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Evaluation of Anti-Fungal Activities of Environmentally Friendly Wood Preservative from Thermal-Induced Lignified Twigs

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Abstract: Enhancing the decay resistance of *Populus tomentosa* wood through environmentally friendly methods is crucial for improving its durability and market competitiveness. Lignified twigs (LT), typically unsuitable as timber due to their small diameter, are rich in lignin, which degrades during thermal induction to produce antifungal organic compounds. In this context, the objective of this study was to develop a lignified twig preservative (LTP) by thermal induction from the LT of *Actinidia chinensis* var. *Jinyang*. The antifungal activity of LTP under varying thermal conditions was analyzed, along with its chemical composition. Enzyme activity, cell membrane integrity, and respiratory metabolism in fungi treated with LTP were examined to elucidate antifungal mechanisms. Additionally, the decay resistance of LTP-treated wood was evaluated. Results revealed that LTP produced under N_2 at 220 °C exhibited robust antifungal activity against *Trametes versicolor* and *Gloeophyllum trabeum*, attributed to phenolic compounds such as syringaldehyde, syringone, vanillin, and vanillone. LTP inhibited fungal cellulases, hemicellulases, and ligninases by 30%–60%, disrupted cell membrane functionality, and suppressed respiratory metabolism. Poplar wood treated with LTP demonstrated significantly enhanced decay resistance (mass loss < 10%). This thermal-induced feedback pattern shows great potential for LT in wood preservation.

Keywords: antifungal activity; antifungal mechanism; lignified twigs; thermal induction; wood preservatives

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1. Introduction

Wood is a natural and renewable material that is widely used in architecture, interior decoration, and other fields due to its numerous advantages. These include a high strength-to-weight ratio, excellent heat insulation performance, wide availability, ease of processing, and unique aesthetic qualities. The area of plantation forests is increasing globally to meet the demand for wood and fiber production, reduce the pressure on logging natural forest, and address environmental protection requirements [1]. Poplar (*Populus tomentosa* C.) is a fast-growing tree species widely planted in Chinese plantation forests [2]. The Three-North Shelter Forest Program in China has widely planted poplar trees. Studies indicate that thinning poplar trees older than 30 years is necessary to ensure effective afforestation and carbon sequestration [3]. Additionally, triploid poplar, which shows significant advantages in net photosynthetic rate and growth rate, has been widely researched and promoted [4,5]. Therefore, poplar wood has become an excellent material for alleviating the supply–demand imbalance in timber. However, poplar is susceptible to biological deterioration caused by insects and fungi, with fungal decay being one of the most severe forms. This decay can

have irreversible negative effects on the appearance of poplar, as well as its physical and mechanical properties [6–9].

In the past, wood preservatives containing heavy metals such as copper, chromium, and arsenic were widely used. These preservatives were effective in inhibiting wood decay and played a significant role in enhancing the durability of wood and extending its lifespan [10]. However, in recent years, some researchers have expressed concerns regarding the potential environmental threats posed by these preservatives [11–14]. As a result, there has been a growing focus on exploring environmentally friendly wood preservatives with low pollution and a wide range of sources. The efforts for environmentally friendly solutions have prompted researchers to focus on natural substances derived from a diverse range of botanical sources. It is practically significant to explore plant-derived raw materials as potential sources for wood preservatives. Promising studies have explored the efficacy of essential oils [15,16], tannins [17], alkaloids [18,19], and other organic compounds from various plants for their potential use as wood preservatives [20].

Thermal modification is a wood modification technique that relies on low-temperature (about 200 °C) thermal degradation reactions to alter the composition and properties of wood. Through this process, the resistance of wood to decay experiences a notable enhancement [21]. However, the mechanical properties of thermally modified poplar diminish as the heat treatment becomes more intense [22]. During thermal modification, lignin undergoes cleavage or condensation reactions with itself and other degradation products, resulting in the formation of substances that possess antifungal properties and inhibit the growth of microorganisms [23,24]. At higher temperatures (over 300 °C), wood undergoes intense pyrolysis, producing mixtures with antifungal activity against wood-decaying fungi, such as wood tar [25], wood vinegar [26], and pyroligneous acids [27]. Some researchers have already applied these antifungal mixtures in wood preservation [28]. In our preceding investigation, we successfully derived extracts possessing potent antifungal properties from thermally modified wood [29]. Building on this foundation, we speculated whether lignified materials universally have the potential to yield antifungal substances after thermal induction. Certain dicotyledons without thick trunks, such as *Actinidia Lindl.*, *Salix cheilophila*, and *Caragana korshinskii* Kom., produce a large number of lignified twigs (LT) during regular pruning. These twigs have even smaller diameters than small logs and cannot be processed into wood chips for the production of particleboard or fiberboard. In Shaanxi Province, China, where this study was conducted, extensive cultivation of Actinidia results in the annual production of a significant quantity of such LT during pruning, which are not effectively utilized. In the past, most LT were typically burned or discarded, leading to resource wastage or environmental burden [30]. Consequently, we selected Actinidia as the raw material of our study. In light of the fact that the antifungal ingredient generated during thermal modification mainly originates from the degradation of lignin, it is plausible to consider LT endowed with a heightened degree of lignification as a promising candidate for the development of wood preservatives.

Given the urgent need to enhance the decay resistance of fast-growing poplar wood in an environmentally friendly manner, and that LT are unsuitable for traditional wood applications, this study focuses on developing environmentally friendly wood preservatives from LT using thermal induction processes. These lignified twig preservatives (LTP), acting as feedback to poplar wood, are anticipated to enhance the lifespan of poplar. The antifungal activity and mechanism of LTP were investigated while evaluating the potential effectiveness of the LTP in enhancing the biological durability of treated poplar.

2. Materials and Methods

2.1. Materials

The poplar (*Populus tomentosa* C.) samples were collected from the artificial forest in Huoditang Experimental Forest, Northwest A&F University, located at 33°25′−33°29′ N latitude and 108°25′−108°30′ E longitude, with an altitude ranging from 1420 to 2474 m in Shaanxi Province, China. Thirty-year-old poplar trees were selected as samples, and their sapwood, with an annual ring width of 4 mm and a moisture content of 8%, was cut into wood blocks measuring 20 × 20 × 10 mm^3 (R × T × L).

Lignified twigs (LT) of *Actinidia chinensis* var. *Jinyang* with a diameter of 0.5–2.0 cm were obtained from Shaanxi Province, China. The LT were dried in an oven at 85 °C until their moisture content was below 8%, followed by pulverization using a grinder, and the powder with a size range of 40–60 mesh was sieved and reserved for later use.

