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Rhizosphere effects on soil extracellular enzymatic activity and microbial abundance during the low-temperature dormant season in a northern hardwood forest

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(Declaration of interest)

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30

Abstract

Plant roots alter nutrient cycling, including nitrogen (N) and phosphorus (P) cycling, within the soil surrounding them (rhizosphere) by affecting microbes and enzyme activities. Recent studies have focused on nutrient uptake by plants in low-temperature seasons. This study aimed to reveal the nutrient dynamics in the rhizosphere during low-temperature seasons in a northern hardwood forest in Japan. For this purpose, the potential extracellular enzymatic activity, bacterial, fungal, and archaeal abundances, and soil chemical properties in the rhizosphere of canopy trees and understory vegetation and non-rhizosphere bulk soil were measured at the beginning of the dormant season (November), end of the dormant season (April and May), and middle of the growing season (August) in a northern hardwood forest in Japan. The abundance of fungi was 1.1 to 1.7 times higher in the rhizosphere than in non-rhizosphere bulk soil regardless of the season. The activity of enzymes involved in N- and P-cycles in the rhizospheres was also 1.4 to 4.0 and 1.3 to 1.9 times higher than that in bulk soil, respectively. The concentration of extractable organic N was 1.5 to 2.0 times higher in the rhizosphere than in the non-rhizosphere bulk soil at the beginning and end of the dormant season, respectively, but this trend was not observed in the middle of the growing season for organic N. Since the concentration of nutrients in the rhizosphere is determined by the balance between nutrient uptake by fine roots and root-induced acceleration of decomposition, our results suggest that plant roots would accelerate N and P cycles during the dormant season, even though the amount of nutrient uptake by plants was lower during the season.

Keywords

Rhizosphere effect, Extracellular enzymes, Plant dormant season, Canopy tree, Understory vegetation

1. Introduction

Nutrient availability regulates the primary production of forests and, consequently, ecosystem C sequestration. In temperate forests, N often limits primary production (LeBauer and Treseder 2008). Phosphorus is also a key element, especially in volcanic ash soil such as Andosols (Shoji and Takahashi 2002). Most nutrients are stored in complex organic materials, which plants cannot directly utilize in forest soils (Bormann et al. 1977; Borie and Rubio 2003). Instead, nutrients are transformed by microbial decomposition and mineralization into plant-available forms. Microbes, such as bacteria and fungi, produce diverse extracellular enzymes involved in organic matter decomposition and mineralization (Rodríguez and Fraga 1999; Talbot et al. 2008; Nikitina et al. 2010) and are the main drivers of nutrient cycling in forest soils (Isobe and Ohte 2014).

In the soil surrounding plant fine roots (rhizosphere), the processes of organic matter decomposition and mineralization are accelerated (Cheng et al. 2003; Phillips and Fahey 2006; Dijkstra et al. 2009; Bengtson et al. 2012; Yin et al. 2014). Plants continuously release labile organic compounds called root exudates from their fine roots into the rhizosphere. Since root exudates are the sources of energy and substrates for microbial activity, the activity and population of microbes is higher in the rhizosphere than in the non-rhizosphere bulk soil (Kuzyakov and Razavi 2019; Wang et al. 2019). Therefore, the activity of extracellular enzymes produced by microbes is higher in the rhizosphere (Kuzyakov and Razavi 2019). This alteration of microbial communities, enzyme activity, and, consequently, nutrient cycling within the rhizosphere is called the rhizosphere effect (RE). Although the rhizosphere comprises a limited area of soil, the C and N mineralization accelerated by the RE accounts for one-third of the total surface soil C and N mineralization in temperate forest soils (Finzi et al. 2015). Root exudates, which constitute photosynthetically produced organic C, are among the most important factors for the RE (Shahzad et al. 2015); therefore, most studies on REs have focused on the plant growing season (Huo et al. 2017), when photosynthetic activity is high. These

understandings concerning RE were based on the results in growing season, however, there is a lack of knowledge on the RE in dormant seasons, especially in forest soils.

The long-lasting winter in northern hardwood forests is considered a dormant season for plants. However, studies have shown that tree species, including deciduous trees, take up nutrients from soil even in winter (Andresen and Michelsen 2005; Ueda et al. 2015; Zavišić and Polle 2018). Furthermore, although photosynthetic activity is relatively low in winter (Voříšková et al. 2014), root exudates are released in winter by evergreen tree species (Phillips et al. 2008). Deciduous trees also release a certain amount of root exudates after leaf fall, at least at the beginning of the dormant season (Nakayama and Tateno 2018). Using ^{13}C labeling, Fahey et al. (2013) also reported that young maple trees released approximately 7.5% of the total belowground C allocation as rhizosphere carbon flux during the dormant season. Studies have also reported that microbial communities are active at sub-zero temperatures (Clein and Schimel 1995; Isobe et al. 2018), and organic matter decomposition and mineralization have been observed even under snow cover (Clein and Schimel 1995; Hishi et al. 2014; Isobe et al. 2018). Therefore, during plant dormant seasons, plants could stimulate microbial growth and extracellular enzyme production so as to take up nutrients efficiently from their rhizosphere—the nutrients taken up in winter are used for spring growth (Ueda et al. 2011). Thus, revealing the rhizosphere processes in winter is needed to better understand soil nutrient cycles and plant growth in forests.

In the northern hardwood forests of Japan, the forest floor is covered by a dense understory vegetation of evergreen dwarf bamboo (*Sasa* spp.). The aboveground biomass of dwarf bamboo is lower than that of canopy trees (Fukuzawa et al. 2015), while the fine root biomass of dwarf bamboo is comparable to that of canopy trees (Fukuzawa et al. 2007, 2021; Tateno et al. 2020). Meta-analyses showed that the magnitude of the RE was higher for tree species than for herbaceous species (Huo et al. 2017; Gan et al. 2021b). Only a few studies have directly compared REs of canopy trees and

understory vegetation in forest ecosystems; however, Yuan et al. (2020) reported that understory herbs had a magnitude of RE comparable with tree species in a subtropical forest. Therefore, the contribution of understory vegetation, as well as that of canopy trees, should be considered with respect to the RE on the nutrient cycle. In this study site, the understory vegetation consist of *Sasa nipponica* (Makino) Makino & Shibata. Several culms and leaves of *S. nipponica* survive winter and have higher photosynthetic activity in early spring (Kayama and Koike 2018). This is partly because the leafless season of canopy trees provides more favorable light conditions for understory vegetation as compared to summer (Kudo et al. 2008). Thus, *S. nipponica* has photosynthetic advantages during early winter and early spring, in which canopy trees have no leaves. Considering that a large part of root exudates is recently assimilated C (Kuzyakov and Cheng 2001; Epron et al. 2011; Sanaullah et al. 2012), the RE of dwarf bamboo would be higher and more significant than that of canopy trees at the beginning and end of the canopy tree dormant season. However, as we described above, previous studies on the RE have mainly focused on the growing season.

