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Effects of biochar and litter on water relations of Japanese black pine (*Pinus thunbergii*) seedlings

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Abstract

Biochar is gaining increasing attention in the fields of forest rehabilitation, agriculture, etc. For evaluating biochar application to improve the rhizosphere environment of Japanese black pine (*Pinus thunbergii*) at harsh coastal environments, a pot experiment was carried out with a focus on ectomycorrhizal (ECM) symbiosis and water relations of seedlings. Another characteristic recently observed at coastal forests is the invasion of the locust (*Robinia pseudoacacia*), which can potentially increase soil nitrogen. In this study, four treatments were examined (biochar addition, litter addition, biochar and litter addition, and no addition [control]) to determine the effects of biochar and/or nitrogen-rich locust (*Robinia pseudoacacia*) litter application. Although effects of biochar and litter addition was not observed on ECM symbiosis rate and species composition, treatments with biochar addition, maintained xylem water potential (XWP) of needles for up to two weeks without irrigation, independent of litter addition. As biochar increased relative fine root biomass (fine root biomass/total root biomass), it can be considered that biochar was able to maintain needle XWP through increasing the relative amount of fine roots that can obtain water. Overall, these findings suggest that biochar application can help to maintain water relations of Japanese black pine by enhancing fine root growth.

Keywords: *Robinia pseudoacacia*, Xylem water potential, ectomycorrhizal symbiosis, fine roots

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1 Introduction

Coastal sandy areas represent harsh growing conditions, such as drought conditions, lack of nutrients and high salinity, all of which challenge newly planted seedlings. Field surveys have shown that Japanese black pine (*Pinus thunbergii*, hereafter referred to as black pine) are associated with ectomycorrhizal (ECM) species (Kataoka et al. 2008; Matsuda et al. 2009), which are suggested to play a vital role in improving water and nutrient uptake from the soil (e.g. Smith and Read 1997), thereby enabling survival under harsh costal environments. Although a number of studies have examined the relationship between ECM symbiosis and soil nutrient conditions (e.g. Marschner and Dell 1994; Choi et al. 2005; Zhang et al. 2017), the role in water uptake and effects on water relations is much less clear (Lehto and Zwiazek 2011) . Several studies, by comparing mycorrhizal and non-mycorrhizal seedlings, have showed that ECM symbiosis improves seedling water relations (Dixon et al. 1980; Yin et al. 2018). Morte et al. (2001) reported that leaf water potential was decreased by decreased soil moisture, however, the decrease was higher for non-mycorrhizal than for mycorrhizal *Pinus halepensis* (Aleppo Pine) suggesting mycorrhizal seedlings may be more tolerant to drought stress through enhanced water uptake. Furthermore, not only ECM infection but ECM species composition may also affect seedling performance under water stress conditions (Kipfer et al. 2012).

Biochar, an impure form of elemental carbon with a highly porous structure and generated via incomplete biomass combustion (Preston and Schmidt 2006) , is gaining increasing attention in the fields of forestry, agriculture (Peng et al. 2019), and especially forest rehabilitation (e.g. Lehmann and Joseph 2015). Studies suggest that biochar has beneficial effects on plant growth and mitigates climate change through enhancing carbon sequestration in managed ecosystems (Makoto et al. 2011b; Gale et al. 2017) as well as natural regeneration after forest fires (Thomas and Gale 2015). Moreover, positive effects of biochar on ECM symbiosis and fine root growth have also been reported (Ogawa 2007; Makoto et al. 2011a) via increases in the availability of soluble/insoluble nutrients (e.g., Lehmann et al. 2003; DeLuca and Sala 2006; Makoto et al. 2011b) and improvements to the hydrological properties of sandy soils (Głąb et al. 2016). Biochar could thus work to enhance the rhizosphere environment and ECM symbiosis, potentially improving water relations and nutrient conditions of black pine seedlings at costal environments.

Another factor affecting ECM symbiosis at coastal forests is the presence of the woody legume: black locust (*Robinia pseudoacacia*), which is in some cases planted as a “fertilizer tree” in coastal forests of northern Japan (e.g. Masaka 2013). However, as it can spread rapidly, invasion by the locust is, in some coastal forests, a recent common concern. It was reported by Taniguchi et al. (2007b) that the survival of black pine seedlings growing in Locust dominant sites was significantly reduced by factors such light intensity. Furthermore, *Locust* forms a symbiotic relationship with N-fixing rhizobia (Rice et al. 2004; Marozas et al. 2015), and therefore can increase soil nitrogen (N) through the symbiosis and by shedding high N content litter (Landgraf et al. 2005). Edaphic changes by the presence of black locust trees are subsequently thought to affect ECM infection and ECM species composition (Aučina et al. 2007; Taniguchi et al. 2007a) . Hence, environmental changes caused by Black locust may affect ECM species composition and formation of black pine seedlings, especially at coastal forests.