The chemical reagents involved in this study are as follows: The potato dextrose agar (PDA) was purchased from Aoboxing Biotechnology Co., Ltd. (Beijing, China). The potato dextrose broth (PDB) was purchased from Huankai Microbial Technology Co., Ltd. (Guangzhou, China). The methyl vanillate (>98%) was purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). The bovine serum albumin V (BSA, MW 68000), isovanillic acid (Reference Standard), phosphate-buffered saline (PBS), and syringic acid (Reference Standard) were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). The 2,6-dimethoxyphenol (>99.92%) and coniferyl aldehyde (>95%) were purchased from Bide Pharmatech Co., Ltd. (Shanghai, China). The dimethyl sulfoxide (DMSO, AR, >99%) and DL-tartaric acid (AR, >99.5%) were purchased from Chron Chemicals Co., Ltd. (Chengdu, China). The manganese sulfate (AR, >99%), sodium acetate trihydrate (AR, >99%), citric acid monohydrate (AR, >99.5%), and sodium citrate dihydrate (AR, >99%) were purchased from Guanghua Sci-Tech Co., Ltd. (Guangzhou, China). The propidium iodide (PI, >94%) was purchased from Ranjeck Technology Co., Ltd. (Beijing, China). The microcrystalline cellulose (50 μm), salicin (>99%), and 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, >99%) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The acetosyringone (>98%) was purchased from Dibai Biotechnology Co., Ltd. (Shanghai, China). The 3,5-dimethoxy-4-hydroxybenzhydrazide (≥96.84) was purchased from Haohong Scientific Co., Ltd. (Shanghai, China). The xylan (from corn, >95%), vanillin (AR, >99%), syringaldehyde (>98%), acetovanillone (>98%) and sinapaldehyde (>98%) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). The D (+) glucose standard solution (Reference Standard) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The carboxymethyl cellulose (AR, CP 300–800 MPa·s) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The DL-tartaric acid (AR, >99.5%) and sodium tartrate dibasic dihydrate (AR, >99%) were purchased from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). The acetone (AR, >99.5%) was purchased from Xilong Science Co., Ltd. (Shanghai, China). This version preserves the information about the chemical purities while removing unnecessary details about suppliers and locations.

Trametes versicolor (L.) Lloyd represents white rot fungi, while *Gloeophyllum trabeum* (Pers.: Fr.) Murr. represents brown rot fungi, both of which were isolated from fruiting bodies on decayed wood and were identified and preserved by the China Forestry Culture Collection Center. PDA medium was disinfected for 30 min at 121 °C and then let to cool to 80 ± 10 °C. PDA cooled to 80 ± 10 °C was distributed into Petri dishes and let to cool to 25 °C. A 4.5 mm diameter disc was excised from the strains preserved by freezing of *T. versicolor* and *G. trabeum*. Then the disc was placed on the PDA medium. And then the dishes were cultivated in the incubator with a dark environment at 80 ± 5% RH and 28 ± 2 °C for 7 days for future use.

2.2. Optimization of the Thermal Induction Process

2.2.1. Thermal Induction of LT and Preparation of LTP

Ground-dried LT powder was spread on a tray and then placed in the oven with a circulating heating medium and subjected to a thermal induction for 6 h at the specified temperature and atmospheric pressure. The heating mediums were air and N_2 and the temperatures were set to a gradient of 180 °C, 200 °C, and 220 °C. Subsequently, the thermal-induced LT was left cool to 60 °C and stored in a desiccator. The 30 g of thermal-induced LT powders were mixed with 900 mL of acetone and subjected to ultrasonic treatment with an FS-1200N ultrasonic cell crusher (Shengxi, Shanghai, China) in 600 W for 10 min. The mixture was filtered and the filtrate was concentrated in a rotary evaporator (R502B, Senco, Shanghai, China) at 45 °C with a vacuum of 0.09~0.1 MPa after being collected by filtration. The solid mixture obtained after concentrating and removing all acetone is the lignified twig preservative (LTP). The preservative obtained comprises the air 180, 200, and 220 °C LTP (LTP-A180, LTP-A200, LTP-A220), the N_2 180, 200, and 220 °C LTP (LTP-N180, LTP-N200, LTP-N220), and the extracts from LT without thermal induction (UT).

2.2.2. Antifungal Activity Test

Due to the common practice of wood thermal treatment being conducted in the air, the antifungal activity of LTP-A220 was initially validated in preliminary experiments. It was found that the inhibition rate of LTP-A220 against *T. versicolor* and *G. trabeum* was 100% at concentrations of 4 mg/mL and 6 mg/mL, respectively. Therefore, the antifungal activity of all LTP obtained under different conditions was evaluated at a concentration of 2 mg/mL to determine the optimal thermal induction conditions. The PDA was disinfected for 30 min at 121 °C and then let to cool to 80 ± 10 °C and the DMSO solution of LTP obtained under different conditions was added into the medium, resulting in a final concentration of 2 mg/mL in PDA, with a DMSO concentration of 1.5% (*v*/*v*). These suspensions were distributed into Petri dishes and let to cool. Disks with a diameter of 4.5 mm were cut from the actively growing edge of each of the two test fungi cultures. These disks were placed on each dish containing LTP and cultivated in darkness at 28 °C and 85% relative humidity for 21 days. The control group consists of PDA containing only 1.5% DMSO (0 mg/mL) and three replicate samples were included in each group. Colony diameters were measured on the 2nd, 4th, 6th, 8th, 11th, 14th, and 21st days, and the inhibition rate was calculated according to Equation (1).

$$IR = (d_1 - d_2)/(d_1 - d_0) \times 100\% \quad (1)$$

where

IR—inhibition rate (%)
d_0—the diameter of the initial disc (4.5 mm)
d_1—the colony diameter of control fungi (mm)
d_2—the colony diameter of LTP fungi (mm)

2.3. Composition Analysis of LTP

The composition of LTP-N220 was analyzed, as it exhibited the strongest antifungal activity in LTP under various conditions. The LTP-N220 was diluted in acetone to 3 mg/mL. The solution was filtered multiple times through a 0.22-μm organic nylon filter to remove insoluble particles. Then the solution was analyzed by GC-MS (TRACE1310-ISQLT, Thermo Fisher, Waltham, MA, USA) with a TG-5MS fused silica column (30 m × 0.25 mm id., film thickness 0.25 μm). The carrier gas was nitrogen with a purity of 99.999% at a flow rate of 1 mL/min. The GC-MS parameters were set according to the method of Bi et al. [31].

The obtained GC-MS results were compared with the NIST 2014 MS spectral library to preliminarily identify the components. For substances with a relative content exceeding 1%, the corresponding pure compounds were purchased and subjected to the same GC-MS analysis. The retention time of LTP and pure compounds was compared to verify the identification results of GC-MS. Components with a relative content exceeding 1% were tested for antifungal activity in a concentration of 4 mg/mL, referencing Section 2.2.2.

The optical density (OD) at 540 nm for various concentrations of a D (+) glucose standard solution was measured using a UV-1200 UV spectrophotometer (Mepada, Shanghai, China), following the dinitrosalicylic acid (DNS) method described by Roberto et al. [32] and Xu et al. [33]. A standard curve was generated. The reducing sugar content in LTP under different conditions was determined based on the standard curve ($y = 0.1309x + 0.0289$, $R^2 = 0.9995$, Figure S1).

