Nitrogen source utilization in co-existing canopy tree and dwarf bamboo in a northern hardwood forest in Japan

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Key message: Understory dwarf bamboo mitigated soil N competition with co-existing canopy oak trees by foraging in deeper soils and increasing dependence on N forms that differ from those used by canopy trees.

Abstract:

Nitrogen (N) competition among co-existing plant species utilizing different mycorrhiza types was explored through the investigation of N sources of oak trees and dwarf bamboo. Vertical distribution of fine roots, soil N pools, $\delta^{15}N$ of leaves and possible soil N sources and nitrate reductase activity (NRA) were all quantified. The fine roots of canopy trees were more concentrated in the surface soils than roots of the understory dwarf bamboo. Soil NH_4^+ and extractable organic N (EON) content (based on unit weight) decreased from the organic horizon (O horizon) to the deep soils, the size of the NH_4^+ pool per unit volume increased with soil depth, and the EON was approximately constant. Soil NO_3^- was not detected at any soil depth or was not significant in value, while NO_3^- captured by ion exchange resin (IER) buried at a 10 cm soil depth and net nitrification were observed via laboratory incubation at all soil depths. The $\delta^{15}N$ of the NH_4^+ and EON pools increased with soil depth and the $\delta^{15}N$ of NO_3^- of IER was lower than that of other N forms, except for the $\delta^{15}N$ of NH_4^+ in the O horizon. Furthermore, root NRA tended to be lower in canopy trees than in the understory, implying lower dependency on NO_3^- by canopy trees. The pattern of root distribution and mycorrhizal fungi association of the understory vegetation (as well as the high root NRA) suggested that dependence on N in deeper soils was higher in understory plants than in canopy trees. These findings indicate that understory vegetation mitigate soil N competition against co-existing canopy trees via the use of alternative N sources.

Keywords: Nitrate Reductase Activity, Nitrogen Source Utilization, Northern Hardwood Forest, Mycorrhiza, Nitrogen Isotope

Introduction

Nitrogen (N) is the most important element limiting plant growth in many terrestrial ecosystems (Vitousek and Howarth 1991). Both inorganic N (NO_3^- and NH_4^+) and organic N are possible N sources for plants in forest soils. Co-existing plants compete for soil N, and the vertical distribution of co-existing plant roots differs with the strength of belowground competition for soil resources (Cardinael et al. 2015). Utilization of N forms at different soil depths varies considerably among tree species, even among those growing at the same site (Nadelhoffer et al. 1996; Brearley 2013; Tanaka-Oda et al. 2016; Liu et al. 2018). Furthermore, N source preference varies among species (Nadelhoffer et al. 1996; Högberg 1997; Gherardi et al. 2013; Debiasi et al. 2019). N foraging strategy and N source utilization are therefore key for a better understanding of N competition among co-existing plants.

Mycorrhizal associations also affect N source utilization by plants (Hobbie et al. 2000; Hobbie and Högberg 2012; Mayor et al. 2015). Ectomycorrhizal fungal species are typically stronger decomposers than arbuscular mycorrhiza fungal species due to the wider array of enzymes that they produce (Phillips et al. 2013). Soils dominated by ectomycorrhizal tree species have faster rates of decomposition of soil organic matter than soils dominated by arbuscular mycorrhizal tree species (Phillips et al. 2013), as well as greater carbon accumulation in the soil (Averill et al. 2014). Ectomycorrhizal and non-ectomycorrhizal tree species have different N acquisition capacities according to their decomposition ability: ectomycorrhizal tree species rely primarily on organic N (Chalot and Brun 1998; Hodge et al. 2001; Courty et al. 2010; Smith and Smith 2011). Co-existence of competing plants could be partly explained by their mycorrhizal associate

type.

Quercus crispula Blume (Oak) is a major component of the northern hardwood forests of northern Japan (Hiura 2001) and is associated with both ectomycorrhizal and arbuscular mycorrhizal fungi (Obase et al. 2007). The understory dwarf bamboo species *Sasa nipponica* Makino et Shibata (Sasa) is a major understory species in the northern hardwood forest of eastern Hokkaido (Kayama and Koike 2018; Tateno et al. 2019), and Genus *Sasa* is known to associate with arbuscular mycorrhizal fungi (Fukuchi et al. 2011). The N source utilization patterns of co-existing canopy and understory plant species could differ according to their associated mycorrhizal fungi. However, details about N source utilization by co-existing canopy and understory species with different mycorrhizal associations have not yet been elucidated. We utilized a forest dominated by *Q. crispula* and *S. nipponica* as a model ecosystem to investigate the N source utilization of these co-existing species.

Determining the N source utilized by plants is a complex process, particularly in field conditions (Nadelhoffer et al. 1996; Nordin et al. 2001; Craine et al. 2015). The N isotope ratio in plant tissues reflects the isotopic composition of the N sources taken up by the plants (Nadelhoffer et al. 1996; Högberg 1997; Craine et al. 2015) because the N isotope ratio is rarely altered at the time of root N uptake under conditions of low N availability (Evans 2001). The N isotope ratio of plant tissues is affected by the form of N absorbed (NH_4^+, NO_3^-, or dissolved organic N), the type of mycorrhizal association (ectomycorrhiza or arbuscular mycorrhiza fungi), the presence or absence of symbiotic N fixation, the rooting depth, and the source of the N (i.e. soil N or rainfall N) (Nadelhoffer et al. 1996; Högberg 1997; Hobbie et al. 2000; Hobbie and Högberg 2012; Brearley 2013; Craine et al. 2015). Recent advances in the denitrifier method (Sigman et al. 2001) have

enabled the determination of N isotope ratios from small quantities of samples, which has led to more precise determination of the N isotope ratios of possible N sources, including soil extractable organic N (EON) (Koba et al. 2010).

