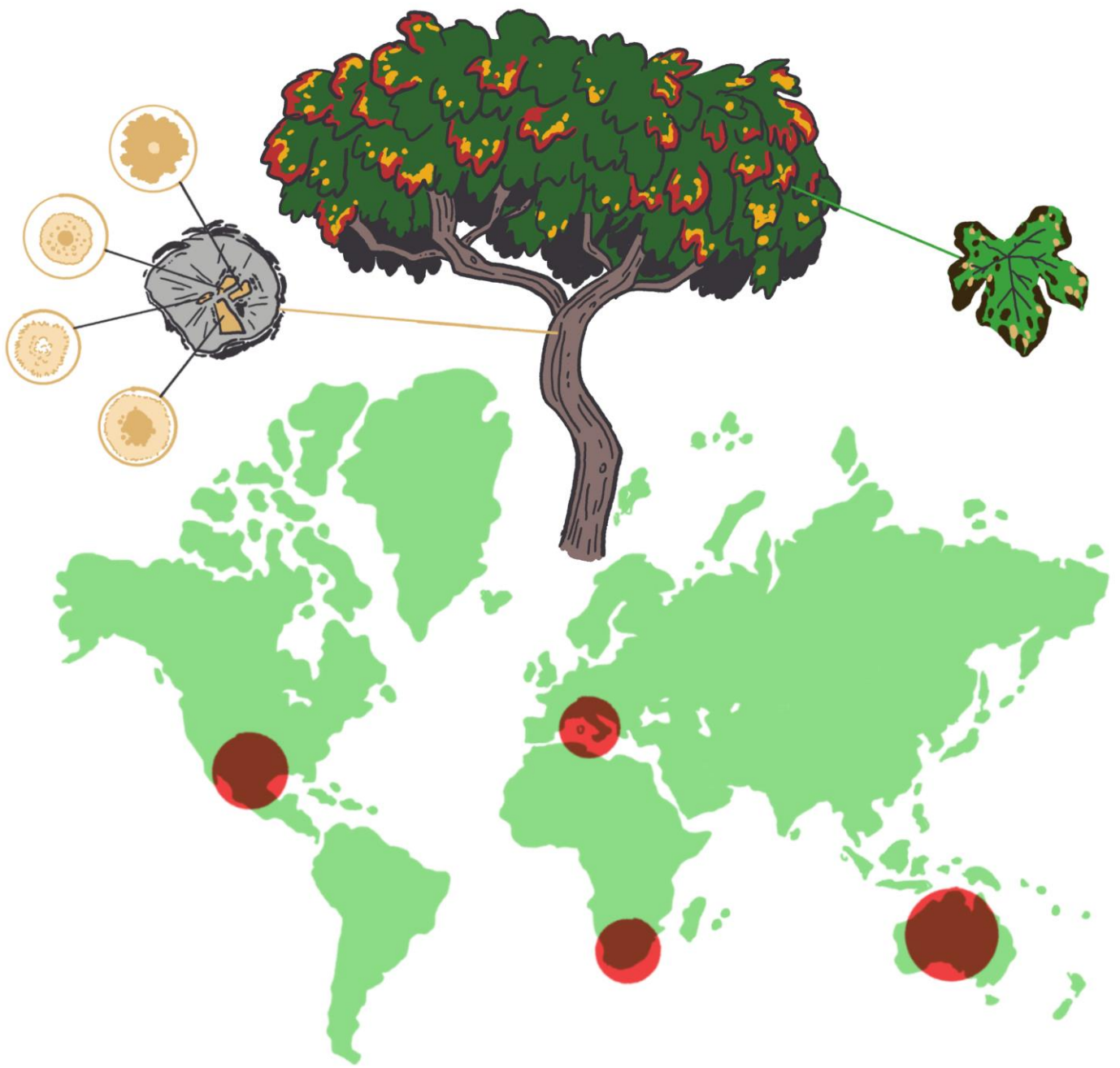


# Enzymatic and Non-Enzymatic Wood Degradation by Basidiomycetes in the Esca Complex of Grapevine

PhD Thesis



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UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

PhD in  
Agricultural and Environmental Sciences  
CYCLE XXXVII

# Enzymatic and Non-Enzymatic Wood Degradation by Basidiomycetes in the Esca Complex of Grapevine

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Anni 2021/2025

Année 2025

N° d'ordre : (attribué par le SCD)

UNIVERSITÉ DE HAUTE-ALSACE  
UNIVERSITÉ DE STRASBOURG

# THESE

Pour l'obtention du grade de  
**DOCTEUR DE L'UNIVERSITÉ DE HAUTE-ALSACE**  
**ECOLE DOCTORALE : Sciences Chimiques (ED 222)**  
Discipline : Sciences de la vie

Présentée et soutenue publiquement

par

**Alessandro PUCA**

Le /2025

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## Enzymatic and Non-Enzymatic Wood Degradation by Basidiomycetes in the Esca Complex of Grapevine

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Riferito a questa tesi :

*“ D’icch  c’  un manca nulla!”*

Il mio amico Nicola.

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## Abstract

Esca Complex of Diseases (ECD) is widespread in viticultural areas worldwide, posing a serious risk to modern viticulture. The role of basidiomycetes in the onset of foliar symptoms has recently been reconsidered, highlighting a link between the reduction in these symptoms and the presence of *Fomitiporia mediterranea* in vines. This pathogen is considered the main decay agent on the grapevine in the European basin and is the most studied among white rot fungi affecting grapevines. However, the global spread of ECD requires a broader perspective, considering other wood-decay fungi associated with grapevines that are much less known than *F. mediterranea*.

Wood degradation is central to many of the hypotheses explaining the development of foliar symptoms, making investigating the biomolecular mechanisms underlying wood decay essential to understanding ECD etiology. With the recent re-evaluation of the distinction between brown and white rot fungi, the first study of this thesis explored the non-enzymatic degradation capabilities of *F. mediterranea* (Chapter II).

To address the knowledge gap regarding other fungal species and to identify commonalities among white rot agents, an investigation of the degradation capabilities of *F. capensis* (South Africa), *F. australiensis* (Australia), *F. langloisii*, *F. polymorpha*, *Tropicoporus texanus* and *Inonotus vitis* (North America), and *Stereum hirsutum* (Europe) was conducted. The analyses focused on lignocellulolytic enzyme activity and the potential implementation of non-enzymatic mechanisms by these fungi (Chapter III). Moreover, the phenotypical characteristics of the fungal collection were investigated regarding the *in vitro* degradation capabilities of grapevine wood and temperature preferences (Chapter IV).

Under nutrient-restricted conditions, *F. mediterranea* was found to produce low molecular weight compounds (LMWC) capable of driving all the steps of the chelator-mediated Fenton (CMF) mechanism. These steps include acidifying the surrounding environment, reducing ferric iron, producing hydrogen peroxide in the process, and ultimately generating hydroxyl radicals.

Importantly, this ability was found to be shared among the studied white rot agents, as all the LMW fractions of their secretomes triggered a radical-generating CMF mechanism. Analyses of the lignocellulolytic activity of fungal secretomes revealed a low production of peroxidase activity, suggesting the importance of the CMF mechanism as a secondary degradation method potentially acting synergistically with the enzymatic pool. This study provides critical insights into the degradation strategies of ECD-associated white rot agents, offering potential new perspectives on studying ECD symptomatology and developing targeted control strategies. The findings emphasize the need to consider the potential role of the CMF mechanism in ECD development. This understanding could potentially be applied to control strategies, as disrupting various phases of the CMF mechanism might represent a novel control strategy on the grapevine. Future research should focus on *in planta* studies to further evaluate the role of wood degradation in ECD symptoms development.

## Riassunto

Il Complesso del mal dell'Esca (ECD) è diffuso nelle zone vitivinicole di tutto il mondo, rappresentando un serio rischio per la viticoltura moderna. Il ruolo dei basidiomiceti nell'insorgenza dei sintomi fogliari è stato recentemente rivalutato, mettendo in evidenza un legame tra la riduzione di tali sintomi e la presenza di *Fomitiporia mediterranea* nelle viti. Questo patogeno è considerato il principale agente di carie della vite nel bacino europeo ed è il più studiato tra i funghi cariogeni che colpiscono la vite. Tuttavia, la diffusione globale dell'ECD richiede una visione più ampia, considerando altri funghi agenti di carie bianca associati alla vite, che sono molto meno conosciuti rispetto a *F. mediterranea*.

La degradazione del legno è centrale per molte delle ipotesi che spiegano lo sviluppo dei sintomi fogliari. Ciò rende essenziale l'indagine dei meccanismi biomolecolari alla base della degradazione del legno per comprendere l'eziologia del ECD. A seguito della recente rivalutazione della distinzione tra agenti di carie bruna e di carie bianca, il primo studio di questa tesi ha esplorato le capacità di degradazione non enzimatica di *F. mediterranea* (Capitolo II).

Per colmare il divario di conoscenze riguardo ad altre specie fungine e identificare eventuali tratti comuni tra gli agenti di marciume bianco, è stata condotta un'indagine sulle capacità di degradazione di *F. capensis* (Sud Africa), *F. australiensis* (Australia), *F. langloisii*, *F. polymorpha*, *Tropicoporus texanus*, *Inonotus vitis* (Nord America) e *Stereum hirsutum* (Europa). Le analisi si sono concentrate sull'attività enzimatica lignocellulolitica e sulla possibile implementazione di meccanismi non enzimatici da parte di questi funghi (Capitolo III). Inoltre, sono state investigate le caratteristiche fenotipiche della raccolta fungina, riguardo le capacità di degradazione in vitro del legno di vite e le preferenze di temperatura (Capitolo IV).

In condizioni di carenza di nutrienti, *F. mediterranea* è stata osservata in grado di produrre composti a basso peso molecolare (LMWC) capaci di guidare tutte le fasi del meccanismo Fenton mediato da chelatori (CMF). Queste fasi comprendono l'acidificazione dell'ambiente circostante, la riduzione del ferro ferrico, la produzione di perossido di idrogeno durante la riduzione del ferro e, infine, la generazione di radicali idrossilici.

Dai risultati è emerso che questa capacità è stata condivisa tra gli agenti di marciume bianco studiati, poiché tutte le frazioni LMW dei loro secretomi hanno innescato un meccanismo CMF generatore di radicali. Le analisi dell'attività lignocellulolitica dei secretomi fungini hanno rivelato una bassa produzione di attività perossidasi, suggerendo l'importanza del meccanismo CMF come metodo di degradazione secondario che potrebbe agire sinergicamente con il pool enzimatico.

Questo studio fornisce approfondimenti critici sulle strategie di degradazione degli agenti di carie bianca associati al ECD, offrendo nuove prospettive nello studio della sintomatologia dell'ECD e nello sviluppo di strategie di controllo mirate. I risultati sottolineano la necessità di considerare il potenziale ruolo del meccanismo CMF nello sviluppo dell'ECD. Questa comprensione potrebbe essere applicata a strategie di controllo, poiché il disturbo di varie fasi del meccanismo CMF potrebbe rappresentare una nuova strategia di controllo dei patogeni. Future ricerche dovrebbe concentrarsi su studi *in planta* per valutare ulteriormente il ruolo della degradazione del legno nello sviluppo dei sintomi del ECD.

## Résumé

Les complexes de champignons associés à l'Esca (ECD) sont largement répandus dans les régions viticoles du monde entier, représentant une menace sérieuse pour la viticulture moderne. Le rôle des basidiomycètes dans l'apparition des symptômes foliaires a récemment été reconsidéré, mettant en évidence un lien entre la réduction de ces symptômes et la présence de *Fomitiporia mediterranea* dans les vignes. Ce pathogène est considéré comme l'agent principal de la dégradation du bois de la vigne dans le bassin européen et est le champignon de pourriture blanche le plus étudié sur la vigne. Cependant la présence au niveau mondiale de l'ECD nécessite d'élargir le modèle d'étude, et de prendre en compte d'autres champignons lignivores associés à la vigne, moins connus que *F. mediterranea*.

Le fait que la dégradation du bois ait un rôle à jouer dans le développement des symptômes foliaires fait partie des hypothèses les plus soutenues à l'heure actuelle. Cela rend indispensable l'étude des mécanismes biomoléculaires sous-jacents à cette dégradation pour comprendre l'étiologie de l'ECD. Faisant écho à la récente réévaluation de la distinction entre les champignons de pourriture brune et blanche, le premier axe d'étude de cette thèse visait à explorer les capacités de dégradation non enzymatique de *F. mediterranea*.

Pour combler le manque de connaissances sur d'autres espèces fongiques et identifier des points communs parmi les agents de pourriture blanche, une investigation des capacités de dégradation de *F. capensis* (Afrique du Sud), *F. australiensis* (Australie), *F. langloisii*, *F. polymorpha*, *Tropicoporus texanus* et *Inonotus vitis* (Amérique du Nord), ainsi que *Stereum hirsutum* (Europe), a été menée. Les analyses se sont concentrées sur l'activité enzymatique lignocellulolytique et la mise en œuvre potentielle de mécanismes non enzymatiques par ces champignons. De plus, une analyse des caractéristiques phénotypiques de la collection de champignons, tels que l'identification de la température optimale de croissance et la capacités de dégradation du bois de vigne, a été conduite in vitro.

En conditions de cultures pauvres en nutriments, il a pu être mis en évidence que *F. mediterranea* produisait des composés de faible poids moléculaire (LMWC) permettant la réalisation de toutes les étapes du mécanisme de Fenton médié par chélateur (CMF). Ces étapes incluent l'acidification de l'environnement, la réduction du fer ferrique, la production de peroxyde d'hydrogène et la génération de radicaux hydroxyles.

Il est important de noter que cette capacité a pu être démontrée chez tous les agents de pourriture blanche étudiés. Toutes les fractions de LMW issues du sécrétome de ces champignons ont permis de déclencher la mise en place d'un mécanisme CMF et d'induire la production de radicaux. Les analyses de l'activité lignocellulolytique des sécrétomes fongiques ont révélé une faible activité peroxydase, suggérant que le mécanisme CMF permet la mise en place d'un processus de dégradation secondaire, agissant potentiellement de façon synergique avec le pool enzymatique.

Cette étude apporte de nouvelles connaissances sur les stratégies de dégradation des agents de pourriture blanche associés à l'ECD, offrant de nouvelles perspectives pour étudier la symptomatologie de l'ECD et développer des stratégies de contrôle ciblées. Les résultats soulignent l'importance de considérer le rôle potentiel du mécanisme CMF dans le développement de l'ECD. Cette compréhension pourrait être appliquée aux stratégies de contrôle. En effet, perturber les différentes phases du mécanisme CMF pourrait représenter une nouvelle stratégie de lutte contre les agents de l'ECD. Les recherches futures devraient se concentrer sur des études in planta pour évaluer davantage le rôle de la dégradation du bois dans le développement des symptômes de l'ECD.

## **Chapter I - Introduction**

### 1. The Grapevine and Viticulture

The earliest traces of wine and viticulture date back to 6,000–5,800 BC during the early Neolithic Period, in an area between the Black Sea and Iran, more than 2000 years before the invention of writing (Estreicher, 2017; McGovern et al., 2017). Throughout history, the wild Eurasian grapevine *Vitis vinifera* sp. *sylvestris* and later its domesticated version *V. vinifera* sp. *sativa* has held immense cultural, economic, and symbolic importance throughout human civilization (Magris et al., 2021). Wine has been deeply involved with many religions (Outreville, 2021). In Egyptian culture, wine was associated with Osiris, the god of the afterlife and agriculture. In Greek mythology, wine was represented by the god Dionysus, later Bacchus, in the Roman pantheon. Wine still holds a significant role nowadays in Judaism, Christianity as well as other religions (Stanislawski, 1975). Domestication of the grapevine from its primary domestication center spread into nearby regions such as Northern Africa and the Mediterranean basin, mainly by Phoenician trade (Riaz et al., 2018). As the early Roman Empire expanded, grapevines were transported to conquered territories throughout Europe, and in the first century, grapevine cultivation reached Britain and was well-established in northern France and Germany (Mullins et al., 2007). In the East, wine cultivation was introduced to Central Asia during the first millennium BCE, and in the second century BCE, domesticated grapes had reached China and Japan (McGovern et al., 2017). The European grapevine was brought to North America following the spread of European colonialism and later also spread to Australia, New Zealand, and South Africa (Estreicher, 2017).

The cultivation of the grapevine had a tremendous impact on the European landscape. In its text “*De Rustica*” dating back to the first century, Columella describes the practice of the *arbustum*, a technique to structure a vineyard where grapevines would have been cultivated following the plant's natural tendencies, as a climbing liana. This would lead to the spread of an early agroforestry system in which the grapevine was “married”, from the Italian name of the practice “*Vite maritata*” to trees such as poplar, maples, elms or olive trees (Olson, 1943). Being a crop with a history as ancient as it is, the grapevine has deep cultural significance in many regions of the world. This is clearly shown by the ample use of the grapevine and wine as symbolic elements in art during the ages. In Egypt, wine had a significant ritual meaning as it was used as an offer to the dead for their journey to the afterlife and a symbol of death and resurrection. Greek and Roman cultures commonly represented frescoes or other artistical pieces with grapes as a symbol of richness and prosperity (Savo et al., 2016).



Figure 1 Dionysus Cup - Greek kylix (drinking cup) attributed to Exekias (circa 540–530 BCE), showing Dionysus reclining on a ship surrounded by dolphins and grapevines, symbolizing the myth of the Tyrrhenian pirates. This decorative piece is held at the Staatliche Antikensammlungen in Munich (Inv. 2044).

Today, the grapevine is among the most widely grown fruit crops globally. In 2022, there were 7.2 mha of estimated vineyard area worldwide and 213.65 million metric tons of grapes produced. Grapes are mainly produced for winemaking purposes (47.9% of 2022 total production) but can also be used as table grapes (44.2%), and dried grapes (8.0%) (OIV 2022). In addition, 94% of global wine production is accounted in 29 countries. As of 2023, Spain is the largest vineyard in the world with a total of 945 kha, followed by France with 792 kha, China with 756 kha, Italy with 720 kha, and Turkey with 410 kha. In 2023, the global wine market was valued at approximately USD 428.96 billion (OIV 2023). In addition to its economic importance as a food and wine crop, the type of tourism defined as oenotourism is gaining increasing attention and leading to important economic growth for most of the historically rich winemaking areas of the world (Hojman and Hunter-Jones, 2012; Molina et

al., 2015). Thus, it is unsurprising that the grapevine is considered an important crop today for economic and cultural reasons.

Nowadays, many factors threaten grapevine cultivation, among which one of the most important is the current climate emergency. The most recent report of the ICCP (2023) predicts that, at best, temperatures will only rise by 1.5°C if no significant action is taken immediately (Calvin et al., 2023). Increasing global temperatures are expected to lead to a rising frequency and intensity of heat waves, as well as changes in precipitation patterns, leading to droughts and heavy rainfall. As the quantity and quality of the yield greatly depends on environmental factors, this will greatly reduce both (Zajac et al., 2022; Sodini et al., 2023). In addition, the impact of the rising temperatures has been predicted to be higher for countries characterized by a Mediterranean climate, like the ones where most of the grapevine cultivation is concentrated (Hannah et al., 2013). This all means that climate change could lead to significant geographical displacement in traditional winemaking areas, leading to a reduction in cultivable areas (Droulia and Charalampopoulos, 2021).

On the other hand, grapevine cultivation is plagued with the susceptibility to a vast range of biological threats in which there are a wide range of microorganisms such as viruses, nematodes, oomycetes, bacteria, and fungi (Wilcox et al., 2015; Travadon et al., 2016). Downy mildew is a disease caused by the obligate biotroph *Plasmopara viticola*, which can be classified as one of the oldest threats to modern viticulture, causing massive economic losses since its first detection in Europe in 1878 (Gessler et al., 2011). Around the same time, the ascomycetes *Erysiphe necator* arrived in Europe from North America, spreading another deadly disease called Powdery mildew (Gadoury et al., 2012). Many more fungal diseases spread on the grapevine, such as anthracnose (*Elsinoe ampelina*) and black rot (*Phyllosticta ampellicida* syn. *Guignardia bidwellii*). In addition, there are quite a few pathogens characterized by a vast number of possible hosts that cause great damage to the grapevine, such as the ascomycete *Botrytis cinerea* and the gram-negative bacteria *Xylella fastidiosa*, not to mention the phytoplasma *flavescence dorée* and the damages it causes in Europe (Rapicavoli et al., 2018; Altieri et al., 2023). Additionally, Grapevine fanleaf and Grapevine leafroll are among the most widespread plant viruses in the world, significantly debilitating the grapevine in each viticulture area where they are present (Reynolds, 2017).

## 2. Grapevine Trunk Diseases, the Rise of an Ancient Threat

Recent decades have seen the rise of another ancient enemy of viticulture: Grapevine Trunk Diseases (GTDs) (Mugnai et al., 1999; Bertsch et al., 2009; Kovács and Sándor, 2016). As suggested by the name, GTDs are defined as all those diseases in which pathogens are mainly located in the woody parts of the vines, such as trunk and cordons, and in which symptoms are a product of the slow direct or indirect activity of fungal pathogens (Bertsch et al., 2009; Kenfaoui et al., 2022). GTDs are difficult to diagnose and are among the most serious threats to worldwide viticulture, as they can cause great losses in terms of productivity by negatively affecting the yield and organoleptic qualities of the final product (Beris et al., 2023). Symptoms associated with this group include vascular symptoms, such as wood streaking and wood cortical necroses, i.e., wood cankers, as well as canopy symptoms on leaves, shoots, and berries. Thus, GTDs affect the grapevine on two different time dimensions: annual vegetative organs, such as leaves and fruits, can be attacked, greatly influencing final profit, while a continuous influence on the whole vine physiology occurs due to the relentless slow colonization by xylem-inhabiting fungi (Schilling et al., 2021; Travadon et al., 2024). This complicated symphony of symptoms can put great stress on affected vines, causing a reduction in the profitable lifespan of vineyards (Hillis et al., 2016; Guarnaccia et al., 2023). This reduction in productivity and longevity ultimately translates into significant financial implications for the winemaking and table grape industries. Furthermore, most of the yearly symptoms are characterized by a year-by-year fluctuation as they are strictly related to environmental, climatic, and agronomical factors (Surico et al., 2000; Murolo and Romanazzi, 2014). Over the last three decades, an increase in the incidence of these diseases has been recorded globally (Bertsch et al., 2013; Beris et al., 2023). The rise in the severity of GTDs is to be included in the bigger picture of the increasing impact of fungal trunk diseases affecting trees and fruit crops, especially if cultivated (Gramaje et al., 2016; Guarnaccia et al., 2023). There are many hypotheses as to why this is happening, both biotic and abiotic. Agricultural practices such as high-density planting, pruning methods, grafting as well as the widespread use of mechanization can greatly increase the susceptibility of woody plants to trunk diseases (Kovács and Sándor, 2016; Travadon et al., 2016; Gramaje et al., 2018; Beris et al., 2023; Rosace et al., 2023a). Additionally, global warming and climate change can cause great stress on plants, creating better conditions for diseases to spread and can influence pathogens life cycle, increasing inoculum production (Bostock et al., 2014). Lastly, the rising impact of GTDs in some countries has also been associated by some authors with the ban on the use of sodium

arsenite in Italy in 1977, in France in 2001, and in other European countries in 2003 as a control strategy (Spinosi et al., 2009), although there has been an increase in all wine-growing countries, even where it was not used. This topic will be further discussed in later chapters.

Nowadays, GTDs incidence is quite important, and they are to be considered of great economic importance, especially considering the rise in economic impact in the last 30 years (Gramaje et al., 2018). In France, GTDs incidence has reached an estimated average of close to 13% annually in unproductive vineyards (Bruez et al., 2013). A similar incidence can be found in Spain, where after the ban on sodium arsenite in 2003, the percentage of GTDs symptoms in vineyards has grown from 1.8% to 10.5% in less than a decade (Rubio and Garzón, 2011). In Italy, the incidence of GTDs was assessed to be from 8% to 19%, varying in different regions and cultivar (Mugnai et al., 1999; Fontaine et al., 2016). North America also faces challenges, with most Californian vineyards experiencing infection from one or more GTD agents, resulting in significant economic losses, surpassing \$260 million annually in California alone (Siebert, 2001). Plants affected by GTDs are often replaced. It is estimated that the cost of a 1% annual replacement rate exceeds 1.5 billion dollars (Hofstetter et al., 2012).

### 2.1. The main Grapevine Trunk Diseases

The GTDs group is composed of different diseases such as Eutypa dieback, Diaporthe dieback, Phomopsis cane and leaf spot, Botryosphaeria diebacks, and Esca Complex of Diseases (ECD – see section 3). These diseases are more commonly observed in mature vines, typically starting around 8 years old or even younger, with their incidence increasing as the vineyard ages (Romanazzi et al., 2009; Hofstetter et al., 2012). On the other hand, on younger vines, the main GTDs are Petri disease and diebacks caused by Botryosphaeriaceae, black foot agents, *Fusarium*, or, rarely, *Verticillium* species (Mugnai et al., 1999; Guerin-Dubrana et al., 2019; Claverie et al., 2020). GTDs that affect younger grapevine plants will not be addressed in the context of this introduction. Many fungi associated with these diseases can have quite a taxonomical distance (Mondello et al., 2018b). Most of these fungi are ascomycetes, and some basidiomycetes can slowly colonize the vine xylem, and all of them are capable of living in the wood, giving latent infections that end up causing damage following inciting factors such, for example, water stress as it happens in Botryosphaeria dieback (Slippers and Wingfield, 2007). Usually, these species have wide host ranges that include woody crops such as grapevine, kiwifruit, stone fruits, and other fruit trees, as well as wild woody plants and forest trees. This

is an important factor, as forest trees can act as a pathogen reservoir and cause a spillover effect in vineyards (Guarnaccia et al., 2023).

### **Eutypa dieback**

Eutypa dieback was formerly grouped with other GTDs associated with dieback and known as "Dead Arm Disease". Later, it was distinct in Eutypa Dead Arm and, finally, Eutypa Dieback. This disease is mainly caused by the pathogen *Eutypa lata*, which was first described by Carter as *E. armeniacae* on *Prunus armeniaca* (Carter, 1957). In addition, other species of the *Diatrypaceae* family belonging to the genera *Anthosoma*, *Cryptosphaeria*, *Cryptovalsa*, *Diatrype*, *Diatrypella*, and *Eutypella* have been described as involved with the disease (Bertsch et al., 2013; Kenfaoui et al., 2022; Guarnaccia et al., 2023). Symptoms of this disease appear in spring and include leaf chlorosis and damping off, dwarfing of shoots and shortening of internodes, and a general bushy appearance of the infected vines (Claverie et al., 2020). *E. lata* was first isolated from grapevine in 1978, but it was already known as a common inhabitant of other woody hosts, and today, it has been reported on more than 90 plant species (Moller and Kasimatis, 1978; Bolay and Carter, 1985). Eutypiose is a threat that impacts most viticultural areas of the world, sparing semi-desert areas (<250 mm rainfall per year) (Bolay and Carter, 1985; Travadon et al., 2024).

### **Diaporthe dieback**

Diaporthe dieback is another ubiquitous GTD. It is caused by agents of the genus *Diaporthe*, which was previously known by the name of the asexual form *Phomopsis* (Úrbez-Torres et al., 2013). The primary cause of the disease is *Diaporthe ampelina*, previously known as *Phomopsis viticola*. Additionally, since the advent of molecular identification, many other species from the same genus have been reported to be associated with the disease. (Azevedo-Nogueira et al., 2022). *D. ampelina* occurs in every viticultural area of the world and is historically associated with another disease called Phomopsis cane and leaf spot, or cortical necrosis of grapevine (also known in Europe as excorioses) (Úrbez-Torres et al., 2013).

### **Phomopsis cane and leaf spot**

Phomopsis cane and leaf spot primarily affects young grapevine shoots and leaves by forming small, black spots on the basal internodes that ultimately enlarge, resulting in distorted growth and shoot dieback (Baumgartner et al., 2013). The same species can cause Diaporthe dieback, infecting wood through pruning wounds and causing cankers associated with a lack of bud break (Úrbez-Torres et al., 2013).

### **Botryosphaeria dieback**

The last of the GTDs that was previously included under the “Dead Arm Disease” umbrella is Botryosphaeria dieback. This GTD is regarded as the most significant for its global impact, spread, and complexity (Bertsch et al., 2013; Guan et al., 2016; Reveglia et al., 2021). The main symptoms include wood necrosis and the formation of elongated cankers. Botryosphaeria dieback is caused by many members of the *Botryosphaeriaceae* family, among which the species *Neofusicoccum parvum* is one of the most widespread and virulent in global vineyards (Belair et al., 2022; Kenfaoui et al., 2022).

The group's last and maybe most famous GTD is Esca, which will be addressed in the following chapter. Therefore, considering the complex etiology of GTDs, the importance of long, latent infections, the slow disease development, the huge impact of environmental factors on symptom expression, and the lack of effective curative treatments, researching GTDs can be quite challenging.

### 3. Esca Complex of Diseases

#### 3.1. Historical Overview

Esca, or ECD, is probably the oldest GTD among the group. The term “Esca” comes from Latin, where it meant “food” or “aliment,” as it was a way to refer to decayed wood and/or fruiting bodies of basidiomycetes that could be used as fuel for fire (Mugnai et al., 1999). Apoplexy, i.e. the fast and sudden wilting of single branches or the whole vine, was first described on the grapevine by Ravaz (1898), who later also linked this symptom to the presence of white rot, identified at the time as caused by *Polyporus ignarius* (later renamed as *Fomes ignarius* or *Phellinus ignarius*), on the vines in warm countries (Ravaz, 1909). A few years later, the pathologist Lionello Petri reported the presence of brown wood streakings in young grafted vines and mother plants, also succeeding in reproducing the symptoms by inoculating the isolated fungi, two belonging to the *Cephalosporium* genus and one *Acremonium* (Petri, 1912). Later, Vinet (1909) pointed out that apoplexy is not unique to warm countries but is also common in the west of France. Vinet also confirms Ravaz's observations, indicating *Stereum hirsutum* as the responsible decay agent. In 1926, a book published by Pierre Viala gave the first in-depth description of Esca (Viala P., 1926). Viala's work outlines a framework of symptoms associated with the disease, including progressive weakening of the vines, foliar symptoms (Figure 2), wilting of the shoots, and apoplectic strokes. In his monography, Viala also addresses grapevine decay, indicating that *S. necator* is the main decay agent on the grapevine. This fungus was later reported to be totally indistinguishable from a morphological point of view from *S. hirsutum* (Rives, 1926). Additionally, Viala presents a geographical distribution of the disease throughout the Mediterranean basin, highlighting that older vines are more susceptible to these issues. In the middle of the 20th century in California, Luigi Chiarappa determined that the pathological condition observed in American vines was the same as the condition described in Europe as Esca. The symptoms he referred to are “black measles”, another name to indicate shoot dieback, foliar symptoms as well as fruit symptoms (Chiarappa Luigi, 1959). Chiarappa also discussed the presence of decayed wood in the vines, indicating the effectiveness of treatments such as trunk surgery and spraying sodium arsenite to reduce the incidence of foliar symptoms. Additionally, Chiarappa successfully isolated several strains of *Cephalosporium*, and by inoculating them, he could reproduce brown streaking symptoms on the infected vines. He also reproduced wood rot in vitro by inoculating the isolated *P. ignarius*. At the end of the century, Chiarappa will conduct additional pathogenicity trials in California to demonstrate that *P. ignarius*, and not *S. hirsutum*, was responsible for the wood

rot associated with Esca. (Chiarappa, 1997). Later, Larignon and Dubos (Larignon and Dubos, 1987) hypothesized that *Phialophora*, *Cephalosporium*, and *Eutypa* species were the first to colonize the wood and that decay would only appear later. In 1994, *Phialophora parasitica* was isolated from vines, showing signs of dieback (Ferreira et al., 1994). The modern definition of Esca-associated ascomycetes began with differentiating the *Phaeoacremonium* genus from *Acremonium* and *Phialophora* (Crous et al., 1996). Shortly after, two distinct species of Esca-associated ascomycetes were classified within this genus: *P. chlamydosporum* and *P. aleophilum* (Mugnai et al., 1999). *P. chlamydosporum* was later renamed *Phaemoniella chlamydospora* based on morphological characteristics such as culture conditions, conidia dimensions, and pigments (Crous and Gams, 2000). The redefinition of *P. aleophilum* occurred a decade and a half later when molecular tools led to its renaming as *P. minimum* (Gramaje et al., 2015).



Figure 2 Grapevine tiger striped leaves, illustrated in *Ampélographie: Traité général de viticulture* by Pierre Viala and Victor Vermorel (1901–1910).

### 3.2. The Modern Definition

The question of whether Esca was a disease complex or a complex of diseases was first proposed at the beginning of the century (Mugnai et al., 1999; Graniti et al., 2000). In the work, Esca is defined as the group of different syndromes, such as:

- Brown wood streaking: present on rootstocks, mother plants, and rooted cuttings, often without external symptoms.
- Petri grapevine decline: the decline of young grapevines (2-6 years old) caused by infected propagation material.
- Young esca: black or brown wood-streaking and xylem gummosis in vines in grapevines independently from wood rot development, usually in vines younger than 8-10 years old.
- White rot: decay caused by basidiomycetes.
- Esca proper: development of white rot simultaneously or consecutively to the development of brown wood streaking on mature vines (8-10 years and older).

The question arises from the fact that several factors, both biotic and abiotic, concur in the development of Esca syndromes, thus indicating that Esca is a disease complex. At the same time, at least two distinct diseases, such as white rot and brown wood streaking, can coexist on the same plant, thus indicating Esca as a complex of diseases. Later in the decade, Surico (2009) will re-address the question, indicating Esca as a complex of four diseases. The diseases in question are brown wood streaking, Petri disease, grapevine leaf stripe disease (GLSD), and Esca (also known as Esca *sensu stricto*). In addition, the condition in which Esca and GLSD coexist on the same plant is indicated as “Esca proper”. With the definition of GLSD, Surico replaced the term “young esca,” which was previously used to refer to the development of foliar symptoms, stressing the need for a new name for a disease in which the real agents had not been detected.

A detailed description of the foliar symptoms comes from Mugnai et al., (1999). Symptoms were defined as developing with time. Initially, internal or along-margin spots of chlorotic/light green tissues are formed. Soon, affected areas expand, coalescing in large patches in the interveinal leaf tissue. In addition, tissues gradually necrotize, leaving ultimately only a small, narrow corridor of visually unaffected tissue along the main veins. Distortion of the leaves, as well as drying of the lamina, can also occur. Accurate descriptions were also reported by Lecomte et al. (2012) in which a new classification for the disease was proposed.

Here it was suggested that symptoms such as the appearance of symptomatic leaves and a total apoplexy collapse of the vine could indicate varying degrees of disease severity linking the two symptoms. In his study, Lecompte also highlighted a previously neglected symptom known as “wood stripes.” These are superficial discolorations found on the outer ring of the wood just beneath the bark, which can be examined by peeling back the bark and indicating sap flow disruption.

GLSD symptoms are described as “fluctuant,” meaning they do not appear consistently every year (Bertsch et al., 2013). Today, vines with tiger-striped symptoms are widespread in all vine-growing regions of the northern hemisphere but infrequent in the southern hemisphere (Fischer et al., 2005; Bertsch et al., 2009; Halleen and Mostert, 2011; Mundy et al., 2020; Del Frari et al., 2021; Kenfaoui et al., 2022). The incidence of GLSD symptoms has been reported to be increasing in recent decades, possibly in relation to climate change (Kovács and Sándor, 2016; Beris et al., 2023; Guarnaccia et al., 2023). It has been proven that there is a correlation between environmental factors such as rainfall and temperature with GLSD expression (Serra et al., 2018; Monod et al., 2023). In addition, frost and associated damages to the vines seem to have an increasing effect on the development of foliar symptoms, as observed after the great frost that occurred in Tuscany in 1985 (Surico et al., 2004).

Although studied for a long time, no clear cause for the development of foliar symptoms has been identified at the time of writing this thesis. This has been made more difficult by the lack of successful Koch postulates, i.e. reproduction of leaf symptoms in artificial conditions, which can be obtained in the other wood disease causing leaf symptoms, *Eutypa dieback*. Nonetheless, several hypotheses have been proposed (Mugnai et al., 1999; Agrelli et al., 2009; Bortolami et al., 2019; Claverie et al., 2020; Del Frari et al., 2022). As listed:

- **Toxin Hypothesis:** fungal toxins can move through the vascular system of perennial wood, ultimately reaching the canopy. This translocation causes phytotoxicity, which leads to foliar symptoms.
- **Byproducts of Fungal Degradation Hypothesis:** molecules produced by the degradation activity of microorganisms in perennial wood can travel through the vascular system to the leaves, resulting in the development of GLSD.
- **Toxins + Byproducts:** a combination of the previous two hypotheses.

- **Competition-induced metabolites hypothesis:** symptoms result from phytotoxic secondary metabolites released due to interactions between esca-associated fungi and/or other components of the wood microbiome, such as bacteria. Foliar symptoms could develop due to the phytotoxic effect of the metabolites or as a reactive oxidative burst caused by the presence of these molecules.
- **Hydraulic dysfunction hypothesis:** foliar symptoms arise due to reduced water transport capacity caused by fungal colonization and blockage of xylem vessels
- **Edge hypothesis:** this hypothesis affirms that all vines infested by esca-associated fungi are continuously exposed to molecules such as toxins, wood degradation byproducts, and competition-induced metabolites. Foliar symptoms only manifest when the concentration of these molecules exceeds a certain threshold which is influenced by various factors, including the plant's health status, environmental conditions, and agronomical practices.

Among all hypotheses, the toxin hypothesis is the most well-investigated one, and several compounds such as polyphenols, tannins, enzymes, as well as low-molecular-weight compounds have been proposed as potentially-diffusible-foliar-symptoms-inducing molecules (Pacetti et al., 2022; Schilling et al., 2022; Moretti et al., 2023).

### 3.3. Agronomical Practices to Control ECD

Protecting grapevines can be challenging, particularly in relation to GTDs. Grapevines are climbing woody vines characterized by long and wide xylem vessels and tracheids considerably larger than those found in other woody plants. This feature is an adaptation to their climbing nature, allowing for efficient water and nutrient transport over long distances (Brodersen et al., 2013; Keller, 2020). The diameter of these vessels can vary depending on the cultivar and the growing season, reaching sizes of up to 120  $\mu\text{m}$  (Pouzoulet et al., 2014; Foglia et al., 2022). Unfortunately, larger vessels can facilitate the spread of pathogens, resulting in faster colonization. Thus they have been directly linked to pathogen susceptibility (Casieri et al., 2009; Gerin et al., 2024). In addition, the xylem architecture of the grapevine can lead to hydraulic vulnerability since they are prone to easier cavitation (Jacobsen et al., 2015). The implementation of the CODIT model (Compartmentalization of Damage/Dysfunction in Trees) can be observed in the grapevine in the occurrence of pathogen attacks. This model was

originally developed to explain tree responses to wounding and infection by Hartig (1878) and later expanded (Shigo and Marx, 1977). The model describes a defense system that consists of the deployment of four walls aimed at containing possible threats to the woody tissues:

- Wall 1: physical barriers such as tyloses, gels, and gummosis are deployed in the vessels to restrain pathogen advancement.
- Wall 2: restriction in the radial direction by production of secondary metabolites and lignified fibers.
- Wall 3: lateral spread limitation by forming along the rays, radially arranged parenchyma cells within the wood.
- Wall 4: the final barrier, formed by the cambium. New wood is being produced, effectively isolating the attacked area.

Although the CODIT model is present and functioning in grapevines, it is less effective than in trees. Several factors contribute to this difference. First, as mentioned earlier, the size of the vessels can significantly affect the ability of the plant to occlude them, which serves as a protective measure against pathogens (Pouzoulet et al., 2014, 2019).

In addition, a significant range of molecular defense mechanisms supports the structural changes in grapevines resulting from pathogen attacks. The interaction between grapevines and pathogens triggers the activation of pathways that lead to a defense response against these threats. Currently, three types of interactions have been recognized.

The first defense lines are plasma membrane-localized pattern recognition receptors. They detect the presence of pathogens by detecting pathogens' structural components, known as PAMPs (Pathogen-Associated Molecular Patterns), and initiate signaling for the induction of PAMPs-triggered immunity (PTI). This immune response aims to prevent microorganisms from accessing the plant's cells. PTI leads to an unspecific physiological and molecular response in plant tissues, resulting in a variety of defensive measures (Reverberi et al., 2022).

The effector-triggered immunity (ETI) represents the second layer of defense. This defense line is based on the recognition by specific plant resistance proteins of specialized effectors, secreted by the pathogen to silence the PTI response. This recognition generally leads to a hypersensitive response, mainly but not only associated with programmed cell death, that leads to the limitation of pathogen spread (Agrios, 2005).

Recently, a new group of signals has been proposed specifically for GTDs. As this group of diseases is associated with fungi that can become pathogens as a reaction to plant stress, molecules that induce this shift in behavior from endophytes to pathogens have been called surrender signals (Khattab et al., 2023).

Pathogen-triggered or effector-triggered immunity, as in other plants, leads to the activation of defense mechanisms (Li et al., 2025). Among the most relevant for ECD, the activation of the mitogen-activated protein kinases (MAPKs) induces a reprogramming of primary and secondary metabolism (Jones and Dangl, 2006; Li et al., 2025). Recently, a difference in the upregulation of several genes associated with the coding of MAPK proteins was observed in grapevines following infection by *P. minimum* and *P. chlamydospora*. Furthermore, the metabolomic responses to the two infections were also distinct, indicating that grapevines may have different mechanisms for perceiving these pathogens (Romeo-Oliván et al., 2024). Induction of genes involved in stilbene syntheses also varies according to the pathogen identity inoculated in grapevine's woody tissues (Pierron et al., 2016). The difference in the accumulation of stilbene compounds and MTI activation was found among different grapevine varieties with different susceptibility to ECD (Lambert et al., 2013b).

Nowadays, the first method, and arguably one of the most effective ones, to control ECD and GTDs in general is the use of non-contaminated plant material. As a matter of fact, nurseries that produce infected material can be an important vector for the spread of fungal pathogens (Gramaje and Armengol, 2011). Nursery practices greatly influence the vine microbiome and the presence of GTDs associated pathogens (Garcia et al., 2024b). Thus, to mitigate the risk of GTDs pathogens contamination, nurseries have implemented various treatments during propagation processes. Hot water treatment is a common practice, used to disinfect scions and rootstocks before grafting and to sanitize dormant vines (Halleen and Fourie, 2016). Ozone-enriched water is another relatively new method employed to eliminate pathogens from propagation material (Pierron et al., 2015; Romeo-Oliván et al., 2022). Additionally, *Trichoderma*-based biocontrol agents are applied to suppress pathogen development and enhance vine resistance (Mondello et al., 2018b). Other strategies include fungicidal dips or coatings and improved sanitation protocols during cutting, grafting, and storage.

Although healthy propagation material is critically important for ensuring phytosanitary safety, additional practices are necessary to protect vines from GTDs, as infections can still occur in the vineyard. Pruning is an ever-occurring practice implemented on the grapevine,

creating numerous wounds that airborne fungal spores can easily colonize. In the grapevine, vessels get occluded with tyloses during summer and gels during winter (Sun et al., 2008). It has been observed that tyloses, in addition to suberins, are more effective in controlling pathogen spread than gels (Pouzoulet et al., 2013). Recent studies indicate that susceptibility to GTDs fungi persists for months after pruning. (Rosace et al., 2023b). There is no consensus on the best time for pruning because spore release periods can vary significantly depending on pathogens, climate, and geographical location. Therefore, vines should be pruned when the level of inoculum is low, i.e. not close to rainy periods, and - ideally - the speed of wound healing is rapid (Gramaje et al., 2018). For wound protection, many options are available. Many fungicides have been reported to be effective (Mondello et al., 2018a). These fungicides are of both synthetic and natural origin and have been tested in different fields. Unfortunately, they have a short persistence. This means that in areas like European vineyards, where the rainy season in spring leads to a prolonged increase in inoculum production, these measures do not offer the long-lasting protection needed to address the extended wound susceptibility (Gramaje et al., 2018).

