Exploring the Role of *Fomitiporia mediterranea* in the Development of Grapevine Leaf Stripe Disease Symptoms

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My thanks, friends of the County Scientific Association,

For this modest boulder,

And its little tablet of bronze.

Twice I tried to join your honored body,

And was rejected,

And when my little brochure

On the intelligence of plants

Began to attract attention

You almost voted me in.

After that I grew beyond the need of you

And your recognition.

Yet I do not reject your memorial stone,

Seeing that I should, in so doing,

Deprive you of honor to yourselves.

(Perry Zoll, Spoon River Anthology (1915) - Edgar Lee Masters)

Preface

This dissertation is presented in two chapters and three annexes (published manuscripts). The first chapter introduces the theme of grapevine trunk diseases focusing on the Esca complex, how to manage this disease and provides an overview of *Fomitiporia mediterranea*, one of the pathogens associated with the Esca complex of diseases, the main subject of this manuscript. The second chapter, called "Results and Discussion", summarizes the results published in the first and third annexes and, for the first experiments, provides supplementary results achieved on-field during a third year of survey.

The first manuscript was published in the Journal of Fungi in 2021 (volume 7, number 521); the second was published in Phytopatologia Mediterranea, also in 2021 (volume 60, issue 2, pp. 351–379); and the third was published in the journal Frontiers in Microbiology in 2022 (13:844264, doi: 10.3389/fmicb.2022.844264).

Abstract

The adverse effects of Esca on the world's viticultural heritage are widely known. Authors such as Ravaz and Viala meticulously described the effects of this disease on the vineyards of the Mediterranean basin at the beginning of the 20th century and contributed to its definition. The evolution of "Esca" into the "Esca complex" stems from the efforts of scientists worldwide. Over the last century, disease management strategies have evolved to include the phasing out of the substances that are most dangerous to human and environmental health. The arsenic-based products initially used in the vineyard for the control of moths have been fundamental in limiting the foliar symptoms of Esca. The banning of these products resulted in a lack of Esca control strategies; consequently, abandoned techniques have been re-evaluated in more modern times, aided by the availability of more efficient equipment. Trunk surgery was historically applied for the removal of decayed wood on many tree species as well as grapevine. The first study in this thesis consolidates data on the efficacy of trunk surgery by testing three levels of decayed wood removal. Here, the published results of two years of survey (Annex I) are augmented by the findings of a third year of survey. A parallel study on the effect of the technique on the vine microbiota confirmed that *Fomitiporia mediterranea* (Fmed) is the species most affected by the removal of decayed wood, as also highlighted by other approaches. The results reinforced the hypothesis, formulated at the beginning of the 20th century, that the presence of decayed wood can lead to the expression of foliar symptoms. Using a multidisciplinary approach, a study was subsequently conducted on Fmed-associated wood degradation processes (Annex III) focusing on the enzymatic pathway of the fungus. Wood cell structure degradation was initially assessed by microscopy. Then, vine wood degradation was reproduced *in lignum* and the levels of each structural polymer were quantified. Finally, the activity of the main ligninolytic enzymes was measured and their molecular modulation was assessed. Our findings helped to consolidate on-field data that were not present in the literature when the experiments were designed and highlighted the importance of the complete removal of decayed wood in trunk surgery. Analysis of the Fmed enzymatic pathway contributed to the knowledge of the fungus, which has recently gained considerable research interest for its role in the Esca complex. The results of the analysis suggested that, as reported for other pathosystems that include *Basidiomycota* species, a distinct pathway which acts synergistically with the enzymes could be necessary for wood degradation to occur, thus opening up a new line of research on grapevine wood decay mechanisms.

Riassunto

Gli effetti negativi del mal dell'Esca sul patrimonio viticolo mondiale sono ampiamente conosciuti. Autori come Ravaz e Viala hanno meticolosamente descritto gli effetti di questa malattia sui vigneti del bacino del Mediterraneo all'inizio del XX secolo e hanno contribuito alla sua definizione. Il processo che ha portato da "Esca" a "complesso Esca" è il risultato degli studi di scienziati di tutto il mondo. Nell'ultimo secolo, le strategie di gestione delle malattie nel vigneto si sono evolute prevedendo una graduale eliminazione delle sostanze più pericolose per la salute umana e l'ambientale. I prodotti a base di arsenico inizialmente utilizzati in vigna per il controllo delle tignole sono stati fondamentali anche per limitare i sintomi fogliari dell'Esca. Il successivo divieto di questi prodotti ha lasciato un vuoto tra le strategie di controllo dell'Esca e di conseguenza alcune tecniche abbandonate in passato sono state rivalutate in tempi più moderni, grazie soprattutto alla disponibilità di attrezzature più efficienti. La dendrochirurgia è stata una tecnica utilizzata storicamente per la rimozione del legno cariato su molte specie arboree e sulla vite. Il primo studio di questa tesi consolida i dati sull'efficacia della dendrochirurgia, testando tre livelli di rimozione del legno cariato. I risultati ottenuti con due anni di indagine (allegato I) sono implementati in questa tesi dai risultati di un terzo anno di monitoraggio. Uno studio parallelo sull'effetto della tecnica sul microbiota della vite ha confermato che *Fomitiporia mediterranea* (Fmed) è la specie più colpita dall'asportazione del legno in decomposizione, come evidenziato anche da altri approcci. I risultati hanno rafforzato l'ipotesi, formulata all'inizio del '900, che la presenza di legno in decomposizione possa portare all'espressione dei sintomi fogliari. Utilizzando un approccio multidisciplinare, è stato successivamente condotto uno studio sui processi enzimatici di degradazione del legno associati a Fmed (allegato III). La degradazione delle strutture cellulari del legno è stata inizialmente valutata mediante microscopia. Quindi, il processo di carie del legno di vite è stato riprodotto *in lignum* ed è stata quantificata la diminuzione di ciascun polimero strutturale. Infine, è stata misurata l'attività dei principali enzimi ligninolitici ed è stata valutata la loro modulazione molecolare. I nostri risultati hanno contribuito a consolidare dati di campo che non erano presenti in letteratura al momento della progettazione sperimentale e hanno evidenziato l'importanza della completa rimozione del legno cariato nella dendrochirurgia. L'analisi della via enzimatica di Fmed ha contribuito ad accrescere la conoscenza del fungo. Il quale ha recentemente ottenuto un notevole interesse da parte del mondo scientifico per il suo ruolo nel complesso Esca. I risultati dell'analisi hanno suggerito, come riportato per altri patosistemi che includono

specie di Basidiomiceti, che potrebbe essere necessario un processo distinto in grado di agire sinergicamente con gli enzimi affinché si verifichi la degradazione del legno, aprendo così una nuova linea di ricerca sui meccanismi di decadimento del legno.

Résumé

Les effets néfastes de l'Esca sur le patrimoine viticole mondial sont connus depuis toujours, et d'autant plus depuis l'ère moderne de la viticulture. Des auteurs comme Ravaz et Viala ont décrit avec précision les effets de cette maladie sur les vignobles du bassin méditerranéen au début du XXe siècle et ont contribué à sa définition. L'évolution d'Esca en complexe de l'Esca est le résultat de nombreuses études effectuées par des scientifiques du monde entier, qui ont su adapter les techniques d'investigation aux nouvelles technologies et ainsi obtenir des résultats toujours plus précis. Tout au long du dernier siècle, les techniques de gestion de la maladie ont également évolué en tenant compte non seulement de leur efficacité mais aussi de leur durabilité. Les produits à base d'arsenic, initialement utilisés dans le vignoble pour lutter contre les mites, ont joué un rôle fondamental dans la limitation des symptômes foliaires de l'Esca. L'interdiction de ces produits a fait émerger un manque de stratégie de contrôle de l'Esca et a conduit à la réévaluation, grâce à la disponibilité d'outils plus efficaces de techniques abandonnée. Entre ces, le curetage du tronc, historiquement adoptée pour l'élimination de l'amadou sur de nombreuses espèces d'arbres et sur la vigne. La première étude présentée dans cette thèse montre des données sur l'efficacité du curetage du tronc en testant trois niveaux d'élimination de l'amadou : les résultats obtenus pour les deux premières années de suivi ont donné lieu à une publication (annexe I) et ont été complétés dans ce manuscrit par les résultats de la troisième année de suivi. Parallèlement, l'étude de l'effet du curetage sur le microbiote de la vigne a permis de confirmer que *Fomitiporia mediterranea* (Fmed) est l'espèce la plus affectée par l'élimination de l'amadou comme souligné par d'autres auteurs. Les données obtenues permettent de renforcer l'hypothèse - déjà formulée au début du XXe siècle – selon laquelle la présence d'amadou pourrait être liée à l'expression de symptômes foliaires. L'objectif de la seconde partie de la thèse (annexe III) concerne le processus de dégradation du bois par Fmed, et a été menée pour caractériser l'activité de dégradation du bois par le champignon selon une approche multidisciplinaire. La dégradation des structures cellulaires du bois a été dans un premier temps décrite par des observations microscopiques. Par la suite, la dégradation du bois de vigne a été reproduite

in lignum et la diminution des polymères constitutifs du bois a été quantifiée. Enfin, l'activité des principales enzymes lignolytiques et leur modulation moléculaire a été mesurée. L'ensemble de cette étude a permis de confirmer des observations faites sur le terrain et absentes de la littérature scientifique lors de la conception de l'expérimentation. L'importance d'une élimination complète de l'amadou lors du curetage du tronc a été mise en évidence et des résultats pertinents sur les effets de la technique sur le microbiote de la vigne ont été publiés. L'étude de la voie enzymatique de dégradation du bois par Fmed a contribué à la connaissance de ce champignon qui, ces dernières années, a suscité un intérêt croissant de la part de la communauté scientifique pour son rôle dans le complexe de l'Esca. De plus, sur la base des résultats obtenus en analysant la voie enzymatique de Fmed, il a été possible de supposer qu'une autre voie de dégradation du bois complémentaire est nécessaire pour que la pourriture du bois se produise, comme cela se passe dans d'autres systèmes pathogènes, ouvrant ainsi une nouvelle voie de recherche basée sur la voie non enzymatique.

Introduction

Human population growth represents the most earth-impacting megatrend forecast for this century. The global population is estimated to reach 9.73 billion in 2050 and 10.87 billion in 2100 (Gerland et al., 2014). Compared with the population of 6.15 billion in 2000, this would represent an increase of 60% over the next 50 years (United Nations, 2019). This massive phenomenon trolling with itself a great increase of food needs will require dedicating a large part of arable land to the production of commodities such as wheat, corn, and rice on which the world's population most depends. The agriculture of the future will develop in a context of climate change and a scarcity of available water, and although technological progress has allowed an increase in yields per unit of crop surface, a land-use conversion will undoubtedly be necessary (FAO, 2009; Le Mouël et al., 2018). At the same time, increasing global temperatures due to climate change have negatively affected harvestable yields and induced a marked reduction in areas suitable for the cultivation of traditional crops (Olesen and Bindi, 2002; Fraga et al., 2012; Droulia and Charalampopoulos, 2021). Taken together, these factors open up a scenario in where new arable lands will likely play a key role in the agricultural production of the future. However, this is not easily applicable to viticulture. Wine is a territory-linked product, and its value is largely associated with the multifactorial concept of "terroir", a combination of socioeconomic factors that include the human factor, making it unlikely that wine production will relocate to new areas (Van Leeuwen and Seguin, 2006; Gladstones, 2011; White, 2020). Consequently, strategies that will allow the continuation of the production of high-quality wines must be implemented in current wine-growing regions worldwide.

There is another plague that has undermined viticulture since, or perhaps even before, its inception, and undoubtedly challenges its economical sustainability, namely, Grapevine Trunk Diseases (GTDs) (Mugnai et al., 1999; Bertsch et al., 2013). The phytosanitary aspect associated with grapevine cultivation comprises annual diseases that affect the green organs and fruits, for which many control tools are available to winegrowers, as well as diseases affecting the perennial organs of the vine, characterized by a continuously increasing incidence (Wilcox et al., 2015). The latter are known as GTDs and represent an important challenge for viticulture in the 21st century. Like annual diseases, GTDs negatively affect grapevine productivity in terms of yield, alcohol content, and aroma (Mugnai et al., 1999; Calzarano et al., 2004b; Lorrain et al., 2012). Moreover, GTDs can markedly reduce vineyard lifespan, thereby representing a long-term challenge for viticulture sustainability (Bertsch et al.,

2013). GTDs include Eutypa dieback, Phomopsis dieback, Botryosphaeria dieback, and the Esca complex, which involve one or more wood-inhabiting fungi. Symptoms associated with Eutypa dieback and Phomopsis dieback have been described as "black-dead arm" (BDA), a term that sporadically included symptoms that are now associated with Botryosphaeria dieback (Gramaje et al., 2018). In 1914, Reddick (1914) provided a description of vine dead-arm disease, caused by *Cryptosporella viticola* (syn. *Diaporthe ampelina*; family: Diaporthaceae), the symptoms of which included the presence of bare arms and crinkled, yellowish-colored leaves (Reddick, 1914). During the following years, many other dead arm-like symptoms were reported by various authors (Pine, 1958; Chamberlain et al., 1964; Lehoczky, 1972). All these contributions led to the definition of BDA, which initially referred specifically to *Diplodia mutila* infections of wood canker, but without the association of characteristic leaf symptoms (Lehoczky, 1974). Later in the early 2000s, other authors reported BDA symptoms in French vineyards, identifying a mild and a severe form of the disease, and redescribed the symptomatology including foliar symptoms (Larignon and Dubos, 2001; Larignon et al., 2001). Larignon e Dubos (2001) incorrectly ascribed other botryosphaeriaceous pathogens, *Botryosphaeria dothidea* and *Diplodia seriata*, as well as different symptoms, to the disease that had been described as BDA by Lehoczky in 1972. The broad set of symptoms and the uncertain pool of pathogenic agents associated with BDA made the diagnosis of the disease difficult and symptoms were often confused with those described for other diseases [well resumed by Urbez-Torres (2011)]. Eutypa dieback, Phomopsis dieback, and Botryosphaeria dieback have now all been more clearly defined.

The main causal agent of Eutypa dieback was identified as *Eutypa lata* (Pers: Fr.) Tul & C. Tul. (=*E. armeniacae* Hansf. & M.V. Carter) (Carter, 1991), a fungus that is distributed worldwide, but mainly in Australian and American vineyards (Carter, 1991; Sosnowski et al., 2009, 2011; Pitt et al., 2013; Ayres et al., 2017). This could be due to the lower aggressiveness of European fungal populations, which are suggested to be low producers of eutypine or wood-hydrolytic enzymes (Péros and Berger, 2003). Phomopsis dieback is the least relevant of the GTDs in terms of crop losses, and induces severe symptoms mainly in grape-growing regions characterized by a humid, temperate climate through the growing season, where it results in up to 30% yield loss (Úrbez-Torres et al., 2013). Phomopsis dieback is caused by *Diaporthe ampelina* Berk. & M.A. Curtis (syn. *Phomopsis viticola* Sacc.), which also causes a different disease called cane and leaf spot disease (Erincik et al., 2001; Úrbez-Torres et al., 2013) and is known under the name "excoriose" in Europe. Botryosphaeria

dieback includes symptoms such as wedge-shaped cankers, wood necrosis, progressive bud-break failure, and vine dieback and is caused by fungi belonging to the *Botryosphaeriaceae* family (Urbez-Torres, 2011). Different *Botryosphaeriaceae* species can be isolated at the same time from grapevine showing Botryosphaeria dieback symptoms and these species are often isolated alongside other GTD-related pathogens from the same vine (Luque et al., 2009; Urbez-Torres et al., 2011). Botryosphaeria dieback is currently the most significant of the GTDs worldwide. The Esca complex, the subject of this thesis, refers to a complex of diseases belonging to the GTDs that mainly affect European vineyards.

1. "Esca"

The description of the symptoms associated with the Esca complex dates to the beginning of the 20th century. Pierre Viala, in his book "Recherches sur les maladies de la vigne: Esca. Annales Des Epiphyties" (1926), begins by saying that "Esca is a very ancient disease of the grapevine, the characteristics and causes of which have only recently been described". Further on, the author states that the disease was endemic everywhere, namely throughout the Mediterranean basin, including Syria, Palestine, Greece and its islands, and southern Italy, especially the Apulia region. Viala indicated that vines aged between 15 and 25 years were the most susceptible to Esca and that the incidence of the disease increased with increasing vine age. The author listed some symptoms associated with Esca, such as apoplexy, progressive weakening of the vine, mosaic discoloration or yellowing of the leaves, and partial wilting of the shoots, a symptom that often ends with the apoplexy of the whole vine. Images in Viala's book (Figure 1) represent the same foliar and wood symptoms described for Esca (*sensu lato*) in more recent times (Larignon and Dubos, 1997; Mugnai et al., 1999; Lecomte et al., 2012). Before Viala's book, a preliminary definition of what was then called "Esca" was officially reported at the end of the 19th century, namely, "apoplexie" (Rolland, 1873) or "folletage" (Ravaz, 1898). These two terms were used as synonyms to describe the sudden death of vines that occurred during the summer season.

The author L. Chiarappa also refers to a communication of this symptom - called "sunstroke"- concurrently reported in California by an anonymous author in 1895 (Anonymous, 1895; Chiarappa, 2000). Over the following years, and until 1926, evidence was added in support of the syndrome called "apoplexie", leading to the definition of Esca as a disease (Ravaz, 1898, 1906, 1909, 1919; Pavlou, 1906). Fundamental was the contribution of Ravaz, who first associated wood rot with the presence of *Polyporus igniarius* [syn. *Fomes*

igniarius (L. ex Fr.) Kick and *Phellinus igniarius* (L.) Quél] mycelia in the diseased tissues (Ravaz, 1909). A second causal agent, *Stereum necator* Viala., was first proposed to be a main pathogen by Vinet, and was later better described by Viala (Vinet, 1909; Viala, 1926). A new symptom was introduced by Viala in 1926 and was associated with the previously described wood (wood rot) and crown (apoplexy) symptoms. The foliar symptoms (Figure 1, left) were described as a light form of the disease, characterized by foliage deterioration while the apoplexy was associated with the acute form of Esca. At this time, "Esca disease" referred to the phenomenon of apoplexy resulting from the presence of fungal-induced wood decay, i.e., what had once been described as a physiologic disorder associated with an "abrupt change in the bottom water level" now became thought of as a parasitic-based disease.

Figure 1 - Depiction of tiger striped leaves of grapevine (left) and white rot decay in sectioned vine trunk (right) (Viala, 1926).

After few decades, studies were conducted also in California on the same disease there called Black measles, due of the frequent symptoms on the berries, showed that the disease was inconstant over years even on the same vine (Hewitt, 1957). Further trials carried out on the same vineyard allowed to

satisfy Kock's postulates on wood symptoms by inoculating and re-isolating *Cephalosporium* sp. and *Fomes igniarius* from Black measles-diseased vines, reproducing vascular streaking and wood rot respectively (Chiarappa, 1959). In that study, the author established a correlation between internal wood decay and the leaf and berry black-measles symptoms. Later on, the role of *S. necator* in grapevine wood degradation was excluded and it was suggested that the presence of the fungus in the samples studied by Viala was probably due to a contamination of vine wood by carpophores developed on wooden poles present in vineyards (Baldacci et al., 1962). *S. necator* would have therefore only acted as a saprophyte in grapevine wood. The study of Chiarappa reported a wood symptom that had been previously highlighted by Petri in 1912, who described brown wood streaks, linked to abundant gum and tyloses formation in wood vessels. In that study, the isolation of fungi belonging to genus *Cephalosporium* and *Acremonium* has been performed from vine wood of mother vines in a nursery affected by pruning-induced disorders, associable to what French authors called apoplexy at that times (Petri, 1912). Esca and Black measles as a synonyms were finally better redefined during in the last decade of 20th century and then described as Esca complex of diseases during the first decade of 21th century (Larignon and Dubos, 1997; Mugnai et al., 1999; Surico, 2009).

1.1. From *Cephalosporium* spp. to *P. chlamydospora* and *P. minimum*

Petri reported two unidentified species of *Cephalosporium* and one of *Acremonium* as the causal agents of the black streaks in the wood of vine plants (Petri, 1912). Chiarappa reported that these internal symptoms were present in vines that also showed external symptoms of Esca/black measles. In 1994, *Phialophora parasitica* was also associated with internal wood symptoms (Ferreira et al., 1994). Other authors subsequently established that the genus *Cephalosporium* differed from *Phialophora* spp. -the causal agent of wood discoloration that had also been isolated by Chiarappa- and *Acremonium* spp. representing the distinct genus *Phaeoacremonium* (Crous et al., 1996). Two species of this genus were then assigned to Esca when it was finally described as a complex of diseases, namely, *P. aleophilum* and *P. chlamidosporum* (Mugnai et al., 1996, 1999). The latter was subsequently redefined as *Phaeomoniella chlamydospora* based on morphological characteristics (Crous and Gams, 2000). *P. aleophilum* was recently reclassified as *Phaeoacremonium minimum* after a molecular-based revision (Gramaje et al., 2015).

1.2. From *Polyporus igniarius* to *Fomitiporia mediterranea*

Since Ravaz and Viala, other basidiomycete fungi have been associated with Esca but only *Polyporus igniarius* was successively confirmed as a pathogen able to colonize and degrade vine wood as mentioned above. At the end of the 1990s, studies conducted in Italy and France confirmed the results obtained by Chiarappa in 1959, namely, the presence of *Phellinus punctatus* (Fr. Kast.) Pilat (syn. *Phellinus igniarius*) (Mugnai et al., 1996; Larignon and Dubos, 1997) in Esca-infected vine wood. At the same time, the nomenclature of the genus *Phellinus* was revised again and the basidiomycete associated with the decayed wood of Esca-infected vines was renamed *Fomitiporia punctata* (Fr.) Murrill (Fischer, 1996; Mugnai et al., 1999). Further genome-based analysis performed on strains isolated from the decayed wood of grapevine led to the definition of the species *Fomitiporia mediterranea* (Fmed) (Fischer, 2002).

1.3. From Esca to the Esca complex of diseases

According to the definition proposed by Surico (2009), Esca is currently considered a complex of four different diseases: brown wood streaking, grapevine leaf stripe disease (GLSD) (previously young Esca), Petri disease, and Esca. The term "Esca proper" is used to describe vines that show both foliar GLSD symptoms and white rot. Although GLSD mainly affects vineyards in the Mediterranean area, the Esca complex, in the form of at least one of the associated diseases, is now widespread in all the main vine-growing regions of the world together with the other GTDs, and their incidence is increasing (Scheck et al., 1998; Ridgway et al., 2002; Halleen et al., 2003; Edwards and Pascoe, 2004; Bertsch et al., 2013; Guérin-Dubrana et al., 2019; Ye et al., 2021). GLSD symptoms typically appear inconstantly over the years, i.e., while symptoms in wood occur perennially after their first appearance, foliar symptoms may not. Foliar symptoms associated with diseases of the Esca complex are well characterized and consist of a green band with a chlorotic perimeter around the main veins, while the central interveinal area appears reddish or yellow, depending on the grapevine variety (Figure 2 A) (Mugnai et al., 1999; Lecomte et al., 2012). Although infection of the wood by the relevant pathogens is a prerequisite for foliar symptoms to occur, other factors also play a regulatory role. Although foliar symptoms are more often expressed in vines older than 10 years (Reisenzein et al., 2000), cases of GLSD in younger vines are increasingly being reported (Romanazzi et al., 2009). The older the vine and the greater the number of pruning wounds, the more wood symptoms are detectable (Figure 2 B). Moreover, each variety of vine is

characterized by a different susceptibility to GLSD symptom expression (Marchi, 2001; Quaglia et al., 2009; Murolo and Romanazzi, 2014; Moret et al., 2019, 2021) and wood degradability (this thesis). Finally, the environment, training system, rootstock, weather, and training practices also influence symptom expression (Marchi et al., 2006; Calzarano et al., 2018; Lecomte et al., 2018; Fischer and Ashnaei, 2019; Kraus et al., 2019; Songy et al., 2019a).

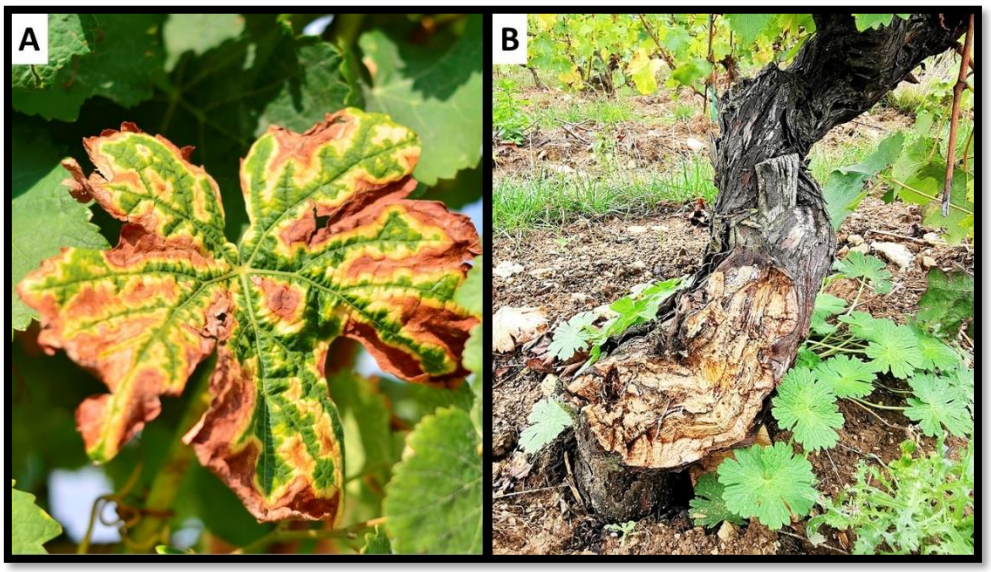

Figure 2 – Typical foliar symptom of grapevine leaf stripe disease (A), a disease within the Esca complex, and internal symptoms represented by white rotten wood (B). As reported by Viala, large-diameter wounds are frequently associated with wood decay (Viala, 1926).

2. Esca management: old tools and current strategies

Since the recognition of Esca as a disease by winegrowers, several methods of disease control have been adopted. An ancestral practice, initially adopted in Greece and southern Italy and subsequently in many wine-growing regions of the Mediterranean basin, consists of splitting the trunk of symptomatic vines into two and inserting a stone to keep the two sides separate (Mugnai et al., 1999; Rumbos and Rumbou, 2001). Although the technique appeared to be useful, it was ineffective in the long term. Defenses against Esca-associated pathogens have evolved over time, first for the control of the disease in the vineyard, and then along the entire supply chain, including nurseries where the young vines are produced. A valid tool that allowed Esca control in European vineyards for more than half of the 20th century were arsenic-based products.

2.1. Arsenite

Very interesting is the millennial link that binds humans to the use of arsenite. Arsenic is a semimetal element ubiquitous in nature, in both inorganic and organic forms and since ancient times, arsenite was used both as a poison and as a precursor to what we nowadays call drug. In ancient Greece arsenic-based compounds were already known. Hippocrates, considered the father of medicine, already at the time used arsenite to heal ulcers and abscesses (Waxman and Anderson, 2001; Riethmiller, 2005). The name arsenic was probably derived from the Greek "arsenikon", meaning powerful or strong. Also The Romans made use of arsenite and the culture of its use lasted throughout the Middle Ages up to the modern era (Meek, 1955). "Cantarella", also known as "the Gift of Borgias" was a white powder made from arsenite and frequently used by Borgia's family to commit political murders during the Renaissance in Italy (Karamanou et al., 2018). Based on this historical knowledge on the effects of arsenite, at the beginning of the 18th century, arsenite was included as active ingredient in products used to protect potatoes from attack by the Colorado potato beetle (*Leptinotarsa decemlineata*) (Riley, 1876). From the 19th century and then during the beginning of 20th century, the use of arsenite spread in agriculture and construction as insecticides, rat poisons, herbicides and wood preservatives, as well as pigments in paints and wallpapers (Quaintance and Scott, 1912; Schultz and Thompson, 1925; Gosselin et al., 1984). This meant that arsenite found many applications also in viticulture, mainly as insecticide and fungicide: against grapevine moths (*Eupoecilia ambiguella* and *Lobesia botrana*) (Capus and Feytaud, 1909), anthracnose (Vergnes, 1957), Phomopsis cane and leaf spot (Hewitt, 1951) and

Esca (Ravaz, 1922; Bonnet, 1926; Viala, 1926). During the period of the world wars and later during the Vietnam war, the study and use of arsenite (Agent Blue) continued in order to improve its use as a herbicide in warfare (Stellman et al., 2003; Bencko and Foong, 2017). In the second half of the 20th century the war industry was converted and like many other war-linked development, arsenite found its widest use as a crop-protection product (Rui and Battel, 1963; Svampa and Tosatti, 1977; Bencko and Slámová, 2007). The toxicity of arsenite was discussed at length. In the region of the Kaiserstuhl and Moselle regions in Germany, people used to produce a wine for their own use from the marc. Grape marcs were suspended in water and pressed a second time for wine production. This method led to a deep wash of grape skin which contained residues of arsenite and the resulting product contained significant quantity of arsenite residues (H-H Kassemeyer personal communication). Study performed on the inhabitants of wine-producing areas where arsenite was used against grape moths showed a higher incidence of lung, skin and liver cancer compared to populations resident in wine-producing regions where arsenic compounds were not used (Tataryn, 2021). Based on this evidence, Germany banned the use of arsenite in viticulture in 1942 (Trappmann, 1948). In 1977, arsenite was also phased out from viticulture in Italy (Ferrari, 1995) while in Europe , to the best of our knowledge, it remained in use only in France, Spain and Portugal. France was the last European country phasing-out arsenite in 2001. Long after Italy and Germany banned arsenite, France continued to support its use in viticulture and public opinion continued to be influenced for many years by pro-arsenite claims and advertising (Figure 3) (Boubals, 1995). Larignon and Dubos (1997) state that at the time the Black measles disease in France was less relevant compared to the other Mediterranean countries because it was controlled with sodium arsenite. Europe completely banned arsenite for phytosanitary use in 2003 (Council Directive 91/414/EEC). Arsenite was withdrawn from agricultural use due to its high risks for human health and its negative impact on the environment (Spinosi et al., 2009). The efficacy of arsenite against GTDs did not find a comparable substitute among sprayable products allowed nowadays in viticulture. A recent study showed how arsenite not only acts directly on inhabiting microorganisms but also activate grapevine defense response (Songy et al., 2019b). Moreover, analysis conducted on the vine microbiota showed that arsenite predominantly affected fungi, mainly *Fomitiporia mediterranea*, compared to bacteria, allowing saprobic fungi to colonize the decayed wood otherwise occupied by this fungus (Bruez et al., 2021b). This is reinforced by the evidence showing that arsenite accumulates mostly in the decayed wood of the vine trunk (Larignon et al., 2008; Goddard et al., 2017).

Figure 3 - Color poster, (original dimension 69 x 59 cm), published by Gabriel Griffié and sons establishments (Villemoustaussou, Aude department, France) in 1936. This iconographic document represents the trademark deposited at commercial court of Carcassonne by the company. The title quotes: "Sodium arsenite - superior insecticide". The short note: "Dear! Remember, this is the best insecticide. With it no more moths, leaf beetle and other insects. No more Folletage, Court-noué (Eutypa) etc.". At the time the poster was designed, the harmful properties of sodium arsenite were not known. So, it is not surprising to see, represented in the promotional material, an elderly winegrower teaching his grandson the virtues of the insecticide: knowledge is transmitted from generation to generation. The grandfather shows with his right hand the name of the product and lists the benefits of sodium arsenite. Source: XVIIe-XIXe siècle - Les Archives départementales de l'Aude https://archivesdepartementales.aude.fr

2.2. Trunk renewal and re-grafting

The arsenite ban resulted in the absence of an effective treatment against Esca. This lack of disease control strategies pushed research to find new solution able to reduce the leaf stripe foliar symptoms expression and the effect of some foliar treatments based on vines defense inducers were studied: natural products such as propolis, chitosan, vegetal extracts such as *Allium sativum*, or brown seaweed extract mixed with mineral nutrients have been tested, and have shown variable levels of pathogen control and symptom remission (Palma-Guerrero et al., 2008; Calzarano et al., 2014, 2017; Cobos et al., 2015; Calzarano and Di Marco, 2018). Furthermore, different disease management practices have been adopted in many wine-growing areas of the world to safeguard the vineyard heritage (Becker, 2018). One technique adopted to counter the Esca-linked decline in the vineyard, which is also used in the management of Eutypa and Botryosphaeria dieback, is trunk renewal (Calzarano, Di Marco, *et al.*, 2004; Sosnowski *et al.*, 2011; Smart, 2015). GLSD symptomatic vines are marked during the growing season, usually using tape, to allow their reidentification during the winter when they lose their leaves. For these vines, a watershoot is raised for one or two years and, during the following winter, the old trunk is eliminated to allow the development of the new one (Egger et al., 1997; Savocchia et al., 2014). A variant of the technique is based on cordon renewal for spur pruned, cordon-trained vines, leaving the trunk standing. This technique is applied when trunks are apparently healthy inside and few or no large-diameter wounds are present (Smart, 2015; Gohil et al., 2019). The success rate of trunk renewal is variable. Additionally, when the portion of decayed wood is very extensive and affects the entire trunk of the vine, regrafting is often also adopted.

Although regrafting allows overcoming the problem of watershoot availability, it requires the intervention of qualified personnel. The trunk is cut 4–5 cm below the pre-existing grafting point and split in two. Then, a bevel graft is applied, whereby a V-shaped scion is inserted at each end of the open cut; after one year, only one newly formed trunk will be retained (Figure 4) (SICAVAC, 2013). Regrafted vines can recover half their yield and quality within one year and normal production in two years after the treatment due to efficient and mature rootstock (SICAVAC, 2013). In comparison, it takes four years for a newly planted vine to achieve a normal yield. For both trunk/spur renewal and regrafting techniques, row management must be adapted and agronomical practices modified accordingly. New shoots from grafted buds or new watershoots are easily damaged and cannot be managed using mechanical tools. Moreover, not all diseased vines produce a watershoot every year and,

in such cases, the renewal process can take longer (Sosnowski et al., 2011). Finally, white rot or Esca-associated wood discoloration commonly also affects the rootstock in older vines; in these cases, neither trunk renewal nor regrafting can be employed (Calzarano et al., 2004a; Maher et al., 2012; Elena et al., 2018). Vine wood can be colonized by pathogenic fungi through wounds made in the nursery during the grafting process and in the vineyard by pruning (Aroca et al., 2010; Van Niekerk et al., 2011; Zanzotto et al., 2013; Gramaje and Di Marco, 2015; Martínez-Diz et al., 2020). Unlike trees, grapevine cannot compartmentalize wounds, rendering vine wood very susceptible to infection (Shigo, 1984; Sun et al., 2008). Many active ingredients have been tested to limit wood infection by fungal pathogens, such as synthetic, inorganic active ingredients and biocontrol agents (BCAs).

Figure 4 – Three-year-old Sauvignon blanc regrafted vine made by SICAVAC technicians in the Val de Loire region of France. After the first season in which the grafted buds have vegetated, only one shoot is retained to form the new vine trunk

2.3. Protecting wounds against infection in the nursery and vineyard

Benomyl, captan, didecyldimethylammonium chloride, and carbendazim are effective in reducing infections on graft cuts during vine grafting in the nursery (Fourie and Halleen, 2006; Gramaje et al., 2009; Gramaje and Armengol, 2011). Carbendazim combined with flusilazole was reported to be effective against *P. chlamydospora* and *P. minimum*; however, the results obtained with other tested triazoles were unsatisfactory (Serra et al., 2011; Mondello et al., 2018). Dithiocarbamates ziram and thiram also produced encouraging results against tracheomycotic fungi if used in combination with hot water soaking treatment before grafting in the nursery (Eskalen et al., 2007). Preventive strategies under vineyard conditions are essential for reducing the risk of new infections by wood-inhabiting pathogens. Fungi associated with the Esca complex mainly spread *via* the airborne route throughout the vineyard (Surico et al., 2000). Although several studies have proposed different vectors for the transmission of fungal spores, including cutting tools used for pruning, the contribution of these vectors is minimal compared with the spread *via* atmospheric agents, such as rain and wind, under the regulating effect of temperature (Cortesi et al., 2000; Gramaje and Di Marco, 2015; González-Domínguez et al., 2020).

Cortesi (2000) highlighted how the pruning wounds of contiguous plants were infected by different strains of *Fomitiporia punctata* and suggested that other host species could contribute as inoculum sources when present close to vineyards. In the same study, symptomatic vines were only rarely spatially aggregated, confirming the main role of the airborne route in the dissemination of pathogen spores. In further support of this possibility, results obtained regarding the genotypic variation of *F. punctata* (probably representing Fmed) in the vineyard underlined the relevance of sexual reproduction via basidiospores (Jamaux-Despréaux et al., 2003). To protect pruning wounds in the vineyard, many classes of active ingredients have been tested. Although triazoles proved ineffective for the suppression of GLSD symptoms if used alone, positive results were obtained when cyproconazole was used together with iodocarb as a wound protectant (Di Marco et al., 2000; Rolshausen et al., 2010b). Treatments performed with fosetyl-Al reduced GLSD symptoms as well as the levels of vascular infection by Esca complex-associated pathogens (Di Marco et al., 2011a; Díaz and Latorre, 2013). Strobilurins, and particularly pyraclostrobin, were efficient at controlling pathogens both *in vitro* and in wound protection trials *in vivo* (Mondello et al., 2018). A mixture of pyraclostrobin and boscalid is used in the Tessior® system, a tool recently developed by BASF (Ludwigshafen am Rhein, Germany) for the protection of

pruning wounds, which involves the use of a polymer to extend the persistence of the product (Kuhn et al., 2017). Among inorganic compounds, trials on wound protection using boron have yielded positive results, although the prophylactic use of this product has remained limited (Rolshausen et al., 2010a). In contrast, the use of copper-based products, brushed on pruning wounds of large diameter, is a very commonly used practice, even though it is well-known that copper has a short persistence on the surface of plants owing to the effects of weather. Studies conducted on the use of copper to limit pathogen infection in wood failed to identify a satisfactory long-term protective effect (Di Marco et al., 2011b).

Extensive research focus has been devoted to the use of BCAs as pruning wound protection tools (Di Marco et al., 2002; Kotze et al., 2011). *Trichoderma* spp. (Ascomycota) have been the most frequently tested BCAs against Esca complex-associated pathogens (Di Marco et al., 2004b; Di Marco and Osti, 2007; Mutawila et al., 2015). The use of *Trichoderma*-based products in nurseries has given inconsistent results, most likely owing to the high variability of the trial conditions used (Fourie and Halleen, 2004; Di Marco and Osti, 2007; Pertot et al., 2016). Members of this fungal genus limit pathogen infection by competing for space and nutrients, as well as by producing volatile or low-molecular-weight compounds (LMWCs) with fungistatic or antibiotic activity (Ghisalberti and Sivasithamparam, 1991; Vinale et al., 2008; Kotze et al., 2011; Woo et al., 2014). The application of *Trichoderma* spp. to reduce wound infection in the vineyard produced positive results only if treatments were repeated for at least three consecutive years and the protection was applied on pruning wounds from the early stage of vine growth, thereby confirming the preventive efficacy of the treatment (Bigot et al., 2020; Di Marco et al., 2022). The efficacy of *Trichoderma*-based products can also vary over the years according to the species or strain tested (Mondello et al., 2018). BCAs other than *Trichoderma* spp. have also been tested for their efficacy in controlling Esca associated pathogens. The ability of some *Epicoccum* spp. to suppress *P. chlamydospora* development has recently been investigated both *in vitro* and *in planta* (Del Frari et al., 2017). Trials on *Pythium oligandrum* suggested that it may exert protective effects against wood infection by *P. chlamydospora* through the induction of plant resistance mechanisms (Yacoub et al., 2016).

2.4. Trunk surgery

Trunk surgery is an ancient technique applied to many tree species with the aim of removing decayed and rotten wood. In Italy, the practice is historically called “slupatura” and, more recently, "dendrochirurgia". The name slupatura derives from “lupa”, which is the name assigned to the wood decay of the olive tree. No document to date has reported on the efficacy of this technique; however, its use is widespread throughout Italy and produces good results in terms of vigor recovery. In the book *Tuscan Olive Tree*, the authors refer to the method and describe it in two chapters (Pisani Barbacciani, 2012; Scaramuzzi, 2012). Trunk surgery is also performed on chestnut, walnut, and ornamental plants of landscape or monumental interest (Catena et al., 1991). In the past, when used on chestnut trees, the technique was sometimes followed by the burning of the exposed surface after the removal of the decayed wood. However, this practice is now discouraged (Maltoni et al., 2016). In France, where it is known as “curetage”, trunk surgery was already applied in grapevine before the 20th century. In 1921, Lafon described the improvements made to the Guyot pruning system and the practices of recovering plants partially affected by apoplexy developed by Mr. E. Poussard (Lafon, 1921). Forty years later, other authors documented the practice of cleaving trunks of vines displaying Esca symptoms and leaving the wound exposed to air and light (Rui and Battel, 1963). Pollastro et al. (1999) listed the abandonment of the practice of “slupatura” among the causes of the wide diffusion of Esca disease in the Apulia region (Pollastro et al., 1999). Technological advancement has led to the development of economical, light, and handy tools. In the past, trunk surgery carried out on an olive tree using manual cutting tools was extremely time-consuming. Today, the technique requires between 5 and 10 minutes when applied on vines, making this practice economically feasible in many viticultural contexts. Trunk surgery is applied using electric or gas chainsaws equipped with a carving blade (Figure 5) (Thibault, 2015). The carving blade allows to face the wood even with the blade shoulder and, therefore, to scrape the surface. To be applied, trunk surgery requires wide-diameter vines trained with the Guyot or alberello pruning system. Trunk surgery is not convenient for application on spur-pruned cordon. The technique starts with the opening of a cavity in the upper part of the trunk for exploration purposes. Once the inner rotten tissue of the trunk is brought to light, the tip of the blade follows the diffusion of the decayed wood until its complete removal. It has been widely reported that it is not necessary to remove the brown wood that delimits the sound wood and the decayed wood, and may even weaken the vine (Thibault, 2015).