Here, we aim to reveal how plants in a northern hardwood forest affect rhizosphere processes at the beginning and end of the dormant season, which is the leafless season for canopy trees. For this purpose, we measured microbial abundance and extracellular enzyme activity involved in the nutrient cycle in the rhizospheres of canopy trees and understory vegetation as well as in the non-rhizosphere bulk soils. For the canopy tree *Quercus crispula* Blume, leaf-fall usually occurs in late October, and leaf flush occurs in late May to early June (Nakayama and Tateno 2018; Tateno et al. 2019). In this study, we defined June to October as the growing season and November to May as the dormant season. Specifically, we defined November and late April to early May as the beginning and end of the dormant season, respectively. The soil is covered by snow from mid-November to April, and the soil is frozen from December to May (Hosokawa et al. 2017); therefore, we did not collect samples from the period when the soil was frozen during the mid-dormant season. Thus, we collected samples at the beginning

96 and end of the dormant season and mid-growing season. We hypothesized that (1) both *Q. crispula*
97 and *S. nipponica* have a positive RE on enzymatic activity and microbial abundance at the beginning
98 and end of the dormant season as well as during the mid-growing season because plants would release
99 root exudates even during the dormant season. (2) The magnitude of the RE is higher for *S. nipponica*
100 than for *Q. crispula* at the beginning and end of the dormant season but lower during the mid-growing
101 season because *S. nipponica* has advantages for photosynthetic activity in these seasons.

2. Materials and Methods

2.1 Study site

This study was conducted in a cool-temperate deciduous forest in eastern Hokkaido, Japan (43°24.2' N, 144°38.5' E), managed by the Shibecha Branch of the Hokkaido Forest Research Station, Field Science Education and Research Center, Kyoto University. The soils at this site are Andosols (IUSS Working Group WRB 2015) with a clay-loam to loamy texture. The mean annual air temperature and precipitation (1986–2015) at a meteorological station located approximately 9 km south of the study site were 6.3 °C and 1,189 mm, respectively. The maximum annual snow depth at the study site was approximately 70 cm (Hosokawa et al. 2017). At 0 and 5 cm depth, the soil temperature was approximately zero degrees under snow cover (Isobe et al. 2018; Watanabe et al. 2019) but the soil was frozen throughout the winter (Watanabe et al. 2019). The mean maximum soil freezing depth under 70 cm snow was 9.7 cm (Watanabe et al. 2019). Seasonality of the soil water content, chemical properties, and microbial biomass C and N in this study site can be found elsewhere (Isobe et al. 2018, Watanabe et al. 2019). The daily average air temperatures and daily precipitation measured at the meteorological station during the experiment are shown in Fig. 1.

Sampling was conducted in the four plots (15 m × 15 m) established in our previous study (Nakayama and Tateno 2018). In the plots, the canopy tree was *Q. crispula*, and the forest floor was densely covered with dwarf bamboo (*S. nipponica*). The plots were separated from each other by at least 30 m. The fine root density of *Q. crispula* at a depth of 0–10 cm in the plots was 132.9 ± 47.8 g m⁻² (Nakayama and Tateno 2018), and that of *S. nipponica* was 88.0 ± 55.9 g m⁻². The soil bulk density (0–10 cm) and the dry weight of organic horizon were 0.38 ± 0.08 Mg m⁻³ and 1.19 ± 0.08 kg m⁻², respectively (Nakayama and Tateno 2021). More detailed information on the study plots can be found in previous reports (Nakayama and Tateno 2018, 2021).

The soil temperature at 5 cm soil depth in the plots was measured once every 30 min during

126 the experiment using a temperature sensor with a data logger (TR-52i; T & D Corporation, Nagano,
127 Japan). The sensor was removed on November 26, 2019 and April 28 and May 22, 2020, for data
128 collection and a battery change and reburied on the same day. The daily average soil temperatures in
129 the study plots are shown in Fig. 1.

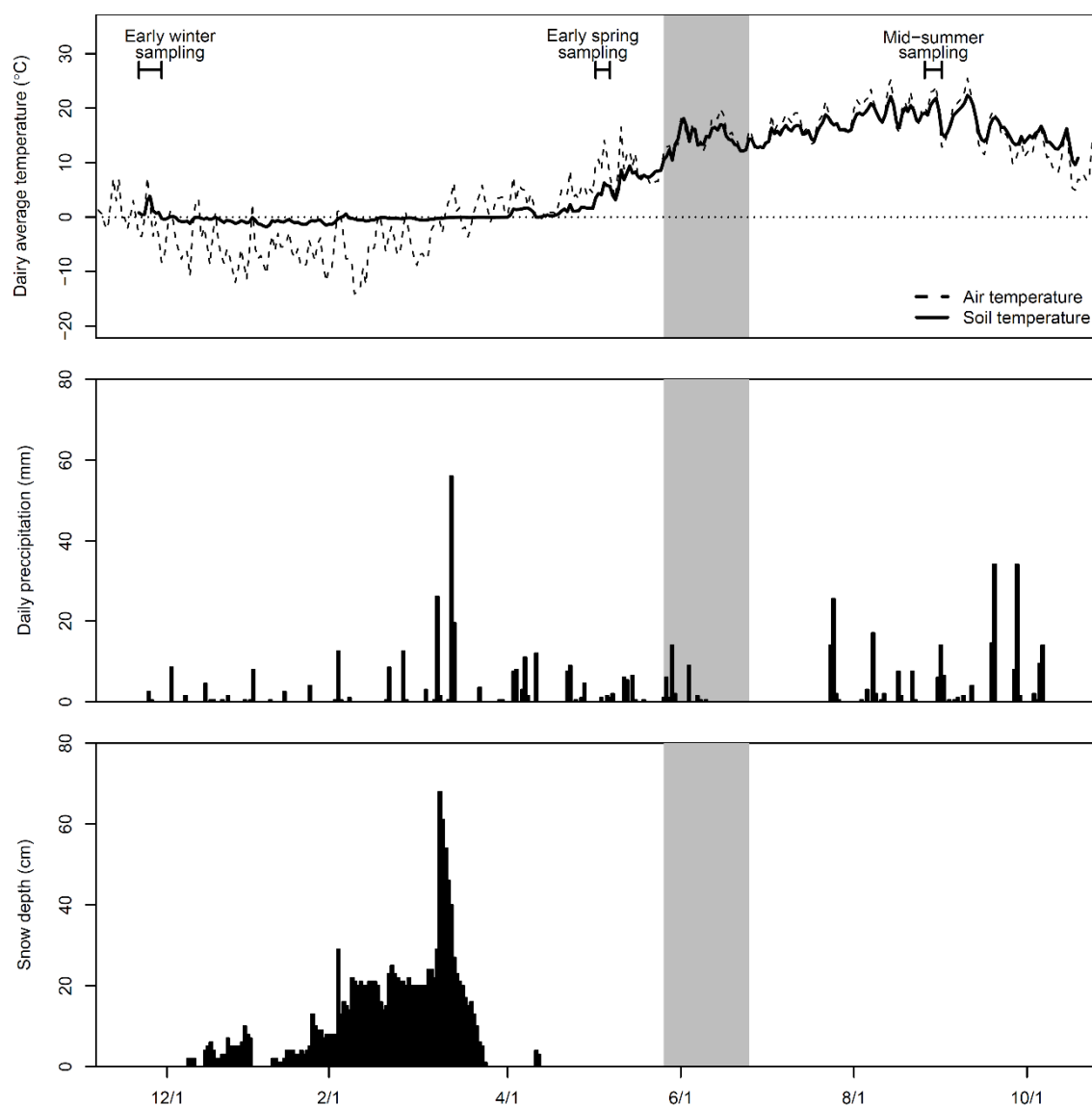


Fig. 1 Seasonal fluctuations in the daily averages of soil (5 cm-depth) and air temperatures, daily precipitation, and snow depth during the experiment. Soil temperature was measured at the study plots, and air temperature, daily precipitation, and snow depth were measured at a meteorological station 9 km from the study site. The shaded area represents the approximate period from the beginning of leaf flushing to full expansion in *Q. crispula* (data was shown in Tateno et al. 2019)