2.4. Determination of Cellulase, Hemicellulase, and Ligninase Activity

2.4.1. Cultivation of Crude Enzyme Solution

PDB was disinfected for 30 min at 121 °C and then let to cool to 80 ± 10 °C. The PDB was mixed with 0.2 g of poplar wood powder per 20 mL and supplemented with LTP-N220 of 1.5 mg/mL. The concentration levels of LTP-N220 were determined based on the antifungal activity test described in Section 2.2.2. The control medium contained 1.5% DMSO. Mycelia (0.2 g) of *T. versicolor* or *G. trabeum* were scraped from the growing medium and cultivated in a PDB medium containing LTP-N220 at 27 °C with shaking for 5 days. After 5 days, the liquid culture was retrieved and centrifuged at 8000 rpm for 10 min at 4 °C. The resulting supernatant was collected as the crude enzyme solution and stored at 4 °C for further use.

2.4.2. Cellulase Activity Determination

Cellulase activity was determined following the protocol outlined by Roberto et al. [32]. For endoglucanase, sodium carboxymethyl cellulose was utilized as the substrate, while microcrystalline cellulose and salicin served as substrates for exoglucanase and β-glucosidase, respectively. The substrate, crude enzyme solution, and buffer solution were mixed and incubated at 37 °C in a water bath for 60 min to initiate enzymatic hydrolysis. The concentration of glucose released from each enzymatic hydrolysis was calculated using the DNS method described in Section 2.3. Six replicates per fungus were conducted for each treatment, and the mean values were calculated.

2.4.3. Hemicellulase Activity Determination

The activity of xylanase was assessed using xylan as the substrate, following the DNS method described in Section 2.4.2. Six replicates were conducted for each treatment of each fungus, and the results were averaged. One international unit (IU) of enzyme activity is defined as the amount of enzyme capable of producing 1 μmol of reducing sugar (glucose or xylose) from the corresponding substrate within 1 min. Enzyme activity (IU) was calculated using Equations (S1) and (S2).

2.4.4. Ligninase Activity Determination

For manganese peroxidase and laccase, manganese sulfate and ABTS were used as reaction substrates. The enzyme activity was determined by measuring the change in optical density (ΔOD) at 238 nm (manganese peroxidase) and 420 nm (laccase) intervals of 1 min [34]. The enzyme activity unit (IU) for both enzymes was defined as the amount of enzyme required to produce a change of 0.01 units of OD within 1 min, calculated using Equation (S3).

2.5. Cellular Membrane Function of Decay Fungi

2.5.1. Mycelial Morphology

Disks with a diameter of 4.5 mm were cut from the actively growing edges of *T. versicolor* and *G. trabeum* cultures. Subsequently, they were placed on PDA medium supplemented with 1.5 mg/mL LTP-N220. Control plates were prepared using PDA media without LTP-N220. Two ethanol-cleaned coverslips were positioned on either side of the disc. Once mycelium had colonized the coverslip, the coverslips were carefully fetched out along with the attached mycelial mat for subsequent microscopic examination.

2.5.2. Leakage of Nucleic Acids and Proteins

Each 10 mL of PDB was supplemented with 15 mg of LTP-N220 and 0.2 g of healthy decay fungi mycelium and was cultivated for 5 days at 27 °C with shaking. The PDB without LTP-N220 was set as the control group. The culture medium was withdrawn and subjected to centrifugation, yielding a supernatant that was subsequently subjected to UV spectrophotometry at 260 nm. The quantity of nucleic acids in the culture medium was determined [31] through changes in optical density (ΔOD260 nm). Three replicates were conducted for each treatment of each fungus, and the results were averaged.

Optical density (OD) of different concentrations of a BSA were measured at 595 nm on a UV-1200 UV spectrophotometer according to the Coomassie brilliant blue (G250) method [32,33]. The standard curve was plotted with the equation $y = 0.2559x - 0.442$ and an R^2 value of 0.9996 (Figure S1). The protein concentration in the culture medium was determined using the G250 method and the standard curve. Each analysis was replicated 6 times per fungus per treatment and values were averaged.

2.5.3. Propidium Iodide (PI) Staining

The PI staining method was adapted from [35] with slight modifications. PDB of 4 mL containing 1.5 mg/mL LTP-N220 was added into *G. trabeum* and *T. versicolor* cultures actively growing to approximately halfway up the PDA medium. The culture was incubated with shaking at 28 °C and was removed after 12 h. The fungal mycelia were washed three times with PBS buffer to remove residual PDB. The mycelia were stained with a 2 μg/mL PI solution in the dark for 5 min, followed by a wash with PBS buffer. The stained mycelia were observed under a BX-50 biological microscope (OLYMPUS, Shinjuku City, Japan). The mycelia samples were illuminated by laser from the U-HGLGPS fiber-coupled fluorescence light source after passing through a FITC filter.

2.6. Respiratory Metabolism

The aerobic respiration level of *G. trabeum* and *T. versicolor* affected with LTP-N220 was determined by adding 0.2 g of mycelium collected from the actively growing culture of each fungus to 20 mL of PDB culture medium with 1.5 mg/mL of LTP-N220 in a closed electrolytic cell. The PDB without LTP-N220 was set as the control group. After allowing the reaction to equilibrate for 15 min, the dissolved oxygen concentration in the closed electrolytic cell was recorded every 15 s using a JPSJ-606L dissolved oxygen meter (Yidian, Shanghai, China). This monitoring continued for a total of 60 min.

2.7. Decay Resistance Test

2.7.1. Immerse Poplar with LTP-N220

The poplar wood samples ($R \times T \times L = 20 \times 20 \times 10$ mm^3) were dried in an oven at 105 °C until their mass remained constant (M_1, with a 0.01 g accuracy). The samples were immersed in an LTP-N220-acetone solution of 30 mg/mL and sustained a 15-min vacuum treatment (−0.08 MPa), and then sustained a pressurization at 0.8 MPa for 30 min. After

the pressurization ended, the samples were removed and dried at 60 °C until their mass remained constant. Subsequently, the aforementioned immersion treatments were repeated. After two rounds of immersion, the samples were dried to an oven-dry state at 105 °C, and their masses were recorded (M_2).

2.7.2. Decay Resistance Test

The sample subjected to LTP-N220 immersion and the control samples of poplar were each prepared. Six replicates were utilized in each set. These samples were positioned atop the mycelium of each decay fungi cultured for 7 days, with two samples per Petri dish. Subsequently, all samples were cultured for 8 weeks at 28 °C and a relative humidity of 80%. Upon completion of the incubation period, the mycelium was removed from the samples, which were then dried and weighed (M3). The mass loss (ML) of the samples was calculated using the Equation (2) [36]:

$$ML = (M_2 - M_3)/M_2 \times 100\% \quad (2)$$

where,

ML—mass loss after decay (%)
M_2—mass of the sample after immersion (g)
M_3—mass of sample after decay test (g)

2.7.3. Electronic Microscope Observation

The poplar samples were cut into sizes of 2 × 2 × 1 (R) mm^3 and dried for 24 h at 45 °C. Subsequently, the dried samples were coated with gold twice and observed under a scanning electron microscope (SEM SU5000, Hitachi, Chiyoda City, Japan). The acceleration voltage was set as 15 kV. The changes in poplar wood samples treated with LTP-N220 before and after the decay were focused.

