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Manganese affects grapevine wood degradation by *Fomitiporia mediterranea*: *in vitro* evidence

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Summary. Grapevine trunk diseases (GTDs) are severe constraints affecting *Vitis vinifera* (grapevine) cultivation, causing vine decline, yield losses, and major economic impacts. Within the Esca disease complex, the white-rot basidiomycete *Fomitiporia mediterranea* is frequently associated with wood degradation. Despite its recognized involvement in disease development, the influence of abiotic factors on the wood-degrading activity of this pathogen has not been studied. Effects of manganese (Mn^{2+}) availability on *F. mediterranea* growth, biomass production, wood degradation, and manganese peroxidase (MnP) activity of this fungus were assessed under controlled laboratory conditions, using increasing manganese concentrations and young (3-year-old) and old (25-year-old) wood from *V. vinifera* 'Cabernet Sauvignon'. Manganese availability had a concentration-dependent effects on *F. mediterranea*. Intermediate Mn^{2+} concentrations, particularly 80 µM, promoted colony growth, biomass accumulation, and wood mass loss, whereas greater concentrations (320 µM) reduced growth and wood degradation. Qualitative observations showed formation of dark pigmented zones in wood, which became progressively more pronounced as Mn concentrations increased. MnP activities were greatest at intermediate Mn^{2+} levels, and declined at the greatest concentration, a trend consistent with *F. mediterranea* growth and wood degradation patterns. These results indicate that Mn^{2+} availability can modulate *F. mediterranea* degradative activity and may influence enzymatic processes involved in grapevine wood decay. Future studies should assess micronutrient bioavailability as an abiotic factor for reducing progression of the Esca complex in grapevines.

Keywords. Esca complex, wood decay, white-rot basidiomycete, micronutrients.

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INTRODUCTION

The Esca complex of diseases (ECDs) causing grapevine trunk diseases (GTDs) is widespread and economically damaging. This complex includes different diseases in different vine life stages, including: Brown wood streaking, Petri Disease, Grapevine Leaf Stripe disease (GLSD). The term Esca

applies wood white rot of grapevines. This disease has slow progression, and is difficult to manage, and has impacts on vineyard longevity, causes substantial grape yield losses, causes death of vines, as well as major economic repercussions across wine-producing regions, especially in Europe (Gramaje *et al*., 2018; Guerin-Dubrana *et al*., 2019).

The wood diseases in ECD are associated with a range of symptoms, and with several pathogenic fungi. The most frequently cited of these include two vascular ascomycetes, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Gramaje *et al*., 2015), and as the white-rot basidiomycete *Fomitiporia mediterranea* (Fischer *et al*., 2002). *Fomitiporia mediterranea* is one of the most frequently isolated pathogens associated with Esca (wood rot), and with GLSD, i.e. disease causing typical tiger stripe foliar symptoms and with unique epidemiology (Mugnai *et al*., 1999; Del Frari *et al*., 2019; Bruez *et al*., 2020). Recent studies on grapevine wood microbiome have indirectly confirmed the central role of *F. mediterranea* within wood decay and leaf stripe disease (Bruez *et al*., 2021; Pacetti *et al*., 2021).

Ecologically, *F. mediterranea* is a highly adaptable species, with a broad host range and distribution across diverse regions and climatic conditions (Moretti *et al*., 2021). Across its geographical range, *F. mediterranea* is strongly associated with *V. vinifera*. However, this association may partly reflect the economic importance of grapevine cultivation, which has led to increasingly detailed study of this pathogen compared to associations other hosts (Moretti *et al*., 2021). Presence of the pathogen is detected at host plant necrosis margins, between white-rot and non-necrotic tissues, as well as in adjacent non-necrotic but recently colonized wood (Fischer, 2002; Péros *et al*., 2008; Surico, 2009; Bruez *et al*., 2017; Elena *et al*., 2018; Bruez *et al*., 2021; Pacetti *et al*., 2021). Due to the extended periods required for wood colonization and degradation, *F. mediterranea* has been primarily detected in grapevines older than 8 to 10 years, while it is only occasionally found in vines younger than this (Sánchez-Torres *et al*., 2008; Mugnai *et al*., 2010). *In vivo* pathogenicity studies on wood-decaying basidiomycetes in grapevines remain scarce (Chiarappa, 1997; Sparapano *et al*., 2000a, 2000b).

Several studies have demonstrated that *F. mediterranea* produces high levels of manganese peroxidase (MnP). Based on a genomic study, MnPs were the only class II peroxidases present in the *F. mediterranea* secretome (Floudas *et al*., 2012). Furthermore, and compared with other white-rot fungi, including the model species *Trametes versicolor*, *F. mediterranea* produces large amounts of MnPs (Schilling *et al*., 2023). In mycelium exposed to wood substrates, increased basal expression of MnP genes has been related to laccase genes (Pacetti *et al*., 2022). Enzyme secretion is also substrate-dependent, with preference for grapevine wood, where *F. mediterranea* has increased MnP and laccase secretion, enhanced wood degradation, and increasingly efficient detoxification of grapevine extractives compared with beech extracts (Schilling *et al*., 2022; Schilling *et al*., 2023). These results highlight specific traits of *F. mediterranea* during grapevine wood colonization compared with other woody substrates and white-rot fungi.

The catalytic mechanism of MnP shares several features with other heme-containing peroxidases, including lignin peroxidase and horseradish peroxidase (Dunford, 1991; Wariishi *et al*., 1991; Kirk and Cullen, 1998). Like other class II peroxidases, MnP operates through formation of the reactive intermediates Compound I and Compound II (Wariishi *et al*., 1988; Kishi *et al*., 1994). However, MnP has a distinctive functional characteristic: it preferentially utilizes Mn^{2+} as its electron donor. Although Compound I can also be reduced by alternative substrates, Compound II shows a strong dependency on Mn^{2+}, while reduction by other electron donors occurs only at low rates (Wariishi *et al*., 1988; Brown *et al*., 1990). Consequently, manganese availability is a key factor regulating MnP functionality and catalytic efficiency.

