Interaction between plant colonizers and ectomycorrhizal fungi through nitrogen transfer in the early stages of volcanic succession

(火山遷移初期段階における窒素移動を介した定着植物と菌根菌の相互作用)

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Contents

Summary

The vegetation composition and successional pattern are affected by the strategy of each plant through nutrient acquisition. Catastrophic disturbances represented by volcanic eruption result in nitrogen (N)-limited ecosystems. Thereafter, the replacements of species and vegetation occur with the N dynamics. Clarifying N dynamics between the early and the late colonizers is essential to understand the restoration and conservation of ecosystems. On terrestrial N cycles, mycorrhizal fungi have been focused, because they often promote N transfer from the substrates to the plants.

The effects of a pioneer species, *Salix reinii* (willow), and mycorrhizal fungi on invasive *Larix kaempferi* (larch) were studied in a greenhouse (Chapter 1). In the experiment, the willow patches affected the shoot-to-root biomass allocation on the larch seedlings and mycorrhizal fungi supplied N efficiently to larch seedlings. The *in-situ* effects were examined at the two elevations (ridge and hillslope) on Mount Koma, northern Japan, by the dynamics of stable N isotope ratio (Chapter 2). The willow shrubs mostly determined N in the tephra. The larch flexibly changed the N dependence on mycorrhizal fungi, depending on N in the tephra, while the willow depended steadily. The study was extended to more woody native plants, *Gaultheria miqueliana*, *Betula platyphylla*, *B. ermanii*, *Populus sieboldii*, *Alnus maximowiczii* and *Quercus mongolica*, on the southern slope (Chapter 3). The results suggested that most native trees had the common characteristics of N dependence on mycorrhizal fungi. With the litter and shade provided by larch, the willow decreased in cover with the decrease of mycorrhizal relationship. The larch interacted more flexibly with willows and mycorrhizal fungi, depending on N in the tephra, than the native woody plants. This research provided the new insights on the mechanisms of biological invasion and on the conservation and restoration of ecosystems.

General introduction

Nitrogen (N) dynamics is crucial to determine the function of terrestrial ecosystems (Chapin et al. 2011). Soon after large-scale disturbances, such as volcanic eruptions, landslides and glacier retreats, the habitat is generally poor in nutrients, in particular, N. The N distribution changes spatio-temporally with progressing succession. Plants use two pathways to obtain N from the substrates, directly from the substrates and indirectly through the mycorrhizal fungi (Hobbie and Hobbie 2006). Plants in N-limited ecosystems obtain nitrogen effectively with mycorrhizal fungi (Simard et al. 2002). The utilization of two N transport pathways varies with successional stages even on a plant species. To understand the mechanisms of succession, therefore, I focused on the N transfer pathways of various plants along succession. The direct N uptake is not sufficient for the growth in N-limited habitats. I predicted that the N dependence of plants on mycorrhizal fungi was high when N in the substrates was low, and *vice versa*.

Mount Koma (1,131 m elevation), located in the southern part of Hokkaido Island, Japan, is one of the active volcanoes worldwide. The eruption in 1929 produced enormous tephra, mostly consisting of volcanic ash and pumice (Takeuchi and Nakamura 2001) and destroyed the former vegetation completely. The succession has proceeded from the bottom, and thereafter the mountain develops a wide range of successional stages from bare ground to forest, depending on the elevation (Photos 1 to 4). I utilized these vegetation types to detect relationships between plants and mycorrhizal fungi at landscape level.

In disturbed habitats, facilitation (positive interspecific interaction) has a role on progressing succession, as well as plant-fungus interaction (Callaway 2007). On Mount Koma, the facilitation is promoted by an early pioneer willow, *Salix reinii* Franch. et Savat. (Tsuyuzaki et al.

2012). Shrub patches formed by *S. reinii* improve microclimate, tephra moisture and fertility, in particular, N, and provide seedbeds (Uesaka and Tsuyuzaki 2004), suggesting that early colonizers forming shrub patches support N for the late colonizers. Therefore, N transfer of plants was examined at the habitat level. However, difficulties overlay to clarifying multiple interactions between the plants, fungi and substrates in the field (Hobbie and Högberg 2012). Prior to the field surveys, therefore, greenhouse experiments were conducted to evaluate the tripartite interactions between larch, willow and mycorrhizal fungi through the substrates.

Biological invasion often occurs in disturbed areas, including post-erupted volcanoes (Nakamura 1985). Nowadays, the biological invasion of *Larix kaempferi* (Lam.) Carr. (Japanese larch), which is non-native to Hokkaido (Nagamitsu et al. 2014), is conspicuous on the southern slope on Mount Koma (Kondo and Tsuyuzaki 1999). The seedlings of *L. kaempferi* establish well in the shrub patches (Akasaka and Tsuyuzaki 2005), showing that the facilitation by willow patches promotes the biological invasion. Most of all species in *Larix* develop symbiosis with mycorrhizal fungi (Qu et al. 2003), most of which are host-specific *Suillus* fungi developing extra-radical mycelia (Photo 5) (Read 1991). Few generalist mycorrhizal fungi, which do not show high host preferences, grow on the larch roots (Nara 2006; Usami et al. 2007; Kayama et al. 2015). I observed the effects of mycorrhizal fungi on the larch establishment with N status in the substrates to confirm if the effects were changed.

The native forests are composed mostly of deciduous, broad-leaved forests, consisting of species in genera *Salix* (willow), *Betula* (birch), *Populus* (poplar), *Alnus* (alder) and *Quercus* (beech) (Yoshioka 1966). These trees develop facultative and generalist mycorrhizal fungi (Szuba 2015, He et al. 2016). Therefore, differences in N uptake between the exotic larch and native trees were expected in relation to N status in the tephra. The results explained why the

invasion of larch suppressed the establishments of native trees. In addition, the effects of larch litter on plants and fungi were investigated, because a large amount of litter was produced in the forests and seemed to affect the vegetation development.

There were two exceptional woody species on N uptake, alder (*Alnus maximowiczii* Callier, Betulaceae) developing nitrogen-fixing actinobacteria and wintergreen (*Gaultheria miqueliana* Takeda, Ericaceae) developing ericoid mycorrhiza. *G. miqueliana* Takeda (Ericaceae) forms shrub patches, as well as *S. reinii*, and develops ericoid mycorrhizal fungi (Fukuchi et al. 2011). This plant established well on Mount Koma, although the distribution was different from the distribution of *S. reinii*. This plant is inhibitive to the cohabitants because of the evergreen (Uesaka and Tsuyuzaki 2004). The behavior of *G. miqueliana* was examined to characterize *S. reinii* by the comparisons between the two species.

The N dependence of plants on ectomycorrhizal fungi was quantified by a two-pool fungal model, using stable N isotope ratio (Hobbie and Agerer 2010). I hypothesized that (1) *S. reinii* patches promoted the growth of *L. kaempferi* seedlings on N-limited tephra, (2) mycorrhizal fungi accelerated N transfer particularly for *L. kaempferi*, (3) the N dependences of plants on mycorrhizal fungi were related to the invasiveness, and (4) litter and shade provided by the larch deteriorated N quality in the tephra with decreasing *S. reinii* cover. Comparing with native woody plants, the invasion of *L. kaempferi* was attributed to the tact that the larch used the two N transfer pathways as the situation demands. The litter and shade of larch affected the successional sere after the establishment. These findings were discussed with the worldwide biological invasions of conifers.

Photo 1. View of southern slope on Mount Koma in 2013

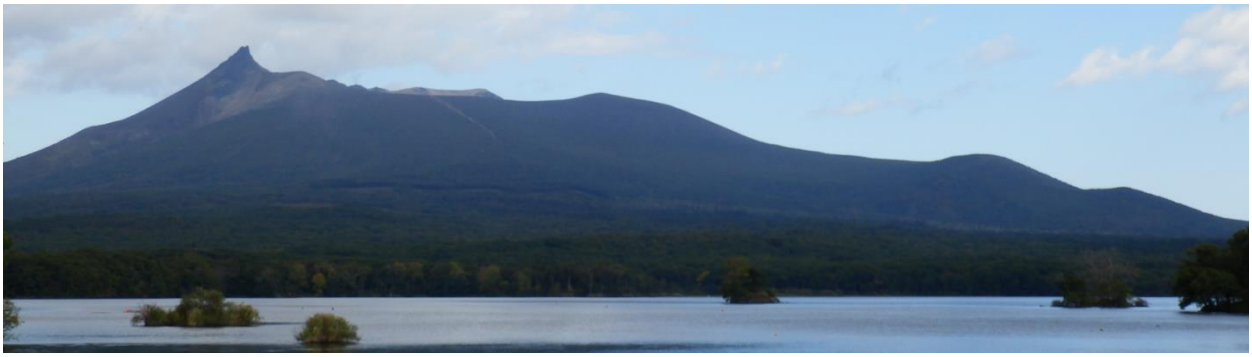
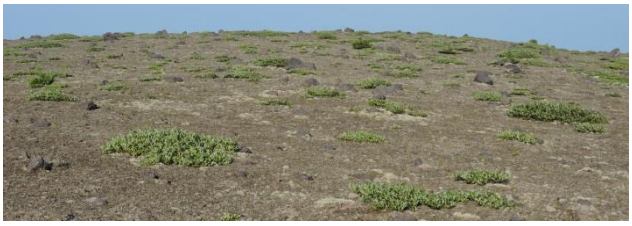


Photo 2. South-flank view on Mount Koma

(a) in 1991 (by Shinji Takarada, Geological Survey of Japan) (b) in 2016

Photo 3. Elevational landscape views on Mount Koma in 2013

(a) 930m (b) 890m



(c) 680m (d) 480m (e) 380m

Photo 4. Representative habitats in 2013

(a) Bare ground

(b) *Larix* understory

(c) *Gaultheria miqueliana* patch

(d) *Salix reinii* patch

Photo 5.
Development of mycorrhizal fungi at 680 m elevation on Mount Koma
(a) Mycorrhizal root of *Larix kaempferi* and *Salix reinii*
(b) Mycorrhizal mycelia
(c) Sporocarp and rhizomorph of *Suillus cavipes*

(a) (b) (c)

Chapter 1

Effects of *Salix reinii* and mycorrhizal fungi on the growth and nitrogen acquisition of *Larix kaempferi* seedlings in greenhouse experiment

1.1 Abstract

Pot experiments were conducted with seeds and seedlings of *Larix kaempferi* (Lam.) Carr. (larch), to investigate the effects of willow (*Salix reinii* Franch. et Savat.) shrub patches and mycorrhizal fungi on the biomass and nitrogen (N) acquisition of larch seedlings. To examine the effects of the above-ground (shade) and below-ground of willow shrubs, two experiments were conducted. A fungicide was applied to investigate the effects of mycorrhizal fungi. Because the seedling biomass became low in the pots shaded by the willow patches, all other experiments were carried out in full light. Shoot/root (S/R) biomass ratio was 2−3 times higher when current-year larch seedlings were grown in the presence of willow patch than in the absence of willow patch. Therefore, larch seedlings used their resources more effectively by growing with willow patch.

Although larch and willow reduced inorganic N in the tephra, the larch acquired inorganic N more effectively by the symbiotic relationships with mycorrhizal fungi. With the positive mycorrhizal effect, foliar N% of larch seedlings was 42% higher than that of larch seedlings grown in weaken mycorrhizal relation by the application of fungicide. This effect was shown only in larch grown with willow and was not observed without willow. In contrast with larch, foliar N% of willow and inorganic N concentration in willow tephra were not affected by fungicide. Instead, higher electrical conductivity (EC) and lower pH in willow tephra than in larch tephra suggested higher ionic contents in the rhizosphere of willow than in that of larch. The willow shrubs affected the resource allocation of larch seedlings and mycorrhizal fungi expanded nitrogen pool of larch seedlings in the presence of willow, whereas the effect of mycorrhizal fungi on N status of willow was not observed in greenhouse experiment.

1.2 Introduction

Primary succession starts immediately after large-scale disturbances, such as volcanic eruption and glacier retreat, which result in denuded habitats, and proceeds slowly because of least nitrogen (N) in the substrates (Schulze et al. 2005). Willows as pioneers in the early stages of succession often facilitate the late colonizers by quantitatively and qualitatively improving N status in the substrates (Michelsen et al. 1998; Hobbie et al. 2000; Kuzovkina and Volk 2009).

After the 1929 eruptions on Mount Koma in Hokkaido, Japan, the substrates have been ameliorated by one of the willows, *Salix reinii* Franch. et Savat. (Tsuyuzaki et al. 2012) forming patches. Shrub patches often facilitate the establishment of the late colonizers (Marchese 2003). However, the shrub patch is a safe site not only for natives but also for exotics, in particular, biologically-invasive species. In fact, *Larix kaempferi* (Lam.) Carr. (larch) is now predominant on the southwestern slope of Mount Koma, immigrating from the transplantation on the bottom of the mountain during 1953 and 1963 (Kondo and Tsuyuzaki 1999). Ecological succession is often determined by the earliest colonizers because they regulate the subsequent biotic and abiotic conditions (Callaway 2007). These studies suggest that the mechanisms of biological invasion utilizing willow shrub patches should be clarified for predicting the succession and conservation of ecosystems.

Not only plant-to-plant interactions but also plant-fungus interactions determine ecosystem structures (Smith and Read 2010; Rodriguez et al. 2012; Kayama et al. 2015). Willows are facultatively-mycorrhizal (Meyer 1973). Larches are host-specific, obligatory mycorrhizal (Meyer 1973) and allocate low biomass to the roots (Vogt et al. 1986; Simard et al. 2012), suggesting that the interactions promote the biological invasion. The distribution of mycorrhizal

fungi differs greatly between the habitats (Titus and Tsuyuzaki 2002), suggesting that *S. reinii* alters the activities of mycorrhizal fungi.

Because the effects of shrub patches are arisen from above-ground and below-ground interactions to the cohabitants, including *L. kaempferi* and mycorrhizal fungi, the two effects should be separately evaluated. However, the separation has difficulties in field. To evaluate the two effects separately, therefore, greenhouse experiments were conducted. I hypothesized that, when the shade was weak, *S. reinii* promoted the growth of *L. kaempferi* seedlings by the cooperation with mycorrhizal fungi through N uptake. To test this hypothesis, I measured (1) the biomass, including allometry, of larch seedlings grown with or without *S. reinii* and (2) N status in the substrates and leaves of *L. kaempferi* and *S. renii* with or without the reduction of mycorrhizal fungi.

1.3 Materials and methods

Field sampling

Seeds, plants and tephra were sampled on Mount Koma located in the southern part of Hokkaido Island, Japan (42°03′N, 140°40′E; 1,133 m a.s.l.) (Yoshioka 1966). The mountain erupted catastrophically in 1929. On the mountain, *S. reinii* facilitates the cohabitants, including *L. kaempferi*, by ameliorating the substrates (Akasaka and Tsuyuzaki 2009). The climate is classified into cold-temperate. In 2011 and 2012 when the samplings were done, the annual precipitation was 942 mm. The annual mean, maximum and minimum temperatures were 8.3,

32.6, and −17.2 ℃ at the Mori Climatological Observatory which is located 9 km from Mount Koma at 10 m elevation.

The larch seeds, *S. reinii* patches and tephra were sampled at around 680 m elevation on southwestern hillslope in late October 2011. The tephra was sampled below the *S. reinii* shrubs to obtain the tephra affected by the shrubs. The sizes of sampled willows were approximately 2,000 cm^2 in patch area to reduce the patch size effects. The tephra was sieved through a 2-mm mesh. Then, these samples were brought to the laboratory.

Larch seedlings (5−7 cm in height) and willow patches (10−15 cm in height) were sampled in May 2012. These samples were used to investigate the variations of chemical properties of tephra after the growth of two species in each pot for 150 days. These samples were also used to investigate the effect of mycorrhizal fungi on the N acquisition of larch and willow.

The seeds were stored at 4 ℃ until use. The plant samples were transplanted on tephra in the greenhouse of Hokkaido University. All the pots were placed on a stainless steel table at 1 m in height. Water was sprinkled twice in the morning and afternoon every day until saturated. The greenhouse was kept at 25−30 ℃ in daytime and 20−25 ℃ at night.

The growth of larch seedlings

All the experiments were carried out in the greenhouse of Hokkaido University in 2012 (Fig. 1-1). The combined effects of the above- and below-ground of *S. reinii* shrubs on the larch seedlings were examined on vermiculite (VermiLite®, Taiheiyo Coal Services & Transportation Co. Ltd, Japan) or tephra (Fig. 1-1a). The pot sizes were 10 cm in depth and 60 cm × 40 cm in surface area when the willows were planted to allow the root development and were 16 cm in depth and 25 cm × 20 cm in surface area when willow was not planted. To each pot, seven seeds

of larch were sown in early January 2012. Ungerminated seeds were removed 30 days after the experiments. The seeds were sown beneath and outside the shrubs in the pots. The growth of seedlings in those different light conditions was compared. The effects of below-ground of willows were examined by excluding the effects of above-ground (shade) apart from the seedlings. The larch seedlings were harvested after 114 days of starting the experiment. The seedlings were weighed on three organs, root, leaves and stem. Shoot implies the leaves and stems in this study. Photosynthetic active radiation (PAR) was measured 40 times beneath and outside shrubs in a daytime of early May 2012, by a PAR sensor (3415FSE, Spectrum Tech., Aurora, IL, USA).

Variations of chemical properties in tephra

The larch seedlings and willows, sampled from Mount Koma in late May 2012, were planted in each pot (7 cm in depth and 5 cm × 5 cm in surface area) filled with tephra in early June 2012 (Fig. 1-1b). The plants were harvested in mid-November after the growth for 150 days. The inorganic N, electrical conductivity (EC) and pH were measured on the tephra and were compared with those of intact tephra and supplied water.

The effects of fungi on N in the leaves and chemical traits in the tephra

A mesocosm experiment was carried out to investigate the effects of mycorrhizal fungi on N in the leaves and chemical traits in the tephra by the application of fungicide. Larch seedlings and willow shrubs, sampled from Mount Koma in late May 2012, were planted to each pot in early July 2012 (Fig. 1-1c). The two types of two-chambered pots were prepared: the two chambers were separated by a wall, or connected by a 41-μm nylon mesh (4 cm in diameter, Millipore,

Ireland) that allowed the movements of mycorrhizal mycelia between the chambers (Teste et al. 2006). The size of each chamber was 9.5 cm in depth and 6.5 cm × 5 cm in surface area. In addition, a fungicide was applied to half the number of pots to investigate the effects of fungi on the seedling growth by the reduction of mycelial growth. The fungicide was propiconazole (25% solution, Chiruto®, Syngenta Japan). The 0.5 g of fungicide was diluted by 1 L of deionized water (Teste et al. 2006). The propiconazole reduces the growth of mycelium by 55%. The fungicide was applied 5 times at three day intervals in mid-August 2012. The foliar N was measured 100 days later. The concentrations of inorganic N, EC, and pH of tephra were concurrently measured.

Measurements of the chemical characteristics in tephra and N in leaves

Inorganic N (NH_4^+, NO_2^-, and NO_3^-), EC and pH in the tephra were measured. Inorganic N was extracted from the tephra by a 2 N KCl solution before drying. NH_4^+, NO_2^-, and NO_3^- concentrations were measured using a QuAAtro colorimetric analyzer (Bran+Luebbe, Norderstedt, Germany). After air-drying the tephra for one week, suspensions were made by 5 g of tephra mixed with 50 ml of deionized water. The pH and EC of the suspensions were measured by a multiple-water checker (WA-2017SD, PE-03K7 Sato Shouji Inc., Japan). Foliar N in the larch seedlings and willow shrubs were measured by a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific, Yokohama, Japan).

Statistical analysis

Prior to the statistical comparisons between two groups, the normality and homoscedasticity of the data were checked by Shapiro–Wilk test and *F*-test, respectively. When these two conditions

were satisfied, parametric *t*-test was used. If not, non-parametric Wilcoxon rank-sum test was used. By this way, the biomass and shoot/root (S/R) ratio of larch seedlings were compared between the willow and non-willow pots on the vermiculite and between the vermiculate and tephra. The effects of fungicide on N in the leaves and chemical properties in the tephra were also compared by the way.

Prior to the statistical comparisons between more than or equal to three groups, the normality and homoscedasticity of the data were checked by Shapiro-Wilk test and Levene's test, respectively. The inorganic N, EC and pH were compared between the intact tephra, larch-planted tephra (hereafter, larch tephra), willow-planted tephra (willow tephra) and supplied water by Tukey's HSD *post-hoc* test after one-way analysis of variance (ANOVA) or pairwise comparisons after Kruskal-Wallis test, depending on the parametricity of the data.

All statistical analyses were performed using the R program (ver. 3.1.2) (R core team 2014).

