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Growth and Nutrient Acclimation of Evergreen Oak Seedlings Infected with *Boletus reticulatus* in Infertile Colluvial Soil in Warm Temperate Monsoon Asia: Evaluation of Early Growth

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Abstract: Soil erosion after harvesting of forest plantations can create infertile colluvial soil, therefore, seedlings used for site reforestation should be equipped against nutrient-poor edaphic conditions. The oak genus is a suitable candidate for such reforestation efforts. Oak is an ectomycorrhizal (ECM) tree genus known to grow under infertile environments. In this study, the initial stage of tree growth in three species of oak seedlings inoculated with a spore suspension of ECM fungus was monitored to evaluate the acceleration of seedling growth and nutrient uptake. I selected *Quercus acuta* Thunb., *Quercus glauca* Thunb., and *Quercus salicina* Blume, as these are common, evergreen, broad-leaved woody species commonly found in Southwestern Japan. The seedlings were inoculated with *Boletus reticulatus* and planted in infertile colluvial soils collected from a site that had undergone soil erosion. I also compared the ecophysiological characteristics of the potted seedlings planted in colluvial soil and normal forest soil. After six months of cultivation, *Q. glauca* with the ECM showed the highest growth rate in the fertile forest soil and had leaves with a higher nutrient content. In contrast, root dry mass increased slightly in *Q. acuta* and *Q. salicina* planted in colluvial soil. In all species, the seedling's ECM colonization rate in colluvial soil was lower than that in forest soil, yet the increase in nutrient uptake in the former was not obvious. The contents of K and Ca in the roots of *Q. acuta* and *Q. salicina* increased with *B. reticulatus* infection. I concluded that the inoculation with a *B. reticulatus* spore suspension effectively accelerated the growths in all three *Quercus* species. *Q. glauca* favored a fertile environment, and *Q. acuta* and *Q. salicina* suitably acclimated to both soil types. Thus, these species were selected as potential future candidates for reforestation in such eroded sites.

Keywords: ectomycorrhizal fungus; soil erosion; nutrient physiology; oak; photosynthetic capacity

1. Introduction

To mitigate soil erosion and degradation in forest ecosystems, reforestation with native species is an essential practice for the conservation of forest ecosystems [1,2]. Soil erosion is one of the driving factors of soil degradation. The recent increase in monsoon rainfall in East Asia due to climate change, have resulted in higher rates of erosion and corresponding soil degradation [3]. In Southwest Japan, increasing rates of slope failure were observed in mountainous areas, as a result of torrential rainfall [4,5]. This area of Japan also experienced an increase in the abandoned sites of reforestation after clear-cutting the harvests of commercial plantations of Sugi-cedar (*Cryptomeria japonica* (Linn.fil) D. Don) [6]. Severe soil erosion and landslides have become more common at these sites [7,8], while colluvial soils accumulated by slope failure showed low fertility [5,9].

Following Japan's national strategy of biodiversity conservation, demand for reforestation species has increased the population of indigenous broad-leaf species in this region [1]. Therefore, species chosen for reforestation should be an adequate native species rather than economically important conifers. Evergreen oaks are widely distributed in the warm temperate zone across Eastern Asia [10,11], of which seven species are found on the Kyushu island of Japan [12]. According to these species' distribution pattern across Southwest Japan, *Quercus acuta* Thunb., *Quercus glauca* Thunb., and *Quercus salicina* Blume can thrive in infertile habitats [12]. Therefore, I expect that three *Quercus* species showed good reforestation results on sites with high soil erosion.

Oak species are known to form symbiotic relationships with ectomycorrhizal (ECM) fungi, which protect their hosts from water and nutrient stresses [13–18]. The development of the external hyphae of ECM fungi can accelerate to absorb a greater amount of phosphorus (P), nitrogen (N), and water in woody species [19–21]. Based on this, researchers have hypothesized that ECM fungi inoculation can accelerate growth and nutrient uptake in oak seedlings [14,15,17], as well as increase host acclimation under infertile field conditions [14,17]. Therefore, Kayama and Yamanaka [22] confirmed growth acceleration of Fagaceae seedlings inoculated with ECM fungi in infertile soil. They successfully grew *Quercus glauca*, *Q. salicina*, and *Castanopsis cuspidata* seedlings inoculated with ECM in infertile acid soil collected from a site of slope failure. Their ECM inoculation method was applied to two species (*Q. glauca* and *Q. salicina)* at the site of land degradation [9], enabling the researchers to establish the availability of hyphal fragments of ECM fungi (*Astraeus hygrometricus* and *Scleroderma cepa*). Incubated mycelia were used as the inoculant; as the isolation and preparation of hyphal fragments require special techniques. However, this inoculation method lacked convenience for the local actors involved in reforestation efforts. Thus, I employed a simple method—using spore suspension. Previous studies reported that seedling growth was accelerated by applying a spore suspension of ECM fungi [14,23,24].

In Southwest Japan, *Boletus reticulatus* Schaeff. (Japanese summer cep) is a typical ECM fungus that forms a symbiotic relationship with oak species in evergreen broad-leaved forests [25–27]. Large fruiting bodies of *B. reticulatus* are abundant in the *Quercus* forests in July [28]. Therefore, a high density of spore suspension could be obtained from one harvest, which accelerates the growth of woody species [28]. Research is yet to investigate the inoculation of *B. reticulatus* to oak species [15,18]. Previous research indicates that *B. reticulatus* can increase tolerance against manganese (Mn) [29], an element known to induce toxicity in acidic soils [30]. As this experiment used acidic soil, I expect that *B. reticulatus* might show a high compatibility in acidic environment. Moreover, the inoculation test was performed using the *Boletus* sp., and the method of inoculation by use of spore suspension was available [24]. Seedlings of *Shorea balangeran* was grown successfully by inoculation of the *Boletus* sp. in acidic and infertile soil [24]. Based on previous research work, I hypothesize that seedlings of *Q. acuta*, *Q. glauca*, and *Q. salicina* can inoculate by the use of spore suspension of *B. reticulatus*. Seedlings of three *Quercus* species are predicted to accelerate their growth in infertile colluvial soils, by the inoculation of *B. reticulatus*. ECM infection and activity are usually regulated by the nutrient availability [15,31]; however, there was no information on the acclimation capacity of nutrients for *B. reticulatus* [26].

This study aimed to examine the nutritional and physiological status of seedlings of three *Quercus* species inoculated with *B. reticulatus* and assessed their suitability for future reforestation. Seedlings were planted in fertile forest soil and infertile colluvial soil to compare the acclimation capacity of nutrients for *B. reticulatus*. The seedlings' ecophysiological traits were examined, including (1) the growth characteristics of the seedlings, (2) percentage of ECM colonization, and (3) concentrations of elements in various plant organs. From these results, the suitability of oak seedlings with inoculating *B. reticulatus* for reforestation in eroded soils was examined.