Beyond *F. mediterranea*, numerous studies on other white-rot fungi have shown that manganese availability plays a central role in enhancing ligninolytic performance and wood substrate degradation. The supplementation of fungal cultures with manganese has been reported to increase lignin depolymerization and the enzymatic hydrolysis of lignocellulosic materials, indicating that manganese acts as a cofactor and as an inducer of MnP expression and activity (Fu *et al*., 2021). Investigations into the regulation of ligninolytic enzymes in white-rot fungi have also shown that manganese presence can upregulate MnP production, further strengthening the link between manganese availability and fungal lignin-degrading capacity (Scheel *et al*., 2000). Ecological studies have provided additional evidence supporting the influence of manganese on wood component degradation. Berg *et al*. (2007) reported a linear relationship between manganese concentration in foliar litter and annual mass loss during the late stages of decomposition, highlighting the broader role of manganese in lignocellulose decay processes. Experimental studies have also shown that manganese supplementation enhances MnP production in the white-rot fungus *Pleurotus ostreatus*, a well-known wood decay agent,

further confirming the stimulatory effect of manganese on MnP-mediated degradation pathways (Slavens *et al*., 2019). Additional evidence comes from studies on other wood-rotting fungi. Investigations of decayed wood have shown that black spots or black zones, which are closely associated with advanced wood degradation, contain high concentrations of manganese compared with adjacent decayed tissues. In contrast, surrounding healthy wood lacked significant manganese accumulation. The oxidized manganese buildup was detected exclusively in black zones within delignified wood, indicating close association between manganese accumulation and lignin degradation processes (Blanchette, 1984).

Taken together, these observations support the overarching hypothesis that manganese bioavailability modulates the wood-degrading performance of white-rot fungi. Consequently, the primary objective of this study was to determine whether a similar manganese-driven stimulatory effect occurs in the Esca-associated pathogen *F. mediterranea*. Investigating this relationship could help to clarify the involvement of other potential abiotic factors involved in the expression of the GLSD foliar symptom.

A long-standing hypothesis in Esca pathogenesis suggests that host plant foliar symptoms are triggered by toxic fungal byproducts or phytotoxic metabolites translocated from decayed trunk wood to grapevine canopies (Mugnai *et al*., 1999). Previous studies have shown that plant nutritional status influences foliar symptom expression (Calzarano *et al*., 2009; Calzarano *et al*., 2023; Dell'Acqua *et al*., 2025). Manganese is of particular interest due to its dual role in plant nutrition and fungal ligninolytic metabolism. Among the abiotic factors influencing disease expression, water availability has been consistently identified as the main key driver, with rainfall patterns positively correlated with the manifestation of foliar symptoms (Surico *et al*., 2000; Calzarano *et al*., 2018). This relationship is particularly relevant because soil manganese availability and plant uptake are strongly influenced by rainfall and soil moisture dynamics (Radaelli and Calamai, 2001; Marschner, 2011). Together, these observations suggest a potential mechanistic link between environmental conditions, nutrient availability, and fungal activity in wood tissues. Therefore, the primary hypothesis of the present study was that increased manganese bioavailability enhances the degradative and metabolic activity of *F. mediterranea*, which could contribute to processes potentially linked to foliar symptom expression.

To test this hypothesis, the present *in vitro* study aimed to determine whether manganese bioavailability levels stimulate fungal growth, ligninolytic activity, and wood degradation, and to assess whether host tissue age modulates these responses. Whether increased degradative capacity under elevated manganese conditions correlates with enhanced MnP production was also assessed. This characterization would establish the necessary baseline for future *in vivo* and field-based studies that aim to link wood-decay severity with grapevine canopy symptom manifestation.

MATERIALS AND METHODS

Fungus isolate identification and culture conditions

One isolate of *Fomitiporia mediterranea* (Fmed It 17) was used in all experiments. This isolate was obtained from symptomatic *Vitis vinifera* wood collected in the municipality of Gambassi Terme (Tuscany, Italy). The isolate was first identified based on morphological characteristics and subsequently confirmed through molecular analyses. DNA extraction, PCR amplification, and sequencing were carried out the University of Florence internal sequencing service CIBIACI (www.cibiaci.unifi.it). DNA was extracted following the 2× cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990), using 10–15 mg of lyophilized material. The primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used for amplification of the ITS region (White *et al*., 1990), while the primer pair LR0R (5′-GTACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′) was used to amplify the LSU region (Rehner and Samuels, 1994). PCRs were carried out in 25 µL reaction volumes using 0.4 µM of each primer, 1.5 mM $MgCl_2$, 1× colourless reaction buffer, 0.5 mM dNTPs, and 1 unit of GoTaq® G2 DNA Polymerase (Promega Corporation). The PCR cycling protocol was carried out on a Bio-Rad thermal cycler under the following conditions: DNA denaturation, 1 min at 94 °C, primer annealing, 1 min at 55 °C; primer extension, 2 min at 72 °C. Negative control reactions omitting DNA were included in all amplification sets to monitor potential contamination by exogenous DNA. Subsequently, the PCR products were visualized on a 1% agarose gel stained with ethidium bromide, using a Gel Doc Bio-Rad system; a 1 kb Plus DNA Ladder (Thermo Fisher Scientific) was used as the molecular weight marker for bp dimensions. Amplicons were then purified by NucleoFast 96 PCR Plate clean-up system (Macherey-Nagel), and were sequenced with forward and reverse primers on an ABI 3130XL genetic analyser (Applied Biosystems). The newly generated sequences were then read and edited using Chromas v.1.45 software, and consensus sequences were compared using the Basic Local Alignment Search Tool (BLASTn) reveal-

ing up to 99% sequence similarity with reference sequences. Sequences from isolate Fmed It 17 used in this study were deposited in the GenBank database under accession numbers PZ489312 for the LSU region and PZ493570 for the ITS region.

The fungal mycelium was stored at −80 °C until used. Before each experiment, the isolate was reactivated by subculturing on potato dextrose agar (PDA; 18 g L^{-1}) plates and incubating for 2 weeks at 26 °C in the dark. All assays were carried out using 0.5 cm^2 mycelium plugs as inoculum and incubated at 26 °C in complete darkness under constant laboratory humidity conditions.

Experimental design

The research was divided into two main experimental sections:

- *Part I* assessed the effect of manganese on *F. mediterranea* growth, biomass production, and wood degradation, comparing young (3-year-old) and old (25-year-old) vine wood from *Vitis vinifera* 'Cabernet Sauvignon'. The objective was to determine the manganese (Mn^{2+}) concentration at which the fungus exhibited growth responses, and whether wood age influenced fungal growth.
- *Part II* focused only on old grapevine wood, as this is the natural host typically infected under vineyard conditions, and also showed the most consistent degradation patterns in Part I. Wood degradation and mycelium colonization were evaluated using *Vitis vinifera* wood discs. The fungus was also grown in submerged culture to determine whether enhanced degradation correlated with increased MnP production.

All experiments were carried out using three biological replicates, each comprising three technical replicates.

Part I: Fungal growth rate, mycelium biomass production, and wood degradation assays

This first part of the study aimed to assess effects of manganese on fungal growth, biomass production, and wood degradation, comparing young (3-year-old) and old (25-year-old) vine wood from *Vitis vinifera* 'Cabernet Sauvignon'.

