Study on the effects of elevated CO_2, O_3 and high nitrogen loading on the rhizosphere dynamics of deciduous trees

（落葉樹の根圏動態に対する高CO_2とO_3及び高窒素負荷の影響に関する研究）

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CONTENTS

ABSTRACT

Recently, rapid economic growth, industrialization and urbanization have caused a series of environmental pollutions mainly due to tremendous energy consumption. Subsequent increase in atmospheric carbon dioxide (CO_2) concentration, nitrogen oxide deposition and tropospheric ozone (O_3) are considered to incite environmental change, threatening forest ecosystems. In northeast Eurasia and Asia, birch and larch represent essential components as well as being promising species for afforestation. Since northern Japan is mostly covered with volcanic ash and pumice soil, ectomycorrhizal (ECM) symbiosis is fundamental to sustain the growth of these trees. This symbiotic relation directly affects rhizosphere activities, such as fine root development. In this study, I tried to elucidate the response of fine root dynamics, species richness of ECM fungi under elevated CO_2 and O_3 as well as high nitrogen loading, aiming to obtain basic information for future afforestation with birch and larch species under changing environments.

At elevated CO_2 concentration, plants usually enhance the growth of aboveground parts and allocate more photosynthates to belowground. This allocation increases the respiration of both coarse and fine roots. Fine-root dynamics play an important role in carbon (C) cycling of belowground and influence C sequestration to the soil. In chapter 2, to investigate the effect of elevated CO_2 on fine-root dynamic of Japanese white birch (*Betula platyphylla* var. *japonica*), I monitored their dynamics using the Free Air CO_2 Enrichment (FACE) facility of Hokkaido University for three years (2011-2013). Elevated CO_2 was maintained at 500 μmol/mol which simulating the situation around 2040 according to IPCC prediction, and currently the ambient air

contains 380-395 μmol/mol of CO_2. Mini-rhizotron (MR) instrument was used: all MR tubes were set up with planting seedlings in 2010. The image scanning in the field started in 2011, one year later, to avoid the gap between tube and soil. Except for the CO_2 treatment, I also applied two soil types, i.e., brown forest (BF) soil and volcanic ash (VA) soil, which is widely distributed in northern Japan. Live fine-root length (LRL), fine-root production (FRP), mortality (FRM) and root lifespan were analyzed after tracing images by the Win-Rhizotron software. LRL was estimated as the total length in each area unit. FRP (FRM) was calculated according to the annual length-based method. It equals to the annual length-based root production (mortality) to live root length. Fine root lifespan was determined by fine root longevity using the Kaplan-Meier survival function. LRL increased under elevated CO_2 in the first year but showed no significant increase thereafter in BF soil. However, in VA soil, it decreased with CO_2 enrichment for all three growing seasons. Independent of treatments and soils, turnover of FRP and FRM ranged from 0.25 to 1.69 yr^{-1}. The turnover of both FRP and FRM was relatively lower under elevated CO_2 in the first two years, and increased from the third growing season by elevated CO_2 in both soils. Elevated CO_2 increased fine-root lifespan in BF soil during the first year and in VA soil during the three years. But the response of root longevity to elevated CO_2 differed according to root diameter classes.

CO_2 is the basic C source for photosynthesis related to plant growth, while O_3 is a phytotoxic air pollutant of major concern for forest decline. In general, elevated CO_2 decreases stomatal conductance, which may reduce harmful effect of O_3 via stomatal function. How does this combination of CO_2 and O_3 effect growth of the underground parts of larch? For tree growth and fine root dynamics, ECM symbiosis is a vital issue. In chapter 3, to clarify the combined effects of elevated CO_2 and O_3 on tree growth

and ECM symbiosis, I used the Open Top Chamber (OTC) system to estimate the response of hybrid larch (F_1) (*Larix. gmelinii* var. *japonica* × *L. kaempferi*) for two years (2011 and 2012). Treatments consisted of i) charcoal-filtered ambient CO_2 (almost no O_3, 385 μmol/mol), ii) 60 nmol/mol O_3, iii) high CO_2 (600 μmol/mol), and iv) their combination. Elevated CO_2 increased the ECM colonization rate but not the diversity of ECM types. Higher net photosynthetic rates at the growth CO_2 level increased the biomass of underground parts and stems, which, in turn, increased the ECM colonization rate. Elevated O_3 negatively affected the ECM colonization rate and more strongly, species abundance. The growth of F_1 was restricted, and the biomass was reduced by O_3. However, specific ECM species, such as *Suillus grevillei* was selected as the one of the capable species that flourishes under enhanced O_3. ECM efficiently absorbed Phosphorous (P) and other elements.

Moreover, the ECM symbioses with host plants greatly depend on C gain and allocation. Although N is an essential element for plant growth, the recent increase of N deposition surely brings an imbalance and is another critical factor of changing environment. N deposition usually increases tree growth, as it is an essential nutrient. How does N deposition affect the ECM symbiosis with host plants, especially in relation to another important nutrient, such as P? In chapter 4, to estimate the ECM symbiosis under different levels of N deposition with P efficiency, I planted the seedlings of three larch species in pots and placed them outdoors in open air, i.e., Japanese larch (JL: *Larix. kaempferi*), Dahurian larch (DL: *L. gmelinii* var. *japonica*) and F_1. Four nutrient levels were applied, using two levels of N (0 and 100 kg $ha^{-1}yr^{-1}$) and two levels of P (0 and 50 kg $ha^{-1}yr^{-1}$). After two years of nutrient application, seven types of ECM were identified to colonize the three larch species. The ECM colonization rate was reduced by 19.8 % for DL, and increased by 39.4 % for JL,

63.7 % for F_1 in high N condition, respectively. P application positively affected the ECM colonization for the three larches. ECM diversity was not significantly affected by N or P treatment except DL. ECM community structure of JL significantly differed among the nutrient regimes, but this was not the case with DL or F_1. Increasing N load obviously reduced P concentration in needles of the parents, but F_1 was not affected.

In summary, elevated CO_2 did not accelerate root turnover in infertile soil condition, especially at the beginning of CO_2 enrichment. Root dynamics of birch seedlings indicated great activity in the third year, most possibly due to mycorrhizal symbiosis. Under elevated CO_2 and O_3, I selected F_1 as the best ECM-colonized species, and found the ECM symbiosis extremely assisting seedling growth under external stress. Uptake of essential elements such as P via ECM symbiosis remained the same or accelerated under elevated O_3 in F_1. ECM community structure greatly changed, and ECM species belonging to genus *Suillus* predominated. Comparing the ECM symbiosis of F_1 seedlings with its parents in terms of N and P treatment, F_1 was considered to retain ECM diversity even when exposed to changes in the levels of N and P. In particular, under high N loading, P in the needle was reduced for DL and JL, but not affecting F_1. This might be attributed to the specific ECM symbiosis between *S. grevillei* and F_1, where the symbiotic relationship remained before and after the nutrient treatments.

From the view of larch afforestation, coping with P deficiency and/or infertile soil conditions is important for seedlings under changing environment, such as elevated CO_2, O_3 and N loading. The birch and F_1 are promising species and is a good candidate for reforestation under such conditions. However, it requires the vital partner, i.e., ECM, especially at the seedling stage. In conclusion, for survival under

changing environment in future, it is necessary to develop the ECM inoculation method for seedlings.

Keywords: Elevated CO_2, Ectomycorrhiza, Nitrogen deposition, Ozone, Root dynamic

Chapter 1
GENERAL INTRODUCTION

1.1 Changing environment

Forest ecosystems are threatened by changing environments due to recent anthropic activities (e.g. Karnosky et al. 2003a; Izuta 2006; Matyssek et al. 2013). This involves, increasing atmospheric CO_2 (CO_2) and tropospheric zone (O_3) concentrations, nitrogen deposition have become the most phytotoxic air pollutants, which are critical factors of changing environment (e.g. Cubasch et al. 2001; Matyssek et al. 2012; Koike et al. 2013).

The concentration of atmospheric CO_2 is increasing continuously, due to intensive deforestation and the use of fossil fuels: in 2013 the concentration reached up to ≈ 400 μmol mol^{-1} (Meehl et al. 2007; Mauna-Loa HP). Moreover, according to modeling results from the last century, the ground surface O_3 has been increasing sharply, and the pollution will extend to a larger region in Asia in the next decade (e.g. Lelieveld and Dentener 2000), especially in East Asia (Akimoto 2003). Numerous studies attempted to investigate the effect of elevated CO_2 and O_3 on forest ecosystem. For instance, Free Air CO_2 Enrichment (FACE) and Open Top Chamber (OTC) systems were developed throughout the world (e.g. Klamer et al. 2002; Karnosky et al. 2003a; Lukac et al. 2003; King et al. 2005; Eguchi et al. 2005; Koike 2006; Norby and Zak 2011; Koike et al. 2013).

The majority of the researchers have found that the growth and root system of plants increases under elevated CO_2 accompanied by higher water and nitrogen use efficiency of plants (e.g. Qu et al. 2004; Koike et al. 2010; Norby and Zak 2011). This is because, usually at elevated CO_2, plants slightly close their stomata (Morison 1998). To deeply understand the response, a number of studies have evaluated the effects of elevated CO_2 on the plant's physic-ecological process (Ceulemans and Mousseau

1994; Curtis and Wang 1998; Norby and Zak 2011). Their general conclusion is that usually, elevated CO_2 leads to a temporal increase of photosynthesis and consequently accelerates plant and root biomass. Moreover, the carbon gain and allocation to the below-ground increased, and this indirectly changes the colonization rate of ectomycorrhizal (ECM) fungi and species composition according to the mutualistic relationship with host trees (Wang et al. 2015).

