INSTITUTO SUPERIOR DE AGRONOMIA
Universidade de Lisboa

FRONTIERS IN THE CONTROL OF PATHOGENIC FUNGI ASSOCIATED WITH THE ESCA DISEASE COMPLEX

GIOVANNI DEL FRARI

SCIENTIFIC ADVISORS:

Professor Doutor Ricardo Manuel de Seixas Boavida Ferreira

Professora Doutora Maria Helena Mendes da Costa Ferreira Correia de Oliveira

THESIS PRESENTED TO OBTAIN THE DOCTOR DEGREE IN **BIOLOGY**

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MicroWine A Marie Curie Initial Training Network

Knowledge is of the mind
Wisdom is a state of no-mind

Osho Rajneesh

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Abstract

The ‘esca disease complex’ is a term used to identify several syndromes associated with a fungal infection of the wood of *Vitis vinifera* L. or other *Vitis* species. The pathogenic agents induce the appearance of internal symptoms in the perennial organs, such as brown wood streaking, wood necrosis and wood decay; and they are believed to be indirectly involved in the manifestation of external symptoms in annual organs as well. Overall, an esca infection induces lower vigor, productivity, quality of the yield and sorter life span of affected plants. Despite several points regarding the etiology and epidemiology of this disease complex remain controversial, due to the recent increase in the disease’s reports and worldwide incidence, there is an urgent need to find effective control strategies both to prevent the spread of esca-associated pathogens and to treat infected vines. To tackle this issue, three strategies were investigated upon: biological control, endotherapy and foliar spray with selected fungicides. In the first case, wood endophyte *Epicoccum layuense* was identified as a potential biocontrol agent. This ascomycete reduced the manifestation of wood symptoms and the wood colonization of two esca pathogens, under greenhouse conditions. Endotherapy, performed in rooted grapevine cuttings, using glutaraldehyde, hydrogen peroxide or silver nanoparticles proved effective in reducing the presence of pathogen *Phaeomoniella chlamydospora*, while it was less efficient against *Phaeoacremonium minimum*. Lastly, the inoculation of a consortium of wood endophytes (skopobiota) and the foliar spray using blad-containing oligomer were effective treatments against early infections by *P. chlamydospora*, reducing approximately 10-fold its abundance in wood, when compared with a positive control. A fourth line of research investigated the wood mycobiome of esca-infected plants, using next-generation sequencing. This study revealed the complexity of the fungal communities living in the wood, also introducing new genera and species not previously reported in grapevines, which is a prerequisite for understanding how control strategies affect the wood mycobiome, under field conditions.

Key words Biological control, endotherapy, esca disease, fungicides, mycobiome.

Resumo

O "complexo de doenças da esca" é um termo usado para identificar várias síndromes associadas a uma infeção fúngica da madeira de *Vitis vinifera* L. ou outras espécies de *Vitis*. Os agentes patogénicos induzem o aparecimento de sintomas nos órgãos perenes, como estrias castanhas na madeira, necrose e deterioração da madeira, e parecem estar também indiretamente envolvidos na manifestação dos sintomas em órgãos anuais. Em geral, uma infeção pela esca induz menor vigor, produtividade, qualidade da produção e tempo de vida das plantas afetadas. Apesar de vários pontos relativos à etiologia e epidemiologia deste complexo de doenças permanecerem controversos, o recente aumento da doença e incidência a nível mundial conduziu a uma necessidade urgente de encontrar estratégias de controlo efetivas, tanto para prevenir a disseminação dos agentes patogénicos associados à esca, como para tratar as videiras infetadas. Numa tentativa de abordar a questão do controlo da esca, três estratégias distintas foram investigadas: controlo biológico, endoterapia e pulverização foliar com determinados fungicidas. No primeiro caso, o endófito de madeira *Epicoccum layuense* foi identificado como um potencial agente de biocontrole. Este ascomicete reduziu, em condições de estufa, a manifestação dos sintomas da madeira e a colonização da madeira por dois agentes patogénicos. A endoterapia, realizada em estacas de videira enraizadas, utilizando glutaraldeído ou peróxido de hidrogénio ou nanopartículas de prata, mostrou-se eficaz na redução da presença do agente patogénico *Phaeomoniella chlamydospora*, mas foi menos eficiente contra o *Phaeoacremonium minimo*. Por fim, a utilização dum consórcio de endófitos da madeira (skopobiota) e a pulverização foliar com um oligómero contendo blad foram dois tratamentos eficazes contra a infeção precoce por *P. chlamydospora*, reduzindo aproximadamente 10 vezes a sua presença na madeira, quando comparado com um controle positivo. Uma quarta linha de pesquisa investigou o micobioma da madeira de plantas infetadas com esca, usando metodologias de sequenciação de última geração. Este estudo revelou uma enorme complexidade das comunidades fúngicas que vivem na madeira, introduzindo também novos géneros e espécies não referidas anteriormente em videiras, o que é um pré-requisito importante para a compreensão

do impacto das estratégias de controle sobre o micobioma da madeira em condições de campo.

Palavras chave Controlo biológico, endoterapia, esca, fungicidas, micobioma.

Resumo alargado

As doenças de tronco da videira (Grapevine Trunk Diseases; GTDs) constituem atualmente uma das principais ameaças à viticultura mundial. São causadas por uma matriz filogeneticamente diversa de fungos que, em geral, originam sintomas semelhantes nos órgãos perenes das videiras (*Vitis* spp.). As plantas afetadas pelas GTDs sofrem com menor vigor, qualidade e quantidade das uvas e menor tempo de vida, de que resultam perdas económicas consideráveis. O complexo de doenças da esca é uma das principais GTDs e inclui múltiplas síndromes causadas por três agentes patogénicos principais: *Phaeomoniella* (Pa.) *chlamydospora*, *Phaeoacremonium* (Pm.) *minimum* e *Fomitiporia mediterranea*. Os dois primeiros podem afetar a madeira das vinhas desde a mais tenra idade e induzir o aparecimento de sintomas, como descoloração, estrias castanhas, gomose e necrose. Contrariamente, o terceiro agente patogénico afeta principalmente as videiras adultas e é o principal responsável pela decomposição da madeira (podridão branca). Os sintomas de uma infeção permanecem invisíveis, no interior da madeira, até ao aparecimento ocasional de sintomas nas folhas (conhecidos por "tiger stripes") ou nos cachos, que indicam um estágio avançado de infeção. A invisibilidade dos sintomas internos e o nicho em que estes agentes patogénicos atuam, i.e., na madeira, dificultam quer a identificação quer a administração do tratamento às plantas infetadas.
O saneamento do material de propagação em viveiros e a aplicação de tratamentos no campo são as principais abordagens para lidar com infeções. Têm sido identificados vários ingredientes ativos e agentes de controle biológico capazes de inibir o crescimento e / ou a germinação conidial de agentes patogénicos fúngicos envolvidos no complexo de doenças da esca, mas, até o momento, a sua aplicação in planta não levou à formulação de uma estratégia de controle sólida.

Os objetivos deste projeto de pesquisa foram investigar o potencial de três estratégias de controle no combate à *Pa. chlamydospora* e / ou *Pm. minimum*. A primeira estratégia envolveu o uso de um ascomicete, *Epicoccum* sp., como agente de controle biológico; o segundo envolveu a aplicação direta de tratamentos químicos na madeira de videiras jovens por meio de endoterapia; o terceiro objetivo pretendeu averiguar se a pulverização foliar de ingredientes ativos poderia ter um impacto no sucesso da colonização da madeira por *Pa. chlamydospora*. Na quarta linha de pesquisa recorreu-se a uma metodologia de sequenciação de última geração para conseguir uma melhor compreensão do micobioma da madeira de videiras adultas infetadas por esca. Este conhecimento é um pré-requisito necessário para a compreensão do impacto das estratégias de controle sobre o microbioma da madeira, sob condições de campo.

A estratégia de controle biológico centrou-se no estudo de *Epicoccum*, um género de ascomicetes frequentemente associado à endosfera da videira. Uma triagem realizada *in vitro*, por meio de cultura dupla, revelou que todos os testes com *Epicoccum* spp. inibiram o crescimento dos agentes patogénicos *Pa. chlamydospora* e *F. mediterranea*, enquanto apenas alguns deles inibiram *Pm. minimum*. *Epicoccum layuense* E24, identificado como o antagonista mais eficiente, foi testado em estacas de videira com raízes das cultivares Cabernet Sauvignon e Touriga Nacional, em condições de estufa, contra *Pa. chlamydospora* e *Pm. minimum*. Este estudo revelou que *E. layuense* E24 poderia colonizar com sucesso a madeira das videiras, após a inoculação, e não prejudicou o crescimento da videira nem induziu o aparecimento de sintomas nas folhas ou madeira. Além disso, as videiras colonizadas por *E. layuense* E24 mostraram uma diminuição considerável na sintomatologia da madeira causada pelos agentes patogénicos inoculados (em 31 - 82%, dependendo do agente patogénico / cultivar de videira), bem como uma redução na sua frequência de re-isolamento (60 - 74%). Estes resultados sugerem que *E. layuense* E24 é um candidato promissor para utilização no controle biológico, devido à sua interação antagónica com alguns agentes patogénicos fúngicos associados à esca.

A segunda estratégia de controle testada incidiu sobre controle químico. Neste estudo, cinco produtos químicos (prata elementar, fosetil-Al, glutaraldeído, peróxido de hidrogénio e oligómero contendo Blad) foram testados *in vitro*, sendo que os primeiros quatro também foram avaliados *in planta*, por meio de endoterapia, contra *Pa.*

chlamydospora e *Pm. minimum*. Os resultados mostram que todos os produtos químicos testados foram eficazes *in vitro* para evitar o crescimento de ambos os agentes patogénicos, embora em diferentes concentrações. A endoterapia das videiras (cv. Touriga Nacional) mostrou-se eficaz contra *Pa. chlamydospora* com todos os produtos químicos testados, com reduções na frequência de re-isolamento deste agente patogénico que variaram entre 90.9 e 94.7% (tratamento com glutaradeído), 68.4 e 95.5% (peróxido de hidrogénio), 68.4 e 77.3% (prata elementar) e 57.9 e 59.1% (fosetil-Al), quando comparados com o controle (tratamento com água). O único tratamento significativamente eficaz contra *Pm. minimum* foi o glutaraldeído, proporcionando uma redução de 75.0 – 83.3% na frequência de re-isolamento do fungo. Esses resultados sugerem que a endoterapia de videiras jovens, durante os estágios iniciais da infeção, pode ser uma estratégia de controle eficaz, especialmente contra o agente patogénico da madeira, *Pa. chlamydospora*.

No terceiro estudo, usámos a metodologia de sequenciação de última geração Illumina® para avaliar os efeitos da pulverização foliar de videiras com fungicidas inorgânicos (oxicloreto de cobre e enxofre), sintéticos (penconazol e fosetil-alumínio) e naturais (oligómero contendo blad), comummente usados contra o míldio e o oídio. Os objetivos desta investigação foram: (i) o micobioma residente da madeira, (ii) a colonização precoce por um consórcio de endófitos da madeira (skopobiota), (iii) o sucesso da colonização da madeira por *Pa. chlamydospora* e (iv) a interação *in planta* entre *Pa. chlamydospora* e skopobiota, em condições de estufa, em estacas de videira Cabernet Sauvignon enraizadas. Os resultados obtidos sugerem que o micobioma residente é resiliente, permanecendo quase inalterado após cada um dos tratamentos fungicidas. Por outro lado, o sucesso precoce da colonização dos endófitos que compõem o skopobiota variou em resposta aos fungicidas, com abundância relativa de alguns táxons super- ou sub-representados, quando comparados com o controle. A colonização da madeira por *Pa. chlamydospora* envolveu mudanças significativas na composição do micobioma e, além disso, foi muito afetada pela pulverização foliar com o oligómero contendo blad, o que diminuiu a abundância relativa daquele agente patogénico em mais de 12 vezes (4.9%), quando comparado com o controle (60.7%) e outros tratamentos. A presença do agente patogénico também diminuiu consideravelmente quando co-inoculado na planta com o skopobiota, alcançando

abundâncias relativas entre 13.9 e 2.0%, dependendo do tratamento fungicida aplicado. Este estudo mostra que os fungicidas pulverizados para prevenir infeções de oídio e míldio têm efeito sobre fungos não-alvo, incluindo alguns que colonizam a endosfera das videiras, com consequências imprevisíveis para o ecossistema do micobioma da madeira. São sugeridas duas possíveis estratégias de controle para combater *Pa. chlamydospora*, a saber, a pulverização foliar com oligómero contendo blad e o uso de skopobiota, apesar de mais estudos serem necessários para confirmar esses resultados.

No último estudo, foi utilizada uma metodologia de sequenciação de última geração Illumina® para investigar o micobioma da madeira de videiras de uma vinha com um historial de esca. Os três principais objetivos foram: (i) caracterizar a composição do micobioma; (ii) compreender a dinâmica espacial das comunidades fúngicas em diferentes áreas do tronco e das varas; (iii) avaliar a possível relação entre micobioma e sintomas foliares. Foi detetada uma diversidade inédita de fungos (289 taxa), incluindo cinco géneros descritos pela primeira vez em associação com a madeira da videira (*Debaryomyces*, *Trematosphaeria*, *Biatriospora*, *Lopadostoma* e *Malassezia*) e numerosas espécies. Os fungos associados à esca, *Pa. chlamydospora* e *Fomitiporia* sp. dominam a comunidade fúngica, e numerosos outros fungos associados com síndromas lenhosas também são encontrados (por exemplo, *Eutypa* spp., *Inonotus hispidus*). A análise espacial revelou diferentes abundâncias de taxa, a presença exclusiva de determinados fungos em áreas específicas das plantas e uma especificidade nos tecidos. Por fim, a composição do micobioma do tecido lenhoso nas proximidades das folhas que manifestavam sintomas foliares de esca, bem como nos ramos contendo folhas sintomáticas, foi muito semelhante à das plantas que não exibiram sintomatologia foliar. Esta observação apoia o consenso atual de que os sintomas foliares não estão diretamente relacionados com a comunidade fúngica na madeira adjacente. Este trabalho contribui para o entendimento da ecologia microbiana da madeira da videira, oferecendo *insights* e uma visão crítica sobre o conhecimento atual da etiologia da esca.

Em conclusão, as três estratégias de controle testadas revelam potencial para combater infeções causadas por fungos traqueomicóticos, *in planta*, em condições de estufa. Os tratamentos mais promissores são o uso de *Epicoccum layuense* E24 contra

Pa. chlamydospora e *Pm. minimum*; a endoterapia em videiras jovens para combater *Pa. chlamydospora*; e a pulverização foliar com oligómero contendo blad e / ou skopobiota no controle de infeções precoces pelo mesmo agente patogénico. É agora necessário prosseguir com a investigação para aprofundar o entendimento sobre estas estratégias de controle quando aplicadas sob condições de campo e em viveiros, examinando também se uma abordagem integrada, na qual uma combinação de duas ou mais dessas estratégias, pode levar a um controle ainda mais efetivo.

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General abbreviations

AW Annual Wood

BCA Biological Control Agent

BCO Blad-Containing Oligomer

BWS Brown Wood Streaking

eDNA environmental DNA

Fmed *Fomitiporia mediterranea*

GLSD Grapevine Leaf Stripe Disease

GTDs Grapevine Trunks Diseases

ITS Internal Transcribed Spacer

MIC Minimum Inhibitory Concentration

NGS Next-Generation Sequencing

Pch *Phaeomoniella chlamydospora*

PDA Potato Dextrose Agar

PDB Potato Dextrose Broth

Pmin *Phaeoacremonium minimum*

PW Perennial Wood

RA Relative Abundance

SDW Sterile Distilled Water

CHAPTER 1

GENERAL INTRODUCTION

1.1 Past and present challenges in grapevine pathology

Viticulture, as we know it today, went through several challenges since its birth, dated between 6000 and 4000 BCE, in the Southern Caucasus region. Nevertheless, the cultivation of grapevine, *Vitis vinifera* (L.), for wine or table grapes production has spread over the millennia to almost all continents, and it is nowadays considered one of the most important crops (Terral *et al.*, 2010; Maghradze *et al.*, 2016).

The recent book "Compendium of Grape Diseases, Disorders, and Pests, Second Edition" (Wilcox *et al.,* 2015), lists twenty-nine diseases caused by fungi and oomycetes, three caused by bacteria, six caused by phytoplasmas, seven caused by viruses or virus-like agents, eight nematode-transmitted virus diseases, seven nematode parasites and five insects that cause disease-like symptoms, for a total of 65 diseases. Moreover, the introduction of grapevines in non-native environments, the vast areas of monocultures and the clonal reproduction, have all together contributed in complicating the approach to pathogens and pests control. It is evident how scientists, in the forefront phytopathologists, microbiologists and agronomists, have been making extensive efforts in order to support viticulture in the endless fight against grapevine's old and new challenges.

In recent history, three major outbreaks of grapevine pathogens put at serious risk viticulture: *Erysiphe necator*, causal agent of powdery mildew; *Plasmopara viticola*, causal agent of downy mildew; the Phylloxera (*Daktulosphaira vitifoliae)* invasion (Ferreira *et al.*, 2006). These pathogens and pest were introduced in Europe from America in the mid-19th century and found in *V. vinifera* a susceptible host. Over the following decades, these pathogenic agents spread unrestricted and caused extensive damage to vineyards, leading to huge crop losses and nearly wiping out grapevines from some European regions (Granett *et al.*, 2001; Gessler *et al.,* 2011). Nevertheless, thanks to research, the threat of these pathogens is nowadays kept in check, mainly using fungicides and other agronomical practices such as grafting.

However, while science was focusing on the aforementioned dangers, new clusters of pathogens have silently emerged, becoming what, by some, is considered the fourth major outbreak in grapevine pathology's history: the pathogens responsible for grapevine trunk diseases (Bertsch *et al.*, 2013; Bruez *et al.*, 2013; Fontaine *et al.*, 2016).

1.2 Grapevine trunk diseases

Grapevine trunk diseases (GTDs) are the next big challenge in grapevine pathology. Unlike the three previously mentioned pathogens and pest, the fungi responsible for GTDs are not native to the Americas and they are believed to be part of the grapevine pathosystem for millennia. In fact, some early reports describe grapevines with trunk diseases-like symptoms during the ancient Greeks age (Mugnai *et al.*, 1999). Despite this, more reliable sources describing GTDs are found in French scientific literature starting from the late nineteenth century (Surico, 2009). From these reports to our days, wood diseases are found in all grape-growing regions of the world and their presence and incidence seems to be steadily increasing (Graniti *et al.*, 2000; Fontaine *et al.*, 2016). Over the last ten years, several surveys tried to give a figure on the percentage of vineyards/plants affected by trunk diseases and, although the estimates are limited by the elusiveness of the symptoms, grapevine trunk diseases are known to affect 10.5% of the vineyard's area in Spain, 13% in France, between 8 to 19% in Italy (Fontaine *et al.*, 2016). Despite these records, the current extent of infections by GTDs pathogens remains uncertain. Infections develop, undetected, in the wood and external symptoms are not visible before several years from the moment an infection has taken place (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016). For this reason, pathogens keep spreading silently and nearly unrestricted. In fact, externally asymptomatic but infected grapevines contribute to the spread of pathogens to other vines, before any control strategy can be performed (e.g. vine removal; Mugnai *et al.,* 1999). A worldwide estimate of the economical damage caused by GTDs was calculated by Hofstetter *et al.* (2012), revealing that the annual cost of replacing grapevines that died due to grapevine trunk diseases exceeds 1.13 billion euro.

1.2.1 Causal agents

Grapevine trunk diseases are caused by fungi belonging to both the Ascomycota and Basidiomycota and, despite the wide array of organisms involved, the similarities in the syndromes' etiology and epidemiology led to this common classification. Recent reviews include in the GTDs cluster five main disease: Botryosphaeria dieback, Eutypa dieback, the Esca disease complex, Phomopsis dieback and Black foot; with the former

three being the most concerning (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016; Gramaje *et al.*, 2016).

Several causal agents have been associated with each of these diseases and a brief list of the most frequent fungi follows. In the case of Botryosphaeria dieback, many members of the Botryosphaeriaceae family have been linked with this syndrome, among the most frequently isolated from symptomatic plants we find: *Diplodia seriata* De Not., *Neofusicoccum parvum* (Pennycook & Samuels), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.), *Botryosphaeria dothidea* (Moug. ex Fr.) and *Lasiodiplodia theobromae* (Pat.) (Úrbez-Torres, 2011; Bertsch *et al.*, 2013). Members of the family Diatrypaceae are considered responsible for the syndrome Eutypa dieback, with *Eutypa lata* (Pers.) being the most recurrent causal agent, while less frequent pathogens are fungi belonging to the genera *Eutypella*, *Diatrypella*, *Cryptosphaeria* and *Diatrype* (Bertsch *et al.*, 2013; Sosnowski *et al.*, 2013).

Three main pathogens are associated with the esca disease complex. Tracheomycotic ascomycetes *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) and *Phaeoacremonium minimum* (Tul. & C. Tul.), and basidiomycete *Fomitiporia mediterranea* M. Fisch, which are involved in one or more of the syndromes included in this disease complex. Despite these three fungi are the most recurrent, other species of wood pathogens, especially within the basidiomycetes, have been isolated from esca symptomatic plants (Mugnai *et al.*, 1999; Surico, 2009).

1.2.2 Infections and symptomatology

According to current understanding, GTDs causal agents can infect plants both at the nursery level and in the field. In the first case, the fungal pathogens are already present in the propagation material (rootstock and/or scion), or can be found in nurseries' tools or equipment used during the grafting process and storage of the cuttings (e.g. grafting machinery, hydration tanks, cold rooms; Zanzotto *et al.*, 2001; Whiteman *et al.*, 2002; Retief, McLeod and Fourie, 2006; Gramaje *et al.*, 2018). In the case of field infections, fungal conidia and/or mycelium reach open wounds in the wood, usually produced during the pruning season, via several media, such as air, water droplets, arthropods and human intervention (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016).

Once landed on fresh pruning wounds, conidia germinate and make their way into the plant's xylem, where the plant – pathogen interaction begins, giving rise to the first symptoms. The manifestation of an infection occurs on several levels and varies among GTDs, although it is most often characterized by an initial vascular discoloration, followed in time by the appearance of wood necrosis (central or sectorial 'wedge-shaped'; Figure 1.1 A, B) which increases in size. Occasionally wood decay, most commonly white rot is present, primarily but not exclusively, in esca infected plants (Figure 1.1 C; Mugnai *et al.*, 1999). Despite their slow progression, these symptoms keep advancing in the wood, compromising the hydraulic system of the plant.

Secondary symptoms are occasionally exhibited by plants affected by GTDs, as well. They become visible at shoots level (e.g. stunted growth, shorter internodes), leaves (e.g. chlorosis, tiger stripes) and berries (e.g. black spotting), often discontinuously, and their appearance occurs several years after the infection has taken place (Mugnai *et al.*, 1999; Sparapano *et al.*, 2001; Bertsch *et al.*, 2013). Despite GTDs pathogens are not considered to be directly responsible for such secondary symptoms, fungal secondary metabolites (e.g. toxins) and byproducts of the degradation of the wood, are believed to play a role in their development (Mugnai *et al.*, 1999; Evidente *et al.*, 2000; Tabacchi *et al.*, 2000; Andolfi *et al.*, 2011). Overall, plants affected by GTDs suffer from decline, dieback, reduced vigor, lower quantity and quality of the yields and an earlier death (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016).

Figure 1.1. Grapevine stems affected by wood pathogens. A- Central necrosis, symptom associated with the Botryosphaeria dieback syndrome; B- Sectorial 'wedge-shaped' necrosis, associated with *Eutypa lata* infection; C- Necrosis and wood rot, symptoms of the esca disease. Sources: A- http://grapepathology.blogspot.com/p/trunk-diseas-projects_6.html (Retrieved on 22-11-2018); B- www.lodigrowers.com/labratory-testing-for-grapevine-diseases/ (Retrieved on 22-11-2018); C- https://it.wikipedia.org/wiki/Mal_dell%27esca_della_vite (Retrieved on 22-11-2018).

1.2.3 Control strategies

To date, it remains unclear the reason(s) behind this sudden epidemic of wood diseases, which seems to have started in the eighties. Among the several hypotheses, Rubio and Garzón (2011) listed some factors that might be related to this recent increase in GTDs: (i) the ban of sodium arsenite, the sole chemical control effective against some GTDs; (ii) the increased number of infected but asymptomatic vineyards, which left untreated keep spreading the infections; (iii) newly grafted plants coming from nurseries, where the presence of GTD fungi has often been identified; (iv) agricultural practices(Rubio, J.J.; Garzón, 2011).

Over the last 20 years, the scientific community has made considerable efforts with the aim of identifying effective control strategies capable of tackling the GTDs issue (Mondello *et al.*, 2017). Research ranged from the test of active ingredients to be used as antifungal agents against GTDs pathogens, to novel delivery ways of such chemicals (e.g. endotherapy; Darrieutort and Pascal, 2007; Mondello *et al.*, 2017). Other studies focused on the use of biological control agents (BCA) both fungal and bacterial (Mutawila *et al.*, 2011; Haidar *et al.*, 2016; Yacoub *et al.*, 2016). On the side of the nurseries, the aims were to (i) limit infections by implementing 'best practices' such as the sanitation of tools, tanks and storage rooms; (ii) treat rooted cuttings with techniques such as the hot water treatment (HWT), to deal with infected plant material (Fourie and Halleen, 2004; Gramaje *et al.*, 2018). Field trials focused primarily on agricultural practices such as the application of dressings to pruning wounds, in order to prevent new infections from taking place (Kotze *et al.*, 2011; Amponsah *et al.*, 2012; Díaz and Latorre, 2013; Sosnowski *et al.*, 2013).

Despite several positive achievements, no definitive control strategy has been identified for any of the GTDs and, to date, an integrated approach that combines nurseries sanitation with agricultural practices, such as pruning wound protection, offer the best chances to prevent these diseases from spreading (Gramaje *et al*., 2018).

In conclusion, grapevine trunk diseases are considered the current biggest challenge in grapevine pathology for the following reasons:

(i) There is still no clear explanation on the causes behind this dramatic increase in GTDs presence;

(ii) There are two battlefronts, the one to prevent infections from taking place (both in nurseries and in the field) and the one that deals with treatments for infected plants;
(iii) Applying treatments in the wood is a difficult task;
(iv) GTDs are spreading silently, as newly infected plants cannot be detected, and nearly unrestricted.

1.3 The esca disease complex

From early research...

Esca can be considered one of the oldest diseases of cultivated grapevine (*Vitis vinifera*), with reports dating back from the ancient Greeks times and from the Latin world. Medieval works also describe grapevines affected by symptoms attributable to this disease (Mugnai *et al.*, 1999).

Early research, dating back to the beginning of the 20th century, associated two basidiomycetes with esca infections, namely *Stereum hirsutum* and *Phellinus (Fomes) igniarius*, with L. Petri being able to reproduce some internal wood symptoms with *Cephalosporium* sp. and *Acremonium* sp. A confirmation of this understanding came in the mid nineteen hundreds, when researchers assessed the role of *Cephalosporium* sp. and *P. igniarius* in producing wood symptoms, including wood decay (Mugnai *et al.*, 1999). Up until this time, esca was considered 'a simple disease of old vines', with symptoms such as white rot present in the trunk and branches, and associated with wounds produced on the plant (e.g. yearly pruning; Graniti *et al.*, 2000). Symptoms were described to appear also in leaves and berries, from June to September, and apoplexy, namely the sudden wilting of the plant, was associated with environmental factors (e.g. heat, water availability, wind; Surico *et al.*, 2006). Researchers had to change their mind starting from the late 1980's, when an upsurge in the disease incidence in Southern Europe led to an increased study of the etiology and epidemiology of the disease (Mugnai *et al.*, 1999; Graniti *et al.*, 2000). The research carried out through the 90'ies raised more questions than it settled. In the eyes of some researchers, esca was a disease complex, with several factors and

microorganisms acting in association or succession. A second branch of thought saw esca as two distinct diseases, one causing the white rot (caused by *Fomitiporia punctata*) and the other being responsible for other wood symptoms such as brown wood streaking and gummosis. A third opinion supported the view that esca was exclusively caused by species of *Phaeoacremonium*, and that the presence of *F. punctata* played a secondary role (Graniti *et al.*, 2000).

…to current understanding

A redefinition of the nomenclature of 'esca' and the esca-associated syndromes was proposed by G. Surico (2009), as a review of past knowledge joined with more recent understanding. This nomenclature gained popularity among leading researchers, becoming accepted by the majority of the scientific community.

Surico established five separate syndromes, identified as such according to three main factors: the age of the plant, the symptomatology expressed in the wood and/or other plant tissues (e.g. leaves, bunches), and the pathogenic fungi that led to the expression of such symptoms. The five syndromes are:

(i) Brown wood streaking of rooted cuttings (BWS);
(ii) Petri disease;
(iii) Grapevine leaf stripe disease (GLSD);
(iv) Esca;
(v) Esca proper;

with the first three of the list belonging to the group "Grapevine Phaeo-tracheomycosis complex", a category created to identify plants of different age, which express similar symptoms and are infected by the same pathogen(s).

These syndromes will be further described, focusing on the pathogens involved, the symptoms they cause and the current control strategies.

Esca-associated syndromes presence in the world

It is difficult to estimate the presence and extent of infections for these five syndromes in the world. This is because wood infections are not visible unless a destructive approach is used and foliar symptoms may or may not appear a number of years after

the wood infection has taken place. In addition, foliar symptoms are discontinuous and appear with no predictable pattern (Li *et al.*, 2017).

Concerning the syndrome **BWS**, several studies revealed the presence of tracheomycotic pathogens in propagating material in nurseries, revealing that either the rootstock/scion coming from the mother field were infected, or that the nursery environment and processes (e.g. hydration, cold storage, disbudding, grafting) were sources of infection (Ridgway *et al.*, 2002; Fourie and Halleen, 2004; Gramaje *et al.*, 2018). Despite that, no large-scale surveys were conducted to assess the presence on wood pathogens in nurseries.

The same applies to **Petri disease**, for which no large-scale epidemiological study has been performed.

On the other hand, several surveys tried to estimate the presence of **GLSD**, although in studies pre-dating the current nomenclature it was considered as 'esca', still referring to the appearance of the 'tiger stripes' symptomatology in leaves. Observations made in Kremstal (Austria) in the 1990s (Reisenzein *et al.*, 2000) revealed a percentage of infected plots (n= 46) greater than 70% when grapevines were above 10 year old. In the 2000s, a French survey recorded the presence of foliar symptoms referable to GLSD (or black dead arm disease) in 66% of vineyards in Alsace, 68% in Bourgogne, 73% in Bordeaux, between 81-85% in Charentes and above 95% in Jura (n= 329) (Bruez *et al.*, 2013). In Portugal, a survey carried out during 2011/2012 in the Dão wine region revealed that GLSD was present in 88% of the sampled vineyards (n= 62) (Sofia *et al.*, 2013). However, no large-scale data is available for what concerns the past or current presence of GLSD in Spain, Australia, Italy or other wine-producing countries.

Concerning **esca** and **esca proper**, also in these cases it is difficult to assess the presence of wood rot as it is externally asymptomatic. In fact, absence of GLSD foliar symptoms in plants located in a field with disease history does not mean plants are non-infected or non-symptomatic in the wood (Liminana *et al.*, 2009; Díaz and Latorre, 2014). Few studies examined, destructively, the presence of wood rot in vineyards with esca proper symptoms, such as the one by Pollastro *et al.* (2000), demonstrating that while only 17% of grapevines showed leaf symptoms, 84% of them presented deteriorated wood.

1.3.1 The Phaeo-tracheomycosis complex

Pathogenic fungi

There is a multitude of tracheomycotic fungi that affect the wood of grapevines. The ascomycetes included in the 'Phaeo-tracheomycosis complex' are those most frequently isolated from the wood of plants affected by BWS, Petri disease and GLSD, namely *Phaeomoniella* (*Pa.*) *chlamydospora* (Phaeomoniellales, Phaeomoniellaceae) and members of the *Phaeoacremonium* (*Pm.*) genus (Diaporthales, Togniniaceae), with *Pm. minimum* being the most represented (Figure 1.2; Surico, 2009; Gramaje, Urbez-Torres and Sosnowski, 2018). They are known as plant pathogens, although some studies reported their presence in asymptomatic wood (Zanzotto *et al.*, 2001). Both *Pa. chlamydospora* and *Phaeoacremonium* spp. have been isolated from numerous hosts, mainly woody plants, and soil (Rooneye *et al.*, 2001; Mostert *et al.*, 2006; Díaz and Latorre, 2014). Moreover, propagules are dispersed by several means, such as wind, rain, arthropods and human intervention (Edwards *et al.*, 2001; Eskalen *et al.*, 2007).
Their pathogenicity is the result of both the colonization of the functional woody tissues and the release of several toxic metabolites (e.g. scytalone, isosclerone; Evidente *et al.*, 2000; Abou-Mansour *et al.*, 2004; Andolfi *et al.*, 2011).

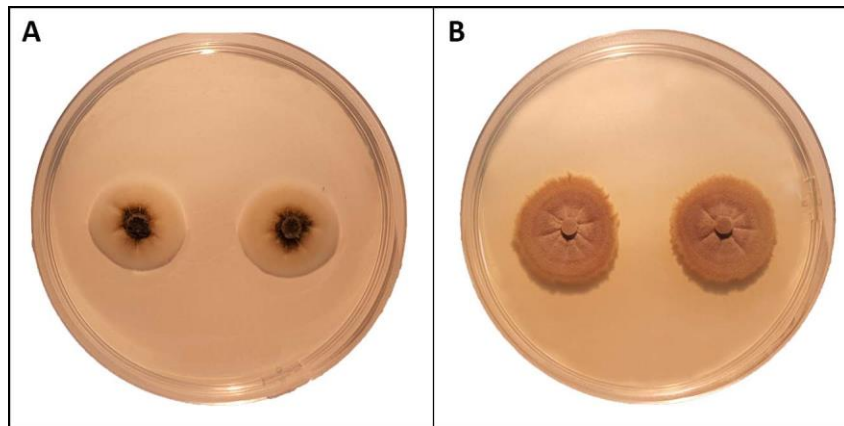

Figure 1.2. Cultures of *Phaeomoniella chlamydospora* (A) and *Phaeoacremonium minimum* (B) grown for 14 days on potato dextrose agar medium.

1.3.1.1 Brown wood streaking of rooted cuttings and Petri disease

Symptomatology

Once conidia land on open wounds (e.g. during pruning season) they germinate and the fungal mycelium starts colonizing the xylem of the plant. Histological studies have shown that the wood colonization differs between *Pa. chlamydospora* and *Pm. minimum*. In fact, the first one is found primarily in the lumen of xylem vessels and in xylem fibers (Valtaud *et al.*, 2009; Pouzoulet *et al.*, 2017), while the second can colonize bark, pith, phloem, xylem fibers and vessels, vessel-associated cells, rays, metaxylem and protoxylem (Valtaud *et al.*, 2009; Pierron *et al.*, 2015).

The plant responds to an infection in several ways, including the synthesis of phytoalexins, the overexpression of genes related with the production of pathogenesis-related proteins (Ferreira *et al.*, 2007; Martin *et al.*, 2009; Fischer *et al.*, 2016) and the formation of tyloses. However, the only internally-visible plant response consists in the discoloration of the wood and the appearance of brown wood streaking (when seen in longitudinal sections), also observed as brown dots (in cross sections; Figure 1.3). This symptom is shared among all the syndromes included in the Phaeo-tracheomycosis complex.

The presence of brown wood streaking is the only internally-visible symptom associated with the BWS syndrome. When observed in cross sections it may be scattered randomly, clustered or forming ring-like structures in the xylem (White *et al.*, 2011; Figure 1.3 B, C). This disease is most often associated with rooted cuttings, in particular with the rootstock of the propagation material (Surico, 2008; Surico, 2009). Vines affected by BWS present damaged xylem vessels, as a result of fungal infection, but they do not express any external symptoms.

Petri disease is associated with young vines, it may occur as an evolution of BWS or it may originate in the field (Surico, 2008). In Petri disease, previously called 'black goo' or gummosis, the vessels affected by brown streaking are occasionally associated with the oozing of a black viscous fluid, a gum, primarily composed by pectin and polymerized phenolics, but whose role and composition remain to be fully understood (Mostert *et al.*, 2006). Other symptoms associated with Petri disease are a darkened central pit, leaf chlorosis, with plants suffering losses in yield, stunted growth and decline in vigor (Surico, 2008).

Figure 1.3. Symptomatology associated with the syndromes brown wood streaking of rooted cuttings and Petri disease. Longitudinal sections of 2 years old rooted cutting presenting brown wood streaking (A). Cross sections showing scattered (B) or clustered (C) black dots.

Control strategies

The majority of the studies concerning the control of the esca-associated syndromes focus on fighting tracheomycotic pathogens, in order to produce infection-free plant material in nurseries and prevent new infections from occurring in the field (Gramaje *et al.*, 2018). For these purposes, a multitude of natural products, chemicals of synthesis and BCAs have been tested against mycelial growth and conidial germination of *Pa. chlamydospora* and *Pm. minimum* (Mondello *et al.*, 2017). In addition, cultural practices or other agronomical practices are considered a valid tool to be employed as control strategies.

Among the successful active ingredients and BCAs tested *in vitro*, only a few made it to the following step of nursery and field trial, where even fewer proved valid for the purpose.

(i) *In nurseries*. The use of fungicides on nurseries' tools, equipment and facilities is common practice, although Chinosol (hydroxyquinoline sulfate), reported to be the most utilized, has proven ineffective against *Pa. chlamydospora* and *Pm. minimum* (Gramaje *et al.*, 2009). The hot water treatment seems the most effective method to disinfect dormant canes during the propagation process. It consists in treating plant material with hot water at 50 °C for 30 min. However, some studies have shown that vines may be negatively affected in terms of survivability and overall vigor (Waite and

May, 2005). On the side of BCAs, the incidence of both tracheomycotic pathogens in rootstock cuttings was reduced by soaking the propagation material in ascomycete *Trichoderma* formulations (Fourie and Halleen, 2004; Mondello *et al.*, 2017). Oomycete *Pythium oligandrum* was tested under greenhouse conditions, it was able to colonize the root systems of grapevine cuttings and its presence was effective in reducing the wood necroses caused by *P. chlamydospora* (Yacoub *et al.*, 2016). Also bacteria have been tested as possible BCAs, with species of *Bacillus* spp. being some of the most promising, although none of them is currently employed in nurseries (Alfonzo *et al.*, 2012; Haidar *et al.*, 2016).

(ii) *In the field*. The treatment of pruning wounds is considered the best strategy to prevent new infections from occurring in the field. Tests revealed that chemicals such as thiophanate-methyl and copper oxychloride worked against *Phaeoacremonium* spp. (Gramaje *et al.*, 2018); while boron, pyraclostrobin and boscalid, and a mixture of cyproconazole and iodocarb proved effective against *Pa. chlamydospora*. Promising results were achieved especially using BCAs, where several species/strains of *Trichoderma* (e.g. *T. harzianum*, *T. atroviride*, *T. asperellum* and *T. gamsii*) were able to control tracheomycotic pathogens protecting pruning wounds (Di Marco *et al.*, 2004; Mutawila *et al.*, 2011; Pertot *et al.*, 2016). All these field trials resulted in a reduction of the incidence of infections but, to date, full protection of pruning wounds has not been achieved.

The control strategies through cultural/agronomical practices focus primarily on preventing infections from occurring on rootstock mother vines. It is suggested to grow them on trellis, to minimize the chance of soil-borne pathogens from reaching the wood; to avoid using sprinkling irrigation, to reduce conidial dispersal and their germination rates (Gramaje *et al.*, 2018). In addition, some studies identified rootstocks less susceptible to tracheomycotic fungi (Eskalen *et al.*, 2001). The pruning timing and technique, as well as the training system of grapevines seem to have an impact on the chance of infections and development of wood necrosis. Late pruning (February-March) and minimizing the amount and size of pruning wounds are advised (Travadon *et al.*, 2016; Gramaje *et al.*, 2018).

According to recent reviews, the best approach currently available is to adopt an integrated disease management strategy that combines nursery sanitation practices

and pruning wound protection, possibly leading to long lasting pathogen-free grapevines (Mondello *et al.*, 2017; Gramaje *et al.*, 2018).

1.3.1.2 Grapevine leaf stripe disease

Symptomatology

Grapevine leaf stripe disease (GLSD) is associated with different symptoms in the wood, such as brown streaking, black spots and wood necroses, and with a main external symptom, the characteristic appearance of the so-called "tiger stripes", in which the leaves present interveinal chlorotic areas and/or reddening of leaves, followed by necrosis (Figure 1.4; Surico, 2008). Another symptom occasionally expressed in GLSD affected plants is dark spotting on the skin of berries, which develops as a results of necrosis of epidermal and hypodermal cells, but whose triggering factor(s) remain unknown (Mugnai *et al.*, 1999).