Figure 5 - The application of trunk surgery on a Guyot-trained vine. An electric chainsaw is used to completely remove the decayed wood. The operation starts by creating a cavity at the top of the trunk for exploration purposes. Once the spongy tissue representing the decayed wood is identified, the decay is completely removed with the tip of the chainsaw blade.

As described above, studies carried out on the mode of action of arsenite have found that the active ingredient that accumulated in the decayed wood mainly targeted Fmed, the basidiomycete fungus responsible for wood decay. Trunk renewal, regrafting, treatment with arsenite, and trunk surgery have proven to be the most effective tools for Esca complex control in mature vineyards to date, and all these strategies acted by removing or sterilizing the decayed wood. This fundamental evidence formed the basis for the trials presented in the first annex of this thesis.

3. *Fomitiporia mediterranea*, the wood decay fungus

Since the definitive identification of Fmed as the causative species of the wood decay seen in grapevine affected by the Esca complex, several studies on the fungus, including work undertaken under the incorrect denomination *Fomitiporia punctata*, have been conducted to unravel as many aspects as possible regarding its pathogenic characteristics and its mode of wood attack. Although Fmed (initially *Phellinus igniarius*) was historically considered to be the *Basidiomycota* species responsible for white rot in vine wood in the Mediterranean area, in recent years several other basidiomycetes have been identified that cause symptoms associated with the Esca complex in vines (White et al., 2011; Brown et al., 2020). This was possible because of an innovative metagenomic approach that enhanced the detection of species that could not otherwise be isolated using classical methods (Del Frari et al., 2019; Bruez et al., 2020; Pacetti et al., 2021). Furthermore, it is important to underline that although Fmed is the predominant species affecting grapevine in Europe and Mediterranean regions, in other areas of the world, several species belonging to the genus *Fomitiporia* have been identified in the decayed wood of vines. These include *F. australianensis* M. Fisch., J. Edwards, Cunning and Pascoe, found in Australia (Fischer et al., 2005); *F. polymorpha* M. Fisch., found in California (Fischer and Binder, 2004; Brown et al., 2020); *F. capensis* M. Fisch., M. Cloete, L. Mostert, F. Halleen, identified in South Africa (Cloete et al., 2014); *F. ignea* and *F. langloisii* (Brown et al., 2020), found in Texas; *F. punicata* Y.C. Dai, B.K. Cui & Decock, detected in China (Ye et al., 2021); and *F. erecta* A. David, Dequatre & Fiasson, which was found in Spain (Fischer and González García, 2015). A comprehensive review regarding many aspects of Fmed is presented in Annex II of this manuscript.

3.1. The intraspecific variability of *Fomitiporia mediterranea*

Although *Vitis vinifera* is the host on which Fmed has been most frequently isolated - likely due to the economic relevance of grapevine cultivation - this fungus is also known to degrade the wood of other species in the Mediterranean region (Table 1). The host specificity of Fmed strains has been investigated to obtain data regarding the evolution and diffusion of the genus. Elena *et al*. (2006) suggested a host specificity for strains isolated from *Citrus* spp., while other experiments involving the inoculation of vine-isolated Fmed strains on different species confirmed the results obtained by the former author (Markakis et al., 2017). A comparison between Fmed strains isolated from grapevine and *Olea europaea* is presented in Annex III. The expression of genes encoding wood-degrading enzymes in Fmed strains was induced using grapevine wood sawdust, with the results showing that genes of the strain isolated from olive tree (235.01) were the most upregulated. The Fmed range extends throughout all the subtropical viticultural areas of southern Europe and North Africa and reaches those of central Europe (Moretti et al., 2021). Based on the Geiger climate classification and subsequent implementation, the Fmed climatic range varies from "arid and semi-arid" to "cool temperate" (Geiger, 1961; Kottek et al., 2006; Beck et al., 2018). Secondary hosts (other than grapevine) likely contributed to Fmed diffusion throughout the Mediterranean basin and central Europe (Fischer, 2002, 2006).

A notable number of repetitive sequences in the Fmed genome, namely, microsatellites and transposable elements (TEs), are suggested to be responsible for generating strain polymorphisms, as frequently observed for *Basidiomycota* spp. (Moretti et al., 2021). The complete sequencing of the genomes of several *Basidiomycota* spp. highlighted that Fmed sequences had the greatest TE coverage of all the species analyzed (Floudas et al., 2012). A more accurate analysis of the proportions of the Fmed genome that comprise its enzymatic pool is provided later in this manuscript.

Table 1 - Hosts other than *Vitis vinifera* from which *Fomitiporia mediterranea* was isolated in Europe and in countries of the Mediterranean basin.

Host	Country	References
Acer negundo	Italy	(Fischer, 2002)
Actinidia spp.	Italy	(Fischer, 2002; Di Marco et al., 2004a)
Albizia julibrissin	Greece	(Markakis et al., 2017)
Cistus sp.	Italy	(Girometta et al., 2020)
Citrus spp.	Greece, Italy	(Elena et al., 2006; Roccotelli et al., 2014)
Corylus avellana	Italy	(Pilotti et al., 2010; Girometta et al., 2020)
Fagus sylvatica	Italy	(Girometta et al., 2020)
Fortunella japonica	Greece	(Markakis et al., 2017)
Hedera helix	Italy	(Girometta et al., 2020)
Lagerstroemia indica	Italy	(Fischer, 2002)
Laurus nobilis	Slovenia	(Fischer, 2006)
Ligustrum vulgare	Italy	(Fischer, 2006)
Olea europaea	Greece, Italy	(Fischer, 2002; Carlucci et al., 2013; Markakis et al., 2017, 2019)
Platanus x acerifolia	Italy	(Pilotti et al., 2005)
Prunus dulcis	Spain	(Olmo et al., 2017)
Punica granatum	Greece	(Markakis et al., 2017)
Pyrus communis	Greece	(Markakis et al., 2017)
Quercus ilex	Italy	(Fischer, 2006)
Quercus robur	Italy	(Girometta et al., 2020)
Quercus rubra	Italy	(Girometta et al., 2020)
Robinia pseudoacacia	Italy, Germany	(Fischer, 2006; Schmidt et al., 2012; Girometta et al., 2020)
Salix alba	Italy	(Girometta et al., 2020)
Ulmus spp.	Iran	(Mirsoleymani and Mostowfizadeh-Ghalamfarsa, 2018)

3.2. Wood decaying processes

Digestible compounds - sources of carbon for wood-inhabiting microorganisms - are stocked into the parenchyma cells of wood and accessing them requires overcoming multiple barriers. Fungi developed their arsenal of ligninolytic enzymes over millions of years of evolution (Janusz et al., 2013). These enzymes participate not only in wood decomposition but also in fruiting body production and physiological processes (Tsai et al., 1999; Sakamoto et al., 2005). Considering the enormous variety in the composition of wood worldwide and the equally large microbial diversity that can attack it, degradation strategies must be analyzed individually. Nevertheless, several generalizations can be made regarding the composition of wood and the activity of the main ligninolytic enzymes. During the entire 20th century, since the study of wood degradation became of primary interest, three main systems of wood degradation by microorganisms have been defined: white rot, brown rot, and soft rot. White rot fungi can selectively delignify wood (preferential or selective white rot) or simultaneously degrade lignin and the other wood polymers throughout decayed wood (simultaneous or non-selective white rot) (Blanchette, 1984, 1991; Daniel, 2003). In preferential white rot, lignin and hemicellulose are "preferentially" degraded and a large concentration of cellulose is normally left (Blanchette et al., 1987; Eriksson et al., 1990). Under preferential delignification, the decay zone progresses from the cell membrane-side outwards, eventually affecting middle lamellar regions of adjacent cells, leaving fibers weakly structured (Daniel, 1994, 2014).

Simultaneous white rot fungi colonize cell lumen and cause cell wall erosion, in which wood tissue appears widely fragmented and contains voids that are filled by mycelia (Hatakka and Hammel, 2011). *Phanerochaete chrysosporium*, *Phlebia radiata*, and *Trametes versicolor* are the best characterized 'simultaneous white rot' species. These fungi can degrade cellulose, hemicellulose, and lignin at a similar rate, from the cell lumen outwards (Blanchette et al., 1988; Niku-Paavola et al., 1988; Tien and Kirk, 1988; Karhunen et al., 1990; Kersten and Cullen, 2007). Selective and non-selective decaying behaviors vary not only among white rot species but also within isolates of the same species. Moreover, some fungi, such as *Heterobasidium annosum*, appear to be capable of generating both types of attack on the same sample, depending on the environmental conditions (Eriksson et al., 1990; Blanchette, 1991). The degradation pattern that results in wood displaying a brownish, fractured, cubic appearance is called brown rot, characterized by the degradation of all wood carbohydrates, including crystalline cellulose. The chemically modified lignin that remains after brown

rot degradation is gradually modified and metabolized to humic substances (Flaig, 1964; Ertel and Hedges, 1985). Brown rot fungi are limited in terms of enzymes when compared with white rot fungi as their genomes do not encode ligninolytic peroxidases; nevertheless, they can efficiently degrade lignin using non-enzymatic mechanisms (Goodell et al., 2017). Notably, wood degradation by brown rot fungi can be detected far in advance of the zone of hyphal growth at the cellular level, and carbohydrates are depolymerized much faster than they can be utilized (Chen, 2014; Goodell et al., 2020).

The final wood degradation pattern involves soft rot agents. Soft rot is a wood decay engendered mainly by some Ascomycota and occurs under conditions of excessive moisture. For soft rot, two distinct morphological types of attack have been identified (Daniel, 2014). Type I degradation is characterized by cavities within the secondary cell wall aligned with cellulose microfibrils, while Type II degradation involves the thinning of the cell wall, like that observed for some white rot decay; unlike with white rot decay, however, with soft rot, the middle lamella remains intact. The wood attack usually occurs on the portion of the wood exposed to the environment. Soft rot fungi degrade both cellulose and hemicellulose, but they can also degrade lignin by producing laccase, as evidenced by the cavities they induce on cell structures (Goodell, 2020). The commonly used terms white rot, brown rot, and soft rot are based on the outward appearance of the degraded wood and are no longer completely explanatory in light of the many studies carried out (Goodell et al., 2020). Indeed, as highlighted by numerous studies, the boundaries among the different types are not always clear and many fungal species do not fall into these definitions, underlining the inadequacy of the above-mentioned paradigm (Floudas et al., 2012; Riley et al., 2014).

3.2.1. Wood composition and degrading enzymes

The wood of angiosperms consists mainly of heartwood and sapwood. The first is composed of parenchymal cells and disused vessels. Sapwood is bordered by the cambium (an active meristematic tissue), followed more externally by the bark, but these latter represent only a small proportion of wood in adult plants (Wilson and White, 1986). Heartwood is composed of hollow cells (vessels), cells with a three-layered secondary wall (fibers), and living, thin-walled cells (parenchyma cells). The wood of non-standing vines such as lianas, including grapevine, is morphologically different compared with standing trees. Parenchyma morphology and quantity are variable across species and can influence wood sensibility to white and brown rot decay

(Schwarze, 2007; Gutiérrez et al., 2009). Pith is usually more developed in grapevine wood compared with that in other angiosperms and represents a greater proportion the younger the woody structures are (i.e., in one-year-old shoots). In grapevine trunks, wood decay and discoloration were frequently observed to develop more rapidly through the pith, thus implicating this tissue in wood susceptibility to decay (Mugnai et al., 1999).

The diameter of vessels in grapevine wood ranges from 60 to ≥160 μm, greater than in other angiosperms, and was shown to be an important factor in vine wood colonization by Esca-associated pathogens (Ellmore et al., 2006; Pouzoulet et al., 2014, 2017, 2020). In general, the composition of wood includes two major complex polymeric components: carbohydrates (65% – 75%) and lignin (18% – 35%). Minor amounts of other materials, mostly in the form of organic extractives and inorganic minerals (ash), are also present in wood (usually 4% – 10%). Overall, wood is composed of 50% carbon, 6% hydrogen, 44% oxygen, and traces of diverse metal ions (Pettersen, 1984). Cellulose is composed of a chain of β-1,4-bonded anhydroglucose units characterized by a variable degree of polymerization and is insoluble at normal temperatures (Figure 6). Cellulose in the primary wall has a lower degree of polymerization compared with that in the secondary cell wall and is thought to be polydisperse (Pettersen, 1984). The S2 layer of the secondary cell wall often differs from the S1 and S3 layers in that it has a lower microfibril angle, i.e., the orientation of S2-layer microfibril is parallel to the axis of the fiber (Donaldson, 2008). Cellulose in the wood cell walls of woody plants usually accounts for 35% to 50% of dry weight (Chen, 2014). Our experiments (Annex III) showed that cellulose represents nearly 35% of grapevine wood dry weight, a non-negligible difference when compared with values reported by other authors (45.4%) (Agrelli et al., 2009). Because of its high degree of polymerization and linear orientation, cellulose is responsible for the high tensile strength of wood.

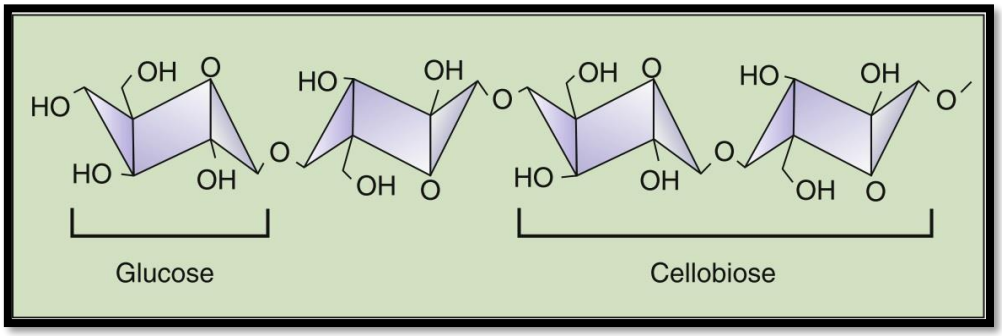

Figure 6 - Molecular chain structure of cellulose (Chen, 2014).

Carbohydrate-active enzymes (CAZymes), such as endoglucanases (EGs) (EC 3.2.14), cellobiohydrolases (CBHs) (EC 3.2.1.91), β-glucosidases (EC 3.2.1.21), and cellobiose dehydrogenases (CDHs) (EC 1.1.99.18), comprise the enzyme set used by microorganisms to digest cellulose. EGs catalyze the cleavage of cellulose along the main chain of its glucose units, CBHs attack the chain at the endpoints, and β-glucosidases act in concert with CDHs to split cellobiose molecules (Daniel, 2014). Auxiliary activity (AA) proteins, represented by eight families of ligninolytic enzymes and two of lytic polysaccharide monooxygenases (LPMOs), and non-catalytic modules named Carbohydrate-Binding Modules (CBMs), are also associated with CAZymes as they contribute to polysaccharide degradation (Boraston et al., 2004; Levasseur et al., 2013). Unlike cellulose, hemicellulose is a heteropolymer composed of varying amounts of different saccharide molecules (Yang, 2008). Although the content and structure of hemicellulose vary with plant species, the basic structure comprises a main chain with branching, secondary chains of glucans (xylan, xyloglucan, glucomannan, manna, galactomannan, and callose, among others) (Chen, 2014).

Hemicellulose is predominantly composed of glucose, mannose, galactose, xylose, arabinose, and galacturonic acid residues (Pettersen, 1984). In wood structures, hemicellulose is intimately merged with cellulose fibrils and can be hydrolyzed by a myriad of hemicellulase enzymes mainly belonging to the CAZymes (Kirk and Cullen, 1998; Daniel, 2014). Hemicellulose acts as a matrix for cellulose and acts as a link between fibrous cellulose and amorphous lignin. Lignin is the second most abundant polymer in woody plants after cellulose; however, it is less abundant than hemicellulose in grapevine wood, accounting for between 12% and 15% of organic polymer content in healthy grapevine wood based on current knowledge (Annex III) (Agrelli et al., 2009).

Lignin is the generic term for a large group of aromatic polymers consisting of three main units: the guaiacyl (G) unit (conyferyl alcohol), the syringyl (S) unit (sinapyl alcohol), and the p-hydroxyphenyl (H) unit (p-coumaryl alcohol) (Figure 7) (Vanholme et al., 2010). β-O-4-aryl ether bonds are the most commonly observed inter-monomeric linkages in lignin polymers (Pandey and Kim, 2011). Generally, gymnosperms mainly contain G-lignin, while angiosperm dicotyledons mainly contain GS-lignin (Wei and Song, 2001). The S/G ratio varies in different hardwood species and higher S-unit contents are associated with a greater susceptibility to white rot decay (Schwarze, 2007; Hatakka and Hammel, 2011; Santos et al., 2012). This polyphenolic compound acts as a cementing material for wood fibers.

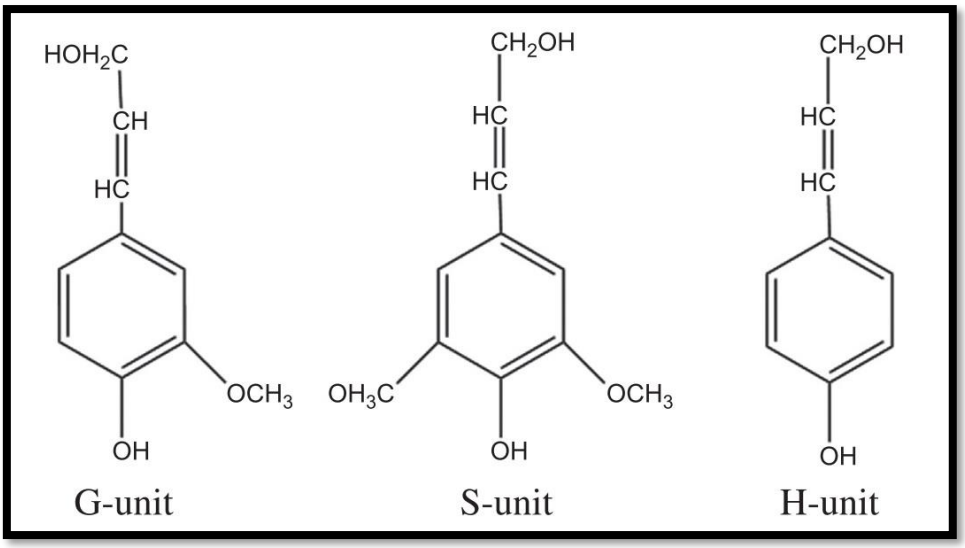

Figure 7 - The three lignin units found in wood: the guayacyl (G) unit, the syringyl (S) unit, and the p-hydroxyphenyl (H) unit (Schilling et al., 2021).

In cell structures, lignin primarily accumulates in the cell corner middle lamella (70% mass ratio), compound middle lamella (50%), and, to a lesser extent, the secondary cell wall, which being far greater than the middle lamella, contains an higher absolute quantity of lignin (Chen, 2014). Lignin is degraded *via* random oxidative reactions driven by a set of extracellular enzymes that includes i) Class II peroxidases (PODs), such as manganese peroxidases (MnPs; EC 95 1.11.1.13), versatile peroxidases (VPs; EC 1.11.1.16), and lignin peroxidases (LiPs; EC 1.11.1.14); and ii) laccases (EC 1.10.3.2; p-diphenol: di-oxygen oxidoreductases) in conjunction with the above-mentioned AA redox enzymes (Daniel, 2014). PODs are heme-containing glycoproteins that require H_2O_2 as the oxidant substrate. MnPs oxidize Mn(II) to Mn(III) and require an organic acid for Mn chelation. Laccases are copper-containing oxidases that generate phenoxy radicals through the oxidation of phenolic rings. Laccases alone cannot depolymerize native lignin but at least modify its structure (Bajpai, 1999). Catabolic activity of laccases leads to the generation of free radicals, which directly oxidize lignin. The first group of radicals is usually produced by laccases from natural methoxyhydroquinones (Janusz et al., 2020). Next, these radicals initiate Fenton reaction leading to the production of different reactive oxygen species (Guillen et al., 2000; Wei et al., 2010).

3.2.2. Grapevine wood degradation by *Fomitiporia mediterranea*

Fmed is still considered to be a white rot agent and its enzymatic pool was shown to consist of a wide range of CAZymes, including CDH, glycoside hydrolase, LPMOs, CBM1 enzymes, and at least 16 PODs by *in silico* analysis (Riley et al., 2014). No lignin peroxidases were identified in the Fmed genome even though preliminary tests suggested the possibility of LiP-associated activity (Floudas et al., 2012; Cloete et al., 2015). Laccase and MnP enzymes were also characterized by *in vitro* analysis, along with other cellulolytic enzymes (Bruno and Sparapano, 2006; Abou-Mansour et al., 2009; Morgenstern et al., 2010). As mentioned here and elsewhere, the white/brown rot paradigm does not exhaustively describe every species. Although Fmed is defined as a white rot species, it cannot therefore be excluded that it may adopt wood degradation mechanisms characteristic of brown rot. As demonstrated for some basidiomycete species that had been defined as brown rot agents, a preliminary non-enzymatic degradation process is necessary for the cellulolytic enzymes to reach the internal structures of the cell walls (Goodell et al., 1997; Arantes and Milagres, 2009). Accordingly, it can be assumed that the wood degradation strategies of Fmed may not be based solely on enzymatic reactions. In support of this possibility, some genes identified in the Fmed genome code for proteins involved in non-enzymatic processes in other brown rot species, namely, multicopper oxidases (MCOs), copper radical oxidases (CROs), a benzoquinone reductase, an iron permease (FTR), and a ferroxidase (Fet3) (Floudas et al., 2012; Sista Kameshwar and Qin, 2020). The results obtained through specific experiments on the enzymatic pathway of Fmed are presented in Annex III of this thesis. Meanwhile, further studies on the non-enzymatic processes that may support wood degradation are necessary to confirm the abovementioned hypothesis.

Aims

The purpose of the present study was to gain new insights into the factors involved in the expression of foliar symptoms associated with the Esca complex, focusing on the role of wood-degrading fungi. As previously mentioned, many factors can influence the expression of GLSD symptoms, and most have yet to be identified. The link between the presence of wood decay and GLSD symptoms is hotly debated and many hypotheses have been proposed (Claverie et al., 2020). Although some studies initially failed to identify a relationship between the extent of wood decay and the frequency of symptom expression (Calzarano and Di Marco, 2007), over the last few years, an increasing number of reports have shown that a relationship does indeed exist between the two factors. Maher et al. (2012) observed that the amount of white rot in vine trunks was significantly proportional to the probability of foliar symptom expression. This relationship was also supported by results obtained by Bruez et al. (2014), who reported that 79% of GLSD-symptomatic vines also presented white rot inside the trunk. Data recently presented by C. Moisy at the conference "Journées Nationales des Maladies du Bois de la Vigne" (Reims, France, November 17/18, 2021) clearly highlighted a correlation between the extent of white rot and GLSD symptom expression by using a very precise method (Moisy, 2021). Several of the treatments that had also been previously used to control the expression of symptoms associated with the diseases of the Esca complex actually acted on decayed wood. Trunk renewal remains the most frequently used and easily applicable of the approaches currently employed in the vineyard. Trunk surgery has also produced promising results, but further investigation is required to confirm its efficacy. Accordingly, a first step in this research was to assess the efficacy of this technique and understand the reasons for its efficacy by applying three levels of rotten wood removal from vines in a 14-year-old Cabernet Sauvignon vineyard. Three symptom types were treated, namely, GLSD symptoms, wilted-shoot symptoms, and apoplexy. Furthermore, having observed that trunk surgery led to a reduction in GLSD symptoms, the impact of the technique on the vine microbiota was investigated to identify putative microbiological factors involved in the triggering of the foliar symptoms. The analysis led to a correlation between the removal of Fmed-infected tissue and the reduction of foliar symptom development. Based on this evidence and published results, a multidisciplinary approach was utilized to assess the wood-degrading ability of the fungus. The mode of wood degradation at the cellular level was documented through observations *in planta* using epi-fluorescence microscopy. Subsequently, other aspects associated with Fmed-mediated wood degradation were described using *in lignum* and *in vitro* tests.

Results and Discussion

The findings obtained from the on-field and laboratory experiments carried out during this research allow the reevaluation of the role of Fmed in the diseases associated with the Esca complex, mainly in Esca proper where GLSD symptoms and wood decay are both present. Evidence obtained through the removal of decayed wood from the vine trunk demonstrated that a decrease in the abundance of Fmed mycelia in vine wood was associated with a lower expression of foliar symptoms, indicating that Fmed indeed has an important role in the development of the disease. However, reports indicating that the expression of GLSD symptoms is not associated with the presence of Fmed suggest that the factors that induce the expression of GLSD symptoms are not exclusive to Fmed (Edwards et al., 2001; Romanazzi et al., 2009). The results of an exploratory study on the wood-degrading activity of Fmed suggested that mechanisms other than the enzymatic pathway could be involved in wood decay. Non-enzymatic pathways, commonly reported to be active in other wood decay-inducing pathosystems, have also been identified in other species associated with the Esca complex (Osti and Di Marco, 2010). Consequently, the results reported here may contribute to supporting future studies in this direction.

1. Trunk surgery efficacy and the impact on vine trunk microbiota (Annex I)

Trunk surgery has been rediscovered in recent times owing to the technological development that now allows the technique to be increasingly applied to grape production in a cost-effectively. To date, relatively few studies have investigated the efficacy of trunk surgery in reducing the expression of GLSD symptoms, and the available results do not mention the long-term effects (Bruez et al., 2021a; Cholet et al., 2021; Annex I). In this study, several aspects of the technique were investigated. The removal of three levels of decayed wood was used to determine whether complete removal was necessary to achieve the remission of foliar symptoms. Furthermore, testing the removal of three levels of decayed wood allowed to establish if a minimal intervention, aimed at inducing mechanical stress in the vine, was enough to limit the expression of foliar symptoms. Professionals who apply this technique remove only the decayed wood tissue, leaving behind the median (brown) and sound (healthy) wood (Dal, 2020). Moreover, to highlight the impact of trunk surgery on the vine trunk microbiota, samples from median and sound tissues, sampled from treated and untreated vines, before and after treatment application, were subjected to genomic massive sequencing. The results obtained reinforce those

previously reported, namely, that in the short term, trunk surgery allows a significant reduction in the expression of GLSD symptoms.

Annex I reports the results obtained on the efficacy of trunk surgery in a two-year-long survey (Figure 8). Here, these results are reinforced with data from an additional year of survey (Figure 9). The application of trunk surgery on apoplectic vines led to an increase in the number of dead vines, whereas untreated vines could revegetate after one or two years. Three years after the first symptom survey, no difference was recorded in the number of dead vines between the treated and untreated groups of apoplectic vines, irrespective of the level of treatment. Thus, trunk surgery is not recommended for vines showing apoplexy, most likely due to the damage induced by the technique to the already weak vascular system that is characteristic of apoplectic vines. The effects on vines presenting with wilted shoot symptoms were also evaluated, and the results obtained were inconsistent over the three years. Complete decay removal led to a reduction in the surveyed symptoms, whereas the incomplete removal of decayed wood did not influence the wilted shoot symptom. Nevertheless, these results were not reproduced every year. On vines showing GLSD symptoms, there was a clear reduction in symptom re-expression in a manner that was proportional to the quantity of decayed wood removed. Foliar symptom expression was significantly lower in vines treated with complete surgery compared with that in untreated vines over all the surveyed years. A slight increase in the number of dead vines was observed among both treated and untreated vines displaying GLSD symptoms; however, the percentage of dead vines was comparable between the two groups after three years.

Alpha-diversity analysis provided a first significant overview of the effects on the trunk microbiota following the removal of decayed wood. In untreated vines, fungal alpha-diversity was higher in median wood than in sound wood at T0 (May), whereas similar values were recorded after three months (T3). Conversely, fungal alpha-diversity in treated vines increased between T0 and T3 in both median and sound wood, which was opposite to that seen in untreated vines. This suggests that the median wood had been colonized by many saprobic species following exposure to air after surgery. Bacterial alpha-diversity was higher at T0 than at T3 in both median and sound wood and the slight decrease recorded at T3 was similar for both treated and untreated vines. These results indicated that treatment had no effect on bacterial species richness in any wood type. The beta-diversity of treated and untreated vines was also compared using non-metric multidimensional scaling (NMDS) analysis. The results showed that trunk surgery promoted the selection of specific taxa, suggesting that the excluded microorganisms may have a role in

symptom development. The results also highlighted that the most representative fungal taxa detected in decayed wood consisted mainly of pathogenic species, among which Fmed and *P. chlamydospora* were the most abundant, in line with previous reports (Mugnai et al., 1999; Bruez et al., 2016; Elena et al., 2018; Del Frari et al., 2019). Regarding bacteria, members of the *Pantoea* genus were the most affected. The abundance of *Pantoea* spp. and that of the yeast-like fungus *Aureobasidium pullulans* increased considerably in both analyzed wood tissues after treatment, suggesting that they could play a positive role in plant–microorganism interactions. In general, the results showed that a strong correlation exists between the removal of decayed wood and the remission of GLSD symptoms. The removal of decayed wood was also associated with a shift in the microbiota in the wood of treated vines. In particular, the results highlight that Fmed was the fungal species most affected by trunk surgery in all types of wood tissue evaluated. Our findings not only support that there is a correlation between wood decay and foliar symptom expression but also provide a valid reference for the vine production sector regarding the most effective methods for applying trunk surgery and achieving reliable results.

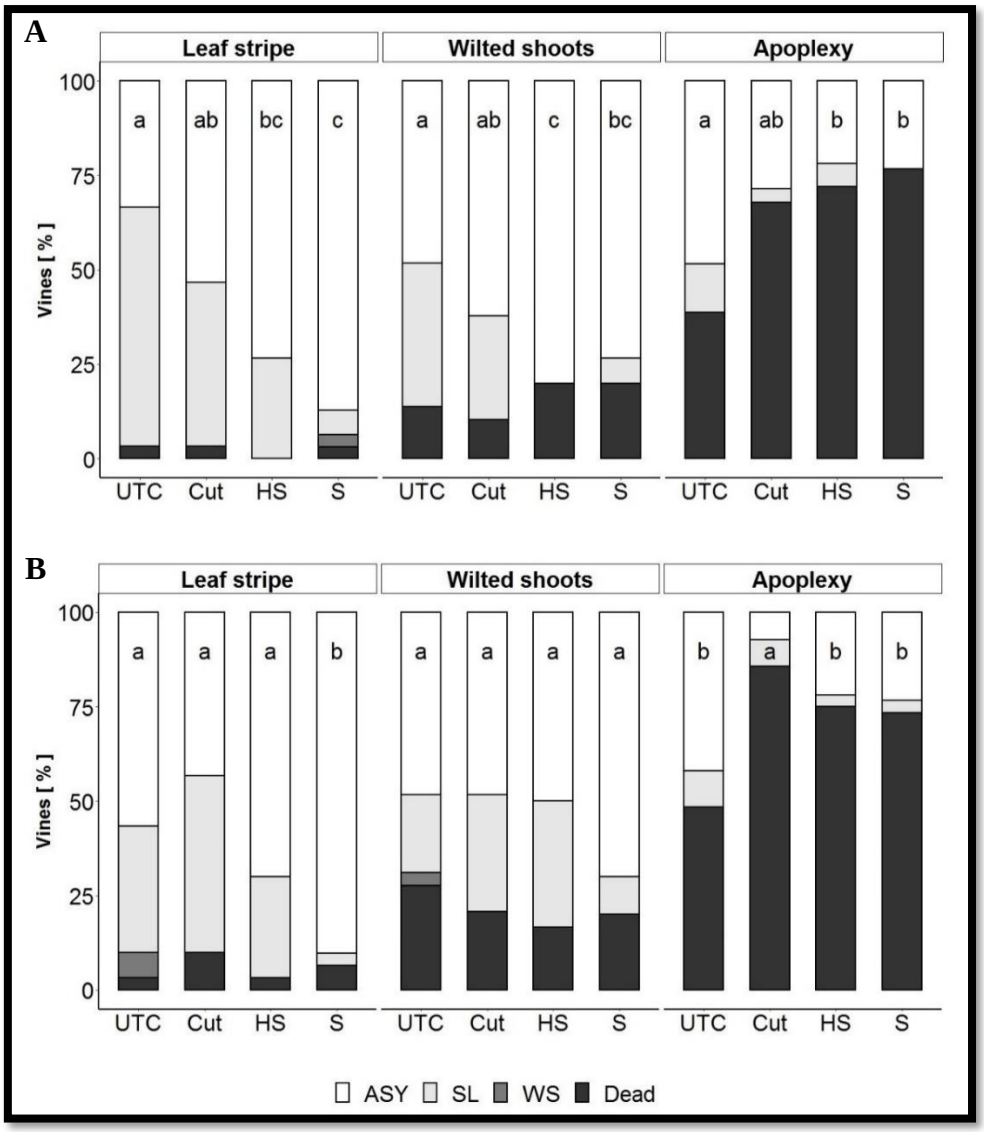

Figure 8 – Extract of Annex I. The health status of vines surveyed in the two years after treatment. (A) Winter-treated vines surveyed after one year (2019) and (B) two years (2020). Histograms are grouped according to the symptoms recorded before treatment (survey of 2018). Black and gray shading represent symptoms reported in 2019 and 2020 for both treated and untreated plants; asymptomatic vines (ASY), striped leaf vines (LS), wilted cane vines (WS), and dead vines (Dead). The number of plants is reported as frequency. On the x-axis, untreated control (UTC), trespassing cut (Cut), half surgery (HS), and complete trunk surgery (S) are the treatments reported and repeated per 2018-monitored symptom type. Different letters at the top of the bars represent statistically significant differences between treatments, considering all symptom types at once, according to Pearson's chi-square test ($\alpha = 0.05$); p-values were compared with Bonferroni-adjusted p-values.

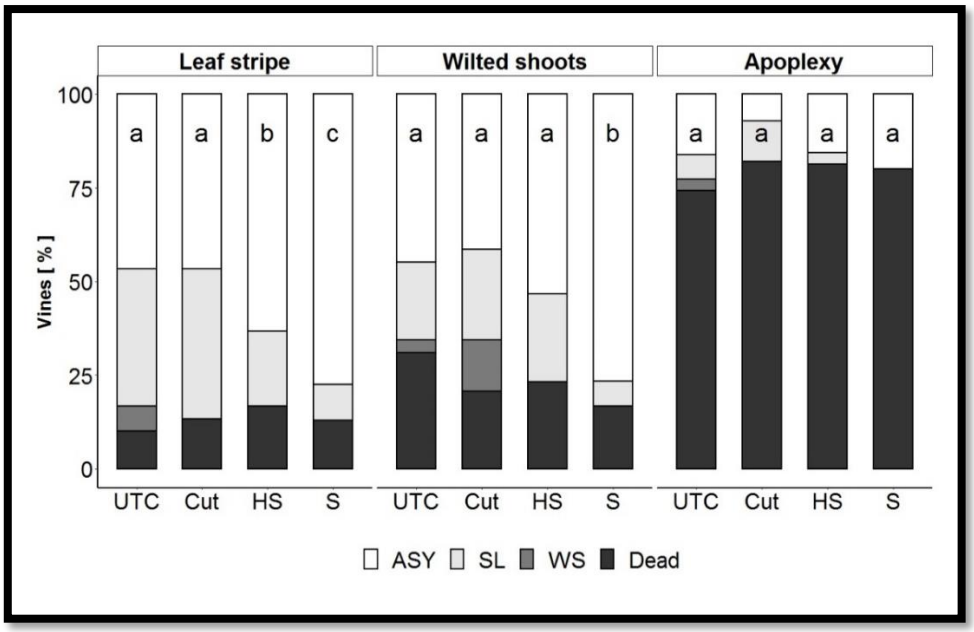

Figure 9 – Results obtained from an additional year of survey with respect to the data published in Annex I and presented in (Figure 8). The health status of surveyed vines three years after treatment (2021). Histograms are grouped according to the symptoms recorded before treatment (survey of 2018). Black and gray shading represent symptoms reported in 2021 for both treated and untreated plants; asymptomatic vines (ASY), striped leaf vines (LS), wilted cane vines (WS), and dead vines (Dead). The number of plants is reported as frequency. On the x-axis, untreated control (UTC), trespassing cut (Cut), half surgery (HS), and complete trunk surgery (S) are the treatments reported and repeated per 2018-monitored symptom type. Different letters at the top of the bars represent statistically significant differences between treatments, considering all symptom types at once, according to Pearson's chi-square test ($\alpha = 0.05$); p-values were compared with Bonferroni-adjusted p-values.

2. The contribution of *Fomitiporia mediterranea* to wood decay (Annex III)

Although some authors regard the white/brown/soft rot paradigm as insufficient to describe wood-rotting variability, Fmed is still considered a white rot fungal agent (Schwarze, 2007; Riley et al., 2014; Goodell et al., 2020). Many studies have sought to characterize the activity of the main Fmed-degrading enzymes and have confirmed that Fmed contains a complete white rot-type enzymatic pool (Abou-Mansour et al., 2009; Morgenstern et al., 2010; Cloete et al., 2015). Tests of pathogenicity were also conducted on vine wood, but the results obtained were inconsistent, resulting in a knowledge gap, especially regarding the Fmed mode of wood degradation (Larignon and Dubos, 1997; Sparapano et al., 2000, 2001). The current definition of white rot for Fmed is based mainly on genomic evidence and on the enzymatic pool of the fungus. Moreover, in-depth studies on the interaction between Fmed and wood, especially vine wood, have been conducted to reproduce the symptom of decayed wood and not to describe the decay processes. The present study (Annex III) highlights the degradation events both by employing a microscopic approach, which documented the damage to cellular structures, and by measuring the content of each of the different wood-forming polymers during degradation. Epi-fluorescence microscopy-based analysis performed on white-rotten vine wood portions provided evidence of cell degradation with characteristic signs of white rot and, to some extent, also soft rot degradation. The initial stage of degradation was characterized by cavities and fissures in the S2 layer of the secondary cell wall of libriform fibers while the middle lamella, the primary cell wall, and the S1 and S3 layers of the secondary cell wall were apparently undamaged or only slightly degraded. In advanced decomposition, all the layers of the cell wall of vessels, tracheids, and libriform fibers were decomposed, including the lignified parts such as the middle lamella, the primary cell wall, and, finally the walls of the parenchyma cells. All these characteristics are commonly associated with white rot degradation (Schwarze, 2007; Daniel, 2014). In contrast, the diamond-shaped cavities observed in the secondary cell wall closely resembled those formed by soft rot agents (Anagnost et al., 1994; Schwarze, 2007).

Although the observed degradation pattern is suggestive of selective white rot behavior, the results obtained through the analysis of residual polymers show non-negligible cellulose consumption, which is more characteristic of simultaneous white rot (Blanchette et al., 1987; Eriksson et al., 1990). We found that as much as 50% of hemicellulose, 30% of cellulose, and 10% of lignin of grapevine wood could be mineralized after 90 days by Fmed and that the polymer degradation ratio varied significantly between strains and

grapevine varieties. Moreover, the analysis performed on the composition of undegraded wood polymers allowed a comparison of the composition of vine wood with that of the better-known hardwoods and softwoods. Our findings on polymer mineralization underline the importance of vine variety in wood degradation susceptibility, showing that the variety that exhibits the most extensive foliar symptoms in the field is also the most susceptible to wood degradation, in line with the proportionality between GLSD symptoms and wood necrosis reported by other authors and in the Annex I (Maher et al., 2012; Bruez et al., 2014; Moisy, 2021). The activity of the main wood-degrading enzymes of Fmed, i.e., laccase and MnP, was also assessed *via* experiments based on substrate oxidation monitoring the changes in absorbance using spectrophotometry. Intraspecific differences among the strains were highlighted and the higher enzymatic activity in general was found for the Fmed strain isolated from *Olea europaea*.
To support these data, we further investigated the molecular regulation of genes encoding for six laccases and three MnPs in fungal cultures grown in the presence or absence of wood sawdust in the medium. The results clearly showed that the expression of all the studied genes was upregulated when Fmed was grown in the presence of vine wood sawdust, thereby confirming the intraspecific variability observed in the enzyme activity test.

