Effects of simulated nitrogen deposition on ectomycorrhizae community structure in hybrid larch and its parents grown in volcanic ash soil: the role of phosphorous

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Abstract

With the rapid industrial development and modern agricultural practices, increasing nitrogen (N) deposition can cause nutrient imbalance in immature volcanic ash soil commonly found in Japan. Larch species, widely distributed in northeast Eurasia, are associated with ectomycorrhizal (ECM) fungi which play a critical role in nutrient acquisition for their hosts. In this study, we investigated species richness and diversity of ECM fungi associated with a hybrid larch (F_1) and its parents, Dahurian larch (*Larix gmelinii var. japonica*) and Japanese larch (*L. kaempferi*), under simulated N deposition (0 and 100 kg $ha^{-1}yr^{-1}$) with/without phosphorous (P) (0 and 50 kg $ha^{-1}yr^{-1}$). Seedlings planted in immature volcanic ash with low nutrient availability were subjected to the N and P treatments for fifteen months. We found that response of ECM community structure to the increased nutrient availability depended on host genotypes. Nutrient addition significantly affected ECM structure in Japanese larch, but no such significant effect was found for Dahurian larch. Effects of the nutrient addition to ECM fungal community in F_1 was intermediate. F_1 was tolerant to high N loading, which was due to consistent, relatively high association with *Suillus* sp. and *Hebeloma* sp. F_1 showed heterosis in relative biomass, which was most apparent under high N treatments. This co-variation of ECM fungal community structure and F_1 biomass in response to N loading suggest that ECM community structure might play an important role in host growth. The present findings indicate effects of N deposition on ECM fungal community structure can depend on larch species, thus it is challenging to predict general trends.

Key words: Community structure, Ectomycorrhizal fungi, Nitrogen, Phosphorus, Volcanic ash soil

1. Introduction

Nitrogen (N) deposition has been increasing since pre-industrial era as a result of industrial development and overuse of N fertilizer (Park et al., 2002; Galloway et al., 2008; Morino et al., 2011). Although N is often known as a limiting nutrient in terrestrial ecosystems (LeBauer et al., 2008), chronic deposition of atmospheric N leads to accumulation of N in the soil. Once N accumulates above a specific threshold, it can suppress root growth and induce imbalances in plant nutrients (Qu et al., 2003; Crowley et al., 2012). Furthermore, it may induce phosphorus (P) limitation in forest ecosystems (Gradowski and Thomas, 2006; Du et al., 2016). Long-term N deposition can negatively impact ecological properties and processes (Liu et al., 2011), such as P and N cycling (Du et al., 2016), soil chemistry (Vitousek et al., 1997), biological diversity (Clark and Tilman, 2008), flux of greenhouse gases (Song et al., 2013), and forest declines (e.g. Aber et al., 1989; Smith et al., 2009).

Japan is a part of the Pacific 'Ring of Fire', therefore, forest soil is often derived from volcanic ash (Schmincke, 2004). A broad area in northern Japan is covered by immature volcanic ash, which has relatively low nutrients (Kayama et al., 2015). In volcanic ash soil, N deposition may lead to a nutrient imbalance, causing P deficiency (Frey et al., 2004; Wang et al., 2016). In such nutrient-poor soils, ectomycorrhizal (ECM) fungi establish symbioses with plant roots and play a critical role in nutrient acquisition (Smith and Read, 1997, 2008; Qu et al., 2010; Agerer, 2012). The ECM colonization of seedlings can occur from existing mycelial networks (Jonsson et al., 1999) or from resistant ECM propagules present in the soil, mainly in the form of spores (Buscardo et al., 2010). For example, the ECM propagule availability was limited in volcanic desert of Mount Fuji, where mycelial networks dominated dwarf willow roots

(Nara, 2006a), whereas ECM propagules were the dominant vectors for the initial colonization of Douglas-fir seedlings in a harvested forest (Teste et al., 2009). As the major root symbionts in the forest ecosystems, ECM can improve the survival ability of trees by absorbing and providing water and essential plant nutrients from soils (Read and Smith, 1998). In general, under infertile conditions, roots associated with ECM fungi can take up significantly higher amounts of water, P, and N (Aquino and Plassard, 2004; Alves et al., 2010; Wang et al., 2016) compared to roots with no ECM (Buscot et al., 2000; Wallander, 2000; Alves et al., 2010; Qu et al., 2010; Wang et al., 2016).

Soil N availability can affect ECM fungal communities. For example, N availability was a major determinant of ECM fungal communities across north-western Europe (Cox et al. 2010). Root tip abundance and mycelial production of ECM fungi decreased with increasing N deposition (Ostonen et al., 2011; Kjøller et al., 2012). A meta-analysis revealed that mycorrhizal abundance decreased 32% and 15% with P and N addition, respectively (Treseder, 2004). There is also a great deal of studies regarding effects of changing environment, such as warming, CO_2 and O_3, on mycorrhizal communities (e.g. Treseder 2004; Treseder et al. 2016; Wang et al. 2016). However, we still have limited ecological understanding about how ECM fungal community respond to chronic N deposition in nutrient-poor soils (Veresoglou et al., 2015; Weemstra et al., 2016).

Larches (*Larix* sp.) are ectomycorrhizal deciduous conifers which occur in a vast area of the Eastern Eurasian continent (Qu et al., 2004, 2010; Abaimov, 2010; Wang et al., 2016). Dahurian larch (*Larix gmelinii* (Rupr.) Rupr.) is a representative dominant tree species in the Eurasian area (Abaimov et al., 2000; Kayama et al., 2009; Mao et al., 2010), and has also been intensively planted for afforestation in northeastern China (Zhang et al., 2000; Zhao et al., 2011). Similarly, Japanese larch (*Larix kaempferi* (Lamb.) Carr.), which is a native species to northern Japan, has been planted widely in Japanese forests. However, it suffers from abiotic and biotic stresses, such as cold

105 weather, wild animal grazing and shoot blight disease (Ryu et al., 2009). To overcome
106 these stresses, a new hybrid larch F_1 (hereafter, F_1) has been developed by hybridizing
107 Dahurian and Japanese larch species (Ryu et al., 2009). It has been shown that ECM
108 fungal colonization increased growth of Japanese and F_1 larches by 1.5-2.0 fold in
109 nutrient-poor soil (Qu et al., 2010). However, it is hitherto unknown which ECM species
110 contributes to this observation, and how characteristics of ECM fungal community in F_1
111 differ from those of its parents. Baxter and Dighton (2005) reported that host trees with
112 a diverse ECM species are regarded as equally efficient in mediating abundant P
113 acquisition as trees with fewer ECM species. On the other hand, growth and
114 photosynthetic function of larch seedlings colonized by multiple ECM species were
115 superior to those of seedlings colonized by only one ECM species (Qu et al., 2004;
116 Choi, 2008). It is therefore important to investigate the ECM diversity under N
117 deposition scenarios and provide new insights that could be applied in management
118 practices.

119 Despite the importance of larch as plantation species in Eurasia (e.g. Abaimov,
120 2010), only a few studies have investigated the symbiotic relationship between ECM
121 fungi and larch species regarding N deposition (Qu et al., 2003; Choi et al., 2005;
122 Shinano et al., 2007), and even less have focused on ECM symbiosis in immature
123 volcanic ash soil (Leski and Rudawska, 2012). Wang et al. (2016) found that ECM
124 composition of F_1 was altered when the atmosphere was enriched with ozone and carbon
125 dioxide. Notably, specific ECM species assisted F_1 and compensated the ozone-induced
126 injury. Furthermore, earlier studies have shown that F_1 shows a better growth
127 performance than its parents (Ryu et al., 2009; Agathokleous et al., 2017), however,
128 ECM colonization and its role concerning these three larch species under N deposition
129 still remains underexplored.

