Flavobacterium* (11.2)		6.7
'161-49 C'	*Aridibacter* (16.7) Ud_Acidobacteria (16.1) *Gp5* (11.4)	*Gp6* (15.4) *Aridibacter* (15.2) *Blastocatella* (13.4)	*Gp10* (19.0) *Sphingomonas* (15.9) *Mycobacterium* (15.6)	Ud_Sphingomonadales (23.4) *Aeromicrobium* (21.0) *Povalibacter* (14.5)	

[#] Rootstock-pairs showing dissimilarity in phyla distribution higher than 10%.

[†] Rootstock-pair showing the lowest dissimilarity observed in phyla distribution.

¥ Ud: unidentified.

(C) SIMPER analysis determined the fungal phyla contributions in Aldea and Olite vineyards.

ALDEA	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		8.2	16.2[#]	6.6[†]	25.3[#]
'140 Ru'	Ascomycota (29.3) Basidiomycota (24.1) Zygomycota (17.8)		9.8	22.7[#]	21.0[#]
'1103 P'	Ascomycota (6.3)	Basidiomycota (30.8) Zygomycota (9.2)		25.9[#]	12.8[#]
'41 B'	Basiodiomycota (22.9) Ascomycota (12.8) Glomeromycota (7.0)	Basidiomycota (22.4) Zygomycota (16.8) Ascomycota (8.4)	Glomeromycota (19.4) Basiodiomycota (12.4) Ascomycota (6.1)		29.7[#]
'161-49 C'	Ascomycota (27.5) Zygomycota (13.5)	Basidiomycota (35.5)	Glomeromycota (22.1)	Glomeromycota (19.5) Zygomycota (13.5) Ascomycota (13.5)	
OLITE	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		2.2	1.9	3.2	1.5[†]
'140 Ru'	Basidiomycota (8.1)		1.7	2.9	3.3
'1103 P'	No significant phyla	No significant phyla		5.6	8.0
'41 B'	No significant phyla	No significant phyla	No significant phyla		9.2
'161-49 C'	Basidiomycota (8.8)	No significant phyla	No significant phyla	Basidiomycota (5.5)	

[#] Rootstock-pairs showing dissimilarity in phyla distribution higher than 10%.

[†] Rootstock-pair showing the lowest dissimilarity observed in phyla distribution.

(D) SIMPER analysis determined the fungal genera contributions in Aldea and Olite vineyards.

ALDEA	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		7.2	7.1	7.8	12.7[#]
'140 Ru'	Ud_Auriculariales (19.0) [¥] *Geopyxis* (15.5) *Psathyrella* (15.1)		6.5	6.6	16.1[#]
'1103 P'	Ud_ Pleosporales (17.5) *Sporormiella* (15.9) *Articulospora* (15.3)	*Clonostachys* (15.5) *Lecanicillium* (14.6) *Scutellinia* (10.3)		6.3[†]	12.9[#]
'41 B'	Ud_Giomeraceae (13.1) *Gongronella* (13.0) *Geopyxis* (13.0)	*Clonostachys* (14.4) *Lecanicillium* (12.5) *Psathyrella* (9.1)	*Clonostachys* (8.1) Ud_Glomeraceae (8.1) Ud_Nectriaceae (8.0)		17.5[#]
'161-49 C'	*Geopyxis* (16.5) Ud_Glomeraceae (16.1) *Psathyrella* (15.6)	*Clonostachys* (9.4) *Cryptococcus* (9.1) *Davidiella* (9.0)	*Clonostachys* (9.2) *Cryptococcus* (9.2) *Lecanicillium* (9.2)	*Cryptococcus* (8.8) *Davidiella* (8.7) *Lecanicillium* (8.7)	
OLITE	**'110 R'**	**'140 Ru'**	**'1103 P'**	**'41 B'**	**'161-49 C'**
'110 R'		4.1	8.2	8.5	8.7
'140 Ru'	*Gymnopus* (5.6) *Spizellomyces* (4.6) *Pseudogymnoascus* (4.3)		4.2	2.7[†]	7.1
'1103 P'	*Calcarisporiella* (8.7)	*Geopyxis* (14.3) *Calcarisporiella* (11.7) *Scytalidium* (10.5)		12.8[#]	8.8
'41 B'	*Clavaria* (11.2) *Scytalidium* (6.7) *Penicillium* (4.5)	*Geopyxis* (17.5) *Scytalidium* (14.0) *Gymnopus* (13.8)	Ud_Basiodiomycota (6.1) Ud_Ceratobasidiaceae (5.5) *Calcarisporiella* (4.5)		4.1
'161-49 C'	Ud_Auriculariales (11.2) *Spizellomyeces* (10.3) *Geopyxis* (6.6)	*Scutellinia* (13.4) Ud_Auriculariales (13.2) *Gymnopus* (5.7)	*Scutellinia* (10.1) Ud_Basidiomycota (7.6) *Gymnoascus* (6.7)	Ud_Auriculariales (12.5) *Gymnoascus* (7.6) *Scytalidium* (5.9)	

[#] Rootstock-pairs showing dissimilarity in phyla distribution higher than 10%.

[†] Rootstock-pair showing the lowest dissimilarity observed in phyla distribution.

¥ Ud: unidentified.

Supplementary Table 3.9. Bacterial OTUs that were unique in each of the sample type.

	Rootstock				
Vineyard	'110 R'	'140 Ru'	'1103 P'	'41 B'	'161-49 C'
Aldea					
	Gp21	*Mobiluncus*	*Terriglobus*	*Pilimelia*	*Turicella*
	Pyrinomonas	*Planosporangium*	ud-Dermacoccaceae	*Gordonia*	ud-Demequinaceae
	Phytomonospora	*Plantactinospora*	*Pseudoclavibacter*	*Xylanibacterium*	*Barrientosiimonas*
	Crossiella	*Actinokineospora*	*Enteractinococcus*	*Micropruina*	*Dietzia*
	Atopobium	*Prevotella*	*Zhihengliuella*	ud-Prolixibacteraceae	*Amnibacterium*
	Vibrionimonas	*Rubrivirga*	*Rugosimonospora*	*Capnocytophaga*	*Krasilnikovia*
	Thermosporothrix	ud-Alicyclobacillaceae	*Tessaracoccus*	*Empedobacter*	*Brooklawnia*
	Elusimicrobium	*Thermicanus*	*Thermocatellispora*	*Epilithonimonas*	*Thermobispora*
	Halobacillus	*Abiotrophia*	*Bacteroides*	*Kyrpidia*	*Imperialibacter*
	Marininema	*Acetatifactor*	*Paludibacter*	*Geomicrobium*	*Filimonas*
	Hespellia	*ClostridiumXlVb*	*Tannerella*	*Piscibacillus*	*Falsibacillus*
	Peptoniphilus	ud-Lentisphaerae	*Bhargavaea*	*Gemella*	*Guggenheimella*
	Faecalibacterium	*Starkeya*	*Oxobacter*	*Jeotgalicoccus*	*Anaerovorax*
	Leptotrichia	Pandoraea	*Eisenbergiella*	*Desemzia*	*Lachnoanaerobaculum*
	Albidovulum	*Tepidiphilus*	*Pelotomaculum*	*Dolosigranulum*	*Stomatobaculum*
	Sandarakinorhabdus	*Simonsiella*	*ClostridiumIV*	*Streptococcus*	*Halobacteroides*
	Burkholderia	ud-Desulfobacteraceae	*Oscillibacter*	*Anaerobacter*	ud-Halobacteroidaceae
	Kingella	*Alishewanella*	*Anoxybacter*	*Natronincola*	*Ignavibacterium*
	Rivicola	ud-Chromatiaceae	*Megasphaera*	*Tepidanaerobacter*	*Pelagibacterium*
	Desulfohalobium	*Vulcaniibacterium*	*Camelimonas*	*Dehalobacter*	*Hansschlegelia*
	ud-Desulfohalobiaceae	*Leptonema*	*Prosthecomicrobium*	ud-Peptococcaceae2	*Aquamicrobium*
	Corallococcus	*Leptospira*	*Rhodomicrobium*	*Pseudobacteroides*	*Phreatobacter*
	Leclercia	*Spirochaeta*	*Methyloligella*	*Dialister*	*Defluviimonas*
	Marinomonas	ud-Spirochaetaceae	*Stella*	*Fusobacterium*	*Falsirhodobacter*

ud-Oceanospirillales	*Fervidobacterium*	*Nitrospirillum*	*Cereibacter*	*Rhodobacter*
Alkanindiges		*Anaplasma*	*Elioraea*	*Acidisoma*
Moraxella		*Advenella*	*Oceanibaculum*	*Defluviicoccus*
Aspromonas		*Malikia*	*Tistlia*	*Pigmentiphaga*
Acholeplasma		*Undibacterium*	*Limnobacter*	*Sulfurisoma*
Subdivision5_genera_incertae_sedis	ud-Desulfovibrionaceae	*Thiobacter*	*Halobacteriovorax*	
		ud-Desulfovibrionales	*Chitinibacter*	*Desulfocapsa*
		Ignatzschineria	*Snodgrassella*	*Desulfuromonas*
		Cloacibacillus	ud-Nitrosomonadaceae	ud-Syntrophaceae
		ud-Synergistaceae	*Azonexus*	*Campylobacter*
			Georgfuchsia	*Raoultella*
			Sulfuricurvum	ud-Methylococcaceae
			Sulfurimonas	*Luteibacter*
			Aggregatibacter	*Limisphaera*
			ud-Pasteurellaceae	
			ud-Pseudomonadales	

Olite

Actinospica	*Flaviflexus*	ud-Cryptosporangiaceae	*Citricoccus*	*Pyrinomonas*
Stackebrandtia	*Frigoribacterium*	*Rubricoccus*	*Zhihengliuella*	*Pseudoclavibacter*
ud-Glycomycetaceae	*Enteractinococcus*	ud-Paenibacillaceae2	*Capnocytophaga*	*Polymorphospora*
Amnibacterium	*Micropruina*	ud-Aerococcaceae	*Asinibacterium*	*Rugosimonospora*
Plantibacter	*Microbispora*	*Flavonifractor*	*Solitalea*	*Brooklawnia*
ud-Bifidobacteriaceae	*Atopobium*	*Pseudoflavonifractor*	*Thermicanus*	*Odoribacter*
ud-Deinococcales	*Alicyclobacillus*	*Vallitalea*	*Saccharibacillus*	*Algoriphagus*
ud-Enterococcaceae	*Aeribacillus*	*Undibacterium*	*Chungangia*	*Nibrella*
Lactococcus	*Geomicrobium*	*Psychrobacter*	*Alloiococcus*	*Imperialibacter*
Anaerosolibacter	ud-Bacillales_incertae_sedis	*Spirochaeta*	*Anaerobacter*	ud-Flammeovirgaceae

Sporanaerobacter	*Aerococcus*	Subdivision5_genera_incertae_sedis	ud-Clostridiaceae2	*Filimonas*
Garciella	*Fonticella*		*Stomatobaculum*	*Heliimonas*
Desulfitispora	*Tepidanaerobacter*		*Peptoniphilus*	*Arcticibacter*
ud-Peptococcaceae1	*Guggenheimella*		*Butyricicoccus*	ud-Chloroflexaceae
Ralstonia	*Acetatifactor*		ud-Erysipelotrichaceae	ud-Deinococcaceae
Hydrogenophilus	*Blautia*		*Ignavibacterium*	*Elusimicrobium*
Arcobacter	*Sporotomaculum*		*Oligosphaera*	*Candidatus Endomicrobium*
Marinobacter	ud-Peptococcaceae2	ud-Hyphomonadaceae	*Kyrpidia*	
Shewanella	*Gemmiger*		*Pandoraea*	*Halobacillus*
Kosakonia	*Hydrogenoanaerobacterium*	*Massilia*	*Marininema*	
Aggregatibacter	*Oscillibacter*		*Hafnia*	*Abiotrophia*
Leptonema	*Syntrophomonas*		*Klebsiella*	ud-Carnobacteriaceae
	Gluconobacter		ud-Oceanospirillales	*Oxobacter*
	Castellaniella		*Xiphinematobacter*	ud-Clostridiaceae3
	Sphaerotilus			*Anaerovorax*
	Azoarcus			*Anaerobacterium*
	Dechloromonas			*Anoxybacter*
	Celerinatantimonas			*Selenomonas*
	Methylobacter			*Camelimonas*
	Aspromonas			*Rhodomicrobium*
	SR1_genera_incertae_sedis		*Aquamicrobium*	
				Alsobacter
				Phreatobacter
				Methyloligella
				Falsirhodobacter
				Rhodobacter
				Acidisoma

		Magnetospirillum
		Orientia
		Sandarakinorhabdus
		Advenella
		Inhella
		Malikia
		Roseateles
		Paraherbaspirillum
		Thiobacillus
		ud-Neisseriaceae
		Halobacteriovorax
		Corallococcus
		ud-Syntrophaceae
		Campylobacter
		ud-Methylococcaceae
		Leptospira
		Anaeroplasma
		Limisphaera

Supplementary Table 3.10. Fungal OTUs that were unique in each of the simple type.

