1 (Title)

2 Rhizosphere effects on soil extracellular enzymatic activity and microbial abundance during the low-

3 temperature dormant season in a northern hardwood forest

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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

24 1. Introduction

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

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

48 understandings concerning RE were based on the results in growing season, however, there is a lack
49 of knowledge on the RE in dormant seasons, especially in forest soils.
50 The long-lasting winter in northern hardwood forests is considered a dormant season for
51 plants. However, studies have shown that tree species, including deciduous trees, take up nutrients
52 from soil even in winter (Andresen and Michelsen 2005; Ueda et al. 2015; Zavišić and Polle 2018).
53 Furthermore, although photosynthetic activity is relatively low in winter (Vořiškova et al. 2014), root
54 exudates are released in winter by evergreen tree species (Phillips et al. 2008). Deciduous trees also
55 release a certain amount of root exudates after leaf fall, at least at the beginning of the dormant season
56 (Nakayama and Tateno 2018). Using ^{13}C labeling, Fahey et al. (2013) also reported that young maple
57 trees released approximately 7.5% of the total belowground C allocation as rhizosphere carbon flux
58 during the dormant season. Studies have also reported that microbial communities are active at sub-
59 zero temperatures (Clein and Schimel 1995; Isobe et al. 2018), and organic matter decomposition and
60 mineralization have been observed even under snow cover (Clein and Schimel 1995; Hishi et al. 2014;
61 Isobe et al. 2018). Therefore, during plant dormant seasons, plants could stimulate microbial growth
62 and extracellular enzyme production so as to take up nutrients efficiently from their rhizosphere—the
63 nutrients taken up in winter are used for spring growth (Ueda et al. 2011). Thus, revealing the
64 rhizosphere processes in winter is needed to better understand soil nutrient cycles and plant growth in
65 forests.
66 In the northern hardwood forests of Japan, the forest floor is covered by a dense understory
67 vegetation of evergreen dwarf bamboo (*Sasa* spp.). The aboveground biomass of dwarf bamboo is
68 lower than that of canopy trees (Fukuzawa et al. 2015), while the fine root biomass of dwarf bamboo
69 is comparable to that of canopy trees (Fukuzawa et al. 2007, 2021; Tateno et al. 2020). Meta-analyses
70 showed that the magnitude of the RE was higher for tree species than for herbaceous species (Huo et
71 al. 2017; Gan et al. 2021b). Only a few studies have directly compared REs of canopy trees and

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

85 Here, we aim to reveal how plants in a northern hardwood forest affect rhizosphere processes
86 at the beginning and end of the dormant season, which is the leafless season for canopy trees. For this
87 purpose, we measured microbial abundance and extracellular enzyme activity involved in the nutrient
88 cycle in the rhizospheres of canopy trees and understory vegetation as well as in the non-rhizosphere
89 bulk soils. For the canopy tree *Quercus crispula* Blume, leaf-fall usually occurs in late October, and
90 leaf flush occurs in late May to early June (Nakayama and Tateno 2018; Tateno et al. 2019). In this
91 study, we defined June to October as the growing season and November to May as the dormant season.
92 Specifically, we defined November and late April to early May as the beginning and end of the dormant
93 season, respectively. The soil is covered by snow from mid-November to April, and the soil is frozen
94 from December to May (Hosokawa et al. 2017); therefore, we did not collect samples from the period
95 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.

102 2. Materials and Methods

103 2.1 Study site

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

117 Sampling was conducted in the four plots (15 m × 15 m) established in our previous study
118 (Nakayama and Tateno 2018). In the plots, the canopy tree was *Q. crispula*, and the forest floor was
119 densely covered with dwarf bamboo (*S. nipponica*). The plots were separated from each other by at
120 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
121 m^{-2} (Nakayama and Tateno 2018), and that of *S. nipponica* was 88.0 ± 55.9 g m^{-2}. The soil bulk density
122 (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},
123 respectively (Nakayama and Tateno 2021). More detailed information on the study plots can be found
124 in previous reports (Nakayama and Tateno 2018, 2021).