Preparation of wood substrates

Fungal growth assays. Different wood-based substrates were prepared. For the mycelium growth rate assays, grapevine sawdust was used in a low-nutrient substrate consisting of PDA at 3 g L^{-1}. Two types of *Vitis vinifera* 'Cabernet Sauvignon' wood were included:

- Young wood: shoots collected from 3-year-old nursery plants
- Old wood: trunk wood obtained from a 25-year-old vineyard in Bolgheri, Italy

Wood material was cut, mechanically ground to sawdust, and sieved through a 0.2 mm mesh to obtain a uniform particle size. For each plate, 0.5 g of dry sawdust was added to 20 mL of PDA (3 g L^{-1}).

Mycelium biomass assays. The same sawdust used for the growth assays (above) was used. Petri plates each contained 20 mL of water agar supplemented with 2 g of dry sawdust. A sterile cellophane film was placed on the agar surface to facilitate clean removal of the fungal mycelium at the end of the incubation period.

Wood degradation assays. Intact wood splints were prepared from the young and old grapevine material. Splints were cut to approx. 5 cm in length and 0.3 cm in diam., then dried at 105 °C for 48 h to obtain constant dry weight of 0.25 g per splint. The splints were then placed on water agar plates amended with the respective Mn^{2+} treatments.

Manganese treatments

Mn^{2+} (as $MnSO_4$) was added to the substrates according to the different experimental treatments (Figure 1). For the *F. mediterranea* growth assays, the fungus was grown on a low-nutrient sawdust agar medium (PDA 3 g L^{-1}) formulated to reduce the influence of readily available nutrients from the culture medium, to promote fungal utilization of the lignocellulosic substrate. The experimental design included a no-sawdust control, a 0 µM Mn^{2+} control, and Mn-amended treatments at 80, 160, or 320 µM.

For the mycelial biomass assays conducted on water agar supplemented with sawdust, the treatments consisted of a 0 µM Mn^{2+} control, and Mn^{2+} concentrations of 80, 160, or 320 µM, along with an additional control of water agar without sawdust supplement.

For the wood degradation assays, intact wood splints were exposed to Mn^{2+} concentrations of 0, 80, 160, or 320 µM, together with a control condition in which wood was incubated without fungal inoculation. All treatments were conducted in triplicate.

Application protocols

Fungal growth assays. A mycelium plus agar plug (6 mm diam.) from a *F. mediterranea* PDA culture was

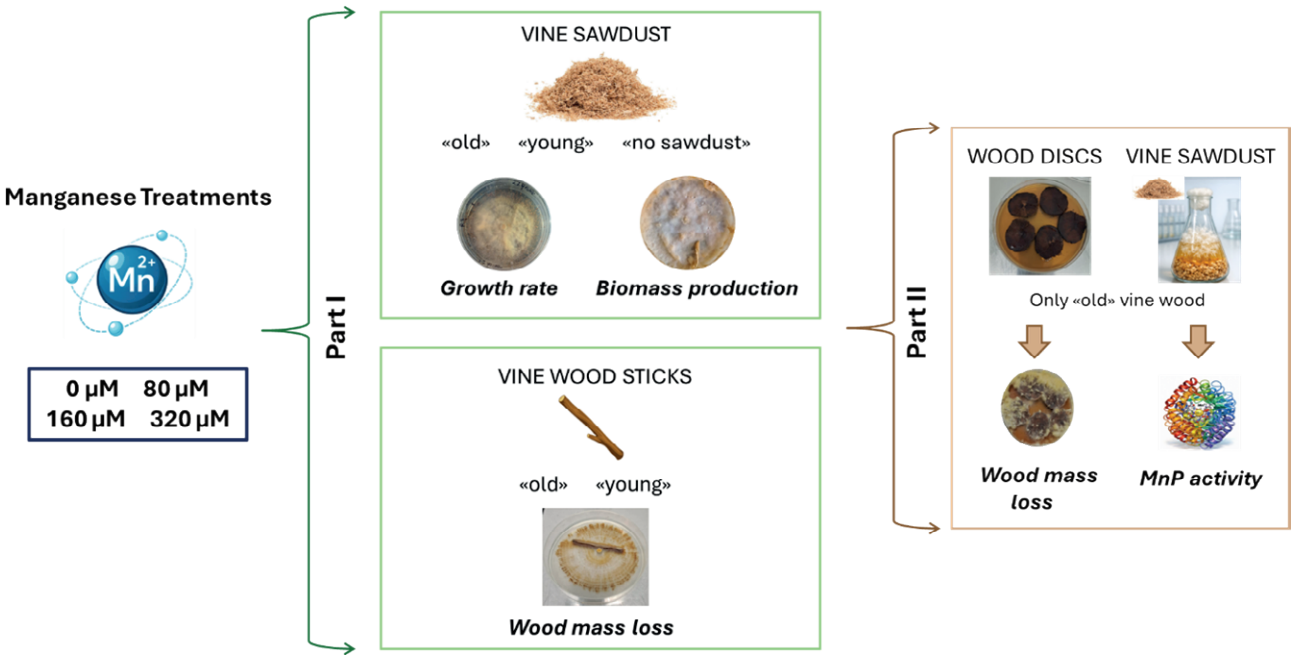

Figure 1. Experimental design.

placed at the centre of each Petri dish with sawdust prepared as described above. Plates were then incubated at 26 °C in complete darkness for 18 d. Digital images of resulting colonies were captured every 3 d, and each colony area was measured using ImageJ. Colony growth rates were expressed as the increase in colony area over time ($mm^2\ d^{-1}$).

Mycelium biomass assays. A mycelium plus agar plug (6 mm diam.) from a *F. mediterranea* PDA culture was placed at the centre of each Petri dish on the cellophane film, prepared as described above. Plates were then incubated for 1 month at 26 °C in the dark. Mycelium growing on the cellophane film was carefully scraped off, dried at 65 °C to constant weight, and weighed, to determine fungal dry biomass.

Wood degradation assays. A mycelium plug (6 mm diam.) from a *F. mediterranea* PDA culture was placed in contact with each wood splint on the agar surface. Wood splints were incubated for 1 month at 26 °C in the dark. After incubation, the splints were re-dried at 105 °C to constant weight. Wood degradation was quantified as mass loss, calculated as:

$$\text{Wood mass loss (\%)} = [(W_0 - Wt) / W_0] \times 100$$

where W_0 is the initial dry weight of the wood samples, and Wt is the dry weight after incubation.

Part II – Experiments on vine wood disks and manganese peroxidase production

This part of the study focused on old grapevine wood, as this represents the natural host typically infected under vineyard conditions, and showed the most consistent degradation patterns in Part I of this study. Wood degradation and mycelial colonization were evaluated using *Vitis vinifera* 'Cabernet Sauvignon' wood.

