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Growth Characteristics of Ectomycorrhizal Seedlings of *Quercus glauca*, *Quercus salicina*, *Quercus myrsinaefolia*, and *Castanopsis cuspidata* Planted in Calcareous Soil

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Abstract: To verify the acclimation capacity of evergreen Fagaceae species on calcareous soil, we compared ecophysiological traits between *Quercus glauca* Thunb., *Q. salicina* Blume, *Q. myrsinaefolia* Blume, and *Castanopsis cuspidata* (Thunb.) Schottky as typical woody species from southwestern Japan. We also examined the inoculation effects of the ectomycorrhizal (ECM) fungi *Astraeus hygrometricus* and *Scleroderma citrinum*, and planted seedlings in calcareous soil collected from a limestone quarry. We measured growth, ectomycorrhizal colonization, photosynthetic rate, and concentrations of nutrients in plant organs for *A. hygrometricus*-inoculated, *S. citrinum*-inoculated, and non-ECM seedlings. Six months after planting on calcareous soil, seedlings of the three *Quercus* species inoculated with *A. hygrometricus* were larger than non-ECM seedlings, especially *Q. salicina*, which showed the greatest increase in dry mass. The dry mass of *C. cuspidata* seedlings was inferior to that of the three *Quercus* species. In the nutrient-uptake analysis, phosphorus, manganese, and iron uptakes were suppressed in calcareous soil for each Fagaceae species. However, seedlings of Fagaceae species that showed better growth had increased concentrations of phosphorus in roots. We concluded that seedlings of *Q. salicina* and *Q. glauca* inoculated with *A. hygrometricus* were best suited to calcareous soil and were considered as useful species for the reforestation in limestone quarries.

Keywords: oak; ectomycorrhizal fungi; limestone; photosynthetic capacity; nutrient physiology

1. Introduction

Limestone occurs throughout East Asia [1]. Calcareous soil, which is formed by weathering of limestone, shows the pH range 7.5–8.5 owing to the buffering effects of the large amounts of calcium carbonate [2,3]. Under such conditions, the uptake of micronutrients (e.g., iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn)), which exhibit low availability at high pH, is suppressed [3,4]. Additionally, carbonate in calcareous soil causes lime-induced chlorosis as a consequence of Fe deficiency [2,3]. Moreover, phosphorus (P) is bound by calcium (Ca), and the uptake of P is also suppressed [3,5]. Accordingly, some plant species exhibit poor growth on calcareous soils [5–8]. Limestone has been quarried at many sites worldwide [9], but once quarrying has finished, natural revegetation of such sites is slow [10,11]. A limestone quarry site in Japan is currently being rehabilitated to a forest stand [12]. The calcareous soil at the quarry has a high pH and large amounts of Ca [12]. Seed from a range of plant species from an adjacent natural vegetation site was dispersed across the quarry site; however, growth of germinated plants was slow [13]. Thus, limestone quarry sites require artificial reforestation for environmental conservation of such sites.

Some plant species have high adaptability to calcareous soil because these species can obtain micronutrients and P from such soil [7]. Evergreen broad-leaved forests are distributed across warm-temperature zones in southern parts of China and southwestern Japan [14–16], and several *Quercus* species are distributed widely across limestone regions [17–19]. In particular, *Q. glauca* Thunb. is abundant on calcareous soils in Japan and China [17–23], but, in contrast, the genus *Castanopsis* is not distributed generally on such soils [18,20,24]. However, the distributions of *Quercus* and *Castanopsis* in East Asia have not been widely examined for their ability to grow on calcareous soil.

Fagaceae species can form a symbiotic interaction with ectomycorrhizal (ECM) fungi [25,26]. On calcareous soil, exudation of organic acids from ECM fungi and the dissolution of insoluble P is important for the uptake of P [27,28]. In the case of Fe, exudation of organic acids, such as citric acid, results in the chelation of Fe, leading to more available for plant uptake [29]. The communities of ECM fungi in calcareous soil can differ from those in non-calcareous soil [30,31], although cosmopolitan ECM fungi, such as genera *Astraeus* and *Scleroderma*, are distributed on calcareous soil [32,33].

The removal of limestone at a quarry site disturbs the topsoil. The abundance of the species of ECM fungi was poor at a disturbed site compared with an undisturbed site [34]. It is predicted that when non-ECM seedlings of Fagaceae species are planted into limestone, colonization of ECM fungi may be poor. When woody species inoculated with ECM fungi are planted on calcareous soil, several species exhibit tolerance to these nutrient-limiting soil conditions [33]. The beneficial effects of inoculation of ECM fungi for plant growth on sites with a poor diversity of ECM fungi have been shown previously [35]. However, few studies have examined the inoculation of seedlings with ECM fungi prior to growing in calcareous soil. We expected that evergreen Fagaceae species inoculated with ECM fungi may be effective for growth on limestone quarry sites. The aim of our research was to verify the acclimation capacity of *Quercus* and *Castanopsis* on calcareous soil. Moreover, we examined the inoculation effects of ECM fungi and compared these between *Quercus* and *Castanopsis* species. We examined the ecophysiological traits of seedlings of *Quercus* and *Castanopsis*, specifically (1) the growth characteristics of the seedlings; (2) symbiosis of ECM fungi on short roots; and (3) concentrations of elements in plant organs. These parameters were compared between ECM-inoculated and non-inoculated seedlings. Our findings may be applied in the artificial reforestation of limestone quarries using Fagaceae seedlings inoculated with ECM fungi.

2. Materials and Methods

2.1. Plants and ECM Fungi

We used *Quercus glauca* Thunb., *Quercus salicina* Blume, *Quercus myrsinaefolia* Blume, and *Castanopsis cuspidata* (Thunb.) Schottky. The three *Quercus* species are distributed across limestone regions in southwestern Japan, whereas *C. cuspidata* is not present in these regions [17–19,21]. Acorns of the four Fagaceae species were collected from an evergreen broad-leaved forest located in the central part of Kyushu, Southwestern Japan in December 2007 for *Q. glauca* and *Q. salicina*, and in December 2008 for *Q. myrsinaefolia* and *C. cuspidata*. To eliminate differences in genetic characteristics caused by edaphic factors, the forest-collected acorns of the four Fagaceae species were from an area with non-calcareous soil. The acorns were stored in a refrigerator at 4 °C prior to sowing.

Information about species of ECM fungi distributed in calcareous soil in Japan has rarely been reported [36]. However, reports from other countries indicate that *Astraeus hygrometricus* (Pers.) Morgan and *Scleroderma citrinum* Pers. can form symbiotic interactions in calcareous soil [31,32]. In addition, *A. hygrometricus* and *S. citrinum* are typical ECM fungi from Fagaceae forests in Japan [37,38], and we found previously that these species could form symbiotic relationships with Fagaceae species [39,40]. Thus, we chose *A. hygrometricus* and *S. citrinum* for the inoculation of Fagaceae seedlings.

In July 2008, fruiting bodies of *A. hygrometricus* and *S. citrinum* were collected from a slope with exposed mineral soil on the edge of a secondary evergreen, broad-leaved forest. This site was the same

as that for the collection of acorns. Several fragments (3–7 mm) were cut off from the fruiting bodies and placed on agar medium. The medium consisted of 10 g·L^{-1} glucose, 10 g·L^{-1} malt extract, and 1 g·L^{-1} bactopeptone [41]. Fragments of *A. hygrometricus* and *S. citrinum* were incubated at 25 °C for 2 weeks in darkness. We confirmed the developed mycelial colony of *A. hygrometricus* and *S. citrinum* based on descriptions from previous literatures [38,41]. We used isolated mycelia for inoculation.

2.2. Preparation of Pot Experiments

Acorns of Fagaceae species were germinated in June 2008 for *Q. glauca* and *Q. salicina*, and in June 2009 for *Q. myrsinaefolia* and *C. cuspidata*. The acorns were surface sterilized with 30% H_2O_2 for 20 min, and rinsed 4 times with sterilized distilled water. Acorns were placed on a sterilized perlite and akadama (red ball earth, originated from subsoil in Kanto region, Japan) soil (1:1) in a free-draining container (width × length × depth = 31 cm × 21 cm × 8 cm). After germination, inoculation of ECM was performed in August of the same year.

Isolated mycelia of *A. hygrometricus* and *S. citrinum* were cultured in 40 mL of Ohta liquid medium [42] in 100 mL flasks at 24 °C for 2 weeks in darkness. The incubated mycelia of *A. hygrometricus* and *S. citrinum* were aseptically fragmented using a stirrer (DX, As One Co., Osaka, Japan) at 7000 rpm for 20 s. The suspension of hyphal fragments was poured into a 50 mL plastic tube and centrifuged at 2320 g for 10 min to collect the hyphal fragments, which were then mixed with sterilized distilled water and centrifuged at 2320 g for 10 min. After the supernatant was removed, the procedure was repeated. The hyphal fragments were resuspended in sterilized distilled water, and 48 seedlings each of *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* were inoculated with *A. hygrometricus* or *S. citrinum*. Prior to the inoculation, seedlings were removed from their containers, and the soil was washed from their roots with sterilized distilled water. On the exposed roots of seedlings, 1 mL of hyphal suspension, which contained 1 mg of hyphal fragments, was added. In addition, 24 seedlings each of *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* were prepared similarly but without inoculation of ECM fungi (non-ECM controls).