1.4 Results

The shade effects of willow shrubs on the growth of larch seedlings

PAR was significantly higher outside the shrub than inside (one-way ANOVA, $P < 0.001$), showing shade was derived by the overstory willow patches (Fig. 1-2). The larch seedlings on vermiculite showed three times higher total biomass under un-shade, i.e., in full light (0.114 ± 0.008 g, $n = 3$) than under shade (0.037 ± 0.004 g, $n = 2$) (*t*-test, $P < 0.01$). The larch seedlings showed lower biomass in root, shoot and stem under willow shade than in light (*t*-test, $P < 0.05$), while they did not change S/R ratio between the two light conditions (*t*-test, $P = 0.08$).

The effects of willow shrub on the growth of larch seedlings

To clarify the effects of *S. reinii* on the belowground, the effects of shade on the aboveground was excluded by placing larch seedlings outside the shrub (Fig. 1-1). The shoot growth of larch seedlings was higher with the willow (0.092 ± 0.001 g, n = 3) than without the willow (0.043 ± 0.006 g, n = 3) on the vermiculite (t-test, $P < 0.01$) (Fig. 1-3). The root biomass of seedlings without the willow was 0.033 ± 0.004 g and was not different from that with the willow (0.023 ± 0.002 g) ($P = 0.07$).

The S/R ratio of seedlings was higher with the willow (4.06 ± 0.16, n = 3) than without the willow (1.30 ± 0.05, n = 3) (t-test, $P < 0.001$) on the vermiculite. This high S/R ratio at the presence of willow was also shown on the tephra (2.88 ± 0.37 with the willow vs. 1.43 ± 0.21 without the willow, n = 3, t-test, $P < 0.01$). The total biomass of larch seedlings on the tephra was higher without the willow than with willow, which was caused by the higher root biomass of larch seedlings in the absence of willow ($P < 0.001$). Although the response of biomass of larch seedlings to the substrates was different, larch seedlings allocated their resources more to the shoot with the willow on both the substrates, which allowed larch seedlings to use their resources effectively in nutrient-poor conditions.

Chemical traits in tephra

The total biomass of larch seedlings was lower on the tephra than on the vermiculite (t-test, $P < 0.05$), independent of the willow patches (Fig. 1-3), indicating that the tephra contained lower nutrients than the vermiculite. When the larch seedlings and willows sampled from Mount Koma were separately grown in full light for 150 days, inorganic N in *Larix* (larch) tephra and

Salix (willow) tephra was lower than inorganic N in intact tephra (one-way ANOVA, $P < 0.001$) (Fig. 1-4). The tephra contained more ammonium ions than nitrate ions on both the pots of larch and willow (Wilcoxon rank-sum test, $P < 0.001$). The EC was comparable in larch tephra and willow tephra but was lower in larch tephra than in intact tephra (one-way ANOVA, $P < 0.001$). The influence of supplied water on inorganic N and EC in tephra was likely to be negligible. The pH in larch tephra and willow tephra was intermediate between pH of the intact tephra and supplied water.

The effects of fungi on N in tephra and leaves

When the larch seedlings grew with the willow separated by a 41μm mesh, the larch tephra showed higher concentrations of total inorganic N with fungicide (5.88 ± 0.37 mg/kg, $n = 4$) than without fungicide (3.71 ± 0.24 mg/kg, $n = 7$) (Wilcoxon rank-sum test, $P < 0.01$) (Fig. 1-5). The higher inorganic N resulted from higher NH_4^+ (*t*-test, $P < 0.01$) and NO_3^- (Wilcoxon rank-sum test, $P < 0.05$).

However, foliar N% of larch became less when fungicide was applied (1.00 ± 0.10 N%) than the case that fungicide was not applied (1.42 ± 0.07 N%) (*t*-test, $P < 0.01$). This indicated less effective N uptake by larch seedlings with less mycorrhizal relation. However, when larch seedlings were grown without willow, inorganic N concentrations (Wilcoxon rank-sum test) and foliar N (*t*-test) were not affected by the application of fungicide ($P > 0.11$), which indicated that the growth of larch seedlings with willow might be favorable to N acquisition under the influence of mycorrhizal relation.

On the willows, inorganic N in the tephra and total N in the leaves were not affected by the fungicide and not by larch seedlings (*t*-test, $P > 0.10$) (Fig. 1-5). However, willow foliar N%

(2.06 ± 0.08 N%, n = 26) was higher than larch foliar N% (1.21 ± 0.06 N%, n = 20) (t-test, P < 0.001) regardless of fungicide and growth with each other. This implied that willows could effectively absorb N with their dense root system even with less mycorrhizal relation and that larch seedling did not affect N acquisition of willow.

EC and pH in the tephra for both the species were not affected by fungicide application whether each species grew separately or with the other species (Fig. 1-5). EC in the willow tephra (606 ± 27 μS/cm, n = 26) was higher than that of larch tephra (512 ± 37 μS/cm, n = 20) (t-test, P < 0.5) and pH of willow tephra (5.72 ± 0.04) was lower than that of larch tephra (5.86 ± 0.05) (t-test, P < 0.5). These suggested that the soil formation occurred more in willow rhizosphere than larch rhizosphere.

1.5 Discussion

The effects of willow shrub on the resource allocation of larch seedlings

Most *Larix* species are shade-intolerant (Walters and Reich 2000), suggesting that the growth and survival rates are highly influenced by solar radiation. The density of *L. kaempferi* seedlings is high in *S. reinii* patch on Mount Koma despite the shade (Akasaka and Tsuyuzaki 2005). This preference of larch seedlings to shrub patches is attributed to the improvement of microclimate, nutrient, and water (Tsuyuzaki et al. 2012). The strategy of resource allocation of plant colonizers in nutrient poor ecosystem is crucial for the successful recruitment. On Mount Koma, larch seedlings allocate more resources to the roots on the bare ground than in the willow patch (Akasaka and Tsuyuzaki 2005). Greenhouse experiment made it clear that the willow resulted in

the higher S/R ratio of larch seedlings on both vermiculite and tephra. The lower biomass of larch seedlings on the tephra than on the vermiculite implied unfavorable condition for the growth of larch seedlings on the tephra. Therefore, the growth of larch seedlings with *S. reinii* patch leads to the better performance of larch seedlings on Mt. Koma.

The nitrogen limitation

S. reinii shrubs accumulate carbon and nitrogen in the tephra by the litter accumulation inside the patch due to the growth form of shrub (Uesaka and Tsuyuzaki 2004). However, another trait of willows is efficiency in acquiring and using N in the substrates to the extent to be used for N removal in N-polluted region (Yang et al. 2015). When *S. reinii* grew during May and June on Mount Koma or in the similar condition like greenhouse, inorganic N might be exhausted, as shown in the greenhouse experiment in which larch and willow were grown for 150 days since late May. Given that *Larix* species produced the low root biomass, mycorrhizal root system was crucial to the acquisition of N in N-limited habitats. This is because mycorrhizal fungi take advantage of longer exploration and increased surface area of mycorrhizal mycelium as well as higher enzymatic capability for N uptake (Schimel and Bennett 2004).

The effects of mycorrhizal fungi on N acquisition of larch seedlings

Larches are obligatory-mycorrhizal whereas willows are facultative-mycorrhizal. The larch makes species-specific mycorrhizal symbiosis with *Suillus* species which belong to the exploration type of long distance (Read 1991; Hobbie and Agerer 2010). Larch is expected to be more sensitive to fungicide. When the larch seedling grew with willow, the higher concentration of inorganic N in larch tephra and lower larch foliar N% after the application of fungicide reflect

the deteriorated efficiency in N acquisition of larch with reduced mycorrhizal influence. Both the willow and mycorrhizal fungi together promote the N nutrition of larch seedlings.

Fig. 1-1 Scheme of greenhouse experiments for evaluating (a) the effects of *S. reinii* patches on *L. kaempferi* seedlings. The effects of shade by willow patches are excluded by separating approximately 30 cm. (b) the effects of *S. reinii* and *L. kaempferi* on the tephra chemistry. (c) the effects of mycorrhizal fungi on N status of *L. kaempferi* seedlings and tephra by fungicide and compartment pot. The chambers in each pot was blocked by a wall or connected by a 41-μm nylon mesh (4 cm in diameter).

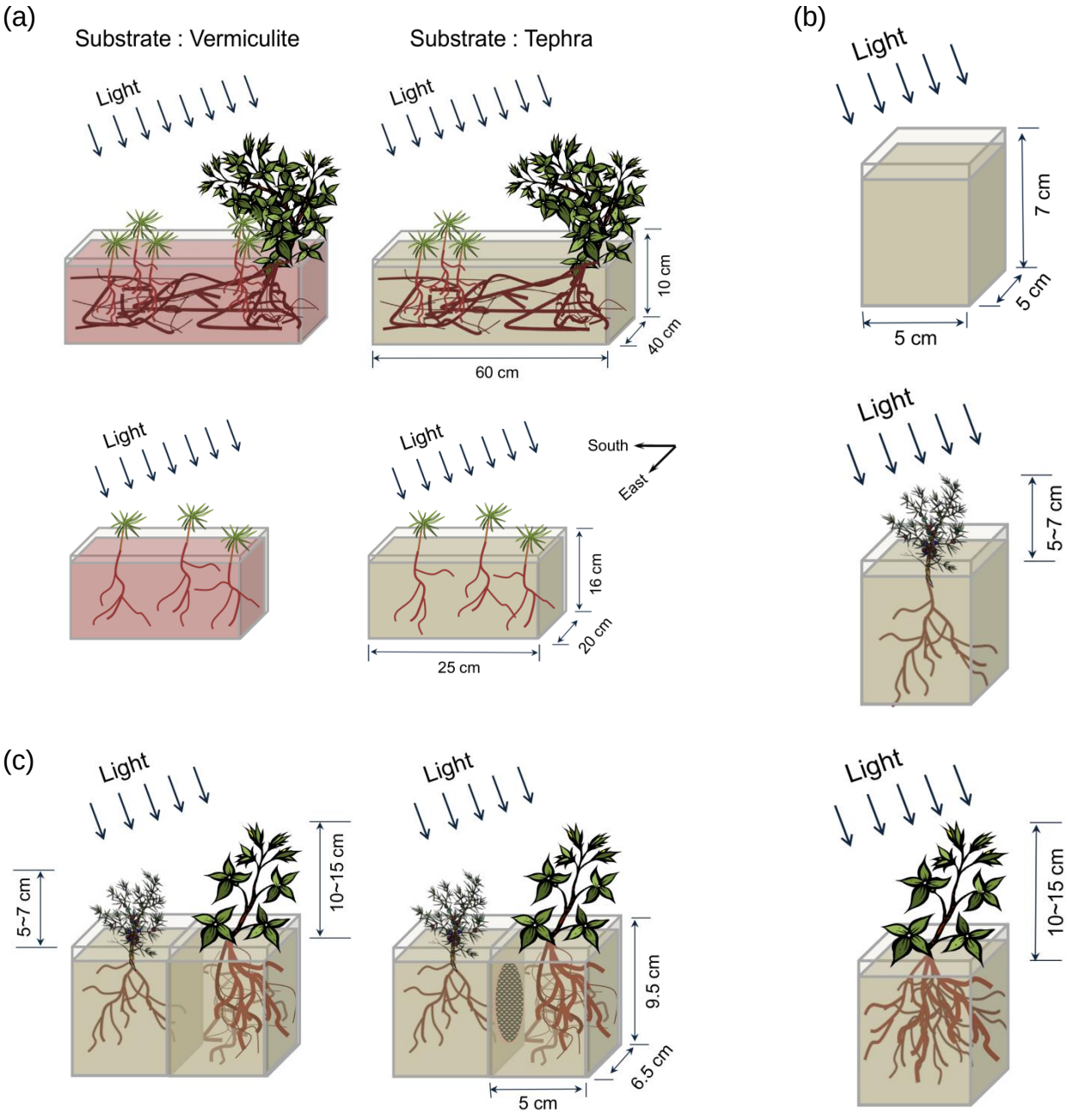


Fig. 1-2 The effects of shade by willow patches on the biomass of *L. kaempferi* seedlings in different light conditions. (a) PAR under *S. reinii* patch (S, n = 40) and in light (L, n = 40) on the vermiculate substrates. (b) biomass (g, dry weight) on above- and below-ground (n = 2 for S and n = 3 for L). (c) shoot/root (S/R) ratio. Mean (bars) is shown with standard error. Significant differences (t-test): n.s. = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

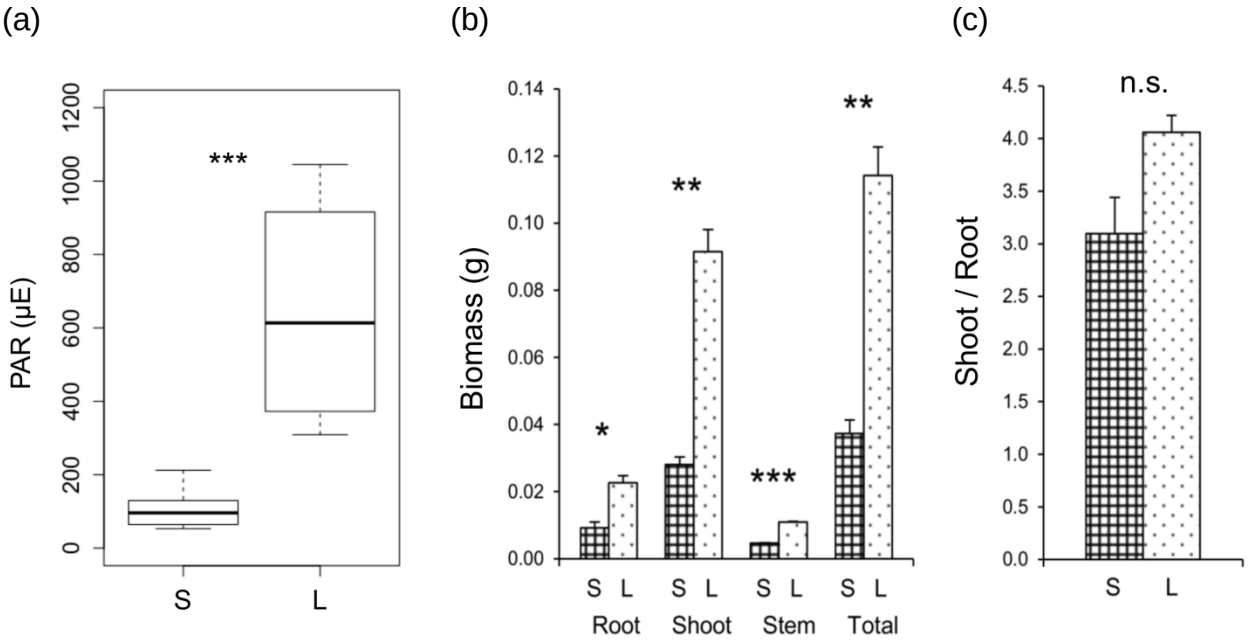
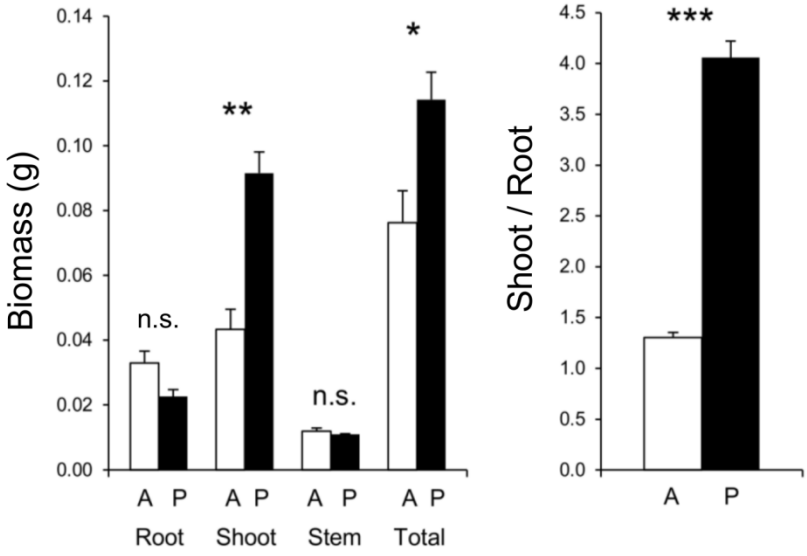


Fig. 1-3 The effects of willow, eliminating shade, shrubs on the biomass and S/R ratio of larch seedlings. (a) on vermiculite and (b) on tephra. Each sample size is three. A: willow absent and P: present. Mean (bars) is shown with standard error. Significant differences are investigated between A and P by *t*-test: n.s. = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

(a)



(b)

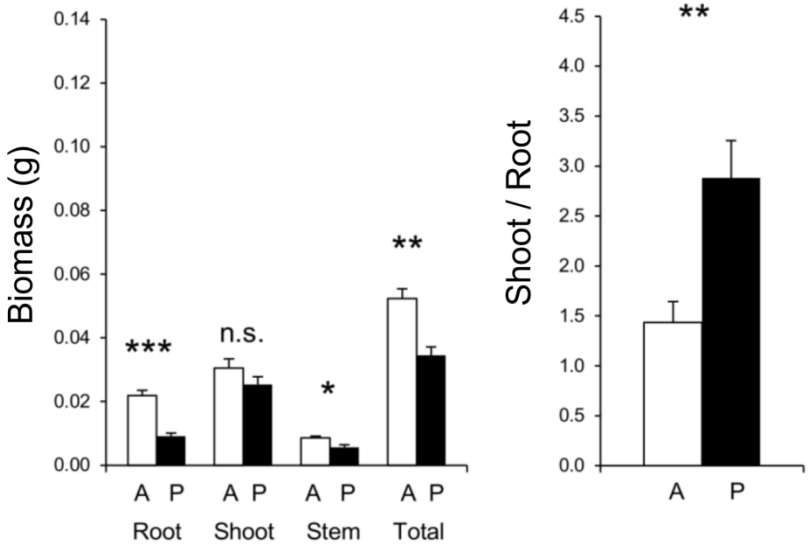


Fig. 1-4 The concentrations of inorganic N, EC and pH in intact tephra (n = 3), supplied water (n = 2), tephra 150 days after larch seedlings grew (*Larix* tephra, n = 19) and tephra 150 days after willows grew (*Salix* tephra, n = 7). Different lower-case letters indicate significant differences investigated by Tukey's HSD test after one-way ANOVA (the P-values are shown in each graph).