Wood disc degradation assay

Wood discs (approx. 3 cm diam., 0.5 mm thick) were obtained from the same 25-year-old *Vitis vinifera* stems. Five wood discs were placed in each large Petri dish (145 mm diam.), with three biological replicate plates per treatment. Total dry weight of the wood per treatment was standardized to approx. 16.5 g by selecting discs of equal total weight for each replicate. Water agar was used as the growth substrate. Mn^{2+} treatments assessed were 0, 80, 160, or 320 µM, together with a control treatment of wood incubated without fungal inoculation. The Petri dishes were incubated at 26 °C in complete darkness for 2 months. After incubation, the wood discs were dried and weighed to determine mass losses, following the procedures described above.

Enzymatic MnP assay

Liquid cultures of *F. mediterranea* were incubated for 21 d at 26 °C with orbital shaking (180 rpm) in 250-mL Erlenmeyer flasks each containing 100 mL of salt medium containing $MnSO_4$ at 0, 80,160 or 320 µM, as well as other salts (Rüttimann *et al*., 1992). The medium in each flask was supplemented with 3.5 g of *Vitis vinifera* 'Cabernet Sauvignon' sawdust, and was inoculated with three 0.5 cm diam. plugs taken from a 3-week-old PDA culture, together with a control condition in which wood was incubated without fungal inoculation. Three technical replicates of 10 mL from each of the three biological replicates were collected and processed through a four-step filtration procedure: first through a 50 µm Nilex sheet, followed by 1.0, 0.45, and 0.2 µm regenerated cellulose membrane filters. Fungal dry biomass was determined by drying the collected mycelium for at least 48 h at 103 ± 1 °C. The protein contents of the filtrates were determined spectrophotometrically, using a NanoDrop (BioSpec-nano, Shimadzu, Kyoto, Japan), assuming that absorbance of 1 OD corresponded to 1 mg mL^{-1} protein (Pacetti *et al*., 2022). The secretome was then diluted to a final protein concentration of 0.2 mg mL^{-1} for enzymatic assays.

The secretomes obtained from liquid cultures were used to assess their MnP activities, using the method of Mathieu *et al*. (2013). MnP activity was determined based on oxidative coupling between 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) and p-dimethylaminobenzaldehyde (DMAB), catalyzed by peroxidases (PODs) in the presence of H_2O_2, and forming a purple indamine complex with an extinction coefficient of ε_{590} = 32,000 M^{-1} cm^{-1}. Assays were carried out at 28 °C in 96-well microplates and using a Tristar two plate reader (Berthold Technologies GmbH & Co.). Each reaction mixture (200 µL total volume) consisted of 140 µL of reagent solution, 50 µL of liquid culture secretome (0.2 mg mL^{-1}), and 10 µL of 1 mM H_2O_2 to initiate the reaction. The reagent solution contained 50 mM DMAB and 1 mM MBTH in a sodium lactate/sodium succinate buffer (100 mM; pH 4.5), supplemented with either 1 mM $MnSO_4$ (Solution A, to measure manganese-dependent activity) or 2 mM EDTA (Solution B, to measure manganese-independent activity). Specific MnP activity was calculated by subtracting the enzyme activity measured in Solution B from that measured in Solution A. Two types of controls were included using, respectively, heat-inactivated samples and H_2O_2-free mixtures. Volumetric activities (U L^{-1}) measured during the assays were converted to specific enzyme activity expressed as U mg^{-1} protein, by dividing the values by the protein concentration of the secretome, where 1 U corresponds to 1 µ mol of substrate oxidized per min.

Statistical analyses

All statistical analyses were carried out using IBM SPSS Statistics software (version 29). For the fungal growth assays and manganese peroxidase activity experiments, three biological replicates were used, each consisting of three technical replicates. Before statistical analyses, normality and homogeneity of variances were assessed, respectively, using Shapiro-Wilk tests ($P > 0.05$) and Levene's tests ($P > 0.05$). Variables meeting these assumptions were analysed using a General Linear Model (GLM) approach.

Data of fungus colony areas (mm^2), manganese peroxidase activity (U mg^{-1}), and wood discs mass loss (%), were analysed using one-way analyses of variance (one-way ANOVA) with Type III sums of squares, considering treatment as a fixed factor. Mean comparisons were made using Tukey's honestly significant difference (HSD) *post hoc* test at $P < 0.05$.

Data of wood biomass losses, fungal biomass production, and colony areas were additionally analysed using two-way factorial ANOVA within a univariate General Linear Model (Univariate GLM) framework, with Type III sums of squares. In these analyses, Mn concentrations added to culture medium (0, 80, 160, or 320 µM) and wood age (young wood, old wood) were considered as fixed factors. The interaction between Mn concentration and wood age was also included in the model to evaluate potential combined effects on the experimental variables. Mean comparisons were carried out using Tukey's HSD *post hoc* test at $P < 0.05$, and statistical significance was set at $P \leq 0.05$.

RESULTS

Part I: effect of Mn^{2+} on growth rate, biomass production, and wood degradation assays

Fungus growth rate

The no-sawdust control treatment (not shown in Figure 2), where *F. mediterranea* was inoculated onto water agar, no growth of the fungus was observed.

One-way ANOVA was first carried out to evaluate differences among individual treatments within each substrate (Figure 2). The growth area of *F. mediterranea* colonies was evaluated after 18 d of incubation. Fungal growth on PDA, without sawdust supplementation,

was consistently less than in the corresponding sawdust-amended treatments, indicating that, in addition to the limited nutrients provided by PDA, the fungus was able to utilize sawdust as a nutrient source. For the young wood substrate, the 80 µM Mn^{2+} treatment resulted in the greatest mean colony area, which differed ($P \leq 0.05$) from the other Mn^{2+} concentrations within the young wood treatments, and from all the other treatments. Similarly, in the old wood substrate, the 80 µM Mn^{2+} treatment also gave the greatest mean colony area, which differed ($P \leq 0.05$) from the corresponding 0 µM Mn^{2+} control. Therefore, in the young and old wood substrates, the 80 µM Mn^{2+} treatment gave greater fungal growth compared to the respective controls. In contrast, excessively high manganese concentrations (320 µM) inhibited fungus growth, with the 320 µM Mn^{2+} treatment giving the lowest growth values in young and old wood substrates.

Since the independent effects of substrate type (young wood, old wood, and no sawdust) and manganese concentration on the dependent variable fungal growth area were statistically significant, a two-way ANOVA was subsequently carried out to assess whether an interaction between these two factors also affected fungus growth. A two-way ANOVA showed a main effect ($P < 0.001$) on fungus growth area for substrate type and manganese concentration. A significant interaction ($P < 0.001$) between substrate type and manganese concentration was also detected, indicating that the effect of manganese on fungal growth varied depending on the substrate.