	Rootstock				
Vineyard	'110 R'	'140 Ru'	'1103 P'	'41 B'	'161-49 C'
Aldea					
	Astraeus	*Bipolaris*	*Amaurodon*	*Camarosporium*	*Ascosphaera*
	Debaryomyces	*Canalisporium*	*Amphinema*	Chaetomiaceae_ud	Capnodiales_ud
	Glomerales_ud	*Colletotrichum*	*Cadophora*	*Cyathus*	*Cladophialophora*
	Hymenoscyphus	*Crepidotus*	*Geotrichum*	*Dothiorella*	*Devriesia*
	Incertae_sedis_12_ud	*Cytospora*	Incertae_sedis_26_ud	*Haematonectria*	Morchellaceae_ud
	Lecythophora	*Guehomyces*	*Inocybe*	*Hebeloma*	*Mycenastrum*
	Leotiomycetes_ud	*Gymnopus*	*Phanerochaete*	*Lyophyllum*	Teratosphaeriaceae_ud
	Lycoperdaceae_ud	*Hypocrea*	*Pholiota*	*Oidiodendron*	Hyaloscyphaceae_ud
	Neophaeosphaeria	*Lacrymaria*	*Pilaira*	*Ophiosphaerella*	Incertae_sedis_25
	Pyrenochaeta	*Neofusicoccum*	*Pilidium*	Phaeosphaeriaceae_ud	*Filobasidiales*
	Rinodina	*Pisolithus*	*Podospora*	*Scleroderma*	
	Sarcinomyces	Polyporales_ud	Pyronemataceae_ud	*Tomentella*	
	Sphaeropsis	*Pringsheimia*	*Sarocladium*	Tricholomataceae_ud	
	Teloschistaceae_ud	*Thecaphora*	*Stagonospora*	*Typhula*	
	Tulostoma	Helotiaceae_ud	*Ascobolaceae*	Mycosphaerellaceae_ud	
	Teloschistaceae_ud	*Strophariaceae*	*Diatrypaceae*	Verrucariaceae_ud	
	Ascomycota_ud	*Filobasidiaceae*	*Pleurotaceae*	*Pezizaceae*	
	Volvariella	Incertae_sedis_12	*Ambisporaceae*	*Verrucaria*	
		Pezizales	*Cantharellales*		
		Verticillium	*Wallemia*		
		Xenasmatella	*Zygosaccharomyces*		
		Xylariaceae_ud			
Olite					
	Auricularia	*Alnicola*	*Arthroascus*	Ambisporaceae_ud	*Ascochyta*

Corticiales_ud
Dissoconium
Eutypa
Geopora
Glarea
Leotiomycetes_ud
Leveillula
Lycoperdaceae_ud
Paurocotylis
Phaeocytostroma
Phialocephala
Septoglomus
Stagonospora
Thanatephorus
Thelonectria
Tulostoma
Orbiliomycetes

Ampelomyces
Annulohypoxylon
Athelia
Calocybe
Cystolepiota
Eupenicillium
Fibroporia
Gymnoascaceae_ud
Heterobasidion
Neostagonospora
Plectania
Pluteus
Polyscytalum
Pringsheimia
Pseudovalsaria
Pyrenochaetopsis
Sphaerulina
Tapinella
Diatrypaceae
Marasmiaceae
Incertae_sedis_28
Archaeorhizomycetes
Cephalothecaceae
Diversisporaceae
Valsaria

Atheliaceae_ud
Battarrea
Bulleromyces
Ceratobasidium
Clavicipitaceae_ud
Crocicreas
Didymosphaeria
Entyloma
Gloeophyllum
Hemimycena
Lachnum
Lalaria
Millerozyma
Monacrosporium
Pilaira
Psathyrellaceae_ud
Saccharomycetaceae_ud
Sebacinales_ud
Sphaeropsis
Stropharia
Blumeria
Helotiaceae_ud
Incertae_sedis_2_ud
Incertae_sedis_26
Hymenochaetales
Onygenaceae
Sordariaceae
Volutella

Amphinema
Backusella
Caloplaca
Crepidotus
Cristinia
Eucasphaeria
Gliomastix
Lachnella
Mycena
Neoerysiphe
Neofusicoccum
Parasola
Parasola
Rosellinia
Simplicillium
Teloschistaceae_ud
Tylospora
Microbotryomycetes
Ustilaginales
Verticillium

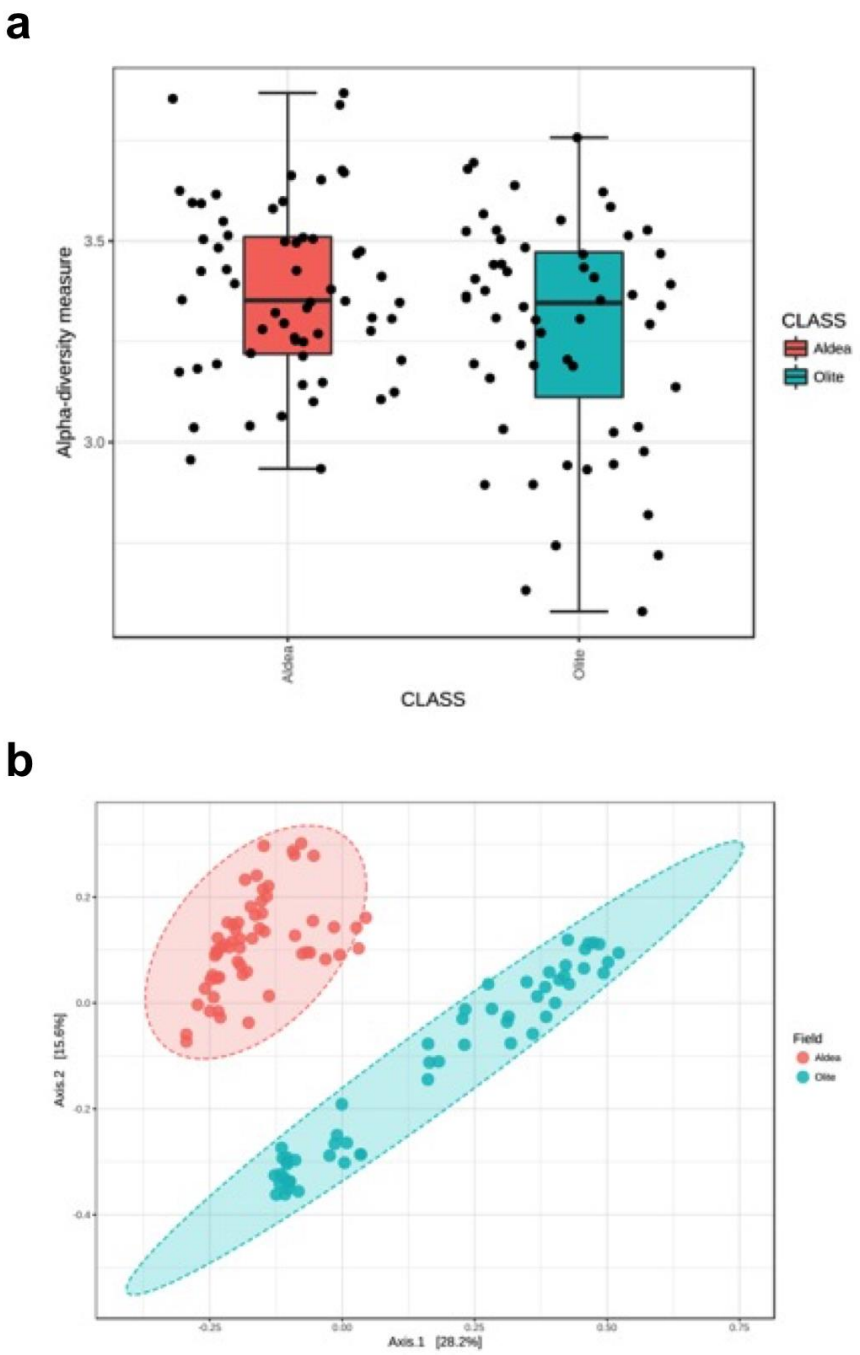
Polyporaceae_ud

Byssomerulius
Cephalotheca
Cistella
Claroideoglomus
Cochliobolus
Exobasidiomycetes_ud
Gnomonia
Hypoxylon
Incertae_sedis_12_ud
Incertae_sedis_2_ud
Leucopaxillus
Lichtheimia
Lophodermium
Monographella
Montagnulaceae_ud
Mycenastrum
Mycosphaerellaceae_ud
Myriodontium
Nemania
Oudemansiella
Phlebia
Plagiostoma

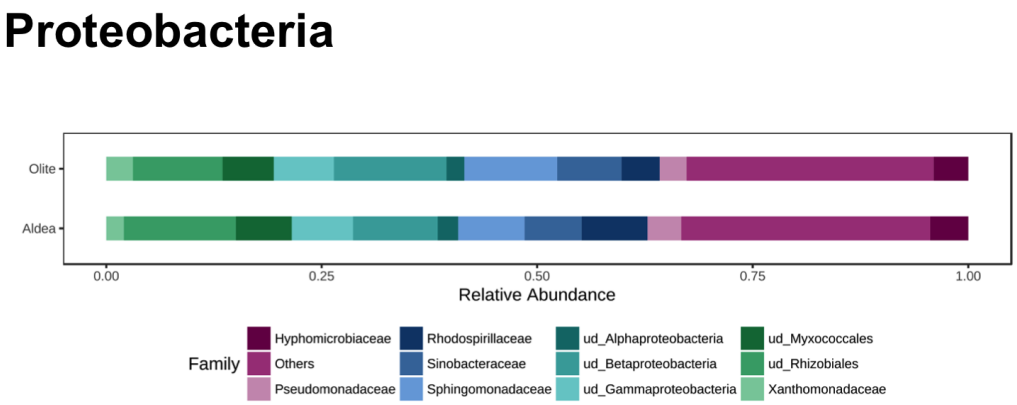
Pseudeurotiaceae_ud
Rhizopogon
Sporisorium
Trametes
Truncatella

