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Degradative Activity of Five Basidiomycota Strains on Sweet Chestnut Wood

Livio Torta [1], Vito Armando Laudicina [1,*], Sara Paliaga [1], Marika Lamendola [1], Patrizia Cancemi [2], Andrea Laschi [1] and Luigi Badalucco [1]

[1] Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo, 90128 Palermo, Italy; livio.torta@unipa.it (L.T.); sara.paliaga@unipa.it (S.P.); marika.lamendola@unipa.it (M.L.); andrea.laschi@unipa.it (A.L.); luigi.badalucco@unipa.it (L.B.)

[2] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90128 Palermo, Italy; patrizia.cancemi@unipa.it

* Correspondence: vitoarmando.laudicina@unipa.it; Tel.: +39-091-2389-7074

Abstract

Basidiomycetes can colonize sweet chestnut (*Castanea sativa* Mill) xylem, causing white or brown rot and losses in wood quality. The aim of this study was to assess the degradative potential of five Basidiomycota strains (*Armillaria mellea* (Vahl) P. Kumm. (Am), *Fistulina hepatica* (Shaeff.) With. (Fh), and *Laetiporus sulphureus* (Bull.) Murrill (Ls), and two strains of *Ganoderma resinaceum* Boud.) on three chestnut woods differing in chemistry. The woods differed in nitrogen content (0.3%–1.0%), carbon/nitrogen (C/N) ratio (43–150), and phenolic-related traits. In a 39-day laboratory assay, the five fungal strains were inoculated on three chestnut woods and compared for colonization time, extracellular enzymatic activity, and C mineralization. Fungal colonization strongly depended on fungus × wood interaction: *L. sulphureus* colonized all woods within 6 days, whereas the two *G. resinaceum* strains required 9–33 days depending on wood type; *A. mellea* and *F. hepatica* colonized only selected woods (up to 39 days). Enzymatic screening indicated laccase activity mainly in *G. resinaceum* (and to a lesser extent *A. mellea*), while *L. sulphureus* expressed cellulolytic activity but no laccase. Over 39 days, total C mineralization peaked under *G. resinaceum* on the two Sicilian woods (up to 270–300 mg CO_2–C g^{-1} dry wood), whereas the Tuscan wood (highest C/N and phenolic content) markedly inhibited most strains; only *L. sulphureus* increased mineralization in this wood (85 mg CO_2–C g^{-1} dry wood). These findings indicate that wood chemistry, especially C/N ratio and phenolic traits, strongly modulates strain-specific decay patterns. Overall, these results highlight the need for an integrated biological–biochemical approach to evaluate fungal decay potential and to inform both the selection of more durable chestnut woods for wood products and the identification of efficient strains to accelerate lignocellulosic biomass composting.

Keywords: basidiomycota; chestnut; wood degradation; c mineralization; enzymatic activity; fungus × wood interaction

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1. Introduction

The sweet chestnut (*Castanea sativa* Mill.) is the only native species of its genus in Europe and is able to grow throughout the whole continent up to Mediterranean regions, ranging from Morocco to southern England and Belgium, to Turkey and Eastern Europe, to the Caspian Sea. The main European chestnut forests are concentrated in Italy, France and the Iberian Peninsula [1].

In the middle of the last century, the species was seriously threatened with extinction due to the parasite attack of the "alien" fungal pathogen *Cryphonectria parasitica* (Murrill) M.E. Barr, causal agent of the chestnut blight that decimated both wild and planted populations. The natural diffusion within the infected areas of hypovirulent strains of the same invasive pathogen induced the partial recovery from the disease, and recently, the sweet chestnut has been widely spread by coppicing existing woods and replanting [2].

Chestnut trees are generally cultivated for several purposes: fruiting, ornamental, soil conservation and timber production. Chestnut woods are widespread in Europe, covering more than 2.50 million hectares, of which 2.20 million hectares are pure chestnut forests [3]. In Italy, they cover over 780,000 ha, with more than 147,800 ha dedicated to fruit production and 605,000 ha to wood production [4]. This last use (managed as coppice and high forest) is the most important because of the intrinsic resistance of chestnut wood to decay and insect attacks [5]. However, some fungal species belonging to the Basidiomycota, agents of wood decay, are able to colonize the host xylem, decomposing mainly lignin and cellulose, thus causing white rot or brown rot, respectively [6], and leading to heavy loss of production. The brown rot fungi, degrading the cellulosic components of woody tissues, do not alter the more stable lignin structure, whereas white rot fungi, generally producing both cellulolytic and ligninolytic degrading enzymes, have the potential to degrade the entire wood structure [7]. The degradative activity is due to the array of the lignocellulolytic enzymes that are deeply different among fungal species and also among strains of the same species [8–10]. On the other hand, each host tree can express different resistance–susceptibility to colonization by wood-decay fungi, as consequence of genetic and ecological factors [11].

The most common wood-decay fungi of chestnut are *Daedalea quercina* (L.) Pers., *Fistulina hepatica* (Schaeff.) With. and *Laetiporus sulphureus* (Bull.) Murrill, agents of brown rot, whereas the polyphagous *Armillaria mellea* (Vahl.) P. Kumm. and *Ganoderma* spp. cause white rot [7,12,13]. Moreover, *A. mellea* is a primary pathogen of broad-leaved and coniferous trees, while *F. hepatica* has gained relevance within woods and is believed to be responsible for Chestnut Red Stain (CRS) in *C. sativa* stumps and trunks [14]. Once the host is infected, both fungal species can remain latent for a long time (even for several years) and produce symptoms and signs only in the presence of suitable environmental conditions and in stressed plants [15–18]. Although all these fungi can induce severe damage to chestnut wood in both standing plants and wooden artifacts, their ecological role is fundamental in the recycling of the tree biomass and, in particular, of highly stable lignin. Therefore, considering the substantial aspects of the stability of standing trees and the technological characteristics of the wooden material, as well as the turnover of soil organic matter through the carbon cycle, it becomes relevant to answer the question that Schwarz et al. [12] posed regarding the progression rate of the deterioration and degradation of chestnut wood. Moreover, the carbon flux from decaying woody debris represents a crucial uncertainty within global carbon climate models, so more detailed information is needed for improving model outputs [19,20].

A meta-analysis of 36 studies from all forested continents revealed that nitrogen, phosphorus, and C:N ratio of wood correlate with decomposition rates of angiosperms [20].

The degradative activity of wood-rotting fungi can be evaluated and quantified by several methods, such as the rate of the fungal colonization on wood samples [21], changes in physical and chemical wood parameters (elemental composition, functional groups, and total polyphenol content [22–24]), the characterization of the fungal enzyme arrays [25] and the assessment of the mineralization rate of the organic matter within the tree biomass [21,26].

Assessing the degradative activity of fungi against different chestnut woods is important because it can provide insights about the durability of wood, which, in turn, depends on the aggressiveness of fungi and the resistance of chestnut wood [27]. From a practical point of view, such information is of great importance because it could help select both chestnut wood clones that are more resistant to fungal decomposition activity and, conversely, the most aggressive fungi to speed up the composting process of wood pruning residues [28,29]. Recent studies [30–32] on *Castanea sativa* have shown that chestnut wood quality is influenced by stand and site conditions, and that biotic alterations in chestnut wood are associated with measurable changes in technological properties and the lignin/cellulose balance. In parallel, current research on wood-decay fungi indicates that host preference and degradation efficiency depend not only on the white rot/brown rot distinction but also on the fungal capacity to attack angiosperm-specific cell-wall components. These findings support the need for substrate-specific studies on the interaction between chestnut wood chemistry and fungal functional traits.