130 In this study, we investigated how F_1 larch and its parents responded to N addition

under different P loadings. We specifically attempted to answer the following questions: (1) Are there any effects of N and P application on colonization and species richness of ECM fungi? (2) Do ECM communities respond to N and P differently among the three larches? (3) Does ECM community structure vary with the host larch species? In order to answer these questions we conducted an experiment using F_1 and its parent species planted in immature volcanic ash soil under two levels of N in combination with or without P loading. We expected that response of ECM fungi of F_1 to increased nutrient levels would be similar to that of Dahurian larch due to maternal inheritance of growth characteristics (Abaimov, 2010).

2 Materials and Methods

2.1 Plants and soil materials

The experiment was conducted in Sapporo Experimental Forest of Hokkaido University, Japan (N43.07, E141.38, 15m above sea level.) from 2009 to 2011. The snow-free period lasted from early May to early November. The average temperature and relative humidity at the experimental site were 20.2 ^{o}C and 74.3%, respectively during the growing season from May to October.

Three-year-old seedlings of the three larches species were planted in 15-L pots. Japanese and Dahurian larches were provided from a nursery near Bibai, Hokkaido. Hybrid larch F_1 was provided by Forestry Research Institute, Hokkaido Research Organization located in Bibai. All seedlings were kept in low temperature cabinets in a storeroom until planting. The seedlings were examined for initial ECM colonization, diameter and height. The diameter was measured by taking two cross-wise measurements using a digital caliber, while the height was measured using a measuring tape with 1-mm gradient. The two cross-wise measurements were averaged. The seedlings of the three larch species were similar. The average (±SD) initial stem basal

157 diameter was 10.13 (± 0.35) mm and the height was 30.75 (± 3.24) cm.

158 Kanuma-pumice and Akadama soils were selected for this experiment. Each pot
159 was filled with a mixture (1:1) of these soils. The physical and chemical properties of
160 the soil were reported by Eguchi et al. (2008, Supplementary Information Table S1). The
161 soil pH and NH_4^+ concentration after the nutrient treatments are shown in Table 1. These
162 soils have low nutrient concentrations, and thus ideal for a nutrient-loading experiment
163 and it is also ideal for mycorrhizae observation.

164 *2.2 Nutrient treatments*

165 The nutrient addition experiment was set up in a completely randomized design.
166 Two levels of N (0 and 100 kg $ha^{-1}yr^{-1}$; zero corresponding to N control) were combined
167 with two levels of P (0 and 50 kg $ha^{-1}yr^{-1}$), resulting in four treatments: a) control
168 (P0N0); b) high N (N100P0); c) high P (N0P50); and d) high P+N (N100P50). Each
169 treatment had six replicates for each larch species (Total 6×4×3=72 seedlings). The
170 amount of 100 kg N $ha^{-1}yr^{-1}$ was determined based on reports that N deposition in some
171 Asian regions has reached, exceeded or predicted to exceed 50 kg ha^{-1} yr^{-1} (e.g.
172 Galloway, 2004, 2008; Li et al., 2013; Xu et al., 2015). Ammonium nitrate solution
173 (NH_4NO_3) and potassium phosphate monobasic (KH_2PO_4) were used as nutrient
174 sources.

175 To prevent nutrient leaching from heavy rain, a matched tray was set in the bottom
176 of each pot, and the collected water was returned to the same pot. Irrigation was applied
177 manually, and N and P were applied every two weeks beginning one month after
178 planting. Treatments period lasted from June 2010 to October 2011, except during the
179 winter period (from November to April).

180 *2.3 Colonization rate and diversity of ECM*

181 The ECM taxa were assessed using both morphological and molecular analyses
182 before planting and after harvesting. At the end of the second growing season (October,

183 2011), the entire seedling was harvested and the roots were covered with wet tissue
184 papers, stored in plastic bag, and transferred to laboratory where they were kept in a
185 dark refrigerator at 4 ^{o}C. Within three days, the harvested roots were washed, and then
186 gently cleaned using a paintbrush. The detailed sampling methodology can be found in
187 Wang et al. (2015). Finally, a microscope (Olympus szx-ILLK100, Japan) was used to
188 observe the ECM colonization. ECM taxa were classified based on morphological
189 characteristics (graphical abstract), including color, texture, ramification, root tip shape,
190 and emanating hyphae (Grand and Harvey, 1982; Agerer, 1987-1993). The taxonomic
191 classification based on the morphology was verified by molecular analyses described
192 below.

193 Ribosomal DNA (rDNA) of ECM fungi was extracted from the root tips colonized
194 by ECM fungi using a DNeasyTM Plant Mini Kit (QIAGEN). Internal transcribed
195 spacer (ITS) was amplified via PCR using a primer set of ITS1-F
196 (CTTGGTCATTTAGAGGAA GTAA) and ITS4-B
197 (CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns, 1993; Bellemain et al.,
198 2010). PCR was performed using 50 µL assays: 5 µL of 10× PCR buffer, 5 µL of 25 mM
199 $MgCl_2$, 4 µLof a 0.2 mM dNTP mixture containing, 0.5 µL of taq DNA polymerase, 1
200 µL each of 10 µM forward and reverse primers, 2 µL of a genomic DNA template (40
201 ng μL^{-1}) and 31.5 µL of PCR-grade water. The PCR thermal profile consisted of an
202 initial denaturation and enzyme activation step of 95 °C for 2 min, followed by 40
203 cycles of 95 °C for 20 sec, 50 °C for 40 sec and 72 °C for 30 sec. PCR products were
204 evaluated for amplification and their lengths by agarose gel electrophoresis, and purified
205 with the PCR purification Kit (Labopass Cosmogenetech co, Ltd). Amplicons were
206 sequenced by a BigDye Terminator v3.1/1.1Cycle Sequencing Kit (Applied Biosystems,
207 USA). Finally, the ECM sequences were assigned to taxa using the basic local alignment
208 search tool (BLAST) at the GenBank database of NCBI. A phylogenetic tree was

209 constructed based on the sequences using MEGA 5 (Tamura et al., 2013). All the
210 sequences were deposited at NCBI. The accession numbers and taxonomic information
211 are listed in Table 2.

212 The colonization rate of ECM (CRE) was determined based on the formula: CRE*i*
213 (%) = ER/(ER+NR) × 100, where ER and NR denote the number of ectomycorrhizal
214 and non-ectomycorrhizal root tips, and *i* an ECM type (Choi et al., 2005; Shinano et al.,
215 2007). The diversity of ECM was calculated using Shannon's diversity index (Keylock,
216 2005): $H' = -\sum^{S}_{i=1} Pi\log Pi$, where S denotes the total number of types of ECM, and *Pi* is
217 the proportion of the *i*th ECM type (Pielou, 1966).

218 *2.4 Plant biomass and nutrient elements concentration in needles*

219 The harvested seedlings were separated into needles, branches, stems and roots,
220 and put into an oven at 80 °C for 48h (roots for one week) to assess dry biomass.
221 Aboveground dry mass was calculated as the sum of needles, branches and stem dry
222 mass. Total dry mass was calculated as the sum of aboveground and root dry mass. The
223 root biomass to shoot biomass (R/S) was also calculated.

224 A couple of needles were randomly selected from each seedling for nutrient
225 analyses. Dried needles were ground to fine powder, and digested by HNO_3, HCl and
226 H_2O_2. The samples were analyzed by an inductively coupled plasma-atomic emission
227 spectrometer (ICP-AES, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc.,
228 Massachusetts, USA).

229 *2.5 Statistical analysis*

230 The threshold of significance was set to $\alpha = 0.05$, and $\alpha < 0.1$ was considered as
231 marginally significant due to the high variability and relatively small sample size (N =
232 6). The data of ECM colonization rates, ECM diversity index, biomass, R/S and element
233 concentrations were transformed to obtain normal distribution using a Box-Cox power
234 transformation, as described by Agathokleous et al. (2016). ECM colonization rates and

diversity, biomass, R/S and elements concentration were analyzed via two-way ANOVAs. Bonferroni post-hoc multiple comparisons were conducted when main effects were found significant. To assess differences in community structure of the ECM fungi associated with the three larch species in the different nutrient treatments, abundance counts of the ECM taxa of the seedlings were analyzed via distance-based redundancy analyses (Legendre and Anderson, 1999). The ECM composition data were abnormally distributed, however typical data transformations could not correct the distribution. Nonparametric Kruskal-Wallis tests were used to test the effects of the interaction Treatment × Taxa (number of experimental conditions = 12). For some of the ECM fungal taxa, statistical tests could not be applied because of too infrequent presence, similar to the study by Leski et al. (2008). For all the applied Kruskal-Wallis tests, the *P*-values ranged between 0.111 and 0.950. The statistical analyses were conducted with the software R version 2.15.0 (R Development Core Team, 2012) and STATISTICA v.10 (© StatSoft Inc.).

