forests

MDPI

Article

Establishment of *Pinus massoniana–Lactarius hatsudake* Symbiosis

Zhineng Wei [1,2,†], Lin Liu [1,2,†], Yidan Lei [1,2], Sisi Xie [2], Jiangming Ma [1,2], Yibo Tan [3,4], Nianwu Tang [5], Zhangqi Yang [3,6,*] and Chenbing Ai [1,2,6,*]

1 Key Laboratory of Ecology of Rare and Endangered Species and Environmental Protection (Ministry of Education, China) & Guangxi Key Laboratory of Landscape Resources Conservation and Sustainable Utilization in Lijiang River Basin, Guangxi Normal University, Guilin 541004, China; weizhineng2024@163.com (Z.W.); 13481369652@163.com (L.L.); leiyidan1999@163.com (Y.L.); mjming03@gxnu.edu.cn (J.M.)
2 College of Life Sciences, Guangxi Normal University, Guilin 541004, China; xiesisi2424@163.com
3 Guangxi Forestry Research Institute, Nanning 530002, China; tybrun@126.com
4 Xing'an Guilin Lijiangyuan Forest Ecosystem Observation and Research Station of Guangxi, Guilin 541316, China
5 Guangxi Key Laboratory of Plant Conservation and Restoration Ecology in Karst Terrain, Guangxi Institute of Botany, Guangxi Zhuang Autonomous Region and Chinese Academy of Sciences, Guilin 541006, China; tangnianwu@163.com
6 Guangxi Key Laboratory of Superior Timber Trees Resource Cultivation, Guangxi Forestry Research Institute, Nanning 530002, China
* Correspondence: yangzhangqi@163.com (Z.Y.); chenbingai@mailbox.gxnu.edu.cn (C.A.); Tel.: +86-13978858085 (Z.Y.); +86-18900728696 (C.A.)
† These authors contributed equally to this work.

Abstract: *Lactarius hatsudake* is a common ectomycorrhizal edible mushroom in *Pinus massoniana* forests, and has important ecological and potential economic values. However, there are only a few reports on the establishment of *Pinus massoniana–Lactarius hatsudake* symbiosis. Here, we isolated a new strain of *L. Lactarius hatsudake* (GX01) from a local masson pine forest and established its ectomycorrhizal symbiosis with the *P. massoniana*. Potato dextrose agar (PDA) medium was optimal for the growth of *L. hatsudake* GX01. The saffron-to-brown ectomycorrhiza formed by *L. hatsudake* GX01 are usually bifurcated or coralloid shape, with a rod and a smooth surface, without emanating hyphae. The characteristic mantle and Hartig net structures of ectomycorrhizae were confirmed by microscope and scanning electron microscope (SEM). *L. hatsudake* GX01 can significantly promote the formation and development of lateral roots of *P. massoniana* seedlings during the early interaction. This study thus lays the foundation for subsequent study of the symbiotic molecular mechanism and application of *P. massoniana–L. hatsudake* symbiosis.

Keywords: ectomycorrhiza; *Pinus massoniana*; *Lactarius hatsudake*; symbiosis establishment

Citation: Wei, Z.; Liu, L.; Lei, Y.; Xie, S.; Ma, J.; Tan, Y.; Tang, N.; Yang, Z.; Ai, C. Establishment of *Pinus massoniana–Lactarius hatsudake* Symbiosis. *Forests* **2024**, *15*, 578. https://doi.org/10.3390/f15040578

Academic Editor: Stefan Arndt

Received: 3 February 2024
Revised: 5 March 2024
Accepted: 13 March 2024
Published: 22 March 2024

1. Introduction

Ectomycorrhiza (ECM) is a symbiosis consisting of a covering around the finest feeder roots of conifers and some vascular plants by ectomycorrhizal fungal mycelium, which characteristically form hyphal sheath (i.e., mantle structure) on the surface of feeder roots and the Hartig net structure by wrapping hyphae tightly in between epidermal or cortical cells of feeder roots [1]. The symbiosis of ectomycorrhiza worldwide involves approximately 6000 plant species and over 20,000 fungal species [2,3]. Ectomycorrhizal fungi (EMF) play important roles in promoting nutrient absorption, improving water metabolism, enhancing salt tolerance, and enhancing disease resistance for host plants [4,5]. Therefore, EMF have great potential in promoting tree growth and afforestation, improving tree stress resistance, and enhancing forest ecological stability [5–7].

The EMF also have economic importance by providing non-wood forest products, i.e., edible fruiting bodies [8,9]. Currently, the *Lactarius* spp., *Boletus* spp., *Tricholoma* spp., *Tuber* spp., and *Cantharellus* spp. are the most popular group and are traditionally eaten by local people and traded in markets worldwide [10,11]. A survey showed that around ten thousand tons of *Boletus edulis* were traded annually in the province of Yunnan, China [12]. A total of 20,337 tons of *Tricholoma matsutake* were exported in the past 15 years [13]. Considering the appropriate annual yield and the considerable high price, the economic value of EMF can undoubtedly exceed the economic profit traditionally obtained from timber-oriented forestry [14,15]. Utilizing forest resources to develop the EMF-based mushroom industry can effectively promote income growth for local people and benefit rural vitalization.

Lactarius belongs to the family Russulaceae in the order Russulales, and forms ectomycorrhizal relationships with conifers and some broad-leaved trees [16]. Around 40 species worldwide in the genus of *Lactarius* are edible [17]. The fruiting bodies of *L. hatsudake* contain many bioactive compounds, such as fungal polysaccharides, unsaturated fatty acids, and phenolic compounds (i.e., gallic acid, pyrogallol, and chlorogenic acid), which exhibit anticancer functions and alleviate symptoms of diabetes [18–20]. Although the fruiting bodies of *Lactarius* are almost always collected from the wild, mycosilviculture, as an alternative strategy, has been developed to provide edible mushrooms in the past few decades [11,17,21]. By improving inoculation methods, mycorrhizal synthesis between *Pinus radiata* and *Lactarius deliciosus* was achieved, and the fruiting bodies of *L. deliciosus* were successfully obtained within 18 months after transplantation [22]. Since then, the yield of the fruiting bodies of *L. deliciosus* in this mycosilviculture plantation has been continuously increased [23]. The effects of different inoculation methods on the mycorrhizal synthesis between edible *Lactarius* species and *Pinus pinaster* or *Pinus sylvestris* under standard greenhouse conditions were evaluated [24]. Edible fruiting bodies of *Lactarius akahatsu* were obtained within 9 months after the transplantation of ectomycorrhizas between *L. akahatsu* and *Pinus densiflora* [25]. Ectomycorrhiza synthesized between *Lactarius indigo* (Schw.) Fr. and the neotropical species of *Pinus* spp. were also reported [25]. Recently, the symbionts synthesized between *Lactarius* spp. and *Pinus* species native in China was reported [17].

Pinus massoniana is a typical host tree species for many EMF. It is worth mentioning that *P. massoniana* is the most important native afforestation and timber tree species in southern China, with a wide distribution area, and a large storage capacity, owing to its strong adaptability [26,27]. At present, the planting area of *P. massoniana* in Guangxi Zhuang Autonomous Region is 1.7829 million hectares [28]. Recently, superior families of *P. massoniana* with significant traits in large-diameter timber production, growth increments, resin yields, and chemical composition have been bred [29]. It is widely accepted that mycosilviculture practice can increase the fruiting body production of various EMF [17,30]. However, only few studies on the mycorrhizal synthesis between edible *Lactarius* species and *P. massoniana* have been reported [17,31,32]. Critical morphological description and molecular evidence were still lacking in these studies on the symbionts synthesized between *L. hatsudake* and *P. massoniana* [31,32]. Therefore, mycorrhizal synthesis between superior families of *P. massoniana* and native *L. hatsutake* strains will deepen our knowledge on the mutual mechanisms underlying this symbiont, and lay a foundation for the establishment of mushroom orchards through mycosilviculture in the near future.

In this study, a native strain of *L. hatsudake* was isolated, and the optimal growth medium and pH value of this strain were identified. Mycorrhizal synthesis between *P. massoniana* in superior families with the trait of growth increments and this *L. hatsudake* strain was tried by using a vegetative inoculum approach. The morphological character of *P. massoniana–L. hatsudake* symbiont were described in detail. The effect of inoculating *L. hatsudake* on the lateral roots of *P. massoniana* seedlings was also observed at the early interaction stage. The new strain and its mycorrhizal synthesis with a superior masson pine thus lays a foundation for subsequent study of the symbiotic molecular mechanism and mycosilviculture application of *L. hatsudake*.

2. Materials and Methods

2.1. Isolation of Lactarius Hatsudake Strain

Fresh fruiting bodies of *L. hatsudake* were collected in *P. massoniana* forest at Guilin National Forest Park in Guangxi Zhuang Autonomous Region. Immature fruiting bodies without insect infection were selected for the following isolation of dikaryotic mycelium in pure culture. The debris and soil particles on the surface of fruiting bodies were removed by using tweezers and damp tissue. Then, the base of the stipe was cut off, followed by disinfection of the surface of fruiting bodies with a tissue soaked in 75% (*v*/*v*) ethanol. The fruiting bodies were broken open inside a super-clean bench. Then, approximately 27 mm^3 of tissue within the internal junction of the stem and cap was cut with a sterile scalpel and placed on potato dextrose agar (PDA) medium in petri dishes. Cultures were incubated at 25 °C in the dark, with a relative humidity (RH) of 75%. The growth of the isolate was checked every 3 days.