Based on this background, we hypothesized that: (H1) the five Basidiomycota strains differ in degradative activity because of their distinct functional traits; (H2) chestnut woods differing in chemistry differ in susceptibility to fungal attack, with higher phenolic content and wider C/N ratio reducing degradation; (H3) more specifically, we expected that strains expressing stronger lignocellulolytic activity would show faster colonization and greater C mineralization, whereas woods with higher phenolic/extractive content and a higher C/N ratio would be less susceptible to degradation [30,33,34]. Accordingly, the objective of this study was to compare five Basidiomycota strains on three sweet chestnut woods by integrating colonization rate, extracellular enzymatic activity, and C mineralization.

2. Materials and Methods

2.1. Fungal Strains and Chestnut Wood Samples

The five fungal strains employed in this study were selected from a collection maintained at the Laboratory of Plant Pathology of the Department of Agricultural, Food and Forest Sciences of the University of Palermo (Italy). Specifically, one strain each of *A. mellea* (Am), *F. hepatica* (Fh), and *L. sulphureus* (Ls), and two strains of *Ganoderma resinaceum* Boud. (GrP and GrCh; Figure 1, Table 1) were tested. The selected strains were intended to represent a functionally relevant spectrum of chestnut-associated or broadly wood-decaying Basidiomycota, including taxa with different ecological roles and degradative strategies. In particular, *F. hepatica* was included because of its current relevance to Chestnut Red Stain in *C. sativa*, *L. sulphureus* as a representative brown rot fungus, *A. mellea* as a pathogenic wood-decay basidiomycete, and two strains of *G. resinaceum* to assess possible intraspecific variability in degradative behavior.

Table 1. Identification and provenance of basidiomycetes used in this study.

Species	Code	Host	Provenance	Wood Rot	GenBank Accession No.
F. hepatica	Fh	*Quercus pubescens*	Monreale (PA), Sicily	Brown	MT581893
A. mellea	Am	*Ficus microcarpa*	Palermo, Sicily	White	MT581894
G. resinaceum	GrP	*Celtis australis*	Pisa, Tuscany	White	MT581895
G. resinaceum	GrCh	*Salix babylonica*	San Giuseppe Jato (PA), Sicily	White	MT581896
L. sulphureus	Ls	*Eucalyptus camaldulensis*	Monreale (PA), Sicily	Brown	MT581897

All the colonies were grown on the universal agarized culture medium Potato Dextrose Agar (PDA) and incubated in the dark at 25 °C for 7–14 days, depending on the growth rate of each strain.

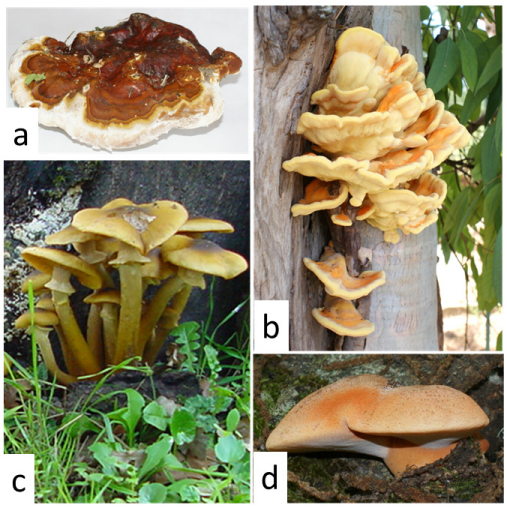

Figure 1. Fruiting bodies of the fungal strains used in this study: (**a**) *Ganoderma resinaceum* Boud; (**b**) *Laetiporus sulphureus* (Bull.) Murrill; (**c**) *Armillaria mellea* (Vahl) P. Kumm.; (**d**) *Fistulina hepatica* (Shaeff.) With.

Three chestnut wood samples per location from shoots that were more than 5 years old were taken from coppiced trees grown in Ficuzza (province of Palermo, Sicily; CSF), the Nebrodi Mountains (province of Messina, Sicily; CSN) and Sammommè (province of Pistoia, Tuscany; CSS). Each wood sample consisted of 3 sub-samples, i.e., radial sections 5 cm in diameter and 3 cm thick, or sawdust obtained by milling and sieving the wood samples at 2 mm.

2.2. Chestnut Wood Characterization

Total carbon, nitrogen, sulfur and hydrogen contents were determined using a Perkin-Elmer 2400 CHNS/O elemental analyzer (PerkinElmer Scientifica Italia s.r.l, Milan, Italy). The total phenol content was assayed using Folin–Ciocalteu reagent according to Singleton et al. [35]. Briefly, 1 g of powdered chestnut wood was stirred with 10 mL of methanol for 30 min. The suspension was kept for 24 h at 4 °C, filtered through Whatman No. 4 filter paper, evaporated under vacuum to dryness, dissolved in 1 mL of methanol, brought up to 10 mL with water, and stored at 4 °C until use. Then, 10 mL of 1:10 (v/v) Folin–Ciocalteu reagent was added to 2 mL of diluted sample. The mixture was shaken and allowed to stand for 6 min before adding 8 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) solution. The absorbance was read at 760 nm after 90 min of incubation at 23 °C. The total phenol content was expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE g^{-1}). The calibration curve range was 0.05–0.5 mg of gallic acid mL^{-1}. A Perkin-Elmer Spectrum Two FTIR spectrometer equipped with an attenuated total reflectance (ATR) device was used for acquiring the spectra (i.e., transmittance vs. wavenumber) of chestnut wood samples [36]. A few milligrams of each powdered sample was used in order to acquire the spectra in the wavenumber range 4000–450 cm^{-1}, with a resolution of 4 cm^{-1} and 8 scans.

Wood chemistry analyses (elemental composition, total phenols, and ATR-FTIR spectra) were used to test H2, i.e., whether differences among chestnut woods could explain differences in fungal performance, whereas colonization assays, extracellular laccase and Carboxymethyl cellulase (CMCase) determinations, and C mineralization measurements were used to test H1 and H3 by linking fungal growth, functional traits, and whole-wood degradation.

2.3. Fungal Growth Rate

The fungal colonization, expressed as number of days (growth rate) needed to colonize the entire surface of the radial sections of each wood sample (Ø 5 cm × 1.5 cm thick; Figure A1), was evaluated in a moist chamber. In Pyrex glass Petri dishes (10 cm in diameter and 2 cm in height), 3 layers of bibulous paper were placed, with a slide on top. Then, 5 mL of distilled water was added, and finally, each radial section of each wood sample was placed into the moist chamber, over the slide. The plates were sterilized in an autoclave at 121 °C (1 atm) for 20 min. Then, the autoclaved wood samples were sterilely inoculated by placing a plug (6 × 6 mm) of each fungal strain colony at the center of the sample. Wood samples inoculated with disks of sterile PDA were used as controls. All samples were then incubated in the dark at 25 °C. Every 3 days the diameter of the colonies grown on the surface of the wood samples (average of two orthogonal diameters and of three replicates) was measured, using a stereoscopic microscope (Zeiss 47 50 52–9901), until colonization of the wood surface was complete [7,37].