2. Materials and Methods

2.1. Preparations of Acorns of Oaks and ECM Fungus

Quercus acuta, *Q. glauca*, and *Q. salicina* were selected for this study; all three are widely distributed in the evergreen broad-leaved forests of Southwestern Japan [32]. In December 2006, acorns of *Q. glauca* were collected from a secondary evergreen broad-leaved forest (32°15′ N, 130°39′ E, 450 m a.s.l.) located in Southwestern Kyushu, Japan. Acorns of *Q. acuta* and *Q. salicina* were collected from a natural evergreen broad-leaved forest (32°08′ N, 130°30′ E, 500 m a.s.l.) in Kyushu Island, Japan, in December 2006. All collected acorns were stored in a refrigerator at 4 °C, before sowing for the experiment.

Boletus reticulatus was selected as an ECM fungus inoculum. This fungus had a large amount of fruiting bodies in evergreen broad-leaf forest in Southwest Japan. *B. reticulatus* fruiting bodies were collected from an oak forest at the Kyushu Research Center, FFPRI, in June 2007. Collected fruiting bodies were observed in terms of the morphologies of pileus, hymenophore tubulate, and stipe; these were then compared to that of *B. reticulatus* [33]. All fruiting bodies were identified as *B. reticulatus*. The pilei of *B. reticulatus* were cut into 3 × 3 cm cross-sections and the samples were placed in cloth bags (0.2 mm mesh, Toray, Tokyo, Japan). Each bag was immersed in sterilized distilled water and the samples were squeezed to remove spores. The spore density was counted by use of hemocytometer and microscope (TE2S-1, Nicon, Tokyo, Japan), and the value of the suspension was 6.8×10^5 spore mL^{-1}. The morphology of the spore was corresponded to the description of *B. reticulatus* [27,33].

2.2. Soil Collection and Analysis

I collected brown forest soil (F: dystric cambisols) and colluvial soil (C: alisols) from the same site (32°16′ N, 130°38′ E, 580 m a.s.l.) in April 2007. The forest soil was collected from a horizon of a plantation of *Cryptomeria japonica*, while the colluvial soil was collected from a collapsed slope located near the *C. japonica* plantation, where the trees were harvested between 2001 and 2002 [7].

Both soil samples were sieved (2 mm) on-site, collected in 12 sandbags (10 kg per a sandbag). I evaluated the samples' soil properties, including pH and the concentrations of carbon (C), nitrogen (N), exchangeable-phosphorus (P), base cations, aluminum (Al), manganese (Mn), and iron (Fe). Soil samples were collected from five sandbags. Soil pH was measured using a pH meter (HM25R, DKK-TOA Co., Tokyo, Japan). Ten grams of fresh soil were mixed with 25 mL of distilled water and shaken for 1 h, before obtaining a reading [34]. After the measurement, samples were dried at 105 °C for 24 h, then used to determine the respective concentrations of C and N, using a CN analyzer (MT-600, Yanako New Science Inc., Kyoto, Japan). Exchanged P was extracted using Bray No. 2 solution [35], by shaking for 1 min. The concentration of P was determined by the molybdenum blue method [36], using a spectrophotometer (UV-2500PC, Shimadzu, Kyoto, Japan). The concentrations of exchangeable-base cations [calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na)] and Mn were determined as follows—a 2.5 g dry soil sample was mixed with 50 mL of 1 M ammonium acetate solution and shaken for 1 h [35]. The concentrations of the base cations were then analyzed using an atomic absorption spectrophotometer (Z-2310, Hitachi High-Technologies Co., Tokyo, Japan). Solutions for the measurement of exchanged Fe and Al were prepared by adding 50 mL of 1 M KCl to 2.5 g of dry soil and shaken for 1 h [35]. The concentrations of Mn, Al, and Fe were determined using an ICP analyzer (SPS 4000, Seiko Instruments Inc., Chiba, Japan).

Although organic substances were destroyed, the collected soil was sterilized using an autoclave (KTS-2346B, Alp Co., Tokyo, Japan) at 120 °C for 20 min to remove other indigenous mycorrhizal species, before planting the seedlings.

2.3. Pot Experiments

Acorns for each of the three *Quercus* species in pots were planted in April 2007. Before sowing the acorns, they were sterilized with 5% H_2O_2 for 10 min and rinsed three times with sterilized distilled water. The pots were then filled with the sterilized forest and colluvial soils (depth, 6.5 cm; diameter,

5.5 cm; volume, 220 mL). In total, 90 pots were filled for each soil type. I sowed 60 acorns of *Q. acuta*, *Q. glauca*, and *Q. salicina*, and 30 acorns for each species were buried separately in forest and colluvial soils. Acorns were germinated in a naturally-illuminated phytotron (3S-135A; Koito Electric Industries Ltd., Shizuoka, Japan) at the Kyushu Research Center (FFPRI). Each pot was irrigated with 10 mL of distilled water per day.

After germination (May 2007), the seedlings were transplanted to individual pots (depth, 30 cm; diameter, 15 cm; volume, 3.7 L) filled with sterilized forest and colluvial soil. Among the germinated seedlings, 40 healthy seedlings were selected for each species, and these were planted on the two types of soils. The seedlings were transported into two phytotron rooms, and the number of seedlings per room was 20 for each species (Figure 1). These seedlings were raised until the end of October 2007. The remaining seedlings were sampled at this time and dry mass was weighed.

