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Effects of Different Ectomycorrhizal Fungal Inoculates on the Growth of *Pinus tabulaeformis* Seedlings under Greenhouse Conditions

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Academic Editors: Jarmo Holopainen and Timothy A. Martin
Received: 10 August 2016; Accepted: 1 December 2016; Published: 14 December 2016

Abstract: The tree species *Pinus tabulaeformis* Carr. (*P. tabulaeformis*) is commonly planted in China due to its economic and ecological value. In order to identify one or more ectomycorrhizal (ECM) fungal species for future *P. tabulaeformis* afforestation, we investigated the effects of five ECM fungal species: *Laccaria laccata*, *Boletus edulis*, *Gomphidius viscidus*, *Suillus grevillei*, and *Suillus luteus* on the growth of *P. tabulaeformis* seedlings under greenhouse conditions. The growth parameters of *P. tabulaeformis* seedlings were evaluated 90 days following fungal colonisation. The majority of seedlings were significantly affected by ECM inoculation. Mycorrhizal inoculated seedlings were taller, had more lateral roots, and a greater biomass compared with the non-mycorrhizal (CK) seedlings. With the exception of *G. viscidus*, inoculated seedlings exhibited higher phosphorus, potassium, and nitrogen content compared with the CK seedlings. In addition, ECM colonisation increased the enzymatic activity of catalase, acidic phosphatase, protease, and the urease content in the rhizosphere soil. Our study showed that *Laccaria laccata*, *Suillus grevillei*, and *Suillus luteus* may be useful for improving the growth and cultivation of *P. tabulaeformis* seedlings. Furthermore, we observed that *S. luteus* inoculation increased the gas exchange parameters of *P. tabulaeformis* seedlings under field conditions.

Keywords: ecto-mycorrhizal fungi; enzymatic activity; *Pinus tabulaeformis* Carr.; seedling growth

1. Introduction

Pinus tabulaeformis is widely cultivated in arid and semi-arid regions of China and has a strong adaptability to dry and barren habitats. Due to its versatility and resiliency, it is considered an important tree species for forest restoration in Northwestern China. Moreover, it provides other important ecosystem functions, including enrichment of headwaters, soil fertility, and water conservation [1,2].

Reduced amounts of root hairs on the *Pinus* species have contributed to the evolution of mycorrhiza, which are particularly important for the absorption of water and nutrients [3]. Ectomycorrhizal (ECM) fungi can ameliorate the growth of seedlings by improving water and nutrient uptake from the soil [4]. Without ECM fungi, *P. tabulaeformis* seedlings may not develop normally [5]. Ectomycorrhiza also play an important role in protecting plants against environmental stress, such

as drought, pathogenic agents, and heavy metal pollution [6]. Artificial inoculation techniques may mitigate the adverse effects of fertilisation and other nursery treatments on seedling mycorrhiza development, and increase the field performance of out-planted seedlings [6].

Inoculating *P. tabulaeformis* seedlings with compatible ECM fungi has been shown to be beneficial for the growth and stress-resistance of the host seedlings [7–9]. However, previous studies have primarily focused on the effects of *Boletus* and *Suillus* species. Therefore, the effects of ECM species belonging to other genera, such as *Laccaria*, on *P. tabulaeformis* are unknown. The response of plants to inoculation largely depends on the host plant and fungal species [10]. Consequently, the selection of appropriate ECM fungi is a critical step towards establishing successful nursery inoculation programs [11,12]. The selection criteria are based on the physiological and ecological characteristics of fungal species and strains [13].

Our objective was to identify one or more ECM fungal species for future afforestation. Therefore, we compared the inoculated effects of five ECM fungal species: *Boletus edulis*, belonging to *Boletaceae*; *Gomphidius viscidus*, belonging to *Gomphidiaceae*; *Laccaria laccata*, belonging to *Tricholomataceae*; *Suillus grevillei* and *Suillus luteus* belonging to *Suillaceae*. Although these ECM fungal species have shown clear effects on *P. tabulaeformis* seedlings or other *Pinus* species in other studies [14–16], the different experimental conditions make it hard to draw clear comparisons. In our study, we evaluated the effectiveness of five ECM fungi by observing the characteristics of the seedlings [17], the uptake of major elements [17], and the enzyme activity (catalase, acidic phosphatase, protease, and urease) present in the rhizosphere soil. Although the colonization of certain ECM fungi has been beneficial under greenhouse conditions, the effects of ECM fungi inoculation on *P. tabulaeformis* seedlings should also be verified under field conditions. To study whether ECM fungi inoculum application under field conditions could benefit the photosynthetic properties of the host plants, as well as the appropriate dosage and the humidity of inoculum, we conducted a field test of *S. luteus* inoculum on planted *P. tabulaeformis* seedlings.

2. Materials and Methods

2.1. Preparation of Seedlings and Fungal Material

P. tabuliformis seeds were obtained from the seed plantation of the Research Institute of Forestry at the Chinese Academy of Forestry in Hebei, China. All seeds were surface-sterilised in 10% hydrogen peroxide (H_2O_2) for 10 min and then washed with sterile distilled water three times. Seeds were then soaked in 45 °C sterile distilled water for 24 h and germinated on glass Petri dishes (diameter: 9 cm) containing moist sterilized vermiculite (121 °C, 2 h) at 25 °C until germination. They were then transplanted into plastic pots (20 cm in depth, 20 cm in diameter) containing 2 kg of sterilised substrate. The substrate used in the pot experiment consisted of turfy soil (Klasmann-Deilmann GmbH, Geeste, Germany), sand, and pearlstone mixed at a V:V ratio of 1:1. The properties of the substrate were: total N, 11.0 $g \cdot kg^{-1}$; total P, 9.4 $g \cdot kg^{-1}$; total K, 4.2 $g \cdot kg^{-1}$; available N, 795.08 $mg \cdot kg^{-1}$; available P, 10.18 $mg \cdot kg^{-1}$; available K, 92.49 $mg \cdot kg^{-1}$; organic matter, 407.2 $g \cdot kg^{-1}$; and pH, 5.75. The plant growth substrate was sterilised using an autoclave at 0.14 MPa and 121 °C for 2 h before use.