2.4. Analysis of Laccase Production and Potential Activity

The laccase production of the five basidiomycetes was determined by a qualitative test, which was carried out on fungal colonies, according to Pointing [38] with some modifications. In brief, on the surface of each fungal colony, three drops of 0.1% *w*/*v* syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde, in 95% ethanol) were added. The change of color of the drops from pale yellow to purple, due to the oxidation of syringaldazine, indicated the production of laccase. The potential activity of laccase was assessed by a quantitative test conducted through a solid submerged fermentation on both wheat bran and chestnut wood [39]. Plugs (6 × 6 mm) of each fungal strain colony were transferred into 250 mL Erlenmeyer flasks containing a liquid medium made of 33 mL of phosphate buffer (50 mM, pH 6) and either 1 g of wheat bran, or 1 g of chestnut sawdust as the solid substrate. Uninoculated flasks were used as controls. The flasks were incubated at 25 °C in the dark. On days 7 and 14 after the start of incubation, the liquid fraction of each flask was filtered through a sterile cheesecloth and centrifuged (4 °C, 10,000 rpm, 5 min); then the crude extracts were used for the laccase activity determination. The laccase activity was spectrophotometrically determined by monitoring the absorbance increase from oxidation of syringaldazine at 530 nm (ε = 65 mM^{-1} cm^{-1}) every 15 s until the increase was linear. Aliquots of 0.8 mL of sodium acetate buffer solution (50 mM, pH 4.5), 0.1 mL of syringaldazine (0.1% *w*/*v*) and 0.1 mL of crude extract [40,41] were placed in 1 mL quartz cuvettes (path length 1 cm). One laccase enzymatic unit (Ue) was defined as the amount of enzyme that catalyzed the conversion of 1 µmol of syringaldazine per 1 mL of reaction mixture per minute.

2.5. Analysis of Cellulase Production

A plate assay method using 0.1% (*w*/*v*) of carboxymethylcellulose (CMC) with 0.5% (*w*/*v*) agarose was used for screening the cellulase production by fungal strains [42] grown on chestnut wood or wheat bran. The crude extracts were centrifuged, and then 20 µL aliquots were pipetted onto filter paper dishes and deposited on the plates. Uninoculated liquid medium was used as a control. The plates were incubated at 37 °C for 24 h, and the cellulase activity was indicated as clear halos after staining with 1% Congo red solution for 30 min and washing several times with 1 M NaCl. The magnitude of the cellulase activity was calculated as the volume of the halos (i.e., integration of optical density over the spot area) using ImageMaster 2D Platinum 6.0 software (Amersham Biosciences, Sweden), as previously reported [43–46].

2.6. Mineralization of Chestnut Wood

The amount of CO_2 emitted from sawdust inoculated with the fungal strains was used as a measure of wood decomposition rate by fungi. Aliquots of 2.5 g (dry weight) of chestnut sawdust were weighed into 200 mL glass bottles, filled with distilled water up to 50% of the water-holding capacity, sealed with a rubber stopper and sterilized in an autoclave at 120 °C for 20 min at 1 atm. After sterilization, the sawdust was inoculated by plugs (1 cm in diameter) of each fungal colony and then incubated at 25 °C ± 1 in the dark (Figure A2). Samples without fungal inoculation were used as controls. Throughout the 39 days of incubation, the CO_2 emitted during 24 h of incubation was measured on days 2, 4, 7, 9, 11, 15, 17, 24 and 39 by sampling an aliquot of gas from the headspace of bottles with a syringe and injecting it into a gas-chromatograph (TRACE GC, Thermo Scientific, Milano, Italia) equipped with a thermal conductivity detector (TCD). The total C mineralized over 39 days of incubation was calculated through the linear interpolation of two neighboring measured fluxes and the numerical integration over time [47], as reported in the following equation:

$$\text{Total C mineralized} = \sum_{i=1}^{n-1} (r_i + r_{i+1}) \times \frac{d_i}{2}$$

where i and i + 1 are the first and the last of two close CO_2–C rate measurements, n is the last day of measurement of CO_2–C rate, r is the CO_2–C rate expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, and d is the number of days between two consecutive CO_2 rate measurements.

2.7. Statistical Analysis

The reported data are the arithmetic means of three replicates; wood data refer to oven-dry wood (70 °C) weight. Total carbon, nitrogen, hydrogen, sulfur, C/N ratio, and polyphenols were subjected to one-way ANOVA (with wood provenance as the factor). Also, data on potential laccase activity on wheat bran were subjected to one-way ANOVA (with fungal strain as the factor). One-way ANOVA was followed by Fisher's multiple comparison test (LSD intervals, Least Significant Difference, at $p < 0.05$) for the separation of the means. Colonization rate, cellulase and laccase activity on chestnut wood samples and total C mineralized data were subjected to two-way ANOVA (fungal strain and wood provenance). Post hoc tests (Tukey at $p < 0.05$) were carried out among fungal strains within the same wood provenance and among wood provenances within the same fungal strain. Before performing parametric statistical analyses, normal distribution and variance homogeneity of the data were checked by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Residual maximum likelihood variance components were also performed to determine which of the two factors or their interaction accounted for the majority of the variation in each of the measured variables. All statistical analyses were performed using Statgraphics Centurion version 15.0 (Statpoint Inc., USA, 2005).

3. Results

3.1. Main Characteristics of Chestnut Woods

The three chestnut samples had similar concentrations of carbon (44.3% on average), hydrogen (6.3% on average) and sulfur (0.3% on average) by weight (Table 2). In contrast, the nitrogen concentration, ranging from 0.3 to 1.0%, was significantly different, being higher in CSN and lower in CSS. As a consequence, the C/N ratio was also significantly different among the samples, according to the following order: CSS > CSF > CSN (Figure 2).

Table 2. Elemental composition (mean ± standard deviation) of the three chestnut woods [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Different lower-case letters within a given variable indicate significant differences at $p < 0.05$.

Chestnut Wood Sample	Carbon %	Hydrogen %	Nitrogen %	Sulfur %
CSF	44.2 ± 0.9 a	6.6 ± 0.8 a	0.6 ± 0.1 b	0.4 ± 0.1 a
CSN	43.5 ± 1.1 a	6.1 ± 0.0 a	1.0 ± 0.1 a	0.3 ± 0.1 a
CSS	45.1 ± 0.8 a	6.2 ± 0.1 a	0.3 ± 0.1 c	0.3 ± 0.0 a

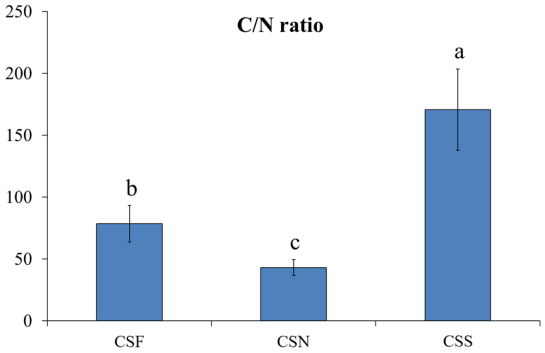

Figure 2. Carbon to nitrogen ratio (C/N) of the three chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Reported values are means ± standard deviations (n = 3). Different letters indicate significant differences at $p < 0.05$.