2.2 Soil sampling and treatment

Mineral soils at a 0–10 cm-depth (A horizon) were collected using a shovel from each plot after removing the organic horizon by hand. Soil sampling was conducted for 4 days during each sampling season, November 21, 23, 27, and 29, 2019 (beginning of the dormant season; hereafter, early winter), April 29, May 1, 3, and 5 (end of the dormant season; hereafter, early spring), August 23, 25, 28, and 30, 2020 (mid-growing season; hereafter, mid-summer). Soil samples were placed on ice in the field and refrigerated at 4 °C until processing.

In the laboratory, soil samples were put on a 4 mm mesh sieve. The fine roots on the sieve were carefully picked up by hand and forceps and separated into three types, *Q. crispula* and *S. nipponica* roots and others, including dead roots and roots of other species, based on their morphological traits (e.g., mycorrhizal type, color, and branching pattern). The ‘other’ roots constituted a small proportion and were discarded. Next, the fine roots of *Q. crispula* and *S. nipponica* were gently shaken, after which the soil adhering to their roots was collected as the rhizosphere (Phillips and Fahey 2006; Wang et al., 2019) and considered as the canopy tree rhizosphere and understory rhizosphere, respectively. Soil that did not attach to the root and passed the 4 mm mesh sieve was considered non-rhizosphere bulk soil. Thus, we obtained three types of samples (bulk soil, tree rhizosphere, and understory rhizosphere) from one field sampled soil block. We note that although this method of separation is commonly used in the field study of rhizospheres (Badaluco and Nannipieri 2007), and the adhesive soil would be strongly affected by the attached fine roots, the tree and understory roots may partly affect the bulk soil and other rhizospheres.

After sieving and separation, the samples were divided into wet, oven-dry, and frozen subsamples. Oven-dried subsamples were dried at 60 °C for more than 72 h. Wet and frozen subsamples were stored at 4 and -20 °C, respectively, until further processing.

2.3 Soil chemical analysis

To measure the gravimetric water content of the samples, the oven-dried subsamples were weighed before and after oven-drying. Total N and C contents of oven-dried samples were then measured using an elemental analyzer (Sumigraph NC-900; Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Next, 2 g of dried soil was extracted in 5 mL of deionized water, and then the pH of the extracts was measured using a pH meter (HORIBA D-51; Horiba, Ltd., Kyoto, Japan).

A portion of the frozen subsample was extracted in 2 M KCl (extracted from 1 g of the wet weight of the frozen subsample into 5 mL of 2 M KCl) to measure the concentration of total extractable N (TEN), NO_3^- -N, NH_4^+ -N, and extractable organic N (EON). We note that there is a possibility that the concentrations of N would differ from that in fresh soils because the frozen sample was used for the extraction. The concentrations of NO_3^- -N and NH_4^+ -N were colorimetrically measured using the Griess assay and indophenol blue method (Miranda et al. 2001), using a microplate reader (Synergy HXT; BioTek, Winooski, VT, USA), at wavelengths of 540 and 636 nm, respectively. To measure the concentration of TEN, 1 mL of 2 M KCl extracts was mixed with 1 mL of deionized water and 200 μL of alkaline potassium persulfate solution and then autoclaved (121 °C, 20 min). Thereafter, the TEN concentration was measured as NO_3^- -N concentration using the aforementioned method. The EON concentration was calculated by subtracting the sum of inorganic N (NO_3^- -N and NH_4^+ -N) from TEN.

2.4 Extracellular enzymatic activity measurements

The enzyme assays were conducted within 24 h of sampling using wet subsamples. The activities of six extracellular enzymes (Table A.1) involved in C, N, and P cycles were measured following the method described by Saiya-Cork et al. (2002) with a few modifications. For the hydrolytic enzyme assays, for β -xylosidase, β -glucosidase, acid phosphatase, and β -1,4-N-acetylglucosaminidase (NAGase), 1.0 g of the wet soil sample was suspended in 100 mL of 50 mM sodium acetate (pH 5.0).

The suspensions were mixed well using a magnetic stirrer for 1 min. Subsequently, 800 μ L of aliquot was dispensed into sample wells (four replicate wells per sample per enzyme) and quenching standard wells of 96-well deep-well plates. For the quenching standard wells, 200 μ L of 4-methylumbelliferone (MUB) was added (concentrations: 0, 1, 2, 4, 10, 20, 40, and 100 μ M). In the negative control well, 800 μ L of acetate buffer was added. Subsequently, 200 μ L of a 200 μ M-substrate was added to the sample wells, after which the deep-well plates were incubated at 20 $^{\circ}$ C in the dark for 1 h. After incubation, the deep-well plates were centrifuged for 3 min at max speed ($600 \times g$) (Bell et al. 2013). Thereafter, 250 μ L supernatant was transferred into a 96-well black microplate; then the fluorescence was measured using a microplate reader (Synergy HXT, BioTek). The wavelengths of excitation and emission were 360 and 460 nm, respectively.

For the oxidative enzyme assays, for phenol oxidase and peroxidase, 800 μ L of soil suspensions and 200 μ L of 5 mM L-3,4-dihydroxyphenylalanine (DOPA) as the substrate were added to sample wells. Negative control wells contained 800 μ L of acetate buffer and 200 μ L of DOPA, and blank control wells contained 800 μ L of soil suspensions and 200 μ L of acetate buffer. For the peroxidase assay, 40 μ L of 0.3% H_2O_2 was further added to each well. The deep-well plates were incubated in the dark at 20 $^{\circ}$ C for 10 h. The deep-well plates were then centrifuged for 3 min at max speed, and 250 μ L of the supernatants were transferred into a flat-bottom clear microplate. Enzyme activity was quantified by measuring absorbance at 450 nm using the same microplate reader.

2.5 DNA extraction and quantification of microbial gene abundance

Soil DNA was extracted from 0.25 g wet weight of frozen soil subsamples using a DNA extraction kit (DNeasy PowerSoil Pro Kit; QIAGEN, Hilden, Germany), following the manufacturer's protocol. The extracted DNA solution was stored at -20 $^{\circ}$ C until further analysis.