2.8. Statistical Analysis

A *t*-test was conducted to assess the effects of LTP on the enzyme activity, leakage of nucleic acids and proteins of decay fungi, as well as the decay weight loss rate of LTP-treated poplar wood. The results are reported as mean ± standard deviation of the repeated measurements. Significance differences between average value are denoted as follows: 'ns' for no significant difference ($p \geq 0.05$), '*' for significant difference ($0.05 > p \geq 0.01$), and '**' for highly significant difference ($p < 0.01$). Post-hoc multiple comparisons of the inhibition rate of LTP with different thermal-induced conditions and main components of LTP to decay fungi were performed using Tukey's test. Significant differences between means are indicated by different letters (e.g., A, B, C) at a confidence level of 95% (*p*-value 0.05).

3. Results and Discussion

3.1. Antifungal Activity of Lignified Twigs Preservatives (LTP)

The inhibition rate (RI) of LTP-A220 against *G. trabeum* and *T. versicolor* was 100% at concentrations of 6 and 4 mg/mL respectively (Figure 1a). Remarkably, the LTP exhibited antifungal activity similar to extracts from the heartwood of *Tectona grandis* L. f. [37] and silverskin of coffee [18]. The inherent cost-efficiency of LT positions it as a promising contender for utilization as a raw material for wood preservation.

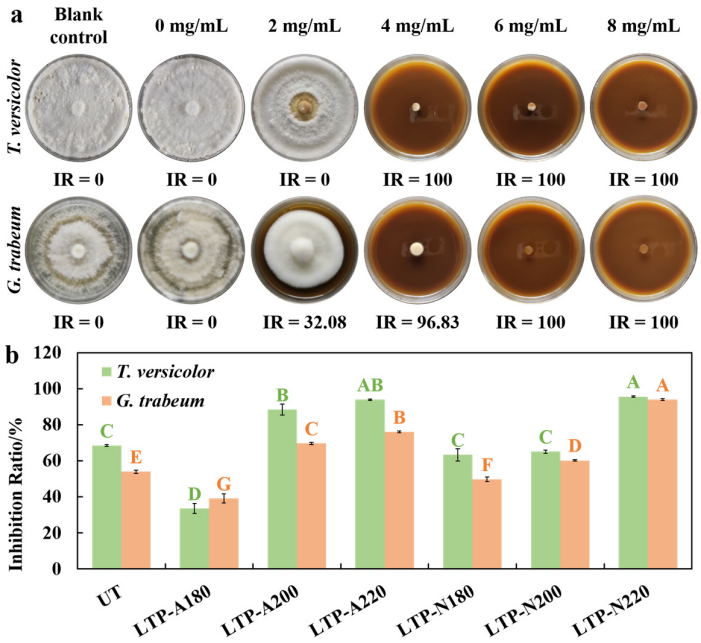


Figure 1. The growth situation of decay fungi upon a PDA with LTP-A220 at concentrations of 2, 4, 6, and 8 mg/mL on 14th day (**a**); The inhibition rate (IR) of different LTP against *T. versicolor* and *G. trabeum* at a concentration of 2 mg/mL on 6th day (**b**). Note: Significant differences between means are indicated by different letters (e.g., A, B, C. . .) at a confidence level of 95% (*p*-value 0.05).

LT were subjected to thermal induction under various conditions to enhance their antifungal activity. Subsequently, the growth of decay fungi was monitored in a medium supplemented with various LTP of 2 mg/mL (Figure 1b). All the LTP exhibited antifungal activity against both fungal species; even the extracts of LT that had not undergone thermal induction exhibited certain antifungal activity. This is attributed to the presence of various secondary metabolites in the entire *Actinidia Lindl*. plant, such as triterpenes, flavonoids, and phenols, among others, many of which exhibit antimicrobial properties [38]. However, at certain intermediate conditions (e.g., 180 °C with an oxygen-rich environment), some native antifungal compounds such as terpenes and flavonoids undergo partial pyrolysis, oxidation, and volatilization before new antifungal substances are generated in significant quantities, leading to a transient decrease in overall activity. It is worth noting that after undergoing thermal induction under certain conditions, this antifungal activity is significantly enhanced. The LTP using air and nitrogen as heating media exhibited an increased inhibitory effect on decay fungi as the treatment temperature increased. Analogously, the decay resistance of thermally modified poplar (*Populus beijingensis*) and eucalyptus (*Eucalyptus grandis*) exhibited an increased trend with the increase in treatment temperature [39,40]. These phenomena are consistent with the phenomenon of increased lignin degradation with increasing temperature in a nitrogen environment [40]. The heightened degradation of the LT at elevated treatment temperatures may lead to the generation of a greater quantity or potency of antifungal substances. The differences in treatment medium also influence the degradation pathways of LT, leading to variations in degradation byproducts. The presence of oxygen in the treatment medium can lead to the further oxidation of already-formed antifungal substances into compounds with weaker antifungal activity, resulting in LTP-N220 exhibiting a slightly superior inhibitory effect compared to LTP-A220 and demonstrating the highest antifungal activity among the six LTPs.

3.2. Composition Analysis of LTP

A gas chromatography-mass spectrometry (GC-MS) system was employed to perform a component analysis of LTP-N220 which exhibited optimal antifungal activity, and the results of gas chromatography are depicted in Figure S2. A part of the major components in LTP-N220 were authenticated by contrasting with the MS library, and their standard substance gas chromatography are depicted in Figure S3. It can be observed that the retention times of the major components in LTP-N220 coincide with those of corresponding standard substances, validating the accurate identification of these components based on mass spectrometry analysis. Table 1 presents the major components found in LTP-N220, with relative contents exceeding 1%, primarily consisting of phenolic compounds, long-chain alkanes, sugars, and other substances. In all components, 1, 2, 3, 5, 6, 9, 10, 11, 12, 13, and 15 may possess antifungal activity. Therefore, the antifungal activity of these components was validated using the growth rate method at a concentration of 4 mg/mL, consistent with the LTP-N220 assay.

Table 1. Components with a relative content exceeding 1% in the LTP-N220.

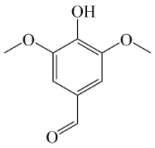
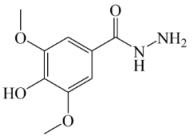
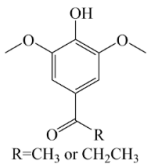
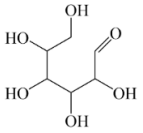
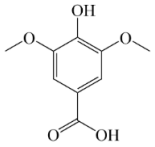
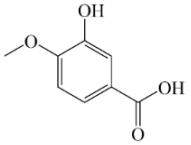
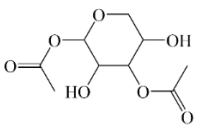
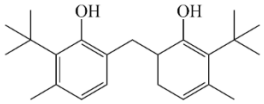
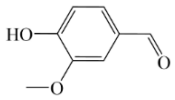
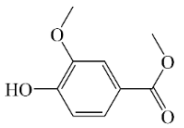
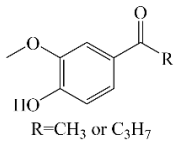
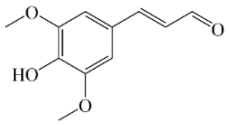
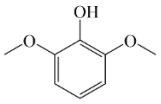
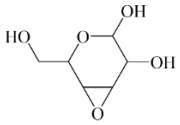
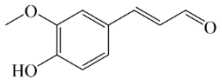
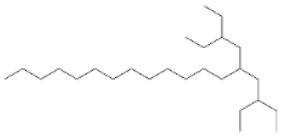
Number	R. Time/min	Ingredient	Structural Formula	Peak Area/%
1	16.26	syringaldehyde		15.78
2	17.54	Syringic acid hydrazide		12.82
3	17.14/18.18	Syringone		8.86
4	14.07	Glucose		7.79
5	18.13	Syringic acid		7.28
6	15.05	Isovanillic acid		6.5
7	13.02	1,3-Di-O-acetyl-à-á-d-ribopyranose		5.58
8	24.03	2,2′-methylenebis(6-tert-butyl-5-methyl-phenol)		3.50