Fungus biomass production and wood degradation

Table 1 shows effects of Mn^{2+} treatments on fungus biomass production and wood degradation. The negative controls (not shown in the table), consisting of *F. mediterranea* grown on water agar without sawdust or wood sticks, and incubated without fungus inoculation, did not show detectable fungus growth or wood degradation. Two-way ANOVA showed that no statistically significant differences were detected between old and young wood at any of the manganese concentrations, either for fungus biomass production ($P = 0.822$) or wood mass loss ($P = 0.367$), showing that biomass production and wood degradation were similar for the two wood types (Table 1).

The highest amounts of fungus biomass and wood mass loss were from the 80 µM manganese treatment. Fungus biomass at 80 µM was greater than from all other treatments, regardless of wood type. Wood mass loss was also greatest at 80 µM, although no differences ($P > 0.05$) were detected between the 80 and 160 µM treatments. At 320 µM manganese, fungal biomass produc-

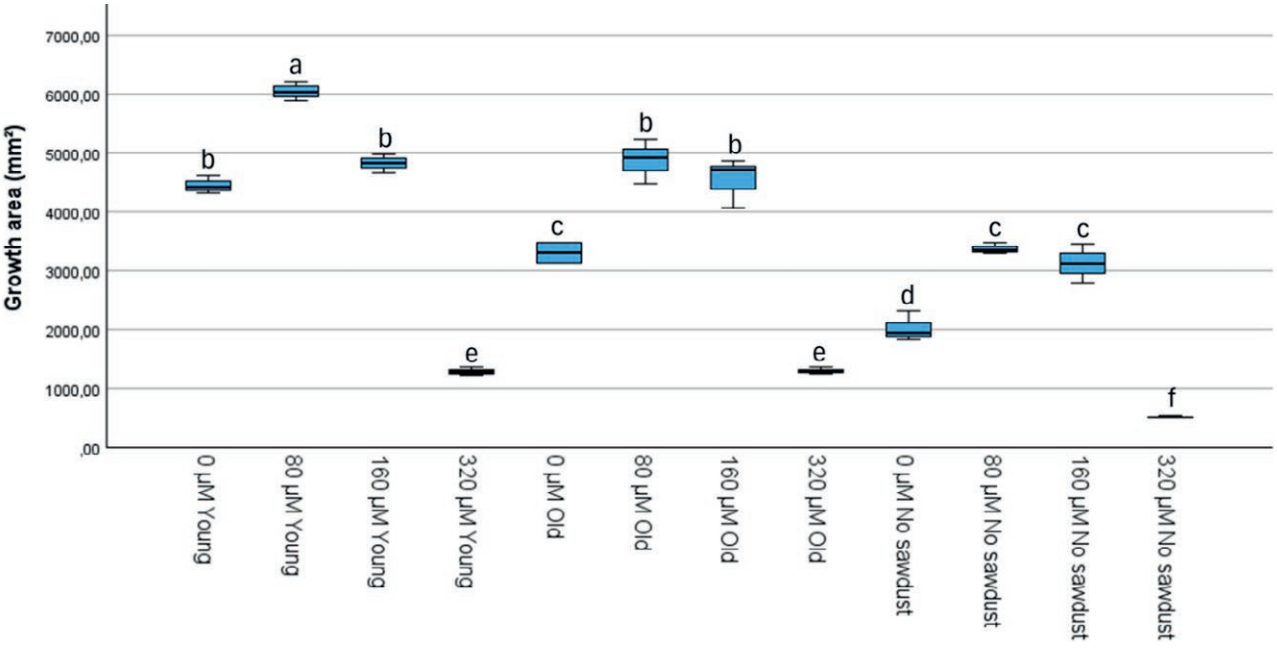

Figure 2. Mean fungal growth area (mm²) of *Fomitiporia mediterranea* after 18 d incubation on young and old *Vitis vinifera* sawdust in PDA (3 g L^{-1}) at different manganese concentrations. Box plots show median values, interquartile ranges, and minimum and maximum values. Different letters accompanying means indicate differences ($P \leq 0.05$) among treatments according to Tukey's honestly significant difference (HSD) tests.

Table 1. Mean *Fomitiporia mediterranea* biomass production (g) and wood stick dry mass loss (%) for young and old wood at different manganese concentrations. Data are means ± standard deviations. Means in each column accompanied by different letters differ ($P \leq 0.05$) among manganese concentrations, as shown by Tukey's honestly significant difference (HSD) tests.

Mn^{2+} µM	Mean fungus biomass (g)		Mean wood mass loss (%)	
	Old	Young	Old	Young
0	0.349 ± 0.034 c	0.312 ± 0.091 c	17.026 ± 3.447 b	17.173 ± 1.936 b
80	0.772 ± 0.054 a	0.758 ± 0.086 a	33.373 ± 2.341 a	29.360 ± 2.865 a
160	0.581 ± 0.060 b	0.619 ± 0.095 b	30.226 ± 2.294 a	27.772 ± 4.233 a
320	0.241 ± 0.033 c	0.228 ± 0.040 c	14.533 ± 2.610 b	16.666 ± 1.368 b

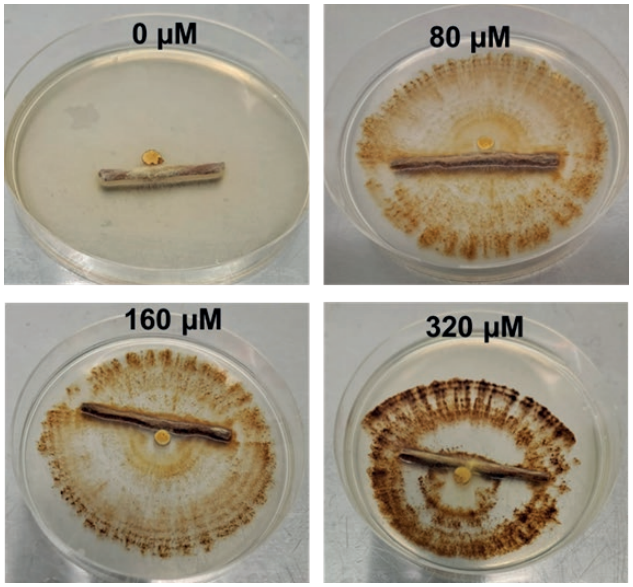

Figure 3. *Fomitiporia mediterranea* colonies on *Vitis vinifera* wood sticks, after 18 d incubation, on agar containing different manganese concentrations.

tion and wood degradation were low, and were not different ($P > 0.05$) from the 0 µM treatment.