Tropospheric or ground-surface ozone (O_3) is recognized as one of the phytotoxic air pollutants (Karnosky et al. 2003; Matyssek et al. 2012, 2013; Agathokleous et al. 2015). The current concentrations of O_3 have a significant adverse effect on yield of crops, forest growth and species composition compared to the effects of elevated CO_2 (Ashmore 2005; Hoshika et al. 2013; Watanabe et al. 2013; Yamaguchi et al. 2011, 2013). Most experiments of O_3 effects on tree growth are based on observations of above-ground parts, but much little is known about below-ground processes (Andersen 2003; Karnosky et al. 2003; Matyssek et al. 2013). Exposure of trees to O_3 modifies the allocation of carbon to roots, which disrupts root metabolism and influences the activity of rhizosphere organisms (Scagel and Andersen 1997).

Hence, mycorrhizal fungi may reduce overall retention of carbon (C) in the plant-fungus symbiosis by increasing C in roots. On the other hand, by means of root production and mortality, C sink retention is also reduced through belowground respiration (e.g. Rygiewicz and Andersen 1994). Therefore, the fine roots ($D < 2.0$ mm) production and their lifespan must be taken into account under changing environments. Since the colonization of ECM fungi usually occurs with only fine root tips (Smith and Read 1998), this rapid turnover of root growth and death plays an essential role in the function of C cycling (Fig. 1.1).

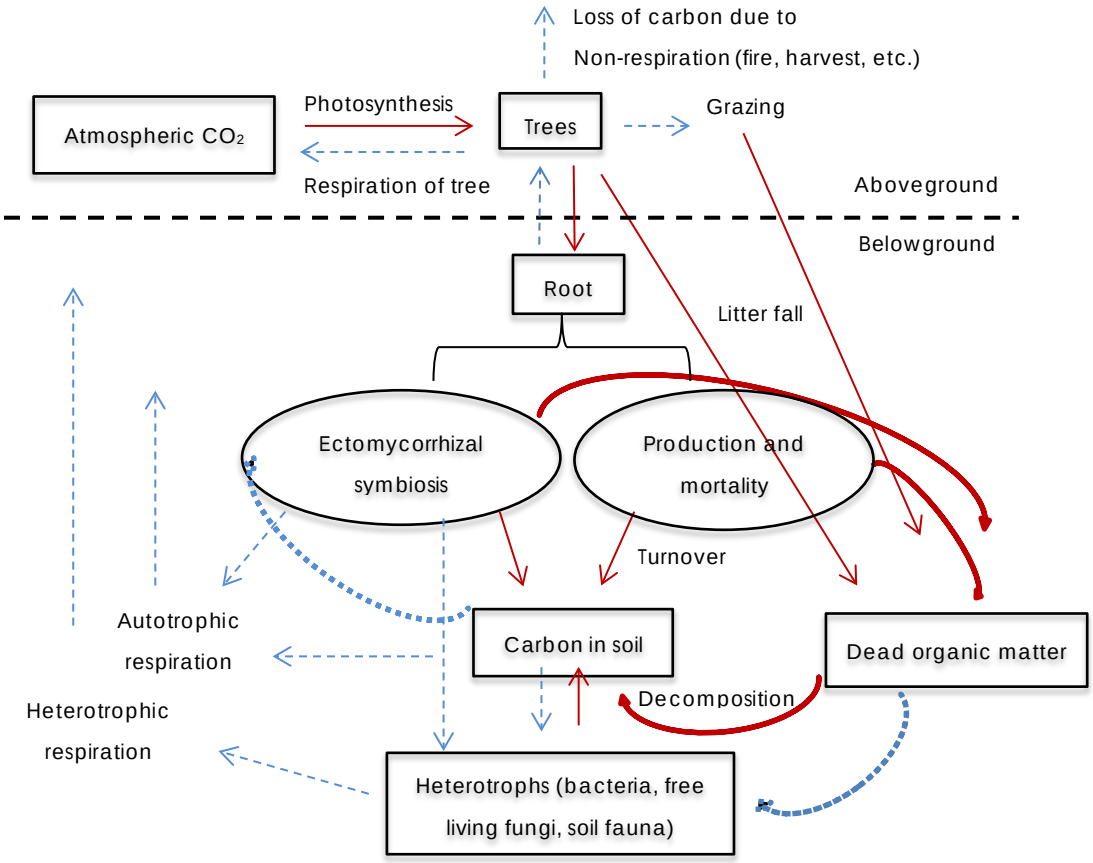

Figure 1.1 Schematic overview of the important role of ectomycorrhizal symbiosis and root dynamics in forests carbon cycling. Carbon pools are represented with boxes and circles, carbon fluxes by arrows (Solid line denotes the increase and dash line denotes the reduction) and processes are marked with text only (illustrated partly based on the idea of Fransson 2012).

During this couple of decades, nitrogen (N) deposition has been dramatically increasing in East Asia (Galloway et al. 2004; 2008). N is an essential element for plant photosynthesis and growth (e.g. Evans 1989) and is also often a limiting element of production in forest ecosystems (e.g. Schulze et al. 2005). As an essential macro element in forest ecosystems, increased N deposition usually leads to enhancement of CO_2 fixation in vegetation. Previous results showed that increased N deposition enhanced forest growth and increased carbon sequestration in soil (Hunter and Schuck

2002; Hungate et al. 2003； De Vries et al. 2006).

The effect of N deposition on belowground of trees was also detected. Since ectomycorrhizal fungi (EMF) exchange nutrients extracted from soil for carbohydrates delivered by plant roots, increased soil N supply may affect host plant-EMF interactions in any ecosystem by altering the abiotic soil environment (Lilleskov et al. 2002; Nakaji et al. 2001; 2004) or by changing the plant's allocation of resources to roots (Treseder and Allen, 2000; Helmisaari et al. 2008). Many studies documented that N deposition changed relationship between host plants and mycorrhizal fungi. For example, a study on a spruce forest across a stand-scale N deposition gradient (from 27 to 43 kg N ha^{-1} yr^{-1}) revealed that with increasing N deposition, ECM root tip abundance and mycelial production decreased five and 10-fold, the change of ECMF community and the species richness decreased (Kjoller et al. 2012). According to the study of Lilleskov et al. (2002), with N loading, composition of ECM species shifted from N-uptake to N-tolerant species, and given that under high-N, low-P and acidified conditions, the ECM species changed to favor types specialized for P uptake. However, there is limited report on N deposition with/without P loading effect on ECM symbiosis (e.g. Kalliokoski et al. 2009; Qu et al. 2010; Leppälammi-Kujansuu et al. 2012).

1.2 Root dynamics under changing environment

Roots account for 50 % of the total biomass of forests (e.g. Kubiske and Godbold 2001). Simultaneously, about 50 % of global net primary productivity (NPP) of terrestrial ecosystems comes from forest ecosystems (Field et al. 1998). But is it going to be the same several years later? It seems, it will not be the same: the scenario is that

changes will occur under elevated CO_2 and O_3 conditions (Karnosky et al. 2005; Norby et al. 2005; Grantz et al. 2006; Norby and Zak 2011) and the NPP of belowground may exceed 50 % of the total NPP (Kubiske and Godbold 2001). Consequently, this may lead to unknown and unpredicted implications to the ecosystem's sustainability. Therefore, deep understanding of interactive response of forest NPP to elevated CO_2 and O_3 will determine the terrestrial C balance (Karnosky et al. 2003a; Pregitzer and Talhelm 2013), and more important is the changes in belowground NPP, as they represent C inputs below the soil surface, where a relative amount of C may eventually be formed.

1.2.1 Fine root biomass under elevated CO_2

In various forest types exposed to elevated CO_2, NPP usually increases both above- and belowground (e.g. King et al. 2005). However, plants prefer to allocate C to roots other than to shoots when grown under elevated atmospheric CO_2, and therefore belowground function of forest ecosystems may change significantly, such as root production, respiration and longevity (Pritchard et al. 2001; Karnosky et al. 2003).

Some general patterns appear for the response to elevated CO_2, including increase in C allocation to belowground, particularly to fine root production and biomass (Nowak et al. 2004; Jackson et al. 2009; Lukac et al. 2009), and increase in soil carbon inputs (Jastrow et al. 2005; Lichter et al. 2008; Hoosbeek and Scarascia-Mugnozza 2009). Concurrent with greater root production, these same experiments have shown increase in soil respiration as well (King et al. 2004; Pregitzer et al. 2006).

Elevated CO_2 increases root growth; especially fine root growth and this can be

said to a range of species and experimental conditions. For example, Lukac et al. (2003) concluded that as a result of FACE treatment, standing root biomass of a *Populus* plantation increased by 47-76 % together with an increase of fine root biomass by 35-84 %. In another report, Pregitzer et al. (2008) found that belowground carbon allocation was positively affected by elevated CO_2. However, we have few information on the effect of elevated O_3 on belowground NPP of woody plants (Matyssek et al. 2012; 2013). Elevated CO_2 increases not only root biomass but affects root diameter and specific root length (SRL). Non-destructive measures show that elevated CO_2 increases fine root biomass through increase in the number and length of fine roots (Tingey et al. 1997; Thomas et al. 1999). However, influence on fine root growth was not positively consistent. Using mini-rhizotron tubes, Thomas et al. (1999) found that the effect of CO_2 on fine root growth was delayed until the second growing season. Therefore, I followed previous experiments to set up the root systems.

1.2.2 Fine root biomass under elevated O_3

The studies focusing on the effects of elevated O_3 on individual fine root biomass are still limited (Grebenc et al. 2007; Wang et al. 2015). Almost neither sufficient information nor consistent results are given from studies concluded. Usually the experiments were conducted with elevated CO_2 and O_3 combined (e.g. Kasurinen et al. 1999; Karnoskey et al. 2003; Pregitzer et al. 2008).

One famous experiment result found at the aspen community of Aspen FACE in Michigan, U.A.S. is that, annual fine-root production and mortality positively correlated with elevated O_3 (Karnosky et al. 2003; Pregitzer et al. 2008; Pregitzer and

Tlhelm 2013): soil respiration was the greatest at elevated CO_2 and CO_2+O_3 mixed conditions, and soil respiration correlated with increase in fine root biomass. With combined fumigation of CO_2 and O_3, they found an increase in belowground carbon allocation after 10 years of exposure. They also found that fine root biomass is actually enhanced by elevated O_3, and especially mixed CO_2+O_3 treatment.