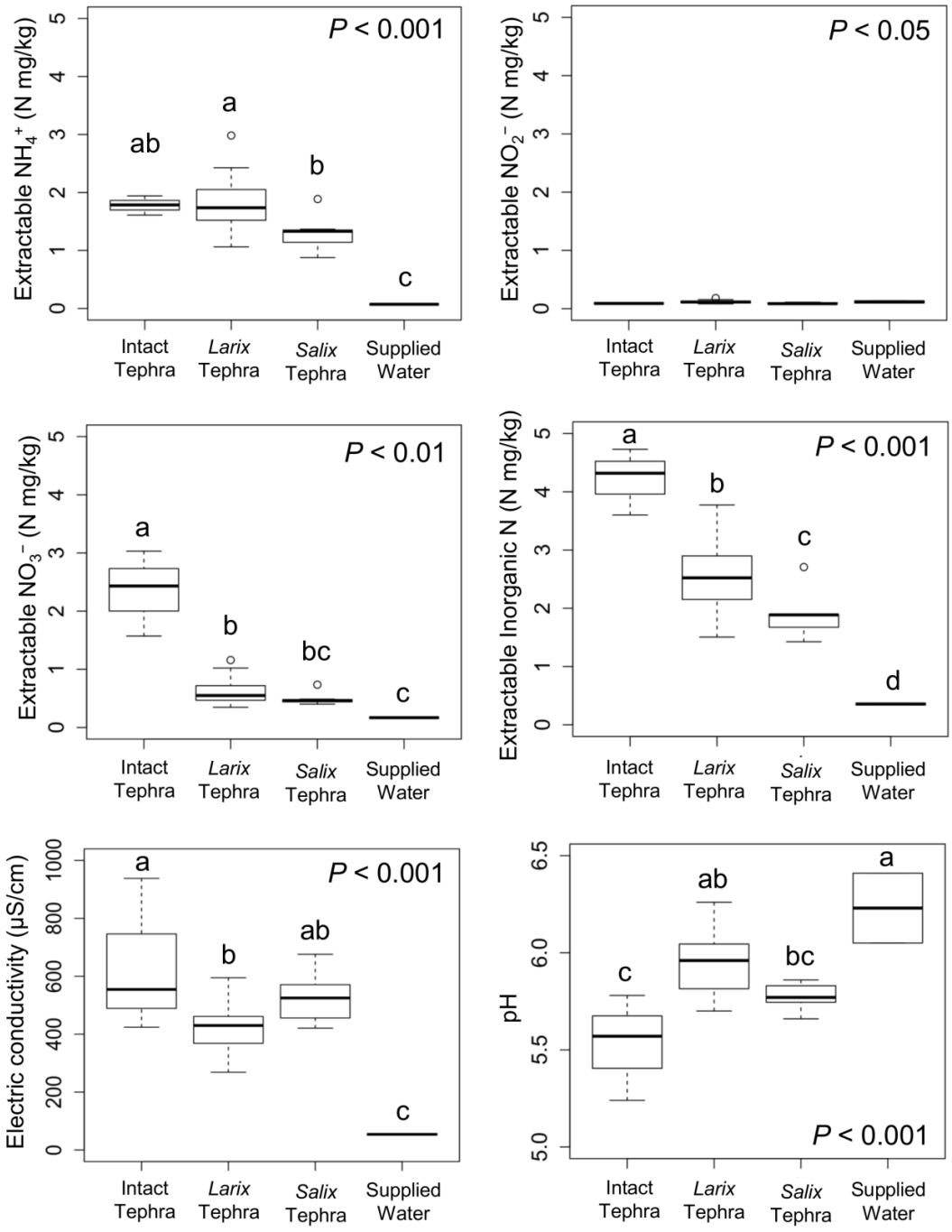
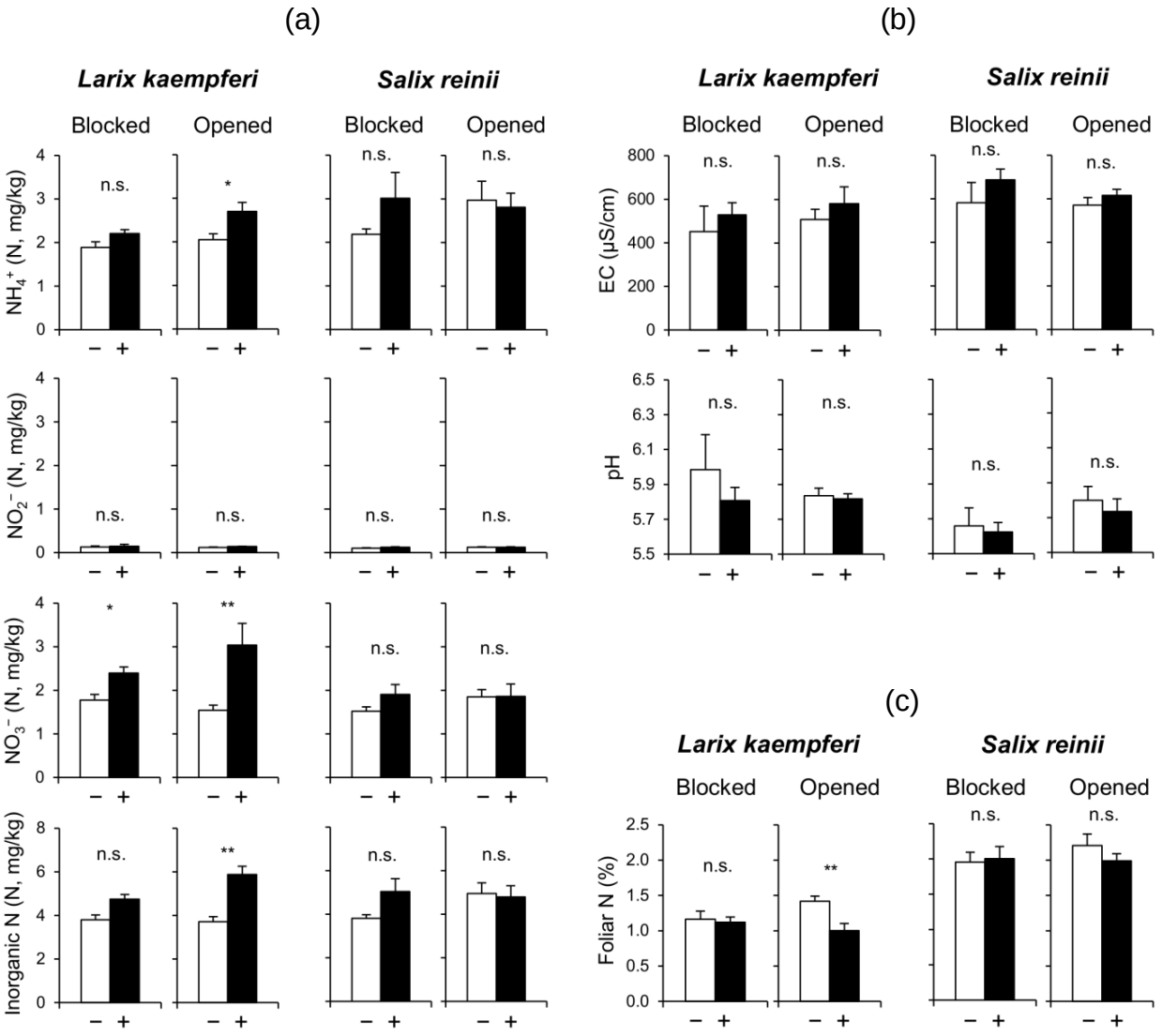


Fig. 1-5 The concentrations of (a) NH_4^+, NO_2^-, NO_3^- and inorganic N in tephra, (b) EC and pH in tephra and (c) foliar N% in the *L. kaempferi* seedlings and *S. reinii* shrubs grown on tephra for 100 days with (indicated by +) and without (-) fungicide applications. Pot type: chambers "blocked" by walls and "opened" and connected by 41µm nylon mesh. Mean (bars) is shown with standard error (n = 4−9). Significant differences are investigated by t-test: n.s. = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.



Chapter 2

Differences in nitrogen redistribution between early and late plant colonizers through ectomycorrhizal fungi on the volcano Mount Koma

2.1 Abstract

Relationships involving the transfer of nitrogen (N) among *Salix reinii* (willow), *Larix kaempferi* (larch), and mycorrhizal fungi were investigated in a ridge and hillslope on the volcano Mount Koma in northern Japan using a two-pool fungal model. This model estimated N transfer among the examined taxa by measuring changes in the stable isotope ratio of N ($\delta^{15}N$). Although N content in tephra was low at both the sites, it was higher on the ridge than on the hillslope, and higher in the willow patch than on bare ground or in the larch understory. The non-mycorrhizal sedge (*Carex oxyandra*) exhibited non-significant differences between the two sites regarding $\delta^{15}N$ for N obtained from tephra. Larches developed a relationship with larch-specific *Suillus* mycorrhizal fungal species in the roots, and had a lower foliar $\delta^{15}N$ on the hillslope than on the ridge. The larch $\delta^{15}N$ increased during the growing season, while the willow $\delta^{15}N$ remained stable. The dependence of larch on mycorrhizal fungi for N uptake was 3−5% on the ridge and 56−76% on the hillslope in autumn. Therefore, larches exhibited a flexible symbiotic relationship with mycorrhizal fungi for obtaining N. Over 45% of the N taken up by willow plants was obtained from mycorrhizal fungi at both the sites. In conclusion, willow plants promoted N deposition in tephra through the litter supply, and formed a stable relationship with mycorrhizal fungi. This enabled successful revegetation with larch plants, which exhibited flexibility in terms of N uptake (i.e., dependent on mycorrhizae or from tephra).

2.2 Introduction

When woody plants grow in the presence of mycorrhizal fungi, they can efficiently absorb nutrients, particularly nitrogen (N) and phosphorus, through the mycorrhizae (Simard et al. 2002; Read and Perez-Moreno 2003). The mycorrhizal fungi obtain carbon compounds not synthesized in mycorrhizae from the host plants (Hobbie and Hobbie 2006). Mycorrhizal associations provide other benefits to the plants, including bioremediation and stress alleviation (Finlay 2008). These symbiotic relationships have evolved for more than 90% of terrestrial plants after the Ordovician period (Cairney 2000).

In N-limited ecosystems, such as those during the early stages of vegetation succession, the establishment of woody plants is enhanced by N uptake through ectomycorrhizal fungi (Read and Perez-Moreno 2003; Schimel and Bennett 2004). Additionally, the activities of ectomycorrhizal fungi are often determined by the availability of N in substrates (Wallander and Nylund 1991, 1992). This suggests that N-mediated interactions among substrates, plants, and fungi may have a crucial role in determining the successional patterns (Leake et al. 2004; Hobbie and Högberg 2012). The dependence of woody plants on ectomycorrhizal fungi for N uptake has been evaluated using a one-pool fungal model, based on the stable isotope ratio of N ($\delta^{15}N$) in soil substrates, mycorrhizal sporocarps, and plants (Hobbie and Hobbie 2006; Nave et al. 2013). However, the model overestimates the contribution of mycorrhizae to the uptake of N by plants, and methods for accurately measuring the representative fungal $\delta^{15}N$ have been an issue. Therefore, Hobbie and Agerer (2010) proposed a two-pool fungal model that reduced the overestimation of the dependence of plants on mycorrhizae by separating the aboveground and belowground fungal $\delta^{15}N$.

After the complete elimination of the local vegetation by the 1929 Mount Koma eruption in northern Japan, vegetation succession has progressed along with the improvement of the microclimate, including temperature, water, and nutrient levels, involving *Salix reinii* Franch. et Savat. (willow) shrub patches (Uesaka and Tsuyuzaki 2004; Tsuyuzaki et al. 2012). An exotic tree species, *Larix kaempferi* (Lam.) Carr. (larch), conspicuously recruited seedlings using willow patches, and currently comprises the predominant forest species (Akasaka and Tsuyuzaki 2009). The biological invasion of larch trees followed the development of willow shrub patches. Therefore, willow and larch are the early and late colonizers on Mount Koma, respectively.

Larches, including *L. kaempferi*, develop ectomycorrhizal relationships, particularly with host-specific *Suillus* fungal species (Qu et al. 2003; Nara 2006), whereas willows, including *S. reinii*, are colonized by various non-host-specific, generalist ectomycorrhizal fungi (Taylor et al. 1997; Nara 2006). Therefore, larches and willows are likely to exhibit different responses to N in substrates, which may help explain why the biological invasion proceeds.

In this study, I examined the following three hypotheses: (1) interactions between the early plant colonizer and mycorrhizal fungi are strong at all sites, and the improved N status in tephra is a consequence of a steady supply of high-quality litter; (2) the late plant colonizer switches from obtaining N from tephra to obtaining it through fungi, according to the N content in tephra, because the late colonizer requires more N than the early colonizer; and (3) the temporal replacement of plant species in ecosystems lacking N is promoted by the adaptation of larch to the obligatory symbiosis with mycorrhizal fungi.

2.3 Materials and methods

Study area

Mount Koma is an active andesite stratovolcano in southern Hokkaido, Japan (42°03′N, 140°40′E; 1,133 m above sea level). The climate in this region is categorized as cold-temperate, with a mean annual precipitation and temperature of 1,081 ± 71 mm and 8.0 ± 0.1 ℃ (mean ± standard error), respectively, from 2005 to 2014. These data were obtained from the Mori Climatological Observatory, which is located 9 km from Mount Koma at a 10 m elevation. From 2012 to 2014, the minimum daytime temperature during January (coldest month) was −10.2 ± 0.3 ℃, while the maximum daytime temperature in August (warmest month) was 26.1 ± 0.5 ℃. During the 3 years that were surveyed, the precipitation averaged 158 ± 14 mm during the early plant growing season from May to July. Therefore, drought conditions were common in this period. In contrast, the average precipitation from August to November was 643 ± 153 mm, which was sufficient for the local vegetation.

The volcanic eruption in 1929 completely destroyed the nearby broad-leaved forests (Yoshioka 1966), but the vegetation has recovered from the base of the volcano to the top in the subsequent years. The tephra consisted mostly of ash and pumice (Kondo and Tsuyuzaki 1999). Additionally, large boulders and gravel were overlaid on the ground in some places. Larch was brought in from a plantation forest at the foot of the volcano between 1953 and 1963, and became the predominant vegetation on the southwestern slope (Kondo and Tsuyuzaki 1999). Willow was the early colonizer, while larch was the late colonizer (Akasaka and Tsuyuzaki 2009) because the willow shrub patches facilitated the establishment of larch (Titus and Tsuyuzaki

2002). Both of the woody species developed ectomycorrhizal relationships (Tsuyuzaki et al. 2005).

To compare the effects of N in the substrates and mycorrhizal fungi on the establishment of larch and willow, two sites were selected on the southern slope. One site was established on a ridge at an elevation of 875–885 m, while the other site was on a hillslope at an elevation of 650–680 m. The tall tree and willow shrub populations were analyzed in seven randomly established 10 m × 10 m plots at each site in early June 2015. The height and diameter at 10% total height of all trees were measured in each plot. The leader stem volume of tall trees was calculated under the assumption that the stem was conical. The long and short axes of all willow patches were measured, and the cover of each willow patch was calculated with the assumption that it was oval. The number of mycorrhizal sporocarps is used to represent mycorrhizal fungal biomass (Hasselquist and Högberg 2014). To estimate the mycorrhizal biomass, larch-specific mycorrhizal sporocarps were counted in five 10 m × 10 m plots at each site in mid-October 2013.

Field samples and measurements

Foliar samples of larch and willow were collected from 32 and 18 plots (5 m × 5 m) on the ridge and hillslope, respectively. Plots were separated by more than 10 m. There were 18 or fewer leaf samples for each species in each month, with the number of leaf samples depending on leaf phenology (i.e., there were fewer samples during the leaf flushing and defoliation periods). The leaf samples of each species were randomly collected when possible. Habitat type (i.e., bare ground, larch understory, or willow patch) was recorded for each foliar sample to analyze the habitat effects on foliar $\delta^{15}N$. Willow patches mainly consisted of a single willow plant, likely because of the shade created by the patches. Therefore, the leaves of host patch willow plants

were collected and used to represent the willow patch. Samples were collected at 1 to 2 month intervals from early August 2012 to early November 2014. To detect the dependence of plants on fungi for N uptake, $\delta^{15}N$ values for non-mycorrhizal plants are required (Hobbie and Hobbie 2006). This is because $\delta^{15}N$ should be related to mycorrhizal status regardless of the plant growth form (e.g., forb, wood, or grass) (Michelsen et al. 1998). *Carex oxyandra* (Franch. et Savat.) Kudo (sedge) is a typical non-mycorrhizal plant on Mount Koma (Titus and Tsuyuzaki 2002). Therefore, its green leaves were collected at 1- or 2-month intervals from early August 2013 to early October 2014. The foliar samples were oven-dried overnight at 60 ℃ on each sampling day, and then transferred to our laboratory within 2 days. Thereafter, samples were oven-dried for 48 h, ground using a mortar and pestle with liquid nitrogen, dried again for 2 days, and then stored in a sealed plastic box containing silica gel until used. I confirmed that differences in plant size and habitat for samples collected in 2012 and 2013 did not affect foliar $\delta^{15}N$. Leaves (collected in 2014) from two to six plants were pooled to prepare samples for the measurement of foliar N% and $\delta^{15}N$.

Mycorrhizae and tephra were sampled in the autumn of 2013 and 2014 at the two study sites. Three *Suillus* ectomycorrhizal species [i.e., *S. cavipes* (Opat.) Smith & Thiers, *S. grevillei* (Klotz.) Sing., and *S. laricinus* (Berk.) Kuntze] were observed at both the sites, and are considered major mycorrhizal species for larches on Mount Koma (Usami et al. 2007; Kayama et al. 2015). *Suillus cavipes* was the predominant species. These three species develop symbiotic associations with larch plants (Duddridge 1986, Read 1991), and do not colonize *S. reinii* roots (Nara 2006). Their sporocarps and, when possible, rhizomorphs were collected from early August to mid-October in 2013 and 2014 to assess $\delta^{15}N$ (Fig. 2-1). The rhizomorphs were sampled from the base of sporocarp stipes to identify species. After confirming that the temporal variations in

$\delta^{15}N$ were not significant in the *S. cavipes* sporocarps collected from early August to mid-October 2013, the $\delta^{15}N$ of mycorrhizal fungi was analyzed using pooled samples collected from early August to mid-October in 2013 and 2014. For $\delta^{15}N$ measurements, mycorrhizal samples were treated in the same way as the foliar samples.

Soil originating from tephra was collected in a soil tin (surface area: 20 cm^2; depth: 5 cm) from bare ground, larch understory, and a willow patch (0−5 cm depth) at each site in early November in 2013 and 2014. The concentrations of total N, inorganic N, and ^{15}N in the soil substrates were measured (Schimel and Bennett 2004). Foliar $\delta^{15}N$ often increases when roots penetrate deeper into soil (Hobbie and Ouimette 2009). To examine the effect of soil depth on $\delta^{15}N$, soil samples were collected from three layers (0−5 cm, 15−20 cm, and 25−30 cm depths) on the hillslope in early August 2013. Before drying the soil samples collected at a depth of 0−5 cm, inorganic N was extracted using a 2 N KCl solution after the soil was sieved through a 2-mm mesh. The NH_4^+, NO_2^-, and NO_3^- concentrations were measured using a QuAAtro colorimetric analyzer (Bran + Luebbe, Norderstedt, Germany). The soil samples which were not used for extraction were oven-dried at 60 ℃ for 7 days, and then sieved through a 100-μm mesh. Fragments (including litter) that did not pass through the mesh were excluded from the analysis. Samples were stored in the same way as for the foliar samples. Although plant size (e.g., height of larch and patch area of willow) varied greatly among the collected samples, preliminary measurements confirmed that size did not affect foliar $\delta^{15}N$.

Modeling and evaluating the dependence of larch and willow on mycorrhizae for N uptake

The concentrations of N and ^{15}N in foliage, mycorrhizae, and tephra were measured using a Delta V Plus continuous flow isotope mass-spectrometer (Thermo Fisher Scientific, Yokohama,

Japan) coupled with a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific). $\delta^{15}N$ was calculated as follows (Coplen 2011):

$$\delta^{15}N = (R_{sample}/R_{standard}) - 1$$

where R_{sample} refers to the $^{15}N/^{14}N$ molar ratio in a sample, and $R_{standard}$ refers to $^{15}N/^{14}N$ in the air. The dimensionless $\delta^{15}N$ value is conventionally expressed as per mil (‰).

To evaluate the dependence of larch and willow on mycorrhizae for N uptake, I used a modified two-pool fungal model (Hobbie and Agerer 2010). This model was modified by separating the N sources available to fungi and plants (Fig. 2-1). This was done because the organic matter degradation rates differ between mycorrhizal fungi and plants (Read and Perez-Moreno 2003).

The following mass balance equations [(1) to (3)] were derived from the isotopic fractionation between mycorrhizae and plants, as well as the mixing of N isotopes in plants under a steady-state open system (Hobbie and Agerer 2010).

$$\delta^{15}N_{my} = \delta^{15}N_{av(my)} + \Delta_f \times T_r \quad (1)$$

$$\delta^{15}N_{pl(my)} = \delta^{15}N_{av(my)} - \Delta_f \times (1 - T_r) \quad (2)$$

$$\delta^{15}N_{pl} = f \times \delta^{15}N_{pl(my)} + (1 - f) \times \delta^{15}N_{av(pl)} \quad (3)$$

$$f = (\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}) / (\Delta_f - \delta^{15}N_{my} + \delta^{15}N_{av(pl)}) \quad (4)$$

where $\delta^{15}N_{my}$ refers to $\delta^{15}N$ in mycorrhizae; $\delta^{15}N_{av(my)}$ and $\delta^{15}N_{av(pl)}$ refer to $\delta^{15}N$ for the N source available to mycorrhizae and plants, respectively; $\delta^{15}N_{pl(my)}$ refers to $\delta^{15}N$ for the N transferred from mycorrhizae to plants; Δ_f refers to the intrinsic fractionation of $\delta^{15}N$ between N in mycorrhizal fungi and N transferred to plants *via* mycorrhizal fungi; T_r refers to the ratio of N

transferred from mycorrhizal fungi to plants to the total N absorbed by the mycorrhizal fungi from the N source; *f* refers to the dependence of plants on mycorrhizae for N uptake [i.e., (N transferred from mycorrhizae to plants) divided by (N in plants)]. Therefore, $\delta^{15}N_{pl}$, $\delta^{15}N_{av(pl)}$, and $\delta^{15}N_{my}$ were measured to determine *f*. Δ_f was set as 9‰ and 10‰, because this was the estimated range in the field (Hobbie and Horton 2007, Nave et al. 2013). A Δ_f of 10‰ results in the most conservative estimated *f* value. $\delta^{15}N_{pl}$ and $\delta^{15}N_{av(pl)}$ were determined using the foliar $\delta^{15}N$ of ectomycorrhizal plants and non-mycorrhizal *C. oxyandra*, respectively.