3. Results

3.1 Taxonomic identification of ECM fungi

Total seven ECM taxa (Type A to G) were identified after morphological and molecular analyses (Table 2). Before the nutrient addition was initiated, four ECM types, *Suillus visidus*, *S. grevillei*, *Inocybe* sp. and *Thelephora* sp. (Type A, B, D and F), were found in roots of the seedlings (Table 3), and three additional ECM species were observed in the roots after the treatments: *Russula* sp. (C), *Hebeloma* sp. (E) and *Tomentella* sp. (G). Taxonomically, the seven observed types were Hymenomycetes of Basidiomycotina, and were divided into two orders, Agaricales and Aphyllophrales. Agaricales included three families: Boletaceae (Type A, B), Russulaceae (Type C) and Cortinariaceae (Type D, E). Aphyllophrales involved only one family, *Thelephoraceae*

(Type F, G) (Fig. 1a, Smith and Read, 2008).

3.2 ECM community composition

Taxon B, which colonized the roots of Dahurian larch, was replaced by other taxa (C, D, E, F and G) after the treatments (Fig. 1, Table 2, 3). In Japanese larch, the number of ECM taxa increased from one (F) to five (A, C, E, F, and G), after the treatments. In F_1, type D was replaced by types A, C, E and G (Fig. 1, Table 2, 3). Overall, taxon G established symbioses with Dahurian larch in all the treatment conditions. Similarly, taxon A colonized the roots of F_1 in all the treatment conditions. Japanese larch roots were generally dominated ECM taxa A, C, G and E (Fig. 1).

In Dahurian larch, four taxa (G, F, D, A) were observed in N0P0 treatment (Fig. 1). In N0P50 treatment, colonization by taxa A and F became negligible, while taxa E and C became dominant compared to N0P0. Taxa E, D and C shared a similar contribution to ECM composition, whereas type G was dominant. In N100P0 treatment, type D (present in N0P0) was replaced by type C. The major composites of ECM community in N100P0 were taxon G (45 %) and C (32 %) followed by taxon A (20 %) and then taxon F at a small proportion (3 %). In N100P50 treatment, the majority of taxon A (dominant in N0P0) was replaced by taxa E and C; thus ECM composites in N100P50 consisted of taxa G, F, E, D, C and A, where taxa G and C accounted for more than 60 % (Fig. 1).

In Japanese larch, taxa G (3 %), F (23 %), E (48 %) and A (18 %) were the components of the ECM community in N0P0 treatment (Fig. 1). Interestingly, type E was not present at N0P50 and N100P0 treatments. The ECM community of N0P50 treatment was composed by taxa G (39 %), F (21 %) and A (40 %), whereas N100P0 was composed by taxa G (9 %), C (25 %), and A (67 %). In N100P50 treatment, taxon C accounted for 72 % of the ECM community composition; taxa G, F and E accounted for 3, 18 and 7 %, respectively.

In F_1 larch, taxa F (7 %), E (31 %), C (26 %), B (3 %) and A (33 %) composed the

ECM community in N0P0 treatment (Fig. 1). In N0P50 treatment, taxa F, E and C were not present, whereas taxon G was present. Therefore, the ECM community in N0P50 was composed of taxa G (5 %), B (20 %) and A (76 %). In N100P0 treatment, the ECM community was composed by taxa G (12 %), F (10 %), E (33 %), C (2 %) and A (43 %), whereas in N100P50 treatment, the ECM community was composed by taxa G (7 %), E (50 %), C (18 %), B (3 %) and A (22 %). Overall, taxon B was only identified at F_1 and not at the parental larches after the treatment.

3.3 ECM colonization and diversity

The ECM colonization rate and species diversity in response to N and P treatments varied among the three larches (Fig. 2a). Independent from the treatments, the greatest colonization rates were observed in Dahurian larch, followed by Japanese larch and finally F_1 (Fig. 2a). Across all the larch species, the colonization rates did not differ significantly between N0P50 and N100P0 or between N0P0 and N100P50 (Fig. 2a). While colonization rate did not significantly differ among N0P50, N100P0 and N100P50, it was greater in N0P50 and N100P0 than in N0P0 (Fig. 2a). The interaction between species and treatment was also significant (Fig. 2a). Treatment with N100P0 caused an increase in colonization rates in Japanese larch and F_1, compared to N0P0, but not in Dahurian larch (Fig. 2a). Within each larch taxon, N0P50 and N100P50 had no significant effects on the colonization rate, compared to N0P0 (Fig. 2a).

Diversity of ECM fungi depended both nutrient addition treatments and larch species, supported by a significant interaction effect (Fig. 2b). Overall, means of N0P0, N0P50 and N100P0 did not differ significantly each other, but were significantly lower than the mean of N100P50 (Fig. 2b). Diversity index was similar among the larch species within N0P0 and N0P50 (Fig. 2b). However, at N100P0, Japanese larch did not have a significantly different diversity index from F_1 but had lower index than Dahurian larch. At N100P50, Japanese larch and F_1 had a similar diversity index, which was lower

than that of Dahurian larch (Fig. 2b). Diversity index of Dahurian larch in N100P50 was greater than in N0P0, N0P50 and N100P0 (Fig. 2b).

3.4 Community structure of ECM species

There was a significant difference in the ECM community structure among the nutrient treatments of Japanese larch (Fig. 3b), but no significant differences in the ECM community structure of Dahurian (Fig. 3a). F_1 showed a marginal significant effect of the nutrient treatments in Cap1 (P=0.06; Fig. 3c).

A significant difference was found among the three larch species at N0P0 and N0P50 treatments (Fig. 4a, b). At N0P0, the ECM community structure differed significantly between Dahurian larch and the others (Fig. 4 a). In N0P50 treatment, a significant difference was detected between Dahurian larch and F_1 larch (Fig. 4b). The ECM community structure at N100P0 and N100P50 treatments did not differ significantly among the larch species (Fig. 4c, d).

3.5 Biomass and root to shoot (R/S) ratio

The dry mass (DM) of the different plant segments significantly varied among the larches (Fig. 5, uppercase letters above species acronyms). The greatest aboveground DM was observed at F_1, followed by Japanese larch and then Dahurian larch, whereas the greatest root DM was observed at F_1, followed by Dahurian larch and then Japanese larch (Fig. 5). Branch + stem DM and total DM were similar between parental larches, and F_1 had the largest values (Fig. 5). R/S did not differ between Dahurian and hybrid larch, and showed a higher value compared to the R/S of Japanese larch (Fig. 5).

The nutrient treatment had significant effects on aboveground DM, root DM and total DM (Fig. 5). The only significant difference in aboveground DM and total DM was between N0P0 and N0P50 where the mean of N0P50 was lower than the mean of N0P0 (Fig. 5). Root DM was not statistically different among N0P50, N100P0 and N100P50 treatments, however all these treatments had a smaller value compared to N0P0.

339 Species × Treatment interaction was significant only in needle DM and root DM,
340 however there was no significant difference in multiple comparisons of needle DM (Fig.
341 5). In root DM, F_1 larch was greater than the parental larches in N0P0 and N100P0.
342 However, there was not such a difference in N0P50. In N100P50, while parental larches
343 both had lower root DM compared to F_1, but the difference between Japanese and F_1
344 was only significant.