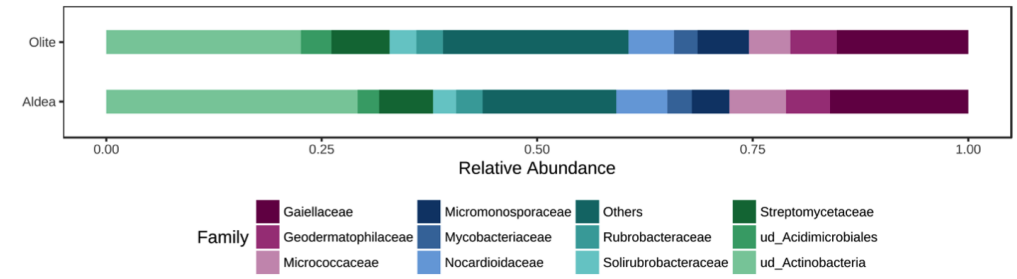
	Williopsis	Tubeufiaceae_ud
		Valsaceae
		Incertae_sedis_25
		Ustilaginaceae
		Entylomatales
		Vuilleminia
		Wallemia
		Xylodon

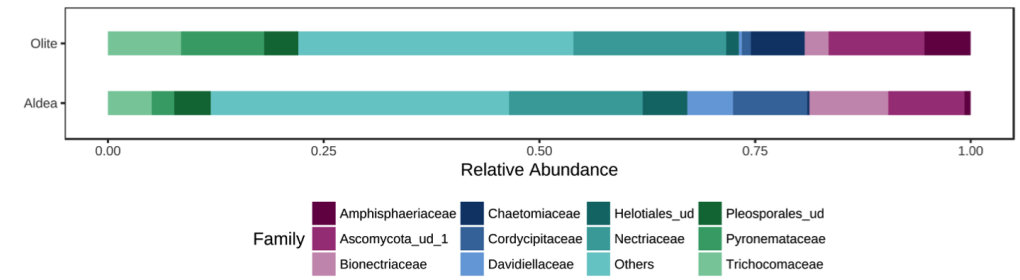
Supplementary Figures



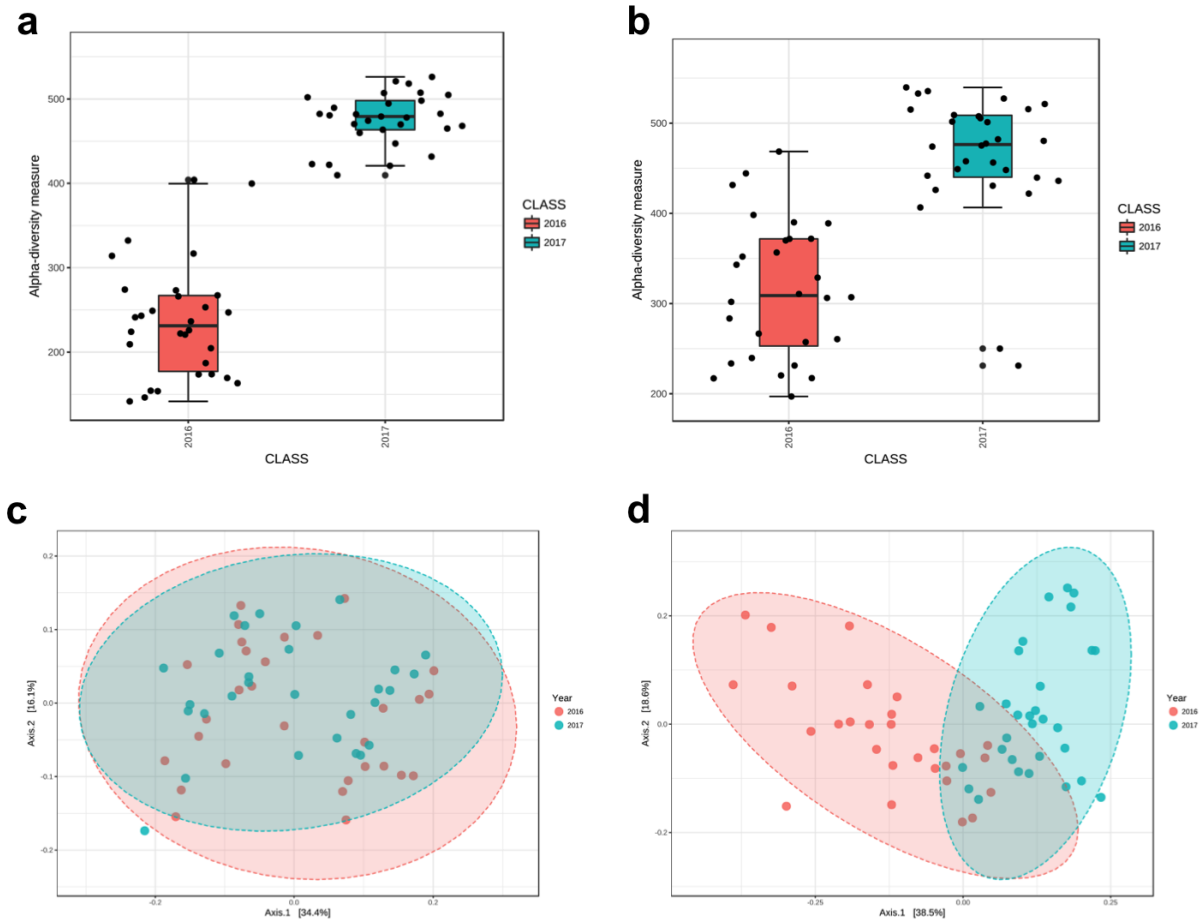
Supplementary Figure 3.3. *Boxplot illustrating the differences in Shannon diversity measures of the fungal communities between vineyards **(a)**. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the fungal communities between vineyards **(b)**.*



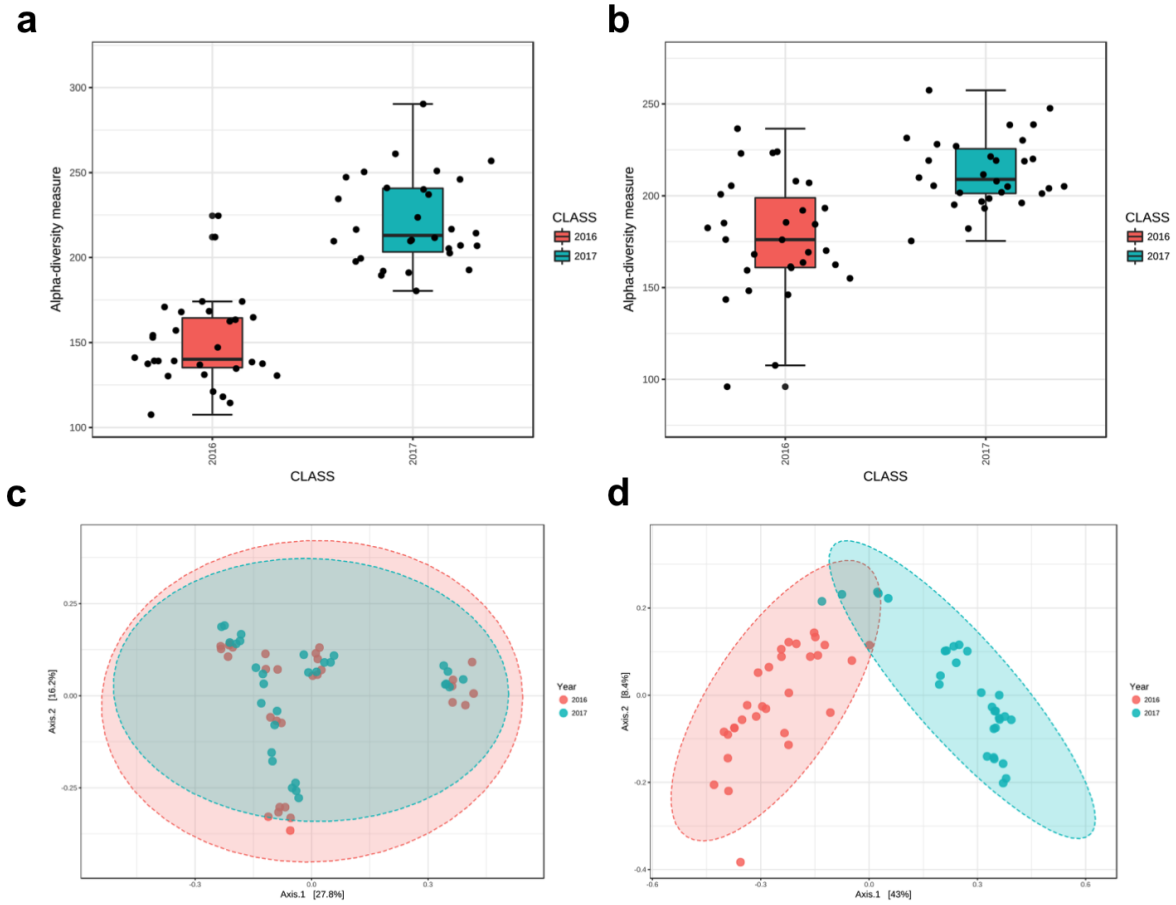




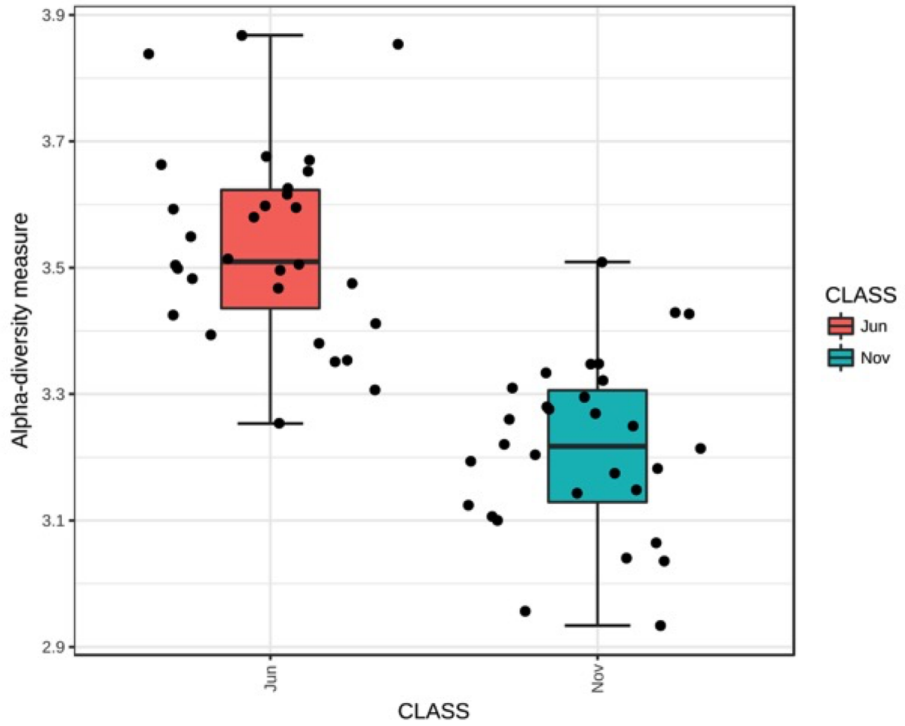
Supplementary Figure 3.4. *Relative abundance of the most abundant families within the phyla Actinobacteria, Proteobacteria and Ascomycota in both vineyards representing OTUs showing more than 1% relative abundance of all reads and present in at least 2/3 of replicates. Families representing less than 1% of the total reads are grouped in 'Others'.*



Supplementary Figure 3.5. *Boxplot illustrating the differences in Chao1 richness measures of the bacterial communities between years of sampling in the grapevine rootstocks in Aldea* ***(a)*** *and Olite* ***(b)*** *vineyards. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the bacterial communities among grapevine rootstocks in Aldea* ***(c)*** *and Olite* ***(d)*** *vineyards.*



Supplementary Figure 3.6. *Boxplot illustrating the differences in Chao1 richness measures of the fungal communities between years of sampling in the grapevine rootstocks in Aldea* ***(a)*** *and Olite* ***(b)*** *vineyards. Principal Coordinate Analysis (PCaA) based on Bray Curtis dissimilarity metrics, showing the distance in the fungal communities among grapevine rootstocks in Aldea* ***(c)*** *and Olite* ***(d)*** *vineyards.*



Supplementary Figure 3.7. *Boxplot illustrating the differences in Shannon diversity measures of the fungal communities between sampling dates in the grapevine rootstocks in Aldea vineyard.*

CHAPTER 4.