The common larch-specific *Suillus* fungal species (i.e., *S. cavipes*, *S. grevillei*, and *S. laricinus*) were used to evaluate $\delta^{15}N_{my}$ (Finlay 1989; Usami et al. 2007; Kayama et al. 2015). Because sporocarps correspond to less than 1% of the total biomass (Deckmyn et al. 2014), $\delta^{15}N$ in rhizomorphs ($\delta^{15}N_{rh}$) was measured as $\delta^{15}N_{my}$. Mycelia on *S. reinii* plants were not sampled [i.e., fungi from the genera *Inocybe*, *Laccaria*, *Hebeloma*, *Cortinarius*, and *Thelephora* (Nara 2006)] because they were too thin and firmly attached to the substrate.

The *f* value in Eq. (4) was not determined for willow because $\delta^{15}N_{my}$ was not measured. Instead, the numerator in Eq. (4) was used to estimate the dependence of willow on mycorrhizae for N uptake at the lowest level. I considered the following two periods during our investigation of the relationships between plants and mycorrhizal fungi; early June to mid-July when leaves grew (early season) and early August to mid-October when leaf development slowed and mycorrhizal sporocarps were observed (late season).

Statistical analysis

The N% and $\delta^{15}N$ values for *L. kaempferi*, *S. reinii*, and *C. oxyandra* leaves were compared among months and between sites with their interactions using two-way analysis of variance

(ANOVA). Total inorganic N in tephra was compared between sites and among habitats using two-way ANOVA. The tree species composition and N status in tephra, mycorrhizal fungi, and plants were compared between the two sites using a *t*-test, and among habitats at each site by one-way ANOVA. The normality and homoscedasticity of the data were confirmed using the Shapiro–Wilk test and *F*-test, respectively. If the two statistical conditions were satisfied, I used the *t*-test. If not, the Wilcoxon rank-sum test was used. For comparisons of multi-level factors, the normality and homoscedasticity were confirmed by the Shapiro–Wilk test and Levene's test, respectively. If these two conditions were satisfied, the Tukey's honestly significant difference (HSD) test was completed after one-way ANOVA indicated there were significant differences among groups. If not, pairwise comparisons using the Wilcoxon rank-sum test were completed after the Kruskal–Wallis test produced significant results.

Pearson's correlation (*r*) and analysis of covariance (ANCOVA) were used to investigate the relationships between N% and $\delta^{15}N$ in larches, willows, and sedges within and between sites. All statistical analyses were conducted with the R program (ver. 3.1.2) (R Core Team 2014).

2.4 Results

Site conditions

Because of the geographical characteristics of the stratovolcano, the ridge was less steep than the hillslope, and was closer to the crater (Table 2-1). A quarter of the ground surface on the ridge and hillslope was covered with willow patches. The willow patches were shorter than 50 cm, and the largest willow patch area was 3.85 m^2 and 6.16 m^2 on the hillslope and ridge,

respectively. The willow patch cover did not differ between the two sites (*t*-test, $P = 0.85$). The tallest larch tree on the hillslope and ridge was 5.56 m and 2.80 m, respectively. The larch stem density on the ridge was a quarter of that on the hillslope ($P < 0.01$). Larches were the predominant trees at the two sites, but the total stem volume was 24 times higher on the hillslope than on the ridge (*t*-test, $P < 0.001$) because there were more short stems on the ridge. There were few *C. oxyandra* plants scattered throughout both the sites, with less than 1% total cover. More than 20 vascular plant species were established at both the sites, but the total cover was less than 25%. The common plant species included *Gaultheria miqueliana* Takeda, *Salix integra* Thunb., *Miscanthus sinensis* Anders., and *Anaphalis margaritacea* Benth. et Hook. Mosses and lichens were established in patches, of which the total cover was 40−50% on the northeastern slope. In total, 20−30% of the ground surface consisted of bare ground.

Nitrogen distribution in substrates

On the hillslope, the total N content in tephra was 0.051 ± 0.003% (mean ± standard error; $n = 10$) on bare ground, 0.047 ± 0.005% ($n = 11$) in the larch understory, and 0.109 ± 0.020% ($n = 13$) in the willow patch in early November in 2013 and 2014 (Fig. 2-2). On the ridge, N% in tephra averaged 0.080 ± 0.007% ($n = 9$) on bare ground, 0.083 ± 0.007% ($n = 10$) in the larch understory, and 0.273 ± 0.046% ($n = 10$) in the willow patch. Overall, the tephra contained less N on the hillslope than on the ridge at the landscape level (*t*-test, $P < 0.01$), even though the willow patch cover did not differ between the two sites (Table 2-1).

At the habitat level, on the hillslope, N% in the substrates below the willow patch was approximately double that of the substrates on bare ground and in the larch understory (Wilcoxon rank-sum test, $P < 0.001$). On the ridge, N% was three times higher in the willow

patch than on bare ground and in the larch understory ($P < 0.001$). Therefore, the willow patch contributed considerably to N% in the tephra, but the larch understory did not contribute to N depositions in the tephra. The $\delta^{15}N$ for the tephra on the hillslope was lower in the willow patch (−2.11 ± 0.18‰) than on bare ground (−0.27 ± 0.14‰) or in the larch understory (−0.71 ± 0.14‰) (HSD test, $P < 0.001$). The $\delta^{15}N$ for the tephra on the ridge was also lower in the willow patch (−3.58 ± 0.25‰) than on bare ground (−0.79 ± 0.18‰) or in the larch understory (−1.21 ± 0.14‰) ($P < 0.001$). The $\delta^{15}N$ for the tephra of each habitat was lower on the ridge than on the hillslope (*t*-test, $P < 0.05$).

The NH_4^+, NO_2^-, and NO_3^- concentrations in the tephra at depths of 0 cm and 5 cm on the hillslope were as follows: 0.92 ± 0.13 mg/kg tephra, 0.17 ± 0.01, and 0.55 ± 0.07 on bare ground ($n = 6$); 0.75 ± 0.11, 0.15 ± 0.01, and 0.48 ± 0.05 in the larch understory ($n = 5$); and 1.06 ± 0.15, 0.15 ± 0.01, and 0.63 ± 0.13 in the willow patch ($n = 5$), respectively. The concentrations on the ridge were as follows: 1.08 ± 0.20 mg/kg tephra, 0.20 ± 0.04, and 0.64 ± 0.11 on bare ground ($n = 4$); 1.24 ± 0.22, 0.17 ± 0.03, and 0.49 ± 0.03 in the larch understory ($n = 5$); and 1.22 ± 0.21, 0.17 ± 0.01, and 0.53 ± 0.05 in the willow patch ($n = 5$), respectively. Therefore, the inorganic N content in the tephra on the hillslope as the sum of NH_4^+, NO_2^-, and NO_3^- averaged 1.64 ± 0.09 mg/kg tephra ($n = 6$) on bare ground, 1.37 ± 0.16 ($n = 5$) in the larch understory, and 1.84 ± 0.27 ($n = 5$) in the willow patch. The concentration of inorganic N on the ridge was 1.92 ± 0.31 mg/kg tephra ($n = 4$) on bare ground, 1.89 ± 0.20 ($n = 5$) in the larch understory, and 1.91 ± 0.21 ($n = 5$) in the willow patch. The concentration as the sum of NH_4^+, NO_2^-, and NO_3^- was not significantly different between sites ($P = 0.10$) and among habitats ($P = 0.50$) without the interaction between site and habitat (two-way ANOVA, $P = 0.55$).

Nitrogen status in plants

The $\delta^{15}N$ for *C. oxyandra* plants averaged −3.11 ± 0.08‰ on the ridge and −2.96 ± 0.08‰ on the hillslope, and did not vary among months (two-way ANOVA, $P = 0.70$) or between sites ($P = 0.65$) with no interactions ($P = 0.13$) (Fig. 2-3). Therefore, the $\delta^{15}N$ of inorganic and soluble organic N in substrates was the same between the two sites throughout the growing seasons.

The N% in larch leaves was higher on the ridge than on the hillslope throughout the seasons (two-way ANOVA, $P < 0.001$) (Fig. 2-3). The N% was highest in the early season at the two sites, and subsequently decreased or fluctuated slightly. The interaction between month and site was significant ($P < 0.001$), meaning the intensity of temporal variations in N% differed between sites. The N% in willow leaves was also highest during the early season, and then gradually decreased afterward ($P < 0.001$). The N% in non-senescent green leaves of sedge plants increased after early September, likely because of the relocation of N. The N% in willow and sedge plants did not differ between sites ($P = 0.09$ and 0.94, respectively).

Foliar $\delta^{15}N$ for larch was higher on the ridge than on the hillslope during each census (two-way ANOVA, $P < 0.001$), while foliar $\delta^{15}N$ for willow did not differ between the two sites ($P = 0.57$). There were no interactions between month and site for both the species ($P > 0.1$). These results implied the dependence of larches on mycorrhizal fungi for N uptake was greater on the relatively N-poor hillslope. The $\delta^{15}N$ for larch gradually increased throughout the seasons at both the sites (i.e., on the hillslope: Pearson's $r = 0.63$, $P < 0.001$; on the ridge $r = 0.48$, $P < 0.001$). Larch leaves began to senesce in mid-October. During senescence, larches did not exhibit isotopic fractionation during N relocation, as indicated by a stable $\delta^{15}N$ (*t*-test, $P > 0.32$) (Fig. 2-3). The seasonal changes in $\delta^{15}N$ for larch leaves suggested the mutual relationships with mycorrhizal fungi decreased from the early to late seasons.

The N% in larch was not correlated with $\delta^{15}N$ at each site throughout the seasons (Table 2-2). However, N% was positively correlated with $\delta^{15}N$ when the data for the two sites were pooled (ANCOVA, $P < 0.001$). The site effect was significant ($P < 0.001$) without interactions between N% and site ($P > 0.13$). Therefore, N% and $\delta^{15}N$ for larch plants were lower on the hillslope than on the ridge. In contrast, N% and $\delta^{15}N$ for willow and sedge plants did not exhibit any correlations within or between sites (Table 2-2). Therefore, as the larch succession proceeded, the larch plants flexibly changed how they obtained N, while the willow and the sedge plants did not.

The $\delta^{15}N$ for tephra increased with increasing soil depths in all three habitats (Kruskal-Wallis test, $P < 0.001$) and with decreasing N% in tephra (Pearson's $r = -0.67$, $P < 0.001$) (Fig. 2-4). The excavations for soil sampling confirmed that the roots of the three examined species were distributed and overlapped in tephra at depths up to 30 cm. Additionally, they were densely distributed at depths up to 15 cm, but there were no obvious or noticeable differences in distribution. Therefore, the root depth effects on foliar $\delta^{15}N$ were likely to be similar among species. Although the soil $\delta^{15}N$ differed between habitats, the foliar $\delta^{15}N$ of each species was unaffected by habitats [willow on bare ground and in the larch understory ($P > 0.48$ at both the sites) (*t*-test or Wilcoxon rank-sum test); larch on bare ground and in the willow patch ($P > 0.11$); sedge on bare ground and in the willow patch ($P > 0.29$)].

Distribution and $\delta^{15}N$ of larch-specific fungi

In total, 178 and 262 sporocarps of all fungal species were counted at five 10 m × 10 m plots on the ridge and hillslope, respectively, in mid-October 2013. These were equivalent to 3,560 ± 1,039/ha on the ridge and 5,240 ± 759/ha on the hillslope. Three larch-specific mycorrhizal

species were detected (i.e., *S. cavipes*, *S. grevillei*, and *S. laricinus*). The sporocarp densities were 440 ± 50/ha and 3,760 ± 370/ha on the ridge and hillslope, respectively, indicating they were nine times higher on the hillslope (*t*-test, $P < 0.001$). Therefore, the biomass of larch-specific mycorrhizal fungi should be greater on the hillslope.

The $\delta^{15}N$ for the *S. cavipes* sporocarp caps did not fluctuate from early August to mid-October 2013 (one-way ANOVA, $P = 0.98$). The $\delta^{15}N$ for the *Suillus* species sporocarp caps was significantly higher on the ridge than on the hillslope (*t*-test, $P < 0.001$) (Table 2-3), as was the internal fractionation (Δ_i) ($P < 0.01$). Additionally, the $\delta^{15}N$ for the belowground rhizomorph did not differ between the two sites ($P = 0.68$).

Effects of mycorrhizae on larch and willow plants

The two-pool fungal model estimates for the dependence of larch plants on mycorrhizal fungi for N uptake during the late season were 56−76% on the hillslope and 3−5% on the ridge at Δ_f = 9−10‰ (Table 2-4). The value of ($\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$) for larch plants was significantly different between the two sites during the early season (*t*-test, $P < 0.001$), which suggested the dependence was higher on the hillslope than on the ridge even during the early season. The one-pool fungal model estimated the dependence was 95 ± 5% on the hillslope and 14 ± 17% on the ridge at Δ_f = 10‰, and 176 ± 10% on the hillslope and −34 ± 44% on the ridge at Δ_f = 9‰ in the late season. These results indicated the one-pool fungal model was inappropriate for estimating dependence. The value of ($\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$) for willow plants was 4.5−5.0‰ at the two sites, and did not differ between sites during both the seasons (one-way ANOVA, $P = 0.10$) (Table 2-4). Because $\delta^{15}N_{my}$ was higher than $\delta^{15}N_{av(pl)}$, the dependence on mycorrhizal fungi for N uptake was possibly over 45−55% at Δ_f =9−10‰.

2.5 Discussion

Nitrogen availability in tephra

Nitrogen levels in tephra were higher on the ridge than on the hillslope of Mount Koma. Montane forests in Hokkaido generally contain 0.4–1.0% of the total N in the soil (Morishita et al. 2004; Urakawa et al. 2014). However, N content in tephra on Mount Koma was below 0.3% even in the willow patches. The inorganic N content on Mount Koma was extremely low, relative to levels in natural forests in Japan (Shibata et al. 2011). The plants on Mount Koma were N-limited, even though 85 years had passed since the 1929 volcanic eruption. Therefore, effective uptake of N is likely crucial for the establishment of plants (Uesaka and Tsuyuzaki 2004; Tsuyuzaki et al. 2005). The larch foliar $\delta^{15}N$ differed between the two sites, whereas the willow $\delta^{15}N$ was consistently low.

The willow patches had higher N levels in the substrates, which was in contrast to the soil under larch plants. Willow litter is easily decomposed because of the relatively low abundance of tannin-protein complexes (Schofield et al. 1998). Early colonizers often improve the N status of substrates in N-limited ecosystems by supplying decomposable litter (Matson 1990; Li et al. 2007). The willow patches were important suppliers of N for the late colonizers, such as *L. kaempferi*. The uptake of N by willow plants was likely highly dependent on mycorrhizal fungi.

Nitrogen levels in tephra did not differ between the larch understory and bare ground, suggesting that the larch litter did not contribute to N depositions. Larch litter is decomposed

slower than broad-leaved litter, including willows (Yang et al. 2013), although the effects of the litter decomposition rate on N levels in tephra were not determined.

δ^{15}N variations in plants and fungi

The two examined woody plants and sedge developed their roots in soil up to a depth of 30 cm, with dense distribution up to a depth of 15 cm. On the volcano Mount Usu, which is close to Mount Koma, nutrient contents in tephra are low, and root development is limited in the shallow soil (Haruki and Tsuyuzaki 2001). It has been a few centuries since the catastrophic eruption of Mount Tarumae in northern Japan, and more than 80% of the root biomass of the local woody trees and understory plants occurs in tephra within a depth of 0–15 cm because of low nutrient levels in deep tephra layers (Sakai et al. 2007). These observations suggest the roots of *S. reinii* and *L. kaempferi* (woody plants) as well as sedge are highly overlapping and, therefore, the root depth effects should be similar among species. As was the case for the root depth effects on foliar δ^{15}N, habitat effects seemed to be weak, as the foliar δ^{15}N of each species was unrelated to the habitats. This may also result from the root development characteristics in nutrient-poor ecosystems. Most of the plants on nutrient-poor volcanoes develop their roots horizontally rather than vertically (Haruki and Tsuyuzaki 2001; Sakai et al. 2007). Their roots may often expand well beyond their original habitats to mask any habitat effects.

The generalist mycorrhizal associates of larches on Mount Koma are mostly from the genera *Inocybe*, *Tomentella*, *Russula*, and *Cenococcum* (Usami et al. 2007; Kayama et al. 2015). However, the contribution of these fungi to N uptake by larch plants appears to be low, because δ^{15}N for larch was equivalent to δ^{15}N for non-mycorrhizal sedge, and δ^{15}N for willow was lower on the ridge during the late season. Of host-specific and host-nonspecific mycorrhizal associates

of larch, *Suillus* species, including three species detected on Mount Koma, have an important role in transferring N to larch plants (Qu et al. 2003, Kayama et al. 2015). Therefore, *Suillus* mycorrhizal fungal species should enable the growth of larches in the N-poor sites on Mount Koma.

The $\delta^{15}N$ for willow did not fluctuate greatly, indicating N uptake from the fungi was stable. In contrast to larches, the pioneer *S. reinii* plants established a relationship with N-insensitive and host-nonspecific ectomycorrhizal fungi (Wallander and Nylund 1992; Nara and Hogetsu 2004), and were not colonized by *Suillus* fungal species (Nara 2006). Changes in the dependence of host trees on fungi are likely to occur throughout vegetation succession. There were fewer changes in $\delta^{15}N$ for the pioneer trees (i.e., *Salix* and *Populus* species) than for the seral or climax trees (i.e., *Picea* and *Tsuga* species from the family Pinaceae) over 20 to 225 years of a primary succession after glacier retreat in Glacier Bay of southern Alaska, USA (Hobbie et al. 2000). Furthermore, pioneer trees, including willows, provide decomposable litter and increase N levels in substrates. Species that produce decomposable litter can increase N abundance in substrates (Uesaka and Tsuyuzaki 2004; Callaway 2007).

The internal fractionation of $\delta^{15}N$ between sporocarps and rhizomorphs differed between the two sites. The $\delta^{15}N$ for protein and amino acids in mycorrhizal fungi is approximately 10‰ higher than the $\delta^{15}N$ for chitin (Taylor et al. 1997), suggesting that $\delta^{15}N$ for *Suillus* species sporocarps, and the associated internal fractionation, should result from the allocation of proteins and their related compounds.

Nitrogen dependence of larch and willow plants relative to species replacement

The one-pool fungal model overestimated the contribution of mycorrhizal fungi to N uptake by plants or resulted in unrealistic estimates beyond the boundary condition (0–100%). Therefore, the current discussion is based on the two-pool fungal model. The estimated dependence of larches on ectomycorrhizal fungi for N uptake was 3–56%, and for willows, it was likely over 45%. The interactions between the early plant colonizer (i.e., *S. reinii*) and mycorrhizal fungi were strong. The willow patches contained higher N concentrations in tephra because of the availability of high-quality litter. The N dependence of willow was likely consistently high. This should influence the species composition of early colonizers soon after catastrophic disturbances, such as volcanic eruptions. The late colonizer (i.e., *L. kaempferi*) flexibly shifted the N transfer routes depending on N levels in tephra. Late colonizers generally need more N than the early colonizers (Van Auken 1985). The N dependence (*f*) of larches indicated that N uptake was highly reliant on host-specific mycorrhizal fungi on the hillslope where N content was low, and was less dependent on fungi on the ridge where N abundance was relatively high. This observation should have important implications for species replacement and establishment.

Fig. 2-1 Pathways for estimating N transfer in a modified two-pool fungal model for an open ecosystem (Hobbie and Agerer 2010). N_I: N flux from N source 1 in tephra to the initial mycorrhizal fungi. N_{II}: from the initial mycorrhizal fungi to plants. N_{pl}: N taken up by plants from tephra and the initial mycorrhizae. $\delta^{15}N_{av(my)}$: $\delta^{15}N$ for N source 1 available to mycorrhizae. $\delta^{15}N_{av(pl)}$: N source 2 available to plants. $\delta^{15}N_{pl(my)}$: N transferred to plants from the initial mycorrhizae. $\delta^{15}N_{my}$: of mycorrhizal fungi. $\delta^{15}N_{pl}$: of plants. $\delta^{15}N_{sp}$: of sporocarps. $\delta^{15}N_{be}$: of belowground mycorrhizal fungi. $\delta^{15}N_{rh}$: of the rhizomorph beneath the tephra. Δ_f (= $\delta^{15}N_{my} - \delta^{15}N_{pl(my)}$): intrinsic fractionation occurring during N transfer from mycorrhizae to plants. Δ_i (= $\delta^{15}N_{sp} - \delta^{15}N_{be}$): internal fractionation between the belowground biomass of mycorrhizae and sporocarps.