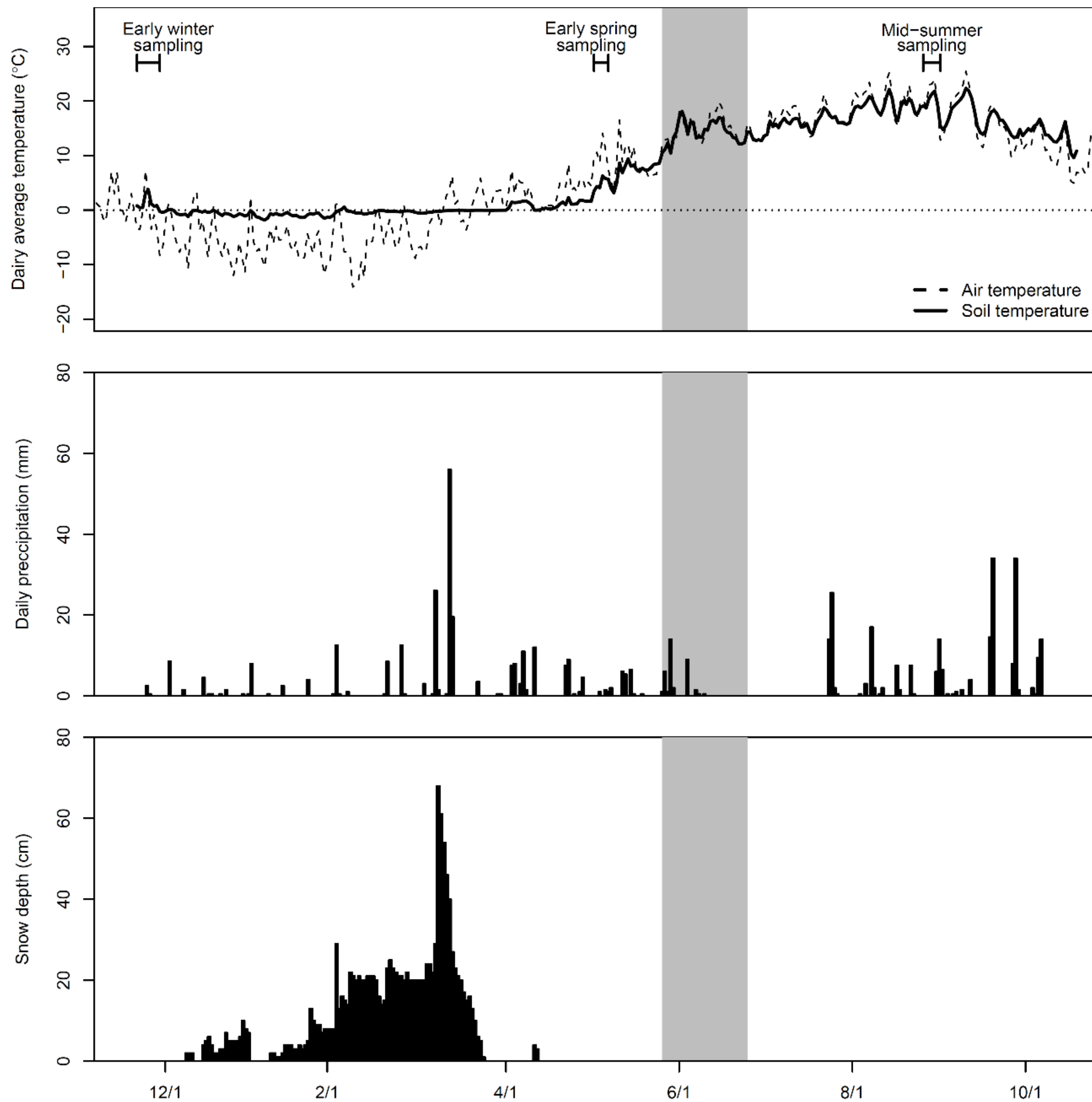
125 The soil temperature at 5 cm soil depth in the plots was measured once every 30 min during

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.