Biocontrol agents (BCAs) can offer extended protection against fungal colonization of fresh wounds. While many BCAs have been selected and formulated for this purpose, their application takes time as these agents need to establish themselves on the wounds. Thus, a potentially vulnerable window may appear, during which the wound is unprotected. However, once established, BCAs can provide longer-lasting protection. (Mondello et al., 2018b). Among bacteria, *Bacillus subtilis* is the most extensively studied BCA. This bacterium is capable of protecting wounds, with varying levels of efficacy depending on the specific pathogen and BCA strain (Kotze et al., 2011a). The most successful and commercialized BCA-based products against GTDs use fungal strains belonging to *Trichoderma* species (Di Marco et al., 2004; Mondello et al., 2018b; Pollard-Flamand et al., 2022; Makhathini Mkhwanazi et al., 2024). *Trichoderma* is a well-known genus often employed for biocontrol, as the selected species can compete with pathogens for space and nutrients while also producing a specific cocktail of antibiotic compounds in their secretome, which includes both the volatitome and the non-volatile fractions. Additionally, nowadays, new *Trichoderma* strains are also tested for their compatibility with pesticides and biostimulants (Kotze et al., 2011b; Zañfano et al., 2024). Other species other than *Trichoderma* have also been tested. Recently, the efficacy of *Epicoccum* spp. to efficacy contain *P. chlamydospora* has been investigated (Del Frari et al., 2019). Furthermore, testing of non-pathogenic strains of species of *Fusarium* has been carried

out, and of *Pythium oligandrum* show how Oomycetes can also be a great asset as BCAs (Yacoub et al., 2016, 2020; Mondello et al., 2018b).

There is a substantial body of research investigating the effects of nutrient application on the expression of GLSD foliar symptoms. An application of a mixture of calcium and magnesium and seaweed, mainly as a carrier, has been shown to be able to reduce GLSD expression (Calzarano et al., 2014; Calzarano and Di Marco, 2018). Application of this mixture also led to the accumulation of phytoalexins, such as resveratrol, in leaves (Calzarano et al., 2017). On the contrary, a following study demonstrated that fertilization with macro- and, particularly, micronutrients via canopy spray significantly increases symptom expression (Calzarano et al., 2023). This phenomenon was accompanied by a marked reduction in calcium and magnesium content in the leaves of symptomatic vines.

These results have been linked to the hypersensitive reactions that would occur in leaves due to metabolite translocation, as suggested by several hypotheses regarding the formation of GLSD. The application of a seaweed mixture can enhance the overall physiological state of the treated vines. Additionally, calcium and magnesium would help modulate and reduce the hypersensitive response by acting as defense elicitors. Moreover, fertilizing with macronutrients can lead to increased vigor in the vines, resulting in a greater amount of translocated metabolites (Calzarano et al., 2009, 2014, 2017, 2021, 2023; Calzarano and Di Marco, 2018). This concept can be considered in light of other GLSD hypotheses, as improving host defenses might raise the threshold for GLSD development, as proposed in the edge hypothesis (Del Frari et al., 2022).

Regarding the use of compounds that can be used on vines, nowadays, following the ban on sodium arsenite, many new practices are being investigated to control GTDs. Research has shown that nanomaterials made of silver and copper can significantly inhibit the growth of several fungi associated with GTDs (Štůšková et al., 2023). In addition, their efficacy as nanocarriers for the control of fungi and bacteria associated with GTDs was assayed both *in vitro* and *in planta* showing promising results (Sánchez-Hernández et al., 2022).

Many natural compounds are in the process of being evaluated as possible compounds to control ECD. Chitosan is a non-toxic polymer that displayed the ability to inhibit the mycelial growth of many GTDs fungal agents, among which *P. chlamydospora*, *P. minimum*, and *F. mediterranea* were present (Nascimento et al., 2007; Buzón-Durán et al., 2021). Both garlic acid extract and vanillin demonstrated high efficacy *in vitro* and in the field when applied to

pruning wounds, particularly when combined with chitosan oligosaccharide (Cobos et al., 2015).

Although some aspects of ECD remain unclear, in particular, the mechanism leading to foliar symptoms or GLSD, various curative methods have been developed in a struggle that is as old as viticulture. The association with wood colonization, necrosis, and disruption has always been recognized and therefore one of the earliest methods to control the disease, employed in Asia Minor and Greece, involved splitting the trunks of vines showing foliar symptoms and placing a stone in the hole to keep it open (Mugnai et al., 1999). Vines subjected to this treatment remained asymptomatic for some time, although foliar symptoms reappeared at some point. Moreover, although this method was sporadically reposed throughout history, it was only recently scientifically investigated in the trials carried out by Pacetti et al. (2021) on trunk renewal, where the application of a longitudinal cut, as in the “split and stone” technique, showed only a temporary and negligible reduction in the development of symptoms.

A more recent and effective technique involves the use of sodium arsenite. Since its introduction in agriculture during the 18th century, arsenite has been widely used as an herbicide and for pest control (Bencko and Yan Li Foong, 2017). In viticulture, it was initially employed to manage insects and has been used to control GTDs since the early 20th century (Viala P., 1926; Mondello et al., 2018b). While incredibly effective at reducing leaf stripe symptom manifestation, its high toxicity to humans, animals, plants, and the environment led to its complete ban in European vineyards in 2003, quite some time after its ban in the United States or other countries. Several authors state that after its ban, no effective chemical control has been available against GTDs (Mondello et al., 2018b; Lecomte et al., 2021, 2022a; Beris et al., 2023). Although nowadays banned, sodium arsenite could still help researchers develop new ways to control GTDs. Many efforts have been made to understand how sodium arsenite is effective against trunk pathogens and GTDs in general. Recent research has shed light on its fungicidal activity, revealing how sodium arsenite significantly impacts the abundance of fungi, specifically the decay agent *Fomitiporia mediterranea*, in wood, leaving bacterial communities relatively untouched (Bruez et al., 2021). In addition, sodium arsenite is also able to induce the upregulation of detoxification-associated genes, meaning that it could help the grapevine to cope with pathogen’s attack (Songy et al., 2019). This hypothesis was further strengthened by a later study that showed changes in the abundance of certain phytotoxins in sodium arsenite-

treated vines, suggesting potential effects on pathogen metabolism or plant detoxification processes (Trouvelot et al., 2023).

Trunk renewal involves removing the infected trunk and training a healthy water shoot to replace it. This method is the most invasive among the ones available for ECD management, as it involves the substitution of the whole trunk. However, it is highly recommended as the most economically rewarding when a vine shows GLSD symptoms. It is also the most applied method in *Eutypa dieback* (Sosnowski et al., 2011; Mondello et al., 2018a; Baumgartner et al., 2024).

The process of removing decayed wood from tree trunks or cordons is known as “trunk surgery” (referred to as “*slupatura*” in Italian and “*curettage*” in French). This technique utilizes a small electric chainsaw to effectively eliminate decayed wood. The procedure aims to completely remove white rot, leaving behind only healthy, non-degraded wood. This technique has proven effective in reducing the reoccurrence of leaf stripe symptoms and enhancing the overall physiological health of treated vines for several years after the treatment (Cholet et al., 2021). The vine usually recovers quickly, but its vigor afterward is typically lower than that of healthy vines. Despite this, the quality of the berries is comparable to that of healthy vines (Cholet et al., 2021; Pacetti et al., 2021).

A key advantage of trunk surgery over trunk renewal is that treated vines quickly regain their productivity while preserving the quality associated with their age, whereas trunk renewal requires 2-3 years to regain productivity. This technique begins with an initial inspection of decayed areas by making a preliminary cut where the dead wood is prominent or located under the largest pruning wounds. Next, the white-rot-affected tissues are removed, leaving only discolored wood and sound wood. On Sauvignon Blanc, one year after treatment, a recovery rate of 85% for the reduction of foliar symptoms was observed, with an average annual decline in efficacy of 8% (Lecomte et al., 2022b). Another study conducted on 14-year-old Cabernet Sauvignon revealed a significant reduction in GLSD, which was linked to the extent of decayed wood removal. Additionally, the study suggested a correlation between the decreased expression of foliar symptoms and the abundance of *F. mediterranea* in the wood (Pacetti et al., 2021).

### 4. Global Diversity of ECD-Associated Basidiomycetes

During the 20<sup>th</sup> century, there was already a lot of literature on the involvement of basidiomycetes in ECD (Ravaz, 1909; Viala P., 1926; Chiarappa Luigi, 1959; Larignon and Dubos, 1987, 1997; Chiarappa, 1997). The lengthy history and extensive distribution of grapevines across various geographical regions have resulted in the development of many terms to describe the presence of white rot, i.e. soft, spongy wood tissue decayed (Figure 3). For example, the term “*amadou*” is used in French, while “*yesca*” in Spanish, “*Esca proper*” or “*Esca sensu stricto*” are also common names for this condition. As for the definition of the fungal species causing white rot in grapevines, research primarily focused on the European context until the early 21st century when global interest in pathogens that colonize wood led to the recognition of several other basidiomycete species responsible for wood rot in grapevines.



Figure 3 Tangential section of a grapevine trunk (rootstock point), showing tissues decayed by the pathogen *Fomitiporia mediterranea*.

### 4.1. The *Fomitiporia* Genus and Other Basidiomycetes

The list of white-rot agents associated with ECD grew at the end of the 19<sup>th</sup> century and continues to expand. In 1898, Ravaz (1898) identified the agent of apoplectic strokes as *Polyporus igniarius*. This fungus was later renamed *Fomes igniarius* or *Phellinus igniarius*. Larignon and Dubos, in 1997, associated the white rot present in several grapevines collected in France with *Phellinus punctatus*. Later, another survey of ECD-infected vineyards in northern Italy identified *Fomitiporia punctata* (formerly *P. punctatus*) as the main white rot agent responsible for wood decay (Cortesi et al., 2000). In this work, strains of this pathogen were collected from grapevines as well as other woody hosts. Years later, Fischer (2002) analyzed the strains collected from *Vitis* and other woody hosts. By utilizing molecular identification and examining the phenotypical characteristics of the mycelia, he renamed the strains isolated from grapevines to *F. mediterranea*. Although *F. mediterranea* and *F. punctata* are identical in mycelium anatomy and very similar in fruiting bodies, the former has been observed to be heterothallic, requiring outcrossing, while the latter is known to be homothallic. Further analyses revealed two types of mycelium for *F. mediterranea*: type B mycelium, characterized by a cottony to wooly appearance, a yellowish-brown color, and fast growth, and type S mycelium, which has aerial hyphae, medium pigmentation, and slower growth (Fischer, 2002, 2006).

Nowadays, *F. mediterranea* is the main basidiomycete associated with ECD in the Mediterranean basin, as well as other regions in continental Europe (Figure 4 - Ciccarone, 2004; Fischer, 2006). In addition, it is, without a doubt, the most well-investigated ECD-associated basidiomycete. This pathogen has been demonstrated to act as a primary pathogen on grapevines and can colonize many other woody hosts (Sparapano et al., 2000). While *F. mediterranea* primarily affects grapevines, it also infects other hosts, such as *Acer negundo*, *Actinidia* spp., *Albizia julibrissin*, *Cistus* spp., *Citrus* spp., *Corylus avellana*, *Fagus sylvatica*, *Fortunella japonica*, *Hedera helix*, *Lagerstroemia indica*, *Laurus nobilis*, *Ligustrum vulgare*, *Olea europaea*, *Platanus × acerifolia*, *Punica granatum*, *Prunus dulcis*, *Quercus ilex*, *Pyrus communis*, *Quercus robur*, *Quercus rubra*, *Robinia pseudoacacia*, *Salix alba*, and *Ulmus* spp. This shows the fungus's ability to thrive on numerous alternative hosts, regardless of the presence of a vineyard. In addition, *F. mediterranea* has been proven to be able to quickly colonize new hosts as it was found to affect plants of *A. deliciosa* in southern Europe only approximately 50 years after the introduction of this crop (Di Marco et al., 2003). The ability

of *F. mediterranea* to colonize various woody hosts could be a crucial reservoir for the fungus, as it takes years for fruiting bodies to develop, and they are rarely found in vineyards and olive orchards (Cortesi et al., 2000; Elena et al., 2006; Fischer, 2006; Fischer and González García, 2015b; Markakis et al., 2017). The adaptation of *F. mediterranea* to the grapevine has been recently highlighted as the fungus was found to be able to effectively degrade grapevine extractives such as flavonoids and stilbenes, especially when compared to another white rot agent, *Trametes versicolor* (Schilling et al., 2023).

*Fomitiporia mediterranea* is able to produce both intracellular and extracellular detoxification processes for the deactivation of grapevine extractives. For intracellular detoxification, the system mainly relies on cytochrome P450s and glutathione transferases, for which associated genes are abundant in the genome of the fungus (Floudas et al., 2012; Morel et al., 2013; Morel-Rouhier, 2021). Extracellular detoxification appears to involve various mechanisms, including the production of laccases and peroxidases, which have been demonstrated to oxidize and modify resveratrol and phenolic compounds, as well as glycoside hydrolases (Schilling et al., 2022; Bchini et al., 2024). Additionally, the pathogen has been found in various climatic conditions, demonstrating its adaptability to varying temperatures (Moretti et al., 2021).

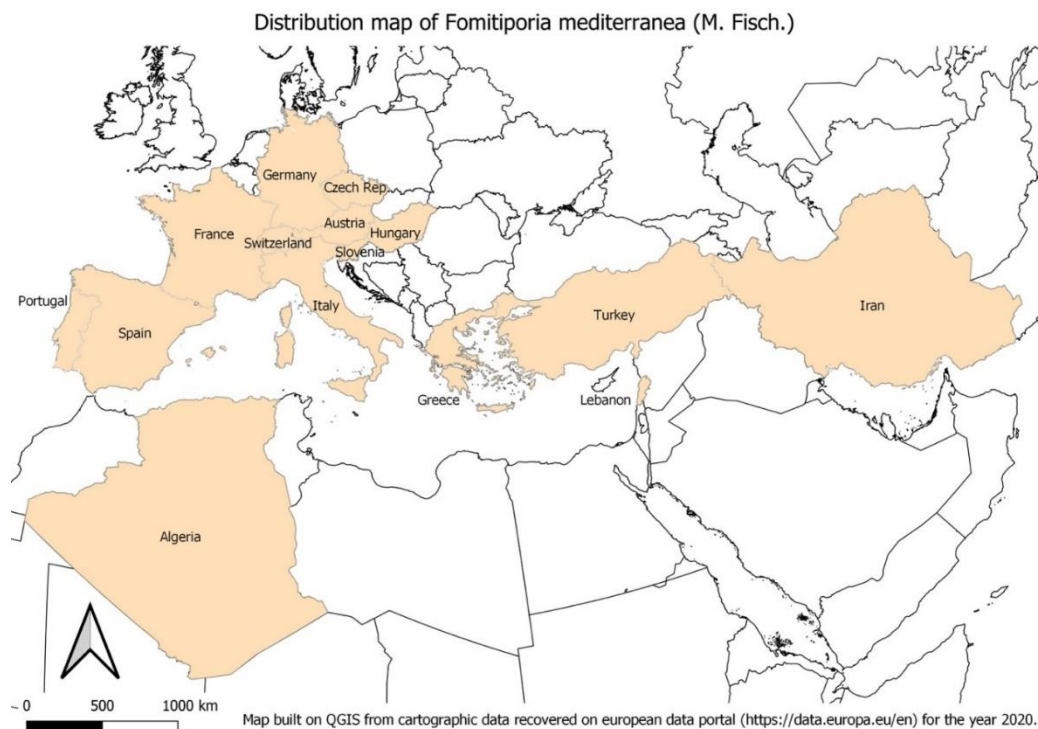


Figure 4 Distribution of *Fomitiporia mediterranea* based on published reports of fungus isolation from the grapevine. From Moretti et al. (2021).

*Fomitiporia capensis* M. Fisch., M. Cloete, L. Mostert, F. Halleen was isolated in South Africa from decayed wood of grapevines showing external symptoms associated with ECD (Halleen and Mostert, 2011; Cloete et al., 2014, 2015a). *F. capensis* has been found to be quite widespread in vineyards in the Western Cape region of South Africa. It was hypothesized that this prevalence might be because this region is the oldest wine-producing area in South Africa or that the fungus originates from the “Fynbos”, the particular natural vegetation found there (Cloete et al., 2014). This fungus was found to be morphologically similar to *F. punctata* as well as *F. aethiopica*, another species of *Fomitiporia* of non-defined host range characterized by the lack of setae, but with larger pore size in its fruiting body (Cloete et al., 2014). The optimal temperature identified for this species is 25°C. As the first description of this fungus dates to the last decade, its host range is still being investigated. In addition to the grapevine, *F. capensis* has also been described on *Quercus*, *Psidium* (Cloete et al., 2015a), a native South African forest host *Gonioma kamassi* (Tchoumi et al., 2020), and only recently on apple trees (Jacobs et al., 2024). Like *F. mediterranea*, the number of fruiting bodies collected during surveys for *F. capensis* is relatively low compared to the number of mycelial cultures obtained. Thus, like for *F. mediterranea*, it is thought that non-*Vitis* hosts may serve as reservoirs for this pathogen (Cloete et al., 2014).

In Australia, *Fomitiporia australiensis* M. Fisch., J. Edwards, Cunningt. and Pascoe has been associated with ECD (Fischer et al., 2005). This fungus was isolated from decayed tissue on grapevine as well as rotten wood of the native bush *Dodonaea viscosa* (Fischer et al., 2005). Among all *Fomitiporia* species, *F. australiensis* is probably one of the less-known as there are few instances of its appearance in the literature. The species can grow at temperatures between 15 and 30 °C, developing two types of mycelia: one that grows quickly and another that grows slowly. This is interesting, as this characteristic is similar to the type-S/type-B mycelium that has been described for *F. mediterranea* (Fischer, 2002).

At the beginning of the decade, an important survey conducted in North America characterized the diversity of basidiomycetes in ECD-symptomatic grapevines in the area. *F. polymorpha* M. Fisch., 2004 was found to be the most common basidiomycete isolated from grapevines showing ECD-associated foliar symptoms in California as it was found in 90% of sampled vineyards (Brown et al., 2020). This fungus was isolated from decayed wood of *V. vinifera* as well as *Salix hindsiana*, and was previously described on a series of

hosts such as *Cercis*, *Cupressus*, *Fraxinus*, *Quercus*, *Pittosporum*, and *Prunus dulcis* (Fischer and Binder, 2004). Again, as for previous species, the presence of basidiocarps on surveyed vines has been described as rare, as only one basidiocarp has been identified as belonging to this fungus, suggesting the importance of alternative sources of inoculum (Brown et al., 2020).

In the same study, the species *Tropicoporus texanus* A.A. Brown, D.P. Lawr. & K. Baumgartner was described for the first time. This species, isolated from different varieties of grapevine, was found to be widespread in Texas. Isolation was performed from white-rot decayed vines' wood that showed foliar symptoms. Inoculations of this pathogen, either alone or in combination with ECD ascomycetes, have resulted in the development of black-streaking symptoms in greenhouse experiments. The lesions formed were larger when co-inoculated with *P. chlamydospora* (Brown et al., 2020). Recent genome sequencing revealed several potential virulence factors in *T. texanus*. In addition, like other white-rot fungi, it possesses genes encoding lignin peroxidases and carbohydrate-active enzymes (Garcia et al., 2024a).

*Fomitiporia langloisii* was first found on at least 33% of surveyed vines in Texas (Brown et al., 2020). This species was the very first to belong to the *Fomitiporia*, as the genus was originally erected to describe it (Murrill, 1908). For a long time, this species has been considered a synonym of *Phellinus*. Recently, as happened to *F. mediterranea*, it has been recognized as a distinct species rather than just a synonym of *F. punctata* through molecular identification. (Decock et al., 2007). This species was isolated on a range of different grapevine cultivars. Host range of *F. langloisii* includes *Quercus* sp. In addition to *V. vinifera*. As this fungus has been wrongly identified for a long time, there is limited information on this fungus in the literature, but previous studies have classified *F. langloisii* as a pathogen given that inoculations on vines led to the development of brown lesions (Brown et al., 2020).

Another basidiomycete recently found in Californian and Texan vineyards is *Inonotus vitis* A.A. Brown, D.P. Lawr. & K. Baumgartner sp. nov. The fungus was recently isolated from decayed wood of vines showing external symptoms of ECD. This species belongs to the *Inonotus* genus, one of the two largest genera within *Hymenochaetales*. Despite this species being recently described, other *Inonotus* species have been described as pathogens

associated with ECD on the grapevine. *I. hispidus* is recognized as a primary pathogen on the grapevine, though it is less impactful than *F. mediterranea*. This is particularly noted in the northern hemisphere, especially in Europe, where it has been recorded in both Central Europe and Spain (González et al., 2009). As it often happens in white rot agents in grapevine, many other host plants have been described for this species, such as *Malus*, *Fraxinus*, *Quercus*, *Salix*, *Robinia*, *Pistacia*, *Acer*, *Ficus*, *Fagus*, and, interestingly, conifers such as *Abies* (Fischer and González García, 2015b). *Inonotus setulosus-croceus* was first reported on the grapevine and on *Salix* in South Africa (Cloete et al., 2015a). Other investigations on the recently described *I. vitis* could help shed light on its pathogenicity, which needs to be evaluated. Despite that, similarly to *T. texanus*, genome analysis has shown the presence of potential virulence factors in the genome of this fungus (Garcia et al., 2024a).

*Stereum hirsutum* has been reported as the agent responsible for the development of wood decay on the grapevine since the beginning of the last century (Ravaz, 1909; Viala P., 1926). Many authors regard this fungus as complex. It belongs to the order *Russolales*, *Steraceae* family, and can be found in various hardwood and softwood species. *S. hirsutum* is considered a cosmopolitan species, having been discovered in Europe, the Middle East, East Asia, North America, and the Southern Hemisphere, including Australia, New Zealand, and Jamaica (Tura et al., 2008). This basidiomycete primarily functions as a saprobe or weak parasite, acting as a white-rot agent and, at best potentially, playing a secondary role in ECD (Mugnai et al., 1999; Cortesi et al., 2000; Fischer and González García, 2015b). However, this fungus has been identified as the primary agent of decay in Romanian vineyards (Muntean et al., 2022). A recent sampling conducted in Minnesota and Wisconsin on vines showing an array of symptoms, including decay, reported the frequent isolation of another *Stereum* species, *S. complicatum* in addition to other basidiomycetes such as *Trametes versicolor* and *Cerrena unicolor* (DeKrey et al., 2022).

Field surveys conducted in the Hebei and Ningxia provinces in China led to the association of the white-rot agent *F. punicata*, originally described only on *Punica granatum*, with wood discoloration of GLSD symptomatic vines (Ye et al., 2021). In South America, *I. jamaicensis* and *Fomitiporella* spp. have been reported as white-rot agents on the grapevine associated with the disease called “Hoja de malvon”, considered by some authors as a synonym of ECD (Gatica et al., 2000; Fischer, 2006; Lupo et al., 2006), but

they actually appear to be a synonym of Esca proper, as they cause no foliar symptoms or are different from GLSD. Regarding the *Fomitiporella* genus, *Fomitiporella vitis* has been found on ECD-diseased vines in South Africa (Cloete et al., 2015a).

Despite the extensive literature published on ECD-associated white rot agents, it is crucial to acknowledge that many potential species involved in ECD may not yet have been described.

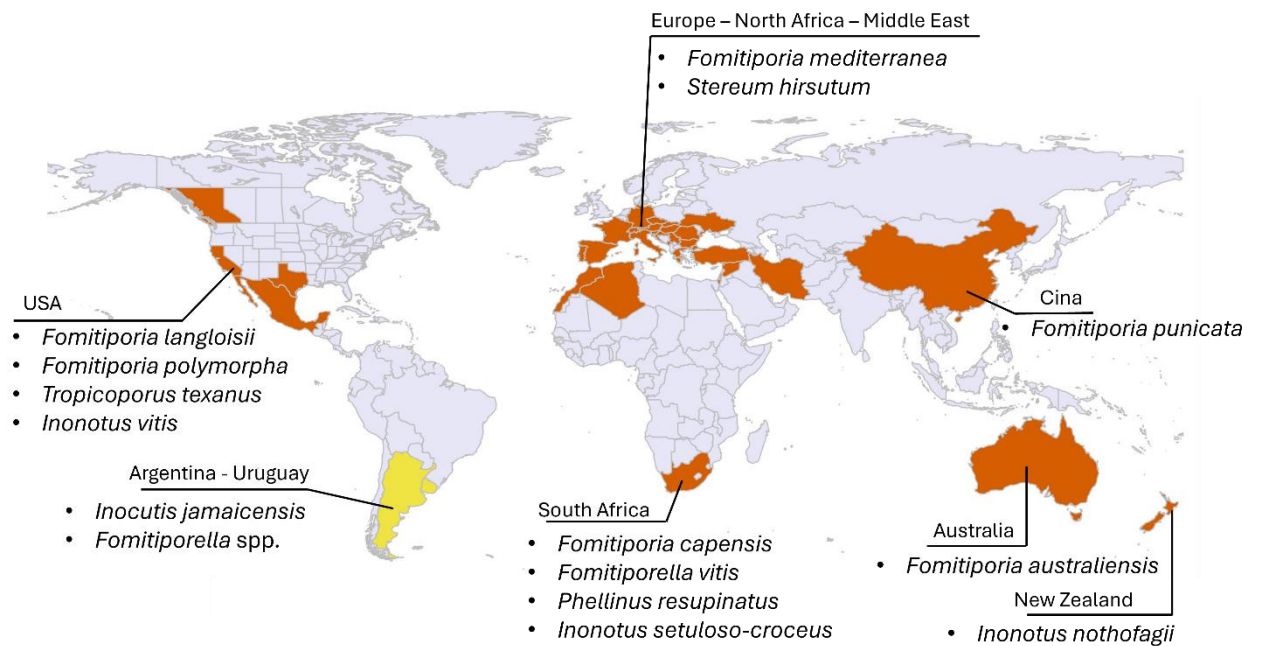


Figure 5 Global distribution of Esca Complex Disease, highlighted in orange, and similar diseases such as Hoja de Malvón, highlighted in yellow, with indications of associated basidiomycetes

### 5. Wood Degradation of ECD basidiomycetes

As previously noted, several decay agents affect grapevines. Wood degradation on the grapevine happens for the same simple reason as why fungal degradation exists: the need to obtain essential nutrients for survival for fungi (Goodell, 2020). Nutrient acquisition associated with fungal wood degradation is a process in which fungi are able to produce enzymes as well as an array of other molecules that can break down complex polymers like hemicellulose, cellulose, and lignin. The result is the production of simpler compounds, such as oligosaccharides and sugars, that can indeed be absorbed for growth (Anand and Rani, 2024).

#### 5.1. The Target of Fungal Degradation: Grapevine Wood

Wood is a biological material with a complex structure and chemical composition. It is classified as a lignocellulosic substance, which means its structure is primarily made up of three main polymers: cellulose, hemicellulose, and lignin. Wood consists of secondary xylem tissue, which includes parenchyma cells as well as conduits such as vessels and tracheids (Rowell and Rowell, 2005; Schwarze, 2007). As a woody liana, grapevine wood differs from wood from tree species. The vine strategy involves climbing on supports rather than standing upright, and thus, grapevine wood lacks the need for stability as other tree species (Schilling et al., 2021). Grapevine wood is less dense than other wood species because it has long and wide xylem vessels and tracheids that allow efficient transport of water and nutrients over long distances (Brodersen et al., 2013; Keller, 2020). The diameter of the vessels can vary depending on the cultivar and the growing season, ranging from 60 to 120  $\mu\text{m}$  (Pouzoulet et al., 2014; Foglia et al., 2022). Vessels and tracheids are distributed to form an axial continuum that allows for efficient liquid transport (Kassemeyer et al., 2022). Grapevine wood contains all the main constituents of Angiospermae lignocellulosic biomass.

Cellulose is a homopolysaccharide composed of chains of anhydroglucose units linked by  $\beta$ -1,4-glycosidic bonds, forming linear glucan chains (Bari et al., 2018). These glucan chains are connected by hydrogen bonds, forming elementary microfibrils that get organized in specific structures. The inner core of these fibrils is tightly packed, known as crystalline cellulose, while the outer part is more loosely arranged and referred to as the amorphous portion of cellulose (Goodell et al., 2020; Niemz et al., 2023). The cellulose content in grapevine wood can vary depending, among other things, on the cultivar, and the woody part considered, and

has been found to range from 31.9 to 45.4% (Agrelli et al., 2009; Pacetti et al., 2022; Čabalová et al., 2023).

In the cell wall, cellulose can be found intimately merged with hemicellulose, which acts as a matrix for the compound. Hemicellulose is a heteropolymer made out of short and branched chains of various sugar units such as xylose, mannose, galactose, arabinose, and other pentose and hexose sugar monomers. Its composition is more amorphous than cellulose, and it varies depending on the wood type. In softwoods, such as the grapevine, the main constituents are glucuronoxylans and glucomannans (Goodell et al., 2020; Niemz et al., 2023). Hemicellulose content in the grapevine can vary, but it is generally lower than that of cellulose content. In *V. vinifera* cv. "Sangiovese," hemicellulose accounts for 20.3% of the wood (Agrelli et al., 2009). In other varieties, such as Teroldego and Gewurztraminer, the content was higher, with 34.3 and 35.7%, respectively (Pacetti et al., 2022).

Lignin is an aromatic-based heteropolymer that is a key structural component of plant cell walls. It is formed through the oxidative coupling of *p*-hydroxycinnamyl alcohol monomers: coniferyl alcohol, also known as Guaiacyl (G)-unit, sinapyl alcohol, or Syringyl (S)-unit and the *p*-coumaryl alcohol, or *p*-Hydroxyphenyl (H)-unit (Schilling et al., 2021; Niemz et al., 2023). The ratios of S, G, and H units in lignin vary by plant species. This variation significantly impacts wood degradation, as higher S/G ratios are linked to increased resistance (Schwarze, 2007; Skyba et al., 2013). Grapevine wood contains less lignin proportion than other tree species, and vine shoots S/G rate has been calculated to be between 1.45 and 1.55 (Markakis et al., 2019; Pereira et al., 2023).

Besides these three major components, other components can be found in the wood cell wall in a portion that is, cumulatively, less of an average of 6% of the total composition, depending on the species (Goodell et al., 2020). Pectins are a group of complex heteropolysaccharides composed of various sugar units that can be found in the middle lamella, primary cell wall, and secondary cell walls. Pectins play an essential role in the fungal degradation of wood cell walls, serving as a more accessible source of carbon, second only to sugars, fatty acids, and starches (Schwarze, 2007; Goodell, 2020). In addition, extractives can diffuse through wood cell walls, particularly through G-units of lignin. Extractives are small molecules with various antifungal properties, as they inhibit the activity of degradative enzymes

and disrupt fungal cell walls and plasma membranes, among other functions (Schilling et al., 2021). In addition, other inorganic materials, such as ash, are present in the wood (Niemz et al., 2023).

All these compounds are assembled in the formation of the wood cell wall, which is composed of multiple layers (Figure 6). From the outermost part to the innermost, the wood cell wall consists of the middle lamella, a primary wall, and a secondary wall. The middle lamella is the thinnest external layer, predominantly composed of lignin and pectin. It provides rigidity and cements adjacent cells together. The primary cell wall is more elastic as it is mainly composed of pectin, hemicellulose, and amorphous cellulose. This layer allows cell expansion. The secondary wall is the thickest of all cell walls. This layer provides mechanical resistance and consists of three sub-layers (S1, S2, and S3), all composed of lignin, cellulose, hemicellulose, and pectin but with different inclinations of microfibrils (Côté, 1968; Bari et al., 2018; Pournou, 2020; Niemz et al., 2023). The S1 layer has less cellulose and lignin compared to the S2 layer, with cellulose microfibrils arranged at a high angle (50-70°) relative to the longitudinal axis of cell walls. This layer serves as a transitional element between the primary cell wall and the more rigid components of the secondary cell wall. Its high angle of microfibrils provides resistance to deformation in the wood cell wall (Kassemeyer et al., 2022). The S2 layer is the thickest of the secondary wall and is mainly composed of crystalline cellulose. Here, microfibrils are arranged in a helical pattern and are oriented nearly parallel to the cell elongation axis (Timell, 1967). The S2 layer contains most of the total lignin within the wood cell wall. This, along with the orientation of the microfibrils, provides major mechanical strength and structural support (Timell, 1967; Mellerowicz and Sundberg, 2008). The S3 layer is located adjacent to the cell lumen. In this thin layer, the microfibrils are nearly parallel to the cell axis and are oriented in a flat helical pattern. This layer can sometimes remain intact even when all other components of the cell wall have been degraded, particularly in cases of brown rot (Goodell, 2020).

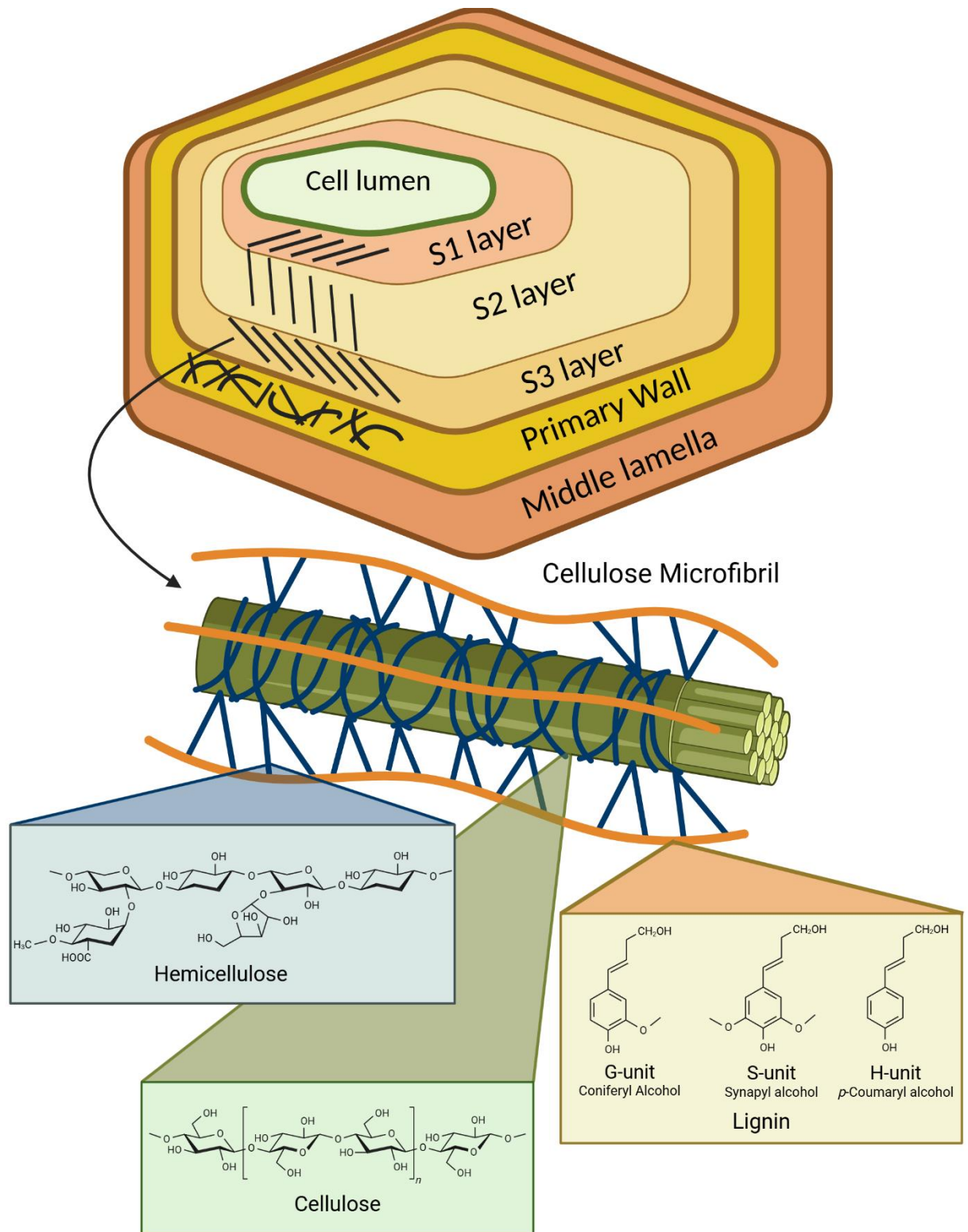


Figure 6 Schematic illustration of the wood cell wall and its principal components. (Created by Puca A. using BioRender.com)

### 5.2. Decay Types and Degradation Mechanisms

Fungi can attack different layers and components of the wood cell wall, ultimately giving different results from fungal wood degradation. Historically, wood decay agents have been divided into white rots, brown rots, and soft rots based on the type of decay they cause and the type of polymeric structure they can degrade (Morrell, 2002; Goodell et al., 2020).

#### 5.2.1. White rot fungi

White rot agents are a group of decay fungi that possess a wide arsenal of enzymes capable of directly degrading celluloses and hemicelluloses, as well as by acting mineralization through oxidation and lignin. They can be further divided into simultaneous and selective pathways. Simultaneous white rot agents are the most common type of white rot fungi. They degrade all the cell wall polymers at the same rate, while selective ones show a preference for lignin and hemicellulose, leaving crystalline cellulose relatively undegraded. In either case, white rot wood degradation leaves a spongy cellulose-enriched matrix as a result of decay activity (Schwarze, 2007; Fackler et al., 2011). White rot wood degradation is directly linked to enzymatic degradation as these fungi utilize a diverse array of enzymes to degrade wood, a capability best characterized in this group but shared to some extent with other decay types.

As for holocellulose, key enzymes for degradation include carbohydrate-active enzymes (CAZymes) such as glycoside hydrolases (GHs),  $\beta$ -glucosidases, cellobiose dehydrogenases (CDHs), and Auxiliary Activities (AAs) enzymes. GHs can hydrolyze glycosidic bonds in polysaccharides and are highly specific to sugar units and linkage types. There are many families of GHs which can have a more specialized function. For example, GH7 and GH6 have cellobiose hydrolase functions, while GH11 is a type of xylanase (Floudas et al., 2012; Goodell, 2020; Garcia et al., 2024a). Among the GH family, endoglucanases can hydrolyze internal bonds on cellulose chains, disrupting the structure and allowing the synergic activity of other enzymes, such as exoglucanase, in the same family or  $\beta$ -glucosidases to further degrade the polysaccharides (Baldrian and Valášková, 2008; Liu et al., 2011). CDHs are oxidoreductases involved in the degradation of cellulose, hemicellulose, and lignin. CDHs is a nonhydrolytic enzyme that efficiently oxidizes cellobiose and other sugars, generating reactive radicals that can further assist in polysaccharide breakdown (Henriksson et al., 2000). AA enzymes are another family of CAZymes that includes lytic polysaccharide monooxygenases (LPMOs), which oxidatively cleave cellulose and hemicellulose. AA enzymes complement hydrolytic enzymes by disrupting the recalcitrant structure of cellulose through oxidative mechanisms

(Goodell, 2020; Pournou, 2020). Among CAZymes, 7 families of hemicellulose enzymes have been described in the basidiomycetes' genome (Riley et al., 2014).

Lignin degradation in white rot fungi is a biochemically complex system that involves several groups of enzymes (Figure 7). These extracellular enzymes belong to the AA family and are classified as laccases (EC 1.10.3.2) and class II peroxidases (PODs), further classified as manganese peroxidases (MnPs, EC 1.11.1.13), lignin peroxidases (LiP, EC 1.11.1.14), and versatile peroxidases (VPs, EC 1.11.1.16) (Hatakka, 1994; Martínez, 2002; Daniel, 2014). Initially, laccases were only associated with lignin-degrading white-rot fungi, and the production of these enzymes was used as a diagnostic character to identify basidiomycetes (Floudas et al., 2012). Laccases are multicopper phenol oxidases capable of directly degrading phenolic lignin. For non-phenolic lignin, laccases require the presence of radical mediator compounds able to catalyze oxidation (Bourbonnais and Paice, 1990; Yang et al., 2017; Goodell, 2020; Pham et al., 2024). In a natural setting, these mediators can be ortho-diphenols, para-diphenols, methoxysubstituted phenols, diamines, and benzenethiols (Niemz et al., 2023).

Manganese peroxidases are non-specific oxidoreductases able to act in a catalytic cycle that leads to the oxidation of phenolic and, mostly through mediators, non-phenolic aromatic compounds (Hofrichter, 2002). MnPs are heme-containing peroxidases that can oxidize  $Mn^{2+}$  to  $Mn^{3+}$  in the presence of hydrogen peroxide that acts as an oxidizing agent. Afterward,  $Mn^{3+}$  can be chelated by many organic acids, such as malonate, lactate, and oxalate, and permeate the lignocellulose structure. Here, chelated  $Mn^{3+}$  acts as a diffusible oxidant leading to the oxidation of aromatic rings into more accessible molecules for other ligninolytic enzymes and CAZymes (Qin et al., 2017).

Lignin peroxidases are heme peroxidases with high redox potential, making them highly efficient in lignin degradation. Similar to laccases, LiPs are able to oxidase both phenolic and non-phenolic lignin. However, unlike laccases, LiPs do not require mediators to directly oxidize non-phenolic lignin, which constitutes the majority of lignin's structure (Tien and Kirk, 1988; Houtman et al., 2018; Cui et al., 2022). VPs share the same characteristics as LiPs regarding lignin degradation but have additional activity associated with MnPs, possessing interaction sites that allow them to oxidize substrates via both mechanisms (Camarero et al., 1999). In addition to the use of enzymes, recent research has shown that white rot fungi can also employ a non-enzymatic mechanism to degrade wood cell walls, mainly before enzymatic action (Goodell, 2020). This system, classically associated with brown rot fungi, is explained in the following paragraph.

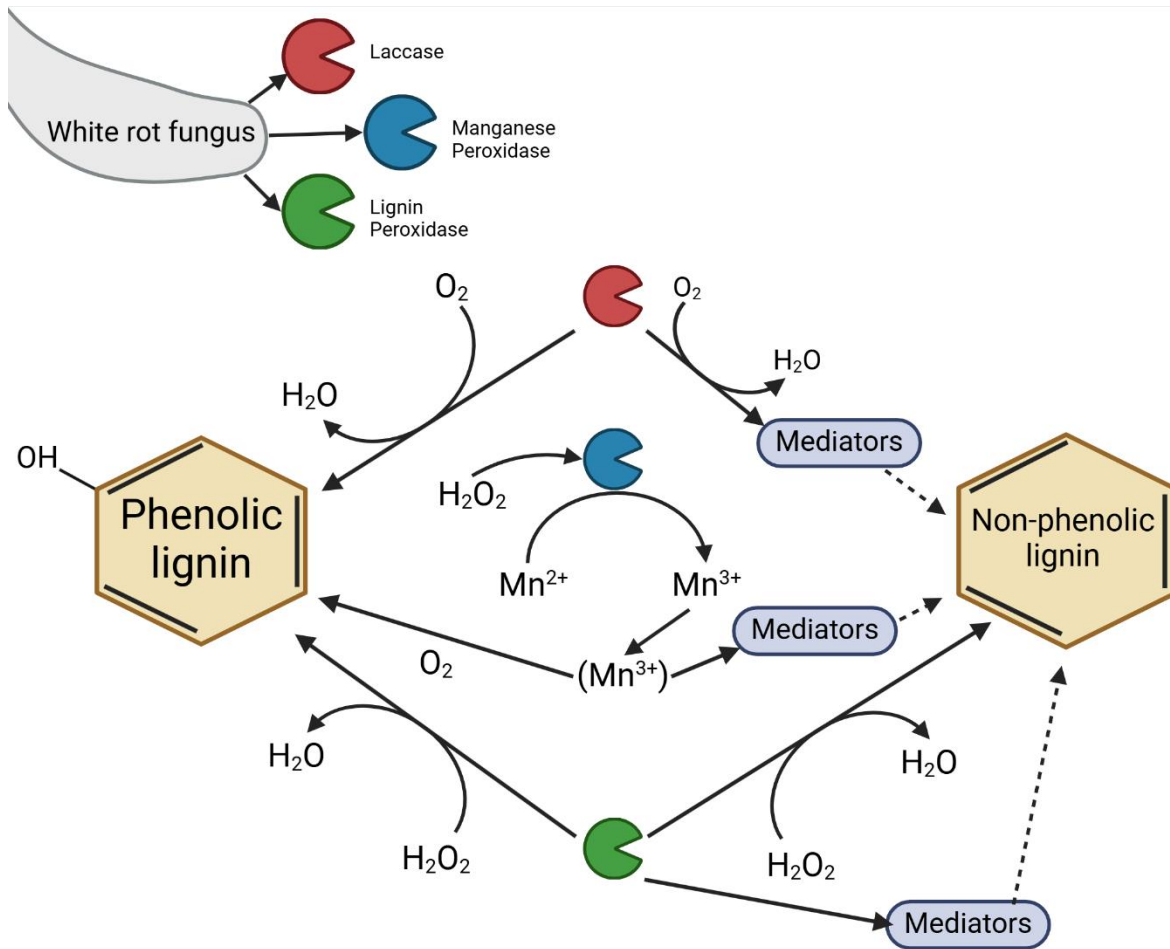


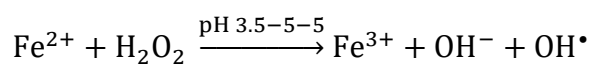
Figure 7 Main lignocellulolytic enzymes involved in white rot wood degradation and their schematic representations. Direct oxidation by enzymes is indicated with solid arrows, while dashed arrows indicate the need for mediators to achieve full oxidation (Created by Puca A. using BioRender.com)

### 5.2.2. Brown rot fungi

Brown rot fungi possess a smaller array of enzymes than white rot agents, as they do not possess peroxidase enzymes for lignin degradation and have a smaller array of CAZymes. Brown rot fungi are efficient in deconstructing polysaccharides such as cellulose and hemicellulose and attacking lignin through oxidative mechanisms, which lead to depolymerization and modification before rapid repolymerization. Thus, they primarily utilize carbohydrate polymers of wood cell walls but are able to affect all three components. However, they cannot metabolize lignin and thus undergo depolymerization, oxidation, and heavy repolymerization by their attack (Eriksson et al., 1990; Goodell, 2020). Ultimately, brown rot

wood degradation leads to the formation of a fragmented, cubical-appearing mass that is brown in color, resulting in significant loss of the properties of the lignocellulosic material.