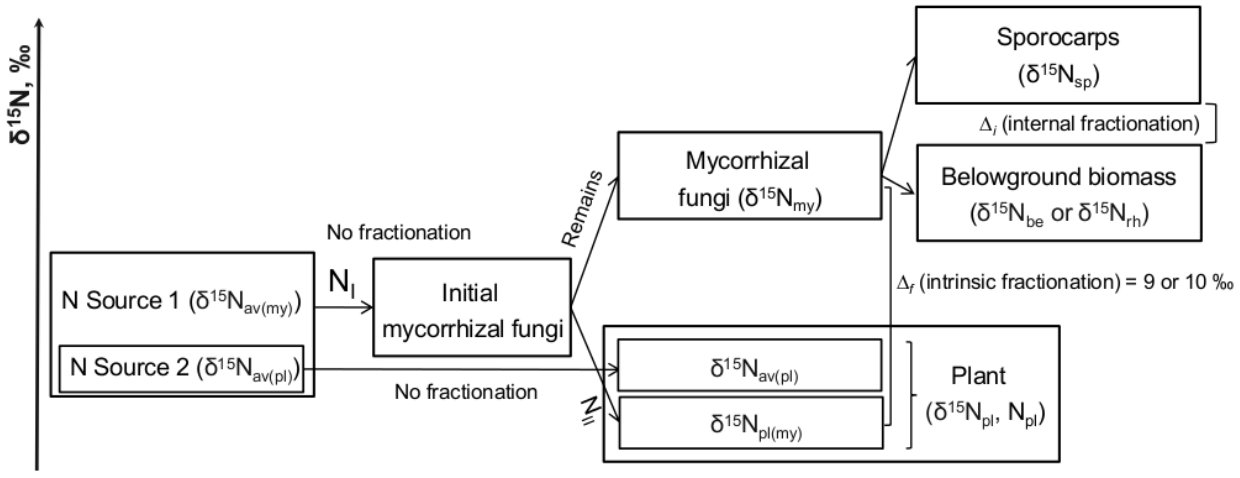


Fig. 2-2 Variations of N (%, dry weight) and $\delta^{15}N$ (‰) for tephra between depths of 0 cm and 5 cm in three habitats on the ridge (triangles) and hillslope (circles) in early November. The three habitats were bare ground (blank), larch understory (solid), and willow patch (shaded). There were 9–13 samples in each habitat at each site. Mean values (symbols) are provided with standard error bars. Data for the samples collected in 2013 and 2014 are combined and examined because of no significant differences between the years.

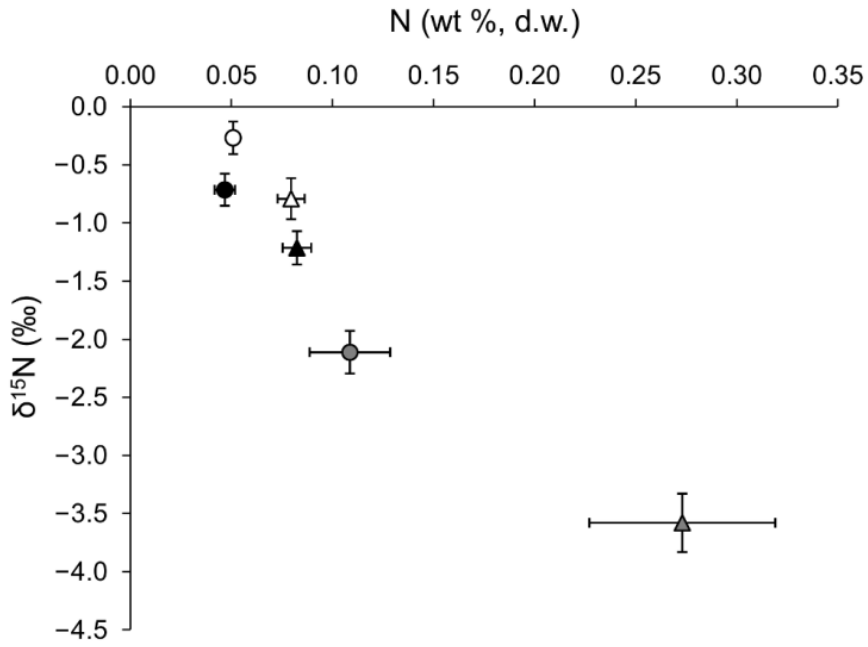


Fig. 2-3 Seasonal variations of N (%, dry weight) and $\delta^{15}N$ (‰) for the leaves of larch (a and d), willow (b and e), and sedge (c and f) on the ridge (solid triangles) and hillslope (blank circles) from early August 2012 to early November 2014. The "e" and "m" labels on the months mean "early" and "mid-", respectively. Mean values (symbols) are provided with standard error bars. Numbers at the bottom of the graphs refer to sample sizes. Significant differences were investigated among months and between sites with their interactions (cross marks) using two-way ANOVA. Data for the same months of both the years were combined in the analysis.

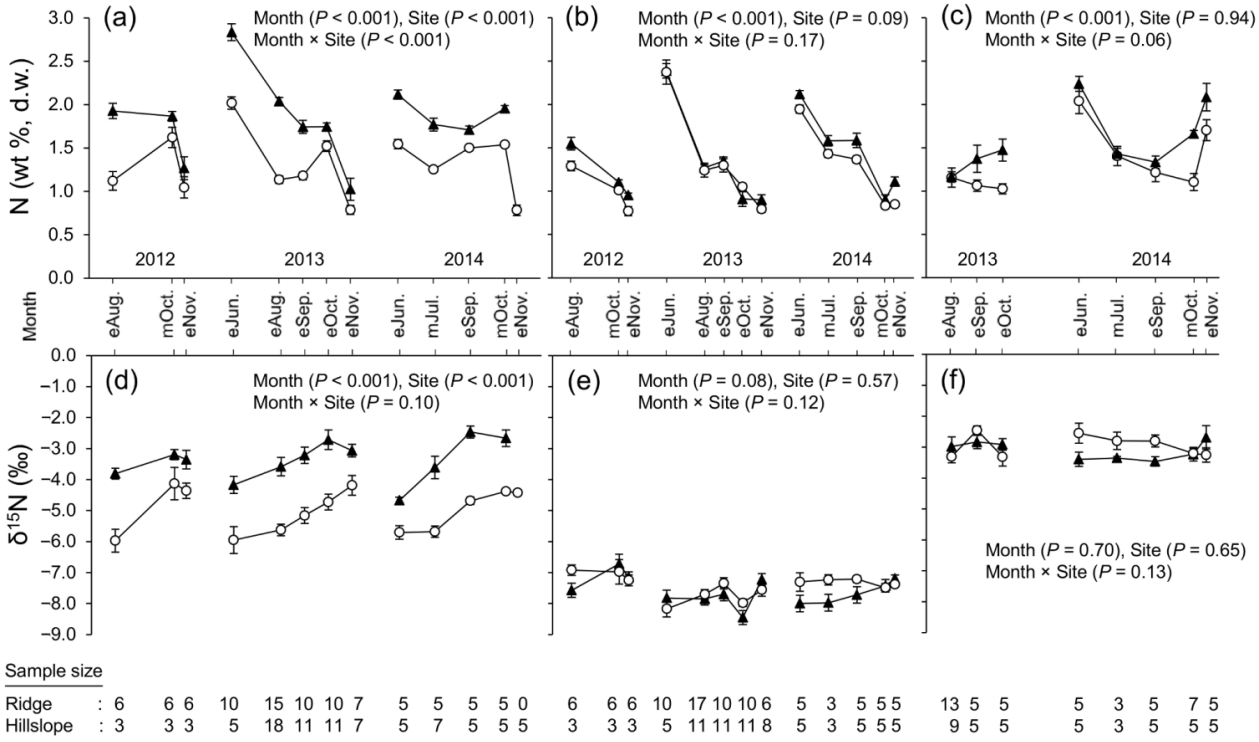


Fig. 2-4 Variations of N (%, dry weight) and δ^{15}N (‰) for tephra at depths of 0–5 cm (circles), 15–20 cm (rhombus), and 25–30 cm (rectangles) in bare ground (blank), larch understory (solid), and willow patch (shaded) on the hillslope in early September 2013. Four or five samples were used for each soil depth in each habitat. Mean values (symbols) are provided with standard error bars. There were significant differences in N% among the tephra depths (one-way ANOVA, $P < 0.001$) and in δ^{15}N among the depths (Kruskal-Wallis test, $P < 0.001$). Pearson's correlation coefficient (R^2) between δ^{15}N and N% is also provided.

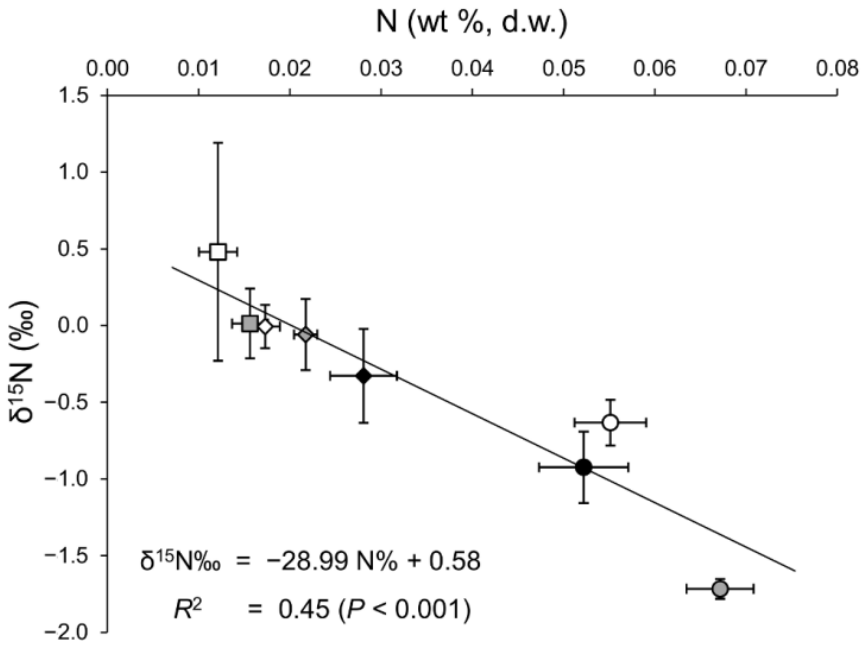


Table 2-1 Characteristics of ridge and hillslope sites, and the composition of tall trees and *Salix reinii* willow shrubs, surveyed on seven 10 m × 10 m plots at each site.

Site	Ridge	Hillslope	*P* value[a]
Elevation (m)	875−885	650−680	
Slope (°)	5.7	13.3	
Distance from crater (m)	850−950	1,725−1,870	
Sampling area (short × long axes, m^2)	100 × 130	40 × 130	
Number of plots for foliage sampling	32	18	
Volume of leader stem (cm^3 100 m^{-2})[b]			
Larix kaempferi	5,627 ± 916	132,347 ± 10,553	< 0.001
Betula ermanii	< 1	6,698 ± 3,310	< 0.001
Betula platyphylla var. *japonica*	< 1	450 ± 155	< 0.001
Alnus maximowiczii	< 10	345 ± 278	< 0.001
Populus sieboldii	< 1	77 ± 39	< 0.001
Stem density (100m^{-2})			
Larix kaempferi	6.67 ± 1.45	25.33 ± 3.48	< 0.01
Betula ermanii	0.05 ± 0.05	1.14 ± 0.26	< 0.001
Betula platyphylla var. *japonica*	0.33 ± 0.07	2.00 ± 0.53	< 0.001
Alnus maximowiczii	0.01 ± 0.01	0.29 ± 0.18	< 0.001
Populus sieboldii	0.23 ± 0.07	5.57 ± 1.25	< 0.001
Willow patch cover (%)[c]	24.21 ± 1.14	23.73 ± 2.16	0.85

- Mean values are provided with the standard errors.
- [a]Differences between the two sites were examined using the *t*-test or Wilcoxon rank-sum test.
- [b]This was calculated using the height and diameter at 10% total height under the assumption that the stems were conical.
- [c]This was calculated using the longest and shortest diameters of each patch under the assumption that they were oval.

Table 2-2 Pearson correlations (*R*) or ANCOVA statistics (*F*) for N (%, dry weight) and $\delta^{15}N$ (‰) for the leaves of larch, willow, and sedge during the growing seasons within and between sites in 2012 and 2014.

		Within one site		Between sites (ridge and hillslope)			Sample size	
		Pearson's *R*		ANCOVA (*F*)[a]				
Species	Season	Ridge	Hillslope	N%	Site	N% × Site	Ridge	Hillslope
Larch	Early	−0.07 (0.76)	0.02 (0.93)	18.64 (< 0.001)	24.98 (< 0.001)	0.06 (0.81)	20	17
	Late	−0.13 (0.30)	0.13 (0.28)	50.33 (< 0.001)	88.03 (< 0.001)	2.30 (0.13)	70	71
Willow	Early − Late	−0.03 (0.76)	−0.05 (0.65)	0.48 (0.49)	1.75 (0.19)	0.00 (0.95)	94	78
Sedge	Early − Late	−0.25 (0.11)	0.06 (0.72)	1.60 (0.21)	0.57 (0.45)	2.12 (0.15)	43	37

- The significance (*P* value) is indicated in parentheses.
- "Early" and "Late" seasons refer to periods from early June to mid-July and from early August to early November, respectively.
- [a]ANCOVA was used to confirm the effects of N% and site on foliar $\delta^{15}N$ with interactions between N% and site. The *F* ratio was adjusted for covariate N%.

Table 2-3 $\delta^{15}N$ for sporocarp caps ($\delta^{15}N_{cap}$) and belowground rhizomorphs ($\delta^{15}N_{rh}$) for *Suillus* species during early August and mid-October. $\delta^{15}N_{rh}$ was estimated according to differences in $\delta^{15}N$ between the sporocarp cap and rhizomorph.

	$\delta^{15}N_{cap}$ (‰)			$\delta^{15}N_{cap} - \delta^{15}N_{rh}$ (‰)			$\delta^{15}N_{rh}$ (‰)[a]		
Species	Ridge	Hillslope	*P*	Ridge	Hillslope	*P*	Ridge	Hillslope	*P*
S. cavipes	$4.86^{b} \pm$ 0.30 (12)	$3.78^{b} \pm$ 0.21 (28)	< 0.01	2.56 ± 0.18 (6)	1.52 ± 0.25 (12)	< 0.05	$2.33^{b} \pm$ 0.30 (12)	$2.26^{b} \pm$ 0.21 (28)	0.85
S. grevillei	$6.73^{a} \pm$ 0.36 (5)	$5.93^{a} \pm$ 0.33 (13)	0.13	-	-	-	$4.20^{a} \pm$ 0.36 (5)	$4.41^{a} \pm$ 0.33 (13)	0.72
S. laricinus	$7.04^{a} \pm$ 0.66 (8)	$4.66^{ab} \pm$ 0.35 (5)	< 0.01	2.50 ± 0.32 (2)	-	-	$4.51^{a} \pm$ 0.66 (8)	$3.14^{ab} \pm$ 0.35 (5)	0.15
Total	6.21 ± 0.27	4.79 ± 0.17	< 0.001	2.53 ± 0.18	1.52 ± 0.25	< 0.01	3.68 ± 0.27	3.27 ± 0.17	0.68

- Within a column, different superscripted letters indicate significant differences among species ($P < 0.05$; one-way ANOVA with Tukey's honestly significant difference test or Kruskal–Wallis test with pairwise Wilcoxon rank-sum test).

- Mean values are provided with standard errors. Sample size is indicated in parentheses. - : not determined.

- [a]$\delta^{15}N_{rh}$ was estimated according to differences in $\delta^{15}N$ between the sporocarp cap and rhizomorph.

Table 2-4 Nitrogen dependence (*f*, %) and ($\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$, ‰) of larch and willow plants at the two sites estimated using a two-pool fungal model.

		Larch[a]				Willow
Season	Site	$\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$ (‰)	$\delta^{15}N_{my}$ (‰)	*f* (%) at Δ_f = 9‰	*f* (%) at Δ_f = 10‰	$\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$ (‰)
Early	Ridge	0.78 ± 0.18^{c} (20)	-	-	-	4.52 ± 0.16^{a} (18)
	Hillslope	3.13 ± 0.15^{a} (17)	-	-	-	4.98 ± 0.19^{a} (13)
Late	Ridge	0.10 ± 0.12^{b} (57)	3.68 ± 0.27^{a} (25)	4.50 ± 5.47^{b} (57)	3.11 ± 3.79^{b} (57)	4.67 ± 0.11^{a} (59)
	Hillslope	2.05 ± 0.12^{d} (56)	3.27 ± 0.17^{a} (46)	76.43 ± 4.39^{a} (56)	55.64 ± 3.20^{a} (56)	4.47 ± 0.08^{a} (49)

- Mean values are provided with standard errors. Sample size is indicated in parentheses. -: not determined because $\delta^{15}N_{my}$ was not measured during the early season (early June and mid-July).
- Different superscripted letters in each column indicate significant differences ($P < 0.05$) (Tukey's honestly significant difference test or *t*-test).
- For larches and willows, ($\delta^{15}N_{av(pl)} - \delta^{15}N_{pl}$) was calculated during both the seasons. [a]For larches, N dependence (*f*) was calculated during the late season (early August and mid-October) assuming Δ_f = 9‰ or 10‰.

Chapter 3

Changes in the nitrogen acquisition of trees with the development of vegetation after the catastrophic volcanic eruptions

3.1 Abstract

The N dynamics of woody plants with N status in the soil substrates was evaluated on the southern slope ranging from 380 m to 930 m elevation of Mount Koma (1,131 m), northern Japan, which erupted in 1929, by stable N isotopic ratios. Two shrubs, *Salix reinii* and *Gaultheria miqueliana*, and six trees, *Larix kaempferi*, *Betula ermanii*, *B. platyphylla*, *Populus sieboldii*, *Alnus maximowiczii* and *Quercus mongolica*, were examined. Only *S. reinii* increased total N in the substrates where the vegetation was sparse at the high elevations, showing that N in the substrates was mostly determined by *S. reinii*. $\delta^{15}N$ in *L. kaempferi*, *S. renii* and *G. miqueliana* was related to the vegetation structures, i.e., the difference of $\delta^{15}N$ between non-mycorrhizal sedge and those mycorrhizal plants was higher as the biomass of each species increases. The nitrogen sources of *L. kaempferi* changed from the shrub, *S. reinii*, to mycorrhizal fungi with decreasing *S. reinii* shrubs and nitrogen in the tephra. N dependence of *L. kaempferi* on mycorrhizal fungi reached over 37% at the 380 m elevation of mountain where *S. renii* almost disappeared with lowered mycorrhizal relationship and tephra N was low. N dependence of *B. ermanii*, *B. platyphylla*, *P. sieboldii* and *Q. mongolica* on mycorrhizal fungi was stable, i.e., 25−50% for *Betula* and *Populus* species throughout elevations and around 62% for *Quercus* species at 380 m elevation. Therefore, the plastic dependence of *L. kaempferi* on mycorrhizal fungi for obtaining N should promote the high biological-invasiveness.

3.2 Introduction

Large disturbances, including volcanic eruption, often lead to nitrogen-limited ecosystems where succession in the early stages is controlled by nitrogen dynamics (Halvorson and Smith 2009). Since nitrogen (N) status in the substrates changes after disturbances, plants need to obtain nitrogen effectively (Simard et al. 2002). Plants have two routes for N uptake, directly from the soil substrates and indirectly from mycorrhizal fungi assisting N transfer. In a nutrient-poor arctic tundra ecosystem, more than half of nitrogen in plants are derived from mycorrhizal fungi and 8−17% of photosynthates of plants are used to support mycorrhizal relation (Hobbie and Hobbie 2006). Therefore, N transfer routes and N dependences of plants on mycorrhizal fungi should be shifted with time (Schimel and Bennett 2004). Most pioneer shrubs, such as dwarf willow and ericaceous plants, are mycorrhizal (Cripps and Eddington 2005; Read et al. 2004). Since they function as a N supplier for the late colonizers (Michelsen et al. 1998; Uesaka and Tsuyuzaki 2004; Tsuyuzaki et al. 2012), their roles on revegetation should be clarified.

Mount Koma located in Hokkaido Island, northern Japan, catastrophically erupted in 1929. The climax is considered to be broad-leaved, deciduous forests, such as *Betula ermanii* Cham., *Betula platyphylla* var. *japonica* Hara, *Populus sieboldii* Miquel, *Alnus maximowiczii* Callier, and *Quercus mongolica* var. *grosseserrata* Rehd. et Wils., although the vegetation has not attained the climax. On the mountain, a deciduous shrub, *Salix reinii* Franch. et Savat., is the most dominant pioneer after the eruption. *S. reinii* has facilitative effects on the late colonizers by improving edaphic and microclimatic conditions (Uesaka and Tsuyuzaki 2004). The predominance and facilitation of *S. reinii* as the first pioneer species is derived somehow by the ectomycorrhiza (Tsuyuzaki et al. 2012), although the mechanisms has not been clarified well.