We hypothesized that co-existing canopy tree species and understory species in a cool-temperate forest in northern Japan would utilize different N forms and from different source and soil depths as a strategy to mitigate N competition. We investigated the N source utilization of co-existing canopy tree species and understory species by measuring the vertical distribution of fine roots and quantifying the soil N pool to elucidate the N foraging mechanisms, determine foliar $\delta^{15}N$, and identify possible soil N sources including EON, which is less reported (Houlton et al. 2007; Takebayashi et al. 2010; Koba et al. 2010). We also measured nitrate reductase activity (NRA) for both species for use as an indicator of NO_3^- uptake and use by the plants (Lee and Stewart 1978).

Materials and methods

Study site

The study was conducted in a cool temperate deciduous natural forest in the Shibecha Branch of the Hokkaido Forest Research Station, Field Science Education and Research Center, Kyoto University, Japan (43° 24.2' N, 144° 38.5' E). The study site was dominated by an Oak canopy layer and the understory was dominated by dwarf bamboo Sasa (Nakayama and Tateno 2018; Tateno et al. 2019). The diameter at breast height of canopy trees of study site were about 30 cm and the height of Sasa was between 80–100

cm (Tateno et al. 2019). The annual litterfall production of the study site was 493.3 g m^{-2} $year^{-1}$ and the biomass of current year shoots and overwinter shoots of Sasa was 219.7 and 60.0 g m^{-2} $year^{-1}$, respectively (Tateno et al. 2019). The photosynthetic rates of current year shade shoots of genus *Sasa* species tend to be higher in the late autumn, when the forest canopy is more open than in mid-summer (Lei and Koike 1998). However, the N content of above-ground current shoots of Sasa reached a maximum from July to August before decreasing gradually until the end of November at this study site (Tateno et al. 2019). Likewise, the N content of current shoots in oak trees reached a maximum value from July to August (Tateno et al. 2019).

The mean annual precipitation and air temperature (1986–2015) were 1189 mm and 6.3 ℃, respectively, according to data from a meteorological station about 9 km from the study site (43° 19.46' N, 144° 36.8' E). The soils of the study site were characterized as andosols (IUSS Working group WRB 2015). Details of soil properties of the study site have been extensively reported elsewhere (Hosokawa et al. 2017; Isobe et al. 2018; Watanabe et al. 2019).

Plant sampling

We established a 20 × 20 m study plot for the sampling of soils and plants. Seven healthy Oak canopy trees within the plot were selected for determining the N isotope ratio from the leaves. On July 30, 2016, twigs with at least ten current year shoots, were harvested from the crown of each tree using a pole pruner. Three Sasa current shoots were concurrently collected from seven points close to the Oak sampling trees within the study plot, and were composited into one sample for each point. The samples were maintained

146 at a cool temperature during transport to the laboratory and separated into leaves and
147 twigs. Leaves were immediately washed with ion-exchanged water to avoid dry
148 deposition effects on the N isotope ratio. Leaf samples were milled after oven drying at
149 60 °C and stored until isotope measurement.
150
151 **Fine root sampling**
152
153 Soil samples along five soil profiles were collected with a cylindrical soil corer (20 cm^2
154 in area) at depths of 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, and 45-
155 50 cm on August 23, 2017. Five samples (20 × 20 cm) from the O horizon were also
156 collected in the area just above each soil sampling profile. Roots in the soil cores and the
157 collected O horizon were wet-sieved using 0.5 mm and 2 mm mesh. Fine roots (< 2 mm
158 in diameter) were hand sorted with tweezers under a stereoscopic microscope based on
159 their morphology (existence of ectomycorrhiza and/or branching pattern), color, and
160 surface condition to separate by species (Oak and Sasa) and condition (living or dead
161 root). Fine roots of other species were not identified within the samples. After sorting, the
162 roots were dried at 70 °C for 72 h and the dry mass was measured. During the fine root
163 sorting, we measured the ECM mycorrhizal infection rate (%, mycorrhizal root tips / total
164 root tips × 100) of Oak by counting all infected and non-infected root tips in the samples
165 under a stereoscopic microscope.
166 To compare with the isotopic value and the quantity of a given N source, the fine
167 root mass densities (mg cm^{-3}) of the O horizon, and the 0-10, 10-30, and 30-50 cm layers
168 of mineral soils were calculated for each soil profile by summing up the fine root mass
169 divided by the sampling area and each depth interval. For the O horizon, the average depth

value of the FH (fermented and humic) horizon (*i.e.* O horizon excluding surface litter horizon) in this study site (2.9 ± 0.9 cm, average ± SD, n = 5) was used for fine root mass densities, because fine roots were not found in the litter horizon.

Soil sampling and chemical analysis

For isotope measurements of soil N species, we collected soil from the four centers of the 10 × 10 m subplots in the study plot (20 × 20 m) using a soil auger at depths of 0-10, 10-30, and 30-50 cm from four soil profiles on July 30, 2016. The O horizon just above the soil sampling profiles was also collected. The quantities of NO_3^-, NH_4^+, and total extractable N were determined by extraction from 8 g of wet soil with 80 ml of 0.5 M K_2SO_4 solution by shaking for 1 h. After shaking, solutions were centrifuged (3000 rpm for 10 min) and then filtered by glass fiber filters (GF/F; Whatman). The K_2SO_4 and glass fiber filters were pre-combusted at 450 ℃ for 4 h before use to remove contamination. Extracts were stored in a freezer (−20 ℃) until chemical and isotope analysis. Soil moisture content was measured using sub-soil samples.

For measurement of the pool size and net change rates of NH_4^+, NO_3^-, and EON during laboratory incubation, we collected soil at depths of 0-10, 10-20, 20-30, 30-40, and 40-50 cm from six soil profiles using a cylindrical soil core (20 cm^2 in area) on August 23, 2017. Samples were extracted using 2 M KCl solution and shaken for 1 h. Sub-samples were incubated aerobically at 25 ℃ for 28 d in an incubator. Incubated soils were also extracted using 2 M KCl solution and shaken for 1 h. The net change rates of NH_4^+, NO_3^-, and EON were calculated by subtracting the pool size of each N species after the incubation period (28 d) from the initial pool size and dividing by the incubation period.