Several authors have proposed that brown rot fungi have lost most of their ligninolytic enzymes through evolution to instead depend on a non-enzymatic degradation pathway (Floudas et al., 2012; Arantes and Goodell, 2014; Eastwood, 2014). This pathway is known as chelator-mediated Fenton (CMF) chemistry (Xu and Goodell, 2001; Arantes and Goodell, 2014; Goodell et al., 2020). As pointed out in many publications by Goodell, the use of the term CMF instead of just Fenton chemistry highlights the much higher complexity of the process compared to the conventional Fenton chemistry (Xu and Goodell, 2001; Goodell et al., 2006a, 2020; Goodell, 2020). The CMF mechanism is induced by the secretion in the apoplast of two types of extracellular low molecular weight (LMW) compounds: chelators, such as oxalate and oxalic acids, and reductants, such as phenolate, hydroquinone, and aminophenols. In the low pH environment that surrounds the hyphae, oxalate is able to sequester iron (Figure 8). From here, it will naturally penetrate the wood cell wall, finding a higher pH (~5.5-6) and other fungal-produced chelators. In these conditions, iron will be transferred from chelators to the reductants, reducing it and also producing hydrogen peroxide ( $H_2O_2$ ) in the process. At this point, reduced ferrous iron will react with  $H_2O_2$ , ultimately leading to the production of a stream of reactive oxygen species (ROS), specifically hydroxyl radicals ( $\cdot OH$ ). Formation of ROS happens in the wood cell wall rather than near the fungal hyphae, as the production of radicals would be greatly hazardous for the fungus. This will ultimately lead to fast non-enzymatic lignin depolymerization before its subsequent repolymerization as small irregular masses separate from cellulose. This leads to the formation of openings in the wood cell wall that could be further utilized to follow degradation mechanisms. Thus, the CMF mechanism leads to the wood cell wall degradation (Goodell et al., 2006b; Goodell, 2020; Zhu et al., 2022).



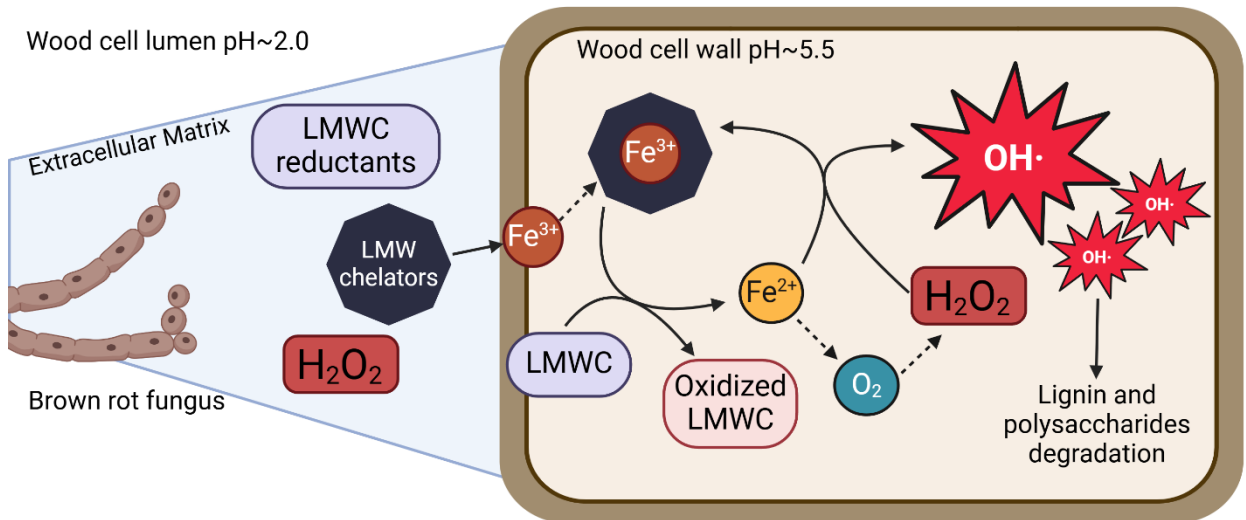


Figure 8 *In situ* generation of  $\cdot\text{OH}$  by CMF chemistry in brown rot wood decay. Readapted from Liu et al. (2020). (Created by Puca A. using BioRender.com)

### 5.2.3. Soft rot fungi

Soft rot fungi are primarily ascomycetes that can degrade wood in moist environments, although they can also thrive in drier conditions. There are two types of soft rot attacks. Type I involves the formation of specific, diamond-shaped cavities that align with the microfibril angle of the S2 layer. Type II is characterized by a more generalized erosion of the S2 layer, starting from the lumen outward (Daniel and Nilsson, 1997). Type II degradation is the more prevalent one among soft rot agents, although some species can produce both patterns. Soft rot agents can utilize cellulose as well as hemicellulose and can also degrade lignin. Regarding this latter component, many species are able to produce laccase (Goodell, 2020).

### 5.3. Enzymatic Wood Degradation in ECD Basidiomycetes

All decay agents associated with ECD are classified as white rot and, as such, employ a wide battery of enzymes for wood degradation. Among all species, *F. mediterranea* is the one for which most literature has been produced, and its enzymatic capabilities have been thoroughly investigated. Multiple *in vitro* experiments conducted in several studies confirm that this fungus possesses a simultaneous degradation pattern (Moretti et al., 2021; Pacetti et al., 2022; Schilling et al., 2022, 2023). Furthermore, *F. mediterranea* is able to employ both CAZymes and PODs to mineralize lignin. As a matter of fact, genomic studies have highlighted the presence of a large variety of enzymes involved with wood degradation in the *F. mediterranea* genome. In a recent work, Garcia, et al. (2024) produced high-quality phased genomes for different ECD-associated white rot agents, among which *F. mediterranea* was also present. Many genes encoding for peroxidases, as well as CAZymes, were found. Specifically, 17 fungal peroxidases were detected, in agreement with the other white rot agents examined. This result confirmed the previous genomic analysis conducted on the fungus, which further classified this group, finding 16 MnPs coding genes, specifically 3 short MnPs, 11 long MnPs, and 2 atypical MnPs, as well as 1 gene associated with the synthesis of generic peroxidases (Floudas et al., 2012). The recently produced genome revealed a variety of CAZymes, including numerous protein-coding genes involved in the enzymatic degradation pathway. Specifically, it identified 18 genes coding for carbohydrate-binding module 1, associated with cellulose-binding activity. Additionally, there were several members of the AA enzymes family, which are connected to polysaccharide breakdown and cellobiose oxidation, respectively (Garcia, Figueroa-Balderas et al., 2024).

As for degradation capabilities more closely related to lignin, in Pacetti et al. (2022), laccase and MnP activities were assessed with spectroscopy measurements and gene expression approaches. All tested strains exhibited strong laccase activity, though differences existed among them. MnP activity was lower than laccase and followed its pattern for intra-strain differences. Gene expression analysis supported these findings. In their work, Schilling et al. (2022) found that 11 of the 16 MnPs in the *F. mediterranea* genome were expressed at one and three months post-inoculation on grapevine sawdust wood cultures. Additionally, they observed the expression of three laccases. The enzymatic activity of this fungus regarding laccase, MnP, and LiP activity was previously assayed using colorimetric assays, resulting in a positive result across the board (Cloete et al., 2015). This work is, to our knowledge, the only investigation of

the enzymatic activity of *F. capensis*, *F. australiensis*, *F. polymorpha*, and other species belonging to the genera *Fomitiporella*, *Inonotus*, and *Phellinus*. The two tested strains of *F. australiensis* showed activity in plate assays for all the tested enzymes, although one strain gave uncertain results for MnPs. *F. capensis* gave overall positive results among all tests for the tested strains. For *F. polymorpha*, recent genome analysis showed similar results to the ones of *F. mediterranea* (Garcia et al., 2024a). Specifically, 20 fungal peroxidases were found alongside 17 CBM1 and many AAs. Although recently identified, genome analyses have been conducted for *T. texanus* and *I. vitis*. Similar to *F. mediterranea* and *F. polymorpha*, numerous fungal peroxidases were detected in the genomes of the basidiomycetes. However, *I. vitis* had only 13 peroxidase-coding genes, making it the lowest among all studied basidiomycetes. In contrast, *T. texanus* exhibited 17 peroxidase-coding genes. Regarding CAZymes, a comparable number of AAs was found, while the number of carbohydrate-binding modules was higher than that found in the studied *Fomitiporia* genus. The genome of *S. hirsutum* possess 5 atypical peroxidases, 1 generic peroxidase, and 15 laccases “stricto sensu” (Floudas et al., 2012). The quantities of CBM1, AAs, and GHs found in the genome are similar to those in other basidiomycetes that have been associated with ECD (Garcia et al., 2024a).

### 5.4. The Unusual Position of *F. mediterranea*: Non-enzymatic Degradation and Soft-rot-like Degradation Pattern

As previously stated and likely evident from this introduction, *F. mediterranea* is the ECD-associated basidiomycete that has been the subject of the most research. Since its recognition from *F. punctata*, many research papers have been published on the fungus, as stated in the recent review of Moretti et al., (2021). In comparison, other ECD-associated basidiomycetes have not garnered as much attention. This discrepancy is likely because *F. mediterranea* is linked to some of the most historically significant wine-growing regions of the world, resulting in a greater focus on it as vineyards attract more interest. Moreover, as environmental, climate and agronomic conditions change in every viticultural area globally, so do the GTDs that threaten grapevines in different regions (Kenfaoui et al., 2022). As such, ECD is not considered the most threatening GTD everywhere in the world, and thus, interest in decay agents follows.

However, *F. mediterranea* has been shown to have many peculiarities, especially as regard grapevine. Based on analysis of the secretome of the fungus and measurement of wood mass loss, the fungus shows a certain degree of adaptation to grapevine wood (Pacetti et al., 2022; Schilling et al., 2022, 2023). Additionally, the basidiomycete effectively detoxifies extractives from grapevine wood, unlike wood from other species (Schilling et al., 2022). As previously stated, the enzymatic degradation capabilities of *F. mediterranea* have been thoroughly investigated using different approaches. Recently, the synergism between the white-rot agent and some bacteria strains of the *Paenibacillus* genus has been proposed to have a role in wood degradation (Haidar et al., 2021, 2024).

The genome of *F. mediterranea* was recently considered in a re-evaluation of the paradigm of white/brown rot. As a matter of fact, the advent of genomics challenges this classical dichotomy, as it was proven that the lignocellulolytic gene pathway is not represented in this “black or white” system (Ohm et al., 2014; Riley et al., 2014). The genome of *F. mediterranea* encodes several reducing-polyketide synthases (R-PKS), terpene synthases, and cytochrome P450 enzymes, which have been associated with LMWC production (Floudas et al., 2012; Garcia et al., 2024a). Production of these small molecules and the iron reduction capability of the supernatants of this fungus’ liquid cultures have already been reported (Tabacchi et al., 2000; Di Marco et al., 2001; Sparapano et al., 2001; Bruno and Sparapano, 2006). Furthermore, lignocellulolytic enzymes are considered to be too large to penetrate the undegraded secondary cell wall (Xu and Goodell, 2001; Arantes et al., 2011; Arantes and

Goodell, 2014; Goodell, 2020; Hou et al., 2020). Thus, the hypothesis that *F. mediterranea* can perform a CMF mechanism in addition to the employment of its enzyme array is defined.

The threat posed by ECD is a global concern, and therefore, it is essential to investigate its associated actors from an international perspective. Besides *F. mediterranea*, there is limited knowledge about other ECD-associated basidiomycetes, particularly regarding their degradation capabilities and phenotypic characteristics. This information is vital for understanding the mechanisms of ECD and developing effective control strategies. In Chapter II, we will evaluate the degradation capabilities—both enzymatic and non-enzymatic—of a collection of basidiomycetes associated with 8 ECD.

## Aims

The increasing importance of ECD and GTDs in vineyards worldwide has become evident over the last few decades, becoming a significant concern in modern viticulture. This awareness has led to a surge in research aimed at understanding the complex mechanisms contributing to the rising severity of ECD. These efforts have recognized *F. mediterranea* as a key player in the complex etiology of ECD, resulting in the emergence of a significant body of literature that focuses on this white-rot pathogen, particularly regarding its role in the development of foliar symptoms. This growing interest has led to a better understanding of *F. mediterranea* degradation capabilities, with a specific emphasis on its enzymatic pathways. While the role of *F. mediterranea*, and decay in general, in ECD etiology, is yet to be fully elucidated, recent studies confirmed that this pathogen is more abundant in vines showing leaf symptoms and agronomical practices aimed at eliminating its presence directly impacts the development of tiger-stiped leaves.

Recent research has underscored the need to reassess the traditional distinction between white and brown rot. *F. mediterranea* occupies a unique position between these categories, sparking investigations into its potential to utilize a chelator-mediated Fenton mechanism for non-enzymatic wood degradation, especially given the absence of lignin peroxidase in its genome. Therefore, the first aim of this thesis was to determine whether *F. mediterranea* could employ a non-enzymatic wood degradation system (Chapter II).

To broaden the scope of research on a global scale, a collection of less-known ECD-associated basidiomycetes was established, largely comprising samples collected from vines exhibiting leaf symptoms. This collection includes five species of *Fomitiporia*: *F. mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, and *F. australiensis*, along with three additional basidiomycetes—two from the *Hymenochaetales* family (*Tropicoporus texanus* and *Inonotus vitis*) and one from the *Russulales* family (*Stereum hirsutum*). Their ability to degrade lignocellulosic biomass was tested to assess both the species lignocellulolytic enzymatic pool and their non-enzymatic degradation abilities (Chapter III).

Considering the differences in degradation capabilities of the studied fungi, primarily linked to the lignocellulosic enzymatic activity in their secretomes, the assessment of *in vitro* ability to degrade dead wood from three different grapevine varieties was conducted in a preliminary study (Chapter IV). To better interpret the results, we conducted a chemical

## Aims

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analysis of the wood prior to fungal degradation. Additionally, the *in vitro* mycelial growth across a range of temperatures was assessed to deepen our understanding of the phenotypical characteristics of the studied strains

**Chapter II** - First Description of Non-Enzymatic Radical-Generating Mechanisms Adopted by *Fomitiporia mediterranea*: An Unexplored Pathway of the White Rot Agent of the Esca Complex of Diseases

## Article

# First Description of Non-Enzymatic Radical-Generating Mechanisms Adopted by *Fomitiporia mediterranea*: An Unexplored Pathway of the White Rot Agent of the Esca Complex of Diseases

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**Citation:** Moretti, S.; Goddard, M.-L.; Puca, A.; Lalevée, J.; Di Marco, S.; Mugnai, L.; Gelhaye, E.; Goodell, B.; Bertsch, C.; Farine, S. First Description of Non-Enzymatic Radical-Generating Mechanisms Adopted by *Fomitiporia mediterranea*: An Unexplored Pathway of the White Rot Agent of the Esca Complex of Diseases. *J. Fungi* **2023**, *9*, 498. <https://doi.org/10.3390/jof9040498>

Academic Editors: Violetta Katarzyna Macioszek and Andrzej K. Kononowicz

Received: 14 February 2023

Revised: 10 April 2023

Accepted: 14 April 2023

Published: 21 April 2023



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**Abstract:** *Fomitiporia mediterranea* (*Fmed*) is the primary Basidiomycota species causing white rot in European vineyards affected by the Esca complex of diseases (ECD). In the last few years, an increasing number of studies have highlighted the importance of reconsidering the role of *Fmed* in ECD etiology, justifying an increase in research interest related to *Fmed*'s biomolecular pathogenetic mechanisms. In the context of the current re-evaluation of the binary distinction (brown vs. white rot) between biomolecular decay pathways induced by Basidiomycota species, our research aims to investigate the potential for non-enzymatic mechanisms adopted by *Fmed*, which is typically described as a white rot fungus. Our results demonstrate how, in liquid culture reproducing nutrient restriction conditions often found in wood, *Fmed* can produce low molecular weight compounds, the hallmark of the non-enzymatic “chelator-mediated Fenton” (CMF) reaction, originally described for brown rot fungi. CMF reactions can redox cycle with ferric iron, generating hydrogen peroxide and ferrous iron, necessary reactants leading to hydroxyl radical ( $\bullet\text{OH}$ ) production. These observations led to the conclusion that a non-enzymatic radical-generating CMF-like mechanism may be utilized by *Fmed*, potentially together with an enzymatic pool, to contribute to degrading wood constituents; moreover, indicating significant variability between strains.

**Keywords:** *Fmed*; CMF; phenolates; ferric iron;  $\bullet\text{OH}$ ; grapevine; GTDs

## 1. Introduction

In Europe, grapevine trunk diseases (GTDs) are a major cause of vineyard decline: they have reached alarming levels of spread and incidence in several countries, with the Esca complex of diseases (ECD) being one of the most harmful and common throughout the Mediterranean region [1–3]. The ECD can considerably reduce the life of a vineyard, causing substantial economic losses in the order of millions of euros per year due to declines in yield and wine quality [4–6].

The ECD is considered to be a complex of multiple diseases, manifesting with internal damage to the wood and systemic damage to leaves and twigs [7]. Notwithstanding the

role played by a broad mycoflora in ECD-affected grapevines, as evidenced by classical and omics approaches [8–11], the pathogens most frequently described in grapevine wood infections (for which Koch’s postulates have been at least partially fulfilled) are the two Ascomycota species *Phaeomoniella chlamydospora* [12] and *Phaeoacremonium minimum* [13], and usually prominent Basidiomycota species, which in Europe is most commonly *Fomitiporia mediterranea* (*Fmed*) [14,15]. The two Ascomycota species have been associated mostly with brown wood streaking, Petri disease, and grapevine leaf stripe disease (GLSD) [16,17], while *Fmed*, like other *Fomitiporia* spp. around the world, is a white rot agent associated with historical “Esca” (*sensu strictu*) and “Esca proper”; the latter being a condition where GLSD and white rot manifest simultaneously [7]. As the primary fungus responsible for white rot in grapevine woody tissue, *Fmed* is revealed to be the primary fungal agent behind the hydraulic, mechanical, and ecophysiological damage by the ECD in vines, stimulating considerable research interest related to vineyard ECD fundamental wood degradation mechanisms [18–20].

Basidiomycota species are the principal wood-decay taxa in nature, even though some Ascomycota and bacterial species are also able to induce or synergistically participate in some types of wood decay [21–24]. According to the dichotomous model of decay types historically proposed by Hartig [25] and still currently used, wood decaying Basidiomycota species can be broadly divided into white rot fungi (WRF) and brown rot fungi (BRF), based on the wood residue left being either bleached fibrous or brownish and cubic material, respectively [26]. In WRF, the breakdown of all wood components (lignin, hemicellulose, and cellulose) happens either progressively or simultaneously, depending on whether lignin is preferentially degraded with respect to holocellulose or not. More precisely, carbohydrate active enzymes (CAZymes) will be active on cellulose and hemicellulose (holocellulose), while laccases and class II peroxidases (Class II-PODs) will be active on lignin, and several auxiliary active (AA) redox enzymes will support cellulolytic and ligninolytic enzyme activity [27–32]. In contrast, in BRF, a reduced suite of CAZymes is typically active, while Class II-PODs are generally inactive or absent. The loss of POD function in the BRF has been documented via progressive genomic depletion as these fungi evolved from a white rot common ancestor [33]. The BRF attack and metabolize holocellulose primarily via non-enzymatic redox mechanisms, and they leave lignin repolymerized in a modified form after initial depolymerization and demethylation [29,34]. These redox mechanisms predominantly involve reactive oxygen species (ROS, mostly hydroxyl radicals) generated by a non-enzymatic pathway involving iron [35]. In the mid-1990’s, Goodell et al. introduced the “Chelator-Mediated Fenton (CMF) reaction” model for the brown rot agent *Gloeophyllum trabeum*, which was then further explored and refined in the following years for other Basidiomycota species [34–40] and recently also for some Ascomycota species [41,42].

The CMF reaction can be described as a mechanistic model in which a series of non-enzymatic chemical events takes place in the fungal extracellular matrix between the wood cell lumen and wood cell wall, involving solubilization of insoluble Fe(oxyhydr)oxide complexes and reduction of iron. This ultimately leads to highly reactive oxygen species production, i.e., hydroxyl radicals ( $\bullet\text{OH}$ ), one of the strongest non-specific oxidants in biological systems [29,43]. The primary stages of the CMF model are (i) acidification of the wood cell lumen micro-environment and extracellular matrix surrounding fungal hyphae (down as low as pH 2) to facilitate iron solubilization and diffusion into the wood cell wall, mainly by secretion of small organic acids (mostly oxalic acid) capable of forming very stable Fe:organic acid complexes; (ii) production of low molecular weight compounds (LMWCs, mostly phenolates) that can bind and reduce iron and are able to diffuse into the wood cell wall through cell wall microvoids; (iii) reduction of iron in the higher pH environment of the wood cell wall (around pH 5.5), with LMWCs redox cycling with molecular oxygen, to yield  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  respectively; and (iv) production of  $\bullet\text{OH}$  radicals via the Fenton reaction (Equation (1)) within the wood cell wall (and at a distance from the fungal hyphae to protect the fungus from oxidative damage). The highly reactive  $\bullet\text{OH}$  radical is then

responsible for oxidative depolymerization of holocellulose and lignin, which can undergo further degradation via CAZymes and, if available, Class-II PODs [29,34,36,43–45].



*Fomitiporia mediterranea* has historically been classified as a WRF [15]. In recent years, research has demonstrated how the binary classification of biomolecular decay mechanisms into either white rot or brown rot fungi is a “reductive monochromatic paradox” that does not represent all the nuances and peculiarities of Basidiomycota decay pathways [46–48]. In particular, as a WRF, *Fmed* presents a unique decay pattern fingerprint that results from a secretome that is unique in several different ways:

- i. CAZymes, laccases, class II-PODs (manganese peroxidases [MnPs] and generic peroxidases [GPs]), and other AAs have been reported in genomic, proteomic, and enzymological studies of *Fmed*, while genes encoding for versatile peroxidases (VPs) and lignin peroxidases (LiP) have been demonstrated to be absent in the *Fmed* genome [20,33,49–51];
- ii. The *Fmed* genome possesses gene copy numbers with suggested involvement in LMWC biosynthesis, such as reducing polyketide synthases (*R-PKSs*), terpene synthases (*TSs*), and cytochrome P450 enzymes, proposed as important in LMWC-mediated brown rot decay mechanisms [33,47,52,53];
- iii. LMWC production in vitro and the iron reduction capability of liquid culture supernatants have been previously reported for *Fmed*, but without separation of the high molecular weight fraction and without obtaining data on the redox cycling ability of the LMW metabolites [54–57];
- iv. Even if described as a simultaneous white rot relying on in vitro wood mineralization assays, at the nanoscale, the wood cell wall decay pattern observed for *Fmed* does not resemble a typical enzymatic white rot decay pattern [20,51,58].

It has been conclusively demonstrated for both WRF and BRF that lignocellulolytic enzymes are too large to readily penetrate intact lignified secondary cell wall material. However, LMW metabolites from fungi are able to diffuse into the cell wall. This suggests that, in WRF, prior non-enzymatic LMWC-mediated degradation may be required or that the fungal extracellular enzymes remain confined to the fungal extracellular matrix [40,59–63]. Moreover, in vitro studies on the non-enzymatic degradation of different carbon sources or recalcitrant compounds used by selective WRF or LiP-deficient WRF showed that ferric ( $\text{Fe}^{3+}$ ) reducing-LMWCs were produced [64,65], efficiently oxidizing lignocellulose constituents in vitro when reacting with iron and  $\text{H}_2\text{O}_2$  [38].

The peculiarities of the *Fmed*-decay pattern have demonstrated the lack of a validated biochemical mechanistic model for *Fmed*-induced white rot. Here we hypothesize that *Fmed* can trigger a non-enzymatic CMF-like pathway, potentially acting together with well-described enzymes. To address our hypothesis, we focused our research only on the low molecular weight *Fmed*-secretome, testing the fundamental steps of CMF chemistry, including: (i) the ability to produce ferric ( $\text{Fe}^{3+}$ ) reducing-LMWCs with ferric reducing activity (FeRA); and (ii) redox cycling capacity of the LMW fraction to generate  $\text{H}_2\text{O}_2$  and  $\bullet\text{OH}$  radicals. We also tested for oxidative degradation in holocellulose and in the sawdust of Gewürztraminer grapevine wood (GW) when exposed to the LMW-*Fmed* extracts in order to verify in vitro the role of non-enzymatic mechanisms in wood constituent degradation. To include more variability, the research was conducted using different *Fmed* strains. A goal of this research is to help fill the knowledge gap on *Fmed* pathogenicity factors through the exploration of potential non-enzymatic mechanisms behind *Fmed*-induced white rot. This work may also help identify new paths for future research to potentially demystify ECD foliar symptom etiology in light of recent findings suggesting a role for white rot fungi in the development of ECD foliar symptoms in the northern hemisphere [19,66–68].

## 2. Materials and Methods

### 2.1. Fungal Strains and Culture Conditions

Six *Fomitiporia mediterranea* strains were used for these experiments, sourced from different mycological collections (Table S1). These strains have been previously used in other publications [20,23,51,69]. In this paper, an easy-to-read key legend has been assigned to each *Fmed* strain to avoid misinterpretation and facilitate the interpretation of the data presented. The strain *Fm1* was originally identified both morphologically and by sequencing of the Internal Transcribed Spacer (ITS) region in 2003 (NCBI GenBank accession number AY529683.1). Strains *Fm2*, *Fm3*, *Fm4*, *Fm5*, and *Fm6* were identified based on morphological aspects and population genetics studies [69]. In addition, strains *Fm2*, *Fm4*, *Fm5*, and *Fm6* were recently rapidly verified by molecular amplification of ITS sequences (% of similarity with *Fomitiporia mediterranea* in blast NCBI). The fungi were grown on 2% malt extract agar in darkness and kept at 27 °C until inoculation of the liquid culture.

### 2.2. Glassware Preparation

Because iron restriction conditions are a prerequisite for the production of ferric ( $\text{Fe}^{3+}$ ) reducing LMWCs, all glassware used throughout the experiments was carefully acid washed three times with 5.15 M nitric acid ( $\text{HNO}_3$ ) to remove any iron residue, then rinsed three times with Milli-Q® water (18.2 M $\Omega$ ·cm).

### 2.3. Liquid Culture Experimental Design, Sampling, and Measurement of Fungal Growth

Liquid cultures were prepared using restricted nutrient and iron-free media to reproduce the nutrient restrictive conditions often found in wood and to stimulate LMW iron-binding compound production [70]. The cultures were prepared as follows: four Erlenmeyer flasks were inoculated for each *Fmed* strain listed in Supplementary Table S1. The liquid medium (200 mL per 500 mL Erlenmeyer flask) was prepared in Milli-Q® water (18.2 M $\Omega$ ·cm) according to Highley [71] with some modifications as follows: 0.25 g L<sup>-1</sup> ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ; Duchefa Biochemie, Haarlem, The Netherlands), 2 g L<sup>-1</sup> monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ ; Carl Roth GmbH, Karlsruhe, Germany), 0.5 g L<sup>-1</sup> magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; Carl Roth GmbH, Karlsruhe, Germany), 0.1 g L<sup>-1</sup> calcium chloride ( $\text{CaCl}_2$ ; Carl Roth GmbH, Karlsruhe, Germany), 0.57 mg L<sup>-1</sup> boric acid ( $\text{H}_3\text{BO}_3$ ; VWR International LLC, Radnor, PA, USA), 0.31 mg L<sup>-1</sup> zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; Sigma-Aldrich, St. Louis, MO, USA), 0.039 mg L<sup>-1</sup> copper sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; VWR International LLC, Radnor, PA, USA), 0.036 mg L<sup>-1</sup> manganese chloride tetrahydrate ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; VWR International LLC, Radnor, PA, USA), 0.018 mg L<sup>-1</sup> ammonium molybdate tetrahydrate ( $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ ; Carl Roth GmbH, Karlsruhe, Germany), and 0.001 g L<sup>-1</sup> thiamine HCl ( $\text{C}_{12}\text{H}_{17}\text{ClN}_4\text{OS} \cdot \text{HCl}$ ; Sigma-Aldrich, St. Louis, MO, USA). The carbon sources were 0.5 g L<sup>-1</sup> glucose (Euromedex, Souffelweyersheim, France) and 50  $\mu\text{m}$  microcrystalline cellulose (Acros Organics, Thermo Fisher Scientific Inc., Geel, Belgium) 1% (*w/v*). Iron was completely omitted. Liquid medium pH was adjusted before autoclaving to 5.5 using sodium hydroxide (NaOH) solution.

After autoclaving and cooling to room temperature, two 5 mm mycelial discs of *Fmed* were scraped from the surface of fully-grown petri dishes, avoiding agar contamination, and these were used for inoculation of the media in the Erlenmeyer flasks. Cultures were grown at 27 °C in darkness and under stationary conditions for a period of 12 weeks. Control flasks were prepared under the same conditions but uninoculated (mock). These negative controls were designated  $C_{(-)}$ .

At the end of incubation, the total mycelium dry weight (DW) was determined by measuring the total dried solid mycelial mass following consecutive filtration steps. For this, an initial filtration using a 1  $\mu\text{m}$ -pore glass fiber prefilter (Merck Millipore Ltd., Cork, Ireland) was carried out, followed by further steps of filtration using Whatman™ cellulose membrane filters of progressively narrower pore size (0.45  $\mu\text{m}$ , then 0.2  $\mu\text{m}$ ), and

finally ultra-filtration with Polyethersulfone (PES) 5 Kda filters (Biomax<sup>®</sup>, EMD Millipore Corporation, Billerica, MA, USA). To facilitate the process, a vacuum pump (Gardner Denver Welch Vacuum Technology Inc., Monroe, LA, USA) coupled with Nalgene<sup>™</sup> filter holders and receivers (Nalgene Nunc International Corporation, Rochester, NY, USA) was used. The filtrate was lyophilized to reduce the liquid volume to approximately one-third and stored at  $-80^{\circ}\text{C}$  until extraction of LMWCs. Filters containing the mycelium were dried at  $60^{\circ}\text{C}$  to obtain the dried mycelial biomass weight.

#### 2.4. LMWC Extraction

In order to extract the phenolic and phenolic-derived LMWCs, the medium was acidified (pH 3) with 1N HCl, and a triple ethyl acetate extraction (1:1 volume ratio) was conducted [70], followed by rotary evaporation under reduced pressure (Laborota 4000, Heidolph Instruments GmbH & Co., KG, Schwabach, Germany). The residual LMW fractions were resuspended in methanol (MeOH, LC-MS quality), filtered through a 0.2  $\mu\text{m}$  syringe filter, then stored at  $-20^{\circ}\text{C}$  for further analysis.

#### 2.5. Determination of Total Phenols

Total phenols in the LMW methanolic extract were determined according to the method described by Perez-Gonzalez et al. [41] with some modifications. Briefly, samples of extract (40  $\mu\text{L}$ ) and the Folin–Ciocalteu reagent (200  $\mu\text{L}$ ) were reacted (5 min at room temperature) before the addition of 5% sodium carbonate (600  $\mu\text{L}$ ) to initiate the reaction. Initial sodium carbonate concentration was reduced, as in the process employed by Cicco and Lattanzio [72], to avoid precipitation. After a further reaction period (90 min at room temperature) in the dark, absorbance values at 765 nm were determined spectrophotometrically (Agilent Cary 8454 UV-Visible, Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia) [73], with gallic acid solutions used as phenolic concentration standards. Methanolic extract fractions were replaced with MeOH-only for blanks, while negative controls ( $C_{(-)}$ ) contained an aliquot of methanolic extract of the liquid mock-inoculated medium (to check background noise).

#### 2.6. LMW Fraction Analysis

LMW methanolic extracts (49  $\mu\text{L}$ ), with 1  $\mu\text{L}$  of a 1 mg/mL methanolic solution of 5-methylsalicylic acid added as an internal standard, were used for HPLC analysis. Ultra-high-performance liquid chromatography (UHPLC) coupled to an Impact II Q-TOF-MS/MS mass spectrometer equipped with an electrospray ESI source Apollo II operating in negative mode (Bruker Daltonics GmbH, Bremen, Germany) was used with a reverse-phase C18 Bruker Intensity Solo HPLC column (2.0  $\mu\text{m}$  particle size, 100 Å pore size, and dimensions of 2.0 mm  $\times$  100 mm) (BRHSC18022100, Bruker Daltonics GmbH, Bremen, Germany). The mobile phases were ultrapure water with 0.1% of formic acid (eluent A) and MeOH with 0.1% of formic acid (eluent B) and were used in gradient expressed as a percentage of B: 2 min at 1.0%, linear gradient during 15 min until 99%, 3 more min at 99%, followed by 5 min in the initial conditions (1% B) for equilibration before a new injection. The total flow rate was 0.25 mL/min, the injection volume was 10  $\mu\text{L}$ , and the oven column and autosampler temperatures were 35 and  $8^{\circ}\text{C}$ , respectively. The MS detector was internally calibrated before starting the batch analysis, and additionally, at the beginning of each injection, spiked with 10 mM sodium formate solution in isopropyl alcohol:H<sub>2</sub>O (1:1, v/v). The ESI parameters were as follows: capillary voltage  $-3500\text{ V}$ , end plate offset  $-500\text{ V}$ , nebulizer gas 29.0 psi, dry gas 8.0 L/min, and dry temperature  $200^{\circ}\text{C}$ . The spectral rate was 8 Hz over a 20–1600 m/z mass range in auto MS/MS scan mode. A collision energy ramp was applied from 20 to 50 eV. Data acquisition was achieved by Bruker Compass HyStar 5.1, and otofControl 5.2 software and data treatment was performed with Data Analysis 5.3 and METABOSCAPE 2021b (Bruker Daltonics GmbH, Bremen, Germany). Relative counts (Rc) of specific LMWC structures were used to solely compare quantities of individual compounds among *Fmed* strains.

### 2.7. Determination of Ferric Iron Reduction Activity (FeRA) in the LMW Fraction

LMW methanolic extracts were tested for ferric iron reduction activity (FeRA), following the protocol of Perez-Gonzalez et al. [41]. A reaction mix at the following final concentration was prepared: 100  $\mu$ M acetate buffer (pH 5.5), 250  $\mu$ M of freshly prepared aqueous FerroZine<sup>TM</sup> (Acros Organics, Thermo Fisher Scientific Inc., Geel, Belgium), and 300  $\mu$ M of freshly prepared FeCl<sub>3</sub>. LMW methanolic extracts, with their LMW total phenol concentration adjusted to 150  $\mu$ M according to Folin–Ciocalteu results. They were used to verify if the differences in iron reduction between strains were due to respective total phenol concentrations or different ratios among phenol types. Blank and control solutions were prepared as in Section 2.5.

All procedures were conducted in the absence of light and at room temperature. Fe<sup>3+</sup> reduction was assayed spectrophotometrically at 562 nm (Agilent Cary 8454 UV-Visible, Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia), monitoring the absorbance after 45 min of incubation with the ferrozine reaction mixture [74], using FeCl<sub>2</sub> solutions as standards.

### 2.8. Determination of Hydrogen Peroxide Levels by Ferrous Oxidation in the Presence of Xylenol Orange (FOX Assay)

LMW methanolic extracts were tested for hydrogen peroxide generation during the oxidation of ferrous iron in the Fenton reaction using the ferrous ammonium–xylenol orange (FOX) assay and the method proposed by Perez-Gonzalez et al. [41], with a few modifications. A stock FOX solution containing Pierce<sup>TM</sup> Peroxide Assay Reagent B (Pierce Biotechnology, Thermo Fisher Scientific Inc., Rockford, IL, USA) was prepared by dissolving ferrous ammonium sulfate directly in H<sub>2</sub>SO<sub>4</sub> to avoid the spontaneous oxidation of ferrous ions at neutral pH. A LMW methanolic extract (buffered in 50 mM MES, pH 5.5) was then mixed with the FOX reagent to yield a final solution of 150  $\mu$ M phenolics (as determined by the Folin–Ciocalteu assay), 100  $\mu$ M of xylenol orange, 250  $\mu$ M of ferrous ammonium sulfate, and 25 mM of H<sub>2</sub>SO<sub>4</sub>. Blank and control solutions were prepared as in Sections 2.5 and 2.7.

The reaction mix was incubated for 30 min in the dark before spectrophotometric determination (Agilent Cary 8454 UV-Visible, Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia) at 560 nm [75]. H<sub>2</sub>O<sub>2</sub> was used for calibration solutions.

### 2.9. Determination of Hydroxyl Radical Activity by Electro Paramagnetic Resonance (EPR)

LMW methanolic extracts from the different *Fmed* strains were evaporated to dryness (45 min at 35 °C) using an Eppendorf Concentrator (Eppendorf AG, Hamburg, Germany), then resuspended either in sodium acetate buffer at pH 5.5 or acidified down to pH 3.5, to check the capacity of the LMWCs to generate hydroxyl radicals both at the pH of the intact wood cell wall (5.5) and in the more acidic environment during decay (3.5). For •OH detection, 5,5-Dimethyl-1-pyrroline N-oxide (DMPO, TCI Europe N.V., Zwijndrecht, Belgium) was used as a spin-trap agent in EPR. The final reaction mixture for each sample was prepared according to the process used by Perez-Gonzalez et al. [41] with a few modifications by adding (in the following order): an aliquot of LMW methanolic extract, H<sub>2</sub>O<sub>2</sub> (0.15 mM), DMPO (100 mM), and finally Fe<sup>3+</sup> (0.15 mM) to start the reaction. An aliquot of the methanolic extract from the uninoculated liquid medium was used as a control to check the background Fenton reaction. After mixing and incubation at room temperature for 5 min (in the absence of light), the mixture was transferred to a 50  $\mu$ L capillary glass tube for EPR analysis. An X-Band ESR spectrometer (Magnettech MS400) was used for these analyses.

The DMPO-OH spectrum was simulated by PEST WinSIM software [76] in order to check the identity of the radical adduct. The simulation parameters were 100% Gaussian line shape, 1.16-G linewidth, and hyperfine coupling constants  $aH = 14.85$  and  $aN = 14.96$  G. Semi-quantification of •OH relative amplitudes were represented by plotting the average amplitude of the tallest peak of DMPO-OH adduct spectra for all *Fmed* strain LMW fractions.

### 2.10. Carbohydrate Oxidative Degradation Assays

To evaluate the oxidative effect of any CMF reactions promoted by  $\text{Fe}^{3+}$ -reducing LMWCs on holocellulose, microcrystalline cellulose and hemicellulose (xylan) sources were used, following the process described by Arantes and Milagres [77]. Grapevine wood sawdust produced from debarked clear wood (free of visible defects) from Gewürztraminer vines (GW-sawdust) was used to evaluate oxidation directly on the wood material. Following procedures from Arantes et al. [38] with some modifications, a mixture of 1% (*w/v*) microcrystalline cellulose, xylan, or GW-sawdust was mixed with 50 mM sodium acetate buffer (pH 5.5), 100  $\mu\text{L}$  of LMW *Fmed* methanolic extract, 40 mM  $\text{H}_2\text{O}_2$ , and freshly prepared 4 mM  $\text{FeCl}_3$ ; the last of which was added at the end to start the reaction. The mixture was incubated at 50 °C for 12 h (hrs). After incubation, 1.5 mL of 1% (*w/v*) 3, 5 dinitro salicylic acid (DNS) reagent was added to each sample, and the mixture was boiled for 5 min and cooled in ice to stop the reaction. For each reaction, a pool of biological replicates was used. Absorbance at 540 nm was measured to assess the release of reducing sugars in the carbohydrates. A control with methanolic extract using a liquid medium mock-inoculated (*Fmed* LMW- $\text{Fe}^{3+}$ -reductants free) was used to check holocellulose oxidation only in the presence of  $\text{Fe}^{3+}$  and  $\text{H}_2\text{O}_2$ .

### 2.11. Statistical Analysis

The experiments were set up in a randomized design. Data distribution was checked using D'Agostino and Pearson and Kolmogorov–Smirnov's tests, and homoscedasticity for normally distributed data was verified using Bartlett's test. To highlight differences between *Fmed* strains, data from DW (g) biomass ( $n = 3$ ) and total phenols ( $n = 3$ ) were analyzed by one-way ANOVA, and groups of means were separated by Fisher's LSD multiple comparison tests ( $p < 0.05$ ). Data from pH in the liquid medium ( $n = 3$ ) were analyzed by Welch's ANOVA. Data from LMWCs Rc ( $n = 4$ ),  $\mu\text{mol L}^{-1}$  of  $\text{Fe}^{2+}$  reduced from  $\text{Fe}^{3+}$  and  $\mu\text{mol L}^{-1}$  of generated  $\text{H}_2\text{O}_2$  ( $n = 3$ ), the relative amplitude of  $\bullet\text{OH}$ -adducts in EPR at pH 3.5 ( $n = 4$ ) were assessed using a Kruskal–Wallis test, and mean ranks were separated according to Dunn's multiple comparison tests ( $p < 0.05$ ). The relative amplitude of  $\bullet\text{OH}$ -adducts in EPR at pH 5.5 ( $n = 3$ ) was analyzed using the unpaired two-tailed *t*-test ( $p < 0.05$ ). Graphs and statistical elaboration were made using Prism8 (GraphPad Software, San Diego, CA, USA).