The results obtained through the multidisciplinary approach presented in this study require some considerations in order to be interpreted. Many studies conducted on other pathosystems regarding wood degradation by basidiomycetes have indicated that enzymes may not be able to reach the innermost region of the cell walls through the pores present in these structures as they may not be large enough for the enzymes to pass through (Evans et al., 1991, 1994; Flournoy et al., 1991, 1993; Paice et al., 1995). Considering this, the cavitation phenomena observed by microscopy in the S2 layer of cell walls at the early stage of degradation could not have occurred. Thus, assuming that the cell wall pore size is a limiting factor is possible to hypothesize that a preliminary activity aimed at widening to the pores could be required. This hypothesis was proposed for brown rot wood degradation: preliminary non-enzymatic reactions, such as the chelator-mediated Fenton system with the contribution of LMWCs with iron-reducing activity, could begin cell wall component deterioration allowing enzymes to reach the core of cell walls (Goodell et al., 1997, 2017). This has already been suggested for vascular pathogens associated with the Esca complex, although never for Fmed (Osti and Di Marco, 2010). Laccases are produced by a wide range of organisms, particularly fungi. The molecular mass of fungal laccases typically ranges from

60 to 70 kDa, with an increment of 10%–30% when glycosylated (Baldrian, 2006; Rivera-Hoyos et al., 2013). The molecular mass of fungal-produced MnPs ranges from 38 to 62.5 kDa (Hatakka, 1994; Hofrichter, 2002). Abou-Mansour et al. (2014) characterized a 60-kDa laccase from Fmed cultures, while no report to date has described the molecular mass of any MnP produced by Fmed. The size of proteins could be calculated by assuming an average specific volume of 0.73 cm^3/g (Erickson, 2009). Thus, the author proposed a formula to calculate the minimal radius (R_{min}) of a sphere that can contain the given mass of a protein:

$R_{min} = 0.066 * M^{1/3}$ (for M in Dalton, R_{min} in nanometer).

Erickson (2009) underlines that since proteins have an irregular surface, the average radius of proteins is always larger than the R_{min} which is theoretically obtained for a perfectly spherical geometry. Adopting this method, basing on above-listed molecular mass values, Fmed laccases and MnPs minimal diameters could range between 5.17 and 5.44 nm, and 4.44 and 5.24 nm, respectively. The measurement of the porosity of the cell walls can be carried out by microscopy or adopting fluid-based methods (Anovitz and Cole, 2015; Jiang et al., 2018). Through mercury intrusion porosimetry, it has been estimated that the mean pore diameter of the cell walls of *Cannabis sativa* averages 510±56 nm (Jiang et al., 2018). An experiment conducted on roots hair cell of *Raphanus sativus*, fiber of *Gossypium hirsutum*, cultured cells of *Acer pseudoplatanus* and palisade parenchyma cells of *Xanthium strumarium* and *Commelina communis*, by solute exclusion technique, reports a cell wall pore diameter ranging from 3.5 to 5.3 nm (Carpita et al., 1979). The wide variability of cell wall pore diameters across the investigated plant species does not allow to speculate on values for the *Vitis vinifera* species, for which no studies were conducted to the best of our knowledge. It is also underlined that the method adopted for pore size estimation can influence obtained results (Jiang et al., 2018). It is therefore essential, in order to support the hypothesis made, to foresee further studies on the size of the cell wall pores of the grapevine. In conclusion, the present study contributes to the knowledge on Fmed as well as on the processes involved in wood degradation providing an overview on the enzymatic pathway of the fungus and suggesting key points on which to focus further investigation.

Conclusions and perspectives

The results obtained in the experiments presented in this manuscript provide evidence of the crucial effect that decayed wood can have on the development of foliar symptoms associated with the diseases of the Esca complex. Fmed is the fungal pathogen most commonly isolated from decayed wood in the Mediterranean area and Europe in general. The factors directly involved in the expression of the foliar symptoms associated with the Esca complex can also be investigated by studying the interaction between this fungus and vine wood. Given the results presented here, an essential next step is to determine which signals reaching the leaves are responsible for the foliar symptoms. A widely accepted hypothesis regarding the expression of GLSD symptoms proposes that foliar symptoms are provoked by the phytotoxic action of one or more metabolites produced by one or more fungi colonizing perennial wood. The toxic metabolites would be released in the vine sap flow and then move up to the leaves and affect cellular metabolism, thereby triggering symptom expression. This is supported by the fact that Esca associated pathogens do not inhabit the green organs of the vine. However, although the identification of a possible toxin-mediated signal is conceivable in the medium term, the development of control tools aiming to neutralize the pathogenic fungi that infect vine wood should not be considered a solution for Esca complex and GTDs control in general, for the future. This must be considered above all in light of the continuing restrictions applied to the broad spectrum active ingredients for the control of fungal diseases. Controlling GTDs, and especially the Esca complex, should be based on agronomic strategies, from the production of grafted material of good quality, through the adoption of efficient prophylaxis protocols in the nursery, and up to a 360-degree management of the vines in the vineyard. Plant pruning will lead to a turning point in the control of GTDs in the short-to-medium term. Pruning that respects the sap flow, such as the Guyot Poussard pruning methods that do not involve large-diameter wounds, can guarantee a longer vineyard lifespan. In the same way, early protection of the pruning wounds has revealed to have a high efficacy in reducing fungal infection and symptoms expression. Finally, fully understanding the changes in plant physiology induced by white decay will contribute to understanding the interaction between wood colonizers and leaf tissue alterations.

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Publications and intervention at conferences

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- Pacetti, A.; Moretti, S.; Pinto, C.; Compant, S.; Farine, S.; Bertsch, C.; Mugnai, L. (2021) Trunk Surgery as a Tool to Reduce Foliar Symptoms in Diseases of the Esca Complex and Its Influence on Vine Wood Microbiota. Journal of Fungi, 7, 521. https://doi.org/10.3390/jof7070521

- Moretti S.; Pacetti A.; Pierron R.; Kassemeyer H-H.; Fischer M.; Péros J-P.; Perez-Gonzalez G.; Bieler E.; Schilling M.; Di Marco S.; Gelhaye E.; Mugnai L.; Bertsch C.; Farine S. (2021) *Fomitiporia mediterranea* M. Fisch., the historical Esca agent: a comprehensive review on the main grapevine wood rot agent in Europe. Phytopathologia Mediterranea 60(2): 351-379. doi: 10.36253/phyto-13021

- Pacetti, A.; Moretti, S.; Perrin C.; Gelhaye E.; Bieler E.; Kassemeyer H-H.; Mugnai L.; Farine S.; Bertsch C. - Grapevine wood-degrading activity of *Fomitiporia mediterranea* M. Fisch.: a focus on the enzymatic pathway regulation. Submitted on 27/12/2021 to Frontiers in Microbiology and accepted with minor revision on 20/01/2022.

Articles under redaction

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Annexes

I. Trunk surgery as a tool to reduce foliar symptoms in diseases of the Esca complex and its influence on vine wood microbiota

II. *Fomitiporia mediterranea* M. Fisch., the historical Esca agent: a comprehensive review on the main grapevine wood rot agent in Europe

III. Grapevine wood-degrading activity of *Fomitiporia mediterranea* M. Fisch.: a focus on the enzymatic pathway regulation

Annex I

Journal of Fungi

MDPI

Article

Trunk Surgery as a Tool to Reduce Foliar Symptoms in Diseases of the Esca Complex and Its Influence on Vine Wood Microbiota

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Abstract: In the last few years, trunk surgery has gained increasing attention as a method to reduce foliar symptoms typical of some of the Esca complex diseases. The technique relies on the mechanical removal of decayed wood by a chainsaw. A study on a 14-year-old Cabernet Sauvignon vineyard was carried out to validate the efficacy of trunk surgery and explore possible explanations behind it. Three levels of treatment were applied to three of the most characteristic symptoms associated with some diseases of the Esca complex, such as leaf stripe symptoms (LS), wilted shoots (WS) and apoplexy (APP). The most promising results were obtained by complete trunk surgery, where the larger decay removal allowed lower symptom re-expression. According to the wood types analyzed (decay, medium and sound wood), different changes in microbiota were observed. Alpha-diversity generally decreased for bacteria and increased for fungi. More specifically, main changes were observed for *Fomitiporia mediterranea* abundance that decreased considerably after trunk surgery. A possible explanation for LS symptom reduction after trunk surgery could be the microbiota shifting caused by the technique itself affecting a microbic-shared biochemical pathway involved in symptom expression.

Keywords: curettage; *Fomitiporia mediterranea*; *Phaeomoniella chlamydospora*; grapevine; decay

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1. Introduction

Wood diseases are a major threat for modern viticulture, and the diseases included within the Esca complex remain the most worrying in Europe and worldwide [1,2]. Esca complex is currently considered a complex of different diseases and syndromes (characterized by several different symptoms) mostly associated with ascomycetes species, namely vascular pathogens as *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) [3] (Pch), *Phaeoacremonium minimum* (syn. *P. aleophilum*) (Tul. & C. Tul.) [4] (Pmin), and basidiomycetes species, which in Europe are mostly represented by the white rot agent [5] *Fomitiporia mediterranea* [6] (Fmed). The role of Botryosphaeriaceous species in some of the Esca complex diseases is also frequently investigated [7,8]. Following the definition proposed by Surico [9], the Esca complex includes four diseases: brown wood

streaking, grapevine leaf stripe disease (GLSD), Petri disease—all associated with vascular pathogens—and Esca, the wood white rot that originally was named as such. When both GLSD foliar symptoms and white rot are present, the term "Esca proper" can be used to describe this condition [9–12]. *Fomitiporia mediterranea* (originally misidentified as *F. punctata*) [13], was shown to be capable of colonizing the wood as a primary pathogen [14,15]. Despite this ability, the assumption that Fmed colonization of wood can be facilitated by a pre-colonization of other pioneer pathogens has not yet been disproved [10,16,17]. The external symptoms detectable in vineyards are the leaf stripe symptoms described by many authors [10,18–20], the apoplectic stroke [21], which originally was considered as synonymous with "Esca disease" [22,23] and the shoots and clusters wilting, which in literature is attributed mainly to Esca complex diseases [11,21,24]. The external symptoms develop more and more frequently as the vines get older and multiple wood pathogens can be associated with the foliar symptoms [25–28]. The association of the same foliar symptoms with such a variable mycoflora and the failure of the Koch's postulates partially explains why the factors triggering the leaf stripe symptoms and wilting have not been fully clarified despite the many hypotheses formulated [29]. During the last two decades, different approaches to manage leaf stripe symptoms and to reduce vine death due to diseases of Esca complex have been tested and applied in the field. After the phasing out of sodium arsenite in the early 2000s, the applied tools for managing leaf stripe symptoms have been diversified, including pruning timing and modes, wound protection, remedial surgery and foliar treatments using biostimulants or chemical products [30–33]. Among the remedial surgery treatments, trunk renewal and trunk surgery are the best-known methods. Trunk renewal was primarily used against Eutypa dieback and Botryosphaeria dieback and is defined as an inexpensive and relatively easy approach to apply [34,35]. Trunk surgery is a long-used approach on fruit trees in the Mediterranean area and has been recently re-discovered. It is also named "slupatura" or "curetage du bois", in Italy or France, respectively. The aim of this technique is to quickly recover productivity of symptomatic plants by keeping their active root systems and therefore maintaining the quality of the product that is linked to the vine age. The technique is carried out using an electric or gas chainsaw with a mounted carving blade. First, to identify the rotten areas, a preliminary cut is done either where dead wood emerges or is found under the biggest pruning wounds. The plant trunk is opened to be inspected. The decay, often located in the upper part of the vine where the larger wounds are applied, is then removed. The actual applicability of the technique is very much linked to the pruning system (Guyot being the ideal pruning system for trunk surgery). Decay removal consists of scraping out the fibrous and bleached decayed wood, leaving the discolored brown wood and the sound wood. It is reported that the removal of brown wood does not affect the result of the technique, and that it is important not to compromise the main sap flow during the trunk surgery, preferring to act only on one side of the plant [36,37].

Trunk surgery is considered more expensive and time-consuming compared to trunk renewal [31] and needs well-trained personnel. Following the description reported in the available literature, the total removal of decayed wood has a great influence on leaf stripe symptom suppression. Moreover, it seems that the decay found in the trunk has less influence on leaf stripe symptom expression than the decay located in the upper part of the vine trunk [36,37]. Despite the promising results brought to light, currently there is a lack of data in the scientific literature about this technique, and only results of some field trials are available in technical magazines and conference proceedings [37–39]. No description of the changes induced in the plant by the rotten tissue removal operation are reported. No explanation has yet been formulated on the changes in the plant–pathogen interaction that bring trunk surgery to reduce foliar symptoms. The exposure of the active wood to air and light could cause a change in the remaining sound wood-microflora composition or activity. Alternatively, the removal of the decayed tissue could reduce the amount of wood degrading enzymes. Approaches for studying changes in the microflora have been

recently utilized more frequently, with metabarcoding as one of the most promising methodologies to screen the entire microbiota [26,40,41]. In this study, we investigate the efficacy of trunk surgery in reducing the external symptoms linked to some diseases of the Esca complex and on the possible role of decayed wood removal on the microbiota in the trunk of Esca complex affected vines. The ultimate goal is to implement the knowledge on the factors involved in the expression of foliar symptoms, for which some hypotheses have already been formulated [29] but without being able to replicate the complete symptomatologic picture.

2. Materials and Methods

2.1. The Vineyard

This study was carried out in a 14-year-old vineyard located in Tuscany, one of the most important winegrowing areas in Italy (42°57′16.9″ N, 11°02′44.3″ E). According to Köppen–Geiger climate classification [42], this area is characterized by a warm temperate climate, with a hot and dry summer [43]. The vineyard was planted in 2004 using *Vitis vinifera* L., cv. "Cabernet Sauvignon", clone R5 omega-grafted onto 161.49 rootstock (*Vitis berlandieri* × *Vitis riparia*) with a density of 4,350 plants/ha at plantation time. The vineyard is located at an altitude of 50 m, exposition west with rows W-E oriented. The training system was a spur pruned cordon for 9 years then it was converted to single Guyot Poussard. This conversion caused several wide-diameter cuts and typical leaf stripe symptoms and apoplectic vines reached 37% of the coetaneous vines standing in the year before this study. An integrated pest management program was adopted to control foliar diseases (e.g., downy mildew, powdery mildew, gray mold) but no chemical or biological treatment against wood diseases was ever applied in the vineyard during the period of study.

2.2. Treatments

To evaluate the efficacy of trunk surgery, in July 2018 the vineyard was mapped, and each vine was classified on the base of the symptoms recorded during the survey. Three symptom types were selected: (i) leaf stripe symptoms (LS), (ii) wilted shoots (WS) and (iii) apoplexy (APP) as shown in Figure 1A, Figure 1B, Figure 1C, respectively. Coding was used to evaluate symptoms before and after trunk surgery. Treatments were performed right after the first survey at the end of July 2018 (summer-treatments) and in February 2019 (winter-treatments). Three level of treatments were tested to prove the effectiveness of the technique and to evaluate the relevance of the complete decay removal: classical trunk surgery (S), with a total removal of white decayed wood; a less invasive version here called half surgery (HS), where only the core of decayed wood tissue in trunk and in branch was removed; a side-by-side trunk trespassing cut (Cut) made with a chainsaw. Treatments were performed only on plants that had shown symptoms during the previous survey. Summer-treatments were performed on 90 LS-symptomatic vines (n = 30 per treatment level). For winter-treatments, all three symptom types were treated with three levels of trunk surgery each: 270 vines were treated (n = 30 per treatment level), while 90 more vines were monitored as untreated control (UTC) per each symptom type (n = 30 per symptom type).

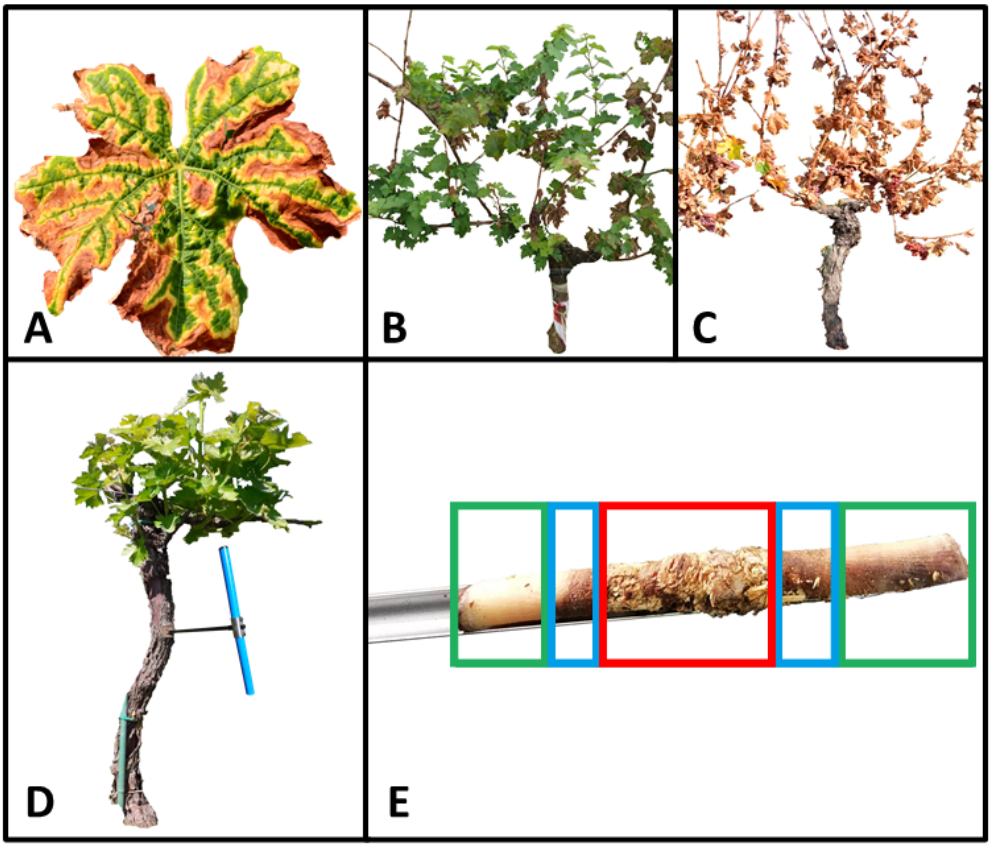

Figure 1. Symptoms monitored during the survey and sampling mode. (**A**) Leaf stripe symptoms (LS) involve leaves that show a green band around the main veins with a chlorotic perimeter while the central interveinal area appears reddish or yellow depending on the variety; in most cases this latter area progressively necrotizes. The necrosis often reaches the leaf margin. The necrotic foliar tissue appears with different colors depending on the variety, from red-brick to dark purple or brown. (**B**) Wilted shoots symptoms (WS) involve entire canes which wilt and necrotize entirely. Leaves wilt and dry up remaining attached to the cane. One or many shoots can dry out and wilt on the same plant, but the vine remains alive. Wilted canes appear shriveled and dry. This symptom is shown mostly during late summer and, if clusters are present, they also dry up remaining attached to the cane. (**C**) Apoplexy (APO) symptom consists in a complete wilt of the vine during summer. All canes, leaves and clusters dry up remaining attached to the canes and the plant apparently dies. During the late part of August or in September or the year after, some of the apoplectic vines resumed some partial and weak growth. (**D**) Sampling procedure using an increment borer, performed on the central portion of the trunk. (**E**) The three types of wood sampled: sound wood (green box), median wood (blue box) and decayed wood (red box).

2.3. Wood Sampling for Microbiota Analysis

To analyze the changes induced by the treatment on the wood-associated microbiota, 3 vines treated by complete trunk surgery (S) were sampled. A specific sampling was performed on vines that had shown foliar LS symptoms in the previous season and were not involved in the on-field trial. Three vines were sampled immediately before treatment in May 2019 (T0), namely at flowering stage (BBCH 60), and 90 days after treatment (T3) (BBCH 83) when foliar symptoms are usually mostly expressed in field, only on the remaining median and sound wood. As control, three more vines were sampled at T0 and T3 without applying trunk surgery. Vines were sampled using an increment borer (which has already been suggested as a suitable tool to study GTDs by Muruamendiaraz and Legorburu [44], at each time point (Figure 1D). Each biological sample was made of three wood cores (subsamples) of 6 mm in diameter per each plant, at each time point. Samples were collected from the central part of the trunk (almost 30 cm above the soil); tools were carefully disinfected in between each sampling with 70% ethanol, to avoid microbiota cross-contamination. In each wood core, three wood types were identified and separated

right after sampling: (i) decayed wood, (ii) median wood (the intermediate discolored, dark brown wood which normally forms between decayed and sound wood) and (iii) sound wood which is the apparently healthy wood (Figure 1E). Once separated by wood type, subsamples were bulked forming the samples to be analyzed and were put in an Eppendorf tube shielded from light and stored at −20°C until processing for microbiota analysis. For microbiota analysis, a total of 30 samples were analyzed for both bacterial and fungal communities, through 16S rRNA gene and ITS gene count data, respectively.

2.4. Microbiota Analysis

2.4.1. Sample Processing

Three biological samples for each type of wood, for treated and untreated conditions and at each time point, were analyzed. For each sample, a total of 25 mg of pulverized wood was used for the DNA extraction using the Danagene Microbiome Soil DNA kit (Danagene, Badalona, Spain), according to the manufacturer's instructions. The yield and purity of the DNA was measured by Qubit and then stored at −20 °C until use.

2.4.2. Library Preparation and Sequencing

PCR reactions were prepared using UV sterilized equipment and negative controls containing sterile water were run alongside the samples. Samples were analyzed for the 16S rRNA gene V4 region, and the ITS gene by amplification of the ITS1 region using WineSeq® custom primers accordingly to the Patent WO2017096385 [45]. After a quality control by gel electrophoresis, each library (16S rRNA gene and ITS genes) was pooled in equimolar amount and subsequently sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using 2 × 301 paired-end reads and according to the Biome Makers implemented protocol. All the data produced and collected were subsequently analyzed through a QIIME-based custom and inhouse bioinformatics pipeline (Patent WO2017096385). A first quality control was used to remove adapters and chimeras [46] and after that, the reads were trimmed out from the point where these did not reach the appropriate quality score. Operational taxonomic unit (OTU) clusters were performed using 97% identity and taxonomy assignment and abundance estimation were obtained comparing OTUs clusters obtained with SILVA database, version 132 [47] and UNITE database version 7.2 [48] as taxonomic references. Results obtained were used to identify fungal and bacteria at species and/or genus taxonomic level.

2.4.3. Statistical Analysis

Results of on-field trials were analyzed by performing contingency table analysis (Pearson chi-square test) and the residual z-scores were studied to establish statistical differences between groups ($\alpha = 0.05$). A 4 × 4 (treatments × surveyed-symptom) contingency table was analyzed per each 2018-recorded symptom type. *p*-values were compared with Bonferroni's adjusted *p*-value after its calculation based on standardized residual [49].

For wood microbiota analysis, the relative abundances of each sample were calculated for all taxa based on the total population identified. Taxa with less than 10 non-zero values were grouped into a single column labelled "Others". The zero counts were replaced for all samples using a Bayesian method (with a Dirichlet multinomial prior), and a multiplicative replacement was performed to maintain the original ratios between the parts of the composition. Sample-wise differences were calculated using the Aitchison distance, and ordination was performed via Kruskal's non-metric multidimensional scaling. Alpha and beta-diversity were analyzed separately for bacteria and fungi using OTUs counts. Alpha diversity was analyzed through Shannon's index [50] and observed richness, which was calculated using three samples as biological repetitions and plotted against wood type groups; time and treatment factors were statistically analyzed using a two-way ANOVA test ($\alpha < 0.05$) and Duncan post-hoc was performed where interaction

between factor results were significant. Decayed wood was not considered in these analyses as decay was removed by the treatment applied. Regarding the beta-diversity, a non-metric multidimensional scaling (NMDS) analysis was performed to highlight clusters of samples for both fungal and bacterial microbiota. All analyses were performed in the R (3.6.3 version) programming environment.

3. Results

3.1. On-Field Results

Regarding the evaluation of the efficacy of trunk surgery on reducing the expression of the three selected Esca symptoms, the vineyard was surveyed in September 2019 and 2020. Total Esca incidence in the whole vineyard in 2019 was 14.3%. In 2020, a similar incidence rate was recorded (14.6%).

3.1.1. Summer-Treated Vines

Summer-treatments were applied only on vines that showed LS symptoms during survey in July 2018. Obtained results are shown in Figure 2A,B. The LS re-expression of the untreated 2018-symptomatic vines was higher in 2019 (63%) than in 2020 (33%). Only one untreated vine died in each of the two surveyed years and two vines showed WS symptoms in 2020. All levels of treatment decreased the LS incidence compared to UTC in 2019 but these results were not confirmed in 2020. The re-expression of LS symptoms increased in Cut and S surgery levels two years after treatment 2020, while—despite LS symptoms did not increase in HS-treated vines—a 10% increase of dead vines was recorded from 2019 to 2020. Twenty-one percent of the total Cut-treated and S-treated vines died in the two years of survey.

3.1.2. Winter-Treated Vines

Vines that showed LS-symptoms in 2018—considering the three treatments as increasing levels of decay removal, data highlight that symptoms occurred less frequently where the quantity of removed decay was greater. This was observed for both 2019 (Cut = 43%, HS = 27% and S = 6.5%) and 2020 (Cut = 47%, HS = 27% and S = 3%) (Figure 2C,D). Despite untreated vines showed less symptoms in 2020 compared to 2019, treated vine symptomatology kept stable over the two years confirming the results obtained.

Vines that showed WS symptoms in 2018—WS symptoms were not re-expressed by untreated vines in 2019 and only one case was recorded in 2020 (Figure 2C,D). Over the two surveyed years, 48% of the untreated vines continued to grow asymptomatically. The rate of dead vines increased from 14% (2019) to 28% (2020). Fifteen percent (15%) of Cut-treated vines showed leaf stripe symptoms in 2019 and 20% in 2020. An increase of 10% of dead vines was recorded from 2019 to 2020 (Cut-treated vines). Vines treated with the complete surgery (S) showed a similar symptoms frame in both 2019 and 2020 (20% dead in both years, 6.7% SL-symptomatic in 2019 and 10% of SL-symptomatic in 2020) and 33% of vines treated with the half surgery showed LS symptoms in 2020 when no symptomatic vine among these vines was recorded in 2019. Twenty percent (20%) of HS-treated vines died in 2019 and one of these vines restarted growing again asymptomatically in 2020.

Vines that showed APO symptoms in 2018—sixty-one percent (61%) of apoplectic untreated vines restarted growing in 2019; 13% of those showed leaf stripe symptoms. A high percentage of untreated vines which showed apoplexy symptoms (APO) in 2018 died during the survey period (39% in 2019 and 48% in 2020) and almost the same percentage kept showing leaf stripe symptoms in both years. The dead vine number increased considerably in all treatments compared to untreated control in both 2019 (Cut = 68%, HS = 72% and S = 77%) (Figure 2C) and 2020 (Cut = 86%, HS = 75% and S = 73%) (Figure 2D).

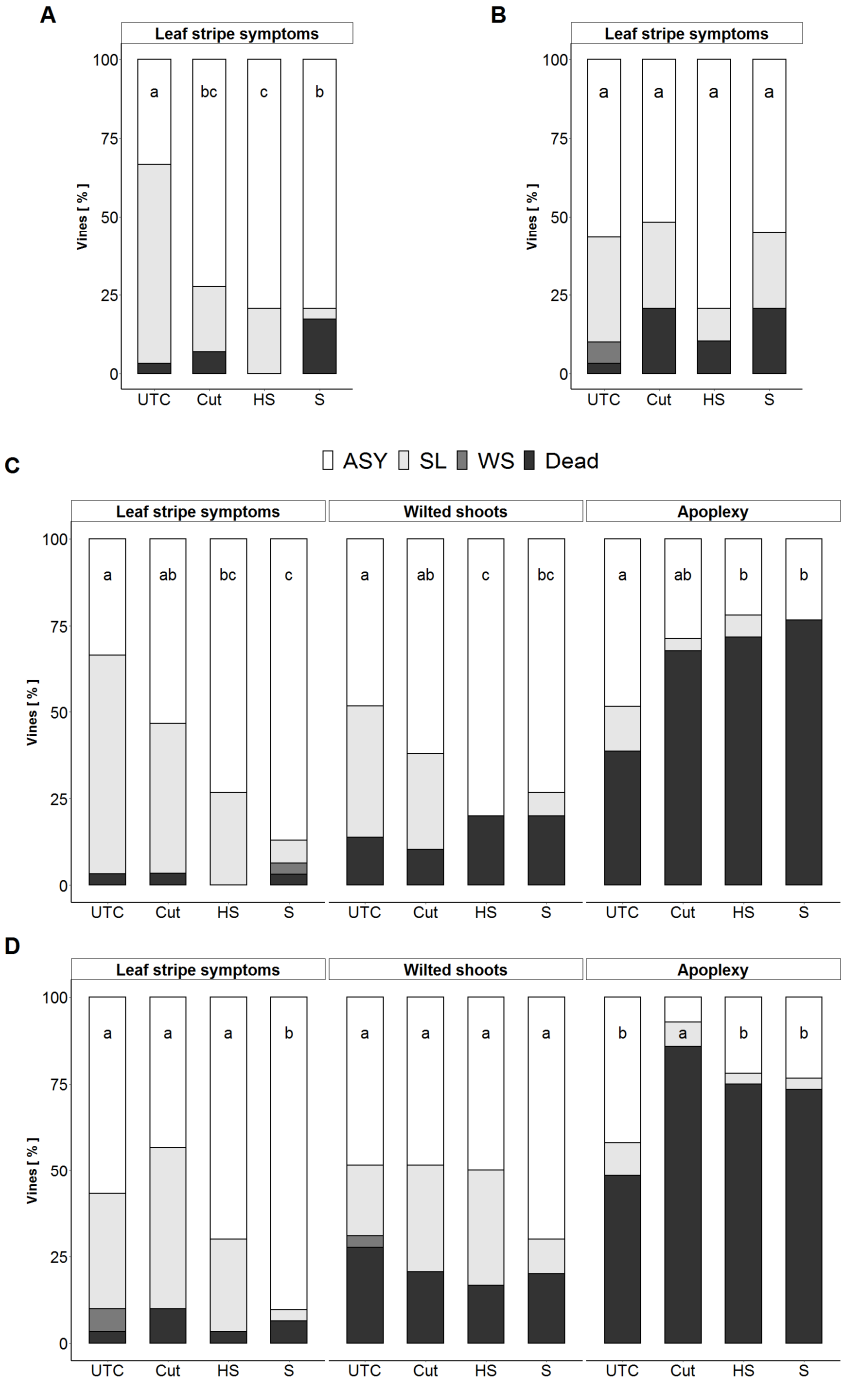

Figure 2. Health status of surveyed vines, one and two years after treatments. (**A**) Summer-treated vines surveyed one year after treatment (2019) and (**B**) two years after treatment (2020). (**C**) Winter-treated vines surveyed one year after treatments (2019) and (**D**) two years after treatment (2020). Histograms are grouped by the symptoms recorded before treatments (survey 2018). Black and grey shades represent symptoms reported in 2019 and 2020 for both untreated and treated plants: asymptomatic vines (ASY), striped leaf vines (LS), wilted cane vines (WS) and dead vines (Dead). The number of plants is reported as frequency. On X-axis, untreated control (UTC), trespassing cut (Cut), half surgery (HS) and complete trunk surgery (S) are the treatments reported and repeated per each 2018-monitored symptom type. Different letters on bars top represents statistically significant differences between treatments, considering all symptom types at once, according to Pearson chi-square test ($\alpha = 0.05$); *p*-value were compared with Bonferroni's adjusted *p*-value.

3.2. Microbiota Overview

The deep sequencing of microbial communities originated a total of 3,180,890 high quality reads. For eukaryotic microorganisms 1,948,262 sequences were obtained and 1,232,628 for prokaryotes. All the high-quality sequence reads were grouped at a genetic distance of 3% and generated a total of 142 OTUs for ITS1, and 5140 for 16S. On average, 117 ± 37 and 921 ± 274 OTUs were obtained for eukaryotes and prokaryotes, respectively. Regarding the taxonomy assignment, a total of 553 fungal and 596 bacterial taxa (genus or species) were identified (Figure 3A,B). Sixty-two fungal taxa and 111 bacterial taxa were found in a relative abundance greater than 1% in at least one sample, while 138 fungal taxa and 200 bacterial taxa were detected with a relative abundance between 0.1% and 1% (Figure 3C,D). Taxa belonging to the phylum *Ascomycota* represent 68% of the eukaryotic microbiota, while 30% belong to *Basidiomycota* and 2% to *Zygomycota* (Figure 3B). The prokaryotic microbiota was represented by 21 phyla of which *Proteobacteria* (37%), *Actinobacteria* (20%), *Firmicutes* (16%) and *Bacteroidetes* (14%) were the most abundant (Figure 3A).

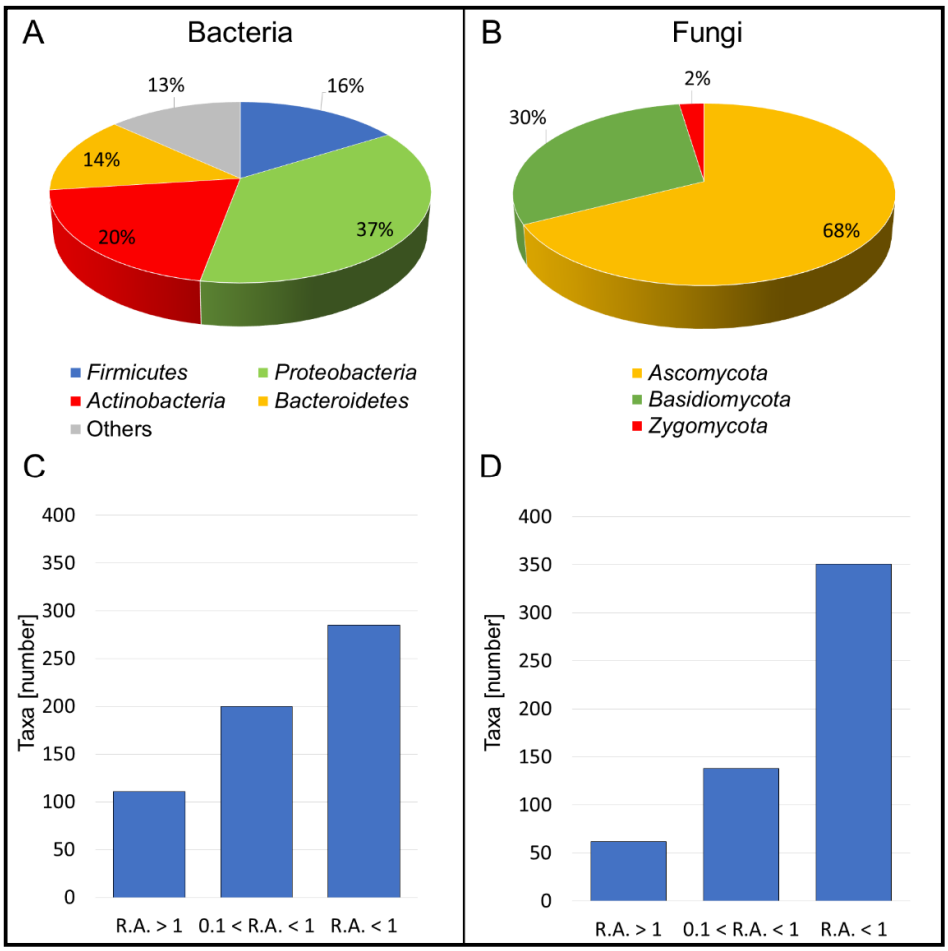

Figure 3. Number of bacterial taxa (**A**) and fungal taxa (**B**) identified referring to SILVA and UNITE taxonomy database according to Materials and Methods description. For both bacteria (**C**) and fungi (**D**), the composition in phyla and the distribution of identified taxa grouped by class of relative abundance (R.A.) are presented.

3.2.1. Microbiota Composition of Wood Types

Fungal microbiota composition was analyzed considering decay, median and sound wood at T0 and median and sound wood at T3 (Figure 4A–C; Appendix, Table A1). Microbiota composition of decayed wood at T0, was mainly represented by *Basidiomycota*,

namely the order *Hymenochaetales* (45%), which consisted almost completely of *Fomitiporia mediterranea*, and *Russulales* (12%), namely *Peniophora* genus (Figure 4A,C). The most abundant *Ascomycota* genera present in decayed wood were *Phaeomoniella* (17%) and *Eutypa* (11%), followed by *Capronia*, which belong to and completely represent the *Herpotrichiellaceae* family (6%) (Figure 4B,C). Among the genus *Phaeomoniella*, only Pch was identified and only *Eutypa lata* was found in the genus *Eutypa*. *Phellinus mori* was detected in relevant abundance (18%) in median wood at T0 where 54% of fungal diversity was represented by Fmed, 9% by Pch and 15% by *Auriculariales* order. At T3, in median wood *Penicillium* spp. and species in *Botryosphaeriaceae* were consistently detected while Fmed was the only *Basidiomycota* still present in significant amounts. Microbiota composition of sound wood was more diverse at T0 than at T3: *Phaeomoniellales* (here only Pch) represented the 23% of diversity at T0. Fmed increased considerably from T0 to T3 in sound wood (from 5% to 55%). As observed in median wood, also in sound wood *Botryosphaeriaceae* were detected only at T3 (Figure 4B). Sound wood at T0 showed the higher microbiota diversity compared to the other wood types and to sound wood itself at T3. Curiously, members of *Saccharomycetales* were found only in sound wood at both T0 and T3. Despite the fact that more than 500 fungal taxa were identified in grapevine trunk wood, more than 50% of the diversity was attributed to only a few species, and mostly associated with pathogenic microorganisms (Supporting Material Table A1).

Regarding the bacterial component of the diversity was more diverse than the fungal component (Figure 5A–C). Herein, *Rhizobiales* was the most abundant order present in decayed wood (22%) at T0. *Sphingomonadales*, *Sphingobacteriales* and *Rhodospirillales* orders represented respectively 13%, 12% and 11% of the bacterial diversity in decayed wood at T0 (Figure 5A). *Proteobacteria* and *Bacteroidetes* were thus the main phyla inhabiting this wood type (20% and 57%, respectively). Regarding the genus level, *Staphylococcus* were present in decayed wood at T0 with an inconsistent abundance but were much more represented in median and sound wood (11% in both wood types) (Figure 5B). *Burkholderia* genus was also more abundant in median and sound wood at T0 (8% and 6%) compared to decayed wood (1%). *Enterobacteriales*, namely *Enterobacter* spp. and *Pantoea* spp. were present in all wood types but in a low abundance at T0; the wood type in which *Enterobacteriales* were more present at T0 was median wood (10%) (Figure 5A,C). Conversely, *Enterobacteriales* represented most of the bacterial diversity at T3 (51% in median and 33% in sound wood). Focusing on the *Enterobacteriaceae* family at T3, *Enterobacter* spp. were the most abundant in median wood (44%), while *Pantoea* spp. were present in sound wood (26%) (Figure 5B). *Burkholderiales* order, represented by *Burkholderia* (*sensu lato*) and *Messilia* genera, grew significantly from T0 to T3, covering the 7% and the 13% in median and sound, respectively. *Rhodospirillales* presence did not change over time in both median and sound wood while *Pseudomonadales*, namely *Pseudomonas* spp., which were not relevant in T0, became representative at T3 in both median (3%) and sound (5%) wood (Figure 5C).

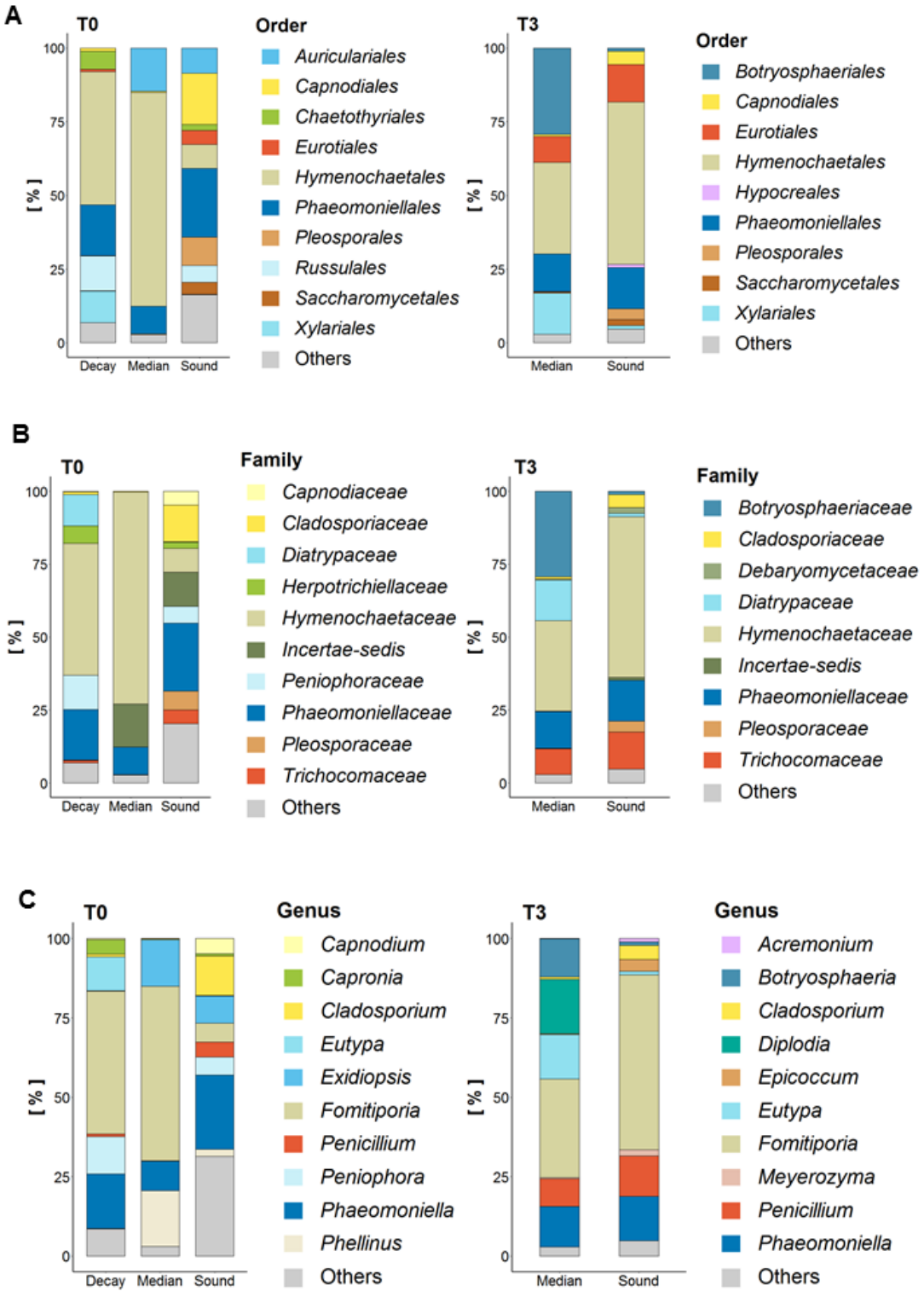

Figure 4. Overview of fungal microbiota composition of untreated vines (UTC) at T0 (May 2019) and T3 (July 2019). Decay, median and sound wood at T0 and median and sound wood at T3 were considered and order (**A**), family (**B**) and genus (**C**) compared. Samples were sampled from LS-diseased vines, which showed symptoms the year before (2018). The means of three biological repetitions per each wood type were calculated. Only taxa with relative abundance higher than 1% are showed.