Results of mixed conditions of elevated CO_2 and O_3 are rarely reported and there is not enough data to prove consistent results (Koike et al. 2012; Matyssek et al. 2013; Wang et al. 2015). However, to comprehend the C cycling of belowground well, another important indicator that must be calculated is the fine root turnover (Nakaji et al. 2008; Leppalammi-Kujansuu et al. 2014).

1.2.3 Turnover of fine root

A substantial amount of the carbon assimilated by plants is transported below ground to produce fine roots (Vogt et al. 1998). The network of tree root system support the fine roots lives and dies rapidly (Hendrick and Pregitzer 1992). The release of carbon fuels to the food web of belowground result in increasing the accumulation in soil organic matter, and may return to the atmosphere. This flux of carbon from vegetation to soil is called fine root “turnover” (Tierney and Fahey 2002). To estimate and to get accurate results of fine root turnover is not easy, thus the understanding of this process is limited and is the key constraint to quantifying terrestrial carbon cycling, vital for predicting the impacts of global environmental changes (Norby and Jackson 2000).

With our current knowledge it is “hard” to forecast the root lifespan of individuals, populations, or ecosystems, and one reason is, the wide variability of the

root turnover (Eissenstat and Yanai 1997). Several methods have been used to calculate rates of root production and mortality (Aerts et al. 1992; Hendrick and Pregitzer 1992; Andersson and Majd, 2005), but turnover rates of fine root obtained seem to vary according to the different methods used (Gill and Jackson 2000; Hertel and Leuschner 2002; Tierney and Fahey 2002). Currently no standard method exists for assessing fine root turnover (Lauenroth 2000; Norby and Jackson 2000).

Currently, mini-rhizotron provides a nondestructive, *in situ* method for viewing roots and is one of the ideal tools available for directly studying roots. By permitting the simultaneous measurement of fine root production and disappearance, mini-rhizotrons provide relatively accurate results which cannot be obtained through other means: by using sequential coring, in-growth cores or even excavation approaches (Majdi 1996; Johnson et al. 2001).

1.3 ECM symbiosis under changing environment

Ectomycorrhizae play an essential role in boreal forest ecosystems: most tree species vitally create a symbiotic relationship with ECM fungi to survive in diverse harsh conditions, such as Siberia, through efficiently nutrient uptake by ECM fungi (e.g. Qu et al. 2010; Jung and Tamai 2012). In nutrient limited soil, ECM are recognized to preferentially supply phosphorus (P) and water to the above-ground parts of host plants rather than their roots (Wallander 2000; Alves et al. 2010). In return, the symbiotic fungi receive carbon from photosynthetic assimilation and about half of the CO_2 efflux from soil originates from symbiotic microbes (Högberg et al. 2001). In comparison with non-mycorrhizal plants of the same species, ECM plants take up more organic P and N, since ECM produces ectoenzymes (Turnbull et al. 1996;

Vander and Sanders 2002; White and Hammond 2008). Is this relationship stable under changing environment? Apparently the answer is negative.

Under the circumstance of the changing environment, ECM richness and community will be altered to adapt and defend against these stresses. It will lead to the changes of symbiosis between ECM fungi and host trees (Wang et al. 2015). Many tree species around the world rely on mutualistic ECM to fulfill their nutrient requirements (Smith and Read 1997). For instance, Landeweert et al. (2001) reported that a major function of ECM symbiosis is the contribution to tree nutrition update by means of mineral weathering and/or mobilization of nutrients from organic matter (e.g. Read and Perez-Moreno 2003), making the host tree dependent on the fungal partner. What kind of changes will happen with this functional partner under changing environment?

1.3.1 Effect of elevated CO_2 on ECM symbiosis

Relationships between ECM symbiosis and host trees can be altered according to the carbon gain and their allocation from the aboveground to belowground. The distinct reflection of the results of the effects are changes in ECM colonization rate and further on their diversity as well as community structure (e.g. Parrent et al. 2006). Usually elevated CO_2 plant increases carbon supply to ECM and the growth of host plants is accelerated (Qu et al. 2004); this results in a higher demand for mineral nutrients and finally to changes in abundance of ECM community.

Elevated CO_2 increases the ECM mass, the infection and colonization, and the quantity of extra-matrical hyphae (Tingey et al. 2000; Langley et al. 2003). In a study of loblolly pine (*Pinus taeda*) forests, richness and diversity of ECM were not

affected by elevated CO_2, but elevated CO_2 altered the relative abundances of particular ECM taxa colonizing fine roots, and increased prevalence of unique ECM species. Finally, a greater ECM community dissimilarity was found among individual *P. taeda* plots (Parrent et al. 2006). On the other hand, the response of the mycorrhizal community to elevated CO_2 varies among tree species. For instance, Lukac et al. (2003), who investigated three *Populus* species in FACE system, stated that the rate of ECM colonization increased only in *P. alba* species.

Besides elevated CO_2, microhabitat characteristics also have effects on colonization of ECM at lava flow of Mt. Oshima-Koma, Hokkaido (Akasaka et al. 2007). After estimating Japanese larch seedlings from three microhabitats in three elevation zones, the results showed that the highest ECM colonization rate was in the most shaded micro-habitat in *Larix* understory than the other two (bare-ground and patch community of *Salix reinii*). Another factor is the N concentration. In a loblolly pine forests, increase of net N mineralization rate was negatively correlated with ECM fungi richness. ECM community composition and structure will change, but the diversity will be maintained with N loading for larch species (unpublished data). High soil N concentrations can also negatively affect ECM diversity (Parrent et al. 2006).

The extra-matrical mycelia (EMM) of ECM make up a large proportion of the microbial diversity and biomass in forest soils. Thus, their response to elevated CO_2 can affect plant nutrient acquisition and carbon movement through forests. So, for extra-radical mycelium study under elevated CO_2, Parrent and Vilgalys (2007) and Godbold et al. (2006) used in-growth bags to assess the response of the extra-radical mycelium. One more study, related with the effects of CO_2 and N fertilization on EMM biomass and community structure in forest plots of *P. taeda*, was conducted by

Parrent et al. (2007): they used sand-filled mesh bags buried in the field, and they found no increase of biomass at elevated CO_2 plots relative to control plots. However, after the analysis of phospholipid fatty acid (PLFA) and DNA sequencing, they found thelephoroid and athelioid taxa were both frequent and abundant as EMM and thelephoroid richness was extremely high. This shows the presence of ECM specific symbiosis under certain conditions. How will this specific symbiosis perform under elevated CO_2 (Fig.1.2)?

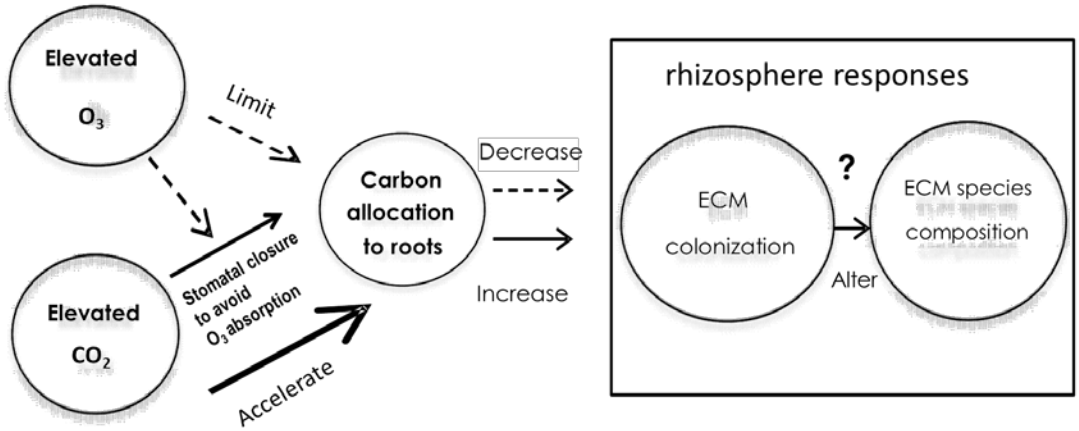

Figure 1.2 Relation between carbon allocation to belowground of a host tree, and the ectomycorrhizal symbiosis under elevated CO_2 and O_3. Arrows shows the carbon driver of different factors (Solid line denotes enhancing factor and dashed line denotes the limiting factors) (Wang XN original)

1.3.2 Effect of elevated O_3 on ECM symbiosis

It is well known that elevated O_3 reduces carbon assimilation and alters the allocation of photosynthates to below-ground (Grantz and Farrar 2000, King et al. 2005). O_3 exposure may slow the specific rate of inorganic N uptake by roots (Haberer et al. 2007), and decrease the standing biomass of fine root and sporocarp production of

fungi (Kasurinen et al. 2005; Andrew and Lilleskov 2009).

The effect of high levels of O_3 on mycorrhizas is not consistent. Some studies have found that high O_3 limits carbon allocation to roots and this is expected to decrease mycorrhizal colonization and alter species-host compatibility (Edwards and Kelly 1992; Smith and Read 1997). Other short-term studies have reported the enhancement of mycorrhizal short-root formations under O_3 exposure (Rantanen et al. 1994; Kasurinen et al. 1999). Furthermore, no effect on mycorrhizae was reported (Kainulainen et al. 2000).

It seems that the effects of elevated O_3 on ECM symbiosis vary according to treatment period. However, there are still limited reports of long-term treatment on O_3 fumigation on forests (Karnosky et al. 2003b; Matyssek et al. 2013). Therefore, this weak point should be taken into account for the next step in research.

1.3.3 Specific symbiosis of ECM under elevated CO_2

Differences in the effectiveness of ECM for improving tree growth and tree nutrition are often species specific (Bruns et al. 2002), or even strain specific (Dell et al. 1994; Agerer 2001). The majority of trees form symbioses with a plethora of fungi species, while some of the latter favor specificity and interact with only one host plant (Krause and Kothe 2006). The reason for this is considered to be that plants can control mycorrhizal colonization by controlling carbon allocation to short or fine roots according to the efficiency of the symbionts (Hoeksema and Kummel 2003). Thus, fungi with low carbohydrate requirements are often favored as symbionts in forest nurseries. Another possible explanation is ascribing this diverse symbiosis to ECM functional diversity.