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

135

136 2.2 Soil sampling and treatment

137 Mineral soils at a 0–10 cm-depth (A horizon) were collected using a shovel from each plot after
138 removing the organic horizon by hand. Soil sampling was conducted for 4 days during each sampling
139 season, November 21, 23, 27, and 29, 2019 (beginning of the dormant season; hereafter, early winter),
140 April 29, May 1, 3, and 5 (end of the dormant season; hereafter, early spring), August 23, 25, 28, and
141 30, 2020 (mid-growing season; hereafter, mid-summer). Soil samples were placed on ice in the field
142 and refrigerated at 4 °C until processing.

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

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

159 2.3 Soil chemical analysis

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

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

176 2.4 Extracellular enzymatic activity measurements

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

182 The suspensions were mixed well using a magnetic stirrer for 1 min. Subsequently, 800 μL of aliquot
183 was dispensed into sample wells (four replicate wells per sample per enzyme) and quenching standard
184 wells of 96-well deep-well plates. For the quenching standard wells, 200 μL of 4-methylumbelliferone
185 (MUB) was added (concentrations: 0, 1, 2, 4, 10, 20, 40, and 100 μM). In the negative control well,
186 800 μL of acetate buffer was added. Subsequently, 200 μL of a 200 μM-substrate was added to the
187 sample wells, after which the deep-well plates were incubated at 20 °C in the dark for 1 h. After
188 incubation, the deep-well plates were centrifuged for 3 min at max speed (600 × g) (Bell et al. 2013).
189 Thereafter, 250 μL supernatant was transferred into a 96-well black microplate; then the fluorescence
190 was measured using a microplate reader (Synergy HXT, BioTek). The wavelengths of excitation and
191 emission were 360 and 460 nm, respectively.

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

200 2.5 DNA extraction and quantification of microbial gene abundance

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

204 Microbial gene abundance quantification by real-time quantitative polymerase chain

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

213 2.6 Statistical analyses

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

227

228 3. Results

229 3.1 Soil chemical properties

230 Regardless of the sampling season, the pH of rhizospheres was significantly lower than that of bulk
231 soil. Although the total C and N concentrations were significantly higher in the rhizospheres, the C/N
232 ratio did not vary among seasons and sampling positions (Table 1). The EON concentrations in the
233 rhizospheres, especially in the tree rhizosphere, were significantly higher than that in bulk soil during
234 the dormant season; however, this trend was not observed in mid-summer (Table 1). The NH_4^+-N
235 concentration was significantly higher in rhizospheres than in bulk soil regardless of the season, and
236 during early winter and mid-summer, the concentration in the tree rhizosphere was higher than that in
237 the understory rhizosphere (Table 1). The soil NO_3^--N concentration in the understory rhizosphere was
238 significantly higher than that in the tree rhizosphere and bulk soil during early winter and mid-summer
239 (Table 1). The difference in NO_3^--N concentration between the tree rhizosphere and bulk soil was not
240 significant (Table 1). The effect of sampling season on soil chemical properties was not significant
241 except for EON.