Table 1. *Cont.*

Number	R. Time/min	Ingredient	Structural Formula	Peak Area/%
9	12.95	Vanillin		2.88
10	14.42	Methyl vanillate		2.38
11	15.35	Vanillone	 R=CH_3 or C_3H_7	2.17
12	19.94	Sinapaldehyde		1.72
13	12.20	2,6-Dimethoxyphenol		1.54
14	14.76	3,4-Altrosan		1.21
15	17.23	Coniferyl aldehyde		1.20
16	27.73	Long-chain alkane		1.02

Media containing components 1, 2, 3, 9, 10, and 11 inhibited the growth of *G. trabeum* and *T. versicolor* in 100% (Figure 2), highlighting the strong antifungal activity of these six main components. Component 9, identified as vanillin, has previously been shown to exhibit antifungal activity against two types of decay fungi at 20 mg/mL in studies conducted by our research group [36]. Component 10, identified as methyl vanillate, has been reported to exhibit inhibitory effects against several fungi at 0.05 mg/mL [41]. These six components are the main substances responsible for the antifungal effects of the LTP-N220. *T. versicolor* grew throughout the entire medium, while the *G. trabeum* did not grow on medium modified with components 12, 13, and 15. Among these components, component 15 is identified as the cypress aldehyde and has been reported in previous studies to be an antifungal compound [42]. It exhibits strong inhibitory effects against fungi and demonstrates significant inhibitory effects in synergy with coumarin [43–45]. This indicates that sinapaldehyde, 2,6-Dimethoxyphenol, and coniferyl aldehyde are also three of the main components in the LTP-N220 that inhibit the growth of *G. trabeum* and play a positive role in enhancing the decay resistance of wood. In addition, on media containing syringic acid (component 5) and isovanillic acid (component 6), although both decay fungi showed some growth, their growth rates were still lower compared to the control group (Table S1). Syringic acid and isovanillic acid also exhibited certain inhibitory effects on the two decay fungi. It was also noted that LTP-N220 contains sugar components (compounds

4 and 7), which are unfavorable to the antifungal activity of LTP. However, measurements of the reducing sugar content in each LTP (Table S2) showed that as the treatment temperature increased, the reducing sugar content in LTP grew consistently, regardless of whether air or nitrogen was used as the heating medium. This contradicts general expectations, which may be because the contribution of reducing sugars to promoting fungal growth is likely minimal and may even be overshadowed by the inhibitory effects of other components.

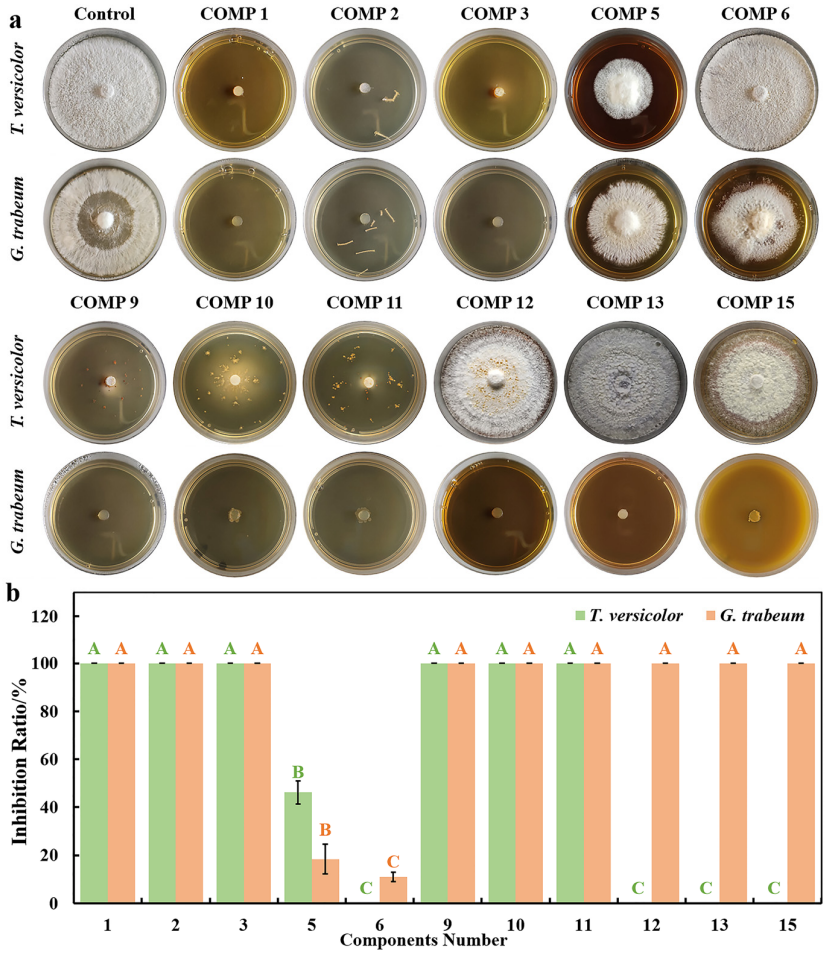


Figure 2. Growth condition of decay fungi upon the PDA with varying compositions at a concentration of 4 mg/mL (**a**) and the inhibition rate (Day 21) of varying compositions against *T. versicolor* and *G. trabeum* (**b**). Note: Significant differences between means are indicated by different letters (e.g., A, B, C) at a confidence level of 95% ($p \leq 0.05$).

The major components of the remaining LTP were also analyzed by GC-MS, and the relative content of the six major antifungal components in six LTPs was statistically determined (Figure 3). The relative content of syringic acid hydrazide and acyl syringone was observed to increase with temperature in LTP using air and nitrogen as the medium. Furthermore, it can be observed from the results that with increasing temperature, there is also an increase in the variety of major antifungal active substances. This validates that as the thermal induction conditions become more severe, there is an increase in both the types and quantities of antifungal active substances. Furthermore, the thermal induction medium significantly influences the stability and retention of antifungal components. In air, the active antifungal components may undergo further oxidation into acids or other compounds with lower antifungal activity, whereas the nitrogen environment reduces the loss of antifungal phenolic compounds. Under the same 220 °C condition, nitrogen-protected LTP retains higher levels of syringic acid hydrazide and methyl vanillate. These

two factors, especially the potential synergistic effects induced by an increase in the variety of antifungal substances, contribute to enhancing the antifungal activity of the LTP.