Microbial gene abundance quantification by real-time quantitative polymerase chain

reaction (qPCR) was performed using a LightCycler 96 System (Roche Diagnostics K.K., Mannheim, Germany), with the intercalating dye SYBR Green I (FastStart Essential DNA Green Master; Roche Diagnostics K.K.). The bacterial and archaeal 16S rRNA genes and fungal internal transcribed spacers (ITS) regions of the rRNA genes were quantified to estimate total abundances of bacteria, archaea, and fungi, respectively. In addition, we estimated the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) by quantifying the bacterial and archaeal ammonia monooxygenase gene (*amoA*), respectively. The subsequent steps and primer set used for qPCR have been described in Nakayama et al. (2021).

2.6 Statistical analyses

Each value for enzyme activity and microbial gene abundances was calculated based on soil dry weight and total C content. To test the difference in soil chemical properties, enzymatic activity, and microbial gene abundance among sampling seasons (early winter, early spring, and mid-summer) and sample positions (bulk soil, tree rhizosphere, and understory rhizosphere), a two-way analysis of variance (ANOVA) with random effects (random effects: sampling date and plots) was used. We conducted multiple comparisons using the *emmeans* function in R with Tukey's adjusted *P*-value to test the difference in soil chemical properties, enzymatic activity, and microbial gene abundance. The magnitude of the RE was calculated as the percentage difference between the paired rhizosphere and bulk soil sample for each variable (based on soil dry weight). To determine whether the magnitude of the RE significantly differed from zero, a *t*-test was used. The differences in the RE magnitude among sampling seasons and between sampling positions (tree rhizosphere vs. understory rhizosphere) were tested using a two-way ANOVA followed by multiple comparisons as described above. All statistical analyses were conducted in R ver. 4.0.5 (R Core Team 2021).

3. Results

3.1 Soil chemical properties

Regardless of the sampling season, the pH of rhizospheres was significantly lower than that of bulk soil. Although the total C and N concentrations were significantly higher in the rhizospheres, the C/N ratio did not vary among seasons and sampling positions (Table 1). The EON concentrations in the rhizospheres, especially in the tree rhizosphere, were significantly higher than that in bulk soil during the dormant season; however, this trend was not observed in mid-summer (Table 1). The $\text{NH}_4^+\text{-N}$ concentration was significantly higher in rhizospheres than in bulk soil regardless of the season, and during early winter and mid-summer, the concentration in the tree rhizosphere was higher than that in the understory rhizosphere (Table 1). The soil $\text{NO}_3^-\text{-N}$ concentration in the understory rhizosphere was significantly higher than that in the tree rhizosphere and bulk soil during early winter and mid-summer (Table 1). The difference in $\text{NO}_3^-\text{-N}$ concentration between the tree rhizosphere and bulk soil was not significant (Table 1). The effect of sampling season on soil chemical properties was not significant except for EON.

3.2 Extracellular-enzyme activity

Although sampling season had a marginally significant effect on the NAGase and peroxidase activity, there was no significant effect of season on enzyme activity (Fig. A. 1 and Table A. 2). The activity of β -glucosidase and NAGase in the tree rhizosphere was significantly higher than that in the understory rhizosphere and bulk soil, but there was no significant difference in the activities between the understory rhizosphere and the bulk soil (Fig. A. 1). The interaction between sampling season and position was significant for acid phosphatase and phenol oxidase (Table A. 2). Therefore, further analyses on the activity of acid phosphatase and phenol oxidase were conducted after considering sampling season and position separately. Sampling position significantly affected acid phosphatase

and phenol oxidase activity regardless of sampling season except for phenol oxidase during early winter (one-way ANOVA with random effects: acid phosphatase, $F = 19.24$ and $P < 0.001$ for early winter; $F = 18.71$ and $P < 0.001$ for early spring; $F = 34.62$ and $P < 0.001$ for mid-summer and phenol oxidase, $F = 1.22$ and $P = 0.307$ for early winter; $F = 8.12$ and $P < 0.01$ for early spring; $F = 18.92$ and $P < 0.001$ for mid-summer). The acid phosphatase activity in the tree rhizosphere was higher than that in bulk soil regardless of the sampling season and was higher than that in the understory rhizosphere during early spring and mid-summer. Furthermore, the acid phosphatase activity in the understory rhizosphere was higher than that in bulk soil during early winter and early spring. The phenol oxidase activity in the tree rhizosphere was higher than that in the bulk soil and understory rhizosphere during early spring and mid-summer, but there was no significant difference in the activities between bulk soil and understory rhizosphere regardless of the sampling season. Sampling season only significantly affected acid phosphatase activity in bulk soil (one-way ANOVA with random effects: $F = 6.10$, $P < 0.05$), and the acid phosphatase activity in the tree rhizosphere during mid-summer was higher than that during early winter. The activity of β -xylosidase and peroxidase did not significantly vary among sampling seasons and positions (Fig. A. 1 and Table A. 2).

The REs on extracellular enzyme activity varied among enzymes and between the tree rhizosphere and understory rhizosphere (Fig 2). The REs were significantly positive on acid phosphatase and NAGase activity regardless of the differences in sampling season and plant species (Fig. 2). The magnitude of the RE tended to be higher for the tree rhizosphere than for understory rhizosphere regardless of the enzymes and seasons except for β -xylosidase and peroxidase during early spring (Fig. 2). However, the difference between the tree rhizosphere and understory rhizosphere was significant only for NAGase (two-way ANOVA with random effects: $F = 4.02$, $P < 0.05$). The difference in the RE magnitude between the tree rhizosphere and understory rhizosphere was marginally significant for phenol oxidase (two-way ANOVA with random effects: $F = 2.48$, $P = 0.091$).

Tukey's HSD test showed that the RE on phenol oxidase in the tree rhizosphere was significantly higher during mid-summer than during early winter and early spring. The effect of sampling season on the RE on acid phosphatase was marginally significant (two-way ANOVA with random effects: $F = 3.50$, $P = 0.065$).

The total soil C-based enzyme activities are shown in Fig. A. 2. Unlike the soil weight-based analysis, the activity of β -glucosidase was not varied among soil positions (Fig. A. 2). The total soil C-based activity of NAGase in the tree rhizosphere was significantly higher than that in bulk soil during the dormant seasons, but the difference was only marginally significant during the dormant season (Fig. A. 2). The difference in the total soil C-based NAGase activity between the tree and understory rhizospheres and between the understory rhizosphere and bulk soil was significant only during early winter (Fig. A. 2). The total soil C-based activity of acid phosphatase varied among sampling seasons and soil positions. It was significantly higher in the tree rhizosphere than in the understory rhizosphere, and in the understory rhizosphere than in bulk soil, and higher during mid-summer than during early winter and spring (Fig. A. 2).