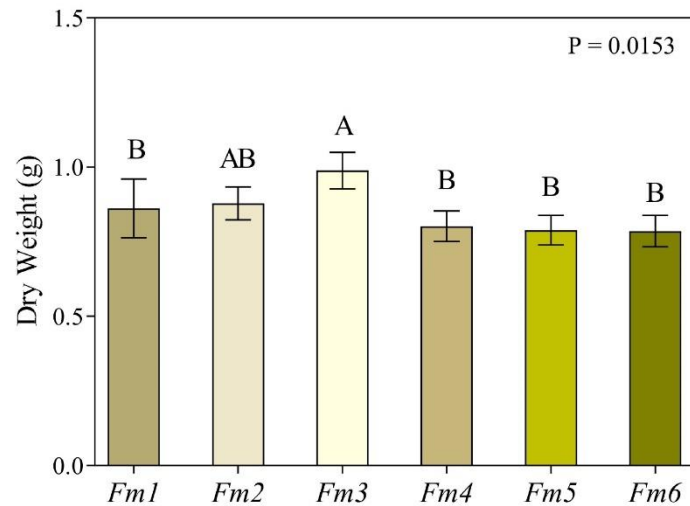
## 3. Results

### 3.1. Fungal Biomass

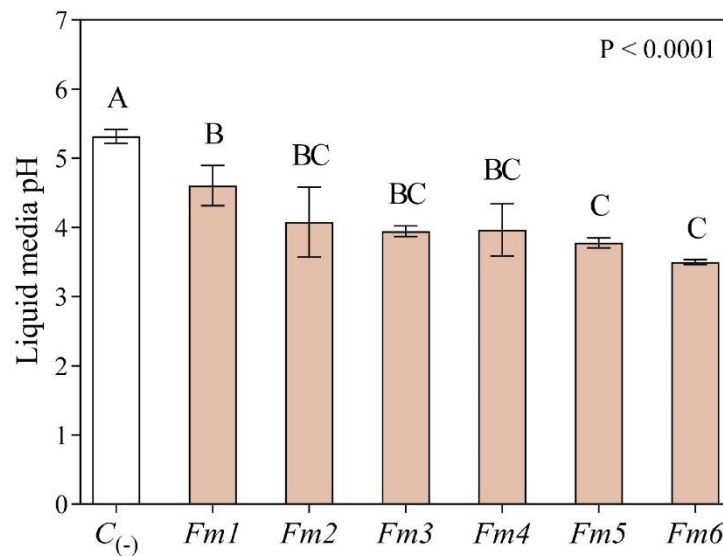
Fungal biomass production measured at the end of the experiment showed that strain *Fm3* produced the highest quantity of dry biomass, while *Fm5* and *Fm6* were the strains that produced the least (−20.56% and −20.23% compared to *Fm3*, respectively). The other strains produced a statistically comparable amount of biomass (Figure 1).

### 3.2. Culture Media Acidification

Culture media pH was measured at the end of the experiment. General acidification with respect to initial pH (5.5) was observed in all liquid cultures, with the lowest pH value recorded for the strains *Fm5* (3.8) and *Fm6* (3.5) (Figure 2). The exception was the negative control ( $C_{(-)}$ ), where the pH of the liquid media remained almost the same (5.3) from the beginning of the experiment. Liquid media from strain *Fm1* culture had the highest pH values (4.6, +21.06% compared to *Fm5*, and +31.43% compared to *Fm6*). *Fm6* was responsible for the greatest amount of liquid media acidification relative to the initial pH, while *Fm1* showed the lowest media acidification.



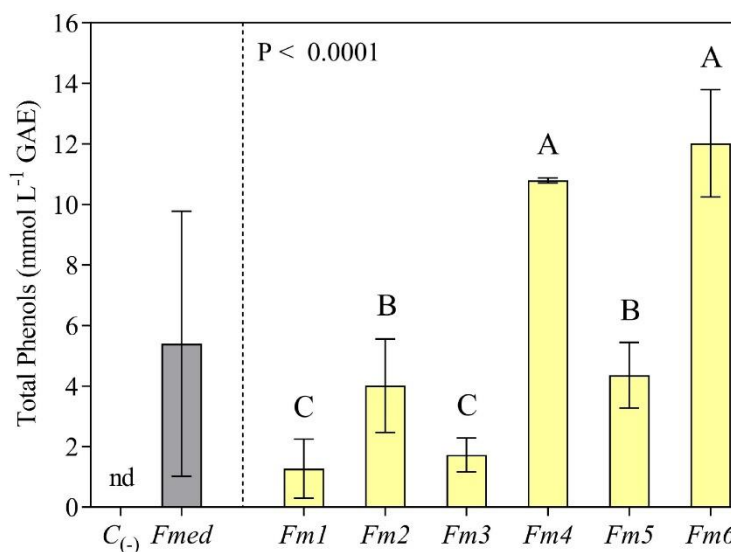
**Figure 1.** Dry weight of filtered liquid culture from 6 strains of *Fmed* (Fm1, Fm2, Fm3, Fm4, Fm5, and Fm6) grown at 27 °C under static conditions for 12 weeks in the dark. Values are the means  $\pm$  standard deviation ( $n = 3$ ). Results were analyzed by one-way ANOVA, followed by Fisher's LSD multiple comparison test ( $p < 0.05$ ). Different letters indicate significant differences according to multiple comparison test results.



**Figure 2.** Liquid media pH at the end of the experiment for the 6 strains of *Fmed* (Fm1, Fm2, Fm3, Fm4, Fm5, and Fm6) and in control ( $C_{(-)}$ ) flasks. Values are the means  $\pm$  standard deviation ( $n = 3$ ). Results were analyzed by Welch's ANOVA ( $p < 0.05$ ). Different letters indicate significant differences according to multiple comparison test results.

### 3.3. Total Phenols Analysis

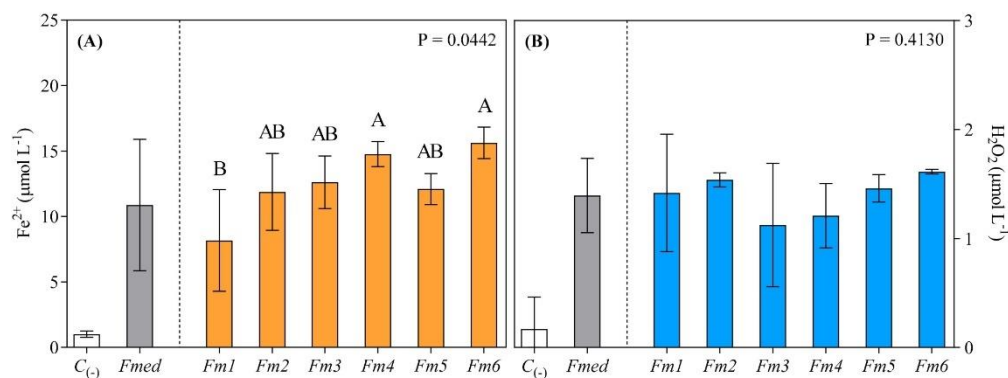
Total phenols were measured in the methanolic LMW extracts, with an average value for *Fmed* of  $5.6 \text{ mmol L}^{-1}$  (Figure 3). The highest values were recorded in strain *Fm6* ( $12.02 \text{ mmol L}^{-1}$ ). The strain with the lowest values ( $1.27 \text{ mmol L}^{-1}$ ) was *Fm1* ( $-89.43\%$  compared to *Fm6*). The other strains produced lower levels of phenols with respect to *Fm6* in the following order: *Fm6* > *Fm4* > *Fm5* > *Fm2* > *Fm3* > *Fm1* (Figure 2).



**Figure 3.** Total phenols from LMW methanolic extracts analyzed spectrophotometrically using a Folin–Ciocalteu assay. Values (expressed as  $\text{mmol L}^{-1}$  gallic acid, GAE) are the means  $\pm$  standard deviation ( $n = 3$ ). Results from the negative control ( $C_{(-)}$ ) and *Fmed* average value are shown on the left (nd = not detected). Results from different *Fmed* strains (*Fm1*, *Fm2*, *Fm3*, *Fm4*, *Fm5*, and *Fm6*) were analyzed by one-way ANOVA, followed by Fisher's LSD multiple comparison test ( $p < 0.05$ ). Different letters indicate significant differences according to multiple comparison test results.

### 3.4. FeRA and Hydrogen Peroxide Generation from LMWC Redox Cycle

FeRA and hydrogen peroxide generated from redox cycling of the methanolic fungal LMW extracts were determined for the different strains of *Fmed*, with all extracts found to reduce ferric to ferrous iron (Figure 4A: average  $\text{Fe}^{2+}$  value for *Fmed* of  $12.52 \mu\text{mol L}^{-1}$ ). All extracts were also found to generate hydrogen peroxide from LMWCs redox cycling (Figure 4B: average  $\text{H}_2\text{O}_2$  value of  $1.4 \mu\text{mol L}^{-1}$ ). Strains *Fm6* ( $15.62 \mu\text{mol L}^{-1}$ ) and *Fm4* ( $14.76 \mu\text{mol L}^{-1}$ ) reduced the most iron, while strain *Fm1* reduced the least ( $8.16 \mu\text{mol L}^{-1}$ ,  $-47.76\%$ , and  $-44.72\%$ , compared to *Fm6* and *Fm4*, respectively). All extracts from the other *Fmed* strains reduced statistically comparable amounts of ferric iron, but all reduced significantly less iron compared to *Fm6* (Figure 4A). LMWCs redox cycling of extracts from different *Fmed* strains generated statistically comparable amounts of  $\text{H}_2\text{O}_2$ , even though the highest absolute values were recorded for strains *Fm1* and *Fm6* ( $1.42$  and  $1.61 \mu\text{mol L}^{-1}$ , respectively) and the lowest for strain *Fm3* ( $1.12 \mu\text{mol L}^{-1}$ ,  $-21.13\%$  compared to *Fm1* and  $-30.43\%$  compared to *Fm6*) (Figure 4B).



**Figure 4.** Ferrous iron (expressed as  $\mu\text{mol L}^{-1}$  of  $\text{Fe}^{2+}$ ) reduced from ferric iron (A) via the fungal secreted LMWCs and  $\text{H}_2\text{O}_2$  (expressed as  $\mu\text{mol L}^{-1}$ ) generated by redox cycling of LMWCs with  $\text{Fe}^{2+}$  and  $\text{O}_2$  (B). Values are the means  $\pm$  standard deviation ( $n = 3$ ). Results from negative controls ( $\text{C}_{(-)}$ ) and *Fmed* average values are shown on the left of each graph. Data from different *Fmed* strains (*Fm1*, *Fm2*, *Fm3*, *Fm4*, *Fm5*, and *Fm6*) were analyzed by the Kruskal–Wallis test, followed by Dunn’s multiple comparison tests ( $p < 0.05$ ). Different letters indicate significant differences according to multiple comparison test results.

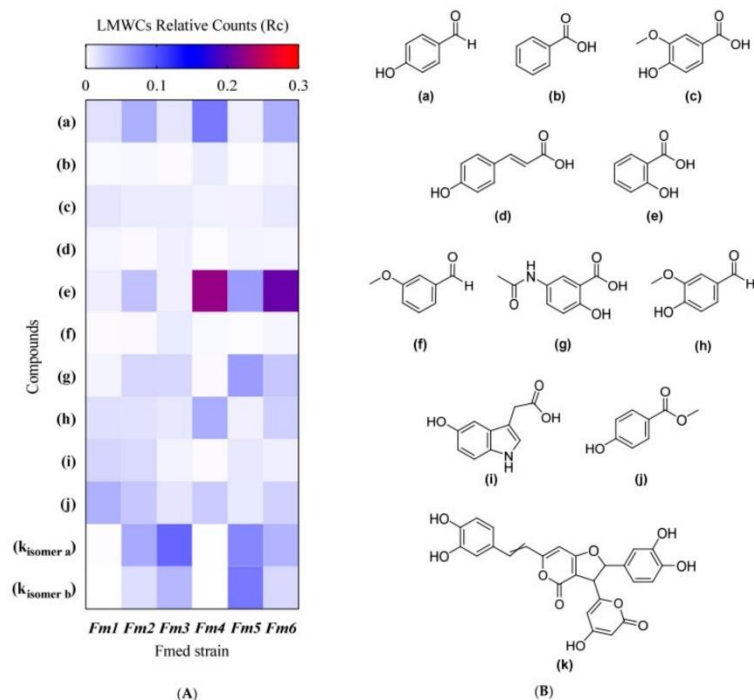
### 3.5. LMWC Fraction Characterization

Several phenolic acids (hydroxybenzoic and hydroxycinnamic acids), phenolic aldehydes, and other aromatic compounds were identified from the LMW methanolic extracts of all *Fmed* strains (Figure 5, Table 1; Appendix A, Figure A1, Tables A1 and A2). Some of the identified LMWCs have previously been reported to chelate and reduce ferric iron (see list of compounds in Table 1). For 3-methoxybenzaldehyde (f), N-acetyl-5-aminosalicylic acid (g), 5-hydroxyindole-3-acetic acid (i), methyl-4-hydroxybenzoate (j), and the phenolic pigment hypholomine B (k), further analyses are necessary to determine their FeRA. No strain-specific compounds were identified. Because standards could not be obtained for most compounds, the Rc of specific LMWC structures can be compared across all samples, but comparative quantification between different compounds in the same sample cannot be performed. Except for hypholomine B (not detected in the extracts of the strains *Fm1* and *Fm4*), all the identified compounds were produced by all strains, although in different amounts. Strain *Fm1* produced the most methyl-4-hydroxybenzoate, with strain *Fm3* producing the most hypholomine B (isomer a), and strain *Fm5* producing the most hypholomine B (isomer b). Strain *Fm4* produced the most 4-hydroxybenzaldehyde and salicylic acid (Figure 5 and Table S2).

### 3.6. Determination of Hydroxyl Radical Activity by Electron Paramagnetic Resonance (EPR)

All LMW methanolic extracts were able to generate  $\bullet\text{OH}$  radicals as detected using electron paramagnetic resonance (EPR) in the presence of the DMPO spin-trapping agent. The DMPO- $\bullet\text{OH}$  adduct displayed its characteristic 4-line spectrum, identified using theoretical simulation (Figure 6A). Note that EPR spectra are typically reported as “relative amplitude” for the Y-axis values. Semi-quantification via spin trapping adduct showed significant differences among *Fmed* strains, with the highest average  $\bullet\text{OH}$  relative amplitude recorded for strain *Fm6* (7669) and the lowest for *Fm1* (−48.05% compared to *Fm6*) and *Fm2* (−29.04% compared to *Fm6*) (Figure 6C). All other strains generated a statistically comparable relative amount of  $\bullet\text{OH}$  radical to the *Fm6* strain. The methanolic extracts of *Fmed* strains *Fm1* and *Fm6*, with the least and most  $\bullet\text{OH}$  produced at pH 3.5, respectively, were also tested for  $\bullet\text{OH}$  generation at pH 5.5. Both were able to generate  $\bullet\text{OH}$ , though

with lower average relative amplitude compared to pH 3.5 (739 for *Fm1* vs. 2056 for *Fm6*) (Figure 6B,D).



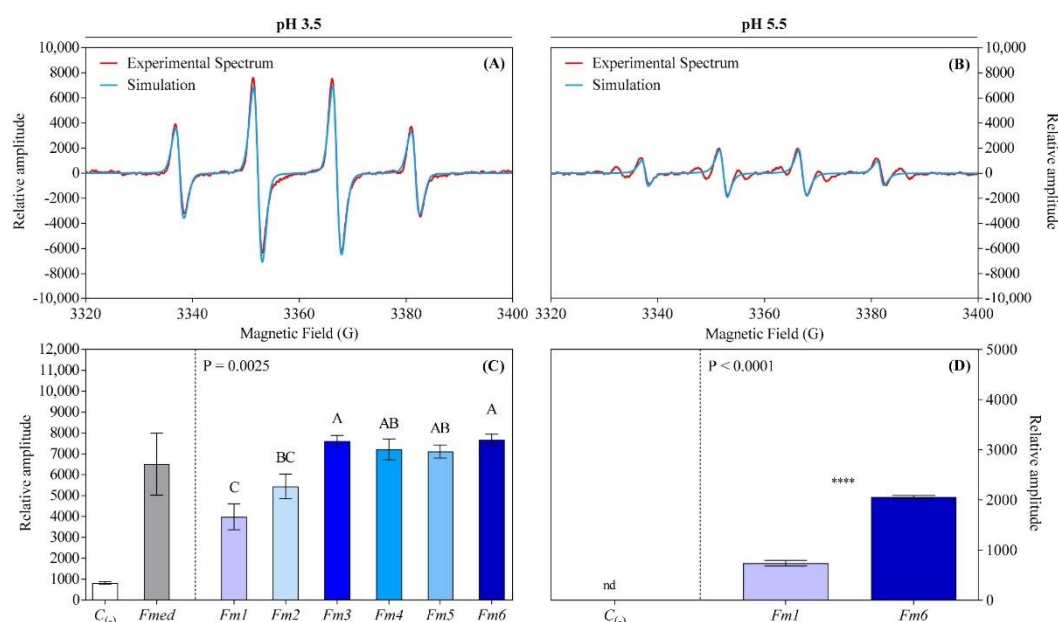
**Figure 5.** Heatmap (A) shows the LMW compounds' average relative counts (Rc) analyzed by UHPLC-MS/MS. Chemical structure (B) of described compounds: (a) 4-hydroxybenzaldehyde, (b) benzoic acid, (c) 4-hydroxy-3-methoxybenzoic acid (vanillic acid), (d) 4-hydroxycinnamic acid (*p*-coumaric acid), (e) 2-hydroxybenzoic acid (salicylic acid), (f) 3-methoxybenzaldehyde, (g) N-acetyl-5-aminosalicylic acid, (h) 4-hydroxy-3-methoxybenzaldehyde (vanillin), (i) 5-hydroxyindole-3-acetic acid, (j) methyl-4-hydroxybenzoate, (k) hypholomine B (isomers a and b). Values of Rc in the heatmap are the means of four replicates ( $n = 4$ ). Statistical analysis results for each compound are shown in Table S2.

### 3.7. Carbohydrate Oxidative Degradation

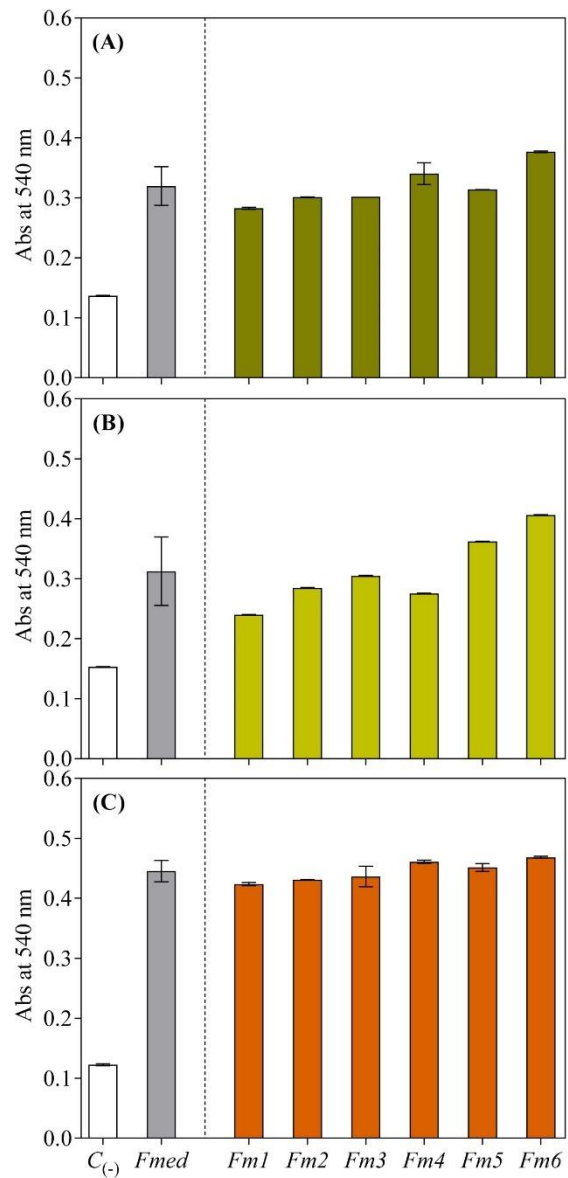
The results from the experiments on the degradation of hemicellulose (xylan), the cellulose model (microcrystalline cellulose) substrate, and GW-sawdust by extracts of LMWCs from the different fungal strains were associated with an increase in reducing sugar concentration after 12 hrs of the oxidative treatments. All the substrates were more degraded by the redox cycling of the LMW methanolic fraction with respect to controls (containing only ferric iron,  $H_2O_2$ , and an aliquot of the methanolic extract coming from the mock-inoculated liquid medium). After 12 hrs, average absorbance values (540 nm) of 0.32, 0.31, and 0.44 were initially obtained for cellulose, hemicellulose, and GW-sawdust, respectively (Figure 7). The *Fm6* strain methanolic extract incubated with cellulose produced the highest absorbance value (0.37), while cellulose incubated with the *Fm1* strain methanolic extract had the lowest value ( $-24.32\%$  compared to *Fm6*). The absorbance of the cellulose mixed with extracts of the other strains fell between the two extremes (Figure 7A).

**Table 1.** Listing of FeRA compounds and annotation levels. Compounds labeled with “+” refer to molecules that have previously been demonstrated to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . Compounds labeled with “/” refer both to unknown and/or described as not able to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The numbers 1, 2, and 3 in the last column indicate the type of annotation carried out: by chemical standard (1), by the spectral library (2), and with correspondence to chemical formulae in the literature (3).

| Compound                                              | FeRA | Type of Annotation |
|-------------------------------------------------------|------|--------------------|
| (a) 4-Hydroxybenzaldehyde                             | /    | 1                  |
| (b) Benzoic acid                                      | /    | 1                  |
| (c) 4-Hydroxy-3-methoxybenzoic acid (vanillic acid)   | +    | 1                  |
| (d) 4-Hydroxycinnamic acid ( <i>p</i> -coumaric acid) | +    | 1                  |
| (e) 2-Hydroxybenzoic acid (salicylic acid)            | +    | 1                  |
| (f) 3-Methoxybenzaldehyde                             | /    | 1                  |
| (g) N-acetyl-5-aminosalicylic acid                    | /    | 2                  |
| (h) 4-Hydroxy-3-methoxybenzaldehyde (vanillin)        | +    | 2                  |
| (i) 5-Hydroxyindole-3-acetic acid                     | /    | 2                  |
| (j) Methyl-4-hydroxybenzoate                          | /    | 2                  |
| (k) Hypholomine B (isomers a and b)                   | /    | 3                  |



**Figure 6.** Hydroxyl radical sample spectrum compared with theoretical simulation (A,B) and relative amplitude (C,D) generated from LMWCs redox cycle with iron, analyzed using spin-trapping in EPR. Values are the means  $\pm$  standard deviation: for the reaction at pH 3.5 (C;  $n = 4$ ), results from negative controls ( $C_{(-)}$ ) and *Fmed* average values are shown on the left. Results from different *Fmed* strains (*Fm1*, *Fm2*, *Fm3*, *Fm4*, *Fm5*, and *Fm6*) were analyzed using the Kruskal–Wallis test, followed by Dunn’s multiple comparison tests ( $p < 0.05$ ). Different letters indicate significant differences according to multiple comparison test results. For the reaction at pH 5.5 (D;  $n = 3$ ), results were analyzed by the unpaired two-tailed *t*-test ( $p < 0.05$ ). Asterisks indicate significance: \*\*\*\*,  $p < 0.0001$ . nd = not detected.



**Figure 7.** Increase in absorbance (measured spectrophotometrically at 540 nm) from the redox cycling of different LMWCs with (A) cellulose, (B) hemicellulose, and (C) Gewürztraminer wood sawdust. Values for each *Fmed* strain are the means  $\pm$  standard deviation of 4 pooled replicates. Results from negative controls ( $C_{(-)}$ ) and *Fmed* average values are shown on the left.

For hemicellulose, the highest absorbance value was recorded when incubated with a methanolic extract from the *Fm6* strain (0.38), while the lowest was when incubated with methanolic extracts from strains *Fm4* and *Fm1* (−28.95 and −39.47% compared to *Fm6*, respectively). The absorbance of all other strain extracts was between the two extremes (Figure 7B).

The same trend was recorded for GW-sawdust when incubated with the methanolic extract from strain *Fm6* producing the highest absorbance (0.40), while the lowest was recorded when incubating with the methanolic extract from the strain *Fm1* (−7.5% compared to *Fm6*). Incubation of the GW-sawdust with all other extracts from other strains produced absorbance values that fell between the two extremes (Figure 7C).

#### 4. Discussion

*Fomitiporia mediterranea* represents the primary white rot fungus (WRF) in European vineyards affected by the Esca complex of diseases. To the best of our knowledge, this article represents the first description of a non-enzymatic CMF-like pathway adopted by a white rot Basidiomycota species involved in ECD, in line with the current reconsideration of the biomolecular mechanisms in the wood decay binary paradox (white vs. brown rot vs. intermediate species). Our results show how different *Fmed* strains were able to produce LMW phenolic or phenolic-derived compounds that were able to redox cycle with iron when cultivated in vitro in nutrient-restricted and iron-limited conditions often found in wood, and at a pH similar to that found in the wood cell wall. Further, under these conditions, the phenolic-derived compounds were shown to generate  $H_2O_2$  and ultimately produce hydroxyl radicals ( $\bullet OH$ ), responsible for lignocellulose constituent oxidation when reacted with holocellulose sources and grapevine wood sawdust. The *Fmed* strain variability was shown to be a factor influencing several stages of the CMF pathway.

When cultivated in a liquid culture designed to reproduce the low nutrient and iron-repressed conditions often found in wood, *Fmed* strains acidified the liquid medium to produce LMWCs, in alignment with previous in vitro observations made by Sparapano et al. [78] and Bruno and Sparapano [57] in static Czapek medium culture. In wood decay induced by Basidiomycota species, the acidification of the microenvironment surrounding fungal hyphae is concomitant with oxalate production as a fundamental step [36,39,45,79,80]. More specifically, for the CMF pathway described for brown rot decay, the low pH and the presence of oxalate are required to chelate and solubilize the unavailable, insoluble Fe(oxyhydr)oxide complexes present in the surrounding microenvironment. This promotes the migration of chelated iron into the fungal extracellular matrix. After the transfer of the iron from oxalate to the fungal phenolic chelators, the iron can be reduced, and this occurs within the higher pH wood cell walls where it is required for the subsequent steps of the CMF pathway [29,36,43]. In this study, levels of oxalic acid or other LMW organic acids typically produced by Basidiomycota species during wood decay (i.e., acetate, gluconate, succinate) were not specifically assessed in *Fmed* secretome. However, it is well known that the *Fmed* genome possesses genes coding for enzymes involved in oxalate metabolism regulation, specifically oxalate oxidases and decarboxylases [33].

In the extensive work on non-enzymatic mediated Fenton (CMF) chemistry in lignocellulose degradation, it is well accepted that LMWC-mediated  $Fe^{3+}$  reduction to  $Fe^{2+}$ , and the reaction of the latter species with  $H_2O_2$  are critical for hydroxyl radical ( $\bullet OH$ ) generation within the wood cell wall microenvironment to initiate lignocellulose degradation. This subsequently may allow extracellular enzymes to further digest wood cell walls or the oligosaccharides diffusing from those cell walls [29,36,40,41]. However, for WRF, the role of LMWCs has not been clear. Even though some LMWCs have been shown to diffuse into wood cell walls in advance of an enzymatic attack, their role has been mostly considered as intimately-associated mediators for extracellular enzyme action, acting as an electron shuttle or metal chelating cofactor to assist with enzymatic degradation [22,63,81–83]. Goodell et al. [84] previously found that many white rot fungi produced iron-reducing compounds when grown on both softwoods and hardwoods, but generally to a lesser extent than classic brown rot fungi. However, the possibility of a LMWC catalytic system in mediated Fenton

reactions playing a direct role in white rot or transitional decay species has received far less consideration. In this context, our study represents an advance both in scientific knowledge for wood decay in general and for ECD pathogenesis.

The LMW methanolic extracts from all *Fmed* liquid cultures tested were able to both chelate and redox cycle with iron, causing a “multiple iron reduction” (MIR) that has previously been reported in other systems [85]. This can function as a catalytic source of  $H_2O_2$  in the presence of reduced iron ( $Fe^{2+}$ ), ultimately leading to  $\bullet OH$  generation. The levels of MIR attained by the different strains displayed significant differences.

The total absence of LiP encoding genes in the *Fmed* genome [33] may suggest that a CMF-like pathway involving  $Fe^{3+}$ -reducing LMWCs could be involved at least partially in lignocellulose degradation found in *Fmed* white rot-affected vines. LiP is one of the Class-II PODs with the highest redox potential, able to oxidize both phenolic and non-phenolic lignin moieties within lignocellulose biomass [29]. Therefore,  $Fe^{3+}$ -reducing LMWCs could represent a “backup strategy” to compensate for the lack of genes encoding for LiP. Future studies on the interactions of the LMW fungal fractions with enzymatic lignocellulolytic components, together with relative gene expression analysis, are necessary to clarify the roles of the fungal metabolites in grapevine wood undergoing decay in *Esca* (*sensu strictu*). Apart from the catalytic role of the LMWCs in the CMF pathway of *Fmed*, the possibility that LMWCs may have a mediating role for extracellular enzymes cannot be ignored. In this regard, naturally produced microbial aromatic metabolites mediating laccase activity have been reported for some WRF [29,86]. Concerning  $H_2O_2$ , our results show an efficient autocatalytic LMWC-mediated molecular oxygen ( $O_2$ ) reduction system responsible for  $H_2O_2$  generation in the *Fmed* methanolic extracts. This is consistent with systems described for non-enzymatic  $H_2O_2$  generation in BRF and, recently, for some Ascomycota species causing soft rot [41,43]. Possible mechanisms explaining a catalytic role for LMW fractions could include (i) the redox cycling of LMWCs with  $O_2$  to yield  $\bullet OOH$ , which can produce  $H_2O_2$  via a dismutation reaction [87]; and (ii) the  $Fe^{2+}$  produced in the MIR phenomena reducing  $\bullet OOH$  generated from  $O_2$  reduction to yield  $H_2O_2$  [88]. Further studies are necessary to elucidate the mechanisms driving  $H_2O_2$  generation in *Fmed* methanolic LMW extracts. Another pathway for the generation of  $H_2O_2$  has been proposed for wood decay, but it involves enzymes (such as alcohol oxidases or methanol oxidases), which are not able to penetrate the wood cell wall during incipient decay [40,60,89]. Thus, reduction of the  $O_2$  present in the cell wall via non-enzymatic reactions remains the most efficient path for the generation of  $H_2O_2$  in situ, where it is needed to produce  $\bullet OH$ . Hydroxyl radicals have a very short half-life (nanoseconds), so they must be generated directly within the cell wall to be active in lignocellulose degradation [29]. Hydroxyl radicals were efficiently generated from the LMW fraction of *Fmed* liquid culture ultrafiltrates, both at pH 5.5 and pH 3.5. These data suggest that  $\bullet OH$  generation from LMWC-mediated mechanisms was possible within the intact wood cell wall (where the pH is slightly acidic, around 5.5) and in more acidic conditions, where the decay was more advanced [41]. Our results could potentially explain the diamond-shaped cavities observed by Pacetti et al. [20] and Kassemeyer et al. [58] in the S2 layer of *Fmed*-infested grapevine wood cell walls, as this phenomenon cannot be explained by the activity of enzymes alone in line with an earlier hypothesis of a non-enzymatic pathway for *Fmed* [19]. More studies in *lignum* are necessary for confirmation.

Ultra-high-performance liquid chromatography coupled with mass spectroscopy analysis allowed the identification of some LMWCs in the ultrafiltered methanolic extracts, including some phenolic acids (hydroxybenzoic and hydroxycinnamic acids), phenolic aldehydes, and other aromatic compounds. The mycotoxin 4-hydroxybenzaldehyde was found previously in the *Fmed* secretome, but no redox cycling ability with iron was attributed to it [54,90]. Other compounds isolated from *Fmed* methanolic extracts were previously found in liquid cultures of BRF, WRF, and soft rot fungi (Ascomycota), including benzoic acid, E4-hydroxycinnamic acid (*p*-coumaric acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 3-methoxybenzaldehyde,

2-hydroxybenzoic acid (salicylic acid), and hypholomine B [38,41,91,92]. The involvement of some phenolic molecules, especially catecholate, in driving mediated Fenton reactions is well known [93,94]. For vanillic acid, *p*-coumaric acid, and vanillin, redox cycling with iron has been individually reported [90]. Interestingly, the only compounds possessing catechol moieties identified in our study (the two isomers of hypholomine B) have been described in *Phellinus baumii* as styrylpyrone pigments with iron chelation capacity that also exhibit antioxidant properties [95]. Future experiments assessing  $\text{Fe}^{3+}$ -reducing capacity and pro-oxidant properties will be of great interest as other LMWCs (such as variegatic acid) with both anti- and pro-oxidant properties, depending on pH and transition metal proximity have been reported in the context of CMF reactions induced by the BRF *Serpula lacrimans* [35,39]. Salicylic acid is well known for its antioxidant properties [96], but it has also been reported to function as a pro-oxidant under some conditions [97]. Salicylic acid has recently been used as a radical scavenger to regulate the CMF reaction induced by the LMW fraction secreted by *Eutypa lata* (Elata), *Phaeoacremonium minimum* (Pmin), and *Phaeomoniella chlamydospora* (Pch) [42]. In our experiments,  $\bullet\text{OH}$  production was still evident even when *Fmed* produced salicylic acid, suggesting that it may function as a pro-oxidant under our conditions or that other LMWCs in the system produce more  $\bullet\text{OH}$  radicals than salicylic acid can scavenge. Other LMWCs (methyl-4-hydroxybenzoate, 5-hydroxyindole-3-acetic acid, and N-acetyl-5-aminosalicylic acid) identified in the *Fmed* methanolic extracts had structures that suggest iron chelation capability. There was also evidence that 5-hydroxyindole-3-acetic acid exhibited both anti- and pro-oxidant properties, and the non-acetylated form of 5-aminosalicylic acid possessed both iron-reducing and pro-oxidant properties, as reported previously [98,99]. However, further experiments with individual model metabolites would be required to demonstrate the redox cycling capacity of these compounds with iron to promote  $\bullet\text{OH}$  generation. Moreover, several metabolites still need to be identified, and the individual contribution of other catecholates, quinones, and enzyme mediators, such as chlorinated alcohols, cannot be discounted.

Degradation of hemicellulosic (xylan) and cellulosic (microcrystalline cellulose) model substrates was associated with an increase in reducing sugar concentration after oxidative treatments, in accordance with previously reported research by Arantes and Milagres [77] and Arantes et al. [38]. Mediated Fenton reactions with the *Fmed* LMW fraction were faster and more efficient in degrading the substrates than reactions with only iron and hydrogen peroxide. Moreover, our results proved the efficiency of the *Fmed* LMW fraction on the degradation of GW sawdust for the first time. However, while demonstrating the efficiency of the *Fmed* LMW fraction in generating  $\bullet\text{OH}$  radicals and increasing the quantity of reducing ends of wood carbohydrates (possibly via hydrogen abstraction), these results remain preliminary without providing information about the depolymerization of the substrates. Future experiments are required to understand the kinetics and timeline of holocellulose depolymerization and possible effects on lignin (demethylation/demethoxylation). This information would be of great interest to clarify not only *Fmed*'s decay pattern(s) but also ECD pathogenesis.

As discussed above, our data represents an advance not only in understanding additional mechanisms associated with a type of white rot decay but also in demystifying “Esca” (*sensu strictu*, currently called “white rot” or “amadou”) pathogenesis, reinforcing the preliminary observations of Di Marco et al. [55] with *Fmed* (formerly classified as *Fomitiporia punctata*) where they describe a positive reaction for the chrome azurol assay (CAS) test in petri dishes. These authors also found iron reduction capacity in their liquid culture supernatant (but without LMW fraction separation) and pioneered the concept that iron (with a subsequent ROS stream) could have a role in “Esca” pathogenesis. Considering the ECD more broadly than just the participation of *Fmed*, other Ascomycota species associated with the complex could potentially also utilize a CMF-like pathway, as demonstrated previously [39], furnishing new insights for understanding typical wood necrosis formation in the ECD. The knowledge gained from our experiments with *Fmed* represents a step forward on the path toward understanding (and potentially controlling) the disease complex. In

agreement with the synergistic microbial hypothesis of Bruez et al. [100], it appears that all the major fungi associated with the ECD in the Mediterranean region share a common biochemical pathway. This pathway, namely a CMF-like reaction, may be activated prior to enzymatic action on the lignocellulose biomass to promote the onset of the diseases and consequent hydraulic and mechanical failures. Interfering with ROS generation may potentially serve to control the diseases' progression, and interestingly, some in vitro trials showed the inhibition of  $\bullet\text{OH}$  generation when the LMW fractions of *Pch*, *Pmin*, and *Elata* were mixed with antioxidants and chelators, including salicylic acid, EDTA, butylated hydroxy anisole (BHA), and butylated hydroxytoluene (BHT) [42].

In general, this study shows that *Fmed* can adopt a non-enzymatic "CMF-like" system to promote pathogenesis and wood necrosis and that significant differences among *Fmed* strains are evident in much of the CMF mechanistic pathway. Indeed, *Fomitiporia mediterranea* is known as a heterothallic species with obligate outcrossing, and because of this, the fungus has remarkable intraspecific variability in vineyards [69]. Interestingly, *Fmed* strain *Fm1*, the only strain originally isolated from a non-*Vitis* host (*Olea europaea* L.), displayed very different behaviour (except for generated  $\text{H}_2\text{O}_2$ ) compared to the other strains, suggesting a possible degree of host-specificity. Host-specificity has previously been discussed for *Fmed* after cross-pathogenicity tests and the consequent formation of different levels of wood discoloration and necrosis, depending on the host of isolation [101,102]. A deep screening of *Fmed* isolates from non-*Vitis* hosts is necessary to confirm how the host-specificity of different strains may contribute to the CMF pathway. Finally, in our research, the *Fmed* strains produced different suites of LMWCs, with strains *Fm1* and *Fm6* being the least and the most active, respectively, relative to triggering a CMF-like pathway. Specifically, *Fm1* methanolic extracts presented the lowest: (i) capacity for acidification; (ii) production of total phenols; (iii) ferric iron reduction capacity, and (iv)  $\bullet\text{OH}$  production. The opposite behavior was observed for *Fm6* in all categories. Surprisingly, the levels of  $\text{H}_2\text{O}_2$  generated do not align with the observations for the other data of the CMF pathway. A possible explanation could involve the ratios between individual compounds in the strains' phenolic profiles. The LMWCs identified did display structures with different multiple coordinating oxygens or different positioning of the hydroxyl groups, suggesting a differential affinity for iron and molecular oxygen in the CMF reaction. Moreover, several compounds in the *Fmed*-methanolic extracts still remain unidentified. It is plausible that some unidentified LMWCs could differentially influence  $\text{H}_2\text{O}_2$  generation, especially if present in different ratios. A similar discrepancy between the results of total phenols, FeRA, and generated hydrogen peroxide has already been observed by Perez-Gonzalez et al. [41] in methanolic extracts from some Ascomycota species. Concerning fungal biomass, at the present stage, it is not possible to formulate a robust hypothesis on its relationship with the CMF pathway for *Fmed*, and research is needed to investigate any potential synergy between primary and secondary metabolism. Differences in growth in an active CMF reaction context may be due to downregulation of primary metabolism when energy is shifted towards phenolic compound biosynthesis, or alternatively, these differences in growth may simply be related to intrinsic nutrient-use efficiency. However, the differences observed at the strain level cannot be assumed to be definitive, as it is known that *Fmed* mycelium can change type during the life span of the fungus, from bleaching (B-Type) to staining (S-Type), with both the physiology and the metabolism of the fungus undergoing change together. It is feasible that such changes could also affect the production of LMW phenolic metabolites in *Fmed*, especially considering that S-Type mycelia induce very strong media pigmentation [15].

## 5. Conclusions

Our experiments demonstrate that *Fomitiporia mediterranea*, a WRF responsible for "Esca" *sensu strictu* (a syndrome of the Esca complex of diseases), possesses the means to generate a CMF reaction by secreting an array of LMW- $\text{Fe}^{3+}$  reducing compounds. The LMWCs produced were able to: (i) redox cycle with iron; (ii) act as an efficient autocatalytic

source for  $\text{H}_2\text{O}_2$  generation; and (iii) generate  $\bullet\text{OH}$  that can participate in holocellulose oxidation. Intraspecific variation appears to be a factor that impacts several stages of the non-enzymatic CMF pathway.

We propose that an early-stage non-enzymatic CMF-type degradation of grapevine wood cell walls, promoted by ECD fungi, is required before action by any fungal CAZyme or ligninase enzymes may occur. Non-enzymatic lignocellulose mineralization may continue either with or without enzymatic depolymerization or synergistically with enzyme action (within the fungal extracellular matrix). Simultaneously, methoxylated phenolic compounds derived from the grapevine wood's degraded lignin may potentially be participating in the previously described multiple iron reduction phenomena, further contributing to  $\bullet\text{OH}$  generation and lignocellulose degradation [85]. In this context, investigating antioxidant sources for treatment to control ECD in the future could be fruitful.

Future experiments in vitro, in lignum, and in planta are required to clarify whether synergy between non-enzymatic and enzymatic mechanisms occurs in *Fmed*-induced grapevine wood decay and to determine the influence of microenvironmental and abiotic factors on CMF reactions. Finally, the involvement of LMWCs (both secreted by *Fmed* and as a "byproduct of wood degradation") must be further explored relative to the development of ECD-foliar symptoms.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof9040498/s1>, Table S1: List of *Fomitiporia mediterranea* strains used during the experiments, originating from different mycological collections. A key was attributed to each strain to facilitate data interpretation.; Table S2: Statistical analysis of LMW phenol-derived compounds determined by UHPLC-MS/MS. Results were analyzed by ruskal–Wallis test (left-hand superscript "a") or one-way ANOVA (left-hand superscript "b") according to data distribution and homoscedasticity, followed by multiple comparison tests ( $p < 0.05$ ), accordingly. Different letters indicate significant differences between *Fmed* strains.

**Author Contributions:** Conceptualization, S.M., B.G., C.B. and S.F.; Data curation, S.M., M.-L.G. and J.L.; Formal analysis, S.M., M.-L.G. and J.L.; Supervision, S.F.; Validation, S.F.; Writing—original draft, S.M.; Writing—review and editing, M.-L.G., A.P., J.L., S.D.M., L.M., E.G., B.G., C.B. and S.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are included in the article and the Supplementary Materials. Further inquiries can be addressed to the corresponding authors.

**Acknowledgments:** We thank Jean-Pierre Péros for providing us with *Fmed* strains LR2, LR71, LR124, and LR85, and Philippe Larignon for providing our laboratory with the strain PHCO36. We also thank Yann Leva from LVBE for his precious technical assistance and Alexandre Verdu from Bruker Daltonics for his help and advice. Professor Goodell appreciates the support from the National Institute of Food and Agriculture, U.S. Department of Agriculture, project number S1075–MAS00503. The authors thank George Pattison for the English language editing.

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

Appendix A

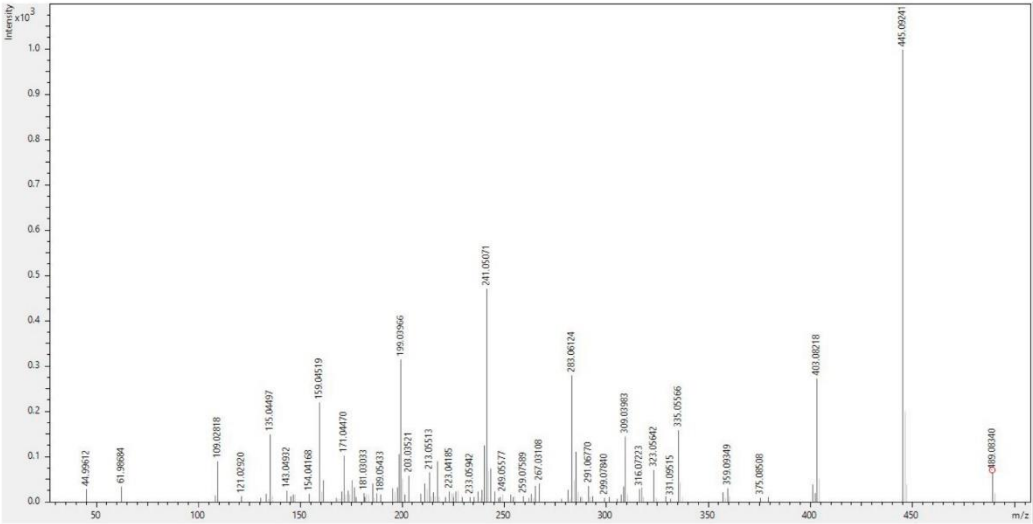


Figure A1. MS/MS spectrum of compound (k) annotated as hypholomine B.