CONTROL

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Effect of white mustard cover crop residue, soil chemical fumigation and *Trichoderma* spp. root treatment on black-foot disease control in grapevine

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Abstract

Background Black-foot disease is one of the main soilborne fungal diseases affecting grapevine production worldwide. Two field experiments were established to evaluate the effect of white mustard cover crop residue amendment and chemical fumigation with propamocarb + fosetyl-Al combined with *Trichoderma* spp. root treatment on the viability of black-foot inoculum in soil and fungal infection in grafted plants and grapevine seedlings used as bait plants.

Results A total of 876 black-foot pathogens isolates were collected from grafted plants and grapevine seedlings used as bait plants in both fields. White mustard biofumigation reduced inoculum of *Dactylonectria torresensis* and the incidence and severity of black-foot of grapevine, but no added benefit was obtained when biofumigation was used with *Trichoderma* spp. root treatments. The effect of white mustard residues and chemical fumigation on populations of *D. torresensis* propagules in soil was inconsistent, possibly due to varying pretreatment inoculum levels.

Conclusion Biofumigation with white mustard plants had potential for improving control of black-foot disease in grapevines. This control strategy can reduce soil inoculum levels and protect young plants from infection, providing grape growers and nursery propagators with more tools for developing integrated and sustainable control systems.

Keywords*:* biocontrol*,* biofumigation, *Brassica* residues, fosetyl-Al, propamocarb, *Vitis vinifera* L.

Introduction

Fungi have many different functions in soils, which include the degradation of dead organic matter into fungal biomass, carbon dioxide and organic acids (decomposers), the formation of beneficial relationship with plants (mutualists), and the penetration into the plant and decomposition of the living tissue, leading to a weakened, nutrient deficient plant, or death (soilborne pathogens) (Wainwright 1988). The most familiar diseases caused by soilborne pathogens in grapevine are rots that affect belowground tissues, namely root and crown rots, and vascular wilts initiated through root infections. Well-known diseases of grapevine are Armillaria root rot, Phytophthora crown and root rot and Verticillium wilt (Bettiga 2013) In addition to these, black-foot disease, which belong to the complex of fungal trunk pathogens of grapevines, (Bertsch et al. 2013) has received special attention by plant pathologists in the last decades due to its implication on the 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 (Halleen et al. 2004; Agustí-Brisach and Armengol 2013; Lombard et al. 2014, Carlucci et al. 2017), with *Dactylonectria torresensis* being the most frequent species associated with diseased vines in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Berlanas et al. 2017). Although these pathogens usually manifest on young vineyards, they have also been frequently isolated from symptomatic or asymptomatic rootstock mother-plants, rooted rootstock cuttings, bench-graft and young grafted vines, being considered the most common pathogenic fungi associated with young nursery vines. Moreover, it is well known that these fungal pathogens are present in nursery soils causing infection of grafted vines after some months of growth for rooting (Agustí-Brisach and Armengol 2013; Halleen et al. 2006).

Characteristic symptoms of black-foot disease include a reduction in root biomass and root hairs with sunken and necrotic root lesions (Halleen et al. 2006). Roots also show abnormal development characterized by shallow growth parallel to the soil surface and become necrotic. In some cases, the rootstock diameter of older vines is thinner below the second tier. Removal of rootstock bark reveals black discoloration and necrosis of wood tissue, which develops from the base of the rootstock (Halleen et

al. 2004; Agustí-Brisach and Armengol 2013). External symptoms show reduced vigour with small sized trunks, shortened internodes, uneven wood maturity, sparse foliage, and small leaves with interveinal chlorosis and necrosis. When young vines are infected, death occurs quickly, nevertheless as the vine ages, infection results in a more gradual decline and death might only occur after a year (Halleen et al. 2004; Agustí-Brisach and Armengol 2013).

Although the disease cycle of black-foot pathogens on grapevines has not been specifically studied, the behaviour of *Cylindrocarpon*-like asexual morphs on other hosts (Booth 1966; Brayford 1993) has indicated that conidia and chlamydospores are likely to be produced on the diseased roots and stem bases of infected vines. The conidia are apparently dispersed in soil water and the chlamydospores can allow the organism to survive in the soil for a number of years (Petit et al. 2011). Agustí-Brisach et al. (2011) reported isolation of *Dactylonectria macrodidyma* complex from 26 of 52 weed species growing in propagation field nurseries and vineyards, with these being pathogenic to grapevine seedlings and, therefore, may provide sources of spores. Previous research reports have shown that contact between these spores and the grapevine roots or callused stem bases results in high rates of infection (Rego et al. 2001; Halleen et al. 2003). Infection can occur through the small wounds made when roots on the callused cuttings break off during the planting process or through the incomplete callusing of the basal ends of the cuttings which expose pith tissues (Halleen et al. 2003; Agustí-Brisach and Armengol 2013).

Fully resistant rootstocks are not available for black-foot pathogens; therefore, management strategies rely primarily on the use of an integrated program including fungicides, biological control agents (BCAs), hot-water treatment (HWT) and cultural practices (Gramaje et al. 2018). Reductions in pesticide use for economic, environmental, and resistance reasons require additional disease management options. Successful biological control of black-foot disease with antagonistic microorganisms is practiced to a rather limited extent, and research, up to date, has shown variable results for preventing fungal infections (Fourie et al. 2001; Halleen et al. 2007; Halleen and Fourie 2016; dos Santos et al. 2016; Álvarez-Pérez 2017). The standard HWT of 50°C for 30 minutes provides 100% control of black-foot, with no reduction in budding or growth rate (Halleen et al. 2007; Gramaje et al. 2010; Bleach

2013; Halleen and Fourie 2016). However, HWT of dormant nursery plants has not yet been embraced as a standard treatment in most European nurseries, due to the confusion in the industry about its efficacy and safety (Gramaje and Di Marco 2015). Crop rotation has been considered an effective method to control numerous soilborne fungal diseases (Mohler and Johnson 2009). Despite its impressive success in other pathosystems, the effectiveness of the standard grapevine nursery practice of a 2 to 4-year rotation system, alternated with a cover crop, to decrease the viability of black-foot pathogens resting spores in the soil, has been questioned by researchers in Portugal (Reis et al. 2013), South Africa (Halleen et al. 2003) and Spain (Berlanas et al. 2017). Biofumigation with *Brassica* spp. has been widely studied in the last 50 years, and research data have confirmed their long recognised allelopathic effects, which may be used for disease management in agricultural systems (Brown and Morra 1997; Rosa and Rodrigues 1999). Thus, *Brassica* biofumigation has shown promising results both *in vitro* and *in planta* to control black-foot disease pathogens in Australia and New Zealand (Bleach 2013; Barbour et al. 2014; Weckert et al. 2014).

In this context, the objective of this research was to evaluate the effectiveness of a registered BCA and a mixture of fungicides used to control other soilborne diseases in Spain, as well as a native plant species in the Brassicaceae family, to decrease the viability of black-foot inoculum in soil and fungal infection in grafted plants and grapevine seedlings used as bait plants.

Materials and methods

Planting material

One-year old dormant grapevine grafted plants of 'Malvasia'/'110 Richter' combination with uniform root distribution of 10 cm length were obtained from a commercial nursery in Spain and used in this experiment. Plants were hot-water treated at 53°C for 30 min to eliminate any existing infections by black-foot disease pathogens (Halleen et al. 2007; Gramaje et al. 2010; Bleach 2013; Halleen and Fourie 2016) and then acclimatized for 24 h at 20°C before plantation. In addition, seeds obtained from grapevine cultivar 'Tempranillo' were planted individually in 220 cm^3 plastic pots containing sterilized peat moss, vermiculite and perlite, and incubated in a growth chamber for 2 months at 25°C before using in the field experiment.

Sites description and experimental design

Experiments were conducted in two field sites located in Mendavia (Navarra, Spain) from December 2015 to February 2017. 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. Plots were less than 1 km apart and had very similar climates. Soil samples were taken for physicochemical properties analysis as described below.

The experimental design was a three-by-two factorial combination of three soil treatments and two grapevine root treatments arranged in a split-plot design with four replications. Main plots consisted of three soil treatments: non treated plot as control treatment, incorporation of white mustard (*Sinapis alba*) cv. 'Braco' biomass residues, and a treatment with propamocarb + fosetyl-Al (Previcur Energy® 47.2+27.6 SL, Bayer Crop Science, 120 mL per 100 litres). Each main plot was 3.1 m long and a raised bed containing two rows wide (0.5 m between row centres with an in-row spacing of 1.5 m). Main plots contained two randomly distributed subplots of two rows each, and were spaced 0.5 m from each other. The subplots consisted of the grapevine root application of *Trichoderma atroviride* T11 + *Trichoderma asperellum* T25 (Tusal® 0.5+0.5 WG, Certis Europe, 2 x 10^8 conidia g^{-1} at 2 g l^{-1}) or water as untreated control.

In December 2015, seeds of white mustard were sown at a dose of 1.5 gr/m^2 and lightly incorporated on the top of the corresponding raised beds. *Brassica* was grown until the plants had reached 50% anthesis, at the end of March 2016, and then all rows were rotary hoed, which chopped and incorporated plant material into the soil. The drip irrigation system was laid on the soil of each raised bed and the raised beds were covered with black polyethylene plastic sheet (PE) before irrigation began to avoid dissipation of volatile compounds (Kirkegaard and Matthiessen 2004) and to ensure complete hydrolysis (Matthiessen and Kirkegaard 2006). The use of PE is a common practice in Spanish grapevine nurseries for weed control. The edges were sealed with soil. At the end of March 2016, propamocarb + fosetyl-Al was applied by drip irrigation to the corresponding raised bed and the raised beds were immediately covered with PE as described above. Control treatment plots were drip irrigated with water and also covered with PE.

One month after covering the raised beds with PE, grafted plants with either *Trichoderma* spp. treatment or untreated control with water were planted through the PE into the treated land subplots according to the standard nursery practice. Treatments consisted in dipping the roots and the basal part of the plants in *Trichoderma* spp. suspensions or water for 24 h at room temperature before planting. The viability of the conidia in the commercial product was checked to be at a minimum of 85% before planting, by plating a serial dilution of the conidia suspension on potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France) and counting the colony forming units (CFUs) after 24-48 h incubation at room temperature. In each subplot, grafted plants were planted in double rows of 12 plants with 10 cm between each plant, giving 24 plants per subplot, 48 per main plot and 576 per field site. In addition, two seedlings were planted through the PE into each treated subplot between the two grafted plant rows, giving 48 seedlings per field site. Seedlings were spaced 25 cm from the grafted plant rows and 0.5 m apart from centre to centre.