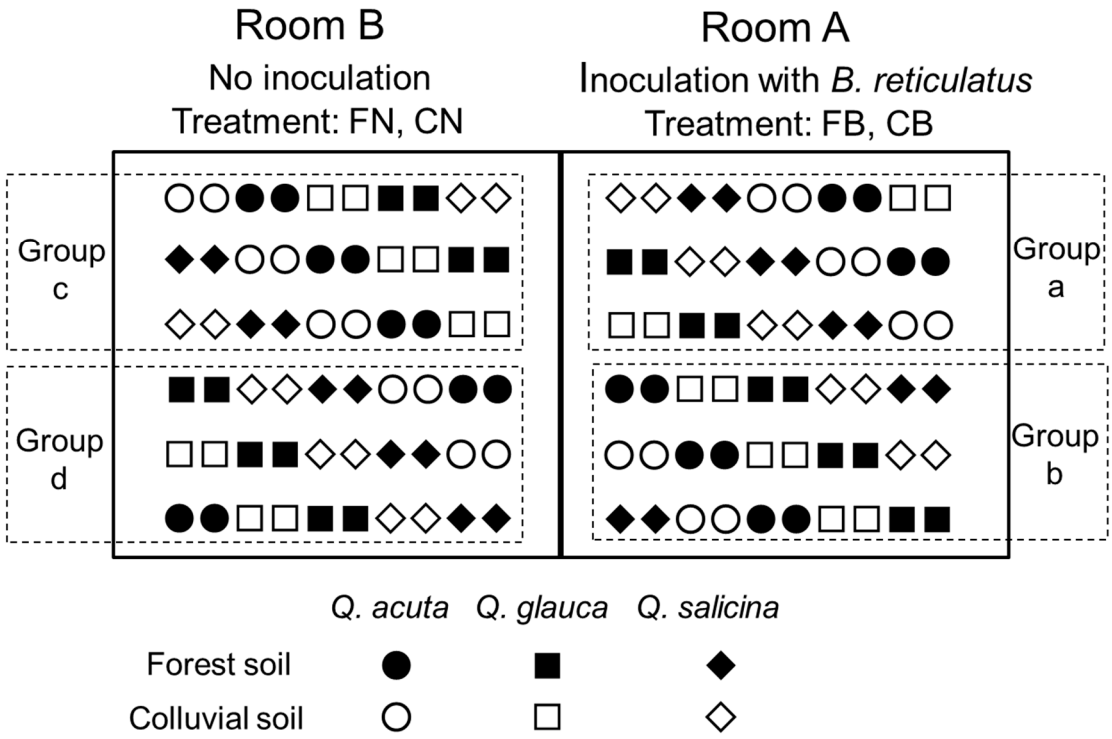

Figure 1. Schematic design of experimental treatment. In **Room A**, inoculation with *B. reticulatus* was performed. In **Room B**, inoculation was not performed. Twenty seedlings of the three *Quercus* species planted on forest or colluvial soils were put in a room.

After transplantation, each pot was irrigated with 100 mL of distilled water daily. The average solar radiation during the experimental period was 16.9 MJ m^{-2} day^{-1} [37]. The air temperature in the phytotron rooms was controlled at 30 °C (day) and 25 °C (night) from July 2007 to September 2007, and at 25 °C (day) and 20 °C (night) from May 2007 to June 2007 and in October 2007. These temperatures were based on air temperature records in Kumamoto City, Japan [37]. The relative humidity was controlled at 75%, also based on the above records. The position of the seedlings of groups 'a' and 'b', or 'c' and 'd' were exchanged every month to prevent the effect of nonuniform environment of solar irradiance in a room (Figure 1), and the phytotron rooms were disinfected using ethanol, simultaneously.

In July 2007, ECM inoculation was performed after transplanting, as follows—10 mL of *B. reticulatus* spore suspension was placed on the surface soil of pots planted with 20 seedlings of the three oak species, in the phytotron room A (Figure 1). Phytotron room B was used to grow control seedlings (Figure 1; those not inoculated with *B. reticulatus*).

Treatments and their abbreviation were as follows—seedlings inoculated with *B. reticulatus* grown in forest soil (Figure 1; FB; Room A), non-ECM seedlings grown in forest soil (FN; Room B), seedlings

inoculated with *B. reticulatus* grown in colluvial soil (CB; Room A), and non-ECM seedlings grown in colluvial soil (CN; Room B). During the experimental period, I measured the dry mass of senescent fallen leaves for each individual plant.

2.4. Photosynthetic Capacity

The photosynthetic rate at light saturation (P_{sat}) at 37.0 Pa CO_2 was measured in all seedlings in October 2007. The third leaf counted from the top of seedling was used to measure P_{sat} and stomatal conductance (gs). Measurements were conducted using a portable gas analyzer (LI-6400, LI-COR Biosciences, Lincoln, NE, USA), under steady-state conditions. The LED light source was adjusted to a saturation light level of 1700 μmol $m^{-2}s^{-1}$ photosynthetic photon flux (PPF).

2.5. Seedling Growth

To determine the growth characteristics of seedlings for each *Quercus* species, I measured the dry masses of leaves, stems and branches, and roots. Ten FB, FN, CB, and CN seedlings from each species were harvested in November 2007. The roots of the harvested seedlings were washed twice with tap water to remove any remaining soil, then washed again with distilled water. The washed seedlings were divided into the respective components mentioned above. Each plant organ type was placed into a separate envelope and oven-dried at 60 °C for four days, then the dry mass of each component was determined.

2.6. Colonization Rate of ECM Fungus

To measure the proportion of roots with ECM fungal colonization, I selected five large clusters of roots from the seedlings, at random, and from over 500 short roots (<5 mm long) diverging from them. To assess the extent of the colonization of the ECM fungus, the seedling roots were harvested in November 2007 and carefully washed to remove any remaining soil under gently flowing tap water. The cluster roots were then soaked in distilled water and observed at 10×–40× magnification, under a stereomicroscope (STZ-40TBIT, Shimadzu, Kyoto, Japan). The ECM short roots of *B. reticulatus* were confirmed by the color, form, and size of the ECM, the presence of rhizomorphs, and the absence of root hairs. The mantle on the ECM root of *B. reticulatus* was plectenchymatous, colorless, and lacking in clamps [26]. The ECM root of *B. reticulatus* for three *Quercus* species [38] was identified by the morphology of the mantle [26]. I did not confirm other types of ECM roots for three *Quercus* species [16]. The number of colonized and uncolonized short roots was counted and the percentage of ECM fungal colonization was calculated, based on the proportion of the colonized short roots to the total number of short roots [39].

2.7. Analysis of Element Concentrations in Plants

I measured the concentrations of N, P, K, Ca, Mg, and Al in the leaves and roots. Dried samples were ground to a fine powder using a sample mill (WB-1; Osaka Chemical Co., Osaka, Japan). The powdered samples were then digested in a Digesdahl® (Hach Company, Loveland, CO, USA), using sulfuric acid and hydrogen peroxide [40]. The concentration of N was determined using the indophenol method [41], with a spectrophotometer. The concentration of P was determined using the molybdenum blue method using a spectrophotometer [36]. The concentrations of K, Ca, and Mg were determined using an atomic absorption spectrophotometer (Z-2310, Hitachi High-Technologies Co., Tokyo, Japan) [35]. The concentration of Al was analyzed using an ICP analyzer (SPS 4000, Seiko Instruments Inc., Chiba, Japan) [35]. I analyzed the standard solutions of each element after every 40th sample, to verify the reliability of the results.

2.8. Statistical Analyses

All parameters were analyzed using analysis of variance (ANOVA) in Kyplot 3.0 (Kyens Lab. Inc., Tokyo, Japan). The dry mass of each organ, P_{sat}, gs, and the element concentrations in the leaves and roots were examined for the effects of two types of soil, inoculation by ECM fungus, and their interaction. The mean values of the soil chemical properties were compared between the forest and colluvial soils. The mean values of ECM fungal colonization of the three *Quercus* species were compared between the FB and CB seedlings.