Unfortunately reports on ECM functional diversity are still limited (Wang et al. 2015). Brearley and Scholes (2005) found ECM fungi isolated from mineral soils of tropical rain forests and are less able to utilize organic sources of nitrogen than mineral sources. For functional diversity of ECM, much more pertinent experiments are urgently required to further understand the response of ECM symbiosis related to changing environments.

1.4 Characteristics of Birch and Larch

White birch (*Betula platyphylla* var. *japonica*) is one of the typical pioneer tree species (e.g. Koike 1995). It widely distributed and is well acclimated in several environmental conditions, its distribution range covers from central Honshu to Far Eastern Asia (including Siberia) (Mao et al. 2010; Shi et al. 2010; Zyryanova et al. 2010). Moreover, this species exists under various conditions, and also has a strong tendency to form a pure stand. White birch is well used in several regions of Hokkaido (Terazawa 2005) as well as in Russia (Zyryanova et al. 2010). They are restricted to distribute in the special habitat, among the marshy edaphic and have shrub habit (the former grows at the special soil originated from peridotite, the latter mainly distributes in wetlands in the eastern Hokkaido).

Larches are considered to be promising species for afforestation and woody resources because of fast growth and high specific gravity of stem (Ryu et al. 2009). They are common components in the northern hemisphere, ranging from China to Japan, Siberian, European and North America and are recognized to be a major carbon sink.

Japanese larch (*Larix kaempferi*) is a native larch species in central, and partly

found in Mt. Manokami, northern Honshu, Japan and was transplanted to Hokkaido Island for timber use. As Dahurian larch (*L. gmelinii*) is found as fossil currently in Hokkaido (Koike et al. 2000), this species and its variety were introduced from most of the temperate forests, such as Siberia, Northeastern China to Hokkaido Island(Kelliher et al. 1997; Wang et al. 2008). Since Japanese larch has good tolerance to cold moist climate and grows rapidly (Matyssek and Schulze 1987a; b; 1988), they were introduced from Honshu mountains areas to northern Japan for reforestation or rehabilitation of bare ground. Due to their high production rate for timber, they were intensively used to reforestation in northern Japan. A variety of Dahurian larch (*L. gmelinii* var. *japonica*) is introduced to Hokkaido with the intention of using it as a breeding material for afforestation. It original distribution area is the Kurile Islands. Besides the fast growth and high yield, it is tolerant to infertile soil. Therefore it has a strong capacity of carbon sink.

Hybrid larch F_1 (*Larix gmelinii* var. *japonica* × *L. kaempferi*) was bred from crossing female Dahurian larch (*L. gmelinii* var. *japonica.*) with a pollen parent of Japanese larch (*L. kaempferi*). The hybrid larch F_1 has more suitable characteristics to the boreal region (Koike et al. 2000; Kuromaru 2008; Ryu et al. 2009). For example, it has much better tolerance to the shoot blight disease, grazing by the Red back vole and deer, and the damage caused by wind and snow. More amazing characteristics and utilities of hybrid larch F_1 are under research (Ryu et al. 2009; Kita et al. 2009).

Although both birch and larch have similar growth traits, such as, light demanding and preference to fertile soil habitat, they can survive in severe environment regions (Mao et al. 2010). Both species are dominant components of boreal forests, and it is vital to evaluate their response to changing environment. To obtain experience for reforesting and contributing trees to adapt to the environment

stresses is an important strategy.

1.5 Objective and structure of study

Recently, CO_2 concentration in the atmosphere has been reported to increase year by year. Though CO_2 is one of the essential components for photosynthesis, along with the increasing tropospheric O_3 concentration and N deposition, the net primary production of forest ecosystems is affected. Our understanding of belowground responses to elevated O_3 and CO_2 as well as N deposition is still not enough due to several limitations (Andersen 2003; Kasurinen et al. 2005).

1.5.1 Hypothesis of the study

In this study, I aim to investigate the effects of elevated CO_2 and O_3, N deposition on deciduous trees (species of birch and larch) in northern Japan.

My questions mainly focus to address (Fig. 1.1):

1) How do the root dynamics change under elevated CO_2, especially in infertile soil condition?

2) What is effect of elevated CO_2 and/or O_3 on plant growth and ECM symbiosis?

3) What is the ECM community structure response to different N and P levels?

1.5.2 Structure of this study

To reveal these questions (1.5.1 questions 1~3), I made three experiments as shown in Fig.1.3. All target plants are light demanding species with high specific gravity, and

are promising tree species for making plantations and re-vegetating open lands and degraded areas in East Asia (Zhang et al. 2000; Mao et al. 2010). The detail effects and their interaction impact of various environmental factors are presented in Fig. 1.4.

At first, I monitored seasonal changes of fine root of Japanese white birch as a model plant grown in volcanic ash and brown forest soil under elevated CO_2 using the free air CO_2 enrichment (FACE). I tried to apply the mini-rhizotoron developed by Nakaji et al (2008) to obtain the essential role of the dynamics of fine root of young fast growing tree species at enhanced CO_2 concentration estimated at 2040 by IPCC.

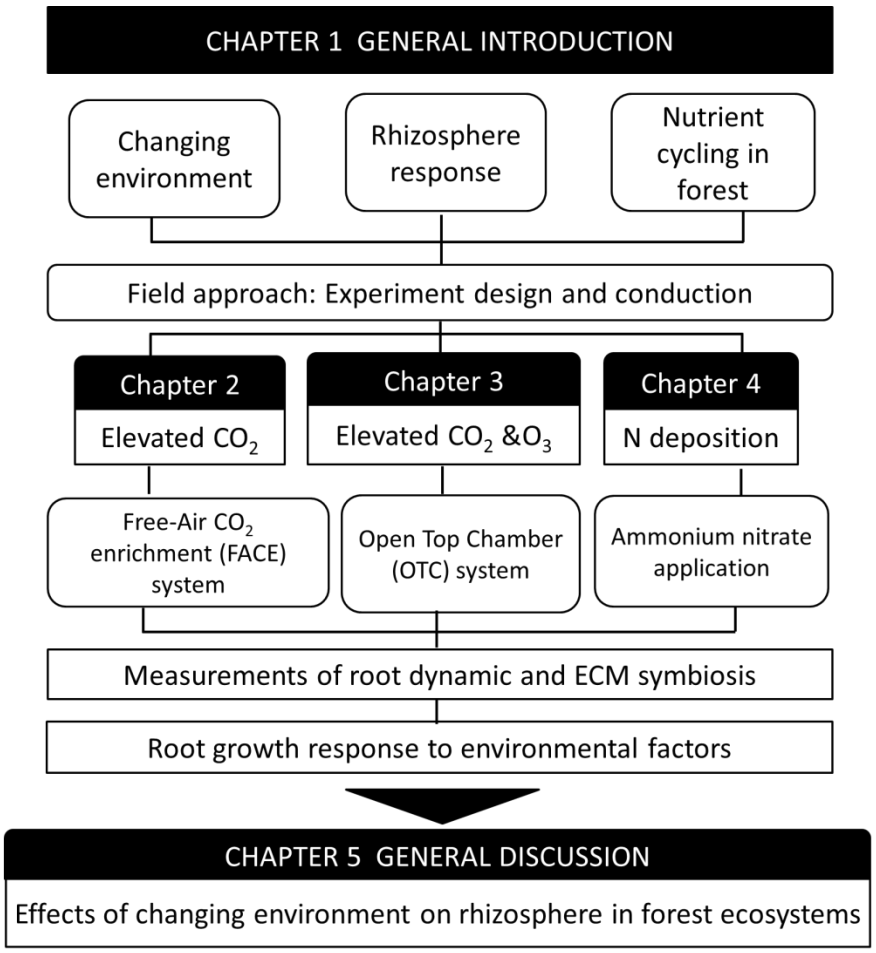

Figure 1.3 Structure of this study

I propose that the current changing environment is affecting the plant-microbe

interaction, especially ectomycorrhizal (ECM) colonization (Chapter 1). Overall, I tried to clarify the performance of colonization rate and species richness of ECM in larch species under different environmental stresses. According to the previous report (Yamakawa 2012), the number of ECM species colonized with Japanese is limited, although its species amount increases with tree size. The infection of ECM with larch is smaller compared with mountain birch (Wang et al. 2012) and white birch (Araki et al. 2013).

In Chapter 2, I found the essential role of dynamics of fine root in different soil types at elevated CO_2 in relation to the colonization of ECM. To reduce the variation of ECM colonization with individual levels, I also studied using a clonal plant, i.e. Hybrid larch F_1 as host plant. I focused on symbiotic relationships between ECM and F_1 grown under elevated CO_2 and/or O_3 grown in brown forest soil (Chapter 3). Then I examined combination effects of high N and Phosphorous loading on ECM colonization and growth of hybrid larch F_1 and its parent larch (Dahurian larch and Japanese larch) grown in immature volcanic ash soils (Chapter 4). Based on my findings, I propose specific traits of ECM on larch as a host plant when grown under various environmental stresses to sustain growth (Chapter 5).