Laccaria laccata was obtained from the Chinese Academy of Agricultural Sciences. *Boletus edulis*, *G. viscidus*, *S. grevillei*, and *S. luteus* were obtained from the Chinese Academy of Forestry in Hebei, China. All of the fungi were stored at a low-temperature preservation slant and maintained at 4 °C. The fungi were sub-cultured once a month using Melin-Norkrans medium (MMN) [18].

For the preparation of the inoculum, a portion of stock culture from each fungus was aseptically transferred to a solid-modified Melin-Norkrans medium (MMN) [18] according to the following modifications: KH_2PO_4 (0.5 $g \cdot L^{-1}$), $(NH_4)_2HPO_4$ (0.25 $g \cdot L^{-1}$), $CaCl_2$ (0.05 $g \cdot L^{-1}$), NaCl (0.025 $g \cdot L^{-1}$), $MgSO_4 \cdot 7H_2O$ (0.15 $g \cdot L^{-1}$), 1% $FeCl_3$ solution (1.2 $ml \cdot L^{-1}$), glucose (15 $g \cdot L^{-1}$), malt extract (10 $g \cdot L^{-1}$), peptone (20 $g \cdot L^{-1}$), citric acid (0.2 $g \cdot L^{-1}$), vitamin B (0.1 $mg \cdot L^{-1}$), and agar (14 $g \cdot L^{-1}$). The pH was adjusted to 5.6 using 1 M HCl, and the medium was autoclaved for 20 min at 121 °C. All fungal

strains were maintained as sub-cultures at 24 °C on the same medium for approximately seven days (until the fungi populated the entire Petri dish). The fungi were subsequently inoculated into solid substrate, according to Brundrett et al. (1996) with some modifications [19]. Inocula were produced by: (1) incubating fungal mycelium from the Petri dish cultures; (2) transferring inoculate into a sterile solid substrate (wood chip-vermiculite mixture, 1:1, w:w); and (3) saturating inoculate in an MMN liquid media in large plastic bags (1 L) containing approximately 500 g of substrate in each bag. The mycelium were incubated at 25 °C for approximately three to four weeks until the substrates were completely covered by fungi and ready to use for inoculation.

The study included six treatments: *Laccaria laccata*; *Boletus edulis*; *G. viscidus*; *Suillus grevillei*; *Suillus luteus*; and a non-mycorrhizal (CK) treatment—all using a sterilised substrate (without fungal hyphae). When the seedlings were about 60 days old, equal-sized seedlings were transplanted into other plastic pots (20 cm in depth, 20 cm in diameter; three seedlings for each pot) containing 2 kg of sterilised soil. Thirty days following transplantation, 20 g of inoculum or sterilised substrate was placed 5 cm below the substrate surface in each mycorrhizal treatment pot. Each treatment had 15 replicates.

2.2. Experimental Design

P. tabuliformis seedlings were grown in a greenhouse from August to November 2010 for 90 days. The experiment was conducted under natural light conditions with a temperature regime of 25 °C day/18 °C night, a 14-h light/10-h dark photocycle, and a relative humidity of 60% day/70% night at the Chinese Academy of Forestry. The containers were irrigated with distilled water to maintain the moisture level at field capacity.

2.3. Plant Measurements and ECM Colonisation

After 90 days of growth in the pots, seedlings were carefully hand-excavated to retrieve a representation of the intact root system. Plant height was measured; the base diameter was measured using Vernier calipers (Mitutoyo, Kawasaki, Japan). The number of lateral roots was calculated by counting the lateral roots that measured more than 5 cm in length.

We randomly selected 10 pots from each treatment, and three seedlings for each pot, to investigate ECM colonisation. The roots were carefully cleaned under water in the laboratory. About 300 1 cm-long root tips were randomly collected from each treatment (30 tips from each pot, 10 tips per seedling) and then clipped and washed using double distilled water. ECM colonization was determined by counting the colonised root tips under a stereomicroscope. EMC roots were evaluated by examining and identifying whether there was the presence of any hyphae or rhizomorphs on the root tips or not. The calculation of ECM colonization was estimated for each sample by examining 300 1 cm-long pieces of root, expressed with the following formula:

ECM colonization rate (%) = Number of mycorrhizal root pieces/Total number of observed root pieces × 100%

The colonization levels were evaluated according to the ECM colonization rate: Level 0: 0%–10%; Level 1: 10%–19%; Level 2: 20%–29%; Level 3: 30%–39%; Level 4: 40%–49%; Level 5: 50%–100% [20].

2.4. Inorganic Nutrient Content

Plant inorganic nutrient content was examined by analysing elements in the roots, shoots, and needles. The samples were oven-dried at 105 °C for 30 min, and then at 80 °C for 24 h until a constant weight was reached. Each sample (0.2 g) was collected by coning and quartering, and then added to a 100-mL Kjeldahl flask containing 5 mL of concentrated sulfuric acid. The mixtures were gently shaken, and then heated until a brown-black colour was observed. After cooling, 5 mL of 30% (w/v) H_2O_2 was added to the solution. The mixtures were then gently shaken and heated again for 20 min. The last step was repeated until the liquid became clear, and the flasks were heated for 10 min until H_2O_2 was eliminated. Distilled water was then added to each flask to reach a final volume of 100 mL.