Another shrub, *Gaultheria miqueliana* Takeda, establishes well on Mount Koma as elevation lowers, which is inhibitive to the cohabitants due mostly to the evergreen leaves and shrub structure casting deep shade (Uesaka and Tsuyuzaki 2004). Comparing N dependences of these two shrub species on mycorrhizal fungi, the effects of pioneer shrub on N dynamics should be revealed.

Recently, the biological invasion of *Larix kaempferi* (Lam.) Carr., which is not originally distributed in Hokkaido, has been conspicuous on Mount Koma, because the enormous seeds has been dispersed from the transplantation conducted on the foot of the mountain between 1953 and 1963 (Kondo and Tsuyuzaki 1999). Therefore, the N dependence of plants on mycorrhizal fungi may differ between an exotic, *L. kaempferi*, and the native broad-leaved trees. In addition, the predominance of *L. kaempferi* alters the behavior of shrubs by changing the distributions of nutrients and solar radiation. As well as the overstory of *L. kaempferi*, the litter of *L. kaempferi* alters the ground surface conditions because larch litters are characterized by slow decomposition due to high contents of antioxidant compounds (Liu et al. 1998, Preston et al. 2009). Because the biological invasions of not only *L. kaempferi* but also the other larch species occur in various cold-temperate regions (Richardson and Rejmánek 2004), the relationships between plants and mycorrhizal fungi should be addressed with vegetation changes (Pringle et al. 2009).

Therefore, I tested two hypotheses by investigating differences in revegetation and N status of plants and tephra along an elevational gradient on Mount Koma; (1) woody shrubs were the major N sources to the soil substrates, which was related to elevation, vegetation and the accumulation of recalcitrant *L. kaempferi* litter. (2) *L. kaempferi* developing N-sensitive mycorrhizae adapted N dependence on mycorrhizal fungi to the variation of N in the substrates

more than native broad-leaved deciduous trees. To demonstrate these hypotheses, N isotopes were used to evaluate the changes in N dependence on mycorrhizal fungi with different elevations that were related to the vegetation development patterns on Mount Koma.

3.3 Materials and Methods

Study site and surveyed plant species

Mount Koma is a stratovolcano located in southern Hokkaido Island, northern Japan (42.063°N, 140.677°E, 1,131 m a.s.l.). Before the 1929 catastrophic eruptions, the vegetation was deciduous broad-leaved forests (Yoshioka 1966, Yoshii 1932). The 1929 eruptions destroyed the former vegetation with the huge amount of the total ejecta ca. 5×10^8 m^3 (Tsuya and Morimoto 1963). Although the revegetation started from the bottom, the revegetation had not reached the climax. In addition, *Larix kaempferi* was afforestated on the south-western slope at 300−400 m a.s.l. between the years of 1953 and 1963 (Kondo and Tsuyuzaki 1999). *L. kaempferi* and two woody shrubs, *Salix reinii* and *Gaultheria miqueliana*, were characterized as the post-eruption vegetation. Besides these species, *Betula ermanii*, *B. platyphylla* var. *japonica*, *Alnus maximowiczii*, *Populus sieboldii* and *Quercus mongolica*, one of the late successional trees, compose the woody vegetation (Table 3-1). These tree species develop ectomycorrhizae (Tsuyuzaki et al. 2005). *G. miqueliana* is colonized by both ectomycorrhizal fungi and ericoid mycorrhizal fungi (Tsuyuzaki et al. 2005). *A. maximowiczii* is associated with ectomycorrhizal fungi and *Frankia* actinobacteria (Tsuyuzaki et al. 2005, Põlme et al. 2013). Non-mycorrhizal *Carex oxyandra* (Franch. et Savat.) Kudo is scattered with less than 1% in cover (Titus and

Tsuyuzaki 2002). Throughout the elevations on Mount Koma, *L. kaempferi* is species-specifically colonized by *Suillus cavipes* (Opat.) Smith et Thiers. with over 45% on root tips (Usami et al. 2007; Kayama et al. 2015).

The climate is categorized into cold-temperate. In 2014 when the sampling was conducted, the mean annual temperature was 8.0 ^{o}C (20.8 ^{o}C of mean maximum in August and −9.6 ^{o}C of mean minimum in January) and total precipitation was 889 mm.

Evaluation of woody vegetation structures

To evaluate elevational differences in woody vegetation among elevations, seven 10 m × 10 m plots were established at each of five elevations, HS3 (365−380 m), HS4 (465−480 m), HS6 (650−680 m), RD8 (875−890 m), and RD9 (930 m) in mid-October 2015 (Table 3-1). The elevations and slopes were determined based on GPS (eTrex® H, Garmin, Taiwan). On the plot code, HS means hillslope and RD means ridge. The third letters indicate the elevation. Six common trees, *L. kaempferi*, *B. ermanii*, *B. platyphylla*, *P. sieboldii*, *A. maximowiczii* and *Q. mongolica*, and two common shrubs, *S. reinii* and *G. miqueliana*, were selected for the measurements. Of these, *L. kaempferi* was not native and the others were native. Stem heights of trees and patch areas of shrubs, taller than 5 cm in height for both, were measured. When the stem was taller than breast height (1.3 m in height), the diameter at breast height (DBH) was measured. When not, the diameter at 10% of height was measured. The stem volume was calculated as the assumption of conical shape. In addition, the lengths of long and short axes of shrub patches were measured and the patch cover (%) was calculated as an oval.

Field samplings of foliage and tephra

Stable N isotope composition of available N in substrate utilized by non-mycorrhizal plants is comparable with stable N isotope composition of non-mycorrhizal plants represented by sedges because isotopic fractionation does not occur during N uptake of non-mycorrhizal plants from substrate (Hobbie and Hobbie 2008). Therefore, the foliage of the woody species and sedge (*C. oxyandra*) was randomly sampled from the five elevations at 1.5-month intervals from early June to mid-October in 2014 to measure N% and $\delta^{15}N$. Although all the foliage samples were collected regardless of light condition, shrubs were in deep or dappled shade cast by *L. kaempferi* overstory at low elevations (HS4 and HS3). Annual leaves defoliated from *L. kaempferi* were sampled in the seven plots at each elevation to obtain *L. kaempferi* litter. The samples were carried to the laboratory within 2 days after drying-out at 60 ℃ overnight on the sampling day, and were dried at 60 ℃ for two days. In the laboratory, the samples were ground by a mortar and pestle with liquid nitrogen and were dried one day. The samples were stored with silica gel in a sealed plastic box until use.

The four habitats were classified, bare ground, *Larix* understory, *G. miqueliana* shrub, and *S. reinii* shrub, based on the dominance (Uesaka and Tsuyuzaki 2004; Akasaka and Tsuyuzaki 2005). Tephra at 0−5 cm in depth was sampled with a soil tin (surface area: 20 cm^2; depth: 5 cm) from four habitats at each of the five elevations between 380 m and 930 m in mid-October 2014. The concentrations of inorganic N in tephra were measured by a half of each sample sieved with a 2 mm mesh after keeping in a refrigerator at −4 ℃. Inorganic N was evaluated by the sum of NH_4^+, NO_2^-, and NO_3^- after extracting inorganic N with 2N KCl solution, using a QuAAtro colorimetric analyzer (Bran + Luebbe, Norderstedt, Germany). The other half was

kept in the same way with the foliage samples for the analysis of N% and δ^{15}N after dried for 7 days and sieved with a 100 μm mesh.

To investigate variations in the δ^{15}N of mycorrhizal fungi on *L. kaempferi*, sporocarp caps and rhizomorphs were sampled from the five elevations in early September and mid-October 2014, which were treated in the same way with foliage samples. Since *S. cavipes* was most dominant on larch roots and was larch-specific (Kwon and Tsuyuzaki 2016, Kayama et al. 2015), this species was selected for the survey.

L. kaempferi litter

To estimate the delayed effects of *L. kaempferi* litter on nitrogen cycle (Liu et al. 1998), the number of layers (m^2/m^2) of annual litter provided by *L. kaempferi* was measured across the elevations. The number of layers of annual *L. kaempferi* litter was calculated from specific leaf area (SLA, cm^2/g) and annual leaf-fall per plot (ALFP, $g/100m^2$). Plot number was seven at each elevation and the area of each plot was 100 m^2. After weighing the dry mass of more than 1,000 *L. kaempferi* litters at each plot, litters were scanned by an image scanner and the projected area was calculated with ImageJ and R statistical software packages to obtain SLA (Katabuchi 2015). ALFP was calculated by the already-proven allometric relationships (Osawa 1990; Miyaura and Hozumi 1988). In the larch stand:

$$w_L = 16.5 \times D_B^{2.16} \quad \dots \text{(Eq. 1)}$$

$$logl_L \; / \; logw_L = 0.94 \sim 1.00 \quad \dots \text{(Eq. 2)}$$

where w_L, is dry foliage mass (g/tree), D_B (cm) is the stem diameter at the height just below the lowest living-branch, and l_L is annual leaf-fall (ALF, g/(tree × year)) for one *L. kaempferi* tree. The annual litter accumulation (ALA) developed by *L. kaempferi* litter was calculated by:

$$\text{ALFP } (\text{g}/100\ \text{m}^2) = \Sigma\ \text{ALF} \quad \text{for all } L.kaempferi \text{ in a plot } (100\ \text{m}^2) \quad \ldots \text{ (Eq. 3)}$$

$$\text{ALA } (\text{m}^2/\ \text{m}^2 \text{ or layers}) = \text{SLA} \times \text{ALFP} \quad \ldots \text{ (Eq. 4)}$$

Additionally, because specific leaf nitrogen (SLN, μg/cm^2) is positively correlated to N availability, SLN of larch litter was calculated from SLA and foliar N concentration and was compared along elevations (Chen and Klinka 1998; Henderson and Jose 2005).

Evaluation of N transfer by isotopes

The foliage, tephra and sporocarp caps and rhizomorphs of *S. cavipes* were analyzed on N% and δ^{15}N, using a continuous flow isotope mass-spectrometer (Delta V Plus, Thermo Fisher Scientific, Yokohama, Japan) coupled with an elemental analyzer (Flash EA 1112, Thermo Fisher Scientific). A sample was made by leaves from 4−5 plants on each species for measuring foliar N% and δ^{15}N. N isotope ratio (δ^{15}N) was calculated as (Coplen 2011):

$$\delta^{15}N\ (‰) = (R_{sample}/\ R_{standard}) - 1$$

where R_{sample} is ^{15}N/^{14}N in a sample with molar ratio, and $R_{standard}$ is ^{15}N/^{14}N in the air.

Based on two fungal-pool model, foliar N% and δ^{15}N in woody plants and non-mycorrhizal sedge were measured along the elevations (Hobbie and Hobbie 2008, Kwon and Tsuyuzaki 2016). For the evaluation of direct N transfer from the substrates to the plants, δ^{15}N of non-mycorrhizal *C. oxyandra* was measured, which was designated as $\delta^{15}N_{av(pl)}$. N dependence of plants on mycorrhizal fungi at the lowest level is set as the difference (Δ_p) between δ^{15}N in the substrates ($\delta^{15}N_{av(pl)}$) and plant ($\delta^{15}N_p$), which is therefore determined by $[\delta^{15}N_{av(pl)} - \delta^{15}N_p]$ for ecto- and ericoid mycorrhizal plants at each elevation. Difference in foliar δ^{15}N between non-mycorrhizal and ectomycorrhizal plants was theoretically derived from N dependence of plants

on mycorrhizal fungi (Hobbie and Hobbie 2008, Kwon and Tsuyuzaki 2016). ($\Delta_P \times 10$) is plant N-dependence (%) on mycorrhizal fungi at the lowest level (Kwon and Tsuyuzaki 2016).

Statistical analysis

To compare the annual leaf-fall of *L. kaempferi*, cover and stem volume on each species, foliar N%, foliar $\delta^{15}N$ and Δ_P among the elevations, multiples comparisons were conducted as follows. The normality and homoscedasticity were confirmed using Shapiro-Wilk test and Levene's test. On one variable such as elevations or habitats, Tukey's HSD post-hoc test was used if one-way ANOVA was significant or pairwise comparisons with Wilcoxon rank-sum test were used if Kruskal-Wallis test was significant. The differences of the inorganic N concentration, N%, and $\delta^{15}N$ in soil were compared among elevations and habitats with their interaction by two-way ANOVA. N% and $\delta^{15}N$ in leaves on each species were compared among elevations and months with their interactions by two-way ANOVA. Relationships between N% and $\delta^{15}N$ in the leaves on each species were examined by Pearson's correlation (r). Statistical significance was $P < 0.05$. All statistical analyses were performed using the R program (ver. 3.1.2) (R core team 2014).

3.4 Results

Distribution of vegetation and litter

Since the craters were developed on the summit area, the distances from crater to the sites increased with decreasing elevation and showed gradual vegetation change (Table 3-1). With

decreasing the elevation and increasing the distance from craters, the trees established well (Table 3-1). *L. kaempferi* was the most abundant tall tree of which stem volumes explained 99 ± 1%, 100 ± 0%, 95 ± 2%, 82 ± 7%, and 92 ± 2% of total tall trees on RD9, RD8, HS6, HS4, and HS3 sites, respectively (Table 3-1). *L. kaempferi* reduced the stem volume with increasing elevations. In particular, the stem volume was abruptly reduced on HS6 and was least on RD8 and RD9. Two shrubs, *S. reinii* and *G. miqueliana*, showed distinct fluctuation patterns of cover. *S. reinii* had the peaks of cover, over 20%, at HS6 and RD8, while *G. miqueliana* gradually increased the cover with decreasing the elevations. At the bottom of the mountain (RD3), the cover of *G. miqueliana* was more than 50%. The five broad-leaved trees, *B. ermanii*, *B. platyphylla*, *P. sieboldii*, *A. maximowiczii* and *Q. mongolica* established with low stem volumes throughout the elevations.

The layers of *L. kaempferi* litter were more at lower elevations, as shown by ALA (Fig. 3-1). The lowest C/N ratio and the highest N%, $\delta^{15}N$, and SLN of *L. kaempferi* litter at RD8 indicated N availability was highest at RD8 site (Table 3-2). Given the variations of C/N ratio, N%, $\delta^{15}N$, and SLN of *L. kaempferi* litter along elevations, N availability increased from RD9 to RD8 and then gradually decreased to the elevation of HS3. Bare ground remained well at the high elevation and shrub litter was confined inside the shrub patch, showing that the litter provided by *L. kaempferi* was less at the high elevation. The ALA (layers) of *L. kaempferi* litter was more than two below 480m elevation (HS4). The field observations confirmed that the ground surface was completely covered with *L. kaempferi* litter when *L. kaempferi* developed its forests below 480m elevation (HS4). Since litter consisting of non-larch leaves of tall trees was not established well in all the sites, the major component of litter was *L. kaempferi* leaves and shrub litters.

N and δ^{15}N in the substrates along elevational changes

Total N in the tephra was higher at the higher elevations (Fig. 3-1). In particular, it abruptly increased in the shrub patches at 890m elevation (RD8). The larch understory showed equal to or lower N% than the bare ground. Therefore, the increases in total N originated from the increase in the shrub patches consisting of the two examined species, *S. reinii* and *G. miqueliana*. These indicated that the determinants of total N were mostly derived from the shrub patches. As *G. miqueliana* was least at the high elevations, the N supply was mostly conducted by *S. reinii* at the high elevations. This also indicated that the litter of *L. kaempferi* did not contribute to N in the tephra throughout the elevations.

Inorganic N in the substrates ranged from 2.2 mg/(kg fresh tephra) to 1.4 mg/(kg fresh tephra) and was not different between the habitats (P = 0.57, two-way ANOVA) (Fig. 3-1). Elevational differences in inorganic N were detected only at HS6 and HS4 sites of which inorganic N was the lowest.

δ^{15}N in the substrates was low at the high elevation, in particular, over 680 m elevation (HS6) (Kruskal-Wallis-test, $P < 0.001$) (Fig. 3-1). In addition, δ^{15}N was lower in the two shrub habitats at the high elevations and was not different between the habitats at the low elevations. This indicated that the positive effect of shrubs on the increase of N in tephra disappeared at the low elevations. Since *S. reinii* was more dominant at the high elevations than *G. miqueliana*, *S. reinii* contributed mostly to the increase of total N in tephra and to the decrease of δ^{15}N in tephra at high elevation.

Seasonal and elevational changes in foliar N and $\delta^{15}N$ of plants

L. kaempferi decreased foliar N% with progressing seasons until early November (Fig. 3-2). As well as *L. kaempferi*, two shrubs, *S. reinii* and *G. miqueliana* seasonally decreased foliar N%. *C. oxyandra* also had high foliar N% in the early seasons. Of the five native trees, three of them, *B. ermanii*, *B. platyphylla* and *P. sieboldii*, decreased foliar N% with seasons. *A. maximowiczii* increased foliar N% gradually until mid-July. *Q. mongolica* did not vary N% among the seasons with low $\delta^{15}N$, at around −8‰.

L. kaempferi showed lower foliar $\delta^{15}N$ at lower elevations and in the early growing seasons (Fig. 3-2), indicating that the role of ectomycorrhizal fungi on plant N uptake became larger with decreasing the elevations in the early growing seasons. *S. reinii* did not change $\delta^{15}N$ throughout seasons with higher $\delta^{15}N$ at the lower elevations, showing that the interaction between *S. reinii* and mycorrhizal fungi became lower as elevation lowered. *G. miqueliana* showed the lowest $\delta^{15}N$ that did not vary across the elevations throughout the seasons. Therefore, the interactions between plants and mycorrhizal fungi were different between these two shrubs, *S. reinii* and *G. miqueliana*.

C. oxyandra had the highest $\delta^{15}N$ of the examined species except N-fixing *A. maximowiczii* and increased with the lowered elevations, synchronized with the fluctuations of $\delta^{15}N$ in the substrates (Fig. 3-1). Therefore, the $\delta^{15}N$ of non-mycorrhizal *C. oxyandra* showed that available N to plants without mycorrhizal relation came to be enriched in $\delta^{15}N$ as elevation lowered, which also indicated that the effect of $\delta^{15}N$-depleted shrub litter diminished as elevation lowered.

Of the five native trees examined, foliar $\delta^{15}N$ did not differ among the elevations (Fig. 3-2). The low foliar $\delta^{15}N$ of these species indicated that these plants started to recruit under the condition of unvaried and high mycorrhizal N transfer. *A. maximowiczii* of the five trees showed

the highest $\delta^{15}N$ ranging from −1‰ and −2‰, showing that this species had the specific N transfer routes different from the other deciduous trees.

Relationships between N% and $\delta^{15}N$ in plant leaves

Foliar N% in *L. kaempferi* was positively correlated to foliar $\delta^{15}N$ throughout the seasons (Pearson correlation coefficients, $0.74 < r < 0.78$, $P < 0.001$) except in early June when the leaves rapidly grew (Fig. 3-3 and Table 3-3). These positive linear correlations showed that the mycorrhizal partnership was enhanced as elevation lowered and N in tephra decreased. In contrast, the native, deciduous trees and shrubs generally did not show significant correlations between foliar N% and foliar $\delta^{15}N$ across the elevations throughout seasons with a few exceptions such as *B. ermanii* in mid-October.

Non-mycorrhizal *C. oxyandra* showed negative correlations between N% and $\delta^{15}N$ from early June to early September ($-0.69 < r < -0.52$, $P < 0.05$) (Fig. 3-3 and Table 3-3). Therefore, as elevation lowered, N acquisition directly from the substrates, reflected on N% and $\delta^{15}N$ in *C. oxyandra*, was limited and higher portion of N came from $\delta^{15}N$-enriched deteriorated N sources, not from $\delta^{15}N$-depleted shrub litters (Fig. 3-1).

$\delta^{15}N$ of plant foliage and Larix-specific Suillus cavipes and the vegetation

The observations of mushroom confirmed that most of the larch-specific fungi belonged to genus *Suillus*, in particular, species *S. cavipes*. However, *S. cavipes* was not observed at the highest elevation (RD9). *S. cavipes* did not fluctuate $\delta^{15}N$ in the sporocarp caps and rhizomorphs along the elevations. $\delta^{15}N$ in the sporocarp caps was 4.32 ± 0.73‰ (mean ± standard error, $n = 11$) on HS3, 4.50 ± 0.41‰ ($n = 9$) on HS4, 4.59 ± 0.37‰ ($n = 9$) on HS6, and 5.07 ± 0.58 ($n = 6$) on

RD8 (one-way ANOVA, $P = 0.85$). $\delta^{15}N$ in the rhizomorphs was 3.44 ± 0.88‰ ($n = 8$), 2.38 ± 0.45 ($n = 8$), 2.50 ± 0.51 ($n = 4$) and 2.52 ± 0.55 ($n = 6$) on HS3, HS4, HS6 and RD8, respectively ($P = 0.62$). Therefore, the fungal $\delta^{15}N$ was assumed to be stable in the surveyed area and was not considered to affect the tendency of plant N-dependence on mycorrhizal fungi along elevations. This suggested that the difference (Δ_P) of $\delta^{15}N$ between mycorrhizal plant and non-mycorrhizal plant, i.e., the N-dependence of mycorrhizal plant on mycorrhizal fungi in minimum level, had the ecological implication due to invariableness of fungal $\delta^{15}N$ along elevations.