Fungal isolation and identification

In February 2017, once grafted plants and seedlings had completed their cycle of vegetative growth and were in a dormant state, they were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing. Total root mass of all planting material was calculated. In order to isolate black-foot pathogens, root sections from 2-3 cm near the base of the rootstock were cut from necrotic areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. The root bark of six small root pieces was removed by carefully scraping it from the wood and the inner tissues were plated on Malt Extract Agar (MEA) supplemented with 0.4 gL^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (MEAS). Two MEAS plates were used per plant. Plates were incubated for 10-15 days at 25°C in the dark and all colonies were transferred to PDA. Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair 1995). The disease incidence (DI) of black-foot pathogens was determined as the mean percentage of grafted plants or seedlings that was infected by these fungi. The disease severity (DS) in infected grafted plants or seedlings was determined as the

mean percentage of root segments (twelve segments per plant) 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* formulation.

Fungal isolates resembling black-foot 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. All fungal species were identified by sequencing part of the histone H3 gene using CYLH3F and CYLH3R primers (Crous et al. 2004; Cabral et al. 2012a, 2012b). 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 collection and fungal quantification

Four soil cores were collected to a depth of 20 cm from each main plot and bulked into a single soil sample per plot. Samples were mixed well, air-dried for one week and sieved (2-mm to 5-mm mesh size) prior to soil physicochemical analyses and culture media plating. Quantification of black-foot pathogens was performed in November 2015, before the beginning of the experiment, and in February 2017, at the end of the experiment, following the procedure described by Berlanas et al. (2017) Soil samples (20 g) were diluted in 100 mL of 0.1% water agar. The mixture was homogenised by shaking the bottle in a reciprocating shaker for 10 min at 250 rpm. One mL aliquots were serially diluted 100 times in 100 mL of 0.1% water. Sterile pipettes were used to transfer 150 µL each of the last dilution onto the surface of a Glucose-Faba Bean Rose Bengal (GFBRB) Agar plate. The soil suspension was spread evenly across the surface of the plate by the use of sterile glass spreading rods. Nine plates were prepared for each soil sample. The experiment was repeated three times. Plates were incubated at 25°C

in the dark for 10 days, and examined daily for development of black-foot pathogens colonies using a stereomicroscope at 7.5 x magnification. Isolates resembling black-foot pathogens were transferred to PDA and identified by molecular methods as described above. Colony counts were converted to CFU per gram of dry soil.

Soil physicochemical properties analysis

Soil samples were tested for electric conductivity (EC) in water and pH with a soil solution ratio of 1:5, soil organic matter (SOM) by dichromate oxidation (Nelson and Sommers 1982), soil texture by laser diffraction particle size (Diffractometer LS 13 320, Beckman Coulter Inc., Brea, Calif.), carbonate total by infrared (Equilab CO-202; Equilab, Jakarta, Indonesia), cation exchange capacity (CEC) by the cobaltihexamine method (Orsini and Remy 1976), assimilable calcium and magnesium by the cobaltihexamine method and by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA) 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 November 2015, 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 arcsine $(DI \text{ or } DS/100)^{1/2}$. Black-foot populations CFU counts from naturally infested soils were transformed to $\log_{10}(x + 1)$, where x is the number of CFUs per g of soil per petri dish.

Each treatment mean (DI, DS, and root mass) was calculated from the corresponding values in each field site over the experiment. For grafted plants, data on DI, DS, and root mass were analysed according to the experimental design in split-plot, including main effect terms for block, soil treatment, root treatment, and terms for the interaction soil-root treatments. For grapevine seedlings, data of DI and DS were analysed using one-way ANOVA. Means were compared by the Student's *t* least significant difference (LSD) ($P < 0.05$). Data of black-foot populations CFU counts were analysed using one-way ANOVA. LSD test was used to determine the differences between black-foot total counts in both fields. 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

Disease incidence, disease severity and root mass in grafted plants

None of the treatments had a negative influence on callus or initial shoot growth. The viability of planting material was estimated to be of 96% at the end of growing season. A total of 664 black-foot pathogens isolates were collected from grafted plants in both fields (315 isolates in Field 1 and 349 isolates in Field 2). The majority of fungal species were identified as *D. torresensis* (82.2%), followed by *D. macrodidyma* (11.9%), *Dactylonectria novozelandica* (5.4%) and *Ilyonectria liriodendri* (0.5%).

There was a significant effect of soil treatment on mean DI values in both fields (Table 4.1). Analysis of variance showed no significant soil treatment-root treatment interactions for DI, suggesting that the effect of the root treatment is independent of the soil treatment (Table 4.1). In both fields, percentages of infected plants (DI) were significantly lower in treatments with white mustard than in the no soil treatment control and chemical fumigation plots (Table 4.2). In plots biofumigated with white mustard, mean DI decreased by 55.3% in Field 1 and 42.2% in Field 2 relative to the no soil treatment control. No significant differences were observed in DI values between treatments with propamocarb + fosetyl-Al and no soil treatment control, and between root treatments in both fields (Table 4.2). White mustard biofumigation regardless of *Trichoderma* spp. root treatment resulted in lower DI than the no soil treatment control and chemical fumigation in both fields (Figure 4.1).

Soil treatments had a significant effect on mean DS in both fields (Table 4.1). Both white mustard residues and chemical fumigation reduced the percentage of DS in both fields (Table 4.2). In plots biofumigated with white mustard, mean DS decreased by 53.5% in Field 1 and 47.6% in Field 2 relative to the no soil treatment control. In plots fumigated with propamocarb + fosetyl-Al, mean DS decreased by 45.5% in Field 1 and 27.8% in Field 2 relative to the no soil treatment control (Table 4.2). Treatment with *Trichoderma* spp. in the roots significantly reduced the percentage of DS only in Field 1 (Table 4.2). White mustard biofumigation and chemical fumigation regardless of

Trichoderma spp. root treatment resulted in lower DS than the no soil treatment control in both fields (Figure 4.1). Neither soil treatment nor root treatment had a significant effect on mean root mass in both fields (Table 4.1). No significant differences were observed in root mass values between soil treatments with and without *Trichoderma* application (Table 4.2, Figure 4.1). Very low levels of *Trichoderma* spp. (<1%) were isolated from roots of plants subjected to *Trichoderma* treatments in both fields.

Table 4.1. Effects of variables on disease incidence, disease severity, and root mass in grapevine grafted plants.

Variables	df[†]	Disease incidence (%)		Disease severity (%)		Root mass (g per plant)	
		MS[‡]	*P* value[§]	MS	*P* value	MS	*P* value
Field 1							
Block	3	353.4	0.3482	127.6	0.2843	28.4	0.0103
A (Soil treatment)	2	1808.2	0.0362	737.0	0.0090	6.2	0.2940
Block x A (error a)	6	297.9		64.6		4.1	
B (Root treatment)	1	145.9	0.4357	380.8	0.0160	1.5	0.3333
A x B	2	250.5	0.3550	53.6	0.4127	1.4	0.4191
Block x B (A) (error b)	33	234.4		59.0		1.5	
Field 2							
Block	3	202.2	0.4788	25.6	0.5865	2.4	0.4305
A (Soil treatment)	2	1356.4	0.0244	318.2	0.0154	2.6	0.4256
Block x A (error a)	6	184.8		35.1		2.6	
B (Root treatment)	1	523.4	0.0859	17.4	0.4088	0.2	0.7959
A x B	2	358.7	0.1327	20.4	0.4491	0.8	0.7349
Block x B (A) (error b)	33	166.9		24.9		2.7	

[†] Degrees of freedom.
[‡] MS = mean square.
[§] Significance level $P < 0.05$.

Disease incidence and severity in grapevine seedlings

A total of 212 black-foot pathogens isolates were collected from grapevine seedlings in both fields (97 isolates in Field 1 and 115 isolates in Field 2). All of them were identified as *D. torresensis*. Analysis of variance showed no significant differences between soil treatments for DI ($P = 0.2301$) and DS ($P = 0.4855$) of grapevine seedlings in Field 1 (Figure 4.2). In Field 2, there was a significant effect of soil treatments on mean DI and DS values ($P < 0.01$). Treatment with propamocarb + fosetyl-Al increased DI of grapevine seedlings by 30% in Field 2 relative to the no soil treatment control and white mustard biofumigation (Figure 4.2). Regarding the DS, mean values decreased by 13.6% and 25.1% in plots biofumigated with white mustard relative to the no soil treatment control and plots fumigated with propamocarb + fosetyl-Al, respectively (Figure 4.2).

Table 4.2. Effects of soil treatments (white mustard biofumigation, chemical and no soil treatment), and root treatment with *Trichoderma* spp. on disease incidence, disease severity, and root mass in grapevine grafted plants.

Soil treatment	Disease incidence (%)		Disease severity (%)		Root mass (g per plant)	
	Field 1	Field 2	Field 1	Field 2	Field 1	Field 2
No soil treatment	34.9 a[†,‡]	39.6 a	23.5 a	18.7 a	8.1 a	5.2 a
White mustard	15.6 b	22.9 b	10.9 b	9.8 b	7.0 a	5.9 a
Propamocarb+fosetyl-Al	32.9 a	38.0 a	12.8 b	13.5 b	6.9 a	5.6 a
Root treatment						
No root treatment	29.6 a	36.8 a	18.5 a	14.6 a	7.5 a	5.6 a
Trichoderma spp.	26.1 a	30.2 a	12.9 b	13.5 a	7.2 a	5.5 a

[†] Mean of four replications (48 grapevine grafted plants in each replication).
[‡] Values followed by the same letters within a column are not significantly different according to the least significant test ($P < 0.05$)

Physicochemical properties of the soil and black-foot pathogens inoculum density

Analyses of variance indicated significant differences for some soil physicochemical properties between fields ($P > 0.05$) (Table 4.3), so both field sites were analysed separately. All black-foot pathogen isolates recovered from soil samples were identified as *D. torresensis*. In Field 1 before soil treatment, the CFUs/g soil in plots selected for no soil treatment and propamocarb + fosetyl-Al treatment were significantly greater than in plots selected for white mustard biofumigation (Table 4.4). The CFUs/g soil 15 months after treatment were significantly greater in propamocarb + fosetyl-Al treatment plots than in no soil treatment and white mustard biofumigation plots. In Field 2 before soil treatment, the CFUs/g soil in plots selected for no soil treatment were significantly the greatest followed by plots selected for propamocarb + fosetyl-Al treatment and for plots selected for white mustard biofumigation (Table 4.4). Fifteen months after treatment, propamocarb + fosetyl-Al treatment plots showed the greatest CFUs/g soil mean followed by no soil treatment and white mustard biofumigation plots.

In Field 1, the CFUs/g soil significantly decreased in no soil treatment plot and increased in propamocarb + fosetyl-Al plots 15 months after treatment (Table 4.4). No significant differences were observed in CFUs/g soil between the two moments in white mustard biofumigation plots. In Field 2, the CFUs/g soil significantly decreased in no soil treatment and white mustard biofumigation plots and increased in propamocarb + fosetyl-Al plots 15 months after treatment (Table 4.4).

Table 4.3. Physicochemical properties of the two soils examined in this study. Values represent the mean±SE.