All seedlings were planted into Ray Leach Containers (164 mL, SC10, Stuewe and Sons, Tangent, OR, USA) filled with stabilized perlite: akadama soil (1:1). The pH of the perlite: akadama soil was 6.1. The seedlings were grown in an enclosed, air-conditioned (25.0–35.0 °C) greenhouse at the Forestry and Forest Products Research Institute (FFPRI), Tsukuba until April 2009 for *Q. glauca* and *Q. salicina.*, and until April 2010 for *Q. myrsinaefolia* and *C. cuspidata*. The greenhouse was sealed to prevent the seedlings from becoming contaminated by airborne spores or hyphal fragments of mycorrhizal fungi. No fertilizers were applied to the seedlings, and each pot was irrigated with 20 mL of distilled water per day. We also confirmed the contamination of ECM root visually, and the control seedlings contaminated with ECM on their roots were eliminated from the trial.

2.3. Soil Collection and Analysis

In January 2009, we collected calcareous soil from the B horizon of a limestone quarry (Todaka Mining Co., Tsukumi, Oita Pref., Japan, 33°03′ N, 131°48′ E, 580 m a.s.l.). The color of the soil was dark red (5YR 3/6), and it showed high viscosity owing to a high content of clay [43]. Collected soil was sieved (2 mm) at the same site and collected in 32 sandbags. After transportation, soil was kept in a storehouse in Kyushu Research Center, FFPRI.

The pot experiments were carried out by dividing them into two groups, in May 2009 for *Q. glauca* and *Q. salicina* and in May 2010 for *Q. myrsinaefolia* and *C. cuspidata*. Before planting for each year, the collected soil was placed in autoclave bags and sterilized using a wet cycle of an autoclave at 120 °C for 20 min. (KTS-2346B, Alp Co., Tokyo, Japan) to remove all other mycorrhizal species. After sterilization, five soil samples were collected from five bags for each year.

We measured the soil properties including pH and the concentration of carbon (C), nitrogen (N), exchanged P, base cations (Ca, magnesium (Mg), potassium (K) and sodium (Na)), Fe, and Mn. Soil

pH was measured using a pH meter (SG2, Mettler Toledo, Zürich, Switzerland); 10 g of soil was mixed with 25 mL of distilled water and shaken for 1 h before the reading was taken [44].

After measuring the soil pH, samples were dried at 105 °C for 24 h for further analysis. Dried soil samples were used to determine the concentration of C and N using a NC analyzer (Sumigraph NC-220F, Sumika Chemical Analysis Service, Tokyo, Japan). Exchanged P was extracted by the Olsen method [45] by shaking for 1 h. P concentration in the soil solution was determined by the molybdenum blue method [46] using a spectrophotometer (UV-2500PC, Shimadzu, Kyoto, Japan).

The concentrations of exchanged base cations and Mn were determined; 2.5 g of dry soil was mixed with 50 mL of 1 M ammonium acetate solution and shaken for 1 h prior to analysis [45]. Solutions for analysis of exchanged Fe were obtained by adding 50 mL of 1 M KCl to 2.5 g of dry soil and shaking for 1 h [45]. The base cations, Fe, and Mn concentrations in the soil solution were analyzed using an inductivity coupled plasma analyser (ICPE-9000, Shimadzu, Kyoto, Japan).

2.4. Establishment of the Pot Experiment

A total of 48 mycorrhizal seedlings and 24 non-mycorrhizal seedlings of the four Fagaceae species were transported to Kyushu Research Center, FFPRI in May. A total of 24 mycorrhizal seedlings and 12 non-mycorrhizal seedlings each of *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* were transplanted into free-draining pots (depth: 30 cm, diameter: 15 cm, volume: 3.7 L) filled with calcareous soil. The remaining seedlings, i.e., 24 mycorrhizal seedlings and 12 non-mycorrhizal seedlings of the four Fafaceae species, were sampled at this time. Fagaceae seedlings were raised for 6 months in a naturally illuminated phytotron (Koito Co., Tokyo, Japan) at Kyushu Research Center, FFPRI. The phytotron had three rooms, and seedlings of *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* were inoculated with *A. hygrometricus* (abbreviated to AH), *S. citrinum* (SC), and non-ECM (NE), the seedlings were then put into the three rooms. Four seedlings of each species inoculated with AH, SC and NE were placed in each room.

After establishment, each pot was irrigated with 60 mL of distilled water per day. The average daily photosynthetically active radiation (PAR) during the experimental period was 39.6 mol·m^{-2}·day^{-1} in 2009, and 37.5 mol·m^{-2}·day^{-1} in 2010, calculated using data on solar radiation from the Japan Meteorological Agency [47] and the ratio of solar radiation to PAR [48]. Air temperature in the phytotron rooms was controlled at 30 °C (day) and 25 °C (night) from July to September, and 25 °C (day) and 20 °C (night) from May to June and in October. These temperatures were based on air temperature in Kumamoto city, Japan [47]. Relative humidity was controlled at 75%. During the experiment, some contamination by airborne spores was noted. The position of seedlings was changed at regular intervals (every month), and the phytotron rooms were disinfected using ethanol at this time.

2.5. Measurement of Seedling Growth

To determine the growth characteristics of seedlings of *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata*, we measured the dry mass of leaves, stems and branches, and roots. Twelve AH, SC, and NE seedlings each for the four tree species were harvested in May (first sampling) and November (second sampling). The roots of harvested seedlings were washed twice with tap water to remove soil, and then washed with distilled water. The washed seedlings were divided into leaves, stems and branches, and root components. Each component was put into its own envelope and oven-dried at 60 °C for 4 days. The dry mass of each component was determined. From the values of dry mass of each component at the first and second samplings, we calculated relative dry mass as follows:

$$\text{Dry mass at second sampling} / \text{Dry mass at first sampling} \times 100 \quad (1)$$

For the relative dry mass, 100 was set as the value at the first sampling. We also measured specific leaf area (SLA [7]) for each species. We used the SLA value to convert the concentration of nutrients into area based values.

2.6. Measurement of the Rate of Colonization of ECM Fungi

To measure the proportion of roots with ECM fungal colonization, we selected five large clusters of roots from each of seedling, and over 500 short roots (<5 mm in length) that diverged from them. This proportion was determined before drying. For assessment of ECM fungi, roots of seedlings were harvested at the first and second samplings and carefully washed free of soil under gently flowing water. Cluster roots were soaked in distilled water and observed at 10–40× magnification using a stereomicroscope (STZ-40TBIT, Shimadzu, Kyoto, Japan). ECM short roots of *A. hygrometricus* and *S. citrinum* were confirmed by the color, form, and size of the ECM fungi, the presence of rhizomorphs and the absence of root hairs [38,40,49]. The numbers of colonized and uncolonized short roots were counted, and we calculated the percentage of ECM fungal colonization from the proportion of colonized short roots to the total number of short roots [50]. We also examined the relationship between growth (total dry mass) and ECM fungal colonization.

2.7. Photosynthetic Capacity

The photosynthetic rate at light saturation (P_{sat}) was measured in all seedlings in October. Third leaves counted from the top of the seedling were used for the measurement of P_{sat} and stomatal conductance (g_s). Measurements were made using a portable gas analyzer (LI-6400, LI-COR Biosciences, Lincoln, NE, USA) under steady-state conditions at an ambient CO_2 concentration of 37.0 Pa. The LED light source was adjusted to a saturation light level of 1700 $\mu mol \cdot m^{-2} \cdot s^{-1} \cdot PPF$.

2.8. Analysis of Element Concentrations in Plants

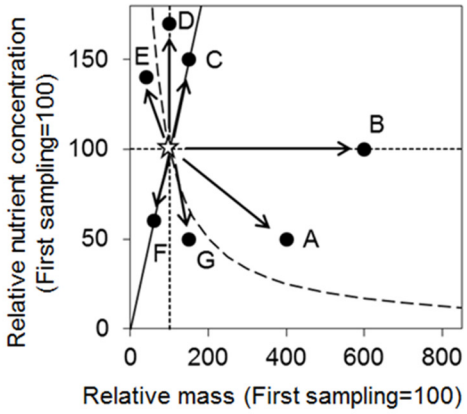
We measured the concentrations of N, P, K, Ca, Mg, Fe, and Mn in leaves and roots at the first and second samplings. Dried samples were ground to a fine powder using a sample mill (WB-1; Osaka Chemical Co., Osaka, Japan). The concentration of N was determined using a CN CORDER analyzer (MT-600, Yanako New Science Inc., Kyoto, Japan). The remaining samples were digested by the HNO_3-HCl-H_2O_2 method [51]. The concentration of P was determined by the molybdenum blue method using a spectrophotometer. Concentrations of K, Ca, Mg, Fe, and Mn were analyzed using an atomic absorption spectrophotometer (Z-2310, Hitachi High-Technologies Co., Tokyo, Japan).

The concentrations of each nutrient at the first and second samplings were calculated as relative nutrient concentration, as follows:

$$\text{Concentration at second sampling} / \text{Concentration at first sampling} \times 100 \quad (2)$$

For the relative concentrations, 100 was set as the value from the first sampling. Based on relative nutrient concentration and relative dry mass, we examined vector analysis in accordance with Valentine and Allen [52] and Scagel [53]. Changes in dry mass and the concentration of nutrients were plotted for AH, SC, and NE seedlings of the four Fagaceae species. The interpretation of vector shifts, as described in Valentine and Allen [52], are shown in Figure 1 as a reference.

We also examined the effects of nutrients on photosynthetic rate. In general, photosynthetic rate is closely related to N [7,54] and P [55,56]. Correlation analysis was performed between P_{sat} and the concentrations of N and P in leaves at the second sampling. For the concentrations of N and P, we used the SLA area-based values.