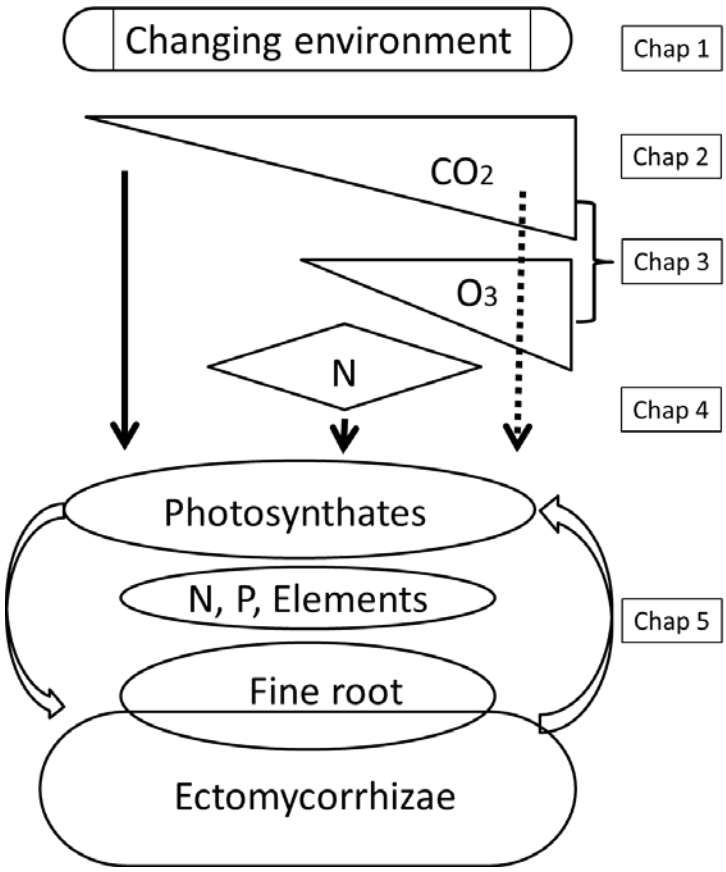

Figure 1.4 Impact scale and interaction effect of different environmental factors.

Note: solid arrow denotes the effects of elevated CO_2, dashed arrow denotes the combined effects of elevated CO_2 and O_3; The different size of shape boxes symbolize influences strength, and overlap of different ellipses indicates the their interaction effects.

Chapter 2

FINE ROOT DYNAMICS OF WHITE BIRCH UNDER ELEVATED CO_2

2.1 INTRODUCTION

The atmospheric carbon dioxide (CO_2) concentration has risen to nearly 30% since the century, resulting from large increases in fossil fuel burning and deforestation (Meehl et al. 2007). The impacts of elevated CO_2 on forest trees and forest ecosystems is currently of great interest, including exchange of energy and materials among soil, aboveground biomass, and the atmosphere (Lal 2005).

The average enhancement of photosynthesis for trees exposed to elevated CO_2 (300 ppm) has been about 60% (Norby et al. 1999). However, the responses vary considerably between species (Naumburg et al. 2001), by position in the crown (Takeuchi et al. 2001), by nitrogen (N) fertility level (Watanabe et al. 2008), by season (Noormets et al. 2001 b), and by co-occurring pollutant concentrations (Noormets et al. 2001a). It is far less certain tree growth and productivity under elevated CO_2. Furthermore, not only the aboveground related to photosynthesis activity, the belowground parts linked with aboveground also required major attention (Scarascia-Mugnozza et al. 2001). Thus, root systems particularly the fine root dynamics should be highlighted in order to thoroughly understand the nutrient cycling under elevated CO_2.

Fine roots were classified generally as $\leq$ 2 mm in diameter based on the definition proposed by Pregitzer et al. (2002). Although fine roots contribute less than 2% of tree biomass in forest ecosystem (Brunner and Godbold 2007), from 33 to 67% of the annual net primary productivity (NPP) in forest ecosystems derives from fine roots (Gill and Jackson 2000). Importantly, despite the small biomass of fine roots relative to aboveground tissues in forest ecosystems, large amounts of carbon (C) and N cycle annually through fine roots, which grow, die, and decompose very rapidly and

have high N concentrations (Hendrick and Pregitzer 1992; Ruess et al. 2003). Therefore, fine root production (FRP) and mortality (FRM) take the vital role of below-ground processes, however, they are less well understood (Norby and Jackson 2000; Aber and Melillo 2001; Fitter 2005). Since fine roots are increasingly recognized as a key to balancing nutrient cycling of trees and ecosystem, and the carbon (C) sequestration to soil (Norby and Jackson 2000; Matamala et al. 2003; Norby et al. 2004), understanding about the effect of CO_2 enrichment on root survivorship is highlighted. However, root longevity and turnover are reported discrepantly, this largely resulted in great uncertainty about terrestrial C cycles (Pritchard et al. 2001a, b; Lichter et al. 2005; Hogberg and Read 2006).

On the one hand, inconsistent findings are reported, as more C being allocated to the roots under elevated CO_2, however negative responses of belowground were happened, because of the enhanced plant growth under elevated CO_2 was reported convergent over time (Arnone et al. 2000; Higgins et al. 2002). The root turnover and longevity are getting mysterious according to this uncertain allocation and root production. On the other hand, even several studies have found production and mortality of fine roots produced by trees growing under CO_2 enrichment are significantly increased (Matamala and Schlesinger 2000; Pregitzer et al. 2000; King et al. 2001; Pritchard et al. 2001), the results are still inconsistent. So far, the stimulation of NPP by CO_2-enrichment at Duke FACE has persisted after more than 8 years amid speculation that nutrient limitations will eventually constrain a positive CO_2 response (Luo et al. 2004a, b; Finzi et al. 2006; Johnson 2006). In particular, NPP is strongly affected by soil nutrient limitation (Oren et al. 2001). Due to the fine roots account for large degree of NPP, fine root dynamics must dramatically affected by soil condition. Recently, as reported, elevated CO_2 accelerated growth and increased plant nutrient

demand and uptake capacity (Bielenberg and Bassirirad 2005). In infertile soil condition or nutrient limitation stress, how the fine roots adjust their dynamics to balance the costs and benefits of the whole plant is rarely addressed.

White birch (*Betula platyphylla* var. *japonica*) is widely distributed and well acclimated itself in several environmental conditions, its distribution range covers from central Honshu to Far Eastern Asia (including Siberia) (Koike 1995, Shi et al. 2010). Moreover, this species exists under various conditions, having a strong tendency to form a pure birch forest. White birch is well used in several regions of Hokkaido (Terazawa 2005) as well as in Russia (Zyryanova et al. 2010) for promising species of green afforestation. To estimate the C cycling of boreal forest in east Aria under elevated CO_2, root dynamics of birch plantation is emphasized as great forest component. Specific in northern Japan, the soil is widely covered by volcanic ash soil which usually has phosphorous (P) deficiency and relative low N concentration (Kayama et al. 2009). Furthermore, P availability is regarded to be a limited factor to tree growth due to several mechanisms, especially with N deposition (Vitousek et al. 2010). Therefore, assessment of future C sequestration should consider the limitations imposed by soil fertility.

In this study, I attempt to understand the root dynamics of Japanese white birch under elevated CO_2 involving two soil types-volcanic ash (VA) soil and brown forest (BF) soil. I hypothesize that 1) In BF soil, elevated CO_2 stimulated plants growths better than VA soil because of the nutrient limitation. Therefore, root length production is increased by elevated CO_2 in BF soil not VA soil. 2) Fine root turnover may be increased with elevated CO_2 due to the increased carbon allocation, and the value in BF soil is higher than VA soil over time. 3) Fine roots have a longer lifespan under elevated CO_2 and also relative longer in VA soil than BF soil, because of a

longer lifespan may lower the cost for root production in nutrient limited soil.

2.2 MATERIALS AND METHODS

2.2.1 Study site and FACE system

The experiment was conducted in Free Air CO_2 Enhancement (FACE) system located in Sapporo Experimental Forest, Hokkaido University, Japan (43° 60′ N, 141°20′ E) (Eguchi et al. 2008; Watanabe et al. 2010). The FACE system was constructed with a size about 7.0 m width and 5.2 m height. The whole-plot treatment consisted of two levels of CO_2 [ambient (380-390 μmol mol^{-1} CO_2) and elevated CO_2 (500 μmol mol^{-1} CO_2)] with three site replications. The tanked CO_2 was supplied mainly in daytime, coving the whole photosynthesis period. Totally I constructed six FACE rings giving a total of six sites for data analysis and including the variance among the sites location.

2.2.2 Plant materials and soil type

The present experiment had a split-plot factorial design and employed the randomized block method. Three-year old Japanese white birches (*Betula platyphylla* var. *japonica*) were planted randomly in each FACE site. There were two soil types -brown forest (BF) soil and pumice included volcanic ash (VA) soil-in each FACE site. The chemical and physical properties of these two types of soil were described by Eguchi et al. (2008). The N content in VA soil was (0.14 mg g^{-1}) lower than BF soil (0.30 mg g^{-1}). Much more significant was P content, P deficiency was severer in VA soil (0.58 mg g^{-1}) than BF soil (4.48 mg g^{-1}).

2.2.3 Mini-rhizotron system

To detect fine root dynamics, Minirhizotrons (fine root observation tubes) and specialized camera or scanner equipment have been widely adopted for *in situ* observation. This technique is a non-destructive method that can be used to monitor the same roots over selected time intervals, which can vary from days to years (Andersson and Majdi 2005). Comparing with ingrowth core or sequential soil core, it takes several advanced points, such as to identify the same roots on successive dates (Hendrick and Pregitzer 1992; Majdi 1996), to quantify the data on root length production, root length mortality, longevity, root density and root diameter (Hendrick and Pregitzer 1996; Majdi and Andersson 2005).

In each FACE site, two birch seedlings were randomly selected as observed target in each soil type and mini-rhizotron tube was installed matching each observed seedling. Totally four birch seedlings were measured by four Minirhizotron (MR) tubes buried beside the seedlings in one FACE site. All the seedlings were planted together with tubes in June 2010. I installed transparent acrylic tubes (0.5 m long with a 5.08 cm inside diameter) at an angle of 45° to the soil surface. I captured digital image in the depth of 15-30cm using a scanner which was exactly right matched the tube size as the schematic was showed by Maeght et al. (2013) (Fig. 2.1) The measurement did not start in 2010, because after installing the tubes, root growth and death at the soil MR tube interface may not be representative of these processes in bulk soil since a lag period of up to a year is required to stabilize the density of fine roots (Joslin and Wolfe 1999). Thus, to avoid the potential gap between soil and tubes, I started the image scanning one year later from April 2011 to October 2013 for an

accurate measurement, and the CO_2 fumigation started from early June each year. I collected three years images excluding the period of snow with a three weeks interval, the size of all the images I obtained is about 21.59 × 19.56 cm^2. These images were used for detecting the fine root dynamics. Fig.2.2 showed the images of one tube rom the first until last session during one year observation.