Each solution was analysed for N, P, and K. The total N content was determined using the Kjeldahl method [21], total P was determined using the Mo-Sb colourimetric method [22], and total K was detected using ammonium acetate extraction-flame photometry [23].

2.5. Soil Sample Preparation and Enzymatic Activity Assays

Soil adhering to the roots was collected and pooled by vigorously shaking the seedlings. Approximately 20 g of each soil sample collected was passed through a 1-mm sieve. All samples were frozen at −80 °C. Urease activity was determined by indophenol colourimetry; protease activity was determined by ninhydrin colourimetry; phosphatase activity was determined by phenyl phosphate disodium colourimetry; and catalase activity was determined by permanganate titration, according to Guan (1986) [24]. These enzymes were chosen for their contribution to nutrient absorption and stress-resistance.

2.6. Field Test Design and Photosynthetic Data Analysis

The field tests were performed in the field of the Beijing Forestry Society, Changping Beijing (40°17′26″ N, 116°25′33″ E) from August to October 2013. *P. tabuliformis* clones were approximately four years old, and were planted in 1 m × 1 m intervals. The height of the plants varied from 70–80 cm. *S. luteus* was selected based on beneficial effects shown in the inoculation trials presented in this paper. The inoculum was the same as previously introduced in this paper.

The seedlings were placed in three blocks and three replications were conducted (Figure 1A); the blocks spanned 10 m × 6 m, for a total area of 60 m2. The distance between the three blocks was at least 3 m. Each block contained 32 plants, which included four plants with eight treatments each (six inoculated, two non-mycorrhizal). Six inoculated treatments were applied for a total volume of 600 mL—a mixture of mycorrhizal inoculum and sterilized mycorrhizal inoculum. (I_1 and I_{W1}: 200 mL inoculum and 400 mL sterilized mycorrhizal inoculum; I_2 and I_{W2}: 400 mL inoculum and 200 mL sterilized mycorrhizal inoculum; I_3 and I_{W3}: 600 mL inoculum and no sterilized mycorrhizal inoculum).

To improve the inoculation method, I_1–I_3 treatments used dry inoculum (inoculated directly), while Iw_1–Iw_3 treatments used moist inoculum (inoculum mixed with l L of water before inoculation). The inoculum was prepared using the same method as the greenhouse trial.

The plants were inoculated in August 2013. To inoculate the plants, four holes were dug approximately 15 cm at four distinct angles from the trunk of the tree (using the tree as the centre of the square)(Figure 1B). To ensure successful inoculation of the roots, the holes were dug as close to the roots as possible and equidistant from the trunk. Inoculum was equally distributed in each of the holes, and then filled with soil. The two non-mycorrhizal plants were treated with 600 mL of sterilised inoculum (mixed or not mixed with water).

Photosynthetic data were collected in October 2013, 60 days after inoculation. By visual inspection, a sample of mature and healthy needles from the top of each plant was collected, and photosynthesis was measured using a portable photosynthesis system (LI-6400, Li-COR Inc., Lincoln, NE, USA) at approximately 12:00 pm. The following photosynthetic parameters were measured using an internal light source with a photosynthetically active radiation (PAR) value of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$: net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), intercellular CO_2 concentration (Ci); the temperature was approximately 18 °C. The physiological water-use efficiency (WUE) was also calculated.

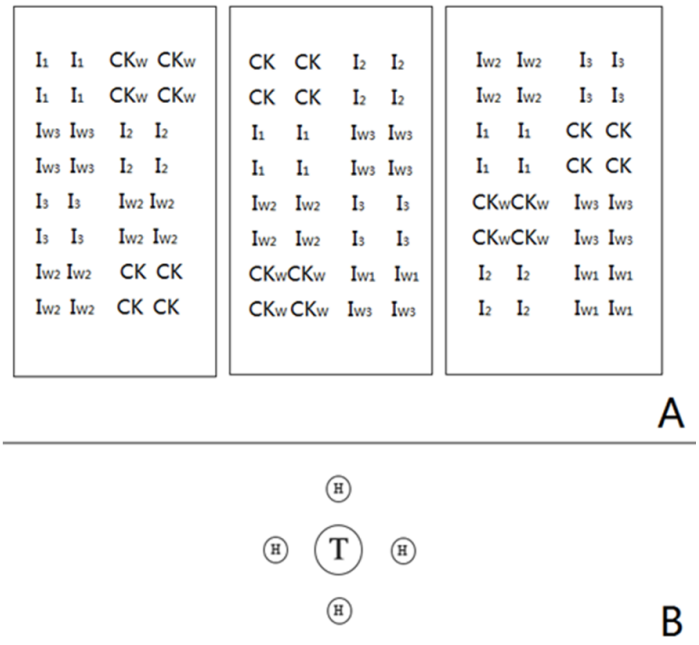

Figure 1. (**A**) The layout of the field test for ectomycorrhizal (EMC) fungi inoculation. I_1–I_3: three inoculated treatments (200 mL, 400 mL, and 600 mL) with dry inoculum; Iw_1–Iw_3: three inoculated treatments (200 mL, 400 mL, and 600 mL) with moist inoculum; CK_W and CK: two non-inoculation treatments using 600 mL moist sterilized inoculum (CK_W) or dry sterilized inoculum (CK). The spacing within the rows was 1 m; the spacing between rows was 1 m; (**B**) The method for the inoculation test: T: inoculated plants; h: holes.