Table A1. In-silico fragmentation of compound (k) annotated as hypholomine B with MetFrag tool [103] embedded in Metaboscope 2021b. 95.7% of the MS/MS spectrum is explained. A comparable MS/MS reference spectrum with the literature was found [104,105].

| <i>m/z</i> Fragment (mDa) | Calculated Formula                                          | Loss                                          | Proposed Structure in Yellow for the Fragment |
|---------------------------|-------------------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 445.092                   | C <sub>25</sub> H <sub>17</sub> O <sub>8</sub> <sup>−</sup> | CO <sub>2</sub>                               |                                               |
| 403.082                   | C <sub>23</sub> H <sub>15</sub> O <sub>7</sub> <sup>−</sup> | C <sub>3</sub> H <sub>2</sub> O <sub>3</sub>  |                                               |
| 335.055                   | C <sub>19</sub> H <sub>11</sub> O <sub>6</sub> <sup>−</sup> | C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>  |                                               |
| 309.040                   | C <sub>17</sub> H <sub>9</sub> O <sub>6</sub> <sup>−</sup>  | C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>  |                                               |
| 283.062                   | C <sub>16</sub> H <sub>11</sub> O <sub>5</sub> <sup>−</sup> | C <sub>10</sub> H <sub>6</sub> O <sub>5</sub> |                                               |

Table A1. Cont.

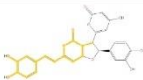
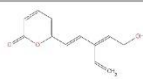
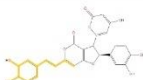
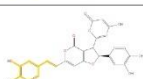
| <i>m/z</i> Fragment (mDa) | Calculated Formula                                         | Loss                                           | Proposed Structure in Yellow for the Fragment                                       |
|---------------------------|------------------------------------------------------------|------------------------------------------------|-------------------------------------------------------------------------------------|
| 241.050                   | C <sub>14</sub> H <sub>9</sub> O <sub>4</sub> <sup>−</sup> | C <sub>12</sub> H <sub>8</sub> O <sub>6</sub>  |  |
| 199.040                   | C <sub>12</sub> H <sub>7</sub> O <sub>3</sub> <sup>−</sup> | C <sub>14</sub> H <sub>10</sub> O <sub>7</sub> |  |
| 159.045                   | C <sub>10</sub> H <sub>7</sub> O <sub>2</sub> <sup>−</sup> | C <sub>16</sub> H <sub>10</sub> O <sub>8</sub> |  |
| 135.045                   | C <sub>8</sub> H <sub>7</sub> O <sub>2</sub> <sup>−</sup>  | C <sub>18</sub> H <sub>10</sub> O <sub>8</sub> |  |

Table A2. Average chromatographic retention time (RT), the mass-to-charge ratio (*m/z*), and mass (*M*) of the identified compounds in the study.

| RT [min] | <i>m/z</i> | <i>M</i> | Compound Name                                         |
|----------|------------|----------|-------------------------------------------------------|
| 8.52     | 121.029    | 122.037  | (a) 4-Hydroxybenzaldehyde                             |
| 11.07    | 121.029    | 122.037  | (b) Benzoic acid                                      |
| 8.58     | 167.037    | 168.042  | (c) 4-Hydroxy-3-methoxybenzoic acid (vanillic acid)   |
| 9.74     | 163.040    | 164.047  | (d) 4-Hydroxycinnamic acid ( <i>p</i> -coumaric acid) |
| 11.35    | 137.024    | 138.032  | (e) 2-Hydroxybenzoic acid (salicylic acid)            |
| 9.30     | 135.045    | 136.052  | (f) 3-Methoxybenzaldehyde                             |
| 7.54     | 194.046    | 195.053  | (g) N-Acetyl-5-aminosalicylic acid                    |
| 9.17     | 151.040    | 152.047  | (h) 4-Hydroxy-3-methoxybenzaldehyde (vanillin)        |
| 9.27     | 190.051    | 191.058  | (i) 5-Hydroxyindole-3-acetic acid                     |
| 10.98    | 151.040    | 152.047  | (j) Methyl-4-hydroxybenzoate                          |
| 11.80    | 489.082    | 490.090  | (k) Hypholomine B (isomer a)                          |
| 12.24    | 489.083    | 490.090  | (l) Hypholomine B (isomer b)                          |

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## Supplementary Material

**Table S1.** List of *Fomitiporia mediterranea* strains used during the experiments, originating from different mycological collections. A key was attributed to each strain, to facilitate data interpretation.

| Key | Strain code | Isolation locality                | Collection from which the strain was sourced                                                               | Host                                          | Sampling date |
|-----|-------------|-----------------------------------|------------------------------------------------------------------------------------------------------------|-----------------------------------------------|---------------|
| Fm1 | 235.03      | Riotorto (Livorno), It            | Fungal collection PVFi of DAGRI, Section of Plant pathology and entomology (University of Florence, Italy) | <i>Olea europaea</i> L.                       | 2003          |
| Fm2 | LR71        | Vendargues (34), Fr               | Fungal collection of INRAE Montpellier, DAAV team of UMR AGAP (INRAE, Montpellier, France)                 | <i>Vitis vinifera</i><br>cv Alphonse Lavallée | 1996          |
| Fm3 | LR2         | Beauvoisin (30), Fr               | Fungal collection of INRAE Montpellier, DAAV team of UMR AGAP (INRAE, Montpellier, France)                 | <i>Vitis vinifera</i><br>cv Carignan          | 1996          |
| Fm4 | LR124       | Villeneuve les Maguelone (34), Fr | Fungal collection of INRAE Montpellier, DAAV team of UMR AGAP (INRAE, Montpellier, France)                 | <i>Vitis vinifera</i><br>cv Carignan          | 1996          |
| Fm5 | LR85        | Ortoffa (66), Fr                  | Fungal collection of INRAE Montpellier, DAAV team of UMR AGAP (INRAE, Montpellier, France)                 | <i>Vitis vinifera</i><br>cv Grenache          | 1996          |
| Fm6 | PHCO36      | Saint-Preuil (16), Fr             | INRA-UMR 1065 SAVE collection (INRAE, Bordeaux, France)                                                    | <i>Vitis vinifera</i><br>cv Ugni blanc        | 1996          |

**Table S2.** Statistical analysis of LMW phenol-derived compounds determined by UHPLC-MS/MS. Results were analyzed by Kruskal-Wallis test (left-hand superscript “a”) or one-way ANOVA (left-hand superscript “b”) according to data distribution and homoscedasticity, followed by multiple comparison tests ( $P < 0.05$ ), accordingly. Different letters indicate significant differences between *Fmed* strains.

| Statistical differences                                        |          |            |            |            |            |            |            |
|----------------------------------------------------------------|----------|------------|------------|------------|------------|------------|------------|
| Compound name                                                  | P value  | <i>Fm1</i> | <i>Fm2</i> | <i>Fm3</i> | <i>Fm4</i> | <i>Fm5</i> | <i>Fm6</i> |
| <sup>b</sup> 4-Hydroxybenzaldehyde                             | < 0.0001 | <i>C</i>   | <i>B</i>   | <i>C</i>   | <i>A</i>   | <i>C</i>   | <i>ABC</i> |
| <sup>a</sup> Benzoic acid                                      | 0.0005   | <i>ABC</i> | <i>ABC</i> | <i>BC</i>  | <i>A</i>   | <i>C</i>   | <i>AB</i>  |
| <sup>b</sup> 4-Hydroxy-3-methoxybenzoic acid (vanillic acid)   | < 0.0001 | <i>A</i>   | <i>B</i>   | <i>B</i>   | <i>C</i>   | <i>C</i>   | <i>A</i>   |
| <sup>b</sup> 4-Hydroxycinnamic acid ( <i>p</i> -coumaric acid) | < 0.0001 | <i>C</i>   | <i>D</i>   | <i>A</i>   | <i>E</i>   | <i>B</i>   | <i>C</i>   |
| <sup>a</sup> 2-Hydroxybenzoic acid (salicylic acid)            | 0.0030   | <i>B</i>   | <i>AB</i>  | <i>B</i>   | <i>A</i>   | <i>AB</i>  | <i>AB</i>  |
| <sup>a</sup> 3-Methoxybenzaldehyde                             | 0.0005   | <i>BC</i>  | <i>ABC</i> | <i>A</i>   | <i>ABC</i> | <i>C</i>   | <i>AB</i>  |
| <sup>b</sup> N-Acetyl-5-aminosalicylic acid                    | <0.0001  | <i>B</i>   | <i>B</i>   | <i>B</i>   | <i>B</i>   | <i>A</i>   | <i>AB</i>  |
| <sup>a</sup> Methyl-4-hydroxybenzoate                          | 0.0010   | <i>A</i>   | <i>AB</i>  | <i>B</i>   | <i>AB</i>  | <i>B</i>   | <i>AB</i>  |
| <sup>a</sup> 4-Hydroxy-3- methoxybenzaldehyde (vanillin)       | 0.0008   | <i>ABC</i> | <i>ABC</i> | <i>BC</i>  | <i>A</i>   | <i>C</i>   | <i>AB</i>  |
| <sup>a</sup> 5-Hydroxyindole-3-acetic acid                     | 0.0006   | <i>A</i>   | <i>AB</i>  | <i>BC</i>  | <i>C</i>   | <i>ABC</i> | <i>ABC</i> |
| <sup>a</sup> Hypholomine B (isomer a)                          | 0.0011   | <i>BC</i>  | <i>ABC</i> | <i>A</i>   | <i>C</i>   | <i>AB</i>  | <i>ABC</i> |
| <sup>a</sup> Hypholomine B (isomer b)                          | 0.0007   | <i>B</i>   | <i>AB</i>  | <i>AB</i>  | <i>B</i>   | <i>A</i>   | <i>AB</i>  |

**Chapter III** - The Tour of the World in Eight White Rot Species: Assessment of Enzymatic and Non-enzymatic Degradation Capabilities of Worldwide Esca Complex of Diseases (ECD) associated Decay Agents.

**The following chapter has been submitted as an original research article.**

**The content has been formatted to align with the structure of this  
dissertation**

**The Tour of the World in Eight White Rot Species: Assessment of Enzymatic and Non-enzymatic Wood Decay Pathways of Worldwide Esca Complex of Diseases (ECD) Associated Basidiomycetes**

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**Keywords:** *Fomitiporia mediterranea*, *Fomitiporia*, Esca, White rot, *Vitis vinifera*, Non-enzymatic degradation, Enzymatic wood degradation.

# Abstract

Esca Complex of Diseases (ECD) is widespread in viticultural areas worldwide, posing a serious risk to modern viticulture. The role of basidiomycetes in the onset of foliar symptoms has recently been reconsidered, highlighting a link between the reduction in these symptoms and the presence of *Fomitiporia mediterranea* in vines. As several basidiomycetes have been associated with wood decay in grapevine, understanding their degradation mechanisms could help unravel the role of wood degradation in ECD. Thus, a collection of ECD-associated basidiomycetes, including *F. capensis*, *F. langloisii*, *F. polymorpha*, *F. australiensis*, *Tropicoporus texanus*, *Inonotus vitis*, and *Stereum hirsutum*, was examined in addition to *F. mediterranea*. Our study investigates the production and activity of lignocellulosic enzymes (particularly class-II peroxidases and laccases), along with the low molecular weight compounds (LMWC) in the secretome of these fungi, to assess their ability to perform the Chelator-Mediated-Fenton (CMF) pathway. Results highlight the production of ligninolytic enzymes with low production of active manganese peroxidases and a virtually total absence of lignin peroxidase activity. Additionally, the study confirms that the CMF mechanism is widespread among these fungi, with all LMW secretomes fulfilling the required steps of the CMF mechanism. This study provides critical insights into the degradation strategies of ECD-associated white rot agents, offering potential new perspectives on studying ECD symptomatology and developing targeted control strategies. The findings emphasize the need to consider the CMF mechanism in the context of fungal wood degradation and its potential role in ECD development.

## 1. Introduction

Grapevine Trunk Diseases (GTDs) represent a great threat to worldwide modern viticulture (Mondello et al., 2018a; Guerin-Dubrana et al., 2019; Kenfaoui et al., 2022). Among GTDs, Esca Complex of Diseases (ECD) is considered one of the most severe, spread, and still least understood threats. Its economic impact has been growing over the last two decades (Bertsch et al., 2009; Gramaje et al., 2018; Mondello et al., 2018b).

Three key symptoms of ECD (Surico, 2009) are the development of tiger-striped foliar symptoms known as Grapevine Leaf Stripe Disease (GLSD), the appearance of white rot

(presenting as exhausted fibrous and bleached wood residue) in the trunk and arms—mainly in older vines—associated with saprophytic and/or parasitic basidiomycetes, and the presence of black streaking in the wood due to vascular ascomycetes. Vines presenting foliar symptoms are widespread in all viticultural regions of the northern hemisphere but rare in the southern hemisphere (Del Frari et al., 2021; Kenfaoui et al., 2022; Mundy et al., 2020; White et al., 2011). White rot, following Surico (2009), is caused by several basidiomycetes pathogens that attack grapevine (*Vitis vinifera* L.) worldwide (Cloete et al., 2015a; Fischer et al., 2005; Moretti et al., 2021). Among these species, *F. mediterranea* and *S. hirsutum* are the most well-known, with *F. mediterranea* (spread in the Mediterranean basin) being the most characterized for pathogenicity mechanisms. In contrast, other species such as *F. capensis*, *F. langloisii*, *F. australiensis*, *F. polymorpha*, *T. texanus*, and *I. vitis* have been recently identified in other areas of the world or have not been studied as much (Brown et al., 2020; Cloete et al., 2014; Fischer et al., 2005). Recent research on their genomic structure shed light on gene pools potentially involved in their pathogenicity, reinforcing the importance in better understanding the functional aspect in their interaction with wood (Garcia et al., 2024).

In the past decades, basidiomycetes associated with ECD have been less investigated than the ascomycetes, as these vascular pathogens were found in vines of all ages with foliar symptoms (Moretti et al., 2021). However, research on methods effective in reducing GLSD shows how these practices also have significant effects on the abundance of white rot and *F. mediterranea* in the wood (Bruez et al., 2021; Cholet et al., 2021; Lecomte et al., 2022; Pacetti et al., 2021). Moreover, the presence of white rot in cordons was defined as the best predictor of the chronic form of GLSD (Maher et al., 2012). This suggests the importance of white rot agents for GLSD development. It also highlights the importance of studying the functional aspects of white rot agents associated with ECD, considering their decay mechanisms arsenal, and filling the gap, especially (but not exclusively) for lesser-known species.

Wood decay agents generally include both basidiomycetes and ascomycetes. The first ones are classified into two categories based on the type of decay they cause: white rot agents and brown rot agents. White rot agents produce a fibrous and bleached-yellowish wood residue, while brown rot agents leave a dark brown residue after lignocellulosic matrix degradation (Blanchette, 1991). White rot agents possess a wide arsenal of enzymes capable of directly degrading celluloses and hemicelluloses and mineralizing lignin through oxidation. This arsenal

includes laccases (EC 1.10.3.2), which are multicopper oxidase enzymes mostly attacking lignin, the carbohydrate-active enzymes (CAZymes) family, active on cellulose and hemicellulose, and class II-peroxidases (PODs), active on lignin. The remarkable production of this second class of enzymes mostly differentiates white rot fungi from brown rot fungi in their decay arsenal. As a matter of fact, in brown rot fungi, the genes encoding for class II-PODs have been theorized to be lost by genomic depletion throughout the centuries (Floudas et al., 2012). Class II-PODs include heme peroxidases such as manganese peroxidases (MnPs; EC 1.11.1.13), lignin peroxidases (LiPs; EC 1.11.1.14), and versatile peroxidases (VPs; EC 1.11.1.16). These enzymes, along with laccase, can directly attack phenolic lignin (Goodell, 2020; Hatakka, 1994; Martínez, 2002). However, non-phenolic lignin can only be directly attacked by high-redox potential enzymes such as LiPs, while for laccases and MnPs, it requires the presence of mediators compounds able to catalyze the oxidation (Martínez et al., 2005; Pham et al., 2024; Yang et al., 2017).

Enzymatic processes are not the only ones that break down the lignocellulose matrix. Since the end of the last century, a complex non-enzymatic decay pathway has been brought to the attention, specifically for brown rot agents, e.g., *Gloeophyllum trabeum* (Arantes and Goodell, 2014; Goodell et al., 2020, 1997a; Xu and Goodell, 2001). It has been proposed by several authors that, due to the loss of their ability to produce most ligninolytic enzymes, brown rot fungi mostly rely on a non-enzymatic wood degradation pathway (Arantes and Goodell, 2014; Eastwood, 2014; Floudas et al., 2012). This pathway is called the “Chelator-mediated Fenton” (CMF) mechanism, which involves a series of reactions that produce highly reactive hydroxyl radicals ( $\cdot\text{OH}$ ). These radicals act on the lignocellulose matrix, initiating its depolymerization (Arantes and Goodell, 2014; Goodell et al., 2020; Xu and Goodell, 2001). The CMF mechanism involves the secretion in the apoplast by the hyphae of two types of extracellular low molecular weight compounds (LMWC): organic acid chelators, such as oxalic acid, and compounds with  $\text{Fe}^{3+}$ -chelating and reducing capability, such as phenolates, hydroquinones, and aminophenols. In the low pH environment that surrounds the pioneer-hyphae, oxalate can solubilize and sequester insoluble Fe(oxyhydr)oxide present in the wood cell wall. From here, the complex oxalate-Fe will naturally penetrate the wood cell wall throughout cell wall microvoids, finding a higher pH ( $\sim 5.5$ -6) and other migrated fungal-produced LMW chelators. In these conditions, iron will be transferred from oxalate to these reducing compounds, leading to its reduction (from  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ ). Then, ferrous iron will react with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), generated in the cell wall via LMWC redox cycling with

molecular oxygen (O<sub>2</sub>) present in the wood cell wall, ultimately leading to the production of a stream of reactive oxygen species (ROS), specifically hydroxyl radicals (•OH), via the Fenton reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \bullet\text{OH} + \text{OH}^-$ ). Those radicals are capable of oxidative breakdown of lignocellulose (Goodell, 2020; Zhu et al., 2022). Eventually, if available, enzymes will participate in wood degradation (Arantes and Milagres, 2007).

In the past few years, the advent of omics sciences challenged the classical white-brown rot dichotomy, as it was proven that it is not possible to correctly summarize the diversity in fungal wood degradation strategies by using this paradigm solely (Ohm et al., 2014; Riley et al., 2014; Shabaev et al., 2022). The CMF pathway similar to the one historically described for brown rot fungi has been shown to exist among other fungal taxa associated with GTDs: ascomycetes (*Phaeoacremonium minimum*, *Phaemoniella chlamydospora*, *Eutypa lata*) and even white-rot basidiomycetes, such as *F. mediterranea* (Moretti et al., 2023; Perez-Gonzalez et al., 2022). It has been suggested that, regardless of the type of decay, non-enzymatic lignocellulose mineralization may initiate the decay process or, in any case, contribute to it. As a matter of fact, enzymes, due to their size, are unlikely to penetrate intact wood cell walls (Tepfer and Taylor, 1981). Therefore, an initial non-enzymatic degradation may be necessary to facilitate further enzymatic action. It has been suggested that this further enzymatic action could occur either within the cell wall or in the fungal extracellular matrix, breaking down diffusing lignocellulose oligomers that were previously generated by the non-enzymatic process (Goodell et al., 2017; Moretti et al., 2023; Perez-Gonzalez et al., 2022, 2023).

Research on the subject highlighted the enzymatic degradation pathway of *F. mediterranea*, showing the ability of multiple strains of this species to produce active laccases and MnPs (Abou-Mansour, 2009; Pacetti et al., 2022). In the work, LiP activity was not investigated as no LiP-encoding genes were found on the available genome version of *F. mediterranea* (Floudas et al., 2012). Finally, the wood decay enzymatic arsenal in *F. mediterranea* has been proposed to act consequentially and/or synergistically to the CMF non-enzymatic pathway, in Moretti et al., 2023. However, *F. mediterranea* remains the only basidiomycetes associated with white rot in ECD with a characterized decay arsenal.

As previously mentioned, ECD is widely found in viticulture regions globally, and various geographically dependent basidiomycetes are associated with it. Therefore, it is essential to compare the (common) features of these different basidiomycetes isolated from plants presenting foliar symptoms to *F. mediterranea*, the basidiomycete predominating in the

Mediterranean basin. Despite recent interest in them (Brown et al., 2020; Garcia et al., 2024), studies on the degradation capabilities have been mainly published for *F. mediterranea*, and only in plate colorimetric assays have been published on other white rot species associated with ECD (Cloete et al., 2015b; Pacetti et al., 2022). Thus, this research aims to investigate degradation capabilities, both enzymatic and non-enzymatic, in a collection of basidiomycetes agents of white rot in ECD present in different regions of the world, with a series of *in vitro* analyses to detail the biochemical level of the decay pathway of these fungi.

## 2. Materials and Methods

### 2.1. Fungal strains

*Fomitiporia mediterranea* strain PHCO36 was obtained from the collection of Laboratoire Vigne, Biotechnologie et Environnement (LVBE) of Université de Haute-Alsace. This French strain was isolated from *V. vinifera* Ugni blanc in the Charente wine region in 1996. *F. capensis* strain 7082 was provided from the collection of Stellenbosch University, South Africa. The fungus was isolated from *V. vinifera* Sauvignon Blanc from the area around Stellenbosch in a sampling campaign from 2001 to 2008. *F. langloisii* strain TX2 (GenBank: MN108107.1, ITS), *F. polymorpha* strain WFB1 (GenBank: MN108110.1, ITS), *Tropicoporus texanus* strain TX9 (GenBank: MN108124.1, ITS), and *Inonotus vitis* strain OC1 (GenBank: MN108118.1, ITS), were provided from the collection of the United States Department of Agriculture, Davis, CA, US. The North American strains were isolated in 2019 from *V. vinifera* Cabernet-Sauvignon in California (Brown et al., 2020). *F. australiensis* strain MUCL 049406 (GenBank: AY624997.1, ITS) was purchased from Belgian Co-ordinated Collections of Micro-Organisms BCCM-MUCL. Genomes of *F. mediterranea* PHCO36, *F. polymorpha* WFB1, *Inonotus vitis* OC1, and *T. texanus* TX9 were recently made available (Accession: PRJNA1099290 in NCBI, (Garcia et al., 2024)). *Stereum hirsutum* strain DP49 (GenBank: AM269810, ITS) was provided by the collection of the University of Turin, Italy (Guglielmo et al., 2007).

### 2.2. Culture Conditions for Enzymatic Assays

To stimulate lignocellulolytic enzyme production, liquid cultures were set up using a modified protocol from Pacetti et al. (2022). Solid precultures were prepared by incubating a 5-mm diameter mycelium plug to 20 ml of Eriksson and Petterson (E&P) medium (Eriksson and Pettersson, 1975) with 1.5% w/v agar. After 20 days of incubation in the dark at 28°C, a quarter of solid preculture was inoculated in 125 ml of E&P medium with 0.625 g of cellulose and 1.25 g of oven-dried sawdust obtained from *V. vinifera* cv. Merlot wood in 250 ml flasks. Two batches of liquid cultures were prepared: one to be harvested after 1 week (1W) and the other after 2 weeks (2W). Cultures were incubated in triplicates (n=3) in the dark at 28°C with an agitation of 125 rpm. At the end of incubation time, cultures were filtered using consecutive filtration steps to a final filtration at 0.2 µm pore size. The solid fraction was then dried at 103±1°C for 48h before being weighed to determine the dry weight (DW). The filtered liquid fraction was stored at -80°C before complete lyophilization. An aliquot of the lyophilized secretome was then resuspended in ultrapure water (18.2 MΩ) and separated from the insoluble part. (Scarpari et al., 2017). The resuspended secretome was diluted to yield a final concentration of 0.2 mg/ml protein content in the following tests after measurements using a NanoDrop (BioSpec-nano, Shimadzu, Kyoto, Japan), assuming 1 OD = 1 mg/ml.

### 2.3. Enzymatic Decay Pathway Analysis: Activity Assays of Resuspended Secretome

To detect and quantify laccase activity in the resuspended secretome of the species, the oxidation of the non-phenolic dye ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt was followed with time. Laccase can oxidize the dye to its more stable preferred state leading to the development of a greenish color. For that, a 100 µl solution containing the following components was prepared in a 96-well microplate (flat-bottom F96 immuno plate, Nunc®, Roskilde, Denmark): sodium acetate buffer pH 4.5 (100 mM), resuspended secretome, ABTS (2 mM). The reaction was carried out at 25°C. Absorbance was measured at 420 nm with a microplate reader (Tristar 2, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) every minute from 10 to 60 minutes of incubation starting from

the addition of ABTS or in the time frame where the enzymatic activity was in the exponential phase. Absorbance measurements were converted into enzyme activity through the extraction coefficient of ABTS ( $\epsilon_{\text{ABTS}} = 36.000 \text{ M}^{-1}\text{cm}^{-1}$ ).

Manganese peroxidase activity was quantified as described by Mathieu et al. (2013). The chemicals MBTH (3-methyl-2-benzothiazolinonehydrazone hydrochloride) and DMAB (para-dimethylaminobenzaldehyde) are catalyzed by POD-II activity if allowed by the presence of  $\text{H}_2\text{O}_2$ , leading to their oxidative coupling (Castillo et al., 1994). This results in the production of indamine, leading to the development of a deep blue-purple color. Two solutions were prepared in a 96-well microplate: one containing MBTH (0.071 mM), DMAB (3.571 mM), sodium lactate-sodium succinate buffer (71.42 mM), and  $\text{MnSO}_4 - 4\text{H}_2\text{O}$  (0.1428 mM) and one containing EDTA (0.2857 mM) instead of  $\text{MnSO}_4$ . Due to the low solubility of DMAB, the chemical was directly dissolved in the volume of the solutions before adding  $\text{MnSO}_4$  or EDTA. 140  $\mu\text{l}$  of the first or the second solution were added to 50  $\mu\text{l}$  of resuspended secretome before adding 10  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  to start the reaction. The reaction was carried out at 25°C and was followed by measuring the absorbance at 590 nm every minute starting from 20 minutes after the addition  $\text{H}_2\text{O}_2$  until 60 minutes or in the time frame where the enzymatic activity was in the exponential phase. The first solution allows for measuring total POD-II and specific MnP activity, while the second refers to total POD-II. Thus, the activity of specific MnP can be obtained by subtracting absorbance values relative to the second solution from the first ones and converting them into enzyme activity using the specific extinction coefficient ( $\epsilon = 32.000 \text{ M}^{-1}\text{cm}^{-1}$ ).

Lignin peroxidase activity was measured with a modified protocol from Mathieu et al. (2013). A solution containing sodium tartrate pH 4.5 (100 mM), resuspended secretome, and Azure B (0.16 mM; Sigma Aldrich Chemicals, Lyon, France) was prepared before adding  $\text{H}_2\text{O}_2$  (1 mM) to start the reaction in a 96-well microplate. LiP causes the oxidation of Azure B in the presence of  $\text{H}_2\text{O}_2$ . Thus, the decrease in absorbance was followed at 651 nm by measuring every minute starting from 5 minutes after  $\text{H}_2\text{O}_2$  addition until 30 minutes.

Three technical replicates were made for each reaction. Solutions without secretome or chemicals were used as controls. All activities are expressed in U/mg where 1 U = 1  $\mu\text{mol}$  of substrate oxidized per minute per unit of protein concentration of the resuspended secretome.

## 2.4. Culture Media and LMW Fraction Extraction for Non-Enzymatic Assays

To promote the production of iron-binding low molecular weight compounds, liquid cultures with low nutrient media to simulate the nutrient-restrictive conditions often found in wood were prepared following Highley (1973). Iron was omitted, and to ensure its total absence, all the glassware used in this experiment was carefully washed with 5.15 M nitric acid ( $\text{HNO}_3$ ) and rinsed with ultrapure water (18.2 M $\Omega$  cm) three times. The low nutrient media solution was prepared with the following composition per liter of ultrapure water (18.2 M $\Omega$  cm): 0.5 g glucose (Euromedex, Souffelweyersheim, France), 0.25 g  $\text{NH}_4\text{NO}_3$  (Duchefa Biochemie, Haarlem, The Netherlands), 2 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.018 mg  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  (Carl Roth GmbH, Karlsruhe, Germany), 0.31 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (Sigma-Aldrich, St. Louis, MO, USA), 0.57 mg  $\text{H}_3\text{BO}_4$ , 0.039 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , and 0.036 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (VWR International LLC, Radnor, PA, USA). 2 g of 20  $\mu\text{m}$  microcrystalline cellulose (Acros Organics, Thermo Fisher Scientific Inc., Geel, Belgium) were added to 500 ml flasks before adding 200 ml of low nutrient media. The pH was adjusted to 5.5 using NaOH. Flasks were then autoclaved before adding 0.001 g thiamine HCl (Sigma-Aldrich, St. Louis, MO, USA). Two 5-mm mycelium plugs were inoculated from the actively growing region of 7-day solid 3% Malt Ager Extract (MEA) cultures from which solid media was carefully removed. Cultures were then incubated in stationary conditions for 6 weeks at 27°C in the dark. As controls, mock-inoculated flasks (containing only a MEA plug without mycelium) were prepared. Four flasks were prepared as replicates for each species, as well as for the control group (n=4). At the end of incubation time, cultures were vacuum-filtered using consecutive filtration steps to a final filtration at 0.2  $\mu\text{m}$  pore size. The solid fraction of the culture was recovered, dried at 103 $\pm$ 1°C for 48h, and weighted to determine the fungal DW. The liquid fraction was reduced through lyophilization to approximately one-third of the initial volume. Next, aliquots of ~10 ml of liquid were transferred to a 15 ml Amicon® ultra centrifugal filter 3 kDa MWCO (UFC9003, Millipore, Merck KGaA, Darmstadt, Germany) and centrifugated at 5000 x g for 60 minutes. The ultrafiltered fraction was then collected and the filter was recharged until the whole liquid mass was processed. Acidification of ultrafiltrate to pH 3 using HCl preceded a triple ethyl acetate (1:1 volume ratio) extraction (Jellison et al., 1991). After this step, the ethyl acetate was removed until complete dryness using a rotary evaporator (Laborota 4000, Heidolph Instruments GmbH & Co., KG, Schwabach, Germany) under vacuum conditions at 40°C at 60 rpm. Finally, the dried solid LMW fraction was resuspended

in 2 ml of LC-MS grade methanol, filtered through a 0.2  $\mu\text{m}$  syringe filter, and stored at  $-20^{\circ}\text{C}$  until analysis.

### 2.5. LMW Fraction Analysis

Methanolic extracts of LMW (25  $\mu\text{L}$ ), along with 2  $\mu\text{L}$  of a 100  $\mu\text{g/mL}$  methanolic solution of 5-methyl salicylic acid as an internal standard, were prepared for chromatographic analysis. As previously described in Moretti et al., 2023 ( $n = 4$ ), ultra-high-performance liquid chromatography (UHPLC) combined with an Impact II Q-TOF-MS/MS mass spectrometer, equipped with an Apollo II electrospray ionization (ESI) source in negative mode (Bruker Daltonics GmbH, Bremen, Germany) was utilized. A reverse-phase C18 Bruker Intensity Solo HPLC column was used (2.0  $\mu\text{m}$  particle size, 100  $\text{\AA}$  pore size, and dimensions of 2.0 mm  $\times$  100 mm) (BRHSC18022100, Bruker Daltonics GmbH, Bremen, Germany). The mobile phases consisted of ultrapure water with 0.1% formic acid (eluent A) and methanol with 0.1% formic acid (eluent B). A gradient elution program was applied: 2 minutes at 1.0% B, a linear increase over 15 minutes up to 99% B, followed by 3 minutes at 99% B, and finally, 5 minutes of re-equilibration at 1% B before the next injection. The total flow rate was maintained at 0.25 mL/min, with an injection volume of 10  $\mu\text{L}$ . The column oven was set at  $35^{\circ}\text{C}$  and the autosampler at  $8^{\circ}\text{C}$ . The mass spectrometer was calibrated internally before batch analysis, and at the start of each injection, it was spiked with a 10 mM sodium formate solution in isopropanol:ultrapure water (1:1, v/v). ESI source parameters included a capillary voltage of  $-3500\text{ V}$ , end plate offset at  $-500\text{ V}$ , nebulizer gas at 29.0 psi, dry gas flow rate of 8.0 L/min, and a dry temperature of  $200^{\circ}\text{C}$ . Spectra were recorded at 8 Hz, spanning a mass range of 20–1600  $m/z$  in auto MS/MS scan mode. A collision energy ramp from 20 to 50 eV was applied. Data acquisition was managed with Bruker Compass HyStar 5.1, and Control 5.2, while data processing was performed using Data Analysis 5.3 and METABOSCAPE 2021b (Bruker Daltonics GmbH, Bremen, Germany), which generated a table of feature intensities, expressed in counts, representing the detected compounds across the samples.

This data is then cleaned: 1) features that are not present in at least 75% of the samples in a modality are eliminated, 2) missing values for a feature are either replaced by 1/1000 of the smallest intensity value when all samples in a modality are missing or by the median in the case where only part of the samples in a modality are missing, 3) features whose intensity in the

samples is not at least three times the intensity in the extraction blanks in the different modality groups are eliminated.

Compound annotation was realized thanks to our own spectral library of commercially available standards for the metabolites with confirmed structure (confidence annotation level 1, according to the annotation confidence system of Schymanski et al., 2014), by comparison of other commercially or free available spectral libraries (Bruker MetaboBASE Personal Library 3.0, Bruker HMDB Metabolite Library-2.0, MassBank NIST library) for probable structure (level 2) or by molecular formula calculation from the exact mass coupled to isotope profile and comparison with literature for tentative candidates (level 3).

### 2.6. Non-enzymatic Decay Pathway Analysis: quantification of Total Phenols, Ferric Iron reduction activity, Hydrogen Peroxide generation by Ferrous Oxidation and Hydroxyl Radical Activity in the LMW fractions

The LMW methanolic extracts were evaluated to determine the total phenols concentration using the Folin-Ciocalteu assay ( $n = 4$ ) (Singleton et al., 1999), assess ferric iron reduction activity with the ferrozine assay ( $n = 4$ ) (Stookey, 1970), and measure the capacity to generate  $\text{H}_2\text{O}_2$  ( $n = 4$ ) (Wolff, 1994) and  $\cdot\text{OH}$  radicals through FOX assay and Electronic Paramagnetic Resonance (EPR) coupled with a spin-trapping agent (5,5-Dimethyl-1-pyrroline N-oxide, DMPO), respectively ( $n = 4$ ). Analyses were carried out using the protocols, instruments, and chemicals described in Perez-Gonzalez et al. (2022) and (Moretti et al., (2023), with few adjustments. For ferric iron reduction activity and  $\text{H}_2\text{O}_2$  generation capacity, the phenolic concentration was adjusted to 150  $\mu\text{M}$ . To quantify  $\cdot\text{OH}$  production through EPR, phenolic concentration was adjusted to 1 mM. Blanks were prepared using only MeOH to replace the LMW fractions, while negative controls were created using an aliquot of the extract from the mock-inoculated cultures. Concentrations were assessed by referring to gallic acid standards for total phenols quantification ( $R^2 > 0.98$ ),  $\text{FeCl}_2$  standard solutions for iron reduction activity ( $R^2 > 0.99$ ), and  $\text{H}_2\text{O}_2$  standard solutions for FOX assay ( $R^2 > 0.98$ ). Hydroxyl radical relative amplitudes semi-quantification were represented by plotting the average amplitude of the tallest peak (second one) of DMPO-OH adduct spectra for all-fungal-strain-LMW fractions. DMPO-OH spectra were simulated using PEST WinSIM software (Duling, 1994) to confirm the

identity of the captured radicals. Simulation parameters were  $aH= 14.85$ ,  $aN=14.9$ , and 100% Gaussian line shape. G.shift parameters were adjusted accordingly to the measured spectra.

### 2.7. Statistical Analysis

Statistical analyses were performed using R (4.4.1 version). The normality of data was checked using the Shapiro-Wilk test and graphical representation tests (histograms, Q–Q, and P–P plots). Levene's test was used in the case of the normal distribution to check for the homoscedasticity of data. Biomass production in cultures for enzymatic assays was analyzed by one-way ANOVA followed by a post-hoc Tukey test for statistically significant differences ( $p < 0.05$ ) between species among weeks and a Student's t-test for differences between weeks among species. Statistically significant differences for the enzymatic assays were assessed using a Mann-Whitney U test to compare secretome activity between different weeks and a Kruskal-Wallis followed by a Wilcoxon signed rank test ( $p$ . adjustment method Bonferroni) to compare different species among weeks. For tests concerning non-enzymatic biochemical pathway, one-way ANOVA was applied when data fulfilled associated requirements, followed by the Tukey post-hoc test to check for differences among groups. For non-normally distributed data, the Kruskal-Wallis test followed by Dunn multiple comparisons tests ( $p$ . adjustment method Bonferroni) were used. Principal Component Analysis (PCA) was performed on the MS/MS features obtained using the `prcomp` function in the Stats package integrated into R software. For PCA, data were normalized using a  $\log_{10}$  transformation before applying Pareto scaling to standardize the variance of features. After assessing the normality of the data, ANOVA ( $p < 0.05$ ) followed by the Tukey post-hoc test was applied to compare different groups. If the assumptions of ANOVA were not satisfied, the Kruskal-Wallis test and Dunn's multiple comparisons test were utilized instead. EPR spectra were aligned using parametric time warping using the `ptw` package (v1.9). All graphical representations were created with R, utilizing the `ggplot2` (v3.5.1) and `cowplot` (v1.1.3) packages. The `rgl` package (v1.3.17) was employed for the 3D PCA visualization. The Upset plot for metabolomics was generated using the `ComplexUpset` package (v1.3.3).

### 3. Results

#### 3.1. Biomass Production in Culture Conditions

Biomass production in culture conditions for enzymatic assays was consistently higher in 2-week-old cultures (Figure 1). Differences between weeks were statistically different only for *T. texanus* and *S. hirsutum*. In 1W cultures, *F. capensis* produced the highest amount of biomass, although different only from *F. mediterranea* and *S. hirsutum*. In 2W cultures, two distinct groups emerged: *F. capensis*, *I. vitis*, *F. polymorpha*, *F. australiensis*, and *T. texanus*, and *F. langloisii*, *S. hirsutum*, and *F. mediterranea* where biomass production was slightly higher than their respective 1W but still quite lower than the first group.

For non-enzymatic conditions, dried weight retained from filtering liquid cultures showed no significant differences among the species, with a mean of  $1.82 \text{ g} \pm 0.1 \text{ g}$  (Supplementary material, Figure 9).

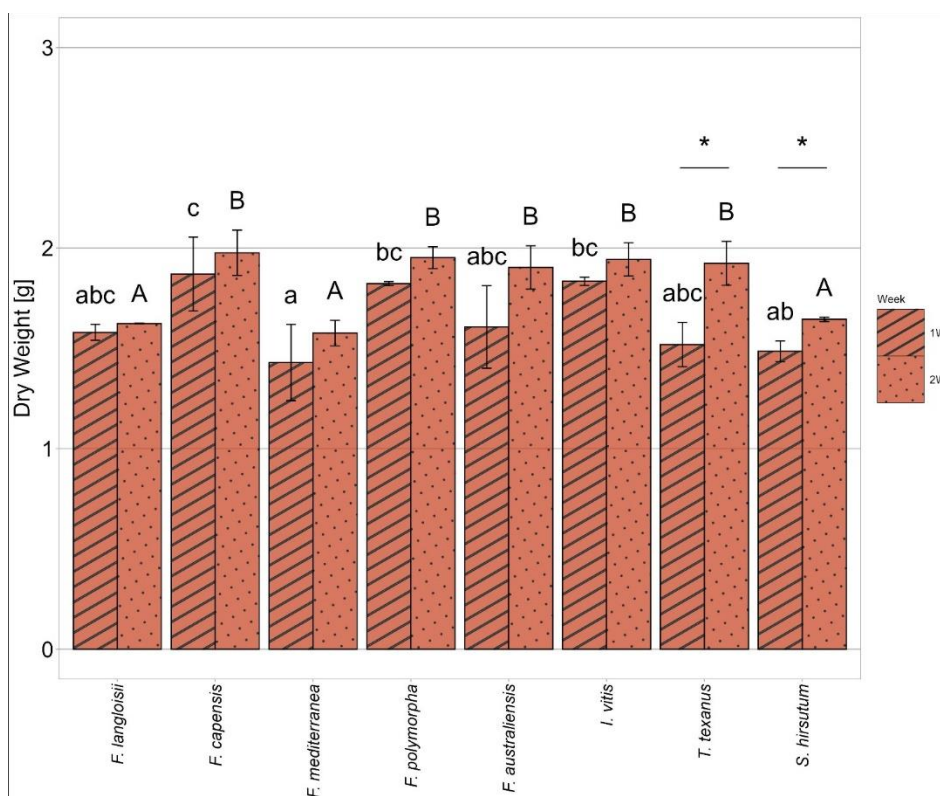


Figure 1 Dry weight of filtered cultures of the 8 ECD WR species (*F. mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, *F. australiensis*, *T. texanus*, *I. vitis*, *S. hirsutum*) for enzymatic assays, after 1W or 2W of growth at 28°C in the dark. Nutrient media consisted of E&P media with the addition of cellulose and grapevine sawdust. The weighted fraction included everything retained by 0.2  $\mu\text{m}$  filtration. Statistical differences among species between weeks were analyzed by *t*-test analysis ( $p < 0.05$ ) and are indicated by \* on top of the graph. Statistical

differences among weeks between species were analyzed by ANOVA followed by post-hoc Tukey test analysis ( $p < 0.05$ ). Lowercase letters indicate the differences between species in 1W, while capital letters refer to 2W.

### 3.2. Enzymatic activities of resuspended secretomes

The laccase activity of *F. langloisii* (Figure 2a) was the greatest for both 1W-old and 2W-old, followed by *F. capensis*. The activity of this enzyme recorded in these two fungi secretomes was much higher than the rest of the species, especially in *F. langloisii* 2W-old, where it was almost 10 times the activity of the third-highest fungus, *F. mediterranea*. All the species showed significant differences from the activity of 1W or 2W. In the case of *F. langloisii*, *F. capensis*, *I. vitis*, and *F. polymorpha*, activity at 2W was higher than at 1W. In the case of *F. australiensis* and *F. mediterranea*, it was the contrary. *T. texanus* and *S. hirsutum* showed almost no activity.

As for MnPs (Figure 2b), overall, the enzyme activity was much lower than laccase's. The highest activity was recorded for *F. australiensis* 2W-old, while the lowest was for *T. texanus* and *S. hirsutum* 2W-old, which were not detected at all in this study. *F. australiensis*, *F. capensis*, *F. langloisii*, *F. mediterranea*, and *F. polymorpha* showed a statistically significant difference between week 1 and week 2. The activity was higher in week 2 for *F. australiensis*, *F. langloisii*, and *F. polymorpha*, while the contrary was true for *F. capensis* and *F. mediterranea*.

As for LiPs activity, no significant differences were found within group 1W or group 2W (Figure 2c). Overall, the LiPs activity was quite small, just detectable in the current experimental setup.