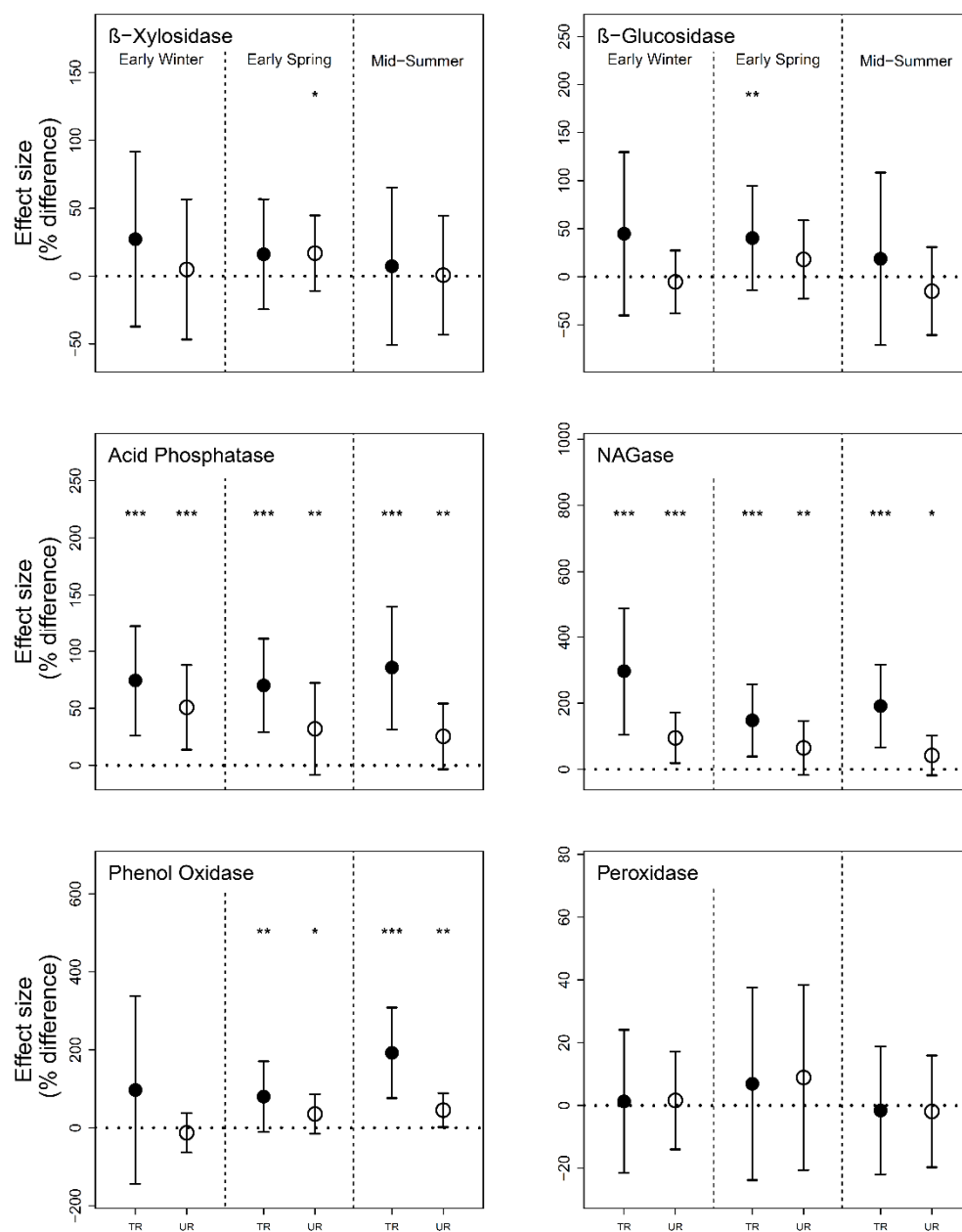


Fig. 2 Rhizosphere effects on the activity of β -xylosidase, β -glucosidase, acid phosphatase, NAGase, phenol oxidase, and peroxidase. The filled and open circles represent the rhizosphere effect for the canopy tree rhizosphere (TR) and understory rhizosphere (UR), respectively. The circles and error bars represent means ($n = 16$) and standard deviation (SD). The symbols (*) indicate that the effect significantly differs from zero. The symbols are as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3.3 Microbial gene abundance

There was no clear difference in bacterial and archaeal 16S rRNA gene abundance among seasons and positions (Fig. A. 3 and Table A. 3). In contrast, fungal ITS gene abundance was significantly higher during mid-summer than during early winter and early spring, and there was a non-significant difference in the fungal gene abundance between early winter and early spring (Fig. A. 3). The fungal ITS gene was also significantly more abundant in the tree rhizosphere and understory rhizosphere than in bulk soil regardless of the season (Fig. A. 3). There was a non-significant difference in fungal ITS gene abundance between the tree rhizosphere and understory rhizosphere.

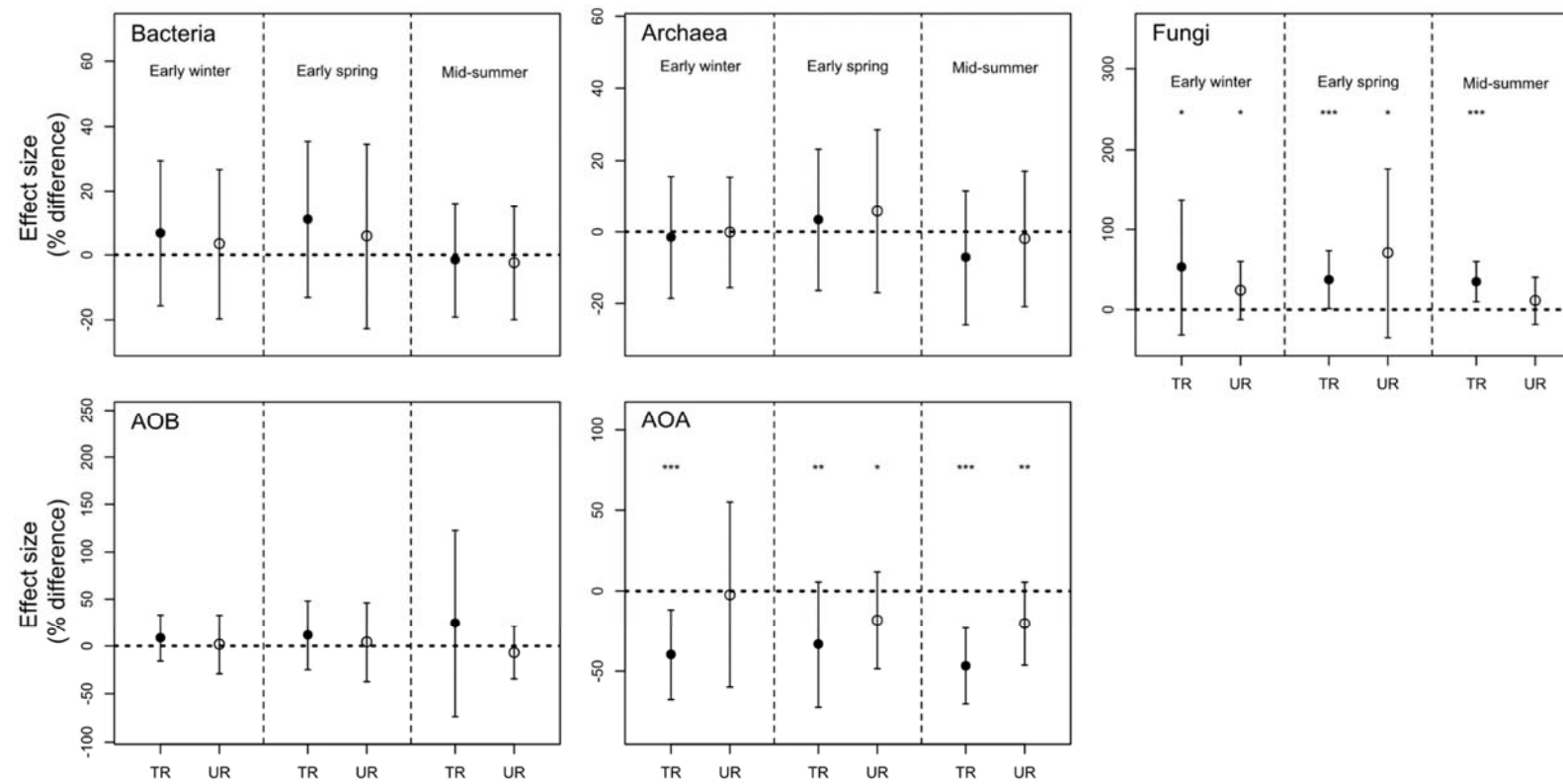
The magnitude of REs on the abundance of bacterial and archaeal 16S rRNA genes did not differ significantly from zero regardless of the season and position, except for the positive and marginally significant REs on the bacterial 16S rRNA gene observed during early spring in the tree rhizosphere (Fig. 3). The REs on fungal ITS gene abundance were significant and positive regardless of the position and season except for the understory rhizosphere during mid-summer (Fig. 3). The magnitude of REs on bacterial and archaeal abundance had no clear trend among seasons and positions (Fig. 3). The magnitude of the RE on fungal ITS in the understory rhizosphere was significantly higher than that in the tree rhizosphere only during early spring (one-way ANOVA with random effects: $F = 4.23$, $P < 0.05$), and the magnitude during early spring was significantly higher than that during mid-summer only in the tree rhizosphere (one-way ANOVA with random effects: $F = 3.92$, $P < 0.05$).