The objective of this study was to evaluate the effects of biochar on black pine seedlings with and without black locust leaf litter, focusing on plant water relations in order to evaluate biochar use at coastal forests to mitigate drought stress. As fine roots play the vital role in absorbing water and nutrients from the soil, and especially, new fine roots are where ECM colonize (Smith and Read 1997), investigations were also made on ECM symbiosis. We hypothesised that addition of biochar would enhance plant water relations by promoting ECM symbiosis and or changing species dominance and enhancing fine root growth. Moreover, it was also hypothesized that the locust litter could work to decrease ECM symbiosis as it can be expected that soil N will increase.

2 Materials and methods

2.1 Plant material

In September 2013, 40 two-year-old black pine seedlings produced in Niigata Prefecture, central Japan, were planted in 17-L pots (27 cm diameter, 30 cm deep) containing river sand to resemble soil conditions of Niigata coastal forests. Sand conditions were infertile, with only trace levels of N, phosphorus, and potassium. The sand was commercially obtained from DALTEC Co., Ltd. (Sapporo, Japan) to uniform soil conditions so that direct effects of biochar and litter on ECM symbiosis could be observed. The sand had a particle diameter of 0.3–2.0 mm. Refer to Table 1 of Makoto et al. (2011a) for sand properties. The seedlings were grown under natural sunlight (90% transmittance of natural sunlight; daytime photon flux density on a fine day: 1300–1600 μmol m^{-2} s^{-1}; cut wavelength: <330 nm; almost no UV-B or C) inside a greenhouse of the Graduate School of Agriculture, Hokkaido University from September 2013 to October 2014. Seedlings were grown in a greenhouse to avoid further natural ECM contamination. The temperature and humidity inside the greenhouse were maintained at 23°C (±8) and 45% (±7), respectively. Seedlings were watered with tap water once a week until mid-October 2014, when irrigation was stopped for XWP measurements.

2.2 Soil treatment

To determine the individual and combined effects of biochar and litter on water relations and ECM infection of black pine seedlings, four different soil treatments were examined: 1) biochar treatment, 2) litter treatment, 3) biochar + litter treatment (both biochar and litter addition), and 4) no litter or biochar treatment (control). The soil treatments began at the start of the experiment in September 2013.

The biochar which was used in this experiment was produced in the laboratory by incinerating Japanese larch (*Larix kaempferi*) at 400°C from Forest Chemistry Lab. of Hokkaido University via residues of harvested timbers of Sorachi Forest Office of Hokkaido Forest Management Bureau, following the method of Makoto et al. (2011a). The produced biochar was crushed to a uniform size using a 5 mm mesh according to methods of Makoto et al. (2010). In total, 25 g of biochar was added at a depth of approximately 5 cm. The methodology of biochar addition followed results of Makoto et al. (2010), where addition of a layer of charcoal in the middle of the soil profile promoted growth of the root system of the seedlings significantly more than did pots without charcoal or with charcoal scattered throughout the soil. The amount of biochar was set at a volume ratio of 1:4 (biochar: sand), which resulted in approximately 5 cm biochar layer. The pH (H_2O) and density of the biochar was 6.0 ± 0.1 and 0.21 ± 0.02 g mL^{-1}, respectively. Refer to Table 1 of Makoto et al. (2011a) for further chemical properties.

In the litter treatments, 7 g of litter, which is equivalent to approximately 20 mg of N when decomposed, was added. This was based on the information that the average N content of black locust leaves is approximately 20~30 mg g^{-1} (Choi et al. 2017). This value was also assumed, considering effects of leaching. The litter used in this experiment was collected from a black locust-dominated area in Sapporo

Experimental Forest of Hokkaido University (Kanetoshi et al. 2009) in autumn, 2013 right before the experiment started. The collected litter was fallen fresh yellow-brown leaves, however including about 20% in volume of pale green leaves as leaves of black locust hardly changes color to yellow due to symbiotic legume activities. The leaves were not yet decomposed. The N content of the locust litter was determined using a NC analyser (NC-900; Sumica-Shimadzu, Osaka, Japan) as 18.5 ± 0.6 mg g^{-1}. The litter was mixed with the sandy soil and then applied as a top layer of approximately 5 cm thick, which resembled *in-situ* conditions obtained from field survey at the Niigata costal forest. Locust litter was used instead of mix planting black pine and black locust together to avoid complications of competition such as light, direct effects on ECM symbiosis, and so-called "Allelopathy effects", as suggested by Nasir et al. (2005) and Taniguchi et al. (2007).