We simultaneously collected three samples from 0-10, 10-20, 20-30, 30-40, and 40-50 cm depths for determination of soil bulk density. Collected soils were sieved using a 2 mm mesh screen and weighed after drying at 105 ℃. Five O horizon samples were also collected from a 20 × 20 cm area to assess pools in the O horizon. Collected samples were weighed after drying at 105 ℃.

The concentrations of NH_4^+ and NO_3^- in the extracts were analyzed colorimetrically by the indophenol blue method and by diazotization after reduction to NO_2^- with zinc powder, respectively (Keeney and Nelson 1982). The total extractable N was converted into NO_3^--N using the persulfate oxidation procedure and analyzed colorimetrically. The EON concentration was calculated by subtracting the inorganic N (NH_4^+-N and NO_3^--N) concentrations from the total extractable N concentration. To compare the isotopic value, the pool size and net change rate of N species in the O horizon, and the 0-10, 10-30, and 30-50 cm layers of mineral soils were weight-averaged for each soil profile using the bulk density and were expressed as soil weight (mgN kg $soil^{-1}$) and volume (gN m^{-3}).

Soil NO_3^- was captured by ionic resin capsules containing about 1 g of mixed ion exchange resins (PST-2, Unibest, Bozeman, MT), which were placed at a depth of 10 cm in the mineral soil. Similar resin capsules have been used to assess *in-situ* net N mineralization and nitrification over a specific time period (e.g. DeLuca et al., 2002). Five resin capsules were placed at a depth of 10 cm in the mineral soil on July 30, 2016 and collected on July 9, 2017. The collected capsules were washed with distilled water to remove adhering soils. The resin capsules were extracted for NO_3^--N analysis by shaking thrice in 10 ml of 2 M KCl for 1 h.

Isotope measurement

The $\delta^{15}N$ values of NO_3^- in the soil and the ion exchange resin capsules extracts were measured by the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002; Koba et al. 2010, 2012). The NO_3^- in the soil and resin extract was converted into N_2O using a denitrifier (*Pseudomonas aureofaciens*) lacking the gene coding for N_2O reductase. The $\delta^{15}N$ value of NH_4^+ in the soil extracts was measured using the denitrifier method after ammonia diffusion (Holmes et al. 1998) followed by the persulfate oxidation method (Koba et al. 2010, 2012) to convert the sample NH_4^+ to N_2O via NO_3^-. The $\delta^{15}N$ values of total dissolved N were determined by analyzing the persulfate-digested samples with the denitrifier method (Koba et al. 2010, 2012). The $\delta^{15}N$ of the N_2O produced by the denitrifier was analyzed using an isotope ratio mass spectrometer (20-22 IRMS equipped with Cryoprep and GC; Sercon Ltd., Cheshire, UK) with the protocol defined by McIlvin and Casciotti (2011). The natural abundance of ^{15}N was reported as $\delta^{15}N$:

$$\delta^{15}N = [(R_{sample} - R_{standard}) / R_{standard}],$$

where $R = {}^{15}N/{}^{14}N$ and the standard is atmospheric N2. Calibrated in-house standards DL-alanine ($\delta^{15}N$ = –1.7‰), glycine ($\delta^{15}N$ = +10.0‰), and L-histidine ($\delta^{15}N$ = –8.0‰); international NH_4^+ isotopic standards USGS25, USGS26, and IAEA-N-2; and international NO_3^- isotopic standards IAEA-NO-3, USGS32, and USGS34 were used for the $\delta^{15}N$ of total dissolved N, NH_4^+, and NO_3^- calibration, respectively. The average standard deviations of replicate measurements of an individual sample for $\delta^{15}N$ of total dissolved N, NH_4^+, NO_3^-, and EON were 0.3, 0.5, 0.2, and 1.5‰, respectively.

The N isotope ratios of the leaves, O horizon, and bulk soils (Bulk) were

241 measured with an on-line C and N analyzer (NC 2500; Thermo Fisher Scientific, Waltham,
242 MA, USA) coupled with an isotope ratio mass spectrometer (MAT252; Thermo Fisher
243 Scientific). The precision of the $\delta^{15}N$ measurement was ± 0.2‰.
244
245 **Nitrate reductase activity**
246
247 On July 9, 2017, leaves and fine root samples were collected from seven healthy Oak
248 trees and seven above-ground shoots of Sasa in the study plot for *in vivo* nitrate reductase
249 activity (NRA) assays. The samples were collected between 11:00 and 13:00 on a sunny
250 day and kept at 4 °C for several hours before the measurement of NRA. The
251 measurements of in vivo NRA followed the procedure described by Koyama and Tokuchi
252 (2003), with some modification: for both species, 20 to 25 leaf disks of 10 mm diameter
253 (about 200 mg) and fine roots (about 200 mg) were used.
254
255 **Statistical analyses**
256
257 Two-way analysis of variance (ANOVA) was used to analyze the effect of tree species,
258 soil depths and the interactions of those variables upon fine root density. For comparison
259 of soil and fine root parameters including pool size and isotopic value of inorganic and
260 organic N sources, and fine root biomass among soil depths and among tree or N species,
261 we used within-subjects ANOVAs for each soil profile, because within-subjects factor
262 with the Holm's test for multiple comparisons. Bartlett's test was conducted to test the
263 homogeneity of variance of the data prior to the ANOVAs. If the *P* values for the Bartlett's
264 tests were < 0.05, the data were log transformed prior to the ANOVA analysis. If the

values were zero or negative, we added an integer to exceed zero before log transformation. For comparisons of leaf N concentration and foliar $\delta^{15}N$ between Oak and Sasa, we used the Student's *t*-test. For comparisons of $\delta^{15}N$ of NO_3^- captured by resin capsules and those of other N species at each soil depth, we used pairwise comparisons using *t*-tests with the Holm's method for the adjustment of *P* values. For comparisons of NRA of leaves and roots between Oak and Sasa, we used the Welch's *t*-test. R software (version 3.3.3; R Development Core Team 2014) was used for all statistical analyses.