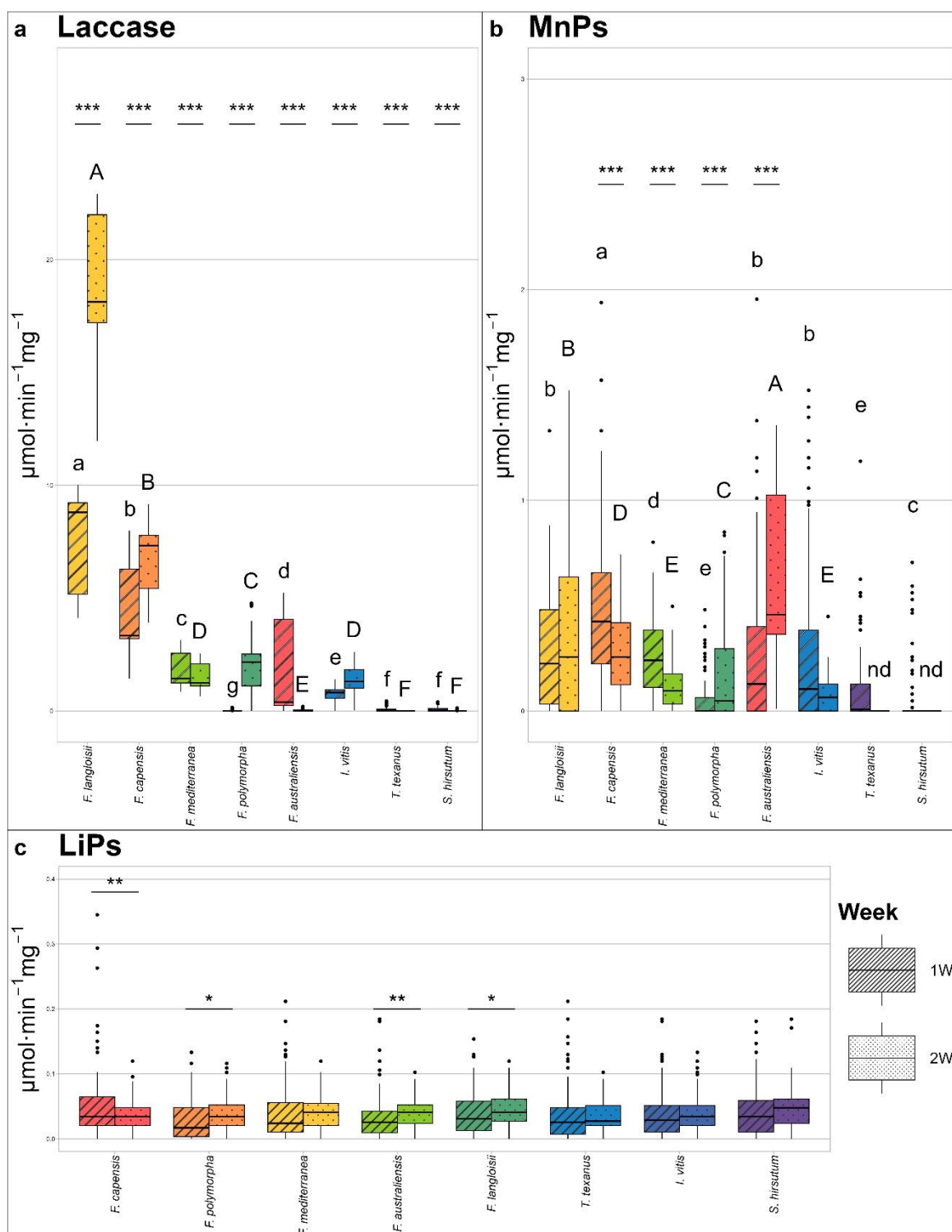


Figure 2 Laccase (a), MnPs (b), and LiPs (c) activities ( $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ) of the secretome of the 8 ECD white rot species after 1W or 2W of growth at 28°C in the dark. Nutrient media consisted of E&P media with the addition of cellulose and grapevine sawdust. Statistical differences among species between weeks were analyzed by the Mann-Whitney U test and are indicated on top of the graph (\* for  $p < 0.05$ , \*\* for  $p < 0.01$ , \*\*\* for  $p < 0.001$ ). Statistical differences among weeks between species were analyzed by Kruskal-Wallis, followed by Wilcoxon signed rank test analysis ( $p < 0.05$ ). Lowercase letters indicate the differences between species in 1W, while capital letters refer to 2W. Non-Targeted Metabolomic Analysis

All species shared the vast majority of extracted compounds (Figure 3b). Within the *Fomitiporia* genus, five unique compounds were identified in *F. australiensis* while two in *F. capensis*. *F. polymorpha* had the highest number of unique compounds in the *Fomitiporia* genus. No unique compounds were found in *F. mediterranea* and *F. langloisii*. Regarding other species, six unique compounds were found for *I. vitis*, twenty-three for *S. hirsutum*, and sixty for *T. texanus* (Figure 3b).

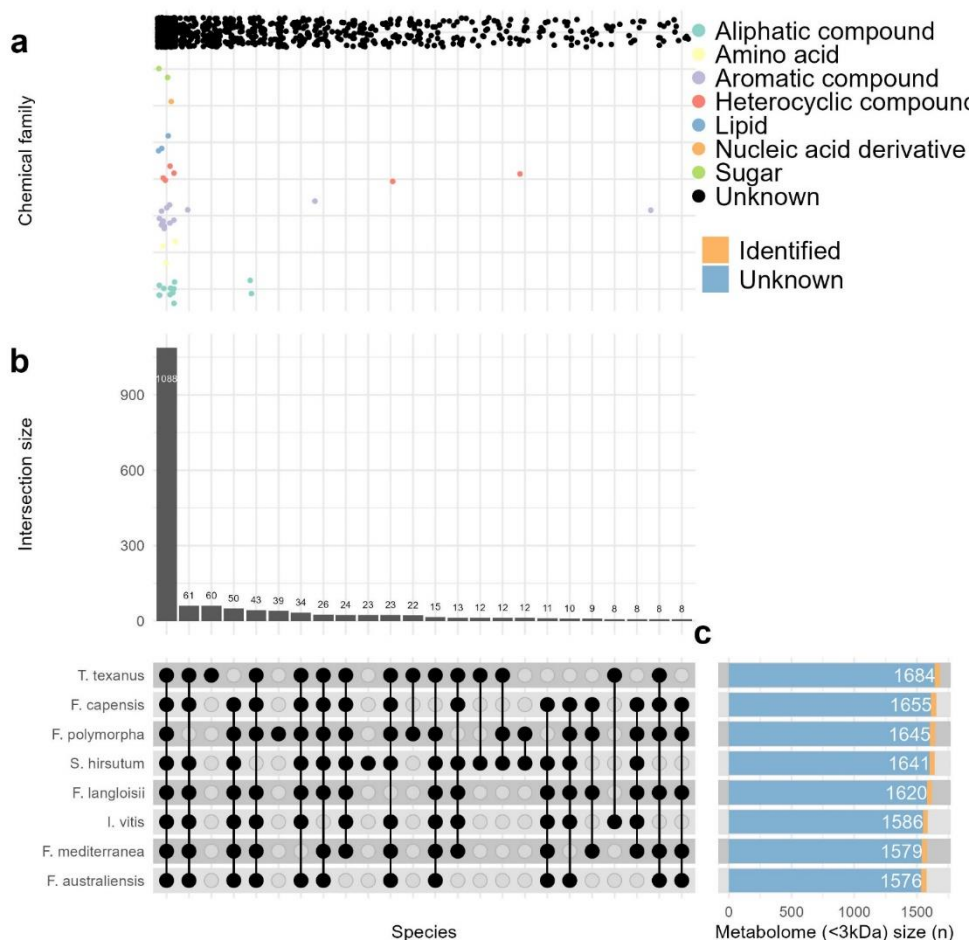


Figure 3 Ultrafiltered Metabolome (< 3 KDa) profiling of the extracted metabolites present in the LMWC methanolic extract of the 8 white-rot ECD species. Extracted metabolites overlap and abundance in each intersection group are illustrated using an UpSet plot (b). The metabolome size, along with the ratio of identified to non-identified compounds, is presented (c), as well as the chemical families of the identified compounds (a).

Principal Component Analysis (PCA) performed on normalized intensities counts of all metabolites from all biological replicates, using PC1 and PC2, revealed an important clustering of the *Fomitiporia* genus, with *F. polymorpha* being the exception being quite distant from other species (Figure 4b). *T. texanus* displayed the greatest distance from the other species. Including PC3, a clear separation emerged between *S. hirsutum* and the *Fomitiporia* cluster

(except for *F. polymorpha*, as previously stated - Figure 4b). Although most of the compounds that explain the variance of these PCs have not been identified, N-Acetylserotonin can be found among the most influential compounds in PC3 (Supplementary Table S3).

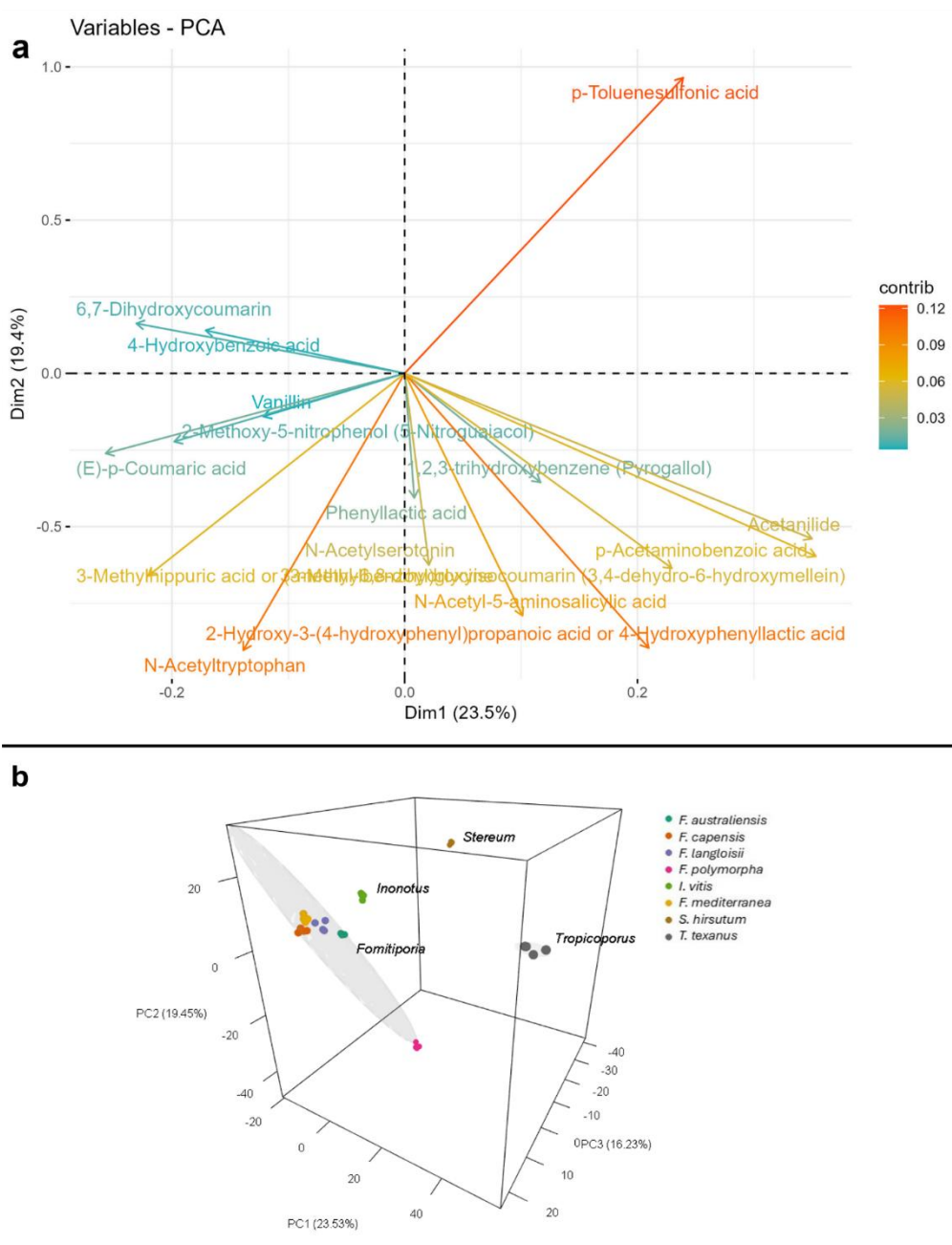


Figure 4 The metabolome of the LMWC methanolic extracts overview using PCA analysis. Normalized intensity count values for detected metabolites were used as input. (a) Contribution of identified metabolites to PC1 and PC2. (b) Data visualization in three-dimensional space was obtained using the first three PCs, explaining the highest percentage of variance. Each point represents a biological replicate, with colors assigned according to species identity. The percentage of variance explained by the three principal components is indicated on the corresponding axis labels. Ellipses represent groupings by genus to highlight species' clustering.

Non-target LMW metabolomics analysis identified numerous aromatic and phenolic compounds, many known for their ability to reduce iron (Figure 5). To simplify, from here on, the definition “FeRa compounds” will include molecules with a proven ferric iron reduction capacity and compounds suspected to be iron-reducing due to their chemical structure displaying coordinating oxygens, multiple hydroxyl groups, amino and methoxyl groups. A broad spectrum of FeRa compounds has been found in the LMW fraction of every species studied, although in different quantities. No specific FeRa compound was identified as species-specific. Most FeRa compounds were consistently present across all groups, with *F. australiensis* being the species that exhibited the most, notably in 4-hydroxycyclohexylcarboxylic acid and coumaric acid. N-acetyltryptophan was only found in *F. australiensis* and *F. polymorpha*, while N-acetylserotonin was scarce in all species but *F. polymorpha* and *S. hirsutum*. Conversely, 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid was low in both *I. vitis* and *S. hirsutum*. Phenyllactic acid was also prevalent in all species, with higher content in *F. australiensis*, *F. polymorpha*, and *S. hirsutum*. Further analyses are necessary to confirm if the identified compounds have FeRa capabilities.

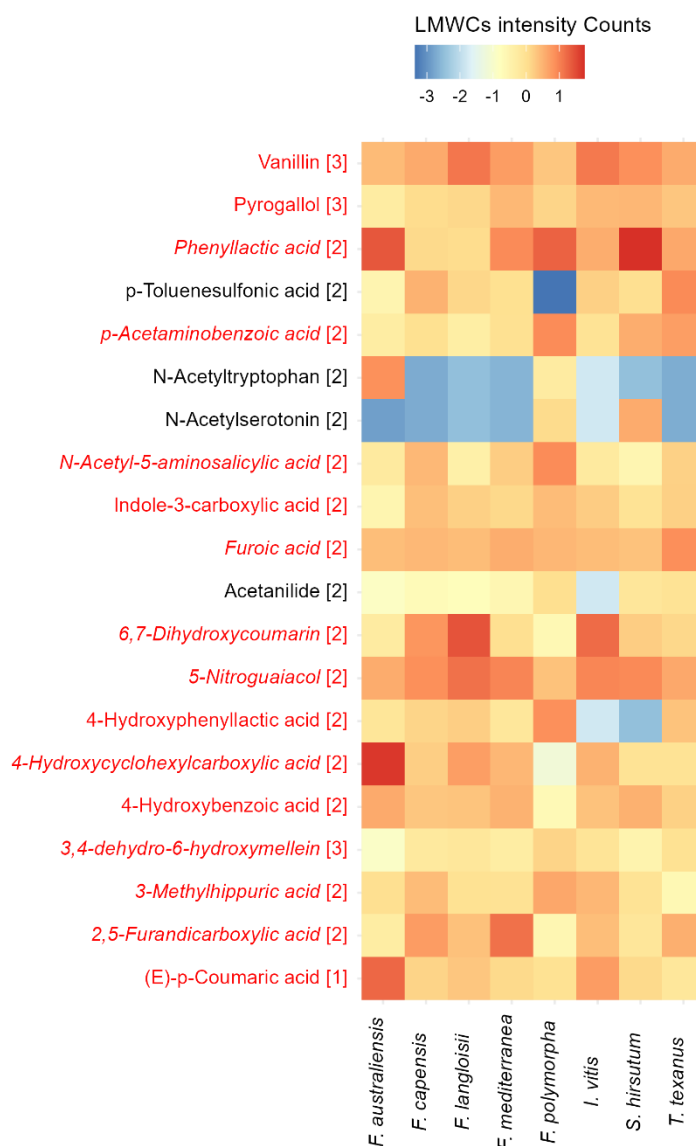


Figure 5 Normalized intensity counts of selected compounds analyzed by UHPLC-MS/MS. Names in red are compounds with FeRa capabilities reported in the literature or hypothesized based on their chemical structure (italics). Heatmap values represent the log<sub>10</sub>-transformed means of four biological replicates, scaled as Z-scores to highlight relative differences across compounds. The numbers in the last column indicate the type of annotation performed: [1] using a chemical standard, [2] based on the spectral library, and [3] by matching with chemical formulae from the literature. Statistical analyses for each compound are shown in Table 3.

### 3.3. Quantification of Total Phenols by Folin-Ciocalteu Assay

Folin-Ciocalteu assay showed that *T. texanus* produced the highest amount of phenols ( $18.07 \pm 3.81 \text{ mmol} \cdot \text{L}^{-1}$  gallic acid equivalent), almost 4 times the amount of the others (Figure 6). All

the species belonging to the genus *Fomitiporia*, *S. hirsutum*, and *I. vitis* produced a statistically comparable amount of phenols.

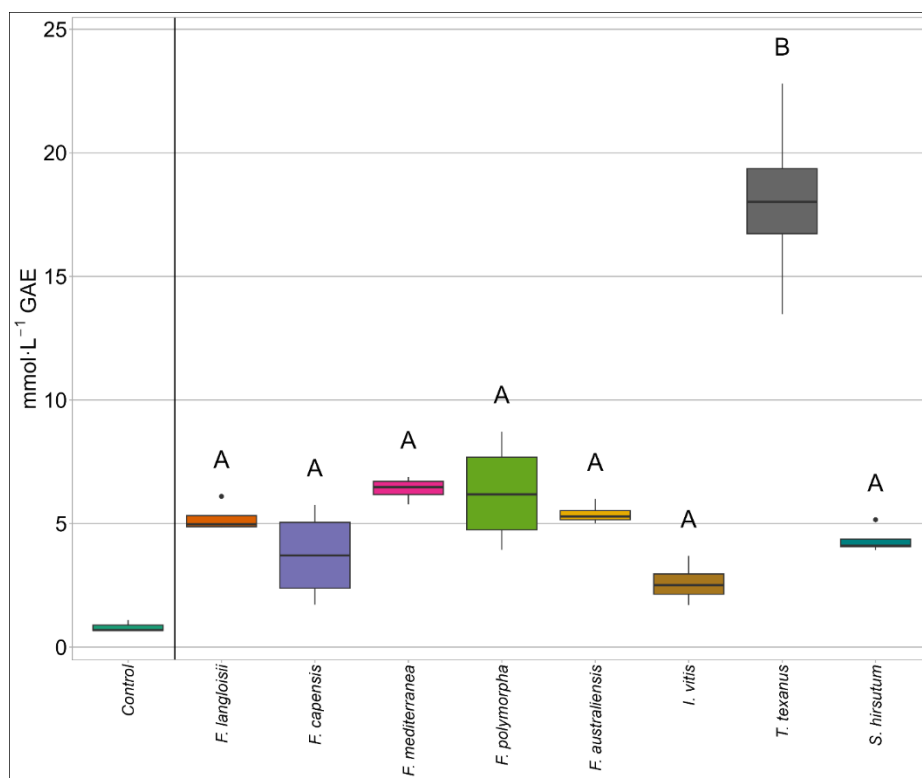


Figure 6 Total phenolic content was measured spectroscopically using the Folin-Ciocalteu assay of LMWC methanolic extracts of the 8 species of the collection of ECD white rot agents (n=4). Results are expressed in mmol·L<sup>-1</sup> gallic acid equivalent (GAE) and were analyzed using the Kruskal-Wallis test followed by a Dunn's test (p. adjustment method Bonferroni,  $p < 0.05$ ).

### 3.4. Iron Reduction and Hydrogen Peroxide Generation from LMWC Redox Cycle

All extracts were able to reduce ferric iron ( $\text{Fe}^{3+}$ ) to ferrous iron ( $\text{Fe}^{2+}$ ) (Figure 7aFigure 7). Most of the species showed comparable activity, with *T. texanus* showing almost double the ability of ferric iron reduction ( $21.53 \pm 2.25 \text{ Fe}^{2+} (\mu\text{mol} \cdot \text{L}^{-1})$ ). *F. capensis* showed the highest variability in ferric iron reduction capabilities within its 4 biological replicates. Hydrogen peroxide production was well detected in all species but low in *T. texanus* (Figure 7b). Extracts of this species showed production of  $\text{H}_2\text{O}_2$  on the lower side. The highest value was detected in *F. capensis* (average FOX value =  $23.52 \pm 13.22 \text{ H}_2\text{O}_2 (\mu\text{mol} \cdot \text{L}^{-1})$ ). *F. langloisii*, *F. polymorpha*, and *S. hirsutum* showed great variability among their biological replicates.

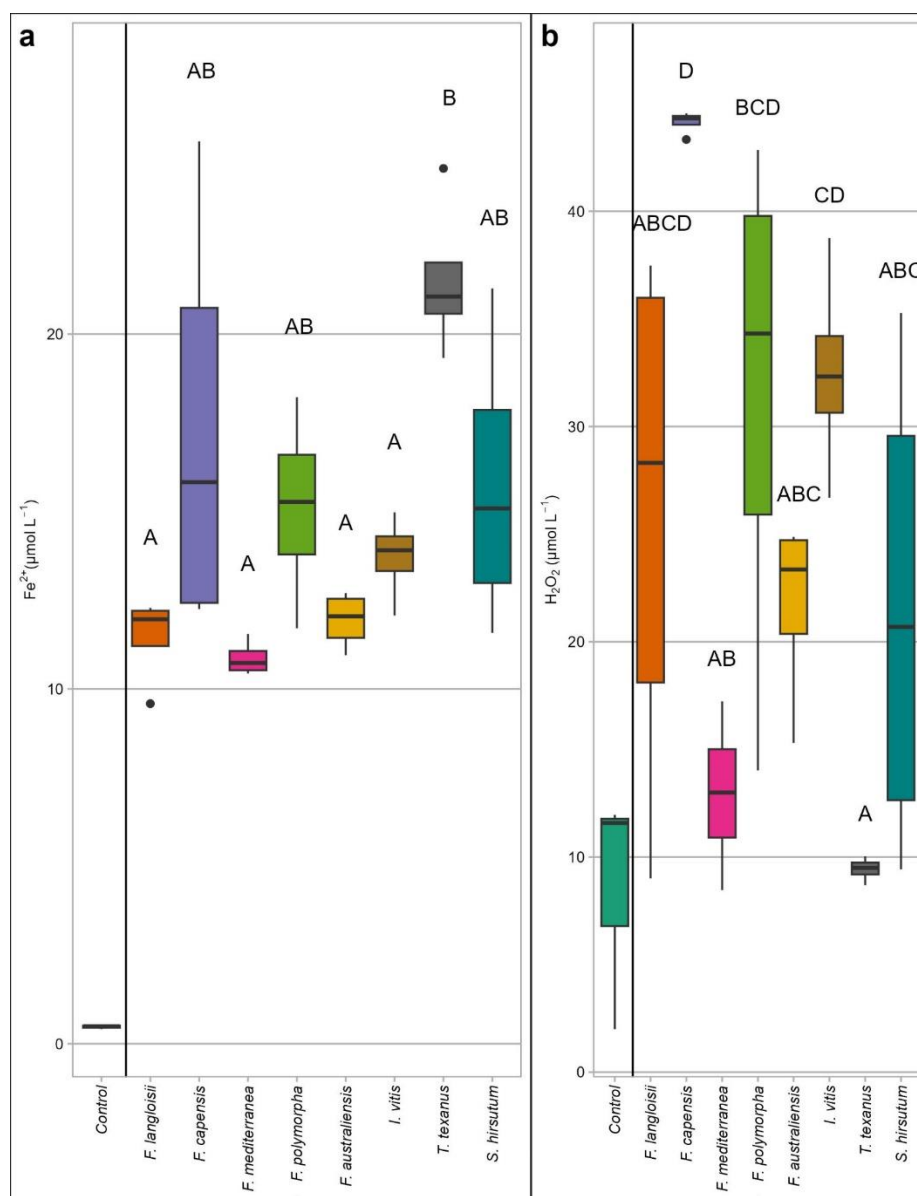


Figure 7 Iron reduction capabilities of the LMWC methanolic extracts of the 8 species of ECD white rot agents (a). Phenols in the solution were standardized according to Folin-Ciocalteu results. Values are expressed in Fe<sup>2+</sup> (μmol · L<sup>-1</sup>). Results of the FOX assay for H<sub>2</sub>O<sub>2</sub> generation during iron reduction (b). Values are expressed in H<sub>2</sub>O<sub>2</sub> (μmol · L<sup>-1</sup>). For both assays, the results showed (n = 4) and were analyzed using one-way ANOVA followed by a Tukey post hoc test.

### 3.5. Spin-trapping Electronic Paramagnetic Resonance (EPR) of Hydroxyl Radicals

Hyperfine couplings and shape of lines arising from the interaction of the spin-trap agent DMPO and radicals generated in the CMF-reaction mix resulted in spectra identifiable with the •OH radical. LMW methanolic extracts from all species were found to be able to produce •OH

radicals in the presence of ferric iron and hydrogen peroxide. Contrary to expectations, the area of the peaks, and thus  $\cdot\text{OH}$  radical production, was generally higher at a higher pH (pH 5.5) than at a lower pH (pH 3.5). At pH 5.5 (described as the intact wood cell wall pH), all species seem to have a statistically comparable production of  $\cdot\text{OH}$ , with the biggest absolute average value recorded for *I. vitis*.

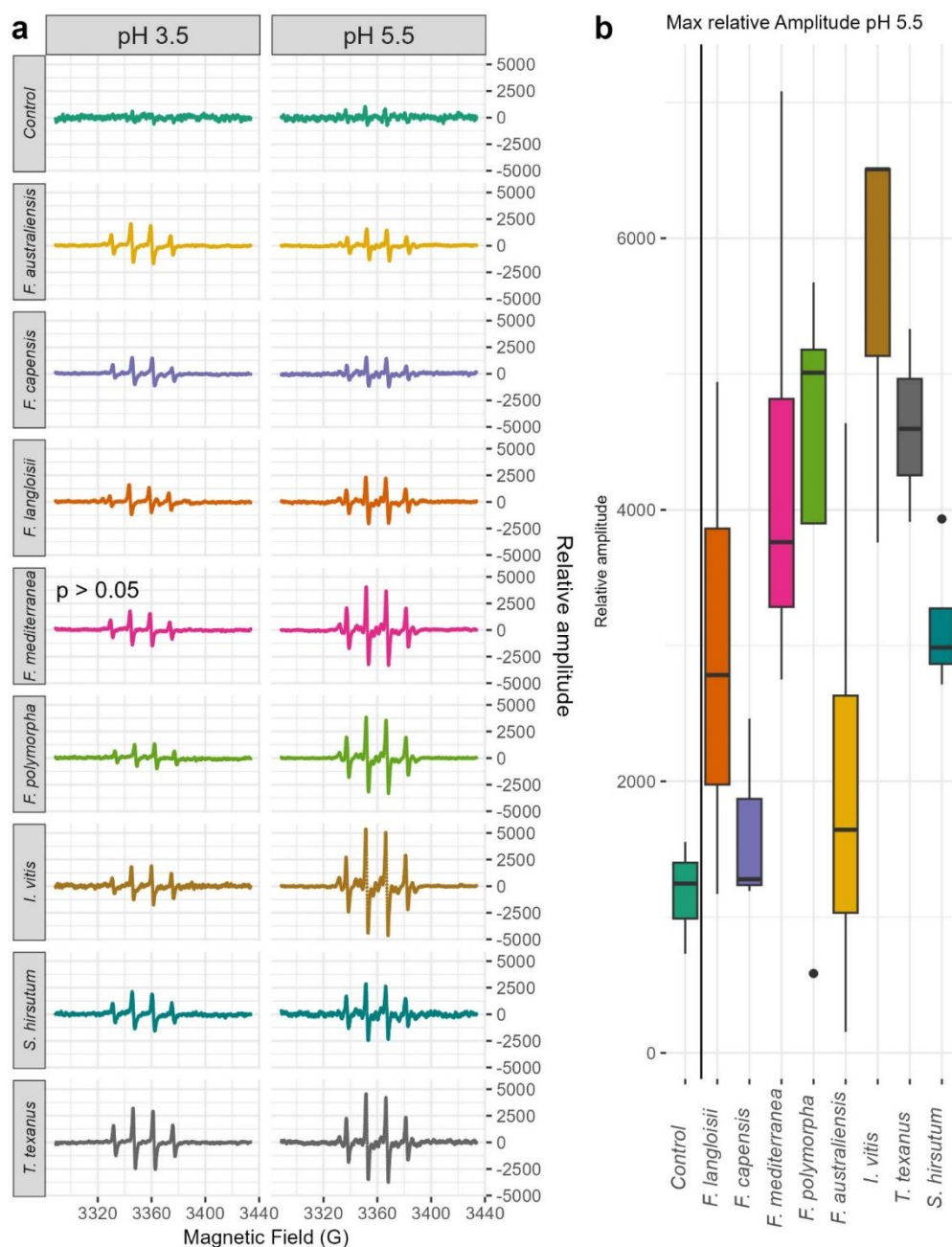


Figure 8 EPR spectra of the DMPO-OH adducts recorded at pH 3.5 and 5.5 for all ECD WR agents (a). Values represented are the means of biological replicates ( $n=4$ ). Maximum values within the interval 3340-3355 magnetic field (G) at pH 5.5 were used to assess differences between species (b) using the Kruskal-Wallis test followed by Dunn's multiple comparison tests ( $p < 0.05$ ).

## 4. Discussion

This research aimed to screen different white rot basidiomycetes fungi associated with Esca Complex of Diseases (ECD) in different world regions for biochemical mechanisms usually involved in the wood decay arsenal. Recent research annotated the presence of different potential virulence factors in at least 5 of the species considered in our study, particularly (but not exclusively) genes encoding for lignocellulolytic enzymes and secondary metabolite biosynthesis (Floudas et al., 2012; Garcia et al., 2024). In this study, we took a further step in the functional characterization of decay mechanisms, considering both enzymatic and non-enzymatic processes typically associated with wood decay. This aligns with recent literature that challenges the association of decay mechanisms with specific rot categories (Moretti et al., 2023; Perez-Gonzalez et al., 2022; Riley et al., 2014; Shabaev et al., 2022). To our knowledge, our manuscript represents the first comprehensive research on the subject, being *Fomitiporia mediterranea* the only ECD-associated white rot fungus studied for both pathways up to now. Our work shows how different white rot-ECD fungi present both enzymatic (specifically laccases and class II-PODs) and CMF-like non-enzymatic radical-generating pathways when cultivated *in vitro* in specific conditions conventionally adopted to study wood decay (Eriksson and Pettersson, 1975; Highley, 1973).

Lignin enzymatic depolymerization in white rot agents is, for the vast majority, attributed to class-II PODs and laccases; therefore, in our study, we focused on Lignin Peroxidases (LiPs), Manganese peroxidases (MnPs), and laccases (Goodell, 2020). The activity of laccase and MnP in the resuspended secretome of *F. mediterranea* was higher after one week of culture compared to two weeks, aligning with previous research that identified 10 days as the optimal harvest time in the same experimental conditions (Pacetti et al., 2022).

Similarly, laccase activity in *F. australiensis* was nearly absent in 2W cultures, while MnP activity increased. In contrast, *F. capensis* showed increased laccase activity from 1W to 2W cultures, accompanied by a decrease in MnP activity. Such shifts in enzyme activity are common during fungal wood degradation, reflecting the changes in substrate composition and shifts in fungal focus toward different components of the wood cell wall (Kuuskeri et al., 2016; Schilling et al., 2022; Skyba et al., 2016). Our results suggest that *F. australiensis* and *F. capensis* may employ distinct strategies for the sequential degradation of wood components, potentially targeting different polysaccharides at specific stages of decomposition.

A clear time-dependent increase in total lignocellulolytic enzymatic activity was exhibited in *F. polymorpha*, as both MnP and laccase activities increased concomitantly in 2W cultures ( $R^2 > 0.95$ ,  $p < 0.005$ , based on Pearson correlation coefficient), although it remained quite weak. This suggests that this fungus may prefer to degrade other polysaccharides before focusing on lignin (Hori et al., 2014; Rudakiya and Gupte, 2017).

*F. langloisii* showed strong activity for laccase in both time points, with particularly high levels at 2W. Recent research has highlighted how *F. mediterranea* shows a preference for lignin and amorphous cellulose (Haidar et al., 2024, 2021). This notable laccase production in *F. langloisii* may indicate a similar degradation pattern, suggesting a comparable lignocellulosic targeting strategy.

The laccase activity of *T. texanus* and *S. hirsutum* was negligible at both time points in this study. Similarly, MnP activity was low at 1W and completely absent at 2W. *S. hirsutum* presents 5 atypical peroxidase-encoding genes in its genome, along with several laccase *sensu stricto* (Floudas et al., 2012). As for *T. texanus*, its genome contains 16 POD-II genes (Garcia et al., 2024). The discrepancy between genomic analyses and our results suggests possible downregulation of genes involved in wood degradations or post-translational modifications rendering the proteins nonfunctional. More investigations on the nature of these protein-encoding genes could help understand *S. hirsutum* and *T. texanus* enzymatic arsenal.

The recorded LiP activity was barely detectable for each studied fungus, but it was significantly different from both control samples ( $p$ -value  $< 0.01$ ). However, no differences were observed within the 1W and 2W cultures among all species and *F. mediterranea*, which has been shown to lack specific genes encoding LiP in its genome and did not produce any enzymes in its secretome (Floudas et al., 2012; Schilling et al., 2022). Thus, it can be speculated that all the basidiomycetes presented in the study are LiP-lacking or not able to express these enzymes in the setting of this study. The dye used in the experimental setup (Azure B) is a triarylmethane dye with a similar structure to lignin (Yang et al., 2021). Besides LiP, this compound has shown little affinity also towards MnP and laccase when in synergy with non-enzymatic oxidative mechanisms (Arantes and Milagres, 2007). As discussed later, the results of this study suggest that the LMW fraction of fungal secretomes can activate an oxidative degradation mechanism potentially acting on the lignocellulosic matrix. This could explain the low levels of LiP activity detected, potentially resulting from the activation of such a synergic system. Moreover, while spectroscopy-based enzyme activity assays are widely used and reliable, they are sensitive to various external factors like pH, light, and temperature (Agrawal and Verma, 2019; Alcalde and Bulter, 2003; Tovar-Herrera

et al., 2018). In addition, as noted by Mathieu et al. (2013), some secretome compounds may precipitate with grapevine sawdust or cellulose, or enzymes may become adsorbed onto the woody matrix. This interaction could lead to artificially low measurements.

Our results represent a first overview of the lignocellulolytic enzymatic array of the studied fungi. Employing proteomic techniques, along with transcriptomic analyses, protein isolation, and structural analysis of the Class II PODs and CAZymes, could provide a deeper understanding of: i) LiP presence or absence in the secretome of ECD-associated white rot agents; ii) time-dependent activation of the CAZymes pathway in relation to class-II PODs and laccase activity.

Recent research on wood decay mechanisms has shown that non-enzymatic radical-generating pathways, potentially associated with the breakdown of the lignocellulose matrix, are more prevalent among wood decay fungi than previously described. Specifically, it has been found that *F. mediterranea*, *Eutypa lata*, *Phaeomoniella chlamydospora*, and *Phaeoacremonium minimum* have the ability to perform a non-enzymatic CMF-like pathway, in addition to their known enzymatic capabilities (Perez-Gonzalez et al., 2022; Moretti et al., 2023). Moreover, Garcia et al. (2024) highlighted the presence of genes associated with secondary metabolisms—such as polyketide synthases, non-ribosomal peptide synthetases, and terpene synthases—in most of the species studied in their research. This finding supports our previous hypothesis regarding the spread of the CMF-like non-enzymatic pathway in white rot fungi other than *F. mediterranea*, as most LMWC linked to the CMF pathway are secondary metabolites of an aromatic nature.

The CMF mechanism is initiated by the secretion of LMW organic acids that can sequester insoluble iron in a low-pH environment. Then, the sequestered iron will transfer into the higher-pH environment of the wood cell wall, where it will be conferred to LMWC-iron-reducing metabolites, primarily of phenolic or aromatic nature. Ultimately, this process leads to LMWCs redox-cycling with iron and molecular oxygen, producing hydrogen peroxide and hydroxyl radicals (Arantes et al., 2011a; Goodell, 2020; Goodell et al., 1997b; Zhu et al., 2022).

As expected, all the white rot agents studied produced phenolic and aromatic compounds. In fact, the production of these classes of compounds by white rot fungi has already been reported in the literature (Arantes et al., 2011b; Pinar and Rodríguez-Couto, 2024). In our study, *T. texanus* produced more phenols than the others. This was visibly

evident as its liquid culture quickly developed a strong yellow color. No specific compound has been identified as having a significant presence in the LMW fraction of this fungus, indicating that many of the phenolic compounds remain to be discovered. Basidiomycetes are known to produce pigments and phenols in response to both abiotic and biotic stimuli (Fink-Boots et al., 1999; Jha, 2024). Since nutrient-limited conditions used for the culture media are stress conditions for fungi, the intense pigmentation observed in LMW methanolic extracts from *T. texanus* liquid culture – together with the high levels of phenols produced – may be linked to a very sensitive stress response triggered in the latter species respect to the others. Further expression studies on secondary metabolism-related genes could help better understand such response regulations.

All methanolic extracts were able to redox-cycle with iron, reducing ferric iron ( $\text{Fe}^{3+}$ ) to ferrous iron ( $\text{Fe}^{2+}$ ) and solidly induce  $\text{H}_2\text{O}_2$  production as a result of the reaction between  $\text{O}_2$  and  $\text{Fe}^{2+}$  mediated by LMWC. The ferric iron reduction (FeRa) activity of *T. texanus* was higher overall than that of other species, despite the number of phenols in the reactions being standardized. However,  $\text{H}_2\text{O}_2$  production was quite low. Methanolic extracts of this fungus were able to generate hydroxyl radicals during EPR experiments. This could suggest that the *T. texanus* LMW fraction might have different individual compound ratios (with different reactivity towards metal ligands and oxygen) compared to the other fungi, therefore enabling it to efficiently redox cycle with iron but to produce a limited amount of  $\text{H}_2\text{O}_2$  during ferric iron reduction (via the reduction of  $\cdot\text{OOH}$  generated from LMWC redox cycling with the molecular oxygen naturally diffused in the wood cell wall), as suggested in Moretti et al. (2023). Hydrogen peroxide produced by LMWC activity in wood cell walls is the primary source of hydrogen peroxide used in the CMF mechanism (Arantes and Goodell, 2014). However, enzymes such as alcohol oxidase, glucose oxidase, MnP, and other peroxidases can also produce extracellular  $\text{H}_2\text{O}_2$  (Kirk and Farrell, 1987; Linke et al., 2014). Since these enzymes cannot penetrate intact wood cell walls, enzyme-produced  $\text{H}_2\text{O}_2$  would only participate in the CMF mechanism during the advanced stage of degradation (Arantes and Goodell, 2014; Tepfer and Taylor, 1981; Zhu et al., 2017). Thus, non-enzymatic degradation caused by *T. texanus*, as well as other fungi, could be amplified by subsequent synergic enzymatic action.

In wood cell walls, hydrogen peroxide reacts with reduced iron, leading to the cascade production of reactive oxygen species via the Fenton reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$ ), particularly hydroxyl radicals ( $\cdot\text{OH}$ ), which ultimately promote a rapid non-enzymatic breakdown of wood cell walls (Goodell, 2020).

All species efficiently generated hydroxyl radicals at both pH tested in our experimental setup (3.5, 5.5). Unexpectedly, the radical generation was found to be higher at a pH of 5.5. Previous studies indicated a higher activity for ECD-associated species at lower pH levels, including *F. mediterranea* (Moretti et al., 2023; Perez-Gonzalez et al., 2022). This discrepancy could be explained by the difference in LMW fraction composition. As a matter of fact, some FeRa compounds, such as variegated acid, demonstrate improved iron-reduction capabilities at higher pH levels (Zhu et al., 2017). Further identification of compounds in the LMW fraction and analysis of their FeRa capabilities could shed light on the specific contributions and pH preferences of individual LMWCs to the overall efficiency of the CMF mechanism.

Nonetheless, hydroxyl radical generation at both 3.5 and 5.5 pH showed the potential ability of LMWC extract to initiate a full CMF mechanism inside the intact wood cell wall (incipient decay) and at a more advanced decay stage (Moretti et al., 2023; Perez-Gonzalez et al., 2022).

A wide range of LMWCs was identified through UHPLC MS/MS. As expected, most of the compounds were shared across species, likely as being part of basal metabolism. Several species-specific metabolites were found in the extract of *T. texanus*. Excluding *S. hirsutum*, this fungus is the most phylogenetically distant species; thus, this result could be linked to evolutionary divergence (Brown et al., 2020).

The ultrafiltered methanolic extracts contained a variety of phenolic and phenol-derived compounds. Although the FeRa capacity of some of these has not yet been demonstrated, the presence of multiple hydroxyl groups, coordinating oxygen atoms, methoxyl groups, and amino groups in their chemical structure suggests a potential ability to chelate and reduce iron ions (Aguiar and Ferraz, 2007; Antolovich et al., 2004). Known FeRa compounds that were identified include vanillin, 4-Hydroxybenzoic acid, (E)-p-Coumaric acid, and pyrogallol, all confirmed as FeRa by previous research (Aguiar and Ferraz, 2007; Arantes et al., 2011b; Pan et al., 2022). Confirmed and suspected FeRa compounds were widespread across species, indicating the presence of a common core of iron-reducing phenols, as well as other non-phenolic aromatic compounds. Phenols also play a pivotal role in oxidative stress response as they participate in excess ROS detoxification (Christen et al., 2005; Goodell, 2020; Schilling et al., 2022). Coumarins are widely used in the medical field for their antioxidant properties and are proven to have iron-reduction capabilities (Catapano et al., 2018; Moretti et al., 2023; Shen et al., 2022). The fact that in our study, OH radicals were

still generated suggests that the concentration of possible antioxidants could not be sufficient to efficiently scavenge radicals. Moreover, the biochemical environment of this experimental setup may have favored the prooxidant activity of these compounds. As a matter of fact, in the literature, these compounds are described as a “double-edged sword” (prooxidant and antioxidant) depending on environmental conditions such as pH and proximity to redox metals (Castañeda-Arriaga et al., 2018). As a result, these compounds may have acted as prooxidants instead.

The analyses on the LMW secretome performed in this study confirm that all studied fungi can induce a CMF-mediated mechanism. However, more compounds still remain unidentified, and identifying them and confirming the iron reduction capabilities of the identified individual metabolites could provide further insights into white rot non-enzymatic wood degradation.

Overall, our findings represent an important advancement in understanding functional pathways associated with wood degradation in ECD- white-rot agents. These pathogens are responsible for the widespread disease called Esca *sensu stricto* or “amadou”. Production and activity of lignocellulosic enzymes such as laccases and manganese peroxidases have been evaluated. In addition to other studies, our research shows that a non-enzymatic CMF pathway is widespread among ECD-associated fungal pathogens, both basidiomycetes and ascomycetes (Moretti et al., 2023; Osti and Di Marco, 2010; Perez-Gonzalez et al., 2022). Microscopy observations on *F. mediterranea* naturally or artificially infected wood have revealed that the formation of soft-rot-like cavities in the S2 layer of the wood cell wall (Kassemeyer et al., 2022; Pacetti et al., 2022). It has been suggested that the activity of CMF chemistry in an early stage of degradation might be the reason for the development of such cavities (Moretti et al., 2023). Future exploration of our findings might potentially involve microscopic observation of wood infected by other ECD white rot species could help determine whether this phenomenon is consistent across all grapevine-associated white rot species, especially since many white rot fungi are known to undergo a soft-rot phase during early stages of degradation (Schwarze, 2007).

Other potential applications of our findings may involve disease control. The inhibition of growth of the mycelia of *E. lata*, *P. minimum*, and *P. chlamydospora* was achieved by successfully disrupting the CMF system using antioxidant and iron-chelating compounds (Sebestyen et al., 2022). Biological control agents (BCAs) used for GTDs control are known to produce siderophores that lead to competition for iron (Mesguida et al., 2023).

Looking for CMF-disruption capabilities in BCAs could help develop new ways to control GTDs pathogens.

Wood-decay enzymes allow GTDs fungi to break down plant cell walls, access nutrients, and colonize the host. Thus, the ability to produce these enzymes is regarded as a crucial virulence factor (Morales-Cruz et al., 2015). Since enzymes have been shown to have difficulty penetrating the intact secondary S2 layer of the wood cell wall, the ability to induce an early CMF-based degradation could represent another important pathogenicity factor to be considered.

The involvement of fungal degradation, both enzymatic and non-enzymatic, in ECD might be a topic that needs to be further investigated to better understand the role of fungal wood degradation and its eventual connection to foliar symptoms development, an aspect of GLSD in ECD, which remains unclear (Bortolami et al., 2019; Del Frari et al., 2021; Hofstetter et al., 2012).

## 5. Conclusions

The aim of this study was to evaluate the lignocellulose matrix degradation capabilities of white rot agents collected from various viticulture areas of the world in vines showing ECD symptoms (including foliar symptoms) by assessing their biochemical pathways associated with lignocellulose matrix mineralization. Results showed that the resuspended secretome of the fungal collection could express laccase and manganese peroxidases activity.

All tested fungi were able to initiate a CMF mechanism, indicating that this mechanism is quite widespread in GTDs-associated basidiomycetes, in addition to being common among the ascomycetes (Moretti et al., 2023; Osti and Di Marco, 2010; Perez-Gonzalez et al., 2022).