Total DNA was isolated from fresh fruiting body tissue and the hyphae of the isolate grew for 4 weeks using the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech Co., Ltd., Shanghai, China). The primer pair of ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used to amplify the fungal rDNA internal transcribed spacer (ITS) [10]. PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each PCR mixture (total volume 50 µL) contained 25 µL of 2× SanTap PCR Master Mix (Sangon Biotech Co., Ltd., Shanghai, China), 2 µL of 10 µmol/L forward and reverse primers, 2 µL of genomic DNA, and 19 µL of nuclease-free water. PCR was performed with an initial denaturation at 95 °C for 5 min, and then 40 cycles of denaturation at 95 °C for 60 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension phase for 10 min at 72 °C. The PCR products were separated on 1.0% (*w*/*v*) agarose gel and then sent to Sangon Biotech (Shanghai, China). DNA sequences were compared with the available sequences from the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool. Based on its morphological characters and ITS sequence alignment, this isolate was named as *Lactarius hatsudake* GX01.

2.2. Comparison Medium Composition and Acidity on the Growth of L. hatsudake GX01

The growth of *L. hatsudake* GX01 on 5 media with different compositions were evaluated in this study. The detailed compositions of these media were described in previous studies and listed in Table 1 [33,34].

Table 1. Composition of nutrients in different media.

Compound	Formula 1 (PDA)	Formula 2	Formula 3	Formula 4 (MMN)	Formula 5
Potato extract (g)	200	200	200	-	-
Glucose (g)	20	25	10	10	20
Yeast extract (g)	-	3	2	-	0.2
KH_2PO_4 (g)	-	3	1	0.5	0.34
Peptone (g)	-	-	4	-	2
$MgSO_4$ (g)	-	-	1	0.15	0.15
NaCl (g)	-	-	-	0.025	0.025
$CaCl_2$ (g)	-	-	-	0.05	0.05
VB_1 (mg)	-	-	-	0.1	0.1
Malt extract (g)	-	-	-	3	-
$(NH_4)_2HPO_4$ (g)	-	-	-	0.25	-
$FeCl_3$	-	-	-	0.012	-
NH_4Cl (g)	-	-	-	-	0.56
MES (g)	-	-	-	-	1.95
Inositol (g)	-	-	-	-	0.05
Morizet (mL)	-	-	-	-	0.2
1% ferric citrate (mL)	-	-	-	-	3
Agar (g)	20	20	20	20	20

Circular areas (6 mm in diameter) of the hyphae of *L. hatsudake* GX01 which grew well on a PDA plate for 30 days were punched out and placed upside down on the center of these 5 solid media in plates as described in Table 1. The growth of *L. hatsudake* GX01 was determined by measuring the colony diameter using the cross-over method at an interval of 3 days after the inoculation. The optimal pH for growth was further assessed by using the same method after the best growth medium was determined. A series of the selected optimal growth medium with pH values of 4.0, 5.0, 6.0, and 7.0 were made. Five replicates were set up for each growth medium. Cultures were incubated at 25 °C in the dark, with a RH of 75%.

2.3. Seed Disinfection Treatment

The seeds with the trait of growth increments were collected from second-generation seed orchards of *Pinus massoniana* at Nanning Forestry Research Institute, Nanning, Guangxi Zhuang Autonomous Region. Seeds were stored at 4 °C for later use. The efficiencies of 0.5% (*w*/*v*) $KMnO_4$ solution or 30% (*v*/*v*) H_2O_2 on the *P. massoniana* seed disinfection were evaluated individually. Full and complete *P. massoniana* seeds were selected and placed in sterile beakers on the super-clean bench, then disinfectant was added for surface disinfection with different times. At least 100 *P. massoniana* seeds were used for each treatment. After disinfection, the waste liquid was discarded and seeds were successively rinsed with sterile water three times, for 5 min each time. Subsequently, these disinfected seeds were transferred into a petri dish and soaked in sterile water, and placed in an artificial climate chamber for germination to determine the germination percentage, the infection rate, and the ratio of germ-free seedlings (25 °C, 70% RH).

2.4. Mycorrhizal Synthesis

The *P. massoniana* seeds with the trait of growth increments were surface-disinfected with 30% (*v*/*v*) H_2O_2 solution, then rinsed three times in sterilized water to wash off any remaining disinfectant. Seeds were sown evenly in damp vermiculite (sterilized by autoclaving at 121 °C for 1.5 h before use) for germination. Regular watering during germination maintained sufficient humidity of the vermiculite. After growing cotyledons, the seedlings were transplanted to a mixed matrix of vermiculite and perlite (1:1 in volume), and cultured in an artificial climate chamber (each day under 6500 lux light, 25 °C temperature, 70% RH, 14 h; under 0 lux light, 25 °C temperature, 70% RH, 10 h). After two months of growth, *P. massoniana* seedlings with well-developed lateral roots and consistent growth were selected for mycorrhizal synthesis. *L. hatsudake* GX01 agar cultures grew around one month with optimal medium and acidity and were collected by removing excessive agar, and then mixed thoroughly with sterilized water to make the ectomycorrhizal inoculum. The substrates used for mycorrhizal synthesis were composed of vermiculite, nutrient soil, and perlite (with a ratio of 3:2:1 in volume) (sterilized by autoclaving at 121 °C for 2.0 h before use). After rinsing the roots with sterile water, *P. massoniana* seedlings were transplanted to a seedling pot (8.0 cm × 8.0 cm × 12.5 cm in size). Each seedling pot was inoculated with 50 mL of ectomycorrhizal inoculum using the "three-layer method", which means adding 1/3 amount of the ectomycorrhizal inoculum respectively at 1/3, 2/3, and 3/4 height of the substrate within each pot. The control plants were not inoculated with ectomycorrhizal inoculum. All *P. massoniana* seedlings were cultured in an artificial climate chamber (each day under 6500 lux light, 25 °C temperature, 70% RH, 14 h; under 0 lux light, 25 °C temperature, 70% RH, 10 h).

2.5. Morphological Observations of Ectomycorrhizae

One month after inoculation, five *P. massoniana* seedlings were randomly examined for verification of the formation of ectomycorrhizae. The morphological characters of ectomycorrhizae were observed and photographed under a stereomicroscope (S8APO, Leica Microsytems, Germany). Anatomical cross sections were prepared from fresh root material and followed by treatments according to methods described in a previous study

with minor modifications [35]: the root tips of *P. massoniana* were sampled and embedded in agarose water solution for solidification. Then, root samples were sliced in a shaking microtome and placed in a 10% KOH solution at 90 °C for 30 min of transparency. Then, the remaining solution was cleaned with distilled water and root samples were stained with 0.03% (*w*/*v*) chlorazole black at room temperature for 12 h. The anatomical characters of ectomycorrhizae were observed and photographed under an Eclipse E100 light microscope (Nikon, Tokyo, Japan). Anatomical cross sections were prepared from fresh root material and also observed by scanning electron microscope (SEM) (JSM-6490LV, JEOL, Tokyo, Japan). Both mycorrhizal and nonmycorrhizal root tips of *P. massoniana* were cut and fixed in formaldehyde–acetic acid–ethanol (FAA) fixative for 7 days. After fixation, the fixative was discarded and the root samples were continuously treated with 50%, 70%, 85%, and 95% ethanol solutions (1 h for each concentration), followed by dehydration with anhydrous ethanol twice (1 h each time). Then, they were continuously treated with ethanol and tert butanol mixtures with ratios of 3:1, 2:2, and 1:3, for 10 min each, and treated with 100% tert butanol for 10 min. Finally, these samples were vacuum freeze-dried and observed by SEM (JSM-6490LV, JEOL, Tokyo, Japan).

2.6. Molecular Analysis of the Ectomycorrhizae

DNA was isolated from fresh ectomycorrhizal root using the the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech Co., Ltd., China). PCR amplification of the ITS sequence of ectomycorrhizal root were identical with those described in Section 2.1. Phylogenetic analysis of the ITS sequence was conducted using MEGA 5.2 with the neighbor-joining method [36].

2.7. Effect of L. hatsudake on the Lateral Roots of P. massoniana Seedlings

The preparation of PDA medium and cultivation of the *L. hatsudake* GX01 inoculum was identical with those described in Section 2.2. The seed disinfection treatment was described in Section 2.3. *P. massoniana* seedlings that had sprouted for one week were selected and *L. hatsudake* GX01 was added for mycorrhizal synthesis according to Section 2.4. The *P. massoniana* seedlings group (i.e., control group) without *L. hatsudake* GX01 inoculum was also set up. The differences in the development of roots between the mycorrhizal synthesis group and the control group at 3, 4, 5, and 6 weeks after the infection were compared.

2.8. Data Analysis

Microsoft Office (Microsoft Office 2016, Microsoft, Redmond, WA, USA) was used for data organization. The statistical software SPSS version 26.0 (Statistical Product and Service Solutions, SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Significant different tests ($\alpha = 0.05$) of mycelial growth in media with different formulas and pH were analyzed by using one-way analysis of variance, LSD, and Duncan multiple comparison methods. Significant difference tests ($\alpha = 0.05$) for the effect of *L. hatsudake* on lateral root formation of *P. massoniana* seedlings were investigated using independent sample *t*-tests. The figures were plotted using the OriginPro 8.5 version (OriginLab Corporation, Northampton, MA, USA). Data are presented as means of five replications.