Field	pH	P mg/100g	K mg/100g	S mg/100g	Mg mg/100g	Mn mg/100g	Fe mg/100g	Ca mg/100g
1	8.25[†]±0.05a[‡]	1.56±0.22b	21.7±0.54a	7.82±0.33a	21.6±0.62a	2.39±0.04b	3.11±0.23b	3608.6±106.0a
2	8.30a	2.53±0.19a	17.5±1.79a	6.02±0.52a	20.2±0.27a	6.42±1.49a	4.96±0.46a	2173.9±282.7b

Na mg/100g	SOM%	Clay%	Sand%	Silt%	CO_3Ca	CEC mekv/100g	EC mS/cm	Assim. Ca mekv/100g	Assim. Mg mekv/100g
1.60±0.11a	1.02±0.03 a	26.2±0.29a	34.4±1.09a	41.4±0.91a	29.8±1.15a	8.65±0.27a	0.19a	10.3±0.02a	0.72±0.03a
2.22±0.17a	1.14±0.04 a	26.4±0.43a	26.6±1.09b	43.95±0.71a	21.8±2.5b	10.7±1.03a	0.18a	12.9±0.06a	0.89±0.02a

[†]Average of 4 replicates.

[‡] Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$).

Table 4.4. Inoculum density of *Dactylonectria torresensis* in the different soil treatments prior to or at 15 months after the treatments were applied in fields 1 and 2.

Treatment Plot	Mean estimated population density (CFU / g soil)			
	Field 1		Field 2	
	Before soil treatment	15 months after treatment	Before soil treatment	15 months after treatment
No soil treatment	333.3[†] ± 94.1aA[‡]	166.7 ± 68.0bB	1156.6 ± 183.3aA	666.7 ± 128.9bB
White mustard	166.7 ± 68.0bA	166.7 ± 68.0bA	333.3 ± 94.1cA	166.7 ± 68.0cB
Propamocarb + fosetyl-Al	333.3 ± 94.1aB	500.0 ± 112.5aA	666.7 ± 128.9bB	1000.0 ± 147.4aA

[†] Average of 3 replicates of 9 plates each.

[‡] Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$). Small letters are for comparison of means in the same column. Capital letters are for comparison of means in the same row within each field.

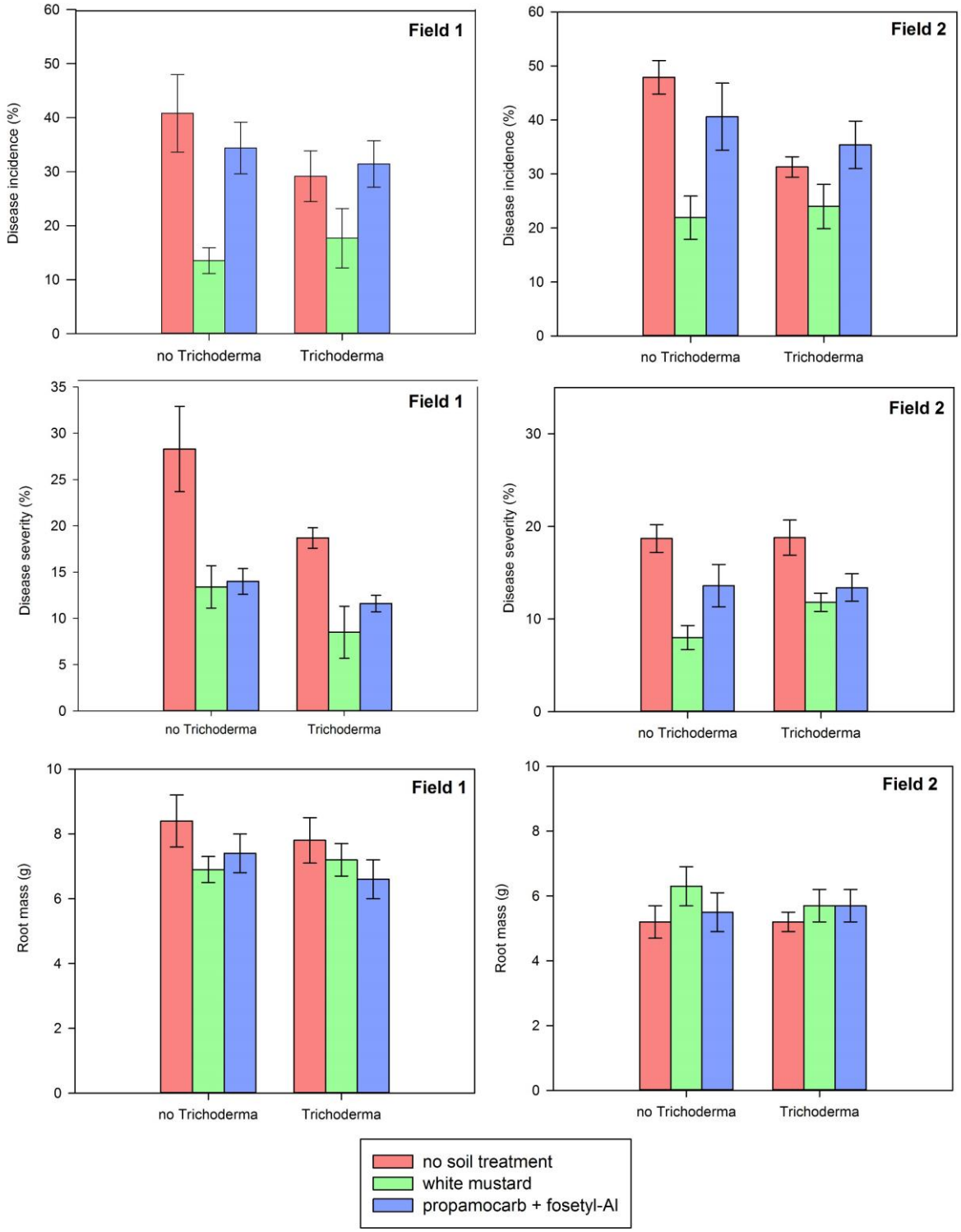

Figure 4.1. *Disease incidence (%), disease severity (%), and root mass (g per plant) of grafted plants in the different treatments in Fields 1 and 2. Values are the mean of four replications and vertical bars are the standard errors of the mean.*

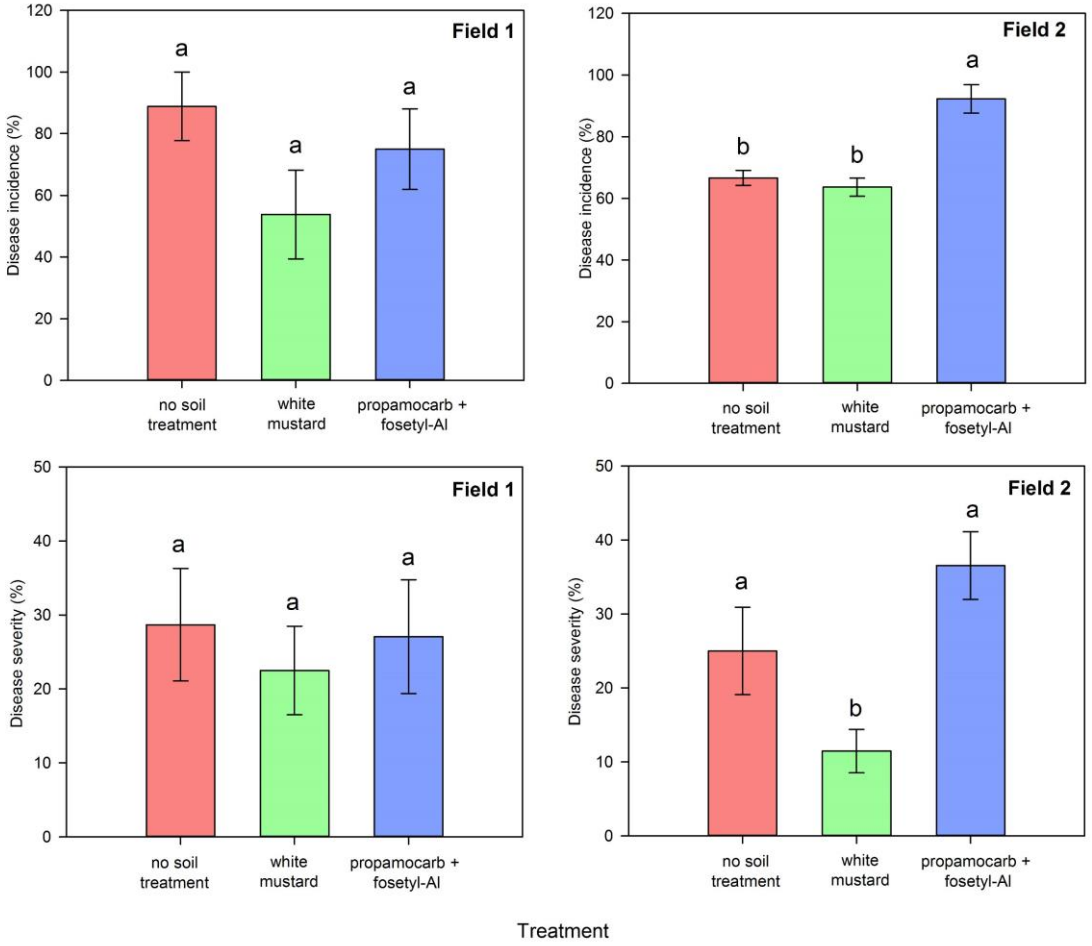

***Figure 4.2**. Disease incidence (%), and disease severity (%) of grapevine seedlings in the different treatments in Fields 1 and 2. Values are the mean of four replications and vertical bars are the standard errors of the mean.*

Discussion

This study represents the first approach to evaluate the effect of combined soil and root treatments to control black-foot disease of grapevine under field conditions. We used *Sinapis alba* as *Brassica* biofumigant because it is native and well adapted to grow naturally in Northern Spain, and wild forms can be found surrounding grapevine nursery fields and vineyards. The soil amendments were done on samples taken at 50% anthesis, as this is the plant development stage when glucosinolate levels peak (Rosa and Rodrigues 1999). This *Brassica* has shown potential as a cover crop in vineyards and as a rotation crop in rotation programmes that include annual crops, such as planting material in grapevine nurseries (Kruger et al. 2013). We used propamocarb + fosetyl-Al because it is widely used for the control of other soilborne diseases in horticultural Spanish nurseries and, given the lack of chemicals registered

against black-foot disease, its efficacy against black-foot under field conditions had not been demonstrated yet. Manufacturers are more likely to proceed with a label extension of a product, which is less expensive and faster, than registration of a new chemical for use on grapevines. We used *T. atroviride* + *T. asperellum* because, at the time of this study, this product was the only biocontrol agent registered in Spain for control of other soilborne diseases. The potential of *T. atroviride* strain SC1 to control Petri disease in nurseries, another trunk disease associated with young vines, has been recently demonstrated (Pertot et al. 2016).

White mustard biofumigation was highly effective in reducing the DI and DS of black-foot pathogens. Preliminary results from different studies have demonstrated the effectiveness of *Brassica* residue incorporation in the reduction of black-foot incidence (Bleach 2013; Barbour et al. 2014). In Australia and New Zealand, biofumigation using Indian mustard seed meal (*Brassica juncea*) significantly improved the yield and growth parameters in diseased vines (Weckert et al. 2014), reduced disease incidence when rootstock cuttings were planted into artificially infested soil (Bleach 2013), and significantly reduced black-foot inoculum in amended soils (Barbour et al. 2014). White mustard biofumigant contains low content of the aliphatic glucosinolate sinigrin, but is rich in aromatic glucosinolates (Ríos et al. 2016). Further research is needed to test a broad spectrum of brassicaceous plant species with different glucosinolate profiles and well adapted to grow in a particular environment, in order to select potential biofumigants for black-foot disease of grapevine.