Considering this, we propose that this degradation pathway may be a key factor in fungal adaptation to the grapevine, thus representing a potential pathogenicity factor for GTDs.

With this in mind, future research should focus on finding ways to disrupt fungal degradation, such as developing new solutions for disease control or investigating aspects that could help search for tolerant varieties. Host specificity and intrastrain differences could also provide a deeper understanding of the degradation capabilities of grapevine white-rot decay agents.

Investigating the role of CMF mechanisms in decay agents in grapevines could enhance our understanding of fungal degradation, helping to unveil new factors related to the movement of metabolites to the leaves and the development of foliar symptoms.

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## Supplementary material

Table 1 List of compounds detected through non-target metabolomic analysis

| Compound                           | Exact<br>Mass | RT<br>(min) | m/z       | Molecular<br>Formula                                        | Identification<br>Level |
|------------------------------------|---------------|-------------|-----------|-------------------------------------------------------------|-------------------------|
| C5 Sugar                           | 150.053       | 01.27       | 149.04538 | C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>               | 3                       |
| C4 Sugar                           | 120.042       | 01.28       | 119.03487 | C <sub>4</sub> H <sub>8</sub> O <sub>4</sub>                | 3                       |
| 2-Hydroxy-2-methylsuccinic acid    | 148.037       | 01.68       | 147.02974 | C <sub>5</sub> H <sub>8</sub> O <sub>5</sub>                | 2                       |
| Succinic acid                      | 118.026       | 02.89       | 117.01916 | C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>                | 1                       |
| Succinic acid                      | 118.026       | 03.08       | 117.0192  | C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>                | 1                       |
| 3-Hydroxy-3-methylglutaric acid    | 162.053       | 03.70       | 161.04537 | C <sub>6</sub> H <sub>10</sub> O <sub>5</sub>               | 2                       |
| Thymine                            | 126.043       | 04.01       | 125.03536 | C <sub>5</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub> | 2                       |
| 2-Methylmaleic acid                | 130.026       | 04.13       | 129.01916 | C <sub>5</sub> H <sub>6</sub> O <sub>4</sub>                | 2                       |
| 6-Hydroxynicotinic acid            | 139.027       | 04.56       | 138.0195  | C <sub>6</sub> H <sub>5</sub> NO <sub>3</sub>               | 2                       |
| Glutaric acid                      | 132.042       | 04.97       | 131.03477 | C <sub>5</sub> H <sub>8</sub> O <sub>4</sub>                | 2                       |
| 2,5-Furandicarboxylic acid         | 156.006       | 05.16       | 154.99853 | C <sub>6</sub> H <sub>4</sub> O <sub>5</sub>                | 2                       |
| 3-Hydroxyisovaleric acid           | 118.063       | 05.23       | 117.05551 | C <sub>5</sub> H <sub>10</sub> O <sub>3</sub>               | 2                       |
| Pyrogallol                         | 126.032       | 05.90       | 125.02436 | C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>                | 3                       |
| Furoic acid                        | 112.016       | 06.09       | 111.00839 | C <sub>5</sub> H <sub>4</sub> O <sub>3</sub>                | 2                       |
| Malic acid                         | 134.021       | 06.16       | 133.01402 | C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>                | 2                       |
| Pantothenic acid                   | 219.111       | 06.33       | 218.10332 | C <sub>9</sub> H <sub>17</sub> NO <sub>5</sub>              | 2                       |
| 3-Methylglutaric acid              | 146.058       | 06.54       | 145.05041 | C <sub>6</sub> H <sub>10</sub> O <sub>4</sub>               | 2                       |
| 2-hydroxy-2-methyl-butyrac acid    | 118.063       | 06.65       | 117.05551 | C <sub>5</sub> H <sub>10</sub> O <sub>3</sub>               | 2                       |
| p-Toluenesulfonic acid             | 172.019       | 06.67       | 171.01197 | C <sub>7</sub> H <sub>8</sub> O <sub>3</sub> S              | 2                       |
| 4-Hydroxyphenyllactic acid         | 182.058       | 06.67       | 181.05051 | C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>               | 2                       |
| 4-Hydroxycyclohexylcarboxylic acid | 144.078       | 06.68       | 143.07117 | C <sub>7</sub> H <sub>12</sub> O <sub>3</sub>               | 2                       |

### Chapter III

|                                       |         |       |           |                                                               |   |
|---------------------------------------|---------|-------|-----------|---------------------------------------------------------------|---|
| <b>N-Acetylvaline</b>                 | 159.089 | 06.69 | 158.08207 | C <sub>7</sub> H <sub>13</sub> NO <sub>3</sub>                | 2 |
| <b>N-Acetyl-5-aminosalicylic acid</b> | 195.053 | 06.74 | 194.04574 | C <sub>9</sub> H <sub>9</sub> NO <sub>4</sub>                 | 2 |
| <b>3-Isopropylmalic acid</b>          | 176.068 | 07.23 | 175.06095 | C <sub>7</sub> H <sub>12</sub> O <sub>5</sub>                 | 2 |
| <b>2-Isopropylmalic acid</b>          | 176.068 | 08.06 | 175.06103 | C <sub>7</sub> H <sub>12</sub> O <sub>5</sub>                 | 2 |
| <b>Phenylacetyl glycine</b>           | 193.074 | 08.41 | 192.06639 | C <sub>10</sub> H <sub>11</sub> NO <sub>3</sub>               | 2 |
| <b>4-Hydroxybenzoic acid</b>          | 138.032 | 08.58 | 137.02425 | C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>                  | 2 |
| <b>Acetanilide</b>                    | 135.068 | 08.65 | 134.06093 | C <sub>8</sub> H <sub>9</sub> NO                              | 2 |
| <b>6,7-Dihydroxycoumarin</b>          | 178.026 | 08.65 | 177.01914 | C <sub>9</sub> H <sub>6</sub> O <sub>4</sub>                  | 2 |
| <b>p-Acetaminobenzoic acid</b>        | 179.058 | 08.67 | 178.05073 | C <sub>9</sub> H <sub>9</sub> NO <sub>3</sub>                 | 2 |
| <b>Vanillin</b>                       | 152.047 | 09.01 | 151.0399  | C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>                  | 3 |
| <b>5-Hydroxyindole-3-acetic acid</b>  | 191.058 | 09.10 | 190.05082 | C <sub>10</sub> H <sub>9</sub> NO <sub>3</sub>                | 2 |
| <b>N-Acetylserotonin</b>              | 218.107 | 09.23 | 217.10014 | C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub> | 2 |
| <b>3-Methylhippuric acid</b>          | 193.074 | 09.71 | 192.06636 | C <sub>10</sub> H <sub>11</sub> NO <sub>3</sub>               | 2 |
| <b>(E)-p-Coumaric acid</b>            | 164.047 | 09.71 | 163.03989 | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>                  | 1 |
| <b>Phenyllactic acid</b>              | 166.063 | 09.73 | 165.05548 | C <sub>9</sub> H <sub>10</sub> O <sub>3</sub>                 | 2 |
| <b>N-Acetylphenylalanine</b>          | 207.089 | 09.92 | 206.08212 | C <sub>11</sub> H <sub>13</sub> NO <sub>3</sub>               | 2 |
| <b>Hydroxysuberic acid</b>            | 190.084 | 09.98 | 189.07667 | C <sub>8</sub> H <sub>14</sub> O <sub>5</sub>                 | 2 |
| <b>N-Acetyltryptophan</b>             | 246.103 | 10.05 | 245.09576 | C <sub>13</sub> H <sub>14</sub> N <sub>2</sub> O <sub>3</sub> | 2 |
| <b>Indole-3-carboxylic acid</b>       | 161.047 | 10.3  | 160.04019 | C <sub>9</sub> H <sub>7</sub> NO <sub>2</sub>                 | 2 |
| <b>5-Nitroguaiacol</b>                | 169.038 | 10.61 | 168.03041 | C <sub>7</sub> H <sub>7</sub> NO <sub>4</sub>                 | 2 |
| <b>Hydroxysebacic acid</b>            | 218.115 | 10.98 | 217.10792 | C <sub>10</sub> H <sub>18</sub> O <sub>5</sub>                | 2 |
| <b>3,4-dehydro-6-hydroxymellein</b>   | 192.042 | 12.04 | 191.03476 | C <sub>10</sub> H <sub>8</sub> O <sub>4</sub>                 | 3 |
| <b>Azelaic acid</b>                   | 188.105 | 13.47 | 187.09732 | C <sub>9</sub> H <sub>16</sub> O <sub>4</sub>                 | 1 |

Table 2 List of identified compounds and their relative contribution to each of the considered principal components.

| <b>Compound</b>                   | <b>PC1</b> | <b>PC2</b> | <b>PC3</b> |
|-----------------------------------|------------|------------|------------|
| <b>Pyrogallol</b>                 | 0.00556    | -0.0186    | -0.0221    |
| <b>p-Toluenesulfonic acid</b>     | 0.0114     | 0.0505     | 0.0143     |
| <b>4-Hydroxyphenyllactic acid</b> | 0.00998    | -0.0469    | 0.0361     |

|                                       |          |          |          |
|---------------------------------------|----------|----------|----------|
| <b>N-Acetyl-5-aminosalicylic acid</b> | 0.00482  | -0.0414  | 0.00309  |
| <b>4-Hydroxybenzoic acid</b>          | -0.00814 | 0.00735  | -0.0098  |
| <b>6,7-Dihydroxycoumarin</b>          | -0.011   | 0.00853  | 0.00323  |
| <b>Acetanilide</b>                    | 0.0167   | -0.0283  | -0.0161  |
| <b>p-Acetaminobenzoic acid</b>        | 0.0168   | -0.0312  | -0.0261  |
| <b>Vanillin</b>                       | -0.00579 | -0.00737 | -0.017   |
| <b>N-Acetylserotonin</b>              | 0.00099  | -0.0328  | -0.0632  |
| <b>3-Methylhippuric acid</b>          | -0.0105  | -0.0346  | -0.0107  |
| <b>(E)-p-Coumaric acid</b>            | -0.0122  | -0.0137  | -0.00383 |
| <b>Phenyllactic acid</b>              | 0.000392 | -0.0213  | -0.0398  |
| <b>N-Acetyltryptophan</b>             | -0.00661 | -0.0473  | -0.00531 |
| <b>5-Nitroguaiacol</b>                | -0.00943 | -0.0117  | -0.00659 |
| <b>3,4-dehydro-6-hydroxymellein</b>   | 0.0109   | -0.0333  | -0.00357 |

Table 3 Statistical analysis of selected LMW-identified compounds determined by UHPLC-MS/MS. Statistical differences were assessed using the Kruskal-Wallis test, followed by Dunn's test for non-normally distributed data, and ANOVA, followed by Tukey's post-hoc test for normally distributed data.

| <b>Compound</b>         | <b>P<br/>valu<br/>e</b> | <b><i>F.<br/>mediterranea</i></b> | <b><i>F.<br/>capensis</i></b> | <b><i>F.<br/>langloisii</i></b> | <b><i>F.<br/>polymorpha</i></b> | <b><i>F.<br/>australiensis</i></b> | <b><i>T.<br/>texanus</i></b> | <b><i>L.<br/>vitiensis</i></b> | <b><i>S.<br/>hirsutum</i></b> |
|-------------------------|-------------------------|-----------------------------------|-------------------------------|---------------------------------|---------------------------------|------------------------------------|------------------------------|--------------------------------|-------------------------------|
| Vanillin                | < 0.001                 | B                                 | AB                            | A                               | A                               | AB                                 | AB                           | AB                             | AB                            |
| Phenyllactic acid       | < 0.001                 | AB                                | B                             | B                               | A                               | AB                                 | AB                           | AB                             | A                             |
| p-Toluenesulfonic acid  | < 0.01                  | B                                 | AB                            | AB                              | C                               | AB                                 | A                            | AB                             | AB                            |
| p-Acetaminobenzoic acid | < 0.001                 | AB                                | AB                            | C                               | A                               | AB                                 | AB                           | BC                             | AB                            |
| N-Acetyltryptophan      | < 0.001                 | /                                 | /                             | /                               | /                               | A                                  | /                            | /                              | B                             |

### Chapter III

|                                |         |    |     |    |    |    |    |    |     |
|--------------------------------|---------|----|-----|----|----|----|----|----|-----|
| N-Acetylserotonin              | < 0.001 | /  | /   | /  | A  | /  | /  | /  | B   |
| N-Acetyl-5-aminosalicylic acid | < 0.01  | AB | AB  | B  | A  | AB | AB | B  | B   |
| Acetanilide                    | < 0.001 | B  | AB  | B  | A  | AB | AB | /  | AB  |
| 6,7-Dihydroxycoumarin          | < 0.001 | C  | ABC | A  | BC | BC | BC | AB | ABC |
| 4-Hydroxybenzoic acid          | < 0.01  | AB | AB  | AB | B  | A  | AB | B  | AB  |
| 3-Methylhippuric acid          | 0.01    | B  | AB  | AB | A  | AB | B  | AB | AB  |
| 3,4-dehydro-6-hydroxymellein   | < 0.001 | B  | AB  | AB | A  | B  | A  | AB | AB  |
| 5-Nitroguaiacol                | < 0.05  | ns | ns  | ns | ns | ns | ns | ns | ns  |
| Hydroxyphenyllactic acid       | < 0.001 | B  | AB  | AB | A  | AB | AB | /  | /   |
| Pyrogallol                     | 0.01    | AB | B   | B  | A  | B  | AB | AB | AB  |
| "(E)-p-Coumaric acid           | < 0.001 | B  | AB  | AB | AB | A  | B  | AB | AB  |

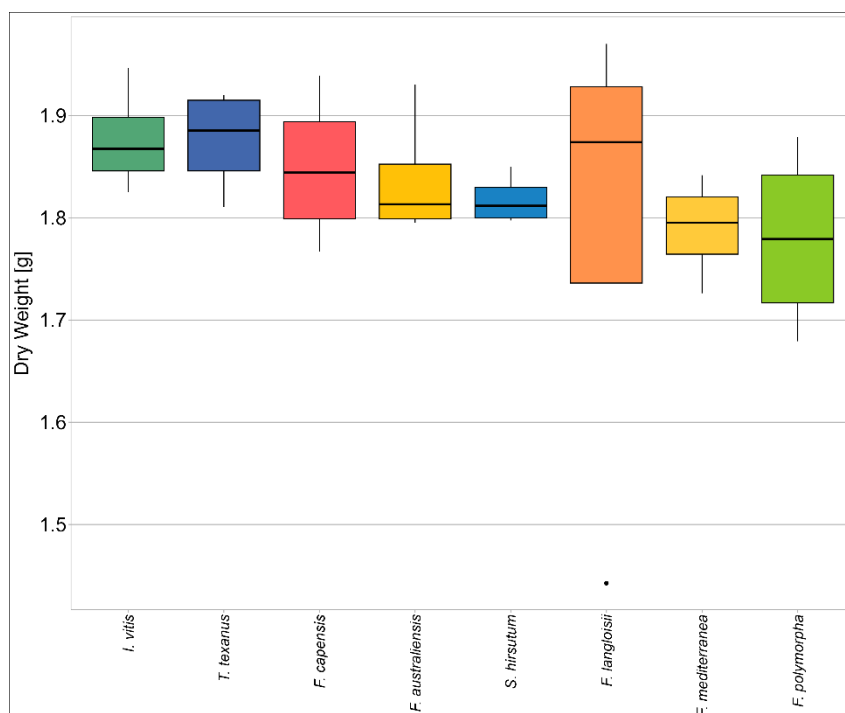


Figure 9 Dry weight of filtered cultures of the 8 ECD WR species (*F. mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, *F. australiensis*, *T. texanus*, *I. vitis*, *S. hirsutum*) for non-enzymatic assays, after 6W of growth at 27°C in the dark. Nutrient media consisted of Higley media with the addition of cellulose. The weighted fraction included everything retained by 0.2 µm filtration.

# **Chapter IV - Multispecies Analysis of Phenotypic Characteristics of White Rot Agents in the Esca Complex of Diseases**

The following preliminary analyses (chemical analyses of wood) were performed during the COST Action CA20132, Urban Tree Guard—Safeguarding European urban trees and forests through improved biosecurity (UB3Guard), which was carried out from 07/09/2022 to 28/09/2022 in Sweden at Linnaeus University, Department of Forestry and Wood Technology, Lückligs plats 1, 351 95 Växjö, Sweden.

**The data presented in the following chapter is intended for inclusion in a research article, which will be prepared and submitted upon completion of further analyses, including ongoing histological observations.**

## **Multispecies Analysis of Phenotypic Characteristics of White Rot Agents in the Esca Complex of Grapevine**

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# Abstract

In light of recent advancements in understanding the degradation capabilities of white rot agents in the context of Esca Complex of Diseases (ECD) and their reconsidered importance in the syndrome, this preliminary study investigates the *in vitro* degradation capabilities and temperature preferences of key white-rot agents linked to ECD. Temperature-based growth measurements revealed that most *Fomitiporia* species achieve optimal growth between 25°C and 30°C, with *F. australiensis* exhibiting the highest growth rate overall. Wood decay analyses demonstrated lower mass loss in Syrah wood than Merlot and Cabernet Sauvignon across strains of *F. mediterranea*, *F. polymorpha*, *S. hirsutum*, and *F. australiensis*. Chemical composition analyses of grapevine wood suggest that Syrah's higher resistance to fungal degradation is likely linked to its higher lignin content and lower holocellulose content. Results from this preliminary study provide insights into the growth dynamics and wood degradation potential of ECD-associated fungi.

**Keywords:** Fmed, grapevine trunk diseases, white rot, *Hymenochaetales*, *Vitis vinifera*, *Fomitiporia*

## 1. Introduction

Esca Complex of Diseases (ECD) is one of the most prevalent and destructive threats to worldwide vineyards (Mondello et al., 2018b; Guerin-Dubrana et al., 2019; Kenfaoui et al., 2022). The ECD-affected vines are characterized by an array of different symptoms that lead to a reduction in the quality and quantity of the yield and significant economic losses (Baumgartner et al., 2019). Symptoms of ECD include black streaking in the wood, foliar symptoms of Grapevine Leaf Stripe Disease (GLSD), and the development of decayed wood on trunks and cordons (Mugnai et al., 1999; Graniti et al., 2000; Calzarano et al., 2021). Although there seems to exist a degree of susceptibility to ECD, no resistant varieties are known as of today exists (Bertsch et al., 2013).

A wide number of white rot agents, mainly within the *Hymenochaetaceae* family, are linked to ECD (Brown et al., 2020; Cloete; Fischer, 2006; Gramaje et al., 2018). Among them,

*Fomitiporia mediterranea* was recognized in 2002 as the main decay agent in European vineyards (Fischer, 2002). In addition, *Stereum hirsutum* is another white rot fungus present in Europe that has been associated with ECD, although its role in its etiology remains debated (Muntean et al., 2022). Other *Fomitiporia* species have been linked to grapevine white rot in vines showing GLSD symptoms. *F. australiensis* has been linked to ECD symptoms in Australia and *F. capensis* in South Africa (Fischer et al., 2005; Cloete et al., 2014). In North America, species such as *Tropicoporus texanus*, *F. langloisii*, *F. polymorpha*, and *Inonotus vitis* have been identified in symptomatic vines (Brown et al., 2020).

Recent research has highlighted differences in the enzymatic and non-enzymatic degradation capabilities of ECD-associated basidiomycetes (Moretti et al., 2023; Pacetti et al., 2022; The work in this thesis). Building on these findings, this study aims to investigate the *in vitro* degradation capabilities of these fungi using wood of different grapevine cultivars associated with different susceptibility to ECD. Additionally, to deepen our understanding of their biology, we examined their growth across a range of temperatures. These findings will provide valuable insights into the ecological roles and pathogenic potential of ECD-associated basidiomycetes, particularly in the context of the growing body of research on their involvement in ECD and GLSD development (Bruez et al., 2021; Del Frari et al., 2021; Pacetti et al., 2021).

## 2. Materials and Methods

### 2.1. Fungal strains

*Fomitiporia mediterranea* strain PHCO36 was isolated in 1996 from *Vitis vinifera* Ugni blanc in the Charente wine region. This strain was provided by the Laboratoire Vigne, Biotechnologie et Environnement at the Université de Haute-Alsace. Additionally, strain 7082 of *F. capensis* was sourced from the collection at Stellenbosch University in South Africa. This strain was isolated from *Vitis vinifera* Sauvignon Blanc in the vicinity of Stellenbosch. The strains *F. langloisii* TX2 (GenBank: MN108107.1), *F. polymorpha* WFB1 (GenBank: MN108110.1), *Tropicoporus texanus* TX9 (GenBank: MN108124.1, ITS), and *Inonotus vitis* strain OC1 (GenBank: MN108118.1, ITS), are from the University of California collection. North American strains were isolated in 2019 from *Vitis vinifera* Cabernet Sauvignon in California. The strain MUCL 049406 of *F. australiensis* (GenBank: AY624997.1, ITS) was acquired from the Belgian Co-ordinated Collections of Micro-Organisms BCCM-MUCL. Cultures were grown on 3% Malt Extract Agar medium (MEA, Oxoid®, Hampshire, UK) at 25°C in the dark before use. *Stereum hirsutum* strain DP49 (GenBank: AM269810, ITS) was provided by the collection of the University of Turin, Italy.

### 2.2. Wood decay test

Wooden cubes measuring 1 cm x 1 cm x 0.5 cm were cut from the most external part of the wood of the trunks of 2 10-year-old grapevines cv. Merlot, 1 <10-year-old grapevine cv. Cabernet Sauvignon and one 9-year-old grapevine cv. Syrah. Cubes showing symptoms of decay, black stripes, or fungal contamination were excluded. The grapevine varieties were chosen as they represent a gradient of susceptibility to GLSD (Merlot and Syrah, tolerant to generally tolerant; Cabernet Sauvignon, susceptible) (Borgo et al., 2016; Cardot et al., 2019; Chacón-Vozmediano et al., 2021; Christen et al., 2007; Csótó et al., 2020; Lambert et al., 2013; Murolo & Romanazzi, 2014; Travadon et al., 2013). Wooden cubes were oven-dried at 80°C for 72h and weighted to get the initial mass ( $W_0$ ). Thereafter, samples were placed in flasks and fully submerged in distilled water before sterilization by autoclaving twice with water at 121°C for 45 minutes and one time without water. Water was replenished if needed. (Rees et al., 2021). Sterilized cubes were then placed in the middle of 90-mm Petri dishes with the addition of 1.5% water agar to fix them in place. Inoculations were carried out by placing a 5-mm mycelium plug

at a distance of 1,5 cm from the wooden block. Plates were then incubated in the dark at 25°C for 30 days. Three replicates were performed for each combination of strain/grapevine wood variety. After incubation, wooden blocks were removed from the dishes, and any remaining mycelium and substrate were removed using a scalpel. Cubes were then oven-dried until a stable weight was achieved to obtain the final weight ( $W_F$ ). Wood weight loss was obtained with the following formula:

$$\text{Weight loss \%} = \frac{(W_0 - W_F)}{W_0}$$

### 2.3. Holocellulose content

Uninoculated wood cubes were ground using a hammer mill (Polymix PX-MFC 90 D) set at 6000 rpm with a 250-425  $\mu\text{m}$  sieve size. Thorough cleaning of the mill was performed between samples to prevent cross-contamination. Later, the powdered samples were oven-dried at  $103 \pm 1^\circ\text{C}$  for 48 hours. 2g of dried samples were added to a 125 ml Erlenmeyer and submerged with 80 ml of water. The flasks were incubated in a warm water bath set at  $70^\circ\text{C}$  with gentle agitation. To initiate the delignification process, 1 g of sodium chlorite and 500  $\mu\text{l}$  of glacial acetic acid were added every hour, starting at time zero, for 8 additions (8 hours). The flasks were then left shaking for a total of 24 hours. Afterward, samples were removed from the water bath and cooled down to room temperature before pouring acetone to stop the reaction. Holocellulose was then filtered using oven-dried paper filters and a vacuum pump. The holocellulose content was calculated by subtracting the dry weight of the extract from the total dry weight of the sample. (Rowell and Rowell, 2005). Analyses were conducted in triplicates.

### 2.4. Fourier-Transform Infrared (ATR-FTIR) Analysis

Chemical characterization of non-inoculated wood was performed using Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy (Perkin Elmer Frontier, Waltham, MA, USA) tuned with a Bruker Invenio R spectrometer fitted with a diamond ATR unit. Uninoculated wood cubes were ground using a hammer mill (Polymix PX-MFC 90 D) set at 6000 rpm with a 250-425  $\mu\text{m}$  sieve size. Thorough cleaning of the mill was performed between samples to prevent cross-contamination. Later, the powdered samples were

oven-dried at  $103 \pm 1^\circ\text{C}$  for 48 hours. Dried powder was spread over the entire lens area, and a similar pressure was applied to each measurement. Spectra were recorded at room temperature in the range of 4000 to  $550\text{ cm}^{-1}$ , averaging 32 scans with a resolution of  $4\text{ cm}^{-1}$  and 9 technical replicates were performed. After correction, spectra were normalized on the most intense band of the C-O, C-C, and C-C-O stretch vibration of lignin, cellulose, as well as hemicellulose at  $1026\text{ cm}^{-1}$  (Xu et al., 2013). The lignin index (LI) was calculated by dividing lignin intensity at  $1505\text{ cm}^{-1}$  and CH/CH<sub>3</sub> groups at  $1369\text{ cm}^{-1}$  (Cavallaro et al., 2018). The total crystallinity index (TCI) was determined from the ratio of band-heights  $1369/2900\text{ cm}^{-1}$  (Åkerholm et al., 2004). The lateral order index (LOI) was calculated to compare the cellulose crystallinity of the varieties by using the intensity at indication points  $1422\text{ cm}^{-1}$  and  $895\text{ cm}^{-1}$  (Colom et al., 2003). Results of chemical characterization are listed as supplementary materials.

### 2.5. Growth Experiment

Strains were first precultured on 3% malt extract agar (MEA) plates in the dark at RT ( $\sim 23^\circ\text{C}$ ) for up to two weeks before use. Inoculations were carried out by placing a 5-mm diameter mycelium disc taken from the edge of each preculture colony in a fresh 90 mm petri dish containing 20 ml of MEA. The inoculated plates were then randomly placed in thermostats at seven different temperatures, i.e., 15, 20, 25, 30, 35, and  $40^\circ\text{C}$ , in the dark. 4 replicates were produced for each species at each temperature. The mycelium growth area was quantified by taking pictures under identical conditions every 3-4 days over 4 weeks or until the mycelium reached the edge of the plate. ImageJ software (v1.53k, National Institutes of Health, USA) was used to measure the area. Standardized daily growth rate (SDG,  $\text{cm}^2/\text{day}$ ) was then calculated using the following formula:

$$\text{Standardized daily growth rate (SDG)} = \frac{\text{Total mycelium area} - \text{Initial plug area}}{\text{Day } n}$$

Biomass production was assessed under the same temperature and culture conditions, with the addition of a sterilized cellophane disc placed on top of the MEA substrate before inoculation. Plates were incubated in the dark for 14 to 16 days. Afterward, mycelial biomass was collected by scraping the surface using a sterile scalpel. Harvested mycelia were oven-dried at  $80^\circ\text{C}$  for 48-72 hours until two consecutive weight measurements gave a consistent

result. Standardized daily biomass accumulation (mg/day) was calculated by dividing the total dry biomass (mg) by the number of incubation days.

### 2.6. Statistical analysis

Statistical analysis was performed using R version 4.4.1. The normality of data was checked using the Shapiro-Wilk test and visual inspection (histograms, Q-Q plots, and P-P plots). Levene's test was used to evaluate homoscedasticity when data followed a normal distribution. One-way ANOVA was conducted on normally distributed data, followed by Tukey's Honestly Significant Difference test for post-hoc comparisons. The Kruskal-Wallis test was applied for non-normally distributed data, followed by Dunn's multiple comparisons test, both with Bonferroni correction. Principal Component Analysis (PCA) was performed on ATR-FTIR spectral intensity data using the `prcomp` function in R. All graphical representations were generated with the `ggplot2` package.

### 3. Results

#### 3.1. Wood decay test

Wood mass loss of cubes caused by fungal degradation of the basidiomycetes collection ranged from 4% to 14% of the total mass of the wooden cubes after one month of incubation at 25°C (Figure 1). Across *F. mediterranea*, *F. polymorpha*, *S. hirsutum*, and *F. australiensis*, mass loss for Syrah was lower compared to Cabernet Sauvignon and Merlot. In the case of *S. hirsutum*, the mass loss of Syrah was less than half the other 2 varieties, the lowest value among all instances. For *F. capensis* and *I. vitis*, degradation of Syrah was lower than Merlot but not statistically different. No statistically significant differences were observed between Cabernet Sauvignon and Merlot degradation.

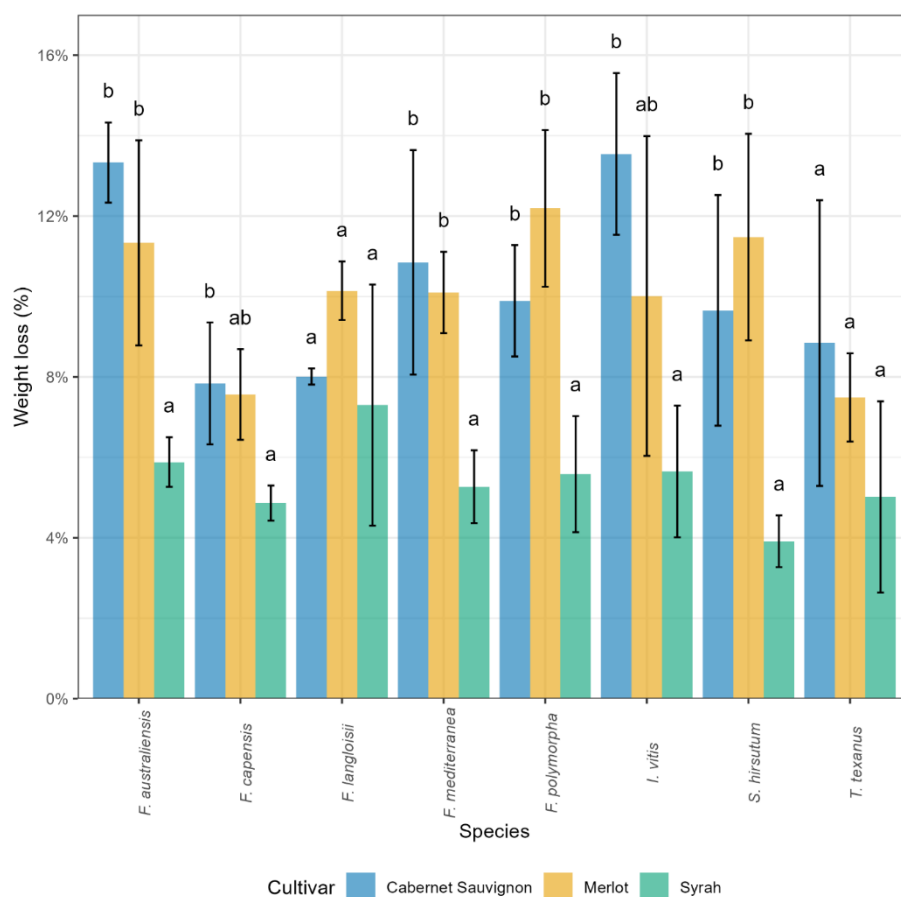


Figure 1 Syrah, Cabernet Sauvignon, and Merlot wood weight loss after 30 days of fungal degradation by the basidiomycetes collection. Weight loss is represented as the means with the standard deviation of replicates. Statistical significance was investigated using one-way ANOVA followed by Tukey's Honestly Significant Difference test (p. value <0.05).

### 3.2. Chemical analysis of wood

Holocellulose content, as well as LI, TCI, and LOI of grapevine wood varieties, is indicated in Table 4. Merlot had the highest holocellulose content (53.19%), followed by Cabernet Sauvignon (41.43%) and Syrah (38.30%).

| Varieties          | Holocellulose %                    | Lignin Index    | Total Crystallinity Index | Lateral Order Index |
|--------------------|------------------------------------|-----------------|---------------------------|---------------------|
| Cabernet Sauvignon | 41.43 ( $\pm 8.32$ ) <sup>ab</sup> | 0.68 $\pm$ 0.11 | 0.85 $\pm$ 0.11           | 1.31 $\pm$ 0.3      |
| Merlot             | 53.19( $\pm 4.01$ ) <sup>b</sup>   | 0.67 $\pm$ 0.1  | 1.09 $\pm$ 0.32           | 1.16 $\pm$ 0.39     |
| Syrah              | 38.30( $\pm 2.88$ ) <sup>a</sup>   | 0.83 $\pm$ 0.19 | 1.77 $\pm$ 0.84           | 0.85 $\pm$ 0.24     |

Table 4 Holocellulose content, Lignin index, and Lateral Order Index of grapevine varieties Cabernet Sauvignon, Merlot, and Syrah. Holocellulose content is indicated as the mean of three replicates. Values in brackets indicate the standard deviation. Small letters show the statistical differences (ANOVA, Tukey HSD test, p. value <0.05). The Lignin index is calculated from the results of FTIR spectroscopy as the ratio between the intensity at 1505 and 1369  $cm^{-1}$ . Lateral Order Index is calculated by dividing the intensity at 1422 and 895  $cm^{-1}$ . Total Crystallinity Index is calculated by dividing the intensity at 1369 and 2900  $cm^{-1}$ . Reported values are the means of replicates with indication of standard deviation.

FTIR spectroscopy was used to analyze the chemical composition of the grapevine varieties analyzed. Indications about peak position and correspondence with the group and vibration mode are listed in Table 5.

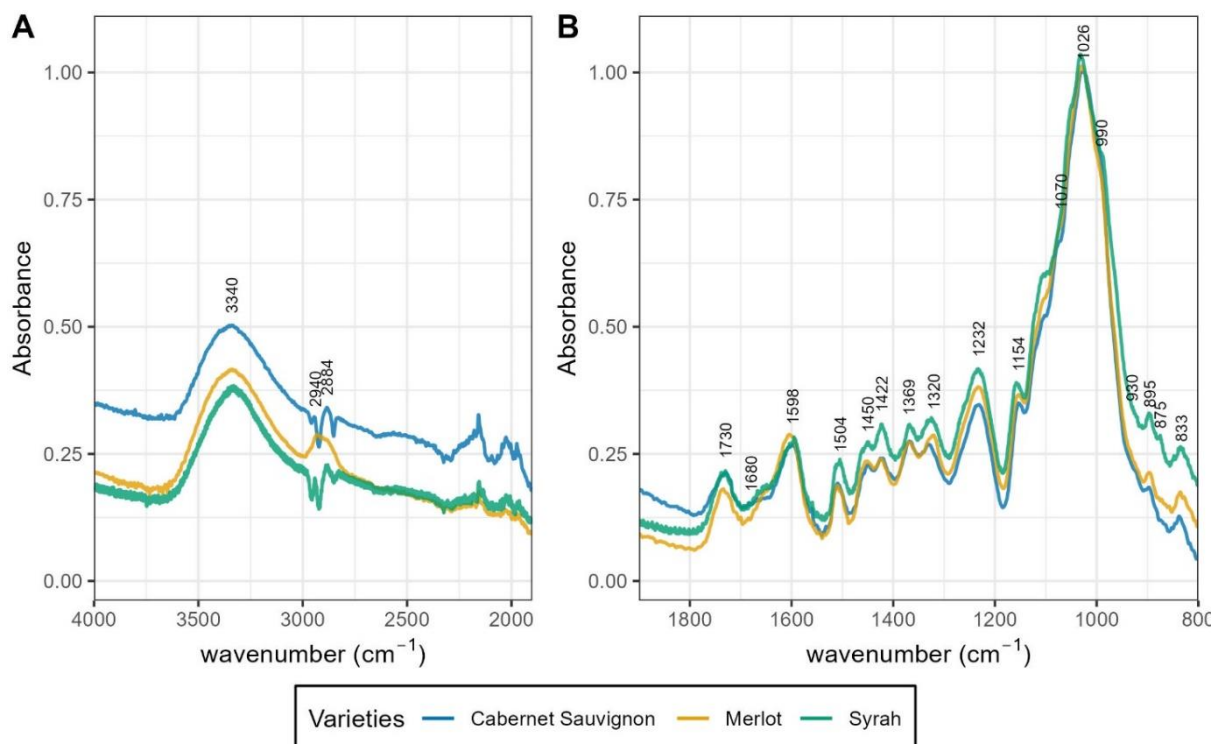


Figure 2 Fourier-transform infrared spectroscopy (FTIR) spectra of grapevine wood of the varieties Cabernet Sauvignon, Merlot, and Syrah with a distinction between functional group (A) and fingerprinting (B) regions.

In the functional group region (Figure 2), the peak at 3340 cm<sup>-1</sup> was assigned to the -OH stretching vibrations, and no major differences were observed between the three cultivars. The peaks at 2940 and 2884 cm<sup>-1</sup> are associated with symmetric and asymmetric stretches of -CH<sub>2</sub>. Unlike Cabernet Sauvignon and Syrah, Merlot did show a wider absorption encompassing both peaks. In general, similar absorption peaks and vibrations were detected in the samples, indicating their almost identical chemical characteristics. Table 2 presents a detailed explanation of FTIR absorption bands.

Table 5 Infrared bands and related molecular bonds. The major wood cell wall component linked is indicated as a related structure (Lignin = Li, Hemicellulose = HC, Cellulose = C). When specified, indications about the associated lignin unit are indicated in brackets.

| Wavenumber (cm <sup>-1</sup> ) | Correspondence                                          | Related structure | Reference                       |
|--------------------------------|---------------------------------------------------------|-------------------|---------------------------------|
| 833                            | p-hydroxyphenyl (H) units lignin                        | Li (H)            | (Pereira et al., 2023)          |
| 875                            | Glycosidic linkage                                      | HC                | (Xu et al., 2013)               |
| 895                            | C-O-C aromatic ethers, symmetric stretch                | Li, C             | (Levasseur-Garcia et al., 2016) |
| 930                            | Glycosidic linkage                                      | C, HC             | (Xu et al., 2013)               |
| 990                            | C-O stretching in cellulose                             | C                 | (Traoré et al., 2016)           |
| 1026                           | C-O-C stretch                                           | Li, C, HC         | (Ghavidel et al., 2021)         |
| 1051                           | C-O-C aromatic ethers symmetric stretch (pyranose ring) | C, HC             | (Santos et al., 2022)           |
| 1070                           | C-O primary alcohol stretching                          | C                 | (Backa et al., 2001)            |

|             |                                                                        |               |                                                                                                                                               |
|-------------|------------------------------------------------------------------------|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| <b>1088</b> | C-O stretch vibration of ester groups                                  | Li            | (Pandey, 1999; Morales et al., 2022)                                                                                                          |
| <b>1154</b> | C-O-C vibration in the structure of cellulose and hemicellulose        | HC, C         | (Levasseur-Garcia et al., 2016; Sunardi et al., 2018; Santos et al., 2022)                                                                    |
| <b>1232</b> | Syringyl ring and C=C stretch in lignin and xylan                      | Li (S)        | (Traoré et al., 2016; Santos et al., 2022)                                                                                                    |
| <b>1320</b> | C-O vibration in syringyl and guaiacyl rings, C-H cellulose            | Li (S), C, HC | (Traoré et al., 2016; Pereira et al., 2023)                                                                                                   |
| <b>1369</b> | CH deformation of cellulose and hemicellulose.                         | C, HC         | (Levasseur-Garcia et al., 2016; Traoré et al., 2016; Sunardi et al., 2018; Santos et al., 2022)                                               |
| <b>1422</b> | CH- deformation; asymmetric in -CH <sub>3</sub> and -CH <sub>2</sub> - | C             | (Traoré et al., 2016; Santos et al., 2022; Čabalová et al., 2023; Pereira et al., 2023)                                                       |
| <b>1450</b> | C=C and C-H bond O-H in-plane deformation                              | Li, HC        | (Levasseur-Garcia et al., 2016; Santos et al., 2022)                                                                                          |
| <b>1504</b> | Aromatic skeletal vibration of guaiacyl rings                          | Li (G)        | (Čabalová et al., 2023; Ghavidel et al., 2021; Levasseur-Garcia et al., 2016; Pereira et al., 2023; Santos et al., 2022; Traoré et al., 2016) |

|             |                                                                                 |           |                                                                                                                     |
|-------------|---------------------------------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------------------------------|
| <b>1598</b> | Aromatic skeletal vibration of S-unit lignin and C=O stretch                    | Li (S)    | (Levasseur-Garcia et al., 2016; Pereira et al., 2023; Santos et al., 2022; Traoré et al., 2016)                     |
| <b>1680</b> | C=O vibration in the carboxylic group of phenolic acids (p-hydroxybenzoic acid) | Li        | (Santos et al., 2022)                                                                                               |
| <b>1730</b> | C=O stretch in unconjugated ketones, carbonyls and in ester groups, Xylan       | Li, HC    | (Chen et al., 2010; Ghavidel et al., 2020; Levasseur-Garcia et al., 2016; Santos et al., 2022; Traoré et al., 2016) |
| <b>2884</b> | -CH <sub>2</sub> - symmetric stretch                                            | C, HC     | (Cogulet et al., 2016)                                                                                              |
| <b>2940</b> | -CH <sub>2</sub> - asymmetric stretch                                           | Li, C, HC | (Ghavidel et al., 2021)                                                                                             |
| <b>3340</b> | -OH stretch                                                                     | Li, C, HC | (Ghavidel et al., 2021; Santos et al., 2022)                                                                        |

PCA analysis was conducted on the fingerprint region of grapevine varieties. The first two components accounted for 75.31% of the total variance of the dataset (PC1: 53.06%, PC2: 22.25%). With the addition of the third component, the percentage of total variance explained amounts to 88.52%. Using the first two components, a clear grouping emerges, separating Merlot from Syrah and Cabernet Sauvignon (Figure 3 A). However, samples in Syrah did not show grouping tendencies.

For PC1, the highest positive scores were observed, from highest to lowest value, at 988, 1025, 1067, 1088, 1158, 1136, 1383, and 870 cm<sup>-1</sup>. This means that PC1 mainly differentiates samples based on variations of polysaccharides such as cellulose and

hemicellulose (Figure 3 B). Although that is the case, the presence of lignin-specific signals at  $1088\text{ cm}^{-1}$  indicates variations in lignin composition or the presence of ester groups. In PC2, the most contributing loadings are 1015, 991, and 1087, followed by 1470, 1373, 1070, 1027, 1385, and  $941\text{ cm}^{-1}$ . These loadings indicate that PC2 is associated with all major components of wood cell walls, with a prevalence of cellulose and hemicellulose. In PC3, we can find 1036, 1018, 1594, 1063, 1323, 1667, 854, 1474, 950, and  $862\text{ cm}^{-1}$ . The presence of 1594, 1323, 1474, and 1667 highlights lignin variability, especially with syringyl and guaiacyl units. Further information from the spread plot in Figure 3 C shows how PC1 is strongly associated with cellulose crystallinity, while PC2 focuses on hemicellulose and amorphous cellulose. The scatterplot in Figure 3 D shows significant differences between Syrah and the other two varieties for PC1. For PC3, no statistical differences were observed between Merlot and Syrah, while Cabernet Sauvignon was statistically different. No statistical significance was found in PC2. Among all PCs, Syrah showed the most variation.