The chestnut sample from Tuscany showed the highest content of total polyphenols, whereas that from Ficuzza had the lowest (Figure 3). ATR-FTIR spectroscopy was used to assess differences among wood samples in terms of their chemical functional groups. As shown in Figure 4, regions A and B of the spectra suggested that the investigated woods had different relative distributions of the main chemical functional groups. Absorbance in the spectral range between 2820 and 2980 cm^{-1} (region A) was attributed to aliphatic saturated C-H stretching with methylene groups in cellulose. Such absorbance peaks were more intense in the Ficuzza wood compared to the other two chestnut samples. Region B was characterized by three main signals at 1730, 1640 and 1590 cm^{-1}. The signal at 1730 cm^{-1} was attributed to C=O carbonyls in ester groups and acetyl groups in xylan, that at 1640 cm^{-1} to absorbed O–H and conjugated C–O in polysaccharides, and that at 1590 cm^{-1} to skeletal vibrations from the C–C [48].

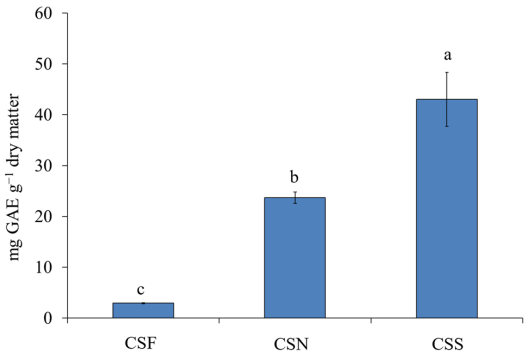

Figure 3. Polyphenol content of the three different chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Reported values are means ± standard deviations (n = 3). Different letters indicate significant differences at $p < 0.05$.

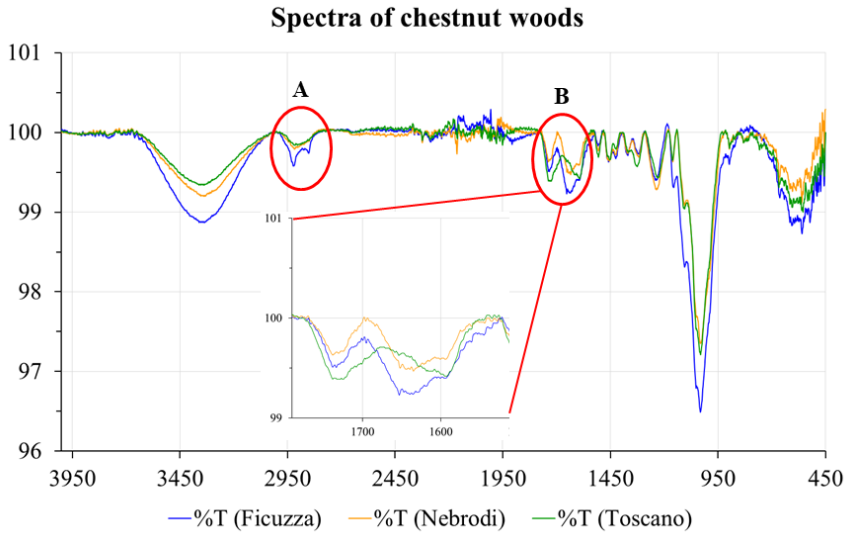

Figure 4. ATR-FTIR spectra of the three different chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Reported values are means ± standard deviations (n = 3).

3.2. Fungal Colonization Rate

The three fungal strains that grew on all the chestnut wood samples, Ls, GrP and GrCh, showed different colonization rates (Figure 5). In particular, Ls colonized the entire surface of the three samples in 6 days, while GrP and GrCh completed the colonization in 9, 27 and 33 days and 30, 27 and 18 days, respectively, on CSF, CSN and CSS. Am completed the colonization of the CSF and CSS wood samples in 39 days, while Fh, which grew only on the CSF wood sample, colonized the entire surface after 33 days of incubation. Therefore, the CSF wood sample was colonized by all the fungal strains, CSS showed resistance against Fh, and CSN against Fh and Am (Table 3).

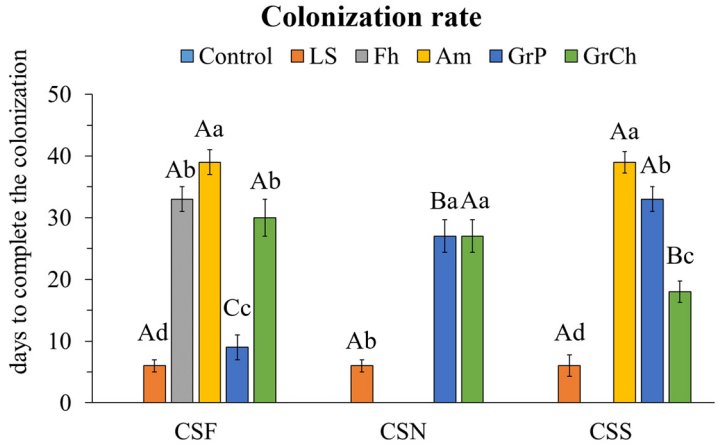

Figure 5. Colonization rate, i.e., number of days needed for *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls) and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains to colonize the entire surface of a radial section (Ø 5 cm × 1.5 cm) of each wood sample [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Different capital letters indicate significant differences ($p < 0.05$) among chestnut wood samples within the same fungal strain. Different lower-case letters indicate significant differences among fungal strains within the same chestnut wood sample. Reported values are means ± standard deviations (n = 3). The higher the height of the histograms, the lower the colonization rate of the wood radial section. The absence of a histogram indicates no fungal colonization.

Table 3. Susceptibility of the chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)] to colonization by *Armillaria mellea* (Vahl) P. Kumm. (Am), *Fistulina hepatica* (Shaeff.) With. (Fh), *Laetiporus sulphureus* (Bull.) Murrill (Ls), and *Ganoderma resinaceum* Boud (two strains: GrP and GrCh) strains: −, no colonization; +, colonization completed in more than 21 days; ++, colonization completed in 11–20 days; +++, colonization completed in less than 10 days.

Chestnut Wood Samples	Fungal Strains				
	GrCh	GrP	Ls	Am	Fh
CSF	+	+++	+++	+	+
CSN	+	+	+++	−	−
CSS	++	+	+++	+	−

The fungal colonization rate was affected by both tested factors individually, but especially by fungal strain (37%); as a consequence, the interaction between wood provenance and fungal strain explained the greatest amount of colonization rate variance (56%) (Table 4).

Table 4. Percentage of explained variance by experimental factor (wood provenance and fungal strains) and their interaction for each investigated parameter. **, and *** indicate significance at $p \leq 0.01$, and $p \leq 0.001$, respectively; NS, not significant.