2.7. Statistics

If not otherwise specified, data are presented as means ± standard deviation. The data from the experiments in the greenhouse were analysed with a one-way analysis of variance (ANOVA) using SPSS software (ver. 19.0; SPSS Inc., Chicago, IL, USA); the data from the photosynthetic parameters of the field test were analysed by two-way analysis of variance (ANOVA) and a one-way ANOVA. In the two-way ANOVA, the model included the sample of inoculum (dosage), the humidity of the inoculum (humidity), and the interaction between the dosage and the humidity (dosage*humidity); we used SPSS (ver. 19.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. ECM Colonisation

Pinus tabulaeformis seedlings were successfully colonised following the ECM inoculation treatment (Figure 2); the fungal species colonised the plant roots at a rate of 66% or greater. The CK samples showed very low colonisation. Among the ECM inoculates, *S. luteus* (93.2% ± 2.7%) and *L. laccata* (85.9% ± 4.8%) exhibited the highest colonisation rates, while the *B. edulis* treatment demonstrated the lowest (66.0% ± 6.3%). Colonisation ability exhibited the following pattern: *S. luteus* > *L. laccata* > *S. grevillei* > *G. viscidus* > *B. edulis* > CK (Table 1).

Table 1. ECM colonisation rate (%) *P. tabulaeformis* seedlings: *L. laccata*, *S. luteus*, *S. grevillei*, *B. edulis*, *G. viscidus*, and CK.

Treatment	ECM Colonisation Rate (%)	Colonisation Level
L. laccata	85.9 ± 4.8 b	5
S. luteus	93.2 ± 2.7 a	5
S. grevillei	78.7 ± 4.5 c	5
B. edulis	66.0 ± 6.3 d	5
G. viscidus	71.0 ± 5.2 d	5
CK	4.2 ± 0.8 e	0

The different letters denote significant differences ($p < 0.05$) between the treatments, according to Duncan's multiple range test. The error bars refer to standard deviation.

3.2. Plant Growth and Biomass

Excluding the *G. viscidus* treatment, both the height and base diameter of the inoculated plants were higher than the CK plants. The *L. laccata*, *S. luteus*, and *S. grevillei* had a significant effect on plant height and lateral root numbers (Figures 2 and 3). *S. luteus* had the greatest effect on plant height (45.85% more than CK samples), and *L. laccata* had the greatest effect on plant base diameter (14.53% more than CK samples). The *B. edulis* and *G. viscidus* had limited effects on plant biomass and growth.

Inoculation with ECM can potentially increase the number of lateral roots and biomass (Table 2). The *S. luteus*, *L. laccata*, and *S. grevillei* treatments significantly increased the number of lateral roots on the seedlings by 15.69%, 12.75%, and 9.80%, respectively, compared with the CK samples. Conversely, the *B. edulis* treatment resulted in 4.90% less lateral root development than the CK samples.

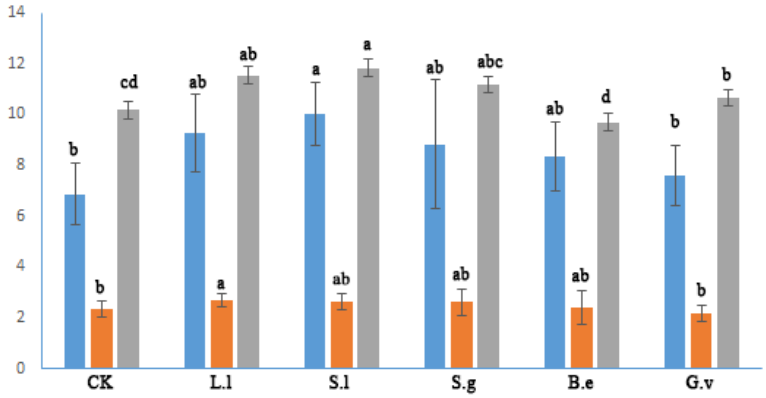

Figure 2. Effects of ECM inoculation on *P. tabulaeformis* seedling growth: *L. laccata* (L.l), *S. luteus* (S.l), *S. grevillei* (S.g), *B. edulis* (B.e), *G. viscidus* (G.v), and treated with steriled substance (CK). Colours denote plant characteristics: **blue** (plant height, cm); **orange** (plant base diameter, mm); **grey** (number of lateral roots). The different letters in the same colours denote significant differences ($p < 0.05$) between the treatments, according to Duncan's multiple range test. The error bars refer to standard deviation.

Table 2. Effects of ECM treatments on the biomass of *P. tabulaeformis* seedlings grown in soil inoculated with: *L. laccata*, *S. luteus*, *S. grevillei*, *B. edulis*, *G. viscidus*, and treated with sterilized substance (CK). The different letters denote significant differences ($p < 0.05$) between treatments, according to Duncan's multiple range test.

Treatments	Biomass (Dry)/g	Aboveground (Dry)/g	Underground (Dry)/g	Root:Shoot Ratio
Nonmycorrhizal	1.3 ± 0.3 b	0.8 ± 0.1 b	0.5 ± 0.1 b	0.6 ± 0.1 ab
L. laccata	1.5 ± 0.1 ab	0.9 ± 0.1 ab	0.6 ± 0.1 a	0.7 ± 0.1 a
S. luteus	1.7 ± 0.2 a	1.0 ± 0.1 a	0.6 ± 0.1 a	0.6 ± 0.1 ab
S. grevillei	1.5 ± 0.2 ab	0.9 ± 0.1 ab	0.6 ± 0.3 ab	0.7 ± 0.1 a
B. edulis	1.3 ± 0.3 bc	0.8 ± 0.1 ab	0.4 ± 0.1 b	0.5 ± 0.1 b
G. viscidus	1.0 ± 0.1 c	0.6 ± 0.1 c	0.4 ± 0.1 b	0.7 ± 0.1 a