345 *3.6 Element concentration in distal organs of seedlings*

346 Independent from treatments, concentrations of Zn, Mn and Fe were significantly
347 different among the larch species, on the other hand, P concentrations were not (Fig.6
348 uppercase letters above larches acronyms). No significant differences were found in
349 concentration of carbon and N among the nutrient treatments (data not shown). Dahurian
350 and Japanese larches had a similar Zn and Mn concentrations. Concentrations of Zn and
351 Mn of the parent larch species were greater and smaller than those of F_1, respectively.
352 Dahurian and Japanese larch also had similar Fe concentrations, however Fe
353 concentration of Japanese larch was significantly lower than that of F_1 larch.

354 Nutrient treatments were a significant factor only for P concentrations (Fig. 6). The
355 only significant difference in P concentrations was between N0P50 and N100P0
356 treatments, where P concentrations were lower at N100P0 than at N0P50.

357 The treatment × species interaction was not significant for any element
358 concentrations. Although there were proportionally large differences in some cases,
359 associated coefficients of variation were also high.

360

361 **4. Discussion**

362 We demonstrated that increased N levels could significantly alter ECM community
363 structure, but such alteration also depended on the larch species. When no N was added,
364 there was distinct difference in ECM community structure among the three larch

species. However, when N availability was increased, such difference was not detected among the three larch species. F_1 showed heterosis in biomass, especially when N levels were increased, and its ECM community structure appeared tolerant to the N loading. This co-variation of ECM fungal community structure and F_1 heterosis suggest that F_1 can perform better under high N deposition environments than its parents, and associated ECM community can play an important role in host plant growth. We note that, as a pot experiment, the study scale is limited, especially with woody species (Kawaletz et al., 2014). Therefore, the role of propagules or mycelial networks is limited, we can not definitely confirm the reason resulted in this phenomenon. Nonetheless, studies of this kind are still valuable to improve the understanding of belowground processes under a nitrogen deposition scenario (Rasheed et al., 2017).

The ECM species and colonization rates changed over the course of the two-year treatment. Larch species tend to establish a symbiosis with *Suillus* sp. under field conditions (Zhou et al., 2000; Zhou and Hogetsu, 2002). At the beginning, the dominant ECM species of Dahurian larch was *Suillus* sp., however, at the end of experiment, Dahurian larch hosted up to six ECM species. Only *Thelephora* sp. colonized Japanese larch before treatments, but five ECM species colonized Japanese larch at the end of the experiment, and showed a higher variety of ECM community structure among different treatments. Additionally, *S. grevillei* colonized solely with F_1, both before and after the treatments, but not at the parental larches. It has been previously reported that larch (*L. laricina*) seedlings were greatly colonized by *S. grevillei* (Samson and Fortin, 1986). This strong symbiotic relationship was also reported by Qu et al. (2003), who detected that *S. grevillei* colonized larch seedlings faster than *S. laricinus*, and F_1 was more easily colonized than Japanese larch. However, *Suillus* sp. did not colonize first with Japanese larch, due to the order of a common succession for symbiotic ECM fungi under natural conditions (Nara et al., 2003, 2006b; Yamakawa, 2012). After the treatment, *Thelephora*

sp. was found at all the larches. This could indicate that *Thelephora* sp. is a generalist ECM genus of larches. *Thelephoraceae* is one of the most represented families of the ECM community in boreal and Mediterranean forests (Miguel et al., 2016). *Thelephora* sp. was also found in red pine (*Pinus densiflora*) seedlings in a natural regenerating area of a mature forest (Ma et al., 2010) and in European larch (*Larix decidua* Mill.) seedlings in forest nursery conditions (Leski and Rudawska, 2012). Obase et al. (2007) investigated the association between ECM species and woody plants in areas devastated by the eruption of Mt. Usu volcano in 2000. They revealed that among 15 identified ECM species, *Thelephoraceae* had high relative colonization, constituting the majority of the ECM colonization in the roots of the woody plants. It has also been reported that *Thelephora terrestris* showed traits of dominance in spruce (*Picea engelmannii* × *P. glauca*) seedlings in clearcut plots, where the soil nutrient concentrations was relatively low (Walker et al., 2016).

Compared to N0P0, no effects of N and P addition on the ECM colonization rate of Dahurian larch was observed. However, single N loading (N100P0) increased colonization rate in Japanese larch and F_1. This increase was lost by P addition (N100P50), suggesting that P availability offset the effect of N on ECM colonization. This may be a trade-off of ecological importance between host-plant and ECM fungi: When plants have access to enough P, the plants may not provide as much photosynthates to associated ECM fungi for taking up nutrients. Overall, ECM colonization rates of F_1 was more similar to Japanese larch than to Dahurian larch. Regardless of P availability, high level of N can stimulate more dichotomous architecture of root elongation with more root tips, therefore, a greater amount of ECM can potentially colonize (Fitter and Stickland, 1991; Taub and Goldberg, 1996). In our study, no significant effect of N loading on the ECM colonization rate of Dahurian larch was observed at N100P0. It has been previously reported that first-order roots of

Dahurian larch colonized by ECM fungi were reduced by 17 % with 100 kg N $ha^{-1}yr^{-1}$ treatment, which is in agreement with our results (Sun et al., 2010).

ECM diversity in Dahurian larch was significantly affected by N100P50 treatment, but the community structure of Dahurian larch was not affected by treatments. In contrast, while nutrient treatments did not affect the ECM diversity in Japanese larch and F_1, a marked difference in community structure among treatments was found. This phenomenon reflects less flexibility of ECM symbioses in response to increased nutrient availability at Dahurian larch, and higher flexibility in Japanese larch and F_1. This observation suggests host genetic effects on ECM community structure.

The ECM community structure was different among the three larch species in N0P0 and N0P50. However, the difference of ECM community structure was reduced by high N loading suggested by no significant difference in community structure among the three larch species under N loading (N100P0 and N100P50). This difference at low N treatments was likely due to host genetic effects. It has been found that ECM community varies among host genotypes in *Populus angustifolia* (Lamit et al., 2016), Scot's pine (Leski et al., 2010) and Norway spruce (Velmala et al., 2012). Our results of three larch species displayed host genetic effects on ECM community structure, and the effects of F_1 were closer to Japanese larch.

Nitrogen loading reduced P concentration in needles of the parental larches, although not statistically significant due to large coefficient of variation. On the other hand, N loading did not reduce P concentration in needles of F_1. Such benefits of unaffected P uptake in F_1 may be the result from some dominant ECM species, such as taxon B, which colonized only F_1, and in particular taxa A (*S. visidus*) and E (*Hebeloma* sp.) which dominantly colonized F_1 at N100P0 and N100P50. The exploration types of *Suillus* sp. and *Hebeloma* sp. are "long distance" and "medium-fringe" respectively (Agerer, 2001). A study revealed that ECM species *Hebeloma cylindrosporum*

443 hydrolyzed organic P in podzols (Louche et al., 2010). Another study on *Hebeloma*
444 *cylindrosporum* and its natural host plant *Pinus pinaster* suggested that P accumulation
445 in whole plants mainly originates from fungal P uptake (Torres Aquino and Plassard,
446 2004). The plasticity of ECM short root can affect P uptake through alterations in
447 specific root length (SRL), specific root area (SRA) and root tissue density (Lambers et
448 al., 2006). Changes in these morphological root traits are related to species-specific
449 impacts of ECM fungi (Ostonen et al., 2009). Thus, ECM composition of F_1 may
450 provide a high capacity and efficient uptake of P under high N condition (Goldstein,
451 2013). Although there were large differences in needle P concentration between F_1 and
452 Japanese or Dahurian larches in N100P0 and N100P50, the coefficient of variation was
453 large. Thus the differences observed were not statistically significant.