Although the bacterial *amoA* gene abundance did not vary among seasons and positions (Fig. A. 3 and Table A. 3), the archaeal *amoA* gene abundance was significantly lower in the tree rhizosphere than in bulk soil (Fig. A. 3 and Table A. 3). There was a non-significant difference in the abundance of the archaeal *amoA* gene between the tree and understory rhizospheres and between the understory rhizosphere and bulk soil (Fig. A. 3).

The magnitude of the RE on the bacterial *amoA* gene abundance did not differ from zero

regardless of the season and position (Fig. 3). In contrast, significant and negative REs on the archaeal *amoA* gene abundance were observed regardless of the season and position except for the understory rhizosphere during early winter (Fig. 3). Furthermore, the RE on the archaeal *amoA* gene abundance tended to be more negative for the tree rhizosphere than for the understory rhizosphere regardless of the season; however, the difference in the gene abundance between the tree rhizosphere and understory rhizosphere was not significant (two-way ANOVA with random effects: $F = 0.76$, $P = 0.385$).

The total soil C-based microbial abundances are shown in Fig. A. 4. Unlike the soil weight-based analysis, the fungal ITS abundance did not vary significantly among soil positions (Fig. A. 4). Fungal ITS had significantly higher abundance during mid-summer than during the dormant seasons (Fig. A. 4), similar to the soil weight-based fungal abundance. The soil C-based abundances of bacterial and archaeal 16S in both tree and understory rhizospheres were significantly lower than that in bulk soil (Fig. A. 4). The soil C-based bacterial *amoA* gene abundance was significantly lower in the understory rhizosphere than in bulk soil (Fig. A. 4). The soil C-based archaeal *amoA* gene abundance in the tree rhizosphere was significantly lower than that in the understory rhizosphere and bulk soil (Fig. A. 4).



336 **Fig. 3** Rhizosphere effects on the abundance of bacterial 16S rRNA, archaeal 16S rRNA, fungal ITS region, bacterial *amoA* gene, and archaeal *amoA* gene.

337 The filled and open circles represent the rhizosphere effect of the canopy tree and understory vegetation, respectively. The circles and error bars represent

338 means ($n = 16$) and standard deviation (SD). The symbols (*) indicate that the effect significantly differs from zero. The symbols are as follows: * $P <$

339 0.05, ** $P < 0.01$, and *** $P < 0.001$.

4. Discussion

Recently, the importance of nutrient cycling processes during plant dormant seasons has been highlighted (Isobe et al. 2018). However, the RE in the plant dormant season remains unclear. The present study demonstrated a significant positive and negative RE on enzymatic activity and microbial gene abundance during plant dormant seasons (Fig. 2). These findings highlight the importance of winter nutrient cycling processes surrounding the fine roots in forest ecosystems, although there are limitations; the method for separating the rhizosphere limit standardization of the results (Badalucco and Nannipieri 2007), and the study investigated only one dormant and growing season.

4.1 Seasonal differences in rhizosphere effects on enzymatic activity and nutrient cycles

Our results did not contradict the hypothesis (1); that is, both *Q. crispula* and *S. nipponica* have positive RE on the enzymatic activity at the beginning and end of the dormant season as well as during the mid-growing season (Fig. 2). The activities of enzymes involving N- and P- cycling were higher in the rhizosphere even after analyzing the total soil C-basis (Fig. A. 2). Previous studies have reported that plants can release a certain amount of labile C from their roots even during the dormant season in the forest ecosystems (Phillips et al. 2008; Fahey et al. 2013; Nakayama and Tatenos 2018). Labile root exuded organic C is one of the main drivers of REs on enzymatic activities (Meier et al. 2017; Wang et al. 2019). Thus, the proportions of labile C would be higher in the rhizosphere regardless of seasons because of root exudation, which might stimulate enzymatic production within the rhizospheres. However, there is still a lack of knowledge about root exudation, particularly its extent in winter; therefore, further studies focused on root exudates during the dormant season are needed to reveal the RE mechanisms.

In this study, we measured the potential activities of enzymes. Schimel et al. (2017) reported

that the potential activities of enzymes, especially NAGase and acid phosphatase, could persist for several months partly as a result of the binding with minerals. Further, the potential activities could largely differ from the actual *in-situ* activities because of the mobility of substrates (Schimel et al. 2017) and lower temperature (Baldrian et al. 2013). Therefore, there was a possibility that such enzymes were produced during the growing season and the potential but not actual activities of the enzymes persisted overall during the dormant season. However, significant microbial activities and nutrient cycling were observed under the low-temperature condition (Freppaz et al. 2007; Isobe et al. 2018). Though research using metatranscriptome and *in-situ* measurement of decomposition and mineralization is needed for a clear understanding of the mechanisms of REs on enzyme activities, the differences in the potential activity of enzymes could imply that the rates of nutrient cycling processes were stimulated within the rhizosphere in the dormant season compared to the bulk soil.