242 3.2 Extracellular-enzyme activity

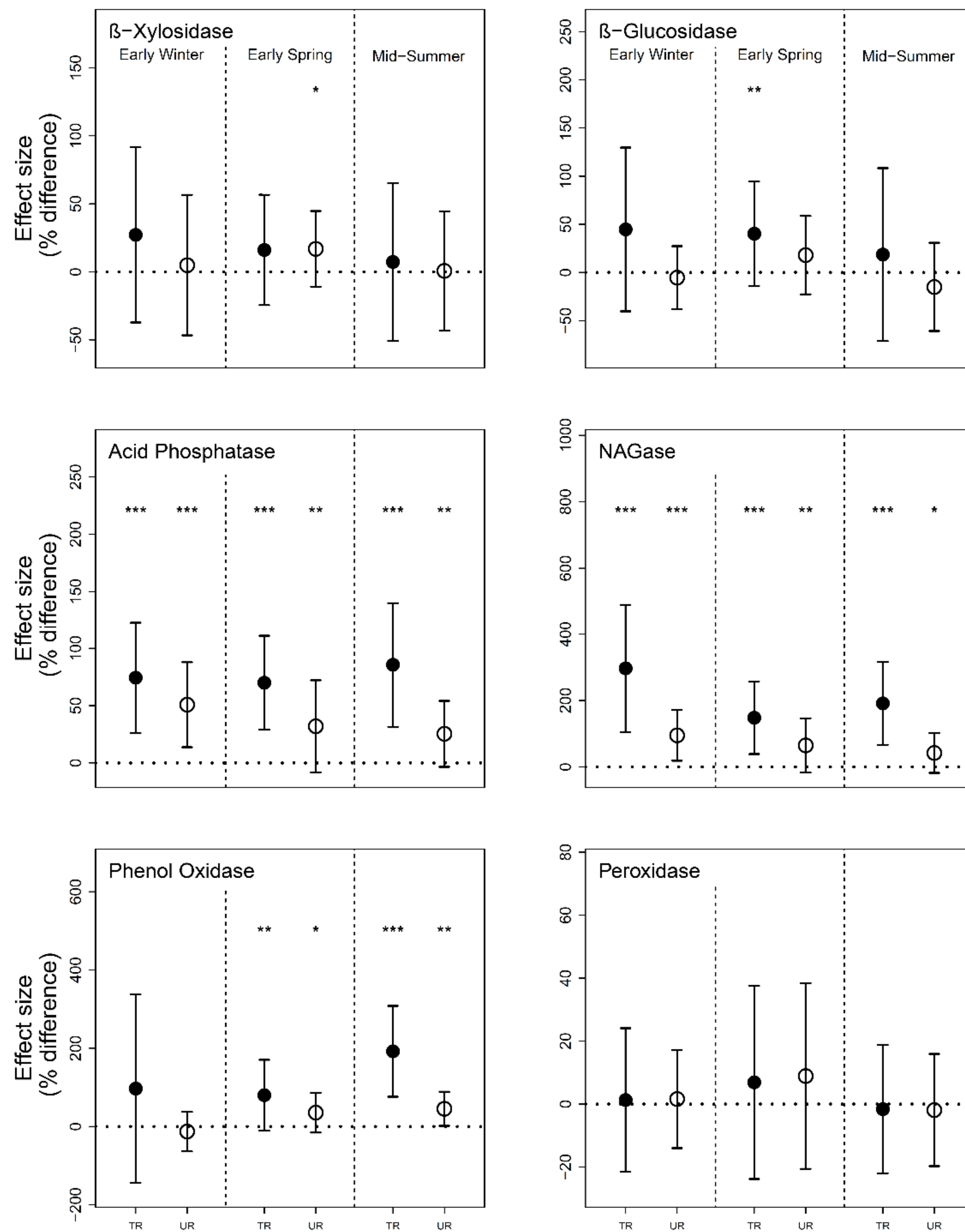
243 Although sampling season had a marginally significant effect on the NAGase and peroxidase activity,
244 there was no significant effect of season on enzyme activity (Fig. A. 1 and Table A. 2). The activity of
245 β-glucosidase and NAGase in the tree rhizosphere was significantly higher than that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

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

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

275 Tukey's HSD test showed that the RE on phenol oxidase in the tree rhizosphere was significantly
276 higher during mid-summer than during early winter and early spring. The effect of sampling season
277 on the RE on acid phosphatase was marginally significant (two-way ANOVA with random effects: F
278 $= 3.50$, $P = 0.065$).