454 All the treatments suppressed root DM, only N0P50 suppressed aboveground and
455 total DM of the three larch species. This shows that roots were more responsive to the
456 treatments than aboveground parts. Based on the photosynthetic parameters, it has been
457 previously shown that growth was not enhanced by up to 50 kg $hr^{-1}yr^{-1}$ P application,
458 and N was vital for maintaining needle P concentration (Ryu et al., 2009; Mao et al.,
459 2014). Furthermore, P in needles is an essential macro-element for ATP and NADPH,
460 which relate to light reactions of photosynthesis (e.g., Reich et al., 2009). Therefore,
461 photolysis activity might be inhibited by N0P50, leading to reduced shoot mass. The
462 smaller root dry mass might be resulted from an enhanced root length without a
463 proportional root biomass increase under high P condition (Steingrobe, 2001). Root DM
464 of F_1 was greater than its parents might partly be due to root morphology change with
465 specific ECM community (Rosado et al., 1994; Ostonen et al., 2009). Overall, F_1
466 showed higher dry mass of all measured segments, except needles, than Dahurian and
467 Japanese larches, indicating heterosis in the hybrid offspring (Matyssek and Schulze,
468 1987; Agathokleous et al., 2017). Considering that there was no significant interaction

between species and treatments, it is suggested that heterosis was sustained under changing environments. The sustained heterosis of F_1 has also been previously observed under O_3-polluted environment (Wang et al., 2015; 2016; Agathokleous et al., 2017), and this may result from superiority in growth and photosynthesis (Ryu et al., 2009).

Deficiency of N and P usually results in accumulation of carbohydrates in leaves (Remans et al., 2006; Sánchez-Calderón et al., 2006). Higher amounts of carbon allocation to root may increase R/S (Groot et al., 2003; Sánchez-Calderón et al., 2006). In this experiment, R/S was not significantly affected by treatments or did not differ among larches. However, at only N100P50, a trend of decreasing R/S was observed at F_1. Japanese larch showed lowest R/S ratio across experimental all conditions, indicating lower dry mass of root than aboveground. Furthermore, ECM community structure of Japanese larch highly varied among the four treatments, suggesting that abundance of ECM species mediated the root's role of nutrient uptake.

The needle element concentrations significantly varied among the three larches. Mn concentration of F_1 was the highest. As Mn has an important role of chlorophyll synthesis, and is also a co-factor of photosynthesis and N metabolism (Likar et al., 2013), higher Mn concentration may have resulted in higher dry mass of F_1. It is suggested that the nutrients that are most influenced by mycorrhizae are Fe, Mn, P and Zn (Ducic et al., 2008; Cavagnaro et al., 2010; Orlowska et al., 2011). Mn concentration of F_1 was higher and Zn concentration was lower than in Dahurian and Japanese larch (Fe was also higher but with large coefficient of variation). According to the ECM composition of F_1, *Hebeloma* sp. (taxon E) was the one only species which colonized F_1 at N100P0. Therefore, *Hebeloma* sp. symbiosis might have suppressed the uptake of Zn and increased the uptake of Mn and Fe. Regarding P concentration in needles, neither P nor N did not have significant alterations compared to the control treatment. This may show that ECM symbiosis maintained a balance in nutrient uptake. Higher Zn

concentration in Dahurian and Japanese larches than in F_1, across treatments, can be attributed to higher P uptake in F_1, as it has been shown that excessive P fertilizer application can reduce Zn uptake and lead to Mn accumulation (Christensen and Jackson, 1981; Barben et al., 2007). In our study, it was implied that the ECM associations of F_1 could contribute in reducing metal toxicity stress (Finlay, 2008).

5. Conclusions

Nitrogen and P fertilization affected the colonization and species richness of ECM, and ECM communities responded to N and P differently among the three larch species. ECM community structure varied among larch species due to the strong host genetic effects. Species-specific diversity was found in the relationship between ECM and the host larches. Specifically, *Hebeloma sp*. may have indirectly contributed to F_1 biomass production by absorbing P nutrient and regulating the uptake of excess metal elements such as Zn which would have toxic effects. F_1 possesses efficient and stable ECM community with *Hebeloma sp*. being a dominant species, was more tolerant to simulated N deposition than its parents. We conclude that F_1 sustained the phenomenon of heterosis under changing soil environments, and this could be indirectly explained by ecological trade-offs driven by shifts in ECM community. Environmental change may have further consequences on the soil biodiversity and thus long-term studies on ECM biogeographic patterns are needed.

Acknowledgments

We thank Prof. Art Chappelka of Auburn University, U.S.A., for invaluable comments on the study. The first author thanks Tommy W. Chan and Jaana Leppälammi-Kujansuu of the Department of Forest Science, The University of Helsinki, Finland, for discussion on plant-microbe interaction. Ms. Rina Yamakawa, Hokkaido

University, Japan, kindly contributed to the field experiments. Evgenios Agathokleous is a JSPS International Research Fellow (ID No: P17102). The Japan Society for the Promotion of Science (JSPS) is a non-profit organization. This study was partly supported by a JSPS research project (No. 26660119, to Takayoshi Koike). It was also partly supported by National Natural Science Foundation of China (No.31600486), Natural Science Foundation of Hebei Province (No.C2017204041), Youth top-notch talent project of Hebei Provincial Department of Education (No.BJ2017053) and Scientific Research Foundation for Returned Scholars of Agricultural University of Hebei (ZD201606).

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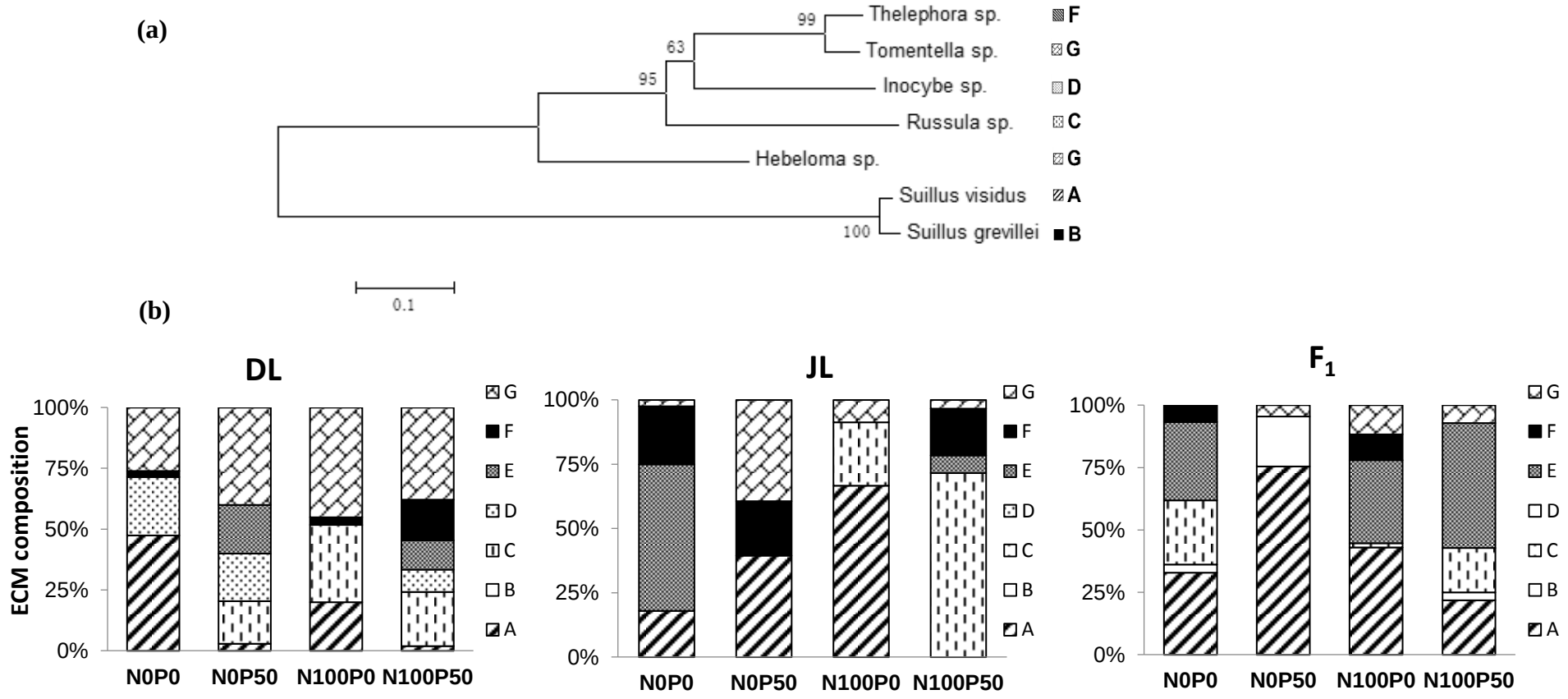
847 **Figures and Tables:**



849

850 **Fig. 1.** (a) A phylogenetic tree of the seven ECM fungal taxa found in this study and (b) composition of ECM fungal species of three larch seedlings
851 with four different nutrient regimes. The horizontal axis is the nutrient regime combined with N (0 and 100 kg $ha^{-1}.yr^{-1}$) and P (0 and 50 kg $ha^{-1}.yr^{-1}$).
852 The different streak symbols correspond to the proportions of ECM fungal types colonizing host seedlings; values are calculated from the
853 colonization rate of ECM fungi in different nutrient conditions. DL, JL and F_1 are the abbreviations for Dahurian larch, Japanese larch and hybrid
854 larch F_1.
855 Note: A *Suillus visidus*, B *Suillus grevillei*, C *Russula* sp., D *Inocybe* sp., E *Hebeloma* sp., F *Thelephora* sp., G *Tomentella* sp.