Vector shift	Concentration	Content	Mass	Interpretation
A	Decrease	Increase	Increase	Nutrient non-limiting **Dilution** due to growth
B	Unchanged	Increase	Increase	Nutrient non-limiting **Sufficiency** on growth
C	Increase	Increase	Increase	**Enhancement** of nutrient uptake, related to change in mass
D	Increase	Increase	Unchanged	**Luxury consumption** Little effect on growth
E	Increase	Decrease	Decrease	**Toxicity** of elevated nutrient Decrease of growth
F	Decrease	Decrease	Decrease	**Nutrient deficiency** due to low availability
G	Decrease	Decrease	Increase	**Efficient use** of very little nutrient

Figure 1. Graphical representation of interpretation comparing changes in dry mass and concentrations of nutrients grown in different media types. The content isograms represent combinations of dry mass and concentration required for constant content per unit dry mass. The arrows indicate the direction in which each interpretation holds (adapted from Valentine and Allen [48] and Scagel [49]). The curve indicated by the broken line shows the border to estimate increase or decrease of content. A plot located above the curve shows increase of content.

2.9. Statistical Analysis

All parameters were analysed for normality and homoscedasticity using the Shapiro–Wilk test with Sigma Plot 12.3 (Systat Software Inc., San Jose, CA, USA,). Some parameters did not show normality and homoscedasticity; therefore, we used nonparametric tests by Kyplot 3.0 (Kyens Lab. Inc., Tokyo, Japan). The data of soil chemical properties were compared between 2009 and 2010 using Mann-Whitney U test. Dry mass of the whole plant and each organ, ECM fungal colonization, P_{sat} and g_s, and concentrations of elements in leaves and roots were examined using a Steel–Dwass test. The mean values of total dry mass and ECM fungal colonization for *Q. glauca*, *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* were compared among the first and second samplings of AH, SC, and NE seedlings. The values of dry mass of each organ, P_{sat}, g_s and concentrations of elements in leaves and roots of the four Fagaceae species were compared among AH, SC and NE seedlings. Different letters are used to indicate statistically significant differences at $p < 0.05$. The mean values followed by the same letter showed no significant differences.

To examine the relationship between total dry mass and ECM fungal colonization, we conducted a Spearman's rank correlation. Additionally, we examined the relationship between P_{sat} and nutrients in leaves by the same method. For the data from the second sampling, we also compared each parameter of the seedlings from each of the three phytotron rooms by a Kruskal-Wallis test. There were no significant differences among the three rooms.

3. Results

3.1. Soil Chemical Properties

Table 1 lists the chemical properties of the calcareous soil. The pH of the calcareous soil was almost neutral (7.01). The Ca concentration was high. Comparing the two experimental years for calcareous soil, there was no significant difference.

Table 1. Chemical properties of calcareous soil used in this experiment (mean ± SD, n = 5).

Year	pH	C ($g \cdot kg^{-1}$)	N ($g \cdot kg^{-1}$)	P [1] ($mg \cdot kg^{-1}$)
2009	7.00 ± 0.03	26.5 ± 2.6	0.962 ± 0.082	59.2 ± 11.3
2010	7.02 ± 0.02	28.6 ± 1.1	0.935 ± 0.089	52.1 ± 10.6
	Ca [2] ($g \cdot kg^{-1}$)	Mg [2] ($mg \cdot kg^{-1}$)	K [2] ($mg \cdot kg^{-1}$)	Na [2] ($mg \cdot kg^{-1}$)
2009	2.64 ± 0.05	62.9 ± 1.3	26.0 ± 1.8	117 ± 6
2010	2.56 ± 0.05	62.1 ± 1.6	25.8 ± 0.8	115 ± 1
	Fe [3] ($mg \cdot kg^{-1}$)	Mn [2] ($mg \cdot kg^{-1}$)		
2009	8.34 ± 0.58	4.93 ± 0.59		
2010	9.52 ± 5.14	4.81 ± 0.68		

Mean values of each parameter were analyzed by Mann-Whitney U test (no significance; $p > 0.05$). [1] Extracted by the Olsen method; [2] Extracted in 1 M ammonium acetate; [3] Extracted in 1 M potassium chloride.

3.2. Growth Characteristics

At the first sampling stage before the experiment, the total dry mass of three *Quercus* species showed little difference among AH, SC, and NE seedlings (Figure 2). For *C. cuspidata*; the total dry mass of AH and SC seedlings showed significant increases compared with NE seedlings.

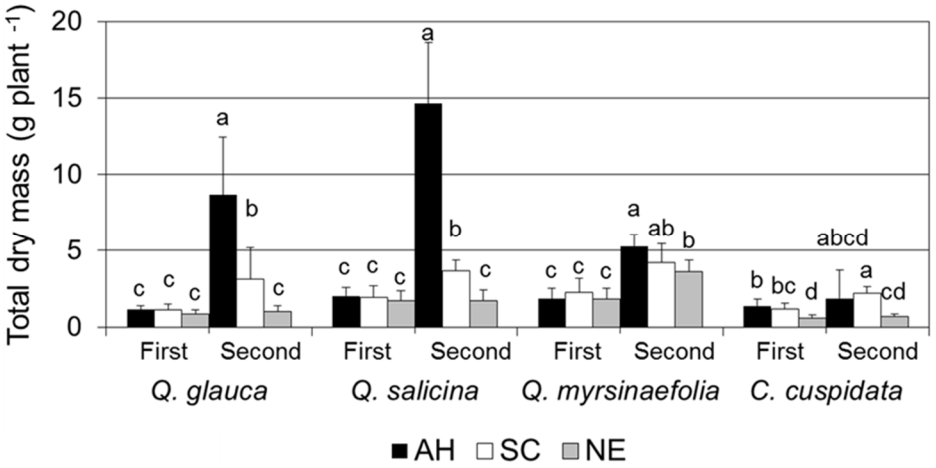


Figure 2. Total dry mass for seedlings of four Fagaceae species grown in a calcareous soil ($g \cdot plant^{-1}$, mean ± SD, n = 12). Different letters indicate significant effects according to a Steel-Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$). AH, seedlings inoculated with *Astraeus hygrometricus*; SC, seedlings inoculated with *Scleroderma citrinum*; NE, non-ectomycorrhizal seedlings.

At the second sampling, the total dry mass was significantly increased in AH and SC seedlings of *Q. glauca*, *Q. salicina*, and *Q. myrsinaefolia*. For NE seedlings of *Q. myrsinaefolia*, the total dry mass increased significantly. In contrast, other NE seedlings did not show a significant increase in the total dry mass in *Q. glauca*, *Q. salicina*, and *C. cuspidata*. AH seedlings of *C. cuspidata* did not show significant increases in the total dry mass.

Comparing AH, SC, and NE seedlings at the second sampling, the total dry mass and dry mass of each organ were significantly higher in AH seedlings of *Q. glauca*, *Q. salicina*, and *Q. myrsinaefolia* than those in NE seedlings (Figures 2 and 3). Additionally, SC seedlings had significantly higher dry mass for each organ compared with NE seedlings of *Q. glauca*, *Q. salicina*, and *C. cuspidata*. For the SC seedlings of *Q. myrsinaefolia* and AH seedlings of *C. cuspidata*, the dry mass of each organ was not higher than that of NE seedlings. Comparing the AH seedlings of the four Fagaceae species, dry mass was highest for *Q. salicina* and lowest for *C. cuspidata*.

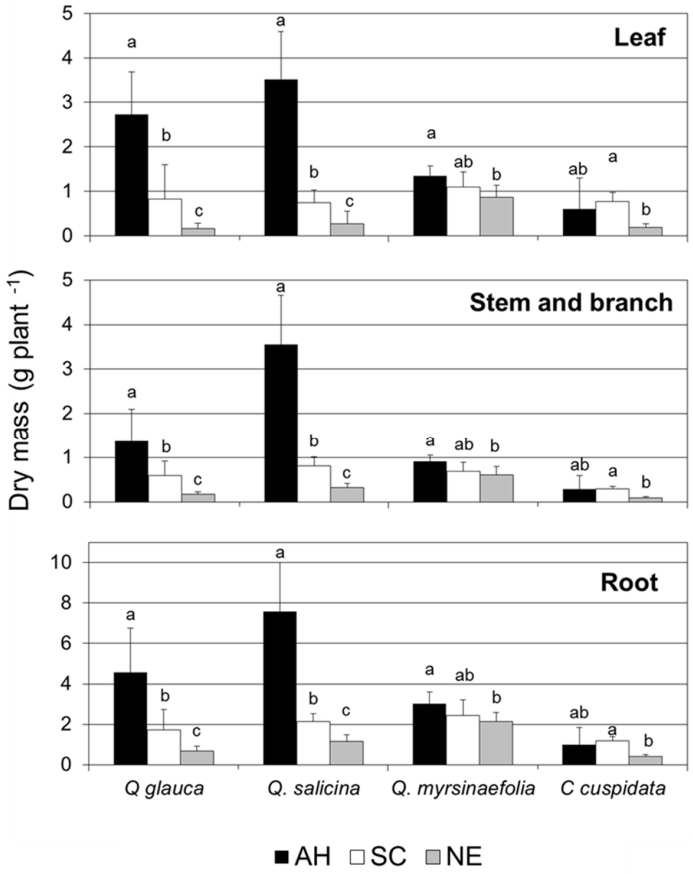


Figure 3. Dry mass of each organ (leaf, stem and branch, and root) for seedlings of four Fagaceae species grown in a calcareous soil at the second sampling ($g \cdot plant^{-1}$, mean ± SD, $n = 12$). Different letters indicate significant effects according to a Steel–Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$).