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



289

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

296 3.3 Microbial gene abundance

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

304 The magnitude of REs on the abundance of bacterial and archaeal 16S rRNA genes did not
305 differ significantly from zero regardless of the season and position, except for the positive and
306 marginally significant REs on the bacterial 16S rRNA gene observed during early spring in the tree
307 rhizosphere (Fig. 3). The REs on fungal ITS gene abundance were significant and positive regardless
308 of the position and season except for the understory rhizosphere during mid-summer (Fig. 3). The
309 magnitude of REs on bacterial and archaeal abundance had no clear trend among seasons and positions
310 (Fig. 3). The magnitude of the RE on fungal ITS in the understory rhizosphere was significantly higher
311 than that in the tree rhizosphere only during early spring (one-way ANOVA with random effects: F =
312 4.23, $P < 0.05$), and the magnitude during early spring was significantly higher than that during mid-
313 summer only in the tree rhizosphere (one-way ANOVA with random effects: $F = 3.92$, $P < 0.05$).

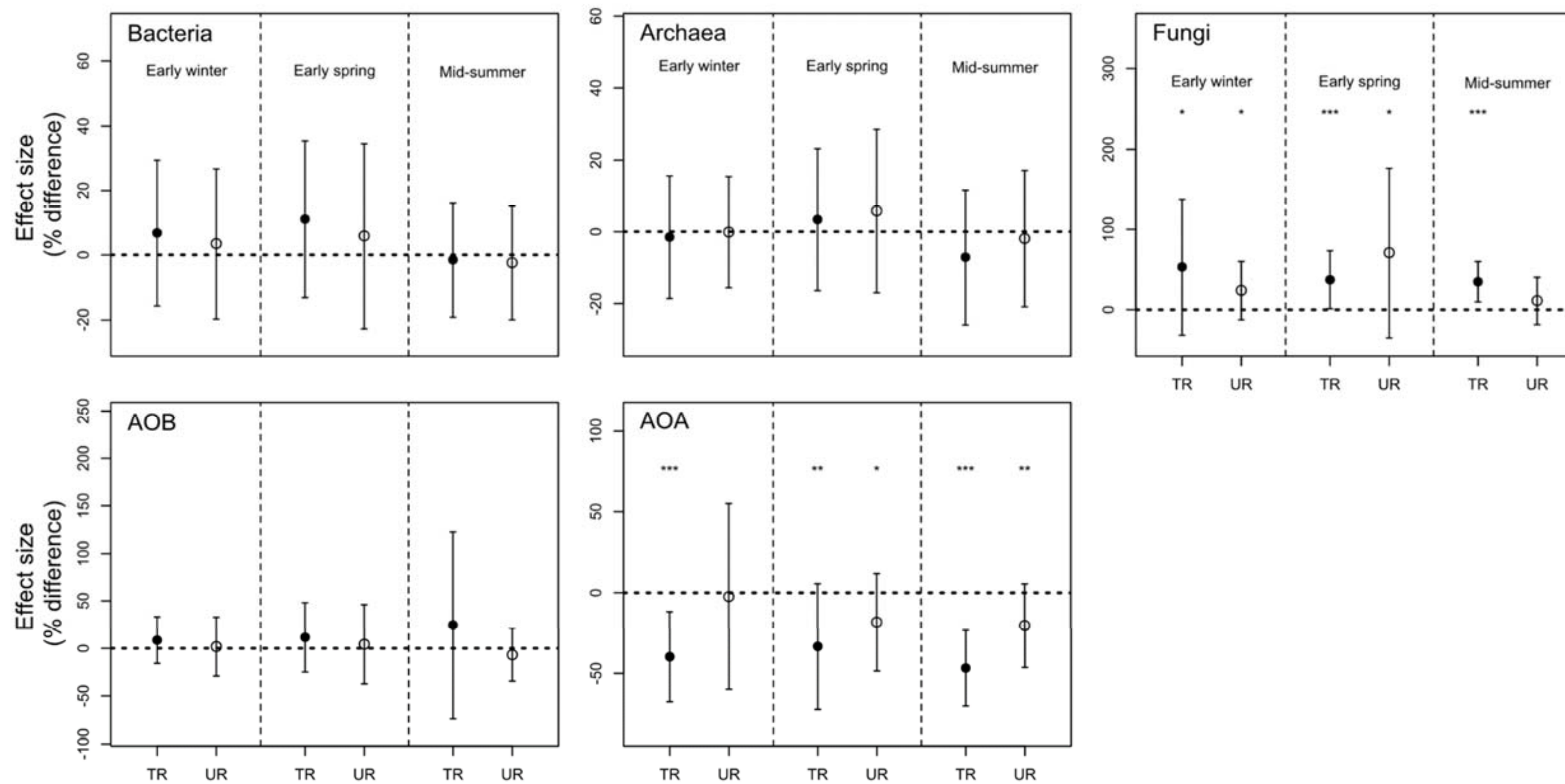
314 Although the bacterial *amoA* gene abundance did not vary among seasons and positions (Fig.
315 A. 3 and Table A. 3), the archaeal *amoA* gene abundance was significantly lower in the tree rhizosphere
316 than in bulk soil (Fig. A. 3 and Table A. 3). There was a non-significant difference in the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