3. Results

3.1. Comparison of Medium Composition on the Growth of L. hatsudake GX01

Fresh fruiting bodies of *L. hatsudake* were collected in *P. massoniana* forest at Guilin National Forest Park in Guangxi Zhuang Autonomous Region (Figure 1). Different colony morphologies were observed for the growth of *L. hatsudake* GX01 on these five media (Figure 2). The color of aerial mycelium of *L. hatsudake* GX01 grown in medium of formula 1 was light yellowish-white, and its growth was tight and had the most aerial mycelium (Figure 2A). The color of mycelium grown with formula 2 and formula 5 was similar to that grown with formula 1, but the aerial mycelium was less than that of formula 1 (Figure 2A,E). The fringe mycelium of *L. hatsudake* GX01 grown with formula 3 was orange-

yellow, while the aerial mycelium inside the fringe mycelium was light orange-yellow, with less aerial mycelium (Figure 2C). In formula 4 (MMN), mycelium grown tightly attached the medium was white, with the least amount of mycelium, and there was no aerial mycelium (Figure 2D).

Figure 1. Fresh fruiting bodies of *L. hatsudake* GX01.

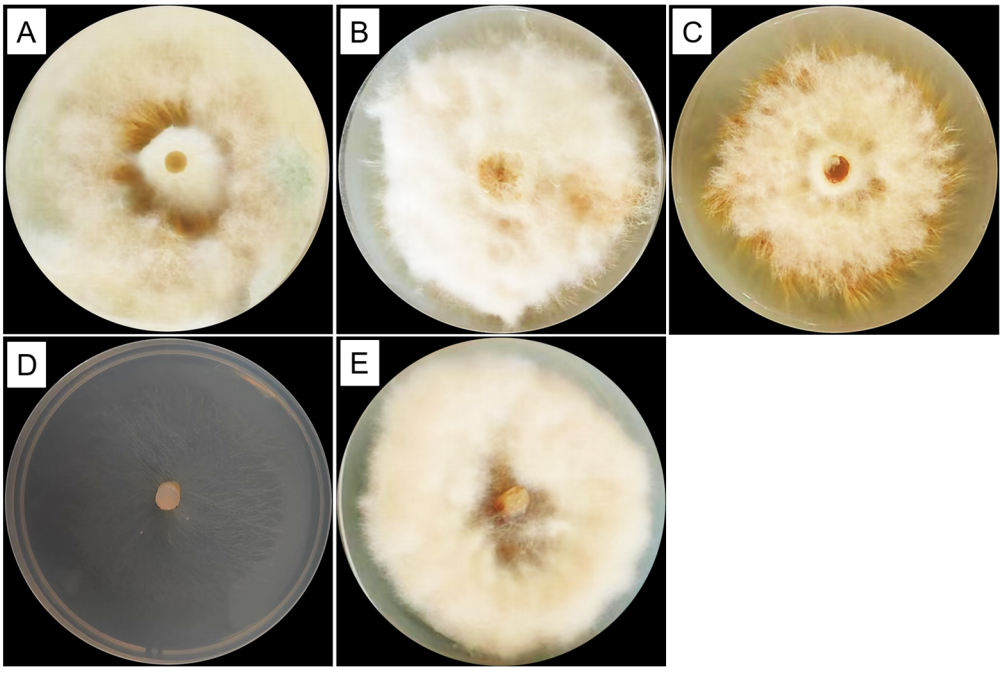

Figure 2. Growth of *L. hatsudake* GX01 with different media. (**A**) Formula I, (**B**) Formula III, (**C**) Formula III, (**D**) Formula IV, (**E**) Formula V.

It can be found that the mycelial growth in formula 3 was the slowest (Figure 3). The mycelial diameter of *L. hatsudake* GX01 colonies grown with PDA and formula 5 became bigger than those grown with other media after the 12th day. The mycelial diameter of *L. hatsudake* GX01 colonies grown with PDA medium started to become larger than that grown with the medium of formula 5 after the 15th day. The average daily growth rate of *L. hatsudake* GX01 grown with formula 1 (PDA) was the fastest, which showed the difference with a significant level ($p < 0.05$) compared with those cultures grown with the other four media (Table 2). The sequence of the daily growth rate of *L. hatsudake* GX01 is formula 1

(PDA) > formula 5 > formula 2 > formula 3 > formula 4 (MMN). Considering the growth rate, the amount of mycelium, and the cost of the medium, formula 1 (PDA) medium was the most suitable medium for *L. hatsudake* GX01 cultivation.

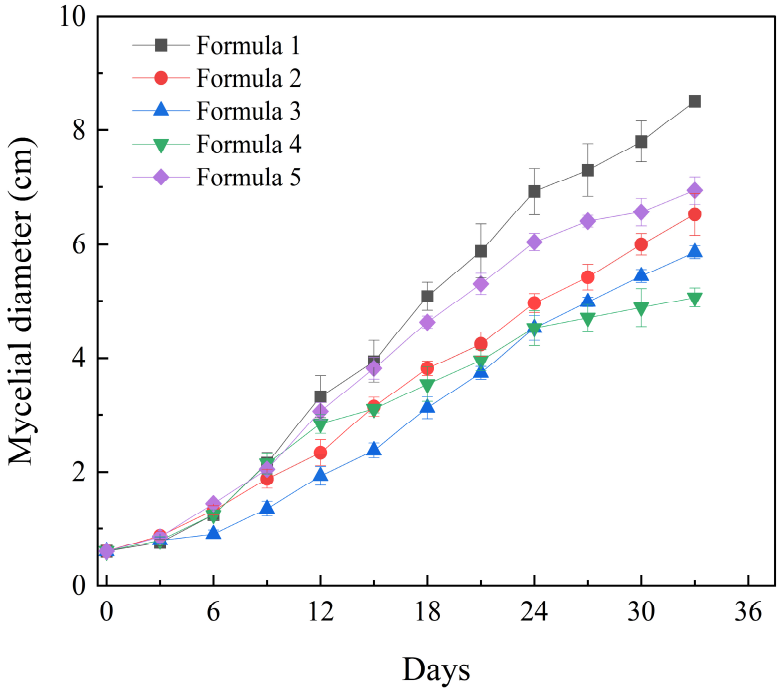

Figure 3. Growth of *L. hatsudake* GX01 in media with different compositions. Data are expressed as the mean ± standard deviation of 5 biological replicates (n = 5).

Table 2. Growth rate of *L. hatsudake* GX01 with different media. Each value is expressed as mean ± SD (n = 5). Different letters indicate significant differences in the means of mycelial diameters in different media ($p < 0.05$, one-way analysis of variance).

Growth Indicator	Formula 1	Formula 2	Formula 3	Formula 4	Formula 5
Diameter (cm)	8.500 ± 0.000 d	5.860 ± 0.110 b	5.060 ± 0.170 a	4.960 ± 0.210 a	6.520 ± 0.360 c
Growth rate (cm/day)	0.258 ± 0.000 d	0.178 ± 0.003 b	0.153 ± 0.005 a	0.150 ± 0.006 a	0.198 ± 0.011 c

3.2. Effects of Acidity on the Growth of L. hatsudake GX01

The effects of acidity on the colony morphology and growth of *L. hatsudake* GX01 on PDA medium was evaluated (Figure 4). Results showed that the stolons of *L. hatsudake* GX01 grown with an acidity of pH 4.0, 5.0, 6.0, and 7.0 were yellowish-brown in color while the aerial mycelia were white in color. The stolon of *L. hatsudake* GX01 was sparser in pH 4.0, while those mycelia cultured in pH 5.0–7.0 were much tighter (Figure 4). Aerial mycelium in pH 4.0 medium was mainly distributed in the center and outer edge of the colony, while aerial mycelium was randomly distributed when grown in pH 5.0. With the pH of the medium increased to 6.0, the aerial mycelium of *L. hatsudake* GX01 was mainly distributed on the outer edge of colony. The *L. hatsudake* GX01 grown in pH 7.0 had the most aerial mycelium, distributed on the stoloniferous mycelium in the whole petri dish. *L. hatsudake* GX01 mycelium grew the slowest in the medium with pH 4.0, reaching a significant difference from those cultured with the other three pH conditions ($p < 0.05$), whereas there was no significant difference in mycelial diameter and growth rate in pH 5.0, pH 6.0, and pH 7.0 (Table 3 and Figure 5). Collectively, the *L. hatsudake* GX01 grown with pH 7.0 was the best regarding the growth rate and amount of aerial mycelium.