3. Results

3.1. Soil Chemical Properties

Table 1 lists the chemical properties of forest and colluvial soils. Every soil examined in this study had a pH of <5. The pH and the concentrations of various elements, such as C, N, P, Ca, Mg, K, Na, Mn, and Fe, in colluvial soil were lower than those in the forest soil. In particular, Ca concentration in colluvial soil was much lower than that in the forest soil, but the concentration of Al was not significantly different between brown forest (F) and colluvial soil (C).

Table 1. Chemical properties of forest and colluvial soils used in this experiment (mean ± SD, $n = 5$). All units (except pH) are in mmol kg^{-1}. Asterisks indicate varying levels of significance in the difference between the two types of soil (ANOVA). ** = $p < 0.01$ and *** = $p < 0.001$. n.s.: No significance.

Element	Forest Soil	Colluvial Soil	Statistical Test
pH	4.67 ± 0.20	4.26 ± 0.14	**
C	5582 ± 573	550 ± 186	***
N	466 ± 36	254 ± 11	***
P	4.85 ± 1.05	0.46 ± 0.14	***
Ca	26.8 ± 9.0	1.8 ± 0.3	***
Mg	3.31 ± 0.75	1.15 ± 0.38	***
K	3.85 ± 0.36	2.86 ± 0.21	***
Na	1.95 ± 0.39	0.59 ± 0.04	***
Al	11.0 ± 3.4	13.6 ± 0.5	n.s.
Mn	1.29 ± 0.57	0.08 ± 0.04	**
Fe	0.32 ± 0.03	0.17 ± 0.07	**

3.2. Growth Characteristics

Percentage germination was 70% for *Q. acuta*, 86% for *Q. glauca*, and 72% for *Q. salicina*. The germination rate was not significantly different between seedlings grown in forest and colluvial soil. After growing for six months, the FB seedlings of the three *Quercus* species showed increased dry masses for each organ, particularly *Q. glauca* (Figure 2). The dry mass of each organ showed significant difference by inoculation of the ECM fungus for the three *Quercus* species, except for the stem dry mass of *Q. acuta* (Table 2, $p < 0.05$). The leaf and root dry masses of *Q. acuta*, and root dry mass of *Q. salicina* did not show significant difference between the two types of soil. By contrast, dry masses of each organ of *Q. glauca* were increased when inoculated by ECM fungus, for only the forest soil. Therefore, dry mass of each organ showed significant interaction between the ECM fungus and the soil types ($p < 0.001$).

FN and CN seedlings showed minimal growth. Moreover, FN and CN seedlings both had a large amount of fallen leaves. The total dry mass of fallen leaves during the experimental period was weighed (Table 3), and was found to be significantly different for the different conditions of inoculation by ECM fungus ($p < 0.001$). *Q. acuta* and *Q. salicina* seedlings also showed significantly larger dry mass of fallen leaves for the forest soil ($p < 0.01$), while in *Q. glauca*, the dry mass of fallen leaves was significantly larger in colluvial soil ($p < 0.01$).

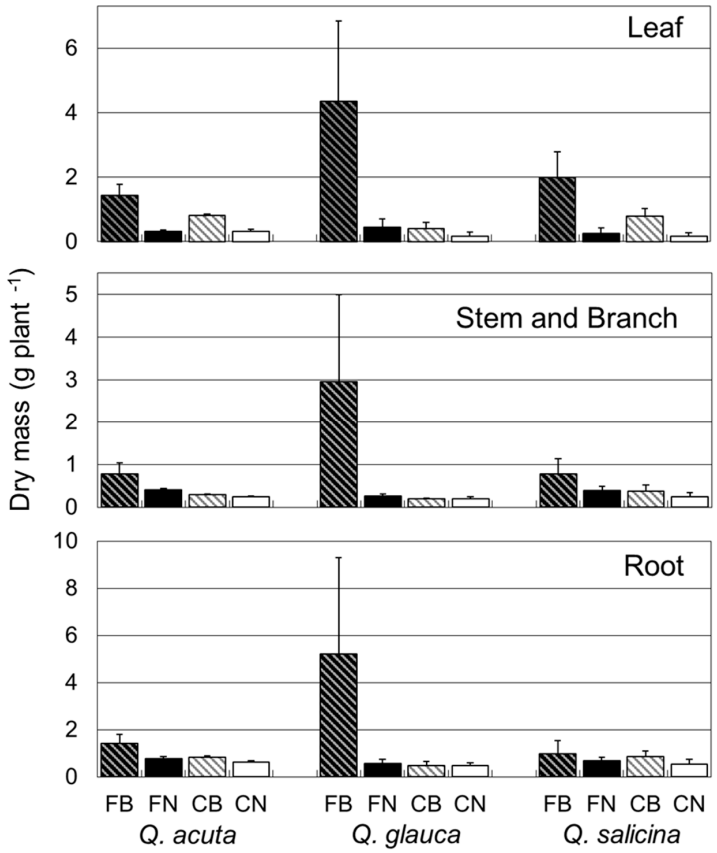

Figure 2. Dry masses of leaf, stem and branch, and root for the three *Quercus* species examined in this study (mean ± SD, $n = 10$).

Table 2. Statistical analysis tests on the dry mass of each organ (leaf, stem and branch, root. and fallen leaf), P_{sat} and gs for the three *Quercus* species. Asterisks indicate significant differences between the inoculation of soil types, ECM fungus, and their interaction (S × E), calculated by ANOVA. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Parameter		Statistical Analysis		
		Q. acuta	*Q. glauca*	*Q. salicina*
Leaf	Soil	n.s.	***	***
	ECM	***	***	***
	S × E	n.s.	***	***
Stem and Branch	Soil	*	***	***
	ECM	n.s.	***	***
	S × E	n.s.	***	*
Root	Soil	n.s.	***	n.s.
	ECM	*	***	**
	S × E	n.s.	***	n.s.
P_{sat}	Soil	***	***	***
	ECM	***	***	***
	S × E	***	***	***
gs	Soil	***	***	***
	ECM	**	***	***
	S × E	n.s.	***	**

Table 3. The dry mass of the fallen leaves of the *Quercus* species seedlings during the experimental period (mean ± SD, $n = 10$). Asterisks indicate significant differences between inoculation of soil types, ectomycorrhizal (ECM) fungus, and their interaction (S × E), as calculated by ANOVA. ** = $p < 0.01$ and *** = $p < 0.001$.