Results

Fine root distribution

The mean ± standard deviation (SD) of total fine root biomass within the top 50 cm of the soil was 259.2 ± 40.8 g m^{-2} and 240.6 ± 59.8 g m^{-2} for Oak and Sasa, respectively. Based on two-way ANOVA, the interaction between tree species and soil depth was significant ($P < 0.0047$). The Oak fine roots were concentrated in the surface soil (0-10 cm depth), and their density decreased sharply with increasing soil depth (Fig. 1). In the O horizon and the surface soil (0-10 cm depth), the fine root densities of Oak were higher than those of Sasa, although the difference was marginally significant in the 0-10 cm soil ($P = 0.007$ and $P = 0.0588$ for the O horizon and 0-10 cm, respectively). For Sasa, the fine root density decreased with the increasing soil depth, as compared of the results for Oak (Fig. 1) The fine root density of Sasa tended to be slightly higher than Oak in the 30-50 cm soils although the difference was marginally significant ($P = 0.0817$).

Mean ± standard deviation (SD) of ECM mycorrhizal infection rates and the

density of mycorrhiza-infected root tips (number of mycorrhizal roots tip / unit volume of soils) of Oak are shown in Table 1.

Soil N isotope and dynamics

The soil weight based NH_4^+ and EON contents were greater in the O horizon and decreased with soil depth (Fig. 2a, 3b). The soil weight based NO_3^- content was not detectable or very low irrespective of soil depths for both sampling dates (Fig. 2a, 3b). The EON pool size was higher than that of NH_4^+ in the O horizon and at the 0-10 cm soil depth (Fig. 2a). The volume-based N pool showed a different pattern on a soil weight basis (Fig. 2b). The pool sizes of NH_4^+ and EON were lower in the O horizon than in deeper soils, although the differences were not significant for EON (Fig. 2b).

The pool sizes of NH_4^+ and NO_3^- per unit soil weight increased and the pool size of EON decreased to a much greater degree in the O horizon than in mineral soils during the incubation period (Fig. 2c). The volume-based net change rate of the NH_4^+ pool was higher in the O horizon (Fig. 2d). Net change rates of the EON pool were almost negative and the difference was not significant among soil depths (Fig. 2d). The net change rate of the NO_3^- pool tended to be the highest at 0-10 cm soil depth and decreased with soil depth, but the differences were not significant (Fig. 2d).

The mean ± SD of soil NO_3^- and NH_4^+ contents captured by the ion exchange resin capsules at the 10 cm depth of the soils were 0.35 ± 0.19 and 0.30 ± 0.22 mgN capsule^{-1} year^{-1}, respectively, indicating that NO_3^- existed in the soil solutions at a similar level as NH_4^+ throughout the year, even though the soil extractable NO_3^- was not detectable or was very low on the two sampling dates.

313 The order of the $\delta^{15}N$ value was EON > Bulk > NH_4^+ for the O horizon and for
314 0-10 cm soils, and while was EON > NH_4^+ > Bulk for the 10-30 cm soils (Fig. 3). The
315 $\delta^{15}N$ values of Bulk, EON, and NH_4^+ ranged from −4.2 to +4.7 ‰, −3.5 to +5.4 ‰, and
316 −8.0 to +4.7 ‰, respectively, all of which increased significantly with soil depth (Fig. 3a).
317 The $\delta^{15}N$ values of NH_4^+ and EON for the 30-50 cm depth could not be measured by our
318 method due to low concentrations. The $\delta^{15}N$ value of NO_3^- also could not be measured
319 due to low concentrations, while the $\delta^{15}N$ values of NO_3^- captured by the ion exchange
320 resin capsules (10 cm in depth) were −8.8 ± 3.6 ‰ (mean ± SD), significantly lower than
321 the value of other N forms, with the exception of the $\delta^{15}N$ value of NH_4^+ of the O horizon
322 (Fig. 3a).
323
324 **Plant leaf N concentration, leaf N isotope ratio, and NRA**
325
326 The mean ± SD of the leaf N concentrations for Oak and Sasa were 2.22 ± 0.20 and 2.15
327 ± 0.13 %, respectively, and the differences between Oak and Sasa were not significant (*t*-
328 test, n = 7, $P = 0.46$). The means ± SD of foliar $\delta^{15}N$ for Oak and Sasa were −3.77 ± 0.83
329 and −2.61 ± 1.16 ‰, respectively (Fig. 3a), and differences between Oak and Sasa were
330 marginally significant (*t*-test, $n = 7$, $P = 0.055$). The values approached the value of EON
331 in the O horizon, but were higher than that of NH_4^+ in the O horizon and lower than that
332 of EON and NH_4^+ at the 10-30 cm depth (Fig. 3a).
333 The mean NRA in the leaves of Sasa was slightly higher than that of Oak, but
334 the difference between Oak and Sasa was not significant (Fig. 4; *t*-test, n = 7, $P = 0.55$).
335 The mean NRA in the fine roots of Sasa was higher than that of Oak, but the difference
336 between Oak and Sasa was not significant (Fig. 4; *t*-test, n = 7, $P = 0.12$).