The first appearance of foliar symptoms occurs years after the infection of grapevine by the wood pathogens (Sparapano *et al.*, 2001) and it is normally exhibited in late spring/early summer. The reasons why leaf symptoms may disappear, from plants which were symptomatic during the previous growing season, have not yet been fully clarified. Some studies suggest that soil (Lecomte *et al.*, 2011), nutrients (Calzarano *et al.*, 2014), fungicides (Di Marco *et al.*, 2011) and environmental factors (Surico *et al.*, 2000; Marchi *et al.*, 2006; Di Marco *et al.*, 2011; Bruez *et al.*, 2013) may play a role in the development of the symptoms, but further research is necessary to support such claims.

To date, the factor(s) directly responsible for the foliar symptoms of GLSD are yet to be found, as tracheomycotic fungi were never found in leaves or berries. Several speculations have been made in the past, but recent evidence is pointing towards the role of fungal metabolites (Abou-Mansour *et al.*, 2004; Andolfi *et al.*, 2011), as well as products from the wood degradation (Evidente *et al.*, 2000; Tabacchi *et al.*, 2000), which are thought to be carried via sap flow from the infected/decaying wood to the leaves and berries. These metabolites and products are thought to trigger a host defense response that results in the expression of foliar symptoms (Andolfi *et al.*, 2011; Bertsch *et al.*, 2013). Also, a recent study provided evidence that the seasonal pattern of development of GLSD symptoms could be related to the phytoalexins levels

in the leaves and the grapevine growth stage (Calzarano *et al.*, 2016). Despite these advances, the full mechanism of the development of foliar symptoms is still unclear.

No grapevine cultivar is considered resistant to GLSD and symptom expression in the leaves may vary by degree and appearance (Köklü, 2000; Marchi, 2001; Murolo and Romanazzi, 2014; Borgo *et al.*, 2016). Similar foliar symptoms are found also in vine plants infected by other wood fungi, mainly species in the Botryosphaeriaceae responsible for black dead arm disease (Larignon *et al.*, 2009).

Figure 1.4. Wood and leaf symptomatology associated with grapevine leaf stripe disease. Cross section of a trunk, the wood presents extensive necrosis, black dots and brown streaking (A). Foliar symptoms, described as 'tiger stripes', expressed on cv Cabernet Sauvignon in July (B), or on cv Fernão Pires in October (C).

Control strategies

Despite the appearance and development of the leaf symptoms remain to be fully elucidated, the application of treatments to fight GLSD focus on chemicals effective against the wood pathogens, and on their delivery method (e.g. foliar pulverization, endotherapy). To date, trials were mostly unsuccessful and only few treatments proved moderately effective in reducing the occurrence of foliar symptoms (Mondello *et al.*, 2017).

Up until the early 2000's sodium arsenite pulverization, few weeks before bud-break, was the only treatment capable of preventing the appearance of foliar symptoms. The mode-of-action of sodium arsenite in this concern remains unclear, as well as its effect

on the wood pathogens. This chemical was banned from vineyards due to its toxicity for both humans and the environment (Di Marco *et al.*, 2000; Bertsch *et al.*, 2013). Among the, moderately, successful trials in reducing the incidence of foliar symptoms, with foliar pulverization, we find a seaweed extract (Calzarano *et al.*, 2014), a copper formulation and fosetyl-aluminium (Di Marco *et al.*, 2011). However, to date, none of these treatments are considered effective enough for their application in vineyards, and the best control strategy lies in the prevention of infections.

1.3.2 Esca and esca proper

Pathogenic fungi

Several basidiomycetes genera have been linked with wood decay of esca symptomatic plants. *Fomitiporia mediterranea* (Hymenochaetales, Hymenochaetaceae) is fungus most frequently isolated from white rot in Europe (Surico, 2009), and this internally-visible symptom has been successfully reproduced with artificial inoculations of this fungus in grapevine (Sparapano *et al.*, 2000), demonstrating that *F. mediterranea* can also act as primary pathogen (Figure 1.5, A). Members of the genus *Fomitiporia* are primarily saprotrophs or parasites, with a worldwide distribution and have been associated with numerous genera of woody plants (Fischer, 2002). Other genera of white rot agents, reported at lower frequencies or most frequently isolated outside Europe are *Fomitiporella*, *Phellinus*, *Inonotus*, *Inocutis*, *Stereum*, *Pletorus* and *Trametes* (White *et al.*, 2011; Bertsch *et al.*, 2013; Cloete *et al.*, 2015).

Symptomatology

There are three main symptoms that identify esca, all of which derive from the presence of an infection by white rot agents. The first and most obvious is the presence of white rot itself, which can be observed either in a destructive way or when large pruning wounds are made in the symptomatic hardwood. White rot is the name used to describe the transformation of the wood into a 'spongy, friable, whitish-yellow mass' (Figure 1.5, C), often delimited by a dark line when observed in cross sections (Surico, 2008). The decay, which normally starts from pruning wounds (Mugnai *et al.*, 1999), is the result of the activity of enzymes involved in cell wall decomposition. Only a few toxic metabolites have been identified from *F. mediterranea* (Andolfi *et al.*,

2011). The second symptom that reveals an advanced infection is the presence of a crack in the trunk, which may occur when the rot reaches the wood surface (Surico, 2008). A third observation can reveal the presence of white rot in grapevines, namely the presence of fruiting bodies of white rot agents on the surface of the trunk or arms of the plants (Figure 1.5, B).

The simultaneous presence of white rot and GLSD foliar symptoms is the condition associated with esca proper.

Esca and esca proper are the two syndromes considered responsible for the event called 'apoplexy', a condition in which the entire plant or only one of its arms suddenly wilt and die. This event generally occurs during summer and it is due to the compromised hydraulic system of the plant. However, environmental factors such as water availability and heath are believed to play a key role (Mugnai *et al.*, 1999).

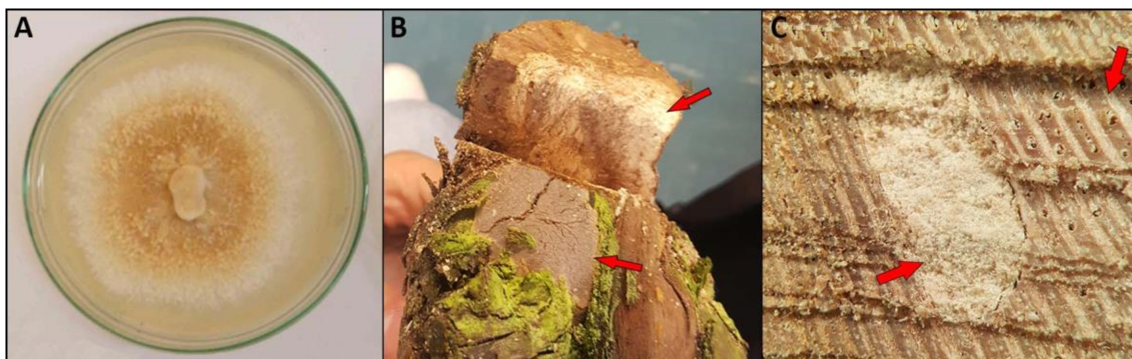

Figure 1.5. White rot agent *Fomitiporia mediterranea* grown on potato dextrose agar medium (A). Trunk of grapevine presenting extensive white rot (top arrow) and a fruiting body of *F. mediterranea* (bottom arrow; B). Section of wood examined under the stereo microscope, top arrow indicates healthy wood, bottom arrow indicates white rot (C). Sources: A- (Rajaiyan *et al.*, 2014)

Control strategies

Tests aiming to control *F. mediterranea,* or other white rot causal agents, are scarce and limited to studies *in vitro*. *F. mediterranea* is inhibited by natural compounds such as chitosan and by chemicals of synthesis such as the combination of pyraclostrobin

and metiram. Both *F. punctata* and *S. hirsutum* were inhibited by the phytoalexins resveratrol and pterostilbene (Mondello *et al.*, 2017).

The only bacterial BCA tested against *F. mediterranea* is *Bacillus amyloliquefaciens*, which proved moderately effective *in vitro*. No fungi were tested as BCA against any white rot agent (Mondello *et al.*, 2017).

A traditional method to treat esca proper consists in splitting the trunk in half and inserting a stone within the two halves of the trunk, therefore exposing permanently the rotted wood to the air (Figure 1.6). This approach, still in practice in rural areas of north-east Italy, seems to delay the reappearance of foliar symptoms, despite no scientific studies have yet verified this claim (Mugnai *et al.*, 1999). More recently, a pruning technique combined with the mechanical removal of the wood rot, which reminds the trunk-splitting technique, has been identified as a promising method to stop the expression of foliar symptoms in plants affected by esca proper (https://simonitesirch.com/). Also in this case, scientific research has not yet investigated the subject.

Figure 1.6. Trunk-splitting technique, also know as 'metodo Armano' (from the Italian: 'Armano's method'), performed on grapevines affected by esca proper. Treated grapevines in a vineyard in northeast Italy (Friuli), in December 2015 (A) and July 2016 (B).

1.4 The microbial ecology of grapevines

1.4.1 The microbiome and next-generation sequencing

The concept of microbiome identifies the community of microorganisms (e.g. fungi, bacteria, viruses) that inhabit a particular environment. All environments, ranging from soil to the human body, present a diverse array of microbes that interact among each other and with their host. The field of microbial ecology, also called environmental microbiology, aims to study such diversity and interactions.

The history of microbial ecology underwent important changes from its beginnings to recent days (Morgan *et al.*, 2017). There are three main approaches to the study of the composition of microbiomes, starting from the earliest to the most recent:

(i) The traditional microbiological approach, which characterizes the first studies of microbial ecology. This method is based on the isolation and cultivation of microorganisms *in vitro*, their identification relying mainly on morphology, microscopy and growth of cultures in different media;

(ii) The molecular biology approach, beginning from the polymerase chain reaction (PCR) revolution. This approach is culture-independent and allows the accurate identification of microorganisms through DNA-based fingerprinting. Common methods included in this category are: single strand conformation polymorphism (SSCP), automated ribosomal intergenic spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE);

(iii) The next-generation sequencing (NGS) approach, being the results of the latest improvements in DNA sequencing and bioinformatics. Metagenomics consists in the direct analysis of the collective genomes of environmental samples, either in their entirety (whole metagenome sequencing) or focusing on a specific category or organisms (amplicon-based sequencing, also called metabarcoding). In metabarcoding, a specific gene marker (amplicon), from fungi or bacteria, is selected and amplified directly from environmental DNA (eDNA) without any step of enrichment or cultivation (Morgan *et al.*, 2017).

The approaches just described are all valid and still applied to this day. Nevertheless, culture-dependent and culture-independent approaches offer different understanding.

The culture-independent approaches are able to identify taxa present in very small abundance and others that are not cultivable *in vitro*; with NGS being able to describe the diversity of complex environmental samples and with greater resolution (Morgan *et al.*, 2017).

1.4.2 The microbiome of the vineyard

In recent years, increasing interest has developed over the understanding of the vineyard's microbiome, using culture-independent approaches. It is not only an interest of the scientific community, but also of vine-growers and wine makers, as the microbial composition of the vineyard may influence several factors, such as the plants health and physiology, the quality of the yield and eventually the fermentation processes that lead to wine (Zarraonaindia and Gilbert, 2015). Therefore, several studies characterized the mycobiome, namely the fungal communities, and the bacterial populations associated with the phyllosphere (Pinto *et al.*, 2014; Singh *et al.*, 2018), rhizosphere and soil (Zarraonaindia *et al.*, 2015; Samad *et al.*, 2017), endosphere (Campisano *et al.*, 2014; Rezgui *et al.*, 2016), grape's skin and flowers (Belda *et al.*, 2017; Morgan *et al.*, 2017), must (Belda *et al.*, 2017; Mezzasalma *et al.*, 2017). This new understanding is yet incomplete: an increasing number of new genera and species are associated with grapevines (Jayawardena *et al.*, 2018), dynamics of spatial and seasonal changes are found (Bruez *et al.*, 2014), microbiomes may vary according to the plants' cultivar and age (Dissanayake *et al.*, 2018), agronomical practices may influence microbiomes as well (Travadon *et al.*, 2016). In conclusion, more research is necessary to advance the present knowledge, especially concerning one of the areas least investigated, namely the endosphere.

1.4.3 The wood mycobiome and GTDs

To date, research on the mycobiome of the endosphere of grapevines, in particular of the wood, have mostly focused on culture-dependent studies. This approach consists in plating on artificial medium pieces of wood and isolating the emerging colonies. These studies identified numerous species of fungi, allowing an understanding of the communities of endophytes, saprophytes and pathogens associated with grapevines (Halleen *et al.*, 2003; González and Tello, 2011; Núñez-Trujillo *et al.*, 2012). A culture-

independent approach was chosen by Pancher *et al.* (2012), using ARISA; while, more recently two studies applied next-generation sequencing to unravel the diversity of the wood mycobiome. Of these last two studies, the first evaluated the saprotrophic communities present in dead vines (Jayawardena *et al.*, 2018), while the second analyzed the endophytes of vines at different ages (Dissanayake *et al.*, 2018). These three culture-independent works contributed to widen the understanding of the wood mycobiome especially, but not exclusively, by introducing organisms that had not been previously reported in association with the grapevine's endosphere.

The study of the microbial ecology of the wood may offer important insights when applied to GTDs as well. In the available literature, there are only three studies which tried to address the composition of the wood mycobiome, in GTDs-affected plants, from this perspective. The first study, by Hofstetter *et al*. (2012), used a culture-dependent approach, revealing an unprecedented fungal diversity of 158 species, including numerous GTDs pathogens. Grapevines apparently healthy and others presenting foliar symptoms of esca had a very similar wood mycobiome composition both in terms of species present and relative frequency. The authors raised doubts in regards to the real role of esca-related pathogens in the expression of foliar symptoms, which is well supported by their evidence, but against the prevalent understanding of the disease. A second study by Bruez *et al*. (2014) used a culture-independent approach (SSCP), with the same objective. Bruez analyzed the mycobiome of non-symptomatic wood of plants with or without foliar symptoms of esca, during the four seasons. The study revealed a shift in the mycobiome of the wood during different seasons, but no differences when comparing symptomatic and non-symptomatic plants, supporting the observations made by Hofstetter. The third study, by the same author and using the same methods, analyzed the mycobiome of old plants with no foliar symptoms of esca, but symptomatic in the wood. The study characterized the mycobiome of old plants, also revealing the presence of many GTDs causal agents, despite the absence of foliar symptoms (Bruez *et al.*, 2016).

The research on microbial ecology of the wood mycobiome greatly improved the understanding of the diversity of the fungal communities inhabiting the endosphere of grapevines, also raising doubts on the etiology of the foliar symptoms. To date, no

studies used NGS to further the understanding of the wood mycobiome and its possible role in the foliar symptomatology expression.

1.5 Aims

This PhD project focused primarily on evaluating control strategies that might find application in the short-to-medium term, in order to confront infections caused by two tracheomycotic fungi directly responsible for the syndromes brown wood streaking of rooted cuttings, Petri disease and grapevine leaf stripe disease, namely *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, in *Vitis vinifera* L. Three research lines were pursued simultaneously, in line with the aims of this research project, each one of which constitutes one chapter of this thesis. The fifth chapter does not deal directly with control strategies, instead the aim of this line of research is to gain an understanding of the microbial ecology of the wood of adult grapevines located in a vineyard with a history of esca proper. This knowledge is a prerequisite for the following investigation (not included in this thesis) aiming to understand how applying any control strategy, under field conditions, may affect the endosphere of grapevines, in regards to both pathogens and non-target fungi (e.g. endophytes, saprobes).

Chapter 1. General introduction. This chapter aims to introduce the reader to grapevine trunk diseases, giving background on the five syndromes associated with the esca complex and their current control strategies. An overview of the concept of mycobiome and its meaning in the vineyard and in the grapevine's endosphere conclude this chapter.

Chapter 2. "*Epicoccum layense* E24 a potential biological control agent of esca-associated fungi in grapevine". In this chapter, we screened several species/strains of *Epicoccum* sp., a genus of ascomycetes commonly associated with grapevines, to assess their potential in biological control. This study had the following aims: (i) identifying *Epicoccum* spp. through molecular analysis, (ii) running *in vitro* dual culture competition tests against three esca-associated fungi and examining their interactions under the microscope, (iii) learning whether *E. layuense* E24 produces symptoms in

grapevines when artificially inoculated, (iv) understanding whether *E. layuense* E24 is capable of antagonizing two esca tracheomycotic fungi *in planta*.

Chapter 3. "Endotherapy of infected rooted grapevine cuttings in the control of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*". In this chapter, we tested the method of endotherapy, namely injecting active ingredients in the wood, as a possible control strategy against two esca-associated fungi, *P. chlamydospora* and *P. minimum*. The aims of this study were two: (i) establishing the efficacy of several chemicals as antifungal agents *in vitro*, (ii) understanding whether endotherapy, using selected chemicals, has the potential to control these two wood pathogens in young grapevines, under greenhouse conditions.

Chapter 4. "Fungicides and the grapevine wood mycobiome. A case study on tracheomycotic ascomycete *Phaeomoniella chlamydospora* reveals potential for two novel control strategies". Application of fungicides, through foliar spray, is a common and necessary practice in vineyards worldwide. The primary aims of this study were to use a next-generation sequencing approach in order to understand whether fungicides applied against powdery and downy mildew agents (i) affect indirectly the wood mycobiome, and/or (ii) have the potential to be exploited in the control of *P. chlamydospora* infections.

Chapter 5. "Characterization of the wood mycobiome of *Vitis vinifera* in a vineyard affected by esca. Spatial distribution of fungal communities and their relation with foliar symptoms". The wood mycobiome in adult grapevines has been studied mainly with culture-dependent approaches. In this study, we used next-generation sequencing (culture-independent approach) in order to gain insights on the microbial ecology of plants found in a field with a history of esca proper. There were three main aims in this study: (i) to characterize the diversity fungi living in the perennial and annual wood of grapevines, (ii) to learn whether different areas of the plants (e.g. graft union, trunk, spurs, canes, etc.) present differences in diversity and abundance of taxa, and (iii) to understand if there is any link between esca foliar symptoms and the fungal communities present in the wood.

Chapter 6. Final remarks. In this last chapter we review the achievements of this PhD project, examining how they may find application in nurseries and/or in the field, also evaluating potential perspectives.

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CHAPTER 2

Epicoccum layuense a potential biological control agent of esca-associated fungi in grapevine

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2. *Epicoccum layuense* a potential biological control agent of esca-associated fungi in grapevine

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Abstract

Epicoccum is a genus of ascomycetes often associated with the mycobiome of grapevines (*Vitis vinifera*). *Epicoccum* spp. are found in the soil, phyllosphere, as well as in the wood, where they interact both with the plant and with other endophytes and pathogens. Wood pathogens involved in the esca disease complex, a grapevine trunk disease, are particularly concerning in viticulture, as current control strategies have proven unsatisfactory. This study investigated the interaction among *Epicoccum* spp. and three esca-associated fungi, with the aim of establishing whether they are suitable candidates for biological control. A screening conducted *in vitro*, by means of dual culture, revealed that all tested *Epicoccum* spp. inhibited the growth of pathogens *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea*, while only some of them inhibited *Phaeoacremonium minimum*. *Epicoccum layuense* E24, identified as the most efficient antagonist, was tested in rooted grapevine cuttings of cultivars Cabernet Sauvignon and Touriga Nacional, under greenhouse conditions, against *P. chlamydospora* and *P. minimum*. This study revealed that the inoculation of *E. layuense* E24 produced a successful colonization of the wood of grapevines; in addition it did not impair the growth of the plants or induce the appearance of symptoms in leaves or in wood. Moreover, grapevines colonized by *E. layuense* E24 showed a considerable decrease in the wood symptomatology caused by the inoculated pathogens (by 31 — 82%, depending on the pathogen/grapevine cultivar), as well as a reduction in their frequency of re-isolation (60 — 74%). Our findings suggest that *E. layuense* E24 is a promising candidate for its application in biological control, due to its antagonistic interaction with some esca-associated fungal pathogens.

Keywords biological control, fungal interaction, grapevine, tracheomycosis

2.1 Introduction

Grapevine trunk diseases (GTDs) are an increasing threat to worldwide viticulture (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016; Gramaje *et al.*, 2018). Affected grapevines (*Vitis vinifera* L.) exhibit lower vigor, reduced productivity and quality of yields, shorter lifespan; which, altogether, cause considerable economic losses (Hofstetter *et al.*, 2012; Gramaje *et al.*, 2018). The causal agents of these diseases are found in diverse groups of both ascomycetes and basidiomycetes, which can colonize the woody tissues of grapevines, interfering with the plant physiology, microbial ecology and activating plant response mechanisms (Bertsch *et al.*, 2013; Bruez *et al.*, 2014). Symptoms of an infection by trunk disease pathogens are often elusive. They are found primarily in the wood, in the form of brown streaking (longitudinal discoloration of xylem vessels), black dots, necrosis and wood decay; occasionally, symptoms may appear in other organs of the plants as well (e.g. leaves and bunches; Mugnai *et al.*, 1999; Surico, 2008).

One of the major GTDs is the esca disease complex which comprises five different syndromes. The first three, (i) brown wood streaking of rooted cuttings, (ii) Petri disease and (iii) grapevine leaf stripe disease (GLSD), are mainly caused by the tracheomycotic pathogens *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams and *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous. These syndromes manifest in vines of different ages, the former affects newly grafted plant material, Petri disease is diagnosed in young vines, while GLDS generally affects adult plants. The fourth syndrome is (iv) esca, which is caused by different basidiomycetes, most frequently *Fomitiporia mediterranea* M. Fisch. This pathogen has the capacity to degrade lignin and induces the appearance of white rot in the wood. Lastly, (v) esca proper, that corresponds to the co-occurrence of GLSD and white rot in the same vine (Surico, 2009; Calzarano *et al.*, 2016). Infections occur primarily in the field, where spores of the pathogens land on fresh pruning wounds and make their way in the wood, as well as via infected propagating material (Mugnai *et al.*, 1999; Fourie and Halleen, 2004; Gramaje *et al.*, 2018). Control strategies, aiming to limit the infection caused by these pathogens, have often proven unsatisfactory, offering only partial protection. Additionally, the regulatory restrictions

that chemicals are facing in most countries around the world are leading researchers towards alternative measures as a priority, in which biological control is included (Mondello *et al.*, 2017; Gramaje *et al.*, 2018). Bacteria, oomycetes and fungi have been screened in order to find suitable candidates to be exploited in the biological control of esca-associated fungi. Among the tested bacteria, *Bacillus subtilis* strains have revealed promising antagonistic traits against GTD pathogens *in vitro* and *in vivo*, both in pruning wound protection and in nurseries (Alfonzo *et al.*, 2012; Haidar *et al.*, 2016; Haidar *et al.*, 2016; Rezgui *et al.*, 2016). On the side of oomycetes, the colonization of the root system by *Pythium oligandrum* triggered the plant defenses and reduced the extent of the wood symptomatology caused by *P. chlamydospora* (Yacoub *et al.*, 2016). Among fungi, tests have been conducted with *Aureobasidium* spp. (González *et al.*, 2012), *Chaetomium* spp. (Spagnolo *et al.*, 2012), *Fusarium lateritium* (Christen *et al.*, 2005), but the most studied species are those belonging to the genus *Trichoderma*, namely *T. atroviride*, *T. harzianum* and *T. longibrachiatum*, which have been used with encouraging results in pruning wound protection or throughout the different steps of grapevine propagation in nurseries (Di Marco *et al.*, 2004; Fourie and Halleen, 2004; Pertot *et al.*, 2016). Despite of these results, there are several other candidates that may reveal potential for biological control against esca-associated pathogens, some of which are assigned to the genera *Epicoccum*, *Cladosporium* and *Alternaria* (Pancher *et al.*, 2012; Bruez *et al.*, 2014).

Fungi belonging to the genus *Epicoccum* are ubiquitous ascomycetes (*Didymellaceae*) frequently isolated from healthy and diseased grapevine wood, being *Epicoccum nigrum* the most referred species (Hofstetter *et al.*, 2012; Pancher *et al.*, 2012; Bruez *et al.*, 2014). However, there are different references to the *Epicoccum* genus, not ascribed to a species, that have been reported from healthy grapevine cuttings (Halleen *et al.*, 2003), pruning wounds (Úrbez-Torres and Gubler, 2011) and mature grapevine plants (Kuntzmann *et al.*, 2010; Choueiri *et al.*, 2014). These fungi have been thought to be endophytes or saprophytes in grapevine wood but their role on healthy or diseased wood, where they can co-inhabit with different GTDs pathogens, has not been investigated.

Several studies proved the antagonistic effect of *E. nigrum* against plant pathogenic agents, and it currently finds application as a biological control agent (BCA) in different

crops (Larena *et al.*, 2005; Koutb and Ali, 2010; Fávaro *et al.*, 2012; El-Gremi *et al.*, 2017; Kosawang *et al.*, 2017). The antagonistic behavior of *E. nigrum* is mainly attributed to the release of secondary metabolites, some of which have antifungal (e.g flavipin, epicorazine and epirodins) and anti-bacterial (e.g. beauvericin) activity (Browne *et al.*, 1987; Dzoyem *et al.*, 2017). *Epicoccum nigrum* is known as a genotypically and phenotypically highly variable species (Arenal *et al.*, 2002) and it has been hypothesized that *E. nigrum* could encompass different species (Fávaro *et al.*, 2011). Recent developments on the taxonomy of *Didymellaceae* fungi are contributing to clarify boundaries between genera in this family, as well as delimiting new species or combinations within the *Epicoccum* genus (Aveskamp *et al.*, 2010; Chen *et al.*, 2017; Jayasiri, 2017; Valenzuela-Lopez *et al.*, 2018). Some of these new species are represented by a restricted number of strains closely related to *E. nigrum* (Chen *et al.*, 2017).

The presence of *Epicoccum* fungi in grapevines is well known, nevertheless, information regarding their interactions with the plants, their role in the microbial ecology of the wood and the interaction with GTDs pathogens is scarce. Moreover, the potential of these fungi to be exploited as BCAs against esca-associated fungi has not yet been assessed. Therefore, the aims of this study are: (i) to identify *Epicoccum* spp. currently isolated from grapevine wood in Portugal, and (ii) to gain an understanding of the type of interaction that occurs among members of the genus *Epicoccum* and esca-associated pathogens both *in vitro* and *in vivo.*

2.2 Materials and methods

2.2.1 *Epicoccum* spp. isolation

Isolates (E17, E20-E24, E27 and E28) were obtained from grapevine woody tissue sampled from canes in a vineyard, cv. Touriga Nacional, located in the Alentejo region (district of Beja), while isolate (E33) was obtained from symptomatic wood in a vineyard, cv. Touriga Nacional, located in the Lisbon region (Table 2.1). Wood chips, 2 mm thick, were surface disinfected by immersion in a NaClO solution (0.05% w/w active chlorine) for 1 min, followed by double-rinsing in sterile distilled water (SDW)

and plated on potato dextrose agar (PDA, BD-Difco Laboratories, Detroit, MI, USA) supplemented with 250 mg l^{-1} chloramphenicol (BioChemica, AppiChem, Germany). Petri dishes were incubated at 25 °C in the dark and regularly checked for the development of *Epicoccum*-like fungi, based on the cultural characteristics of colonies. The isolates were single-spored or purified by cutting off the tip of a hypha, and maintained on slants of PDA, at 5 °C, until use.

2.2.2 *Epicoccum* spp. identification

The total genomic DNA of each isolate was extracted from mycelium of 10 d old cultures grown in PDA, according to Nascimento *et al*. (2001). The internal transcribed spacer regions 1 and 2 and the intervening 5.8S nrDNA region (ITS) were amplified by using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990), the RNA polymerase II second largest subunit (*rpb2*) gene by using the primer pair RPB2-5F2 (Sung *et al.*, 2007) and fRPB2-7cR (Liu *et al.*, 1999) and the partial gene of β-tubulin (*tub2*) by the primers T1 and T2 (O'Donnell and Cigelnik, 1997), respectively. PCR amplifications were performed using 1× PCR buffer with 2 mM $MgCl_2$ (Thermo Scientific, Lithuania), 48 µM of each dNTP, 0.32 µM of each primer, 0.5 units Taq DNA Polymerase (Dream Taq, Thermo Scientific, Lithuania) and 10-15 ng of gDNA in a final volume of 20 µL. The cycle conditions in a iCycler thermocycler (Bio-Rad, USA) were 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 52 °C (for ITS) or 58 °C (for *tub2*) for 30 s, and 72 °C for 1:40 min, and a final elongation at 72 °C for 10 min. For the *rpb2* gene the amplifications followed the protocol of Woudenberg *et al*. (2013). Sequencing was performed by StabVida (Portugal). Sequences generated in this study were deposited in GenBank (accession numbers MH643917 to MH643946), the alignments and trees in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S23108).

Sequences of related *Epicoccum* species were retrieved from GenBank and are listed in Table 2.1. Alignments were performed with MAFFT version 7 ((Katoh *et al.*, 2017); https://mafft.cbrc.jp/alignment/server/) using the L-INS-i method and manually adjusted, if necessary, in MEGA7 (Kumar *et al.*, 2016). Maximum likelihood (ML) and Bayesian analyses were conducted for each locus and on a three-locus dataset combined using the program SequenceMatrix 1.8 (Vaidya *et al.*, 2011). ML was

implemented in the CIPRES Science Gateway V 3.3 (Miller *et al.*, 2010) using RAxML-HPC v.8 on XSEDE (8.2.9) using the GTRCAT model and 1000 rapid bootstrap inferences. Prior to Bayesian inference, the best nucleotide substitution models for each locus according to the Akaike Information Criterion were calculated in jModelTest 2.1.10 (Darriba *et al.*, 2012). According to this software, a Hasegawa-Kishino-Yano model with proportion of invariable sites (HKY+I) was suggested for ITS dataset, a General Time-Reversible model with gamma-distributed rate (GTR+G) for *tub2* and a GTR+G+I model for *rpb2*. The Bayesian analyses were performed in MrBayes v. 3.2.6 (Ronquist *et al.*, 2012). The Markov Chain Monte Carlo sampling was set to 10 million generations, with two independent runs with four chains, one cold chain and three heated chains with a temperature value of 0.2. The tree samples of the two cold chains were compared every 1,000 generations and stopped when the average standard deviation of split frequencies fall below 0.01. Burn-in was set at 25 % after which the likelihood values were stationary and the remaining trees were used to calculate posterior probabilities. Trees from different runs were then combined and summarized in a 50% majority-rule consensus tree. *Didymella exigua* (CBS 183.55) was selected as outgroup.

2.2.3 *In vitro* interactions among *Epicoccum* spp. and esca-associated fungi

Epicoccum spp. isolates were tested in dual culture against three esca associated-fungi, *P. chlamydospora* CBS 161.90 and *P. minimum* CBS 110713 from the CBS culture collection (Westerdijk Fungal Biodiversity Institute, Netherlands) and a local strain of *F. mediterranea*. All possible combinations of pathogen – *Epicoccum* spp. were tested, as well as each fungus alone or against itself. All experiments were done in 90 mm diam. Petri dishes containing 15 mL of PDA in four biological replicates. Mycelial plugs of 4 mm diam. were cut, with a cork borer, from the actively growing margin of each fungal

Table 2.1. Isolates of *Epicoccum layuense* and related species used for phylogenetic analyses. Ex-type isolates are shown in bold type.

Species	Isolate [1]	Host, substrate	Country	GenBank accession numbers		
				ITS	*TUB*	*RPB2*
Didymella exigua	**CBS 183.55**	*Rumex arifolius*	France	GU237794	GU237525	EU874850
Epicoccum cedri	**MFLUCC 17-1058**; KUMCC 17-0140	*Cedrus deodara* (dead land branch)	Italy	KY711170	KY711168	–
E. dendrobii	**CGMCC 3.18359**	*Dendrobium fimbriatum*	China	KY742093	KY742335	–
	LC 8146	*Dendrobium fimbriatum*	China	KY74209	KY742336	–
E. italicum	**CGMCC 3.18361**	*Acca sellowiana*	Italy	KY742099	KY742341	KY742172
	LC 8151	*Acca sellowiana*	Italy	KY742100	KY742342	KY742173
E. layuense	**CGMCC 3.18362**	*Perilla* sp.	China	KY742107	KY742349	–
	LC 8156	*Perilla* sp.	China	KY742108	KY742350	–
	E 20	*Vitis vinifera*	Portugal	MH643918	MH643928	MH643939
	E 21	*Vitis vinifera*	Portugal	MH643919	MH643929	MH643940
	E 22	*Vitis vinifera*	Portugal	MH643920	MH643930	MH643941
	E 23	*Vitis vinifera*	Portugal	MH643921	MH643931	MH643942
	E 24	*Vitis vinifera*	Portugal	MH643922	MH643932	MH643943
	E 27	*Vitis vinifera*	Portugal	MH643923	MH643933	MH643944
	E 28	*Vitis vinifera*	Portugal	MH643924	MH643934	–
	E 33	*Vitis vinifera*	Portugal	MH643925	MH643935	MH643945
E. mackenziei	MFLUCC 16-0335; KUMCC 16-0071	*Ononis spinosa* (dead aerial stem)	Italy	KX698039	KX698032	KX698035
E. nigrum	**CBS 173.73**	*Dactylis glomerata*	USA	FJ426996	FJ427107	KT389632
	CBS 125.82	Human toe nail	The Netherlands	FJ426995	FJ427106	KT389631
	LC 8158	*Poa annua*	USA	KY742111	KY742180	KY742353
	LC 5180	*Lonicera japonica*	China	KY742109	KY742178	KY742351
E. nigrum (ex-type of ***E. mezzettii***)	**CBS 173.38**	*Populus* (pulp of wood, in paper factory)	Italy	MH643926	MH643936	MH643946
	E17	*Vitis vinifera*	Portugal	MH643917	MH643927	MH643938
E. poae	LC 8161	*Poa annua*	USA	KY742114	KY742356	KY742183
	CGMCC 3.18363	*Poa annua*	USA	KY742113	KY742355	KY742182
E. pimprinum	**CBS 246.60**; ATCC 22237; ATCC 16652; IMI 81601	Soil	India	FJ427049	—	FJ427159

[1)] ATCC: American Type Culture Collection, Virginia, U.S.A; CBS: Westerdijk Fungal Biodiversity Institute (formerly CBS-KNAW), Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; LC: Lei Cai personal collection deposited in laboratory, housed at Chinese Academy of Sciences, Beijing, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; KUMCC: Culture collection of Kunming Institute of Botany, Chinese Academy of Sciences, Beijing, China.

colony and placed, facedown, 40 mm apart. Dishes were incubated at 25 °C, in the dark, for 14 d, and photographed every 2 or 3 d. The size of all colonies was measured from the images generated, using Adobe Photoshop Creative Cloud (2015). The percent growth inhibition (PGI) of pathogens was calculated using the formula: PGI (%) = 100 × ((control - treatment)/control), in which the ‘control’ represents the area of each fungal colony growing against itself and ‘treatment’ the area of each colony growing against *Epicoccum* spp.

For microscope observations, a square of agar was removed from the interaction zone of the dual cultures (*E. layuense* isolate E24 and pathogen) and mounted on a microscope slide on a drop of 85% lactic acid (w/w) and overlaid with a cover slip. Images were captured by using a differential interference contrast microscope (Leica DM2500, Germany) equipped with a Leica DCF295 camera. Measurements of conidia were made at 1000x magnification with the Leica Application Suite (LAS) version 3.3.0 software and round to the nearest 0.5 µm. Thirty conidia of *P. chlamydospora* and *P. minimum* were measured and the means, at 95% confidence intervals, were calculated. The spore dimensions are presented as mean values with extreme values in parentheses.

2.2.4 In vivo interactions among E. layuense and esca-tracheomycotic fungi

The experiments conducted *in vivo* focused on one isolate of *E*. *layuense*, namely ‘E24’, selected from the *in vitro* assays as best performing antagonist. The experiments were conducted in grapevine potted plants as follows. In a first experiment (year 2016), the isolate was tested for its pathogenicity in the wood of grapevines. One year-old canes of cv. Touriga Nacional were collected in a vineyard in the Azeitão region (Portugal), in December 2015, and left in a cold-room (4 °C) for two months. Canes were divided into 3-buds-long cuttings, rooted in a warm bench, at 24 °C, and potted in a mixture of peat and sand (1:1 v/v). Before inoculation, cuttings were surface disinfected with 70% ethanol. Discs of mycelium (4 mm diameter) of the isolate E24, were cut with a cork borer from the actively growing edge of a seven-days-old culture growing in PDA, and inoculated in the rooted grapevine cuttings. Mycelium discs were inserted in a wound (4 mm diameter, 4 mm deep) made in the wood with a cork borer, 40 mm below the top shoot (Figure 2.1A). A small piece of sterile cotton, imbibed in sterile distilled

water (SDW) was placed on top of the mycelium plug and tightened to the plant with Parafilm®. Mock-treated plants were inoculated with a sterile plug of PDA. Each treatment consisted of 10 biological replicates.

In a second experiment (year 2017), 3-buds-long cuttings of cvs. Touriga Nacional and Cabernet Sauvignon, rooted and potted in a mixture of peat and sand (1:1 v/v), were provided by VitiOeste nursery (Pó, Bombarral, Portugal). The following fungal combinations were tested: (i) E24 alone, (ii) *P. chlamydospora* CBS 161.90 and (iii) *P. minimum* CBS 110713 alone, (iv, v) combination E24 – each pathogen, and (vi) mock-control; with each combination consisting of 10 biological replicates.

All inoculations with the isolate E24, as well as the mock-control, were carried out as described in the first experiment (mycelial discs). The inoculation of the pathogens occurred one month later, a 50 µl aliquot of a suspension containing approximately 2000 conidia was deposited in a fresh wound (4 mm diameter, 4 mm deep; Figure 2.1B) and covered with Parafilm®. Mock-treated controls were inoculated with 50 µl of SDW. The conidial suspension was prepared by flooding 14 days-old PDA cultures of each pathogen with SDW, and the conidia were dislodged from the mycelium with a sterile glass rod. The suspension was filtered through a double layer of cheesecloth, the conidial concentration was determined using a hemocytometer and adjusted to 1 $\times 10^5$ conidia/mL with SDW.

After inoculation, plants were randomly placed in a greenhouse equipped with fan and pad evaporative system (24±5 °C day/18±5 °C night) and watered three times a week, or when needed. Fortnightly treatments with meptyldinocap (35.7% p/p) or sulfur wettable powder (80% p/p) were carried out to prevent powdery mildew (*Erysiphe necator*) infections.

Three months after the pathogens inoculation, plants were visually inspected for the appearance of foliar symptoms and the length of the shoots was recorded. Afterwards, vines were uprooted, their bark removed and longitudinal sections were made in order to assess the presence and extent of symptoms in the wood (brown streaking), departing from the inoculation points.

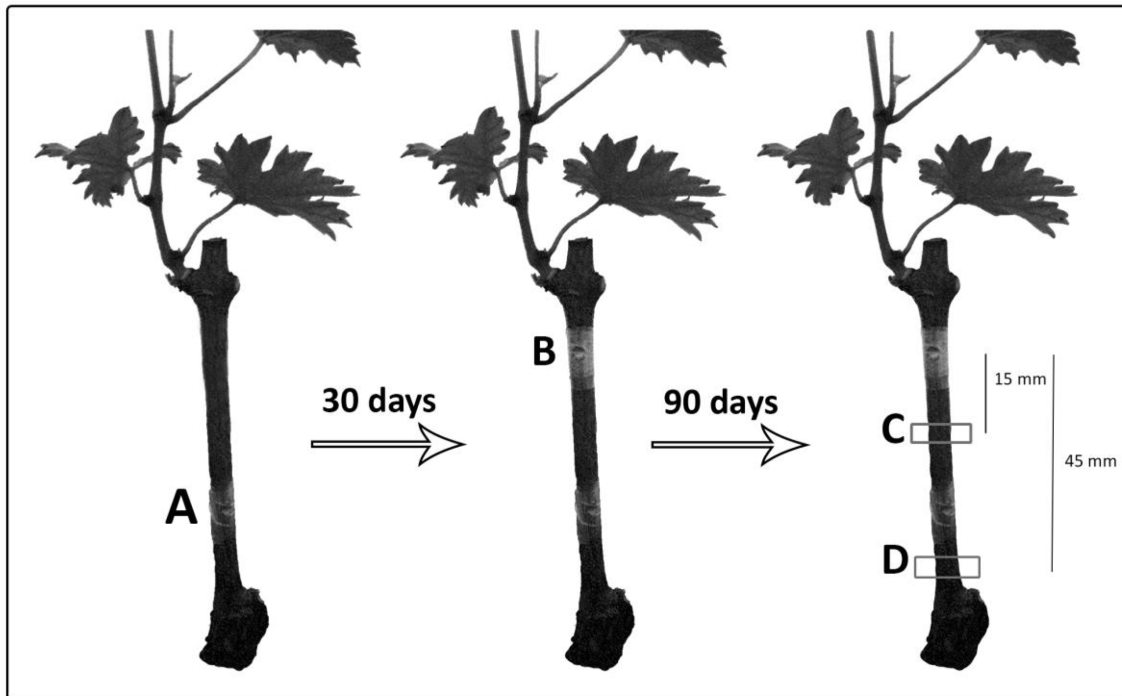

Figure 2.1. Schematic representation of the inoculation and re-isolation areas in grapevine rooted cuttings. (A) E24 inoculation via mycelium plug; (B) pathogen inoculation via spore suspension; (C) re-isolation area 15 mm below the pathogen inoculation; (D) re-isolation area 45 mm below the pathogen inoculation.