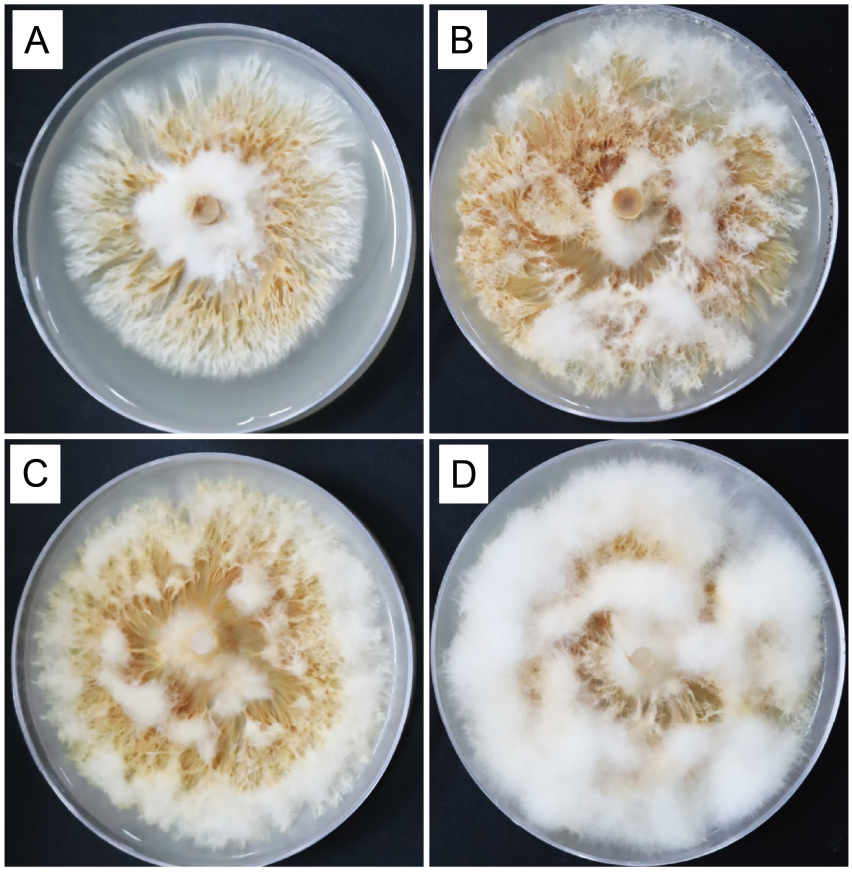

Figure 4. Growth morphology of *L. hatsudake* GX01 in PDA with different acidity. (**A**) pH 4.0, (**B**) pH 5.0, (**C**) pH 6.0, (**D**) pH 7.0.

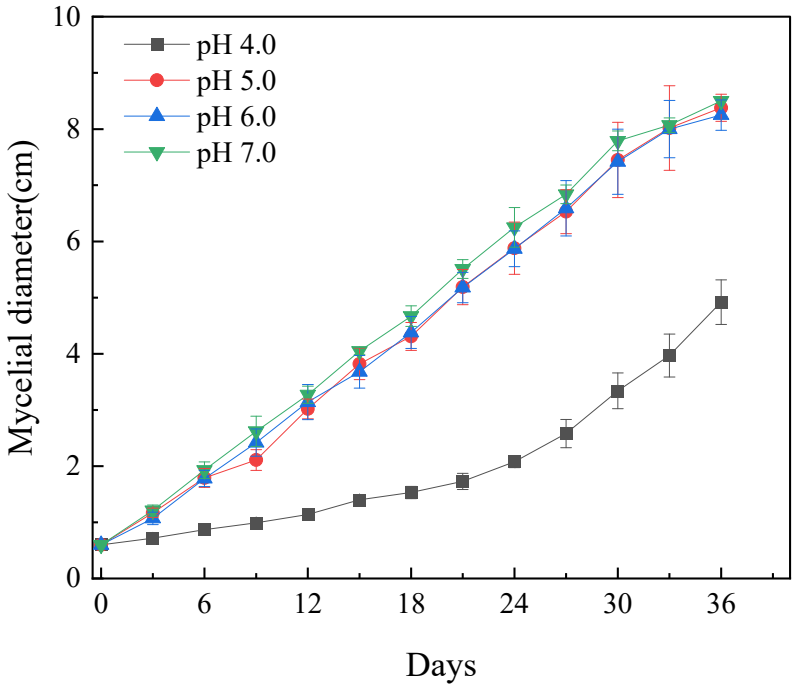

Figure 5. Growth of *L. hatsudake* GX01 in media with different acidity. Data are expressed as the mean ± standard deviation of 5 biological replicates (n = 5).

Table 3. Mycelial diameter of *L. hatsudake* GX01 in different pH media. Each value is expressed as mean ± SD (n = 5). Different letters indicate significant differences in the means of mycelial diameters in different-acidity media ($p < 0.05$, one-way analysis of variance).

Growth Acidity	pH 4.0	pH 5.0	pH 6.0	pH 7.0
Diameter (cm)	4.920 ± 0.400 b	8.380 ± 0.240 a	8.250 ± 0.270 a	8.500 ± 0.000 a
Growth rate (cm/day)	0.137 ± 0.011 b	0.233 ± 0.007 a	0.229 ± 0.007 a	0.236 ± 0.000 a

3.3. Seed Disinfection Treatment

As shown in Table 4, the seed contamination rate decreased, and the sterile seedling rate tended to increase, with the increase in disinfection time for 0.5% (*w*/*v*) $KMnO_4$. With 30% (*v*/*v*) H_2O_2 as the disinfectant, the seed germination rate and sterile seedling rate decreased with the increase in disinfection time. Considering the contamination rate, germination rate, sterile seedling rate and time cost, it is obvious that 30% (*v*/*v*) H_2O_2 had a better disinfection efficiency on *P. massoniana* seeds than 0.5% (*w*/*v*) $KMnO_4$. Disinfection of seeds with 30% (*v*/*v*) H_2O_2 for 4 min was the most suitable treatment for the preparation of sterile seedlings.

Table 4. Seed germination of *P. massoniana* with different disinfection treatments.

Sterilization Effects	0.5% $KMnO_4$				30% H_2O_2			
	15 min	20 min	30 min	40 min	4 min	5 min	10 min	15 min
Contamination rate (%)	79	58	55	33	8	2	3	2
Germination rate (%)	53	60	58	60	85	67	43	41
Germ-free seedling rate (%)	16	34	29	45	77	65	42	41

3.4. Mycorrhizal Synthesis between P. massoniana and L. hatsudake GX01

Based on morphological characters of ectomycorrhizae, ectomycorrhizal seedlings were successfully synthesized within two months after the inoculation by the colonization of *L. hatsudake* GX01 in the roots of *P. massoniana* (Figure 6). The root tips of non-ectomycorrhizal seedlings were covered with root hairs on the surface, with white color in the middle and upper part of the root tips, and pointed tips (Figure 6A–C). The root tips of the ectomycorrhizal roots were enlarged and a pronounced vivid orange in colour, with white hyphae entangled on the mantle surface (Figure 6D–F). No laticifers were found on the mantle surface. No root hair structure was observed for the ectomycorrhizal roots. The ectomycorrhiza were mainly in the shape of bifurcate branches with a clustering phenomenon (Figure 6C). Significant differences in the anatomical cross-sections were observed between the ectomycorrhizal and non-ectomycorrhizal *P. massoniana* roots (Figure 7). The periphery of non-ectomycorrhizal root tips had a root hair structure without a mycorrhizal sheath (Figure 7A). However, a typical mantle structure was observed at the periphery of root tips of ectomycorrhizal seedlings (Figure 7B). No root hair structure was found on the periphery of ectomycorrhizal root tips.

The differences in anatomical cross sections between ectomycorrhizal and non-ectomycorrhizal *P. massoniana* roots were further confirmed by SEM analysis (Figure 8). The root tip of a non-mycorrhizal seedling has root hairs on the surface of the root system, with neither mantle structure nor the mycelium invading between root cells (Figure 8A,B). The ectomycorrhizal root tip has many hyphae tightly wrapped around the periphery of the root tip, forming a typical mantle structure (Figure 8C,D). The hyphae penetrate the epidermis and cortex and invade the intercellular structure, forming a typical Hartig net structure (Figure 8C,D).

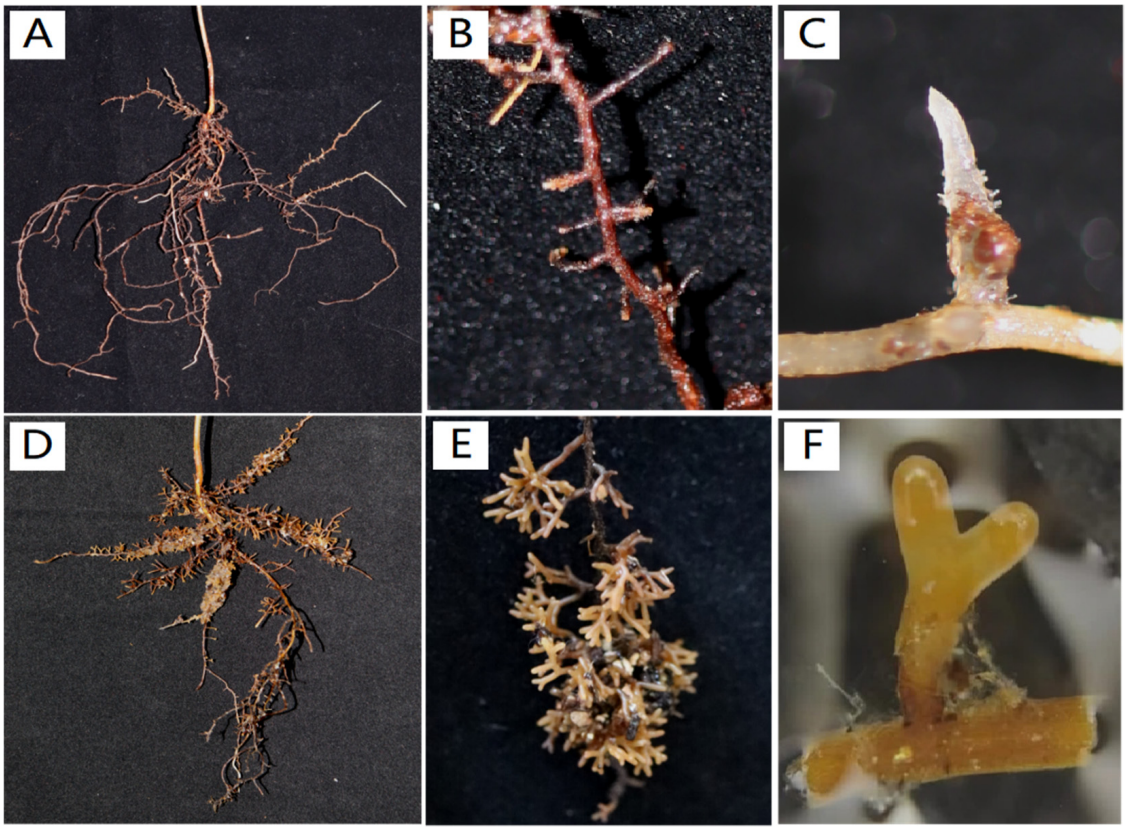

Figure 6. Root morphology of *P. massoniana* seedlings. (**A**–**C**) non-ectomycorrhizal seedlings; (**D**–**F**) ectomycorrhizal seedlings.