SLA of the four Fagaceae species was 85.2 $cm^2 \cdot g^{-1}$ for *Q. glauca*, 95.6 $cm^2 \cdot g^{-1}$ for *Q. salicina*, 87.7 $cm^2 \cdot g^{-1}$ for *Q. myrsinaefolia*, and 162.2 $cm^2 \cdot g^{-1}$ for *C. cuspidata*. There were no significant difference among AH, SC, and NE seedlings for each species.

3.3. The Percentage of ECM Colonization

At the first sampling, the percentages of ECM colonization in roots of AH seedlings were 69% for *Q. glauca* and *C. cuspidata*, 83% for *Q. salicina*, and 55% for *Q. myrsinaefolia* (Figure 4). In SC

seedlings, these values were significantly lower than those of AH seedlings except for *Q. myrsinaefolia*. The percentage of ECM colonization of SC seedlings was around 40% for *Q. glauca*, *Q. myrsinaefolia*, and *C. cuspidate*, whereas this value was 54% for *Q. salicina*.

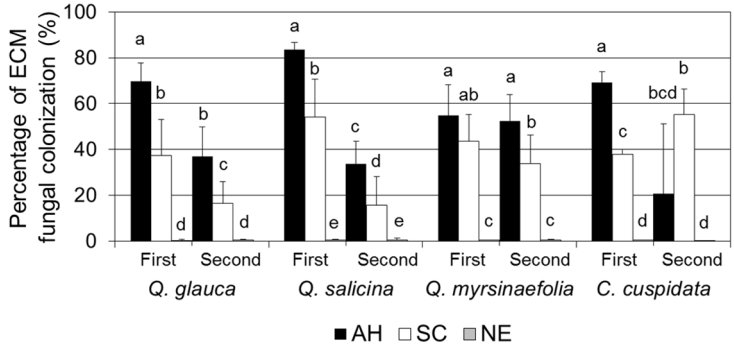


Figure 4. Percentage of ectomycorrhizal fungal colonization for seedlings of four Fagaceae species grown in a calcareous soil (%, mean ± SD, n = 12). Different letters indicate significant effects according to a Steel–Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$).

After the second sampling, the percentages of ECM colonization of AH seedlings decreased significantly for *Q. glauca*, *Q. salicina*, and *C. cuspidata*. In addition, the percentages of ECM colonization decreased significantly for SC seedlings of *Q. glauca* and *Q. salicina*. The percentages of ECM colonization of the three *Quercus* species were significantly higher for AH seedlings than those of SC seedlings. In contrast, the percentage of ECM colonization of SC seedlings increased significantly to 55% for *C. cuspidata*. In half of the AH seedlings of *C. cuspidata*, the percentage of ECM colonization decreased to <1%. For *Q. myrsinaefolia*, the percentage of ECM colonization of AH and SC seedlings did not change after the experiment.

In NE seedlings of the four Fagaceae species, the percentage of ECM colonization was <1% at the two sampling points. In addition, these values of NE seedlings were significantly lower than those of AH and SC seedlings.

In terms of the relationship between total dry mass and the percentage of ECM colonization, we found a significant positive correlation for each Fagaceae species at the second sampling (Table 2, $p < 0.001$). Moreover, *Q. glauca* and *C. cuspidata* showed a significant positive difference at the first sampling ($p < 0.05$).

Table 2. Spearman's correlation coefficients (R) for seedlings of four Fagaceae species grown in a calcareous soil (n = 12).

	Q. glauca		***Q. salicina***		***Q. myrsinaefolia***		***C. cuspidata***	
	R	***p***	***R***	***p***	***R***	***p***	***R***	***p***
	0.826	<0.001 *	0.876	<0.001 *	0.638	<0.001 *	0.776	<0.001 *
Elements	***Q. glauca***		***Q. salicina***		***Q. myrsinaefolia***		***C. cuspidata***	
	R	***p***	***R***	***p***	***R***	***p***	***R***	***p***
N	0.217	0.199	–0.350	0.038 *	–0.394	0.020 *	0.138	0.415
P	0.030	0.859	0.435	0.010 *	0.605	<0.001 *	0.777	<0.001 *

The upper row shows the relationships between total dry mass and ectomycorrhizal (ECM) fungal colonization. The lower row shows the relationship between photosynthetic rate at light saturation (P_{sat}) and area-based concentrations of N and P in leaves at the second sampling. * shows a significant correlation at $p < 0.05$.

3.4. Photosynthetic Capacity

The P_{sat} of *Q. glauca* and *Q. salicina* was significantly higher in AH seedlings than in SC and NE seedlings (Figure 5). In *Q. myrsinaefolia*, the P_{sat} in AH and SC seedlings was significantly higher than that in NE seedlings. The P_{sat} of *C. cuspidata* was significantly higher in SC seedlings than in NE seedlings. Comparing the four Fagaceae species, P_{sat} was highest in AH seedlings of *Q. glauca* and lowest in NE seedlings of *C. cuspidata*.

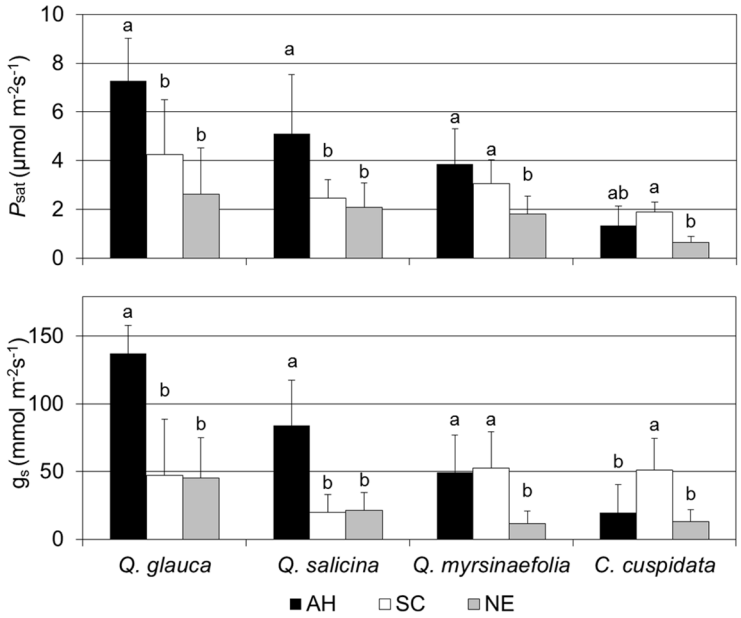


Figure 5. Photosynthetic rate at light saturation (P_{sat}, μmol·m^{-2}·s^{-1}) and stomatal conductance (g_s, mol·m^{-2}·s^{-1}) for seedlings of four Fagaceae species grown in a calcareous soil (mean ± SD, n = 12). Different letters indicate significant effects according to a Steel–Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$). P_{sat} and g_s for *Q. glauca* and *Q. salicina* were measured in October 2009, and for *Q. myrsinaefolia* and *C. cuspidata* in October 2010.

A similar trend was observed for g_s as for P_{sat}, and g_s of *Q. glauca* and *Q. salicina* was significantly higher in AH seedlings than in SC and NE seedlings. g_s in AH and SC seedlings of *Q. myrsinaefolia* was significantly higher than that in NE seedlings. In *C. cuspidata*, the g_s in SC seedlings was higher than that in AH and NE seedlings. In particular, the g_s in AH seedlings of *Q. glauca* was higher compared with other seedlings.

3.5. Element Concentrations in Leaves

The concentrations of elements in leaves at the second sampling are shown in Table 3. The concentration of P was significantly higher in AH seedlings of *Q. salicina* and *Q. myrsinaefolia* than in SC seedlings. In particular, AH seedlings of *Q. salicina* showed the highest P concentration between the four Fagaceae species. In *C. cuspidata*, the concentration of P was highest for SC seedlings. In contrast, other elements did not show high concentrations in AH seedlings despite their greater growth. The concentration of N in AH seedlings was significantly lower for *Q. salicina* and *C. cuspidata* than in SC and NE seedlings. Similarly, the concentration of K was significantly lower in AH seedlings of *Q. glauca* and *Q. myrsinaefolia* compared with in SC and NE seedlings. Moreover, NE seedlings had the highest Mg concentration in *Q. salicina* and *C. cuspidata*.

For the relationship between the concentrations of N or P and P_{sat}, we found a significant positive correlation between the concentrations of P and P_{sat} of *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* (Table 2, $p < 0.05$). In contrast, there were significant negative correlations between concentration of N and P_{sat} of *Q. salicina* and *Q. myrsinaefolia* ($p < 0.05$).

Table 3. Concentrations of elements (N, P, K, Ca, Mg, Fe, and Mn) in leaves of seedlings of four Fagaceae species grown in a calcareous soil sampled at the second sampling (mean ± SD, n = 12).