337

338 **Discussion**

339

340 **Vertical distribution of plant fine roots**

341

342 According to the vertical fine root distribution, most of the fine roots were concentrated
343 in the surface mineral soils for both Oak and Sasa, and the roots of the species overlapped
344 considerably, which likely induced competition for the same N sources between the two
345 species. The extent of the fine root concentration in the surface mineral soils was greater
346 in Oak than in Sasa (Fig. 1). Mycorrhizal associations differed between the two species:
347 Oak was associated with ectomycorrhizal fungi (Obase et al. 2007), but Sasa was not
348 (Fukuchi et al. 2011). Lindahl et al. (2007) suggested that ectomycorrhizal mycelium
349 dominated in the decomposed litter and humus horizon in coniferous boreal forests. The
350 fine root biomass of Oak was concentrated in the surface mineral soil (0-10 cm depth),
351 just below the O horizon (Fig. 1), and mycorrhizal root tips were also found in greater
352 density in surface mineral soils. Anderson et al. (2014) reported that fine-scale
353 distribution patterns along a soil profile were different between mycorrhizal root tips and
354 mycelium. However, these findings were derived from coniferous boreal forests, and may
355 not be directly applicable to a broad-leaved forest dominated by the ectomycorrhizal tree
356 species. We speculated that a considerable portion of the ectomycorrhizal mycelium
357 associated with Oak trees may extend from the roots in surface mineral soils into the O
358 horizon. Compared with Oak, Sasa tended to distribute more fine roots in deeper soils
359 (Fig. 1). This indicates that Sasa may not depend on organic N, because the organic matter
360 decomposition ability of arbuscular mycorrhizal fungi is lower than that of

361 ectomycorrhizal fungi (Courty et al. 2010). However, the mycorrhizal infection of Sasa
362 was not analyzed in this study. Further study of mycorrhizal infection on root tips and the
363 mycelial distribution pattern of both species would help to clarify the detailed
364 mechanisms of N competition and N foraging by mycorrhizal fungi. In spite of these
365 limitations, our results indicate the competitive advantage of Oak in the nutrient rich O
366 horizon and surface mineral soils, whereas Sasa might avoid soil N resource competition
367 by foraging in deeper soils.
368
369 **Isotopic evidence of N utilization of co-existing canopy and understory vegetation**
370
371 The $\delta^{15}N$ value of possible N sources varied widely among N species as well as among
372 soil depths. The $\delta^{15}N$ value of Bulk, NH_4^+, and EON increased with soil depth, consistent
373 with the results of previous studies (Koba et al. 1998, 2010, 2012; Hobbie and Ouimette
374 2009; Brearley 2013). Dependence on shallower N sources results in lower N isotope
375 ratios in plants (Kohzu et al. 2003). Although the difference was marginally significant
376 ($P = 0.055$), the $\delta^{15}N$ value of Oak leaves was slightly lower than that of Sasa, indicating
377 that Oak may depend on shallower N source. Furthermore, N species are also an important
378 determinant for plant $\delta^{15}N$ value (Nadelhoffer et al. 1996; Högberg 1997; Koba et al.
379 2003; Tateno et al. 2005; Houlton et al. 2007; Craine et al. 2015; Liu et al. 2018). The
380 order of the $\delta^{15}N$ value of the soils was EON > Bulk > NH_4^+ at this study site which was
381 consistent with previous reports (Houlton et al. 2007; Koba et al. 2010, 2012).
382 The $\delta^{15}N$ value of soil NO_3^- could not be measured owing to an extremely low
383 pool size, but the $\delta^{15}N$ of soil NO_3^- is typically much lower than that of NH_4^+ and EON
384 (Koba et al. 1998, 2010, 2012; Tanaka-Oda et al. 2016; Liu et al. 2018). The $\delta^{15}N$ value

of NO_3^- captured by the ion exchange resin capsules was lower than that of the other sources. Positive net production rates of NO_3^- were observed (Fig. 2d). NO_3^- was available to plants above and below 10 cm mineral soil depth, and acquisition of soil NO_3^- lead to ^{15}N-depletion in the plants even when the measurable pool sizes of the extractable NO_3^- were very low (Fig. 2a, c). However, the negligible NRA of Oak (Fig. 4) suggests low dependence on NO_3^- at this site, although *Quercus serrata*, *Quercus robur,* and *Quercus petraea* have been shown to have NRA capability in other regions (Truax et al. 1994; Thomas and Hilker 2000; Schmull and Thomas 2000; Takahashi et al. 2005). This may be partly due to extremely low NO_3^- supply rates in this study site because NRA is known as a substrate-induced reaction (Beevers and Hageman 1969). The NRA of Sasa leaves was negligible, but the NRA in the roots of some individuals was far higher than that of Oak, although the difference of mean root NRA between the two species was not significant (P = 0.12). Large variations in root NRA for Sasa may have been due to individual variations in accessibility of the soil NO_3^- just before the sampling timing. This result may indicate that NO_3^- assimilation of Sasa primarily takes place in the roots rather than in the leaves, if the roots can access soil NO_3^- sources. Root NO_3^- assimilation of understory species was also reported by Oliveira et al. (2017), suggesting that it might have an adaptive advantage in shady environments because N assimilation would compete with carbon assimilation in the leaves (Bloom et al. 2010).

Soil NH_4^+ (particularly in the O horizon) was the second ^{15}N-depleted N source; acquisition of this N source can lead to ^{15}N-depletion in plants, while utilization of EON and NH_4^+ in deeper soils can lead to ^{15}N-enrichment in plants. Previous studies have reported that plants can take up organic N as an N source (Näsholm et al. 1998, 2009), while report of $\delta^{15}N$ value of EON have been very limited until recently (Houlton et al.