Treatment	Dry Mass (g Plant^{-1})			Statistical Analysis			
	Q. acuta	*Q. glauca*	*Q. salicina*		*Q. acuta*	*Q. glauca*	*Q. salicina*
FB	0.326 ± 0.312	0.038 ± 0.121	0.308 ± 0.189	Soil	**	**	**
FN	0.623 ± 0.255	0.241 ± 0.090	0.719 ± 0.268	ECM	***	***	***
CB	0.067 ± 0.078	0.192 ± 0.117	0.120 ± 0.132	S × E	n.s.	n.s.	n.s.
CN	0.377 ± 0.216	0.357 ± 0.152	0.550 ± 0.199				

3.3. Percentage of ECM Fungal Colonization

The percentage of ECM colonization was the highest in *Q. glauca* FB seedlings (73.4%, Table 4), while those of *Q. acuta* and *Q. salicina* showed low ECM colonization rates. Contrastingly, the ECM colonization rate in the CB seedlings was lower for *Q. glauca* (16.4%) than for *Q. acuta* and *Q. salicina*. The ECM colonization rate was significantly higher in the FB seedlings of the three *Quercus* species than in the CB seedlings ($p < 0.01$). I also confirmed the ECM colonization rate in several FN and CN seedlings of the three *Quercus* species, although ECM colonization was below 1%.

Table 4. Percentage of ECM fungal colonization of seedlings of the three *Quercus* species (mean ± SD, $n = 10$). Asterisks indicate varying levels of significance in the difference between the two types of soil (ANOVA). ** = $p < 0.01$ and *** = $p < 0.001$.

Treatment	*Q. acuta*	*Q. glauca*	*Q. salicina*
FB	64.6 ± 3.7	75.9 ± 9.1	60.2 ± 14.6
CB	41.9 ± 11.4	16.4 ± 11.8	42.6 ± 10.0
Statistical test	***	***	**

3.4. Photosynthetic Capacity

The value of P_{sat} for all three *Quercus* species was significantly higher in the FB seedlings (Figure 3). The stomatal conductance (gs) of the three *Quercus* species showed a similar trend to P_{sat} and was significantly higher in the FB seedlings. Among the three *Quercus* species, the values of P_{sat} and gs were the highest in the FB seedlings of *Q. glauca*.

The values for P_{sat} and gs for all three *Quercus* species showed significant differences for soil types (Table 2, $p < 0.001$). Moreover, the values for P_{sat} for the treatments of forest soil were clearly increased when inoculated by the ECM fungus, whereas these trends did not confirm under colluvial soil. Therefore, P_{sat} and gs for three *Quercus* species showed significant interaction between ECM fungus and soil types, except for gs for *Q. acuta* ($p < 0.01$).

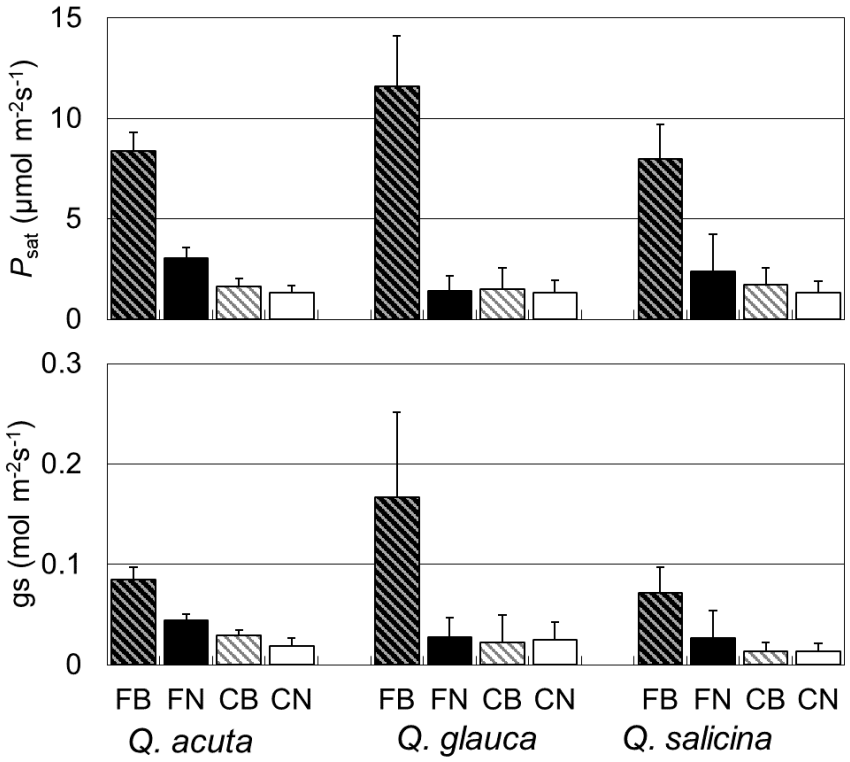

Figure 3. The photosynthetic rate at light saturation (P_{sat}) and stomatal conductance (gs) of the three *Quercus* species examined in this study (mean ± SD, n = 10).

3.5. Element Concentrations in the Leaves

The concentration of N and P in the leaves of three *Quercus* species was higher in the FB seedlings, compared to those from other treatments (Table 5). All elements of the three *Quercus* species, except for Al, showed significant difference between the two types of soil ($p < 0.001$). On the effects of ECM fungus, the concentration of N for *Q. salicina*, the concentration of K for *Q. glauca*, and the concentration of Ca for *Q. acuta* showed significant difference ($p < 0.05$), and these elements were increased by its inoculation. The concentration of P for *Q. acuta* and *Q. salicina* showed significant difference in terms of inoculation by ECM fungus ($p < 0.001$). However, these trends were obvious only for the FB seedlings; therefore, the concentration of P for *Q. acuta* and *Q. salicina* showed significant interaction between the ECM fungus and the soil types ($p < 0.001$). The concentration of Al for *Q. glauca* showed significant difference in terms of inoculation by ECM fungus ($p < 0.01$), and Al was increased when inoculated. However, differences in the concentrations of some elements were not evident, despite a significant difference in the leaf dry mass.

To examine the total nutrient uptake by the leaves, the total element content was examined (Table 6). The contents of N, P, Ca, and Mg for the three *Quercus* species showed significant difference for soil types or inoculation by ECM fungus ($p < 0.05$). However, these contents were markedly high values for FB seedlings, and showed significant interaction between soil types and ECM fungus ($p < 0.05$). The K content for *Q. acuta* showed significant differences for inoculation by ECM fungus ($p < 0.001$), and K was increased when inoculated.