2.2.4 Root image analysis

I used the program WinRHIZOTron (Regent Instruments, Quebec, Canada) to analyze the root in the captured images. It was difficult to distinguish whether one root appeared from the time when I scanned the image. Therefore, roots that were unsuberized and white when observed for the first time were recorded as new, whereas those remaining white or changing to brownish in subsequent viewings were recorded as living. Roots were defined as dead when they turned black and wrinkled and produced no new roots in subsequent viewings. For each tube, I traced the length and diameter of each individual root appeared in the image area. The sum of the length of new roots and the increase in the length of existing roots during each observation interval was calculated as FRP. Parallelly FRM was evaluated as the length of root that disappeared (Tingey et al. 2000; Satomura et al. 2007; Nakaji et al. 2008). Each parameter was obtained from the 12 MR tubes.

Fine root turnover (y^{-1}) can be estimated generally in two ways: (1) as the ratio of annual root length production to average live root length observed; (2) the inverse of median root longevity (Majdi et al. 2005). In this study, I calculated the turnover of FRP and FRM following the first method according to the annual length-based method (Gill et al. 2002),

Production rate (mm cm^{-2}) =$ALRP/LRL_{max}$ or $ALRP/LRL_{mean}$

Mortality rate mm cm^{-2})=$ALRM/LRL_{max}$ or $ALRM/LRL_{mean}$

where ALRP is annual length-based root production and ALRM is annual length-based root mortality. LRL is live root length (standing crop). LRL_{max} and LRL_{mean} denote for the maximum and mean value of LRL during the corresponding year.

I define fine lifespan (median root longevity) obtained from MR, as the time during which 50% of the fine roots die (Andersson and Majdi, 2005; Green et al. 2005). Additionally, the fine root diameters (D) were classified into five orders: D < 0.2mm, 0.2-0.3mm, 0.3-0.4 mm, 0.4-0.5mm and > 0.5mm. Roots of D > 2 mm were not estimated for all parameters in this study.

Due to the plant canopy was closed since 2012 (Hara 2014), thus, I separated the first year data from next two years for calculating and plotting graphs, particularly for the live root length, fine root production and mortality.

2.2.5 Soil texture

According to the report of Eguchi et al. (2008), nutrient concentration was relative lower in VA soil than BF soil, I mainly detected the C and N concentration in soil during 2011 and 2012, the measurement was conducted with NC analyzers (NC-900, Sumica-Shimadzu, Kyoto, Japan).

2.2.6 Statistical analysis

I estimated the FRP and FRM of different treatment (ambient and elevated CO_2) on

different soil types in each year by multiple liner models. The fine root median and mean longevity were analyzed using nonparametric Kaplan-Meier survival function with the factors of diameter class, and different soil and CO_2 treatment. The statistical analysis unit was three FACE replications, all the data were undertaken by SPSS software.

2.3 RESULTS

2.3.1 Soil nutrient concentration

I detected the C and N concentration of two soil types (Table 2.1), VA soil showed lower content than BF soil of both C and N, no significant effect of elevated CO_2 was found on soil nutrient content.

2.3.2 Live fine root length

During the three-year treatments, LRL showed higher amount in the period of early growing season (June to Aug) from 2011 to 2013 (Fig. 2.3). In 2011, LRL under elevated CO_2 was significantly higher than ambient treatment in BF soil. However, the result was contrary in VA soil, LRL showed higher values in ambient than elevated CO_2 condition (Fig. 2.3 a). From 2012 to 2013, there was no distinct effect of elevated CO_2 on LRL in BF soil, and LRL was extreme higher in ambient VA soil than other three conditions (Fig. 2.3b). Elevated CO_2 markedly reduced LRL in VA soil during the three observed growing year.

2.3.3 Fine root production and mortality

In BF soil, fine root production rate did not affect by elevated CO_2 during 2011 except July and August when it was increased by it (Fig. 2.4a). It was unaffected during 2012 and reduced by elevated CO_2 in August of 2013(Fig. 2.4b). In VA soil, no significant difference was found between elevated CO_2 and ambient treatment in 2011(Fig. 2.4a), however it was reduced during the early growing season in 2012 and 2013 (Fig. 2.4b). No clear trend was found for mortality rate in BF soil, and elevated CO_2 tend to reduced it in the late growing season in VA soil (Fig. 2.5).

Turnover of fine root production and mortality differed significantly among the treatments (Table. 2.2). Elevated CO_2 did not affect the production and mortality turnover, but there was an interaction effect of year and CO_2 on mortality turnover. It was reduced by elevated CO_2 in two kinds of soil over time. The effect of soil significantly influenced production turnover, it showed lower trend in VA soil than BF soil. Time had significant effect on turnover and reduced it during the three-year treatment. The interaction effect of soil and year influenced the production turnover. There was no interaction effect of CO_2 and soil, nor CO_2, soil and year. Additionally, the annual length-based root production (ALRP) and annual length-based root mortality (ALRM) of each tube with different treatments had positive correlation with each other (Fig. 2.6).

2.3.4 Fine root longevity

The median fine root longevity was analyzed by different treatments and root diameters. Overall, median root longevity differed with different treatments, and it

was increased under elevated CO_2 in 2011 for BF soil and VA soil (Table 2.3). From 2012 to 2013, median fine root longevity was gradually reduced under elevated CO_2 in BF soil, and the increased effect on median fine root longevity in VA soil was weakened by elevated CO_2.

Median root longevity of different diameter classes showed significant response to different treatments. The fine root (D < 2mm) were not affected by elevated CO_2 in all conditions in 2011 and 2012, but it was increased by elevated CO_2 in BF soil and reduced in VA soil in 2013 (Table 2.4). Roots of diameter between 0.2 mm and 0.3 mm, their longevity were markedly increased by elevated CO_2 except the year of 2013 in BF soil, when longevity was contrary reduced by elevated CO_2. Roots of diameter between 0.3 and 0.4cm showed significantly increased effect on their longevity by elevated CO_2, there was no effect on median longevity of fine root (D > 0.5mm) in 2013.

2.4 DISCUSSION

I found the LRL (live root length or length-based standing crop) significantly influence by elevated CO_2. In BF soil, LRL was higher under elevated CO_2 than ambient, but this trend disappeared from the second year. Contrarily, in VA soil, elevated CO_2 reduced the LRL for three observed years (Fig. 2.1). Generally, elevated CO_2 stimulates plant growth (Norby and Zak 2011) and more carbon is allocated to the roots (Lukac et al. 2003), therefore, elevated CO_2 was also assumed to increase the root/shoot ratio in earlier studies. However, new studies have revealed less pronounced effects (Bielenberg and Bassirirad 2005) or even negative responses of CO_2 (Arnone et al. 2000; Higgins et al. 2002). Present results proved this point and

suggested that the effects of elevated CO_2 diminished over time. On the other hand, photosynthesis down-regulation of birch seedlings occurred during the treatment years (unpublished data), this frequently observed in many seedling and sapling stage of trees (Tissue and Lewis 2010), thus, the unaffected LRL under elevated CO_2 from 2012 in BF soil probably occurred.

The difference results between BF soil and VA soil, suggested the LRL strongly related with soil nutrient condition. My results found the soil N concentration in VA soil was relatively lower than BF soil as reported before (Eguchi et al. 2008). Moreover, this down-regulation is expected to be clearly found in immature volcanic ash soil (Mao 2013), it limited photosyntate allocation to belowground. Importantly elevated CO_2 accelerates plant growth, increases plant nutrient demand and uptake capacity (Bielenberg and Bassirirad 2005). As a result, with higher nutrient demand under elevated CO_2, plant growth especially the belowground was likely restricted or even reduced in VA soil. Thus, a reduced LRL of white birch was found in VA soil.

Additionally, the unclear trend of elevated CO_2 effect on LRL from second year in BF soil, and the negative effect of elevated CO_2 on LRL in VA soil, it potentially derived from the changes of root production and mortality. For instance, changes of higher mortality or lower production under elevated CO_2 can lead to a reduced LRL. However, I did not find any clear trend for root production rate and mortality rate (Fig. 2.4, 2.5), the strongly correlation of ALRP and ALRM in all conditions suggested there was no significant change difference between them, even the correlation was slightly lower in VA soil than BF soil (Fig. 2.6). Therefore, I deduced this might depend on the root turnover and lifespan, following I discussed this point.

Elevated CO_2 did not affect the fine root turnover of production and mortality (Table 2.2), the results indicate no effects of elevated CO_2 on root turnover. It has

been estimated the effects of elevated CO_2 that did not appear to alter the turnover of loblolly pine, despite an increased root length, production and mortality according to Pritchard et al. (2011). Moreover, an interactive effect with year was found that elevated CO_2 reduced FRM turnover over time. It indicated a longer lifespan with CO_2 enrichment as I detected (Table 2.2). Additionally, Eissenstat et al. (2000) concluded that elevated CO_2 may be associated with longer root lifespan, by decreasing the root N concentration and reducing the root maintenance respiration, which was also reported by Arnone et al. (2000) that the longer lifespan was also found. Within year, FRM turnover increased by elevated CO_2 in 2013, therefore there was lower LRL in the third year comparing with first two years during the observation, corresponding to a reduced root lifespan under elevated CO_2 (Table 2.4).

Soil only had significant effect on FRP turnover, not affect the FRM turnover. Overall, VA soil had low capacity for FRP turnover, this might be the reason that lifespan in VA soil was relative higher than BF soil in the same CO_2 condition (Table 2.3). Another possibility is symbiotic effect of ectomycorrhiza (ECM), because the root with ECM symbiosis can live much longer or with lower production than non-colonized roots. This has been recently demonstrated by Bidartondo et al. (2001), who found ECM colonizing the roots (D = 0.3~0.6mm) of Bishop pine (*Pinus muricata*) prolonged the root longevity. Therefore, present result proved with limited nutrient in VA soil, lower turnover and longer lifespan suggested the slower root dynamic in VA soil than BF soil.