Element, Inoculation		*Q. glauca*	*Q. salicina*	*Q. myrsinaefolia*	*C. cuspidata*
N ($mg \cdot g^{-1}$)	AH	10.7 ± 3.4 a	9.4 ± 2.2 b	9.2 ± 2.8 a	9.7 ± 1.8 b
	SC	11.1 ± 1.5 a	13.2 ± 2.3 a	11.2 ± 3.8 a	15.4 ± 3.4 a
	NE	10.5 ± 2.8 a	15.3 ± 2.0 a	11.0 ± 2.41 a	12.2 ± 1.10 a
P ($mg \cdot g^{-1}$)	AH	1.43 ± 0.33 a	1.98 ± 0.30 a	1.81 ± 0.27 a	0.84 ± 0.33 a
	SC	1.40 ± 0.28 a	1.34 ± 0.35 b	1.41 ± 0.12 b	1.11 ± 0.19 a
	NE	1.35 ± 0.52 a	1.41 ± 0.62 ab	0.69 ± 0.29 c	0.30 ± 0.12 b
K ($mg \cdot g^{-1}$)	AH	5.54 ± 0.80 b	4.52 ± 1.12 a	3.91 ± 0.50 b	5.20 ± 2.12 ab
	SC	8.58 ± 1.37 a	5.39 ± 0.83 a	4.78 ± 1.48 b	3.96 ± 0.65 b
	NE	8.65 ± 1.15 a	6.17 ± 2.22 a	7.15 ± 1.18 a	5.81 ± 1.18 a
Ca ($mg \cdot g^{-1}$)	AH	25.3 ± 7.1 a	19.6 ± 6.5 a	26.6 ± 3.3 a	15.9 ± 2.8 a
	SC	19.2 ± 5.8 a	14.9 ± 3.2 a	26.8 ± 5.1 a	16.2 ± 2.7 a
	NE	20.6 ± 4.8 a	14.6 ± 4.2 a	23.8 ± 3.30 a	17.3 ± 5.11 a
Mg ($mg \cdot g^{-1}$)	AH	1.40 ± 0.43 a	1.12 ± 0.35 b	2.30 ± 0.31 a	1.86 ± 0.65 ab
	SC	1.43 ± 0.40 a	1.23 ± 0.20 b	2.47 ± 0.40 a	1.36 ± 0.25 b
	NE	1.88 ± 0.60 a	3.50 ± 2.66 a	2.68 ± 0.41 a	2.18 ± 0.52 a
Fe ($\mu g \cdot g^{-1}$)	AH	102 ± 20 a	77 ± 12 b	85 ± 19 a	155 ± 139 a
	SC	110 ± 44 a	159 ± 51 a	79 ± 17 a	105 ± 26 a
	NE	182 ± 137 a	215 ± 139 a	94 ± 21 a	180 ± 115 a
Mn ($\mu g \cdot g^{-1}$)	AH	204 ± 31 b	202 ± 76 ab	273 ± 65 a	454 ± 239 a
	SC	140 ± 67 b	163 ± 73 b	200 ± 115 a	167 ± 58 b
	NE	434 ± 189 a	363 ± 151 a	206 ± 67 a	507 ± 222 a

Different letters indicate significant effects according to a Steel-Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$). AH, seedlings inoculated with *Astraeus hygrometricus*; SC, seedlings inoculated with *Scleroderma citrinum*; NE, non-ectomycorrhizal seedlings.

3.6. Element Concentrations and Contents in Roots

The concentrations of elements in roots at the second sampling are shown in Table 4. The concentrations of P in *Q. glauca*, *Q. salicina*, and *Q. myrsinaefolia* were significantly higher in AH seedlings than in NE seedlings. AH seedlings also exhibited high concentrations of Fe and Mn in *Q. myrsinaefolia* than in SC seedlings. In *C. cuspidata*, the concentrations of N and P were highest in SC seedlings. The concentration of Ca was significantly lower in SC seedlings of *Q. myrsinaefolia* and *C. cuspidata*. In addition, the concentration of Fe in SC seedlings of *Q. salicina* and *Q. myrsinaefolia* was significantly lower. In NE seedlings of *Q. myrsinaefolia*, the concentration of Mg was significantly higher than in AH and SC seedlings. Across the four Fagaceae species, the concentration of P was the highest for AH seedlings of *Q. salicina*.

Table 4. Concentrations of elements (N, P, K, Ca, Mg, Fe, and Mn) in roots of seedlings of four Fagaceae species grown in a calcareous soil sampled at the second sampling (mean ± SD, n = 12).

Element, Inoculation		*Q. glauca*		*Q. salicina*		*Q. myrsinaefolia*		*C. cuspidata*	
N ($mg \cdot g^{-1}$)	AH	8.3 ± 1.8	a	9.8 ± 1.7	a	7.5 ± 1.9	a	7.5 ± 1.9	b
	SC	7.3 ± 1.3	a	7.6 ± 0.8	b	6.3 ± 1.9	a	12.2 ± 1.4	a
	NE	6.8 ± 1.6	a	7.7 ± 1.6	ab	5.5 ± 1.8	a	5.8 ± 1.1	b
P ($mg \cdot g^{-1}$)	AH	2.34 ± 0.49	a	3.76 ± 0.70	a	2.63 ± 0.74	a	1.20 ± 0.92	ab
	SC	1.84 ± 0.76	ab	1.21 ± 0.30	b	1.95 ± 1.02	ab	2.45 ± 0.75	a
	NE	1.13 ± 0.29	b	1.17 ± 0.29	b	0.97 ± 0.17	b	0.39 ± 0.17	b
K ($mg \cdot g^{-1}$)	AH	2.74 ± 0.42	a	2.65 ± 0.42	a	2.75 ± 0.36	a	2.17 ± 0.50	a
	SC	2.70 ± 1.40	a	2.28 ± 0.45	a	2.21 ± 0.42	a	2.44 ± 0.34	a
	NE	2.07 ± 0.54	a	2.49 ± 0.60	a	2.52 ± 0.42	a	1.96 ± 0.37	a
Ca ($mg \cdot g^{-1}$)	AH	14.8 ± 2.3	a	15.0 ± 2.2	a	13.4 ± 1.0	a	15.3 ± 2.3	a
	SC	13.8 ± 2.2	a	13.8 ± 1.1	a	11.7 ± 1.0	b	12.5 ± 1.2	b
	NE	17.0 ± 2.2	a	15.7 ± 1.6	a	13.5 ± 1.7	ab	15.1 ± 1.81	a
Mg ($mg \cdot g^{-1}$)	AH	1.85 ± 0.21	ab	1.64 ± 0.23	a	1.54 ± 0.18	b	1.46 ± 0.18	a
	SC	1.70 ± 0.59	b	1.62 ± 0.27	a	1.51 ± 0.28	b	1.25 ± 0.12	bc
	NE	2.10 ± 0.32	a	1.71 ± 0.26	a	1.94 ± 0.19	a	1.36 ± 0.16	ab
Fe ($\mu g \cdot g^{-1}$)	AH	0.99 ± 0.49	a	1.45 ± 0.53	a	1.38 ± 0.42	a	0.76 ± 0.87	ab
	SC	0.82 ± 0.33	a	0.76 ± 0.19	b	0.64 ± 0.20	b	0.69 ± 0.26	a
	NE	1.49 ± 0.77	a	1.32 ± 0.33	a	0.98 ± 0.42	ab	0.38 ± 0.18	b
Mn ($\mu g \cdot g^{-1}$)	AH	63 ± 17	b	70 ± 20	a	39 ± 9	a	31 ± 13	a
	SC	73 ± 11	b	67 ± 18	a	28 ± 6	b	33 ± 8	a
	NE	138 ± 78	a	80 ± 20	a	34 ± 10	ab	24 ± 5	a

Different letters indicate significant effects according to a Steel-Dwass test ($p < 0.05$). Mean values followed by the same letter showed no significant difference ($p > 0.05$).

3.7. Vector Analysis between Relative Dry Mass and Nutrient Concentration

The relationships between relative leaf dry mass and relative concentrations in leaves are shown in Figure 6. For the relationship of Ca, uptake of each Fagaceae species showed enhanced or luxury consumption. In contrast, Mn showed dilution or efficient use by each Fagaceae species. For N, most plots showed dilution or sufficiency, whereas those for SC seedlings of *Q. salicina* and SC and NE seedlings of *C. cuspidate* showed enhanced uptake. In the case of NE seedlings of *Q. salicina*, the plot showed toxicity.

For P and Fe in the leaves, most plots of each Fagaceae species showed dilution or efficient use for leaves and roots. For P in leaves of NE seedlings of *Q. glauca* and *Q. salicina*, and Fe in leaves of NE seedlings of *Q. salicina*, the plots showed nutrient deficiency. The trend of K in leaves showed various patterns. Plots of AH seedling of *Q. glauca* and *Q. salicina* showed sufficiency, but the plots of *C. cuspidata* and AH and SC seedlings of *Q. myrsinaefolia* showed dilution. Other plots showed enhanced or luxury consumption. For the relationship of Mg, the plots showed sufficiency or dilution for each Fagaceae species. In the case of NE seedlings of *Q. salicina*, the plot showed toxicity.