Table 5. Element concentration (in μmol g^{-1}) in the leaves of the three *Quercus* species (mean ± SD, n = 10). Asterisks indicate significant differences between inoculation of soil types, ECM fungus, and their interaction (S × E), calculated by ANOVA. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Elements		Concentration (μmol g^{-1})			Statistical Analysis			
		Q. acuta	*Q. glauca*	*Q. salicina*		*Q. acuta*	*Q. glauca*	*Q. salicina*
N	FB	2296 ± 477	2279 ± 328	2136 ± 160	Soil	***	***	***
	FN	925 ± 190	1751 ± 292	1326 ± 517	ECM	***	**	***
	CB	674 ± 238	790 ± 203	1469 ± 236	S × E	***	**	n.s.
	CN	852 ± 247	840 ± 183	877 ± 504				
P	FB	205 ± 126	155 ± 39	138 ± 17	Soil	***	***	***
	FN	47 ± 19	91 ± 109	73 ± 36	ECM	***	n.s.	***
	CB	38 ± 9	42 ± 12	45 ± 10	S × E	***	n.s.	***
	CN	47 ± 8	41 ± 10	49 ± 15				
K	FB	138 ± 36	157 ± 26	164 ± 21	Soil	***	***	***
	FN	156 ± 49	133 ± 29	285 ± 39	ECM	n.s.	*	***
	CB	198 ± 40	235 ± 50	170 ± 30	S × E	n.s.	n.s.	***
	CN	202 ± 52	212 ± 36	202 ± 40				
Ca	FB	305 ± 86	583 ± 165	429 ± 77	Soil	***	***	***
	FN	259 ± 35	452 ± 103	368 ± 68	ECM	*	n.s.	n.s.
	CB	232 ± 60	182 ± 38	212 ± 43	S × E	n.s.	**	n.s.
	CN	194 ± 17	239 ± 53	212 ± 54				
Mg	FB	45 ± 15	109 ± 28	91 ± 12	Soil	***	***	***
	FN	44 ± 11	73 ± 23	73 ± 16	ECM	n.s.	*	n.s.
	CB	26 ± 5	43 ± 8	48 ± 7	S × E	n.s.	***	**
	CN	24 ± 7	54 ± 10	51 ± 10				
Al	FB	35 ± 13	26 ± 10	32 ± 9	Soil	n.s.	n.s.	***
	FN	33 ± 17	21 ± 12	81 ± 20	ECM	n.s.	**	***
	CB	25 ± 6	36 ± 10	41 ± 8	S × E	n.s.	n.s.	***
	CN	29 ± 15	24 ± 6	37 ± 12				

Table 6. Total element content (in μmol $plant^{-1}$) in the leaves of the three *Quercus* species (mean ± SD, n = 10). Asterisks indicate significant differences between inoculation of soil types, ECM fungus, and their interaction (S × E), as calculated by ANOVA. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Elements		Content (μmol $Plant^{-1}$)			Statistical Analysis			
		Q. acuta	*Q. glauca*	*Q. salicina*		*Q. acuta*	*Q. glauca*	*Q. salicina*
N	FB	3334 ± 2253	9666 ± 5407	4336 ± 1820	Soil	**	***	***
	FN	298 ± 109	899 ± 472	329 ± 229	ECM	***	***	***
	CB	539 ± 214	309 ± 188	1178 ± 398	S × E	**	***	***
	CN	270 ± 203	301 ± 240	161 ± 122				
P	FB	263 ± 161	593 ± 183	276 ± 118	Soil	***	***	***
	FN	15 ± 8	48 ± 62	20 ± 21	ECM	***	***	***
	CB	30 ± 9	15 ± 7	36 ± 14	S × E	***	***	***
	CN	15 ± 9	13 ± 8	9 ± 5				
K	FB	201 ± 140	647 ± 296	331 ± 145	Soil	n.s.	***	***
	FN	49 ± 15	71 ± 43	79 ± 54	ECM	***	***	***
	CB	161 ± 48	84 ± 37	132 ± 38	S × E	n.s.	***	**
	CN	62 ± 40	72 ± 50	39 ± 25				
Ca	FB	435 ± 330	2550 ± 1623	870 ± 413	Soil	*	***	***
	FN	86 ± 35	225 ± 120	96 ± 52	ECM	***	***	***
	CB	192 ± 76	65 ± 29	169 ± 76	S × E	*	***	***
	CN	63 ± 38	82 ± 62	42 ± 28				
Mg	FB	59 ± 32	462 ± 256	185 ± 82	Soil	***	***	***
	FN	14 ± 7	39 ± 25	19 ± 13	ECM	***	***	***
	CB	21 ± 6	16 ± 7	38 ± 13	S × E	**	***	***
	CN	8 ± 6	19 ± 14	10 ± 7				
Al	FB	54 ± 47	113 ± 70	65 ± 32	Soil	*	***	***
	FN	11 ± 8	10 ± 7	20 ± 11	ECM	**	***	***
	CB	21 ± 7	13 ± 7	33 ± 11	S × E	n.s.	***	n.s.
	CN	8 ± 6	8 ± 6	7 ± 4				

3.6. Element Concentrations in Roots

The concentrations of P in the roots of all three *Quercus* species showed significant difference for soil types or inoculation by ECM fungus (Table 7, $p < 0.001$). However, the concentration of P showed markedly high values for the FB seedlings, and showed significant interaction between soil types and ECM fungus ($p < 0.001$). Moreover, the concentrations of N, Ca, and Al in the roots of all three Quercus species showed a significant difference between the two types of soil ($p < 0.001$). The concentrations of N and Ca were high for the forest soil, whereas the concentration of Al was high for colluvial soil. The concentrations of N in the *Q. glauca* roots showed significant difference in terms of inoculation by ECM fungus ($p < 0.001$), and the value was increased when inoculated. However, many elements did not show a clear trend in terms of inoculation by the ECM fungus.

Table 7. Element concentration (in μmol g^{-1}) in the roots of the three *Quercus* species (mean ± SD, $n = 10$). Asterisks indicate significant differences between inoculation of soil types, ECM fungus, and their interaction (S × E), as calculated by ANOVA. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Elements		Concentration (μmol g^{-1})			Statistical Analysis			
		Q. acuta	*Q. glauca*	*Q. salicina*		*Q. acuta*	*Q. glauca*	*Q. salicina*
N	FB	1199 ± 244	1004 ± 230	1103 ± 211	Soil	***	***	***
	FN	1115 ± 213	697 ± 189	892 ± 188	ECM	n.s.	***	n.s.
	CB	606 ± 163	638 ± 144	544 ± 168	S × E	n.s.	n.s.	*
	CN	603 ± 200	457 ± 53	553 ± 89				
P	FB	64 ± 24	74 ± 35	67 ± 18	Soil	***	***	***
	FN	24 ± 4	26 ± 12	25 ± 8	ECM	***	***	***
	CB	22 ± 3	21 ± 5	26 ± 5	S × E	***	***	***
	CN	17 ± 4	20 ± 2	23 ± 7				
K	FB	133 ± 40	175 ± 50	146 ± 40	Soil	*	n.s.	*
	FN	109 ± 27	146 ± 21	145 ± 20	ECM	n.s.	n.s.	n.s.
	CB	140 ± 28	155 ± 18	159 ± 28	S × E	n.s.	*	n.s.
	CN	147 ± 36	166 ± 21	176 ± 38				
Ca	FB	119 ± 18	117 ± 9	116 ± 19	Soil	***	***	***
	FN	120 ± 24	141 ± 15	135 ± 25	ECM	n.s.	**	n.s.
	CB	47 ± 11	58 ± 5	54 ± 11	S × E	n.s.	***	n.s.
	CN	33 ± 4	53 ± 8	51 ± 14				
Mg	FB	80 ± 32	122 ± 15	90 ± 26	Soil	***	***	n.s.
	FN	75 ± 11	114 ± 19	94 ± 20	ECM	n.s.	n.s.	n.s.
	CB	56 ± 08	87 ± 13	84 ± 13	S × E	n.s.	*	n.s.
	CN	54 ± 15	100 ± 14	84 ± 19				
Al	FB	314 ± 055	370 ± 85	364 ± 102	Soil	***	***	***
	FN	409 ± 125	323 ± 57	397 ± 96	ECM	n.s.	n.s.	n.s.
	CB	579 ± 117	486 ± 75	545 ± 103	S × E	n.s.	n.s.	n.s.
	CN	571 ± 156	507 ± 62	573 ± 166				