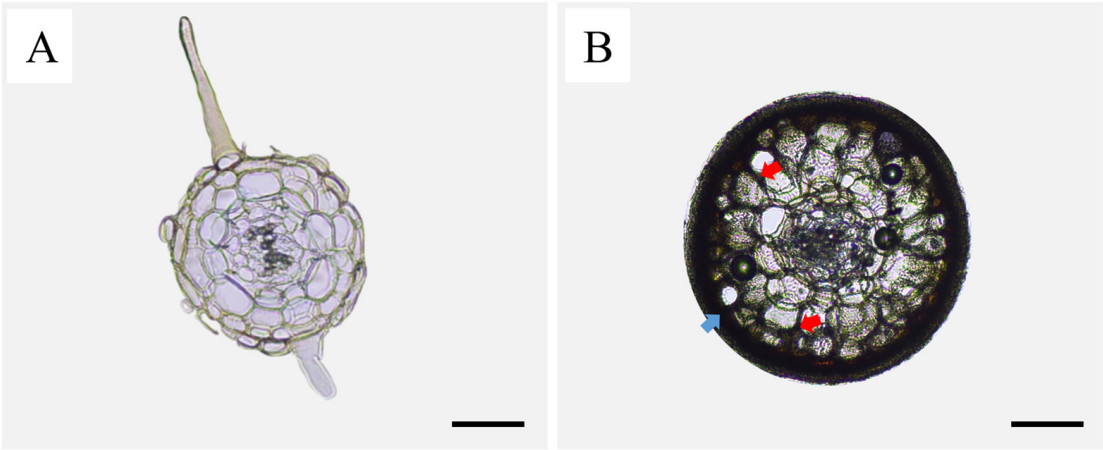

Figure 7. Anatomical cross sections of *P. massoniana* seedling roots. (**A**) non-ectomycorrhizal seedling root; (**B**) ectomycorrhizal seedling root, blue arrow indicates the mantle; red arrows indicate Hartig net structure. Scale bar: 100 μm.

The phylogenetic analysis of *L. hatsudake* GX01 mycelium in pure culture and mycorrhizae colonization was constructed by using MEGA software (version 11.0.13) (Figure 9). The result showed that the ITS sequence of ectomycorrhizal mycorrhizae was identical to that of the initial inoculum, *L. hatsudake* GX01, which proved that the mycorrhizalized seedlings of *P. massoniana* were formed by the colonization of *L. hatsudake* GX01.

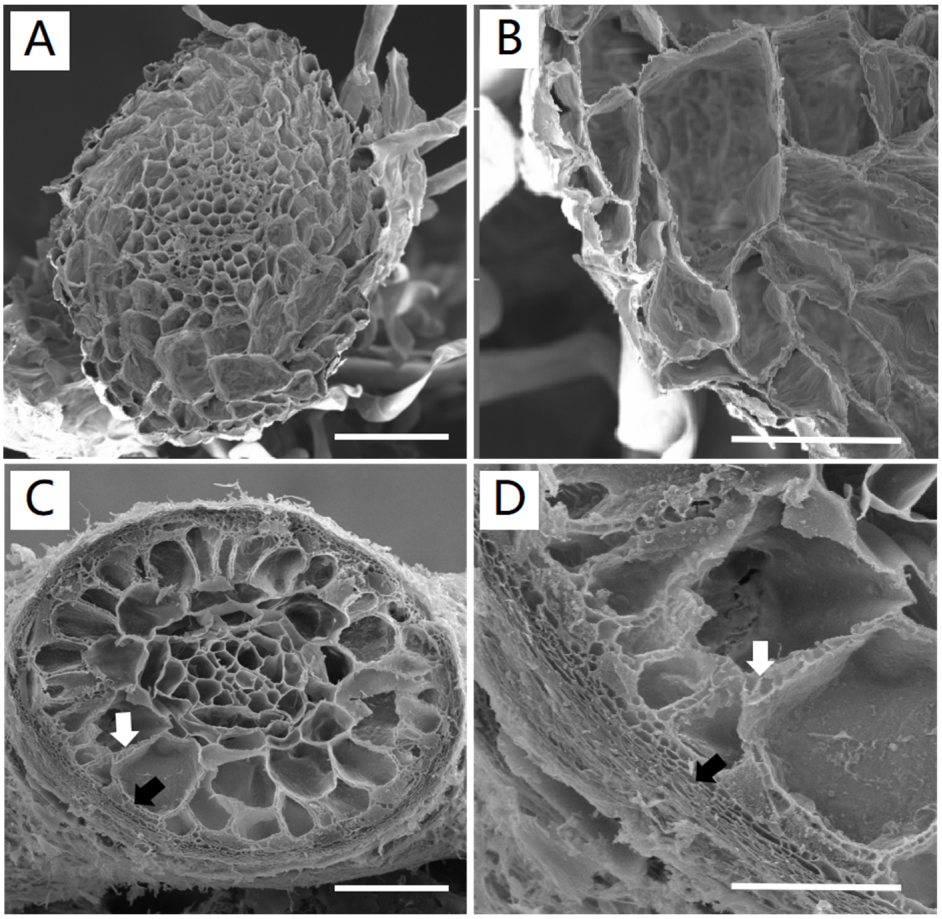

Figure 8. Micrographs of *P. massoniana* seedling root tips captured by scanning electron microscopy. (**A**,**B**) non-ectomycorrhizal seedling root; (**C**,**D**) ectomycorrhizal seedling root, black arrows indicate the mycorrhizal mantle, and white arrows indicate the Hartig net. Scale bar: (**A**,**C**) = 100 μm, (**B**,**D**) = 50 μm).

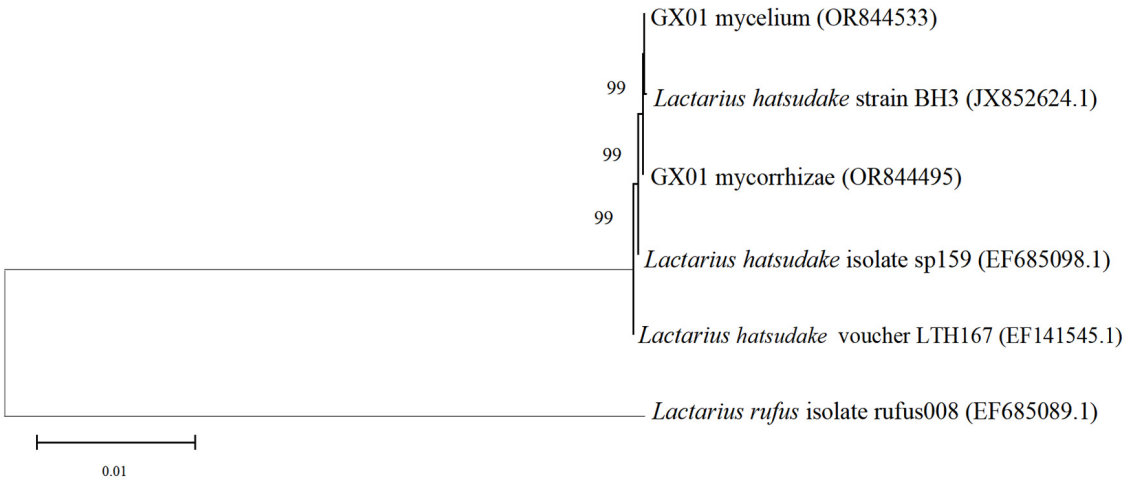

Figure 9. Neighbor-joining phylogenetic analysis of *L. hatsudake* GX01 mycelium and mycorrhizae.

3.5. Effect of L. hatsudake on the Lateral Roots of P. massoniana Seedlings

Inoculation of *L. hatsudake* GX01 greatly improved the formation of lateral roots of mycorrhizal *P. massoniana* seedlings within 4 weeks as compared with that of the non-mycorrhizal control (Figure 10). After 5 weeks of inoculation, *P. massoniana* seedlings

treated with *L. hatsudake* GX01 began to exhibit typical mycorrhizal morphology, i.e., root tip enlargement and branching (Figure 11). No typical mycorrhizal morphology was observed for the root tips of the non-mycorrhizal controls. The number of lateral roots of mycorrhizal *P. massoniana* seedlings started to be significantly higher than that of the control group 4 weeks after inoculation, and reached a significant difference ($p < 0.01$) (Figure 12). An increase of 174.41% in the number of lateral roots of mycorrhizal *P. massoniana* seedling was achieved in 5 weeks as compared with non-mycorrhizal control. After 6 weeks of inoculation, there was a significant difference in the number of lateral roots between mycorrhizal and non-mycorrhizal *P. massoniana* seedlings ($p < 0.01$), and a significant difference in the number of lateral roots could be observed (Figure 12).