The median longevity was increased by elevated CO_2 in both BF soil and VA soil, but the influence was not continuing from the second year in BF soil, and appeared a convergent effect of elevated CO_2 in VA soil (Table 2.3). As it has been reported, plant under elevated CO_2 usually increases water use efficiency and stimulate dramatic

aboveground growth (Qu et al. 2004; Koike et al. 2010). It has been proved that under elevated CO_2, root uptake and provide nutrient resources for CO_2-induced increases in aboveground with a more modest production in fine roots or longer lifespan roots (Housman et al. 2005). In VA soil, the root median longevity was increased consistently under elevated CO_2. One possibility is the nutrient limitation resulted in a lower turnover of FRP and FRM, because the root longevity was inversely related to the duration of the resource supply (Pregitzer et al. 1993). Therefore, a longer root lifespan was found due to limited nutrient availability. Another reason is that plant is preferentially to enhance the growth of aboveground as I discussed above, this may readily occur in nutrient limited condition, because root lifespan would be increased if construction costs relative to maintenance costs are high, or if the nutrient availability is low (Eissenstat et al. 2000).

Root diameter in forest changed during the study with elevated CO_2 (Pritchard et al. 2008), and highlights the importance of taking soil samples during the MR image acquisition, to get SRL and to be able to estimate biomass. This was not done in my study and I am therefore not able to convert root length to biomass. However, the root responses to the treatments of different diameter classes were estimated (see Table 2.4). The fine root median lifespan ($D < 2mm$) significantly affect by different treatments, the thinnest roots ($D < 0.2$ mm) were affected under elevated CO_2 since 2013 after two growing seasons. The other order fine root lifespan was influenced by the beginning and unaffected under elevated CO_2 with the root diameter larger than 0.4 mm since 2013. Talking about the fine root longevity, one thing must be taken into account is the mycorrhiza. Plant usually increases mycorrhizal colonization and decrease root N concentration under elevated CO_2 (Pritchard and Rogers 2000; Tingey et al. 2000). Given that root longevity is negatively correlated with tissue N

concentration (Pregitzer et al. 1998; Wells 1999), I concluded that the mycorrhiza symbiosis was strongly stimulated under elevated CO_2 in the beginning. As a result, fine root performed a longer lifespan with no distinct effect by CO_2 enrichment in present study. Moreover, mycorrhizal colonization under elevated CO_2 is not consistently increasing, Shirano et al. (2007) found that elevated CO_2, the ECM colonized Japanese larch (*Larix kaempferi*) with an increasing rate during the first year treatment, and later equilibrated to a stable lower rate. In my case, the shorter longevity of fine root was likely derived from the decreased ECM colonization during the third year. It was possible ECM assisted birch seedlings to survive in a new soil condition, and the shorter root lifespan revealed a completed necessary aboveground growth, and plants started to develop root system after that establishment (Eissenstat et al. 2000).

Additionally, regardless of the mycorrhiza symbiosis, the different response of different root diameter classes indicated root heterogeneity. The location of a root and branched system of root are potentially to influence the root lifespan (Guo et al. 2004). All of these characters are required to deep understand under changing environment.

2.5 SUMMARY

This study investigated the fine root dynamic responses to elevated CO_2 of white birch regarding to root turnover as well as root longevity with different kinds of soil. Changes in root production and mortality in response to elevated CO_2 could be a key link between plant responses and long term changes in soil organic matter and ecosystem C balance (Norby and Jackson 2000).

In this study, elevated CO_2 reduced fine root length standing crop in VA soil, and

a lower turnover of production and mortality were found comparing with BF soil. This indicated a weak root dynamic of white birch in VA soil. Elevated CO_2 increased root longevity, especially in VA soil during three observed growing seasons, suggested soil nutrient status affecting root longevity strongly. The shorter fine root longevity under elevated CO_2 comparing with ambient in VA soil during the third growing season, suggested the root dynamic is getting higher, thus a C sequestration to soil may happen to increase. The result may due to the changes of mycorrhizal colonization, root specific character, and the position of a root on the branching root system. These factors can't be ignored, thus more effort are required to contribute the root research, to thoroughly understand the response of root dynamic under changing environment.

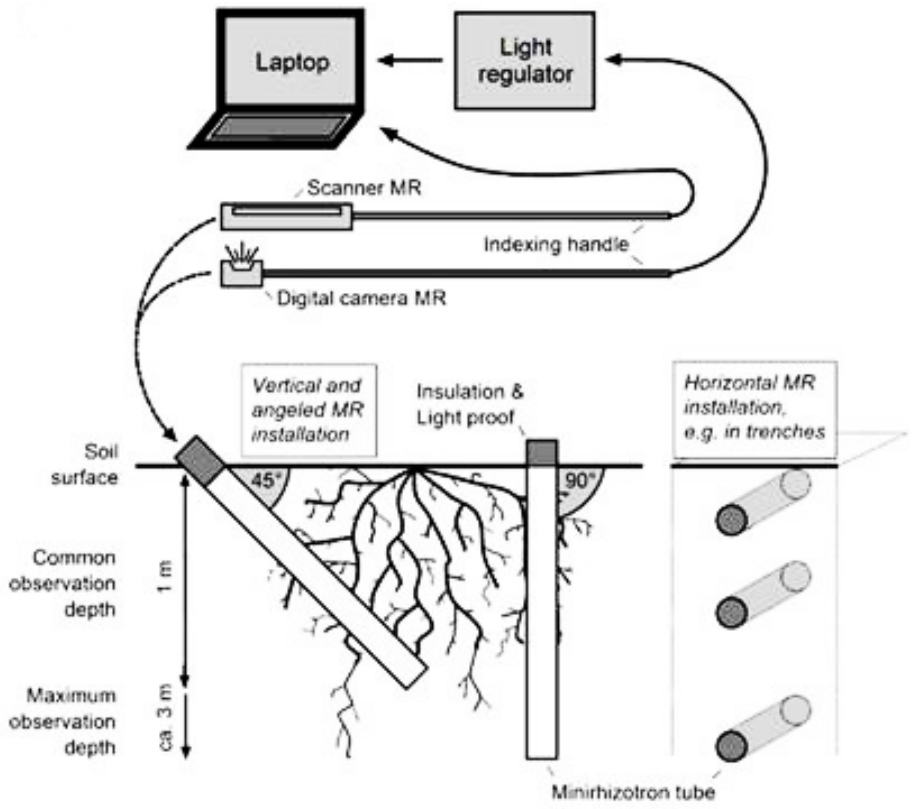

Figure 2.1 Schematic of Minirhizotron (MR) techniques, (Image capture instrument with Digital Camera or Scanner MR) and different options to install the MR tubes (angled or vertical from the soil surface or horizontally from trenches) (Maeght et al. 2013).

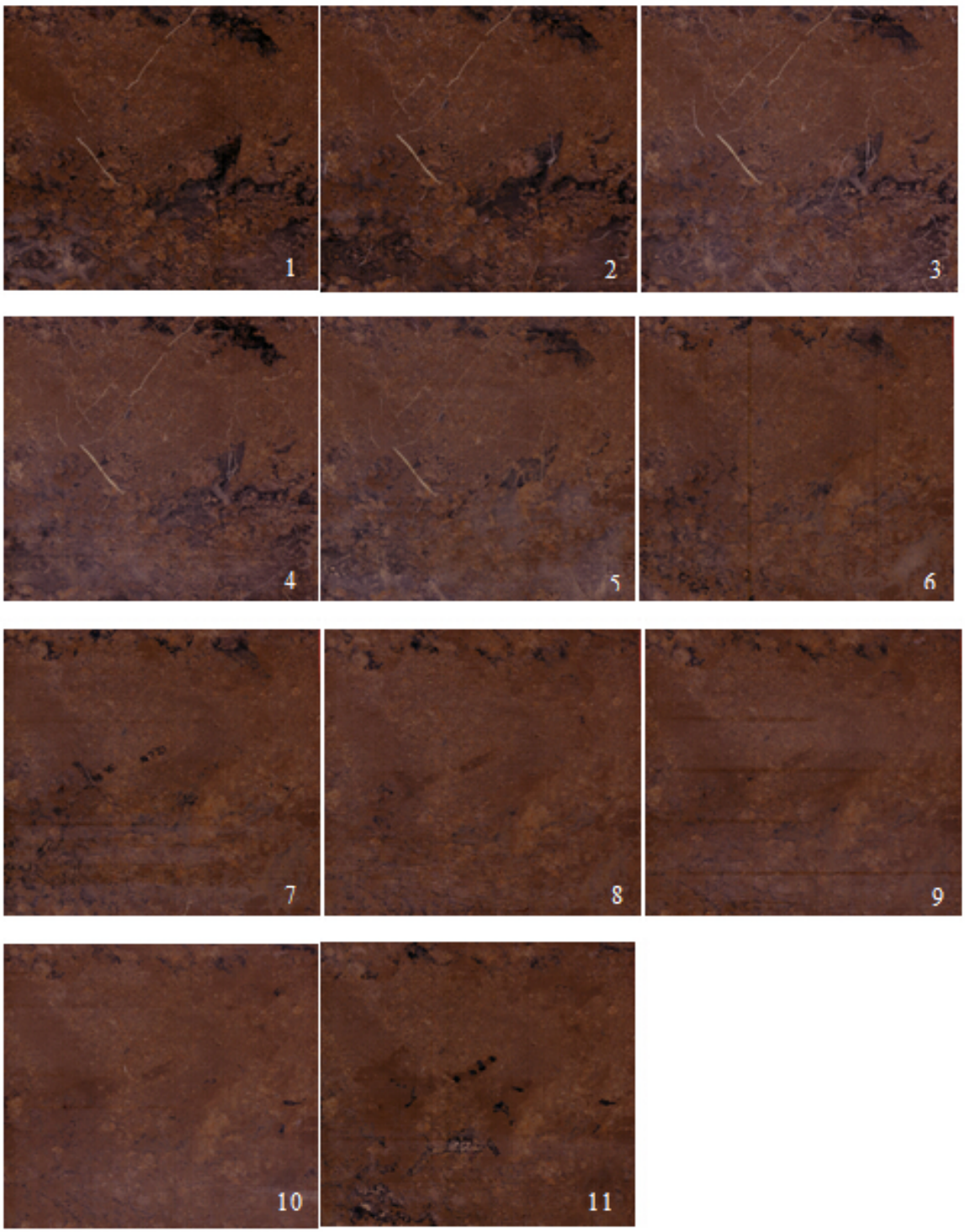

Figure.2.2 Root images captured in one tube from the first session to the last one during one year observation. The accurate size of each image is 21.59 × 19.56 cm^2, the number in each image denotes the sessions, 1-11 indicates the first to last session.