I also examined the total element content in the roots (Table 8). The contents of K and Ca for *Q. acuta* and *Q. salicina*, and the content of N for *Q. salicina* showed significant difference in terms of inoculation by ECM fungus. The contents of these elements were increased when inoculated by the ECM fungus. The contents of each element for *Q. glauca* showed significant difference for soil types or inoculation by ECM fungus ($p < 0.05$). However, the contents of each element showed a markedly high value for the FB seedlings, and showed a significant interaction between the soil types and ECM fungus ($p < 0.01$).

Table 8. Total element content (in μmol plant^{-1}) in the roots of the three *Quercus* species (mean ± SD, n = 10). Asterisks indicate significant differences between inoculation of soil types, ECM fungus, and their interaction (S × E), as calculated by ANOVA. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Elements		Content (μmol Plant^{-1})			Statistical Analysis			
		Q. acuta	*Q. glauca*	*Q. salicina*		*Q. acuta*	*Q. glauca*	*Q. salicina*
N	FB	1880 ± 1995	4852 ± 3460	1099 ± 705	Soil	**	***	***
	FN	863 ± 252	398 ± 171	602 ± 171	ECM	n.s.	***	**
	CB	504 ± 223	317 ± 143	480 ± 196	S × E	n.s.	***	n.s.
	CN	393 ± 232	221 ± 74	295 ± 119				
P	FB	102 ± 99	287 ± 129	71 ± 57	Soil	**	***	**
	FN	19 ± 7	14 ± 5	17 ± 7	ECM	**	***	**
	CB	18 ± 6	10 ± 4	23 ± 8	S × E	*	***	*
	CN	11 ± 4	9 ± 3	12 ± 6				
K	FB	202 ± 191	761 ± 423	157 ± 140	Soil	n.s.	***	n.s.
	FN	83 ± 21	80 ± 21	100 ± 34	ECM	*	***	*
	CB	116 ± 42	76 ± 28	136 ± 31	S × E	n.s.	***	n.s.
	CN	89 ± 26	77 ± 15	91 ± 32				
Ca	FB	165 ± 136	629 ± 516	110 ± 56	Soil	***	***	***
	FN	93 ± 33	78 ± 21	90 ± 18	ECM	*	**	*
	CB	40 ± 19	28 ± 9	47 ± 16	S × E	n.s.	**	n.s.
	CN	20 ± 5	25 ± 4	26 ± 8				
Mg	FB	133 ± 142	625 ± 493	96 ± 76	Soil	*	***	n.s.
	FN	59 ± 20	63 ± 19	64 ± 23	ECM	n.s.	**	n.s.
	CB	47 ± 15	44 ± 21	73 ± 18	S × E	n.s.	***	n.s.
	CN	33 ± 9	48 ± 16	44 ± 17				
Al	FB	447 ± 387	2105 ± 2199	376 ± 288	Soil	n.s.	*	n.s.
	FN	316 ± 109	183 ± 72	268 ± 78	ECM	n.s.	**	*
	CB	478 ± 164	243 ± 108	471 ± 124	S × E	n.s.	**	n.s.
	CN	345 ± 112	239 ± 63	309 ± 136				

4. Discussion

4.1. Effects ECM Inoculation on Oak Seedling Growth

Seedling growth of all three *Quercus* species was accelerated by the inoculation of *B. reticulatus* spore suspension, particularly when grown in nutrient-rich conditions (Figure 2). Interestingly, the effects of inoculation with *B. reticulatus* on oak seedling growth differed between the forest and the colluvial soils. In the fertile forest soil, ECM colonization was high (Table 4) and all three *Quercus* species showed increased growth rate, photosynthetic and gs rate, and P and N concentration in the leaves and roots (Figures 2 and 3; Tables 5 and 7). This suggests that the formation of ECM roots with *B. reticulatus* was accelerated in forest soil, as compared to colluvial soil.

According to Kleczewski et al. [31], mycorrhizal growth and colonization relied on soil N and P availability. Mycorrhizal growth and colonization were highest under intermediate N and P availability conditions, whereby the nutrients still limited plant growth. Oliveira et al. [15] also reported that *Quercus ilex* seedlings showed the highest value of ECM colonization, when grown in a fertile substrate, without additional fertilization. From previous reports, mycorrhizal development in forest soil appeared to be suitable for *B. reticulatus*, given the intermediate nutrient availability. During nutrient absorption, enzymes and organic acids were exuded from the ECM roots [19,20,42], which was thought to facilitate nutrient uptake in nutrient-poor soils.

In my study, nutrient content in the infertile colluvial soil was lower than that in the forest soil, particularly Ca concentration (Table 1). Leaf dry mass of *Q. acuta*, and root dry mass of *Q. acuta* and *Q. saliciana* were increased by the inoculation of *B. reticulatus* grown in the colluvial soil (Figure 2, Table 2); however, growth acceleration was not obvious in the seedlings grown in the colluvial soil, compared to those grown in the forest soil. CB seedlings of all three *Quercus* species showed a

significantly low ECM colonization rate, compared to the FB seedlings (Table 4). The soil of the low availability of soil N or P, mycorrhizal colonization and growth were suppressed because of the limitation of photosynthates (=carbon) from the host plant [31]. The infertile colluvial soil had low concentrations of N and P (Table 1), and much worse, their availability was probably low. As a result, the colonization of *B. reticulatus* in oak seedlings was not accelerated in colluvial soils.

The N concentration in leaf and N content in root for *Q. salicina* was significantly different in terms of inoculation by ECM fungus (Tables 5 and 8), and the CB seedlings confirmed their increase in the colluvial soil. This trend was already confirmed by Kayama and Yamanaka [22], and the seedlings of *Q. salicina* inoculated with *Astraeus hygrometricus* was accelerated in terms of uptake of N, under infertile soil conditions. *Q. salicina* might have an inherent capacity of N uptake, even in adverse soil conditions.