2007; Takebayashi et al. 2010; Koba et al. 2010, 2012). In right of the ectomycorrhizal associations and low NRA (Fig. 4), it is likely that Oak used both NH_4^+ and EON in the O horizon and surface mineral soils. In this study, we did not specify the chemical composition of EON, which can include easily decomposing substrates and recalcitrant substrates; further study is needed to reveal the N source utilization of ectomycorrhizal species through determining the chemical composition of EON. In contrast to Oak, Sasa had high NRA, particularly in the roots (Fig. 4), suggesting that it utilized NO_3^- from surface and deep mineral soils and used NH_4^+ as a counter source of enriched $\delta^{15}N$. Thus, the understory dwarf bamboo, Sasa, mitigated soil N competition against co-existing canopy trees not only by foraging in deeper soils, but also by increasing its dependence on N forms different than those used by the canopy trees.

Limitation of this study and possible effects of N source utilization of plants on ecosystem functions

Due to the simple vegetation composition of our study sites, which primarily contained one species of canopy tree and one species of understory vegetation, the ecosystem-scale implications of our findings may not directly scale to more diverse ecosystems due to variation in the timing of N uptake between different functional types of plant species (Larsen et al 2012). However, our results aid in understanding the N cycling of the typical vegetation in the study area (Watanabe et al. 2019; Tateno et al. 2019).

At this study site, NO_3^- pool sizes were relatively small (Fig. 2 and 3b). However, considerable gross NO_3^- production occurred in forests adjacent to our study site (Hosokawa et al. 2017; Watanabe et al. 2019), as compared with other regions from 38

433 sites across the Japanese archipelago (Urakawa et al. 2015). Furthermore, ammonia
434 oxidizing archaea were found throughout the growing season in these adjacent forests
435 (Isobe et al. 2018). Thus, NO_3^- could be an important N source at this study site, although
436 Oak may not be an effective user of NO_3^- as interpreted in the earlier section on NRA
437 analysis and isotopic evidence. The importance of understory *Sasa senanensis* Rehder for
438 the N leaching process has been reported in the northern hardwood forest ecosystems of
439 northern Japan (Fukuzawa et al. 2006, 2015). For example, Fukuzawa et al. (2006)
440 reported that existence of *S. senanensis* considerably reduced NO_3^- leaching via stream
441 water after forest clear cutting; about 59-88 % of fine root biomass and about 30 % of
442 litterfall was accounted for by *S. senanensis* (Fukuzawa et al. 2013; Watanabe et al. 2013).
443 In this study site, Sasa likely took up NO_3^- as interpreted in the earlier section on root
444 NRA analysis, although further study of the detailed seasonality of NRA in Sasa and Oak
445 is needed to confirm this assertion.
446

447 **Conclusions**

448 Although leaf $\delta^{15}N$ values did not significantly differ between Oak and Sasa, dependence
449 on N source can be assumed to differ between the two species due to differences in their
450 morphological, physiological, and mycorrhizal properties. Based on the results of the
451 vertical distribution of fine roots and the soil N pool, the $\delta^{15}N$ of leaves and possible soil
452 N sources, and the NRA of the fine roots, Oak would primarily use NH_4^+ and organic N
453 in the O horizon and/or surface soils, while Sasa would likely utilize the N in deeper soils
454 and NO_3^-. Our findings therefore indicate that co-existing canopy tree species and
455 understory species in a cool-temperate forest in northern Japan have N sources of different
456 N forms, from different soil depths.

457

458 **Author contribution statement** RT, KF, YI, KK, and SU designed the study and
459 conducted sampling and pretreatments for isotopic measurements. RT and MN conducted
460 soil chemical analyses and NRA measurement, and RT, MN, and KF conducted fine root
461 analyses. MY, YI, and KK conducted isotopic measurements. RT wrote the paper and all
462 authors have critically reviewed the manuscript.

463

464 **Acknowledgements**

465 We thank members of the for Hokkaido Forest station, Field Science Education and
466 Research Center, Kyoto University for cooperation and logistics at the field site and
467 laboratory. We also thank to Dr. Keitaro Fukushima and Ms. Chikae Tatsumi and the
468 members of Center for Ecological Research, Kyoto University and FFPRI for isotope
469 measurements. This work was supported by JSPS-KAKENHI (NO. 26292085,
470 16H04937, and 18H02241). This work was also supported by Joint Usage / Research
471 Grant of Center for Ecological Research, Kyoto University. We would like to thank
472 Editage (www.editage.jp) for English language editing.

473

474 **Compliance with ethical standards**

475 **Conflict of interest** The authors declare that they have no conflict of interest.

476

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665

666 **Table 1** Mean ± SD of mycorrhizal infection ratio and mycorrhizal root tip density of
667 *Quercus crispula* (Oak) along the soil profile. Differences in lowercase letters indicate
668 significant differences among soil layers ($p < 0.05$) based on results of multiple
669 comparison tests with Holm's adjustment.

	Mycorrhizal infection ratio (%)	Mycorrhizal root tip density (cm^{-3})
O horizon	$88.89 \pm 3.38_{a}$	$2.20 \pm 1.06_{bc}$
0-10cm	$69.57 \pm 2.39_{b}$	$8.40 \pm 1.74_{a}$
10-30cm	$61.66 \pm 7.24_{b}$	$1.57 \pm 0.52_{b}$
30-50cm	$77.45 \pm 9.72_{ab}$	$0.76 \pm 0.39_{c}$

670

671 **Figure legends**

672

673 **Fig. 1** Mean ± SD (n = 5) of vertical distribution of fine root density along the soil profile
674 of *Quercus crispula* (Oak) and *Sasa nipponica* (Sasa). Differences in lowercase letters
675 mean significant differences among soil layers for each species ($p < 0.05$) based on results
676 of multiple comparison tests with Holm's adjustment. Symbols with brackets indicate
677 significance levels of the paired t-test for tree species at each depth ($P < 0.01$ and $P < 0.1$
678 for ** and +, respectively).