Figure 10. Effect of *L. hatsudake* GX01 on the root growth of *P. massoniana* seedlings. (Lha−: without inoculation of *L. hatsudake* GX01; Lha+: with inoculation of *L. hatsudake* GX01).

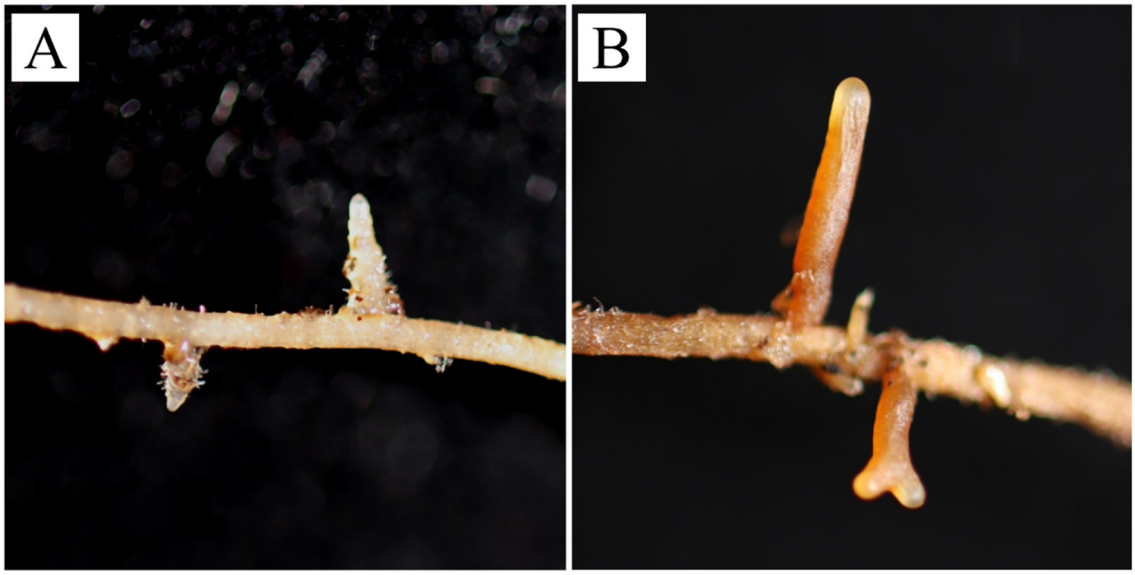

Figure 11. Effect of *L. hatsudake* GX01 on the morphology of lateral roots of. *P. massoniana* seedlings. ((**A**): without inoculation of *L. hatsudake* GX01; (**B**): with inoculation of *L. hatsudake* GX01).

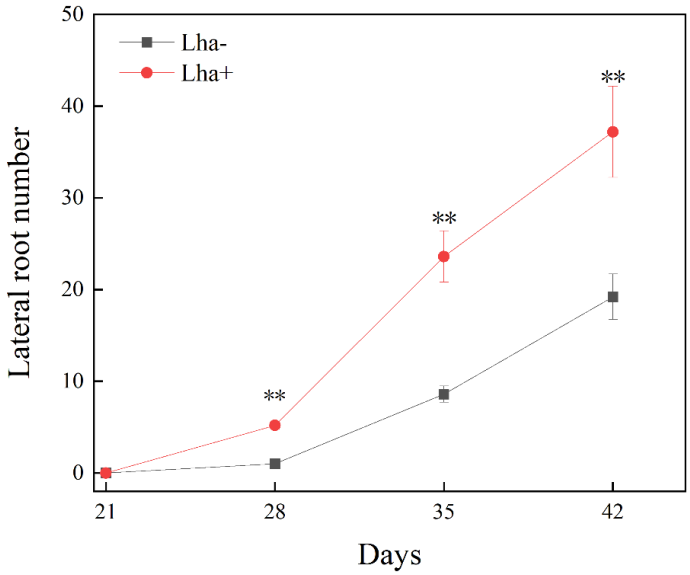

Figure 12. Effect of *L. hatsudake* GX01 on the number of lateral roots in *P. massoniana* seedlings. Lha−: without inoculation of *L. hatsudake* GX01; Lha+: with inoculation of *L. hatsudake* GX01. (Data are expressed as the mean ± standard deviation of 5 biological replicates (n = 5); the "**" indicates significant differences in the means of the lateral root number of *P. massoniana* between Lha− and Lha+ at the same treatment time (**: $p < 0.01$).

3.6. Comparison the Anatomical Cross-Sections of Ectomycorrhizal Root

Differences regarding the mantle thickness and average pore size of mycelium were observed among the different parts of cross-sectional structures of the coral-like ectomycorrhizae (Figure 13). The mantle thickness of ectomycorrhizal roots gradually decreased from the base to the root tip, while the average diameter of *L. hatsudake* GX01 hyphae in the mantle layer gradually increased (Table 5). The results showed that the mantle was only present in coralloidal ectomycorrhizae, and the developmental status was inconsistent at different sites. The main root connected to the base of the coralloidal ectomycorrhizae does not have the mycorrhizal structures (Figure 13B).

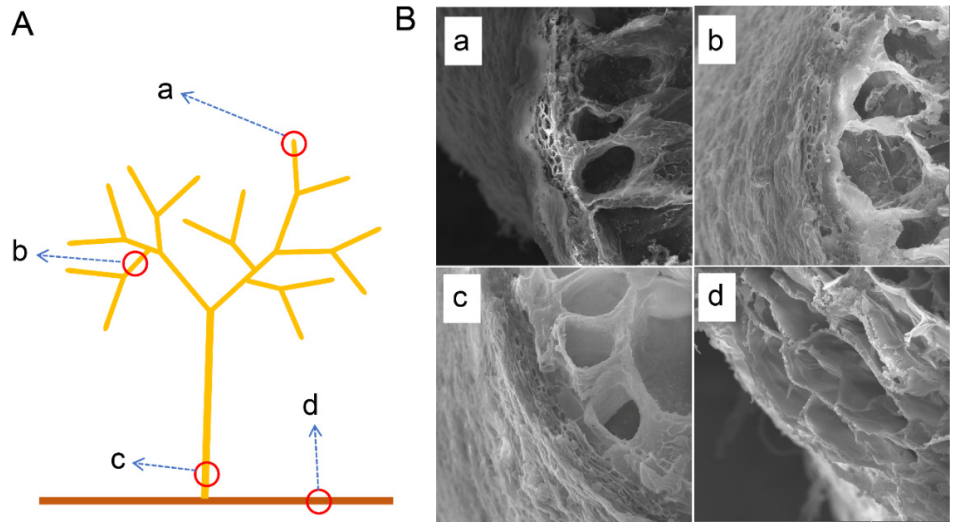

Figure 13. Comparison the anatomical cross sections of ectomycorrhizal root. ((**A**): schematic figure of these anatomical cross sections sampled for SEM; (**B**): micrographs of these anatomical cross sections by scanning electron microscopy); (a: coralloid mycorrhizal root tip; b: bifurcate branching mycorrhizal root base; c: coralloid mycorrhizal root base; d: root connected to coralloid ectomycorrhizal root base).

Table 5. Different mantle thicknesses and mycelial pore diameters in the coral-like mycorrhizae.

Extraction Site	Mantle Thickness (μm)	Mycelium Average Pore Size (μm)
a	9.48	3.29
b	17.23	2.50
c	20.70	2.35
d	ND	ND

a: coralloid mycorrhizal root tip; b: bifurcate branching mycorrhizal root base; c: coralloid mycorrhizal root base; d: root connected to coralloid ectomycorrhizal root base; ND: not detectable.

4. Discussion

The fruiting body of *L. hatsudake* contains rich nutrition, and an anti-cancer effect; it is one of the main commodities in the wild edible mushroom trade market in southwestern China [18,34]. In this study, by analyzing the mycelial growth status of *L. hatsudake* GX01 in different nutrient media, PDA medium was screened out as the optimal medium, and the optimal pH was 7.0. From the perspective of batch production of mycorrhizal *P. massoniana* seedlings at the commercial scale, the use of PDA medium as the base medium can significantly reduce the use of a large number of expensive chemicals and save the cost of production, since its formula only consists of potato, glucose, and agar. The degree of mycorrhizal colonization depends on the inoculation method and plant–fungi interactions [24]. Currently, the ectomycorrhizal fungal inocula used for artificially sustainable mycorrhizal synthesis were mainly prepared by utilizing mycorrhizal strains cultured in liquid medium [37–39]. In the preliminary experiments of this study, it was found that the growth rate of *L. hatsudake* GX01 in liquid medium was not as fast as that in solid medium. Therefore, we chose to cultivate the mycelium in solid medium for preparing the mycorrhizal inoculum. The other two environmental factors of mycelium culture, i.e., temperature and humidity, were set to 25 °C and 75% RH, respectively. In order to better fit the actual production of quantified mycorrhizal *P. massoniana* seedlings, the temperature and liquid culture conditions should be optimized in the near future, to create a more suitable environment for mycelium growth and facilitate the obtaining of more biomass in a shorter period of time.