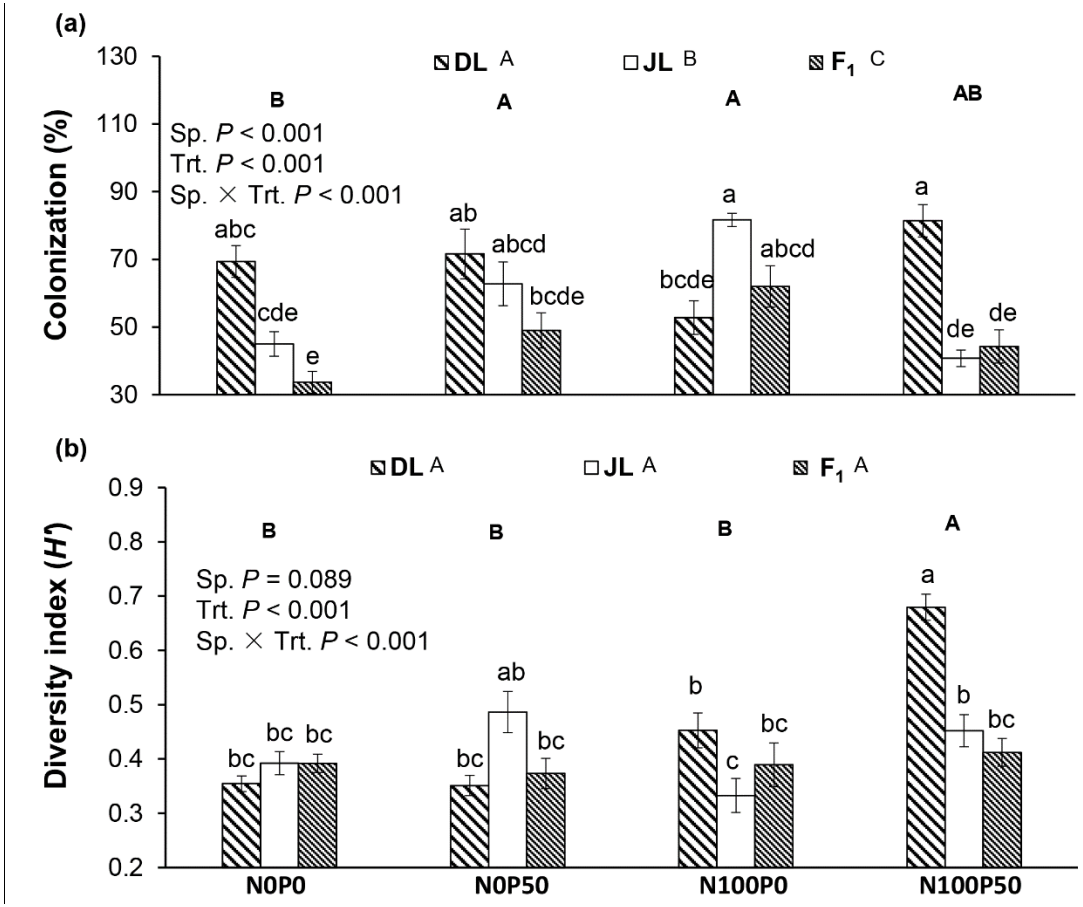


Fig.2. Means (±SE) of ECM colonization rate (a) and species diversity (b) in the roots of three larches treated with different N and P loadings. Each mean is the average of six replications. The horizontal axis is the treatment regime combined with N (0 and 100 kg $ha^{-1}.yr^{-1}$) and P (0 and 50 kg $ha^{-1}.yr^{-1}$). Different uppercase letters above the species name indicate statistically significant differences among species. Different bold uppercase letters above the treatments indicate statistically significant differences among treatments. Different lowercase letters above means indicate statistically significant differences within a significant interaction Species × Treatment (Sp. × Trt.). Differences are marked according to Bonferroni post-hoc test. DL, JL and F_1 are the abbreviations for Dahurian larch, Japanese larch and hybrid larch F_1.

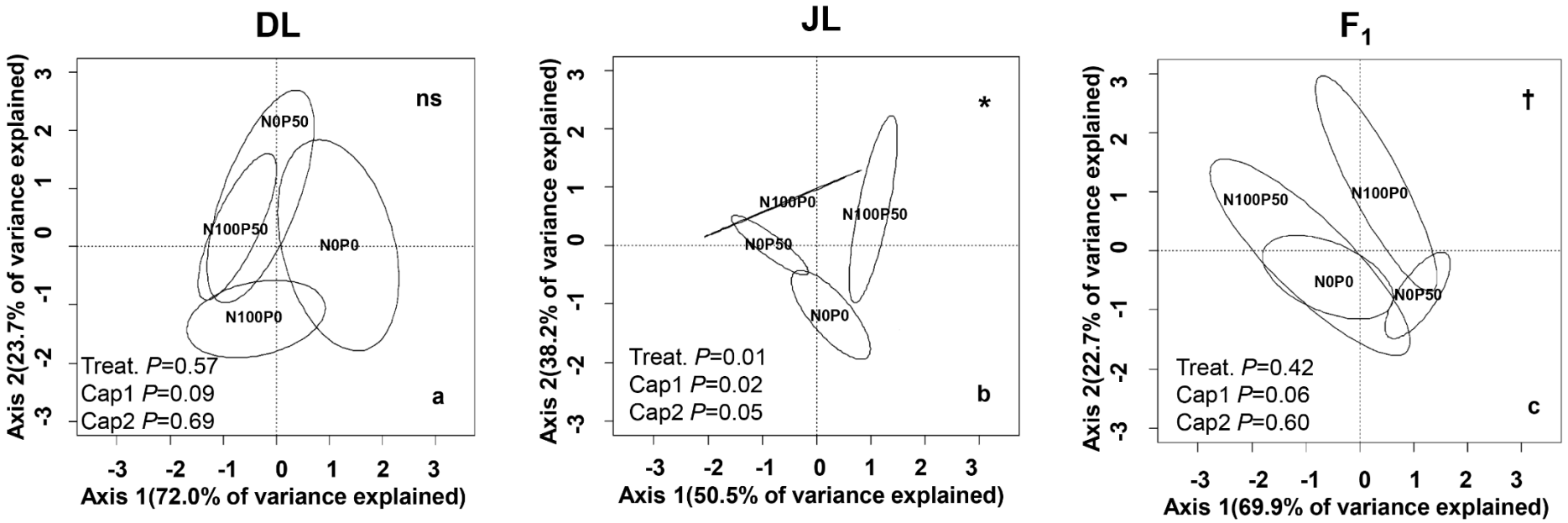


Fig. 3. Community structure of ECM species with nutrient treatments as factors among the three *Larix* species. Treatment (Treat.) is the analysis factor in three larch species. Cap1 and Cap 2 express the significance along the axis 1 and axis 2 with variance explanation in parenthesis, respectively. The asterisk symbols indicating the ANOVA results of axis 1: †, $P \approx 0.05$; *, $P \leq 0.05$; ns, not significant. Differences are marked according to distance-based redundancy analysis,