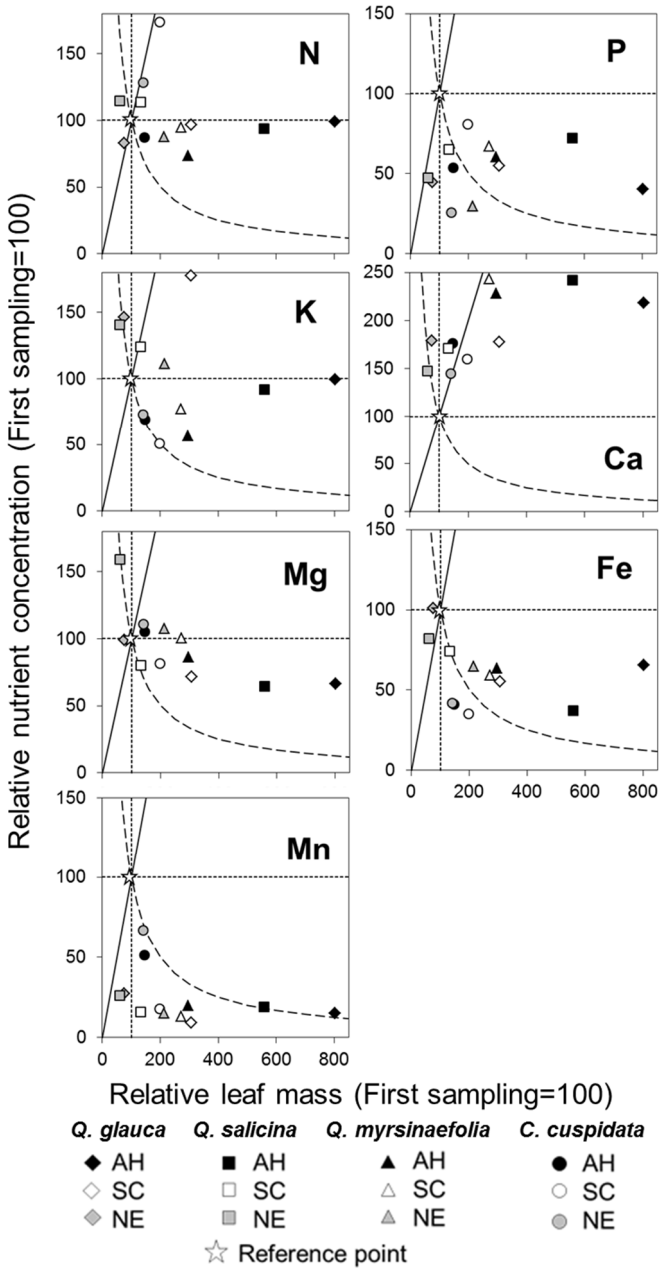


Figure 6. Comparison of relative nutrient concentration in leaves and leaf dry masses for seedlings of four Fagaceae species grown in a calcareous soil. The content isograms represent the combination of dry mass and concentration required for these values at the second sampling relative to the first sampling. A value of 100 represents the dry mass and concentration at the first sampling.

4. Discussion

4.1. Growth and Photosynthesis

After 6 months of cultivation in calcareous soil, we confirmed that symbiosis with ECM fungi was essential for growth acceleration in calcareous soil (Figure 2). Symbiosis with *A. hygrometricus* contributed to growth acceleration in the three *Quercus* species compared with that of *S. citrinum*. In *C. cuspidata*, only *S. citrinum* inoculation showed high values of dry mass of each organ (Figure 3). In general, compatibility with ECM fungi and effects on growth acceleration are different among woody

species [57,58]. Unlike *Quercus* species, *C. cuspidata* may have better compatibility with *S. citrinum*. We have already confirmed that *C. cuspidata* inoculated with *S. citrinum* showed accelerated growth [40].

Moreover, AH seedlings of *Q. glauca* and *Q. salicina* showed the highest values of P_{sat} and g_s (Figure 5). Woody species inoculated with ECM fungi exhibit accelerated water uptake from external hyphae [59]. AH seedlings probably had accelerated water uptake, and stomatal closure did not occur. Accordingly, the P_{sat} and g_s were high in AH seedlings. Similar trends were shown for AH and SC seedlings of *Q. myrsinaefolia*, which had high values for the percentage of ECM colonization (Figures 4 and 5).

Comparing AH seedlings, *Q. salicina* showed remarkable growth acceleration (Figure 2). *Q. salicina* have high acclimation capacity in calcareous soil, and symbiosis with *A. hygrometricus* can take place in calcareous soil. *Q. salicina* grows at high altitudes in the karst region in southwestern Japan [17,18,21]. Our results reflected the distribution of *Q. salicina*.

Compared with *Q. salicina* and *Q. glauca*, growth acceleration by inoculation with ECM fungi was not obvious for *Q. myrsinaefolia* (Figure 2). Acclimation capacity in calcareous soil was lower for *Q. myrsinaefolia* than for *Q. salicina* and *Q. glauca*. In contrast, the total dry mass for *C. cuspidata* was lowest even in SC seedlings (Figure 2). The leaves of *C. cuspidata* yellowed after transplantation, indicating the occurrence of lime-induced chlorosis [3]. In addition, lime-induced chlorosis can readily appear in calcifuge plants [7]. Based on growth and lime-induced chlorosis, *C. cuspidata* was considered as a calcifuge plant. Conversely, *C. cuspidata* inoculated with ECM fungi accelerated its growth in acidic soil [40], indicating that this species has a preference for an acidic environment.

4.2. Nutrient Relationships

For the nutrients in calcareous soil, the concentration of exchangeable Ca was 2.6 $g \cdot kg^{-1}$ (Table 1). The values of soil pH and Ca concentration differ among calcareous soils in Japan [12,13,60–63], with ranges of 5.8 to 8.3 for pH and 0.8 to 12.1 $g \cdot kg^{-1}$ for Ca. Compared with other regions, the soil used for our experiment did not have high soil pH, and did not exhibit an extreme excess of Ca. In contrast, the concentration of N in calcareous soil used in our experiment was lower than those in other regions [60–63]. However, the concentration of N in leaves of the four Fagaceae species was not in the deficient range compared with other woody species grown in calcareous soil [13,63]. For other nutrients, the concentrations of K and Mg were higher than typical forest soil in this region [64]. There was no previous information available on P in calcareous soil in Japan from analyses by the Olsen method. Compared with the data from other countries, the concentration of P was higher in calcareous soil used in our experiment [65,66]. Thus, the concentrations of N, P, K, and Mg in calcareous soil in our experiment were not insufficient.

AH and SC seedlings of the four Fagaceae species did not show high N concentrations in leaves (Table 3). Thus, uptake of N in leaves is not related to ECM colonization. Comparing traits among *Quercus* species, the concentration of N in roots was higher for *Q. salicina* and *Q. glauca* than that for *Q. myrsinaefolia* (Table 4). *Q. salicina* and *Q. glauca* had higher adaptability to various poor habitats [19,67]. *Q. glauca* and *Q. salicina* have high capacity to acquire N from calcareous soil. In contrast, the habitats of natural forests of *Q. myrsinaefolia* include deep, well-drained soil [68]. In the karst region, *Q. myrsinaefolia* has been found on a site with deep calcareous soil [19]. Differences in acclimation capacity for *Q. myrsinaefolia* may originate from the inherent nutrient requirements. There was a similar case for *Picea abies*, which has low adaptability to calcareous soil [69], and suppressed uptake of N in roots increased in calcareous soil [70].

On the uptake of P in roots, NE seedlings showed low P concentrations compared with AH seedlings of *Q. glauca*, *Q. salicina* and *Q. myrsinaefolia* (Table 4). Thus, uptake of P is linked with symbiosis with ECM fungi. On calcareous soil, symbiosis with ECM fungi is important for the uptake of P [27,28]. Symbiosis between *Quercus* species and *A. hygrometricus* produced a high capacity for the acquisition of P.

Meanwhile, we confirmed that the uptake of P, Fe and Mn in leaves was suppressed in each Fagaceae species (Table 3, Figure 6). Uptake of micronutrients (e.g., Fe, Mn, Cu, and Zn) and P is suppressed by low availability at high pH in calcareous soil [3–5,70]. Our results suggested that uptake of Mn is suppressed for the Fagaceae species in calcareous soil. For P and Fe in leaves, AH seedlings of three *Quercus* species exhibited increased contents; however, concentration was decreased and showed evidence of dilution (Figure 6). This trend suggests that the uptake of P and Fe in leaves is suppressed even after inoculation with *A. hygrometricus*. In particular, NE seedlings of *Q. glauca* and *Q. salicina* showed a P deficiency in leaves (Figure 6). We considered that NE seedlings of *Q. glauca* and *Q. salicina* suffered from serious nutrient suppression due to lower availability. *C. cuspidata* showed an obvious decrease of the concentration of Fe in leaves, and showed efficient use of little Fe (Figure 6). When calcifuge woody plants are inoculated with ECM fungi, the effects of the fungi are probably quite limited in calcareous soil.

In contrast, Ca in leaves and roots showed a high value (Tables 3 and 4) compared with seedlings of Fagaceae species grown on acidic colluvial soil [40]. Moreover, many plots showed enhancement of uptake or luxury consumption (Figure 6). However, the actual concentration of Ca in plant organs was almost the same among AH, SC, and NE seedlings for each Fagaceae species (Tables 3 and 4). Thus, we considered that the differences in Ca were not the main factor for growth differences among AH, SC, and NE seedlings.

4.3. Relationships among Variables

In examining the factors affecting differences in growth between AH, SC and NE seedlings, ECM colonization was considered one of the important factors. The percentages of ECM colonization were higher in AH seedlings of the three *Quercus* species and in SC seedlings of *C. cuspidata* (Figure 4). Additionally, there were positive relationships between total dry mass and ECM colonization (Table 2). Thus, different percentages of ECM colonization were probably related to differences in growth.

With respect to the relationships between growth and nutrient uptake, N and P in roots showed enhancement of uptake in AH seedlings of *Q. salicina* (Table 4). The storage of N and P in belowground organs has a significant role in plant growth, and plants with high N and P in roots show high growth rates [71–73]. Based on these results, it appeared that the amount of stored N and P resulted in the high growth of AH seedlings of *Q. salicina*.

The photosynthetic rate showed positive correlations between P_{sat} and the concentrations of P in leaves of *Q. salicina*, *Q. myrsinaefolia*, and *C. cuspidata* (Table 2). In general, the photosynthetic rate is closely related to P concentration [55,56]. Thus, the concentration of P in leaves is considered as an important factor that affects the photosynthetic rate. In contrast, *Q. glauca* did not show a positive correlation between P_{sat} and the concentration of P (Table 2). Other factors that affect differences in P_{sat} include high value of g_s (Figure 5). AH seedlings of *Q. glauca* could absorb water through developed hyphae (Figure 4); as a result, the stomata stay open and absorption of CO_2 through the stomata is also increased.