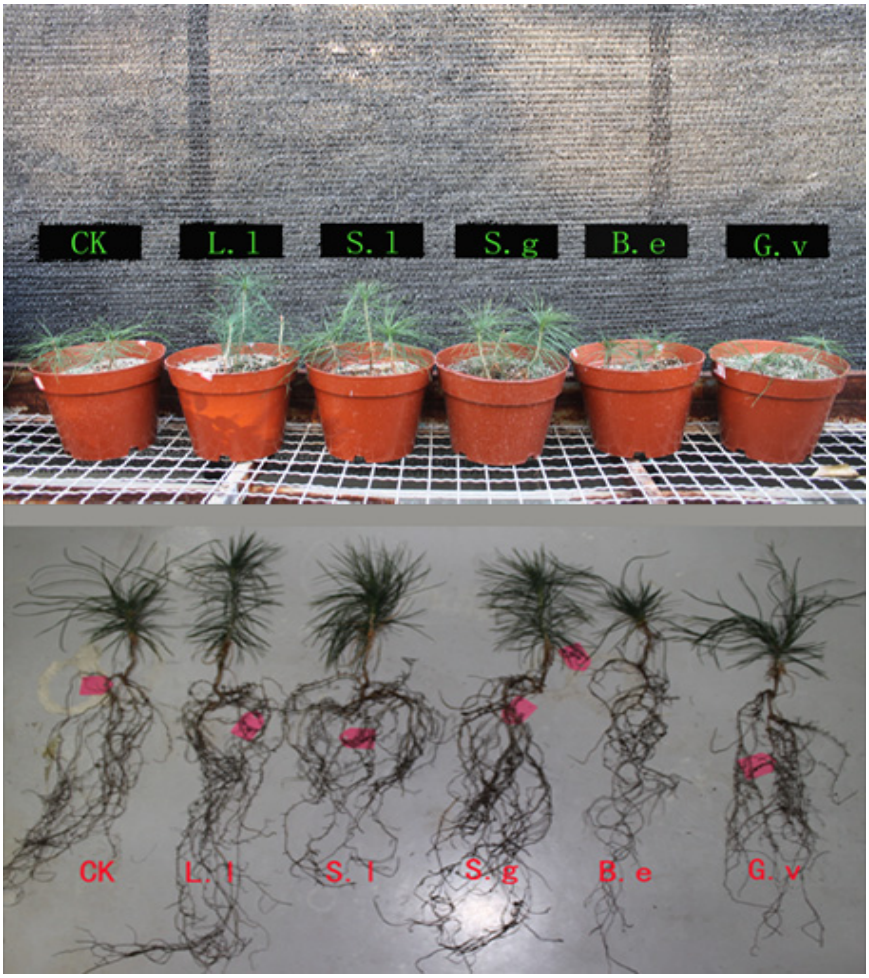

Figure 3. Growth of *Pinus tabulaeformis* seedlings inoculated with *L. laccata* (L.l), *S. luteus* (S.l), *S. grevillei* (S.g), *B. edulis* (B.e), *G. viscidus* (G.v), and non-mycorrhizal (CK); above (above-ground growth) and below (below-ground growth).

3.3. Nutrient Content

The inoculated groups showed improved absorption of N and P. The *S. luteus* treatment resulted in the most efficient K absorption (Table 3), with increases of 22.72%, 42.56%, and 44.97%, in the leaf, stem, and root samples, respectively, compared with the control group. The *B. edulis* treatment contained the highest N content (in the plant leaves), which was 199.51% more than the CK samples. All treatments exhibited increased K absorption, compared with total N and P absorption. The *G. viscidus* treatment contained the greatest K content (in the leaves and stem), and *S. luteus* contained the highest P content (in the leaves). Although the *L. laccata* treatment resulted in a high P content in the leaves, the stems and roots of *L. laccata* contained the greatest N content. The *S. luteus* treatment contained the highest K content in all sampled plant organs.

Table 3. Nutrient content (mg/pot) in the leaves, stems, and roots of *Pinus tabuliformis* seedlings: *L. laccata*, *S. luteus*, *S. grevillei*, *B. edulis G. viscidus*, and treated with sterilized substance (CK) (mean ± SD).

Treatments	N			P			K		
	Leaf	**Stem**	**Root**	**Leaf**	**Stem**	**Root**	**Leaf**	**Stem**	**Root**
CK	16.6 ± 1.7 c	17.7 ± 3.0 b	22.6 ± 2.9 b	6.1 ± 0.1 c	10.9 ± 1.2 c	13.7 ± 3.0 d	10.6 ± 1.9 a	10.1 ± 2.6 ab	11.5 ± 3.0 ab
L. laccata	23.0 ± 2.8 abc	28.2 ± 3.9 a	35.3 ± 6.5 a	8.8 ± 0.2 c	30.1 ± 3.8 a	35.3 ± 9.4 ab	9.7 ± 1.3 ab	13.1 ± 3.2 ab	16.0 ± 4.7 ab
S. luteus	30.9 ± 1.4 ab	23.1 ± 5.1 ab	33.2 ± 2.3 a	25.2 ± 7.8 a	27.0 ± 5.1 ab	21.1 ± 3.8 cd	13.0 ± 2.2 a	14.4 ± 3.4 a	16.7 ± 3.5 a
S. grevillei	25.1 ± 3.3 abc	28.2 ± 4.7 a	34.1 ± 3.7 a	10.5 ± 1.3 bc	24.8 ± 2.9 ab	43.5 ± 5.2 a	11.4 ± 2.0 a	12.3 ± 3.7 ab	14.6 ± 2.4 ab
B. edulis	33.1 ± 14.4 a	27.4 ± 7.0 a	21.0 ± 1.0 b	7.3 ± 1.8 c	28.4 ± 3.9 a	24.5 ± 2.6 c	10.3 ± 2.6 a	11.1 ± 1.8 ab	11.1 ± 2.6 ab
G. viscidus	20.7 ± 1.1 bc	14.7 ± 2.8 b	21.9 ± 1.4 b	15.5 ± 2.2 b	20.9 ± 2.7 b	28.4 ± 2.6 bc	6.7 ± 0.6 b	8.1 ± 1.3 b	10.7 ± 1.4 b

The different letters denote significant differences ($p < 0.05$) between the treatments, according to Duncan's multiple range test.