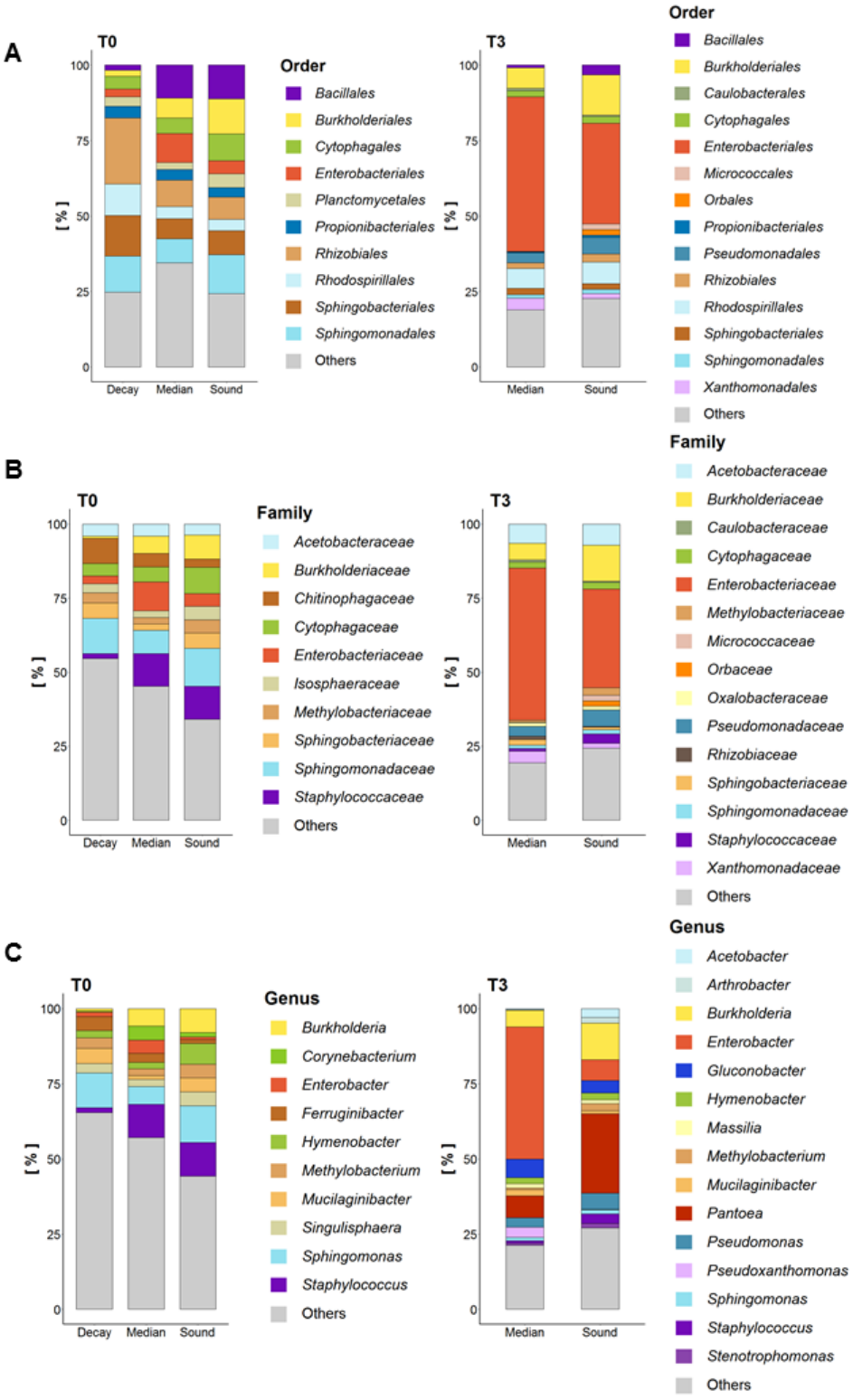

Figure 5. Overview of bacterial microbiota composition of untreated vines at T0 (May 2019) and T3 (July 2019). Decay, median and sound wood at T0 and median and sound wood at T3 were considered and order (**A**), family (**B**) and genus (**C**) compared. Samples were sampled from LS-diseased vines which showed symptoms in the previous year (2018). The mean of three biological repetitions per each wood type was calculated. Only taxa with relative abundance higher than 1% are showed.

3.2.2. Alpha-Diversity Analysis

Fungal alpha-diversity of untreated vines (UTC) was higher in median wood than in sound wood at T0, while in T3 they tended to present similar values as shown in (Figure 6). While from T0 to T3, a natural significant decrease was observed in median wood, compared to T0, the fungal diversity increased slightly in sound wood. Focusing on trunk surgery-treated vines, the fungal diversity increased in median wood from T0 to T3 conversely to untreated control while as for non-treated vines, it increased in sound wood with a similar trend. Shannon's index analysis highlights the inverse behavior in median wood where alpha-diversity of untreated vines decreased from 2.93 to 1.20 while values of trunk-surgery treated vines passed from 1.93 to 2.78 (Figure 6A,B). Moreover, the Observed Richness confirms that phenomena: detected OTUs in untreated vines decreased from 177 to 81, while increased from 111 to 150 in trunk surgery-treated vines in median wood (Figure 6C).

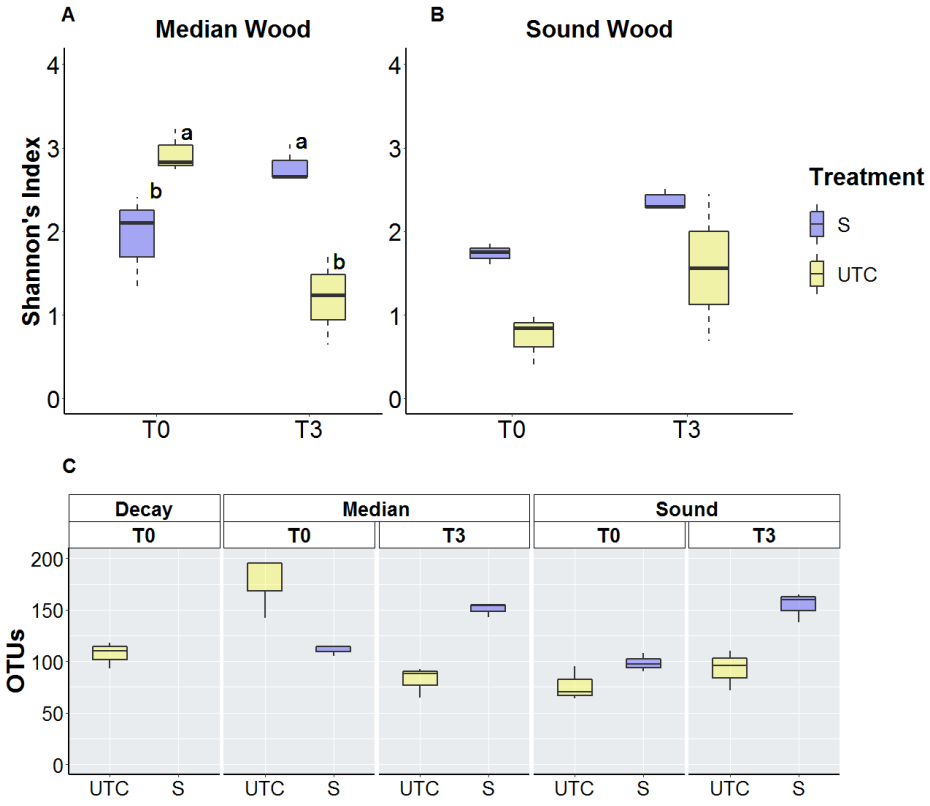

Figure 6. Fungal Alpha-diversity of trunk-surgery treated and untreated vines that showed LS symptoms in the previous year is measured by Shannon's index (**A**,**B**) and by Observed Richness (**C**). Treatments are compared for both median (**A**) and sound (**B**) wood before trunk surgery (T0) and 3 months after (T3). Trunk surgery-treated vines (S) and untreated vines (UTC) are compared. Statistical analysis was performed on Shannon's index values using a 2-way ANOVA test ($\alpha < 0.05$) and Duncan post-hoc was carried out only for median wood where interaction between factors (Time and Treatment) was significant.

Overall, the alpha-diversity of bacterial microbiota decreases from T0 to T3 in both median and sound wood and for both trunk surgery-treated (S) and untreated vines (UTC). Shannon's index values of untreated vines decreased from 5.09 to 4.71 in median wood, and from 5.36 to 4.91 in sound wood. Shannon's index values of trunk surgery-treated vines decreased from 4.11 to 3.46 in median wood and from 2.68 to 2.29 in sound wood (Figure 7A,B). Compared to fungal diversity, bacteria values of detected OTUs were largely higher: at T0, 1314 and 1251 OTUs were identified for untreated vines in median

and sound wood, respectively (Figure 7C). In general, starting from a comparable situation at T0 in median wood, bacterial biodiversity at T3 decreased more in treated vines than in untreated vines, while the genera abundance changed less between treated and untreated in sound wood over time.

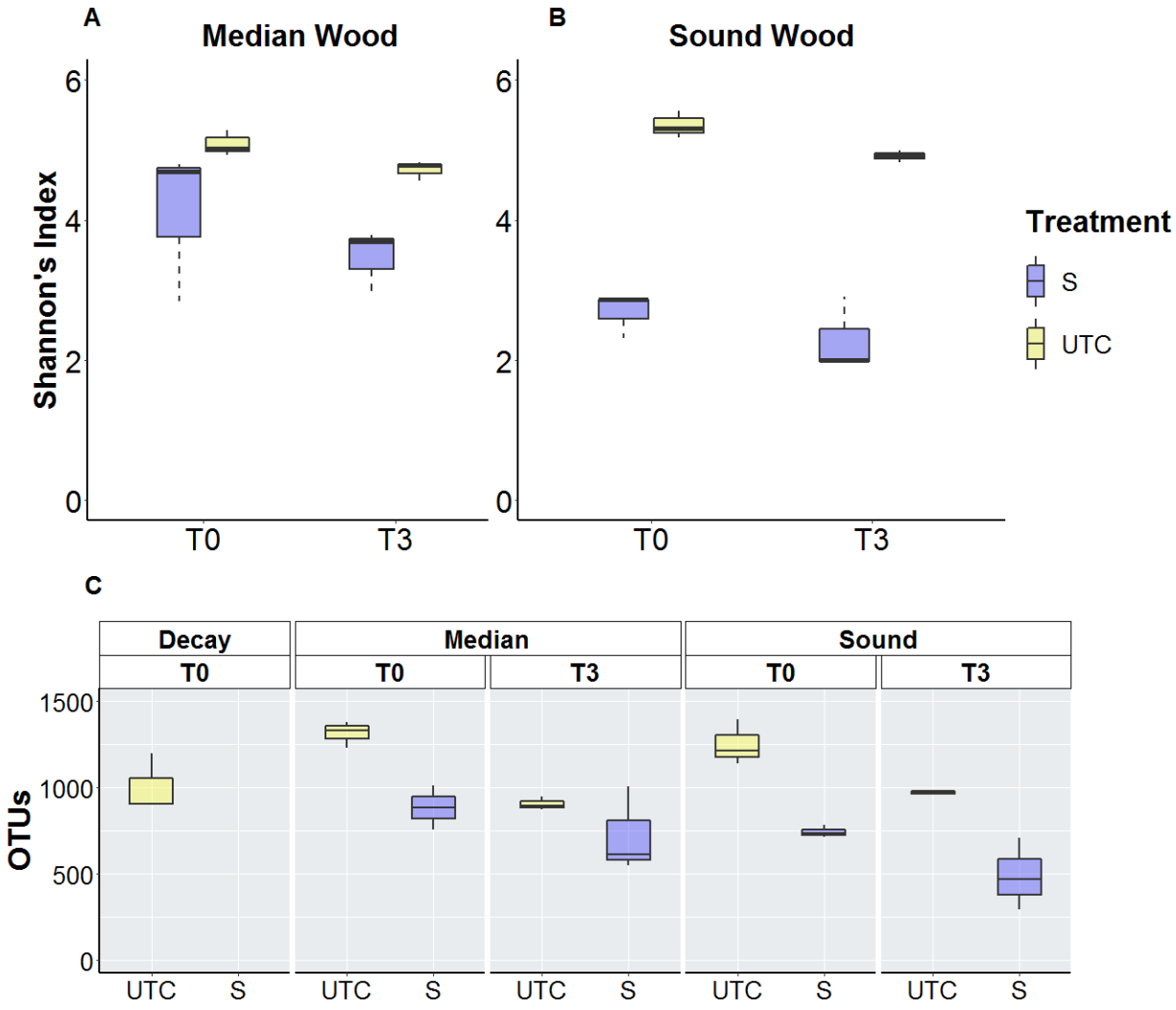

Figure 7. Bacterial alpha-diversity of trunk-surgery treated and untreated vines that showed LS symptoms in the previous year is measured by Shannon's index (**A**,**B**) and by observed Richness (**C**). Treatments are compared for both median (**A**) and sound (**B**) wood before trunk surgery (T0) and 3 months after (T3). Statistical analysis performed on Shannon's index values using a two-way ANOVA test ($\alpha < 0.05$) did not highlight significant differences between T0 and T3 in both median and sound wood.

3.2.3. Beta-Diversity

The analysis of beta-diversity highlighted the homogeneity of fungal and bacterial composition of all samples before trunk surgery treatment at T0 for both median and sound wood (Figure 8). However, after 3 months from trunk surgery (T3), the microbiota composition of the treated vines tended to differ from the microbiota of the untreated ones. Overall, fungal microbiota appeared to be more affected by trunk surgery compared to bacterial microbiota, which was represented mainly in sound wood.

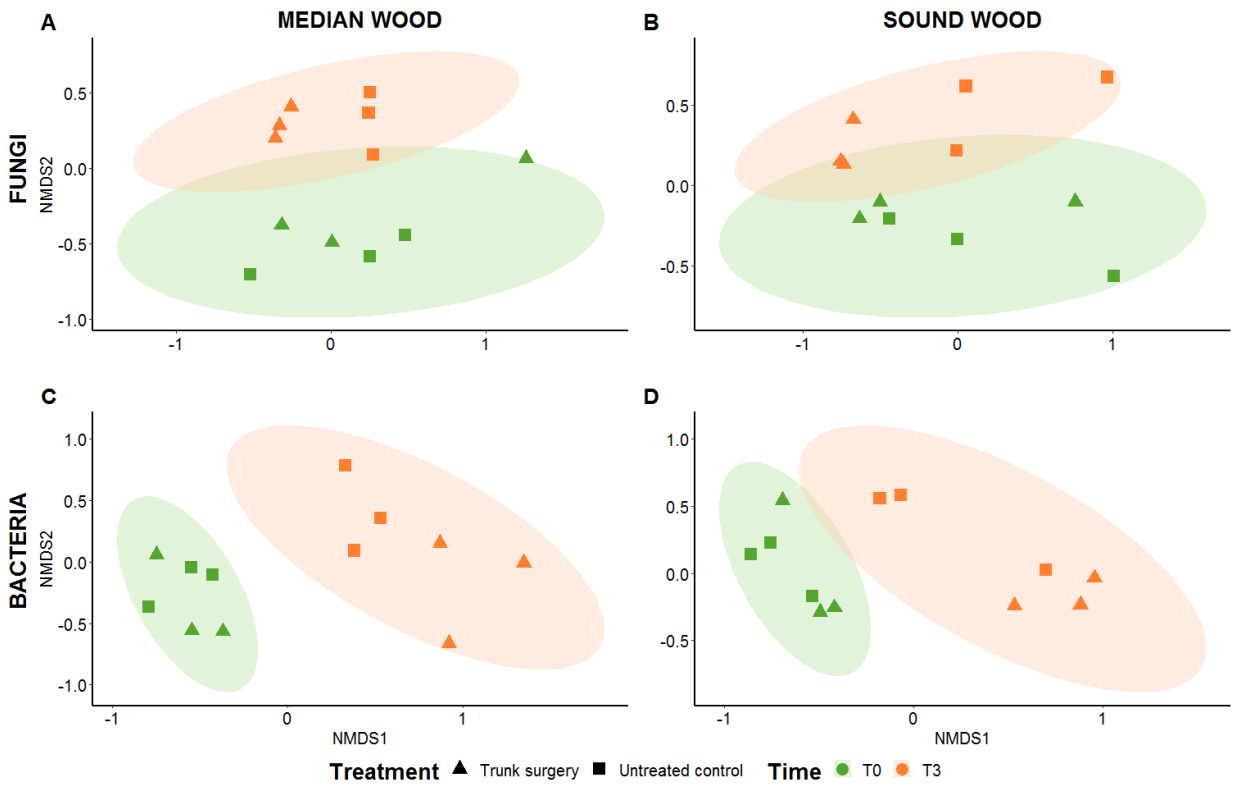

Figure 8. Non-metric multidimensional scaling (NMDS) analysis of fungal (**A**,**B**) and bacterial (**C**,**D**) microbiota. Microbiota of trunk surgery-treated vines and untreated control were analyzed over two timepoints (T0 and T3) considering 674 OTUs for fungi and 5676 OTUs for bacteria. Ellipses indicate 95% confidence intervals fitted into the spatial ordination. Microbial compositions of samples were significantly different where ellipses do not overlap. Significance was calculated performing ANOVA analysis ($p < 0.05$).

3.2.4. Taxa Variation across Time

To better understand the effect of trunk surgery on the microbiota, the relative abundance of the most representative taxa was analyzed over timepoints (T0 and T3). Eleven of the most abundant fungal taxa belonged to *Ascomycota*, while four belonged to *Basidiomycota*. As shown in Figure 9, grapevine pathogens were most abundant in sound wood. Focusing on pathogens involved in the diseases of the Esca complex, *P. chlamydospora* (Pch) was present in both median and sound wood in a similar manner and its presence appeared not to be affected by trunk surgery. Pch abundance did not change (from T0 to T3) comparing trunk surgery treated and untreated vines. Conversely *Fomitiporia mediterranea* (Fmed) was the species that showed a stronger variation, decreasing significantly from T0 to T3 in both treated median wood and treated sound wood. On the other hand, Fmed abundance increased in untreated sound wood and remained high in untreated median wood. The other phytopathogenic species, *Diplodia seriata* and the *Penicillium* genus were more abundant at T3 compared to T0 in both wood types shown to be affected by time more than by trunk surgery. *Eutypa lata* and *Botryosphaeria dothidea* appeared consistently only in median wood at T3. Cryptococcus sp. and *Aureobasidium pullulans* abundance increased significantly in treated vines compared to untreated control after treatment in both median and sound wood.

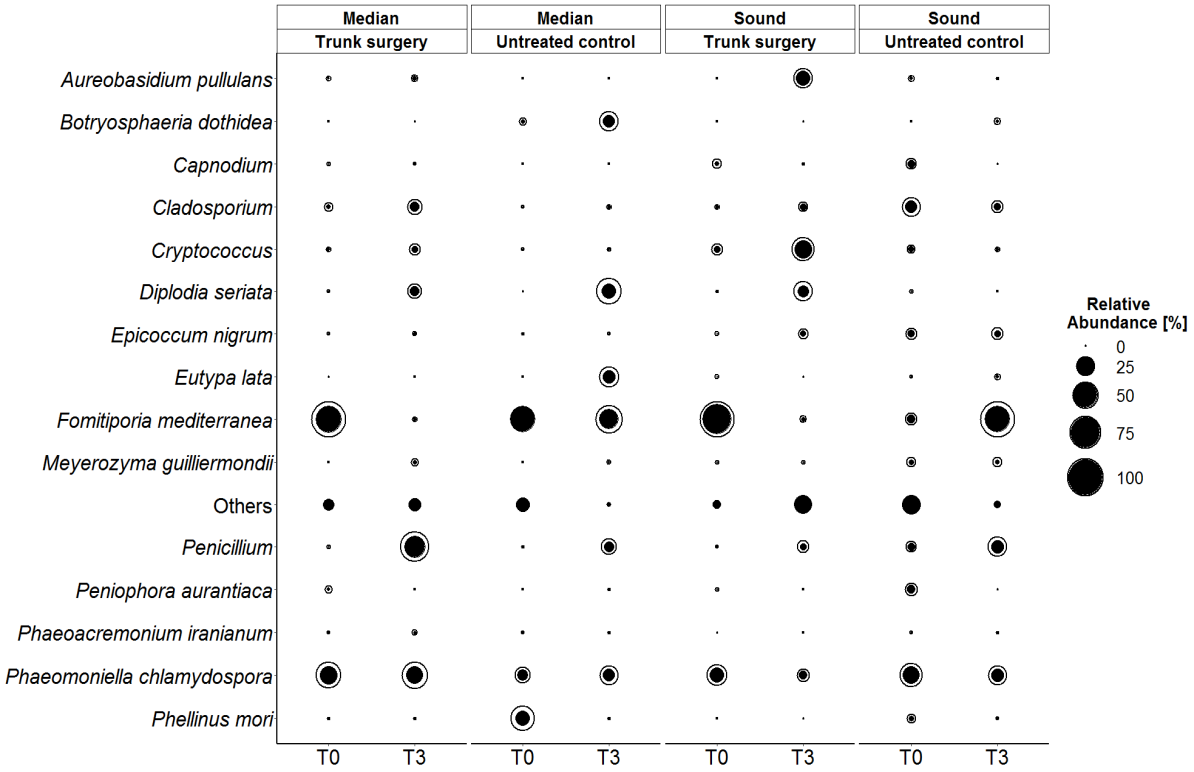

Figure 9. Qualitative changes in fungal diversity grouped by wood types (median and sound) a day before trunk surgery (T0) and 3 months after (T3); only the taxa with a relative abundance greater than 1% and the taxa which were much relevant for grapevine are showed. Solid black circles represent mean relative abundance of three biological repetition and external black line describes + standard deviation (SD): the greater the SD, the bigger is the space between the black line and the black solid circle.

The bacterial genus that has been affected most heavily by trunk surgery was *Burkholderia* (*sensu lato*), i.e., its abundance decreased in both median and sound trunk surgery-treated wood while its abundance did not vary in untreated control. The same was observed for the *Corynebacterium* genus but with lower values (Figure 10). Conversely to the former, *Massilia* sp. and *Pantoea* sp. increased considerably on median and sound treated vine woods. The abundance of members of the *Agrobacterium* genus increased with treatment in median wood but not in sound wood. Conversely, *Hymenobacter* genus decreased only in trunk surgery-treated median wood. As shown in Figure 10, other variations between bacterial microbiota can be noted for many genera over time but no consistent differences between treated and untreated emerged: *Propionibacterium*, *Roseomonas*, *Singulishaera*, *Sphingomonas* and *Staphylococcus* variation were more affected by time than by treatment.

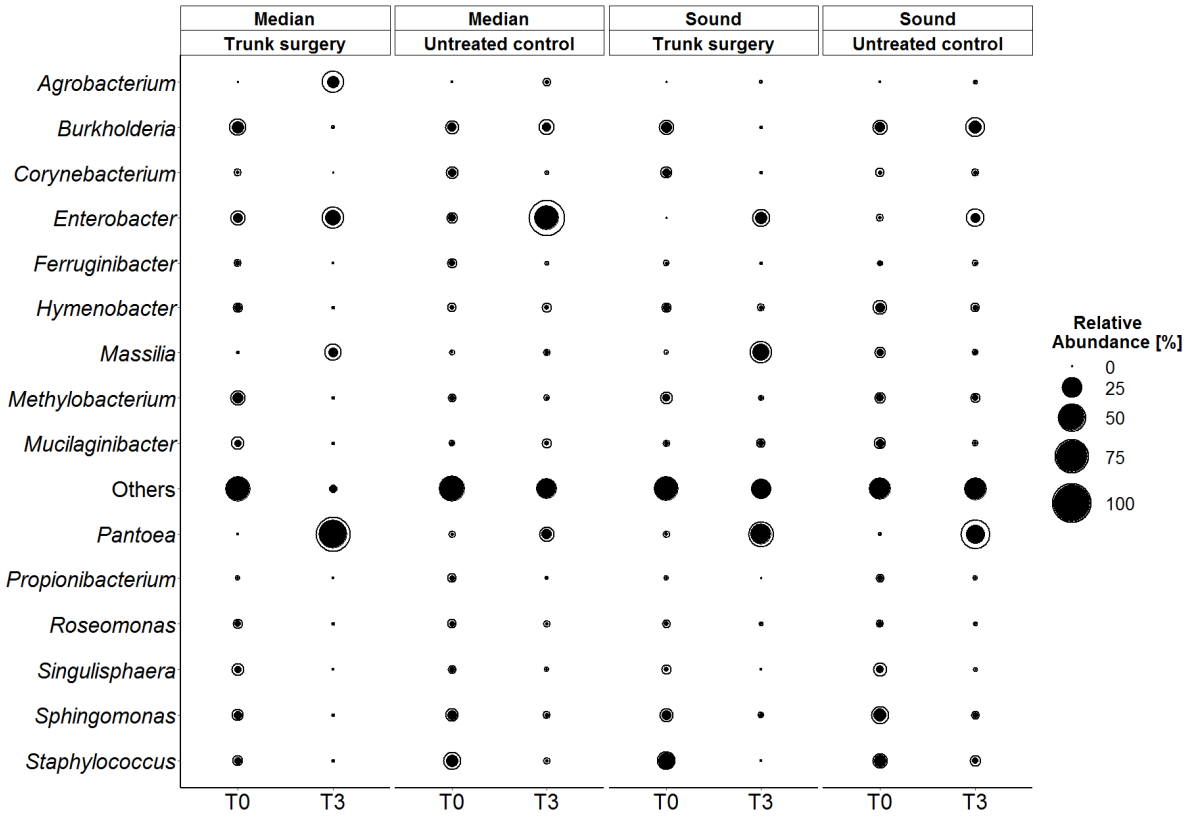

Figure 10. Qualitative changes in bacterial diversity grouped by wood types (median and sound) a day before trunk surgery (T0) and 3 months after (T3); only the genera with a relative abundance greater than 1% are showed. Solid black circles represent mean relative abundance of three biological repetition and external black line circles describe + standard deviation (SD): the greater the SD, the bigger is the space between the black line and the black solid circle.

4. Discussion

So far, only a few studies have been conducted on the efficacy of trunk surgery to reduce the expression of symptoms associated with Esca complex diseases, in particular with leaf stripe symptoms [51], and to our knowledge this is the first study that highlights the effect of the technique on the vine microbiota. It should be noted that the field tests were carried out over two years and characterized by different meteorological conditions (Appendix, Figures A1 and A2), and the consistency of the results obtained underlines their reproducibility. In order to better understand the role of decay removal on reducing foliar symptoms, three levels of treatment were tested. The trial showed that only the complete removal of decayed wood ensured the remission of LS symptoms in both years of trials, 2019 and 2020. Despite trunk surgery is currently being poorly described in the literature, the results obtained confirm the importance of the complete decay removal as suggested by other authors [36,37] and strengthen the hypothesis of a relevant role of decayed wood in foliar symptom expression [52]. Trunk surgery has been applied also on vines with wilted shoot symptoms and apoplexy: in those treatments, results showed, as to be expected, the inefficacy of the technique on reducing WS symptoms and moreover, highlighted a relevant increase of mortality on apoplectic vines probably due to the weakening of the vascular system. Comparing winter and summer treatment applications performed on vines showing LS symptoms in 2018, a higher number of dead vines was recorded in summer S-treatment, which may be due to the conjunction of the high rate of wood removal and the external climatic conditions (high temperatures and evaporation rate). This could be confirmed by the fact that only one Cut-treated vine and no HS-treated

vines died in 2019 over the total of summer treated vines. Grapevine microbiota is influenced by various external factors, such as grape cultivars, farming practices, pedoclimatic conditions or geographical location [40,53,54]. Recently, several studies have been carried out on grapevine microbiota using different methodologies and the most relevant taxa of fungi and bacteria in grapevine wood are more defined [25,26,40,55]. The analysis on microbiota carried out in this experiment is related to a one-year study with the final aim of highlighting the fungal and bacterial taxa that were mainly affected by the trunk surgery technique. Obtained results allowed us to postulate hypothesis in order to carry out further investigations on the factors involved in the microbiota activity on symptoms appearance: a direct activity or a metabolite-driven activity. A first survey highlighted the microbiota composition of three analyzed wood types—sound, median and decayed wood. The most abundant bacterial taxa found in decayed wood at T0 belong to *Rhizobiales*, *Sphingomonadales* and *Sphingobacteriales*. The number of bacterial taxa with a relative abundance higher than 1% decreased over time and the majority of median and sound wood bacterial diversity were represented by few taxa: *Enterobacteriales*, *Burkholderiales*, *Pseudomonadales* and *Rhodospirillales* being the most abundant orders at T3. The most representative fungal taxa detected in decayed wood consisted mainly of pathogenic species, in which *F. mediterranea* and *P. chlamydospora* were the most abundant species found in decayed wood as previously shown by different authors [10,25,26,56]. Pmin, which is a common species often detected in Esca affected vines, was not found at all in the analyzed samples. Within the genus *Phaeoacremonium*, only *P. iranianum* was present in all the studied wood types with a very low relative abundance, a species that has been associated to Esca complex diseases only occasionally [57–59]. The analysis of the microbiota composition before treatment highlights that median wood fungal diversity was represented by a higher number of OTUs than sound wood fungal diversity due to a higher number of taxa in median wood. As a consequence of trunk surgery treatment, the fungal diversity raised in both median and sound wood of the treated vines, while it increased only in sound wood in the untreated control as shown by alpha-diversity analysis. Conversely, bacterial diversity decreased from T0 to T3 in a similar manner in both median and sound wood, without relevant differences between treated and untreated vines. Moreover, clusters observed at T3 in beta-diversity analysis showed how trunk surgery appears to select specific taxa suggesting the opportunity to investigate the role of excluded taxa in symptom development and at the same time on the increase in the population of bacterial species belonging to *Pantoea* spp. and the yeast-like fungus *Aureobasidium pullulans* in the vines showing symptom reduction. Among bacteria, *Burkholderia* (*sensu lato*) decrease in trunk surgery-treated wood (both median and sound wood). *Corynebacterium* decrease too while *Massilia* and *Pantoea* increased. *Burkholderia sensu lato* comprise several genera, such as *Trinikia, Parabulkholderia, Burkholderia, Caballeronia* and *Mycetohabitans* [60]. Some of these genera have been previously detected in grapevine. Some strains of *Burkholderia*, *Messilia* and *Pantoea* have been reported to be endophytic in grapevine plant tissues and associated with biocontrol action against phytopathogens [61,62]. Changes of other taxa abundance were detected suggesting that trunk surgery impacts ecologic niches of these microbial endophytes and that some of them are better adapted to survive in the specific wood tissue. Some other taxa are more affected by time and plant physiology with differences according to plant tissue [55] including trunk [63]. Obtained results highlight that trunk surgery allows for the enhancement of fungal diversity and the selection of specific taxa in median and sound wood. Analyzing the change of the most abundant species over time, Fmed is the fungal specie most affected by trunk surgery beside being the most recurrent species detected in decayed wood before trunk surgery application. Applying trunk surgery, the decayed tissue—which acts as a sort of fungal mycelium reservoir—is removed and Fmed presence decreases significantly in both the remaining median and sound wood showing a correlated behavior with symptom remission. This phenomenon led us to postulate that the Fmed activity in wood colonization had a relevant role in foliar symptom expression in "Esca proper" affected vines. Further investigations could be devoted to

evaluate the possible role of light and air, as well as plant physiological reaction. Moreover, arsenite treatments reduced the expression of LS symptoms and it was found that arsenite concentrates in the decayed wood tissue where it showed a fungal inhibiting activity [64,65]. Considering this evidence, it is reasonable to accept that *Fomitiporia mediterranea* and its metabolism can play a main role in LS symptom expression. Furthermore some of the results obtained suggest that this basidiomycete might not require previous infection in order to extensively colonize the wood as recently highlighted by other authors [66]. On the other hand, the hypothesis that a single fungus causes the chloro-necrotic symptoms on leaves still cannot be accepted, despite it appears strictly related to Fmed presence in the rotten wood, as the same foliar symptoms are well known to be present in vines that have no wood rot or decay and so no basidiomycetes activity [67–69]. This suggests that there are some pathways in common among extremely different pathogens that cause a similar reaction, and therefore symptomatology, on the vine. While Esca in its original definition describes a wood rot, the association with leaf stripe symptoms has been better described as a separate disease, namely GLSD, that clearly appears to be due to various factors, and not to the action of a single pathogen. The idea of synergism between pathogens as a main trigger responsible for disease onset has been recently proposed by Bruez [70]. Recent literature reviews on wood degradation mechanism shows how some white rot, brown rot and soft rot agents share a non-enzymatic iron-dependent mechanism to intake lignocellulose biomass, either acting alone or in synergism with enzymes [71,72]. The involvement of those mechanisms in Esca related fungi has been proposed for Pch and Pmin [73], as well as for Fmed [74]. Furthermore, the evidence on the role of phytotoxic metabolites which could also contribute to the expression of symptoms [75,76] interacting with the aforementioned pathways, or alone, cannot be excluded. Thus, it is possible to hypothesize that the role played by those pathways could be very relevant in causing the foliar symptom expression, better described as a separate disease from white rot within Esca complex of diseases. Further research on the topic is needed to confirm this hypothesis and investigate the role of the metabolites involved in vine physiology.

5. Conclusions

Trunk surgery is a technique applied to reduce leaf stripe symptoms when they occur in vines, showing the whole range of wood symptoms and especially wood decay (the condition that can be described as Esca proper). This study shows the efficacy of the trunk surgery in LS symptom remission suggesting a link between the fungal wood colonization by basidiomycetes and foliar symptoms. This is highlighted by the fact that the higher was the degree of decayed wood removal, the lower was the expression of LS foliar symptom shown. This study strengthens the knowledge on vine microbiota and for the first time describes changes induced on it by trunk surgery technique as well as highlights the link between the decrease of the expression of LS symptoms and the abundance of *Fomitiporia mediterranea*.

Author Contributions: A.P. contributed to the concept/design, samples collecting and processing, data analysis and interpretation, drafting and critical revision of the article. S.M. contributed to samples colleting, drafting and critical revision. C.P., S.C., S.F. and C.B. contributed to critical revision of the article. L.M. contributed to concept/design and critical revision of the article. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available within the article and the supplementary materials.

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Conflicts of Interest: The authors declare no conflicts of interest. Funder had no role in the design of the study, in the collection, analysis or interpretation of data; in writing of the manuscript or in the decision to publish the results.

Appendix A

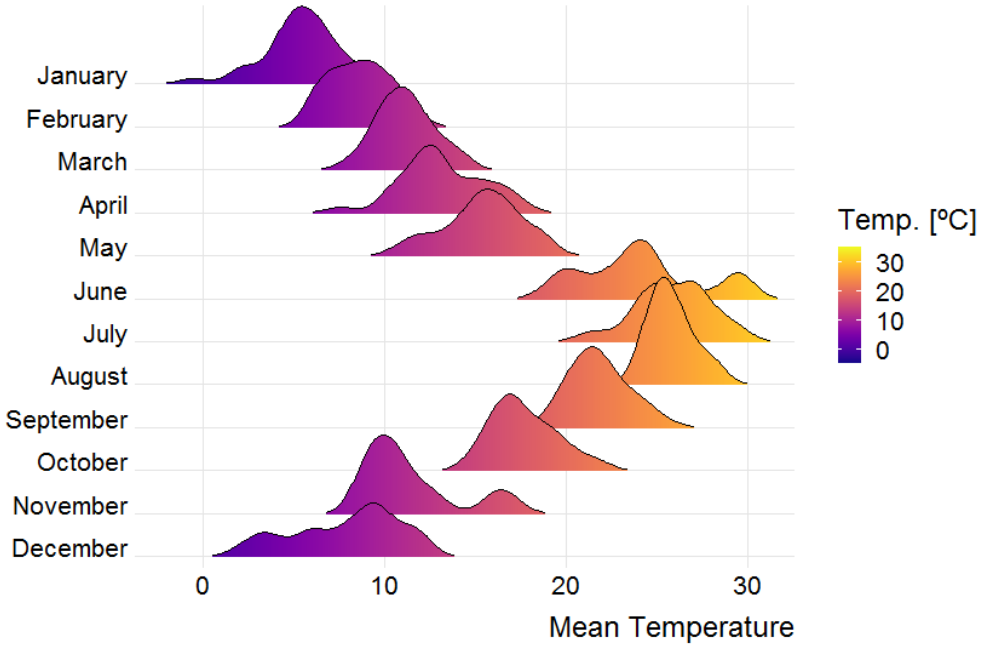

Figure A1. Mean Temperature (Celsius) recorded in the surveyed vineyard by month for 2019.

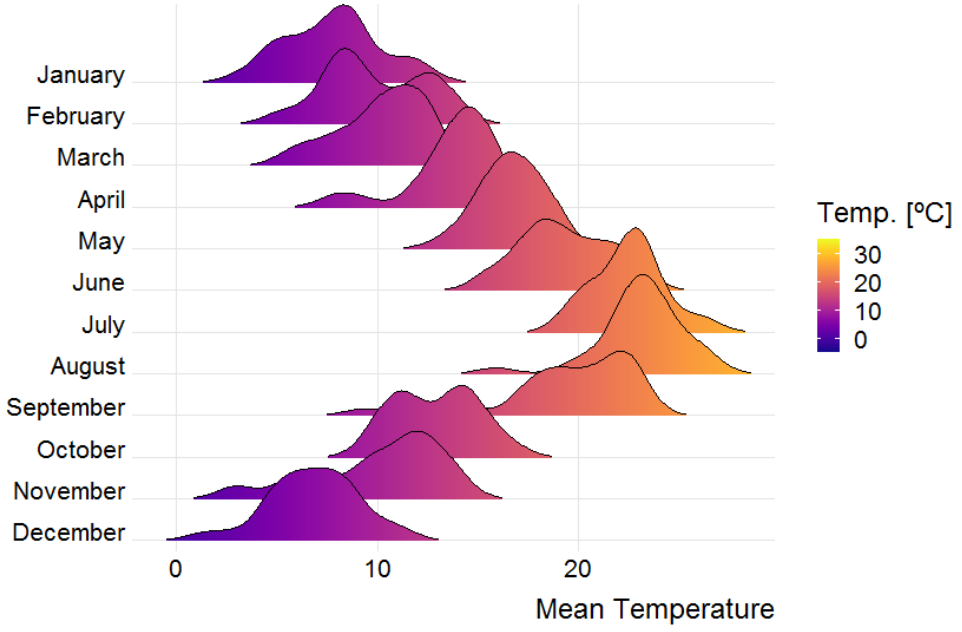

Figure A2. Mean Temperature (Celsius) recorded in the surveyed vineyard by month for 2020.

Table A1. Microbiota of each different type of wood before trunk surgery. Listed species represent 96% of fungal diversity in decayed wood, 85.5% in median wood and 99% in sound wood. Only the species with a RA greater than 1% and the species which were much relevant for grapevine are shown. Wood type: ■ Decay wood, ▲Median wood, ● Sound wood.. Ecology in wood: (E) Endophyte; (P) Pathogen; (S) Saprotroph; (U) Unknown.