679

680 **Fig. 2** (a, b) Soil nitrogen (N) pool on August 23, 2017 and (c, d) net N pool change of
681 dissolved N (NH_4^+, NO_3^-, and extractable organic N (EON)) in soil weight basis and
682 volume basis, respectively. The net N pool changes were calculated by subtracting the
683 pool size of each N species after the incubation period from initial pool size and expressed
684 as per day values by dividing by the incubation period (28 d). Data are presented as mean
685 ± SD (n = 6). Lowercase letters indicate significant differences among soil layers and
686 uppercase letters indicate significant differences among N species ($p < 0.05$) based on
687 results of multiple comparison tests with Holm's adjustment.

688

689 **Fig. 3** (a) Nitrogen (N) isotope ratios of plant leaves, bulk soils and extracts from soils
690 and ion exchange resin capsules buried at 10 cm depth, and (b) soil N pool for extracts
691 from soils of isotope measurement collected on July 30, 2016. Data are presented as mean
692 ± SD (n = 5 and 7) for $\delta^{15}N$ value of resin and leaf samples, respectively. n = 4 for each
693 N pool and $\delta^{15}N$ values of soil extracts at each horizon; n = 3 for $\delta^{15}N$ of NH_4^+ and
694 extractable organic N (EON) at 10-30 cm horizon. Lowercase letters indicate significant

differences among soil layers and uppercase letters indicate significant differences among N species ($p < 0.05$) based on results of multiple comparison tests with Holm's adjustment..

Fig. 4 Boxplot ($n = 7$) of nitrate reductase activity (NRA) in leaves and fine roots of *Quercus crispula* (Oak) and *Sasa nipponica* (Sasa). Each box shows the lower quartile, median and upper quartile. Each whisker extends from each quartile to the outermost point that does not extend beyond the upper or lower quartile by a value more than 1.5 times the interquartile range.

Fig. 1 Tateno et al.

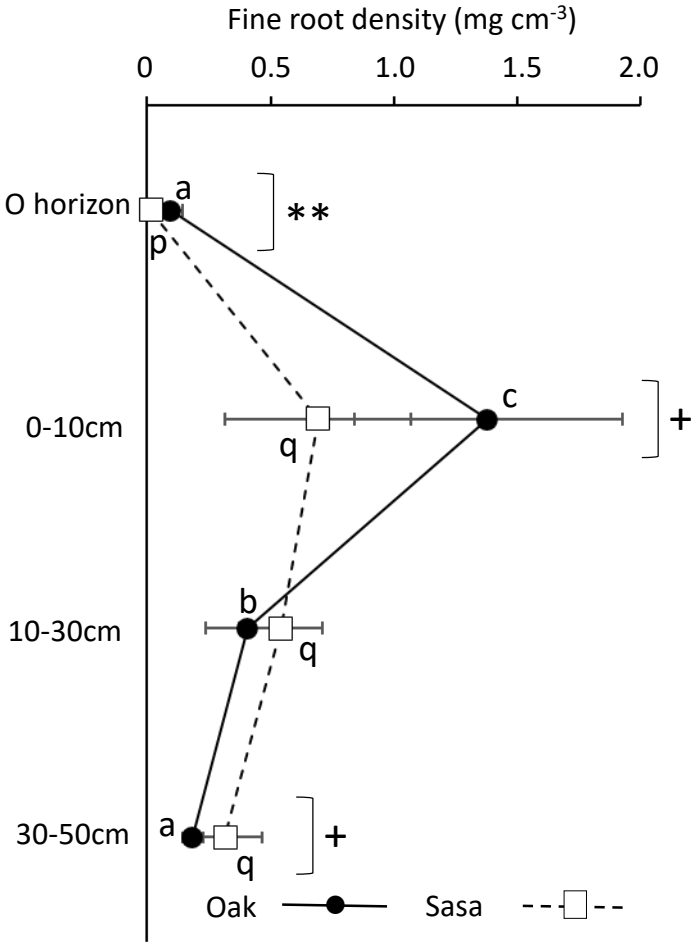

Fig. 2 Tateno et al.