Δ_P in *S. reinii* peaked at the middle elevations (RD8 and HS6) and varied in consistent with *S. reinii* cover along elevations (Fig. 3-4). Another shrub, *G. miqueliana*, gradually decreased the cover from 57 ± 9% at the lowest elevation to 1 ± 0% at the highest elevation. Δ_P and cover of *G. miqueliana* gradually increased from high to low elevations. Because *G. miqueliana* established least at the high elevations, it contributed less to N in the substrates there.

L. kaempferi at two high elevations (RD8 and RD9) showed equivalent foliar $\delta^{15}N$ to that of non-mycorrhizal *C. oxyandra* (t-test, $P > 0.80$) and gradually increased Δ_P as well as the biomass of *L. kaempferi* as elevation lowered (Fig. 3-4). Therefore, the N-dependence on mycorrhizal fungi of *L. kaempferi* was less at the high elevations and became greater at lower elevations. Because *A. maximowiczii* was in strong symbiotic relation with actinobacteria, Δ_P derived from two-pool fungal model could not be applied to *A. maximowiczii*. All the other native trees showed higher than 2.5‰ of Δ_P, indicating that more than 25% of N were obtained from mycorrhizal fungi throughout the elevations.

3.5 Discussion

N status in the substrates

On Mount Koma, N in the substrates, measured by total and inorganic N, was lower at lower elevations where the litter of *L. kaempferi* was well-accumulated. Forests in Hokkaido, including larch forests, contain more than 0.3% on total N and more than 4 mg/kg on inorganic N (Morishita et al. 2004; Shibata et al. 2011). Larch plantations such as *L. olgensis*, *L. kaempferi*, and *L. gmelinii* which are most frequently used for reforestration in Northeast Asia reduce total N and inorganic N in the soils by supplying persistent litter (Yang et al. 2013, Liu et al. 1998). The litter of *L. kaempferi* was decomposed two times slower than the litter of deciduous trees, birch, popular and willow in Sheffield, UK (Cornelissen 1996). Therefore, the litter of *L. kaempferi* should slow the accumulation of N in the tephra. When the ground is completely covered with thick layers of poor quality litter of *L. kaempferi*, it is possible that good quality litter such as shrubs would be prohibited from entering into the soils and subsequently N cycling would be retarded. In support of this possibility, SLN of *L. kaempferi* litter, which shows strong positive correlations with N availability, dramatically decreased at lower elevations (Henderson and Jose 2005).

S. reinii, the major contributor to N deposition in tephra, showed high cover at the top and middle elevations, while *G. miqueliana* established well at the low elevations. *S. reinii* is intolerant to shade whereas *G. miqueliana* is tolerant (Kuzovkina and Volk 2009; Fraser et al. 1993). Therefore, the distributions of the two shrub species were considered to be determined mostly by the overstory shade. Not only on Mount Koma, *S. reinii* is dominant above the treeline where no overstory is developed on Mount Fuji, central Japan (Ohsawa 1984).

N% and δ^{15}N in plants

S. reinii shrub patch increased N in the substrates well, suggesting that the establishment of *S. reinii* patches was a key to the deposition of N in the substrates. However, *S. reinii* did not establish well at the low elevations. In addition, *S. reinii* leaves increased δ^{15}N as elevation lowered, showing that relationships between *S. renii* and mycorrhizal symbionts became weak with lowering the elevations. Several lines of evidence suggested that shade reduces ectomycorrhizal colonization, diversity of colonized mycorrhizal fungi and intraspecific mycorrhizal network, which cause less survival rate and poorer performance of ectomycorrhizal plants than otherwise (McGuire 2007, Druebert et al. 2009; Zhou and Sharik 1997). On Mt. Koma, shade-intolerant willow shrub is likely to be earlier placed under energetically stressful condition due to unfavorable carbon balance than other tall trees and shade-tolerant *G. miqueliana* (Craine et al. 2012). The foregoing suggests that mycorrhizal symbiotic relation for the C-N exchange between willow shrubs and their fungal symbionts is stunted and imbalanced to the extent that willow shrubs cannot sustain their dominance (Zhou and Sharik 1997). Therefore, the shade cast by *L. kaempferi* at the low elevations was considered to inhibit the photosynthesis and mycorrhizal interaction on *S. renii*.

G. miqueliana leaves showed the lowest δ^{15}N of the examined species. Ericoid mycorrhizal plants such as *G. miqueliana* also showed the lowest δ^{15}N among mycorrhizal and non-mycorrhizal plants in a global perspective (Fukuchi et al. 2011, Craine et al. 2009, 2015), particularly, in Lyman Glacier retreat region (Hobbie et al. 2005), and in heath and forest tundra in northern Sweden, north-eastern Greenland, and eastern Siberia (Michelsen et al. 1998). Schulze et al. (1994) suggested that enzymatic activity of ericoid mycorrhizal fungi is higher

than ectomycorrhizal fungi. *G. miqueliana* shrub patch did not contribute to N in the substrates, although the shrubs established well at the low elevations. Therefore, the high cover of *G. miqueliana* was supported by ericoid mycorrhizae, in addition to the shade tolerance.

The Δ_p in *L. kaempferi* was nearly zero at the high elevation and increased gradually with decreasing the elevations, showing that N dependence of *L. kaempferi* on mycorrhizal fungi increased with decreasing N in substrate as elevation lowers. N dependence of *L. kaempferi* on mycorrhizal fungi is less than 5% at the high elevations on Mount Koma, whereas it is over 50% on the middle elevations (Kwon and Tsuyuzaki 2016). *L. kaempferi* develops the specific mycorrhizae of which fungi are in genus *Suillus* (Kwon and Tsuyuzaki 2016). The development of *Suillus* mycorrhiza is enhanced by low N in the substrates (Hobbie and Colpaert 2003, Lilleskov et al. 2011). Therefore, the positive correlation between $\delta^{15}N$ and N% was observed on *L. kaempferi*.

In contrast to *L. kaempferi*, all the broad-leaved trees did not show the significant correlations between $\delta^{15}N$ and N% because their $\delta^{15}N$ was low and unchanged. Species in *Betula* and *Populus* develop generalist mycorrhizal fungi on nutrient poor habitats in the early successional stages of succession (Szuba 2015, Nara 2006). The generalist fungi are not in genus *Suillus* and are insensitive to N availability in the substrates (Wallander and Nylund 1992; Lilleskov et al. 2011). Therefore, the different responses to N in the substrates between *L. kaefmperi* and broad-leaved trees were derived by the characteristics of symbiotic fungi, although all of the trees developed ectomycorrhizae. Because *Q. mongolica* established only at the lowest elevation with low $\delta^{15}N$, the altitudinal changes in N dependence on mycorrhizal fungi were not evaluated. However, the low $\delta^{15}N$ suggested that *Q. mongolica* intensively utilized mycorrhizal N on the N-poor habitats.

A. maximowiczii showed highly enriched foliar $\delta^{15}N$, ranging from −2‰ to −1‰, conspicuously different from the other native trees. Various early studies concluded that biologically N-fixing plants by assimilating atmospheric N_2 into NH_3 through *Rhizobium* and *Frankia* actinobacteria do not significantly fractionate stable N isotopes (Unkovich 2013) and therefore are enriched in $\delta^{15}N$ similar to $\delta^{15}N$ of atmospheric N_2. It is also reported that $\delta^{15}N$ in N-fixing plants ranges from 0‰ to −2‰ in the early successional stages in Alaskan glacial retreat (Hobbie et al. 2000). *A. maximowiczii* develops nodules in root that harbor nitrogen-fixing *Frankia* actinobacteria and is also colonized by ectomycorrhizal fungi in and on the roots (Põlme et al. 2013). Considering highly enriched foliar $\delta^{15}N$, *A. maximowiczii* depends mostly on the actinobacteria for N uptake (Unkovich 2013).

Vegetation and N dependence of plants on mycorrhizae

Salix renii is a shrub pioneer on volcanoes in Japan (Tsuyuzaki and Hase 2005, Marchese 2003). Willows, including *S. reinii*, often become pioneers on nutrient-poor habitats (Kuzovkina and Volk 2009). N dependence (%) of plants on mycorrhizal fungi is estimated by Δ_P multiplied by ten, although this estimation is minimal (Kwon and Tsuyuzaki 2016). The Δ_P variations of *S. reinii*, *G. miqueliana* and *L. kaempferi* matched well with the change of their aboveground abundances. Their patterns clearly showed that *S. reinii* played a vital facilitative role on the recruitment of *L. kaempferi*, although the dominance and positive effects of *S. reinii* declined due to the overstory of *L. kaempferi* and finally vanished at lower elevations. The mycorrhizal association of *L. kaempferi* is obligate. However, the N uptake through mycorrhizal fungi became low at the high elevations in the early successional stages. Δ_P in native broad-leaved trees except alder was high and stable, showing that the considerable amount of plant N was

constantly transferred through mycorrhizal fungi and it was a prerequisite for the initial recruitment of native broad-leaved trees. Therefore, the flexibility in mycorrhizal relation of plants represented by *L. kaempferi* should determine the vegetation development.

Fig. 3-1 Differences in (a) inorganic nitrogen in tephra, (b) N (%) and $\delta^{15}N$ (‰) in tephra, and (c) Range of annual litter accumulation (ALA) on the ground surface by *Larix kaempferi* litter between habitats and/or elevations. On (a) and (b), mean (columns) is shown with standard error (bar). On (a), the number of samples is five. On (b), the number of samples is shown on the bottom of the figure. On (c), mean (circles) of maximum (gray, at $logl_L$ / $logw_L$ = 1.00) and minimum (white, at $logl_L$ / $logw_L$ = 0.94) is shown with standard error (n = 7). Two-way ANOVA were used for the effect of elevations (E) and habitats (H) with their interactions (E × H). Significance level: * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. The differences between habitats at each elevation were confirmed by Tukey's HSD test after one-way ANOVA or Wilcox rank sum test after Kruskal-Wallis test at $P < 0.05$. Habitat: white bar = bare ground, slashed = *L. kaempferi* understory, latticed = *G. miqueliana* patch, and black = *S. reinii* patch.

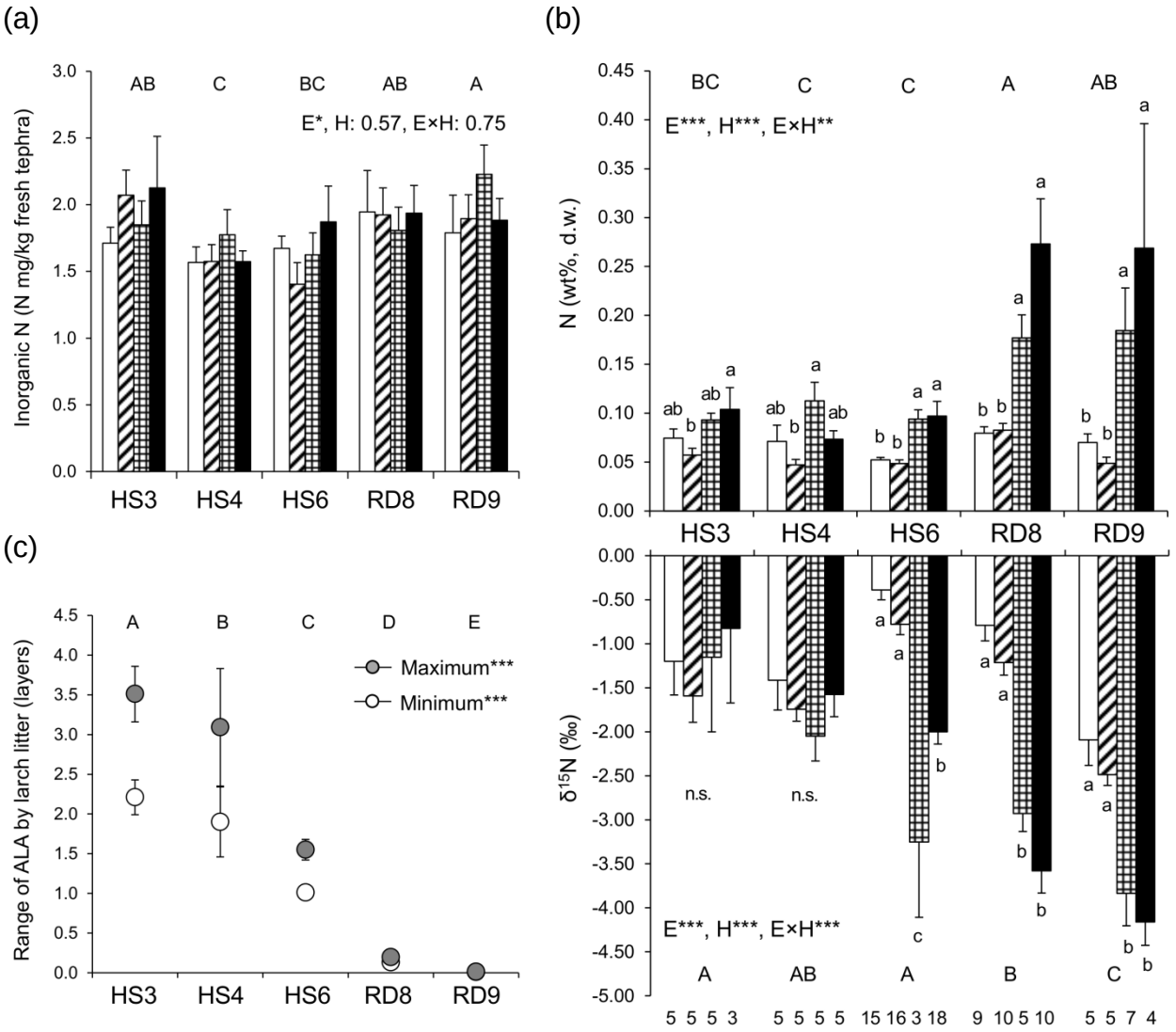


Fig. 3-2 The effects of seasons and elevations on foliar N (%) and $\delta^{15}N$ (‰) in *Larix kaempferi* (*n* = 5 for each bar), *Salix renii* (n = 5), *Gaultheria miqueliana* (*n* = 3−5), and *Carex oxyandra* (*n* = 5). The results of two-way ANOVAs were shown on each panel for the effect of month (M) and elevation (E) with their interactions (M×E), of which significant differences were indicated as follows: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and *p* values for $P > 0.05$. Upper and lower case letters indicate significant mean difference of months and elevations, respectively, by Tukey HSD test after one-way ANOVA or by pairwise comparison after Kruskal-Walls test at $P < 0.05$. The five sites are ordered from the lowest elevation (HS3, white bar) on left to the highest elevation (RD9, black) on right in the figures. Labels "e" and "m" on months mean early and mid-.

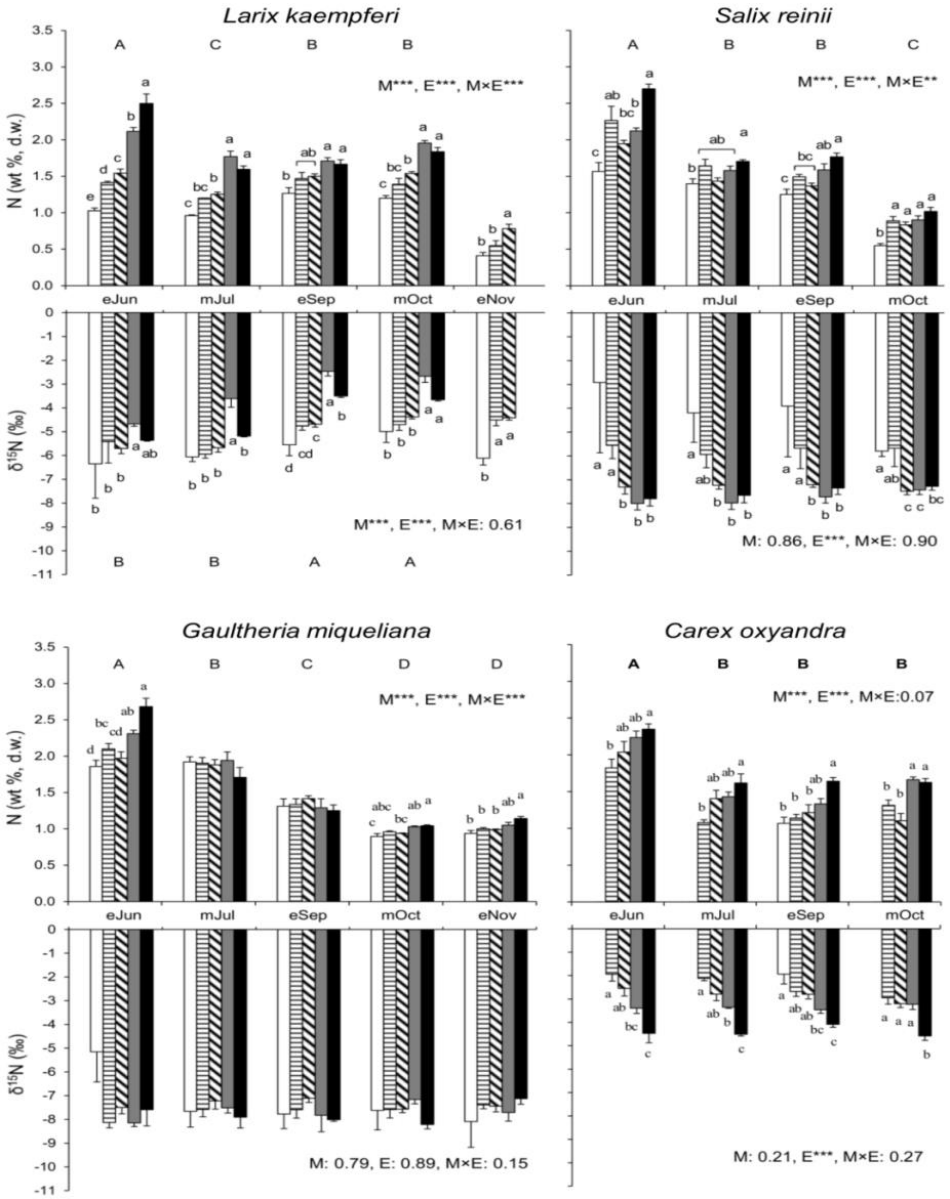


Fig. 3-2 (*continued*) The effects of seasons and elevations on foliar N (%) and $\delta^{15}N$ (‰) in *Betula ermanii*, *B. platyphylla*, *Populus sieboldii*, *Alnus maximowiczii*, and *Quercus mongolica*. Sample size = 3 except 1 on *A. maximowiczii* at RD8.