Species	Phylum	Family	Ecology in Wood	Wood Type	Relative Abundance (%)	References
Acremonium sp.	*Ascomycota*	*Incertae-sedis*	E, P	▲	0.2	[77–83]
Alternaria sp.	*Ascomycota*	*Pleosporaceae*	E, P	▲; ●	0.1; 0.1	[17,78,80,82,84–88]
Aureobasidium pullulans	*Ascomycota*	*Saccotheciaceae*	E, S	■; ▲	0.3; 0.9	[28,80,87,89–94]
Botryosphaeria dothidea	*Ascomycota*	*Botryosphaeriaceae*	E, P, S	■; ●	0.2; 1.4	[28,78,79,91,92,94–109]
Botrytis cinerea	*Ascomycota*	*Sclerotiniaceae*	E, P, S	▲	0.2	[28,80,87,88,91,92,96,110–120]
Capnodium sp.	*Ascomycota*	*Capnodiaceae*	P	■; ▲	0.3; 4.7	[121]
Capronia sp.	*Ascomycota*	*Herpotrichiellaceae*	S	■; ▲	5.2; 0.7	[122]
Cladosporium sp.	*Ascomycota*	*Cladosporiaceae*	E, P, S	■; ▲; ●	0.5; 9.1; 0.2	[28,88]
Cladosporium sphaerospermum	*Ascomycota*	*Cladosporiaceae*	P, S	▲	1.1	[88]
Cryptococcus sp.	*Basidiomycota*	*Cryptococcaceae*	E, S	■; ▲; ●	0.3; 2.8; 0.2	[28,92]
Diplodia seriata	*Ascomycota*	*Botryosphaeriaceae*	E, P, S	■; ▲	0.2; 0.3	[78,79,82,88,91,92,94,97,98,102,104,108,123–131]
Epicoccum nigrum	*Ascomycota*	*Pleosporaceae*	E, S	■; ▲; ●	0.1; 4.3; 0.1	[80,88,91,92,96]
Eutypa lata	*Ascomycota*	*Diatrypaceae*	P	■; ▲	10.8; 0.2	[17,79,103,114,121,130,132–142]
Exophiala sp.	*Ascomycota*	*Herpotrichiellaceae*	E	■; ▲	2.0; 0.7	[28]
Fomitiporia mediterranea	*Basidiomycota*	*Hymenochaetaceae*	P	■; ▲; ●	45.0; 5.9; 55.0	[6,79,82,130,143–146]
Leptosphaeria sp.	*Ascomycota*	*Leptosphaeriacea*	E; S	▲	0.1	[80,88]
Meyerozyma guilliermondii	*Ascomycota*	*Debaryomycetaceae*	E	■; ▲	0.1; 2.3	[147,148]
Penicillium sp.	*Ascomycota*	*Aspergillaceae*	E; P; S	■; ▲	0.9;5.5	[28,80,82,88,92,123]
Peniophora aurantiaca	*Basidiomycota*	*Peniophoraceae*	S	■; ▲	11.7; 5.7	[149,150]
Phaeoacremonium iranianum	*Ascomycota*	*Togniniaceae*	P	■; ▲; ●	1.0; 0.2; 0.1	[57,151–156]
Phaeomoniella chlamydospora	*Ascomycota*	*Phaeomoniellaceae*	P	■; ▲; ●	17.2; 23.3; 9.4	[3,17,78,79,88,107,130,154,157–162]
Phellinus mori	*Basidiomycota*	*Hymenochaetaceae*	U	■; ▲; ●	0.1; 2.3; 17.6	
Pleospora herbarum	*Ascomycota*	*Pleosporaceae*	E	▲	2.2	[92]

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Annex II

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Review

Fomitiporia mediterranea M. Fisch., the historical Esca agent: a comprehensive review on the main grapevine wood rot agent in Europe

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Summary. *Fomitiporia mediterranea* M. Fisch. (*Fmed*) is a basidiomycete first described in 2002, and was considered up to then as part of *Fomitiporia punctata* (P. Karst) Murrill. This fungus can degrade lignocellulosic biomass, causing white rot and leaving bleached fibrous host residues. In Europe *Fmed* is considered the main grapevine wood rot (Esca) agent within the Esca disease complex, which includes some of the most economically important Grapevine Trunk Diseases (GTDs). This review summarises and evaluates published research on *Fmed*, on white rot elimination by curettage or management by treatments with specific products applied to diseased grapevines, and on the relationship between wood symptoms and Grapevine Leaf Stripe Disease (GLSD) in the Esca disease complex. Information is also reviewed on the fungus biology, mechanisms of pathogenicity, and their possible relationships with external foliar symptoms of the Esca disease complex. Information on *Fmed* control strategies is also reviewed.

Keywords. *Fmed*, Basidiomycete, white rot, wood symptoms, foliar symptoms.

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INTRODUCTION

Grapevine Trunk Diseases (GTDs), mainly comprising Botryosphaeria dieback, Eutypiosis and the Esca disease complex, are widespread in vineyards (Mugnai *et al.,* 1999; Bertsch *et al*., 2013; Bruez *et al*., 2013; Mondello *et al*., 2018a). These diseases significantly affect grapevine productivity causing yield losses and quality degradation affecting wine alcohol content and flavour components (Mugnai *et al*., 1999; Lorrain *et al*., 2012; Calzarano *et al*., 2001, 2017).

For several decades the only effective pesticides used to control GTDs were sodium arsenite, to reduce the leaf stripe foliar symptoms in the Esca complex of diseases (Ravaz, 1919; Bonnet, 1926; Rui and Battel, 1963; Svampa and Tosatti, 1977; Del Rivero and García-Marí, 1984), and the fungicides benomyl and carbendazim, to reduce infections by the agents of Eutypiosis and Botryosphaeria dieback, respectively (Magarey and Carter 1986; Ramsdell, 1995). All these pesticides were banned in European countries in the early 2000s because of their potential environmental and/or user toxicities, more than 10 years after GTDs and especially Esca disease complex were becoming acute problems in Europe and in other grapevine growing countries. GTDs have been described as "the biotic stress of the century" for grapevines (Songy *et al*., 2019a), and all wine-growing countries are likely to be affected by these diseases. In France, the National Grapevine Trunk Disease Survey assessed incidence and evolution of GTDs during a 10 year survey period. Up to 13% of productive vines were affected by GTDs in French vineyards (Grosman, 2008; Grosman and Doublet, 2012; Bruez *et al*., 2013). In Italy, incidence of GTDs was between 8 to 19% (Romanazzi *et al*., 2009), and was of average annual incidence of 12% in vineyards younger than 10 years (Abbatecola *et al*., 2000), or up to 63% in 30 year old vineyards (Surico *et al*., 2000).

In several countries within and outside Europe, there has been an upward trend of GTDs since the end of the 20th century (Mugnai *et al*., 1999; Wicks and Davies, 1999; Rubio and Garzón, 2011; Úrbez-Torres *et al*., 2014; Fontaine *et al*., 2016a; Guérin-Dubrana *et al*., 2019; Kraus *et al*., 2019). GTDs have also caused severe economic losses. These have been estimated as up to $US 260 million in California (Siebert, 2001) for GTDs, and $US 2000 to 3000 per hectare for "Esca disease" (Vasquez 2007, in Rubio and Garzón, 2011), and approx. one billion euros in wine production due to GTDs in France (reported in 2014 by IFV, the French Wine Institute). Annual financial costs of dead vine replacements in all wine production countries were estimated to be 1.132 billion euros (Hofstetter *et al*., 2012). These losses have been the major reason that professional winegrowers, research agencies, financial consortia and the scientific community have concentrated on GTDs research in recent decades.

The complexity of symptoms and fungi involved in these diseases is great, and this is particularly true for "Esca disease" (in this paper, when the literature data refer to this generic term – as well as when we will arbitrarily refer to this generic term to avoid nomenclature confusion when critically discussing the literature – we will place quotation marks around the term "Esca disease"). Historically, Esca, a word of indo-european origin meaning "food", tinder for fire, used to indicate "amadou" i.e. white rot (Viala, 1922, on the chapter written by Gard in "Bulletin de la Societé de Pathologie Vegetale", 1922; Montanari, 2010), had been used for a grapevine wood rot disease. Later, the term was associated with foliar symptoms, described as chronic or acute forms (Viala, 1926; Larignon and Dubos, 1997; Letousey *et al*., 2010; Lecomte *et al*., 2012), and was shown to involve several different symptoms associated with different pathogens. It was then proposed as a disease complex involving multiple pathogens including basidiomycetes and/or ascomycetes (Mugnai *et al*., 1999; Surico, 2009; Bertsch *et al*., 2013). These caused separate diseases including: *i*) white rot (Esca) that develops mostly in old vines; *ii*) vascular diseases, widely present in propagation material and young vines (brown wood streaking of grape cuttings and Petri disease); and *iii*) Grapevine Leaf Stripe Disease (GLSD), which has an unusual epidemiology and symptomatology that can be associated with some or all of the wood pathogens, i.e. only vascular and canker agents, or, very often, also wood decay in all possible combinations. The condition where white rot (Esca) and GLSD occur together can be indicated as "Esca proper", in recognition of the original disease description.

The frequency of GLSD foliar symptoms has increased considerably over the last two decades. A preliminary study (Fussler *et al*., 2008) indicated mean incidence increase of 3.25% for "Esca disease" (leaf stripe and apoplexy symptoms) in France between 2003 and 2005. "Esca disease" and Eutypiosis were responsible together for 10% of vine replacements in Alsace (Kuntzmann *et al*., 2010). Also in France, Bruez *et al*. (2013) showed that incidence increase varied according to region. In Austria, Reisenzein *et al*. (2000) estimated a 2.7% annual increase of plants showing "Esca disease" foliar symptoms. In Italy, Surico *et al*. (2006) indicated an increase from 30 to 51% between 2000 and 2006, and Romanazzi *et al*. (2009) showed how disease incidences reached 60 to 80% in many old vineyards of south-

ern Italy. A comprehensive survey for 22 European and Mediterranean vine-growing countries (COST Action; Guérin-Dubrana *et al*., 2019) described GLSD trends in most surveyed countries as "increasing" and/or "worrying", particularly in France, Italy, Spain and Turkey. In Germany, in 12 intensively pruned vineyards of red and white grape varieties and resistant and traditional cultivars, incidence of GLSD increased from 1.9% in 2015 to 3.6% in 2018, and was greatest in 2017 at 4.5% of vines affected (Kraus *et al*., 2019).

Some observations on Esca complex of diseases are now well accepted, despite the complexity, terminology evolution, and difficulties in understanding the interactions of variables that affect symptom expression. These include:

i) The interactions with environmental, pedo-climatic and agronomic factors/practices that can affect symptom expression and disease severity (Marchi *et al*., 2006; Calzarano *et al*., 2018a; Lecomte *et al*., 2018; Fischer and Peighami-Ashnaei, 2019; Songy *et al*., 2019a).

ii) No completely resistant grape cultivar has been reported, but cultivar and clone contributions to symptom expression and severity have been observed and reviewed (Marchi, 2001; Quaglia *et al*., 2009; Murolo and Romanazzi, 2014; Kraus *et al*., 2019; Moret *et al*., 2019; Songy *et al*., 2019a; Moret *et al*., 2021).

iii) The nutritional (especially macronutrient) status of vines can affect foliar symptom expression (Calzarano *et al*., 2009, 2021).

iv) Although the presence of an exceptionally wide mycoflora in Esca- and GLSD-affected vines has been confirmed by meta-barcoding (Del Frari *et al*., 2019a; Niem *et al*., 2020), the pathogens most frequently associated with the wood infections are the two Ascomycota *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) (Crous and Gams, 2000) (*Pch*) and *Phaeoacremonium minimum* (Tul. & C. Tul.) Gramaje, L. Mostert & Crous (Gramaje *et al*., 2015) (*Pmin*) (syn. *Phaeoacremonium aleophilum*), while the most frequently isolated basidiomycete in Europe has been *Fomitiporia mediterranea* M. Fisch. (Fischer, 2002) (*Fmed*). The two Ascomycota species have mostly been associated with the "phaeotracheomycotic complex" (brown wood streaking, Petri disease and GLSD; Bertsch *et al*., 2013), while *Fmed* or the other basidiomycetes causing wood decay (Fischer and González-García, 2015) have only been associated with white rot, Esca and "Esca proper". Nevertheless, decay elimination (curettage) reduced foliar symptoms, and correlations between white rot extent, elimination and foliar symptom expression have been reported (Maher *et al*., 2012; Thibault, 2015; Cholet *et al*., 2019, 2021; Pacetti *et al*., 2021).

Fomitiporia mediterranea (as *Fomitiporia punctata*) was shown to be a primary pathogen by artificial inoculations, either in vineyards or in greenhouse experiments (Sparapano *et al*., 2000a; 2001a). The need for specific successions of fungi in wood colonization to detoxify wood cellular microenvironments from excesses of polyphenols produced by plant reactions has been suggested but was never fully proved (Larignon and Dubos, 1997; Mugnai *et al*., 1997; Amalfitano *et al*., 2000). Microbial combinations between *Fmed*, *Pch* and some bacterial taxa (i.e. *Sphingomonas* spp. and *Mycobacterium* spp.) may have a role in the onset of "Esca disease" in young vines (Bruez *et al*., 2020). Synergism between *Fmed* and bacteria such as *Paenibacillus* spp. for grapevine wood component degradation has also been confirmed (Haidar *et al*., 2021).

Despite the year-to-year fluctuations in incidence, foliar symptom surveys represent simple and non-invasive ways to indirectly assess grapevine wood infection by Esca complex pathogens, and for determining epidemiology, crop losses and health status of vineyards (Guérin-Dubrana *et al*., 2013). Claverie *et al*. (2020) summarised knowledge on foliar symptom outbreak in the "toxins hypothesis" and "hydraulic dysfunction hypothesis". The first describes how phytotoxic compounds produced by "Esca disease"-associated fungi could diffuse through host transpiration stream sap flow to leaves, inducing the typical tiger-striped leaf patterns (Abou-Mansour *et al*., 2004; Bruno and Sparapano, 2006b; Andolfi *et al*., 2011). The second hypothesis explains how impairment of sap flow to leaves, mainly caused by vessel occlusions/pathogen compartmentalization, could lead to cavitation contributing to foliar symptom expression (Pouzoulet *et al*., 2014, 2017, 2019). Recent findings, however, suggest how these two hypotheses can be complementary and not exclusive, due to observed association between foliar symptoms, disruption of vessel integrity and presence of some "Esca disease"-associated pathogens presence in host trunks, which could elicit a distance-response (Bortolami *et al*., 2019). Stem vessel occlusion has been related to exacerbation of foliar symptom expression in the following growth season (Bortolami *et al*., 2021).

Because the two Ascomycota *Pch* and *Pmin* have frequently been associated with foliar symptoms of "Esca disease"-symptomatic plants, considerable research has been carried out on *Pch* and *Pmin* biology and pathogenicity, and a more comprehensive and integrated view on these species was presented by authors such as Valtaud *et al*. (2009), Mostert *et al*. (2006) and Gramaje *et al*. (2015). The same cannot be affirmed for *Fmed*. Despite progress made since taxonomic description of this fungus (Fischer, 2002), knowledge on the pathogen, its wood

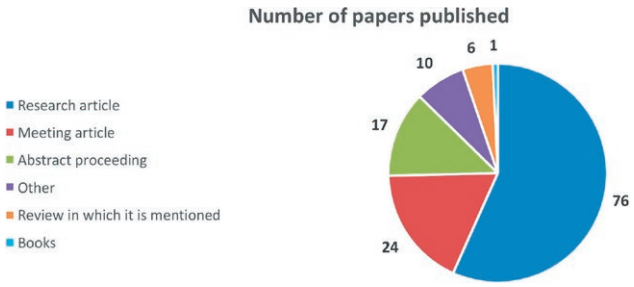

Figure 1. Number of published papers dealing with *Fomitiporia mediterranea* from 2002 (*Fmed* identification date) to 2021, as indexed in the Web of Science™ database (Thomson Reuters).

degradation process and relationship with the other Esca complex diseases, is fragmented. There are few reviews which consider *Fmed* as part of "Esca disease" or GTDs in general, and to our knowledge no comprehensive review on *Fmed* alone has been undertaken (Figure 1).

For this reason, along with contrasting reports of correlations between amounts of white rot necrotic tissues (thus *Fmed* presence) and the leaf stripe symptoms (Maher *et al*., 2012; Bruez *et al*., 2014; Bruez *et al*., 2020; Cholet *et al*., 2021; Pacetti *et al*., 2021), and reports of little or no correlation between these factors (Edwards *et al*., 2001; Calzarano and Di Marco 2007; Romanazzi *et al*., 2009; Mugnai *et al*., 2010), a review of *Fmed* is necessary, in order to collect knowledge of the fungus itself, and to stimulate scientific debate and novel ideas in the context of GTDs.

BASIDIOMYCETES ASSOCIATED WITH ESCA

Knowledge on basidiomycetes associated with "Esca disease" has increased, and Fischer (2006), Fischer and González-García (2015), and Cloete *et al*. (2015a, 2016) provided a comprehensive compendium on the topic. Ravaz (1909) was a pioneer in grapevine basidiomycete identification, with the putative identification of *Phellinus igniarius* (L.) Quél. (at the time *Fomes igniarius* (L.) Fr. and formerly *Polyporus igniarius* (L.) Fr. based on fruit bodies found on diseased grapevines in southern France). Vinet (1909) and Viala (1926) also reported the presence of *Stereum hirsutum* (Willd.) Pers. in French vineyards. These two basidiomycetes were long considered as causal agents of Esca wood decay but pathogenicity was only proven for *P. igniarius* (most likely a *Fomitiporia* sp.) by Chiarappa (1997).

Studies on "Esca disease" and its etiology multiplied in the late 1990s, especially when Larignon and Dubos (1997) isolated *Phellinus punctatus* (P. Karst.) Pilát from Esca wood decay in French vineyards. After studies of the infrageneric structure of *Phellinus* s.l. by Fiasson and Niemelä (1984), Fischer (1996) and Wagner and Fischer (2001, 2002), *P. punctatus* was grouped within *Fomitiporia*, as *F. punctata* (P. Karst) Murrill (= *P. punctatus*). Multiple surveys of Italian vineyards by Cortesi *et al*. (2000) concluded that the main cause of decay in Esca-affected vines was *F. punctata*, later recognized as the new species *F. mediterranea* (Fischer 2002), which is now considered the main white rot agent in "Esca disease" in Europe and Mediterranean regions.

Stereum hirsutum was isolated by Larignon and Dubos (1997) from central decayed grapevine wood inhabited by putative *P. igniarius*, but its role in GLSD is still debated, although it is clearly a white rot basidiomycete agent. Some authors have suggested that this fungus has little or no role in the Esca complex of disease because it is found only rarely in vineyards (Mugnai *et al*., 1999; Cortesi *et al*., 2000; Reisenzein *et al*., 2000; Vicent *et al*., 2001). In any case, *S. hirsutum* may act as a weak facultative parasite, occasionally penetrating the heartwood of host plants and producing very limited infections and decay of the inner host tissues (Fischer and González-García, 2015).

Many other basidiomycetes have been isolated from decaying grapevine wood. White *et al*. (2011) characterised ten possibly novel taxa belonging to *Hymenochaetales* associated with "Esca disease", *i.e.* white rot on GLSD symptomatic vines. For Europe, an annotated checklist of GTD-related basidiomycete taxa has been published (Fischer and González-García, 2015). With the advent of metagenomic approaches, reports of basidiomycetes are increasing (Del Frari *et al*., 2019a; Bruez *et al*., 2020). A recent study by Brown *et al*. (2020) aimed to clarify the relevance of basidiomycete colonisation within the Esca complex of diseases. They isolated many taxa (including new species, such as *Inonotus vitis* A.A. Brown, D.P. Lawr. & K. Baumgartner, *Tropicoporus texanus* A.A. Brown, D.P. Lawr. & K. Baumgartner, and *Fomitiporia ignea* A.A. Brown, D.P. Lawr. & K. Baumgartner) from white rot and black/brown discoloured wood collected from grapevine plants expressing GLSD foliar or shoot symptoms in Californian and Texan vineyards.

More *Fomitiporia* spp. have been associated with "Esca-diseased" grapevines in other regions, including *Fomitiporia australiensis* M. Fisch., Jacq. Edwards, Cunningt. and Pascoe in Australia (Fischer *et al*., 2005), *Fomitiporia polymorpha* M. Fisch. in California (Fischer and Binder, 2004), *Fomitiporia capensis* M. Fisch., M. Cloete, L. Mostert, F. Halleen in South Africa (Cloete *et al*., 2014), *F. ignea* (Brown *et al*., 2020) in Texas, and, more recently, *Fomitiporia punicata* Y.C. Dai, B.K. Cui & Decock in China (Ye *et al*., 2021), originally described

on *Punica granatum* (Dai *et al*., 2008). As well, *Fomitiporia erecta* A. David, Dequatre & Fiasson, and *F. punctata* were mentioned as occurring on grapevine in Spain (Fischer and González-García, 2015). Of these species, *F. australiensis*, *F. capensis* and *F. ignea* were exclusively documented from grapevine.

Geographic distribution and host range of *Fmed* have probably expanded in recent decades. This is supported by the number of host plants in the different regions. In Central Europe the host range is largely limited to *Vitis vinifera*, given the scarcity of reports of the fungus on other hosts: i.e. on *Laurus nobilis* (Fischer, 2006) and on *Robinia pseudoacacia* (Schmidt et. al., 2012). However, *Fmed* occurs on several other hosts in the Mediterranean area (see below). This discrepancy in the reported host range indicates a recent invasion of the fungus into the viticultural regions of Central Europe, possibly associated with climatic changes leading to increased temperatures in this region.

These observations indicate that intrinsic geographic and climate conditions play roles in diffusion of Basidiomycota pathogens and influence spread of *Fomitiporia* spp. in vineyards (Fischer *et al*., 2005; Fischer, 2006; Cloete *et al*., 2014). Geographical variations influence distribution of fungi among different locations (Hofman and Arnold, 2008; Dietzel *et al*., 2019), and climate is a major abiotic factor shaping fungal biogeography (Castillo and Plata, 2016; Větrovský *et al*., 2019). Spatial analyses of "Esca-disease"-related basidiomycete taxa, and comprehensive screening of possible non-*Vitis* host plants, preferably from proximity of vineyards, could help to identify key pedo-agroclimatic factors affecting their diffusion, and could indicate why some *Fomitiporia* spp. are retrieved from some areas but not others.

In Europe information on grapevine white rot agents began to be revised in 2002: Fischer found that strains formerly acknowledged as *F. punctata* collected from grapevines in Italy and Germany differed from strains from other hosts and other geographic areas (Central Europe). Molecular diagnoses (ITS data), pairing tests of single spore isolates, compared with temperature preferences of cultured mycelia allowed description of the new species *Fomitiporia mediterranea* M. Fischer (Fischer, 2002). This is currently considered the main causal agent of white rot in "Esca diseased" grapevines in Europe and in Mediterranean climate areas, while *F. punctata* is more ubiquitous, although a European centred distribution has been suggested by Decock *et al.* (2007).

Due to indistinguishable morphology between *Fmed* and *F. punctata*, but the highlighted phylogenetic differences, previous isolates and findings attributed to *F. punctata* should be reconsidered as possibly assignable to *Fmed* (Fischer, 2002; Ciccarone *et al*., 2004; Fischer, 2006). Recent findings on *P. punctatus* and *P. pseudopunctatus* A. David Dequatre and Fiasson by Polemis *et al.* (2019) and Markakis *et al.* (2019) have reinforced that possible misidentification led to the underestimation of *F. mediterranea* incidence in the Mediterranean region.

This review focuses only on *Fmed* and its role in Esca-related wood degradation, with careful reconsideration of previous reports where the pathogen may have been incorrectly identified.

IDENTIFICATION, TAXONOMY, HOST RANGE, AND SYMPTOMS INDUCED IN GRAPEVINES

Description of fruit bodies and mycelia

Morphology and anatomy of *Fomitiporia mediterranea* (*Hymenochaetaceae*, *Hymenochaetales*, *Agaricomycetes*, *Basidiomycota*) were described by Fischer (2002).

Fomitiporia mediterranea fruiting bodies (Figure 2) are resupinate, inseparable, and hard woody, up to 15 mm thick with yellowish-brown narrow margins, containing subglobose to oval basidiospores. The hyphal system is dimitic, with generative and skeletal hyphae. Detailed descriptions of the hyphal system, fruiting bodies and basidiospores of this fungus were provided by Fischer (2002, 2009) and Fischer and González-García (2015).

Figure 3, A, B and C show, respectively, a tube mouth with outgrowth of vegetative hyphae, outgrowing hyphae from naturally infected grapevine wood and the pore surface of a fruiting body of *F. mediterranea*.

After isolation from infected wood and/or fruit bodies, mycelial isolates may develop into a so-called "bleaching type" (Type B: Fischer, 1987) or a "staining type" (Type S: Fischer, 1987) (Figure 4).

Type B mycelium has a cottony to woolly appearance, and aerial hyphae are yellowish to brown. Medium pigmentation is sparse or absent. Type S-mycelium has sparse aerial hyphae, and medium pigmentation is strong. Colony growth after 14 days at 21°C on Malt Extract (ME) agar in complete darkness was more rapid in bleaching-type isolates (colony diameter = 3.0 to 4.5 cm) than staining-type isolates (1.5 to 2.5 cm). The two mycelium types may alternate over ensuing inoculations. Growth was confirmed between 15 and 35°C, with optimum growth at 30°C (Fischer, 2002, 2006; Fischer and Kassemeyer, 2003). Under laboratory conditions, sporulation is absent in *Fmed*. Spore germination tests with spores from actively sporulating fruit bodies were performed for some *Fmed* strains, indicating very low germination rates (less than 1%) with a high variation in germination times (Fischer, 2002).

Figure 2. Fruiting bodies of *Fomitiporia mediterranea* on a grapevine trunk. Photograph taken in July 2018, from 'Sauvignon Blanc', in a vineyard in Ehrenkirchen, Germany.

Spread by basidiospores is considered the main dispersal form for *Fmed* and this has been described to be mainly via rain and wind (Cortesi *et al*., 2000). In Central European vineyards, fruiting body sporulation is increased after rainy periods and is related to daily temperatures greater than 10°C and relative humidity greater than 80% (Fischer, 2009).

Mating system

Studies of the *Fmed* mating system were also affected by misidentification of the pathogen. Fischer

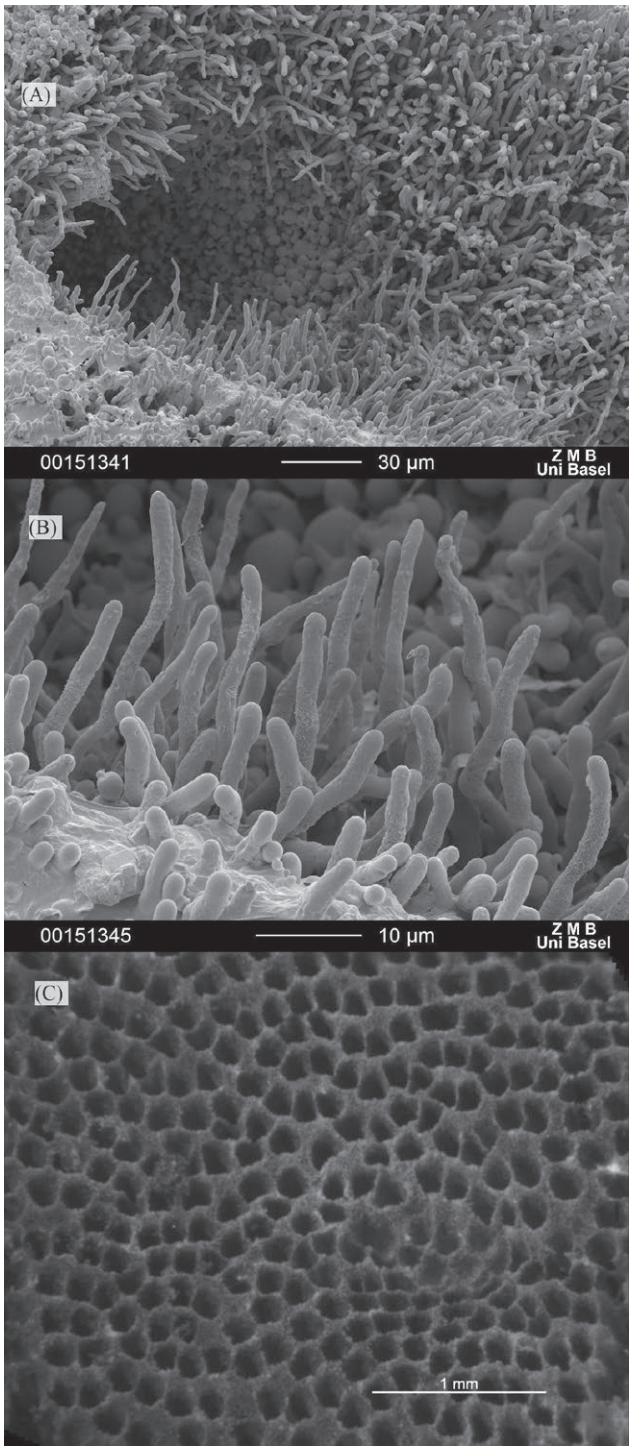

Figure 3. (**A**), Cryo-Scanning-Electron-Microscopy (Cryo-SEM) micrograph (×500 magnification) of an *Fmed* tube mouth showing outgrowth of vegetative hyphae. (**B**), Cryo-SEM micrograph (×2,000) of hyphae in a cross section of a grapevine trunk naturally affected by white rot. (**C**), Stereo micrograph of the surface of an *Fmed* fruiting body (×50) The pores are 5-8/mm. The diseased grapevine specimen was collected in a vineyard in Pfaffenweiler, south-west Germany.

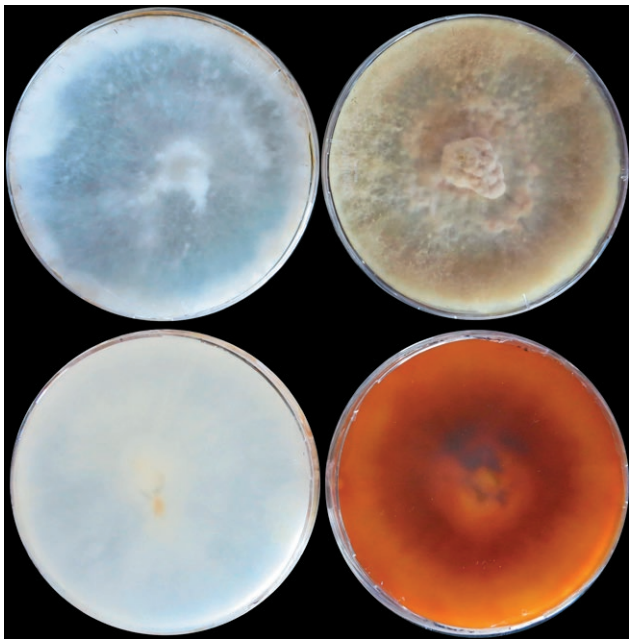

Figure 4. Mycelium cultures of *Fomitiporia mediterranea* on malt extract agar (ME) after 28 days of incubation. "Type B" (left, surface and reverse side) and "Type S" (right, surface and reverse side).

(1996) described *F. punctata* strains (later distinguished from *Fmed*) as homothallic, with no mating types evident in pairings of spores originating from one fruiting body. The time lag between separation of *Fmed* from *F. punctata* by Fischer (2002) and the research of Jamaux-Despreaux and Péros (2003) initially provided conflicting reports of the homothallic mating system described for the fungus. Jamaux-Despreaux and Péros (2003) observed outcrossing populations in France and Italy. This was indicated by high genetic variation within and between vineyards, and random assortment of genetic markers. They therefore suggested possible existence of non-outcrossing populations in other areas. Following correct assignment of species, it was shown that *Fmed* was a heterothallic bipolar species (Fischer, 2002), while *F. punctata* was confirmed as homothallic.

A variety of pairing tests were conducted by Fischer (2002), who demonstrated a range of compatible and incompatible reactions (see Fischer, 2002 for details). Growth of secondary mycelia was stronger in compatible inter-strain than in intra-strain pairings. Mycelia formed from inter-strain pairings could prevail under natural conditions, resulting in high outcrossing rates.

It is now accepted that basidiomycete mating is regulated by different genes, grouped in two types of homeodomain transcription factors (*HD genes*), in pheromones genes and their related receptors and response genes (*PR genes*). These genes can reside in linked or un-linked chromosome loci (James *et al*., 2006; Kües *et al*., 2013, 2015). James *et al*. (2013) highlighted at least four *HD* (two pairs of *HD1* and two pairs of *HD2*), and several apparently functional *PR genes* in the *Fmed* genome, such as 2 *STE3*, several *MAP* kinases and 3 *Prf1*. These authors also suggested that the genes were not linked in a unique mating locus (James *et al*., 2013; Kües *et al*., 2015).

Further pairing tests, perhaps with other strains, could better clarify the dispersal system of the fungus, and identify other possible intersterility groups. This would be further clarified if genetic variability data retrieved from German vineyards were considered. Fischer (2012) found 14 different *Fmed* genotypes out of 15 fruit bodies, all derived from one vineyard. Lentes and Fischer (personal communication) identified 56 genotypes out of 64 isolated mycelia, derived from different vines in two vineyards in the Moselle region of Germany.

Available *Fmed* genome could provide new information for expression analysis of mating-associated genes, especially in response to changing environmental conditions which are increasingly affecting European vineyards. Increasing this knowledge could provide a better understanding on pathogen spread and genetic recombination, since *Fomitiporia* spp. are well adapted to different conditions and climates, as reflected by their biogeographical variability.

Field identification

Compared to white rot presence, fruiting bodies of *Fmed* are very rarely found in vineyards, mostly on the uppermost parts of trunks, near pruning wounds, which are the main sites of infection (Cortesi *et al*., 2000; Fischer *et al*., 2005; Fischer, 2006; Fischer and González-García, 2015). In vineyards in Central Europe, *Fmed* fruiting bodies were only present on 1 to 3% of "Esca-diseased" grapevines that were older than 15 years (Fischer, 2009), and the pathogen was detected as vegetative mycelium in infected hosts. In Germany, a 100:1 ratio is mentioned by Fischer (2006) indicating a low co-occurrence of vegetative mycelium with white rot and fruiting bodies. This was also reported in Tuscan vineyards by Cortesi *et al*. (2000). Because compatibility groups were identified (Fischer, 2002), the occasional occurrence of fruiting bodies in vineyards may be partly explained by the possibly rare contact between sexually compatible basidiospores (Jamaux-Despreaux and Péros, 2003). Dead grapevine trunks are the most favourable substrates for development of fruiting bodies, and the dead trunks are usually removed from vineyards. Another hypothesis to explain the low ratio between white rot and fruiting bodies in non-Central European countries is that the disease

Table 1. Host range and geographic distribution of *Fomitiporia mediterranea* M. Fischer 2002. For each host (and each country), the table includes first reports which used classical isolations, and some of the significant subsequent reports/studies which used molecular classification and metagenomic approaches. References accompanied by "†" refer to studies which used former classifications (as *Phellinus punctatus* or *F. punctata*) which should be carefully reconsidered as representing *F. mediterranea*.

Host	Country	References
Vitis vinifera	Algeria	Berraf and Péros, 2005
	Austria	Fischer *et al*., 2006
	Czech Republic	Baranek *et al*., 2018
	France	†Larignon and Dubos, 1997; †Jamaux-Despreaux and Péros, 2003; Péros *et al*., 2008; Laveau *et al*., 2009; Kuntzmann *et al*., 2010; Ouadi *et al*., 2019; Bruez *et al*., 2020
	Germany	Fischer, 2002; Fischer and Kassemeyer, 2003; Fischer, 2006; Fischer, 2012; Fischer and González-García, 2015; Fischer, 2019
	Greece	†Rumbos and Rumbou, 2001
	Hungary	Rábai *et al*., 2008
	Iran	†Karimi *et al*., 2001; Farashiyani *et al*., 2012; Mohammadi *et al*., 2013; Rajaiyan *et al*. 2013; Amarloo *et al*.,2020; Mirabolfathy *et al*., 2021
	Italy	†Mugnai *et al*., 1999; †Cortesi *et al*., 2000; Ciccarone *et al*., 2004; Romanazzi *et al*., 2009; Quaglia *et al*., 2009; Del Frari *et al*., 2019a; Girometta *et al*., 2020; Pacetti *et al*., 2021
	Lebanon	Choueiri *et al*., 2014
	Portugal	Sofia *et al*., 2006
	Slovenia	Rusjan *et al*., 2017
	Spain	†Armengol *et al*., 2001; Martin and Cobos, 2007; Sánchez-Torres *et al*., 2008; Luque *et al*., 2009; Garcia Benavides *et al*., 2013; Elena *et al*., 2018
	Switzerland	Fischer, 2006
	Turkey	Akgül *et al*., 2015
Acer negundo	Italy	Fischer, 2002
Actinidia spp.	Greece	†Elena and Paplomatas, 2002
	Italy	Fischer, 2002; Di Marco *et al*., 2004a; Di Marco and Osti, 2008; Girometta *et al*., 2020
Albizia julibrissin	Greece	Markakis *et al*., 2017
Cistus sp.	Italy	Girometta *et al*., 2020
Citrus spp.	Greece	Elena *et al*., 2006
	Italy	Roccotelli *et al*., 2014
Corylus avellana	Italy	Pilotti *et al*., 2010; Girometta *et al*., 2020
Elaeagnus angustifolia	Iran	Ahmadyusefi and Mohammadi, 2019
Fagus sylvatica	Italy	Girometta *et al*., 2020
Fortunella japonica	Greece	Markakis *et al*., 2017
Hedera helix	Italy	Girometta *et al*., 2020
Lagerstroemia indica	Italy	Fischer, 2002
Laurus nobilis	Slovenia	Fischer, 2006
Ligustrum vulgare	Italy	Fischer, 2006
Olea europaea	Greece	†Paplomatas *et al*., 2006; Markakis *et al*., 2017; Markakis *et al*., 2019
	Italy	Fischer, 2002 ; Carlucci *et al*., 2013
Platanus x acerifolia	Italy	Pilotti *et al*., 2005
Prunus dulcis	Spain	Olmo *et al*., 2017
Punica granatum	Greece	Markakis *et al*., 2017
Pyrus communis	Greece	Markakis *et al*., 2017
Quercus ilex	Italy	Fischer, 2006
Quercus robur	Italy	Girometta *et al*., 2020
Quercus rubra	Italy	Girometta *et al*., 2020
Robinia pseudoacacia	Italy	Fischer, 2006; Girometta *et al*., 2020
	Germany	Schmidt *et al*., 2012
Salix alba	Italy	Girometta *et al*., 2020
Ulmus spp.	Iran	Mirsoleymani and Mostowfizadeh Ghalamfarsa, 2019

and fungus structures could occur in several alternative hosts within or near vineyards (see Table 1). These sources of inoculum could be important. The discrepancies between occurrence of vegetative mycelia and fruit bodies are often large in lignicolous fungi. While the existence of *F. mediterranea* fruiting bodies may be underestimated (Fischer 2006), precise evaluation of exogenous inoculum sources remains a challenging issue.

Field identification of *Fomitiporia* spp. on grapevines is often complex. Because fruiting bodies are very rare and have very similar morphologies, molecular diagnoses after isolations from infected wood tissues or fruiting bodies are likely to be the most reliable tools for identification of mycelia not clearly assignable to particular species (Ciccarone *et al*., 2004).

Overview of molecular diagnosis, taxonomy and phylogeny

Amplifications and sequencing of ITS regions with or without Large Sub-Unit (LSU) translation elongation factor subunit 1-α (*tef1*) and the second largest subunit of RNA polymerase II (*RPB2*) sequence analysis, have permitted new advances in classification of grapevine basidiomycetes using specific primers. Reports are increasing, differentiating new *Fomitiporia* species (Cloete *et al*., 2016; Chen and Cui, 2017; Brown *et al*., 2020; Chen *et al*., 2021; Ye *et al*., 2021).

Fischer (2002) reported a specific method for identification, based on the nuclear encoded ribosomal DNA region ITS1-5.8S-ITS2 using the primer pair prITS5 and prITS4 (White *et al*., 1990). Compared to other *Fomitiporia* spp., *Fmed* strains showed unique small insertions in both ITS regions: between nucleotides 201 and 206 (AATAAT) in ITS1 and between nucleotides 748 and 745 (CCTTTGA) in ITS2 (Fischer, 2002; 2006; Fischer and Binder, 2004). Since 2006, specific primers based on these insertions have been available for differentiation of *Fmed* from other species such as *F. punctata* and *F. australiensis* (Fischer, 2006). The primer sequences and characteristics are as follows: pr*Fmed*1, 5′ GCA GTA GTA ATA ATA ACA ATC 3′ (GC = 28.6%, TM = 50.1°C); and pr*Fmed*2, 5′ GGT CAA AGG AGT CAA ATG GT 3′ (GC = 45%, TM= 55.3°C). A 550 bp product is only obtained for *Fmed*. Parameters for successful amplification were described by Fischer (2006).

With basidiospores being the main dissemination agent of *Fmed*, considerable genetic variation in *F. punctata* (probably *Fmed*) has been described by Random Amplified Polymorphic DNA (RAPD) markers. This variation has been shown among isolates derived from individual vineyards (Pollastro *et al*., 2000; Jamaux-Despreaux and Péros, 2003). Pollastro *et al*. (2001) successfully developed sequence-characterised amplified region (SCAR) primers suitable as a molecular diagnostic tool for *Fmed*.

The primer pair ITS1 and ITS4 (White *et al*., 1990) have also been successfully used for identification of *Fmed* isolates within an Italian mycological collection based on fresh mycelial isolates (Girometta *et al*., 2020).

Other *Fomitiporia* species recorded from grapevine include: *F. polymorpha*, *F. capensis*, *F. australiensis*, *F. ignea*, *F. erecta*, *F. punctata*, and *F. punicata*. For *F. polymorpha, F. australiensis*, *F. erecta* and *F. punctata*, characterization of the ITS1-5.8S-ITS2 region was sufficient either to describe them as separate species, or to establish phylogenetic relationships with other *Fomitiporia* spp. (Fischer and Binder, 2004; Fischer *et al*., 2005; Fischer and González-Garcia, 2015). Implication of other conserved genetic regions has distinguished these other species above metioned, using the LSU unit ribosomal RNA-enconding regions *tef1* and *RPB2* together with ITS data to describe them (Cloete *et al*., 2014; Brown *et al*., 2020; Ye *et al*., 2021). Nevertheless, species-specific primers are available only for *Fmed* (Fischer, 2006), although unique forward primers paired with ITS4 primers have been designed to successfully detect *F. capensis* (Bester *et al*., 2015).