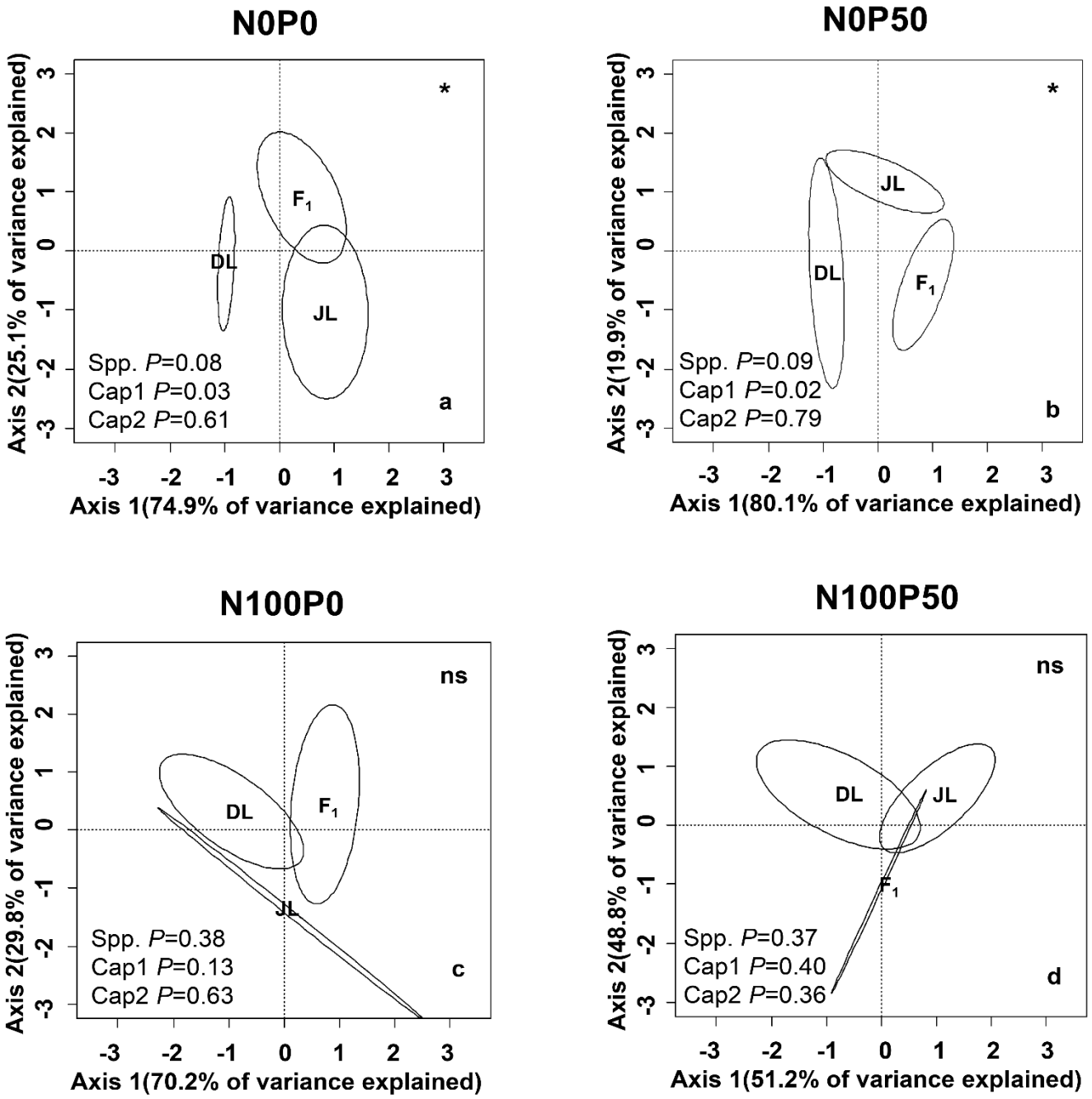


Fig. 4. Community structure of ECM species with the three *Larix* species as factors among the different nutrient regimes. Larch species (Sp.) is the analysis factor in each treatment: DL, JL and F_1 (DL, JL and F_1 are the abbreviations for Dahurian larch, Japanese larch and hybrid larch F_1). Cap1 and Cap 2 express the significance along the axis 1 and axis 2 (variance explanation in parenthesis), respectively. The asterisk symbols in upper-right indicating the ANOVA results of axis 1: *, $P \leq 0.05$; ns, not significant. Differences are marked according to distance-based redundancy analysis,

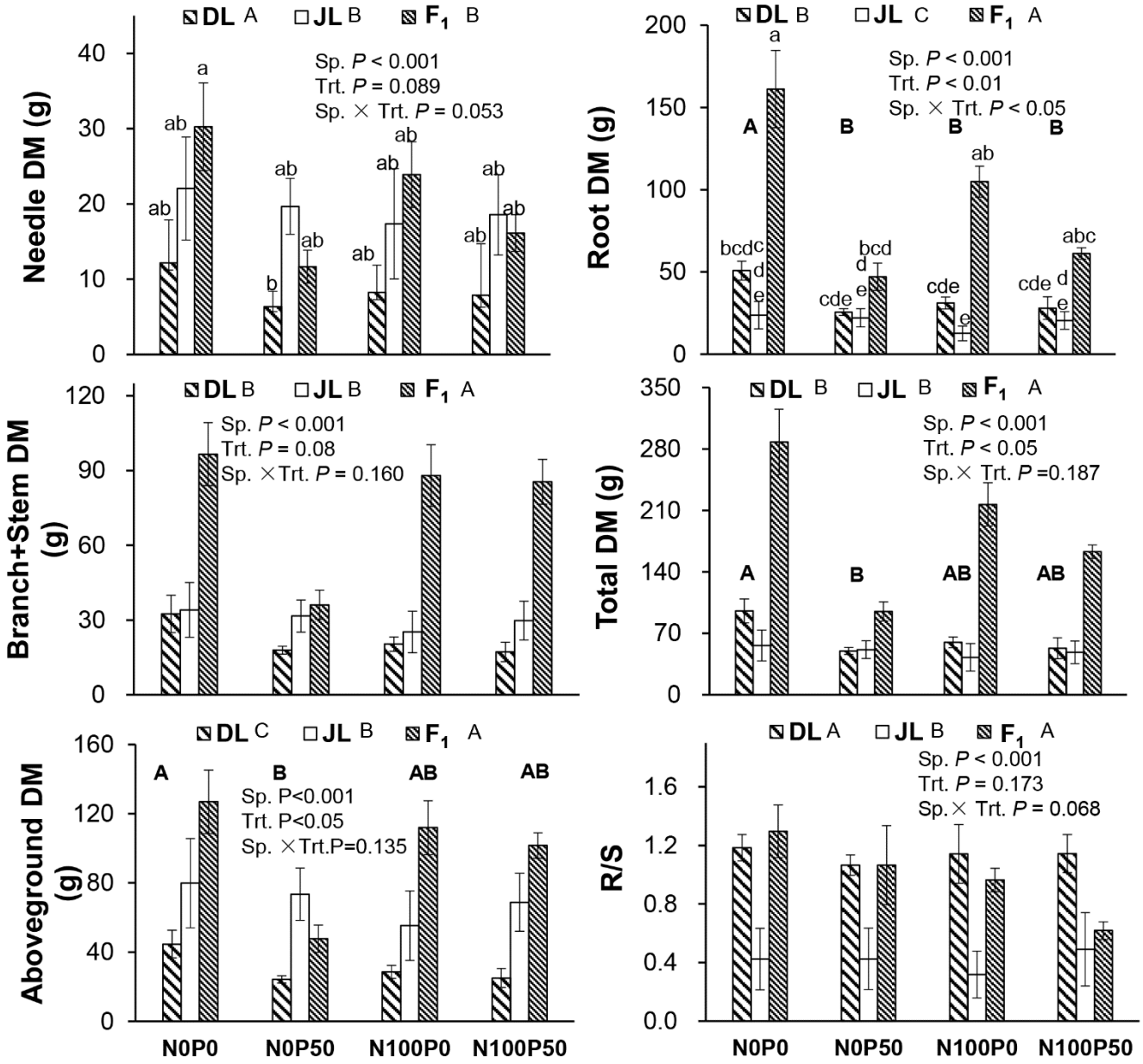


Fig. 5. Means (±SE) of dry mass (DM) of plant tissues (g) and root to shoot (R/S) ratio. Each value is the mean of six replications. Different uppercase letters above the species name indicate statistically significant differences among species. Different bold uppercase letters above the treatments indicate statistically significant differences among treatments. Different lowercase letters above means indicate statistically significant differences within a significant interaction Species × Treatment (Sp. × Trt.). Differences are marked according to Bonferroni post-hoc test. DL, JL and F_1 are the abbreviations for Dahurian larch, Japanese larch and hybrid larch F_1.