The initial mean stem base diameter (SBD) ± standard error under biochar treatment, litter treatment, litter + biochar treatment, and no treatment (control) were 4.1 ± 0.1, 4.9 ± 0.3, 4.1 ± 0.1, and 4.4 ± 0.2 mm, respectively.

2.3 Measurements and analyses

2.3.1 XWP of the needles

Xylem water potential of needles was measured to examine the water relations of black pine seedlings. Four randomly selected seedlings from each treatment group were not given water from October 17, 2014, to examine the XWP of the needles. Needles were sampled before dawn at approximately 2-3 day intervals between October 20 and November 3, 2014 (total six measurements). The XWP of the needles were immediately measured using a pressure chamber (Model-3000; Soil Moisture Equipment Corp., Santa Barbara, CA).

2.3.2 ECM identification

ECM identification was done on five seedlings for each treatment (four of which were measured for XWP and dry weight). After harvest, roots were immediately covered with wet paper towels, placed in a plastic bag, and transferred to the laboratory, where they were stored in a refrigerator at 4°C until further analysis. The roots were carefully washed to remove remaining soil clods and further washed with a paintbrush until all soil was removed. A total of 200–300 root tips per seedlings (1200–1500 root tips were observed per treatment) were randomly selected from the top, middle, and bottom of the root system. This method followed the methods of Shinano et al. (2007) and Wang et al. (2015).

ECM taxa were first classified by microscopic observation (Olympus SZX-ILLK100, Japan) based on their morphological characteristics, such as colour, texture, level of ramification, root tip shape and morphology of the emanating hyphae. The observed root tips were then stored in a freezer at – 20℃ until further analysis. Taxonomic classification based on the morphology was verified by DNA analysis using three to five tips with identical morphotypes.

DNA was extracted using ISOPLANT (Nippon Gene Co., Ltd., Japan). Three cycles of centrifugal separation were carried out at 12,000 × *g* (11,441 rpm, 4ºC) for 15, 4 and 1 minute, respectively. The extracted DNA was amplified by polymerase chain reaction (PCR) using a thermal cycler (Applied Biosystems 2720 Thermal Cycler). PCR was carried out using a primer set that reads the internal transcribed spacer (ITS) region of ribosomal DNA with primer ITS1 (Fungal rDNA [ITS1] PCR Kit Fast; Takara Bio Inc., Japan) under following conditions; one cycle at 94ºC for 3 minutes, 25 cycles at 94ºC for 20 seconds → 50ºC for 20 seconds → 72ºC for 5 seconds and one cycle at 7ºC for 7 minutes. The samples were then stored at 8ºC. DNA samples were confirmed by visual classification, using gel electrophoresis with a gel made from 0.3 - 0.6 g of Agarose S (Nippon Gene Co., Ltd., Japan) and 15 - 30 mL of 0.5 × Tris-borate-EDTA buffer and were refined using the Fast Gene® Gel/PCR Extraction Kit (Nippon Gene Co., Ltd., Japan). The base sequence of each refined sample was decoded by a professional service using an ABI 3730xl DNA Analyser. Finally, obtained ECM sequences were compared with the GenBank database at the DNA Nata Bank of Japan using the basic local alignment search tool (BLAST). If only one species had high sequence homology, the sample was identified as that particular species. However, if several species had high sequence homology, the sample was considered an unidentified species from a particular family.

From the obtained DNA identification, the ratio of dominance (P_S) of each species were calculated as follows:

$$P_S = \frac{Q_{ECM}}{Q_{T-ECM}}, \qquad \text{Eq. 1,}$$

where Q_{ECM} is the number of root tips infected by a particular ECM species in a given treatment and Q_{T-ECM} is the total number of root tips infected by ECM fungi in a given treatment.