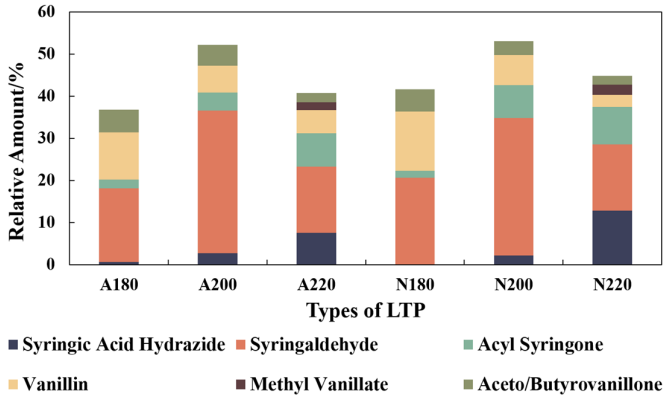


Figure 3. The relative amount of the six major antifungal compounds in the LTP from various thermal-induced process conditions.

3.3. The Enzymatic Activity of Decay Fungi Affected with LTP-N220

Wood-decaying fungi degrade wood through the action of cellulases, hemicellulases, and ligninases. The activities of these enzymes provide an indirect means to evaluate the potential effects of the LTP on fungal physiology. In the culture medium containing 1.5 mg/mL of LTP-N220, the activities of manganese peroxidase (Mnp) and laccase (Lac) exhibit a significant decrease compared to the medium containing 0 mg/mL of LTP (Figure 4). Mnp activity exhibited a decrease of 45.6%, while Lac activity showed a significant decline of 60.3%. These results indicate that LTP-N220 inhibits the activities of two ligninolytic enzymes, thereby impeding the degradation of wood lignin by *T. versicolor*.

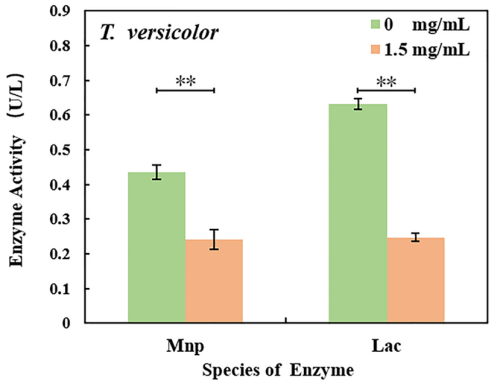


Figure 4. Manganese peroxidase (Mnp) and Laccase (Lac) activity of *T. versicolor* influenced by LTP-N220. Note: Significance differences between means are denoted as follows: '**' for highly significant difference ($p < 0.01$).

The LTP-N220 had no significant effect on the activity of exoglucanases (EC) in *T. versicolor*'s cellulase. However, it shows a significant reduction in the activity of endoglucanases (EG), β-glucosidases (BG), and hemicellulase xylanase (XL) after LTP-N220's addition (Figure 5a). In contrast, the enzyme activity of *G. trabeum* was not significantly inhibited by the LTP-N220 and even exhibited a stimulating effect on the activity of EG in *G. trabeum* (Figure 5b). This may be due to the enhanced stability of *G. trabeum*'s endoglucanase, GTCel12A, in acidic environments (pH = 3) [46]. The presence of organic acids such as syringic acid and isovanillic acid in LTP contributes to its overall acidity, which may favor the activity of *G. trabeum*'s EG.

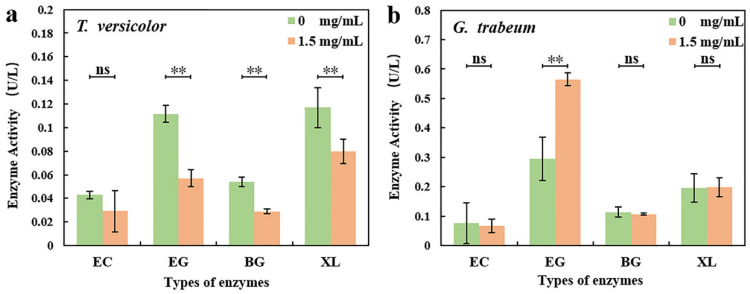


Figure 5. Exoglucanases (EC), endoglucanases (EG), β-glucosidases (BG), and xylanase (XL) activity of *T. versicolor* (**a**) and *G. trabeum* (**b**) influenced by LTP-N220. Note: Significance differences between means are denoted as follows: 'ns' for no significant difference ($p \geq 0.05$), and '**' for highly significant difference ($p < 0.01$).

The results above indicate that the LTP-N220 inhibits the activity of the ligninase, cellulase, and hemicellulase enzymes in *T. versicolor*, comprehensively suppressing its ability to degrade wood. In the case of *G. trabeum*, the inhibition of its activity by the LTP-N220 doesn't seem to be caused by the inhibition of its cellulase and hemicellulase enzyme activities. Although both *T. versicolor* and *G. trabeum* produce cellulases and hemicellulases, these enzymes belong to different glycoside hydrolase families, with distinct structural characteristics and catalytic mechanisms. They also exhibit varying responses to environmental factors such as pH, temperature, and carbon sources. These differences may explain the contrasting enzymatic activities observed under the influence of LTP, where the cellulases and hemicellulases of *T. versicolor* and *G. trabeum* respond differently.

3.4. Cell Membrane Function of Decay Fungi Affected with LTP-N220

A healthy fungal cell membrane separates the cell from its external environment to ensure a relatively stable internal environment. The cell membrane acts as a gateway for controlling the entry and exit of substances in cells, playing a vital role in maintaining cellular pH, and osmotic pressure, and supporting normal life activities [31,47]. Observations under an optical microscope using the slide method reveal the morphology of the decay fungi mycelium, as shown in Figure 6a.

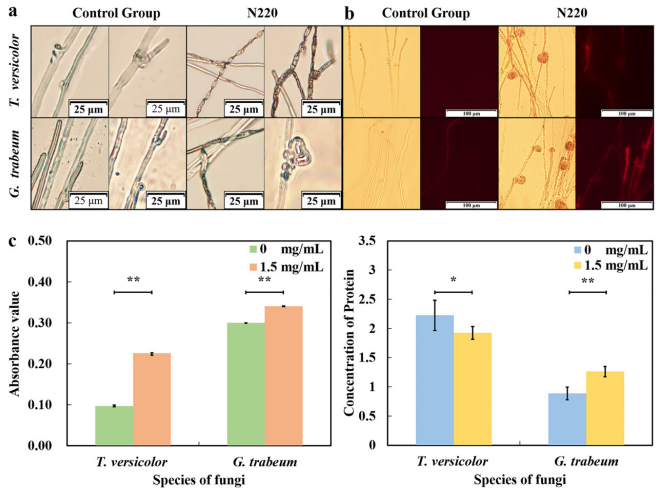


Figure 6. The mycelium of *T. versicolor* and *G. trabeum* (**a**); images of the fungal mycelium under visible light and fluorescence (**b**); and the extracellular nucleic acid and extracellular protein levels of the two decay fungi (**c**). Note: Significance differences between means are denoted as follows: '*' for significant difference ($0.05 > p \geq 0.01$), and '**' for highly significant difference ($p < 0.01$).