Pieces of wood (approx. 4 mm^3) were cut transversally 30 mm above the area A for the first experiment, and areas C and D (Figure 2.1) for the second, to undergo re-isolation. The re-isolation of the pathogens occurred as follows. Four pieces of wood were taken from each level (C and D) of each plant, they were surface disinfected by flame, followed by immersion for 1 min in a NaClO solution (0.35% w/w as active chlorine), double rinsed in SDW (1 min each), dried with sterile filter paper and plated on PDA amended with 250 mg l^{-1} chloramphenicol. Petri dishes were incubated at 25 °C, in the dark, for 21 d. The re-isolation of E24 was achieved as described for its isolation. The percentage of re-isolation was calculated as the proportion of wood pieces from which fungal colonies were recovered over the total number of pieces of wood plated for each plant.

2.2.5 Data analysis

All data were subjected to analysis of variance (ANOVA), and statistically significant means were compared using the Tukey *post hoc* test at a 5% significance level (STATISTICA 8.0). Before analysis, arcsine-square root transformation was performed for data expressed as percentage. For each experiment, the following analyses were carried out: (i) one-way ANOVA model to evaluate the *in vitro* effect of *Epicoccum* spp. on esca-associated fungi ; (ii) two-way ANOVA model to assess the effects of the grapevine cultivar, fungal inoculation (E24, *P. minimum* and *P. chlamydospora*), and their interaction, upon the shoot length and brown wood streaking length; (iii) three-way ANOVA model to assess fungal re-isolation in which, in addition to the grapevine cultivar and fungal inoculation variables, two regions of fungal re-isolation were considered (Figures 2.1C and 2.1D), as well as their interactions. Data recorded from mock-treated plants were used to estimate any infection of rooted grapevine cuttings before inoculation and, therefore, they were not included in the statistical analyses.

2.3 Results

2.3.1 *Epicoccum* spp. identification

The three locus alignment contains 25 ingroup isolates and one outgroup *Didymella exigua* (CBS 183.55). A total of 1422 characters were considered, including alignment gaps (490 for ITS, 596 for *rpb2* and 336 for *tub2*), from which 1154 were conserved and 154 were parsimony-informative (12 for ITS, 111 for *rpb2* and 31 for *tub2*). The tree topologies obtained by ML and Bayesian analysis of individual loci and for the combined alignments were essentially congruent, therefore, only the ML tree is shown, with bootstrap support values (MLBS) and Bayesian posterior probabilities (BPP) indicated at the nodes (Figure 2.2). The ITS loci were the least informative resolving only four out of nine taxa present in the dataset, whereas the other two loci, *rpb2* and *tub2*, were able to resolve all the taxa.

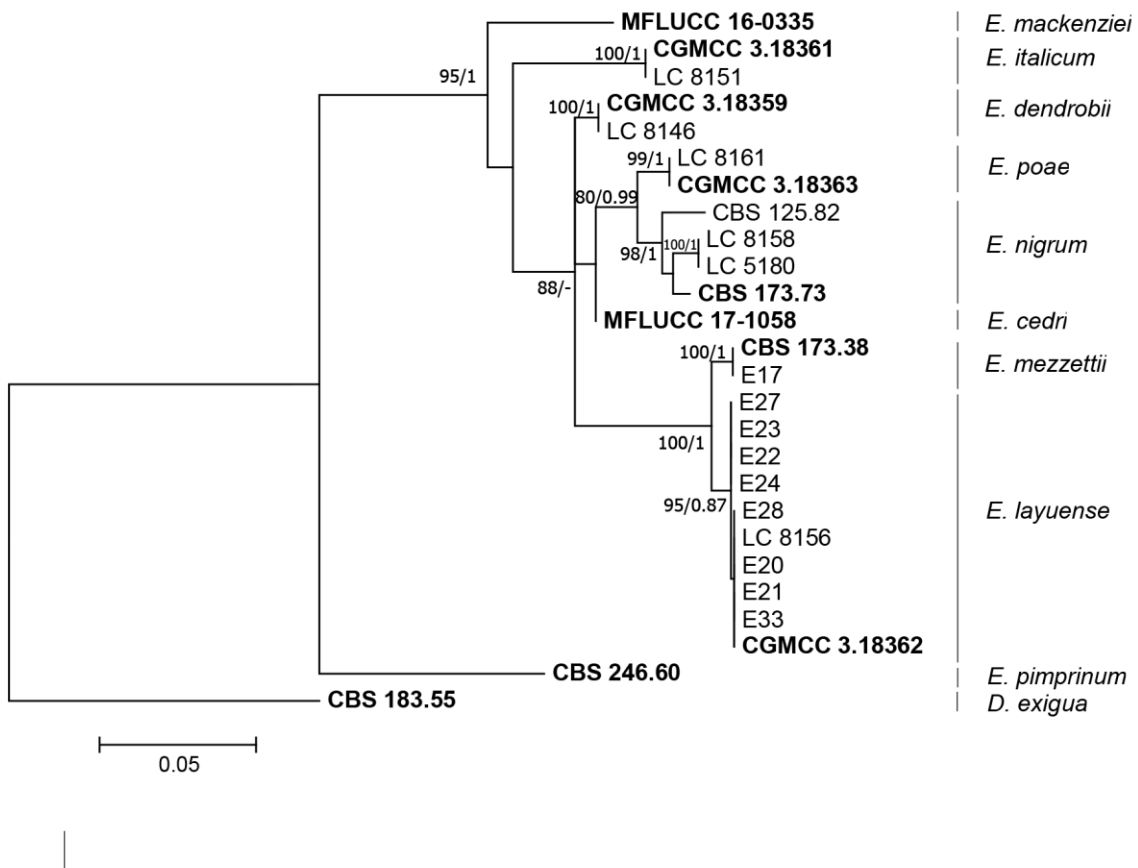

Figure 2.2. Phylogenetic tree inferred from a Maximum likelihood analysis based on a concatenated alignment of ITS, *rpb2* and *tub2* sequences of 25 isolates of *Epicoccum* and one isolate of *Didymella*. The RAxML bootstrap support values (MLBS) and Bayesian posterior probabilities (BPP) are given at the nodes (BPP/MLBS). The tree was rooted to *Didymella exigua (*CBS 183.55). Ex-type cultures are emphasised in bold type. The scale bar indicates 0.1 expected changes per site.

Among the nine isolates of *Epicoccum* obtained from grapevine, eight of them (E20-E24, E27, E28 and E30) cluster within the *E. layuense* clade, and one (E17) clusters with the ex-type strain of *E. mezzettii* (CBS 173.38), whose current name is *E. nigrum*. From

here on, the isolate E17 will be referred as *E. mezzettii*, in order to distinguish it from the others belonging to *E. nigrum* species.

2.3.2 *In vitro* interactions among *Epicoccum* spp. and esca-associated fungi

Epicoccum mezzettii (E17) and all the strains of *E. layuense* inhibited the growth of *P. chlamydospora* and *F. mediterranea,* after 14 d of growth on PDA medium, by the dual-culture method, while only some strains of *E. layuense* (E22-E24 and E33) were able to inhibit *P. minimum* (Figure 2.3).

The growth behavior of the confronting fungi varied depending on the different fungal combinations. *Epicoccum mezzettii* (E17) was the only fungus capable of clearly overgrowing all three pathogens (Figure 2.4 (ii)), while the remaining isolates (E20-E24, E27, E28 and E33), all assigned to *E. layuense,* were able to inhibit the pathogens without a physical colony contact, when observed by the naked eye.

The interaction *Epicoccum — P. chlamydospora* was characterized by a great reduction of the colony size of the pathogen, when compared to both single and dual culture controls (Figure 2.3A). The average reduction was of 71.0%, with the best performer being E24 that reduced the growth of *P. chlamydospora* by 79.9% (Table 2.2), as exemplified in Figure 2.4 (a, d and g).

Microscopic observations of the interaction between *E. layuense* (E24) and *P. chlamydospora* (CBS 161.90) revealed that the growth of *P. chlamydospora* ceases before the contact with the antagonist. When the hyphae of *E. layuense* are within 3-4 mm distance of *P. chlamydospora* hyphae, the growth slackens and the hyphae become sparser, growing below the agar surface without visible aerial mycelium (Figure 2.5A). The *E. layuense* isolate extends its growth towards *P. chlamydospora*, surrounding it, with an intermingling of the hyphae but without overlapping the pathogen (Figures 2.4a and 2.5B). The *P. chlamydospora* isolate reacts to the presence of the antagonist increasing the conidia production along the contact line and the hyphae differentiate swollen cells that remind chlamydospores (Figures 2.5C and 2.5D).

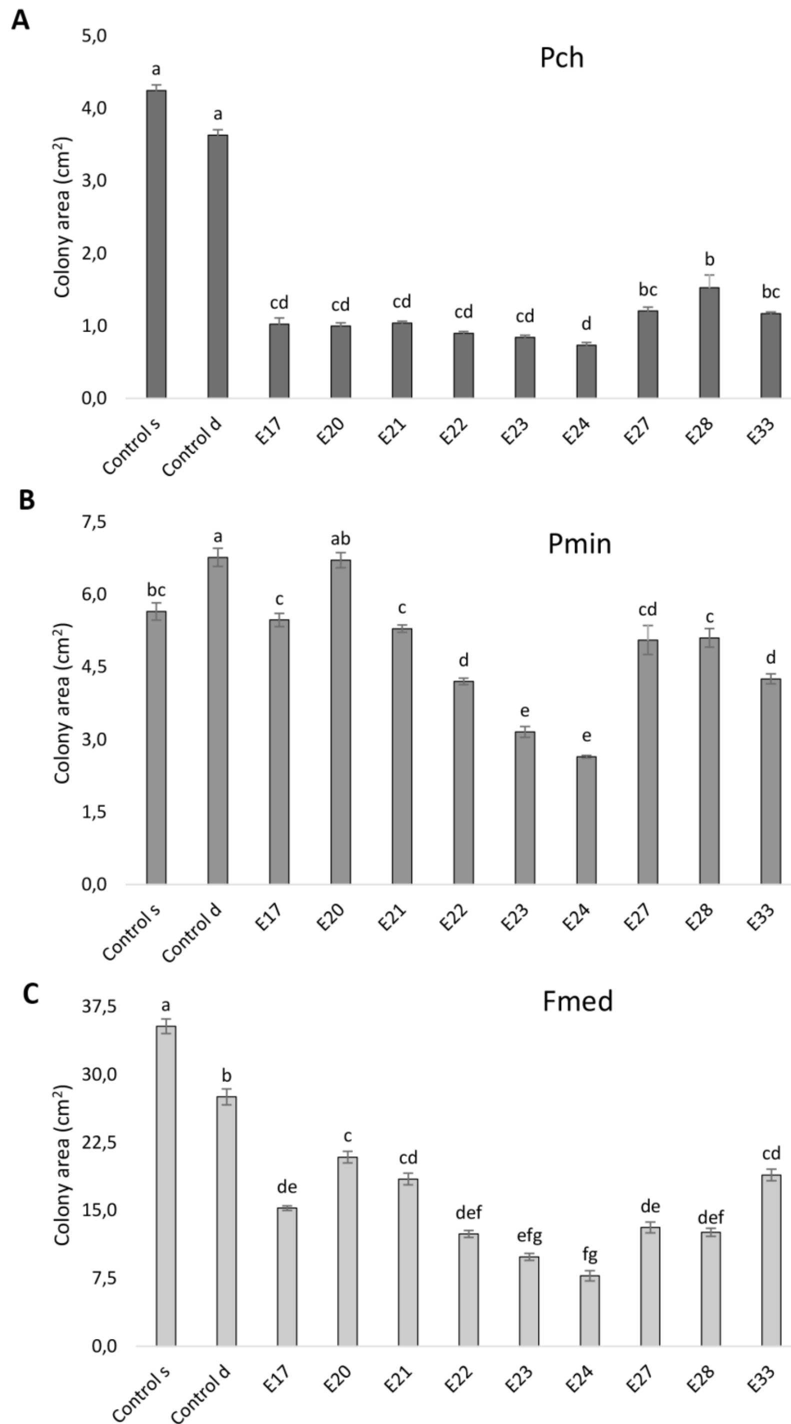

Figure 2.3. *In vitro* inhibition of esca-related fungi by *Epicoccum* spp. isolates. Growth was measured 14 days post-inoculation on PDA medium in dual-culture with (A) Pch, *Phaeomoniella chlamydspora*; (B) Pmin, *Phaeoacremonium minimum* and (C) Fmed, *Fomitiporia mediterranea*. 'Control s' and 'Control d' indicate the colony area of the pathogen in single culture or dual culture, respectively, and 'E' indicates the *Epicoccum* isolate. Error bars represent the standard deviation from the mean; different letters indicate statistically significant differences (Tukey *post hoc* test, p ≤ 0.05).

No differences were observed between the conidia dimensions of *P. chlamydospora* withdrawn from the interaction zone and the conidia of this pathogen growing alone.
All antagonists but one (E20) significantly inhibited the growth of *P. minimum* when compared to the dual culture control (Figure 2.3B). The single culture control grew significantly less than the dual, which suggests a possible growth-promoting interaction between *P. minimum* colonies. Also in this case, the interaction between E24 and *P. minimum* caused the greatest reduction in the colony size of the pathogen, amounting for a 61.0% decrease (Table 2.2; Figure 2.3B). Microscopic details of the interaction *E. layuense* (E24) and *P. minimum* (CBS 110713) reveal that both fungi slacken their growth when they approach each other. The pathogen, sensing the presence of *E. layuense*, alters the growth direction of their hyphae, in an attempt to avoid contact (Figure 2.5H), although at a later stage the intermingling and overlapping of hyphae of both fungi become visible (Figures 2.5G and 2.5I). The conidia of *P. minimum* withdrawn from the interaction zone are larger and narrower ((3.5–)4.5–5.0–5.5(–6.5) × (1.0–)1.0–1.3–1.5(–1.5) µm) than those collected from the single culture of *P. minimum* ((3.0–)3.5–4.1–4.5(–5.5)×(1.0)–1.5–1.5–1.5(–2.0) µm), having a greater length-width ratio ((3.0–)3.5–4.1–4.5(–6.0)) when compared to the control ((1.5–)2.5–2.7–3.0(–3.5)).
Regarding *F. mediterranea, E. layuense* (E24) proved to be one of the most efficient inhibitor, inducing a colony size reduction of 71.8% (Table 2.2; Figure 2.3C). All other *Epicoccum* isolates were effective in inhibiting the mycelial growth of this pathogen, although there were significant differences among them (Figure 2.3C). Microscopic details of the confronting zone between *E. layuense* (E24) and *F. mediterranea* reveal that after six days of incubation the hyphae of both fungi come into contact and the pathogen responds by entangling their hyphae, forming hyphal strands. In addition, degradation of the hyphal tips and formation of clamp connections are observed. On the bottom of the dual culture it is visible a brown streak along the line of contact of fungi corresponding to a multiplication of branches piles up the hyphae to prevent the contact with the antagonist (Figure 2.5M-Q).

Table 2.2. Percent growth inhibition of esca-associated fungi by *Epicoccum* spp.

		PGI (%)[1]		
***Epicoccum* spp.**	**Isolate**	***P. chlamydospora***	***P. minimum***	***F. mediterranea***
E. mezzettii	E17	71.8	19.1	44.6
E. layuense	E20	72.6	0.9	24.2
	E21	71.4	21.8	32.9
	E22	75.3	37.9	55.0
	E23	76.9	53.4	64.2
	E24	79.9	61.0	71.8
	E27	66.8	25.3	52.4
	E28	58.0	24.6	54.3
	E33	67.8	37.2	31.4

[1]Calculated using the formula: PGI (%) = 100 × ((control - treatment)/control) in which the control represents the area of each fungal colony growing against itself and treatment the area of each colony growing against *Epicoccum* spp.

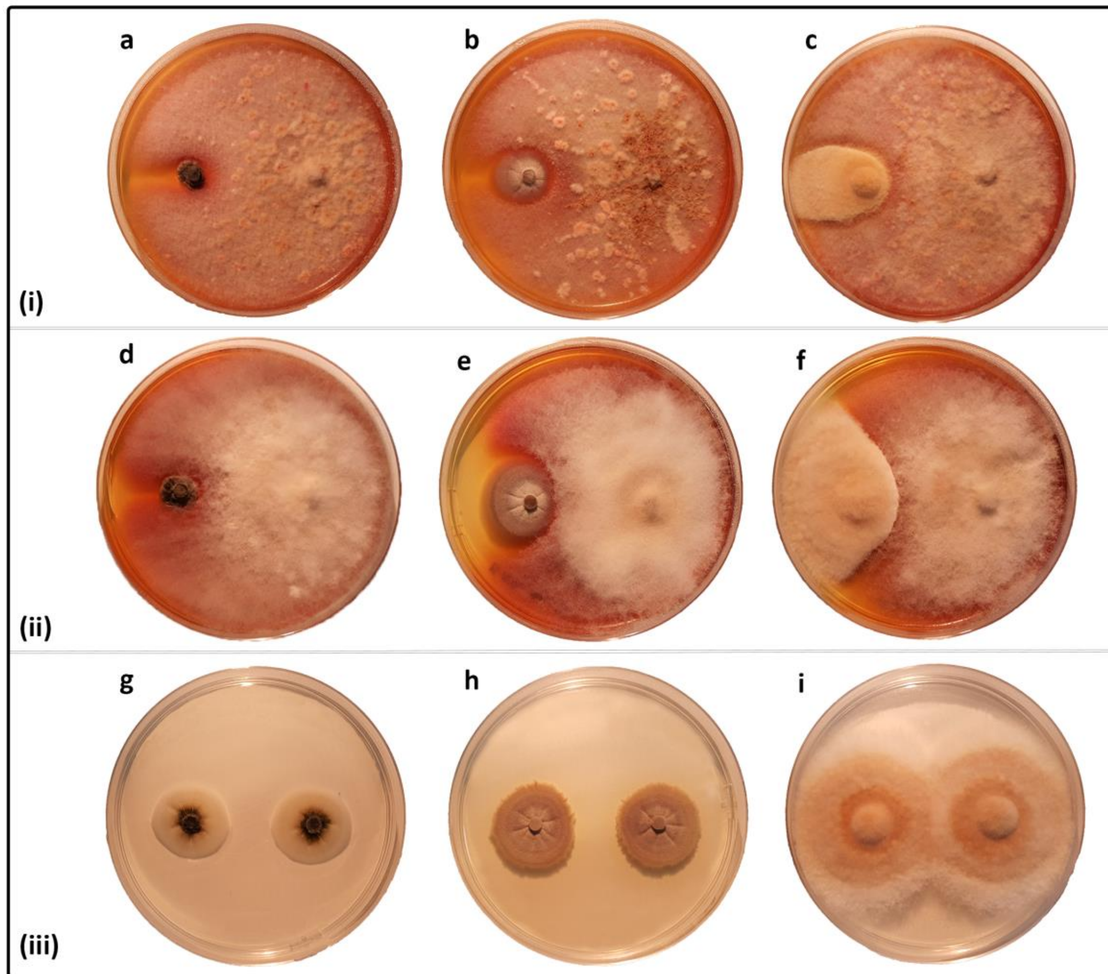

Figure 2.4. Interaction pathogen – antagonist or pathogen — pathogen after 14 d of incubation on PDA. (i) *Phaeomoniella chlamydospora* (a), *Phaeoacremonium minimum* (b) and *Fomitiporia mediterranea* (c) inoculated on the left side, against *Epicoccum layuense* strain E24 inoculated on the right; (ii) the same pathogens against *E. mezzettii* strain E17, (iii) dual cultures of each pathogen.

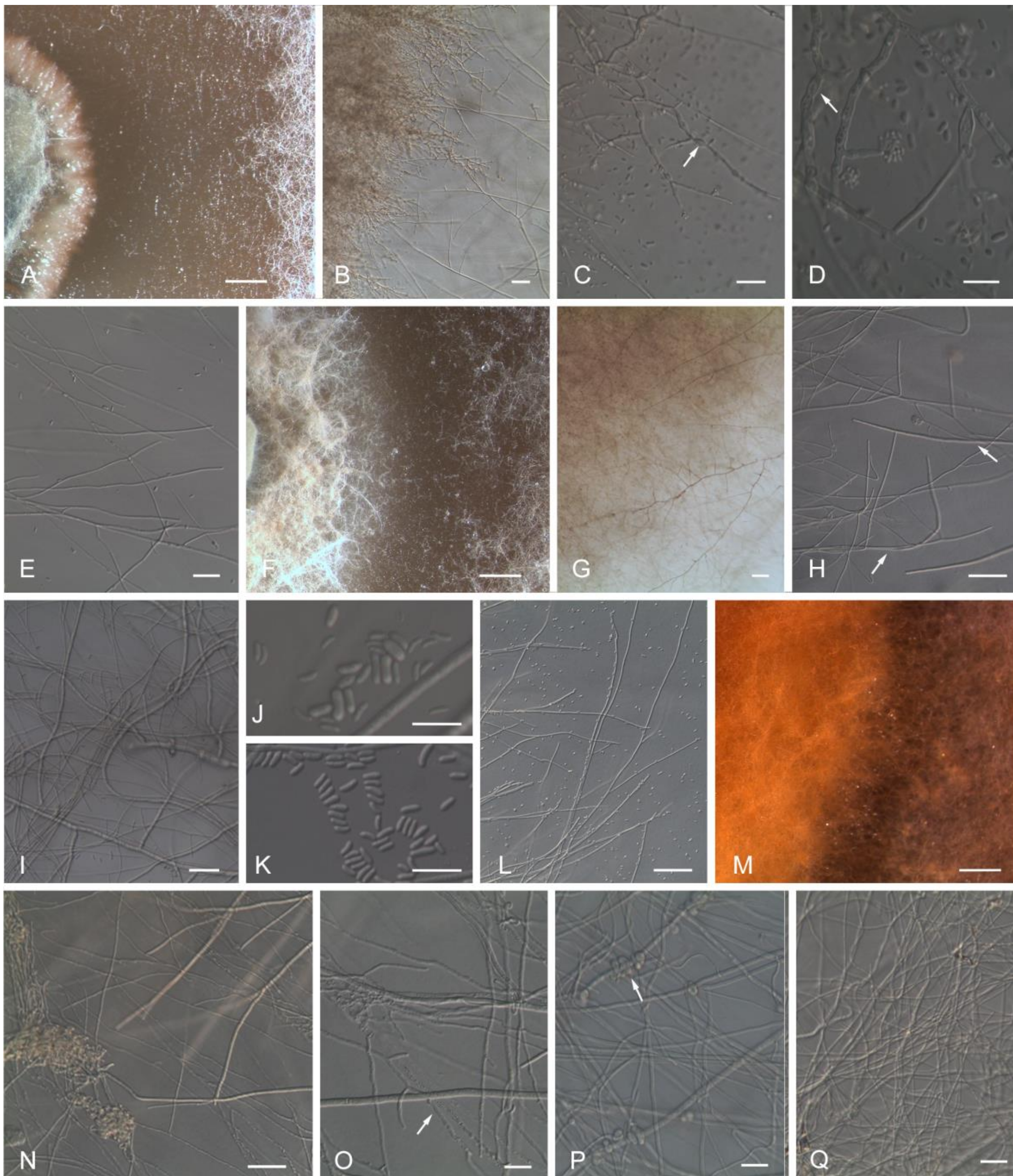

Figure 2.5. Morphological changes of vegetative structures of esca-associated fungi upon interaction with *Epicoccum layuense* isolate E24 in dual culture plates. *Phaeomoniella chlamydospora* isolate CBS 161.90 (left side) and *E. layuense* (right side) at interaction area at day eight (A); hyphae of *P. chlamydospora* with swollen chlamydospores (arrow) and an increasing of sporulation in the contact line with *E. layuense* (B-D) when compared to *P. chlamydospora* growing alone (E) at day ten. *Phaeoacremonium minimum* isolate CBS 110713 (left side) and *E. layuense* (right side) at the interaction zone at day eight (F); intermingled hyphae of both fungi at day ten (G) after the hyphae of *P. minimum* have tried to change their growth direction (left

Figure 2.5 (*continues*). arrow) to avoid the contact with the antagonist (right arrow) at day six (H); agglomerates of *P. minimum* hyphae intermingled with the antagonist hyphae (I), conidia of *P. minimum* from the interaction zone (J) when compared to conidia from the *P. minimum* single culture (K), and hyphae of *P. minimum* growing alone (L), all at day ten. *Fomitiporia mediterranea* (left side) and *E. layuense* (right side) at the interaction zone at day eight (M); agglomerates and strands of *F. mediterranea* hyphae in an attempt to block the advance of *E. layuense* (N); hyphae of *F. mediterranea* denoting plasmolysis (arrow) (O) and clamp connections (arrow) (P); mycelium of *F. mediterranea* growing alone (Q). Scale bars represent A, F, M = 1 mm; B, G, H, L, N =50 µm; C, E, I, O-Q = 20 µm; D, J, K = 10 µm.

3.3.3 *In vivo* interactions between *E. layuense* and esca-tracheomycotic fungi

The first experiment was carried out to infer whether E24 isolate could induce symptoms of a wood disease, in grapevine potted plants of cv. Touriga Nacional. This investigation revealed that, after four months of incubation, inoculated plants did not present any foliar symptoms, such as chlorosis, spotting or tiger stripes pattern, and the shoot length of inoculated plants was not significantly different from the control (Figure 2.6A). The examination of the stem for the presence of wood symptoms, in longitudinal sections, revealed no relevant brown-wood streaking, when compared to the mock-treated plants. The discoloration observed in woody tissue was short in length and related to the wound produced in the inoculation point, being present both in inoculated and mock-treated vines (Figure 2.6B). By the end of the assay, the E24 isolate was consistently yielded from inoculated plants, by means of *in vitro* re-isolation, and never from the control, thus indicating its potential for the inner colonization of grapevine.

The results from the first experiment, revealing that *E. layuense* E24 was unable to produce wood symptoms and affect the grapevine growth, led to a second experiment, performed in 2017, in which two grapevine cultivars were evaluated (Touriga Nacional and Cabernet Sauvignon). In this case, the *in vitro* re-isolations of the inoculated fungi aimed to assess the presence of both pathogens and antagonist in all

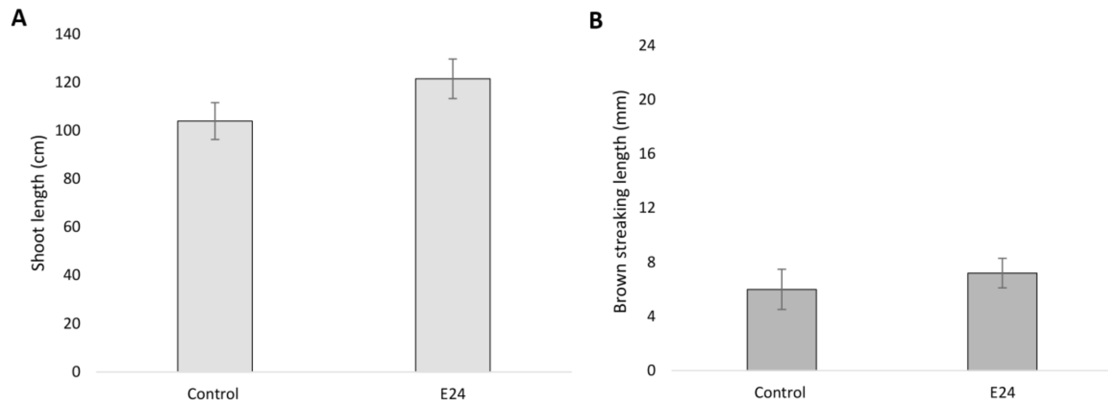

Figure 2.6. Effects of *Epicoccum layuense* E24 on grapevines. Measurements of the shoot length (A) and brown wood streaking length (B), in cv. Touriga Nacional, four months after inoculation. Error bars represent the standard deviation from the means. No significant differences in shoot length or brown streaking length were detected ($p > 0.05$).

combinations, and the re-isolations occurred both 15 mm and 45 mm below the point where the pathogens were inoculated (Figure 2.1).

Results revealed that the artificial inoculation of *E. layuense* (E24) did not produce external symptomatology on both grapevine cultivars, four months after inoculation. The pathogens, *P. chlamydospora* and *P. minimum*, whose inoculation was performed one month after that of the antagonist, were also unable to induce external symptomatology.

The growth of the green shoots, and therefore their final length, was not significantly influenced by the presence of either pathogens and/or antagonist, in both cultivars ($p > 0.05$; Table 2.3).

While the inoculation of E24, *P. minimum* or *P. chlamydospora* did not produce any external symptom on grapevines of both cultivars, the two pathogens induced the appearance of internal symptoms of brown wood streaking that differed significantly in length from those inoculated with E24 isolate and mock-treated plants (Figure 2.7). In both cultivars, the wood symptoms due to E24 inoculation alone were not significantly greater than the controls, which support results from the previous experiment (first year) on the behavior of *E. layuense* on grapevine wood. In Touriga

Nacional, the wood streaking significantly decreased in length due to the E24 — pathogen interaction, for both pathogens, when compared to pathogen-alone inoculation. The same behavior was recorded in Cabernet Sauvignon for both interactions, namely E24 x *P. minimum* and E24 x *P. chlamydospora*. In both cultivars, the inoculation of E24 reduced or completely prevented the negative effects of the pathogens, in regards to the development of brown wood streaks.

Table 2.3. Shoot length (cm) of grapevine potted plants inoculated with water (control) or with *Epicoccum layuense* strain E24, *Phaeoacremonium minimum* (Pmin), *Phaeomoniella chlamydospora* (Pch) and the combination of E24 with Pmin (E24 x Pmin) or Pch (E24 x Pch). Measurements were taken three months after the inoculation of the pathogens.

Grapevine cultivar	Water control	E24	Pmin	Pch	E24 x Pmin	E24 x Pch
Touriga Nacional	77.2 ± 12.1	61.4 ± 12.2	62.7 ± 13.6	75.0 ± 13.7	63.6 ± 16.8	60.3 ± 10.9
Cabernet Sauvignon	89.2 ± 12.2	81.4 ± 19.7	70.6 ± 13.6	79.1 ± 14.0	82.9 ± 18.6	71.7 ± 10.6

The presence of the inoculated fungi, calculated as the frequency of re-isolation, supported the insights gained by the brown wood streaking measurements. A more comprehensive understanding of the pathogen — antagonist interaction was obtained thanks to the two re-isolations areas examined, 15 mm and 45 mm below the pathogens' inoculation point (Figures 2.1C and 2.1D). The presence of *E. layuense* E24 was also assessed in the same areas as for the pathogens.

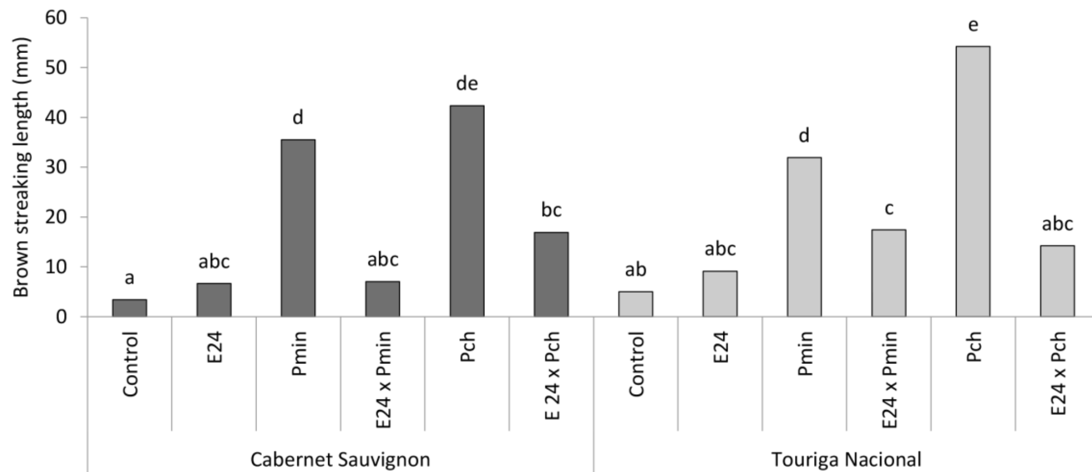

Figure 2.7. Brown wood streaking length in inoculated grapevine potted plants. Grapevines were inoculated with water (control) or with *Epicoccum layuense* strain E24, *Phaeoacremonium minimum* (Pmin) and *Phaeomoniella chlamydospora* (Pch) alone, or combined with E24 (E24 x Pmin and E24 x Pch). Bars followed by the same letter do not differ statistically according to the Tukey's test ($p > 0.05$).

The frequency of re-isolation of E24 in inoculated plants revealed a significant interaction among 'grapevine cultivar', 're-isolation area' and 'fungal inoculation' ($p < 0.05$), in which the 'grapevine cultivar' and the 'fungal inoculation' were responsible for the major effects ($p \leq 0.001$), while no meaningful differences emerged from the variable 're-isolation area'. The average frequency of re-isolation of E24 in control plants of Cabernet Sauvignon was 68.8% and a similar value was recorded in Touriga Nacional (58.8%), highlighting a successful colonization of the woody tissues in the areas near the inoculation points, thus revealing that E24 could colonize indifferently the wood tissues above and below its inoculation point.

Although some variation has been observed when *Epicoccum* E24 re-isolation means are compared, neither *P. minimum* nor *P. chlamydospora* have significant detrimental effect on the antagonist re-isolation (Figure 2.8).

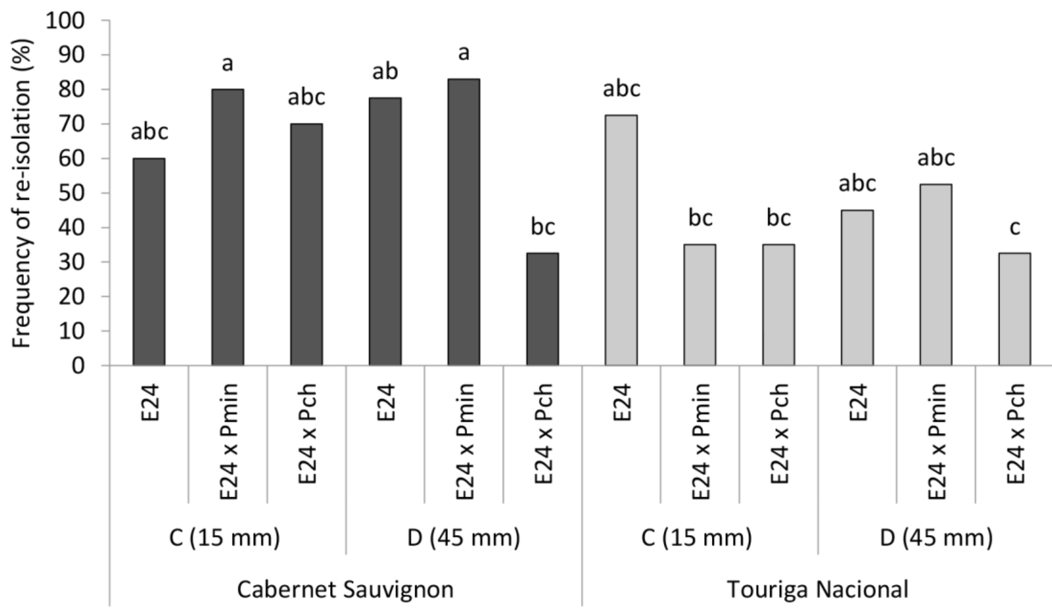

Figure 2.8. Frequency of *Epicoccum layuense* re-isolation from two grapevine cultivars and two areas of re-isolation (C 15 mm and D 45 mm). *E. layuense* was inoculated alone (E24) or in combination with *Phaeoacremonium minimum* (E24 x Pmin) or *Phaeomoniella chlamydospora* (E24 x Pch). Different letters on the top of the bars represent statistical differences according to the Tukey's test ($p \leq 0.05$).

The frequency of re-isolation of pathogens, when inoculated in absence or presence of *E. layuense* E24, is presented in Figure 2.9. The three-way ANOVA revealed a significant interaction among 'grapevine cultivar', 're-isolation area' and 'fungal inoculation' ($p = 0.0029$), with positive effects coming from the 're-isolation area' ($p = 0.0017$) and 'fungal inoculation' ($p < 0.001$).

By examining the 'area of re-isolation' C (15 mm), it is possible to infer that, for both pathogens and cultivars, the frequency of re-isolation of *P. minimum* and *P. chlamydospora* is lower in the presence of E24, although never significantly different from the positive controls (inoculated with the pathogen alone). In contrast, in 'area of re-isolation' D (45 mm) there is a strong decrease in the presence of both pathogens in both grapevine cultivars. The re-isolation of *P. minimum*, when co-inoculated with E24, was reduced by 83.3% in Cabernet Sauvignon and 95.2% in Touriga Nacional. A similar trend was registered for *P. chlamydospora*, where the decrease in the frequency of re-isolation was of 85.7% in Cabernet Sauvignon and 88.9% in Touriga Nacional.

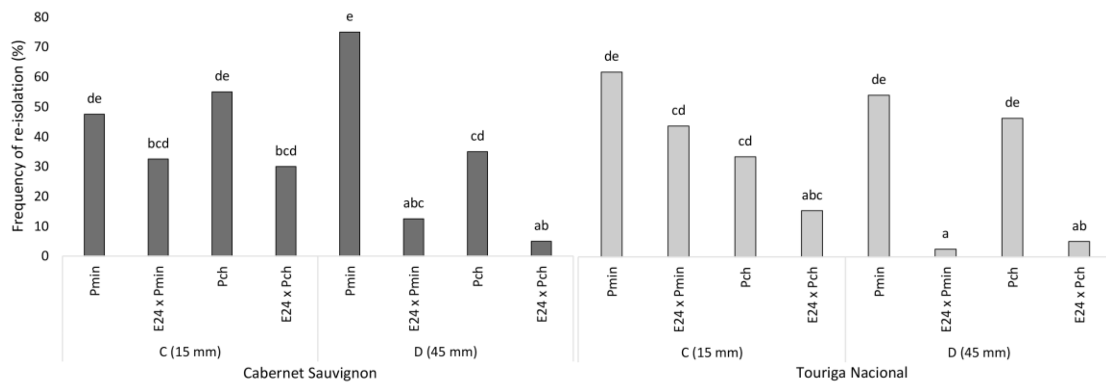

Figure 2.9. Frequency of re-isolation of inoculated pathogenic fungi. *Phaeoacremonium minimum* (Pmin) and *Phaeomoniella chlamydospora* (Pch) were either inoculated alone, or in combination with *Epicoccum layuense* E24 (E24 x Pmin, E24 x Pch). Re-isolations occurred at 15 mm (C) and 45 mm (D) below the pathogens' inoculation point in cultivars Cabernet Sauvignon and Touriga Nacional. Different letters indicate statistically significant differences after a Tukey's *post hoc* test ($p \leq 0.05$)

2.4 Discussion

2.4.1 *Epicoccum* spp. identification

In this study, a collection of *Epicoccum* spp. obtained from wood of grapevine plants in Portugal was identified morphologically and by gene sequencing (ITS, *rpb2* and *tub2*). Results revealed two *Epicoccum* species within the collection, *E. layuense* and *E. mezzettii*, being *E. layuense* the most represented. This species is phylogenetically closely related to *E. nigrum* and *E. poae* and it was recently reported from leaves of *Perilla* sp. in Tibet, China (Chen *et al.*, 2017). In turn, *E. mezzettii*, represented by the type strain CBS 173.38 and E17, although considered synonym of *E. nigrum* (Mycobank), proved to be a different species.

E. nigrum, apparently not represented in our collection, is the most reported species of the genus, in grapevine wood. However, *E. nigrum* was for long considered a single variable species, encompassing high diversity (Fávaro *et al.*, 2011), and it is possible that other closely related species (e.g. *E. layuense* or *E. mezzettii*) have been

misidentified as *E. nigrum*, before the taxonomic reassessment of the genera within *Didymellaceae* (Chen *et al.*, 2015, 2017).

2.4.2 *Epicoccum layuense* E24 and grapevines

Strains of *E. nigrum* are known as potential biological control agents (BCAs) in grapevine, against fungal aerial diseases, as well as in other pathosystems (Madrigal *et al.*, 1994; Koutb and Ali, 2010; Fávaro *et al.*, 2012; Li *et al.*, 2013), but their role as a grapevine wood endophyte, along with that of other closely related species, has not been addressed. This study reveals the inability of *E. layuense* E24 to impair the growth of grapevines and to induce leaf and wood symptoms on inoculated plants of cvs Touriga Nacional and Cabernet Sauvignon, within the timings of the experiments. These results support the current understanding that *Epicoccum* spp. are not associated with any grapevine disease. Long-term studies are necessary to confirm this conclusion, as wood pathogens grow slowly and plants may remain externally asymptomatic for a number of years after an infection has taken place (Sparapano *et al.*, 2000).