Variables	Wood Provenance (W)	Fungal Strains (F)	W × F
	%	%	%
Colonization rate (days)	7 **	37 ***	56 ***
Laccase	NS	93 ***	NS
CMCase	NS	38 ***	56 ***
Total C mineralized	28 ***	35 ***	36 ***

3.3. Laccase Activity

The qualitative test for laccase production suggested that GrP and GrCh had the highest laccase activity, as confirmed by the fast and clear change in color from pale yellow to purple. Am showed a lower laccase production, confirmed by the slow change in color from pale yellow to light purple. Ls and Fh did not lead to any color changes, thus suggesting the absence of laccase activity (Figure A3).

With regard to the quantitative test, the spectrophotometric analyses carried out after 7 days of incubation on wheat bran and on chestnut wood sawdust revealed no laccase production. At day 14, laccase activity was observed for GrP, GrCh and Am but not for Ls and Fh (Figure A4). In particular, the highest laccase activity was registered for GrP, followed by GrCh and then Am (Figure 6), thus confirming the results of the qualitative analysis conducted using syringaldazine.

On chestnut sawdust, GrP and GrCh produced laccase with no significant differences between them within the same chestnut wood sample, whereas the strain GrP showed the highest activity when inoculated on the CSN wood sample (Figure 7). The other fungal strains did not show any laccase activity, since no significant differences were observed between them and the respective control. The production of laccase was affected only by fungi (93% of variance explained; Table 4).

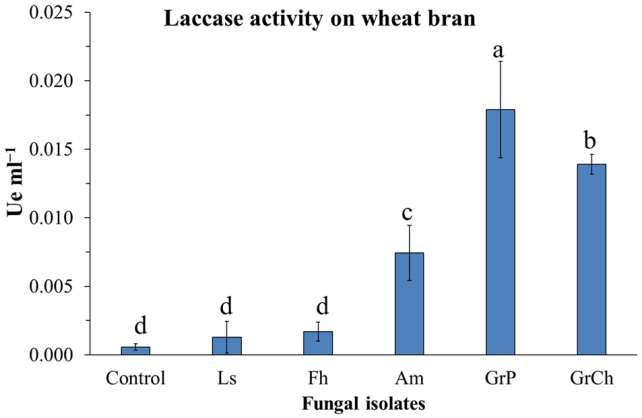

Figure 6. Potential laccase activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains during submerged fermentation using wheat bran as a substrate. The assay was carried out on day 14 of incubation. Different letters indicate significant differences at $p < 0.05$. Reported values are means ± standard deviations (n = 3).

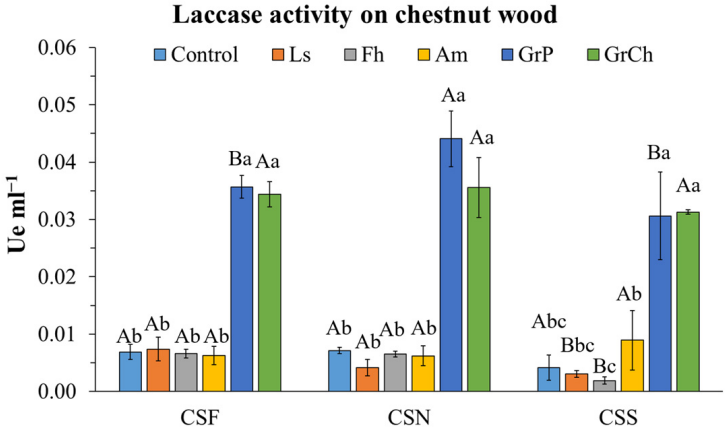

Figure 7. Laccase activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains grown on chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Different capital letters indicate significant differences ($p < 0.05$) among chestnut wood samples within the same fungal strain. Different lower-case letters indicate significant differences among fungal strains within the same chestnut wood sample. Reported values are means ± standard deviations (n = 3).

3.4. Cellulase Activity

The Congo red test showed that the five fungal strains had different CMCase activity on wheat bran (Figure A5). Ls, GrP and GrCh showed the highest activity, followed by Am and, finally, Fh (Figure 8). The CMCase activity depended on fungal strain taken alone (38% of variance explained) but not on wood provenance alone; however, the two factors combined explained 56% of the variance among the fungi (Table 4).

On the CSF wood sample, the highest CMCase activity was shown by Am, whereas the activity of the other fungi was less than half that of Am (Figure 9). On CSS wood, the highest activity occurred with Ls, whereas no activity was found for Fh; the CMCase activity of the other fungal strains was half that of Ls. The CMCase activity was similar among the five fungal strains on the CSN wood sample, with the exception of GrP, which did not show any activity.

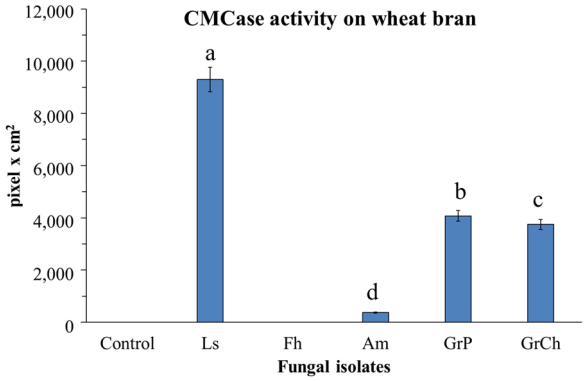

Figure 8. Potential carboxymethyl cellulase (CMCase) activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains during submerged fermentation using wheat bran as a substrate. The assay was carried out on day 14 of incubation. Different letters indicate significant differences at $p < 0.05$. Reported values are means ± standard deviations (n = 3).

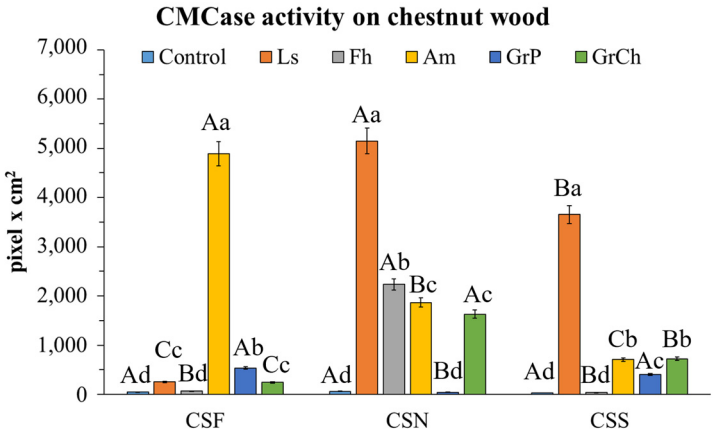

Figure 9. CMCase activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains grown on chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Different capital letters indicate significant differences ($p < 0.05$) among chestnut wood samples within the same fungal strain. Different lower-case letters indicate significant differences among fungal strains within the same chestnut wood sample. Reported values are means ± standard deviations (n = 3).

3.5. Mineralization Rate of Chestnut Wood

The mineralization rate showed significant differences among either chestnut wood types or fungal strains (Figure 10). As a consequence, also total C mineralized was affected, almost to the same extent, by both tested factors as well as by their interaction (Table 4).