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

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

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

335



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 *** $P < 0.001$.

340 4. Discussion

341

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

349

350 4.1 Seasonal differences in rhizosphere effects on enzymatic activity and nutrient cycles

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

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

364 that the potential activities of enzymes, especially NAGase and acid phosphatase, could persist for
365 several months partly as a result of the binding with minerals. Further, the potential activities could
366 largely differ from the actual *in-situ* activities because of the mobility of substrates (Schimel et al.
367 2017) and lower temperature (Baldrian et al. 2013). Therefore, there was a possibility that such
368 enzymes were produced during the growing season and the potential but not actual activities of the
369 enzymes persisted overall during the dormant season. However, significant microbial activities and
370 nutrient cycling were observed under the low-temperature condition (Freppaz et al. 2007; Isobe et al.
371 2018). Though research using metatranscriptome and *in-situ* measurement of decomposition and
372 mineralization is needed for a clear understanding of the mechanisms of REs on enzyme activities, the
373 differences in the potential activity of enzymes could imply that the rates of nutrient cycling processes
374 were stimulated within the rhizosphere in the dormant season compared to the bulk soil.

375 The results of N concentrations might support that at least the N cycle would be accelerated
376 within the rhizosphere. In the plant dormant season, the concentration of EON within rhizospheres
377 was higher than that in non-rhizosphere bulk soils, although this trend was not observed in the mid-
378 growing season (Table 1). The amount of plant-available N in the soil is determined by the balance
379 between uptake and root-primed decomposition and mineralization (Nakayama and Tateno 2021).
380 Plants and mycorrhiza can take up organic and inorganic N (Schimel and Bennett 2004; Liese et al.
381 2018; Zhang et al. 2019). Ueda et al. (2015) demonstrated that plants, including *Q. crispula*, took up
382 a certain amount of inorganic N during mid-winter in sub-zero temperatures in the northern hardwood
383 forest. Although it is unknown whether deciduous plant species can take up low-molecular-weight
384 organic N during the dormant season, Zhang et al. (2019) reported that both the roots of evergreen
385 conifers and their symbiotic mycorrhiza took up organic N during the dormant season as well as during
386 the growing season. In addition, the organic and inorganic N uptake by herbaceous species during the
387 dormant season has also been reported in a temperate coastal heath (Andresen and Michelsen 2005).