Despite *Epicoccum* spp. are often associated with the endosphere, phyllosphere and rhizosphere of grapevine plants (Cueva *et al.*, 2012; Pancher *et al.*, 2012; Pinto *et al.*, 2014), to date, it is not clear the mode of colonization of the wood. Our results show that the inoculation of a mycelium plug of *E. layuense* produces a successful colonization in one-year-old cuttings of both tested cultivars, also revealing that its antagonist behavior was not influenced by grapevine cultivar.

2.4.3 *In vitro* interaction between *Epicoccum* spp. and esca-associated fungi

The various syndromes that constitute the esca disease complex are an increasing concern in viticulture for their unrestrained spread, which highlights the need to apply immediate and effective control strategies to prevent what may become the 21st century phylloxera (Bruez *et al.*, 2013). Success stories of the application of BCA against grapevine diseases are many (Compant and Mathieu, 2016), and their use could prevent several of the side effects that result from the application of fungicides (Komárek *et al.*, 2010). Grapevine wood is characterized by a complex microbiome (Hofstetter *et al.*, 2012; Pancher *et al.*, 2012; Bruez *et al.*, 2015) comprising hundreds

of species that interact with one another, and the role that some of these organisms play in preventing infections or slowing the spread of grapevine trunk pathogens must be further investigated. In this work, we present an overview of the interactions that lie between *Epicoccum* spp. and three esca-related fungi.

Although *E. layuense* has never been studied as an antagonist, the taxonomic proximity of this species to *E. nigrum* suggests that it may act in a similar way. Several studies demonstrated antibiosis among *E. nigrum* and different plant pathogenic fungi, including hyperparasitism (El-Gremi *et al.*, 2017). The interaction types may vary, ranging from a pure chemical one (Fávaro *et al.*, 2012) to a combination of chemical and physical (Brown *et al.*, 1987; Li *et al.*, 2013).

Our work shows a species-specific interaction of *Epicoccum* spp. over *P. chlamydospora*, *P. minimum* and *F. mediterranea*, where *E. mezzettii* overgrows all three pathogens, while for *E. layuense* there was mutual inhibition. The fast growth of *E. mezzettii* and *E. layuense*, along with the diffusion in the culture medium of pigments, suggest that the antagonism is primarily due to competition for space, nutrients and probably chemical interaction. These claims are also supported by previous reports of *E. nigrum* - pathogen interactions (Brown *et al.*, 1987; Madrigal *et al.*, 1994; Talontsi *et al.*, 2013). The different pathogens behavior reported in response to the presence of *E. layuense* E24, such as (i) the increase in the conidia production along the contact line in *P. chlamydospora*, (ii) the alteration of the growth direction of the hyphae in *P. minimum* and (iii) the formation of hyphal strands, degradation of the hyphal tips and formation of clamp connections in *F. mediterranea*, are considered antagonistic interactions in other studies where different fungi were confronting (Porter, 1924; Molla *et al.*, 2001; Naidu *et al.*, 2016).

However, the in-depth study of these interactions, as well as the chemical nature of the pigments produced by *E. layuense* E24 and by *E. mezzettii*, was not covered in this study and needs to be further investigated.

2.4.4 In vivo interaction between *E. layuense* E24 and esca-tracheomycotic fungi

The *in vivo* study focused on the interaction between *E. layuense* E24 and the two tracheomycotic fungi *P. chlamydospora* and *P. minimum.* These pathogens are the most frequently isolated from symptomatic young grapevines and they are responsible

for the brown wood streaking appearance, which is a parameter of our interest. Therefore, in this part of the study, *F. mediterranea* was not further examined.

In the greenhouse experiment, the inoculation of both pathogens did not induce any significant external symptom, most likely because of the relatively short amount of time that the pathogens had to interact with the plant. Nevertheless, the evident brown streaking, that manifested from the inoculation point, revealed that a pathogenic mechanism had taken place in the wood, and the role of the pathogens in its occurrence was confirmed by *in vitro* re-isolations.

The wood symptomatology caused by *P. chlamydospora*, when interacting with *E. layuense* E24, was consistently lower in both grapevine cultivars, with a 67.5% reduction of the brown wood streaking length in Cabernet Sauvignon and 73.8% in Touriga Nacional. Also for *P. minimum*, a significant reduction of symptoms was observed, although unevenly between cultivars (82% in Cabernet Sauvignon and 31.3% in Touriga Nacional; Figure 2.9). Studies that described a similar reduction in the streaking length induced by *P. chlamydospora*, achieved with other antagonists, include *Pythium oligandrum* inoculated in the root system (40 – 50%; Yacoub *et al.*, 2016), *Trichoderma* spp. in nurseries and in pruning wound protection (67 – 79%; Di Marco *et al.*, 2004; Di Marco and Osti, 2007), *Bacillus pumilus* and *Paenibacullus* sp. (31 – 39%) in co-inoculation assays (Haidar *et al.*, 2016).

The frequencies of re-isolation of *P. chlamydospora* and *P. minimum*, when alone or interacting with *E. layuense*, support the observations gained from the measurements of the brown wood streaking. In fact, the shorter the streaking the lower the frequency of re-isolation of the pathogens (Figures 2.7 and 2.9). The examination of the wood at different distances from the pathogens inoculation point increased the understanding of their wood colonization success. While the pathogens presence is similar to that of the positive controls near the inoculation point (15mm below), it is considerably reduced further down the wood (45mm; Figure 2.9). This suggests that the presence of *E. layuense* E24 slowed-down or completely stopped the spread of the pathogens in the wood. These observations also confirm the general understanding that the brown wood streaking length, although not incited exclusively by *P. chlamydospora* and *P. minimum*, is linked to the re-isolation of these pathogens (Mugnai *et al.*, 1999; Díaz and Latorre, 2014).

Combining the length of brown wood streaking with the re-isolation of pathogens, it is interesting to notice how fast *P. minimum* and *P. chlamydospora* could colonize woody tissues, approximately 10 mm/month for the first and 17 mm/month for the second, under our experimental conditions. These data are particularly worrying when thinking about nursery infections, as pathogens may fully colonize newly rooted cuttings in a matter of months.

The application of biological control agents in perennial plants presents several challenges and its success can be influenced by biotic and abiotic factors. This study examined the potential of *Epicoccum layuense* E24 in the biological control of two esca-associated pathogens, under greenhouse conditions. Further investigation is required to assess whether the success of the wood colonization by *E. layuense* E24 and the antagonism observed vary in time (e.g. aging of the vine) and under field conditions (e.g. seasonality, extreme weather conditions). Infections of newly grafted rooted cuttings or young grapevines have been widely reported (Scheck *et al.*, 1998; Rego *et al.*, 2000; Edwards *et al.*, 2001; Ridgway, Sleight and Stewart, 2002; Fourie and Halleen, 2004); they entail weaker and less productive plants, leading often to death of the infected vines. From our results, it can be hypothesized that the application of *E. layuense* E24, or even its metabolites, in nurseries or young vineyards may help the young plants to cope with early infections, by reducing both the wood colonization by *P. chlamydospora* and *P. minimum*, as well as the brown streaking symptom associated with them. The modes of production, delivery and application of *E. layuense* E24 need to be further investigated. In conclusion, *E. layuense* E24 is a promising candidate for biological control and its future application in nurseries and young vineyards is the natural follow-up of this study, in the ultimate quest of keeping in check these esca-related pathogens.

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CHAPTER 3

Endotherapy of infected rooted grapevine cuttings for the control of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*

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Abstract

The pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* are associated with different syndromes of the esca disease complex affecting grapevine propagation material, and young and adult plants. Infections by these fungi occur in grapevine nurseries and in vineyards, with disease control strategies providing limited protection in both cases. Several chemicals are effective *in vitro* against these two pathogens, but treatment of infected plants, especially endotherapy, has not yet proven satisfactory. Five chemicals (elemental silver, fosetyl-Al, glutaraldehyde, hydrogen peroxide and Blad-containing oligomer) were tested *in vitro*, with the first four also tested *in planta*, by means of endotherapy, against *Pa. chlamydospora* and *Pm. minimum*. All chemicals were effective *in vitro* for preventing growth of both pathogens, at different concentrations. Endotherapy of rooted grapevine cuttings (cv. Touriga Nacional) was effective against *Pa. chlamydospora* for all the tested chemicals, with reductions in the frequency of re-isolation of this pathogen of 91 – 95% (glutaraldehyde), 68 – 96% (hydrogen peroxide), 68 – 77% (elemental silver) and 58 – 59% (fosetyl-Al) when compared with the water-treated experimental controls. The only treatment that was effective against *Pm. minimum* was glutaraldehyde, providing a 75 – 83% reduction in re-isolation frequency. These results indicate that endotherapy of young grapevines during early stages of infection may be an effective control strategy, especially against the wood pathogen *Pa. chlamydospora*.

Key words Tracheomycosis, grapevine trunk diseases, fungicides, chemical control, endotherapy

3.1 Introduction

Phaeomoniella (*Pa.*) *chlamydospora* and *Phaeoacremonium* (*Pm.*) *minimum* are tracheomycotic ascomycetes involved in the esca disease complex, a major trunk disease of grapevines (*Vitis vinifera* L.; Gramaje *et al.*, 2018). They are directly responsible for three syndromes, affecting rooted cuttings (brown wood streaking), young grapevines (Petri disease) and adult plants (grapevine leaf stripe disease, GLSD). They also play a role in the development of the 'esca proper' syndrome, in which their presence occurs simultaneously with wood rotting basidiomycetes (e.g. *Fomitiporia mediterranea*; Surico, 2008; 2009). Primary internal symptoms develop in grapevine wood, where these fungi cause brown wood streaking, black dots and necroses. However, plants may remain externally asymptomatic for a number of years after infections have taken place (Mugnai *et al*., 1999; Sparapano *et al.*, 2001). Overall, affected plants suffer reduced vigour, shortened lifespan, and reduced grape quality and yield (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016).

Over the last 20 years, several studies have identified fungicides, biocontrol agents and natural compounds that are capable of inhibiting growth of *Pa. chlamydospora* and *Pm. minimum in vitro* and in grapevines. However, to date, control methods have only achieved partial protection through integrated management approaches, in which sanitation methods are also of major relevance (Bertsch *et al*., 2013; Gramaje *et al.*, 2018; Mondello *et al.*, 2018). There are two main disease management strategies. The first is against tracheomycotic fungi inside the wood of grapevines, which is a challenging task, as it is difficult to reach the pathogens with fungicides and other compounds. The second is to prevent new infections, due to the widespread occurrence of these pathogens as epiphytes or in the endosphere of grapevine propagation material (Larignon and Dubos, 2000; Rego *et al.*, 2000; Zanzotto *et al.*, 2001; Ridgway *et al.*, 2002; Fourie and Halleen, 2004), as well as in the air (Gramaje *et al.*, 2018), soil (Rooney *et al.*, 2001; Whiteman *et al.*, 2002), arthropods (Edwards *et al.*, 2001) and other plant hosts (Díaz and Latorre, 2014). For the control of tracheomycotic/esca infections in the field, moderate results have been obtained by spraying leaf symptomatic grapevines with a copper formulation (Di Marco *et al.*, 2011a) and with a mixture of calcium chloride, magnesium nitrate and seaweed

extract (Calzarano *et al.*, 2014), achieving reduced expression of the frequency of foliar symptoms. However, trunk injections of active ingredients performed in adult plants with established infections gave mainly negative results (Calzarano *et al.*, 2004; Sentenac *et al.*, 2005; Loskill *et al.*, 2006; Darrieutort and Pascal, 2007). These studies focused on endotherapy, based observations exclusively on the appearance of leaf symptoms to assess treatment effects, and did not examine changes in the presence of pathogens in the wood pre- or post-treatment, leaving questions on the true efficacy of endotherapy.

The aims of the present study were: (i) to test promising chemicals *in vitro* against *Pa. chlamydospora* and *Pm. minimum*, and (ii) to test endotherapy for the control of these pathogens in the wood of artificially infected rooted grapevine cuttings.

3.2 Materials and Methods

3.2.1 Fungal isolates, chemicals tested and plant material

The fungal isolates used in this study were *Pa. chlamydospora* CBS 161.90 and *Pm. minimum* CBS 110713, from the CBS culture collection (Westerdijk Fungal Biodiversity Institute, Netherlands). Stock cultures were maintained in Petri dishes containing potato dextrose agar (PDA; Difco™), at 25 °C, in the dark.

The chemicals examined in this study, and the range of tested concentrations, are listed in Table 3.1.

Rooted cuttings of *Vitis vinifera* L. cv. Touriga Nacional were used in greenhouse experiments. For the first year experiment (EXP 1), one-year-old canes were sampled in a vineyard in the Azeitão region (Portugal), and left in a cold-room (4 °C) for 2 months. Three-bud cuttings were rooted in a warm bench, at 24 °C, and then potted in a mixture of peat and sand. For the second year experiment (EXP 2), three-bud cuttings, rooted and potted in a mixture of peat and sand, were provided by the Viveiros VitiOeste nursery (Pó, Portugal). Rooted cuttings were grown and maintained under greenhouse conditions at an average temperature of 24 °C. Plants were treated fortnightly with meptyldinocap (35.7% w/w) or sulfur wettable powder (80 % w/w) to prevent powdery mildew (*Erysiphe necator*).

Table 3.1. Chemicals tested against *Phaeomoniella chlamydospora* (Pch) and *Phaeoacremonium minimum* (Pmin), the range of concentrations of active ingredient (a.i.) tested *in vitro* and the minimum inhibitory concentration (MIC) of each chemical.

Active Ingredient	Trade Name	Manufacturer	Formulation	*In vitro* conc. max – min (g a.i. L^{-1})	MIC (g a.i. L^{-1})	
					Pch	**Pmin**
Blad-containing oligomer (BCO)	Fracture®	CEV/CONVERDE	20% (v/v) BCO	1.00 – 1.69×10^{-5}	0.037	0.111
Elemental silver	BioBac®	M.H.I Compania de Ingenerie	1000 ppm Elemental silver	0.012 – 2.00×10^{-7}	0.004	0.012
Fosetyl-Al	Aliette Flash®	Bayer	74.6% (w/w) Fosetyl-Al	0.667 – 1.13×10^{-5}	0.222	0.222
Glutaraldehyde*	-	VWR chemicals	25% (v/v) Glutaraldehyde	14.88 – 2.52×10^{-4}	0.061	0.020
Hydrogen peroxide	-	Sigma-Aldrich	30% (v/v) Hydrogen peroxide	11.10 – 1.88×10^{-4}	0.015	0.046

*In this study, the glutaraldehyde solution was activated by increasing its pH to 8, using a solution of $NaHCO_3$ (0.93% w/v; Gorman and Scott, 1977).

3.2.2 Minimum inhibitory concentration (MIC) of the chemicals tested

To determine the lowest concentration of active ingredient required to prevent visible fungal growth, experiments were performed as a modified version of that described by Kuipers *et al.* (1999).

(i) Separate conidia suspensions of *Pa. chlamydospora* or *Pm. minimum* were prepared, by flooding 14-d-old cultures of each pathogen with sterile distilled water (SDW), and dislodging the conidia from the mycelium with sterile glass rods. The suspension of each fungus was filtered through a double layer of cheesecloth, the conidia concentration was determined using a hemocytometer, and then adjusted to 1 × 10^5 conidia/mL with sterile distilled water (SDW).

(ii) The liquid growth medium was a solution of potato dextrose broth (PDB).

(iii) The five chemicals examined in this study as antifungal agents were tested at 11 concentrations, and their respective dilution ranges are presented in Table 3.1.

Each tested concentration was a sequential three-fold dilution of the previous solution, commencing from the most concentrated solution (e.g. 1.000, 0.333, 0.111, 0.037 … 1.69×10^{-5} g a.i. L^{-1}).

Fungal growth occurred in 96-well culture plates (flat bottom). Each well contained equal volumes (80 µL) of pathogen, liquid growth medium and candidate chemical. Positive controls contained a combination of equal volumes (80 µL) of conidia suspension, PDB and SDW; negative controls contained equal volumes (80 µL) of PDB, candidate chemical at its greatest concentration and SDW.

The 96-well culture plates were sealed with Parafilm®, and the cultures left to grow at 25 °C, in the dark, for 72 h, during which the conidia would be mostly germinated (Pierron *et al.*, 2016). Fungal growth was measured spectrophotometrically, targeting turbidity of the solution (optical density [λ = 630 nm]; Kuipers *et al.*, 1999). Samples were analysed in a microplate reader (BIO-TEK Synergy HT) with BioTek GEN5 Data Analysis Software. Each treatment was applied in four replicates, and the experiment was repeated once.

3.2.3 Pathogen inoculation and endotherapy

In greenhouse assays, each grapevine stem was surface-disinfected with 70% ethanol. An artificial wound was then made by drilling a hole in the bark of the stem (4 mm diam. 4 mm depth), below the upper bud (Figure 3.1a).

Inoculation of *Pa. chlamydospora* or *Pm. minimum* was performed by inserting in the wound either a mycelium plug (EXP 1) or a conidia suspension (EXP 2). In the first case, the mycelium plug (4 mm diam.) was cut from the margin of a 2-week-old colony, to minimize the presence of conidia, and was then placed in the stem hole with the mycelium facing the inner part of the stem. The inoculated wound was covered with moist cotton and wrapped tightly to the plant with Parafilm®. In the second experiment, 50 µL of conidia suspension (1×10^5 conidia/mL), prepared from 2-week-old colonies, were deposited in the stem hole, which was then covered with Parafilm® for protection during the incubation period.

Three months after inoculation, each grapevine cutting was injected with 1 mL of candidate chemical solution (or SWD for the controls), with the aid of a modified syringe, into a hole made 3 cm below the pathogen inoculation point. Each hole (4 mm

diam., 5 mm deep; Figure 3.1, b-c) was made using a drill. The drill bit was disinfected with ethanol (70%) and NaClO solution (0.5% w/w active chlorine) after each use. Each treatment consisted of ten biological replicates. Preliminary tests, using a food-colouring agent dissolved in water, showed that the injected liquids diffused throughout the xylem vessels of the plants (data not shown). Fluids were absorbed into the stem wood, on average, within 5 h from the moment the syringes were applied to the rooted cuttings.

Injection of BCO was unsuccessful, probably due to the large size of this oligomer, which prevented the BCO solutions from entering the xylem of the plants. Application of BCO in endotherapy will not be further discussed in this paper.

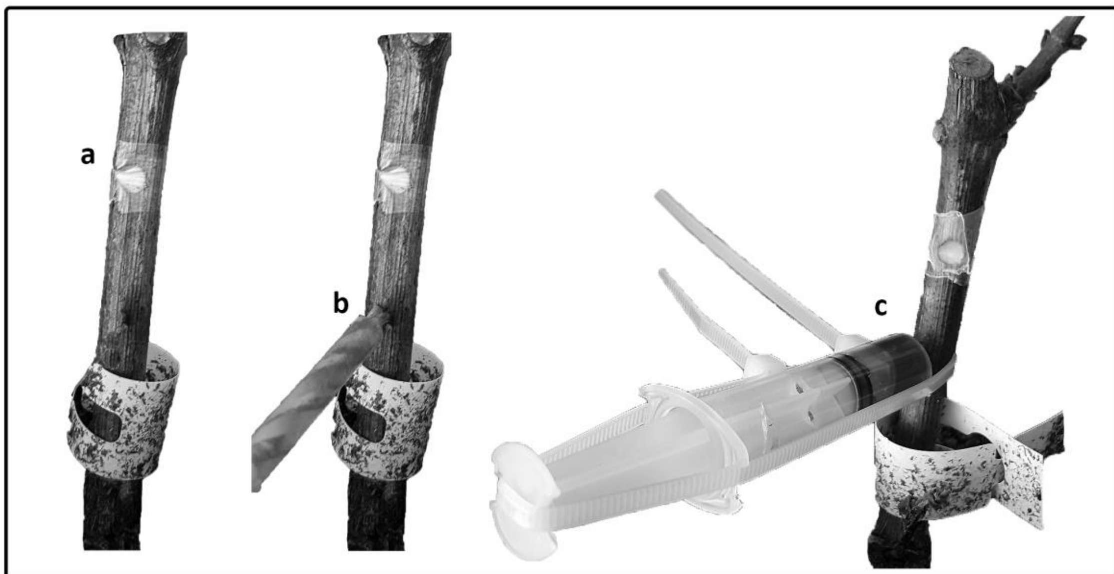

Figure 3.1. Schematic illustration of grapevine cuttings (cv Touriga Nacional). Pathogen inoculation, via mycelium plug or conidia suspension, occurred in point (a). For endotherapy, a drill-made wound (b) was performed 3 cm below point (a) and a modified syringe was placed in the hole (c) forcing the chemicals into the xylem.

3.2.4 Examination of rooted cuttings, and re-isolation of inoculated pathogens

One month after treatment, the green shoot length of each plant was measured, and the plants were inspected for the appearance of symptoms attributable to pathogens

infection or phytotoxicity from the treatments applied, both in leaves and in the stem. Symptoms such as brown streaking and other wood alterations were recorded.

Pieces of wood were collected 1.5 cm below the pathogens inoculation point, they were surface sterilized using flame, immersed in NaClO solution (0.5% w/w active chlorine) for 1 min, then double rinsed in sterile water, and plated onto PDA supplemented with chloramphenicol (250 mg/L) in Petri dishes. Resulting fungus colonies emerging from the wood pieces were identified, and their frequency of re-isolation was calculated as follows: 100 × (number of wood pieces from which a pathogen was re-isolated / total number of pieces plated).

Wounded but non-inoculated and non-treated plants, representing negative controls, were screened for the presence of wood discoloration and background infections of *Pa. chlamydospora* and/or *Pm. minimum*.

3.2.5 Data analyses

Minimum inhibitory concentrations (MICs) were determined as outlined by Kuipers *et al.* (1999). The least concentration of each active ingredient capable of preventing fungal growth was selected as the MIC. The MICs were recorded for both pathogens, and in all treatments these were identical among replicates and between the repetitions of the test, so no averaging was necessary.

All other data were compared using analyses of variance (ANOVA) followed by Tukey´s *post hoc* tests (at $p < 0.05$; GraphPad Prism 7.05). Data obtained from EXP 1 and EXP 2 were analyzed separately, as they used different inoculum types and were carried out in different years. In both cases, three different parameters were examined: shoot length, length of brown wood streaking, and frequency of re-isolation of the inoculated pathogens. For each parameter, a two-way ANOVA was performed, examining the factors ‘pathogen’ (*Pa. chlamydospora* or *Pm. minimum*) and ‘treatment’ (water control, elemental silver, fosetyl-Al, glutaraldehyde or hydrogen peroxide). Data expressed as percentages, as for frequency of re-isolation, were arcsine-square root transformed before analyses.

3.3 Results

3.3.1 Minimum inhibitory concentration

All the chemicals studied prevented *in vitro* growth of *Pa. chlamydospora* and *Pm. minimum*, but at different concentrations (Table 3.1). MICs indicated that *Pa. chlamydospora* is more sensitive than *Pm. minimum* to most chemicals tested, with the exception of fosetyl-Al (identical MICs for this fungicide) and glutaraldehyde (lower MIC for *Pm. minimum*). The *in vitro* inhibitory effects of the tested chemicals against these pathogens is reported in here for the first time.

3.3.2 Examination of grapevine cuttings

Four months after inoculation of the rooted grapevine cuttings with *Pa. chlamydospora* or *Pm. minimum*, and 1 month after treatment with each of the chemicals under study, except for BCO, no leaf symptoms attributable to the wood pathogens were observed. In addition, no phytotoxicity symptoms in leaves were observed for any treatment, except for glutaraldehyde, where 10% of the plants exhibited leaf wilting on the two lower internodes of the green shoots. The shoot lengths of grapevine plants were not statistically affected ($p > 0.05$) by the 'treatment' and 'pathogen' factors analyzed, both for EXP 1 and EXP 2, so data were pooled for presentation in Table 3.2.

For lengths of brown wood streaking, two-way ANOVA followed by Tukey's *post hoc* tests revealed no statistically significant differences ($p > 0.05$) for factors 'pathogen' and 'treatments'. However, the different inoculum types that characterized EXP 1 (mainly fresh mycelium) and EXP 2 (conidia only) produced different lengths of streaking (Table 3.2), which was presumably due to the different wood colonization rates by mycelium and conidia. Negative controls, non-inoculated and non-treated, did not present wood streaking attributable to wood pathogen infections.

Examination of the wood surrounding the chemicals injection point revealed no wood discoloration or necrosis for the injections with water, fosetyl-Al or elemental silver, while different degrees of wood discoloration were evident after treatments with hydrogen peroxide or glutaraldehyde (Table 3.2), highlighting some phytotoxicity effects of these chemical at the tested concentrations.

3.3.3 Re-isolation of pathogens

The frequency of re-isolation of the inoculated pathogens was chosen to determine how effective each treatment had been. Two-way ANOVA showed that the factors 'pathogen' (*Pa. chlamydospora*, *Pm. minimum*; $p < 0.05$) and 'treatments' ($p < 0.01$) had statistically significant effects on the re-isolation of the pathogens, for both EXP 1 and EXP 2, and the two factors interaction effect was also significant ($p < 0.05$) in EXP 2. *Pa. chlamydospora* was affected by all treatments, and its frequency of re-isolation was significantly lower than in the water-treated plants for three of the four treatments. The most effective chemical for the control of *Pa. chlamydospora* was glutaraldehyde, resulting in 90.9 – 94.7% reduction in re-isolation frequency, although this was not significantly different from hydrogen peroxide (68.4 – 95.5% reduction) or elemental silver (68.4 – 77.3%; Table 3.2; Figure 3.2 A, B). The fosetyl-Al treatment also reduced the re-isolation of *Pa. chlamydospora,* although not to a statistically significant extent.

Pm. minimum was unaffected by elemental silver or fosetyl-Al, and only mildly affected by hydrogen peroxide, while a significant reduction in frequency of re-isolation of this pathogen was observed from the glutaraldehyde treatment. In this case, treatment with glutaraldehyde resulted in a 75.0 – 83.3% reduction in re-isolation of the pathogen (Table 3.2; Figure 3.2 C, D).

The examination of negative controls did not reveal background infections of *Pa. chlamydospora* or *Pm. minimum*.

Table 3.2. Mean shoot lengths, extents of brown wood streaking and wood discolouration, and reductions in frequencies of pathogen re-isolation, after injection of different chemicals into rooted grapevine cuttings (cv. Touriga Nacional) that had been inoculated with *Phaeomoniella chlamydospora* (Pch) or *Phaeoacremonium minimum* (Pmin).

Treatment	Conc. (g a.i. L^{-1})	Shoot length* (mm)		Brown wood streaking (mm)				Wood discoloration near injection point* (mm)		Reduction in frequency of re-isolation (%)			
		EXP 1	EXP 2	EXP 1		EXP 2		EXP 1	EXP 2	EXP 1		EXP 2	
				Pch	Pmin	Pch	Pmin			Pch	Pmin	Pch	Pmin
Water control	-	1000 a	928 a	102 b	120 b	62 c	69 c	<1.0	<1.0	-	-	-	-
Blad-containing oligomer (BCO)	-	-	-	-	-	-	-	-	-	-	-	-	-
Elemental silver	0.250	1028 a	938 a	103 b	118 b	59 c	69 c	<1.0	<1.0	68.4	12.4	77.3	0.00
Fosetyl-Al	0.250	1019 a	941 a	-	-	62 c	63 c	<1.0	<1.0	57.9	0.00	59.1	12.5
Glutaraldehyde	0.318	1029 a	933 a	111 b	118 b	64 c	67 c	12.4 d	12.2 d	94.7	75.0	90.9	83.3
Hydrogen peroxide	0.333	990 a	934 a	109 b	108 b	62 c	67 c	6.6 e	7.0 e	68.4	56.3	95.5	37.5

(*) Data presented for 'Shoot length' and 'Wood discoloration near injection point' are an average of the two pathogens inoculation (Pch, Pmin), as no statistical differences ($p > 0.05$) were detected between them. Numbers followed by the same letter, in each column, do not differ statistically according to Tukey's test ($p > 0.05$).

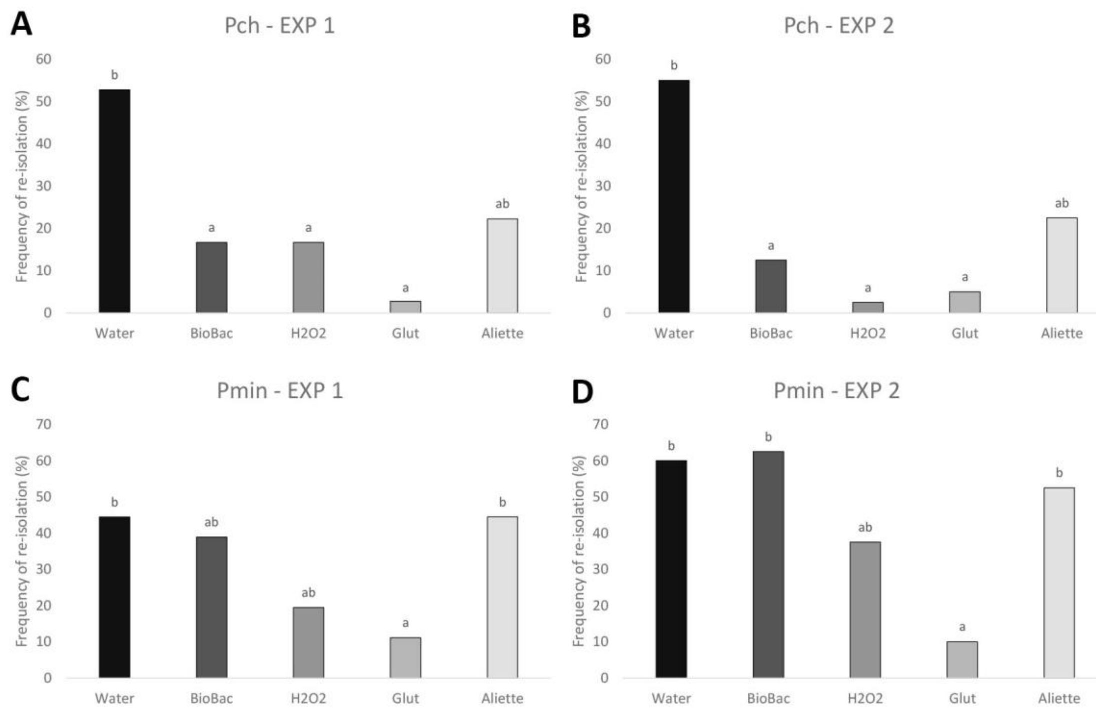

Figure 3.2. Frequency of re-isolation (%) of *Pa. chlamydospora* (Pch; A, B) or *Pm. minimum* (Pmin; C, D), one month after endotherapy with either water (negative control), elemental silver (BioBac), hydrogen peroxide (H_2O_2), glutaraldehyde (Glut) or fosetyl-Al (Aliette). Experiments conducted in year 2016 (EXP 1) used a mycelium plug inoculum (A, C), while that in 2017 (EXP 2) used a conidia inoculum (B, D). Bars accompanied by the same letter do not differ significantly according to Tukey's test ($p < 0.05$).

3.4 Discussion

A considerable number of chemicals and biocontrol agents have been tested against esca-related tracheomycotic pathogens over the last 20 years (Mondello *et al.*, 2018). Many chemicals have been identified as effective *in vitro*, against conidia germination, and for reduction of mycelium growth of *Pa. chlamydospora* and *Pm. minimum*, with follow-up studies on the application of chemicals under greenhouse conditions and in

the field. Despite these attempts, effective control of esca-associated tracheomycotic pathogens has not been achieved.

3.4.1 Screening of chemicals as antifungal agents *in vitro*

The majority of *in vitro* studies performed to test fungicidal activity against *Pa. chlamydospora* or *Pm. minimum* have been conducted on solid growth media, measuring the growth inhibition of inoculated mycelium (Mondello *et al.*, 2018). In the present study, a different approach was selected, to deal with chemicals whose activity is negatively affected by dilution in solid media or long incubation periods (e.g. hydrogen peroxide, glutaraldehyde or BCO).

This study expands the list of chemicals assayed against grapevine trunk diseases (Mondello *et al.*, 2018; Gramaje *et al.*, 2018) with new active ingredients capable of inhibiting visible growth of *Pa. chlamydospora* and *Pm. minimum.*

Blad-Containing oligomer (BCO) is a high molecular weight oligomer (210 kD) extracted from the cotyledons of *Lupinus albus* L. plantlets. It is non-toxic and it has a broad-spectrum fungicidal activity (Monteiro *et al.*, 2015; Carreira *et al.*, 2018), having multi-site activity (Pinheiro *et al.*, 2016; Pinheiro *et al.*, 2017). With these characteristics, it was included in the 2017 FRAC code list as BM 01.

Silver nanoparticles-based chemicals have been shown to be effective against bacteria (Morones *et al.*, 2005) and several plant pathogenic fungi (Kasprowicz *et al.*, 2010; Kim *et al.*, 2012; Patel *et al.*, 2014). Their modes of action are yet to be fully understood but they are known to negatively interact with fungal cell membrane structure and functions (Lamsal *et al.*, 2011).

Fosetyl-aluminium (fosetyl-Al) acts primarily as elicitor of plant defence responses (Di Marco *et al.*, 2011b). This fungicide has also been tested, *in vitro*, against *Pa. chlamydospora*, but no fungicidal activity has been recorded for concentrations up to 5 mg a.i. L^{-1} (Groenewald *et al*., 2000). In the present study we have demonstrated that fosetyl-Al prevents the growth of *Pa. chlamydospora* and *Pm. minimum*, albeit at a higher concentration (222.2 mg a.i. L^{-1}).

No studies describe fungicidal activity of glutaraldehyde against *Pa. chlamydospora* or *Pm. minimum*, although this is a well-known antimicrobial agent (Gorman and Scott, 1977; Baldry, 1983; Migneault *et al.*, 2004).

The use of hydrogen peroxide against *Pa. chlamydospora* and *Pm. minimum* is known, but has not been assayed *in vitro*, and results were inconsistent when this compound was tested as a soak-treatment of propagation grapevine material in nursery conditions (Fourie and Halleen, 2006). Despite unpredictable results, some winegrowers are already using injections of hydrogen peroxide into grapevine trunks to control grapevine trunk diseases, but more testing is required before this technique can be fully recommended (Prezman, 2017).

3.4.2 Endotherapy of infected rooted grapevine cuttings

Despite the promising results *in vitro*, previous *in planta* tests have been discouraging, especially for endotherapy (Calzarano *et al*., 2004; Sentenac *et al.*, 2005; Loskill *et al.*, 2006; Darrieutort and Pascal, 2007). The present study focused on applying some of the tested chemicals as endotherapy treatments, for the control of two grapevine pathogens in their wood environment. Injecting bioactive molecules into plant xylem allows direct interaction between the chemicals and mycelia and conidia of the pathogens, but application to adult plants with established infections finds limitations. The internal xylem surface area is large, the wood is dense and it cannot be easily penetrated by the active ingredients. Moreover, if pathogens are not completely eradicated from plants, they may re-colonize the wood when active ingredient concentrations diminish. Endotherapy of young grapevines allows increased penetration of active ingredients into internal xylem tissues, and control of pathogen infections before the hosts become heavily colonized.

Our results show that colonization by *Pa. chlamydospora* in the wood can be reduced, at least up to one month after treatment, using selected chemicals (Figure 3.2). Hydrogen peroxide and glutaraldehyde, the most efficient chemicals, caused wood discoloration near the injection points, indicating the need to optimize their use concentrations in future trials. The elemental silver-based chemical (BioBac) considerably reduced the pathogen presence and did not cause phytotoxicity, making it a suitable candidate for the control of *Pa. chlamydospora* in plant wood. *Pm. minimum* was more difficult to control, with glutaraldehyde being the only treatment capable of significantly reducing the presence of this pathogen.

Different responses to endotherapy were observed for the two pathogens, with presence of *Pa. chlamydospora* considerably reduced by all treatments, while *Pm. minimum* was unaffected by most of them. This behaviour is probably not attributable to differences in sensitivity to the injected chemicals, as their concentrations were always equal or greater than their *in vitro* MICs. Histological studies unveiled the different wood colonization strategies of *Pa. chlamydospora* and *Pm. minimum*. *Pa. chlamydospora* occurs mainly in the lumen of xylem vessels and in xylem fibers, having limited capacity to degrade cell wall polymers (Valtaud *et al.*, 2009; Pouzoulet *et al.*, 2017). *Pm. minimum* can colonize bark, pith, phloem, xylem fibers and vessels, vessel-associated cells, rays, metaxylem and protoxylem (Valtaud *et al.*, 2009; Pierron *et al.*, 2015). Therefore, endotherapy may be particularly efficient against *Pa. chlamydospora* because the chemicals are transported in host xylem vessels, where the fungus was located. Concerning *Pm. minimum*, although the chemicals may have effectively interacted with this pathogen in the xylem vessels, its presence in several other areas of the stem tissues may have allowed it to remain present in the wood, making endotherapy less efficient against this pathogen.

3.5 Conclusions

Although not addressed in this study, the results obtained indicate that some of the tested chemicals may find applications during the grapevine propagation processes in nurseries, for control of external viable propagules of *Pa. chlamydospora* and *Pm. minimum*. These compounds could be used in plant material, on pruning shears and on grafting machines (Retief *et al.*, 2006; Gramaje *et al.*, 2018), to replace other chemicals reported to be ineffective against *Pa. chlamydospora* and *Pm. minimum*, such as Chinosol (hydroxyquinoline sulfate), one of the most commonly used fungicides in nurseries (Gramaje *et al.*, 2009).

We are aware that the process of injecting chemicals into host plants (endotherapy), as described in this study, is time-consuming and may not find application in large-scale nursery production systems or young vineyards. It is necessary to improve chemical delivery technology, to make it rapid and reliable, as could be the case for the

instrument developed by Montecchio (2013). Testing whether different concentrations of active ingredients, and/or multiple or mixture treatments, may lead to a complete and long-lasting eradication of these important grapevine pathogens.

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CHAPTER 4

Fungicides and the grapevine wood mycobiome. A case study on tracheomycotic ascomycete *Phaeomoniella chlamydospora* reveals potential for two novel control strategies.

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4. Fungicides and the grapevine wood mycobiome. A case study on tracheomycotic ascomycete *Phaeomoniella chlamydospora* reveals potential for two novel control strategies.

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Abstract

Phaeomoniella chlamydospora is a tracheomycotic fungus that colonizes the xylem of grapevines (*Vitis vinifera* L.), causing wood discoloration, brown wood streaking, gummosis and wood necrosis, which negatively affect the overall health, productivity and lifespan of vines. Infections by *P. chlamydospora* may occur both in the nursery, via infected propagation material and/or contaminated equipment, and in the field, though propagules disseminated by several means. Current control strategies to prevent or cope with *P. chlamydospora* infections are frequently ineffective. It remains unclear whether fungicides commonly applied in vineyards against downy and powdery mildew agents affect the wood mycobiome, including wood pathogens such as *P. chlamydospora*.

In this study, we used next-generation sequencing to assess the effects of foliar spray of grapevines with inorganic (copper oxychloride and sulfur), synthetic (penconazol and fosetyl-aluminium) and natural (blad-containing oligomer) fungicides currently used against the downy and powdery mildews. The subjects of our investigation were (i) the resident wood mycobiome, (ii) the early colonization by a consortium of wood endophytes (skopobiota), (iii) the wood colonization success of *P. chlamydospora*, and (iv) the *in planta* interaction between *P. chlamydospora* and the skopobiota, under greenhouse conditions, in rooted grapevine cuttings cv Cabernet Sauvignon.

The data obtained suggest that the resident mycobiome was affected by different fungicide treatments. In addition, the early colonization success of the endophytes composing the skopobiota varied in response to fungicides, with relative abundances of some taxa being over- or underrepresented when compared with the control. The wood colonization by *P. chlamydospora* comported significant changes in the mycobiome composition and, in addition, it was greatly affected by the foliar spray

with blad-containing oligomer, which decreased the relative abundance of this pathogen 12-fold (4.9%) when compared with the control (60.7%) and other treatments. The presence of the pathogen also decreased considerably when co-inoculated into the plant with the skopobiota, which reached relative abundances between 13.9 and 2.0%, depending on the fungicide treatment applied.

This study shows that fungicides sprayed to prevent infections of powdery and downy mildews have an effect on non-target fungi, including some that colonize the endosphere of grapevines, with unpredictable consequences for the wood mycobiome ecosystem. We suggest two potential control strategies to fight *P. chlamydospora*, namely the foliar spray with blad-containing oligomer and the use of skopobiota. Further studies to confirm these results are required.

4.1 Introduction

Protecting grapevines (*Vitis vinifera* L.) from fungal pests by means of fungicides dates back to the late eighteen hundreds, with the accidental discovery that a mixture of copper sulfate and lime, sprayed on vine leaves, was not only able to deter thieves from stealing grapes, but also prevented downy mildew infections (Morton and Staub 2008). Since then, research on fungicides improved the use of copper and sulfur as contact fungicides and introduced additional synthetic broad-spectrum systemic fungicides (e.g. benzimidazoles, triazoles, fludioxonil, organophosphorous compounds) during the mid-late nineteen hundreds (Morton and Staub 2008). Overuse of fungicides in vineyards came at a cost. Several were shown to be phytotoxic to grapevines (Dias 2012; Juang *et al*., 2012), toxic to humans and the environment (Komárek *et al*., 2010), some affected non-target organisms (La Torre *et al*., 2018), while others hampered beneficial insects (Thomson *et al.*, 2000). In addition, the development of pathogen resistance pushed the search of ever more effective chemicals capable of dealing with increasingly resilient species (Verweij *et al*., 2009).