Q. acuta was significantly increased in terms of the concentration of Ca in leaf and the content of K in leaf (Tables 5 and 6). In addition, the seedlings of *Q. acuta* and *Q. salicina* showed significantly higher contents of K and Ca in the roots, for the CB seedlings, compared to the CN seedlings (Table 8). The ECM root of the CB seedlings of *Q. acuta* and *Q. salicina* could accumulate K and Ca in a low environment. Kayama and Yamanaka [22] already reported that the seedlings of *Q. salicina* inoculated with *A. hygrometricus* showed increased K concentration in roots, under infertile soil conditions. K plays an important role in root growth, development of the root system architecture, and cellular functions [43]. In pine trees, mycorrhizal roots interact with *Suillus bovinus* to accumulate Ca in the interfacial apoplast [44]. Ca in apoplast can lead to the elongation of root hairs and secretion of mucilage for the protection of meristems [45]. An increase in root K and Ca can improve root growth and function in seedlings grown under adverse soil conditions. One of the reasons for these trends could be attributed to the relatively high ECM colonization (40%, Table 4), as commonly found in woody legumes [46] and pines in China [47].

In contrast, dry masses of each organ of *Q. glauca* showed significant interaction between soil types and ECM fungus (Table 2), and the CB seedlings did not show any obvious increase when inoculated by *B. reticulatus*. The nutrient contents in the leaves and roots for the seedlings of *Q. glauca* showed significant interaction between soil types and ECM fungus (Tables 6 and 8), and the CB seedlings did not show high values of nutrient contents. The ECM colonization of *Q. glauca* grown in colluvial soil was low (Table 4); as a result, nutrient uptake was probably not boosted.

4.2. Growth Characteristics of Oak Seedlings without ECM Fungus Inoculation

Quercus seedlings under non-ECM (control) conditions did not grow well, even in forest soils (Figure 2). This result suggests that symbiosis with an ECM fungus is essential for seedling growth in these *Quercus* species. FN and CN seedlings showed low values of P_{sat} and gs (Figure 3), a trend that was also found in non-ECM infected seedlings [22]. ECM fungal inoculation generally increased water uptake from the hyphae [21], but as FN and CN seedlings had no hyphae from the ECM fungus, their capacity to absorb water was low, as compared to ECM seedlings. This indicated that the low values of P_{sat} and gs in the FN and CN seedlings result from stomatal closure, to avoid water loss and, as a result, stomatal CO_2 absorption was decreased. By contrast, the FB seedlings with ECM showed high P_{sat} and gs in all three *Quercus* species (Figure 3). As the FB seedlings showed more than 60% of ECM colonization (Table 3), water uptake in the FB seedlings might be supported by the developed hyphae.

The dry mass of fallen leaves increased in non-ECM seedlings compared to ECM seedlings, particularly in *Q. salicina* (Table 3). Generally, woody species re-translocated various nutrients from aged leaves [48]. In fact, leaf N concentration was typically low in the fallen leaves of the evergreen *Quercus* species [49]. Resorbed nutrients might be allocated to the newly developing and remaining leaves [48]; consequently, the differences in the concentration of leaf nutrients were small between the ECM and non-ECM seedlings.

4.3. Specific Differences in ECM Response between the Three Selected Species

Among the three *Quercus* species investigated in this study, the dry mass of each organ was highest in the *Q. glauca* FB seedlings (Figure 2). Among the evergreen *Quercus* species, the seedlings of *Q. glauca* grew the fastest [50]. However, there is limited information available regarding the relationship between the growth of *Q. glauca* and soil fertility. This study further revealed that the growth of the *Q. glauca* seedlings was accelerated in fertile soils. Moreover, the FB seedlings of *Q. glauca* showed particularly high ECM colonization (Table 4), suggesting a facilitation of the ECM root system in fertile forest soils. Contrastingly, the CB seedlings of the *Q. acuta* and *Q. salicina* showed large root dry mass in infertile colluvial soil (Figure 2). Ito et al. [12] previously reported that *Q. acuta* was commonly found in such habitats (e.g., ridge and upper slope) and that *Q. salicina* showed a high adaptation capacity for various edaphic conditions. Based on these findings, *Q. acuta* and *Q. salicina* might have a high inherent ability to adapt to infertile environments, even with a low ECM colonization rate.

4.4. Practical Success of the ECM Inoculation Method

This study found that the relationship with *B. reticulatus* had positive effects on the selected oak seedling, despite previous reports claiming that inoculation with *B. reticulatus* had no effect on the growth of the woody species [47]. Moreover, inoculation with other species of the genus *Boletus* were known to increase host plant height [24,46] and root length [46], in woody seedlings. Osonubi et al. [46] found that inoculation with *B. suillus* increased the biomass of some *Fabaceae* woody species. In my study, the seedlings of three *Quercus* species showed high compatibility with the spore suspension of *B. reticulatus*, together with a significant increase in the host plant dry mass, when inoculated with *B. reticulatus* and grown in fertile soil conditions. Based on these results, the growth of *Quercus* seedlings could be increased when the host oaks were inoculated with *B. reticulatus*.

5. Conclusions

Seedlings of *Quercus acuta*, *Q. glauca*, and *Q. salicina* inoculated with *B. reticulatus*, by spore suspension, showed signs of accelerated growth. The inoculation method using spore suspension of *B. reticulatus* was simple and convenient for the local actors involved in reforestation efforts. This method was expected to contribute to a new method for reforestation in warm-temperature zones of East Asia. As this experiment was carried out under controlled conditions with sterilized soils, to spread this method for a revegetation technology, field experiments are needed to confirm the generalizability of my findings.

I also confirmed that these host plants' symbiosis with *B. reticulatus* was key to acquiring water and nutrients. *B. reticulatus* preferred fertile substance such as the forest soil, and seedlings of the three *Querucs* species inoculated with *B. reticulatus* showed particular growth acceleration in forest soil. Among the three *Quercus* species, *Q. glauca* inoculated with *B. reticulatus* showed the highest growth rate in the forest soil, suggesting that this species was the most suitable for revegetation in areas with little to no soil erosion. In previously eroded infertile colluvial soils, *Q. acuta* and *Q. salicina* inoculated with *B. reticulatus* showed slight increase of root biomass and the contents of K and Ca in the roots, suggesting that these two species might be suitable for reforestation in sites with colluvial soil.

Author Contributions: M.K. conceived the experiments. M.K. raised all of seedlings and inoculated them with ectomycorrhizal fungi. M.K. performed the experiments, measured photosynthetic rates, and analyzed various nutrients. M.K. discussed the results, and wrote the paper. The author has read and agreed to the published version of the manuscript.

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