The total ECM symbiosis (R_E) rate was calculated by the following equation:

$$R_E = \frac{Q_T - Q_N}{Q_T} \times 100, \qquad \text{Eq. 2,}$$

where Q_T is the total number of root tips observed per treatment and Q_N is the total number of root tips not infected with ECM fungi.

2.3.3 N content of the needles and soil

In December 2014, needle and soil samples (taken approximately 5 cm below the surface) were collected from four seedlings, which were measured for XWP and ECM analysis (n=4 for each treatment). The N content (mg g^{-1}) of each sample (n = 4) was then measured using a CN analyser (Vario EL III; Elementar, Ronkonkoma, NY).

2.3.4 Biomass

At the end of the experiment, the dry biomass and SBD were measured in the seedlings which were measured for XWP and ECM fungi identification (n=4). Each plant was sorted into needles, buds, branches, stems, roots, and fine roots (diameter < 2 mm) were separated from coarse roots (diameter > 2 mm). Relative fine root biomass was also calculated by the following equation; fine root biomass/total root biomass × 100. All samples were dried at 70°C for at least 72 h prior to measurement.

2.4 Statistical analysis

The statistical difference between each treatment groups were analysed by one-way ANOVA, if significant, Tukey’s post-hoc test was carried out with a significance level of p <0.05. It was also analysed that the initial seedling size (initial stem base diameter) did not have a significant effect on the final results. The data were assumed to follow a normal distribution. The relationship between needle XWP and the relative fine root biomass was examined using linear regression analysis with a significance level of p < 0.05. All analyses were performed using R (ver. 3.4.3).

3 Results

3.1 XWP of needles

XWP was measured six times at an interval of 2–3 days. Until 10 days after ceasing irrigation, no difference was observed between treatment groups (Fig. 1). However, from 10 days, the biochar treatment and biochar + litter treatment showed a higher XWP value compared to the control and litter treatment. This trend continued till 17 days after stopping irrigation, and the biochar treatment and biochar + litter treatment showed a more gradual decrease than the other two groups.

3.2 ECM identification and species dominance

Wilcoxina mikolae, Amphinema sp., *Russula* sp., *Suillus grandulatus,* and *Rhizopogon roselus* were determined from the DNA identification (Table 1, Table 2). In all four treatments, *Wilcoxina mikolae, Amphinema* sp. and *Russula* sp. were observed. At the biochar treatment, 6 species (including unknown species) were observed, which was the most compared to other treatments. *Suillus grandulatus* was only observed in this treatment and *Rhizopogon roselus* was only observed at the biochar treatment and the biochar + litter treatment.

The ECM symbiosis rate (R_E) for each treatment was as follows; 99.93% in the biochar treatment, 98.50% in the litter treatment group, 99.12% in the biochar + litter treatment group, and 99.01% in the control group (Table 2). Thus, neither litter nor biochar addition influenced R_E. In all treatments, P_S of *Wilcoxina makolae* was the largest value, making it the most dominant species. At the biochar treatment, although not statistically significant, P_S of *Amphinema* sp. was over 20%, meanwhile in the other treatments, it was less than 5%. P_S of *Amphinema* sp. was second highest at the litter treatment. The P_S of other species was all less than 5%.

3.3 Needle and soil N contents

The N content of needles and soil are shown in Table 3. For needle N content, difference between treatments were not detected. On the other hand, for soil N content, it was increased at the biochar + litter treatment compared to the other three treatments. Therefore, although soil N was not increased at the litter treatment, it was increased at the biochar + litter treatment.

3.4 Growth and dry weight

Results of growth and, above- and below-ground dry weight are shown in Table 4 and 5. Concerning the final stem base diameter, the smallest value was the biochar + litter treatment, and the other three treatments showed similar results. A similar trend was also observed for final height.

Needle and branch dry weight were not affected by litter or biochar addition. For bud dry weight, the litter treatment showed the highest value, and the biochar + litter treatment showed the smallest value. Biochar addition did not have any effect on above-ground organ and dry weight. For stem dry weight, the biochar + litter treatment showed the smallest value of all treatments. Concerning roots, biochar addition had a significant increasing effect on fine root dry weight and also relative fine root (fine root dry weight/total root dry weight). However, when both biochar and litter were added, neither fine root dry weight and relative fine root were not increased. Furthermore, although from personal observation, the increase in fine roots were especially observed at the middle part of the pot (5~8 cm from the surface), relatively close to where the biochar was added.

As biochar addition increased fine root dry weight, consequently, the above-/below-ground (fine + coarse root biomass) ratio showed the smallest value at the biochar treatment, however, the value was highest at the biochar + litter treatment (Table 5).