It was only in recent years, with the advent of molecular ecology and its tools, such as metagenomics and metabarcoding, that scientists have begun to understand how fungicides, along with herbicides and insecticides, impact the microbial ecology of

environments with unpredictable medium- and long-term consequences (Komárek *et al.*, 2010; Setati *et al.*, 2012; Karlsson *et al.*, 2014; Morrison-Whittle *et al.*, 2017). The vineyard is an extremely diverse environment, characterized by complex interactions among plants, soil microbes, endophytes and epiphytes, which are associated not only with grapevine health (Zarraonaindia and Gilbert 2015; Jayawardena *et al*., 2018), but also with the concept of *terroir*, as it is a source of yeasts and bacteria especially important in winemaking (Pretorius *et al.*, 1999; Setati *et al.*, 2012).

Concerning plant health, grapevine trunk diseases (GTDs) are currently a major concern in viticulture, considered by some as 'the new Phylloxera' due to their potential destructive power (Bruez *et al*., 2013). Early scientific reports on GTDs are over a century old (Surico 2009) and the reasons underlying their recent worldwide outbreak have not yet been fully addressed. Unlike powdery and downy mildews agents (*Erysiphe necator* and *Plasmopara viticola*) and Phylloxera (*Daktulosphaira vitifoliae*), which were introduced from the Americas and found a susceptible host in *Vitis vinifera* (Jackson 2014), GTD pathogens have presumably been co-evolving with grapevines for centuries, if not millennia (Mugnai *et al.*, 1999).

Copper and sulfur fungicides were introduced approximately two decades before the first scientific reports on GTDs and the recent outbreak began some 30 years ago (Surico *et al*., 2004; Fontaine *et al*., 2016), approximately two decades after the massive introduction of synthetic broad-spectrum systemic fungicides in vineyards. To date, no study has investigated the effect of these fungicides, and that of other natural active ingredients, on the wood mycobiome and its possible implications in GTDs. To address this issue, we used metabarcoding (Illumina® next-generation sequencing; NGS) and tested four hypotheses. (1) Are fungicides commonly sprayed to fight powdery and downy mildews capable of affecting the resident wood mycobiome and/or (2) an inoculated consortium of wood endophytes (skopobiota)? (3) Is *Phaeomoniella chlamydospora*, a wood pathogen associated with several syndromes in grapevine (Surico 2009), affected by such fungicides, when artificially inoculated in wood? (4) Is a skopobiota inoculation potentially exploitable in the control of early infections of *P. chlamydospora*, independently of fungicide treatments?

4.2 Materials and Methods

4.2.1 Experimental setup

Plant and fungal material

The grapevines used in this assay were one-year-old canes of cv Cabernet Sauvignon, provided by Viveiros VitiOeste nursery (Pó, Portugal), that were cut at 3-buds-long size. The cuttings were rooted, potted in a mixture of peat and sand (1:1 v/v) and maintained under greenhouse conditions, at the temperature of 24 °C ± 5 °C day/18 °C ± 5 °C night.

The pathogenic ascomycete tested in this study was *Phaeomoniella chlamydospora* (CBS 161.90), from the CBS culture collection (Westerdijk Fungal Biodiversity Institute, Netherlands). The fungi used to produce the skopobiota, as named by the authors, and consisting in a consortium of grapevine endophytes, were isolated from grapevine wood (cv Cabernet Sauvignon) of asymptomatic plants and identified as *Alternaria* (*Al.*) *alternata* A101, *Epicoccum nigrum* E279, *Cladosporium* sp. C22, *Aureobasidium (Au.*) *pullulans* AU86. All fungi were maintained in Petri dishes with vents, on potato dextrose agar medium (Difco™), at 25 °C, in the dark.

Inocula preparation and delivery

Four different inocula were prepared for delivery into the plants wood, three of them consisting of a suspension of conidia and/or fungal cells and the fourth as a control. Inoculum (i) consisted of a conidia suspension of *P. chlamydospora*, which was prepared by flooding a two-week old colony. The conidia were dislodged from the mycelia with a sterile glass rod and the suspension was filtered through a double layer of cheesecloth. The conidial concentration was determined using a hemocytometer and adjusted to 1×10^5 conidia/mL with sterile distilled water (SDW). Inoculum (ii) consisted of a skopobiota, which was prepared by joining a conidia suspension of ascomycetes *Al. alternata*, *E. nigrum*, *Cladosporium* sp. and cells of yeast *Au. pullulans*. Cultures' growth conditions and conidia/cell suspension preparation occurred similarly to inoculum (i), where the final skopobiota presented a concentration 1×10^5 (conidia-cells)/mL of each one of the four fungi. Inoculum (iii) was prepared by joining a conidia suspension of *P. chlamydospora* and the skopobiota, in order to produce a solution

with a final concentration of 1×10^5 (conidia-cells)/mL of each one of the five fungi. Inoculum (iv) consisted of sterile distilled water.

The delivery of the inocula into the rooted plants was performed as follows. Grapevine cuttings were surface disinfected with 70% (v/v) ethanol and a wound in the wood was produced with the aid of a cork borer (4 mm diameter, 4 mm deep), approximately 1 cm below the green shoot (Figure 4.1 A- (a)). The four inocula were delivered with a micropipette by depositing 50 µL of solution in freshly made wounds, which were immediately sealed with Parafilm®. Each inoculum was delivered in 32 plants (Figure 4.1 B).

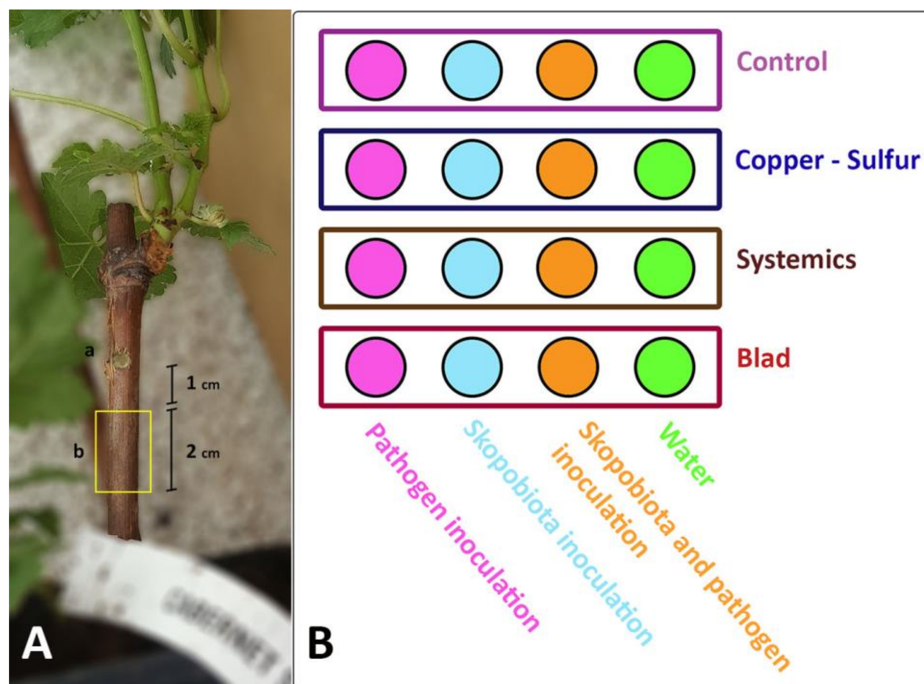

Figure 4.1. A – Grapevine rooted cutting of cv Cabernet Sauvignon. (a) Inoculation point of a conidia suspension of *P. chlamydospora,* skopobiota, *P. chlamydospora* and skopobiota, or water. (b) Two cm of wood, located one cm below the inoculation point, were sampled three months post-inoculation and its mycobiome was examined through metabarcoding. B – Experimental setup. Each circle represents a set of eight plants that underwent the same treatment. Same colors in the circles represent the same inoculum type, while rectangles represent different treatments. All possible combinations of inoculum and fungicide application by foliar spray are shown.

Treatments via foliar spray

Four treatments were tested for each inoculum type. The chemicals used and their application rate are reported in Table 4.1. The spray occurred every fortnight, starting from the 7th day post-inoculation and consisting in a total of six applications per treatment. Plants were sprayed on leaves and stem, with the exception of the control, which was sprayed exclusively on leaves. The chemicals were prevented from dripping onto the soil. All possible combinations of fungal inoculum and foliar treatment were tested, for a total of 16 combinations, each one consisting of eight biological replicates (Figure 4.1 B). The assay ended one week after the last spray (99 days post-inoculation).

Potassium permanganate ($KMnO_4$) was chosen as control treatment due to the need of dealing with natural infections of powdery and downy mildews, which normally occur under greenhouse conditions. This chemical is effective against these pathogens due to its strong oxidative properties (La Torre *et al.*, 2004) and we used the assumption that, when sprayed exclusively on leaves, it would not interact in a significant way with the wood mycobiome. Concerning the treatments 'Copper-Sulfur' and 'Systemic fungicides', the two active ingredients of each treatment were sprayed approximately 3 h apart from one another.

Table 4.1. Chemical treatments sprayed fortnightly on grapevine rooted cuttings. Six applications were performed during three months. Each active ingredient was sprayed separately.

Treatment	Active ingredient	Trade name	Manufacturer	Formulation	Tested concentration
Control	Potassium permanganate[a]	Permanganato de potassio basi	Laboratorios Basi	Capsules 500 mg	1.0 g/L
Copper - Sulfur	Copper oxychloride[b]	Cuprocol®	Syngenta	Copper oxychloride 36.5%	2.5 mL/L
	Sulfur[c]	Microthiol Special Disperss	Epagro	Sulfur wettable powder 80%	6.0 g/L
Systemic fungicides	Fosetyl-Aluminium[b]	Aliette Flash®	Bayer	Fosetyl-Aluminium 80%	2.5 g/L
	Penconazol[c]	Topaze®	Syngenta	Penconazol 10.5%	35 mL/100L
Blad	Blad-containing oligomer (BCO)[a]	Fracture®	CEV/CONVERDE	BCO 20%	2.0 mL/L

[a] treatments that target simultaneously powdery and downy mildews pathogens; [b] treatments that target downy mildew; [c] treatments that target powdery mildew

4.2.2 Assessment of the plant health status

Three parameters were evaluated to assess the health status of the plants at the moment of sampling. First, a visual inspection of the leaves and green shoots to establish whether any symptomatology related to the pathogen or skopobiota inoculation occurred. Second, the shoot length was recorded, to understand if inoculated fungi and/or treatments had an effect of the growth of the plants. Lastly, for each combination of inoculum – treatment, three vines were subject to a destructive inspection to assess the presence and degree of internal wood symptomatology (e.g. brown streaking) visible in the stem of plants, and starting from the inoculation point. Three categories of symptomatology degree were established: category 1, absence of wood symptoms; category 2, presence of brown streaking in the proximities of the inoculation point; category 3, presence of brown streaking that extended several centimeters from the inoculation point (Figure 4.2).

4.2.3 Wood sampling, DNA extraction and amplification

Grapevines were uprooted and the stem processed under laboratory conditions. The bark was sterilized by dipping the stem in 70% (v/v) ethanol followed by flame sterilization, and then removed with the aid of a sterile scalpel. Exactly 2 cm of wood were sampled, 1 cm below the inoculation point (Figure 4.1), for five plants within each combination inoculum – treatment. Samples were frozen, freeze-dried and stored at - 80 °C.

Wood samples were ground to dust using sterile mortars and pestles, aided by liquid nitrogen. An aliquot of ground wood (0.25 g ± 0.01 g) of each sample was added to DNA extraction columns (FastDNA™ SPIN Kit for Soil, MP Biomediacals® LLC) and total DNA was extracted as described by the kit manufacturer. Three negative controls consisted in extraction columns, without wood, that underwent the DNA extraction procedure.

The amplicon chosen for the metabarcoding analysis targets the Internal Transcribed Spacer ITS2 region, and the primer set selected was ITS86F and ITS4R (Op De Beeck *et al*., 2014). For building libraries we used a double-step PCR approach as reported by Feld *et al.* (2016). The full primer sequences including Illumina overhangs are: ITS86F (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGAATCATCGAATCTTTGAA-3’) and

ITS4R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-TCCTCCGCTTATTGATATGC-3').

Every first-PCR reaction contained 12.5 µL of Supreme NZYTaq II 2x Green Master Mix™ (NZYtech™), 0.5 µL of forward and reverse primer from a 10 µM stock, 1.5 µL of sterile water, and 5 µL of template. Each reaction was pre-incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 40 s; a further extension was performed at 72 °C for 10 min.

Second PCR-step for barcoding, MagBio bead fragment-purification and Qubit quantification was performed as reported in Gobbi *et al*. (2018). Final pooling was performed at 10 ng/sample. DNA Sequencing was performed using an in-house Illumina MiSeq instrument and 2x250 paired-end reads with V2 Chemistry.

4.2.4 Bioinformatics

After sequencing, demultiplex was performed using an Illumina MiSeq platform and the raw data were analyzed using QIIME 2 v. 2018.2 (Caporaso *et al.*, 2010), using the same pipeline described in Gobbi *et al.* (2018); denoised reads were trimmed by 15 bp on the left to remove adapters and were then analyzed using DADA2 with the exact sequence variants (EVS) methods (Callahan *et al*., 2017). To minimise barcoding noise, OTU counts below 25 reads were filtered out across all samples. Taxonomic assignments were performed at 99% identity using qiime feature-classifier classify-sklearn with a Naïve-Bayes classifier trained with UNITE (Nilsson *et al*., 2013) v7.2 for ITS. After taxonomy assignment, the dominant features assigned to high taxonomical ranks such as order, class or family were further investigated using BLAST to refine the analyses (Bokulich *et al*., 2018).

4.2.5 Data analysis

Shoot length data was subjected to analysis of variance (two-way ANOVA model) to evaluate the effects of factors 'fungicide treatment' and 'inoculum type'. Significant means were compared using the Tukey post hoc test at a 5% significance level (GraphPad Prism 7.05).

The frequency table and its taxonomy table were combined, converted to biom format in QIIME (Caporaso *et al*., 2010), then merged with a table of metadata into an S4

object and analysed in R (v. 3.4.3) using the following packages: phyloseq, v. 1.22.3 (McMurdie and Holmes, 2013); vegan, v. 2.5.2 (Oksanen *et al*., 2007); DeSeq2 v. 1.22.1 (Love *et al.*, 2014); ggplot2, v. 3.0.0 (Wickham, 2016); metacoder, v. 0.2.1.9005 (Foster *et al.*, 2017); adespatial, v. 0.1.1 (Dray *et al*., 2018); data.table, v 1.10.4.3 (Dowle and Srinivasan, 2017). R code is publicly available at github.com/Marieag/EMG.

The alpha diversity was measured using the Shannon diversity index and Pielou's evenness, and tested with one-way ANOVA with Tukey's HSD (Honestly Significant Difference) post hoc to determine differences among treatments and inoculum types.

We analyzed the beta dispersion to measure between-sample variances in abundance, computing average distances of the individual samples. The resulting ordination was plotted using non-metric multidimensional scaling (NMDS) combined with a Jaccard index matrix. To assess overall inter-group variance, we performed a PERMANOVA, using a Jaccard distance matrix with 999 permutations.

To investigate any systematic changes between treatments, we calculated the differential abundance of any taxon with a relative abundance of more than 0.1% using DESeq2. Twenty-four pairwise analyses were run for every combination of fungicide treatment per inoculum type, log2 fold changes were calculated for each taxon resolved to species or genus level, and tested for significance using a Wald test on the negative binomial distribution of the pairwise treatments. The p-value threshold was set to 0.05, and log fold change threshold to 0.1. In order to illustrate the results and to factor in the relative abundance of unresolved species, we created differential heat trees using MetacodeR. A Wilcoxon Rank Sum test was applied to test differences between the same species in different treatments, and the resulting p-values were corrected for multiple comparisons using FDR, as implemented in MetacodeR. p-Value threshold was set to 0.05.

4.3 Results

4.3.1 Evaluation of the plant health parameters

Visual inspection of the grapevine cuttings, shoots length measurements and wood examination occurred on the day of sampling. The visual inspection revealed no foliar

symptomatology attributable to the wood pathogen *P. chlamydospora* or to the inoculated skopobiota.

The statistical analysis of the green shoot length measurements, performed as a two-way ANOVA, revealed no significant differences for the factor 'inoculum type' ($p = 0.228$), meaning that shoot growth was not influenced by the inoculation with pathogen, skopobiota or the combination of both. However, a positive effect was present when comparing the factor 'treatment' ($p < 0.01$), revealing that BCO and/or systemic fungicides increased the growth of the shoots of inoculated plants (pathogen, skopobiota and pathogen + skopobiota), when compared with one or both the two other treatments (Table 4.2).

Table 4.2. Measurements of shoots length (cm). Grapevines were inoculated with a control (Water), *P. chlamydospora* (Pathogen), a consortium of wood endophytes (Skopobiota), and a combination of both (Pathogen + Skopobiota). Grapevines were also treated with a foliar spray of potassium permanganate (Control), copper oxychloride and sulfur (Copper – Sulfur), fosetyl-Al and penconazol (Systemics), blad-containing oligomer (BCO). Numbers followed by the same letter are not statistically different according to Tukey's *post hoc* test ($P < 0.05$).

	Water	**Pathogen**	**Skopobiota**	**Pathogen + Skopobiota**
Control	92.5 ab	75.9 a	76.8 a	77.4 a
Copper – Sulfur	88.1 ab	77.5 a	96.1 ab	82.8 ab
Systemics	89.4 ab	100.3 b	101.5 b	93.5 ab
BCO	101.3 b	88.6 ab	104.3 b	110.3 b

The examination of the wood revealed different degrees of symptomatology, ranging from absence of symptoms to extensive brown streaking departing from the inoculation point, as shown in Figure 4.2. Plants inoculated with *P. chlamydospora* presented extensive wood discoloration (Category 3; Figure 4.2 C, D), under all spray

treatments; while mild-to-absent symptoms, such as those shown in Figure 4.2 (A, B) were recorded when *P. chlamydospora* was inoculated along with the skopobiota. Plants inoculated with only water or the skopobiota did not show brown wood streaking symptoms, with the exception of the Copper-Sulfur treatment, suggesting that either copper oxychloride or sulfur or the combination of both may contribute to this plant response, independently from the inoculation of the pathogen.

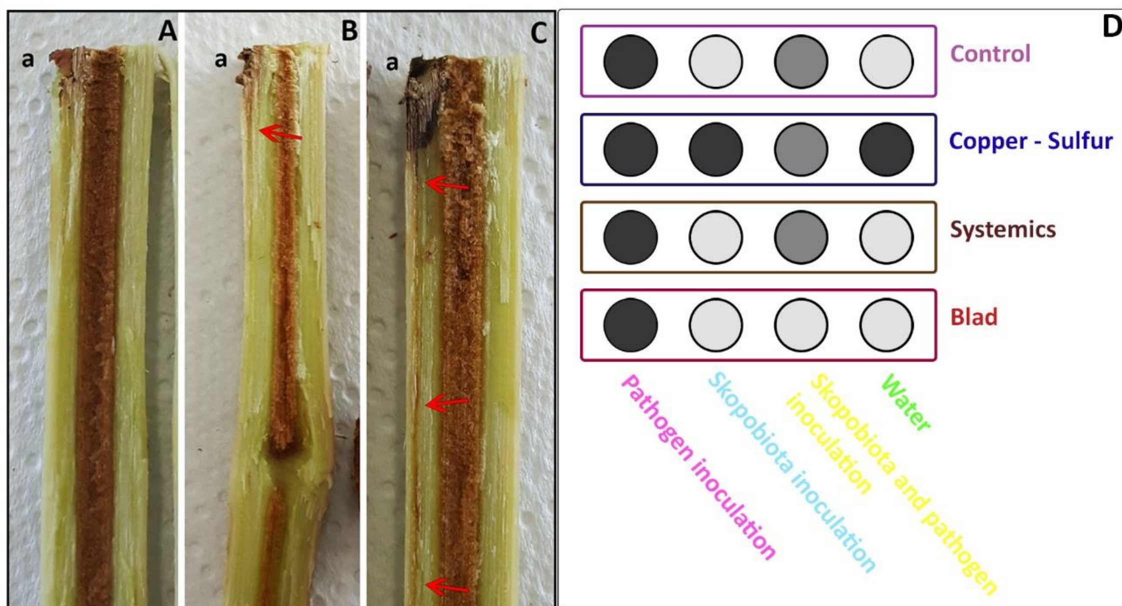

Figure 4.2. Grapevine rooted cuttings were examined revealing different degrees of brown wood streaking symptomatology. Category 1, absence of symptoms (A) departing from the inoculation point (a) downwards; Category 2, presence of mild symptomatology (B); Category 3, extensive symptomatology (C). In (D), each category is presented in a different scale of gray, as an average of the observations (n = 3) for each treatment - inoculum, where Category 1 is shown in light gray, Category 2 in gray and Category 3 in dark gray.

4.3.2 Sequencing dataset description

The dataset containing the NGS data produced and analyzed in this study account for 78 samples. These samples are represented by 6.454.075 high quality reads distributed in 506 unique features. The average number of reads per sample is 82.744, which

allowed an adequate sequencing depth to unravel the complexity of the grapevine cuttings wood mycobiome.

Using BLAST to refine the taxonomic classification, taxon 'Pleosporales incertae sedis' was assigned to *Epicoccum nigrum* in 98.12% of the reads. *Alternaria* sp was retrieved in 16 sequence variants, with the dominant feature correspond to 97.88% of the total. After Blasting this dominant sequence, the assignment was refined to *Alternaria alternata*, with 100% identity. The genus *Cladosporium* sp. appears 12 times in our database for a total of 61288 high quality reads. Blasting the four dominant features, which cover 94.7% of the total amount of reads, they were all assigned to *Cladosporium* sp.

4.3.3 A qualitative overview of the wood mycobiome

The diversity of fungal taxa identified examining all grapevine cuttings (n= 78) amounts to 66 species or genera of both ascomycetes and basidiomycetes. Among these 66 taxa, 30 of them are represented in a relative abundance (RA) greater than 0.1%, while the remaining 36 taxa are considered rare taxa (RA < 0.1%; Tables 4.3 and S4.5). Among the 30 most abundant taxa, several genera of ascomycetes are known to be involved in GTDs (e.g. *Neofusicoccum, Diaporthe, Cadophora*, *Fusarium*), while no pathogenic basidiomycetes were detected (Table 4.3). The most frequent (non-inoculated) taxa are *Debaryomyces* sp., *Cryptococcus* sp., *Malassezia restricta*, *M. globosa, Diaporte* sp*., Acremonium alternatum, Candida sake* and *C. friedrichii*. On the other hand, 16 out of the 30 taxa shown in Table 4.3 were detected in less than 10% of the total number of vines and, among these 16, 8 of them were present only in a single plant (1.3%; RA > 0.1%). This observation highlights the high variability often encountered when comparing individual plants.

The metagenomic analysis detected *Phaeomoniella chlamydospora* in 28% of non-inoculated plants (RA > 0.1%), while the presence of wood endophytes that composed the skopobiota, in non-inoculated grapevines, revealed to be very low. *Alternaria* sp. and *Cladosporium* sp. were present in 8% of the plants, while *E. nigrum* (Pleosporales incertae sedis) and *Aureobasidium pullulans* in 3% of them (RA > 0.1%).

Table 4.3. Taxonomic classification of the most abundant taxa identified to genus or species level in the wood of grapevine rooted cuttings. The relative abundance of the listed taxa was equal or greater than 0.1%. Species between parentheses were determined using BLAST.

Phylum	Family	Species
Ascomycetes	-not available-	*Circinotrichum maculiforme* †
	Botryosphaeriaceae	*Neofusicoccum* sp.*†
	Cucurbitariaceae	*Pyrenochaeta* sp. †
	Davidiellaceae	*Cladosporium* sp.
		Cladosporium sphaerospermum
	Debaryomycetaceae	*Meyerozyma guilliermondii* †
	Diaporthaceae	*Diaporthe* sp.*
	Didymellaceae	Pleosporales Incertae sedis (*Epicoccum nigrum*)
	Dothioraceae	*Aureobasidium pullulans*
	Helotiales	*Cadophora* sp.*†
	Herpotrichiellaceae	*Phaeococcomyces nigricans* †
		Phaeomoniella chlamydospora *
	Hypocreales	*Acremonium alternatum*
		Ilyonectria destructans *†
	Nectriaceae	*Fusarium* sp. *†
	Pleosporaceae	*Alternaria* sp. (*Alternaria alternata*)
	Saccharomycetaceae	*Debaryomyces* sp.
		Candida friedrichii
		Candida parapsilosis †
		Candida sake
		Candida tropicalis †
Basidiomycetes	Cystofilobasidiaceae	*Cystofilobasidium capitatum* †
	Filobasidiaceae	*Naganishia* sp. †
	Malasseziaceae	*Malassezia* sp. †
		Malassezia restricta
		Malassezia globosa
		Malassezia sympodialis †
	Peniophoraceae	*Peniophora* sp. †
	Sporidiobolaceae	*Sporidiobolus* sp. †
	Tremellaceae	*Cryptococcus* sp.

(*) taxa associated with grapevine trunk diseases
† taxa detected in less than 10% (n = 8 or less) of the total number of vines (n = 78)

4.3.4 Alpha diversity

The boxplots in Figure 4.3 show the Shannon diversity and Pielou's evenness in grapevines treated with different fungicides and inocula. The Shannon diversity and evenness of the resident mycobiome of non-inoculated plants did not vary in relation to the fungicide treatment, according to Tukey's HSD ($P > 0.05$). On the other hand, the inoculation of P. chlamydospora comported changes in both diversity and evenness ($P < 0.05$) of the mycobiome, when vines were sprayed with blad (Figure 4.3). This active ingredient induced a reduction in Shannon diversity when compared to control-treated plants, as well as a strong trend when comparing blad-treated plants to those treated with systemic fungicides ($P = 0.058$) . No diversity differences were significant among the other treatments ($P > 0.05$). The evenness of the fungal communities significantly varied when comparing blad-treated with copper-sulfur-treated vines (Figure 4.3), and a strong trend was detected when comparing the former treatment with systemic fungicides-treated vines ($P = 0.051$). The application of the different fungicides did not affect the Shannon diversity and evenness of vines inoculated with either the skopobiota alone or the skopobiota and the pathogen ($P > 0.05$).

4.3.5 Beta dispersion

The Jaccard's index, when visualized in a non-metric multidimensional scaling (NMDS) plot, shows a considerable overlap for different fungicide treatments and inoculum types (Figure 4.4). The PERMANOVA analysis indicates a significant difference between groups ($p = 0.001$) but, looking at the ordination, the difference seems to lie in the clustering of the observations, rather than any distinct difference in sample composition. For example, the fungal communities in BCO–treated plants cluster more tightly than the Control–treated ones, and a similar trend applies to non-inoculated vines when compared with those inoculated with both pathogen and skopobiota.

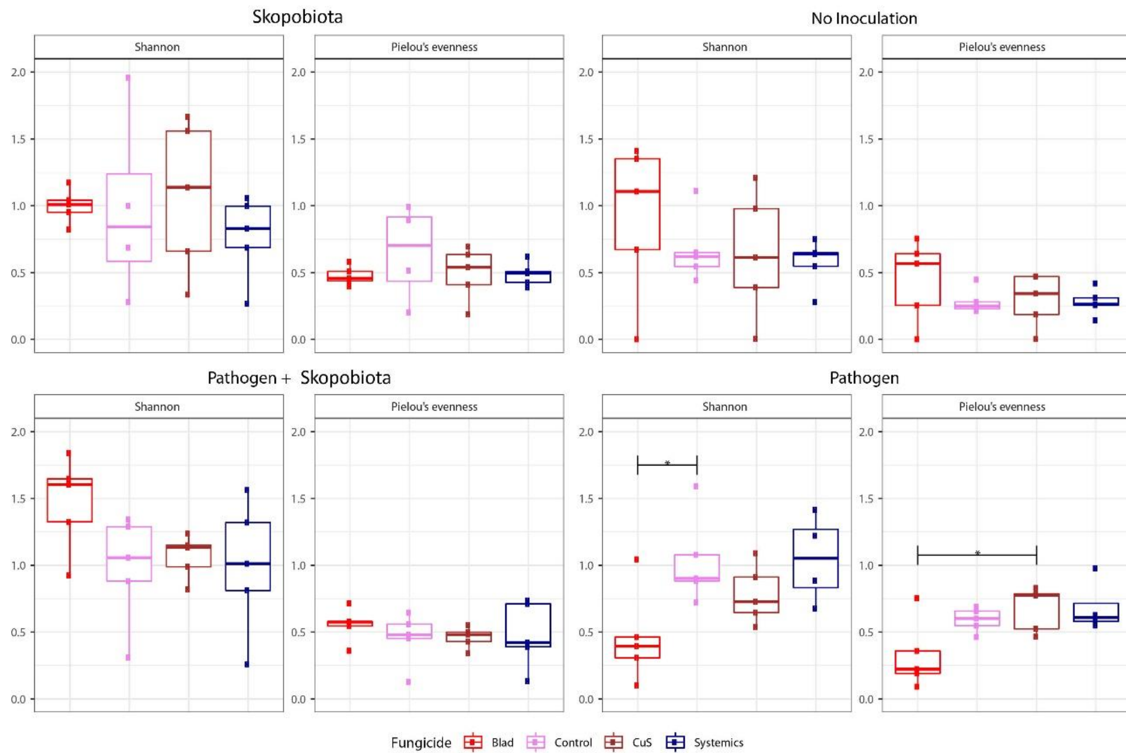

Figure 4.3. Box plots of diversity indexes (Shannon, Pielou's evenness) of the fungal community present in grapevine cuttings added with water (No Inoculation), or inoculated with P. chlamydospora (Pathogen), a consortium of fungal wood endophytes (skopobiota), or both P. chlamydospora and skopobiota. Inoculated plants were sprayed with either blad or potassium permanganate (Control) or copper oxychloride and sulfur (CuS) or penconazol and fosetyl-aluminium (Systemics). n= 5 for each combination inoculum/treatment. The black, horizontal brackets at the top of the figures denote statistical comparisons of the two treatments at each end of the bracket, calculated using one-way ANOVA with Tukey's HSD post hoc. Statistical differences are shown by asterisk, where P < 0.05 = *.

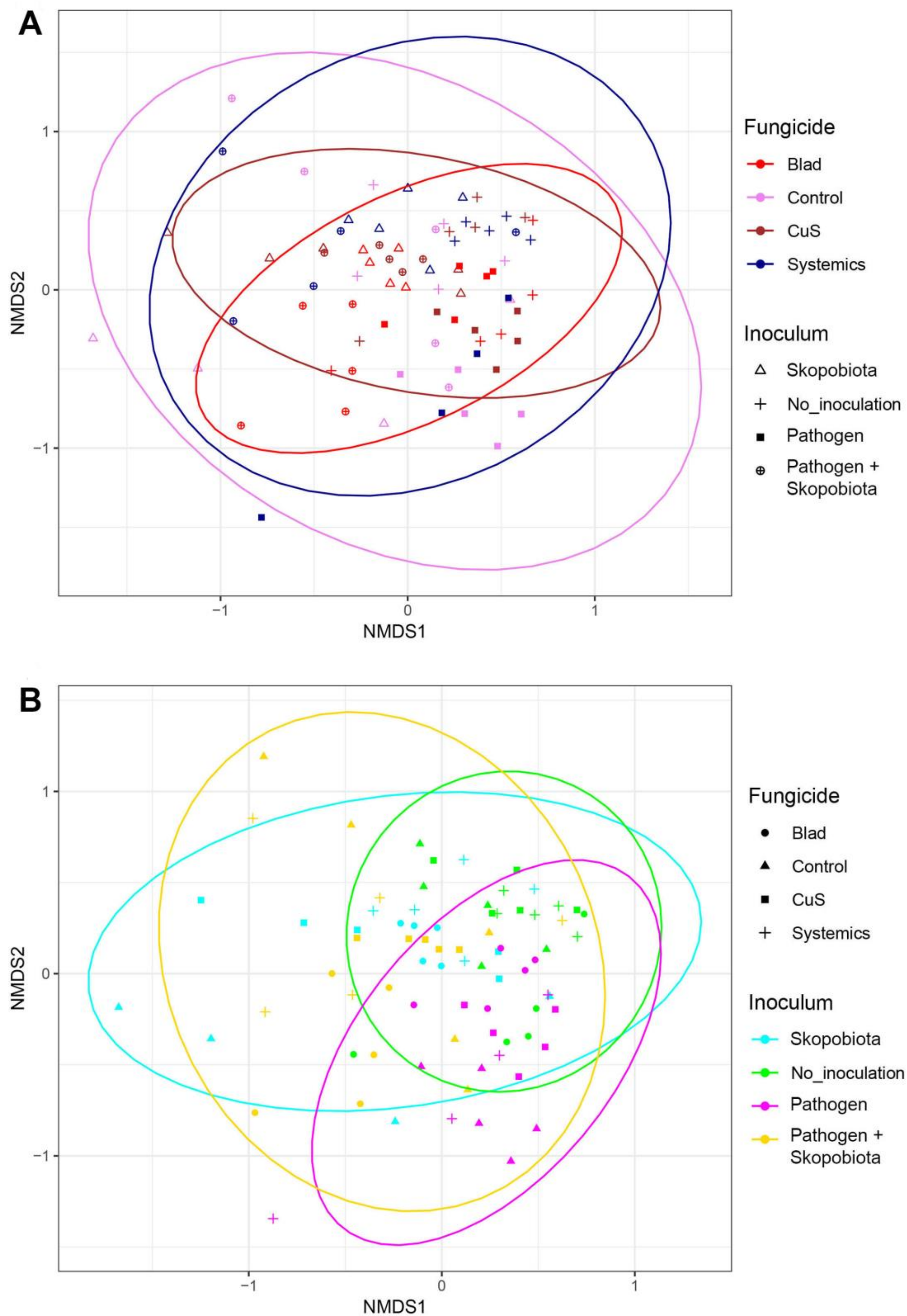

Figure 4.4. Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in grapevine cuttings added with water (No_inoculation), *P. chlamydospora* (Pathogen), a consortium of wood endophytes (Skopobiota), or both *P. chlamydospora* and skopobiota. Inoculated plants were sprayed with either Blad-containing oligomer (Blad) or potassium permanganate (Control) or copper oxychloride and sulfur (CuS) or penconazol and fosetyl-aluminium (Systemics). Ellipses illustrate the multivariate normal distribution of samples within the same fungicide (A) or inoculum (B) group.

4.3.6 Effect of inoculum type and fungicide treatments on taxa abundance

When examining the effect of fungicide treatments on the fungal communities of both non-inoculated and inoculated plants, no differences are detected according to the Wilcoxon test ($p > 0.05$). However, several taxa were found to have significantly different abundances between treatments according to the Wald tests on the pairwise treatment comparisons, and the combined metacoder and DESeq analyses revealed up- and downregulation of numerous taxa under different treatments (Figures 4.5 and 4.6). Nevertheless, some of the differently abundant taxa are found in less than 10% of the total number of plants (Table 4.3), therefore, their differential abundance may not lie in the effect of the treatments. For this reason, these taxa are not going to be further examined.

In non-inoculated vines, *Candida sake* and *C. friedrichii*, present in Control-treated plants, are absent in BCO-treated vines; similarly, *C. sake*, *Malassezia restricta* and *M. globosa* are present in Control-treated plants but absent in Systemic fungicide-treated vines; *Diaporthe* sp. was detected under all treatments with the exception of Systemic fungicides. None of the most frequent taxa differ significantly when comparing Control- with Copper-sulfur-treated grapevines (Figure 4.6).

In inoculated vines, we assumed that a successful colonization only occurred when inoculated fungi were detected in at least 50% of the biological replicates (per treatment/inoculation combination). The inoculation with *P. chlamydospora*, whether alone or in combination with the skopobiota, resulted in a successful wood colonization, 1 cm below the inoculation point, in 87% of the cases. In the pathogen-only inoculation, fungicide treatments affected the relative abundance of *P. chlamydospora* (Figure 4.5, Table 4.4), and that of other taxa. The foliar spray with BCO significantly decreased the relative abundance of this fungus when compared with the control and other treatments ($p < 0.05$).

In grapevines inoculated with the skopobiota, and sprayed with potassium permanganate (Control), only *Epicoccum nigrum* could successfully colonize the wood examined. The abundance of this ascomycete increased when plants were treated with BCO or Systemic fungicides (Table 4.4). *Alternaria* sp. was absent (or present in RA < 0.1%) in control-treated plants, while it was detected, in different abundances, under all other treatments (Table 4.4). The wood colonization by *Aureobasidium pullulans*

was poor-to-absent and it was not detected in 50% or more of the inoculated plants, suggesting that it perhaps was part of the resident mycobiome (Table 4.4). *Cladosporium* sp. was present exclusively in copper-sulfur-treated plants, although not detected in 50% or more of the inoculated plants.

The simultaneous inoculation of *P. chlamydospora* and the skopobiota resulted in a lower colonization success by the pathogen, when compared with the *P. chlamydospora*-only inoculum (Table 4.4), for all treatments except BCO. *Alternaria* sp. was detected under all fungicide treatments, except for the control-treated vines. *E. nigrum* exclusively colonized the wood when vines were treated with systemic fungicides or copper-sulfur. The BCO treatment facilitated the wood colonization by *Au. pullulans* and *Cladosporium* sp., that were not detected under any of the other treatments (Figure 4.6).

Table 4.4. Relative abundance of inoculated fungi. Grapevine cuttings were non-inoculated (No inoculation), inoculated with *P. chlamydospora* (Pathogen inoculation) or a consortium of wood endophytes (Skopobiota inoculation) or a combination of both (Pathogen + Skopobiota inoculation). Grapevines were treated with a control (potassium permanganate; C) or copper oxychloride and sulfur (CuS) or fosetyl-aluminium and penconazol (Sys) or blad-containing oligomer (BCO). Different colors represent different intervals of relative abundance (≤1; 1-5; 5-10; 10-20; >20)

	No inoculation				Pathogen inoculation				Skopobiota inoculation				Pathogen + Skopobiota inoculation			
	C	**CuS**	**Sys**	**BCO**	**C**	**CuS**	**Sys**	**BCO**	**C**	**CuS**	**Sys**	**BCO**	**C**	**CuS**	**Sys**	**BCO**
Phaeomoniella chlamydospora	-	-	-	11.6*	60.7*	64.0*	33.3*	4.9*	17.6*	1.8*	1.8	0.4	13.9*	2.0*	2.9	6.6*
Alternaria alternata (*Alternaria sp.*)	-	-	-	-	-	13.1	-	-	-	22.3*	4.3*	2.1*	5.0	5.5*	11.0*	4.3*
Epicoccum nigrum (Pleosporales incertae sedis)	-	-	-	-	-	-	2.0	-	2.8*	7.6*	24.1*	12.9*	6.4	25.9*	40.4*	-
Aureobasidium pullulans	-	-	0.8	-	-	-	-	-	0.7	-	-	0.2	-	-	-	2.5*
***Cladosporium* sp.**	-	-	-	0.3	0.3	-	0.1	-	-	2.5	-	-	-	0.2	-	2.7*

(*) 50% or more of the biological replicates were successfully colonized by the inoculated fungi

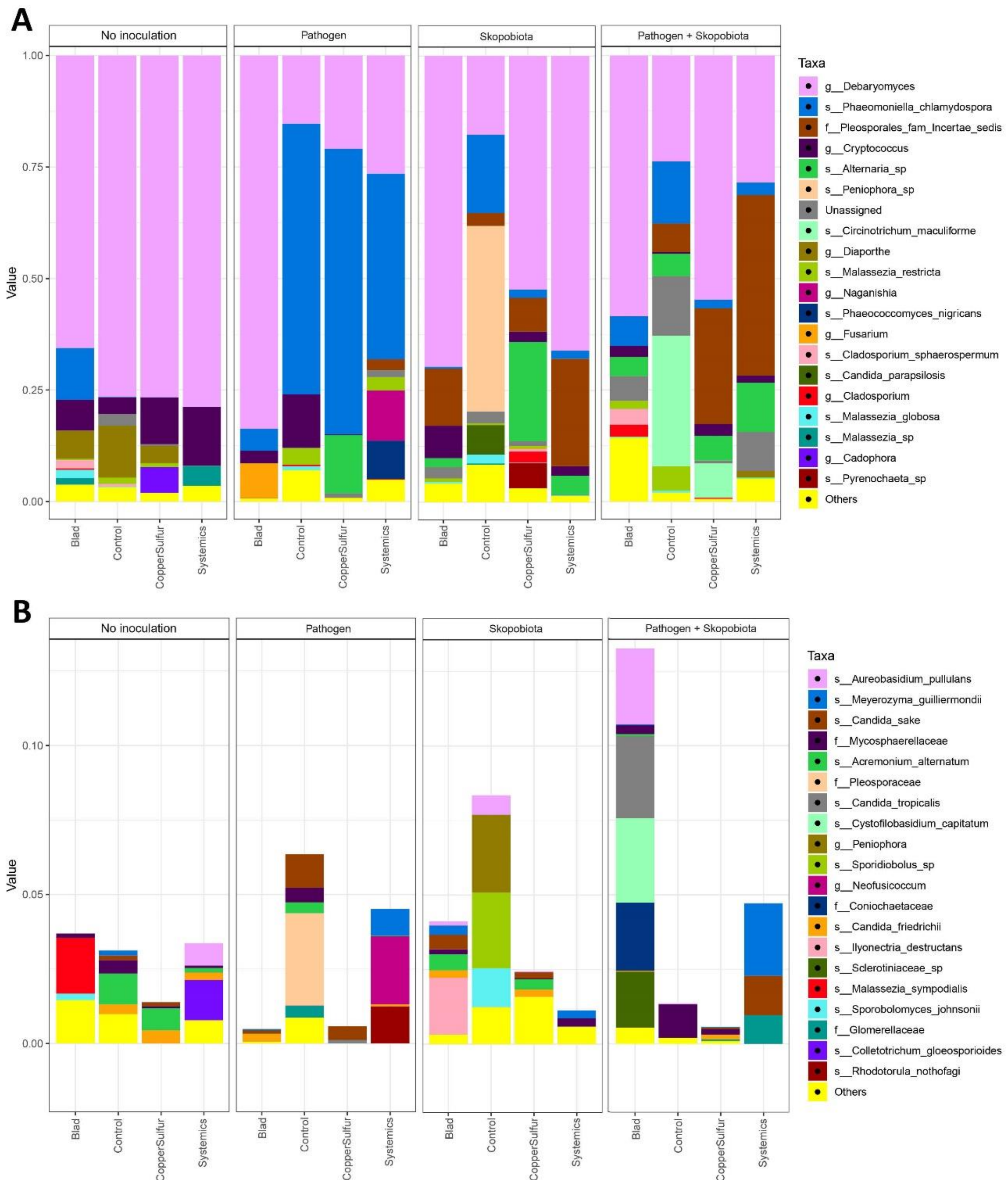

Figure 4.5. Barplots of the relative abundance of the 20 most abundant taxa (A) identified to species (s_), genus (g_) or family (f_) level, found in rooted grapevine cuttings non-inoculated (No inoculation), or inoculated with *P. chlamydospora* (Pathogen) or a consortium of wood endophytes (Skopobiota) or a combination of both (Pathogen + Skopobiota). Grapevines were treated with either blad-containing oligomer (Blad) or potassium permanganate (Control) or copper oxychloride and sulfur (CopperSulfur) or fosetyl-aluminium and penconazol (Systemics). 'Unassigned' are taxa identified to a lower taxonomic level than family or non-identified, 'Others' are taxa not included in the 20 most abundant. In (B) the 20 most abundant taxa within the 'Others' group of (A), are shown.