The use of *Brassica* cover crops are associated with changes in soil microbiome (Buyer et al. 2010; Frasier et al. 2016), sometimes increasing BCAs populations in the rhizosphere (Benítez et al. 2007). For instance, Montfort et al. (2011) reported that *T. atroviride* was relatively unaffected by mustard biofumigation under controlled conditions, which agreed with Galletti et al. (2008) who reported that *in vitro Brassica carinata* seed meal trial combined with *Trichoderma* spp. improved mycelium inhibition of *Pythium ultimum*. In this context, further research is also necessary on the effect of biofumigation on microbial community structure in grapevine production systems under field conditions.

This is the first study to investigate the effect of a chemical soil fumigant applied before planting on black-foot disease of grapevine. Previous studies have been limited

to *in vitro* fungicide screenings (Rego et al. 2006; Halleen et al. 2007; Alaniz et al. 2011), greenhouse experiments (Marais and Hattingh 1986; Schwinn and Staub 1995; Nascimiento et al. 2007) and field experiments with planting material previously soaked with fungicides (Halleen et al. 2007). Chemical soil fumigation with propamocarb + fosetyl-Al was not effective in reducing DI of grafted plants in both fields, but reduced the DS compared to no soil treatments. In grapevine seedlings used as bait plants, the chemical soil fumigant had no effect on DI and DS compared to the control treatment. Rego et al. (2006) reported that plants that had been dipped for 50 min in fosetyl-Al, which is rapidly degraded to phosphorous acid, and then grown for 3 months in substrate infested with *I. liriodendri*, had significantly reduced disease incidence compared to untreated control plants, although this product had not shown *in vitro* efficacy (Rego et al. 2006). Phosphoric acid salts are thought to protect plants from root pathogens by "stimulating host resistance" (Schwinn and Staub 1995) to pathogens such as *Pythium* or *Phytophthora* (Marais and Hattingh 1986).

Fumigation with methyl bromide has been used for decades as a standard treatment for eliminating soilborne disease and pests in replant systems, such as crown and root disease caused by "*Cylindrocarpon destructans*" in strawberry Bonfiflioli 2005; Fang et al. 2011). However, the use of methyl bromide for soil fumigation was banned in many countries from 2008 (EPA, 2008). This has led to a search for alternative strategies to manage soilborne diseases, but so far none of the chemical alternatives identified have the full spectrum of activity and versatility of methyl bromide as a preplant soil fumigant (Martin 2003). Given the reduction in the availability of effective chemical products against grapevine trunk diseases fungi worldwide, sustainable non-chemical alternatives such as biofumigation and the use of BCAs are urgently demanded by growers.

Our study highlighted the limitation of the application of *T. atroviride* and *T. asperellum* as dips before planting to control black-foot pathogens. Several factors could have contributed to our current findings, such as the application method, the time of root exposure to the biocontrol agent or the rootstock susceptibility. For instance, grapevine cultivar has been pointed out to affect the colonization and persistence of *Thichoderma* spp. in the pruning wounds (Mutawila et al. 2011). Infection by these pathogens was shown to occur from nursery soils (Halleen et al.

2003; Agustí-Brisach et al. 2013a, 2013b), and the application of *Trichoderma* spp. into the roots might not be protective enough to impede fungal infection due to an insufficient systemic colonization of the basal ends of rootstocks by the BCA, especially in grapevine nursery soils and young vineyards where black-foot inoculum pressure is frequently high (Reis et al. 2013; Agustí et al. 2014; Berlanas et al. 2017). dos Santos et al. (2016) reported no significant differences in *D. macrodidyma* reisolation from roots previously inoculated with the pathogen and treated with several *Trichoderma* spp. under greenhouse conditions in Portugal, despite the increase of plant growth parameters. Soil amendments with *Trichoderma harzianum* formulations proved inefficient to reduce black-foot disease infection in South African grapevine nurseries (Halleen et al. 2007). By contrast, although low levels of black-foot disease infection were recorded, Fourie et al. (2001) demonstrated that *Trichoderma* soil amendments notably reduced disease incidence in roots of nursery grapevines in South Africa. More recently, Halleen and Fourie (2016) concluded that *T. harzianum* performed better against natural black-foot infections in an integrated strategy within the propagation process combined with chemical and/or HWT, than in repeat treatments of the same product. Other BCAs have been tested against black-foot disease pathogens with promising results (Petit and Gubler 2006; Álverez-Pérez et al. 2017). Inoculation of roots with the mycorrhizal fungus *Rhizophagus irregularis* (syn. *Glomus intraradices*) reduced the disease severity caused by black-foot disease pathogens (Petit and Gubler 2006). On the other hand, application of actinobacteria in grafted plant roots, selected for their *in vitro* antifungal properties against black-foot disease pathogens, resulted in a significant decrease in fungal infection in nursery fields (Álvarez-Pérez et al. 2017).

Our research suggests that factors other than reduction of black-foot soil populations likely contribute to lowering the DI in all the treatments. Additionally, no positive correlation was observed between DI and DS, suggesting that reduction of DI is not a necessary condition for lowering of DS. These results agree with those of other authors when investigating the effect of several strategies for the control of soilborne diseases (Raio et al. 1997; Pinkerton et al. 2000; Berbegal et al. 2008).

Conclusions

The results of our research showed that biofumigation with white mustard plants had potential for improving control of black-foot disease in grapevines. This control strategy can reduce soil inoculum levels and protect young plants from infection, providing grape growers and nursery propagators with more tools for developing integrated and sustainable control systems. Based on the restrictions and difficulties that chemicals are facing in most countries around the world, an integrated management program including hot-water treatments, biofumigation and the application of new BCAs to prevent or at least reduce the development of black-foot disease should be considered a research priority.

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CHAPTER 5.

GENERAL DISCUSSION

During the last few years, extensive research into black-foot disease (BFD) of grapevine has been carried out in Spain with significant advances on its etiology, epidemiology and control (Aroca et al. 2006; Alaniz et al. 2007, 2009, 2010, 2011a, b; Gramaje et al. 2010a; Agustí-Brisach et al. 2011a, 2012, 2013a, b, 2014, 2019; Tolosa-Almendros 2016; Martínez-Diz et al. 2018; Pintos et al. 2018). BFD affects particularly plant nursery stock and young vineyards (Halleen et al. 2006; Agustí-Brisach and Armengol 2012), and has been associated, together with Petri disease, to the young vine decline syndrome in almost all grapevine growing regions worldwide (Gramaje and Armengol 2011).

There has been a continuous changing of the taxonomic reclassification within the *Cylindrocarpon*-like asexual morphs fungi, with the identification and description of many new species from different genera associated with BFD. This fact, together with the still lack of complete information about the ecology and epidemiology of the disease, and the unavailable curative methods for its control, has increased the complexity of this pathosystem.

Different aspects of BFD have been studied in this thesis, but the overall objective was to obtain information about the biology and ecology of the disease, as well as to evaluate different control strategies, in order to improve the disease management. This concluding chapter discusses the results obtained in the previous chapters of the thesis and addresses areas of potential future research that have arisen based on the data generated in this study.

5.1 Biology and ecology

Up to 27 species in the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon* and *Thelonectria* have been reported to cause BFD (Gramaje et al. 2018; Lawrence et al. 2019; Aigoun-Mouhous et al. 2019). In this thesis, 11 known species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* have been identified from asymptomatic nursery stock, with *I. pseudodestructans* and *N. quercicola* reported for the first time in Spain. In addition, two novel species have been characterized, *Dactylonectria riojana* and *Ilyonectria vivaria*, bringing the total number of BFD pathogens isolated from grapevines in Spain to 17. Micromorphological characters, such as conidiophores morphology, macroconidia and

microconidia size and shape, and cultural characters were used to describe new fungal species. However, several studies indicated that such characters alone are not sufficient to differenciate among BFD fungi (Cabral et al. 2012a, c; Lombard et a. 2014, Lawrence et al. 2019), therefore being necessary the use of DNA sequences to get a confident species diagnosis when working with *Cylindrocarpon*-like asexual morphs. Traditionally, while working with ascomycetes, the ITS region, or partial sequences of the *tef1* and *tub2* genes have been used in molecular phylogenetic analysis, either as a single-gene or as a concatenation. Lawrence et al. (2019) studied the accurate species identification of the traditional gene set compared to the use of the *his3* locus, as suggested firstly by Cabral et al. (2012a, c), and confirmed that the use of multigene analysis including the *his3*, *tef1* and *tub2* genes increased the accuracy of *Cylindrocarpon*-like fungal species identification. In Chapter 3.1, *his3*, ITS, *tef1*, and *tub2* were used to confirm the identity of *D. riojana* and *I. vivaria*.

Dactylonectria torresensis was the most common species isolated from grapevines, which agrees with previous studies conducted in Algeria (Aigoun-Mouhous et al. 2019), Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016). Recent findings also indicated that agricultural crops such as *Actinidia chinensis* (Erper et al. 2013), *Eriobotrya japonica* (Agustí-Brisach et al. 2016), *Malus domestica* (Manici et al. 2018), *Olea europea* (Nigro et al. 2019), or forest trees (Mora-Sala et al. 2018) represent other common niches for *D. torresensis*. However, even if *D. torresensis* has a high prevalence in some countries and hosts, it is not the most common fungal species associated with BFD everywhere. For example, *D. macrodidyma* is one of the most prevalent species in South Africa (Langenhoven et al. 2018), New Zealand (Mundy 2015) and Canada (Úrbez-Torres et al. 2014). *I. liriodendri* is also very frequently isolated in New Zealand (Mundy 2015) and Canada (Úrbez-Torres et al. 2014).

Dispersal of BFD pathogens through asymptomatic planting material might have great impact in other regions where the disease is not present or the fungal diversity associated with BFD is still low. This finding also highlights the urgent need to implement early, accurate and specific *in planta* detection and quantification of these fungi to prevent the spread of BFD in grapevine propagation material. The endophyte definition has changed many times over the last years. Recently, Hardoim et al. (2015) defined endophyte based on the colonization niche but not on the function. These authors

therefore considered the existence of both pathogenic and non-pathogenic endophytes (Hardoim et al. 2015). In this study, 13 fungal species associated with BFD colonized the root vascular tissue endophytically without causing any type of external or internal symptom in plants. However, only 5 out of the 13 species showed high degree of virulence. The most virulent species were *D. novozelandica*, *D. alcacerensis*, *D. macrodidyma* and *I. vivaria*, which were isolated, respectively, in only 3.22%, 2.94%, 3.85% and 0.21% of the plants. These results agree with those obtained by Cabral et al. (2012b), who found that minor species such as *I. lusitanica*, *D. estremocensis* and *I. europaea* were more virulent to grapevine than *D. macrodidyma* and *I. liriodendri*, species previously accepted as the main causal agents of BFD. Given these findings, the future prospects on BFD needs to investigate (i) how *Cylindrocarpon*-like asexual morphs colonize the grapevine endorhizosphere and establish themselves inside, and (ii) what triggers latents BFD fungi to transition from a non-pathogenic to pathogenic endophyte, and cause disease symptoms in grapevine.