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

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

411 4.2 Seasonal differences in rhizosphere effects on microbial abundance

412 It is well known that microbial abundance within the rhizosphere is higher than that in bulk soil
413 (Kuzyakov and Razavi 2019). Our results were partly consistent with previous findings, that is, only
414 fungal abundance in the rhizospheres was higher than that in bulk soil—abundances of bacteria and
415 archaea did not differ between the rhizosphere and bulk soils (Fig. 3). In general, the fungal community
416 in the rhizosphere is characterized by mycorrhizal fungi (Baldrian 2017), and their abundance
417 decreases with distance from the rhizoplane (Kuzyakov and Razavi 2019). Mycorrhizal fungi are still
418 active and are affected by tree C supply in winter (Kaiser et al. 2010, 2011). Therefore, the observed
419 higher fungal abundance may be explained by a higher abundance of mycorrhizal fungi within the
420 rhizospheres during the plant dormant season. There is another contrasting explanation for this result.
421 Žifčáková et al. (2017) reported that EcM activity and abundance were much lower in the bulk soil in
422 winter than in summer, as observed in this study site (Nakayama et al. 2021), and the relative
423 contribution of non-mycorrhizal fungi to extracellular enzyme production was higher during winter.
424 Therefore, the higher abundance of fungi in the rhizosphere could come from the higher abundance
425 and activity of non-mycorrhizal fungi under reduced mycorrhizal abundance (Gadgil and Gadgil 1971;
426 Frey 2019). Additionally, the total C was higher in the rhizospheres regardless of the season (Table 1),
427 and the abundance of fungi did not vary among soil positions based on the total soil C-based analysis
428 (Fig. A. 4). Therefore, in the rhizospheres, the availability of C substrate would be higher than that in
429 bulk soil regardless of the season. The third possible hypothesis was that the higher saprotrophic fungal
430 abundance because of higher C availability explains the higher total fungal abundance than the
431 seasonal difference in mycorrhizal activity. To test these contrasting hypotheses, further studies
432 focusing on the fungal communities within the rhizosphere during the plant dormant season are needed.
433 Contrary to our results, bacteria and archaea are typically much more abundant in the
434 rhizosphere than in bulk soil (Kuzyakov and Razavi 2019). However, in the rhizosphere, some bacteria,
435 such as pathogenic bacteria, are suppressed by root exudates (Bais et al. 2006). The significantly lower

436 abundance of AOB and AOA within the rhizosphere (Fig. 3) also suggested the suppression of a part
437 of bacterial and archaeal communities. One possible explanation for these results is that the
438 magnitudes of stimulation and suppression of bacterial and archaeal growth within the rhizospheres
439 were similar. Alternatively, the limited photosynthetic activity, especially the lack of photosynthesis
440 by canopy trees, would also explain the bacterial and archaeal population results within the rhizosphere
441 at the beginning and end of the dormant season. Wang et al. (2019) reported that the amount of root-
442 exuded C positively correlated with the RE on microbial biomass. Although the release of root
443 exudates from tree species in winter or at least at the beginning of the dormant season has been
444 observed in some studies (Phillips et al. 2008; Nakayama and Tateno 2018), the amount of root-exuded
445 C at the beginning and end of the dormant season should be lower because a majority of root exudates
446 are recently assimilated organic C (Epron et al. 2011; Sanaullah et al. 2012; Nakayama and Tateno
447 2018). However, there is limited knowledge on root exudation in winter; therefore, further research on
448 root exudates and microbial community structures within the rhizosphere in the winter dormant season
449 is needed to reveal the relationships among plant roots, root exudates, and rhizosphere microbes.
450 Unlike total bacterial and archaeal abundance, the population of AOA was suppressed within the
451 rhizosphere seasonally (Fig. 3). Studies have shown that plants have some functions that inhibit
452 nitrification steps and growth of microbes responsible for nitrification partly to prevent N loss from
453 ecosystems (reviewed in Moreau et al. 2019). Therefore, plants possibly suppressed the nitrification
454 processes driven by AOA at the beginning and end of the plant dormant season as well as during the
455 mid-growing season.