GrP and GrCh determined the highest mineralization rate (Figure 10) compared to the other fungi when inoculated on CSF, with no significant differences between them. Also, the other three fungal strains increased the mineralization rate, compared to the control; however, they showed different decomposition activities. As a consequence, the total C mineralized was highest for GrCh and GrP, followed by Ls and Am, and finally by Fh (Figure 11).

Also, in CSN, GrCh exhibited the highest mineralization rate, followed by GrP and to a lesser extent by Am, Ls and Fh. As a result, the total C mineralized was the highest in GrCh, followed by GrP, then Ls, and finally Am and Fh, which did not show significant differences between them. The mineralization rate of CSS wood was higher than the control only with *L. sulphureus* (Figure 9). Consequently, the total C mineralized was the highest

with *L. sulphureus*, whereas no differences were observed among the other fungal strains and the control (Figure 11).

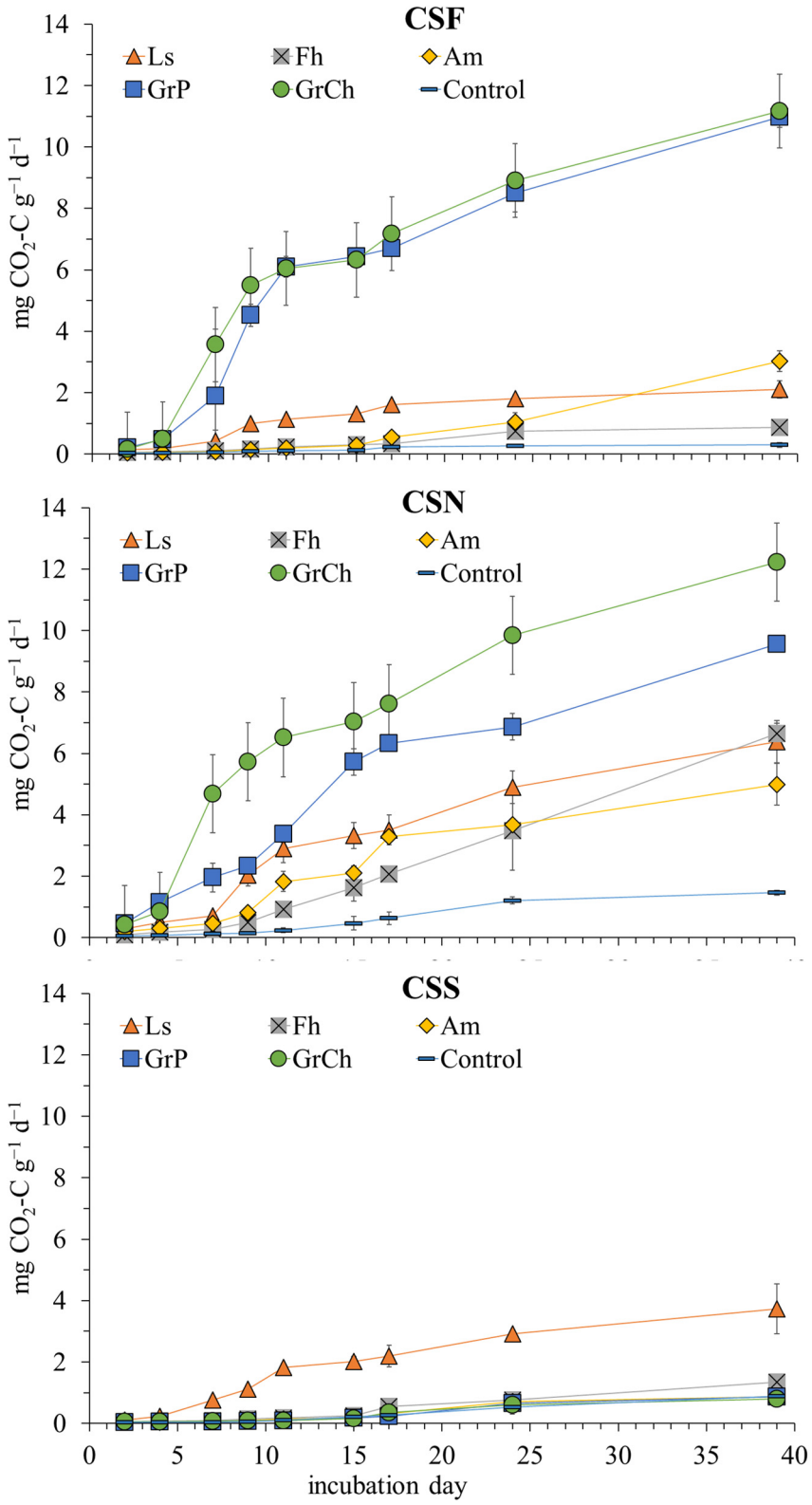

Figure 10. Mineralization rate of chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)] inoculated with *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh), during 39 days of incubation. Reported values are means ± standard deviations (n = 3).

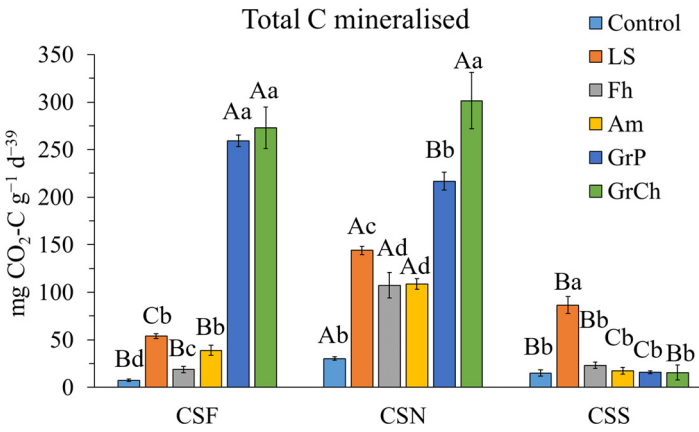

Figure 11. Total C mineralized during 39 days of different chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy] inoculated with *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains. Different capital letters indicate significant differences ($p < 0.05$) among chestnut wood samples within the same fungal strain. Different lower-case letters indicate significant differences among fungal strains within the same chestnut wood sample. Reported values are means ± standard deviations (n = 3).

It is of note that in CSF and CSN, the mineralization rate started to increase exponentially after 7 days only when they were inoculated with the two strains of *G. resinaceum*. Moreover, CSS was the chestnut wood type that exhibited the lowest mineralization rate.

Taken together, the enzymatic and mineralization data indicate that whole-wood C loss was associated with different functional strategies among fungi. In the Sicilian woods, the highest C mineralization was observed for the two *G. resinaceum* strains, which also showed the clearest laccase activity, whereas in CSS, the only marked increase in mineralization was observed for *L. sulphureus* despite the absence of detectable laccase, suggesting a greater contribution of cellulolytic and other non-laccase mechanisms to C turnover in that wood type.

4. Discussion

The integrated approach, including biological, chemical and biochemical assays, used for determining the degradative activity of the five tested basidiomycota strains on various sweet chestnut woods, suggested that wood decay depended on both its chemical features and different enzymatic arrays synthetized by each fungal strain devoted to the degradation of cellulosic or ligninic wood compounds.