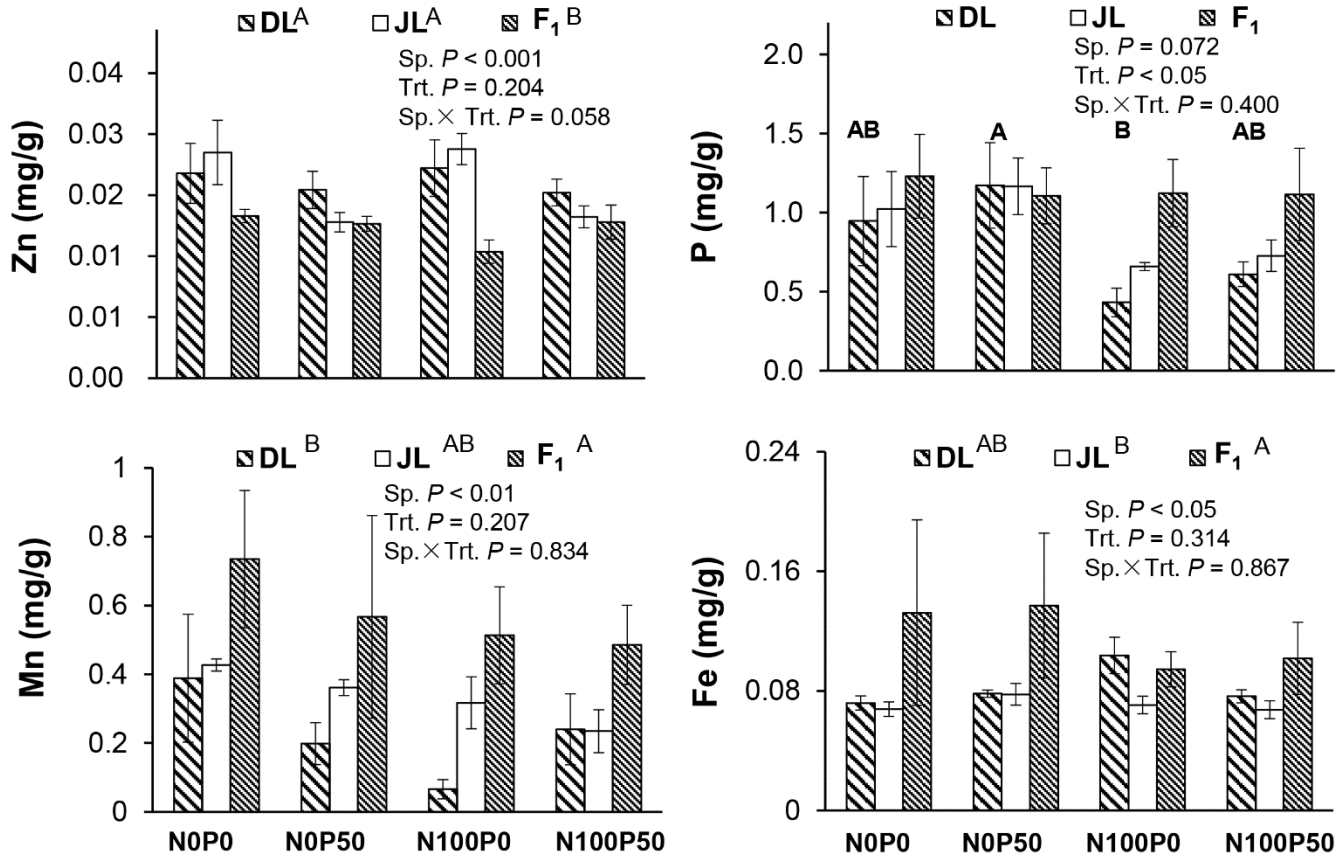


Fig. 6. Means (±SE) of the concentration of nutrient elements in needles of three larch species under the treatments. The horizontal axis is the treatment regime combined with N (0 and 100 kg $ha^{-1}.yr^{-1}$) and P (0 and 50 kg $ha^{-1}.yr^{-1}$). Each value is the mean of six replications. Different uppercase letters above the species name indicate statistically significant differences among species. Different bold uppercase letters above the treatments indicate statistically significant differences among treatments. Different lowercase letters above means indicate statistically significant differences within a significant interaction Species × Treatment (Sp. × Trt.). Differences are marked according to Bonferroni post-hoc test. Each value is the mean of six replications, and the error bar shows SE. DL, JL and F_1 are the abbreviations for Dahurian larch, Japanese larch and hybrid larch F_1.

Table 1

Soil properties at the end of experimental period.

		P0N0	P50N0	P0N100	P50N100	N	P	N+P
DL	pH	4.39(0.11)	4.34(0.11)	4.43(0.11)	4.37(0.11)	n.s.	n.s.	n.s.
	NH_4^+	31.27(3.95)	25.74(3.95)	27.83(3.95)	25.75(3.95)	n.s.	n.s.	n.s.
JL	pH	4.63(0.12)	4.60(0.12)	4.50(0.14)	4.39(0.12)	n.s.	n.s.	n.s.
	NH_4^+	33.43(3.83)	24.43(3.83)	32.26(4.23)	27.26(3.83)	n.s.	*	n.s.
F_1	pH	4.48(0.08)	4.64(0.08)	4.27 (0.08)	4.32(0.08)	*	n.s.	*
	NH_4^+	23.79(3.05)	17.84(3.05)	31.68(3.05)	28.00(3.05)	*	*	n.s.

Each value indicates the average of six replications and standard error is shown in parentheses. ANOVA analysis: *, $P<0.05$; ns, not significant. Unit of NH_4^+ is mg/kg.

Table 2

Molecular identification of ECM species with three larch seedlings after nutrient treatment.

Type ID	Fungal taxon	Accession No.	Length (bp)	Closest BLAST match				
				Score	*e* value	Accession No.	Identities	Species
A	*Suillus visidus*	LC029032	811	1473	0.0	AB971282	773/783(98%)	Fungal sp. YT2014TypeOS
B	*Suillus grevillei*	LC029033	563	1076	0.0	KX230615	559/563(99%)	*Suillus clintonianus* IDENT18
C	*Russula* sp.	LC029034	539	1815	0.0	KY681443	418/419(99%)	*Russula paldosa* HMJAU38014
D	*Inocyb*e sp.	LC029035	620	1203	0.0	HQ604432	613/615(99%)	*Inocybe lacera* isolate2041020-010
E	*Hebeloma* sp.	LC029036	446	1567	*e*-158	LC068995	406/446(91%)	*Hebeloma* sp.2015-1
F	*Thelephola* sp.	LC029037	591	1051	0.0	AB971281	530/530(100%)	Fungal sp. YT2014TypeI
G	*Tomentella* sp.	LC029038	634	1223	0.0	LC145311	620/630(99%)	*Tomentella* sp. T1

Table 3

ECM taxa colonizing three larches before nutrient treatment.

ECM Type	Colonization rate (%)		
	DL	JL	F_1
Suillus visidus (A)	38	-	-
Suillus grevillei (B)	50	-	32
Inocybe lacera (D)	-	-	25
Thelephora sp. (F)	-	67	40

-: not colonized, because the samples were too small to identify. The letter A, B, D and F in parenthesis are the ECM type label.