The concentration of N in leaves showed a negative correlation with P_{sat} for *Q. salicina* and *Q. myrsinaefolia* (Table 2), and our results contradicted the general trend [7,54]. The cause of this negative trend was probably related to the high concentration of N in leaves and the low values of P_{sat} in NE seedlings. In the case of NE seedlings, they had no hyphae of ECM fungi and a lower capacity for water uptake. The stomata of NE seedlings may have closed in order to avoid water stress; as a result, NE seedlings showed lower P_{sat} and g_s and decreased absorption of CO_2 through stomata. Similar trends were shown for NE seedlings of Fagaceae species planted on acidic soil [40].

With respect to the relationship among nutrients for *C. cuspidata* and AH seedlings of *Q. myrsinaefolia*, the concentrations of K were decreased in leaves (Table 3) and were diluted (Figure 6). High Ca content in soil causes ion-antagonism with K [70]. As a result, the suppression of was confirmed for several woody species (*Picea abies* and several Lauraceae species), in which growth was suppressed on calcareous soil [13,70]. Compared with AH seedlings of *Q. glauca* and *Q. salicina*,

growth levels of *C. cuspidata* and AH seedlings of *Q. myrsinaefolia* were inferior. Woody species that show less adaptability to calcareous soil can probably suppress uptake of K.

5. Conclusions

When seedlings of the four Fagaceae species were planted on calcareous soil, the uptake of various nutrients, such as P, Fe, and Mn in leaves was suppressed. However, Fagaceae seedlings inoculated with ECM fungi showed accelerated growth. In particular, Fagaceae seedlings inoculated with ECM fungi had increased concentration of P in roots, and this trait was important for growth acceleration. Additionally, the concentration of P in leaves was an important factor and affected the differences in photosynthetic rate. The most suitable ECM fungi differed among the four Fagaceae species, and higher growth levels were shown from inoculation with *A. hygrometricus* for the three *Quercus* species, and with *S. citrinum* for *C. cuspidata*. Growth levels of the seedlings inoculated with ECM fungi were different among the four Fagaceae species, and *C. cuspidata*, which showed the lowest growth, was considered as a calcifuge plant. From their growth characteristics, we concluded that *Q. salicina* and *Q. glauca* inoculated with *A. hygrometricus* showed the highest growth levels, and these species are most suitable for growth on calcareous soil.

These findings are expected to contribute to new methods for reforestation of limestone quarries in warm-temperature zones in East Asia. In particular, *Q. salicina* and *Q. glauca* inoculated with *A. hygrometricus* may be useful species for reforestation. The effects on growth acceleration without fertilization are advantageous for reforestation in limestone environments. It should be noted that our experiment used sterilized calcareous soil; therefore, further field cultivation experiments with ECM-fungi inoculated plants will be needed.

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References

1. Ford, D.C.; Williams, P.W. *Karst Geomorphology and Hydrology*; Chapman & Hall: London, UK, 1989; p. 620.
2. Haleem, A.A.; Loeppert, R.H.; Anderson, W.B. Role of soil carbonate and iron oxide in iron nutrition of soybean in calcareous soils of Egypt and the United States. In *Iron Nutrition in Soils and Plants*; Abadía, J., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherland, 1995; pp. 307–314.
3. Marschner, H. *Mineral Nutrition of Higher Plants*; Academic Press: New York, NY, USA, 1995; p. 889.
4. Chen, Y.; Barak, P. Iron nutrition of plants in calcareous soils. *Adv. Agron.* **1982**, *35*, 217–240.
5. Tyler, G. Mineral nutrient limitation of calcifuge plants in phosphate sufficient limestone soil. *Ann. Bot.* **1996**, *77*, 649–656. [CrossRef]
6. Jefferies, R.L.; Willis, A.J. Studies on the calcicole-calcifuge habit: II. The influence of calcium on the growth and establishment of four species in soil and sand cultures. *J. Ecol.* **1964**, *52*, 691–707. [CrossRef]
7. Larcher, W. *Physiological Plant Ecology*, 4th ed.; Springer: Berlin, Germany, 2003; p. 513.

8. Zohlen, A.; Tyler, G. Soluble inorganic tissue phosphorus and calcicole-calcifuge behaviour of plants. *Ann. Bot.* **2004**, *94*, 427–432. [CrossRef]
9. Oates, T. Lime and limestone. In *Kirk-Othmer Encyclopedia of Chemical Technology*; Kirk-Othmer, Ed.; John Wiley and Sons: Hoboken, NJ, USA, 2010; pp. 1–53.
10. Ursic, K.A.; Kenkel, N.C.; Larson, D.W. Revegetation dynamics of cliff faces in abandoned limestone quarries. *J. Appl. Ecol.* **1997**, *34*, 289–303. [CrossRef]
11. Duan, W.J.; Ren, H.; Fu, S.L.; Wang, J.; Yang, L.; Zhang, J. Natural recovery of different areas of a deserted quarry in south China. *J. Environ. Sci.* **2008**, *20*, 476–481. [CrossRef]
12. Kayama, M. Growth characteristics of Lauraceae tree species planted on limestone quarry. *Ann. Rep. Kyushu Res. Cen. FFPRI* **2011**, *23*, 16–17. (In Japanese)
13. Kayama, M. Growth characteristics of four Lauraceae tree species planted on calcareous and brown forest soils. *Kyushu J. For. Res.* **2013**, *66*, 63–66. (In Japanese)
14. Kira, T. Forest ecosystems of east and Southeast Asia in a global perspective. *Ecol. Res.* **1991**, *6*, 185–200. [CrossRef]
15. Fang, J.Y.; Song, Y.C.; Liu, H.Y.; Piao, S.L. Vegetation-climate relationship and its application in the division of vegetation zone in China. *Acta Bot. Sin.* **2002**, *44*, 1105–1122.
16. Menitsky, Y.L. *Oaks of Asia*; Science Publishers: Enfield, CT, USA, 2005; p. 549.
17. Yamanaka, T. The limestone vegetation in middle Kyushu. *Res. Rep. Kochi Univ. Nat. Sci.* **1966**, *15*, 1–9. (In Japanese with English summary)
18. Yamanaka, T. The forest and scrub vegetation in limestone areas of Shikoku, Japan. *Vegetatio* **1969**, *19*, 286–307. [CrossRef]
19. Miyawaki, A. *Vegetation of Japan, Chugoku*; Shibundo: Tokyo, Japan, 1983; p. 540. (In Japanese with English summary)
20. Xu, Z.R. A study of the vegetation and floristic affinity of the limestone forest in southern and southwestern China. *Ann. Mo. Bot. Gard.* **1995**, *82*, 570–580.
21. Miyawaki, A. *Vegetation of Japan, Kyushu*; Shibundo: Tokyo, Japan, 1981; p. 484. (In Japanese with English summary)
22. Liu, J.M. The reproductive and regenerative counter measures of the main woody species in Maolan karst forest. *Sci. Silvae Sin.* **2000**, *36*, 115–122. (In Chinese with English summary)
23. Yao, Y.Q.; Zhang, Z.H.; Liang, S.C.; Bi, X.L.; Li, G.R.; Hu, G. Structure of *Cyclobalanopsis glauca* population on karst hills of Guilin. *J. Zhejiang For. Sci. Technol.* **2008**, *28*, 8–11. (In Chinese with English summary)
24. Feroz, S.M.; Hagihara, A. Comparative studies on community ecology of two types of subtropical forest grown in silicate and limestone habitats in the northern part of Okinawa Island, Japan. *Taiwania* **2008**, *53*, 139–149.
25. Newman, E.I.; Reddell, P. The distribution of mycorrhizas among families of vascular plants. *New Phytol.* **1987**, *106*, 745–751. [CrossRef]
26. Wang, B.; Qiu, Y.-L. Phylogenic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* **2006**, *16*, 299–363. [CrossRef]
27. Wallander, H. Uptake of P from apatite by *Pinus sylvestris* seedlings colonised by different ectomycorrhizal fungi. *Plant Soil* **2000**, *218*, 249–256. [CrossRef]
28. Ström, L.; Owen, A.G.; Godbold, D.L.; Jones, D.L. Organic acid behaviour in a calcareous soil: Sorption reactions and biodegradation rates. *Soil Biol. Biochem.* **2001**, *33*, 2125–2133. [CrossRef]
29. Tyler, G. Some ecophysiological and historical approaches to species richness and calcicole/calcifuge behavior—Contribution to a debate. *Folia Geobot.* **2003**, *38*, 419–428. [CrossRef]
30. Van der Heijden, E.W.; Vosatka, M. Mycorrhizal association of *Salix repens* L. communities in succession of dune ecosystems. II. Mycorrhizal dynamics and interactions of ectomycorrhizal and arbuscular mycorrhizal fungi. *Can. J. Bot.* **1999**, *77*, 1833–1841. [CrossRef]
31. Wiemken, V.; Laczko, E.; Ineichen, K.; Boller, T. Effects of elevated carbon dioxide and nitrogen fertilization on mycorrhizal fine roots and the soil microbial community in beech-spruce ecosystems on siliceous and calcareous soil. *Microb. Ecol.* **2001**, *42*, 126–135.
32. Kennedy, K.H.; Maxwell, J.F.; Lumyong, S. Fire and the production of *Astraeus odoratus* (Basidiomycetes) sporocarps in deciduous dipterocarp-oak forests in northern Thailand. *Maejo Int. J. Sci. Technol.* **2012**, *6*, 483–504.