3.4. Enzymatic Activity in the Rhizospheric Soil of Seedlings

Catalase activity in the rhizospheric soil of inoculated seedlings (*S. luteus*, *S. grevillei*, *L. laccata*, and *G. viscidus*) increased by 24.02%, 11.44%, 9.35%, and 23.33%, respectively, compared to the CK samples (Figure 4). The *B. edulis* treatment did not affect catalase activity. ECM treatment significantly increased the rhizospheric soil urease activity of inoculated plants. Ranking of the six inoculates, in terms of urease activity, was *S. grevillei* > *L. laccata* > *S. luteus* > *B. edulis* > *G. viscidus* > CK. The urease activity of the *S. grevillei* treatment was 3.432 NH_3-N mg/g, which was 304.24% more than the CK plant soil. The remaining inoculated treatments exhibited a urease activity of 71.85% to 120.85% greater than that of the CK samples.

The protease activity in rhizosphere soil of the *B. edulis* treatment demonstrated the highest protease activity, which was approximately 69.29% more than the CK samples. The other inoculated treatments exhibited significantly greater protease activity (20.00% to 59.60%) than that of the CK samples. However, the *B. edulis* treatment did not increase the acidic phosphatase activity in the rhizosphere soil. The acidic phosphatase activity of the *L. laccata* treatment was the highest (8.78% greater than CK samples).

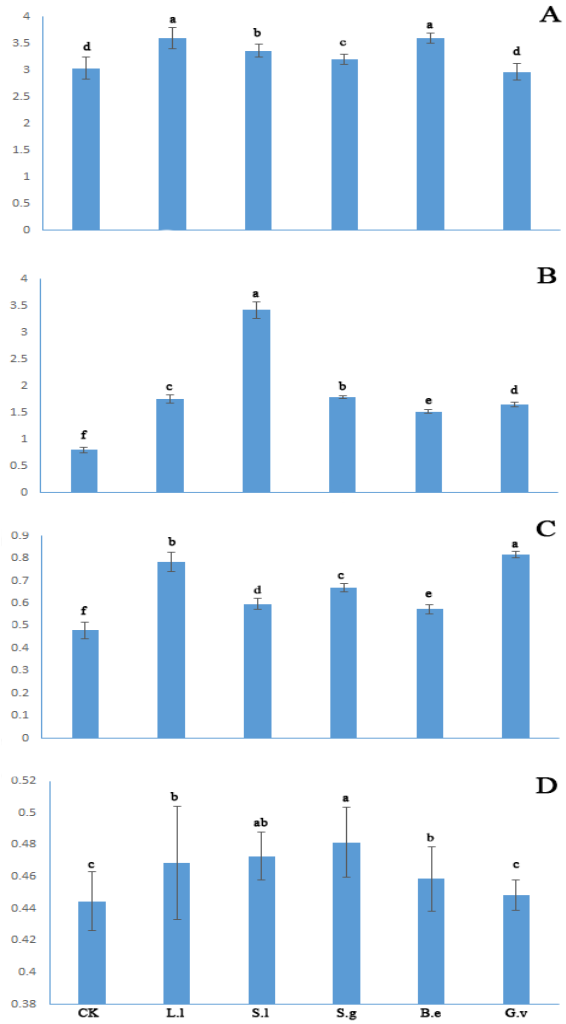

Figure 4. Enzymatic activity in the rhizospheric soil of *P. tabulaeformis* seedlings inoculated with *L. laccata* (L.l), *S. luteus* (S.l), *S. grevillei* (S.g), *B. edulis* (B.e), *G. viscidus* (G.v), and treated with sterilized substance (CK). The letters denote: (**A**) catalase activity, (0.1 mol/L $KMnO_4$ mL/g soil); (**B**) urease activity, (NH_3-N mg/g); (**C**) phosphatase activity, (phenol mg/g); and (**D**) protease activity, (amino-nitrogen g/100 g). The error bars refer to standard deviation.

3.5. Gas Exchange Parameters

The gas exchange parameters are shown in Table 4 All five gas exchange parameters were significantly increased by the use of inoculum; however, most parameters did not increase with the use of added inoculum. Lower usage of inoculum (200 mL) even resulted in higher *Pn*, *Gs* (moist), *Tr* (moist), and *WUE* (dry) values than higher usage of inoculum (400 mL or 600 mL). Inoculated *Pinus tabuliformis* seedlings with moist inoculum showed higher photosynthetic and transpiration rates, but also had higher intercellular CO_2 concentrations than did those with dry inoculum inoculated seedlings (Figure S2).

Table 4. Effects of EMC treatment on gas exchange parameters of *P. tabuliformis* under field conditions: four inoculated treatments (0, 200, 400, and 600 mL) with dry sterilized inoculum; four inoculated treatments (0, 200, 400, and 600 mL) with moist sterilized inoculum.