Host range and geographical distribution

Fomitiporia mediterranea is considered to be a highly adaptable species, based on the diversity of host plants, and occurrence in different regions and climates.

Isolates from grapevine were retrieved from a range of climate conditions, according to the most updated version of the original Köppen-Geiger climate classification map (Geiger, 1961; Beck *et al*., 2018): from "Mediterranean and temperate humid-subtropical climates" (for most of non-Central European isolates of the pathogen), to "arid and semi-arid climate" (for Algerian, Iranian and some of the non-Central European isolates), to "cool temperate climate" (for most of the Central-European isolates)" (see Figure 5 for the detailed geographical distribution of *Fmed*). Throughout its geographical range, *Fmed* shows close affinity with *V. vinifera*. However, this may be due to the economic significance of grapevine in these regions, resulting in detailed field observations for vineyards compared with other woody hosts. The fungus is also found on other host plants outside non-Central European countries, potentially resulting in increased infection pressure on grapevines (see Table 1 for a detailed list of hosts in different countries). Fischer (2002, 2006) postulated that, at least for non-Central European countries, alternative hosts could be foci for development of *Fmed* fruiting bodies, reinforcing the observed high adaptability of the pathogen to mul-

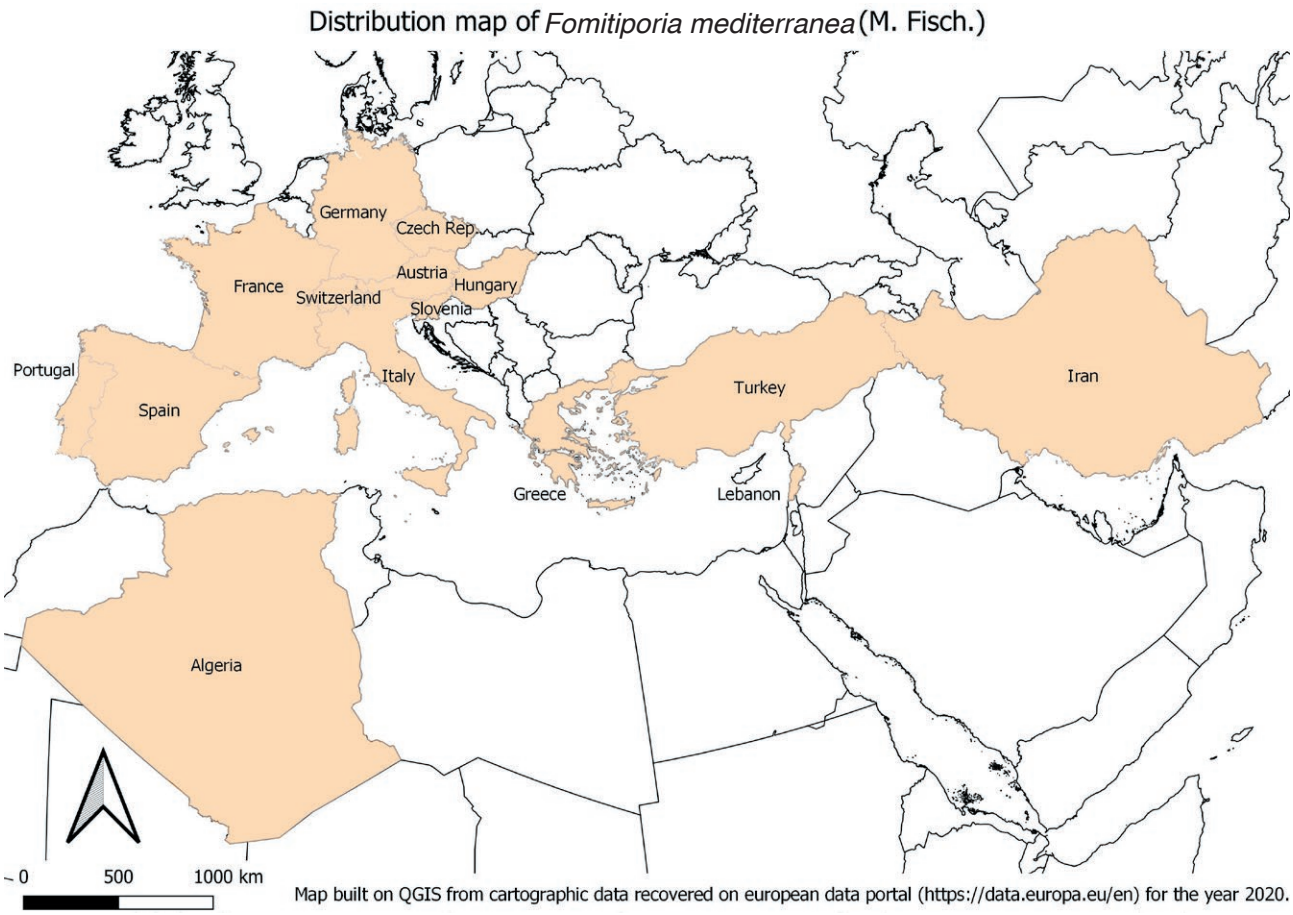

Figure 5. Distribution of *Fomitiporia mediterranea*, based on published reports of isolation of the fungus from grapevine, *Vitis vinifera*. The map was constructed using QGIS software (version 3.10.9-A Coruña).

tiple host species. Although current data show only very few isolations of *Fmed* from Central-European non-*Vitis* hosts, i.e. *Robinia pseudoacacia* in Germany (Schmidt *et al*., 2012) and *Laurus nobilis* in Slovenia (Fischer, 2006), the potential for the fungus to colonize other hosts is demonstrated. Future studies could focus on: *i*) surveys of fruiting body incidence on alternative hosts in the proximity of vineyards (to better assess inoculum sources); *ii*) performing comparative secretome and metabolome assessment of wood from different hosts to increase knowledge on *Fmed* colonization and growth and fruiting body development; and *iii*) determine pathogenicity to grapevine of isolates from different host plants.

Spread in grapevines and vineyards, wood symptoms and their relationships with foliar symptoms

Fomitiporia mediterranea is mainly retrieved from grapevine white rot necrotic tissue, although its presence in necrosis borders between white rot and non-necrotic tissues, and adjacent non-necrotic but recently colonised wood has been demonstrated (Fischer, 2002; Péros *et al*., 2008; Surico, 2009; Bruez *et al*., 2017; Elena *et al*., 2018; Bruez *et al*., 2021; Pacetti *et al*., 2021). The pathogen needs some time to colonise and decay woody tissues, explaining why it is predominantly found in trunks greater than 10 years old, and only to a lesser extent in young trunks (Sánchez-Torres et al., 2008; Fischer, 2009). White rot has also been mostly reported in old vineyards although it can be sometimes found in young vines and very occasionally in young GLSD symptomatic vines (Edwards *et al*., 2001; Mugnai *et al*., 2010).

In the Esca complex of diseases, several diseases have been recognised and have been related to infections by different fungi, and symptom expression can be influenced by many agronomic and environmental factors (cultural practices, host plant age, soil type, weather conditions) (Calzarano *et al*., 2018a; Gramaje *et*

al., 2018; Lecomte *et al*., 2018). Distribution of symptomatic vines within a vineyard poorly indicates the dissemination mode of related fungi, including *Fmed*, but diseased vines can be found grouped along vineyard rows. This supports the hypothesis that human-mediated practices are involved in pathogen spread (Mugnai *et al*., 1999; Guérin-Dubrana *et al*., 2019). Research on *F. punctata* (likely *Fmed*) isolates obtained from different vines showed they belonged to different somatic incompatibility types (Cortesi *et al*., 2000), indicating that each vine was colonised by genetically distinct individuals. Similarly, results for *F. punctata* (likely *Fmed*) (Jamaux-Despreaux and Péros, 2003) on the genotypic differences at vineyard level strongly indicated outcrossing reproduction via basidiospores. These results are not consistent with the hypothesis that *Fmed* is spread through wounds by pruning tools. In addition to this genetic evidence, much epidemiological data has shown that "Esca disease" symptoms were spatially random in vineyards (Cortesi *et al*., 2000; Surico *et al*., 2000; Sofia *et al*., 2006, Li *et al*., 2017), which is consistent with the hypothesis that basidiospores are the likely agents of dispersal for *Fmed* (Cortesi *et al*., 2000; Fischer, 2002). Although sporulation is rare on grapevine trunks, the inoculum could come from the many other hosts of the pathogen.

Typically, *Fmed* induces white rot in innermost grapevine wood (Figure 6). The decay is most often observed in arms, stem heads and trunks, with colonisation starting from pruning wounds and extending along entire trunks, mostly in the central parts but occasionally also at trunk bases, with desuckering wounds as entrance points (Larignon and Dubos 1997; Mugnai *et al*., 1999, 2010; Sparapano *et al*., 2000a, 2001a; Fischer, 2002; Fischer and Kassemeyer, 2003).

Rot diameters vary and decrease from infection origin points to boundaries with healthy wood (Mugnai *et al*., 2010). Rootstocks are rarely affected because vines die before white rot reaches rootstock tissues, although white rot has been reported in rootstock tissues (Maher *et al*., 2012; Elena *et al*., 2018). However, *Fmed*-related white rot is common in rootstock mother plants (Fischer, 2019).

The main symptom induced by *Fmed* on grapevines is spongy yellowish or bleached decay in wood tissues, but the relationship between wood and external foliar symptoms of "Esca diseased" grapevines is debated. To the best of our knowledge, Lafon (in 1921) was the first author to make this association. He assumed that *P. ignarius* (most likely a *Fomitiporia* sp.) present in the decay was the main agent responsible for the apoplectic form of "Esca disease" and leaf dessication (due to sap flow impairment). However, information on *Fmed*,

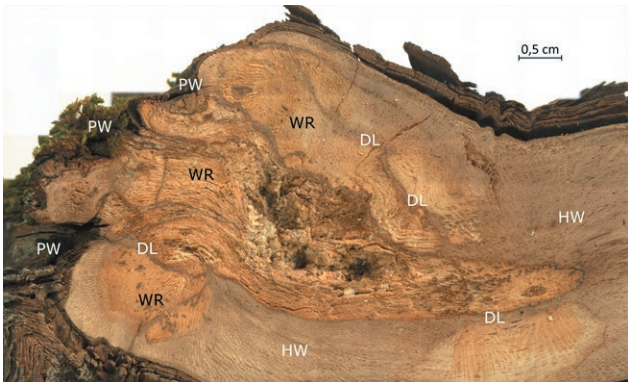

Figure 6. Macroscopic observation by stereo microscopy (×12 magnification) of a cross section (1 cm thick) of the head of a grapevine stem with a large area of white rot (WR) spreading from pruning wounds (PW) into healthy wood (HW). The necrotic zones are separated from the healthy wood by demarcation lines (DL). Specimen collected in May 2021 from a 28-year-old 'Sauvignon Blanc' plant, from a vineyard in Pfaffenweiler, south-west Germany.

grapevine wood symptoms and foliar symptoms needs to be re-examined. Demonstration of *Fmed* to act as a primary grapevine pathogen – after artificial inoculations by mycelial plugs or toothpicks (Sparapano *et al*., 2000a, 2001a) – confirms the importance of this pathogen within the Esca disease complex. However, research on the actual colonization sequence of "Esca disease"-associated fungi in field-grown grapevines is still required to increase knowledge of relationships between wood decay and foliar symptoms. Under field conditions, basidiospores can be infection agents, and artificial basidiospore inoculations in greenhouses would help to determine the colonization ability of *Fmed* and its role in foliar symptoms. However, basidiospores are difficult to obtain, and rarely germinate under laboratory conditions (Fischer, 2002). Therefore reviewing studies from last two decades will provide insights on the impacts of *Fmed* on "Esca disease" foliar symptoms (GLSD).

Different types of necrosis have been described in "Esca diseased" wood (Larignon and Dubos, 1997). White rot necrosis clearly shows the presence of *Fmed*, which has been the main isolation source of this pathogen in Europe (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Bruez *et al*., 2017). Through logistic regression analyses in an old 'Cabernet Sauvignon' vineyard, Maher *et al*. (2012) analysed data from presumably 20 to 25 year-old vineyards, and assumed white rot to be the tissue type most strictly associated with the leaf stripe symptoms. A relationship between *Fmed* and "Esca disease" foliar symptoms (GLSD) was shown if white rot presence overcame a 10% necrosis threshold, in vine grafts and/or cordons. Using a similar approach, Calzarano and Di Marco (2007)

showed that, in 32- and 36-year-old vineyards, there was no relationship between severities of wood deterioration and external foliar symptoms. The leaf-symptomatic vines percentage with discoloration (but no white rot) was 46.2% in 32-year-old vineyards and 7.2% in 36-year-old vineyards. Both of these findings have been supported by other authors. Bruez *et al*. (2014) found white rot tissue in cordons from 79% of GLSD-symptomatic 10-year-old vines. Fischer (2012) found 100% of 366 trunks of 28-year-old 'Traminer' vines to be affected by white rot, but less than 10% had foliar symptoms. Fischer (2019) also reported a possible correlation between the presence of *Fmed* (but also other GTD related fungi) and leaf symptoms in rootstock mother plants. Ouadi *et al*. (2019) observed white rot on 15 to 50% of the total necrotic area of trunks and cordons in 16-year-old 'Cabernet Sauvignon' grapevines expressing foliar symptoms. Edwards *et al*. (2010), after fully dissecting trunks of ten GLSD symptomatic vines (aged 4 to 7 years) detected white rot in only one vine. Mugnai *et al*. (2010) dissected nine symptomatic 5- and 6-years-old vines. They found that four of the vines had no white rot and five had traces of decayed wood, mainly following infections from desuckering wounds (Mugnai *et al*., 2010). These results confirm that white rot, and *Fmed* as the main white rot agent in Europe, becomes increasingly present as vines age, thus becoming increasingly associated with leaf symptoms. The pathogen may play a fundamental role in activating mechanisms leading to the onset of leaf stripe symptoms. However, observations of foliar symptoms not linked to white rot suggest that even if *Fmed* plays a very important role, other factors are also likely to be involved.

In 10-year-old 'Cabernet Sauvignon' cordons expressing foliar symptoms, Bruez *et al*. (2020) using meta-barcoding, proposed the link between the onset of GLSD with white rot and a combination of *Fmed*, *Pch* as well as *Sphingomonas* spp. and *Mycobacterium* spp. They suggested that microbiota interactions in white rot necrotic tissues could induce production of phytotoxic secondary metabolites, or increase some shared metabolic pathway, thereby inducing typical GLSD foliar symptoms. *In vitro* production of fungal secondary metabolites by co-culture with bacteria has been documented (Haidar *et al*., 2016), but occurrence in natural conditions is yet to be assessed.

Cholet *et al*. (2021) and Pacetti *et al*. (2021) have also shown the correlation of white rot with foliar stripe symptoms. Decay elimination (curettage, see below for details) drastically reduced GLSD symptoms during the years following curettage treatment, in 24-year-old 'Sauvignon Blanc' and in 14-year-old 'Cabernet Sauvignon' plants. Another interesting correlation between white rot and foliar symptoms came from the curative side: recently, Bruez *et al*. (2021) shown how after sodium arsenite treatment (see below for details), 25-year-old 'Gewürztraminer', 27-year-old 'Chardonnay' and 40-year-old 'Merlot' plants did not shown any foliar symptoms.

In conclusion, the relationship between outbreak of foliar symptoms and white rot in the Esca complex of diseases is widely supported and linked to *Fmed* in Europe, although this is not exclusive as the fungus is absent in young symptomatic vines. Nevertheless, clarifying the mechanisms involved in this relationship will be a big step towards full understanding of the processes leading to the characteristic symptoms and symptom expression timing of GLSD (the symptom fluctuations is not found in other diseases of perennial hosts). Several hypotheses have been proposed for etiology of leaf stripe symptoms, but to understand the role of *Fmed* in development of the disease, an important and essential first step is to detect the signals reaching the leaves and causing the outbreak of these symptoms. In the case of *Fmed* we suggest the following etiology: *i*) joint action of extracellular enzymes and toxins (from *Fmed* or the entire white rot microflora); *ii*) vascular system destruction caused by lignocellulolytic enzymes; *iii*) the formation of low molecular weight diffusible compounds from *Fmed* or from wood degradation-host infection reactions; and *iv*) a combination of these situations and the wood cellular microenvironment.

GENOMIC INFORMATION

Many fungal *Agaricomycetes* genomes have been sequenced by the Joint Genome Institute (JGI) (http://jgi.doe.gov/fungi) (Grigoriev IV *et al*., 2011). According to these data, 93% of the *Agaricomycotina* sequenced genomes are from *Agaricomycetes*. This could be related to the roles of these fungi in trees decay and to their potential applications in biotechnology (Lundel *et al*., 2014; Hyde *et al*., 2019). In a comparative genomic study, Floudas *et al*. (2012) sequenced the *Fmed* genome. The final draft assembly was obtained by *in silico* combination of Roche (454), Sanger Fosmids, and Illumina data. Information on genome annotation statistics and composition are available at the MycoCosm portal (https://mycocosm.jgi.doe.gov) (Grigoriev IV *et al*., 2014) and in Floudas *et al*. (2012).

Fmed genome size is approx. 63.35 Mbp which accounts for 11,333 predicted complete gene models with start and stop codons. The genome shows a conspicuous repetitive sequence component, mostly represented by microsatellites and Transposable Elements (TEs). It is

generally accepted that repetitive sequences play roles in *Basidiomycota* genome rearrangements and gene mutations, interrupting genome linearity between strains, and producing strain polymorphisms (Castanera *et al*., 2017). This could at least partially explain the level of polymorphism detected between strains of *Fmed* (Pollastro *et al*., 2001). Specifically, 4157 microsatellites were detected, most of them (52.27%) being dinucleotides, followed by tri-, mono-, penta-, hexa- and tetra-nucleotide microsatellites. TE analysis revealed a high proportion of TEs coverage (41.42%), representing the greatest for the sequenced genomes in the study. The biggest portion of TEs was LTR-Gypsy elements (21.33%), followed by LTR-Copia, TIR, DNA-transposons and Helitrons. A consistent number of non-classified TEs were reported (Floudas *et al*., 2012).

Besides constitutive analysis of the genome, Floudas *et al*. (2012) conducted a comparative study with 30 fungal genomes (presenting different ecological strategies), to establish the origin of ligninolytic activity. Through molecular clock analysis of genes encoding Class II Peroxidases (PODs: responsible for lignin degradation), it was possible to date the appearance of ligninolytic activity and the *Agaricomycetes* ancestor (a white rot agent, most likely). The activity probably appeared approx. 290 Ma ago (between the end of Carboniferous and Permian period, Paleozoic era). Subsequently, by Class II PODs-encoding gene expansion through the lineage, five orders of basidiomycetes differentiated, including the *Hymenochaetales* (most likely approx. 237 Ma, during the Triassic period, Mesozoic era). *Fmed* was estimated to have up to 17 Class II PODs-encoding genes in its genome, and these genes can possibly be found clustered with cellobiose dehydrogenase (CDH) encoding genes and other unclassified genes.

Other gene copy numbers expanded in the lineage during *Fmed* genome evolution, such as genes encoding for glycoside hydrolases (GH) families, Fe (III)-reducing glycopeptides (GLP), dye-decolourizing peroxidases (DyP) and laccases (Lac) (Floudas *et al*., 2012). A detailed report on carbohydrate active enzymes (Cazymes) and class II PODs is presented below.

PATHOGENICITY

In vivo white rot basidiomycete pathogenicity studies on grapevine have been rarely documented (Chiarappa, 1997; Larignon and Dubos, 1997; Sparapano *et al*., 2000a, 2000b, 2001a; Gatica *et al*., 2004; Laveau *et al*., 2009; Luque *et al*., 2009; Diaz *et al*., 2013; Akgül *et al*., 2015; Cloete *et al*., 2015b; Amarloo *et al*., 2020; Brown *et al*., 2020), but contrasting results to fulfil Koch's postulates were obtained, and the role of basidiomycetes as causes of grapevine foliar symptoms (GLSD) is not clear. Experiments have been conducted either on young or old grapevine plants, but very few of these studies used *Fmed* as inoculum (Larignon and Dubos, 1997; Sparapano *et al*., 2000a, 2000b, 2001a; Laveau *et al*., 2009; Luque *et al*., 2009; Akgül *et al*., 2015; Amarloo *et al*., 2020).

The first pathogenicity study on *F. punctatus* (probably *Fmed*) was conducted in France by Larignon and Dubos (1997) to determine the pathogen's role in wood decay. After fungus inoculation of healthy 'Cabernet Sauvignon' wooden blocks or rooted canes, they observed formation of typical white rot only in wooden blocks. As expected the fungus only colonised old wood.

Sparapano *et al*. (2000a), inoculating old vines and assessing wound-induced wood discolouration and white rot symptoms, found that inoculation with *F. punctata* (probably *Fmed*) produced the symptoms with different timing dependant on the cultivar, host plant age and portion inoculated. Specifically white rot formation tooks: *i)* approx. 6 months after inoculation for symptoms to occur in trunks, branches and spurs of 6-year-old 'Italia', and 9-year-old 'Matilde' plants; *ii*) 2 years after inoculation of 13-year-old 'Sangiovese' vines spurs and branches; or *iii*) 2 years after inoculation in 2-year-old rootstock Kober 5BB grafted with 'Italia'. No symptoms on leaves were induced. While *F. punctata* re-isolation was successful, no other wood degrading fungi were re-isolated. Sparapano *et al*. (2000a) concluded that *F. punctata* (probably *Fmed*) could act as a primary pathogen, and was able to colonize the grapevine woody tissues without other previous fungal infections when inoculated through wounds.

To gain details on the role of each fungus in "Esca disease", Sparapano *et al*. (2000b, 2001a) studied fungus-to-fungus and fungus-to-plant interactions, both *in vitro* and *in planta* co-inoculations. Sparapano *et al*. (2000b) showed *in vitro* competitive interaction of *F. punctata* (probably *Fmed*) with *P. chlamydospora* and antagonism between *P. aleophilum* (= *P. minimum*) and *F. punctata*. They also observed that each fungus could act as a primary pathogen by *in planta* inoculations. Moreover, the effect of *F. punctata* (probably *Fmed*) on the woody tissue of 'Italia' and 'Matilde' grapevines was limited by *P. aleophilum (= P. minimum)* but not by *P. chlamydospora*. Only *F. punctata* (probably *Fmed*) alone was able to induce white rot. This fungus was re-isolated, but no foliar symptoms were observed in the co-inoculation experiments. Besides confirming the fungus-to-fungus competitive and antagonistic interactions with *Pch* and *P. aleophilum* (= *P. minimum*), Sparapano *et al*. (2001a)

found that *F. punctata* causes wood discolouration followed by limited and localised white rot lesions within 3 years after single-inoculations or all possible co-inoculations, in 5-year-old 'Italia' and 9-year-old 'Matilde' vines, when they were inoculated in the spurs, spreading slightly more rapidly when trunks of plants were inoculated. Fungus re-isolation was always successful, and few foliar symptoms (even though not fully corresponding to the typical tiger stripe pattern) were observed after 2 to 3 years from inoculation in all the inoculation combinations. Non-inoculated plants (experimental controls) did not develop foliar symptoms.

The most recent study of *Fmed* pathogenicity was conducted by Amerloo *et al*. (2020). They inoculated *Fmed* mycelium on 2-year-old rooted grapevine 'Kolahdari' cutting under controlled greenhouse conditions, obtaining wood discolourations (but not white rot) 10 months after inoculation, confirming that white rot formed only on old wood. The proportion of *Fmed* re-isolation was approx. 60%, and no foliar symptoms were recorded. These results were in agreement with findings in rooted cuttings of 'Cabernet Sauvignon' (Laveau *et al*., 2009), 1-year-old 'Macabeo' and 'Tempranillo' plants grafted onto Richter 110 rootstock (Luque *et al*., 2009), and 1-year-old rooted plants of 'Sultana Seedless' (Akgül *et al*., 2015).

Data from pathogenicity tests of *Fmed* and grapevine are still too scarce for postulation of general concepts, especially considering that multiple factors could play a role in wood symptoms appearance (i.e. grapevine cultivar, age, trunk portion). However, the experimental evidence on ability of the pathogen to primarily colonize grapevine wood, and on relationships between white rot presence/amount and external foliar symptoms, require further investigation, especially considering contrasting results obtained from artificial inoculations versus the ones obtained from curative experiments (see above).

Host specificity should also be considered. *Fmed* isolates from different hosts have been used for pathogenicity tests on citrus trees (Elena *et al*., 2006). According to the extent of wood discolouration in citrus trees after inoculation with *Fmed* isolates from *Citrus*, *Vitis, or Actinidia*, a degree of host specificity for *Citrus* spp. was suggested. Other cross-pathogenicity tests conducted by Markakis *et al*. (2017) shown a certain degree of host-specificity in *Fmed*: grapevine-isolates inoculated in wood of pomegranate tree and kumquat tree shown shorter wood discoloration (and no fungal re-isolation) than in pathogenicity test with isolates from the same trees.

Study of host specificity for different *Fmed* isolates could elucidate dissemination modes for *Fmed*.

As indicated above, most wood and/or foliar vine symptoms could be caused by enzymes, toxins and/or other metabolites secreted by the pathogens individually or in combinations spreading through vines from the colonised wood, together with products of host defence reactions (Sparapano *et al*., 1998; Graniti *et al*., 1999; Mugnai *et al*., 1999; Amalfitano *et al*., 2000, 2011; Sparapano *et al*., 2000a, 2000b; Bruno and Sparapano, 2006b; Claverie *et al*., 2020). Recently, wood degradation in grapevine diseases was critically reviewed in comparison with other tree species by Schilling *et al*. (2021), reinforcing our observation that studying enzymatic and non-enzymatic fungal degradation, together with host defence related compounds, could be the key to understanding fungal adaptation to grapevine, and provide insights into wood and foliar symptoms.

Enzymes

White rot in wood is the result of lignin, cellulose, and hemicellulose degradation (either simultaneously or preferentially) by extracellular enzyme activity (Blanchette, 1991). These enzymes include: *i*) carbohydrate-active enzymes (CAZymes), such as endoglucanases (EC 3.2.14), cellobiohydrolases (EC 3.2.1.91, classified in the Glycoside Hydrolase family, GH), β-glucosidases (EC 3.2.1.21) and cellobiose dehydrogenase, CDH (EC 1.1.99.18); *ii*) laccases (EC 1.10.3.2; p-diphenol:di-oxygen oxidoreductases); and *iii*) Class II peroxidases (PODs), such as manganese peroxidases (MnP; EC 1.11.1.13), lignin peroxidases (LiP; EC1.11.1.14) and the versatile peroxidases (VPs, EC 1.11.1.16) (Daniel, 2014). Auxiliary activities (AA) redox enzymes are also considered to be present in white rot agents: eight families of ligninolytic enzymes and two of lytic polysaccharide mono-oxygenases (LPMOs) are associated with CAZymes and Class II PODs, since they may contribute jointly to degradation of polysaccharides (Levasseur *et al*., 2013; Daniel, 2014). Carbohydrate-Binding Modules (CBMs) are non-catalytic modules which were also found to be associated with CAZymes, contributing to polysaccharide degradation activity (Boraston *et al*., 2004). All these enzymes are currently collected for each fungus in the Carbohydrate Active Enzymes database (CAZy database, https://www.cazy.org), including its update for AA (http://www.cazy.org/Auxiliary-Activities.html) (Levasseur *et al*., 2013; Lombard *et al*., 2014).

Enzymes included in the pool of fungal secreted proteins, the secretome, can be involved in *Fmed* pathogenicity. *Fomitiporia mediterranea* and *F. punctata* (probably *Fmed*) secrete ligninolytic enzymes (such as laccases and peroxidases), and cellulolytic enzymes (such as endo-

1,4-β-glucanases and β-glucosidases), for which *in vitro* activities in *Fmed* cultures have been assessed (Mugnai *et al*., 1999; Bruno and Sparapano 2006a). Laccases are known for their oxidase activity on a large set of phenolic compounds, and on non-phenolic compounds in the presence of mediators (Pérez *et al*., 2002). However, the role of laccases in plant-pathogen interactions is still discussed. Their importance in pathogenicity has been suggested for some fungal species, such as the chestnut blight pathogen *Cryphonectria parasitica* (Murrill) M.E. Barr, through tannin detoxification and involvement in several other metabolic pathways, such as fungal morphogenesis and pathogenesis (Singh Arora and Kumar Sharma, 2010). For *Fmed*, Abou-Mansour *et al*. (2009) purified a typical fungal 60kDa laccase from some isolates. This enzyme oxidizes many natural polyphenolic compounds. Complete lignin degradation was not achieved alone, however, but only with the contributions from ligninolytic class II PODs. Three manganese peroxidase genes supplementing laccase activity were characterized in the *Fmed* genome, as *Fmmnp1, Fmmnp2* and *Fmmnp3* (Morgenstern *et al*., 2010). Cloete *et al*. (2015b) highlighted the LiP activity of *Fmed in vitro*. It therefore appears that *Fmed* produces a complete white rot-type enzymatic pool, capable of oxidizing and mineralizing lignin and polysaccharides. In addition, comparative genomic studies supported laboratory data and highlighted a rich enzymatic pool for *Fmed*. Floudas *et al*. (2012) and Riley *et al*. (2014) showed that *CDH* gene copies, several *GH* gene families, *LPMOs* and *CBM* family 1 (*CBM1*) genes were detected for the CAZymes pool. Other AAs were identified for lignin degradation pathways, including multicopper oxidases (MCO), copper radical oxidases (CRO), benzoquinone reductase, iron permease (FTR), and ferroxidase (Fet3) (Floudas *et al*., 2012). The genes encoding for the latter five proteins have been described as genes involved in the non-enzymatic wood degradation caused by some brown rot pathogens (Sista Kameshwar and Qin, 2020). This could support the hypothesis that a similar non-enzymatic iron-dependent system (as described by Goodell *et al*., 1997, for brown rot, and by Osti and Di Marco, 2010, for the *Pch* and *Pmin* soft rots) could also be part of the *Fmed* white rot process (Moretti *et al*., 2019). Low Molecular Weight Compounds (LMWC) Fe^{3+} reductants could also be involved in generating OH radicals through a mediated Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$), as suggested in the Chelator Mediated Fenton (CMF) model proposed by Goodell *et al*. (1997). Three studies support this hypothesis, including: *i*) the *Fmed* draft genome revealed a homologous *SidA* gene responsible for inducing siderophore biosynthesis in *Ustilago maydis* (DC.) Corda (Mei *et al*., 1993; Floudas *et al*., 2012; Canessa and Larrondo, 2013); *ii*) *F. punctata* (probably *Fmed*) produces LMW metabolites *in vitro*, some of which have iron-chelating ability (Sparapano *et al*., 2000b; Di Marco *et al*., 2001); and *iii*) the *Fmed* genome includes genes codifying for reducing-polyketide synthase (R-PKS) which upregulate in some brown rot fungi, and these have been related to LMWC production likely involved in the redox chemistry of non-enzymatic degradation models (Goodell *et al*., 1997; Riley *et al*., 2014; Goodell, 2020). These observations are in line with Riley *et al*. (2014), who observed that the lignocellulolytic gene pathway does not capture the prevailing paradigm of white rot/brown rot wood decay fungi over several *Basidiomycota* genomes. A more nuanced and less dichotomic categorization of rot types could be implemented.

The *Fmed* genome also includes several gene copies codifying for terpene synthase (TS), cytochrome P450 monoxygenase (CytP450) and glutatione transferases (GSTs) (Floudas *et al*., 2012). Together with R-PKS, TS could confer competition advantages against other microorganisms through secondary metabolite production (Riley *et al*., 2014). CytP450 could also be involved in secondary metabolite production, and was originally described with GSTs as part of fungal xenomes, often associated with intracellular detoxification processes against lignin and other secondary metabolites synthesized by plants in reaction to fungus attack (Morel *et al*., 2013). This could confer the "primary" pathogen character reported by Sparapano *et al*. (2000a, 2001a).

Degradative enzymes (such as laccases, peroxidases and tannases) produced by *Fmed* could also degrade antimicrobial substances synthetized by host plants (tannic acid and resveratrol), playing putative roles in host-pathogen interactions. Moreover, detoxification enzymes such as phenol-oxidases and peroxidases were also detected in the contact zones of dual cultures with *Fmed* and *Pch* or *Fmed* and *Pmin*, suggesting detoxification activity by these enzymes against antimicrobial substances secreted by antagonistic fungi (Bruno and Sparapano, 2006a).

In conclusion, studying the complexity of the enzymatic pool, the secretome and xenome, together with possible presence of a non-enzymatic iron-dependent pathway, could provide further insight into *Fmed*-grapevine interactions and "Esca disease" symptomatology.

Phytotoxic compounds, organic acids, and other molecules

Toxin production and translocation to foliage via sap flow has been often proposed as the possible cause of "Esca disease" foliar symptoms (Claverie *et al*., 2020),

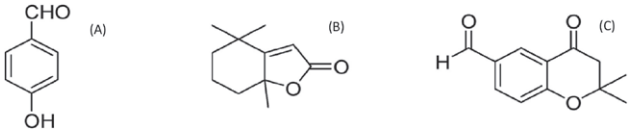

Figure 7. Phytotoxins produced by *Fomitiporia mediterranea*, based on relevant reports. (**A**), 4-Hydroxybenzaldehyde; (**B**), dihydroactinolide; (**C**), 6-Formyl-2,2-dimethyl-4-chromanone. Chemical formulae retrieved by PubChem (https://pubchem.ncbi.nlm.nih.gov/).

and its role in GTDs has recently been reviewed (Masi *et al*., 2018). It has been postulated that an oxidative burst triggered by the toxins in leaves could be more likely involved in foliar symptoms appearance than toxins themselves (Calzarano and Di Marco, 2018b). Toxicity thresholds and possible interference with other foliar susceptibility factors are still unclarified (Claverie *et al*., 2020). Production of low molecular weight metabolites with potential phytotoxicity was recorded (without identification) by Sparapano *et al*. (2000c), but phytotoxins were identified by Tabacchi *et al*. (2000) in *F. punctata* cultures (probably *Fmed*). They detected 4-hydroxybenzaldehyde, dihydroactinolide and 6-Formyl-2,2-dimethyl-4-chromanone (Figure 7), a phytotoxin related to eutypine produced by *Eutypa lata* (Deswarte *et al*., 1996a, 1996b; Andolfi *et al*., 2011). It was suggested that hydroxyl-benzaldehyde and its derivatives (carrying the aldehyde function) play important roles in the toxicity of fungi implicated in "Esca disease". Phytotoxicity was reported only for 4-Hydroxy-benzaldehyde on living protoplasts from *V. vinifera* 'Cabernet Sauvignon' at 10^{-5} and 10^{-6} M, as well as on callus from *V. vinifera* 'Gamay' grown in media supplemented with different concentrations of the metabolite (100, 250, and 500 mM) (Tabacchi *et al*., 2000). Further research is necessary to fully assess phytotoxicity of fungal metabolites and their roles in the diseases of Esca complex.

There is good correlation between fungus pathogenesis and oxalic acid secretion (Dutton and Evans, 1996). This is especially true for wood decay agents, where organic acids (mainly oxalic acid) may facilitate lignocellulosic biomass degradation, due to pH acidification, unstable and toxic divalent metal chelation, and H_2O_2 production (Kuan and Tien, 1993; Shimada *et al*., 1994; Tanaka *et al*., 1994; Urzúa *et al*., 1998). Oxalic acid metabolism is mainly regulated by two enzymes, i.e. oxalate decarboxylase (ODC, EC 4.1.1.2) and oxalate oxidase (OXO, EC 1.2.3.4), both of which catabolize the organic acid and reduce its level, which in high concentrations could be cytotoxic for pathogenic fungi (Svedružić *et al*., 2005; Zhuang *et al*., 2015). For *F. punctata* (probably *Fmed*), despite pH lowering in liquid culture (from 6.8 to 5.3; Sparapano *et al*., 2000c), Liaud *et al*. (2014) observed no organic acid production by *Fmed* in a comparative liquid culture chromatography screening. However, the presence of oxalate decarboxylase/oxidases gene copies in the *Fmed* genome (Floudas *et al*., 2012) indicates that epigenetic regulation of their expression could often occur.

Basidiomycete species are well known to produce pigments in response to abiotic and biotic stimuli, and these pigments act as chemical mediators during interactions between multiple organisms. Among them, terpene polyketide and amino acid derivates are known to be inducible, and to confer competition advantage (Spiteller, 2008, 2015; Halbwachs *et al*., 2016). In co-culture assays with *Hapalopilus rutilans* (Pers.) Murrill, *Fmed* mycelium increased pigmentation earlier compared to axenic culture, via hyperproduction of hypholomine B (Tauber *et al*., 2018), suggesting a role in interaction modulation. Interactions of *Fmed* with other microorganisms has been studied by Bruno and Sparapano (2006a) and Sparapano *et al.* (2000b, 2001b). In dual cultures with *Pmin* on modified Czapek medium, *Fmed* colony margins turned brown, became thicker and aerial hyphae formed ridge-like barriers, but the fungus growth stops at the contact zone. In dual cultures with *Pch*, after agonistic early growth, *Fmed* overgrew *Pch* mycelium (Sparapano *et al*., 2000b). The *Pmin* vs *F. punctata* (probably *Fmed*) antagonistic effect was confirmed in triple cultures with *Pch* in that condition, *Pch* was not overgrown by the *Fmed* mycelium, suggesting a suppressive role of *Pmin* (Sparapano *et al*., 2000b). Sparapano *et al*. (2001b) also studied the possible biochemical motivation of these agonistic and not-agonistic effects: *Pmin* and *Pch* culture filtrates, depending on their dilution in culture media, inhibited or reduced growth of *F. punctata* in Malt Extract Agar (ME). No inhibition of *Fmed* in ME medium was observed for *Pmin* or *Pch* crude organic extracts (ethyl acetate extraction of culture filtrates), purified scytalone from *Pmin* and *Pch* (at 1 mg mL^{-1}), pullulan from *Pch* (at 0.2 mg mL^{-1}) and oligosaccharides up to 2.5 kDa obtained by digestion of *Pch*-pullulan (2 mg mL^{-1}) (Sparapano *et al*., 2001b).

Study of the metabolome and transcriptome of the contact zones of different dual cultures to assess molecular cross-talking between *Fmed* and its competitor, would be worthwhile, to complete the partially studied secretome of this pathogen (Bruno and Sparapano, 2006a).

HOST PHYSIOLOGY CHANGES AND DEFENSE RESPONSES FOLLOWING *F. MEDITERRANEA* INFECTIONS

Data is sparse on changes in grapevine physiology and defence mechanisms specifically related to *Fmed*.

Effects of GTDs on grapevine physiology were reviewed by Fontaine *et al*. (2016b), but no specific responses to *Fmed* colonization and infection have been reported. Nevertheless, research on re-established plant vigour and quality grape production after 3 years from curettage treatments (see below) has demonstrated that white rot (where the main European decay agent *Fmed* is overabundant; Fischer and Kassemeyer, 2003; Bruez *et al*., 2017) probably affects grapevine physiology (Cholet *et al*., 2021). This follows observations by Ouadi *et al*. (2019) on 'Cabernet Sauvignon' plants presenting foliar symptoms of "Esca disease". They linked the abundance of necrotic wood (mainly white rot) in grapevine trunks and cordons with a 30% reduction in vine sap flow circulation, and thus leaf transpiration.

Few experiments have been performed to clarify plant defence mechanisms against *Fmed*. In callus/fungus dual culture experiments, Bruno and Sparapano (2006c) identified a number of phenolic molecules (benzaldehyde derivates, benzoic acid derivates, flavonols, flavonol-3-o glycosides, quercetin 3-rhamnoside, catechins and stilbenes) that were differentially induced in 'Matilde' and 'Italia' grapevines. Other studies have focused directly on vine sap (Bruno and Sparapano, 2006b, d) or brown-red symptomatic wood (Amalfitano *et al*., 2000, 2011; Agrelli *et al*., 2009) of "Esca disease"-symptomatic plants. Several stilbene-phenolic molecules were identified, which are theoretically toxic to *Fmed*, that showed greater sensitivity to phenols than *Pch* or *Pmin* (Amalfitano *et al*., 2001, 2011). Similar results were obtained by Rusjan *et al*. (2017) in wood of leaf stripe symptomatic vines, but with phenolic alterations reflecting both presence of the pathogen and wood condition in different parts of vines (trunks and rootstocks). Rusjan *et al*. (2017) proposed a relationship between the period of presence of the pathogen in different vine portions and their phenolic profiles. However, biomolecule concentration increases observed by Rusjan *et al*. (2017) may not be related exclusively to *Fmed*. Diseased plants are naturally infected by all the "Esca disease"-related fungi (*Pch, Pmin*, *Fmed*), and other possible microbial consortia highlighted by metagenomic approaches. Nevertheless, because a *Fmed-Pch-Pmin* interaction has been demonstrated, a relationship is likely between those compounds and *Fmed* (Sparapano *et al*., 2001b; Bruez *et al*., 2020). For this reason, results from most metabolomic studies in leaves responding to "Esca disease"-associated pathogens should be treated with caution, when attempting to understand exact plant responses to *Fmed* (Goufo *et al*., 2019; Moret *et al*., 2021). Further studies are necessary to precisely determine grapevine metabolite production burst specifically in response to *Fmed* colonization.