33. Lapeyrie, F. The role of ectomycorrhizal fungi in calcareous soil tolerance by "symbiocalcicole" woody plants. *Ann. Sci. For.* **1990**, *21*, 579–589. [CrossRef]
34. Taste, F.P.; Schmidt, M.G.; Berch, S.M.; Bulmer, C.; Egger, K.N. Effects of ectomycorrhizal inoculants on survival and growth of interior Douglas-fir seedlings on reforestation sites and partially rehabilitated landings. *Can. J. For. Res.* **2004**, *34*, 2074–2088. [CrossRef]
35. Marx, D.H.; Maul, S.B.; Cordell, C.E. Application of specific ectomycorrhizal fungi in world forestry. In *Frontiers in Industrial Mycology*; Leatham, G.F., Ed.; Chapman and Hall: New York, NY, USA, 1992; pp. 78–98.
36. Endo, M. Higher fungi of the Hirao-karst topography. *Res. Bull. Cult. Asset Kitakyushu* **1973**, *13*, 53–56. (In Japanese)
37. Yoshimi, S. Taxonomic study of the Japanese taxa of *Scleroderma* Pers. *Jpn. J. Mycol.* **2002**, *43*, 3–18. (In Japanese with English summary)
38. Fangfuk, W.; Okada, K.; Petchang, R.; To-anun, C.; Fukuda, M.; Yamada, A. In vitro mycorrhization of edible *Astraeus* mushrooms and their morphological characterization. *Mycoscience* **2010**, *51*, 234–241. [CrossRef]
39. Makita, N.; Hirano, Y.; Yamanaka, T.; Yoshimura, K.; Kosugi, Y. Ectomycorrhizal-fungal colonization induces physio-morphological changes in *Quercus serrata* leaves and roots. *J. Plant Nutr. Soil Sci.* **2012**, *17*, 900–906. [CrossRef]
40. Kayama, M.; Yamanaka, T. Growth characteristics of ectomycorrhizal seedlings of *Quercus glauca*, *Quercus salicina*, and *Castanopsis cuspidata* planted on acidic soil. *Trees* **2014**, *28*, 569–583. [CrossRef]
41. Akama, K.; Okabe, H.; Yamanaka, T. Growth of ectomycorrhizal fungi on various culture media. *Bull. FFPRI* **2008**, *7*, 165–181. (In Japanese with English summary)
42. Ohta, A. A new medium for mycelial growth of mycorrhizal fungi. *Trans. Mycol. Soc. Jpn.* **1990**, *31*, 323–334.
43. Oita Prefecture. *Fundamental land classification survey, Usuki and Hotojima*; Fuji Micro-service Center: Kumamoto, Japan, 1980; p. 51. (In Japanese)
44. Van Reeuwijk, L.P. *Procedures for Soil Analysis*, 6th ed.; International Soil Reference and Information Centre: Wagningen, The Netherlands, 2002; p. 100.
45. Sparks, D.L.; Page, A.L.; Helmke, P.A.; Loeppert, R.H.; Soltanpour, P.N.; Tabatabai, M.A.; Johnson, C.T.; Sumner, M.E. *Methods of Soil Analysis, Part 3. Chemical Methods*; Soil Science Society of America Inc.: Madison, WI, USA, 1996; p. 1390.
46. American Public Health Association; American Water Works Association; Water Environment Federation. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association: Washington, DC, USA, 1998; p. 1220.
47. Japan Meteorological Agency. Climate Statistics. Available online: http://www.data.jma.go.jp/obd/stats/etrn/index.php (access on 31 October 2010).
48. Iwasaki, T.; Okuyama, T.; Sakuratani, T. Climatic estimation of photosynthetically active radiation (PAR) in Kanto and Tokai districts. *Bull. Natl. Inst. Agro Environ. Sci.* **1986**, *1*, 1–35. (In Japanese with English summary)
49. Mohan, V.; Natarajan, K.; Ingleby, K. Anatomical studies of ectomycorrhizas. III. The ectomycorrhizas produced by *Rhizopogon luteolus* and *Scleroderma citrinum* on *Pinus patula*. *Mycorrhiza* **1993**, *3*, 51–56. [CrossRef]
50. Quoreshi, A.M.; Timmer, V.R. Exponential fertilization increases nutrient uptake and ectomycorrhizal development of black spruce seedlings. *Can. J. For. Res.* **1998**, *28*, 674–682. [CrossRef]
51. Goto, S. Digestion method. In *Manual of Plant Nutrition*; Editorial Committee of Methods for Experiments in Plant Nutrition. Hakuyusha: Tokyo, Japan, 1990; pp. 125–128. (In Japanese)
52. Valentine, D.W.; Allen, H.L. Foliar responses to fertilization identify nutrient limitation in loblolly pine. *Can. J. For. Res.* **1990**, *20*, 144–151. [CrossRef]
53. Scagel, C.F. Growth and nutrient use of ericaceous plants grown in media amended with sphagnum moss peat or coir dust. *HortScience* **2003**, *38*, 46–54.
54. Evans, J.R. Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* **1989**, *78*, 9–19. [CrossRef]
55. Raaimakers, D.; Boot, R.G.A.; Dijkstra, P.; Pot, S.; Pons, T. Photosynthetic rates in relation to leaf phosphorus content in pioneer versus climax tropical rainforest trees. *Oecologia* **1995**, *102*, 120–125. [CrossRef]

56. Bown, H.E.; Watt, M.S.; Clinton, P.W.; Mason, E.G.; Richardson, B. Partitioning concurrent influences of nitrogen and phosphorus supply on photosynthetic model parameters of *Pinus radiata*. *Tree Physiol.* **2007**, *27*, 335–344. [CrossRef]
57. Parladé, J.; Peta, J.; Alvarez, I.F. Inoculation of containerized *Pseudotsuga menziesii* and *Pinus pinaster* seedlings with spores of five species of ectomycorrhizal fungi. *Mycorrhiza* **1996**, *6*, 237–245. [CrossRef]
58. Chen, Y.L.; Dell, B.; Malajczuk, N. Effects of *Scleroderma* spore density and age on mycorrhiza formation and growth of containerized *Eucalyptus globulus* and *E. urophylla* seedlings. *New For.* **2006**, *31*, 453–467. [CrossRef]
59. Lehto, T.; Zwiazek, J.J. Ectomycorrhizas and water relations of trees: A review. *Mycorrhiza* **2011**, *21*, 71–90. [CrossRef]
60. Morita, S.; Nagata, S. The causes and prevention of the leaf dying of the melon in the calcareous soil. *Jpn. J. Soil Sci. Plant Nutr.* **1995**, *66*, 217–222. (In Japanese with English summary)
61. Terai, A. The effects of organic fertilizer application on limestone-derived soils in Miyakojima Islands, Okinawa. *Ryukoku J. Econ. Stud.* **2008**, *47*, 125–138. (In Japanese)
62. Kayama, M. Concentrations of nutrients for trees grown on limestone region. *Ann. Rep. Kyushu Res. Cen. FFPRI* **2009**, *21*, 9–10. (In Japanese)
63. Kayama, M.; Aoki, N.; Hirayama, D.; Tateno, R.; Kawaji, M.; Yoneda, T. Growth characteristics of *Quercus miyagii* and *Q. glauca* var. *amamiana* seedlings planted on two types of soil. *Kyushu J. For. Res.* **2012**, *65*, 64–67. (In Japanese)
64. Maesako, S.; Yonemaru, S.; Sakai, M. Comparative studies of the physical and chemical properties of surface soils in a sugi plantation and it's two adjacent broad-leaved forest stands. *Bull. Kagoshima Prefect. For. Exp. Stn.* **2002**, *7*, 14–22. (In Japanese)
65. Sardans, J.; Rodà, F.; Peñuelas, J. Phosphorus limitation and competitive capacities of *Pinus halepensis* and *Quercus ilex* subsp. *rotundifolia* on different soils. *Plant Ecol.* **2004**, *174*, 305–317.
66. Pascual, S.; Olarieta, J.R.; Rodoríguez-Ochoa, R. Development of *Queucus ilex* is related to soil phosphorus availability on shallow calcareous soils. *New For.* **2012**, *43*, 805–814. [CrossRef]
67. Ito, S.; Ohtsuka, K.; Yamashita, T. Ecological distribution of seven evergreen *Quercus* species. *Veg. Sci.* **2007**, *24*, 53–63.
68. Miyawaki, A. *Vegetation of Japan, Kanto*; Shibundo: Tokyo, Japan, 1986; p. 641. (In Japanese with English summary)
69. Ewald, J. Ecological background of crown condition, growth and nutritional status of *Picea abies* (L.) Karst. In the Bavarian Alps. *Eur. J. For. Res.* **2005**, *124*, 9–18. [CrossRef]
70. Baier, R.; Ettl, R.; Hahn, C.; Göttlein, A. Early development and nutrition of Norway spruce (*Picea abies* (L.) Karst.) seedlings on different seedbeds in the Bavarian limestone alps—A bioassay. *Ann. For. Sci.* **2006**, *63*, 339–348. [CrossRef]
71. Volenec, J.J.; Ourry, A.; Joern, B.C. A role for nitrogen reserves in forage regrowth and stress tolerance. *Physiol. Plant.* **1996**, *97*, 185–193. [CrossRef]
72. Gloser, V. The consequences of lower nitrogen availability in autumn for internal nitrogen reserves and spring growth of *Calamagrostis epigejos*. *Plant Ecol.* **2005**, *179*, 119–126. [CrossRef]
73. Peri, P.L.; Gargaglione, V.; Pastur, G.M. Above- and belowground nutrients storage and biomass accumulation in marginal *Nothofagus antarctica* forests in southern Patagonia. *For. Ecol. Manag.* **2008**, *255*, 2502–2511. [CrossRef]