The results of N concentrations might support that at least the N cycle would be accelerated within the rhizosphere. In the plant dormant season, the concentration of EON within rhizospheres was higher than that in non-rhizosphere bulk soils, although this trend was not observed in the mid-growing season (Table 1). The amount of plant-available N in the soil is determined by the balance between uptake and root-primed decomposition and mineralization (Nakayama and Tateno 2021). Plants and mycorrhiza can take up organic and inorganic N (Schimel and Bennett 2004; Liese et al. 2018; Zhang et al. 2019). Ueda et al. (2015) demonstrated that plants, including *Q. crispula*, took up a certain amount of inorganic N during mid-winter in sub-zero temperatures in the northern hardwood forest. Although it is unknown whether deciduous plant species can take up low-molecular-weight organic N during the dormant season, Zhang et al. (2019) reported that both the roots of evergreen conifers and their symbiotic mycorrhiza took up organic N during the dormant season as well as during the growing season. In addition, the organic and inorganic N uptake by herbaceous species during the dormant season has also been reported in a temperate coastal heath (Andresen and Michelsen 2005).

Thus, it is possible that plants may have utilized EON and inorganic N during the dormant season in the present study. However, the amount of N taken up by plants during the dormant season should be lower than that taken up in the mid-growing season in the present study, as shown for evergreen tree species (Zhang et al. 2019). Therefore, at the beginning and end of the plant dormant season, the lower EON uptake and positive RE on N decomposition could lead to higher EON concentration within the rhizospheres. During the mid-growing season, the higher potential activity of NAGase in the rhizosphere suggested that N cycling would be stimulated by RE similar to that during the dormant season. However, a not higher concentration of EON in the rhizospheres during the growing season could be a result of the higher nutrient uptake activity by plant roots during the growing season.

During the plant dormant season, especially at its beginning and middle, decomposed N is retained by the microbial communities (Isobe et al. 2018). In snowy northern hardwood forests, the freeze-thaw event during the snowmelt season causes microbial cell disruption, releasing EON and reducing microbial abundance (Isobe et al. 2018). At the same time, the released organic matter stimulates microbial growth (Watanabe et al. 2019). In the current study, the magnitude of the RE on bacterial and archaeal abundances at the beginning of the dormant season was more positive than that during the mid-growing season (Fig. 3), although the magnitudes were not significantly different from zero. The abundances of 16S rRNA do not directly represent the microbial biomass, but the abundances of bacterial 16S rRNA and total bacterial phospholipid fatty acids (PLFA), which represent the biomass of bacteria, were correlated significantly and positively (Zhang et al. 2017). Thus, excess bioavailable N transformed by root-induced decomposition and mineralization may be retained by microbes but not by the plants, at least at the beginning and end of the plant dormant season. Further research using ^{15}N -tracers should reveal the fate of the excess N transformed by root-induced decomposition and mineralization during the plant dormant season.

4.2 Seasonal differences in rhizosphere effects on microbial abundance

It is well known that microbial abundance within the rhizosphere is higher than that in bulk soil (Kuzyakov and Razavi 2019). Our results were partly consistent with previous findings, that is, only fungal abundance in the rhizospheres was higher than that in bulk soil—abundances of bacteria and archaea did not differ between the rhizosphere and bulk soils (Fig. 3). In general, the fungal community in the rhizosphere is characterized by mycorrhizal fungi (Baldrian 2017), and their abundance decreases with distance from the rhizoplane (Kuzyakov and Razavi 2019). Mycorrhizal fungi are still active and are affected by tree C supply in winter (Kaiser et al. 2010, 2011). Therefore, the observed higher fungal abundance may be explained by a higher abundance of mycorrhizal fungi within the rhizospheres during the plant dormant season. There is another contrasting explanation for this result. Žifčáková et al. (2017) reported that EcM activity and abundance were much lower in the bulk soil in winter than in summer, as observed in this study site (Nakayama et al. 2021), and the relative contribution of non-mycorrhizal fungi to extracellular enzyme production was higher during winter. Therefore, the higher abundance of fungi in the rhizosphere could come from the higher abundance and activity of non-mycorrhizal fungi under reduced mycorrhizal abundance (Gadgil and Gadgil 1971; Frey 2019). Additionally, the total C was higher in the rhizospheres regardless of the season (Table 1), and the abundance of fungi did not vary among soil positions based on the total soil C-based analysis (Fig. A. 4). Therefore, in the rhizospheres, the availability of C substrate would be higher than that in bulk soil regardless of the season. The third possible hypothesis was that the higher saprotrophic fungal abundance because of higher C availability explains the higher total fungal abundance than the seasonal difference in mycorrhizal activity. To test these contrasting hypotheses, further studies focusing on the fungal communities within the rhizosphere during the plant dormant season are needed.

Contrary to our results, bacteria and archaea are typically much more abundant in the rhizosphere than in bulk soil (Kuzyakov and Razavi 2019). However, in the rhizosphere, some bacteria, such as pathogenic bacteria, are suppressed by root exudates (Bais et al. 2006). The significantly lower

abundance of AOB and AOA within the rhizosphere (Fig. 3) also suggested the suppression of a part of bacterial and archaeal communities. One possible explanation for these results is that the magnitudes of stimulation and suppression of bacterial and archaeal growth within the rhizospheres were similar. Alternatively, the limited photosynthetic activity, especially the lack of photosynthesis by canopy trees, would also explain the bacterial and archaeal population results within the rhizosphere at the beginning and end of the dormant season. Wang et al. (2019) reported that the amount of root-exuded C positively correlated with the RE on microbial biomass. Although the release of root exudates from tree species in winter or at least at the beginning of the dormant season has been observed in some studies (Phillips et al. 2008; Nakayama and Tatenno 2018), the amount of root-exuded C at the beginning and end of the dormant season should be lower because a majority of root exudates are recently assimilated organic C (Epron et al. 2011; Sanaullah et al. 2012; Nakayama and Tatenno 2018). However, there is limited knowledge on root exudation in winter; therefore, further research on root exudates and microbial community structures within the rhizosphere in the winter dormant season is needed to reveal the relationships among plant roots, root exudates, and rhizosphere microbes. Unlike total bacterial and archaeal abundance, the population of AOA was suppressed within the rhizosphere seasonally (Fig. 3). Studies have shown that plants have some functions that inhibit nitrification steps and growth of microbes responsible for nitrification partly to prevent N loss from ecosystems (reviewed in Moreau et al. 2019). Therefore, plants possibly suppressed the nitrification processes driven by AOA at the beginning and end of the plant dormant season as well as during the mid-growing season.

4.3 Differences in the RE between canopy trees and understory vegetation

The positive and negative REs on NAGase and AOA were stronger for the canopy tree *Q. crispula* than for the understory vegetation *S. nipponica* during the growing season (Figs. 2 and 3). This may

be explained by the advantage of photosynthetic activity in canopy trees relative to understory vegetation during the growing season (Kuzyakov and Cheng 2001, 2004). However, in contrast to our second hypothesis, the magnitude of the RE was higher for *Q. crispula* than for *S. nipponica* during the dormant as well as growing seasons (Figs. 2 and 3). At the beginning and end of the plant dormant season, *Q. crispula* had no leaves, whereas *S. nipponica* showed photosynthetic activity (Kayama and Koike 2018; Tateno et al. 2019). Thus, factors other than the advantage of photosynthesis would affect the difference in the magnitude of the RE between *Q. crispula* and *S. nipponica*.