Our results are consistent with recent studies showing that wood-decay performance depends on fungal functional strategy as well as substrate chemistry. The rapid colonization of chestnut wood by *L. sulphureus*, together with detectable cellulolytic activity and no measurable laccase, agrees with recent multi-omics evidence that this species follows a non-canonical brown rot strategy based on carbohydrate-active enzymes and Fenton-related oxidative chemistry on lignocellulosic substrate [49]. By contrast, the comparatively limited performance of *A. mellea* in our assay is compatible with recent evidence that *Armillaria* spp. express an atypical decay repertoire, with stronger investment in pectinases and expansins than in lignin-decaying enzymes [50]. The high laccase activity and C mineralization shown by the two *G. resinaceum* strains are also in line with the recognized extracellular degradative potential of wood-decay *Ganoderma* spp. [51]. From a chestnut-specific perspective, recent studies confirm that *F. hepatica* is a relevant cause of Chestnut Red Stain and wood devaluation in *C. sativa* stands, suggesting that pathogenic relevance in

the field may not necessarily coincide with high short-term mineralization under controlled laboratory conditions [52,53].

The FTIR spectra of the chestnut wood samples suggested that they differed in lignin structure, with the CSS wood being more lignified, as indicated by the more intense band (1730 cm^{-1}) related to xylan [48,54], which is more abundant in hardwoods than softwoods. These results were also confirmed by the lower concentration of N and the higher polyphenol content in CSS compared to the other two chestnut woods [55]. The two *G. resinaceum* strains (GrP and GrCh), known as etiological agents of white wood rot on several species of broad-leaved and coniferous trees [7,12,13], were detected for the first time on chestnut wood in this study. The ascertained high wood degradative activity indicates this fungal species is a potential cause of decay for chestnut timber and related wood products. These two strains showed, on average, higher degradative activity on chestnut woods, as suggested by the lowest colonization rate and the highest mineralization rate compared to the other fungi. These results agree with previous studies [7,56] which reported all the species belonging to genus *Ganoderma* as active wood-decay agents of many forestry and cultivated trees.

The higher degradative activity of GrP and GrCh could be due to the higher laccase activity compared to the other fungi, thus confirming our first hypothesis. The laccase activity of GrP and GrCh was higher than that reported by Elissetche et al. [57] for *G. australe* (0.001−0.002 Ue mL^{-1}) cultivated in submerged conditions with minimal substrate. In contrast, it was much lower than that reported by Sitarz et al. [58] for a strain of *G. lucidum* (2.9−3.1 Ue mL^{-1}) cultivated in submerged conditions, using standard liquid substrate supplemented with sugar cane bagasse as further substrate. Moreover, the two *Ganoderma* strains tested in this study were also able to produce cellulase that may contribute to speeding up the degradation of the wood matrix. However, the degradative activity of the two strains depended on the chemical properties of the chestnut woods, which were able to limit the fungal activity, thus protecting the wood from quick fungal decay [11]. Indeed, the CSS wood had the highest content of polyphenols, which, as reported by Mihara et al. [59], have a remarkable antimicrobial activity. The inhibitory behavior of CSS may be related to the chemical peculiarities of sweet chestnut heartwood, which is rich in extractives, especially polyphenols and tannins, including ellagitannins such as vescalagin and castalagin [60,61]. More generally, recent reviews and durability studies indicate that wood extractives act as natural defense compounds and contribute to decay resistance, whereas extractive depletion is associated with reduced durability and weaker inhibition of fungal attack [33,34,62]. Within this framework, the higher phenolic content and wider C/N ratio of CSS may have contributed to the lower colonization and mineralization shown by most strains, although the specific inhibitory compounds were not directly quantified in the present study. On the other hand, the CSS wood also had higher xylan and a higher C/N ratio compared to CSF and CSN, thus suggesting a greater lignification grade [48,54]. As reported by D'Agostini et al. [63] and Nadeem et al. [64] for some basidiomycota wood-decaying fungi, there is an inversely proportional relationship between C/N ratio and laccase production. This was also observed for CSN and CSS; in fact, the highest laccase activity was recorded on the Nebrodi chestnut wood that had the lowest C/N ratio, whereas the lowest was recorded on Tuscan chestnut wood with the highest C/N ratio.

Finally, it is of note that GrCh and GrP showed different mineralization and colonization rates on the same chestnut wood. The higher mineralization rate of CSN induced by GrCh compared to GrP may be ascribed to the higher cellulase production by the former fungal strain. The colonization rate confirmed this different behavior between the two fungal strains, being the highest with GrCh in CSF and the lowest in CSS. This finding

suggested that different strains of the same fungal species could have different wood degradative activity. Similar findings were reported by Elissetche et al. [57] for *Ganoderma australe* and by Janusz et al. [9] for *Flammulina velutipes* strains. The integrated approach used to investigate the degradative activity of *L. sulphureus* provided contrasting results. On one hand, *L. sulphureus* showed the lowest colonization time, i.e., the highest rate to colonize a radial section of chestnut wood, and a similar mineralization activity across all the chestnut woods. On the other hand, it did not produce laccase but only cellulase on CSN and CSS, and wheat bran. Thus, these results confirmed that *L. sulphureus* is an agent of wood brown rot due to its ability to produce cellulase, but this activity may be affected by the wood type. The chemical analyses carried out on the chestnut woods used in this study did not allow us to assess which factor could inhibit the production of cellulase on a specific wood sample (e.g., CSF). This is an aspect worthy of further investigation. *A. mellea* and *F. hepatica* were the strains that showed the lowest degradation ability, as confirmed by the mineralization rate and total C mineralized, although, depending on the substrate, they were able to synthetize laccase and CMCase, respectively, to same extent.

Such lower degradative ability shown under laboratory conditions can be ascribed to the need of these two fungal species for different growing conditions rather than those maintained during the laboratory tests. Deepening the knowledge related to the degradative activity on chestnut wood by some of the tested species is particularly important, since these decaying fungi could compromise the mechanical characteristics of the wood also used in products. Otherwise, the enzymatic array of each fungal species and, within each species, of various single strains can considerably differ due to environmental, genetic and trophic features [9,57,65]. The variable presence of molecules with antifungal activity, such as polyphenols, tannins, etc., but also heavy metal content such as nickel, lead, or copper, could play a crucial role in inhibiting or stimulating the fungal activity [22–24].

Taken together, the results support H1 and H2, whereas H3 was only partially confirmed. H1 was supported by the clear inter- and intraspecific variation in fungal behavior, especially between the two *G. resinaceum* strains and between *G. resinaceum* and *L. sulphureus*. H2 was also confirmed, as the Tuscan wood (CSS), characterized by higher phenolic content and a wider C/N ratio, was consistently less susceptible to colonization and mineralization than the Sicilian woods. In contrast, H3 received only partial support: although the enzymatic profiles were consistent with the high mineralization shown by *G. resinaceum*, they did not fully explain the rapid colonization and selective mineralization pattern of *L. sulphureus*, indicating that additional oxidative or other unmeasured enzymatic mechanisms likely contributed to wood decay. This interpretation is consistent with the recent mechanistic study of de Figueiredo et al. [49] showing that *L. sulphureus* adopts a broader lignocellulose deconstruction strategy than can be inferred from laccase and CMCase alone, and that *A. mellea* does not always conform to a canonical white rot pattern.