The knowledge about the epidemiology of *Cylindrocarpon*-like asexual morphs in grapevine is inferred from studies conducted in other hosts (Booth 1966; Brayford 1993). These fungi are able to develop chlamydospores, resistant structures that allow them to survive for a long time in the soil. In recent years, the development of new molecular tools has been crucial for the correct detection and identification of *Cylindrocarpon*-like asexual morphs from soil samples (Cardoso et al. 2013; Agustí-Brisach et al. 2014). However, DNA-based methods are unable to distinguish between viable or dead organisms with intact genetic material (England et al. 1997; Demanèche et al. 2001). In this thesis, a semi-selective medium has therefore been developed to identify the active and viable *Cylindrocarpon*-like species directly from soil (Chapter 3.2). Glucose-Faba Bean Rose Bengal Agar (GFBRBA) medium was adapted from Hunter et al. (1980) and selected as the best option among three different media after several tests, including evaluation of mycelial growth and efficacy of inoculum recovery. BFD pathogens, mainly *D. torresensis*, were isolated from soils in mature and young vineyards, nursery fields with grapevine and in rotation. In a previous study, Cardoso et al. (2013) detected *D. macrodidyma* and *D. torresensis* from 5 out of 12 soils in Portugal by planting soil samples on PDA supplemented with chloramphenicol. The fact that viable inoculum of BFD was found in nursery fields during the standard crop rotation

procedure suggests that this approach is unsuccessful for the management of BFD, as others studies had previously reported in Portugal (Rego et al. 2009; Cardoso et al. 2013) and South Africa (Halleen et al. 2003; Langenhoven et al. 2018).

The development of the GFBRA medium has improved the knowledge on the ecology of BFD fungi in soil, and has allowed researchers (i) to characterize the genetic structure of *D. torresensis* populations in soil, and compare them with those collected from grapevine roots and asymptomatic secondary hosts such as weeds (Berlanas et al. 2019), and (ii) to compare the genomes of *D. torresensis* isolated from soil, asymptomatic grapevine roots and weeds (Gramaje et al. 2019).

The effect of the physicochemical properties of the studied soils on BFD pathogen populations was also evaluated. High amount of calcium carbonate in soil favoured the presence of BFD fungi. Carbon availability as well as others nutrients are affected by plants growth and microbial communities, which at the same time are influenced by that nutrient availability (Kaiser et al. 2010). Moreover, several studies suggest that soil physicochemical properties and moisture content can affect the grapevine rhizosphere microbiome (Fernández-Calviño et al. 2010; Corneo et al. 2014; Burns et al. 2015; Zarraonaindia et al. 2015; Holland et al. 2016). Therefore, population structure of specific soil-borne pathogens can also be altered by soil physicochemical properties. Further studies are needed to understand how soil properties affect both grapevine and BFD pathogen health in order to make effective management decisions.

Soil microbiome is not only affected by the soil physicochemical properties, but also by the plants. In fact, the plants, and their genotype, have a key role in the selection of the microbiome that inhabits their rhizosphere and root compartments (Aira et al. 2010; Berendsen et al. 2012; Bazghaleh et al. 2015). Therefore, knowledge on how the microbiome is affected by the plants can help in the management of soilborne pathogens, such as BFD fungi. In grapevine, microbes associated with the plant meanwhile it is growing and producing may influence the organoleptic properties of the wine (Zarraonaindia et al. 2015). On the other hand, grapevine microbiome also affects plant health, stress protection, productivity and plant development (Zarraonaindia and Gilbert 2015). Moreover, grapevine wine production is linked to the *Terroir*, and the knowledge of the biogeography patterns and spatio-temporal dynamics of the grapevine associated microorganisms is fundamental to recreate that characteristic

(Zarraonaindia and Gilbert 2015; Marasco et al. 2018). Several studies also suggests that microbiome components can have an important role as inhibitors of phytopathogenic bacteria and fungi (Haas and Keel 2003; Vacheron et al. 2013; Yu and Hochholdinger 2018). An effect of rhizosphere microbiome reducing pathogen growth has been found in grapevines (Zarraonaindia et al. 2015).

Molecular approaches based on high-throughput sequencing technology (NGS) have progressively replaced molecular markers to characterize microbial communities in nature, including soil samples. They allow the detection and identification of more microorganisms, including species that cannot be obtained in culture (Amann et al. 1995). The new advance in NGS have increased both the resolution and scope of fungal community analyses and have revealed a high diverse and complex microbiota of grapevine soils (Zarraonaindia et al. 2015; Holland et al. 2016; Marasco et al. 2018; Martínez-Diz et al. 2019b). NGS has allowed recovering data not only about the genera linked to pathogenic species, but also about genera associated to biocontrol activities, such as *Trichoderma* spp. or *Bacillus* spp. (dos Santos et al. 2016).

The fungal and bacterial microbiome was deeply studied in the rhizosphere of five rootstocks of young and mature grapevines in Chapter 3.3. A comparison of the relative abundances of sequence reads by NGS and DNA amount of BFD pathogens by qPCR was performed for the first time. The results showed that in the case of the bacterial communities the most common phylum were Proteobacteria and Actinobacteria. Regarding fungal microbiome, Ascomycota was the most abundant phyla. Previous studies conducted on grapevine soil bacterial and 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). Moreover, the root system was able to select specific bacterial and fungal OTUs depending on the genotype. For instance, *Bacillus* spp. were only found in rootstocks '140 Ru' and '161-49 C', and some species of the arbuscular mycorrhizal fungal genus *Glomus* were one of the most differentially abundant taxa for '110 R' rootstock. Some species of both genera have been described as potential biocontrol agents (Tahat et al. 2010; Siahmoshteh et al. 2018). *Cylindrocarpon*-like asexual morphs DNA concentration detected was affected by the year and vineyard, and were found in lower abundance in

‘161-49 C’ rootstock by both high-throughput amplicon sequencing and real-time PCR (qPCR) approaches. The use of ‘161-49 C’ rootstock was previously recommended within an integrated management program for esca and Petri disease pathogens (Gramaje et al. 2010b). However, the use of this rootstock has decreased over the last years due to physiological problems detected in most vineyards in France, and in some regions in Italy and Germany (Spilmont et al. 2016).

Recently, more innovative molecular techniques such as the droplet digital PCR (ddPCR) or NGS based on RNA have been adapted to study the grapevine microbiome, particularly the fungal pathogens associated with grapevine trunk diseases (Úrbez-Torres et al. 2017; Eichmeier et al., 2018; Martínez-Diz et al. 2019a). On one hand, ddPCR detects and provides absolute quantification of lower target concentrations than qPCR (Úrbez-Torres et al. 2017). The protocol to quantify black-foot pathogens from soil and plants have been already established, confirming the usefulness of the technique for a better understanding of soil microbiome (Martínez-Diz et al. 2019a). On the other hand, sequencing of the community mRNA presents an even greater improvement for microbial ecology studies because, unlike other methods targeting DNA, this approach can differentiate between viable and dead microorganisms since it targets the metabolically active fraction of the microbiome (Keer and Birch 2003).

5.2 Control

To date, the control of BFD has been based on the use of chemical products and hot-water treatment (HWT) (Halleen et al. 2007; Rego et al. 2006; Alaniz et al. 2011a). However, due to the difficulties associated with the implantation of HWT as a standard process in nurseries (Gramaje and di Marco 2015), and the reduction of chemical control products due to environmental and public health concerns (Decoin 2001), an alternative to control BFD is needed. In order to fill this gap, the final aim of this thesis was to evaluate other management strategies such as the use of biocontrol agents and biofumigants which can be applied to nursery soils or to graftlings as a pre-planting strategy.

The efficacy of *Trichoderma atroviride* SC1 against Petri disease (Pertot et al. 2016; Berbegal et al. 2019) and BFD (Berbegal et al. 2019) have been proven in nurseries and in newly established vineyards, whereas biofumigation with *Brassica* sp. has shown

promising results against BFD (Bleach 2013). In Chapter 4.1, the effect of white mustard biofumigation and propamocarb+fosetyl-Al applied into the soil, and Tusal® (*Trichoderma atroviride* T11 + *Trichoderma asperellum* T25) applied as a pre-planting method into grapevine grafted roots was evaluated. Soil treatments were also evaluated in grapevine seedlings. In grafted plants, biofumigation with white mustard plants reduced disease incidence by 55.3% in Field 1 and 42.2% in Field 2 when compared to the no soil treatment control. Disease severity was also reduced with white mustard residues, suggesting that biofumigation is a valid alternative to chemical fungicides to reduce soil BFD inoculum levels. Reduction of disease incidence and severity in grafted plants was independent on the application of *Trichoderma* spp. as dips before planting. This finding agrees with previous research showing the limitations of the application of these biocontrol agents into the roots (Halleen et al. 2007; dos Santos et al. 2016). Halleen et al. (2007) dipped the plants for 1 min in the treatment, whereas dos Santos et al. (2016) drenched the commercial substrate with the biocontrol agents 14 days prior planting. Recent research suggested that the dipping of basal ends in dry formulation gives higher colonization than soaking the base of vines during 1 hour or field drenching (van Jaarsveld et al. 2019). Regarding grapevine seedlings, biofumigation reduced BFD severity in both fields of study by 13.6% and 25.1%, respectively. The future direction of research needs to evaluated a wide spectrum of brassicaceous plant species with different glucosinolate profiles in order to select potential biofumigants for BFD of grapevine in different regions.

All the knowledge generated by this thesis is now available to researchers, diagnostic laboratories, grapevine nurseries and growers. The results obtained in this study points to a need for alternative strategies to minimize the impact of BFD pathogens on the long-term sustainability of viticultural production worldwide. Although the emphasis of the thesis was put on a specific pathosystem on viticulture, other agricultural systems could equally benefit from our results.

5.3 References

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CHAPTER 6.

CONCLUSIONS

1. A wide diversity of black-foot disease pathogens were identified from visually symptomless vines and asymptomatic internal wood tissue of grafted vines, bringing the total number of *Cylindrocarpon*-like asexual morphs fungi isolated from grapevine in Spain to 17.
2. Two novel species, namely *Dactylonectria riojana* and *Ilyonectria vivaria*, found on asymptomatic grapevines were characterized.
3. High degree of virulence variability was noticed among the *Cylindrocarpon*-like asexual morphs fungi in Spain, with the prevalent species *D. torresensis* showing low virulence on grapevine seedlings.
4. An early, specific, and accurate detection method of viable propagules of black-foot disease pathogens in soil based on the Glucose-Faba Bean Rose Bengal Agar (GFBRBA) medium was provided.
5. Viable inoculum of *Dactylonectria torresensis* was still present during the rotation cycle in grapevine nurseries as conidia or chlamydospores.
6. Colony Forming Units (CFU) of black-foot pathogens per gram of soil correlated positively with $CaCO_3$ concentration in soils.
7. Grapevine rootstock genotype was the most important factor in shaping the microbiome in a mature vineyard (25-year-old), but not in a young vineyard (7-year-old).
8. Many bacterial and fungal species were found in all rootstocks and in both locations/vineyards, demonstrating the existence of a “core” grape phylogeny that is independent of the growing region.
9. A significant positive correlation was observed between the relative abundance of high-throughput amplicon sequencing reads and the relative abundance of DNA of black-foot disease pathogens in soil.
10. The rhizosphere compartments of “140 Ru” and “161-49 C” rootstocks harboured lower number of black-foot pathogens than the other grapevine rootstocks evaluated (“1103 P”, “110 R” and “41 B”).
11. Biofumigation with white mustard plants showed potential for improving control of black-foot disease in grapevines.
12. The application of a *Trichoderma*-based commercial product as dips before planting was ineffective to control black-foot pathogens.

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