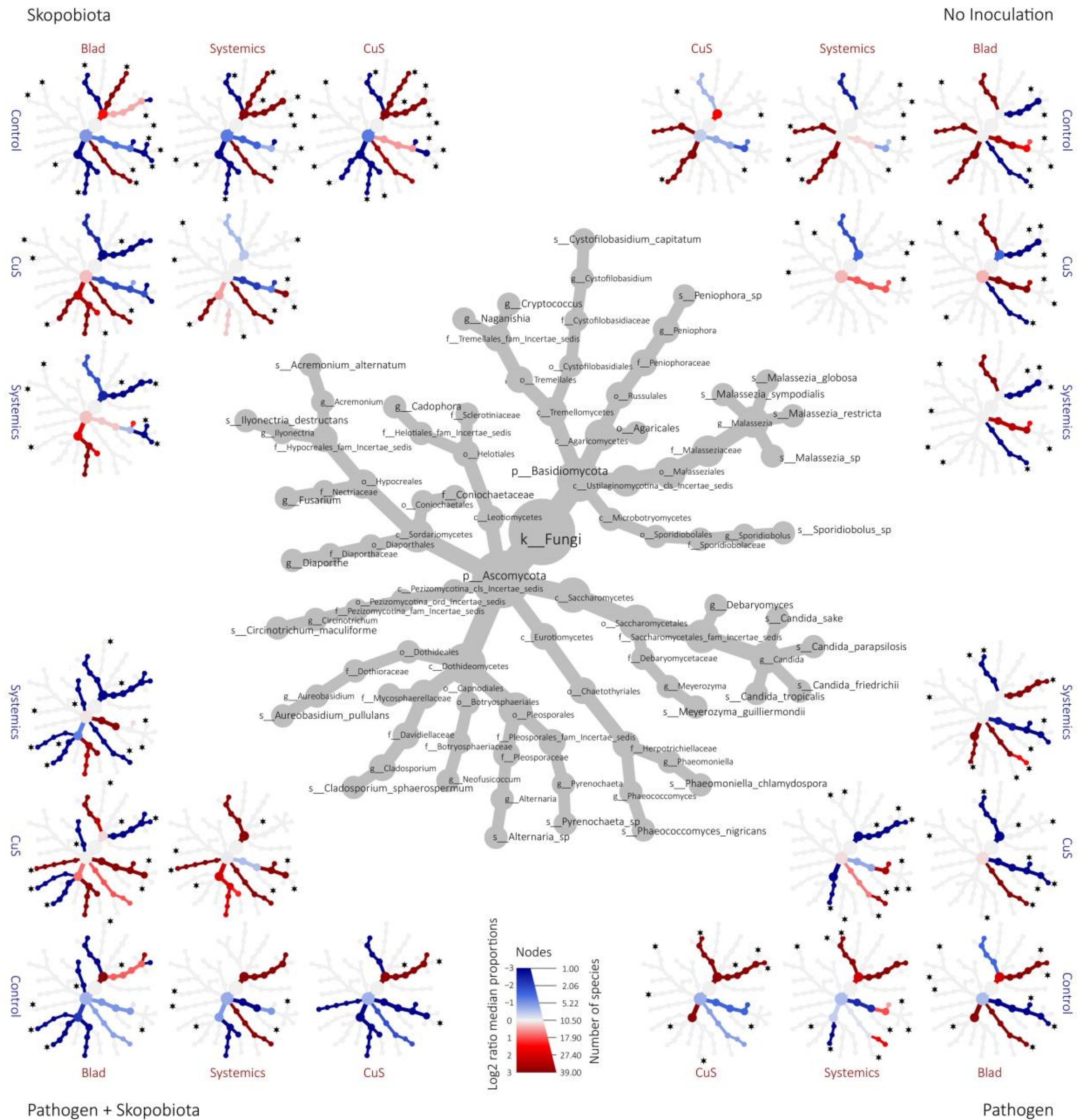

Figure 4.6. Differential heat tree matrixes depicting the change in species abundance between different tissue groups, represented in the dataset with a RA > 0.1%. The size of the individual nodes in the grey cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each treatment, with the colour illustrating the log2 fold change: a red node indicates a lower abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite. A black star next to a node represents the statistical differences according to DeSeq2 ($p < 0.05$).

4.4 Discussion

The authors would like to introduce the concept of 'skopobiota', a neologism built from the Greek word *skopos*, which translates in 'purpose'. A skopobiota represents a diverse array of selected microorganisms who may act synergistically in order to accomplish a predefined purpose in/on a specific environment. In light of the increasing understanding of the importance of microbiomes in the overall 'health' of environments, humans and plants (Alivisatos *et al.*, 2015; Dubilier *et al.*, 2015; Claus *et al.*, 2016), possible applications of skopobiota can be, among many others, to restore diversity in a system (e.g. depleted soils, reclaimed environments from pollution), in human health (e.g. probiotics, restoring gut microbiota) or to be exploited in the control of plant pathogens, as this work suggests.

The skopobiota of this study was conceived with two purposes. (1) To be used as reference, to monitor whether the application of different fungicides favor or inhibits the wood colonization success of any of its component organisms; (2) to test whether it has the potential to be exploited in the control of early infections by *P. chlamydospora*. By simultaneously inoculating the skopobiota and the pathogen, the authors hypothesized that the multiple interactions among organisms would decrease the pathogen's likelihood of successfully producing an infection in the plant. The organisms that constitute this skopobiota are endophytes of grapevine wood, believed to be potential candidates for biological control (Jayawardena *et al*., 2018; Mondello *et al*., 2017; Pancher *et al*., 2012; Pinto *et al*., 2018).

4.4.1 Visual inspection, shoot length and brown wood streaking

The absence of foliar symptoms in the vines inoculated with the skopobiota confirms the current understanding that none of the selected organisms composing the skopobiota, when present in the wood, are pathogenic in grapevines (Jayawardena *et al*., 2018). Concerning *P. chlamydospora,* Sparapano *et al.* (2001) observed that this fungus induced foliar symptoms appearance, albeit only several months/years post-inoculation, therefore the absence of foliar symptoms at the end of the experiment was expected.

The differences in length of the green shoot, only observed in response to fungicide treatments, suggest that both systemic fungicides and BCO positively affect the growth of vines, however, their mechanisms of action remain unclear.

Wood discoloration, in the form of brown wood streaking, is a result of the oxidation and polymerization of phenolic compounds, through the action of phenolases, a plant response to pathogen infections (Agrios 2012) but also to other stresses such as mechanical injuries (Pouzoulet *et al*., 2013). Absence of brown wood streaking in water- and skopobiota-inoculated vines, in plants treated with potassium permanganate, systemic fungicides and BCO, confirms the endophytic nature of the components of the skopobiota. On the contrary, copper-sulfur treated plants manifested extensive brown wood streaking. A recent report revealed that wood necrosis caused by grapevine wood pathogen *Neofusicoccum parvum* was promoted by the presence of high doses of copper in soil (Bruez *et al.*, 2017). Copper is also associated with fungal pigmentation production (Griffith *et al*., 2007), a virulence factor (e.g. fungal melanins; Jacobson, 2000); laccase synthesis, which is involved in wood degradation (Tychanowicz *et al*., 2006); and induction of phytotoxic effects, with changes in the plant's morphology, biochemistry and physiology (Bruez *et al.*, 2017). Overall, these clues suggest that copper may play a relevant role in the development of brown wood streaking, by interacting with plant, endophytes and/or pathogens. The extensive symptomatology recorded in plants inoculated with *P. chlamydospora*, under all fungicide treatments, indicates that the plant readily responded to the infection, and it did so independently of the abundance of *P. chlamydospora* in the wood (e.g. BCO treatment; Table 4.4). Wood pathogens are known to induce the appearance of brown wood streaking, but they are not always found in symptomatic wood, especially that farther from the inoculation point (Mugnai *et al.*, 1999). This suggests that, under some circumstances, the extent of wood streaking may not be correlated with the abundance of the pathogen. Moderate-to-absent wood symptoms were observed when co-inoculating *P. chlamydospora* and skopobiota, suggesting that the interactions among fungi resulted in a weaker or absent plant response to the infection. In fact, *P. chlamydospor*a has been previously reported in wood free from brown streaking symptoms (Rumbos and Rumbou 2001; Abreo *et al*., 2011; Hofstetter *et al*., 2012), and its ecology requires further investigation. In addition, the reduction in

wood streaking extent, due to the presence of fungal antagonists, has been previously reported in grapevines (Mondello *et al.*, 2017)

4.4.2 Effect of fungicides on the resident mycobiome and on the early colonization by endophytes

Previous studies revealed that fungicides application can affect the mycobiome of grapevines and other crops (Karlsson *et al.*, 2014; Morrison-Whittle *et al.*, 2017), although none of them investigated the fungal communities present in the wood. This study shows that some of the fungal taxa composing the resident wood mycobiome of grapevine cuttings cv. Cabernet Sauvignon are affected by fungicides. This observation suggests that active ingredients interacted with fungi either directly or indirectly, for example stimulating plant defenses. In fact, copper oxychloride is known to be absorbed in the xylem of grapevines, when sprayed, although in a much lower concentration than that found on the bark (Di Marco *et al.*, 2011). Further studies are necessary to confirm this preliminary understanding, especially in light of the high variability in taxa composition and abundance that characterize individual cuttings. Moreover, the DNA of dead fungal cells, supposedly affected by fungicides, may persist in the wood, during short-term experiments, hiding the real effect of the treatments.
The effect of fungicides application on the early colonization by fungal endophytes composing the skopobiota reveals that some interaction occurred, and that fungicides have the potential of shaping the wood mycobiome by favoring/inhibiting the early wood colonization of specific fungi. Nevertheless, it remains to be elucidated whether this interaction is (i) skopobiota-fungicide or (ii) fungicide-plant, resulting in a fungicide-plant plant-skopobiota interaction, or (iii) a combination of both. It is important to mention that the colonization success of the endophytes composing the skopobiota may be influenced by several other factors, such as the resident mycobiome, climatic conditions, and health status of the vine, making this a case study rather than a general understanding.

4.4.3 Effect of fungicides on the early colonization of *P. chlamydospora*

The copper-sulfur and systemic fungicides treatments did not affect the colonization success of the pathogen, within the timings of the experiment. A preliminary study in

support of the neutral effect of copper oxychloride on the colonization success of *P. chlamydospora* was presented by Di Marco *et al*. (2011), although no literature is available on the influence of sulfur or other synthetic fungicides. Blad-containing oligomer was the only active ingredient capable of significantly inhibiting the wood colonization success of *P. chlamydospora*. This is the first report of a foliar spray capable of reducing the wood colonization success of this pathogen. BCO is a recently developed non-toxic fungicide with large spectrum activity (Monteiro *et al*., 2015). It is a large size oligomer (210 kDa), known as contact fungicide, also effective against *P. chlamydospora* (Del Frari *et al.*, 2018), and data suggest that it also interacts with plants, for example as growth enhancer. Due to its large size, it is very unlikely that BCO reached the xylem where the pathogen was located, which excludes its contact fungicidal activity. The authors hypothesize that BCO interacted with the plants, for example activating natural defenses, which in turn inhibited the growth of *P. chlamydospora*. Research on the physiological response of grapevine after treatment with BCO is underway.

4.4.4. Use of a skopobiota in the biological control of *P. chlamydospora*

As postulated, the wood colonization success of *P. chlamydospora* was greatly reduced when co-inoculated with a consortium of wood endophytes (skopobiota) that are considered potential antagonists. Some of the possible explanations are found in the multiple inhibitory effects that occur during conidia germination and mycelial growth (e.g. release of metabolites) and by increasing the competition for space and nutrients. In addition, only minor changes in the abundance of the pathogen are observed with the different fungicide treatments, making this skopobiota a very promising tool in biological control. Interestingly, the BCO treatment facilitated the wood colonization by both *Cladosporium* sp. and *Au. pullulans*, while inhibited that of *E. nigrum*, in the presence of *P. chlamydospora*, when compared with the results obtained from the skopobiota-only inoculation. This observation suggests the occurrence of complex interactions in which not only the fungicide, but also the pathogen, play a primary role, resulting in a selective wood colonization. In addition to the fungicides, the pathogen-skopobiota interaction may also be influenced by other factors, such as the plant and/or the resident mycobiome. Further investigation is necessary to confirm these

results also from a quantitative point of view, and to understand if the effect observed by the addition of this skopobiota is long lasting, and it may be successfully applied to plants with an established infection, as a curative strategy.

4.5 Conclusion

This study shows that some common fungicides that have been applied in viticulture for decades (copper-sulfur and systemics) affect the wood colonization success of some endophytes, with unpredictable consequences, both short and long term, on the wood mycobiome ecology. It is reasonable to wonder whether the wood mycobiome composition of grapevines before the fungicide era was considerably different from today's, and whether a diversity loss has occurred. As the diversity of a biological system is positively correlated with its stability (McCann 2000), a loss in endophytic or saprophytic species that antagonize GTD pathogens, such as *P. chlamydospora*, may be a possible explanation for the recent success of these pathogenic fungi. This work only addressed four endophytes, although the wood mycobiome of adult plants is known to be potentially colonized by hundreds of species. In conclusion, restoring or enhancing the diversity of the wood mycobiome, for example using skopobiota, may considerably improve our chances to control GTDs. While research on skopobiota advances, blad-containing oligomer seems a promising tool to confront early infections by *P. chlamydospora*. This active ingredient is non-toxic for the environment and humans, it has growth promoting properties, and strongly antagonizes the wood colonization success of *P. chlamydospora*, making it the most promising control mean currently available.

4.6 Acknowledgements

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4.7 Author contributions

GDF: conceived the study, performed greenhouse experiment, performed wet-lab work, interpreted and discussed the results, wrote the manuscript; AG: performed wet-lab work, performed bio-informatics analyses, wrote the manuscript; MRA: performed statistical analyses and data visualization; HO, LHH, RBF: supervised the study, reviewed the manuscript.

4.8 Supplementary material

Table S4.5. Fungal taxa identified to genus or species level in the wood of grapevine rooted cuttings, at relative abundances (RA) inferior to 0.1 % or 0.01% of the total.

RA < 0.1 %
Buckleyzyma sp.
Cenococcum geophilum
Colletotrichum gloeosporioides
Cryptococcus victoriae
Cyberlindnera jadinii
Cystofilobasidium infirmominiatum
Filobasidium magnum
Hannaella oryzae
Meira nashicola
Naganishia albidosimilis
Neophysalospora eucalypti
Peniophora piceae
Phyllosticta sp.
Pseudozyma sp.
Rhodotorula mucilaginosa
Rhodotorula nothofagi
Saccharomyces cerevisiae
Sporobolomyces johnsonii
Stemphylium sp.
RA < 0.01 %
Ceratobasidium sp.
Cladosporium fusiforme
Coriolopsis gallica
Cryptococcus heimaeyensis
Debaryomyces mycophilus
Debaryomyces prosopidis
Diaporthe phaseolorum
Dioszegia sp.
Erythrobasidium hasegawianum
Hyphodermella rosae
Itersonilia perplexans
Lecanicillium lecanii
Leucosporidium sp.
Phaeotheca triangularis
Pseudotaeniolina globosa
Tilletiopsis pallescens
Tubaria furfuracea

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CHAPTER 5

Characterization of the wood mycobiome of *Vitis vinifera* in a vineyard affected by esca. Spatial distribution of fungal communities and their putative relation with foliar symptoms.

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5. Characterization of the wood mycobiome of *Vitis vinifera* in a vineyard affected by esca. Spatial distribution of fungal communities and their putative relation with foliar symptoms.

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Abstract

Esca is a disease complex belonging to the grapevine trunk diseases cluster. It comprises five syndromes, three main fungal pathogenic agents and several symptoms, both internal (e.g. affecting the stem) and external (i.e. affecting leaves and bunches). The etiology and epidemiology of this disease complex remain, in part, unclear. Some of the points that are still under discussion concern the sudden rise in disease incidence, the simultaneous presence of multiple wood pathogens in affected grapevines, the causal agents and the discontinuity in time of foliar symptoms manifestation. The standard approach to the study of esca has been mostly through culture-dependent studies, yet, leaving many questions unanswered.

In this study, we used Illumina® next-generation amplicon sequencing to investigate the mycobiome of the wood of grapevines in a vineyard with history of esca. We characterized the wood mycobiome composition, investigated the spatial dynamics of the fungal communities in different areas of the stem and in the canes, and assessed the putative link between mycobiome and foliar symptoms.

An unprecedented diversity of fungi is presented (289 taxa), including five genera reported for the first time in association with grapevine's wood (*Debaryomyces*, *Trematosphaeria*, *Biatriospora*, *Lopadostoma* and *Malassezia*) and numerous hitherto unreported species. Esca-associated fungi *Phaeomoniella chlamydospora* and *Fomitiporia* sp. dominate the fungal community, and numerous other fungi associated with wood syndromes are also encountered (e.g. *Eutypa* spp., *Inonotus hispidus*). The spatial analysis revealed different abundances of taxa, the exclusive presence of certain fungi in specific areas of the plants, and tissue specificity. Lastly, the mycobiome composition of the woody tissues in proximity to the canopy that manifested foliar symptoms of esca, as well as in foliar symptomatic canes, was highly

similar to that of plants not exhibiting any foliar symptomatology. This observation supports the current understanding that foliar symptoms are not directly linked with the fungal community in the wood.
This work builds to the understanding of the microbial ecology of the grapevines wood, offering insights and a critical view on the current knowledge of the etiology of esca.

Key words esca disease, fungal diversity, grapevine, metabarcoding, wood mycobiome

5.1 Introduction

The phyllosphere, rhizosphere and endosphere of grapevine (*Vitis vinifera* L.) are characterized by the presence of complex communities of microorganisms that constantly interact with one another and with the plant, affecting it positively, neutrally or negatively (Bruez *et al*., 2014; Pinto *et al*., 2014; Zarraonaindia *et al*., 2015). Until a decade ago, the approach to characterize the mycobiome - namely the fungal community present in/on an organism - of grapevines, focused on culture-dependent studies in which fungi were isolated *in vitro* and identified morphologically and/or molecularly (Morgan *et al*., 2017). This approach remains valid to this day, however it presents several limitations, such as the impossibility of detecting uncultivable fungi, the bias of the cultivation conditions (e.g. growth medium, incubation parameters) and the difficulty of isolating species present in low abundances (Morgan *et al.*, 2017). In recent years, technologies like next-generation sequencing (NGS) have improved in quality and reduced in cost, which, in combination with ever more efficient bioinformatics tools, have allowed the exploitation of this method in the study of the molecular ecology of environmental DNA (eDNA) samples. In particular, DNA metabarcoding approaches have taken the investigations of microbiomes to a new level, surpassing some of the limitations which characterize culture-dependent studies. In fact, NGS studies have revealed a higher diversity of taxa and accurate relative abundances in samples coming from different environments,

including the vineyard (Peay *et al*., 2016; Morgan *et al*., 2017; Jayawardena *et al*., 2018). Despite these recent advances, culture-independent studies describing the microbial endosphere of grapevines are still scarce.

DNA metabarcoding is a promising tool to investigate the microbial communities present in the wood of grapevines, as it may lead to a new understanding of the complexity that characterizes grapevine trunk diseases (GTDs). This cluster of fungal diseases affects primarily the perennial organs of the plants, such as the trunk and roots, however secondary symptoms may be observed in leaves, bunches and shoots. Overall, GTDs cause a loss in vigor, productivity, quality of the yield and lifespan of the plants, with conspicuous economic consequences (Bertsch *et al*., 2013; Fontaine *et al*., 2016; Hofstetter *et al*., 2012). GTDs pathogens are phylogenetically unrelated, belonging to different families, orders and even phyla, although plants infected may reveal similar symptomatology. For example, wood discoloration and necrosis are symptomatology shared by all GTDs (e.g. Figure 5.1 C), whereas the 'tiger stripes' pattern in the leaves can be attributed to both grapevine leaf stripe disease (GLSD; Figure 5.1 F) and black dead arm (Mugnai *et al.*, 1999; Larignon *et al.*, 2009; Bertsch *et al*., 2013). Moreover, the simultaneous presence of several possible causal agents in infected grapevines complicates the outline of a clear etiological pattern (Bruez *et al*., 2016; Edwards and Pascoe, 2004; Mondello *et al*., 2017). This is especially true in the case of esca, a disease complex consisting of five separate syndromes (brown wood streaking of rooted cuttings, Petri disease, GLSD, esca and esca proper) in which, according to current literature, several pathogenic fungi play a role (Surico, 2009). These fungi may infect vines in the field, where conidia or other propagules reach fresh pruning wounds and start colonizing the xylem, or during the propagation process in nurseries (Gramaje *et al*., 2018). The pathogens most frequently associated with the first three syndromes are *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, two tracheomycotic ascomycetes; while the latter two syndromes are associated with the presence of the wood rotting basidiomycete *Fomitiporia mediterranea* (esca), especially common in Europe, or with the simultaneous presence of both tracheomycotic and wood rotting pathogens (esca proper; Surico, 2009). Along with these three players, other wood pathogens including, but not limited to, members of the Diatrypaceae and Botryosphaeriaceae are often found in symptomatic

plants (Bruez *et al*., 2014; Edwards and Pascoe, 2004; Hofstetter *et al*., 2012; Travadon *et al*., 2016). Studies that used the NGS approach to learn more about the grapevine endosphere are scarce (Dissanayake *et al*., 2018; Jayawardena *et al*., 2018) and none of them investigated the mycobiome of GTDs-affected plants.

This study focuses on a vineyard in Portugal, which ranks 11th in the world for wine production, with a total vineyard area of 195 kha (Aurand, 2017). *V. vinifera* cultivar Cabernet Sauvignon is the most cultivated worldwide, with a total vineyard area of 340 kha. It is considered susceptible to trunk diseases (Darrieutort and Pascal, 2007; Eskalen *et al*., 2007) and has already been cited in studies concerning the microbial ecology of wood, phyllosphere and grapes (González and Tello, 2011; Bruez *et al.*, 2014; Morgan *et al.*, 2017; Singh *et al*., 2018). This work aims to investigate the fungal communities present in the wood of grapevines, in a vineyard with history of esca proper. Three main objectives were set: (1) to characterize the mycobiome of the wood of *V. vinifera* cv Cabernet Sauvignon, in a vineyard located in the Lisbon area (Portugal), using Illumina® NGS; (2) to understand the spatial distribution of the communities present in different areas of perennial wood and in annual wood; (3) to understand whether there is a link between the microbial communities of the wood and the expression of foliar symptoms of esca.

5.2 Materials and Methods

5.2.1 The vineyard

Field sampling took place in the experimental vineyard (Almotivo) of the Instituto Superior de Agronomia, in Lisbon (38°42'32.7"N, 9°11'11.5"W). The vineyard has a density of 3333 plants/ha, the soil is classified as vertisoil, it is managed under conventional agricultural practices and there is no irrigation system. The selected cultivar was Cabernet Sauvignon grafted on 140 RU rootstock, 19 years old at the moment of sampling (planted in 1998), trained as Cordon Royat Bilateral and spur pruned. The field has a history of esca, with leaf-symptomatic grapevines accounting for less than 1% of the total plants in all recorded years (2015, 2016 and 2017). The selection of the plants used in this experiment was restricted to a block of 450 m^2.

The immediate surroundings of the vineyard, within a 25 m radius from the perimeter, are characterized by the presence of diverse vegetation. The majority of the species are listed in Table S5.4, found in the supplementary materials.

5.2.2 Sampling and experimental setup

Samples of perennial wood (PW) were taken in a non-destructive way, in April 2017, by means of hand-drilling the plants with a gimlet (Figure 5.1 B). The sampling procedure occurred as follows. The bark, on each sampling point, was removed with the use of a knife and the wounds were disinfected with ethanol (70% v/v); the gimlet was placed perpendicularly on the open wounds and manually forced in the wood until it went through the whole width of the plant. This allowed us to extract cores of wood (5 mm of diameter and approximately 60 mm long) which were immediately placed in sterile 15 mL falcon tubes and temporarily stored in ice (Figure 5.1 C). Samples were then frozen, freeze-dried and stored at -80 °C. After extracting each core of wood, the gimlet was sterilized by dipping it in a sodium hypochlorite solution (0.35 w/w of active chlorine) for 1 minute, followed by a rinse with ethanol (70% v/v) and then double-rinsed with sterile distilled water (SDW), in order to minimize cross-contamination.
Canes grown in the 2017 growing season were sampled, as annual wood (AW), in September, detaching them with pruning scissors, approximately 3 cm above the spur from which they departed (total length of the canes sampled 50 mm; Figure 5.1 F). Each sample was deprived of its bark, frozen, freeze-dried and stored at -80 °C until processing.

Concerning the PW, ten grapevines were sampled in 9 areas each, as shown in Figure 5.1 (B). Five of these plants did not show foliar symptoms of esca during the previous two growing seasons (years 2015 and 2016), while the other five presented leaf ‘tiger stripes’ symptoms, only in one of the two cordons (cordon 2; Figure 5.1 A), during the previous growing season (year 2016). The terms ‘asymptomatic’ and ‘symptomatic’, which will often be encountered in the rest of the text, refer exclusively to the foliar symptomatology and not to the wood symptomatology (unless specifically stated).
The tissue types corresponding to the nine sampling areas are: ‘Graft Union’ (GU), located approximately (3±1 cm) above the soil, on the graft union; ‘Trunk’ (T), (22±1

cm) above GU; ‘Upper Trunk’ (UT), (22±1 cm) above T; ‘Arm 1’ (A1), was located on the cordon, (36±2 cm) away from UT; the sample point ‘Spur 1’ (S1) is located on the cordon, right below the spur, (10±1 cm) from A1; ‘Arm 2’ (A2), located (22±1 cm) to the right of A1; ‘Spur 2’ (S2), (10±1 cm) from A2 (Figure 5.1). Sample points (A1, S1, A2 and S2) belonged to cordon 1, and the canopy departing from this cordon never exhibited foliar symptoms, for all 10 sampled plants. Sampling points (SA, symptomatic arm) and (SS, symptomatic spur) are the equivalent of points (A1) and (S1), but located in cordon 2. In this case, 5 out of the 10 sampled grapevines presented foliar symptoms in the canopy departing from (SS), while the other 5 plants had a non-symptomatic canopy.

Only one tissue type was examined in the AW, namely the ‘Canes’, where 15 canes were sampled from 10 plants. Five of them came from asymptomatic plants, while the other 10 came from symptomatic plants. Within these 10, 5 canes were foliar-symptomatic, while the other 5 were asymptomatic and sampled in cordon where the whole canopy was asymptomatic (Figure 5.1 F).

To address the three objectives of this study the sample points corresponding to different tissue types were combined as follows. (1) To characterize the mycobiome of the wood of the vineyard, all sample points were taken in consideration (n= 80 from wood, n= 15 from canes; Figure 5.1 A). (2) To learn about the spatial distribution of the mycobiome in the different areas of the plants, we used tissue types (GU – S2) (n= 10 per tissue type, total n= 70; Figure 5.1 D), along with asymptomatic canes (n= 10). (3) To understand the link between foliar symptoms expression and mycobiome of PW or AW, we created three groups per category. In the category ‘PW’, group (i) consisted in asymptomatic plants (cordon 1, points ‘A1 and S1’; n= 10), group (ii) consisted in symptomatic plants, sampled in the asymptomatic cordon (cordon 1, points ‘A1 and S1’; n= 10), group (iii) consisted in symptomatic plants, sampled in the symptomatic cordon (cordon 2, points ‘SA and SS’; n= 10) (Figure 5.1 E). The same applied for the category ‘AW’, where group (i) consisted in asymptomatic canes, sampled from asymptomatic plants, group (ii) consisted in asymptomatic canes, sampled from symptomatic plants, group (iii) consisted in symptomatic canes.

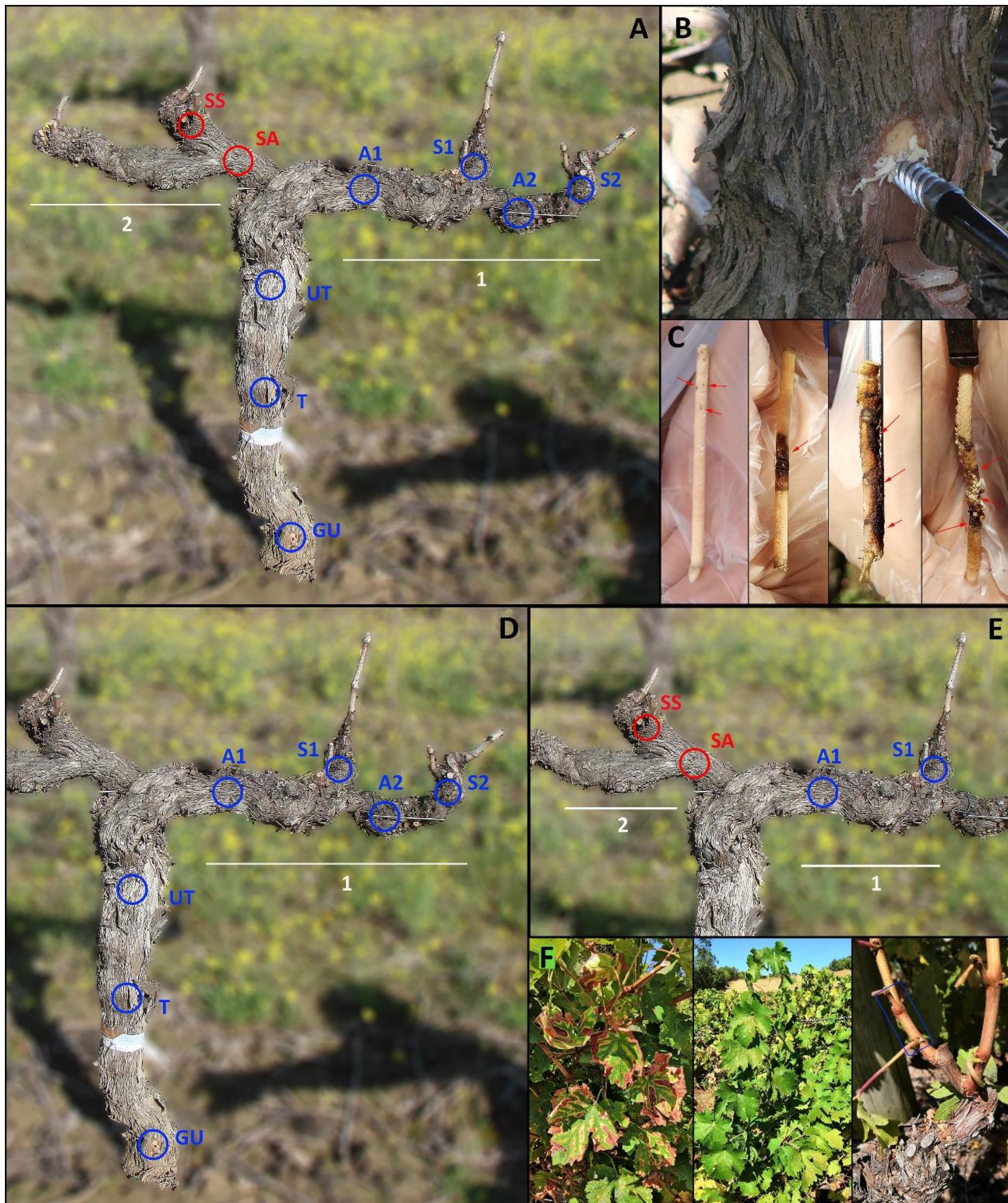

Figure 5.1. Sampling points in the perennial wood or annual wood of grapevine cv Cabernet sauvignon. (GU) Graft union, (T) Trunk, (UT) Upper trunk, (A1) Arm 1, (S1) Spur 1, (A2) Arm 2, (S2) Spur 2, (SA) Symptomatic arm, (SS) Symptomatic spur. Cordon (1) presented canopy with healthy leaves in all ten sampled plants, while cordon (2) presented foliar symptoms in the canopy, departing from SS, in five of the sampled plants (circles and letters in red). (A) Sampling points used to characterize the mycobiome of perennial wood – objective 1-; (B) Sampling procedure involved using a gimlet to drill the wood and extract wood cores; (C) Cores of wood extracted with a gimlet (red arrows indicate wood symptomatology). From right to left: brown wood streaking, wood necrosis, extensive wood necrosis, wood decay-white rot-wood necrosis. (D) Sampling points used to test the spatial distribution of fungal communities – objective 2-; (E) Sampling points used to examine the mycobiome present in the wood in proximity of the canopy with foliar symptomatology (AS, SS) and healthy (A1, S1) – objective 3-; (F) From left to right: symptomatic canes sampled from plants with foliar symptoms, asymptomatic canes sampled from plants with no foliar symptoms in either of the cordons or with foliar symptoms in only one of the two cordons; the sampling area for each cane is indicated by the blue rectangle.

5.2.3 DNA extraction, amplification, library preparation and sequencing

Wood samples were ground to dust using sterile mortars and pestles aiding the process with liquid nitrogen. An aliquot of ground wood (0.25 ± 0.01 g) of each sample was added to DNA extraction columns (FastDNA™ SPIN Kit for Soil, MP Biomediacals® LLC) and total DNA was extracted as described by the kit manufacturer. Three negative controls of the DNA extraction procedure were added.

The amplicon chosen in this study targeted the Internal Transcribed Spacer ITS1 region, and the primer set selected was ITS1F2 - ITS2 (Gaylarde *et al*., 2017) with overhang recommended by Illumina. For building libraries, we have been using a double-step PCR approach as reported by Feld *et al*. (2015). The full sequence of the primers, including Illumina overhangs, is the following: ITS1F2 (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GAACCWGCGGARGGATCA-3’) and ITS2 (5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GCTGCGTTCTTCATCGATGC-3’) (Gaylarde *et al*., 2017).

Each first-step PCR reaction contained 12.5 µL of Supreme NZYTaq II 2x Green Master Mix™ (NZYtech™), 0.5 µL of forward and reverse primers from a 10 µM stock, 1.5 µL of sterile water, and 5 µL of template. Each reaction was pre-incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 40 s; a further extension was performed at 72 °C for 10 min.

Second PCR-step for barcoding, fragment-purification by using MagBio beads and Qubit quantification was performed as reported in Gobbi *et al*. (2018). Final pooling was performed at 10 ng/sample. DNA Sequencing was performed using an in-house Illumina MiSeq instrument and 2x250 paired-end reads with V2 Chemistry.

5.2.4 Bioinformatics

After sequencing, demultiplex was performed using our Illumina MiSeq platform and the raw data were analyzed using QIIME 2 v. 2018.2 (Caporaso *et al.*, 2010) using the same pipeline described in Gobbi *et al.* (2018); denoised reads were trimmed 15 bp on the left to remove the adapters and then they were analyzed using DADA2 with the exact sequence variants (EVS) methods (Callahan *et al.*, 2017). Each ESV appears at least twice in the dataset. Singletons were discarded. Taxonomic assignments were performed at 99% identity using qiime feature-classifier classify-sklearn with a Naïve-

Bayes classifier trained with UNITE (Nilsson *et al.*, 2013) v7.2 for ITS. To test the three hypotheses underlying this work we separate the frequency table in three sub-tables which were tested under different conditions.

5.2.5 Statistics

The frequency table and its taxonomy were combined, converted to biom format in QIIME (Caporaso *et al*., 2010), then merged with a table of metadata into an S4 object and analysed in R (v. 3.4.3) using the following packages: phyloseq, v. 1.22.3 (McMurdie and Holmes, 2013); biomformat, v. 1.10.0 (McMurdie and Paulson, 2018), vegan, v. 2.5.2 (Oksanen *et al*. 2007); ggplot2, v. 3.0.0 (Wickham, 2016); igraph, v. 1.2.2 (Csardi, 2006), MetacodeR, v. 0.2.1.9005 (Foster *et al*., 2017); adespatial, v. 0.1.1 (Dray *et al*., 2018); data.table, v 1.10.4.3 (Dowle and Srinivasan, 2017); and microbiome, v. 1.4.0.R code is publicly available at https://github.com/marieag/EMG.

To assess the alpha diversity, Shannon diversity index and Pielou's evenness were calculated and tested using a one-way ANOVA with post hoc Tukey's HSD (Honestly Significant Difference), determining differences in these indexes between tissue types or tissue groups. We analyzed the β-dispersion to measure between-sample variances in abundance, computing average distances of the individual samples. The resulting ordination was plotted using the non-metric multidimensional scaling (NMDS) combined with a Jaccard index matrix. These ordinations were also performed with a Bray-Curtis dissimilarity matrix. To assess overall inter-group variance, we performed a PERMANOVA, using a Jaccard distance matrix with 999 permutations. In order to illustrate this effect size compared to the relative abundance of the taxa, we created differential heat trees using MetacodeR, illustrating the log2 fold change in species abundance. A Wilcoxon Rank Sum test was applied to test differences between the same species in different tissue groups, and the resulting p-values were corrected for multiple comparisons using FDR, as implemented in MetacodeR. P-value threshold was set to 0.05.

5.3 Results

5.3.1 Sequencing dataset description

This dataset, obtained by sequencing, consists of a total of 95 samples. This includes 80 samples collected from different parts of the PW and 15 from canes (AW). All of them represent a total of 20 plants and each one of these counts as an independent biological replicate. This dataset contains 2805 exact sequence variants which appear a total of 8.184.885 times among all the different samples.

5.3.2 Visual examination of the sampled wood

Before processing the samples for the molecular analysis, wood cores of perennial wood and samples of annual wood were visually examined to assess the presence of symptomatology. Approximately 10% of the wood cores were fully asymptomatic, 75% presented symptoms of tracheomycosis (e.g. brown wood streaking and/or wood necrosis), and 15% showed the presence of white rot, which was always associated with other tracheomycosis symptoms. The examination of the annual wood revealed that 100% of the samples were fully asymptomatic.

5.3.3 The wood mycobiome

The identification of sequences in our dataset revealed an unprecedented diversity. Taxa that were assigned to genus or species level are 289, 50 of them are found in relative abundance (RA) greater than 0.1%, while the remaining 239 are considered rare taxa (RA < 0.1%). Within these 239 taxa, 146 are found in a RA included between 0.1 and 0.01%, and 93 have a RA lower than 0.01%. The full list of taxa is available in the supplementary material (Table S5.5).