The growth mycelial of the normal *T. versicolor* and *G. trabeum* appears smooth, with clear and filled cell lumens and normal development of the germ tube. In contrast, *T. versicolor* and *G. trabeum* grown in the culture medium containing 1.5 mg/mL of LTP-N220 exhibited a significant amount of shriveled and twisted mycelium. This can be attributed to the disruption of the fungal mycelium cell membranes by LTP-N220, resulting in cytoplasmic leakage and subsequent mycelium shriveling. Furthermore, under the influence of the LTP-N220, the fungal mycelium was observed with abnormalities in germ tube development, segmental thinning, and pigment deposition at cell junctions. See Supplementary Figure S4 for images at a lower magnification scale.

Propidium iodide (PI) is a red fluorescent dye capable of staining DNA. It is typically excluded from viable cells but can penetrate damaged cells, hence it is commonly used for assessing cell viability [48]. Based on the results of PI staining (Figure 6b), the fluorescence phenomenon was not observed in the *T. versicolor* control group, with only a few hyphae stained in *G. trabeum*, demonstrating healthy fungal growth in the absence of LTP-N220 influence. On the other hand, the growing mycelium of decay fungi was successfully stained with PI after exposure to LTP-N220, exhibiting a significantly higher fluorescence intensity compared to the control group. At the same time, the terminal ends of the growing mycelium of decay fungi exhibited cytoplasmic leakage upon exposure to LTP-N220. These observations indicate that LTP-N220 causes damage to the cell membrane of wood decay fungi.

When the cell membrane is damaged by toxicants, substances such as proteins and nucleic acids that were originally protected will leak out. The leakage of nucleic acids and proteins serves as one of the indicators for assessing cell membrane permeability. Nucleic acids and proteins are both electrolytes, and as cell membrane permeability increases, so does the leakage of nucleic acids and extracellular protein concentration [49], which can lead to disruptions in normal intracellular metabolism. As depicted in Figure 6c, the leakage of nucleic acids from *T. versicolor* and *G. trabeum* treated with 1.5 mg/mL LTP-N220 is significantly higher compared to the 0 mg/mL treatment group. The concentration of extracellular proteins from *T. versicolor* treated with 1.5 mg/mL LTP-N220 shows a decrease compared to the untreated group. This can be attributed to a reduction in the levels of various enzymes, which are also proteins, in *T. versicolor* after treatment with the LTP-N220, resulting in a lower overall extracellular protein level compared to normally growing *T. versicolor*. In contrast, for *G. trabeum*, despite the lack of significant differences in the activity of most enzymes before and after treatment with LTP-N220, there is a significant increase in the concentration of extracellular proteins compared to the untreated group. The alterations in nucleic acid and protein leakage levels suggest that the treatment with LTP-N220 may have increased the membrane permeability of both *T. versicolor* and *G. trabeum*, thereby impacting membrane functionality and leading to the leakage of intracellular nucleic acids and proteins, potentially affecting the normal activities of wood decay fungi.

3.5. Respiratory Metabolism of Decay Fungi Affected with LTP-N220

Most fungi need to obtain energy through aerobic respiration. Decay fungi convert nutrients into the energy required for life through aerobic respiration. The respiration rate serving is a crucial indicator for assessing the vitality of decay fungi (Figure 7a). The respiratory status of *T. versicolor* and *G. trabeum* after treatment with LTP-N220 is depicted in Figure 7b. The results show that in the closed space, the respiration rate of *T. versicolor* treated with 1.5 mg/mL LTP-N220 is slightly lower than that of the 0 mg/mL treatment. Furthermore, after 30 min, the respiration rate at 1.5 mg/mL tends to stagnate, while the 0 mg/mL group continues to respire even after 60 min. *G. trabeum* treated with 1.5 mg/mL LTP-N220 exhibits significantly lower respiration levels and rates compared to the untreated group (0 mg/mL). The results indicate that the LTP-N220 exerts inhibitory effects on the aerobic respiration of

the two fungi, impeding the efficient conversion of organic matter into the energy needed for the life activities of the two wood decay fungi, thereby weakening or potentially disrupting the life processes of these two wood decay fungi.

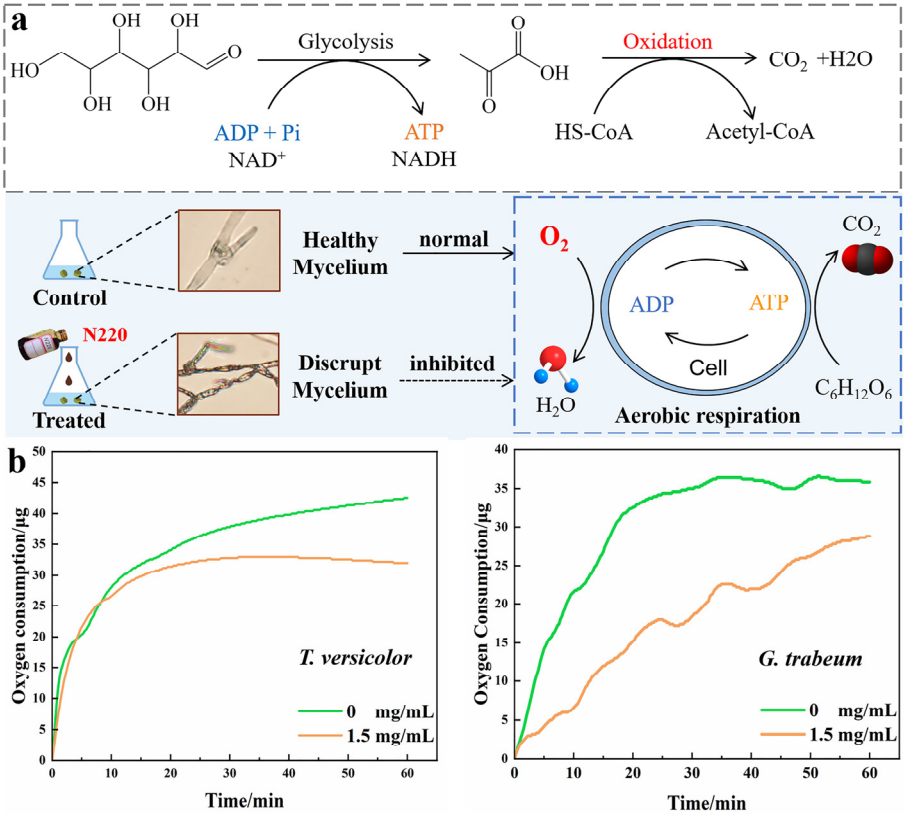


Figure 7. The underlying mechanisms (**a**) and impact (**b**) of LTP-N220 on the respiratory metabolism of decay fungi.