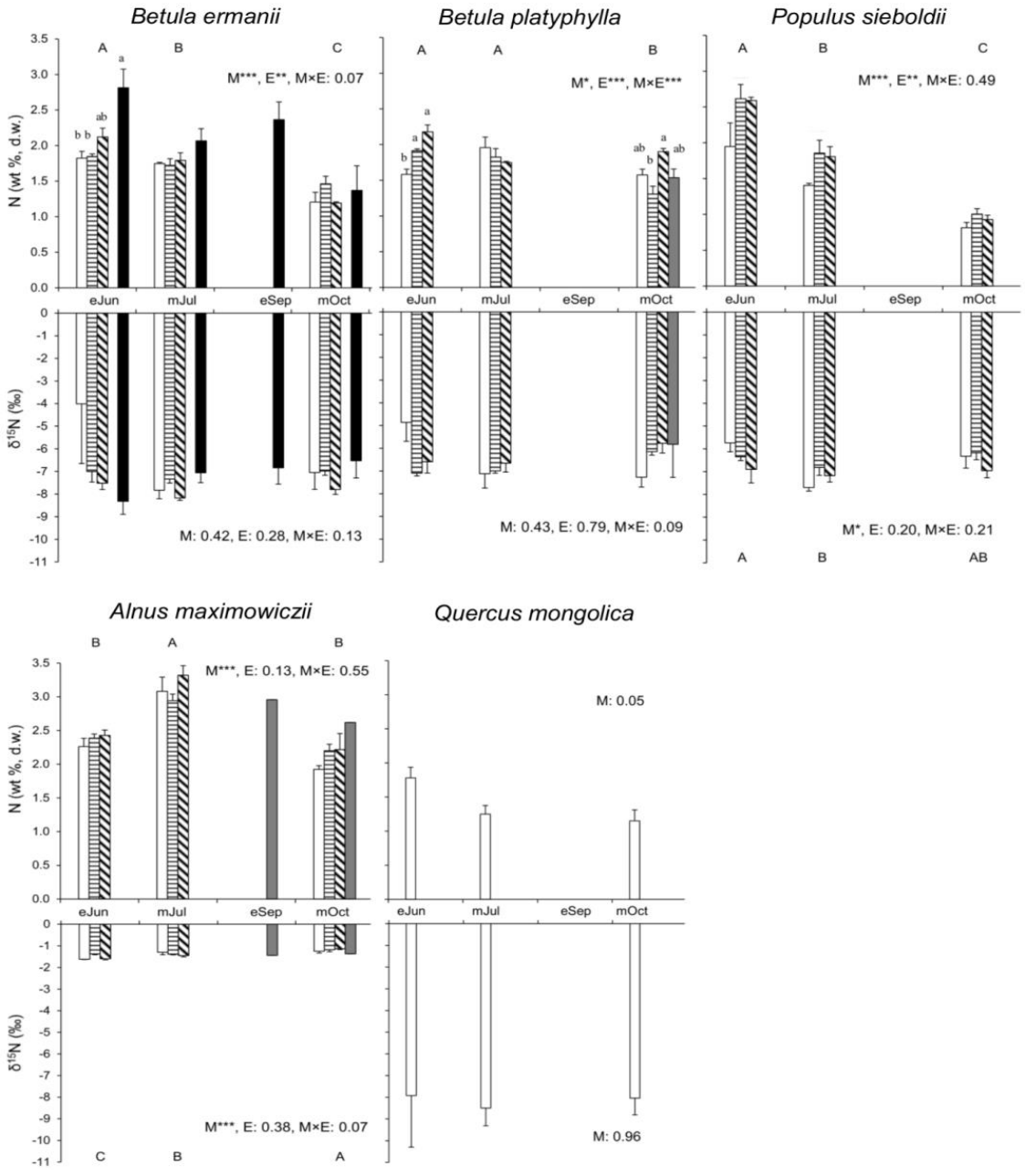


Fig. 3-3 Relationships between foliar N (%) and $\delta^{15}N$ (‰) on *Larix kaempferi* and *Carex oxyandra* in early September. Mean is shown with standard error. Site: white = HS3, lined = HS4, slashed = HS6, gray = RD8 and black = RD9. *r* is Pearson correlation coefficient. ***: significant at $P < 0.001$. The equations indicate linear regressions. For other months and species, refer to Table 3-3.

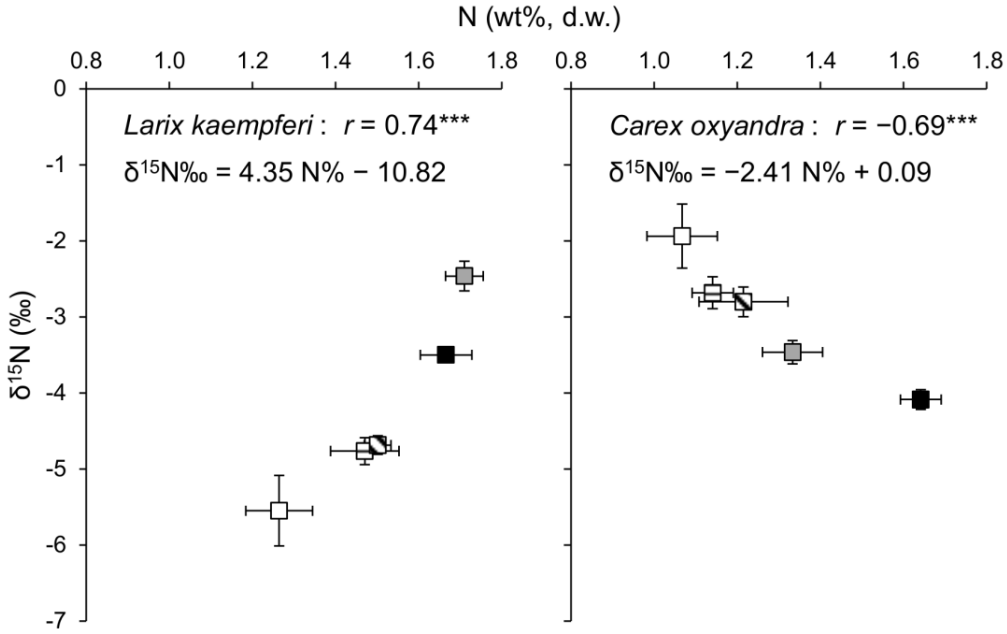


Fig. 3-4 Coverage of shrubs, stem volume of tall trees, and Δ_P ($=\delta^{15}N_{av(pl)} - \delta^{15}N_P$) along elevations. Bars and lines designate stem volume (or cover) and Δ_P, respectively. The plot number for the measurement at each elevation is 7 and the area of each plot is 10 m × 10 m. Upper and lower case letters indicate significant mean difference of stem volume (or cover) and Δ_P, respectively, by Tukey HSD test after one-way ANOVA or by pairwise comparison after Kruskal-Walls test. Sample size for Δ_P is 16−22 for *L. kaempferi*, 16−18 for *S. reinii*, 12 for *G. miqueliana* and 8−12 for other tall trees at each elevation except 3 at RD8 for *B. platyphylla*. For the calcualtion of Δ_P, foliar $\delta^{15}N_P$ data from early June to mid-October were pooled at each elevation for each species. The average of $\delta^{15}N$ of *C. oxyandra* at each elevation was used as $\delta^{15}N_{av(pl)}$. Mean ± SE. Shrub cover and stem vomume were redrawn from Table 3-1.

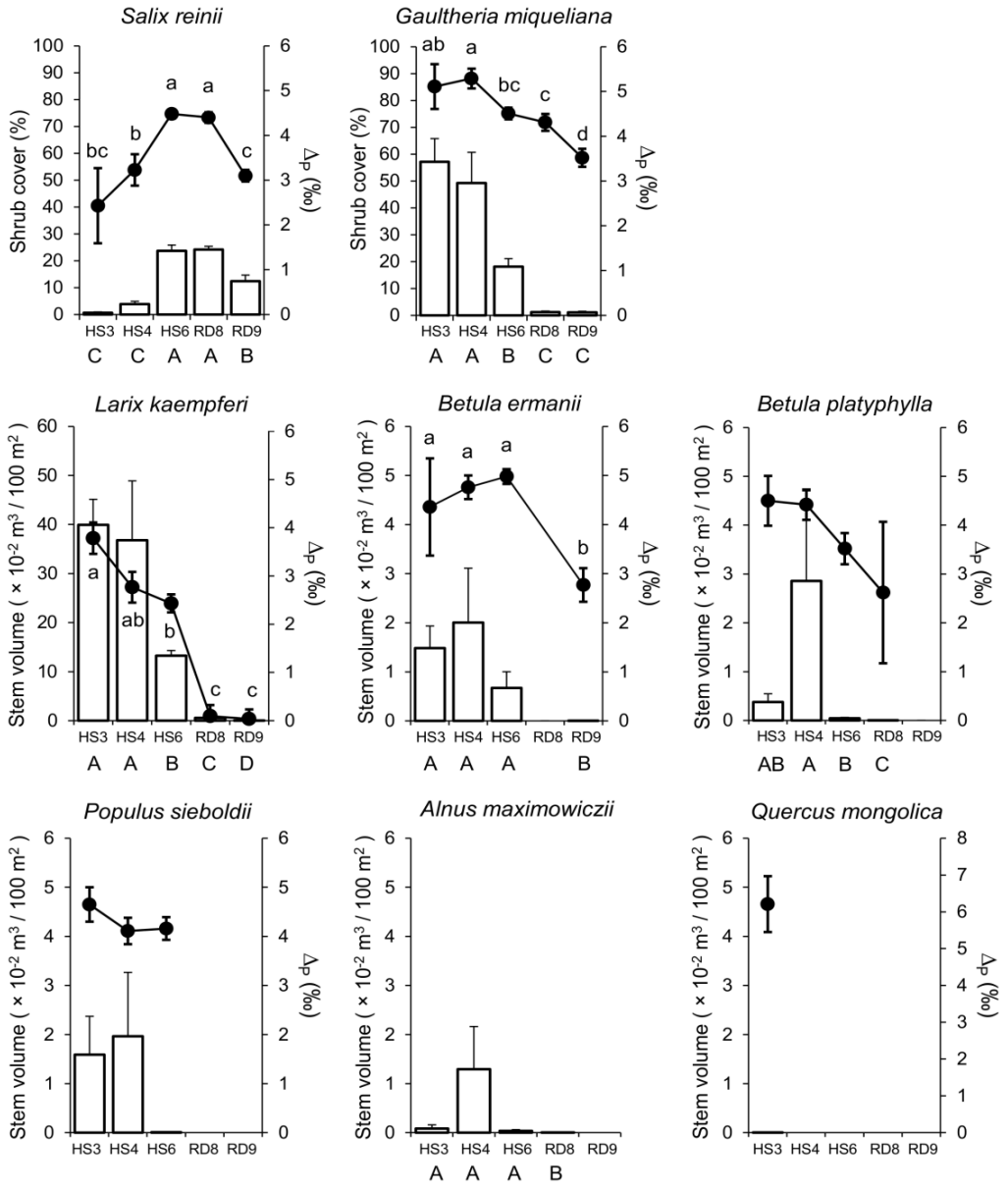


Table 3-1 Site description and vegetation composition.

Site description	HS3	HS4	HS6	RD8	RD9
Elevation (m)	365 − 380	465 − 480	650 − 680	875 − 890	930
Feature	Hillslope	Hillslope	Hillslope	Ridge	Ridge
Slope (°)	5.4	22.0	13.3	5.7	0.6
Distance from crater (m)	3,530 − 3,650	2,600 − 2,700	1,725 − 1,870	850 − 950	50 − 150
Area of study site (m × m)	150 × 250	70 × 100	40 × 130	100 × 130	150 × 250
Shrub cover (%)					
Salix reinii	0.65 ± 0.31^{c}	3.87 ± 1.06^{c}	23.73 ± 2.16^{a}	24.21 ± 1.14^{a}	12.43 ± 2.24^{b}
Gaultheria miqueliana	57.14 ± 8.65^{a}	49.28 ± 11.46^{a}	18.10 ± 3.02^{b}	1.20 ± 0.43^{c}	1.15 ± 0.40^{c}
Stem volume of tall trees ($\times 10^{-2}$ m^3 / 100m^2)					
Larix kaempferi	39.93 ± 5.22^{a}	36.79 ± 12.11^{a}	13.23 ± 1.06^{b}	0.56 ± 0.09^{c}	0.02 ± 0.01^{d}
Betula ermanii	1.48 ± 0.45^{a}	2.00 ± 1.11^{a}	0.67 ± 0.33^{a}	−	< 0.001^{b}
Betula platyphylla	0.38 ± 0.17^{ab}	2.86 ± 1.84^{a}	0.05 ± 0.02^{b}	< 0.001^{c}	−
Populus sieboldii	1.59 ± 0.78	1.96 ± 1.30	0.01 ± 0.00	< 0.001	−
Alnus maximowiczii	0.08 ± 0.08^{a}	1.29 ± 0.87^{a}	0.03 ± 0.03^{a}	< 0.001^{b}	−
Quercus mongolica	0.002 ± 0.001	−	−	−	−

- Lowercase letters indicate significant difference between elevations at $P < 0.05$ (Tukey's HSD test after one-way ANOVA or pairwise comparisons using Wilcoxon rank sum test after Kruskal-Wallis test).
- Plot number surveyed at each elevation for vegetation composition = 7, each plot area = 100m^2, and Mean ± standard error (SE).

Table 3-2 Traits of *L. kaempferi* litter along elevations.

Traits of *L. kaempferi* litter	HS3	HS4	HS6	RD8	RD9
C (wt %)	49.38 ± 0.06^{c}	49.64 ± 0.21^{c}	50.50 ± 0.09^{b}	50.71 ± 0.22^{ab}	51.41 ± 0.27^{a}
N (wt %)	0.42 ± 0.01^{d}	0.47 ± 0.02^{cd}	0.67 ± 0.02^{c}	1.39 ± 0.09^{a}	1.10 ± 0.08^{b}
C/N ratio	118.86 ± 3.17^{a}	105.04 ± 3.25^{b}	75.76 ± 2.27^{c}	36.94 ± 2.23^{d}	47.77 ± 3.23^{d}
δ^{15}N (‰)	−6.54 ± 0.18^{d}	−5.42 ± 0.16^{c}	−4.86 ± 0.20^{bc}	−3.91 ± 0.03^{a}	−4.35 ± 0.08^{ab}
Specific leaf N (SLN, μg / cm^2)	51.5 ± 1.4^{d}	55.2 ± 1.9^{cd}	74.8 ± 2.4^{bc}	136.9 ± 8.6^{a}	89.0 ± 6.5^{b}

- Lowercase letters indicate significant difference between elevations at $P < 0.05$ (Tukey's HSD test after one-way ANOVA or pairwise comparisons using Wilcoxon rank sum test after Kruskal-Wallis test).
- Sample size = 5−7, each plot area = 100m^2, and Mean ± standard error (SE).

Table 3-3 Relationships between foliar N (%) and $\delta^{15}N$ (‰) of woody species and sedge, *C. oxyandra*. *r* is Pearson correlation coefficient. *n* is sample size. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and n.s. for $p > 0.05$.

Species	Month	*n*	Slope	*r*
Larix kaempferi	early June	23	0.62	0.26 n.s.
	mid July	21	2.75	0.77***
	early September	25	4.35	0.74***
	mid October	25	2.61	0.78***
Salix reinii	early June	23	−1.32	−0.22 n.s..
	mid July	15	−2.37	−0.21 n.s.
	early September	25	−3.61	−0.31 n.s.
	mid October	25	−1.42	−0.23 n.s.
Gaultheria miqueliana	early June	15	−2.14	−0.46 n.s.
	mid July	15	1.92	0.49 n.s.
	early September	15	4.17	0.81***
	mid October	15	2.80	0.26 n.s.
	early November	25	5.77	0.46*
Betula ermanii	early June	12	−2.03	−0.36 n.s.
	mid July	12	0.02	0.01 n.s.
	mid October	12	1.84	0.61*
Betula platyphylla	early June	9	−2.81	−0.60 n.s.
	mid July	9	−2.14	−0.59 n.s.
	mid October	12	1.41	0.28 n.s.
Populus sieboldii	early June	9	−0.36	−0.21 n.s.
	mid July	9	0.49	0.26 n.s.
	mid October	9	−0.11	−0.02 n.s.
Alnus maximowiczii	early June	9	0.04	0.05 n.s.
	mid July	9	−0.25	−0.53 n.s.
	mid October	10	−0.01	−0.02 n.s.
Quercus mongolica	early June ~ mid October	9	−1.84	−0.30 n.s.
Carex oxyandra	early June	20	−2.21	−0.57**
	mid July	15	−1.79	−0.52*
	early September	25	−2.41	−0.69***
	mid October	22	−0.83	−0.29 n.s.

General discussion

Nitrogen (N) dynamics determines the patterns of succession and functions of ecosystems. N dynamics is changed greatly after catastrophic disturbances, represented by volcanic eruption, because N is least in the substrates soon after the disturbances. For plant establishment, I hypothesized that N dynamics was tightly linked with the vegetation structures at both small (habitat) and large (landscape) scales. To clarify the spatio-temporal changes, N transfer was monitored with the distinctive vegetation types on Mount Koma (1,131m elevation) in northern Japan that erupted in 1929. Although the climax in this region is deciduous, board-leaved forests, the biological invasion of larch (*Larix kaempferi*) alters the ordinal succession on the mountain (Kondo and Tsuyuzaki 1999). The invasion is promoted by willow shrub patches formed by *Salix reinii* (Akasaka and Tsuyuzaki 2005). Therefore, an objective in this study was clarifying why the biological invasion was promoted by the shrub patches. *S. reinii* shrub patches do not alter phosphorus in the tephra and increase nitrogen on Mount Koma (Uesaka and Tsuyuzaki 2004). *S. reinii* develops mycorrhiza facultatively (Nara 2006, Kuzovkina and Volk 2009). Since N transfer from substrates to plants is often mediated and assisted by mycorrhizal fungi, in particular, in N-poor habitats, the N transfer was focused.

A greenhouse experiment on the growth of larch seedlings was firstly conducted to obtain the relationships between them, by supplying plentiful water not to consider the effects of water deficiency (Chapter 1). The shoot/root (S/R) ratio of larch seedlings is determined by both light and nutrition. In particular, S/R ratio decreases with decreasing N in the substrates. Plants starving N allocate their resources more to the roots for obtaining N from the substrates (Levy et al. 2004; Hermans et al. 2006). The greenhouse experiment indicated that the shade by *S. reinii*

patches decreased the biomass of larch seedlings and did not influence greatly on the S/R ratio. The growth of larch seedlings at the presence of *S. reinii* markedly increased S/R ratio of larch seedlings. The larch seedlings decreased foliar N concentration when mycorrhizal fungi were reduced by a fungicide, showing that N uptake by the larch seedlings was assisted by the mycorrhizal fungi. Therefore, the nutrition of larch seedlings from substrates to larch were altered by the willow patches and the N limitation of larch seedlings was mediated by the mycorrhizal fungi. To obtain the reliability *in situ* to the next step, the field observations were conducted (Chapters 2 and 3).

The regeneration of larch was faster on hillslope at the middle elevation than on ridge at high elevation, while the tephra contained more total N on the ridge than on the hillslope. To evaluate the N dependence of larch on mycorrhizal fungi, I used a two-pool fungal model with stable N isotope ratio ($\delta^{15}N$). The N dependence was less than 5% on the ridge and more than 50% on the hillslope (Chapter 2), showing that the dependence was changed with the N status in the substrates. The N dependence of larch was fluctuated along the elevational gradient (Chapter 3). Therefore, the invasion of larch was supported by the flexibility of N dependence on mycorrhizal fungi at landscape level.

The N uptake of woody plants through mycorrhizal fungi was compared between the exotic larch and the native trees (Chapter 3). The larch developing the host-specific fungi in genus *Suillus* showed, at least, 0% to 37% of N obtained from the mycorrhizal fungi along elevations. The dependence was increased with decreasing the total N in the tephra. The native trees, all of which developed generalist ectomycorrhizal fungi, showed higher than 25% of N dependence irrespective of N in the tephra at all the examined elevations. These results showed that the

strategies of nutrient acquisition through mycorrhizal fungi were different between the exotic and native trees and were related to the establishment patterns.

The two examined species, *Gaultheria miqueliana* (Ericaceae) and *Alnus maximowiczii* (Betulaceae), showed the specific N transfer pathways (Chapter 3). *G. miqueliana* is associated with ericoid mycorrhizal fungi (Fraser et al. 1993). However, this species did not contribute to N deposits in the tephra. *A. maximowiczii* is a N-fixing plant by actinobacteria (*Frankia*) and promotes N deposition (Titus 2009; Vitousek et al. 1987). However, bacterial activities decrease in a *L. kaempferi* plantation, because of antibacterial substances produced by *L. kaempferi* (Chen et al. 2015; Yang et al. 2013). The belowground interactions through the substrates and microbial activities should be related to the establishment patterns of these two woody plants.

At habitat level, *S. reinii* shrub patches showed 2−4 times higher total N in the substrates than bare ground and larch understory at the high and middle elevations, while inorganic N did not differ between their habitats (Chapter 2). *S. reinii* is a pioneer in N-poor habitats on Mounts Fuji and Koma (Ohsawa 1984, Titus and Tsuyuzaki 2003). Producing degradable litter is one of the characteristics of pioneer plants that promote N cycling (Kueffer et al. 2008). Willows, including *S. reinii*, produce degradable litter (Schofield et al. 1998). *L. kaempferi* utilized N in such habitats for the invasion, even though the mycorrhizal relation for N uptake was low in the initial invasion phase.

Shade and litter provided by the larch reduced the dominance of shade-intolerant *S. reinii* with decreasing the mycorrhizal relation. In addition to the biological invasion, the litter consisted mostly of larch itself after the predominance. The larch litter prevented non-larch, decomposable litter from N deposits in the tephra.

In conclusion, *L. kaempferi* invaded to the barren areas, using *S. reinii* patches at habitat scale and using the plasticity of N dependence on mycorrhizal fungi at landscape level. Well-developed *L. kaempferi* trees suppressed the native woody plants, by shade and litter accumulation. The present study clarified that the vegetative structures were distorted by the biological invasion that was promoted by the relationships of mycorrhizal fungi, in particular, in N-poor habitats. These findings are applied to clarify why the biological invasions of conifers, including larches, have occurred globally (Richardson and Rejmánek 2004) and why conifers are often dominant in boreal regions (Gauthier et al. 2015).

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