4 Discussion

The addition of biochar significantly improved water relations of black pine seedlings, where XWP of the needles were maintained for over 10 days regardless of litter addition (Fig. 1). However, in contrast with our hypothesis, addition of biochar and litter had no effect on the rate of ECM symbiosis (R_E) and species abundance (Table 2). That is, high rates of R_E were observed in all treatment groups, including those with litter addition.

It was previously revealed that addition of N caused a decrease in both R_E and the number of ECM species (Wallenda and Kottke 1998). In other studies, it has also been revealed that addition of litter had a greater effect on the relative proportion of ECM symbionts than on species diversity (Aučina et al. 2007). Furthermore, a decrease in R_E was previously observed in a pine stand rich in soil N due to the addition of locust litter (Taniguchi et al. 2007a). However, in the latter study, 0.76 mg N g^{-1} was detected at a depth of

0–5 cm, approximately three- to four-fold more than that observed under our litter treatment (Table 3, approximately 0.20 mg N g^{-1}). We initially calculated that 20 mg N g^{-1} would be added to each pot through litter addition, however, this value assumed that all litter were decomposed, which may not have been the case since this study ran for only one growing season and the given litter was fresh yellow-brown leaves including pale green leaves collected right before the experiment. It is therefore possible that the amount of litter added in our experiment was not sufficient to affect ECM fungal composition and diversity. Furthermore, this may be due to the immobilization of nutrients by microorganism increased by the addition of fresh leaf litter (undecomposed leaf litter). Litter addition did not decrease fine root dry weight, although a decrease in fine roots (or increase in fine root turnover) is often reported with increasing soil N as the necessity of roots decrease with the increase of relative nutrients, which also indicates that the added N amount was relatively small. Nevertheless, it can be assumed that short-term litter accumulation at a soil depth of 5 cm does not negatively affect R_E. However, effects of biochar and litter combined are still not clear as in bud and stem dry weight, and final stem diameter and height showed the smallest value at the biochar + litter treatment. No clear explanation could be made on this result, and further analyses must be made on the combination effect of biochar and litter.

W. mikolae (Ascomycetes) was the dominant ECM species in all four treatment groups (Table 2). This taxon is a generalist that is able to grow under a wide range of environmental conditions and often forms symbiotic relationships with coniferous species (e.g. Baar et al. 1999; Ashkannejhad and Horton 2006). At the initial stage of our experiment, morphological identification also identified *W. mikolae* as the dominant species, suggesting that neither biochar nor litter addition affected species composition.

Although no effects of biochar and litter were observed on ECM symbiosis, significant effects were observed on needle XWP (Fig. 1). The XWP of the seedlings under biochar treatment was consistently high even without watering for 15 days, and independent of litter addition. Similar effects of biochar on leaf water potential were observed under drought conditions in pear (*Pyrus ussuriensis* Maxim.) (Lyu et al. 2016), with a high XWP observed up until 11 days of drought. Meanwhile, the results of our study suggest that this positive effect may last for up to 17 days. Concerning results of fine roots, both fine root dry weight and relative fine root was increased by biochar addition. This trend is supported by previous findings in which biochar enhanced root growth, particularly that of fine roots (e.g. Makoto et al. 2011a). Biochar may have enhanced the soil's aeration property and induced fine root growth as roots of black pine is reported to have a high demand in oxygen and prefer non-excessive moisture conditions (Karizumi 2012). Additionally, since fine roots function to absorb water from the soil, it can be considered that biochar addition was able to maintain needle XWP after ceasing irrigation as seedlings with biochar had relatively more fine roots to obtain water. Accordingly, a significant correlation was observed between relative fine root biomass and XWP at 17 days after stopping irrigation; seedlings with a high relative fine root biomass also showing a high XWP (Fig. 2). These findings suggest that the addition of biochar improves plant water relations by enhancing fine root growth (Prendergast-Miller et al. 2014).

Overall, findings of this study suggest that biochar has positive effects on water relations in black pine seedlings as determined by observations of needle XWP and enhanced fine root growth. As the added amount lead to increase in fine root biomass, it may also be the suggested amount for field application. However, effects of biochar in combination with locust litter amount may need further investigations. Furthermore, long-term and field studies may also be further required to further understand the combined effects of locust litter (and the tree itself) and biochar.

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Declaration on interest

No potential conflict of interest was declared by the authors.

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