Qualitative observations of the *F. mediterranea* colonies detected visible differences among treatments (Figure 3). Compared to the experimental controls without manganese, increased release of dark brown/orange pigmentation was observed with the increasing manganese concentration treatments.

Part II: Effects of Mn^{2+} on grapevine wood discs and manganese peroxidase production

Effects of Mn^{2+} on wood disc degradation

The biomass loss experiment was repeated using old wood and cross-section discs obtained from grapevine

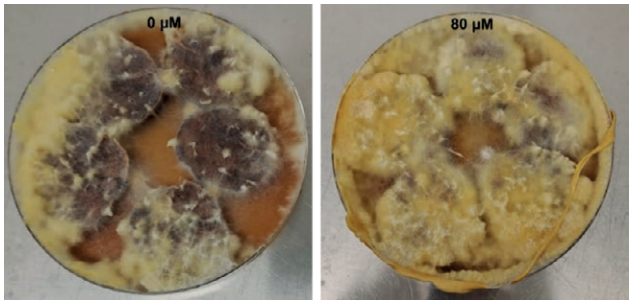

Figure 4. Growth of *Fomitiporia mediterranea* on grapevine wood discs (0 µM Mn^{2+} (left) or 80 µM (right).

branches (Figure 4). Old wood was selected because it is the grapevine tissue preferentially colonized by *F. mediterranea* under natural conditions. In this second experiment, the initial wood mass was increased to determine whether the differences observed in the first part of the study could be repeated under conditions of greater substrate availability.

The non-inoculated control treatment did not result in detectable changes in wood dry biomass at the end of the experiment. The results (Figure 5) showed the same overall trend observed in Part I for the old wood substrate. At 80 µM and 160 µM manganese concentrations, biomass loss was greater ($P \leq 0.05$) than in the 0 µM manganese control treatment, whereas at 320 µM manganese, biomass loss was comparable to that of the control.

Across treatments, highest degradative activity was consistently observed at 80 µM manganese, although no differences ($P > 0.05$) were detected between the 80 and 160 µM Mn^{2+} treatments, further supporting the effect of intermediate manganese concentrations in enhancing fungal degradative activity.

Effects of Mn^{2+} on production of manganese peroxidase

In the negative experimental control (not shown Figure 6, below), consisting of the liquid substrate without

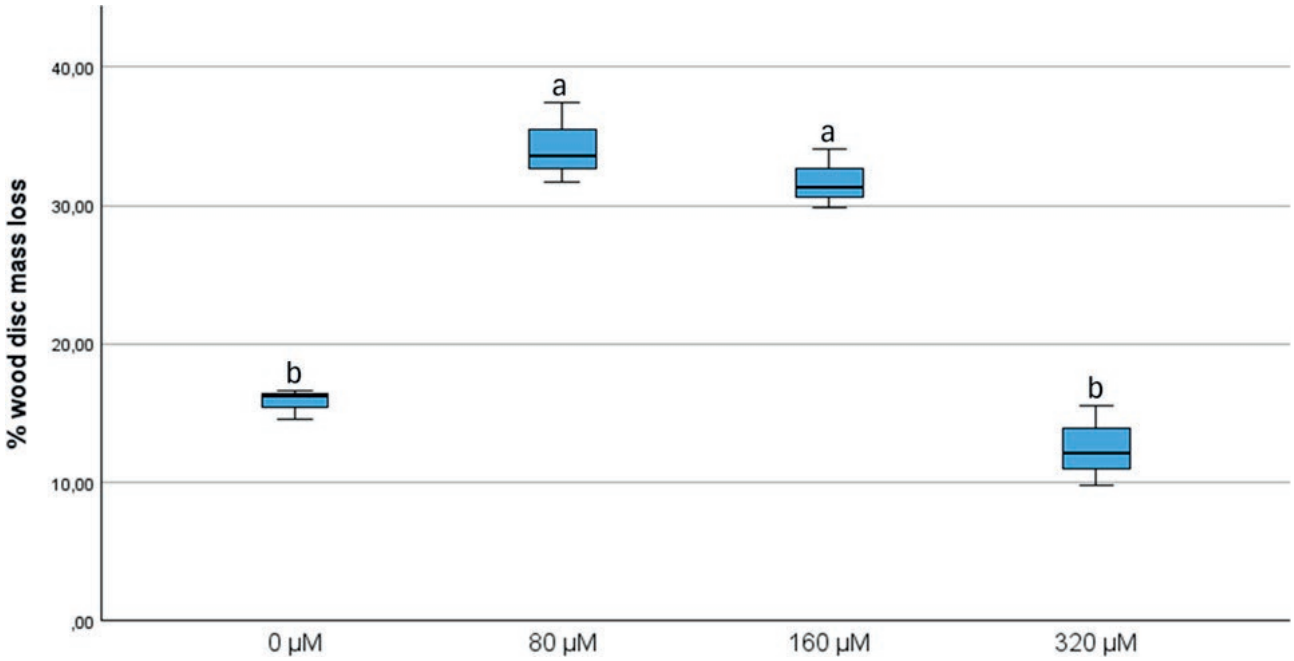

Figure 5. Mean weight losses for old *Vitis vinifera* wood discs in *Fomitiporia mediterranea* cultures at different manganese concentrations. The box plots show median values, interquartile ranges, and minimum and maximum values. Means accompanied by different letters are different ($P \leq 0.05$) according to Tukey's honestly significant difference (HSD) tests.

fungus inoculation, no manganese peroxidase activity was detected. At 80 µM and 160 µM manganese concentrations, however, peroxidase activity was greater than from the control treatment without manganese. Greatest MnP activity was detected at 80 µM Mn^{2+}, as mean specific activity of 4.19 ± 0.284 U mg^{-1} (mean ± SD), followed by the 160 µM treatment, which gave mean activity of 3.855 ± 0.289 U mg^{-1}. Both of these treatments were different from the 0 µM and 320 µM manganese treatments. The lowest MnP activities were recorded from the 0 µM control treatment (0.791 ± 0.218 U mg^{-1}), or from 320 µM Mn^{2+} (1.412 ± 1.561 U mg^{-1}).

DISCUSSION

The Part I results from this study highlight the concentration-dependent role of Mn^{2+} in modulating growth, biomass production, and wood-degrading ability of *F. mediterranea*. Manganese at intermediate concentrations was stimulatory, but inhibitory to the fungus at high concentration, confirming its dual role as an essential micronutrient and a potential toxic element for fungal metabolism, particularly in fungi causing white rot (Gadd, 1994; Baldrian, 2003). Manganese-enriched substrates have been shown to enhance manganese peroxidase production and wood-degrading activity in other white rot fungi, including *Ceriporiopsis subvermispora*, where Mn^{2+} availability directly stimulated ligninolytic enzyme systems and decay efficiency (Benavides *et al*., 2024). These results support the hypothesis that Mn^{2+} plays a key regulatory role in oxidative wood decay processes activated by the white rot pathogen *F. mediterranea*.