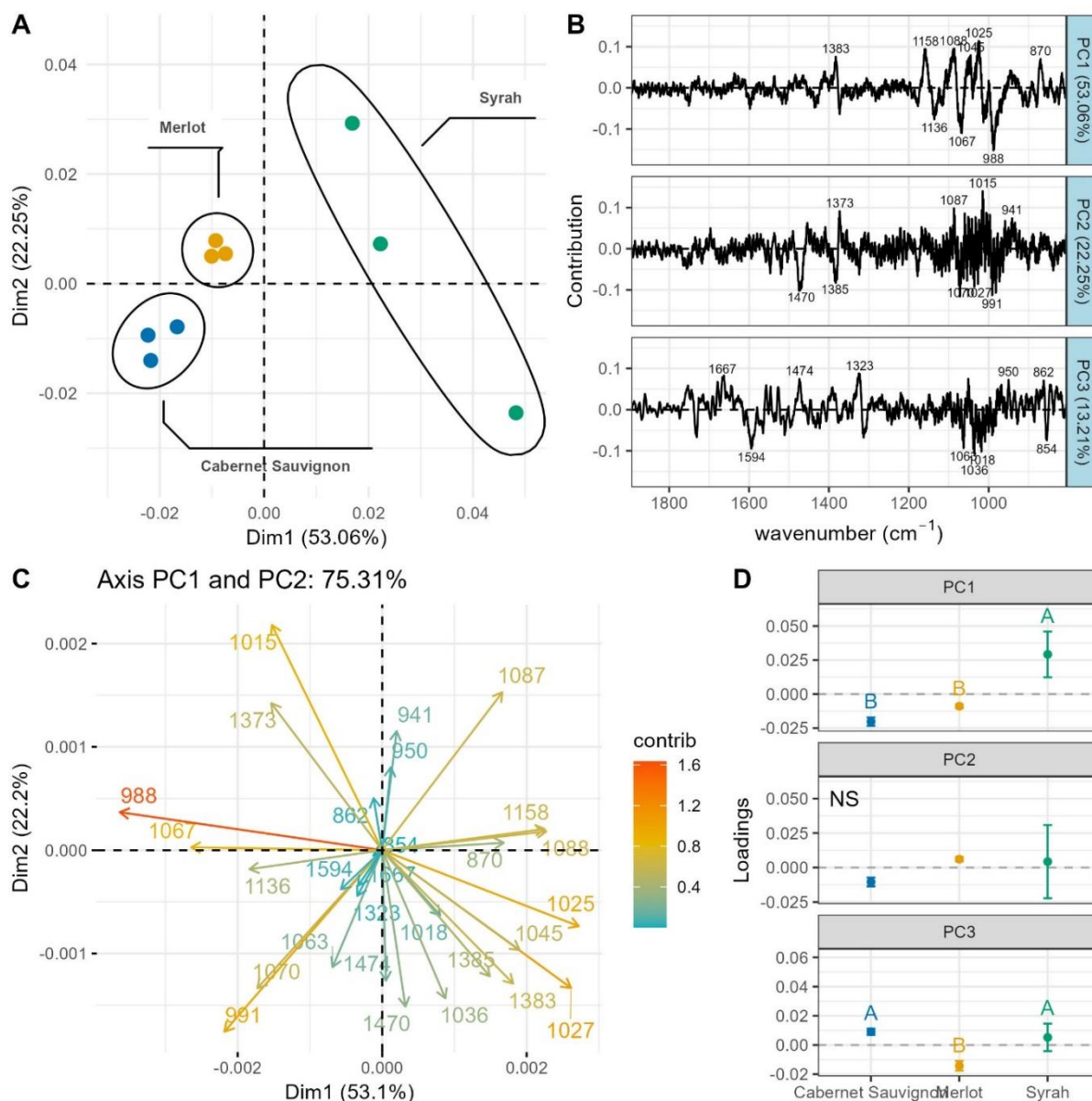


Figure 3 Principal component analysis (PCA) of the first derivative FTIR fingerprinting region (1900-800) spectra of wood of grapevine varieties Cabernet Sauvignon, Merlot, as well as Syrah. (A) scatter diagram of PC1 (53.06 %) and PC2 (22.25 %) showing sample grouping. (B) Loadings profile showing most contributing wave points. (C) Contribution of most important wave points to PC1 and PC2. (D) Loadings graph of PC1, PC2, and PC3. Points indicate the mean ( $\pm$ standard deviation) contribution of features in each variety in each Pcs. Significant differences among groups were investigated using one-way ANOVA followed by the Tukey Honestly Significant Difference test ( $p$ . value  $<0.05$ ) and are indicated as letters. No significant differences were found in PC2 loadings.

### 3.3. Growth Experiment

The mycelial growth rates of the fungal collection under various temperatures ranging from 15°C to 40°C were measured to determine the optimal growth conditions (Figure 1). Temperature significantly influenced mycelium growth for all species, displaying distinct optimal and suboptimal ranges. Overall, basidiomycetes not belonging to the *Fomitiporia* genus exhibited significantly higher SDG rates than species within *Fomitiporia*, except for *F. australiensis*, which demonstrated the highest growth rate noted among all fungi. All *F. mediterranea* replicates presented characteristics of the “bleaching type” mycelium. The comparison of growth among species is presented separately for each temperature in Figure 1 of the supplementary material.

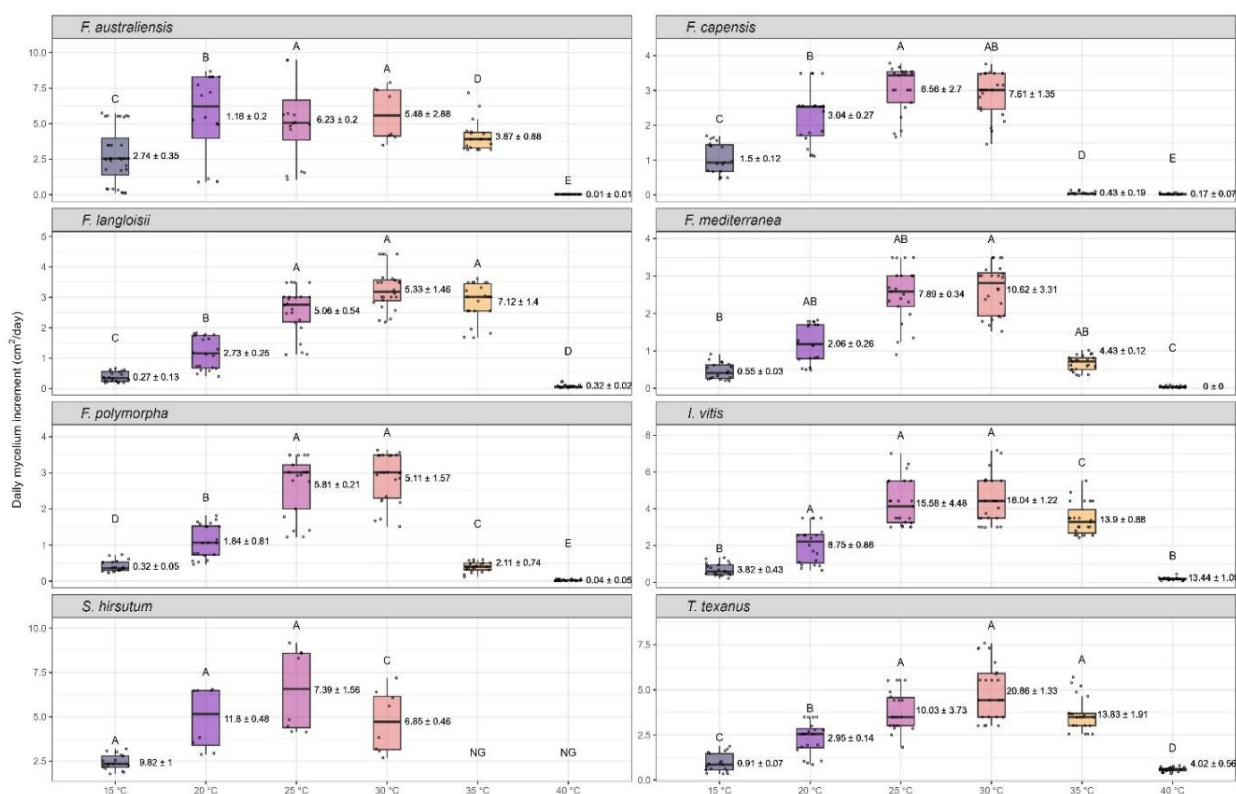


Figure 4 Standardized daily increment of the colony area of *Fomitiporia mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, *F. australiensis*, *S. hirsutum*, *T. texanus*, and *I. vitis*, grown at 15, 20, 25, 30, 35, 40°C. Mycelium increment is shown using boxplots. Statistical differences between daily mycelium increments (cm<sup>2</sup>/day) at different temperatures were assessed using the Kruskal-Wallis test, followed by Dunn's post hoc test ( $p < 0.05$ ). Biomass production is indicated in mg/day on the side of boxplots (mean of three replicates with standard deviation).

## 4. Discussion

For most of the fungal strains examined, except for *F. langloisii*, *T. texanus*, and *I. vitis*, Syrah wood was the most degraded. For the non-*Fomitiporia* basidiomycetes, this result might be linked to the higher SDG detected at 25°C. As for *F. langloisii*, recent research has highlighted the production and activity of carbohydrate-active enzymes (CAZymes) such as laccase and manganese peroxidase in its secretome, suggesting a higher degradation capacity (as previously stated in this thesis).

No notable differences were observed in mass loss among the different species across all tested cultivars. Considering the differences in growth at 25°C between different species, this result indicates that the incubation duration was probably too short to reveal species-specific variations.

For Syrah, a lower holocellulose content was found compared to Cabernet Sauvignon and Merlot. This result is supported by FTIR multivariate analysis, which shows that Syrah contributed more significantly to PC1 compared to Merlot and Cabernet Sauvignon. Notably, the peak at 988 cm<sup>-1</sup>, corresponding to cellulose, was negatively associated with PC1.

In addition, LI was the greatest in Syrah, but no significant differences were found between Syrah and Cabernet Sauvignon on the PC3 values, which is mainly connected with lignin compounds. Although that is the case, recorded absorbance at 1505cm<sup>-1</sup>, 1323 cm<sup>-1</sup> (the peak associated with the syringyl ring), and 833 cm<sup>-1</sup> (for p-hydroxyphenyl units) clearly showed a higher value in Syrah wood, revealing an abundance of the three groups H-S-G of lignin in this variety (Faix, 1991, 1992). As indicated by the high LOI, Cabernet Sauvignon had a higher cellulose crystallinity, while Syrah displayed a relative abundance of the amorphous fraction. However, mass loss in this variety was quite high, suggesting that this is not an important factor in resistance to white rot degradation in the setting of this experiment.

FT-IR is a rapid and nondestructive tool that allows for qualitative and semiquantitative analysis of samples (Xu et al., 2013). However, its precision in qualitative analyses is limited, and data interpretation is not always straightforward (Traoré et al., 2016). The use of complementary methods, such as chromatography analyses, could provide further insights into this study's findings.

The results developed in this preliminary work are insufficient to draw a direct link between resistance to the development of GLSD and wood decay resistance. Other factors, such

as environmental conditions, rootstock, and clones, can also influence the expression of the disease (Murolo and Romanazzi, 2014). In addition, wood composition can greatly change as it can be influenced by the environment (Downes and Drew, 2008).

In previous research, the resistance of various *Olea europea* cultivars to *F. mediterranea* wood degradation was associated with a higher soluble lignin content (Markakis et al., 2019) and higher lignin content in wood has been associated with higher resistance to wood decay (Schwarze, 2007). Thus, it would be reasonable to hypothesize that Syrah's higher resistance to fungal degradation compared to other varieties is linked to its high presence of lignin and lower holocellulose content, as suggested by our results. Further analysis of the wood composition of grapevine varieties can help shedding a light on the role of the chemical composition of the lignocellulosic matrix in wood degradation and susceptibility to GLSD.

Most *Fomitiporia* species showed optimum growth between 25°C and 30°C, consistent with the literature for *F. mediterranea* (Fischer, 2002; Moretti et al., 2021). *F. mediterranea* has been described as the main fungus associated with wood decay symptoms in ECD-affected grapevines in Europe, while *S. hirsutum* is considered a subordinate pathogen (Fischer and González García, 2015a). It is also reported that *S. hirsutum* is the most common ECD-associated fungal species in Romanian vineyards (Muntean et al., 2022). Considering our results, the reason this fungus is as widespread as it is in Romania could be related to the state's colder climate compared to other European wine-growing states, as it does not grow at temperatures higher than 30°C. The Koppen climate zone in south-eastern Europe is shifting to Cfa-Cfb climates instead of the typical colder climate (Mimić et al., 2024). Considering this change, the presence of *F. mediterranea* in Europe may become more important as temperatures rise.

Notably, *F. capensis* exhibited a higher optimum temperature than previous findings (Cloete et al., 2014), likely due to strain-specific adaptations or differences in experimental conditions, such as substrate composition.

*Fomitiporia australiensis* was the white rot agent with the higher variability at each temperature point. The fast growth rate of this fungus was probably related to the growth of its mycelium, which happened mainly inside the substrate and was much less dense than that of other species. This is also shown by the biomass production, which did not follow the same ratio as the SDG. The growth rate of this fungus, as well as the morphology observed in this study, were quite different from the reported information. *F. australiensis* was reported to be

indistinguishable from *F. mediterranea* or the other morphologically identical species *F. punctata* (Fischer et al., 2005). In the present work, the strain of *F. australiensis* grew mostly at a very high rate. Factors such as substrate properties, colony age, and storing conditions are well-known for influencing the epigenetics of basidiomycetes, leading to phenotypical differences (Slepecky and Starmer, 2009). Thus, future investigations involving additional strains of this species could help clarify its morphological and growth characteristics at the species level.

Among the North American species, *T. texanus* and *I. vitis* displayed faster growth rates than *Fomitiporia* species, except for *F. australiensis*. In the future, rising temperatures in North America could favor these fungi over *F. polymorpha*, leading to their replacement in the ecological niche.

*Fomitiporia mediterranea* climatic range has been proposed to vary from “arid and semi-arid” to “cool temperature” based on the Koppen-Geiger climate classification (Moretti et al., 2021). Based on the results of this work, the same definition could be proposed for *F. capensis*, *F. polymorpha*, *F. langloisii*, *F. australiensis*, as well as the other basidiomycetes *T. texanus*, and *I. vitis*. New analyses involving more strains for each species could confirm these results.

## 5. Conclusions

This preliminary study offers valuable insights into the phenotypic characteristics and wood degradation abilities of key white rot agents linked to wood decay associated with ECD. This includes several species of *Fomitiporia* (*F. mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, and *F. australiensis*), as well as other basidiomycetes (*T. texanus*, *S. hirsutum*, and *I. vitis*). The influence of strain specificity on phenotypic characteristics suggests that the results of this study would be enhanced by repeating the analyses with a larger number of strains. Additionally, host specificity has been shown to significantly affect the degradation capabilities of *F. mediterranea*, both enzymatically and non-enzymatically (Elena et al., 2006; Pacetti et al., 2022; Moretti et al., 2023). Therefore, this factor should be taken into account for this species as well as all others.

Chemical analyses of grapevine wood composition provide new insights into the differences among various wood varieties. Utilizing complementary methods could further clarify the compositions of different grapevine wood types.

The findings of this preliminary study already offer insights into the resistance to white rot wood decay, focusing on wood composition and the phenotypic characteristics of ECD-associated decay agents. Expanding on these findings could strengthen them and provide new insights into white rot wood degradation in ECD.

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## Supplementary Material

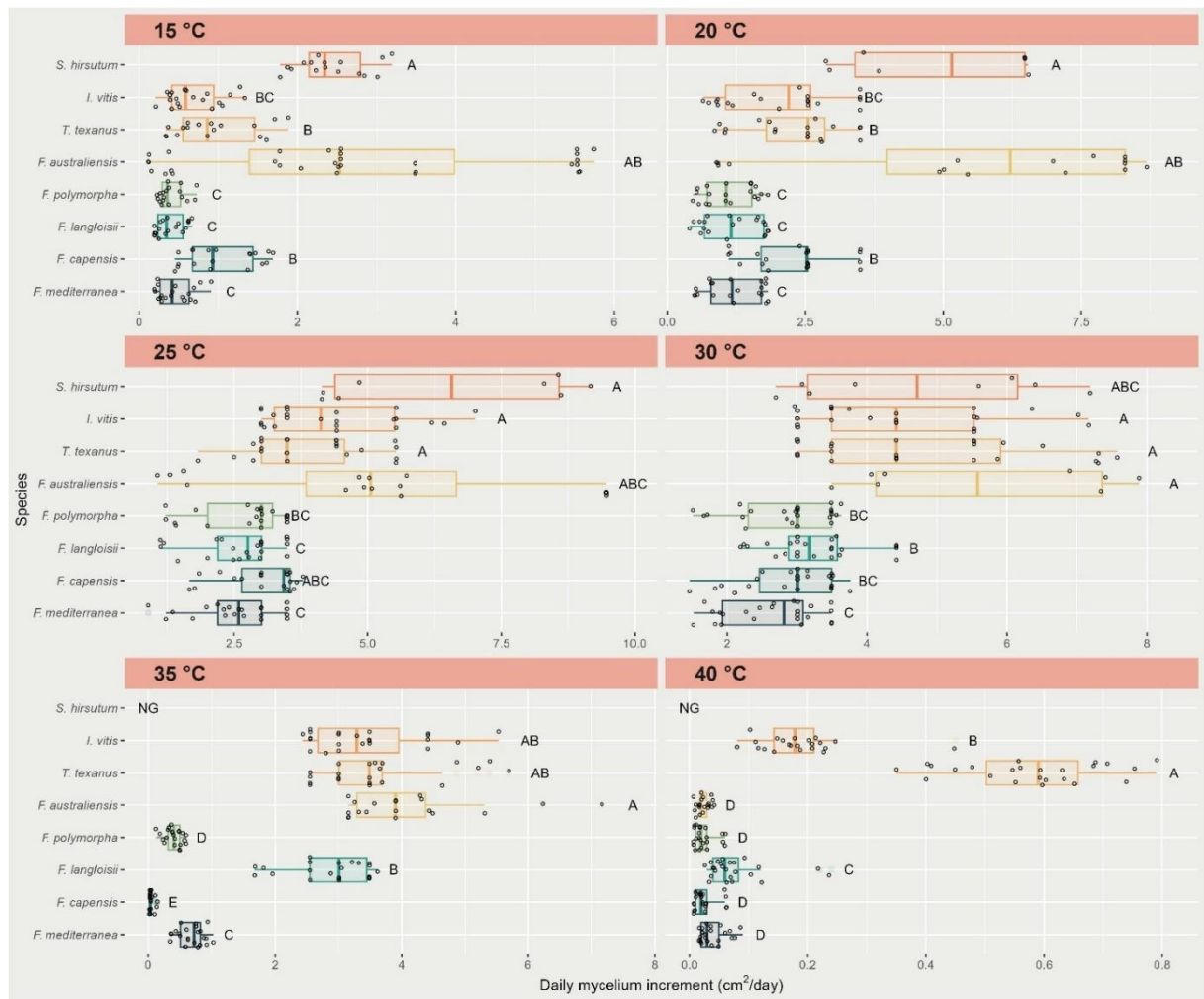


Figure 1 Standardized daily increment of the colony area of *F. mediterranea*, *F. capensis*, *F. langloisii*, *F. polymorpha*, *F. australiensis*, *S. hirsutum*, *T. texanus*, and *I. vitis*, grown at 15, 20, 25, 30, 35, 40 °C. Mycelium increment is shown using boxplots. Statistical differences between species were assessed using the Kruskal-Wallis test, followed by Dunn's post hoc test ( $p < 0.05$ ).



## For General Discussion

The aim of this thesis was to advance understanding of the degradation capabilities of white rot agents isolated from grapevines exhibiting symptoms of the Esca Complex of Diseases (ECD), in most cases presenting foliar symptoms. The final aim was to identify common factors among various *Fomitiporia* species and other basidiomycetes decay agents isolated from viticulture areas worldwide. This understanding may allow us to take a step forward in the potential role of the wood degradation process in ECD development.

The first objective was to investigate the potential involvement of a Chelator-Mediated Fenton (CMF) mechanism in the degradation strategy of the most well-studied decay agent of the grapevine, *Fomitiporia mediterranea*. It was hypothesized that the CMF system was adopted as an alternative system to degrade wood, considering the lack of genes encoding for lignin peroxidases in its genome. As this objective was achieved, the research was extended to other white rot agents, mostly from the same genus, from other viticultural areas of the world, such as *F. capensis* from South Africa, *F. australiensis* from Australia, *F. langloisii*, *F. polymorpha*, and the non-*Fomitiporia* basidiomycetes *Tropicoporus texanus* and *Inonotus vitis* from North America and of *Stereum hirsutum* from Italy. The secretomes of these fungi were also investigated for lignocellulolytic enzymatic degradation activity. Moreover, phenotypical characteristics such as *in vitro* degradation of wood as well as preferred temperature for mycelium growth were assessed for the strains under study.

### 1. Description of a non-enzymatic Fenton-based radical-inducing mechanism by *F. mediterranea*

It was demonstrated that the low molecular weight (LMW) fraction of the secretome of *F. mediterranea* can fulfill all the steps of the CMF mechanism, enabling non-enzymatic wood degradation (Chapter II). All tested strains of *F. mediterranea* could acidify the liquid medium, redox cycle with iron with the production of hydrogen peroxide in the process, and result in  $\cdot\text{OH}$  radicals generation through Fenton chemistry, especially at low pH. Most of the isolated compounds through metabolomic analyses are still unknown, but among the identified ones, many phenolic acids, phenolic aldehydes, and other aromatic compounds were detected. Many of these compounds have known iron reduction capabilities. While no strain-specific

compounds were detected, their relative abundance in the LMW fraction varied. Besides, other experiments in this study revealed a degree of strain variability, suggesting that host specificity may play a role in influencing the ability to employ a CMF system.

## 2. Universality of CMF-chemistry among ECD basidiomycetes

The adaptation of a CMF-like non-enzymatic degradation system was found to be a feature shared by all studied basidiomycetes in the collection (Chapter III). Nevertheless, many differences among species were found. The basidiomycete *T. texanus* produced a high amount of LMW compounds, as shown by the measured total phenols in the extracted fraction, with high redox potential. However, compared to other species, the production of hydrogen peroxide due to ferric iron reduction was lower, although still present. The generation of  $\cdot\text{OH}$  radicals was higher at pH levels that were more similar to the inside of the wood cell wall. Metabolomic analyses of the LMW fractions revealed a core of shared identified iron-reducing compounds, as most of these compounds were present in the secretome of every species.

### Validation of the Experiment Setup

The use of a similar experimental setup for many strains of *F. mediterranea* and different species of basidiomycetes associated with ECD revealed a common ability to induce a non-enzymatic CMF-like pathway for wood degradation. The liquid medium employed in the experiment was specifically designed to simulate *in vitro* the low-nutrient, primarily iron-deficient conditions typically found in wood microcosms (Highley, 1973). Therefore, our results reflect the natural environment as much as possible.

### Variations in *Fomitiporia mediterranea* Results Across the Two Studies

Although the protocols used were slightly different, results for the CMF analyses of the strain Phco36 (FM6 in Chapter II and *F. mediterranea* in Chapter III) were mostly the same, as they only differed in  $\text{H}_2\text{O}_2$  production. This difference could be related to the different sizes of the ultrafiltration as in Chapter II, ultrafiltration was performed with a 5kDa ultrafilter mounted in a filtration unit, while in Chapter III, ultrafiltration was achieved using a 3kDa ultra centrifugal filter. Differences in the used protocols could have led to a difference in LMWCs

isolated (e.g. in ultrafiltered antioxidants), which led to different hydrogen peroxide production. The differences in the identified metabolites profile also highlight this.

On that note, the unexpected higher hydroxyl radicals production observed at pH 5.5 reported in Chapter III could be related to this difference, as it has been demonstrated that different CMF-associated molecules can have improved activities at different pH values (Zhu et al., 2017). As much of the LMW fraction remains unidentified, enhancing this identification, along with evaluating the iron-reduction capabilities of the compounds, could provide insight into the reason for the observed discrepancy. Moreover, as the two experiments were performed within months of each other, a difference in the calibration of the used equipment could partly explain the obtained results. Despite these differences for *F. mediterranea*, our findings remain solid in both studies.

### **Intraspecific Variability Among Strains of *F. mediterranea***

Our findings from Chapter II reveal significant intraspecific variability in the results of the strains of *F. mediterranea* used in our study. One of the first notable differences between *F. punctata* and *F. mediterranea* is that the latter is classified as heterothallic, as important intraspecific variability was observed in vineyards (Fischer, 2002; Jamaux-Despréaux and Peros, 2003). The variability observed in our results may be linked to this genetic diversity.

Furthermore, the strain exhibiting the most distinct behavior was isolated from *Olea europaea*. In another study, analyses of secretome activity and the gene expression of laccase and MnP genes indicated that this specific strain also demonstrated higher activity than *Vitis vinifera* strains (Pacetti et al., 2022). These findings suggest that host-specificity might be an influencing factor for the virulence of this fungus, as it may have contributed to the observed variability (Elena et al., 2006; Markakis et al., 2017).

### **Future research**

Aside from *F. mediterranea*, limited information is available regarding strain-specificity in degradation capabilities for the studied species. Since many of the basidiomycetes studied in Chapter III have also been isolated from a wide range of host species (Cloete et al., 2015; DeKrey et al., 2022; Di Marco et al., 2003; Fischer et al., 2005; Fischer and Binder, 2004) strain

variability and potentially host specificity may influence their wood degradation capabilities as well.

Recent observations at the nanoscale level revealed that in early degradation, this pathogen causes the development of a soft-rot-like degradation pattern, as diamond-shaped cavities have developed in the S2 layer of libriform fibers (Kassemeyer et al., 2022; Pacetti et al., 2022). As we hypothesized in Chapter I, this phenomenon could result from the initial non-enzymatic degradation of wood as LMWC could access the intact wood cell wall and cause the generation of *in situ*  $\cdot\text{OH}$  through multiple iron reduction (Tamaru et al., 2019). To investigate this, further trials involving microscopy observations on early-degraded wood and wood that had been inoculated to replicate early degradation conditions could be performed to see if the same symptoms could appear on the S2 layer. Thus, the claim that diamond-shaped S2 cavities in early degradation are a consequence of early non-enzymatic degradation could be confirmed.

The importance of CMF in the early stages of brown rot wood decay is evident, as genes associated with non-enzymatic CMF degradation are typically activated prior to the expression of CAZymes in brown rot fungi (Ehmcke et al., 2020; Martinez et al., 2009; Zhang et al., 2019, 2016). To the best of our knowledge, no studies have clearly shown that non-enzymatic degradation occurs in the early stages of wood decay before the production of enzymes in white rot wood decay.

Many genes are suggested to be involved in LMWC biosynthesis. Among them, some are present in the genome of *F. mediterranea*, *S. hirsutum*, *F. polymorpha*, *T. texanus*, and *I. vitis* (Floudas et al., 2012; Garcia et al., 2024). Hydrogen peroxide generation has been associated with glyoxal oxidase and aryl-alcohol oxidase (Ferreira et al., 2009; Kersten and Cullen, 2007; Ruiz-Deñás and Martínez, 2009). Cellobiose dehydrogenase (CDHs) have also been associated with the CMF mechanism for their hydrogen peroxide production, although recent research has reconsidered their role in Fenton chemistry as CDHs show little activity at low pH values and have instead been associated with lytic polysaccharide monooxygenases activity (Harreither et al., 2011; Tan et al., 2015). Additional genes proposed to participate in LMWC-mediated decay mechanisms include polyketide synthases (PKSs), reducing PKSs, terpene synthases, and cytochrome P450 enzymes, which were shown to be upregulated in some brown rot fungi and are likely involved in LMWC production involved in Fenton chemistry (Goodell, 2020). Fet3 ferroxidase is a key enzyme in iron homeostasis and is believed to facilitate iron cycling during CMF (Sista Kameshwar and Qin, 2020). Genes such as SidA are responsible for inducing siderophore biosynthesis and are important for iron uptake and

transport (Puja et al., 2023). Considering their iron-chelating capacity, these molecules could also be involved in the CMF mechanism.

### 3. Lignocellulosic enzymatic activity of the secretome of ECD decay agents

The activity of lignocellulosic enzymes of the studied species was investigated (Chapter III). The evaluation of the laccase activity revealed differences among species with a notable high activity of *F. langloisii*. On the other hand, *S. hirsutum* and *T. texanus* showed almost no laccase activity. As for manganese peroxidases, detected activity was overall much lower compared to laccase. Again, no activity for *T. texanus* and *S. hirsutum* was detected in 2-week-old cultures. Lignin peroxidase activity was barely noticeable and, as it was also detected in the secretome of *F. mediterranea*, which is known to not possess LiP encoding genes in its genome (Floudas et al., 2012). Thus, it was hypothesized that the feeble detected activity resulted from the activity of CMF molecules in the resuspended secretome.

#### **Lignocellulolytic enzymatic activity of *S. hirsutum* and *T. texanus***

Genomic information is available for *S. hirsutum* as well as *T. texanus*. The genome of *S. hirsutum* presents 15 laccases “*sensu stricto*” as well as five atypical peroxidases (Floudas et al., 2012). As for *T. texanus*, a variety of CAZymes from the families CBM1, AA9, and AA2 are present in its genome in quantities that are indicative of white-rot agents (Garcia et al., 2024). Although no studies explicitly confirm this, the presence of these enzyme families suggests a classical white-rot mechanism for wood degradation that likely involves laccases. Results from the secretome activity of *S. hirsutum* and *T. texanus* indicate a discrepancy between observed enzymatic activity and genomic information available for these basidiomycetes. As a matter of fact, spectroscopy-based assays are reliable but sensitive to several external factors (Agrawal and Verma, 2019; Alcalde and Bulter, 2003; Mathieu et al., 2013; Tovar-Herrera et al., 2018). Thus, the need for comprehensive genomic and gene expression analyses to clarify this difference arises.

### Enzymatic activity over time

Over time, the expression of lignocellulolytic enzymes in wood decay agents can change in response to substrate composition, environmental conditions, and the stage of wood degradation (Kuuskeri et al., 2016; Sunardi et al., 2016). Our results indicate that the kinetics of enzymatic activity in the secretomes demonstrate changes in the proteomic profiles of the studied fungi over time. The activity of both laccase and MnP for *F. polymorpha* increased from the 1-week-old secretome to the 2-week-old secretome, although it still remained weak. In Garcia et al. (2024) it has been reported that degradation of the lignocellulose matrix leads to the persistence of lignin in the residues (Galarneau et al., in review) for this fungus. Therefore, it can be hypothesized that this fungus exhibits a preference for cellulose and hemicellulose instead of lignin.

In contrast, *F. mediterranea* enzymatic activity decreased from the 1-week-old to the 2-week-old secretome. *F. mediterranea* is a white rot fungus that, based on *in vitro* analysis of mineralization assays, has been described as a simultaneous white rot agent (Schilling et al., 2022). Even though the final product of wood degradation is a white-rot-like decayed matrix, it has been highlighted that the fungus shows a preference for lignin and amorphous cellulose (Haidar et al., 2024, 2021). The decrease in activity for laccase and MnP could be related to their preference for lignin, as the expression of ligninolytic enzymes happened first.

For *F. australiensis*, MnP activity increased while laccase activity decreased from the 1-week-old to the 2-week-old secretome. Conversely, *F. capensis* exhibited an increase in laccase activity and a decrease in MnP activity over the same time frame. These shifts could be an answer to changes in substrate composition as well as fungal focus towards wood cell wall polysaccharides (Kuuskeri et al., 2016; Skyba et al., 2016).

For most fungi studied, their decay type—whether simultaneous or selective white rot—remains unclear. Our results provide a solid basis for forming hypotheses, but further research involving analyses of residual wood polymers after fungal degradation would be necessary. Such studies could help determine the specific type of white rot caused by these fungi, clarifying whether the decay is simultaneous or selective and identifying any preferences for particular wood polymers. Although that is the case, our studies provide a solid information base on the lignocellulolytic enzymatic activity of many ECD-associated white rot agents.

#### **4. Preliminary results on Growth Temperature, Wood Composition, and Degradation Assays**

Preliminary results (Chapter IV) indicate that *Fomitiporia* species and other species, such as *T. texanus* and *I. vitis*, locate their optimal growth temperature at 25-30°C. *Stereum hirsutum*, on the other hand, was shown to be more susceptible to higher temperatures as its optimum was at 25°C. Chemical analyses of wood composition revealed differences among the cultivars Syrah, Merlot, and Cabernet Sauvignon. Wood degradation *in vitro* assays conducted for *F. mediterranea*, *F. capensis*, *F. polymorpha*, *F. australiensis*, and *S. hirsutum*, showed that Syrah was the most resistant to degradation among the three cultivars. This observation is hypothesized to stem from Syrah higher lignin content and lower holocellulose content, which may contribute to its greater resistance to fungal decay. For the other species studied, no significant differences were observed in mass loss due to fungal degradation across the cultivars. This finding is linked to the faster growth rates exhibited by *T. texanus* and *I. vitis*, as well as the high activity of lignocellulosic enzymes identified in the secretome of *F. langloisii* (Chapter III).

#### **General Discussion**

Basidiomycetes developed the ability to fully degrade wood approximately 295 million years ago during the Permian period when they evolved to produce class II-peroxidases enzymes (PODs) (Goodell, 2020). From that point, modern-day white rot agents evolved. Brown rot fungi descended from a white rot ancestor, losing most of their lignin-degrading enzymes and relying instead on a non-enzymatic wood degradation mechanism that uses LMWCs to induce a CMF mechanism to produce ROS species and degrade wood. For brown rot fungi, this mechanism was rewarded by evolution as it is thought to be more energetically efficient (Eastwood, 2014).

Considering the evolutionary history of decay fungi, it is no surprise that the complexity of modern-day decay fungi can be simply explained by the dichotomy brown vs. white rot that has been classically used. In light of this reevaluation, our exploration of the degradation capabilities of the basidiomycetes collection has revealed that the ability to employ a CMF system is quite widespread among ECD-associated basidiomycetes. Interestingly, our finding can be associated with the fact that the ability to induce a non-enzymatic wood degradation has

been found in other species associated with GTDs, such as *Eutypa lata*, *Phaeoacremonium minimum*, and *Phaemoniella chlamydospora* (Osti and Di Marco, 2010; Perez-Gonzalez et al., 2022). In Chapter III, it has been proposed that for *F. mediterranea*, and probably all the other studied species, the implementation of this mechanism could fall among the adaptation strategies that the fungus has shown to the grapevine (Schilling et al., 2022). As the ability to employ a CMF is widespread among fungi belonging to different genera, families, and phyla, the argument that the implication of this degradation mechanism could result in an advantage for grapevine-wood degrading fungi can be made. Further investigation into the mechanisms behind the CMF non-enzymatic degradation system could help shed light on why it is as widespread as it is among GTD fungi.

The ability to produce wood-decay enzymes is considered to be an important virulence factor for GTD fungi as it enables them to break down plant cell walls, access nutrients, and colonize the host (Morales-Cruz et al., 2015). As it has been shown that the non-enzymatic decay pathway is important for many GTD fungi, we propose, based on our findings, that the investigation into the CMF mechanism could reveal new important virulence factors.

Furthermore, Sebestyen et al. (2022) demonstrated the effectiveness of antioxidant and iron-chelating compounds in inhibiting the growth of *E. lata*, *P. minimum*, and *P. chlamydospora* through successful disruption of the CMF system. In the same work, the effects of the two biocontrol agents, *Bacillus subtilis* and *Trichoderma atroviride*, were evaluated against these fungi, as both agents produce iron-chelating and antioxidant compounds. These biocontrol agents proved to be highly effective in controlling the targeted fungi, a result that may be attributed to their secretion of anti-CMF compounds. Siderophore production that leads to competition for iron is a well-known process that occurs in BCAs that are used in GTDs (Mesguida et al., 2023). The implication of siderophores in CMF disruption could be studied as another side-effect of these compounds. In addition, antioxidants acting as ROS-scavenging compounds could neutralize  $\cdot\text{OH}$  production.

Another step that could be targeted is the initial acidification of the environment in the fungal extracellular matrix, as low-pH conditions are needed to start the CMF mechanism. Some *Bacillus* species are able to regulate the pH of their surrounding environment to neutralize it (Tran et al., 2024).

# Conclusions and Perspectives

The findings of this thesis enhance our understanding of white rot wood degradation in basidiomycetes associated with ECD. The first objective was to determine whether *Fomitiporia mediterranea*, a decay agent commonly found in the Mediterranean basin, could induce a non-enzymatic CMF mechanism involved in wood degradation. As of today, the European basin is the most important region for viticulture and grapevine cultivation in the world. Global change in light of the current climate emergency could change the future landscape of viticulture, highlighting the need to broaden the scope when researching global threats such as ECD. Thus, to build on our initial objective, we expanded our research globally by examining various white rot agents from different viticultural regions, addressing the existing gap in knowledge about their degradation capabilities.

Our results indicate that the capacity to initiate a Fenton-chemistry-based non-enzymatic degradation system is widespread among basidiomycetes linked to ECD. Additionally, the analysis of lignocellulosic enzyme activity in the fungal secretome demonstrated weak activity of peroxidases. This supports the hypothesis that a CMF system may serve as a secondary degradation mechanism in place of lignin peroxidases and other peroxidases for white rot decay on the grapevine.

The role of fungal degradation in ECD has been shown to be crucial, as it is considered a direct or indirect cause of the development of GLSD in light of the various hypotheses proposed for the emergence of foliar symptoms. The results of this study enhance our understanding of how fungal degradation occurs in grapevines. The findings indicate that the CMF mechanism is widespread among GTD fungi, suggesting that non-enzymatic wood degradation through this mechanism may be an essential adaptation strategy for these fungi to interact with vines.

Further research should focus on analyzing grapevine wood decay through gene expression and genomic approaches to confirm that the employment of a CMF system before enzymatic degradation, as it has already been shown for brown rot fungi, can also be confirmed for decay fungi of the grapevine, especially for the studied white rot agents.

Moreover, the widespread use of this mechanism among all examined fungi points to its significance for the metabolic functions of these species. This understanding could potentially be applied to control strategies, as disrupting various phases of the CMF mechanism

## Conclusions and Perspectives

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might inhibit fungal growth and overall performance on grapevines. Future research should focus on *in planta* studies to further assess the importance of wood degradation in the development of ECD symptoms.

## Publications, conference presentations, and awarded grants.

### Published articles

- Moretti, S.; Goddard, M.-L.; Puca, A.; Lalevée, J.; Di Marco, S.; Mugnai, L.; Gelhay, E.; Goodell, B.; Bertsch, C.; Farine, S. First Description of Non-Enzymatic Radical-Generating Mechanisms Adopted by *Fomitiporia mediterranea*: An Unexplored Pathway of the White Rot Agent of the Esca Complex of Diseases. *J. Fungi* 2023, 9, 498. <https://doi.org/10.3390/jof9040498>

### Submitted Articles

- Puca, A.; Moretti, S.; Goddard, M. L.; Lalevée, J.; Kassemeyer, H. H.; Farine, S.; Mugnai, L.; Bertsch, C.; The Tour of the World in Eight White Rot Species: Assessment of Enzymatic and Non-Enzymatic Degradation Capabilities of Worldwide Esca Complex of Diseases (ECD) Associated Decay Agents.

### Presentations at Scientific Conferences

*The name of the presenting author is underlined*

- Puca A.; Moretti S.; Farine S.; Bertsch C.; Mugnai L.; Around The World In Eight White Rot Species: Understanding Wood Degradation In Esca Complex Of Diseases; at XXIPPC – XX International Plant Protection Congress, 1-5/07/2024, Athens, Greece;
- Puca A.; Moretti S.; Farine S.; Bertsch C.; Mugnai L.; Unveiling degradation capabilities and phenotypical traits of white-rot agents from grapevine affected by Esca Complex of Diseases in different geographical areas; at SIPaV XXVIII – Italian Phytopathological Society, 18-20/09/2023, Naples, Italy.

## Presentation at Public Outreach events

- Puca A.; Amadou dans le monde: focus sur les dégradations enzymatiques et non-enzymatiques du bois; at Journée d'échanges entre chercheurs et professionnels de la filière viti-vinicole, 19/04/2024, Colmar, France

## Posters at conferences

- Puca A.; Moretti S.; Farine S.; J. Lalevée; Bertsch C.; Mugnai L.; The tour of the world in eight wood rot species: enzymatic and non-enzymatic degradation in Esca Complex of Diseases white-rot agents; at SIPaV XXXI – Italian Phytopathological Society, 9-11/09/2024, Trento, Italy
- Puca A.; Moretti S.; Farine S.; Bertsch C.; Mugnai L.; Basidiomycetes in Esca complex of diseases: phenotypical characteristics and degradation capabilities with a focus on the non-enzymatic pathway; at ICPP – 12th International Congress of Plant Pathology at Lyon, France, 20-25/08/2023
- Puca A.; Witzell J.; The Right Chemistry – can host chemicals regulate the capacity of endophytes to antagonize Dutch elm disease pathogens?; at IUFRO Conference Division 7 – Forest Health Pathology and Entomology, Lisbon 6 – 9/09/2022

## Awarded grants

- COST Action CA20132 “Understanding complex tree diseases using a combination of wood technology and tree pathology” – Short-term scientific mission at Linnaeus University, Forestry and Wood Technology, Växjö, Sweden from 05/09/2022 to 26/09/2022
- “Young Researcher In Training” award, conferred by SIPaV – Italian Phytopathological Society on the 22/06/2023
- SIPaV Contribution for PhD Students and Early-Career Researchers for the Presentation of Scientific Work at the XX International Plant Protection Congress (IPPC) July 1–5, 2024 (Athens, Greece), conferred by SIPaV on the 15/05/2024

# Acknowledgments

Well, that was tough.

Being in a foreign country, learning a new language, and, on top of that, working on a PhD thesis proved to be a bit tough for me. So, if I've made it this far, it's only thanks to the people who were there for me. Being surrounded by all of you has taught me a lot about who I want to be—and who I don't want to be.

First, I want to thank all the jury members for taking the time to read and evaluate my work.

I am also deeply grateful to my PhD committee—Laura Mugnai, Christophe Bertsch, and Sibylle Farine—for their trust, which made this long journey possible.

No amount of words could truly express how grateful I am to Dr. Samuele Moretti. His constant support and human qualities are what made this thesis possible. I sincerely doubt that I would never have made it this far without him, and I'm happy to say that, besides being a true mentor, I'm also lucky to call him a great friend.

Mes remerciements à toute l'équipe du labo 124 et à tout le laboratoire en général. Plus particulièrement, un immense merci à Dr. (!) Alexia Grau, avec qui j'ai eu le plaisir de partager le bureau. Je n'aurais pas pu rêver d'une meilleure prof de français. En plus, j'ai énormément appris de toi sur ce que signifie être doctorant et un vrai scientifique.

Merci aussi à Dr. Loriane Merlen, qui a toujours été un exemple de professionnalisme, de force et, surtout, de générosité. Je n'oublierai jamais l'aide que tu m'as offerte à un moment où j'en avais vraiment besoin.

Un grand merci à Margot Elz pour m'avoir écouté parler vraiment trop. Je suis très heureux de pouvoir t'appeler mon amie (message en code : *Procyon lotor*).

Très certainement, un grand merci à la vélo-gang : Romain Pierron et Lucas Galimand. Merci pour avoir tenté de m'assassiner avec tous ces kilomètres, pour les apéros, et pour les blagues que, probablement, j'ai toujours comprises à moitié. Et, Romain, merci pour avoir été une vraie inspiration pour moi.

Merci à Papa Yann Leva pour avoir fait peur à tous les trucs du labo quand ils ne voulaient pas fonctionner, et pour avoir passé tant de temps à réfléchir avec moi sur les manips.

Merci à Hélène d'avoir été la meilleure guide de Colmar et la meilleure agente immobilière possible quand j'avais besoin.

Toujours en français, parce que c'est une langue que vous comprenez tous : un immense merci à mes super stagiaires (officiels et pas tout à fait) et étudiant qui ont travaillé avec moi : Nicholas Cauda, Elise Lenoir Hulin et Samantha Carlier. Ça a été un plaisir de travailler avec vous, et je vous assure que j'ai énormément appris de vous – peut-être même plus que vous n'avez appris de moi !

Thanks to Samuele Recchia, the super illustrator and great friend, for the incredible cover of this thesis.

Un enorme grazie a tutti i miei amici italiani che mi hanno ascoltato quando ne avevo bisogno e mi hanno sempre detto la loro quando avevo bisogno di un consiglio.

Un grazie a Nicola e Alice, che mi hanno offerto un rifugio per staccare un po', e per raccogliere delle castagne.

Un grande grazie anche alla mia famiglia per avermi continuamente supportato in ogni modo, nonostante la lontananza.

Infine, il più grande grazie a Veronica, l'unica persona che mi è stata accanto dall'inizio alla fine, e che quindi sa davvero che cosa ha significato, e quanto è costato, questo percorso. Mi hai tirato su e ricordato quanto valgo infinite volte. Grazie, non avrei potuto chiedere di meglio.

Infine ringrazio anche la mia mia nipotina, a cui voglio tanto bene.

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