Damage to host hydraulic systems caused by the white rot necrosis could be the most important driver of physiological effects in grapevies, but specific studies are necessary to verify these hypotheses. To the best of our knowledge there have been no reported studies of wood compartmentalization specifically towards *Fmed*. The metabolic changes induced by *Fmed in planta* could generate biochemical markers for presence of the pathogen and wood degrading activity.

CONTROL STRATEGIES WITH A FOCUS ON *F. MEDITERRANEA*

Effective disease management is a major challenge in crop protection, and particularly for disease complexes such as "Esca disease". Efficiency of individual control methods for Esca complex of diseases is limited, and is best managed using integrated disease management, from nursery to vineyard. This includes cultural or remedial practices, vineyard sanitation, and use of pesticide chemicals or biological agents to protect grapevine wounds from pathogen infections (Gramaje *et al*., 2018). Methods to reduce or limit disease incidence, especially against *Fmed* infection, are outlined below.

Disease resistance

Incidence of "Esca disease" symptoms have been reported as cultivar-, rootstock-, and clone-related (Marchi 2001; Fussler *et al*., 2008; Grosman, 2008; Murolo and Romanazzi, 2014; Guan *et al*., 2016; Kraus *et al*., 2019; Moret *et al*., 2019), but they were all related to reduced presence of leaf symptoms, not to wood decay development. Some hypotheses could explain cultivar differences. Rolshausen *et al*. (2008) reported greater lignin levels in 'Merlot' grapevines tolerant to *E. lata* compared to susceptible 'Cabernet-Sauvignon' vines. A similar correlation has been suggested for *Fmed* affecting different olive tree varieties (Markakis *et al*., 2019). Assessment of susceptibility of wild grapevine (*V. vinifera* subsp. *sylvestris*) to *Fmed* could be worthwhile, because this host has been shown to be a promising potential source of resistance to Botryosphaeria dieback (Guan *et al*., 2016). Some data are available on other *Vitis* genotypes used in resistance source trials (Kraus *et al*., 2019). Fischer (2019) also detected regular presence of *Fmed* vegetative mycelium in rootstock mother blocks of rootstocks SO4, 5BB and 125AA in Germany, all of which were a cross population of *Vitis berlandieri* × *Vitis rupestris*.

Grapevine propagation material

The use of a good quality pathogen-free plant material is essential to limit inoculum propagation. Although *Fmed* has been shown to be present in blocks of rootstock mother vines (Fischer, 2009, 2019), and thus the derived plant material could be infected by "Esca disease"-associated fungi before nursery stages or during the propagation processes, *Fmed* has never been isolated from grafted 1-year old cuttings or propagation material. Furthermore, this pathogen has not been reported in grapevine nurseries (Larignon and Dubos 2000; Halleen *et al*., 2003; Zanzotto et al., 2007; Larignon *et al*., 2008a; Aroca et al, 2010; Gramaje and Armengol, 2011; Fischer, personal communication).

Protective and curative disease control methods

Curative control

Removing white rot from diseased grapevines seems to be efficient for reducing leaf stripe symptoms. This old technique, called "curettage" or "trunk surgery", consists of cutting affected vines and removing white rot with small precision chain saws. It provides good results; foliar symptoms are reduced even several years after curetting (Thibault, 2015). Cholet *et al*. (2021) demonstrated how curettage in "Esca disease"-symptomatic plants reduced foliar symptoms during 3 years after treatment, and re-establish vine vigour and grape production. Pacetti *et al*. (2021) confirmed foliar symptom remission in 14-year-old GLSD symptomatic 'Cabernet Sauvignon' vines for the following 2 years after complete trunk surgery, and demonstrated microbiome change induced by the treatment. *Fomitiporia mediterranea* abundance decreased after curettage, in parallel to an alpha-diversity increase in fungal population, suggesting a microbiota shift as a likely explanation for foliar symptom reduction during the post-curettage period.

Plant endotherapy is another promising curative technique against white rot. This includes direct treatment of white rot by drilling a vertical hole in grapevine trunks and injecting specific molecule solutions (typically fungicides), aiming to reduce foliar symptoms. However, due to the complexity of microbial consortia in diseased trunks, and because of the wood peculiar structure in old cultivated vines, specificity of the technique against *Fmed* needs to be refined. This approach is the subject of ongoing research (Gellon *et al*., 2017; Pacetti *et al*., 2019).

Sodium arsenite has been used in viticulture for a long time as the only effective and curative treatment against "Esca disease" (Songy *et al*., 2019b), and studies on modes of action of this compound are increasing. Larignon *et al*. (2008b) suggested *Fmed* as the most sodium arsenite sensitive "Esca disease"-associated fungus, and when Goddard *et al*. (2017) investigated the fate of arsenite within "Esca-diseased" treated plants, they found it concentrated in white rot necroses. Bruez *et al*. (2017) demonstrated that *Fmed* isolations were reduced from white rot necrotic tissue coming from sodium arsenite treated plants. Bruez *et al*. (2021) showed how in sodium arsenite treated 'Gewürztraminer', 'Chardonnay' and 'Merlot' vines (25 to 40 years old) expressing tiger stripe symptoms, the relative abundance of *Fmed* decreased in white rot necroses and necrosis boundaries, confirming *Fmed* as the most sodium arsenite sensitive among GTDs-associated fungi (Larignon *et al*., 2008b). Previously foliar symptomatic plants did not express these symptoms after treatment, suggesting that the positive effect of sodium arsenite on GLSD was from specific toxicity to *Fmed* in white rot necrotic tissues and their boundaries, where other parasitic and saprobic fungi (*Inonotus hispidus* Bull. P. Karst., *Lepiota brunneoincarnata* Chodat & C. Martín) took place, increasing their relative abundance (Bruez *et al*., 2021).

Except for host endotherapy, for which experiments are ongoing, up to now curettage is likely to be the most sustainable physical management method against *Fmed*. More user- and environmentally-friendly chemical curative alternative should be proposed. The long-term efficacy of curative treatments has not been fully assessed. Data on reduction of foliar stripe symptoms provided by these two curative techniques (curettage and sodium arsenite) reinforce the link between GLSD and white rot, suggesting that more studies are required on these disease management approaches.

Preventive control

Protection of grapevine pruning wounds is an essential point to reduce pathogen entry (Eskalen and Gubler, 2001; Eskalen *et al*., 2007). In some European countries, some pesticide products (based on boscalid or pyraclostrobin) and biocontrol products (based on specific strains of *Trichoderma* spp.) are available for protection against GTDs. However, research with these products for management of *Fmed* diseases has not been reported yet.

Beside the authorized and registered products containing boscalid, pyraclostrobin or *Trichoderma* spp., many products or molecules have been tested *in vitro* for the control of GTD pathogens, and these were reviewed by Gramaje *et al*. (2018) and Mondello *et al*. (2018b). For *Fmed*, however, only few reports are available. Chitosan

in *in vitro* tests gave a low EC_{50} value (1.53 mg L^{-1}) for *Fmed* (Nascimento *et al*., 2007). Sensitivity of *Fomitiporia* spp. to chitosan was first reported by Bruno *et al*. (2001). Incorporation of resveratrol in culture media gave a direct antifungal effect against *Fmed* growth (Mazzullo *et al*., 2000). Copper oxychloride and gluconate formulations slightly reduced *Fmed* mycelium growth *in vitro*, with an EC_{50} of 11.242 mg Cu L^{-1} (Di Marco *et al*., 2011). For biological control, sensitivity of *Fmed* to crude protein extracts (CPE) from *Bacillus amiloliquefaciens* AG1 has been recorded as 2.000 AU mL^{-1} (Alfonzo *et al*., 2012). Del Frari *et al*. (2019b) demonstrated with *in vitro* dual culture plates that growth of *Fmed* and other "Esca disease"-associated fungi was inhibited by *Epicoccum* spp., a member of ascomycetes which have been commonly identified in grapevine microbiomes. No clear data have been provided about effects of *Trichoderma* spp. on *Fmed*, in contrast to documented effects of these fungi on the growth of *Pch* and *E. lata* (Di Marco *et al*., 2004b; John *et al*., 2005).

CONCLUSIONS AND FUTURE PERSPECTIVES

We have made careful attempts to collect and review all relevant published information on *F. mediterranea*, to stimulate debate within the GTD scientific community. Approximately 20 years after formal classification of this fungus, it is well established that it induces white rot in the grapevine wood, but details of the relationships between *Fmed* and GLSD essentially remain unknown. The causes and biomolecular mechanisms of white rot, and their relationships with external grapevine foliar symptoms, has yet to be deciphered, especially in light of knowledge and observations reviewed here. To fully describe these processes could be a standing point in the context of GTDs, and will allow viticulture to adopt new solutions for management of grapevine trunk diseases.

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Annex III

frontiers in Microbiology

ORIGINAL RESEARCH
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Grapevine Wood-Degrading Activity of *Fomitiporia mediterranea* M. Fisch.: A Focus on the Enzymatic Pathway Regulation

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Fomitiporia mediterranea is a *Basidiomycetes* fungus associated with some of the Esca complex diseases and responsible for decay in grapevine wood. Its role in the onset of foliar symptoms has recently been reconsidered, mainly after evidence showing a reduction in foliar symptom expression after removal of rotten wood. The study of its degradation pathways has already been approached by other authors, and with this study much information is consolidated. A microscopic observation of degraded wood provides a first approach to the characterization of *F. mediterranea* modalities of wood cellular structure degradation. The decay of grapevine wood was reproduced *in vitro*, and the measurement of each wood-forming polymer loss highlighted characteristics of *F. mediterranea* common to selective white rot and showed how fungal strain and vine variety are factors determining the wood degradation. All these observations were supported by the analysis of the laccase and manganese peroxidase enzyme activity, as well as by the expression of the genes coding 6 putative laccase isoforms and 3 manganese peroxidase isoforms, thereby highlighting substantial intraspecific variability.

Keywords: decay, SEM, cellulose, lignin, grapevine trunk diseases, Esca

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INTRODUCTION

Grapevine trunk diseases (GTDs) are considered "the biotic stress of the century" for the grapevine (Songy et al., 2019), with an increasing economic impact in all grape-growing countries. Among them, the Esca complex of diseases (ECDs) is a huge problem in Europe, where disease incidence (measured by foliar symptoms and vine death) reached "increasing" and/or "worrying" levels in several regions of Italy, France, and Spain (Guérin-Dubrana et al., 2019). This has led to huge economic losses (up to 1 billion euros per year estimated by the French Wine Institute, IFV), justifying the interest in increasing research and knowledge gathering in that area (Claverie et al., 2020). The Esca complex is currently considered a complex of different diseases, characterized by several different symptoms. Even though a quite complex microbiota has been recently shown to be involved in its onset (Bruez et al., 2020), the diseases of the complex are usually associated with the Ascomycota species including *Phaeomoniella chlamydospora* (Pch) (Crous and Gams, 2000), *Phaeoacremonium minimum* (Pmin) (Gramaje et al., 2015), and, in Europe, the basidiomycete

Fomitiporia mediterranea (Fmed), while in other grapevine-growing regions other *Fomitiporia* species can be identified (Fischer, 2002, 2006; Del Frari et al., 2021; Moretti et al., 2021). Pch and Pmin are considered to be responsible for the "phaeotracheomycotic complex" (brown wood streaking; Bertsch et al., 2013), while Fmed is a white-rot agent responsible for wood degradation and decay, historically associated with "Esca" (Gard, 1922) and "Esca proper" (Surico, 2009). Despite Esca being described as a complex of diseases, epidemiological analysis mostly relies on the description of the typical striped leaf symptoms (characterizing grapevine leaf stripe disease following Surico, 2009) and vine apoplexy (sudden wilting of part or the whole vine), given the difficulty of assessing internal wood symptoms in standing vines, i.e., brown or black wood streaking, central or sectorial necrosis, and white rot. However, recently new interest has arisen in the activity and role of the white-rot agent *Fomitiporia mediterranea* (Moretti et al., 2021), on the basis of increasing reports on the direct effect on symptom development following white-rot elimination (Thibault, 2015; Dal, 2020; Cholet et al., 2021; Pacetti et al., 2021). Studies on microbiota composition in wood rot and grapevine leaf stripe disease (GLSD) symptomatic vines (Del Frari et al., 2019; Bruez et al., 2020; Pacetti et al., 2021) are shedding new light on the historically debated—and never fully clarified—relationship between white rot and GLSD-foliar symptom expression (Calzarano and Di Marco, 2007; Maher et al., 2012). Wood is the major structure that gives trees and other woody plants and vines stability for upright growth and maintains the water supply from the roots to all other plant tissues. Woody plant cell walls consist mainly of a lignocellulose complex, which is composed of cellulose, hemicellulose, and lignin heteropolymers, organized in different ratios depending on the woody plant species (Lundell et al., 2010). Lignin generally represents the second most abundant biopolymer of the plant cell wall in hardwood and softwood species (Pettersen, 1984; Howard et al., 2003; Chen et al., 2014), while in grapevine wood it represents the less abundant one in accordance with its liana morphological characteristics (Agrelli et al., 2009). Lignin also has an important defensive role in plant–pathogen interaction (Weng and Chapple, 2010), especially in host resistance against white-rot agents (Schwarze, 2007). Despite its abundance in woody plants, lignin is neither an energy nor a carbon source for fungi if available alone, but, as lignin in the plant cell wall is constitutively merged with carbohydrates, the *in vivo* degradation by a concert of enzyme activity could lead to complete digestion (Kirk et al., 1976; Chen et al., 2014). The processes of depolymerization of cellulose and lignin are interrelated, and they can even boost each other (Westermark and Eriksson, 1974a,b, 1975). The fact remains that to access cell wall carbohydrates, a lignin bypass or degradation mechanism is required (Cragg et al., 2015). Historically, white-rot degradation was described as the result of complete lignin, cellulose, and hemicellulose mineralization driven by extracellular enzymes (Blanchette, 1991). Many families of degrading enzymes are involved in the degradation of white-rot wood such as carbohydrate active enzymes (CAZymes) for cellulose and hemicelluloses or laccases and Class II peroxidases (PODs) for lignin (Daniel, 2014), as well as several auxiliary active (AA) redox enzymes, a class of enzymes currently associated with CAZymes and Class II PODs that sustain ligninolytic enzyme activity, with results present in white-rot fungi (Levasseur et al., 2013). Nevertheless, a comparative genomic study based on CAZymes of 31 fungal species showed that the prevailing paradigm of white versus brown rot does not capture the diversity of wood-decay mechanisms in *Basidiomycetes* (Riley et al., 2014). Fmed is nowadays considered a white-rot agent (Fischer, 2002), and its degrading secretome has been investigated by many authors (Mugnai et al., 1999; Bruno and Sparapano, 2006; Abou-Mansour et al., 2009; Schilling et al., 2021). Besides several identified or targeted enzymes belonging to the CAZyme pool which degrade cellulose (Mugnai et al., 1999; Bruno and Sparapano, 2006; Riley et al., 2014), as a white-rot species Fmed has all the main enzymes described to fully mineralize lignin, enzymes that are involved in the pathogenicity process, specifically (i) Class II PODs, such as manganese peroxidases (MnP; EC 1.11.1.13), and (ii) laccases (EC 1.10.3.2; p-diphenol:di-oxygen oxidoreductases). Three MnP genes were characterized in the Fmed genome, *Fmmnp1*, *Fmmnp2*, and *Fmmnp3* (Morgenstern et al., 2010). Although some authors have highlighted the lignin peroxidase (LiP) activity of Fmed *in vitro* (Cloete et al., 2015b), a comprehensive study based on genome sequencing of wood-degrading fungi reported the absence of LiP (EC1.11.1.14) genes in the Fmed genome (Floudas et al., 2012). Abou-Mansour et al. (2009) purified a typical fungal 60-kDa laccase from some Fmed isolates, even though complete lignin degradation was not achieved by these proteins alone. Currently, the role of laccases in pathogenicity itself is still being debated (Giardina et al., 2010; Singh Arora and Kumar Sharma, 2010). As suggested in a recent review on Fmed (Moretti et al., 2021), studying the pathogenicity factors involved in grapevine wood rot, in order to fill the gaps in information concerning the biomolecular process of white rot, could eventually furnish keys to contribute to the interpretation of the etiology of the leaf stripe symptoms. For that purpose, we first performed a visual analysis of cell wall degradation by epi-fluorescence microscopy, on naturally Fmed-infected grapevine wood, highlighting the degradation of all components of the wood cell wall due to the enzyme activity. In order to characterize this activity, we focused on the lignin-degrading enzymes, the ones that could be responsible not only for causing the white rot itself but also possibly involved in the "by-product of wood degradation" theory on the appearance of striped leaves symptoms postulated by Mugnai et al. (1999) and investigated by other authors (Agrelli et al., 2009; Amalfitano et al., 2011). In particular, we investigated *in vitro* (i) the enzyme activity and kinetics of ligninolytic enzymes; (ii) their molecular regulation; and (iii) the residual wood polymer after fungal degradation.

MATERIALS AND METHODS

Epi-FM on Naturally Infected Wood

For microscopic analysis, trunks of grapevines of cv. Riesling (Kober5bb rootstock), planted in 1997, and cv. Sauvignon blanc (SO4 rootstock), planted in 2009, were used. The sampling sites

were neighboring vineyards on gently west-sloping slopes on loess and loess loam (subsoil Tertiary marls, limestones, and sandstones) in the Batzenberg area of southwestern Germany, south of Freiburg (47.97°N, 7.75 E and 47.95°N, 7.74 E). Using a band saw, longitudinal and transverse sections were taken from the trunks, from which segments of 10 mm × 5 mm × 5 mm from lesions with white rot were excised. The samples were fixed in glutaric aldehyde for more than 24 h and, after being washed three times in deionized water, dehydrated in an increasing concentration of isopropyl alcohol. After embedding in methacrylate resin, semi-thin sections of 3 and 1 μm were prepared with a rotary microtome (LEICA 2065 and LEICA 2044). For further processing, the resin was removed from the sections by rinsing overnight in isopropyl alcohol. Next, the specimens that had been fixed on glass slides were stained in a programmable slide stainer (ZEISS HMS TM Series, Carl Zeiss AG, Oberkochen, Germany) with 2% safranin and 1% acriflavine (12 h), 1% acid yellow (30 min), and 1% methylene blue (5 min) and embedded in Eukitt (O. Kindler, Freiburg, Germany). For epi-fluorescence microscopy (epi-FM), the slides were stained with acridine orange. The microscopic analyses were carried out with a light (bright-field) and epi-fluorescence microscope (ZEISS Axio Imager Z1) equipped with the optical sectioning system Apotome 2 for structural illumination and a digital imaging system (ZEISS Axiocam MR35, ZEN 2.6 pro image processing software by Carl Zeiss AG, Oberkochen, Germany). FM-3D image visualization of the samples stained with acridine orange was performed with the filter combination 38 HE, excitation 460-488 nm, emission 500-557 nm.

Fungal Isolation From Wood Samples and Identification

To attribute microscopic observations to certain fungal species, classic isolations were made by sampling fragments of fungal-colonized wood, contiguous to the tissue sampled for microscopic analysis. In order to establish which fungal species were responsible for the observed wood alterations, isolations were made from 50 wood fragments. The wood fragments were placed on PDA (Potato Dextrose Agar), with streptomycin sulfate at 1 $g^{*}l^{-1}$, for 30 days at 28°C in the dark, and each colony developed on the medium was transferred to a fresh PDA plate for identification. The morphology of the isolated fungi was studied by optical microscopy.

Fomitiporia mediterranea Strains

Four strains of *Fomitiporia mediterranea* (**Table 1**) from a mycological collection, different from that isolated for microscopy, were tested for both *in vitro* and enzyme activity assay. The identity of selected strains was confirmed by culture morphology and ITS sequence data.

Fomitiporia mediterranea Solid and Liquid Cultures

To study enzyme activity regulation and related gene expression, liquid cultures and solid cultures were set up, respectively. Solid cultures of the selected strains of Fmed were grown at 28°C in the dark on 90-mm petri dishes of Eriksson and Pettersson medium (Eriksson and Pettersson, 2005) (E&P) solidified by adding 15 mg of agar per liter. Liquid cultures were prepared using 125 ml of E&P medium supplemented with 0.625 g of cellulose and 1.25 g of *Vitis vinifera* (cv. Gewurztraminer) wood sawdust and inoculated with a section (1/4) of 20-day-old solid culture in a 250-ml flask. Liquid cultures were grown for 10 days at 28°C, shaken at 150 rpm in the dark. Three biological replicates per strain were tested. Three technical replicates of 10 ml of each sample were sampled and filtered by 4-stage filtration: 50 μm Nilex sheet and 1-, 0.45-, and 0.2-μm regenerated cellulose membrane filters. The whole solid mass of each technical replicate was separated from the liquid, dried at 110°C for 48 h, and weighed. The protein content of filtered liquid (hereinafter secretome) was defined by spectrophotometry using a NanoDrop (BioSpec-nano, Shimadzu, Kyoto, Japan) and assuming that 1 OD corresponded to a concentration of 1 mg/ml. The culture secretome was diluted to a final concentration of 0.2 mg/ml for enzymatic assays.

TABLE 1 | *Fomitiporia mediterranea* strains used in laboratory experiments in this study.

Strain	Location	Host	Isolation date
LR71	Vendargues (34), FR	*V. vinifera* cv. Alphonse Lavallée	1996
LR124	Villeneuve les Maguelone (34), FR	*V. vinifera* cv. Carignan	1996
PHCO36	Saint-Preuil (16), FR	*V. vinifera* cv. Ugni blanc	1996
235.01	Riotorto (Livorno), IT	*Olea europaea L.*	2003

Determination of Residual Wood Polymer After Fungal Degradation

To estimate the degradation of each grapevine wood polymers caused by Fmed, cv. Teroldego and cv. Gewurztraminer vine wood blocks were used for *in vitro* assays. Cultivar Teroldego was chosen as a variety rarely showing the leaf foliar symptoms of the Esca complex, while cv. Gewurztraminer was selected as a highly symptomatic variety (Grosman and Doublet, 2012; Bottura, 2018). For each variety, 3 blocks of wood were shaped to 0.5 cm × 5 cm × 2.5 cm (**Figure 1A**) and degraded for 30 and 90 days by 2 of the selected strains of Fmed (LR124 and PHCO36). Each block weighed 10 g with a variation of less than 0.5 g, and the wood did not show necrosis or discoloration. The wood blocks were dried and sterilized at 110°C for 48 h in the oven and then placed in petri dishes on 20 ml of solid E&P medium with four 6-mm plugs from each Fmed 20-day-old culture on E&P (**Figure 1B**). Three more blocks for each variety were also sterilized and used as controls without inoculation. To confirm that the wood degradation activity was carried out by the target fungus, samples were analyzed by SEM before and after colonization. In addition, the grapevine undegraded wood polymer composition was determined on wood blocks not inoculated with Fmed strains and, in order to compare the resulting profiles to better known soft- and hardwood profiles, also three samples of beech (*Fagus sylvatica* L.) and three samples of spruce (*Picea abies* L., H. Karst.), a hardwood and

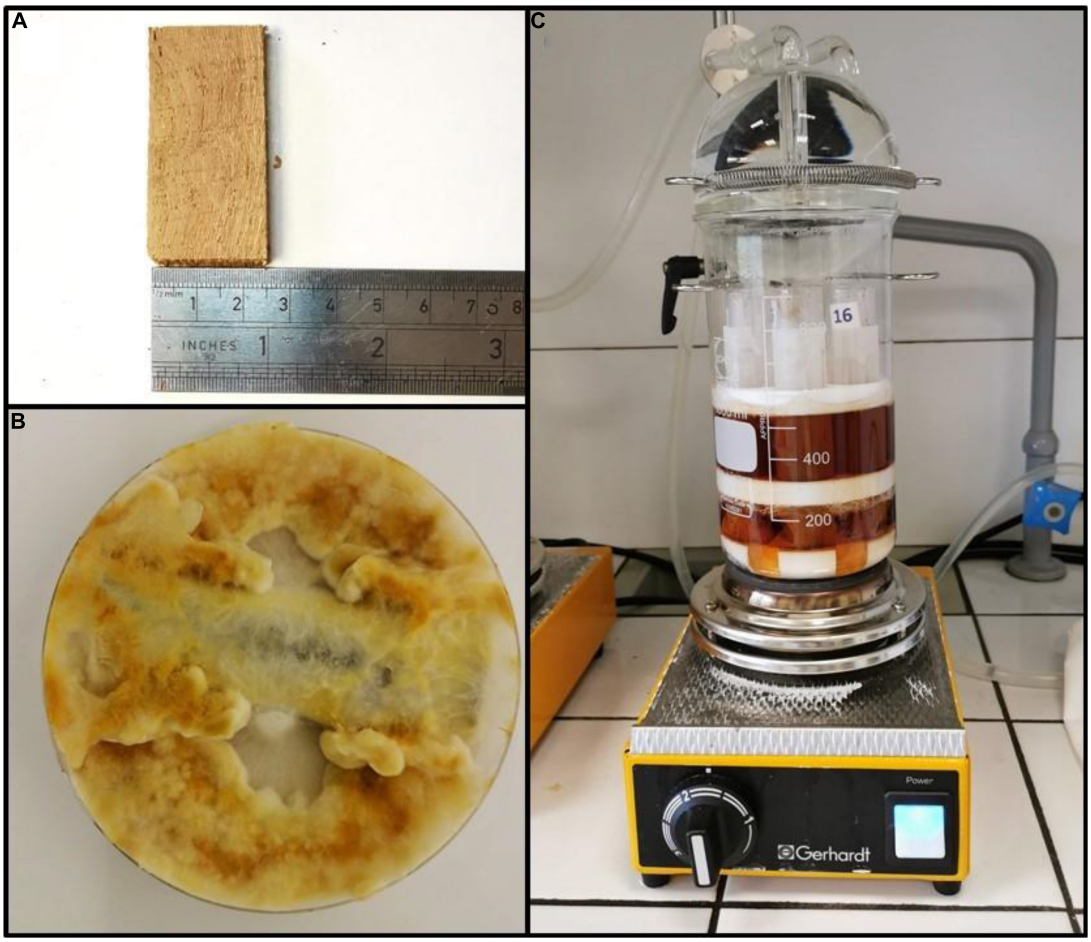

FIGURE 1 | (A) Grapevine wood block before fungal degradation. **(B)** Wood block incubated with Fmed in a petri dish after complete wood colonization. **(C)** Plastic mesh-filter bag with spacers during the detergent bath for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) determination.

a softwood, respectively, were analyzed. After degradation, the fungal mycelium was scraped off from the surface of wood blocks and the residual wood was dried again as before the inoculation. After drying, the wood blocks were lyophilized, then frozen with liquid nitrogen and pulverized using a mixer mill (Retsch MM 400, Retsch GmbH, Haan, Germany). The wood powder obtained was digested using detergents and acid solutions in a 3-step protocol. One gram (± 0.01 g) of the powdered samples was placed in a plastic mesh-filter bag (Fibrebags ADF, C. Gerhardt GmbH & Co.) with a spacer and subjected to 3 consecutive baths (**Figure 1C**). After each bath, samples were dried in order to measure neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) (Van Soest and McQueen, 1973). The neutral detergent solution was composed of sodium dodecyl sulfate (30 g/l), sodium EDTA (18.61 g/l), sodium phosphate monobasic (4.56 g/l), sodium tetraborate decahydrate (6.81 g/l), and triethylene glycol (10 ml/l). The pH of the solution was adjusted to between 6.9 and 7.1. Samples were immersed in this boiling solution for 1 h and washed 5 times in hot water to eliminate the detergent solution. Then, the samples were dried at 105°C overnight and weighed to determine the NDF fraction. The second solution was composed of 20 g of cetyltrimethylammonium bromide (CTAB) dissolved in 11 ml of sulfuric acid (0.5 mol/l). Samples were immersed in this boiling solution for 1 h and washed 5 times in hot water to eliminate the acidic solution. Then, the samples were dried again at 105°C overnight and weighed to determine the ADF fraction. The third solution was composed of 72% sulfuric acid. The samples were then immersed in this solution for 3 h at room temperature and washed 5 times in hot water to eliminate the acidic solution. Finally, the samples were dried one last time at 105°C overnight and weighed to determine the ADL fraction.

Scanning Electron Microscopy on *in vitro* Incubated Wood Blocks

Scanning electron microscopy (SEM) was performed on incubated grapevine wood blocks (for 30 and 90 days) to confirm that the degradation was carried out by the inoculated Fmed strains: the surface of the dried samples was ground and polished with the Leica EM TXP Target Surfacing System. After sputtering with 20 nm gold, the wood structure was analyzed using the

Philips XL30 ESEM environmental scanning electron microscope with an SE detector at an accelerating voltage of 5-10 kV. The image processing was done with DISS5 Software from REM-X GmbH Bruchsal (Germany).

Enzyme Activity of Fmed Secretome

For the evaluation of enzyme activity, the liquid culture experimental setup was used. Laccase activity was indirectly determined by oxidation of ABTS (2.2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to $ABTS^{+}$. Oxidized ABTS turns color from transparent to green and can be measured by spectrophotometry. The absorbance was measured at 420 nm, every 20 s for 250 s from the addition of 25 μl of ABTS (2 mM) to a mix of 25 μl of sodium acetate buffer (100 mM, pH 4.5) and 50 μl of liquid culture secretome (0.2 mg/ml). Absorption values were converted into enzyme activity *via* the extinction coefficient of ABTS ($\varepsilon_{420} = 3.6\ 10^4\ M^{-1} * cm^{-1}$) (Bourbonnais et al., 1995). According to Mathieu et al. (2013), MnP activity assay was based on the reaction of MBTH (3-methyl-2-benzothiazolinonehydrazone hydrochloride) and DMAB (para-dimethylaminobenzaldehyde) catalyzed by PODs in the presence of H_2O_2. The reaction allows the formation of indamine, a purple-colored compound which absorbs at 590 nm.

Tests were carried out in two steps, using two different reagent solutions. The common reagent mix contained 0.5 ml of DMAB (50 mM) and 0.5 ml MBTH (1 mM) in a buffer mix of 5 ml of sodium lactate (100 mM) and sodium succinate buffer (100 mM) adjusted to pH 4.5. For the first reaction, 1 ml of $MnSO_4$ (1 mM)—which is required for manganese-dependent PODs—was added to the common reagent mix (solution A) to measure the specific MnP activity. Secondly, the total POD activity was assessed adding 1 ml EDTA (2 mM) to the common reagent mix instead of manganese sulfate (solution B). MnP activity was calculated by subtracting enzyme activity measured with solution B from that measured with solution A. Since some studies exclude the presence of LiP-encoding genes in the Fmed genome, the enzymatic activity of these enzymes has not been tested (Floudas et al., 2012). Absorbance measures were carried out on a reaction mixture prepared with 140 μl of reacting solution, 50 μl of liquid culture secretome (0.2 mg/ml), and 10 μl of H_2O_2 (1 mM), added to activate the reaction. Absorbance was then converted into enzyme activity *via* the extinction coefficient ($32{,}000\ M^{-1} * cm^{-1}$). The relative activities of each enzyme were determined by spectrophotometry, and all reactions were driven at 28°C maintaining the reaction mix in a 96-well microplate (flat-bottom F96 immuno plate, Nunc®, Roskilde, Denmark), using a filter-based plate reader (Tristar 2, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Two types of control were run by using heat-inactivated samples and H_2O_2-free mix. All specific enzyme activity is expressed as specific activity by U/mg unit, where 1 U = 1 μmol of substrate oxidized per minute; the enzyme activity was measured during assays, and obtained U/l was then divided by the protein concentration of the secretome.

Total RNA Isolation and cDNA Synthesis

Total RNA isolation was performed in 3 technical replicates: 0.2 g of fresh mycelium scraped from each sample of E&P-solid cultures, made by scraping fresh mycelium from the dish surface, frozen in liquid nitrogen, ground, then processed using an extraction kit (Qiagen® AllPrep Fungal DNA/RNA/Protein Kit, Qiagen, Hilden, Germany) and following the manufacturer's instructions with little modification. Quality of RNA was verified by demonstration of intact ribosomal bands in 1.5% agarose gel electrophoresis, in addition to 1.8–2.0 absorbance ratios (A260/280 and A260/230, respectively). First-strand cDNA was synthesized from 1 μg of DNA-free RNA using a reverse transcription mix (iScript™ Reverse Transcription Supermix, Bio-Rad Laboratories, Hercules, USA) following the manufacturer's instructions. The cycle was set up as follows: one 5-min step at 25°C, a reverse transcription step at 46°C for 20 min, and a final step at 95°C for 1 min.

Gene Expression Analysis of Laccase and MnP Genes

The expression level of the *Fomitiporia mediterranea* laccase (*Fmlcc*) and manganese peroxidase (*Fmmnp*) genes were determined by quantitative real-time PCR (qRT-PCR) using a CFX96 system (BioRad, Foster City, CA, United States). Primers for gene-specific amplification of *Fmmnp* were retrieved from the literature (Morgenstern et al., 2010), while that for gene-specific amplification of *Fmlcc* was designed using the Primer3 program[1], retrieving template sequences published on the website of the National Center for Bioinformatic Information[2]. Primer specificity was checked by the Primer-BLAST tool on the NCBI webpage[3]. Transcription elongation factor 1 (*tef1*) was used as housekeeping gene to normalize the expression of target genes (Morgenstern et al., 2010). All the details on template sequences and specific primers are reported in **Table 2**.

PCR reactions were carried out in a reaction mix containing 10 μl of iTaq Universal SYBR® Green Supermix (Bio-Rad, CA, United States), 1 μl of forward and reverse primers (10 μM), and 15 ng of cDNA, in a final volume of 20 μl. Thermal cycling conditions were set up as follows: one 30-s cycle at 95°C, followed by 39 cycles at 95°C for 5 s and a step at 60°C for 20 s. All PCR amplicons were subjected to melt curve analysis from 55 to 95°C to evaluate their specificity, and a negative control was run without a cDNA template. The results obtained for each gene of interest were normalized to the expression of a housekeeping gene (*tef1*). Relative expression ($2^{-\Delta\Delta CT}$) compared to the mean of ΔCT of samples without wood was also calculated as described by Livak and Schmittgen (2001). Data are presented as means ± SE of three biological replicates, each one having three technical replicates per 96-well plate (Livak and Schmittgen, 2001).

Statistical Analysis

Data normality and homoscedasticity were tested before running statistical analysis by the Shapiro–Wilk test and Bartlett test, respectively. The significance of differences in the dry weight of mycelial biomass grown in liquid culture and specific enzyme

[1]https://bioinfo.ut.ee/primer3-0.4.0/

[2]http://www.ncbi.nlm.nih.gov/

[3]https://www.ncbi.nlm.nih.gov/tools/primer-blast/

TABLE 2 | Gene accession numbers and sequences of primer pairs used for qRT-PCR.

Genes	Accession number	Primer sequences	Amplicon length (bp)
Fmmnp1	*HM480274.1*	†*Forward: 5′-ACGGCATTCCAAACGTCCATGAAG-3′*	*197*
		†*Reverse: 5′-GCACCAGGGTCCGTAGAAAGAGTA-3′*	
Fmmnp2	*HM480275.1*	†*Forward: 5′-GGCAATCAATGGTTGCAAACCAGC-3′*	*248*
		†*Reverse: 5′-AATCTGAGTCGCTTGTCCACCG-3′*	
Fmmnp3	*HM480276.1*	†*Forward: 5′-CGTCTTCAATCTGACTTCGCCCTC-3′*	*165*
		†*Reverse: 5′-GAGATCGGAGCAGTCAACGAGC-3′*	
Fmlcc1	*XM_007269683.1*	*Forward: 5′-TGGATCCGTGCTCAACCTTC-3′*	*206*
		Reverse: 5′-AGTGCTAAGTCAACGCCTCC-3′	
Fmlcc3	*XM_007269436.1*	*Forward: 5′-TTGGAGGCGGTACAGACAAC-3′*	*176*
		Reverse: 5′-ACACAGTCCCAGCCAATCAG-3′	
Fmlcc4	*XM_007269469.1*	*Forward: 5′-TCACTCGCATGAAGGAACCC-3′*	*177*
		Reverse: 5′-GTTGAATTGGGTGGTCTGCG-3′	
Fmlcc7	*XM_007261239.1*	*Forward: 5′-TCCTTTCCATCTGCACGGAC-3′*	*225*
		Reverse: 5′-TCCACATCCTCAGCGAACAC-3′	
Fmlcc8	*XM_007262111.1*	*Forward: 5′-AAGAGGCGGCGATGACTATG-3′*	*211*
		Reverse: 5′-TTGACTTGTCGAACCTGGGG-3′	
Fmlcc9	*XM_007263477.1*	*Forward: 5′-ACAAACTGGTCAAGGTGGGG-3′*	*223*
		Reverse: 5′-AGCAAGGATGGAAGTGTCGG-3′	
tef1	*AY885149.1*	†*Forward: 5′-TGGATTGCCACACTGCCCATATTG-3′*	*215*
		†*Reverse: 5′-GGTTTGCCTCATGTCACGCAC-3′*	

Sequences accompanied by "†" were retrieved from published literature (Morgenstern et al., 2010), while the remaining ones were newly designed for this study.

activity between Fmed strains was determined by a Tukey *post hoc* test after one-way ANOVA ($p \leq 0.05$). A two-way ANOVA test ($p \leq 0.01$) was performed for each polymer singularly, in order to establish if strain and grapevine variety significantly influence grapevine wood degradation. Differences between *Fmmnp* and *Fmlcc* expression of Fmed cultures grown in the presence of wood sawdust and cultures grown without wood sawdust were studied by Student's *t*-test ($p \leq 0.05$). Differences are highlighted by an asterisk when significant. The values reported are the averages of at least three replicates ($n = 3$) and are presented as mean values ± standard error (SE) of the mean. All analyses were performed in the R (3.6.3 version) programming environment.

RESULTS

Epi-FM analyses performed on white-rotten wood portions highlighted evidence of cell degradation due to fungal enzyme activity, a common feature in white rot. Microscopic analysis revealed a distinct demarcation zone between the intact xylem and the white rot in which the cell lumina were discolored with dark inclusions. In contrast, the white-rotten region appeared lighter in color because of degradation of the lignocellulose (**Figure 2A**). The presence of tylosis in xylem vessel lumens denoted an active defensive activity of the vine in response to fungal development (**Figures 2A,B**). In the initial stage of the degrading activity, cavities and fissures occurred on the S2 layer of the secondary cell wall of the libriform fiber (**Figure 2C**), leaving apparently undamaged or slightly degraded only the middle lamella, the primary cell wall, and the S1 layer and S3 layer of the secondary cell wall (**Figures 2C,D**). In advanced decomposition, all layers of the cell wall of vessels, tracheids, and libriform fibers were decomposed including the lignified parts such as the middle lamella, the primary cell wall, and finally the walls of the parenchyma cells (**Figure 2D**). A soft rot-like cavity formation was also observed in some cases (**Figure 2C**). In *in vitro* fungal isolation of infected wood observed under the microscope, a high frequency of Fmed isolates (90%) was recorded. Other species isolated belonged mostly to *Penicillium* (6%) and only rarely to species in *Botryosphaeriaceae* (4%) (**Table 3**).

Biomass Production

Strain LR124 produced the highest quantity of biomass, while LR71 was the strain which produced the least (−59.5% compared to LR124). Strains PHCO36 and 235.01 produced a comparable quantity of mycelial biomass (−42.4% and −41.5% compared to LR124, respectively) (**Figure 3**). Differences in growth performance were recorded also by measuring the protein content of the secretome. PHCO36 strain secretome contained 0.36 mg/ml of proteins after filtration, LR124 secretome 0.59 mg/ml, 235.01 secretome 0.54 mg/ml, and LR71 secretome 0.32 mg/ml.