	Inoculum Dosage				*p*-Values (Two-Way ANOVA)		
	0 mL	200 mL	400 mL	600 mL	Humidity	Dosage	Humidity and Dosage
			Pn				
Dry Inoculum	2.14 ± 0.06 c	3.25 ± 0.10 a	2.84 ± 0.08 b	2.90 ± 0.05 b	<0.01	<0.01	<0.01
Moist Inoculum	2.18 ± 0.07 d	6.23 ± 0.08 a	3.94 ± 0.07 c	4.90 ± 0.06 b			
			Gs				
Dry Inoculum	0.028 ± 0.002 c	0.037 ± 0.001 b	0.041 ± 0.001 b	0.052 ± 0.002 a	<0.01	<0.01	<0.01
Moist Inoculum	0.027 ± 0.002 d	0.066 ± 0.004 a	0.047 ± 0.001 c	0.052 ± 0.002 b			
			Tr				
Dry Inoculum	0.25 ± 0.01 b	0.25 ± 0.02 b	0.26 ± 0.02 b	0.29 ± 0.01 a	<0.01	<0.01	<0.01
Moist Inoculum	0.24 ± 0.01 d	0.62 ± 0.02 a	0.34 ± 0.02 c	0.41 ± 0.03 b			
			Ci				
Dry Inoculum	176.33 ± 11.50 c	264.33 ± 8.14 ab	253.00 ± 9.17 b	274.00 ± 11.53 a	<0.01	<0.01	<0.01
Moist Inoculum	178.0 ± 10.44 c	213.33 ± 22.30 b	243.67 ± 20.74 a	215.00 ± 14.00 b			
			WUE				
Dry Inoculum	8.58 ± 0.47 c	12.86 ± 1.13 a	10.83 ± 0.88 ab	9.89 ± 0.54 b	<0.01	<0.01	ns
Moist Inoculum	9.20 ± 0.39 c	10.05 ± 0.20 b	11.72 ± 0.82 a	11.89 ± 0.76 a			

NS: no significant difference; The different letters in the same row denote significant differences ($p < 0.05$) between the treatments, according to Duncan's multiple range test; *Pn*: net photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$); *Gs*: stomatal conductance ($mol \cdot m^{-2} \cdot s^{-1}$); *Tr*: transpiration rate ($mmol \cdot m^{-2} \cdot s^{-1}$); *Ci*: intercellular CO_2 concentration ($\mu mol \cdot mol^{-1}$); *WUE*: water-use efficiency ($mol \cdot m^{-2} \cdot s^{-1}$).

4. Discussion

ECM fungal species have a wide range of hosts and exhibit preferences for particular plants. The characteristics of ECM and host plants play an important role in colonisation. In our study, the five ECM treatments resulted in successful fungal colonisation of the plant roots of *P. tabulaeformis* seedlings 90 days following inoculation. Differences in the colonisation rates may be due to host specificity to *P. tabulaeformis*.

In our study, *S. luteus* had great effects on the growth and biomass of the *P. tabulaeformis* seedlings, which is similar to the results from previous studies. In a study of EMC inoculation, Huang et al. (2006) showed that *B. edulis* could increase the biomass of *P. tabulaeformis* seedlings under both salt stress and non-stress conditions [15]. However, in our study, *B. edulis* did not increase the biomass of host plants. This may be due to the short time of colonization. *L. laccata* inoculation also increased the height and biomass of host plants, which indicated that *L. laccata* inoculation may positively affect *P. tabulaeformis*. Although *G. viscidus* successfully infected the *P. tabulaeformis* seedlings it had a negative effect on the aboveground growth when compared to the CK treatment. In fact, mycorrhizal inoculation is not always beneficial to host plants; the growth of host plants may be negatively affected or not affected by mycorrhizal colonisation [25–28]. These results indicated poor mutualism between *P. tabulaeformis* and *G. viscidus*. However, Jie et al. (2011) showed that *G. viscidus* inoculation could stimulate the growth and mineral intake of *P. tabulaeformis* seedlings in saline soil [29], which indicates

that ECM fungi may have different effects under stressful and non-stress conditions. In future studies, we will focus on the effects of ECM fungi inoculation under stressful conditions. The pot experiment showed that *S. luteus*, *S. grevillei*, and *L. laccata* significantly promoted the growth of *P. tabulaeformis* seedlings after 90 days of inoculation, and should be considered for use as inoculum in *P. tabulaeformis* afforestation. The mycorrhizal dependency of *L. laccata*, *S. luteus*, and *S. grevillei* has been measured at 117%, 130%, and 117%, respectively, according to Gerdemann (1974) [30]. Our results demonstrated a low mycorrhizal dependence, possibly because the mycorrhizal species were not specific to the particular host plant or that the experimental time (90 days) was insufficient.

The absorption of several minerals improved after the ECM infection. In all plant species, nitrogen (N) is an indispensable macroelement found in nucleic acids, proteins, phospholipids, and in other plant components [31,32]. N plays a crucial role in chlorophyll synthesis; increased plant intake of N is conducive to photosynthesis and the synthesis of carbohydrates [32,33]. In our field test, we found that inoculation with ECM increased net photosynthesis and water use efficiency, which was potentially due to the fact that the external mycelia of the ECM promoted the uptake and transport of NH^{4+} and NO^{3-} to the host plant [34]. Phosphorus (P) is one of the most important minerals [35]. P is a component of many compounds, such as nucleic acids, ATP, and phospholipids, and it plays a role in metabolism [36–38]. ECM promotes P absorption from soil in two ways: (1) the hyphae absorb nutrients directly from the soil and transport those nutrients to their host plants to support growth and development; and (2) inoculation changes root shape and various biochemical characteristics of the host plant, thereby enlarging the scope of the rhizosphere and improving the absorption ability of the roots [38]. Potassium (K) exists primarily in plants as an ion and is found in areas with strong metabolic activity. It is involved in the formation of proteins and in photosynthesis, as well as in resistance to stress [37,39].

In our study, *L. laccata* and *S. grevillei* increased the N content in all three of the organs and the P content in the stem and root. However, none of the inoculated treatments significantly promoted K absorption in the seedlings. *G. viscidus* treatments even showed a significant decrease in K content in the leaf. This result indicated that the five ECM fungi colonisations may mainly improve N and P absorption. Similar results were reported in other study that found that *P. tabulaeformis* seedlings inoculated with *G. viscidus* or *B. edulis* could enhance the absorption of P, but not K [29].