The qualitative overview of the wood mycobiome will focus mainly on the 30 most abundant taxa in PW and the 12 most abundant in AW, which account for 79.1 and 80.8% of the total RAs respectively (Table 5.1), while the remaining percentages represent unidentified taxa or fungi found in lower abundances. Within this group of taxa, five genera and nine species of fungi are described for the first time in association

with the grapevine wood mycobiome, while the remaining 18 taxa have already been reported (Table 5.1).

The community encountered in perennial wood is characterized by the presence of both ascomycetes and basidiomycetes (66.7% and 27.7% RA), with high abundances of tracheomycotic pathogen *Phaeomoniella chlamydospora* (25.8%) and white rot agent *Fomitiporia* sp. (14.6%), two organisms directly associated with esca proper and other esca-related syndromes. Among all sampled wood cores (n= 80), *P. chlamydospora* was present in 68 of them (85%; RA> 0.1%), while *Fomitiporia* sp. in 58 (64%; RA> 0.1%) or 14 (17.5%; RA> 35%). Other GTD pathogens among the 30 most abundant taxa are *Eutypa lata* (0.7%) and *E. leptoplaca* (0.9%), within the Diatrypaceae. More members of this family are *Anthostoma gastrinum* (0.9%), a potential wood pathogen, as well as *E. flavovirens*, *Eutypella citricola* and *Cryptovalsa ampelina*, identified as rare taxa. Members of the Botryosphaeriaceae (e.g. *Diplodia pseudoseriata*, *Neofusicoccum parvum*, *N. australe*), *Ilyonectria* sp. and *Neonectria* sp. are also found, although represented only as rare taxa (Table S5.5). Decay agents, such as *Fomitiporia* sp., *Fomitiporia mediterranea* (0.2%) and *Inonotus hispidus* (0.3%), were also identified in this study, along with several others represented in minor abundances (e.g. *Fomitiporella* sp.). Among the endophytes and saprophytes, *Alternaria* sp. (3.2%), *Cladosporium* sp. (1.9%), *Aureobasidium pullulans* (0.4%) and *Psathyrella* sp. (0.5%) are the most abundant. Several other genera or species, identified for the first time in association with grapevine wood, amount to 14 taxa out of the 33 most abundant in PW or AW (Table 5.1).

Annual wood is also colonized by both ascomycetes (76.3%) and basidiomycetes (18.8%). The most abundant taxa are endophytic and saprophytic fungi, with *Alternaria* sp. (14.6%), *Ramularia* sp. (9.4%) and *Cladosporium* sp. (8.2%) being among most abundant, as well as other species reported for the first time (e.g. *Debaryomyces prosopidis*; Table 5.1). Only two wood pathogens are present in moderate abundances in AW, namely *P. chlamydospora* (3.9%) and *Diaporthe* sp. (0.8%), while other pathogenic agents are found in minor abundances (RA < 0.2%; e.g. *Neofusicoccum australe*).

Table 5.1. List of most abundant taxa, identified to genus or species level, found in grapevine wood. The list includes the 30 most abundant taxa found in perennial wood and the 12 most abundant in annual wood, for a total of 32 taxa. The numbers between brackets represent the relative abundance of that Phylum or Family in the perennial wood or annual wood (PW% - AW%) based on the table created to address objective (1). The ecology of the identified taxa in wood of grapevines or of other plants is shown based on available literature (E= endophyte, S= saprophyte, P= pathogen, na= unknown ecology). The presence of taxa in different tissue types is based on the table created to address objective (2), (+) indicates presence (RA ≥ 0.1%), (-) indicates absence or presence in RA < 0.1%. Meaning of GU, T, UT, A1, A2, S1, S2 and C as in the legend of Figure 5.1.

Phylum	**Family**	**Species**	**Relative abundance (%)**		**Ecology in wood** [*]	**Presence in different tissue type**			
			PW	AW		GU / T / UT	A1 / A2	S1 / S2	C
Ascomycetes (66.7 – 76.3)	Biatriosporaceae (0.6 – 0)	*Biatriospora mackinnonii*[‡]	0.6	-	E [a]	- / - / -	- / -	- / -	-
	Bionectriaceae (0.4 – 0)	*Clonostachys rosea*	0.4	-	E, S, P [b]	- / - / -	- / -	- / +	-
	Davidiellaceae (2.0 – 8.2)	*Cladosporium* sp.	1.9	8.2	E, S [b]	+ / + / +	+ / +	+ / -	+
	Diaporthaceae (<0.1 – 0.8)	*Diaporthe* sp.	<0.1	0.8	E, S, P [b]	- / - / -	- / -	- / -	+
	Diatrypaceae (2.6 – 0)	*Anthostoma gastrinum*[†]	0.9	-	S, P [c, d]	+ / + / +	+ / +	+ / -	-
		Eutypa lata	0.7	-	P [b]	- / - / -	- / -	- / +	-
		Eutypa leptoplaca	0.9	-	P [b]	- / - / +	- / -	+ / +	-
	Dothioraceae (0.4 – 4.0)	*Aureobasidium pullulans*	0.4	4.0	E, S [b]	- / + / +	+ / +	- / -	+
	Glomerellaceae (<0.1 – 0.7)	*Colletotrichum* sp.	<0.1	0.4	P [b]	- / - / -	- / -	- / -	-
	Herpotrichiellaceae (27.1 – 3.9)	*Exophiala xenobiotica*[†]	0.5	-	na	- / - / -	- / +	+ / +	-
		Phaeomoniella chlamydospora	25.8	3.9	P [d]	+ / + / +	+ / +	+ / +	+
	Hypocreales (0.3 – 0.2)	*Acremonium* sp.	0.2	-	E [e]	- / - / -	- / -	- / +	-
	Lophiostomataceae (3.8 – 0)	*Angustimassarina acerina*[†]	0.5	-	S [f]	- / - / +	+ / +	+ / +	-
		Lophiostoma sp.	2.7	-	E, S [b]	+ / + / -	+ / -	+ / -	-
		Lophiostoma cynaroidis[†]	0.3	-	E [g]	- / + / -	- / -	- / -	-
		Lophiotrema rubi	0.3	-	na	+ / - / -	- / -	- / -	-

Table 5.1. (*continue*).

Phylum	Family	Species	Relative abundance (%) PW	AW	Ecology in wood *	Presence in different tissue type GU / T / UT	A1 / A2	S1 / S2	C
	Massarinaceae (0.5 – 0)	*Massarina* sp.	0.5	-	E [h]	+ / + / +	- / -	+ / -	-
	Mycosphaerellaceae (3.3 – 9.4)	*Ramularia* sp.	3.1	9.4	na	+ / + / +	+ / +	+ / +	+
	Pleomassariaceae (2.9 – 0)	*Trematosphaeria pertusa*‡	2.9	-	S [i]	+ / + / -	- / +	+ / +	-
	Pleosporaceae (3.9 – 14.8)	*Alternaria* sp.	3.2	14.6	E [b, j]	+ / + / +	+ / +	+ / +	+
	Saccharomycetaceae (10.9 – 31.8)	*Debaryomyces prosopidis*‡	10.4	31.5	na	+ / + / +	+ / +	+ / +	+
	Xylariaceae (0.7 – 0)	*Lopadostoma meridionale*‡	0.3	-	S [k]	- / - / -	- / -	- / -	-
		Lopadostoma quercicola‡	0.4	-	S [k]	+ / - / -	- / -	- / -	-
Basidiomycetes (26.7 – 18.8)	Filobasidiaceae (0.2 – 0.2)	*Filobasidium magnum*†	0.1	0.2	na	+ / - / -	- / +	- / -	-
	Hymenochaetaceae (15.2 – 0)	*Fomitiporia* sp.	14.6	-	P [d]	+ / + / +	+ / +	+ / +	-
		Fomitiporia mediterranea	0.2	-	P [d]	- / + / -	+ / -	- / -	-
		Inonotus hispidus	0.3	-	S, P [l]	+ / - / -	- / +	- / -	-
	Malasseziaceae (0.3 – 0.3)	*Malassezia restricta*‡	0.2	0.2	na	+ / - / -	- / -	- / -	+
	Psathyrellaceae (0.5 – 0)	*Psathyrella* sp.	0.5	-	S [m]	+ / - / -	- / -	- / -	-
	Sporidiobolaceae (0.6 – 0)	*Rhodotorula mucilaginosa*†	0.4	<0.1	S [n]	- / + / +	+ / +	- / -	-
	Tremellaceae (6.4 – 8.3)	*Cryptococcus* sp.	2.7	7.6	E, S [b]	+ / + / +	+ / +	+ / +	+
		Cryptococcus heimaeyensis†	0.3	-	-	- / - / +	- / -	- / -	-
		Cryptococcus victoriae†	2.9	0.7	-	+ / + / +	+ / +	+ / +	+
		TOTAL	**79.1**	**80.8**					

* References. [a](Kolařík *et al.*, 2017), [b](Jayawardena *et al.*, 2018), [c](Haynes, 2016), [d](Gramaje *et al.*, 2018), [e](González and Tello, 2011), [f](Thambugala *et al.*, 2015), [g](Xing *et al.*, 2011), [h](Casieri *et al.*, 2009), [i](Suetrong *et al.*, 2011), [j](Pancher *et al.*, 2012), [k](Jaklitsch *et al.*, 2014), [l](González *et al.*, 2009), [m](Bruez *et al.*, 2016), [n](Haňáčková *et al.*, 2017).

‡First report of genus and species in grapevine wood.

†First report of species in grapevine wood.

The core mycobiome, namely the taxa shared between PW and AW, is constituted by 44 taxa. Only 10 taxa are unique to AW and the remaining 235 are unique to PW. All the 10 unique taxa found in AW are considered rare taxa, as their RAs are lower than 0.1% of the total, while among the many taxa unique to PW we find organisms belonging to the Hymenochaetaceae, Lophiostomataceae, Pleomassariaceae, Xylariaceae and several others (Table S5.5).

5.3.4 Diversity and spatial distribution of the mycobiome

Alpha diversity

The Shannon (H') and Pielou's (J') indexes vary significantly among tissue types, according to Tukey's HSD (Figure 5.2, Table 5.2). The Shannon diversity analysis reveals that one sample point, namely the Upper Trunk, differs from both the spur points S1 and S2 ($P < 0.05$). The spur tissue is also significantly different from canes ($P < 0.05$). No differences are observed when comparing GU, T, UT, the two sample points in the arm (A1 and A2) and canes. Exact p-values of the significant differences are available in Supplementary Table S5.6.

Different tissue types also vary in mycobiome evenness, with fungal communities of canes, GU, T and UT being more evenly distributed than those of the spurs ($P < 0.05$; Figure 5.2). All other tissue types examined do not differ in evenness (Figure 5.2).

Beta diversity

The Jaccard's index, when visualized in a non-metric multidimensional scaling (NMDS) plot, shows a considerable overlap for different tissue types (Figure 5.3 A). The PERMANOVA indicates significant difference between groups ($p < 0.001$), but looking at the ordination, the difference is arguably in the clustering of the observations, rather than a distinct difference in sample composition. A pattern emerges when examining each tissue type separately (Figure 5.3 B). We observe a reduction in between-sample variability starting from the GU to T and until UT. The UT variability is very similar to that of both the Arm points (A1 and A2), while the Spurs (S1 and S2) have higher between-sample variability, when compared to the Arms, but similar to one another. Concerning the AW, the variability of this tissue type is very low (Figure

5.3 B). Bray Curtis test revealed a similar profile and significant differences ($p < 0.001$; data not shown).

Table 5.2. Shannon diversity (H’) and Pielou’s evenness (J’) indexes of the spatial distribution analysis (objective 2) and the mycobiome analysis in the wood associated with symptomatic canopy (objective 3).

	Shannon (H’)	Pielou's evenness (J’)
Spatial distribution (objective 2)		
Graft Union	1.85	0.60
Trunk	1.73	0.57
Upper trunk	1.92	0.61
Arm 1	1.26	0.40
Spur 1	1.08	0.34
Arm 2	1.58	0.49
Spur 2	1.09	0.37
Canes	1.93	0.61
Foliar symptomatology (objective 3)		
Perennial wood		
Asymptomatic arm in asymptomatic plant	1.29	0.41
Asymptomatic arm in symptomatic plant	1.05	0.33
Symptomatic arm	1.25	0.39
Annual wood		
Asymptomatic cane in asymptomatic plant	2.04	0.63
Asymptomatic cane in symptomatic plant	1.81	0.59
Symptomatic cane	2.09	0.68

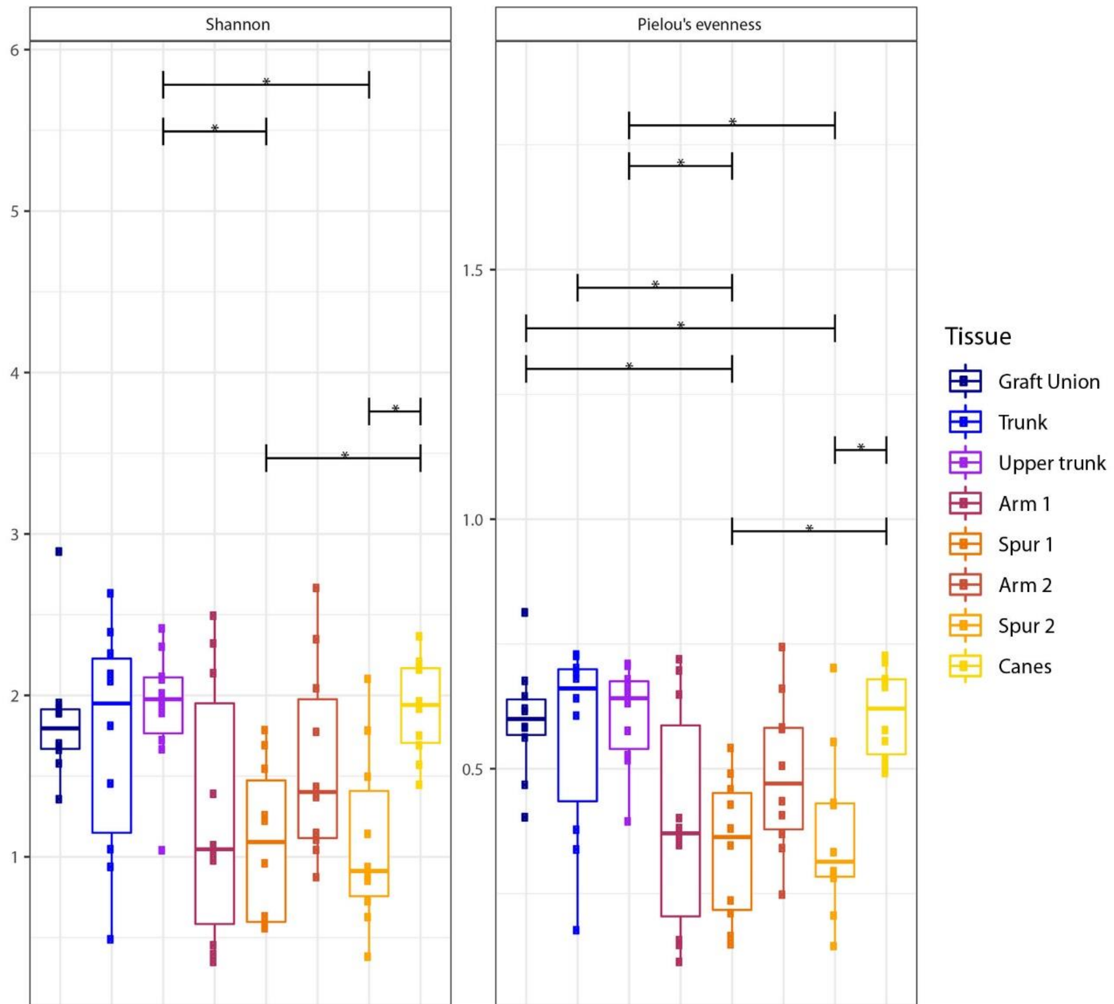

Figure 5.2. Box plots of diversity indexes (Shannon, Pielou's evenness) of the fungal communities present in different sampling areas of the perennial wood (Graft Union, Trunk, Upper trunk, Arm 1, Spur 1, Arm 2, Spur 2) and canes. Vertical boxes denote the median, the upper and lower quartiles, and the extremes of data. The black, horizontal brackets at the top of the figure denote statistical comparisons of the two tissues at each end of the bracket, calculated using a one-way ANOVA with post-hoc Tukey's HSD. Statistical differences are shown by asterisks, where $P < 0.05$ = *.

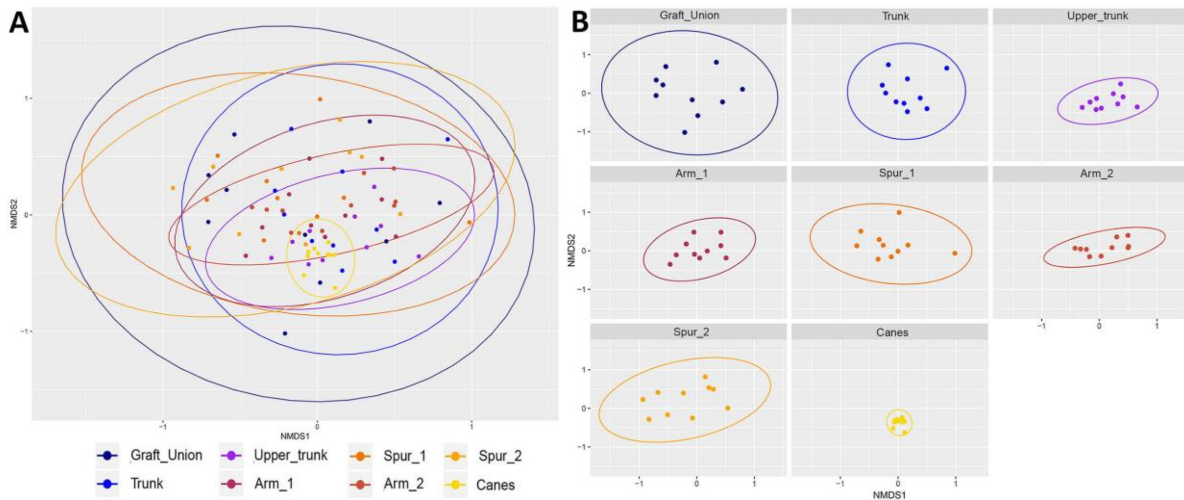

Figure 5.3. Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in different tissue types in grapevine. (A) Shows all samples ordinated together, while (B) is the same data, split up per tissue group. Ellipses illustrate the multivariate normal distribution of samples within the same tissue group. The groups are colour-coded to correspond with Figure 5.2.

Mycobiome composition and differentially represented taxa

The bar plot in Figure 5.4 shows the relative abundance of the 20 most abundant taxa in different tissue types, giving an overview of the presence/absence of taxa and their differential representation. For example, *Eutypa lata* and *Acremonium* sp. are present exclusively in the wood below the spurs, while *Trematosphaeria pertusa* was detected in the same tissue type and in the graft union. *Fomitiporia* sp., very represented in most of the perennial wood, is nearly absent in the graft union. This tissue contains three unique taxa, *Psathyrella* sp., *Lophiotrema rubi* and *Lopadostoma quercicola*, as well as other taxa which are present in higher abundances when compared to the rest of the plant, such as *Lophiostoma* sp. and *Massarina* sp. The heat trees shown in Figures 5.5 and 5.6 give a thorough view of the differently abundant taxa, when comparing each tissue type, for taxa with RAs > 0.1% (n= 50). The majority of the statistical differences observed, for both ascomycetes and basidiomycetes, concern the comparison between the Canes (AW) and all other tissue types of the perennial wood. Among the ascomycetes, the AW presents lower abundance of *P.*

chlamydospora and Diatrypaceae, and higher abundance of *Aureobasidium pullulans*, *Debaryomyces prosopidis*, *Diaporthe* sp., Capnodiales, depending on the woody tissues compared.

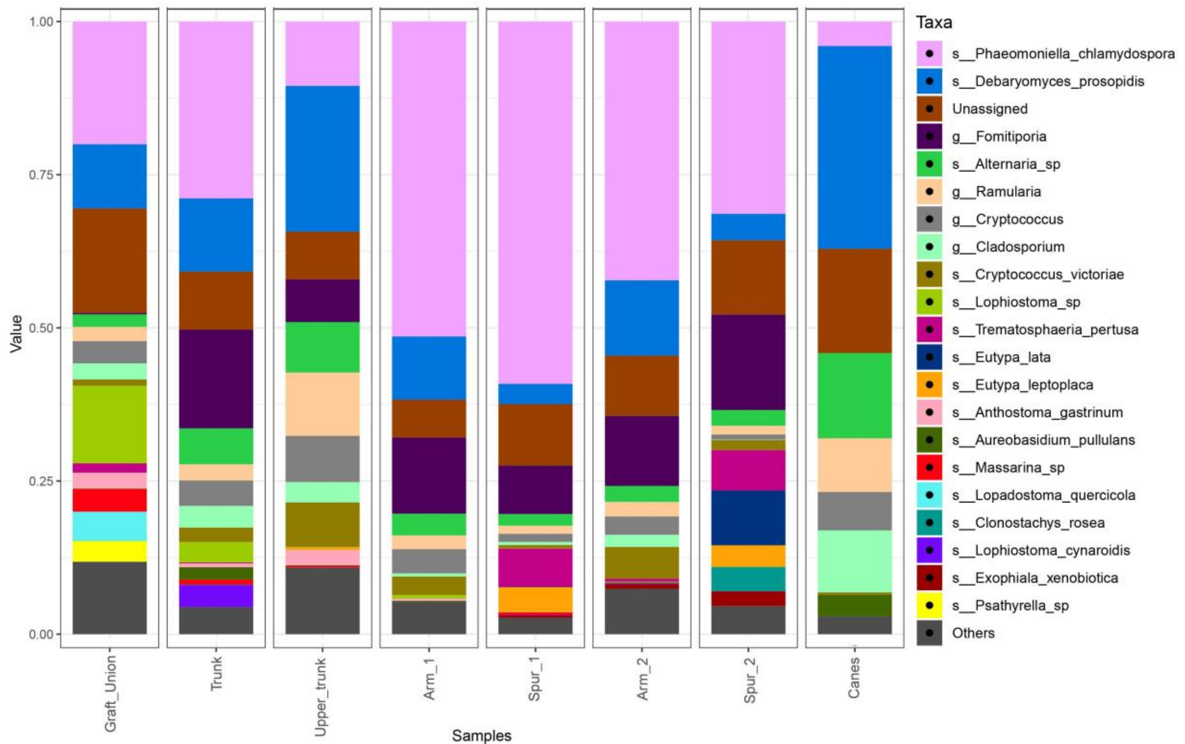

Figure 5.4. Barplots of the relative abundance of the 20 most abundant taxa identified to species (s_) or genus (g_) level, found in different sampling areas of the stem and in the canes of grapevine. 'Unassigned' are taxa identified to a lower taxonomic level than genus, 'Others' are taxa not included in the 20 most abundant.

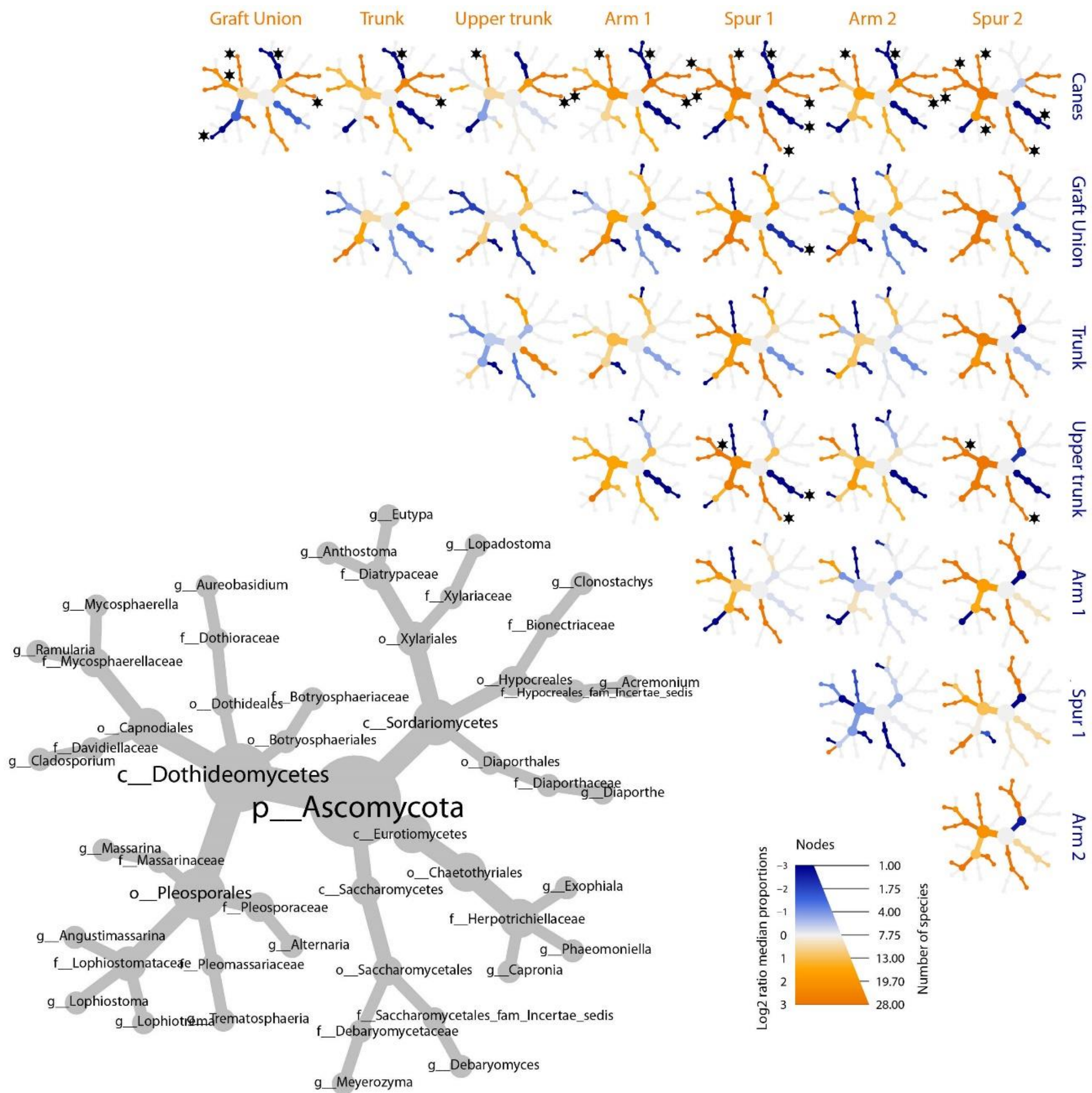

Figure 5.5. Differential heat tree matrix depicting the change in taxa abundance between different tissue groups, for ascomycetes, represented in the dataset (RA > 0.01%). The size of the individual nodes in the grey cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue group: an orange node indicates a higher abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite. Taxa identified as statistically differently represented, according to the Wilcoxon test, are tagged with a black star.

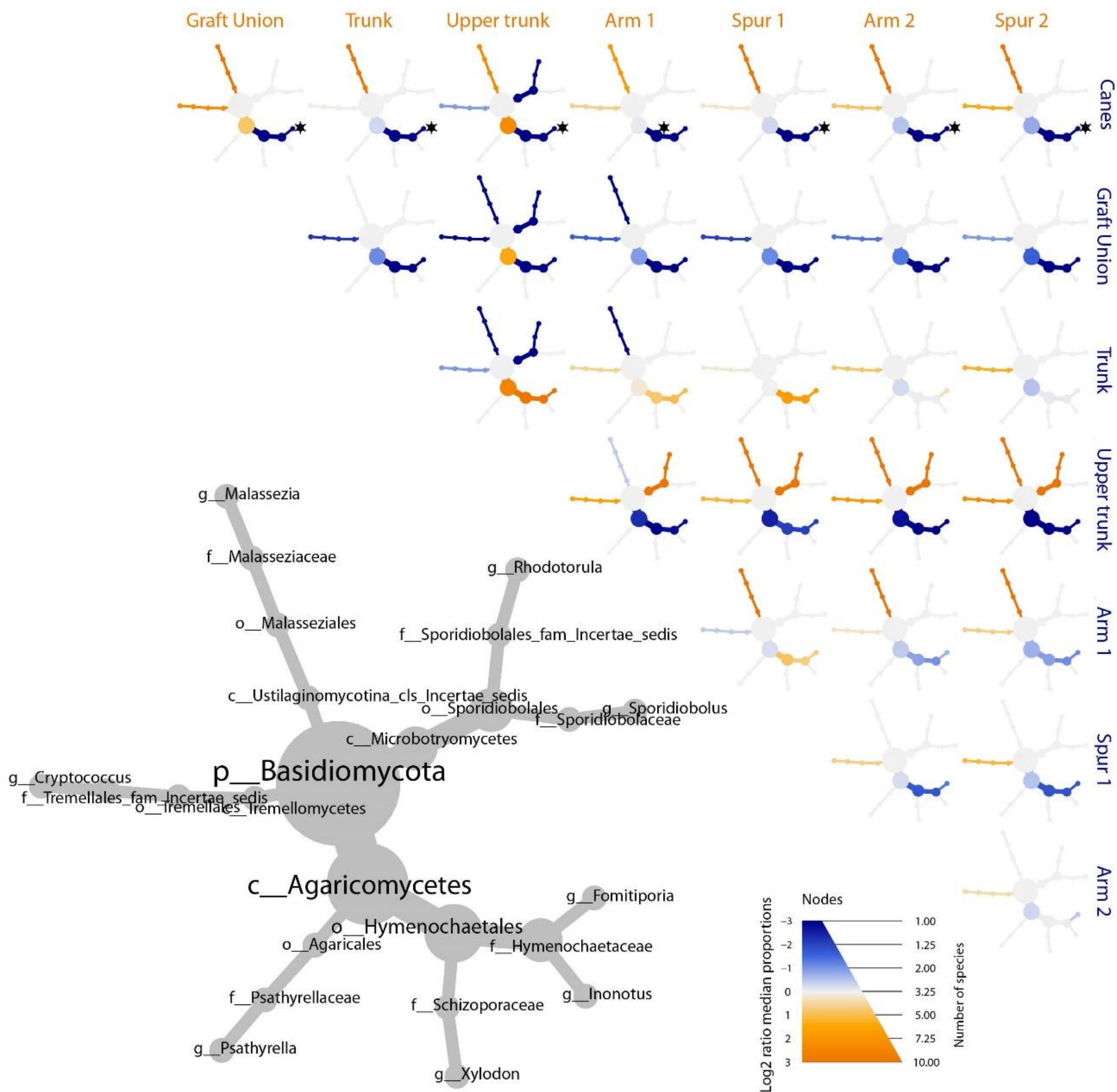

Figure 5.6. Differential heat tree matrix depicting the change in taxa abundance between different tissue groups, for basidiomycetes, represented in the dataset (RA > 0.01%). The size of the individual nodes in the grey cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue group: an orange node indicates a higher abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite. Taxa identified as statistically differently represented, according to the Wilcoxon test, are tagged with a black star.

5.3.5 Mycobiome and foliar symptoms

Alpha and beta diversity

The examination of the mycobiome of the wood in the proximity of the canopy that exhibited foliar symptoms of esca revealed no significant differences in term of diversity (H') and evenness (J'), when compared with the fungal communities in the wood in proximity of non-symptomatic canopy, both in symptomatic or asymptomatic vines ($p > 0.05$; Table 5.2). The same results were obtained when comparing the mycobiome of the annual wood (canes) presenting foliar symptoms or non-symptomatic leaves, in both symptomatic and asymptomatic plants. The Jaccard indexes, when plotted in a NMDS matrix, reveal overlapping communities with a very similar between-sample variability, suggesting an overall similarity in beta diversity. In fact, the PERMANOVA analysis revealed no statistical differences among the three tissue groups in both perennial wood ($p = 0.067$) and annual wood ($p = 0.429$). Boxplots of the diversity indexes and NMDS of beta dispersion are available in the supplementary materials (Figures S5.9 and S5.10).

Mycobiome composition and differentially represented taxa

The mycobiome of the perennial wood in proximity of symptomatic or non-symptomatic canopy is characterized by high abundances of *P. chlamydospora*, *Fomitiporia* sp. and *Debaryomyces prosopidis* (Figure 5.7). The most frequent taxa of known GTD-associated pathogens are presented in Table 5.3.

The MetacodeR analysis revealed no statistical differences among taxa for their differential abundance in the three tissue groups compared in perennial wood and in annual wood. Nevertheless, trends of change are present for some taxa, and they are shown in the heat trees in Figure 5.8.

The perennial wood in proximity of non-symptomatic canopy in non-symptomatic plants presents higher abundances of *Ramularia* sp., *Cladosporium* spp., *Alternaria* sp., *Debaryomyces* sp., *Cryptococcus* sp. and, to a lower extent, *P. chlamydospora*; while *Fomitiporia* spp. is under-represented, when compared with the wood near symptomatic canopy.

When comparing the PW in proximity of non-symptomatic and symptomatic canopy, both collected from a symptomatic vine, similar trends are observed. In fact, *Ramularia* sp., members of the Pleosporales and *Aureobasidium pullulans* are over-represented in the former, along with a minor over-representation of the other taxa previously mentioned. Also, in this case a trend of underrepresentation is observed for *Fomitiporia* spp.

Some differences are also observed when comparing the PW near asymptomatic canopy coming from either asymptomatic or symptomatic plants. In the former we observe an over-representation of *Alternaria* sp., *Debaryomyces* sp. and *Cryptococcus* spp., and an under-representation of *Aureobasidium pullulans* and *Anthostoma* sp.

Annual wood communities are characterized by similar relative abundances, among the most abundant taxa, for all three groups analyzed. Several trends of variation are shown in Figure 5.8, involving primarily genera *Malassezia*, *Cryptococcus*, *Acremonium* and *Diaporthe*.

Table 5.3. Relative abundances of genera or species of fungi known to be involved in GTDs, encountered in the perennial wood (PW) or annual wood (AW), relative to objective (3). Three groups of wood were examined, namely the PW in proximity of symptomatic canopy (SYM); asymptomatic canopy, but in vines that presented foliar symptoms (ASYM-SYM); asymptomatic canopy, in vines that did not present foliar symptoms (ASYM). The same groups were examined in AW, namely canes with manifested foliar symptoms (SYM); asymptomatic canes, but in vines that presented symptomatic canopy (ASYM-SYM); asymptomatic canes, in vines that did not present symptomatic canopy (ASYM). Taxa listed are found in RA > 0.01% of the total dataset, other known wood pathogens (RA < 0.01%) are not included.

Genus/Species	**Pathogens' RA in PW**			**Pathogens' RA in AW**		
	SYM	ASYM-SYM	ASYM	SYM	ASYM-SYM	ASYM
Ascomycetes						
*Anthostoma gastrinum**	1.3	0.7	-	-	-	-
Diaporthe sp.	-	-	<0.1	0.6	<0.1	1.2
Eutypa lata	0.2	0.1	<0.1	-	-	-
Eutypa leptoplaca	0.7	3.7	<0.1	-	-	-
Neofusicoccum parvum	0.1	-	-	-	-	-
Neofusicoccum australe				-	0.2	-
Phaeomoniella chlamydospora	21.8	50.1	43.1	3.5	3.4	4.9
Basidiomycetes						
Fomitiporia sp.	34.9	24.1	7.7	-	-	-
Fomitiporia mediterranea	0.9	<0.1	0.1	-	-	-
Total Ascomycetes	22.7	53.9	43.1	4.1	3.6	6.1
Total Basidiomycetes	35.8	24.1	7.8	-	-	-
TOTAL	**58.5**	**78.0**	**50.9**	**4.1**	**3.6**	**6.1**

*genus *Anthostoma* has been associated to pathogenicity in grapevine wood, but not *A. gastrinum,* being this the first report of this species in grapevine wood. The RAs of this taxon were not calculated in the Total(s).

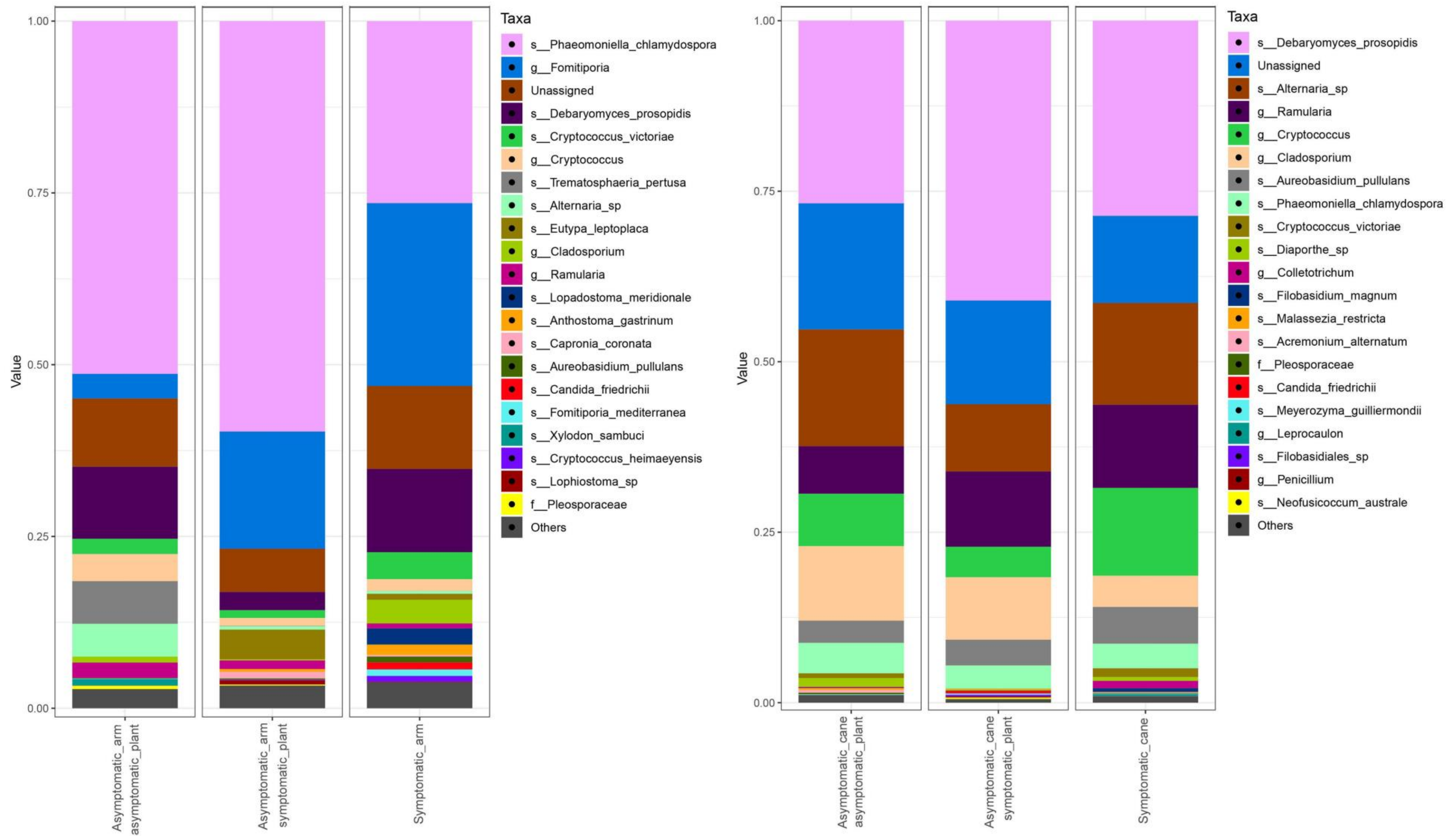

Figure 5.7. Barplots of the relative abundance of the 20 most abundant taxa identified to species (s_), genus (g_) or family (f_) level. 'Unassigned' are taxa identified to a lower taxonomic level than family. 'Others' are taxa not included in the 20 most abundant. (Left) Communities found in the PW in proximity of symptomatic canopy ('Symptomatic_arm') or of asymptomatic canopy, either in symptomatic plants ('Asymptomatic_arm symptomatic_plant') or in asymptomatic plants ('Asymptomatic_arm asymptomatic_plant'). (Right) Communities found in the canes with manifested foliar symptoms ('Symptomatic_cane') or asymptomatic, but coming from symptomatic ('Asymptomatic_cane symptomatic_plant') or asymptomatic plants ('Asymptomatic_cane asymptomatic_plant').

Figure 5.8. Differential heat tree matrix depicting the change in species abundance between different tissue groups, represented in the dataset with a (RA > 0.01%). The size of the individual nodes in the grey cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue group, with the colour illustrating the log2 fold change: a red node indicates a higher abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite.