ECM fungi have been reported to be more or less host plant-specific, but most ECM fungal species are associated with a wide range of hosts, or at least with several species within the same genus [40,41]. Therefore, the combination of mycorrhizal symbionts is diverse, and there likely exist some differences in their morphology. The coral-like ectomycorrhizae of the *P. massoniana* and *Suillus luteus* are bifurcated in shape and have a light brown to dark brown color, with a large amount of hyphae covering the surface [42]. The mantle of the ectomycorrhizae formed between *Lactarius cuspidoaurantiacus* and *Alnus acuminata* was bright yellow to yellow-orange or brown in color, and had a smooth surface with only a few tangled hyphae [43]. The morphological characteristics of ectomycorrhizaes synthesized between *Lactarius* and *Pinus* species (13 distinct combinations of five *Pinus* and five *Lactarius* species) were extensively compared in a recent study [17]. Although these ectomycorrhizaes shared overall similarities in both shape and color, remarkable differences were also observed in terms of the occurrence of laticifers, rhizomorphs, and "spiky" cistidiae [17]. Critical morphological characteristics were lacking in previous studies on the symbionts synthesized between *L. hatsudake* and *P. massoniana* [17,31,32]. Therefore, mycorrhizal synthesis between superior families of *P. massoniana* and native *L. hatsudake* GX01 in this study will increase our knowledge on this symbiosis and lay a foundation for the establishment of mushroom orchards through mycosilviculture in the near future.

There are differences in the field persistence of ectomycorrhizal symbionts for different EMF–plant combinations. Mycorrhizal synthesis of the seedlings of *Pinus pinaster* and *Pinus sylvestris* by inoculation of *Lactarius deliciosus* achieved high colonization rates of 95% and 94%, respectively [24]. However, a sharp decrease in mycorrhizal colonization was detected on transplanted seedlings after 4 months' growth in the greenhouse. The colonization

rate of *P. pinaster* and *P. sylvestris* decreased respectively to 49% and 2% [24]. Gomes et al. documented that mycorrhizae formed by *L. deliciosus* and *Arbutus unedo* L. were able to persist for 9 months after plant domestication [38]. The *P. massoniana–L. hatsudake* symbiont established in this study showed a high rate of mycorrhizal colonization even after 12 months of transplantation (unpublished data). The capacity of this *P. massoniana–L. hatsudake* symbiont in maintaining long field persistence of ectomycorrhizal rate make it a model for the study of the symbiotic molecular mechanism underlying EMF–plant interaction, as well as a promising symbiont for the establishment of trial mushroom orchards in the near future. It is worth mentioning that evaluation of the field persistence of this synthesized ectomycorrhizal symbiont in a trial mushroom orchard is necessary since it will likely suffer from the multiple influences of biotic (e.g., fierce competition from other ectomycorrhizal fungi) and abiotic (harsh climate, stress) conditions.

EMF have a significant impact on the growth and development of plant root systems. Under drought stress, mycorrhizalized seedlings significantly increased the biomass of roots compared to non-mycorrhizalized seedlings [44]. The root length of mycorrhizal seedlings was much higher than that of non-mycorrhizal seedlings under salt stress [45]. The total root length, surface area, average diameter, root volume, and number of root tips of mycorrhizal seedlings were higher than those of non-mycorrhizal seedlings under aluminum stress [46]. Feng et al. found that volatile organic compounds (VOCs) and exudates released by *Suillus bovinus*, an EMF, could significantly promote the development of the roots of *P. massoniana* [35]. It has been shown that the ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation through auxin transport signaling [47]. Ditengou et al. found that the ectomycorrhizal fungus *Laccaria bicolor* promoted the formation of lateral roots in plants through the release of bioactive substances, i.e., sesquiterpenes [48]. The results of this study showed that the addition of *L. hatsudake* GX01 to the seedling substrate could significantly improve the formation of lateral roots in *P. massoniana* seedlings, thereby expanding the area of root contact and nutrient uptake and promoting seedling growth and development. For *L. hatsudake* GX01, whether its secretion or the released VOCs play a dominant role in the induction of lateral roots of *P. massoniana* seedlings during ectomycorrhizal synthesis remains to be further investigated in subsequent experiments.

During the establishment of ectomycorrhiza, fungal mycelium invades the root of the host plant from the root crown cells and invades upward into the epidermis, and after attaching to the epidermal cells, the mycelium multiplies to form a series of mantle layers [49]. Felten et al. (2009) demonstrated that as the co-cultivation time increased, the fungal mycelium wrapped around the root tip gradually increased, and the thickness of the mantle gradually increased [47]. We also observed similar results: a gradual accumulation of mycelium at the site of initial exposure to EMF (i.e., the base of the coralline ectomycorrhizae) and a gradual increase in the thickness of the mantle layer as the root tip extended outward (Figure 13, Table 5).

5. Conclusions

This study systematically investigated the mycorrhizal synthesis of *L. hatsudake* GX01 colonized in a commercially important pine species (*P. massoniana*). The morphological characteristics of ectomycorrhizal *P. massoniana* seedlings colonized by *L. hatsudake* GX01 are usually orange-yellow to brown in color, and rodlike, bifurcated, or coralloid in shape with dichotomous branches. The mycorrhizal *P. massoniana* seedlings developed many more lateral roots than the control (non-mycorrhizal seedlings), which might act as a form of afforestation and trending technology in China. Differences regarding the mantle thickness and average pore size of mycelium were observed among the different parts of cross-sectional structures of the coral-like ectomycorrhiza. The pipeline and relevant methods used in this study were insightful for the further development of mycorrhizal synthesis of *Lactarius* species and numerous other edible ECM fungi with pine species. Mycorrhizal synthesis between superior families of *P. massoniana* and native *L. hatsudake* GX01 in this study lay a foundation for a model for study of the symbiotic molecular

mechanism underlying EMF–plant interaction, as well as the establishment of mushroom orchards through mycosilviculture in the near future.

Author Contributions: Z.W.: Writing—original draft, Investigation, Software, Methodology. L.L.: Methodology, Investigation. Y.L.: Validation, Investigation. S.X.: Software, Data curation. J.M.: Funding acquisition, Resources. Y.T.: Resources. N.T.: manuscript revision and suggestion. Z.Y.: Conceptualization, Funding acquisition, Resources. C.A.: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Guangxi Key Research and Development Program (AB22080072, AB21220057); the Guangxi Science & Technology Project (AD21220074); the Research Funds of The Guangxi Key Laboratory of Landscape Resources Conservation and Sustainable Utilization in Lijiang River Basin, Guangxi Normal University (Grant No.: LRCSU21Z0316); the Guangxi Key Laboratory of Superior Timber Trees Resource Cultivation (22-B-01-01); the scientific research capacity building project for Xing'an Guilin Lijiangyuan Forest Ecosystem Observation and Research Station of Guangxi under Grant No. GXSTP 22-035-130-02; the Xing'an Guilin Lijiangyuan Forest Ecosystem Observation and Research Station of Guangxi (LJF-2022KF01).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

References

1. Tedersoo, L.; Bahram, M. Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. *Biol. Rev.* **2019**, *94*, 1857–1880. [CrossRef] [PubMed]
2. Rinaldi, A.C.; Comandini, O.; Kuyper, T.W. Ectomycorrhizal fungal diversity: Seperating the wheat from the chaff. *Fungal Divers.* **2008**, *33*, 1–45.
3. Brundrett, M.C. Mycorrhizal associations and other means of nutrition of vascular plants: Understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil* **2009**, *320*, 37–77. [CrossRef]
4. Brundrett, M.C.; Tedersoo, L. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* **2018**, *220*, 1108–1115. [CrossRef] [PubMed]
5. Martin, F.; Kohler, A.; Murat, C.; Veneault-Fourrey, C.; Hibbett, D.S. Unearthing the roots of ectomycorrhizal symbioses. *Nat. Rev. Microbiol.* **2016**, *14*, 760–773. [CrossRef] [PubMed]
6. Van Tichelen, K.K.; Colpaert, J.V.; Vangronsveld, J. Ectomycorrhizal protection of *Pinus sylvestris* against copper toxicity. *New Phytol.* **2002**, *150*, 203–213. [CrossRef]
7. Luo, Z.B.; Wu, C.; Zhang, C.; Li, H.; Lipka, U.; Polle, A. The role of ectomycorrhizas in heavy metal stress tolerance of host plants. *Environ. Exp. Bot.* **2014**, *108*, 47–62. [CrossRef]
8. Bonet, J.A.; de-Miguel, S.; Martínez de Aragón, J.; Pukkala, T.; Palahí, M. Immediate effect of thinning on the yield of *Lactarius* group deliciosus in Pinus pinaster forests in Northeastern Spain. *For. Ecol. Manag.* **2012**, *265*, 211–217. [CrossRef]
9. De Román, M.; Boa, E. The marketing of *Lactarius deliciosus* in northern Spain. *Econ. Bot.* **2006**, *60*, 284–290. [CrossRef]
10. Sun, X.; Feng, W.; Li, M.; Shi, J.; Ding, G. Phenology and cultivation of *Suillus bovinus*, an edible mycorrhizal fungus, in a Pinus massoniana plantation. *Can. J. For. Res.* **2019**, *49*, 960–968. [CrossRef]
11. Guerin-Laguette, A. Successes and challenges in the sustainable cultivation of edible mycorrhizal fungi—Furthering the dream. *Mycoscience* **2021**, *62*, 10–28. [CrossRef]
12. Zhao, Y.; Chai, H.; Chen, W. Discussion on the current development status and sustainable development technologies of China's wild edible fungi industry. *Edible Med. Mushrooms* **2021**, *29*, 372–379. (In Chinese)
13. Zhao, C.; Sun, D.; Hua, R.; Dong, J.; Liu, Y.; Zhou, X.; Tai, L. Study on development status and sustainable development countermeasures of *Tricholoma matsutake* industry. *Edible Fungi China* **2023**, *42*, 103–109. [CrossRef]
14. Palahí, M.; Pukkala, T.; Bonet, J.A.; Colinas, C.; Fischer, C.R.; Martínez de Arago'n, J.R. Effect of the inclusion of mushroom values on the optimal management of even-aged pine stands of Catalonia. *For. Sci.* **2009**, *55*, 503–511.
15. Martínez de Aragón, J.; Riera, P.; Giergiczny, M.; Colinas, C. Value of wild mushroom picking as an environmental service. *For. Policy Econ.* **2011**, *13*, 419–424. [CrossRef]
16. Lee, H.; Wissitrassameewong, K.; Park, M.S.; Verbeken, A.; Eimes, J.; Lim, Y.W. Taxonomic revision of the genus *Lactarius* (Russulales, Basidiomycota) in Korea. *Fungal Divers.* **2019**, *95*, 275–335. [CrossRef]
17. Wang, R.; Guerin-Laguette, A.; Huang, L.L.; Wang, X.H.; Butler, R.; Wang, Y.; Yu, F.Q. Mycorrhizal syntheses between *Lactarius* spp. section Deliciosi and *Pinus* spp. and the effects of grazing insects in Yunnan, China. *Can. J. For. Res.* **2019**, *49*, 616–627. [CrossRef]