Although *G. viscidus* colonised *P. tabulaeformis* seedlings successfully, it decreased the growth of *P. tabulaeformis* seedlings. Schroeder et al. (2005) proposed that the positive growth effects of mycorrhiza occur when the benefits of increased nutrient absorption exceed the total carbon cost [40]. Our study indicated that limited nutrient absorption may be responsible for the reduced growth of *G. viscidus* inoculated seedlings. Due to nutritional deficiencies, the host plant cannot compensate for the increase in carbon cost.

Rhizosphere soil enzymatic activities are sensitive to perturbations associated with ECM inoculation; monitoring of the rhizosphere soil enzyme activities provides insight on plant mineralisation of important nutrient elements, such N, and P, as well as information on soil microbial activity [41,42].

Phosphatase enzymes can be synthesised by the colonised tree roots and mycelium, and may help to promote P uptake. In our study, with the exception of the *B. edulis* treatment, phosphatase enzyme activity of the inoculated treatments was significantly greater than that of the CK samples; this eventually led to improved P uptake [43]. The *G. viscidus* exhibited the highest phosphatase enzyme activity. However, the P content in the roots and stems was significantly less than that of the *S. luteus*, *S. grevillei*, and *L. laccata* treatment. This may be due to the P not having been given to host plants but to the fungi [27].

ECM fungi can synthesise many different hydrolytic enzymes (e.g., protease, chitinase, and glucosidase) that attack recalcitrant forms of soil organic matter and often increase the N supply to plants [42,44]. In our study, all ECM treatments significantly increased rhizospheric protease activity, and the protease activity of the *S. grevillei* treatment was higher than that of other treatments. The *S. grevillei* treatment also increased significantly the N content in all three plant organs, compared with the CK samples. This indicated that the *S. grevillei* treatment may have released more proteases

into the rhizosphere soil, thereby improving N intake. We also observed a significant increase in rhizospheric urease activity by the *S. grevillei* treatment. Urease is required to break down urea to liberate N into a usable form for plants [45]; thus, urease activity is related to N intake. Our study indicated that urea may be an important N source for the *S. grevillei* treatment. With the exception of the *B. edulis* treatment, we also observed a significant increase in catalase activity in the inoculated treatments. Catalase is involved in the regulation of reactive oxygen species (ROS) production during ECM establishment [46]. Increases in temporal catalase activity may serve a protective function for host plant roots against ROS overproduction, which can induce cell death. Therefore, catalase activation may be enhanced by ECM fungi.

The improvement in host plant photosynthesis by ECM inoculation has been reported in many studies, and it is postulated that photosynthetic improvement is associated with nutrient element absorption [47,48]. In our study, we demonstrated that *S. luteus* inoculation can significantly enhance photosynthesis in *P. tabulaeformis* seedlings under field conditions. However, Correa et al. (2006) found that increased photosynthesis of *Pinus pinaster* L. did not reflect a higher biomass compared with CK plants and that higher plant growth was not observed with photosynthesis changes [27]. Thus, despite ECM inoculation enhancement of photosynthesis in host plants, ECM may not increase biomass production. In future studies, we will measure data that is indicative of plant growth. In our study, the higher inoculum volumes (600 mL and 400 mL) of inoculation did not show significantly greater effects than the lower treatment, which indicates that 200 mL of inoculum may be enough for inoculation. In addition, either moist or dry inoculum could be chosen for the inoculation. It is interesting that moist inoculum increased the net photosynthetic and the transpiration rate of inoculated seedlings significantly more than did the dry inoculum.

5. Conclusions

All the five ECM fungi successfully inoculated the *P. tabuliformis* seedlings in greenhouse conditions after 90 days inoculation; *Laccaria laccata*, *Suillus grevillei*, and *Suillus luteus* showed significant effects in improving the growth and the N and P absorption of *P. tabuliformi* seedlings. ECM colonisation also increased the enzymatic activity of catalase, acidic phosphatase, protease, and the urease content in the rhizosphere soil. Furthermore, a field test proved that *S. luteus* inoculation increased the gas exchange parameters of *P. tabulaeformis* seedlings under field conditions, which indicated that *Suillus grevillei* may be used in *P. tabulaeformis* afforestation.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/7/12/316/s1, Figure S1: Colonisation of *P. tabulaeformis* roots under microscopic examination: *Laccaria laccata* (L.l), *Suillus luteus* (S.l), *Suillus grevillei* (S.g), *Boletus edulis* (B.e), *Gomphidius viscidus* (G.v), and non-mycorrhizal (CK), Figure S2. Effects of different dosages (0, 200, 400, and 600 mL) moist (orange) or dry (blue) inoculum on gas exchange parameters of *P. tabuliformis* under field conditions: Pn: net photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$); Gs: stomatal conductance ($mol \cdot m^{-2} \cdot s^{-1}$); Tr: transpiration rate ($mmol \cdot m^{-2} \cdot s^{-1}$); Ci: intercellular CO2 concentration ($\mu mol \cdot mol^{-1}$); WUE: water-use efficiency ($mol \cdot m^{-2} \cdot s^{-1}$). * Significant difference ($p < 0.05$, $\alpha = 0.05$, ANOVA).

Acknowledgments: The authors appreciate the financial support from Beijing Municipal Science and Technology Commission Program (Z151100002115006); the National Key Basic Research Development Project Program of China (No.2016YFC0502504); National Science and Technology Support Program (No.2012BAC09B03).

Author Contributions: Yun Li conceived and designed the experiments; Nan Lu, Meng Yu, Zijing Luo, Yue Feng, and Sen Cao performed the experiments; Nan Lu, Meng Yu, and Ming Cui wrote the paper together.

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

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