5.4 Discussion

5.4.1 Characterization of the mycobiomes in PW and AW

The diversity of the fungal communities living in the stem of woody plants, characterized by NGS approaches, is largely unknown. Research performed over the past decades on the grapevine mycobiome, using both culture-dependent and independent approaches, revealed over 900 fungal taxa (Jayawardena *et al*., 2018). The richness of the wood mycobiome in esca infected vineyards was estimated to be 88 taxa in Montpellier, France (Travadon *et al*., 2016); 85 species in Bordeaux, France, from 108 single strand conformation polymorphism (SSCP) profiles (Bruez *et al.*, 2014); and 150 operational taxonomic units (OTUs) in Switzerland (Hofstetter *et al*., 2012). This study, the first using a NGS approach, reveals an even greater richness (289 taxa), adding numerous fungi to the known list of 900+ known taxa. Several factors may play a role in shaping the fungal community composition, such as location, cultivar and age of the plants (Dissanayake *et al*., 2018; Travadon *et al*., 2016). This is the primary reason why we expect that using NGS to assess the diversity of the wood mycobiome in other vineyards will considerably increase the number of fungal species found in association with grapevine wood.

Interestingly, 239 out of the 289 taxa (80%) are rare taxa. The ecology of several of them is known (Jayawardena *et al*., 2018), while that of several others needs to be assessed, nevertheless the extent to which they contribute to the grapevine-mycobiome and fungus-fungus interactions remains to be elucidated. Grapevines perennial wood seems to function as a reservoir of fungal diversity, where species that are found in minor abundances may thrive under specific environmental conditions (e.g. extreme weather conditions, mechanical injuries), leading to positive or negative effects for the plant's wellbeing.

Unsurprisingly, within this massive richness, numerous fungi associated with GTDs were identified, both as frequent and rare taxa. This agrees with previous reports showing that esca-affected plants may host numerous other wood pathogens (Edwards and Pascoe, 2004; Rumbos and Rumbou, 2001). However, *Phaeoacremonium* spp., a genus of tracheomycotic fungi often involved in esca-associated syndromes

(Mostert *et al*., 2006), was not detected. The vineyard under study, as well as other esca-symptomatic fields examined in previous works (Bruez *et al*., 2014; Hofstetter *et al*., 2012; Travadon *et al*., 2016), are dominated by wood pathogens (e.g. *P. chlamydospora* or Botryosphaeriaceae), and the presence of decay agents was confirmed in all studies. On the other hand, the wood mycobiome of healthy vineyards, characterized by other authors, is dominated by endophytes and occasionally saprobes, some of which hold potential for biological control of wood pathogens, with genera *Trichoderma*, *Aureobasidium*, *Acremonium*, *Cladosporium*, *Alternaria* and *Epicoccum* being predominant (Dissanayake *et al*., 2018; González and Tello, 2011; Pancher *et al*., 2012). Despite the fact that wood pathogens were encountered, in minor abundance, also in healthy vineyards (González and Tello, 2011; Pancher *et al*., 2012), decay agents were not reported. Unfortunately, previous studies did not report data on rare taxa, therefore it is not possible to establish if other wood pathogens – including decay agents – were present but unable to thrive (e.g. antagonistic interactions with other fungi, strong plant defenses) or if they were completely absent.

The differences observed between PW and AW (e.g. alpha diversity, beta dispersion, taxa composition and abundance), some of which have also been observed in a previous study in grapevines (Hofstetter *et al*., 2012) and other plants (Qi *et al*., 2012), can be due to two main factors. First factor is the time that fungi had to colonize the perennial wood (up to 19 years in the present study), which is also subjected to yearly pruning, leaving wounds that are the optimal entry point for colonizers. The annual wood had approximately 5 months of age from bud-burst to sampling and no wounding occurred in the shoots (e.g. summer pruning). A second factor that explains these differences is the tissue-specificity, as considerable anatomical, biochemical and physiological differences characterize perennial wood and annual wood, which may prevent some fungi, such as decay agents, from colonizing the latter. In fact, the absence of decay agents in annual wood (e.g. *Fomitiporia* sp., *Inonotus hispidus*, *Fomitiporella* sp.) observed in this study, and also reported in canes and nursery propagation material in other studies (Casieri *et al*., 2009; Halleen *et al*., 2003; Hofstetter *et al*., 2012; Rumbos and Rumbou, 2001), suggests that the infection by these pathogens occurs exclusively in older wood and under field conditions.

A brief introduction of the five genera identified for the first time in grapevine's wood follows.

Debaryomyces. Members of the genus *Debaryomyces* (Saccharomycetales, Saccharomycetaceae) are yeast-like ascomycetes found in several ecological niches. There are 93 species in the genus, with *D. hansenii* being the most widely studied. *D. hansenii* has been isolated from shoots of *Sequoia sempervirens* and from the soil under the tree (Middelhoven, 2003), as well as from white and brown rot of several woody species (González *et al*., 1989). *Debaryomyces* sp. was also detected in slime fluxes of *Prosopis juliflora*, a deciduous woody plant, as well as in insects associated with it (*Drosophila carbonaria* and *Aulacigaster leucopeza*; Ganter *et al*., 1986). Molecular analysis identified some of these strains as a new species, *D. prosopidis* (Phaff *et al*., 1998), which is the only record of this organism in literature. Concerning the vineyard, *Debaryomyces* sp. has been identified on grape berries (Jara *et al*., 2016) and during wine fermentation (Varela and Borneman, 2017).

*Trematosphaeria***.** Members of the genus *Trematosphaeria* (Pleosporales, Pleomassariaceae) are saprotrophs or hemibiotrophs of terrestrial woody plants, with some exceptions reported from freshwater or marine habitats (Tanaka *et al.*, 2005). *T. pertusa*, from this study, is known to grow on the surface of decaying terrestrial wood (Suetrong *et al*., 2011), however, it has also been retrieved from wood of *Fagus sylvatica* and *Pinus sylvestris* submerged in a river (Kane *et al.*, 2002). Terrestrial woody hosts of *T. pertusa* include also *Fraxinus excelsior*, *Fagus* sp. and *Platanus* sp.

*Lopadostoma***.** Members of the genus *Lopadostoma* (Xylariales, Xylariaceae) are ascomycetes currently considered saprotrophs (Anthony *et al.*, 2017). Members of the Xylariaceae are typically saprobes, despite some members of this family are endophytes and others are plant pathogens (Mehrabi and Hemmati, 2015). To date, due to the lack of studies on this topic, the ecology of the genus *Lopadostoma* remains to be fully understood. There are 12 species described in this genus, most of which have been isolated from hosts *Quercus* spp. or *Fagus sylvatica* (Jaklitsch *et al*., 2014).

*Biatriospora***.** The genus *Biatriospora* (Pleosporales, Biatriosporaceae) contains species that have been isolated as endophytes of both terrestrial and marine-associated plants (Kolařík *et al.*, 2017), as well as from lichens, as an endolichenic fungus (Zhou *et al*., 2016). Known hosts of this genus, in temperate climate, are *Ulmus spp.* and *Acer*

pseudoplatanus, along with several other hosts from tropical climates (Haňáčková *et al.*, 2017; Kolařík *et al.*, 2017). *Biatriospora* sp. is a source of bioactive compounds (heptaketides) with antifungal properties (Zhou *et al*., 2016) and preliminary studies suggest some potential in biological control (Haňáčková *et al*., 2017). *B. mackinnonii*, from this study, has been isolated from terrestrial plant material and it has also been linked with human mycetoma, a skin disease (Ahmed *et al.*, 2014; Kolařík *et al.*, 2017).

*Malassezia***.** Members of the genus *Malassezia* (Malasezziales, Malasezziaceae) are yeast or yeast-like basidiomycetes isolated from numerous niches. Fourteen species belong to this genus and most of them are obligatory lipophiles (Sommer *et al*., 2015). *M. restricta*, from this study, has been identified as endophyte of both woody and herbaceous plants (e.g. *Eucalyptus* sp., *Populus deltoids*, *Spiranthes spiralis*, *Solanum tuberosum*) as well as found on orchid roots, and in soil (Abdelfattah *et al*., 2016; Connell and Staudigel, 2013; Paulo *et al*., 2017).

It is important to mention the genera *Cryptococcus*, *Angustimassarina* and *Exophiala*, found in this study, which have been previously associated once to grapevine's wood, either as saprobes or endophytes, by a culture-independent study (Jayawardena *et al*., 2018). Lastly, species of *Ramularia* have been isolated from leaves (both as epiphytes and endophytes) of several hosts, including *Platanus* sp., *Prunus cerasus* and *Vitis vinifera* (Bakhshi and Arzanlou, 2017), although never in association with wood.

Among the genera and species that were found associated with grapevines for the first time in this study, some have previously been detected in the endosphere or phyllosphere of other woody plants that were found in the proximities of the vineyard used in the present study (Table S5.4). For example, *Lopadostoma* spp. and *Anthostoma gastrinum* were reported in *Quercus* (Haynes, 2016; Jaklitsch *et al*., 2014), *Rhodotorula mucilaginosa* and *Trematosphaeria pertusa* in *Fraxinus* (Haňáčková *et al*., 2017; Suetrong *et al*., 2011), and *Malassezia restricta* in *Eucalyptus* (Paulo *et al*., 2017). This suggests that the fungi present in the endosphere and phyllosphere of the flora in proximity of a vineyard might influence the composition of the mycobiome of grapevines wood, acting as a reservoir of multi-host fungi, with wind, rain and insects being possible vectors for mycobiome exchange.

5.4.2 Spatial distribution of the fungal communities

The concept of spatial distribution and tissue specificity in woody and herbaceous plants is not new in microbial ecology (Kovalchuk *et al*., 2018; Paulo *et al*., 2017; Singh *et al*., 2017; Wearn *et al*., 2012). However, studies that investigated the spatial distribution of fungal communities that colonize different areas or tissues in the wood of adult grapevines are scarce (Bruez *et al*., 2014; Travadon *et al*., 2016), and none of them used NGS approaches. When examining the relative abundances of identified taxa with NGS, it is important to remember that the ITS marker, our target amplicon, can be found in multiple copies in the genome of fungi and the number of copies may vary considerably from species to species (Schoch and Seifert, 2012), which inherently leave fungal relative distributions with great uncertainties.

From a qualitative point of view, the communities of various areas in PW shared numerous taxa, overall in agreement with the findings of Bruez *et al*. (2014), although some tissue types were more variable than others. Also, considerable differences are evident from a quantitative point of view. High variability was observed among individual plants, as already noted in grapevines (Travadon *et al*., 2013).

The graft union and the wood below the spurs host unique taxa or taxa that are found only in low percentages (RA < 0.1%; Table 5.1) in other parts of the plant. Supposedly, the root system (in this study *Vitis berlandieri* x *V. rupestris*), whose endosphere is influenced by the soil microbiome (Zarraonaindia *et al*., 2015) and by the rootstock used in the propagation process, harbor unique fungi that have a limited capacity of colonizing the stem of *V. vinifera*. Interestingly, *Fomitiporia* sp., a species that is well represented in all PW tissues (8.9% < RA < 22.9%), is nearly absent in the graft union (0.6%; Figure 5.4). Artificial inoculations of *F. punctata* in rootstock Kober 5BB (*V. berlandieri* x *V. riparia*) led to low re-isolation percentages of this pathogen (8% of inoculated plants; Sparapano *et al.*, 2000), and we are not aware of any other literature that describes the presence of *Fomitiporia* sp. in the root system of naturally infected vines.

The spur tissue is located in proximity of pruning wounds, which are considered the main entry point of GTDs-associated fungal pathogens (Bertsch *et al*., 2013). Through this woody tissue some colonizers successfully spread throughout the cordon and trunk (e.g. *P. chlamydospora* or *Fomitiporia* sp.), while others may be restrained by the

antagonistic interactions with the resident mycobiome and/or plant defenses (e.g. *Eutypa* spp., *Clonostachys rosea*, *Acremonium* sp.; Table 5.1).

The remaining tissues, namely the trunk, upper trunk and arm points, do not differ by any of the parameters measured in this study (e.g. alpha and beta diversity, MetacodeR; Figures 5.2, 5.3, 5.5 and 5.6), where unique taxa among the most represented fungi are nearly absent (Table 5.1), and relative abundances differ primarily in the representation of *P. chlamydospora* (Figure 5.4).

The results of this study show that the wood mycobiome of grapevines may vary and, in order to have a representative understanding of the diversity and abundance of the fungal communities, multiple sampling areas are recommended. We propose four samples per plant. The first two are the graft union and the wood below one spur, where some taxa are uniquely represented and abundances vary considerably; the third is any point of the trunk or arm; and the forth is annual wood.

5.4.3 Fungal communities and foliar symptomatology

The outcome of the statistical analyses of the mycobiome of both perennial wood and annual wood highlight that the fungal communities were not affected by the manifestation of foliar symptoms, or *vice versa*. The manifestation of “tiger stipes” foliar symptoms, always associated with an advanced stage of infection by tracheomycotic pathogens, is in large part cryptic due to its discontinuity and unidentified causal agents (Mugnai *et al.*, 1999). Greenhouse and field trials failed to reproduce such symptoms with artificial inoculations of *Pa. chlamydospora* and/or *Pm. minimum* (Zanzotto *et al*., 2007; Gramaje *et al*., 2010), except for one study in which esca-like foliar symptoms were replicated in a very small percentage of inoculated plants (Sparapano *et al*., 2001). Other microbial ecology studies of the wood from foliar-symptomatic and asymptomatic vines showed that there are no differences in the fungal community composition, finding similar abundances of wood pathogens (Bruez *et al*., 2014; Hofstetter *et al*., 2012). Nevertheless, tracheomycotic fungi are currently believed to be directly or indirectly responsible for foliar symptoms as they are frequently isolated from symptomatic wood (Surico, 2008). The two arguments supporting this hypothesis are that the translocation of (1) fungal toxins, (2) byproducts of the wood degradation or (3) a combination of both, via xylem sap, from

the perennial wood to the leaves, are responsible for the appearance of leaf symptoms (Mugnai *et al.*, 1999). While other studies showed that some toxins or culture filtrates of tracheomycotic fungi could lead to leaf discoloration and necrosis, solid evidence of the replication of the "tiger stripes" pattern is lacking (Abou-Mansour *et al*., 2004; Andolfi *et al*., 2011; Evidente *et al*., 2000; Sparapano *et al*., 2000). No evidence is currently available in support of the second hypothesis, namely the role of byproducts of wood degradation.

The results of the present study are, to some extent, in agreement with the findings of Hofstetter *et al.* (2012) and Bruez *et al.* (2014). In fact, the fungal communities present in the PW in proximity of canopy that had exhibited symptoms were overall similar to those found in plants with healthy canopies (in symptomatic or asymptomatic plants). When considering trends, *Pa. chlamydospora* is more abundant in the wood near non-symptomatic canopies, while *Fomitiporia* sp. is the most represented taxa in the wood near symptomatic canopy. This observation suggests a higher wood decay activity, which likely leads to a greater presence of byproducts of wood degradation, therefore supporting the second hypothesis for foliar symptoms manifestation. Regardless, it does not explain the manifestation of foliar symptomatology in grapevines not infected by *Fomitiporia* sp. (Edwards *et al*., 2001).

The similarity in the community structure of symptomatic and non-symptomatic canes is an additional evidence in support of the current understanding that fungi present in AW are not directly linked with the foliar symptomatology.

Foliar symptoms' manifestation remains cryptic, however, it is important to note that this study, as well as the one by Bruez *et al*. (2014), analyzed the microbial communities of the perennial wood several months after the plant had exhibited foliar symptoms. It is not known whether the pathogens abundance in the moment pre-/during/post-symptomatology vary. In fact, the study by Bruez *et al*. (2014) revealed that alterations in the wood mycobiome may occur in different seasons, therefore further research is needed in this direction. However, this may not apply to the annual wood, as foliar symptoms were still present, and manifested only two months before wood tissue sampling.

5.4.4 Further considerations

In this study, the mycobiome of the canes (AW) is composed principally by endophytes and saprobes, with the exception of pathogens *P. chlamydospora* and *Diaporthe* sp., whose presence did not induce the appearance of wood symptoms. Concerning the former, this observation suggests that this fungus may fall along a continuum 'from mutualist to saprophyte or latent pathogen' (Wearn *et al*., 2012), as also proposed by other authors (Hofstetter *et al*., 2012; Rumbos and Rumbou, 2001), and whose pathogenicity is triggered by external factors. Interestingly, also *Fomitiporia* sp. was often identified in woody tissue that did not present the typical white rot symptom. This fungus may live asymptomatically in wood when found in low abundances, and produce white rot when its presence increases considerably. Hypothetically, the wood cores showing this symptom (15% of the total) may be the same in which *Fomitiporia* sp. was present in a RA > 35% (17.5%).

The presence of *P. chlamydospora*, and that of other wood pathogens, in vineyards around the world might be currently blatantly underestimated due to the elusiveness of internal symptoms. The Almotivo vineyard (this study) presented an incidence of foliar symptoms manifestation of ca. 1%, for three consecutive years, nevertheless, 100% of the sampled plants were colonized by both *P. chlamydospora* (in PW and AW) and *Fomitiporia* sp. (in PW). It is already known that foliar symptoms are not a reliable parameter to assess the health status of a vineyard (Pollastro *et al*., 2000), although it is the only one currently employed. It is of utmost importance to develop tools that allow vine growers to assess the real extent of infections in the wood, and apply appropriate control measures.

5.5 Conclusion

The characterization of the grapevine wood mycobiome, using NGS, in a vineyard affected by esca proper is an important step that lays the foundations for future studies to compare microbial community structures of vineyards affected by esca or other GTDs. Some parameters that may influence the mycobiome composition and which may be of interest to investigate upon are: the flora surrounding the vineyard, the climatic conditions and seasonality, geographical location and year. Moreover,

useful comparisons can be made between cultivars and vineyard management strategies (e.g. conventional, organic, biodynamic). Eventually, a meta-analysis of the mycobiome that takes into account several of these parameters may reveal a pattern that could elucidate some of the obscure points that still prevent a full understanding of the etiology of this disease complex.

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5.7 Authors contribution

GDF: conceived the study, performed field sampling, performed wet-lab work, interpreted and discussed the results, wrote the manuscript; AG: performed wet-lab work, performed bio-informatics analyses, interpreted the results, wrote the manuscript; MRA: performed statistical analyses and data visualization; HO, LHH, RBF: supervised the study, reviewed the manuscript.

5.8 Supplementary material

Table S5.4. Classification of the woody plants encountered in the proximity of the vineyard used in the present study.

Phylum	Class	Order	Family	Genus	Species
Magnoliophyta	Liliopsida	Liliales	Agavaceae	*Agave*	*A. americana*
-	-	-	Asphodelaceae	*Aloe*	*A. arborescens*
-	Magnoliopsida	Apiales	Pittosporaceae	*Pittosporum*	*P. undulatum*
-	-	Caryophyllales	Cactaceae	*Opuntia*	*O. ficus-indica* *
-	-	-	-	-	*O. stricta*
-	-	Fabales	Fabaceae	*Ceratonia*	*C. siliqua*
-	-	-	-	*Cercis*	*C. siliquastrum*
-	-	Fagales	Fagaceae	*Quercus*	*Q. rotundifolia*
-	-	Lamiales	Oleaceae	*Fraxinus*	*F. angustifolia*
-	-	-	-	-	*F. australis* *
-	-	-	-	*Olea*	*O. silvestrys* *
-	-	-	-	*Phillyrea*	*P. latifolia* *
-	-	-	-	-	*P. media* *
-	-	Myrtales	Myrtaceae	*Eucalyptus*	*E. camaldulensis*
-	-	-	-	-	*E. globulus*
-	-	Proteales	Platanaceae	*Platanus*	*P. occidentalis*
-	-	Rhamnales	Rhamnaceae	*Rhamnus*	*R. alaternus*
-	-	Rosales	Rosaceae	*Eriobotrya*	*E. japonica*
-	-	-	-	*Prunus*	*P. dulcis*
-	-	-	-	-	*P. persica*
-	-	-	Moraceae	*Maclura*	*M. pomifera*
-	-	Urticales	Ulmaceae	*Celtis*	*C. australis* *
Pinophyta	Pinopsida	Pinales	Cupressaceae	*Cupressus*	*C. sempervirens*
-	-	-	Pinaceae	*Pinus*	*P. pinea*

(*) denotes the most abundant species in the proximities of the Almotivo vineyard.

Table S5.5. List of taxa identified to genus or species level present in the dataset including all sampling points for all tissue types –objective (1)-. Three groups are created to separate taxa present in a relative abundance (RA) greater than 0.1%, included between 0.1% and 0.01%, and lower than 0.01%. The taxa with a RA < 0.1% are considered rare taxa. Taxa followed by (*) are part of the core mycobiome – shared by perennial wood and annual wood –, taxa followed by (†) are unique to annual wood, taxa not followed by any symbol are unique to perennial wood.

RA > 0.1% (n= 50)	**0.01% < RA < 0.1% (n= 146)** (continues)	*Wallemia muriae* *
Acremonium sp.	*Cyphellophora* sp.	*Xanthoria* sp.
Acremonium alternatum *	*Cystobasidium pinicola*	*Xylodon sambuci*
Alternaria sp. *	*Cystobasidium* sp.	*Zymoseptoria* sp.
Angustimassarina acerina	*Cystofilobasidium capitatum*	**RA < 0.01% (n= 93)**
Anthostoma gastrinum	*Cystofilobasidium macerans*	*Acremonium fusidioides*
Aureobasidium pullulans *	*Devriesia pseudoamericana* *	*Agaricus blazei*
Biatriospora mackinnonii	*Devriesia* sp.	*Alternaria brassicae*
Candida friedrichii *	*Dioszegia hungarica*	*Alternaria eureka*
Capronia coronata	*Erythrobasidium hasegawianum*	*Alternaria metachromatica*
Cladosporium sp. *	*Eucasphaeria capensis* *	*Apiotrichum domesticum*
Clonostachys rosea	*Eutypa flavovirens*	*Arthrinium* sp.
Colletotrichum sp. *	*Eutypella citricola*	*Arthrobotrys superba*
Cryptococcus sp. *	*Exidia japonica*	*Ascochyta medicaginicola*
Cryptococcus heimaeyensis	*Exobasidium* sp. †	*Aspergillus amstelodami*
Cryptococcus victoriae *	*Exophiala oligosperma*	*Aspergillus ochraceus*
Debaryomyces sp. *	*Fellomyces* sp.	*Beauveria bassiana*
Debaryomyces prosopidis *	*Fomitiporella* sp.	*Bjerkandera adusta*
Diaporthe sp. *	*Funneliformis geosporum*	*Candida etchellsii*
Eutypa lata	*Fusarium poae*	*Candida mycetangii*
Eutypa leptoplaca	*Fusarium solani*	*Candida zeylanoides*
Exophiala sp.	*Ganoderma australe*	*Capronia pulcherrima*
Exophiala xenobiotica	*Ganoderma lucidum* †	*Catenulostroma hermanusense*
Filobasidium globisporum	*Ganoderma resinaceum*	*Cladosporium salinae*
Filobasidium magnum *	*Gibellulopsis chrysanthemi*	*Colacogloea* sp.
Fomitiporia sp.	*Holtermanniella takashimae*	*Cryptococcus aureus*
Fomitiporia mediterranea	*Hyphodontia alutaria*	*Cryptosphaeria subcutanea*

RA > 0.1% (n= 50) (continues)	**0.01% < RA < 0.1% (n= 146)** (continues)	**RA < 0.01% (n= 93)** (continues)
Fusarium sp. *	*Hyphodontia radula* *	*Cryptovalsa ampelina*
Glomerella acutata *	*Knufia epidermidis*	*Curvularia* sp.
Guehomyces pullulans	*Knufia perforans*	*Curvularia tsudae*
Inonotus hispidus	*Laetiporus sulphureus*	*Cyphellophora europaea*
Lopadostoma meridionale	*Leprocaulon* sp. †	*Cyphellophora reptans*
Lopadostoma quercicola	*Meira nashicola* *	*Cystofilobasidium infirmominiatum*
Lophiostoma cynaroidis	*Microdiplodia* sp.	*Cytospora* sp.
Lophiostoma sp.	*Minimedusa polysp.ora*	*Debaryomyces mycophilus*
Lophiotrema rubi	*Mortierella* sp.	*Dioszegia zsoltii var. yunnanensis*
Malassezia globosa *	*Mortierella minutissima*	*Diplodia pseudoseriata*
Malassezia restricta *	*Mrakia* sp.	*Engyodontium album*
Massarina sp.	*Mucor* sp.	*Epicoccum pimprinum* †
Meyerozyma guilliermondii *	*Mycena metata*	*Erysiphe necator*
Mycosphaerella tassiana *	*Naganishia albidosimilis*	*Erythrobasidium elongatum*
Penicillium sp. *	*Neodevriesia capensis*	*Erythrobasidium* sp.
Peniophora sp. *	*Neoerysiphe galeopsidis*	*Exophiala bergeri*
Phaeomoniella chlamydospora *	*Neofusicoccum* sp. *	*Fellomyces penicillatus*
Psathyrella sp.	*Neofusicoccum parvum*	*Fellomyces polyborus*
Ramularia sp. *	*Neofusicoccum australe* *	*Filobasidium wieringae*
Rhinocladiella sp.	*Neonectria* sp.	*Fuscoporia ferruginosa*
Rhodotorula mucilaginosa *	*Occultifur* sp.	*Gymnopus barbipes*
Sporidiobolus sp.	*Orbilia* sp.	*Hannaella* sp.
Trematosphaeria pertusa	*Papiliotrema flavescens*	*Hanseniaspora* sp. †
Vishniacozyma carnescens	*Paraconiothyrium* sp. *	*Heterobasidion irregulare* †
	Paraphaeosphaeria parmeliae	*Hyphoderma nudicephalum*
0.01% < RA < 0.1% (n= 146)	*Penicillium citreonigrum*	*Ilyonectria liriodendri*
Absidia sp.	*Peniophorella pubera* *	*Itersonilia pannonica* †
Acremonium brunnescens	*Petriella* sp.	*Knufia tsunedae*
Annulohypoxylon sp.	*Phacidiella eucalypti*	*Kondoa aeria*
Apiotrichum sp.	*Phaeomoniella* sp.	*Kurtzmanomyces* sp.
Articulospora sp.	*Phallus impudicus*	*Lachancea thermotolerans*
Ascobolus sp.	*Phanerochaete* sp.	*Lycoperdon ericaeum*
Aspergillus sp.	*Phialemoniopsis ocularis*	*Magnaporthe grisea*
Aspergillus conicus	*Phialophora cyclaminis*	*Malassezia* sp. *
Aspergillus penicillioides *	*Phialophora verrucosa*	*Malassezia sympodialis*
Aspergillus proliferans	*Physcia* sp.	*Mariannaea superimposita*

0.01% < RA < 0.1% (n= 146) (continues)	**0.01% < RA < 0.1% (n= 146)** (continues)	**RA < 0.01% (n= 93)** (continues)
Bensingtonia sp.	*Pleospora fallens*	*Mollisia cinerea*
Bipolaris sp.	*Podospora* sp.	*Monographella cucumerina*
Blumeria graminis	*Pyrenochaeta* sp.	*Monographella nivalis*
Boeremia exigua	*Pyrenochaeta keratinophila*	*Mortierella alpina*
Buckleyzyma sp.	*Pyrenochaeta unguis-hominis*	*Mucor hiemalis*
Caloplaca obscurella	*Pyrenophora tritici-repentis*	*Mucor saturninus*
Candida sp.	*Ramicandelaber* sp.	*Naganishia albida*
Candida palmioleophila	*Ramichloridium cucurbitae*	*Naganishia randhawae*
Candida parapsilosis	*Rhizomucor pusillus*	*Neodevriesia simplex*
Candida sake	*Rhizopus microsp.orus*	*Occultifur externus*
Candida tropicalis *	*Rhodotorula* sp.	*Papiliotrema pseudoalba*
Candida orthopsilosis	*Rhodotorula graminis*	*Paraphoma fimeti*
Capnodium sp.	*Rhodotorula nothofagi*	*Parasola conopilus*
Capronia sp.	*Rhodotorula diobovata*	*Periconia pseudobyssoides*
Cenococcum sp.	*Saccharomyces cerevisiae*	*Periconia* sp.
Ceratobasidium cornigerum	*Sakaguchia dacryoidea*	*Phialemoniopsis curvata*
Ceratobasidium sp.	*Sarcoporia* sp.	*Phlebia acerina*
Circinotrichum maculiforme *	*Sarocladium subulatum* *	*Phlebiopsis gigantea* †
Citeromyces matritensis	*Scheffersomyces spartinae*	*Pseudocercospora* sp.
Cladophialophora chaetospira	*Schizophyllum commune*	*Ramularia stellenboschensis* †
Cladophialophora sp.	*Sclerostagonospora* sp.	*Rhodotorula ingeniosa*
Cladosporium delicatulum *	*Scytalidium* sp.	*Rhodotorula terpenoidalis*
Cladosporium fusiforme	*Sistotremastrum* sp. *	*Rhodotorula toruloides*
Cladosporium sphaerospermum *	*Solicoccozyma terrea*	*Sclerostagonospora cycadis*
Clathrus ruber	*Sporobolomyces oryzicola*	*Scopuloides rimosa*
Clitopilus sp.	*Stemphylium* sp.	*Setophaeosphaeria badalingensis*
Colletotrichum acerbum *	*Tetracladium* sp.	*Sistotremastrum guttuliferum*
Colletotrichum gloeosporioides *	*Torulaspora delbrueckii*	*Sterigmatomyces halophilus*
Coprinellus micaceus	*Trametes hirsuta*	*Taphrina deformans*
Cryptococcus aerius	*Trichaptum abietinum*	*Trametes versicolor*
Cryptococcus frias	*Trichoderma* sp. *	*Trichosporon asahii*
Cryptococcus uniguttulatus	*Trichoderma harzianum* †	*Uncispora sinensis*
Cutaneotrichosporon sp.	*Veronaea compacta*	*Valsaria insitiva*
Cutaneotrichosporon cyanovorans	*Verticillium* sp. *	*Verrucocladosporium dirinae*
Cyberlindnera jadinii	*Wallemia* sp.	*Yamadazyma triangularis*

Table S5.6. One-way ANOVA with post-hoc Tukye's HSD of woody tissue types to assess differences in the Alpha diversity (Shannon index, Pielou's evenness) of fungal communities. For each row, column (A) is significantly different from column (B).

Index	A	B	P value
Shannon	Canes	Spur_1	0.023
	Canes	Spur_2	0.027
	Upper_trunk	Spur_1	0.024
	Upper_trunk	Spur_2	0.028
Pielou's evenness	Canes	Spur_1	0.005
	Canes	Spur_2	0.016
	Graft_Union	Spur_1	0.010
	Graft_Union	Spur_2	0.029
	Trunk	Spur_1	0.034
	Upper_trunk	Spur_1	0.006
	Upper_trunk	Spur_2	0.020

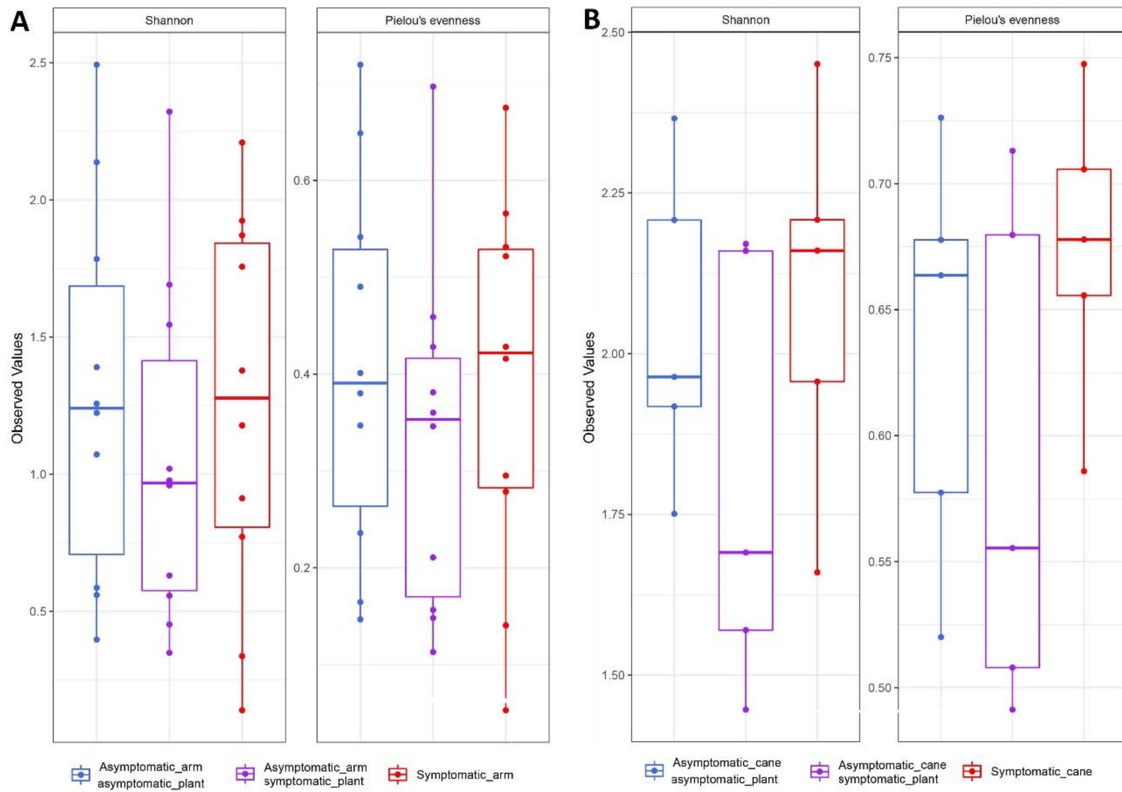

Figure S5.9. Box plots of diversity indexes (Shannon, Pielou's evenness) of the fungal communities present in (A) perennial wood or (B) annual wood. (A) Communities found in the wood in proximity of symptomatic canopy ('Symptomatic_arm') or of asymptomatic canopy, either in symptomatic plants ('Asymptomatic _arm symptomatic_plant') or in asymptomatic plants ('Asymptomatic_arm asymptomatic_plant'). (B) Communities found in the cane with manifested foliar symptoms ('Symptomatic_cane') or asymptomatic, but coming from symptomatic plants ('Asymptomatic_cane symptomatic_plant') or asymptomatic plants ('Asymptomatic_cane asymptomatic_plant').

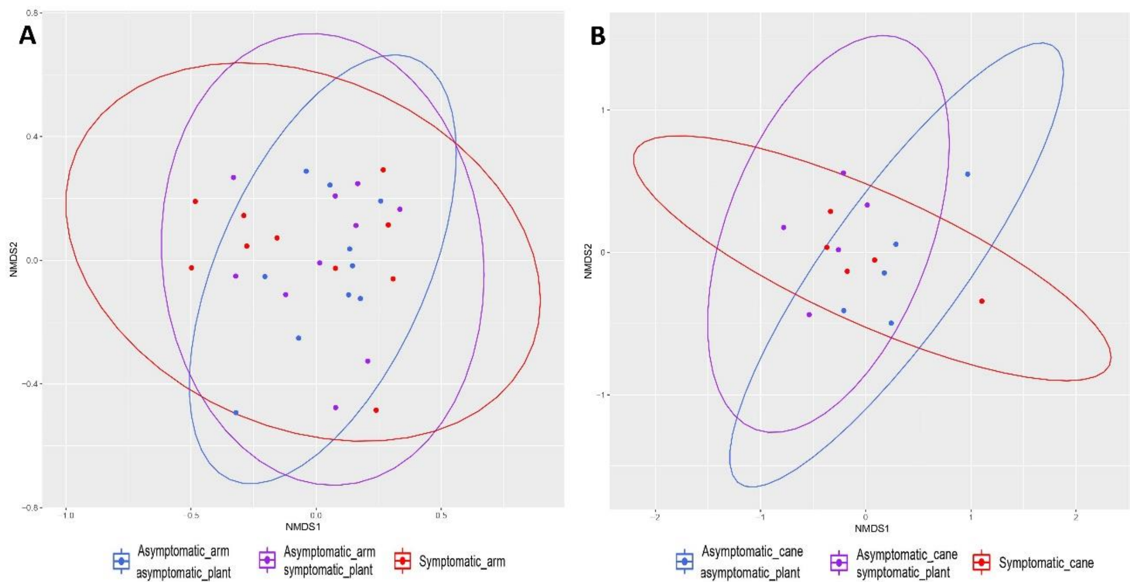

Figure S5.10. Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in different tissue types in grapevine. (A) Communities found in the wood in proximity of symptomatic canopy ('Symptomatic_arm') or of asymptomatic canopy, either in symptomatic plants ('Asymptomatic _arm symptomatic_plant') or in asymptomatic plants ('Asymptomatic_arm asymptomatic_plant'). (B) Communities found in the cane with manifested foliar symptoms ('Symptomatic_cane') or asymptomatic, but coming from symptomatic plants ('Asymptomatic_cane symptomatic_plant') or asymptomatic plants ('Asymptomatic_cane asymptomatic_plant'). Ellipses illustrate the multivariate normal distribution of samples within the same tissue group.

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CHAPTER 6

FINAL REMARKS

6. Final remarks

Over the last 20 years, the interest in grapevine trunk diseases has been steadily increasing. Esca is perhaps the most complex, among GTDs, and several aspects of its etiology remain to be fully elucidated. Reports on the influence of climate and weather conditions on the manifestation of foliar symptoms often contradict each other (Calzarano *et al.*, 2018; Serra *et al.*, 2018), and weak correlations were found when comparing pruning and training systems (Travadon *et al.*, 2016; Lecomte *et al.*, 2018). The much declared 'trend of increase' in the disease's incidence is not uniformly reported in literature (Reisenzein *et al.*, 2000; Redondo *et al.*, 2001; Bruez *et al.*, 2013; Guérin-Dubrana *et al.*, 2013; Li *et al.*, 2017; Calzarano *et al.*, 2018), and the causal agent(s) behind the foliar symptomatology manifestation are not unanimously agreed upon. We currently lack a fast and reliable mean for assessing the extent of field infections (Pollastro *et al.*, 2000), as symptoms are mostly hidden in the wood, as well as detection methods for infected plant material in nurseries. Some authors even put in doubt the very role of tracheomycotic fungi in the esca symptomatology expression (Hofstetter *et al.*, 2012), especially, but not exclusively, in light of the presence of esca-associated fungi in asymptomatic wood (Rumbos and Rumbou, 2001).

Trials conducted over the last 15 years, aiming to find effective control strategies to deal with infected vines or prevent infections from occurring, did not lead to the formulation of a solid strategy (Mondello *et al.*, 2017). This may not be necessarily due to the lack of effectiveness of treatments, but instead due to the lack of reliable parameters to assess their efficacy. For example, endotherapy and foliar spray treatments have been mostly evaluated by the reduction of foliar symptoms manifestation, however, the link between this symptom and the action of pathogens in the wood remains unclear. Despite it is true that foliar symptomatic plants correspond to an advanced stage of infection in the wood, plants with a similar wood symptomatology may not exhibit foliar symptoms. Moreover, the identification of treatments that prevent the manifestation of foliar symptoms, such as the case of sodium arsenite (Fontaine *et al.*, 2016) or the more recent mixture developed by Calzarano and Di Marco (2018), does not necessarily mean that the fungi in the wood have been effectively controlled.

For this reason, the first three lines of research of this PhD project focused on assessing the efficacy of treatments by using as parameter the reduction in presence/abundance of pathogens, rather than foliar symptomatology. By controlling pathogens in the wood, we are able to prevent, or at least delay, their negative effects on the plants physiology and microbial ecology, on the hydraulic systems of the stem and, as a consequence, on the manifestation of foliar symptoms and apoplexy.

Each of the three tested strategies proved effective against early infections by *Phaeomoniella chlamydospora* and/or *Phaeoacremonium minium*, under greenhouse conditions. In biological control, both *Epicoccum layuense* E24 and the skopobiota were capable of reducing the wood symptoms expression and of antagonizing the wood colonization success of *P. chlamydospora* and/or *P. minimum*. The endotherapy with silver nanoparticle, hydrogen peroxide and glutaraldehyde was particularly effective against early infections by *P. chlamydospora*. In addition, this pathogen was greatly inhibited when plants were sprayed with Blad-containing oligomer. Despite these three control strategies were effective when applied alone, their joint action may produce enhanced results. For example, a young grapevine with an established infection may be treated with endotherapy, strongly decreasing the pathogens abundance in the wood. Subsequently, the plant may be inoculated with *E. layuense* E24 or a skopobiota, antagonizing the pathogen from the re-colonization of the wood. Meanwhile, vines can be sprayed with Blad-containing oligomer, another mean for weakening *P. chlamydospora*'s wood colonization success, while also being effective against other common grapevine pathogens.

The study on the wood mycobiome of adult grapevines, in a vineyard with history of esca, contributed to the ecological understanding of the fungal communities that may play a role in the whole disease complex. The richness of taxa is considerably greater than what previously believed, and the differences observed in the spatial distribution of fungal communities are crucial information for planning future studies. These results lay the foundation for future investigation on the effect of control strategies on the wood mycobiome, whether the aim is to fight GTD-associated pathogens or to monitor the effect of treatments on non-target fungi, at community level.

The natural continuation of the work pursued during this PhD lies in the optimization of tested concentrations of active ingredients, means and timings of treatment application, and the investigation in a medium-to-long term perspective. Eventually, these ameliorated control strategies may be applied in nurseries and young or established vineyards. Here, in conjunction with other control strategies suggested in the literature, such as pruning wound protection, nursery sanitation and 'best practices' in the field, the checkmate on the esca disease complex will take place.

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