18. Yang, Q.; Zhang, X.; Qin, H.; Luo, F.; Ren, J. Phenolic acid profiling of *Lactarius hatsudake* extracts, anti-cancer function and its molecular mechanisms. *Foods* **2022**, *11*, 1839. [CrossRef] [PubMed]
19. Wang, L.; Li, Z.; Zhu, M.; Meng, L.; Wang, H.; Ng, T.B. An acidic feruloyl esterase from the mushroom *Lactarius hatsudake*: A potential animal feed supplement. *Int. J. Biol. Macromol.* **2016**, *93*, 290–295. [CrossRef] [PubMed]
20. Miyazawa, M.; Kawauchi, Y.; Matsuda, N. Character impact odorants from wild mushroom (*Lactarius hatsudake*) used in Japanese traditional food. *Flavour Fragr. J.* **2010**, *25*, 197–201. [CrossRef]
21. Tomao, A.; Bonet, J.A.; Martínez de Aragón, J.; de-Miguel, S. Is silviculture able to enhance wild forest mushroom resources? Current knowledge and future perspectives. *For. Ecol. Manag.* **2017**, *402*, 102–114. [CrossRef]
22. Wang, Y.; Hall, I.R.; Dixon, C.; Hance-Halloy, M.; Strong, G.; Brass, P. The cultivation of *Lactarius deliciosus* (saffron milk cap) and *Rhizopogon rubescens* (shoro) in New Zealand. In Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3–6 July 2021; pp. 511–523.
23. Guerin-Laguette, A.; Cummings, N.; Butler, R.C.; Willows, A.; Hesom-Williams, N.; Li, S.H.; Wang, Y. *Lactarius deliciosus* and *Pinus radiata* in New Zealand: Towards the development of innovative gourmet mushroom orchards. *Mycorrhiza* **2014**, *24*, 511–523. [CrossRef]
24. Parladé, J.; Pera, J.; Luque, J. Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. *Mycorrhiza* **2004**, *14*, 171–176. [CrossRef]
25. Yamada, A.; Ogura, T.; Ohmasa, M. Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by in vitro mycorrhizal synthesis I. Primordium and basidiocarp formation in open-pot culture. *Mycorrhiza* **2001**, *11*, 59–66. [CrossRef]
26. He, Y.; Ma, J.; Chen, G. Potential geographical distribution and its multi-factor analysis of *Pinus massoniana* in China based on the maxent model. *Ecol. Indic.* **2023**, *154*, 110790. [CrossRef]
27. Meng, J.; Lu, Y.; Zeng, J. Transformation of a degraded *Pinus massoniana* plantation into a mixed-species irregular forest: Impacts on stand structure and growth in southern China. *Forests* **2014**, *5*, 3199–3221. [CrossRef]
28. Xu, Q.; Wu, G.; Zeng, R.; Wei, S.; Zhang, X.; Zhang, W.; Chen, J. Developing stand form-height tables of *Pinus massoniana* plantations in Guangxi. *Guangxi For. Sci.* **2023**, *52*, 575–580. (In Chinese)
29. Yang, Z.; Xia, H.; Tan, J.; Feng, Y.; Huang, Y. Selection of superior families of *Pinus massoniana* in southern China for large-diameter construction timber. *J. For. Res.* **2018**, *31*, 475–484. [CrossRef]
30. Savoie, J.-M.; Largeteau, M.L. Production of edible mushrooms in forests: Trends in development of a mycosilviculture. *Appl. Microbiol. Biotechnol.* **2010**, *89*, 971–979. [CrossRef]
31. Xue, Z.; Ying, G.; Lv, M.; Li, L.; Gao, F. Studies on mycorrhizal formation of *Pinus massoniana* inoculated *Lactarius hatsudake*. *Edible Fungi China* **2014**, *33*, 18–19. (In Chinese)
32. Tan, Z.; Eric, D.; Airong, S.; Fu, S. Successful cultivation of *Lactarius hatsutake*—An evaluation with molecular methods. *Acta Edulis Fungi* **2008**, *15*, 85–88. (In Chinese)
33. Marx, D.H. The Influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **1969**, *59*, 153–163.
34. Wang, R.; Alexis, G.-L.; Yu, F. Optimum media for hyphal growth and mycorrhizal synthesis of two *Lactarius* species. *Mycosystema* **2020**, *39*, 1346–1355. (In Chinese)
35. Feng, W.; Sun, X.; Ding, G. Morphological and transcriptional characteristics of the symbiotic interaction between *Pinus massoniana* and *Suillus bovinus*. *J. Fungi* **2022**, *8*, 1162. [CrossRef]
36. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
37. Navarro García, A.; del Pilar Bañón Árias, S.; Morte, A.; Sánchez-Blanco, M.J. Effects of nursery preconditioning through mycorrhizal inoculation and drought in *Arbutus unedo* L. plants. *Mycorrhiza* **2010**, *21*, 53–64. [CrossRef] [PubMed]
38. Gomes, F.; Suárez, D.; Santos, R.; Silva, M.; Gaspar, D.; Machado, H. Mycorrhizal synthesis between *Lactarius deliciosus* and *Arbutus unedo* L. *Mycorrhiza* **2015**, *26*, 177–188. [CrossRef]
39. Li, M.; Wang, H.; Zhao, X.; Feng, W.; Ding, G.; Quan, W. Effect of ectomycorrhizal fungi on the drought resistance of *Pinus massoniana* seedlings. *J. Fungi* **2023**, *9*, 471. [CrossRef]
40. Kumar, J.; Atri, N.S. Studies on ectomycorrhiza: An appraisal. *Bot. Rev.* **2017**, *84*, 108–155. [CrossRef]
41. Dickie, I.A. Host preference, niches and fungal diversity. *New Phytol.* **2007**, *174*, 230–233. [CrossRef]
42. Pan, X.; Zhang, J.; Xue, Z.; Liang, J.; Chen, Y.; Liu, Y. Synergistic effect of phytohormone-producing ectomycorrhizal fungus *Suillus luteus* and fertilizer GGR6 on *Pinus massoniana* growth. *J. Plant Interact.* **2022**, *17*, 643–655. [CrossRef]
43. Montoya, L.; Bandala, V.M.; Garay-Serrano, E. The ectomycorrhizas of Lactarius cuspidoaurantiacus and *Lactarius herrerae* associated with *Alnus acuminata* in Central Mexico. *Mycorrhiza* **2015**, *25*, 457–467. [CrossRef] [PubMed]
44. Alvarez, M.; Huygens, D.; Fernandez, C.; Gacitua, Y.; Olivares, E.; Saavedra, I.; Alberdi, M.; Valenzuela, E. Effect of ectomycorrhizal colonization and drought on reactive oxygen species metabolism of *Nothofagus dombeyi* roots. *Tree Physiol.* **2009**, *29*, 1047–1057. [CrossRef]
45. Bullaín Galardis, M.M.; López Sánchez, R.C.; Fall, F.; Eichler-Löbermann, B.; Pruneau, L.; Bâ, A.M. Growth and physiological responses of ectomycorrhizal *Coccoloba uvifera* (L.) L. seedlings to salt stress. *J. Arid. Environ.* **2022**, *196*, 104650. [CrossRef]

46. Li, K. Response of Mycorrhizal *Pinus massoniana* Seedlings to Aluminum Stress. Master's Thesis, Guizhou University, Guiyang, China, 2019.
47. Felten, J.; Kohler, A.; Morin, E.; Bhalerao, R.P.; Palme, K.; Martin, F.; Ditengou, F.A.; Legué, V.r. The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in *Poplar* and *Arabidopsis* through auxin transport and signaling. *Plant Physiol.* **2009**, *151*, 1991–2005. [CrossRef]
48. Ditengou, F.A.; Müller, A.; Rosenkranz, M.; Felten, J.; Lasok, H.; van Doorn, M.M.; Legué, V.; Palme, K.; Schnitzler, J.-P.; Polle, A. Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat. Commun.* **2015**, *6*, 6279. [CrossRef] [PubMed]
49. Horan, D.P.; Chilvers, G.A.; Lapeyrie, F.F. Time sequence of the infection process eucalypt ectomycorrhizas. *New Phytol.* **2006**, *109*, 451–458. [CrossRef]