The Mn^{2+} concentrations used in the present study were selected to match experimentally relevant ranges previously reported in white rot pathogen systems. Mancilla *et al*. (2010) investigated a comparable gradient of Mn^{2+} concentrations, and identified an intermediate range as optimal for MnP induction, providing a methodological and biological basis for the concentrations adopted here. Among the manganese treatments applied, the 80 µM concentration promoted the greatest *F. mediterranea* growth rates and biomass production, indicating an optimal Mn^{2+} availability for metabolism and enzyme activation in this pathogen. The increased growth observed on young host wood could be related to differences in wood structure and chemical composition, including low lignin recalcitrance and high accessibility of carbohydrates (Blanchette, 1991).

Unlike mycelial radial growth, *F. mediterranea* biomass accumulation did not differ between young and old wood, indicating that manganese availability played a more dominant role than wood age, although wood age strongly influences characteristics, such as increased

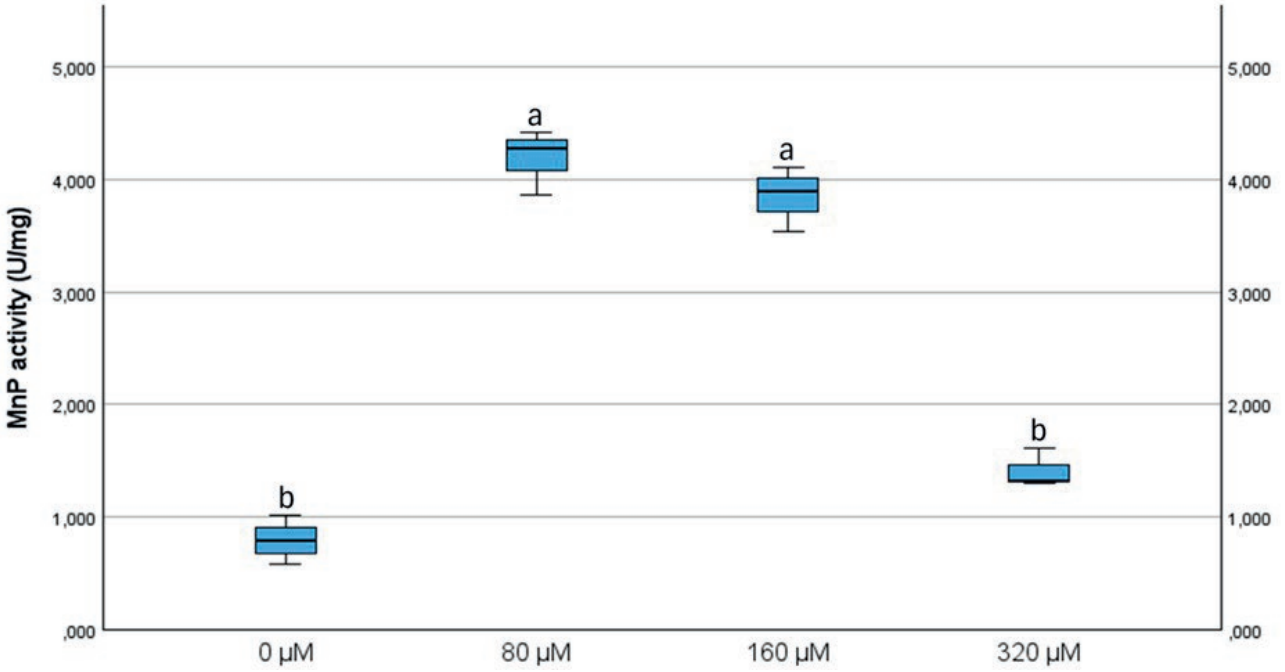

Figure 6. Mean manganese peroxidase activity in submerged *Fomitiporia mediterranea* cultures at different manganese concentrations and enriched with old *Vitis vinifera* sawdust. The box plots show median values, interquartile ranges, and minimum and maximum values. Different letters indicate differences ($P \leq 0.05$) among treatments according to Tukey's honestly significant difference (HSD) tests.

lignin content and structural complexity (Fengel and Wegener, 1984; Blanchette, 1991), and regulates mycelium production. Despite the lack of statistical significance, biomass production tended to be greater in old than in young wood. This is consistent with field observations, where grapevine trunk diseases primarily affect old grapevines. The observed stimulatory effect of manganese on fungus growth and biomass accumulation was similar to effects reported in previous research, where Mn^{2+} played a key role in support of oxidative metabolism and ligninolytic enzyme systems in white rot fungi, and promoted mycelium development (Hatakka, 1994; Hofrichter, 2002).

The wood degradation assays further indicated the stimulatory role of manganese at intermediate concentrations. At 80 µM and 160 µM Mn^{2+}, wood mass losses were greater than in the control treatment (no added manganese), confirming that manganese enhanced *F. mediterranea* degradative capacity. The absence of statistically significant differences between these two concentrations indicates a saturation effect, similar to that reported elsewhere. This indicates that manganese-dependent enzymes, such as MnP, reach maximum activity within limited Mn^{2+} concentration ranges (Hatakka, 1994).

At the greatest manganese concentration tested (320 µM), both growth rate and biomass production of *F. mediterranea* were reduced compared to the manganese-free control, indicating toxic effects. Excess Mn^{2+} has been shown to overwhelm fungal homeostatic mechanisms, causing ion imbalance, oxidative stress, and interference with essential cellular processes, as has been demonstrated in *Saccharomyces cerevisiae* and suggested for filamentous fungi (Yang *et al*., 2005; Robinson *et al*., 2021). This inhibitory threshold may also have ecological relevance for ECDs. Blanchette *et al*. (1984) reported localized Mn^{2+} accumulation within black zone lines that separate decayed from apparently healthy host wood tissues. The inhibitory effects observed at high Mn^{2+} concentrations in the present study indicate that localized Mn^{2+} accumulation could influence fungal activity within these transition zones. However, this interpretation requires direct *in planta* validation.

Qualitative observations also showed increasing release of dark brown/orange pigmentation in grapevine wood with increasing Mn^{2+} concentrations**.** Based on microscope observations and on the occurrence of Mn-rich dark zone lines previously described in decayed wood tissues (Blanchette *et al*., 1984), it is likely that this pigmentation is associated with manganese oxide compounds generated through fungal-mediated Mn^{2+} oxidation**.** Similar phenomena have been described in white rot fungi, and are often linked to manganese peroxidase

activity, which oxidizes Mn^{2+} into the highly reactive Mn^{3+} involved in ligninolytic processes (Li *et al*., 2022). However, further chemical and spectroscopic analyses are required to confirm the precise nature of these compounds.