One possible factor that may result in the RE differences between the canopy tree and understory vegetation irrespective of seasons is mycorrhiza type. Huo et al. (2017) reported that trees tended to have higher REs than herbaceous species in a meta-analysis using RE data obtained during the growing season. They surmised that the difference between trees and herbaceous species is a result of differences in mycorrhiza, i.e., EcM (trees) vs. AM (herbaceous species). In general, EcM fungi have a greater capacity for producing extracellular enzymes than AM fungi (Frey 2019), and the enzymatic production activity of symbiotic mycorrhizal fungi are also important factors for the RE on enzymatic activity (Phillips and Fahey 2006). Therefore, the difference in mycorrhizal type could explain the higher RE in the tree rhizosphere on the enzymatic activity during leafless seasons.

The negative rhizosphere effect on the abundance of AOB and AOA was stronger in the tree rhizosphere than in the understory rhizosphere (Fig. 3). EcM symbiotic species suppress nitrification more than AM symbiotic plants (Phillips et al. 2013; Tatsumi et al. 2020). Most mineralized N at the present study site undergoes nitrification processes in laboratory incubation without living roots and mycorrhiza (Nakayama et al. 2021). Thus, our results of nitrifiers and inorganic N concentrations (Fig. 3 and Table 1) suggest that *Q. crispula* had a stronger nitrification suppression effect than *S. nipponica*. Plants take up each of the low-molecular-weight organic N, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$ (Jones et al. 2005; Liese et al. 2018); however, there is a source preference for plant N uptake (Nordin et al. 2001).

Ectomycorrhizal symbiotic trees prefer organic N and $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ compared with AM symbiotic species (Phillips et al. 2013; Liese et al. 2018). Tateno et al. (2020) reported that *Q. crispula* preferred $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ and *S. nipponica* utilized both types of inorganic N at the present study site. Therefore, our results implied that canopy tree species (EcM symbiotic plant) suppressed the AOA population more strongly and, consequently, nitrification processes within the rhizosphere than those in the understory vegetation (AM symbiotic plant), regardless of the season.

Another possible explanation for the difference in REs between *Q. crispula* and *S. nipponica* was their root traits rather than the symbiotic mycorrhiza. Recent studies have reported that the magnitudes of REs varied with respect to the plant traits rather than the symbiotic mycorrhizal types (Chen et al. 2018; Han et al. 2020; Gan et al. 2021a). For example, Cheng et al. (2018) reported that the differences in REs between EcM and AM were not remarkable because there were significant interspecific variations even within the same mycorrhizal types (Cheng et al. 2018). Han et al. (2020) reported that plants traits, such as root C concentration and specific root length, were significantly correlated to the magnitude of the RE by investigating broadleaved trees in the forest in the continental temperate monsoon climate. Although a recent meta-analysis showed that mycorrhizal types affected the RE on soil gross N mineralization (Gan et al. 2021b), the difference in REs between *Q. crispula* and *S. nipponica* would come from the variations of root or other traits of these two species. In the present study, there is a limitation that only one AM and EcM symbiotic plant species each was investigated; thus, studies using diverse plant species are needed to clarify the difference in the RE between AM and EcM symbiotic plants, especially during the dormant seasons.

5. Conclusions

In the present study, we demonstrated that plants in the northern hardwood forests accelerated nutrient cycling within the rhizosphere during the non-growing season. Although recent studies have shown

that the nutrients taken up by plants in winter are important for subsequent spring growth, our results also suggest that nutrient uptake by plants and the abundance of their root symbiotic mycorrhiza was less during the non-growing season compared with the growing season. Therefore, our results implied that both canopy trees and understory vegetation hasten nutrient cycling processes, including decomposition and mineralization at the beginning and end of the dormant season. However, a large part of the nutrients transformed by root-induced decomposition would not be taken up by plants and was possibly retained in the microbes. These retained nutrients would be released later during the subsequent growing season and utilized for plant growth.

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714 Table 1 Chemical properties of soils.

	pH	C (%)	N (%)	C/N ratio	Extractable nitrogen (mg-N kg ⁻¹ dry soil)			
					EON	NH ₄ ⁺ -N	NO ₃ ⁻ -N	
Early winter								
Bulk soil	5.2 ± 0.3 ^a	11.0 ± 2.2 ^c	0.76 ± 0.16 ^c	14.5 ± 0.6	67.8 ± 17.9 ^c	10.0 ± 8.7 ^b	1.0 ± 2.3 ^b	
Tree rhizosphere	4.7 ± 0.2 ^b	14.2 ± 1.5 ^a	0.96 ± 0.12 ^a	14.8 ± 0.7	130.4 ± 42.6 ^a	18.7 ± 7.7 ^a	1.6 ± 1.6 ^b	
Understory rhizosphere	4.8 ± 0.3 ^b	12.1 ± 1.8 ^b	0.84 ± 0.15 ^b	14.5 ± 0.7	105.3 ± 24.9 ^b	14.4 ± 8.1 ^{ab}	4.7 ± 4.9 ^a	
Early spring								
Bulk soil	5.1 ± 0.2 ^a	11.7 ± 1.4 ^b	0.79 ± 0.10 ^b	14.7 ± 0.9	68.6 ± 20.1 ^b	13.8 ± 11.8 ^b	2.0 ± 2.8	
Tree rhizosphere	4.7 ± 0.2 ^b	14.3 ± 1.2 ^a	0.97 ± 0.10 ^a	14.8 ± 0.9	114.8 ± 19.4 ^a	25.7 ± 14.8 ^a	2.8 ± 3.5	
Understory rhizosphere	4.8 ± 0.2 ^b	13.3 ± 1.6 ^a	0.90 ± 0.11 ^a	14.8 ± 0.8	96.5 ± 31.7 ^a	27.9 ± 17.0 ^a	3.5 ± 4.3	
Mid-summer								
Bulk soil	5.0 ± 0.2 ^a	10.2 ± 1.9 ^c	0.70 ± 0.14 ^c	14.6 ± 0.6	63.5 ± 12.8	5.8 ± 4.7 ^c	0.2 ± 0.5 ^b	
Tree rhizosphere	4.5 ± 0.2 ^b	14.0 ± 2.0 ^a	0.96 ± 0.15 ^a	14.6 ± 0.7	77.9 ± 30.0	17.6 ± 8.9 ^a	0.4 ± 0.5 ^b	
Understory rhizosphere	4.6 ± 0.2 ^b	11.9 ± 1.8 ^b	0.82 ± 0.14 ^b	14.5 ± 0.6	78.7 ± 15.6	12.5 ± 6.6 ^b	2.2 ± 3.2 ^a	

715 Note: EON represents extractable organic nitrogen. The values are mean ± standard deviations. Different lowercase letters indicate a significant
716 pairwise difference between soil positions at each sampling season.

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