3.6. Decay Resistance of LTP-N220-Immersed Poplar

The average mass gain rate of poplar samples reached 12.02 ± 0.37% after immersion twice with a 30 mg/mL acetone solution of LTP-N220. From the tangential section of the treated wood, it is also evident that the LTP adheres to the cell walls (Figure 8f). Following an 8-week decay period by the decay fungi, the poplar wood samples in the control group exhibited significant decay (Figure 8a,b), with mass loss rates of 36.39% and 26.17% for *T. versicolor* and *G. trabeum*, respectively (Figure 8e). Additionally, for untreated poplar wood (Figure 8i), extensive fungal mycelium growth can be observed within the cellular cavities of the samples, accompanied by cellular wall disruption (Figure 8j,k). In contrast, the samples treated with the LTP-N220 showed no significant changes in appearance after 8 weeks of exposure to decay fungi, except for a slightly lighter color (Figure 8c,d). Unlike the untreated group (Figure 8b), no pores or damage caused by decay were observed (Figure 8c,d). Moreover, compared to the untreated group, the samples exhibited significantly lower mass loss after 8 weeks of exposure to the two decay fungi, measuring only 8.35% and 6.35%, respectively. Compared to the untreated samples, the decay levels were reduced by 77.1% and 75.7% for *T. versicolor* and *G. trabeum*, respectively. The higher decay resistance against *T. versicolor* may be attributed to the inhibitory effect of LTP on its enzymatic activity. Intact cell walls and less fungal mycelium were observed in treated samples after decay, yet the presence of the LTP could still be observed (Figure 8g,h). The results demonstrate that the LTP obtained through thermal induction at 220 °C using N_2 as the heating medium can significantly improve the decay resistance of wood.

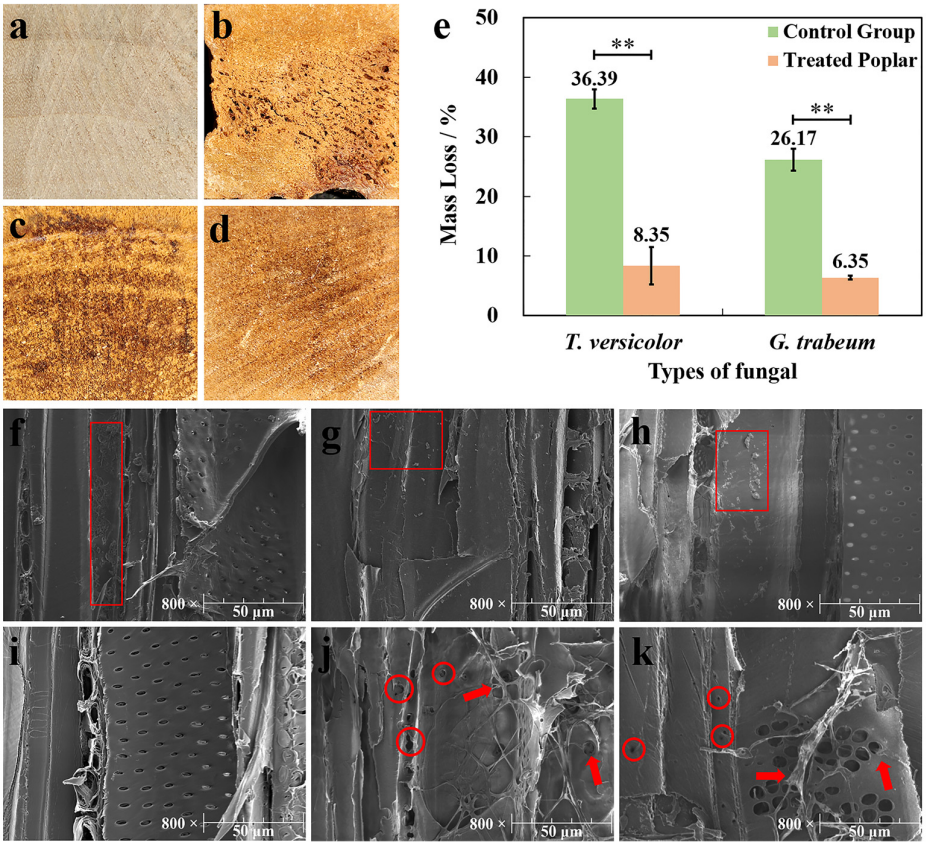


Figure 8. The appearance of samples including untreated poplar wood (**a**), decayed poplar wood (**b**), LTP-N220-immersed poplar wood (**c**), and treated poplar decayed by *G. trabeum* (**d**). The mass loss of LTP-N220-immersed poplar after exposed to *T. versicolor* and *G. trabeum* (**e**). The electron microscopy images of LTP-N220-immersed poplar wood (**f**), treated poplar wood decayed by *T. versicolor* (**g**) and *G. trabeum* (**h**), poplar wood (**i**), and poplar wood decayed by *T. versicolor* (**j**) and *G. trabeum* (**k**). The rectangular areas are LTP; the circular areas are cell walls damaged by decay fungi; and the regions indicated by arrows are the mycelium of decay fungi. Note: Significance differences between means are denoted as follows '**' for highly significant difference ($p < 0.01$).

4. Conclusions

The wood preservative produced from lignified twigs by thermal induction, especially thermal induction under N_2 at 220 °C (LTP-N220), contains a bioactive phenolic compound with antifungal properties such as syringaldehyde, syringone, syringic acid hydrazide, vanillin, methyl vanillate, and vanillone. The decay resistance of poplar (*Populus tomentosa* C.) wood treated with LTP-N220 was enhanced significantly. The LTP-N220 inhibits the activity of cellulases, hemicellulases, and ligninases in *T. versicolor*. It impairs the function of fungal cell membranes and inhibits the respiratory metabolism of both *T. versicolor* and *G. trabeum*, thereby resulting in the inhibition of their growth. These findings underscore that the thermal induction feedback pattern, making use of non-timber LT on fast-growing poplar wood, shows great potential in wood preservation.

Looking ahead, ongoing efforts will center on further enhancing the leach resistance of LTP-based treatments and examining their antifungal efficacy across a broader range of wood species. Such endeavors aim to build upon the present findings, reinforcing the potential of this sustainable approach and paving the way for more versatile wood preservation strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f16010119/s1, Table S1. Colony Diameter of Decay Fungi

on PDA Medium with LTP-A220; Figure S1. Standard curves for glucose (a) and protein (b); Equations (S1)–(S3), relevant calculations of enzyme activities; Figure S2. Gas chromatogram of the LTP-N220; Figure S3. Gas chromatography of standards for some components in LTP-N220; Figure S4. The mycelium of normally growing *T. versicolor* (a) and *G. trabeum* (c), as well as the mycelium of *T. versicolor* (b) and *G. trabeum* (d) affected by LTP-N220; Table S2. Reducing sugar content of LTP prepared with air and nitrogen as heating medium at 180, 200 and 220 °C; Table S3. Average fungal colony diameter of decay fungi on PDA or media with varying compositions.

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Data Availability Statement: The datasets generated during and/or analyzed in this study are available from the corresponding author upon reasonable request.

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