The second part of the present study was designed to further investigate the role of Mn^{2+} in grapevine wood degradation, and to assess the possible link between biomass loss and manganese peroxidase activity, using experimental conditions that resemble those encountered by *F. mediterranea* during wood colonization in grapevine tissues. Consistent with the results obtained in Part I, manganese supplementation enhanced wood degradation at intermediate concentrations of 80 µM and 160 µM Mn^{2+}, whereas no enhancement was observed at 320 µM Mn^{2+}. These results confirm the concentration-dependent effects of Mn^{2+} observed in Part I, while extending these observations to natural grapevine wood conditions, and on an increased experimental scale. Given that *F. mediterranea* is considered a key species associated with ECDs, and that its wood degradation by-products have been suggested as contributors to foliar symptom development (Mugnai *et al*., 1999), these results further support the potential involvement of Mn^{2+}-dependent mechanisms in GLSD. Increased Mn^{2+} availability probably enhances fungus degradative activity, and consequently favours production or release of metabolites potentially associated with GLSD foliar symptom expression.

The analyses of manganese peroxidase production provided a mechanistic explanation for the observed wood degradation patterns. MnP activity increased with increasing manganese concentrations, and reached significant levels at 80 µM and 160 µM Mn^{2+}, followed by a decline at the greatest Mn^{2+}concentration assessed. This bell-shaped response mirrored the trends observed for *F. mediterranea* growth, biomass production, and wood degradation, indicating the functional link between Mn^{2+} availability, MnP expression, and fungus degradative capacity. Manganese peroxidase is a key enzyme in white rot fungi, catalyzing the oxidation of Mn^{2+} to Mn^{3+}, which acts as a low-molecular-weight diffusible oxidant capable of attacking lignin and other phenolic compounds at a distance from hyphae (Hatakka, 1994; Hofrichter, 2002). The enhanced MnP activity observed at intermediate manganese concentrations provides a direct explanation for the increased biomass loss measured in both parts of the present study. Similar manganese-dependent regulation of MnP activity has been reported in other white rot fungi, including *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*, where Mn^{2+} availability is a limiting factor for efficient ligninolytic activity (Couto *et al*., 1998; Kannaiyan *et al*., 2015). Mn^{2+} has been shown to be an essential requirement for stimulation of extracellular MnP production in several white rot fungi, including *C. subvermispora*, *Trametes versicolor*, *P. chrysosporium*, and *Pleurotus ostreatus*, further supporting the central role of manganese in regulating ligninolytic enzyme systems (Brown *et al*., 1991; Ruttiman-Johnson *et al*., 1992; Johansson *et al*., 2002). For ECDs, MnP-mediated Mn^{2+} oxidation and the associated production of diffusible Mn^{3+} chelates may contribute to the spatial dynamics of lignin modification and wood cell wall degradation in infected grapevine tissues. These oxidative processes could influence the extent and pattern of wood decay caused by *F. mediterranea*, so factors influencing Mn^{2+} concentration could influence wood decay process and its consequences for host plant leaves. However, given the complexity of Esca aetiology, and the involvement of multiple fungal species and host responses, this interpretation should be considered as a working hypothesis rather than a direct causal link.

At 320 µM Mn^{2+}, the decline in MnP activity likely reflected interference with enzyme production and/or stability, consistent with the inhibitory effects observed on grapevine growth and biomass. As discussed for Part I (above), this may result from disruption of Mn^{2+} homeostasis, where excessive intracellular Mn^{2+} impairs oxidative metabolism (Robinson *et al*., 2021).

Some limitations of the present study should be acknowledged. Firstly, all experiments were conducted under controlled *in vitro* conditions, which, while allowing precise manipulation of Mn^{2+} availability, do not capture the full complexity of *in planta* environments, including host immune responses, competing microorganisms, and dynamic soil chemistry. Secondly, while the Mn^{2+} concentrations used were selected based on a comparable study of *Ceriporiopsis subvermispora* (Mancilla *et al*., 2010), the direct correspondence to manganese bioavailability in grapevine wood under natural conditions remains to be determined. Thirdly, the present study used one isolate of *F. mediterranea*, and intraspecific variability in Mn^{2+} sensitivity and MnP production capacity cannot be excluded. These limitations underscore the need for follow-up *in vivo* and field-based research to validate and extend the results reported here.

The results reported here provide important insights into the mechanisms underlying grapevine wood colonization and degradation by *F. mediterranea*, the white rot agent of ECDs. This pathogen preferentially colonizes old wood, where manganese availability may locally favour MnP-mediated lignin degradation,

thereby facilitating pathogen spread and the progressive development of white rot. A possible implication of these results is that soils with high manganese availability may enhance plant uptake of this element, and consequently promote manganese accumulation in woody tissues, as manganese is readily absorbed by roots and translocated through host xylem, particularly under acidic soil conditions or when Mn^{2+} bioavailability is high (Kabata-Pendias, 2000; Fageria *et al*., 2010; Marschner, 2011).

On this basis, it can be hypothesized that soil manganese availability probably influences the development of grapevine wood colonization by modulating MnP activity, and, therefore, wood degradation efficiency in Mn-dependent trunk pathogens such as *F. mediterranea*, although this remains to be confirmed by dedicated *in planta* experiments.

CONCLUSIONS

The combined results of both experimental parts of this study demonstrate that Mn^{2+} plays a key, concentration-dependent role in regulating the growth, enzymatic activity, and wood-degrading capacity of *F. mediterranea*. In addition, the use of different wood types and experimental procedures demonstrated that while substrate characteristics (e.g., young vs. old wood) can influence pathogen growth dynamics, manganese availability is a dominant factor controlling decay efficiency.

The present study was conducted using one isolate of *F. mediterranea*, which is an inherent limitation. Intraspecific variability in MnP and laccase activity has been documented in *F. mediterranea*, and associated with differential expression of peroxidase-encoding isoforms (Pacetti *et al*., 2022). Therefore, the absolute enzymatic values recorded in the present research should not be generalized for the species. Other isolates may exhibit different baseline MnP activities and different responses to manganese supplementation. Future research including multiple isolates with contrasting enzymatic profiles is required to determine whether the stimulatory effects of manganese on MnP production and wood degradative capacity observed in this study are a conserved physiological response across *F. mediterranea* strains or are an isolate-dependent trait.

Overall, these results highlight the need to investigate micronutrient bioavailability and the influencing factors of this bioavailability, as potential abiotic drivers of wood colonization in Esca complex progression, with manganese emerging as a key factor influencing pathogenesis.

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