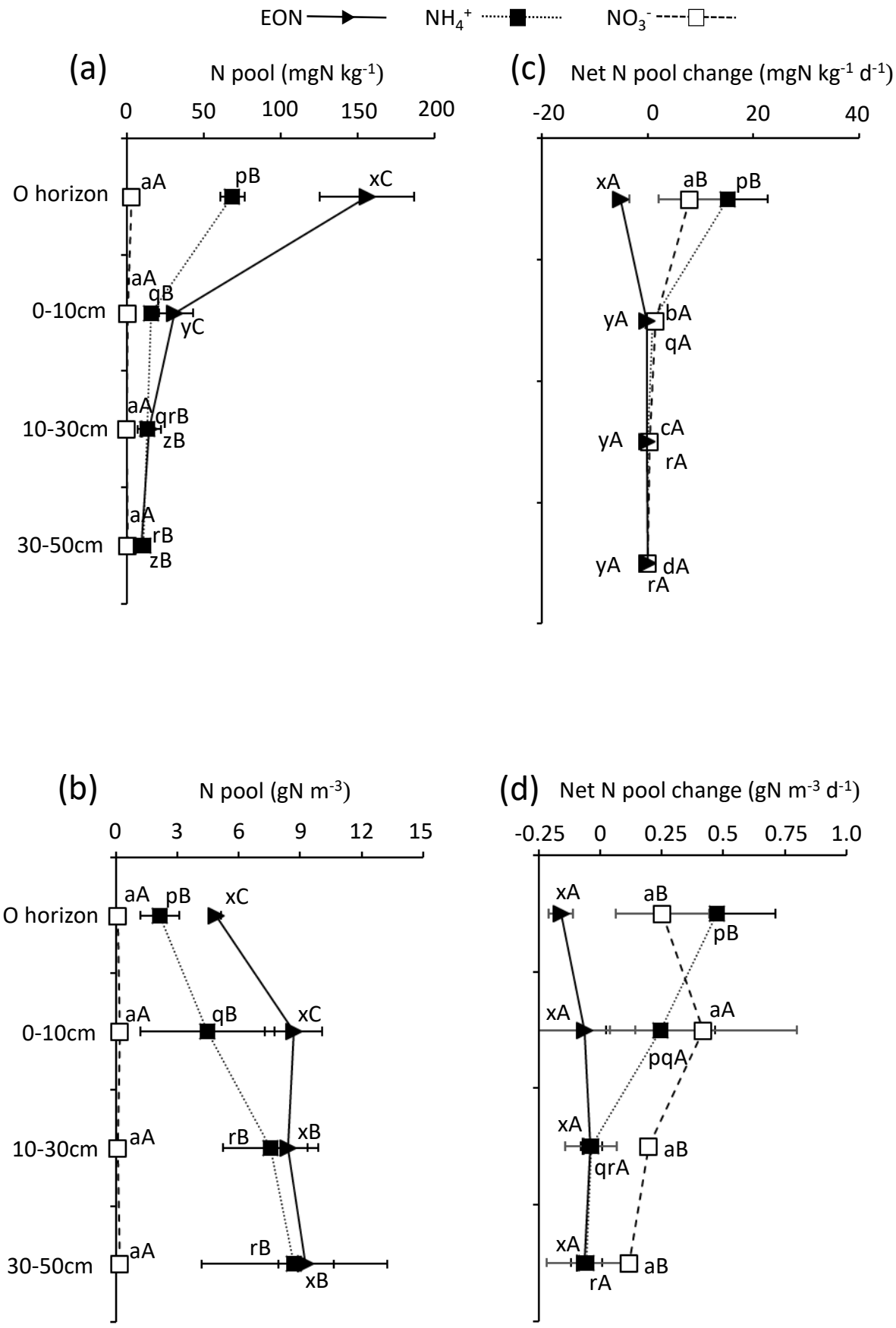

Fig. 3 Tateno et al.

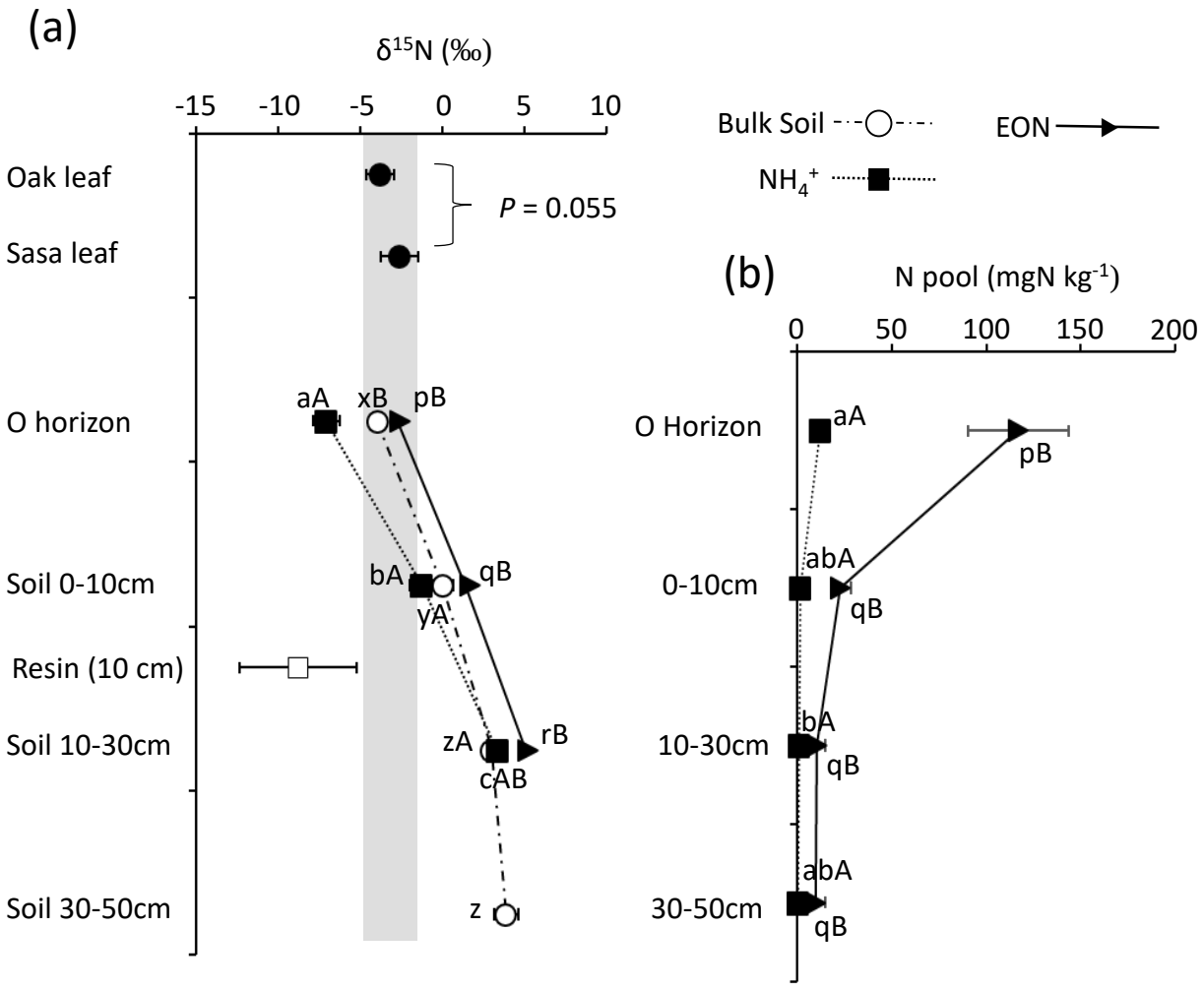

Fig. 4 Tateno et al.