In vivo Wood Degradation and Residual Polymer Analysis

SEM analysis confirms the progress of Fmed colonization of the wood blocks subjected to degradation. Samples were sterilized before incubation with Fmed and, as shown in **Figures 4A,B**, all the structures were free of fungal mycelium. After 30 days' incubation, the vessels were partly colonized by the Fmed mycelium (**Figures 4C,D**). 90 days after inoculation, all the wood structures were widely colonized by Fmed (**Figures 4E,F**). Based

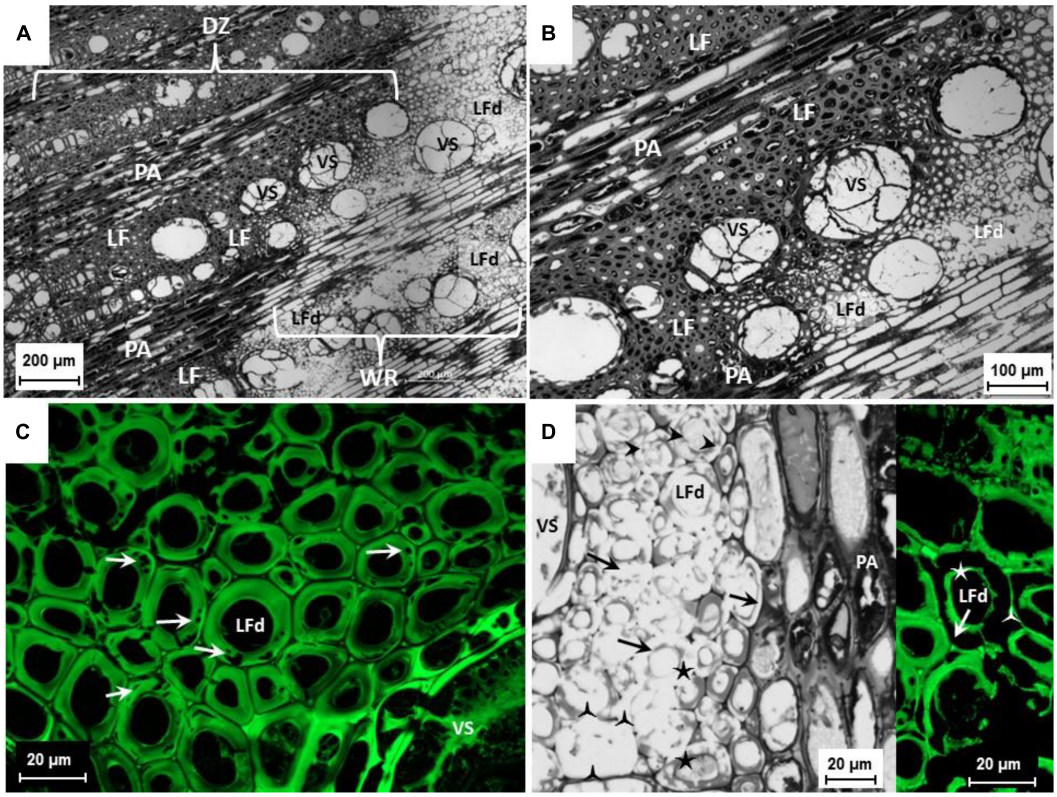

FIGURE 2 | (A,B) Cross section of a symptomatic cv. Sauvignon blanc trunk. Lesion affected by white rot, vessels (VS) obstructed by tylosis; demarcation zone (DZ) identifiable by dark inclusions in the libriform fibers (LF) and parenchymal cells (PA) of the pith rays; white rot (WR) with decomposed libriform fibers (LFd). Bright-field Plan-Neofluar 5x **(A)** and bright-field Plan-Apochromat 10x **(B)**. **(C)** Cross section of a symptomatic cv. Sauvignon Blanc trunk. Lesion affected by white rot, vessels (VS) obstructed by tylosis; libriform fibers (LFd) with beginning of cell wall decomposition, S2 layer of the secondary cell wall with cavities caused by Fmed (arrows). Epi-FM image, excitation 460-488 nm, emission 500-557 nm; C-Apochromat 63x. **(D)** Cross section of a symptomatic cv. Riesling trunk. Lesion affected by white rot, vessels (VS) obstructed by tylosis; libriform fibers (LFd) with beginning of the cell wall decomposition, S2 layer of the secondary cell wall with cavities caused by Fmed (arrow heads) and advanced decomposition of the S2 layer (arrows), only middle lamella with primary cell wall and S1 layer (ג) as well the S3 layer (★) of the secondary cell remained. Bright field, C-Apochromat 40x. On the right extract, libriform fibers (LFd) with advanced cell wall decomposition, S2 layer of the secondary cell wall totally decomposed, only middle lamella with primary cell wall and S1-layer (ג) as well the S3 layer (★) of the secondary cell remained.

on these observations, it was assumed that the degradation of samples was carried out entirely by Fmed.

The weight loss of the wood blocks due to the degradation that occurred in the petri dishes, at 30 and 90 days, was calculated as the difference between the dry weight of the blocks measured before and after degradation. Weight loss after 30 days was less than 5% and comparable for both strains on both varieties. After 90 days' degradation, both strains had been able to degrade the wood of the cv. Gewurztraminer more than the cv. Teroldego. LR124 degraded 9% of cv. Teroldego wood and 16% of cv. Gewurztraminer wood. The PHCO36 strain degraded 7% of cv. Teroldego wood and 10% of cv. Gewurztraminer wood. After 90 days, the LR124 strain had degraded more wood than the PHCO36 strain, as could already be observed after 30 days (**Figure 5**).

In addition to the loss in weight of the samples due to fungal degradation, the quantity of structural polymers of undegraded wood and the decrease of the different wood-forming polymers of wood incubated with Fmed were also analyzed. The lignin content of grapevine wood (15.3% cv. Teroldego and 15.7% cv. Gewurztraminer) was similar to the lignin content of beech (17.7%) and lower than the content in spruce (32.6%). In general, lignin represented the less abundant polymer in grapevine wood, similarly to hardwood, while cellulose and hemicellulose were present in grapevine wood in comparable proportions. The cellulose content of the grapevine wood tested (33.7% cv. Teroldego and 34% cv. Gewurztraminer) resulted lower than the beech (45.9%) and spruce (44.6%) content while hemicellulose represented 34.3 and 35.7% (cv. Teroldego and cv. Gewurztraminer, respectively) of grapevine wood, compared to 32.5% of hardwood and 18.6% of the softwood samples

TABLE 3 | Percentage of fungal taxa isolated from wood fragments sampled from grapevine wood tissue adjacent to the tissue area sampled for microscopy.

Fungal taxa	Incidence (%)
Fomitiporia mediterranea	90
Penicillium spp.	6
Botryosphaeriaceae	4

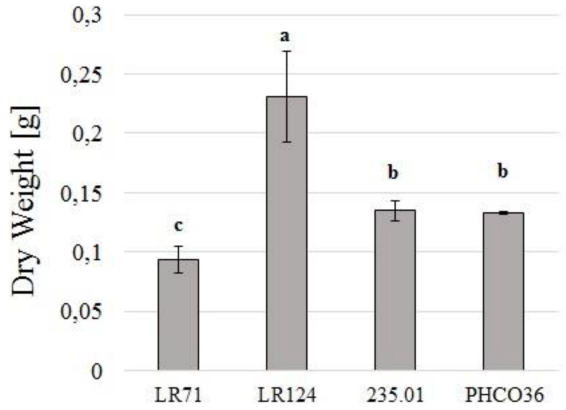

FIGURE 3 | Dry weight of filtered liquid culture of 4 strains of Fmed (PHCO36, LR124, 235.01, LR71) grown at 28°C for 10 days in the dark. Cultures grown in 125 ml of Eriksson and Pettersson's medium in the presence of cellulose and grapevine wood sawdust. Dried matter including everything was retained by 0.2-μm filtration. Statistical differences were calculated by ANOVA analysis ($p \leq 0.05$), and letters above histograms indicate different statistical groups determined by Tukey's test; error bars on histograms represent the standard error of the mean.

(**Figure 6**). The soluble fraction, i.e., pectin, proteins, sugars, and lipids, measured in grapevine wood represented a relevant percentage (16.7% cv. Teroldego and 14.7% cv. Gewurztraminer) compared to the quantity measured in spruce (4.2%) and beech (3.9%) wood.

Afterward, the decrease of soluble compounds, hemicellulose, cellulose, and lignin was evaluated on the incubated samples. In general, as shown in **Figure 7**, the content of soluble compounds increased because of the fungal enzyme activity on the wood components. Hemicellulose was the most degraded polymer by both Fmed strains. After 90 days, the LR124 strain degraded more hemicellulose than the PHCO36 strain on cv. Gewurztraminer wood (−51%) while on cv. Teroldego wood almost the same quantity was degraded by both strains (−36% LR124 and −37% PHCO36). On cv. Gewurztraminer wood, cellulose was degraded more by the LR124 strain (−32% after 90 days) than the PHCO36 strain (−26% after 90 days) as on cv. Teroldego wood (-21% LR124 and −18% PHCO36). Lignin was degraded more by the PHCO36 strain (−8%) on cv. Teroldego wood than the LR124 strain (-5.6%), while a comparable amount was degraded on cv. Gewurztraminer wood (−9.7% LR124 and −9.6% PHCO36). In general, after 90 days' degradation, more hemicellulose was degraded on cv. Gewurztraminer wood (mean −47%) (**Figures 7A,B**), the variety that shows more symptoms of Esca, than on cv. Teroldego wood (mean −37%) (**Figures 7C,D**), the variety that shows fewer symptoms of Esca. The same observation can be made for cellulose (−27% on cv. Gewurztraminer and −19% on cv. Teroldego) and lignin (−9.6% on cv. Gewurztraminer and −7% on cv. Teroldego). Polymer degradation after 90 days of fungal growth was significantly affected by both fungal strain and grapevine variety ($p \leq 0.01$).

Enzyme Activity of Extracellular Protein Extracts

Laccase and MnP-specific activity values (**Figure 8**) evidence that in the tested strains, 235.01 showed the greatest overall specific enzyme activity. The specific laccase and MnP activities of the LR124 strain were among the highest (**Figures 8A,B**), lower than those of strain 235.01, but not statistically different. The specific laccase activity of strain LR71 was the lowest, while the weakest specific MnP activity was recorded for PHCO36. Overall, the enzyme activity of the PHCO36 strain was the weakest despite it not being statistically different from that of the LR71 strain in the laccase assays. Generally, laccase activity was higher than MnP activity for all tested strains.

Gene Expression

With the aim of supporting a specific enzyme activity test, an *in vitro* assay was performed by qRT-PCR to assess the differential ability to express laccase- and MnP-coding genes, in the presence or absence of grapevine wood sawdust (**Figures 9**, **10**). The relative normalized expression of laccase- and MnP-coding genes, regardless of wood sawdust presence, confirms the intra-specific differences observed by enzyme activity tests. However, the data showed how the presence of wood sawdust induces an upregulation of all genes, without changing the trend in basal expression. The laccase-coding gene less affected by the presence of wood sawdust resulted as *Fmlcc7*. Strain 235.01 showed the highest level of transcript abundance of all the laccase-encoding genes compared to the other strains, when cultivated in the presence of wood sawdust. The expression level of the laccase-coding gene by the LR124 strain resulted higher than LR71 for all isoforms except *Fmlcc7*. The PHCO36 strain showed the weakest *Fmlcc* and *Fmmnp* expression in general: 4 laccase-encoding genes of the PCHO36 strain were not upregulated by the presence of sawdust, compared to their basal expression. Moreover, the data show how *Fmlcc3*, *Fmlcc8*, and *Fmlcc9* seem to be the most upregulated laccase-encoding isoforms, in the presence of sawdust, considering all strains (**Figure 9**).

Concerning MnP-encoding genes, strain LR124 showed the highest level of *Fmmnp1* and *Fmmnp2* transcript abundances in the presence of wood sawdust, while strain 235.01 showed the highest level of expression of the *Fmmnp3* gene. Relevant variations in the expression of all the tested MnP-encoding genes were observed: the *Fmmnp1* gene and *Fmmnp3* gene majorly contributed to *Fmmnp* expression compared to *Fmmnp2*, and as recorded for laccase-encoding genes, the presence of wood sawdust induced a higher level of gene expression (**Figure 10**). As recorded for laccase-encoding genes, the PHCO36 strain was shown to be the strain with the weakest MnP-encoding gene expression. For each tested gene, the significance of the difference in gene expression between culture grown with and without sawdust was statistically confirmed ($p < 0.05$).

DISCUSSION

The results obtained in the present study show which wood cellular structures are mainly concerned by the degrading activity

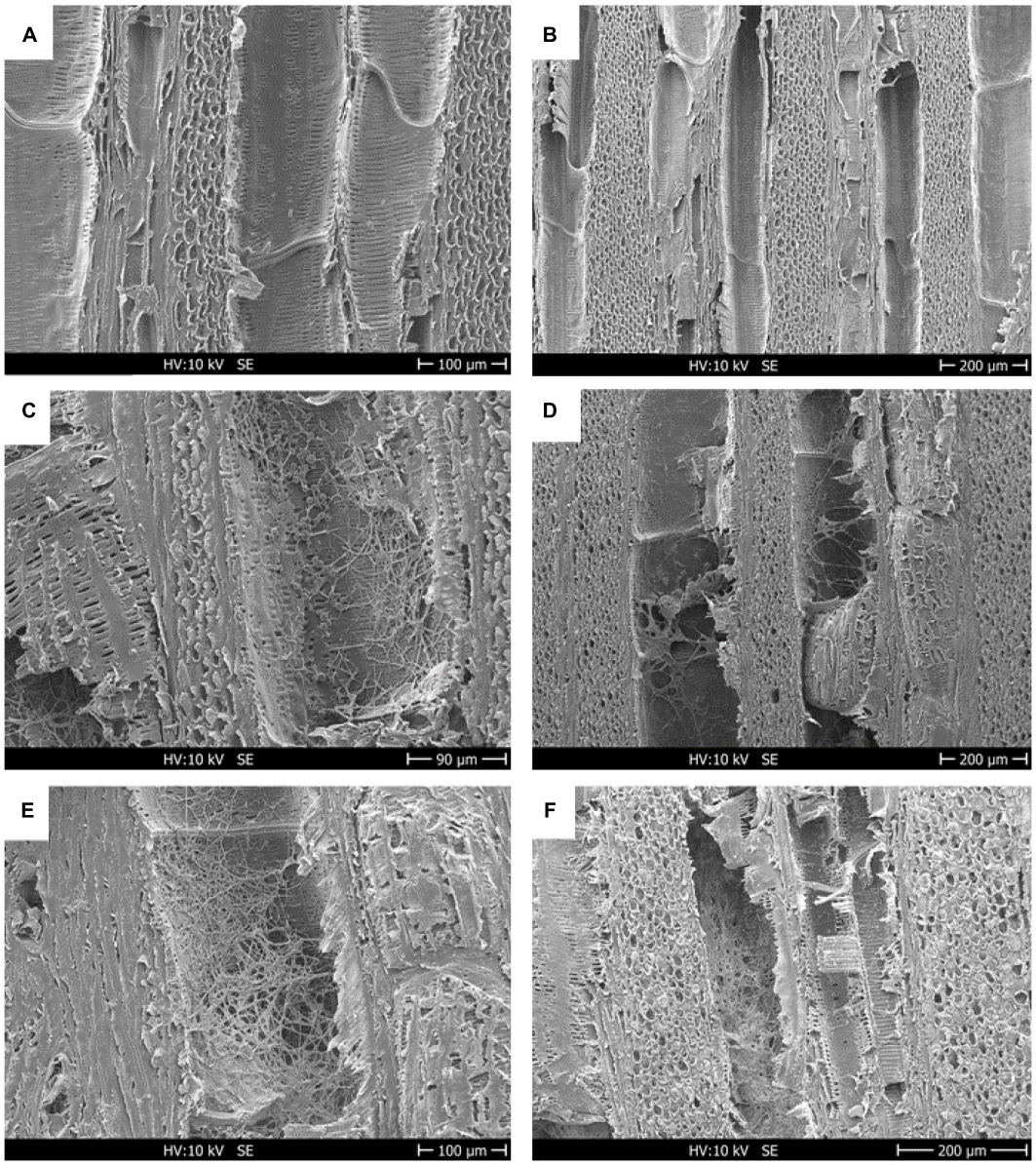

FIGURE 4 | Scanning electron micrographs of cv. Gewurztraminer wood showing the longitudinal face colonized by the white-rot fungus Fmed. Before inoculation by an Fmed-solid culture plug, the wood structures were completely free of mycelium **(A,B)**; a diffuse colonization is appreciable after 30 days from inoculum **(C,D)**, and wider development of the fungus was detected after 90 days **(E,F)**. After 30 and 90 days, vessels and parenchyma cells were colonized by Fmed hyphae. Scale: **(C)** = 90 μm; **(A,E)** = 100 μm; **(B,D,F)** = 200 μm.

of Fmed. The epi-fluorescence microscopy analysis denoted a degrading pathway between selective white-rot and soft-rot characteristics. The degradation of grapevine wood was replicated *in vitro*, and the decrease in each wood-constituent polymer was measured by incubating sterilized vine wood blocks with different Fmed strains: grapevine variety and Fmed strain were shown to be factors influencing the degrading process. Based on wood-constituent consumption, as all the measured polymers were degraded simultaneously (**Figure 7**), the classification as selective white rot cannot therefore be confirmed. Furthermore, the intraspecific variability in laccase and MnP enzyme activity, and finally the gene regulation of these enzymes, was documented. Microscopy observations on the structure of vine wood cells naturally infected by fungal decay showed the common signs of white-rot biodegradation also described by other authors (Eriksson et al., 1980; Schwarze, 2007; Liew et al., 2011). All the woody structure alterations observed in this study were mainly attributed to Fmed after classic fungal isolation on an artificial substrate, which confirmed a very high incidence of Fmed in the rotten vine wood. High incidence of Fmed in the grapevine decayed wood is commonly reported (Larignon and Dubos, 1997; Maher et al., 2012). Fmed is a white-rot

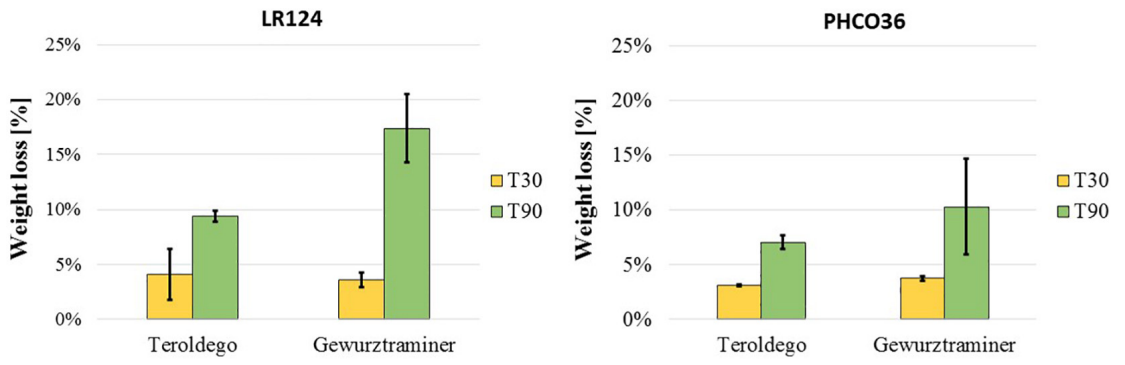

FIGURE 5 | Grapevine wood weight loss after fungal degradation. Two grapevine varieties (cv. Teroldego and cv. Gewurztraminer) were compared. Weight loss is shown as the difference between wood blocks weight before and after degradation. For degradation, wood blocks were inoculated with 2 strains of Fmed (LR124 and PHCO36) and degraded at 28°C in the dark for 30 and 90 days. Error bars on histograms represent the standard error of the mean.

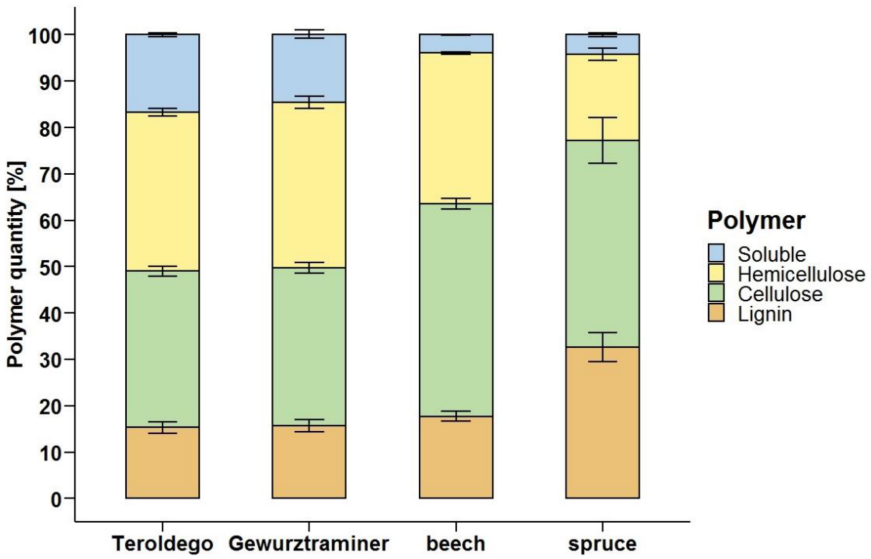

FIGURE 6 | Grapevine (cv. Teroldego and cv. Gewurztraminer), beech, and spruce undegraded wood composition. Comparison of grapevine wood polymer composition to soft- (spruce) and hardwood (beech) profiles. Polymer content measures were performed on non-inoculated sound wood samples.

fungus holding the most efficient wood-degrading enzymatic pool (Floudas et al., 2012; Riley et al., 2014). The observed alteration in grapevine wood structure, mainly at secondary cell wall level and on the median lamella, due to the activity of Fmed, possibly suggests a selective white-rot behavior. In fact, when selective delignification occurs, the decay process starts from the cell membrane side outward and can also affect the middle lamella regions of adjacent cells, leaving fibers weakly structured (Daniel, 1994, 2014). Moreover, the decomposition pattern observed also displays some characteristics of soft rot degradation. The diamond-shaped cavities highlighted by the epi-fluorescence microscopy observations on the S2 layer of secondary cell walls are similar to those formed by soft rot, namely, type I soft rot degradation (Anagnost et al., 1994; Schwarze, 2007; Daniel, 2014). The activity of the Ascomycota species in the formation of the soft rot decay cannot be excluded, although very few colonies other than Fmed have been isolated from the sampled wood. The decay pattern observed on the S2 layer has previously been reported for some other *Basidiomycetes*, but it is known among several wood-degrading Ascomycetes (Schwarze et al., 1995; Martínez et al., 2005). The analysis of the residual polymers carried out on grapevine wood samples after 30 and 90 days' degradation showed that hemicellulose was the wood component most extensively degraded, followed by cellulose and lignin, in that order. The results obtained suggest that as much as 50% of hemicellulose, 30% of cellulose, and 10% of lignin of grapevine wood could be mineralized after 90 days under optimal conditions. The polymer degradation ratio varied significantly between strain and grapevine varieties. Residual polymer analysis showed a non-negligible cellulose consumption, which is uncommon for selective white rot. In selective white rot, lignin and hemicellulose are "preferentially" (selective white

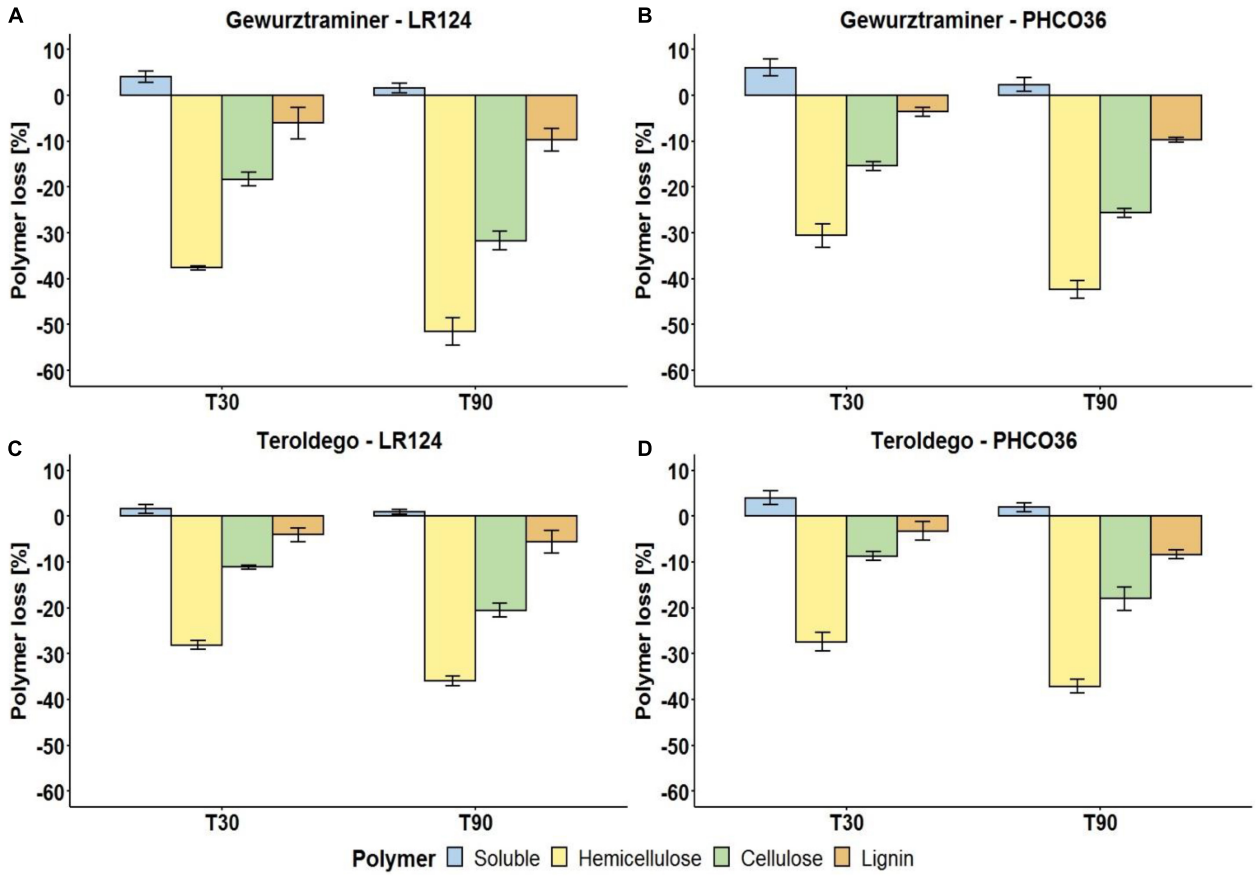

FIGURE 7 | Degradation of wood polymers of two grapevine cultivars [cv. Gewurztraminer **(A,B)** and cv. Teroldego **(C,D)**] by two strains of Fmed [LR124 **(A,C)** and PHCO36 **(B,D)**] after 30 and 90 days. Degradation is presented as a percentage of polymer loss, compared with the untreated wood, 30 and 90 days after inoculation with the fungus. Error bars on histograms represent the standard error of the mean. ANOVA analysis ($p \leq 0.01$) shows that strain and grapevine variety are factors which significantly influence all polymer degradation after 90 days.

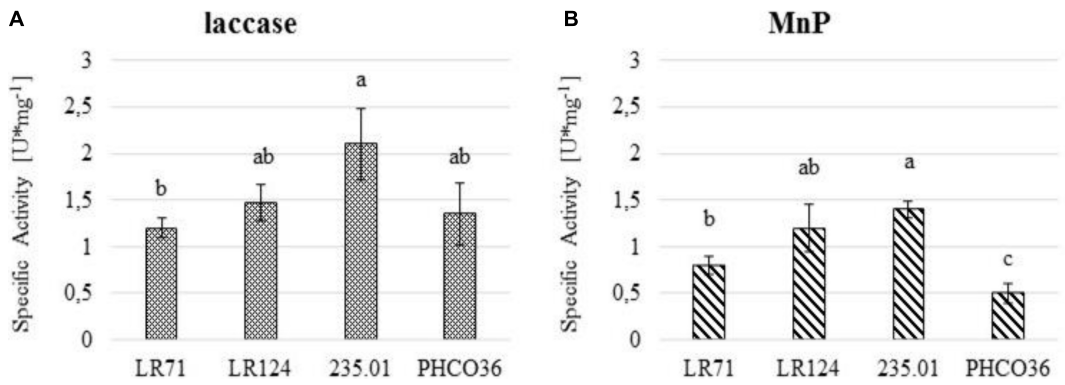

FIGURE 8 | (A) Specific laccase and **(B)** manganese peroxidase (MnP) activity of 4 strains of Fmed measured by spectroscopy. Fmed liquid culture filtered secretome was used to measure the specific enzyme activity. U/mg unit represents 1 μmol of substrate oxidized per minute per mg of protein. ANOVA was performed to determine significant differences ($p \leq 0.05$), and Tukey's test was performed for statistical group identification. Error bars on histograms represent the standard error of the mean.

rot is also named preferential white rot) degraded and a large concentration of cellulose is normally left (Blanchette et al., 1987; Eriksson et al., 1990). Thus, based on the wood polymer consumption, Fmed polymer-relative consumption appears to be more associable with simultaneous white rot than with selective white rot (Blanchette, 1991; Daniel, 2003). Otherwise,

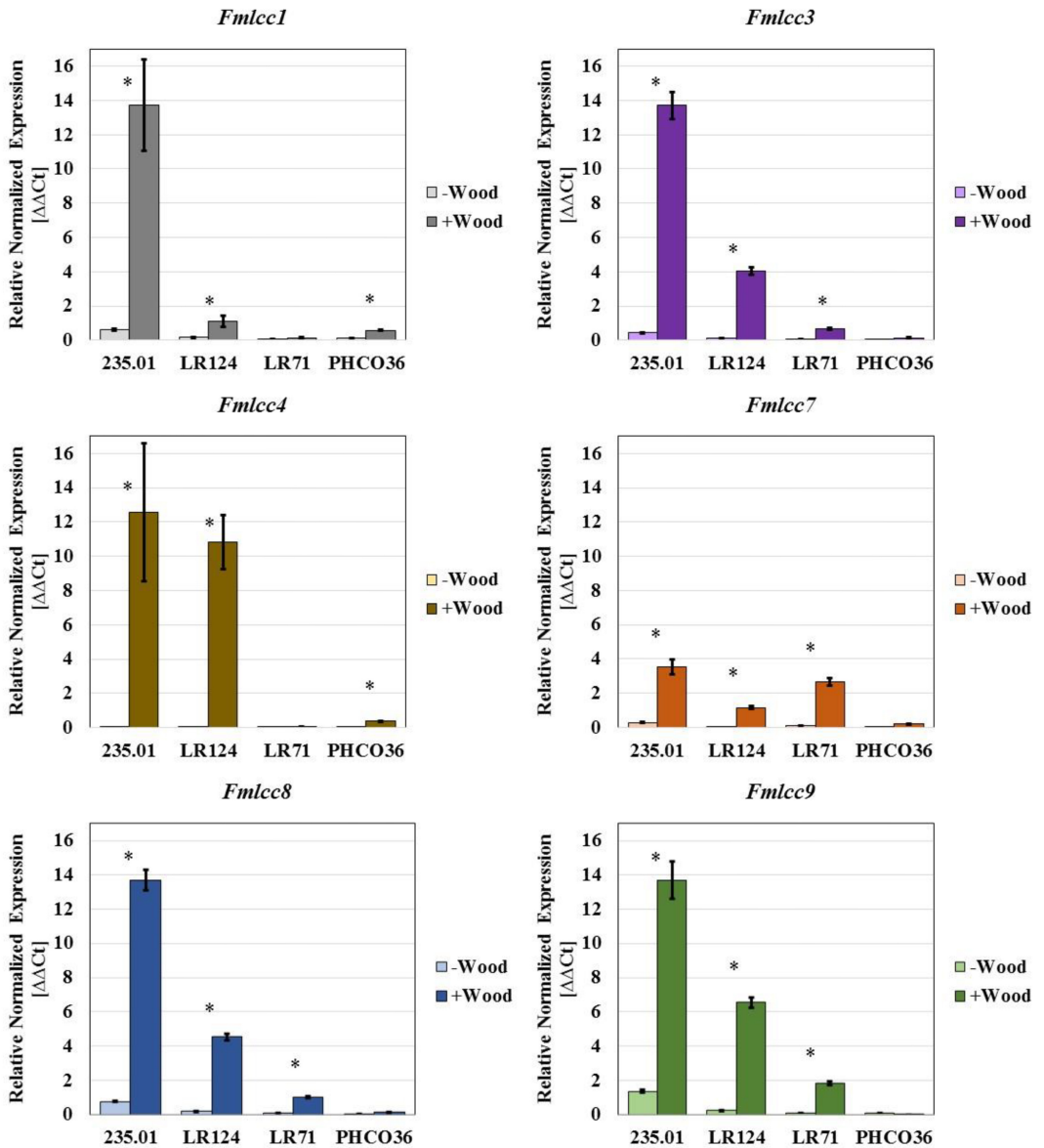

FIGURE 9 | Gene expression of six laccase isoforms measured in 4 different strains of Fmed. Data are expressed as relative normalized expression of genes with cv. Gewurztraminer wood sawdust (+ Wood) and without sawdust (–Wood) compared to the mean of ΔCT of samples grown without wood sawdust. The asterisk denotes significant difference in Student's *t*-test ($p \leq 0.05$) between gene expression with and without wood sawdust. Error bars on histograms represent the standard error of the mean.

this may have occurred because *in vitro* assays reproduce an unreal environment with a high availability of oxygen and a high moisture level. Under aerobic conditions (a quite rare circumstance inside a woody plant (*sensu lato*) solid trunk), white-rot fungi can completely mineralize lignin and wood polysaccharides through the production of hydrogen peroxide as a extracellular oxidant, to CO_2 and H_2O (Sánchez, 2009; Daniel, 2014). Also the high moisture level of the substrate in the petri plate could have influenced the fungal degrading activity (Brischke and Rapp, 2008). This analysis also supplemented data on the polymer constitution of grapevine wood, which had only been reported once in the literature (Agrelli et al., 2009). Afterward, intraspecific differences among the strains were first highlighted by the analysis of laccase and MnP activity and then confirmed *via* gene expression of 6 laccase- and 3 MnP-encoding genes. The reported high number of laccase and MnP isoforms could be potentially explained by the high presence of repetitive sequences in the Fmed genome. In fact, it is generally accepted that transposable elements and microsatellites are responsible for rearrangements and gene mutation in *Basidiomycetes* (Castanera et al., 2017). Laccase activity was slightly predominant compared to MnP activity according

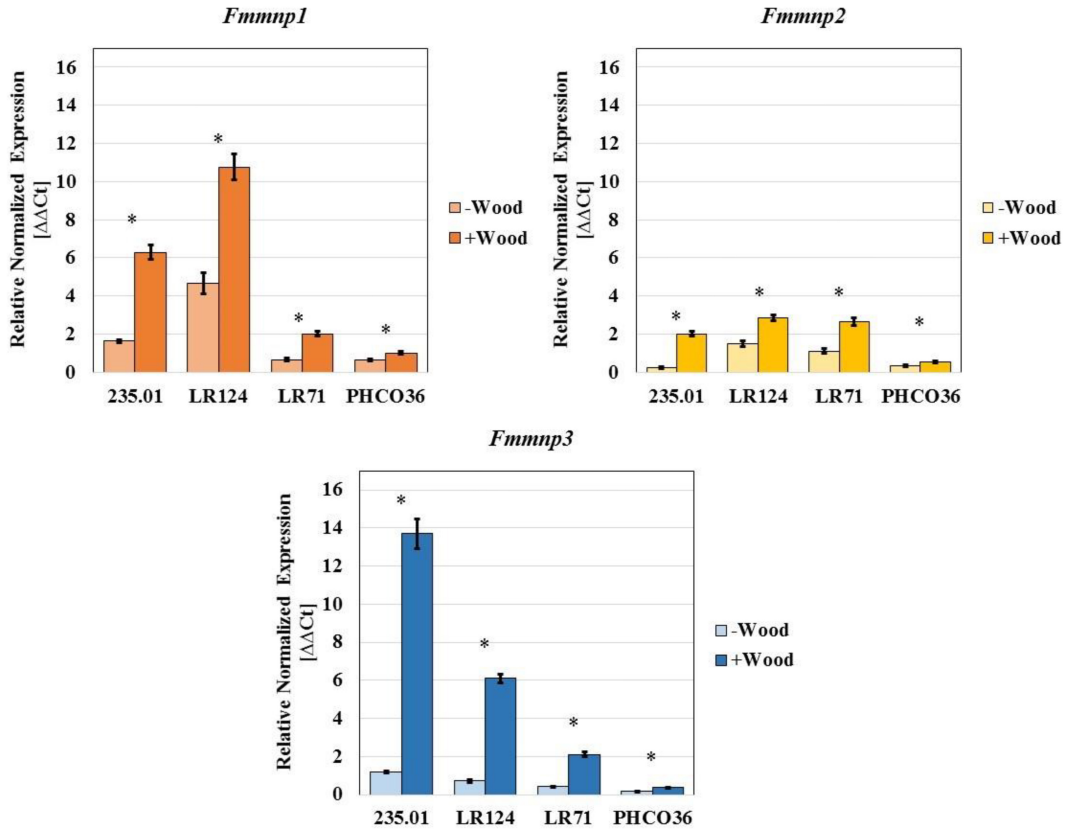

FIGURE 10 | Gene expression of three manganese peroxidase isoforms measured in 4 different strains of Fmed. Data are expressed as relative normalized expression of genes with cv. Gewurztraminer wood sawdust (+ Wood) and without sawdust (–Wood) compared to the mean of ΔCT of samples grown without wood sawdust. The asterisk denotes significant difference in Student's *t*-test with $p \leq 0.05$ between gene expression with and without wood sawdust. Error bars on histograms represent the standard error of the mean.

to the results obtained on different white-rot *Basidiomycetes* (Mathieu et al., 2013). The white-rot wood-degrading enzymatic pool, the oxidative and extracellular ligninolytic system which depolymerizes lignin, is solidly represented by laccase, MnP, and LiP. Laccases are phenoloxidase that oxidize phenolic and non-phenolic compounds in the presence of specific mediators (Bourbonnais et al., 1995; Call and Mücke, 1997; Gianfreda et al., 1999; Majcherczyk et al., 1999). On phenolic compounds, one-electron oxidation generates phenoxy-free-radical products, which can lead to polymer cleavage. MnPs catalyze the Mn(II) oxidation to Mn(III) after its chelation by organic acid, in the presence of H_2O_2 (Kuwahara et al., 1984; Glenn and Gold, 1985). Mn(III) oxidated by MnPs can oxidize phenolic compounds but not non-phenolic units of lignin (Pérez et al., 2002). Upon completion, the hydrolytic system of fungi, responsible for cellulose and hemicellulose degradation, is formed by a wide range of enzymes: hemicellulose biodegradation needs the joint action of several enzymes such as xylan esterases, ferulic and p-coumaric esterases, α-l-arabinofuranosidases, and α-4-O-methyl glucuronidases (Kirk and Cullen, 1998), while cellulases, namely, endoglucanases (EGs) and cellobiohydrolases (CBHs), synergically degrade cellulose. The degradation of lignin-based cellular structures observed by epi-fluorescence microscopy and the polymer loss measured in this study can therefore both be attributed to the enzymes mentioned above (Goodell, 2020). LiP activity was not considered in this study, based on evidence that excludes the presence of LiP-encoding genes in the Fmed genome (Floudas et al., 2012). In order to gain further details on the fine regulation of grapevine wood degradation process by Fmed, the gene expressions of MnP and laccase-encoding genes were studied. According to the literature, the use of a gene expression stimulator (sawdust in our case) allowed an upregulation of studied genes (Mansur et al., 1998; Giardina et al., 2000; Ohga and Royse, 2001; Hakala et al., 2006; Sakamoto et al., 2009). Regardless of wood sawdust presence, the results highlight a basal expression of the MnP-encoding genes higher than that of laccase. Furthermore, the expression of the targeted genes highlighted remarkable intraspecific variability, in line with the observed genetic population variability (Pollastro et al., 2001; Jamaux-Despréaux et al., 2003). The measurement of the enzyme activity *in vitro* and the correlated gene expression are consistent with results previously obtained by other authors (Abou-Mansour et al., 2009; Morgenstern et al., 2010; Cloete et al., 2015a). In conclusion, if we assume that the pores of the cell walls may not be large enough to allow the studied enzymes to reach the core of the secondary cell wall, as proposed by

other authors for other pathosystems, the cavitation phenomena observed in the S2 layer of the secondary cell walls cannot be justified solely by the activity of the enzymes (Evans et al., 1991, 1994; Flournoy et al., 1991, 1993; Paice et al., 1995). Therefore, a lack of link between the degradative potential of the tested enzymes and the evidence observed *via* microscopy emerges as a general result from this multidisciplinary experiment. Based on this, we can thus hypothesize that a preliminary non-enzymatic activity, i.e., the Fenton-type chemical reactions, allowed the enlargement and the damage of the cell wall pores in order to allow the enzymes to produce the observed degradation effects (Goodell et al., 1997, 2017, 2019; Arantes and Milagres, 2009; Moretti et al., 2019). The role of low molecular weight compounds (LMWC) postulated by these authors is also crucial in other white-rot fungi, since they can also act as mediators of specific lignin-degrading enzymes such as laccase and MnP (Faison et al., 1986; Jellison et al., 1991; Dutton et al., 1993; Eggert et al., 1996; Ten Have and Teunissen, 2001; Arantes and Milagres, 2007). During this preliminary phase, bacterial activity could also potentially influence wood attack (Haidar et al., 2021). Considering what was observed in this study and taking into account the information just introduced, further investigations on the wood-degrading mechanisms caused by *Fomitiporia mediterranea*, as well its interaction with the wood microbiota, are required to shed light on factors possibly involved in foliar symptom expression.

CONCLUSION

Over the years, several studies have focused on putative factors that can trigger the Esca-associated leaf stripe symptoms: metabolomic, metagenomic, biochemical, and chemical approaches have been adopted. Much evidence has been presented over the last few years, but even today many aspects of the ECD continue to be unclear. The complex etiology attributed to the leaf symptom development, namely, GLSD, makes it even more difficult to determine which factors may actually influence the generation of symptoms. This study highlighted the most important metabolic aspects of one of the main fungal species associated with the Esca complex. The role of *Fomitiporia mediterranea* in Esca diseases has recently been investigated in more depth, and the results presented here not only strengthen knowledge on the enzymatic processes that lead to the degradation of vine wood but also pave the way for a new line of research, focused on the processes of synergistic enzymatic and non-enzymatic degradation of wood, which could be involved in triggering the foliar symptoms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AP contributed to the study design, conducted the laboratory trials, analyzed the data, and wrote the original manuscript. SM contributed to the study design, assisted with the laboratory trials, and improved the manuscript. CP assisted the laboratory trials. EB conducted the microscopy trials. H-HK provided the naturally infected wood samples and revised the manuscript. EG revised the manuscript. LM revised and polished the manuscript. SF and CB designed the study. All authors contributed to the manuscript and approved the submitted version.

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