Overall, from an ecological point of view, these results suggested that the selection of the most appropriate chestnut wood for products has to take into account not only the intrinsic chemical properties of the wood but also all the potential variability of the decay fungal agents, as well as within the same fungal species. On the other hand, the selection of fungal strains with the highest degradative activity may be useful to speed up the composting process of lignocellulosic biomasses.

5. Conclusions

This study showed that the degradative activity of Basidiomycota on sweet chestnut wood is strongly influenced by both fungal identity and substrate characteristics. The five strains displayed distinct colonization, enzymatic, and mineralization patterns, including clear intraspecific variation between the two *G. resinaceum* strains. Differences among

the three chestnut woods further indicated that wood chemistry, particularly phenolic content and C/N ratio, plays an important role in modulating fungal performance and decay intensity. At the same time, the relationship between enzymatic activity and C mineralization suggests that the measured laccase and CMCase activities captured only part of the underlying degradative mechanisms. Overall, the results highlight the value of an integrated approach combining wood chemistry, colonization dynamics, enzyme assays, and C mineralization to assess fungal decay potential in chestnut wood. These findings may be useful both for the selection of more durable chestnut wood for timber applications and for the identification of fungal strains with greater potential for controlled lignocellulosic biomass conversion.

Author Contributions: Conceptualization, L.T. and V.A.L.; methodology, L.T. and V.A.L.; investigation, L.T., V.A.L., S.P., M.L., P.C. and A.L.; resources, L.T. and V.A.L.; data curation, L.T. and V.A.L.; writing—original draft preparation, L.T. and V.A.L.; writing—review and editing, L.T., V.A.L., S.P., M.L., P.C., A.L. and L.B.; supervision, L.T. and V.A.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

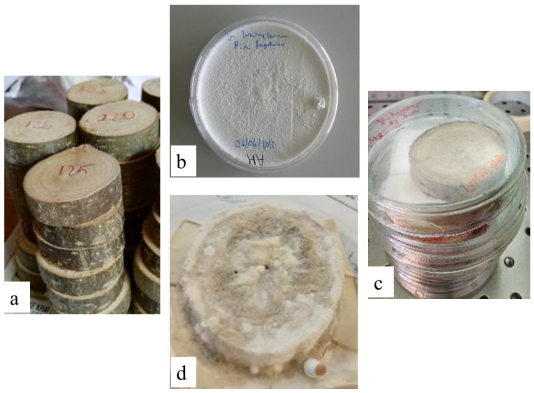

Figure A1. Inoculation of chestnut wood: (**a**) radial section of chestnut wood samples (Ø 5 cm × 1.5 cm); (**b**) *G. resinaceum* (GrP) 7-day-old colony; (**c**) moist chambers with inoculated radial section of a chestnut wood sample; (**d**) complete colonization of a chestnut wood sample by *G. resinaceum* (GrP).

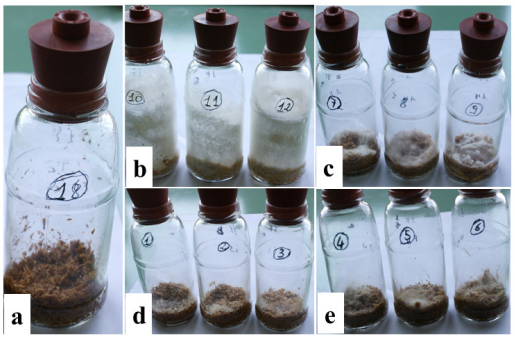

Figure A2. Glass bottles used for the determination of carbon mineralization. Different mycelial growth of the tested fungal strains inoculated on chestnut wood samples from Nebrodi (CSN): (**a**) control; (**b**) *G. resinaceum* (GrP); (**c**) *A. mellea*; (**d**) *L. sulphureus*; (**e**) *F. hepatica*.

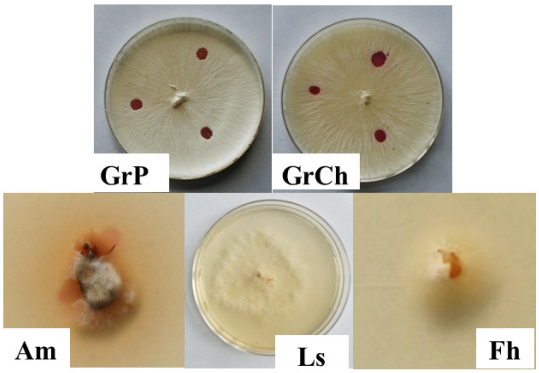

Figure A3. Qualitative test to detect laccase production using syringaldazine by *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains. Color change of syringaldazine from yellow to purple indicates positive results: the higher the color intensity, the greater the production of laccase (GrP and GrCh > Am). The absence of color changes indicates no laccase production (Ls and Fh).

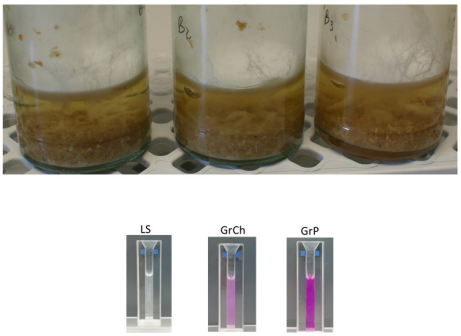

Figure A4. Fermentation in solid submerged substrate (wheat bran) for the determination of potential laccase activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) strains. Syringaldazine oxidation in different extracts after 14 days of incubation is also shown: negative reaction for Ls and positive reaction for GrCh and GrP.

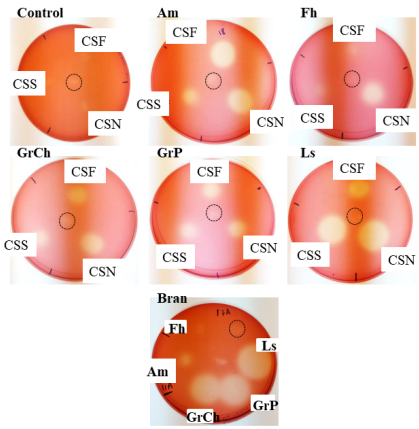

Figure A5. Plate tests for carboxymethylcellulase activity of *Armillaria mellea* (Am), *Fistulina hepatica* (Fh), *Laetiporus sulphureus* (Ls), and *Ganoderma resinaceum* (two strains: GrP and GrCh) grown on chestnut wood samples [CSF, sample from Ficuzza (province of Palermo, Sicily, Italy); CSN, sample from the Nebrodi Mountains (province of Messina, Sicily, Italy); CSS, sample from Sammommè (province of Pistoia, Tuscany, Italy)]. Uninoculated liquid medium (33 mL of phosphate buffer, 50 mM, pH 6 and either 1 g of wheat bran or 1 g of chestnut sawdust as solid substrate) was used as a control. Dashed circles indicated the response of the phosphate buffer on carboxymethylcellulase substrate.

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