456 4.3 Differences in the RE between canopy trees and understory vegetation

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

459 be explained by the advantage of photosynthetic activity in canopy trees relative to understory
460 vegetation during the growing season (Kuzyakov and Cheng 2001, 2004). However, in contrast to our
461 second hypothesis, the magnitude of the RE was higher for *Q. crispula* than for *S. nipponica* during
462 the dormant as well as growing seasons (Figs. 2 and 3). At the beginning and end of the plant dormant
463 season, *Q. crispula* had no leaves, whereas *S. nipponica* showed photosynthetic activity (Kayama and
464 Koike 2018; Tateno et al. 2019). Thus, factors other than the advantage of photosynthesis would affect
465 the difference in the magnitude of the RE between *Q. crispula* and *S. nipponica*.
466 One possible factor that may result in the RE differences between the canopy tree and
467 understory vegetation irrespective of seasons is mycorrhiza type. Huo et al. (2017) reported that trees
468 tended to have higher REs than herbaceous species in a meta-analysis using RE data obtained during
469 the growing season. They surmised that the difference between trees and herbaceous species is a result
470 of differences in mycorrhiza, i.e., EcM (trees) vs. AM (herbaceous species). In general, EcM fungi
471 have a greater capacity for producing extracellular enzymes than AM fungi (Frey 2019), and the
472 enzymatic production activity of symbiotic mycorrhizal fungi are also important factors for the RE on
473 enzymatic activity (Phillips and Fahey 2006). Therefore, the difference in mycorrhizal type could
474 explain the higher RE in the tree rhizosphere on the enzymatic activity during leafless seasons.
475 The negative rhizosphere effect on the abundance of AOB and AOA was stronger in the tree
476 rhizosphere than in the understory rhizosphere (Fig. 3). EcM symbiotic species suppress nitrification
477 more than AM symbiotic plants (Phillips et al. 2013; Tatsumi et al. 2020). Most mineralized N at the
478 present study site undergoes nitrification processes in laboratory incubation without living roots and
479 mycorrhiza (Nakayama et al. 2021). Thus, our results of nitrifiers and inorganic N concentrations (Fig.
480 3 and Table 1) suggest that *Q. crispula* had a stronger nitrification suppression effect than *S. nipponica*.
481 Plants take up each of the low-molecular-weight organic N, NH_4^+-N, and NO_3^--N (Jones et al. 2005;
482 Liese et al. 2018); however, there is a source preference for plant N uptake (Nordin et al. 2001).

483 Ectomycorrhizal symbiotic trees prefer organic N and NH_4^+-N to NO_3^--N compared with AM
484 symbiotic species (Phillips et al. 2013; Liese et al. 2018). Tateno et al. (2020) reported that *Q. crispula*
485 preferred NH_4^+-N to NO_3^--N and *S. nipponica* utilized both types of inorganic N at the present study
486 site. Therefore, our results implied that canopy tree species (EcM symbiotic plant) suppressed the
487 AOA population more strongly and, consequently, nitrification processes within the rhizosphere than
488 those in the understory vegetation (AM symbiotic plant), regardless of the season.

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

503 5. Conclusions

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

506 that the nutrients taken up by plants in winter are important for subsequent spring growth, our results
507 also suggest that nutrient uptake by plants and the abundance of their root symbiotic mycorrhiza was
508 less during the non-growing season compared with the growing season. Therefore, our results implied
509 that both canopy trees and understory vegetation hasten nutrient cycling processes, including
510 decomposition and mineralization at the beginning and end of the dormant season. However, a large
511 part of the nutrients transformed by root-induced decomposition would not be taken up by plants and
512 was possibly retained in the microbes. These retained nutrients would be released later during the
513 subsequent growing season and utilized for plant growth.
514

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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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