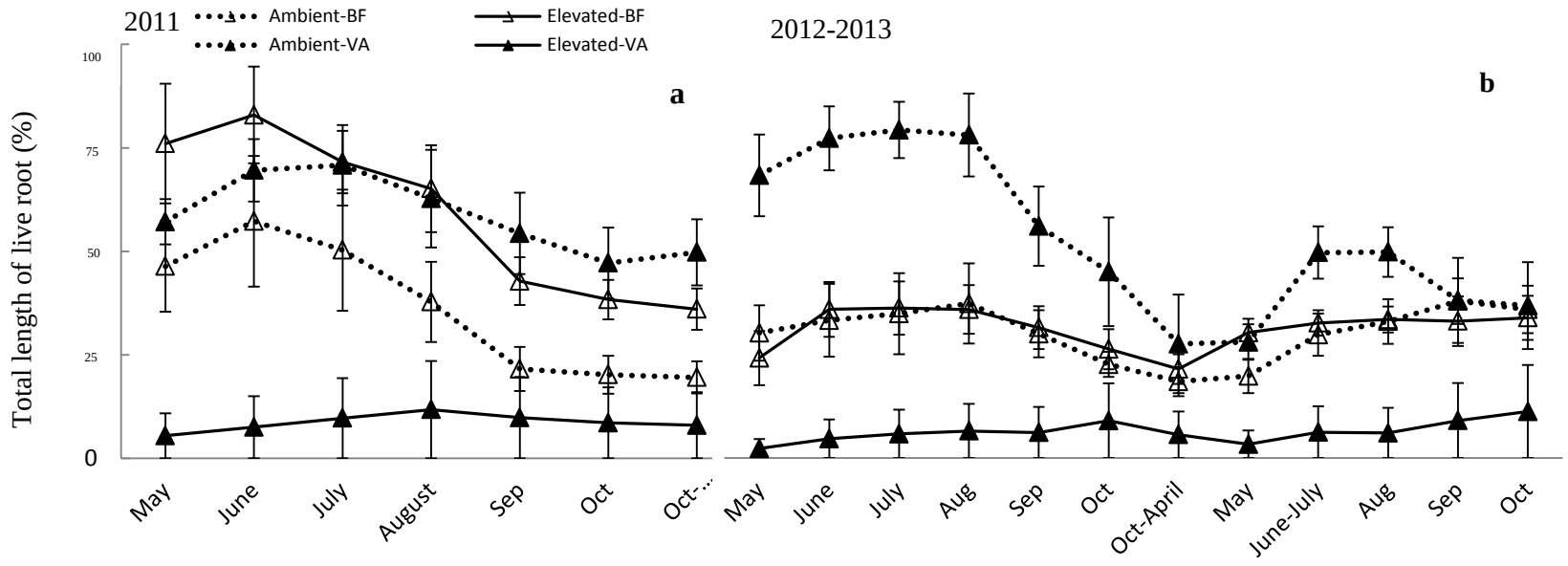

Figure 2.3 Relative length of live fine root (standing crop) of white birch seedlings growing under elevated (500 lmol mol^{-1}) and control (370 lmol mol^{-1}) [CO_2] on volcanic ash (VA) and brown forest (BF) soil.

The maximum absolute vale of vertical axis of (a) and (b) is 16 mm/cm^2 and 3.5 mm/cm^2, respectively.

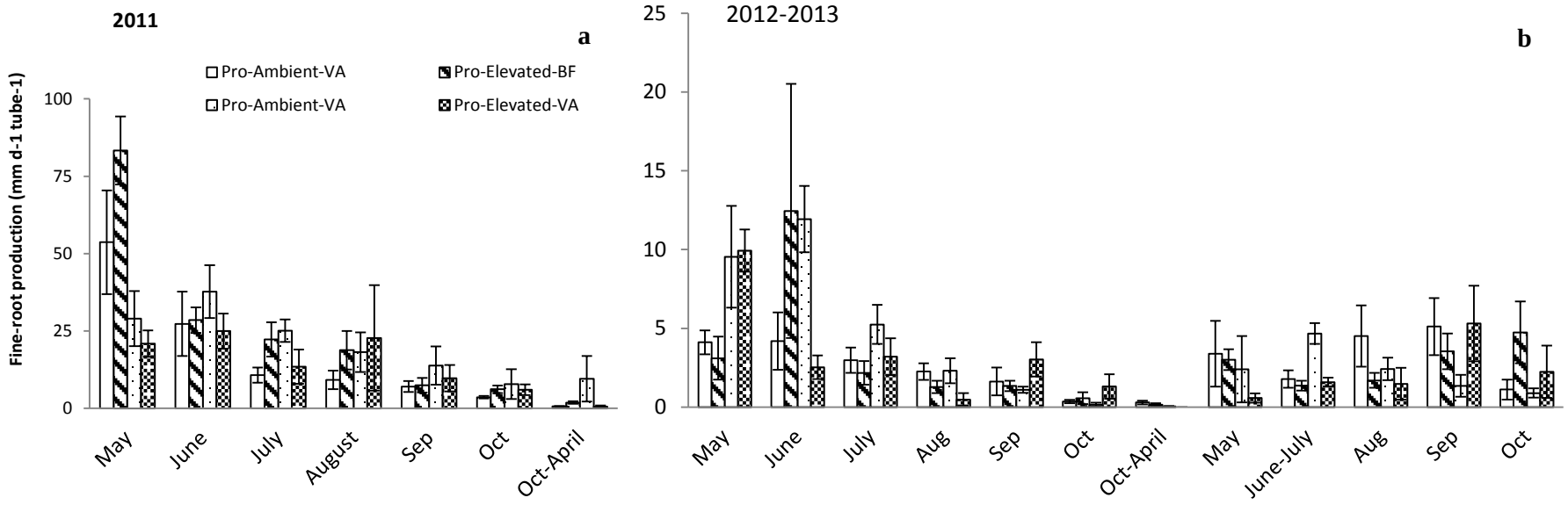

Figure 2.4 Fine root length production rate of birch seedlings growing under elevated and ambient [CO_2] on volcanic ash (VA) and brown forest (BF) soil.

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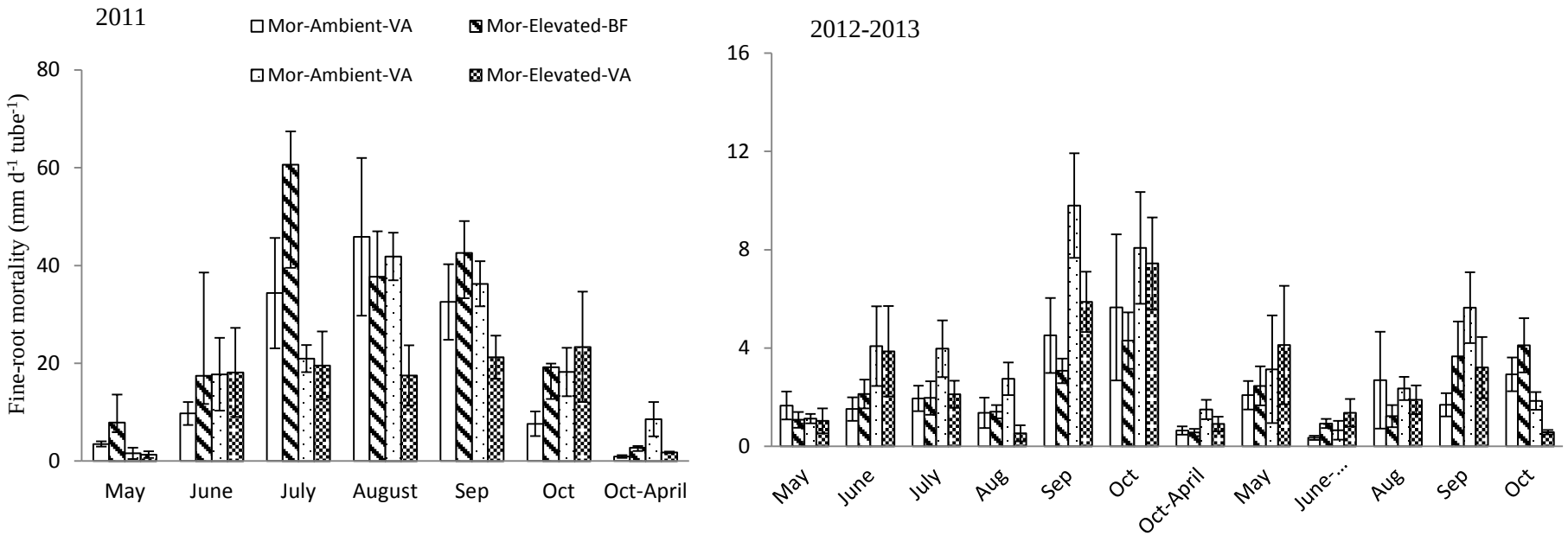


Figure 2.5 Fine root length mortality of birch seedlings growing in elevated and ambient [CO2] on infertile volcanic ash (VA) and fertile brown forest (BF) soil.

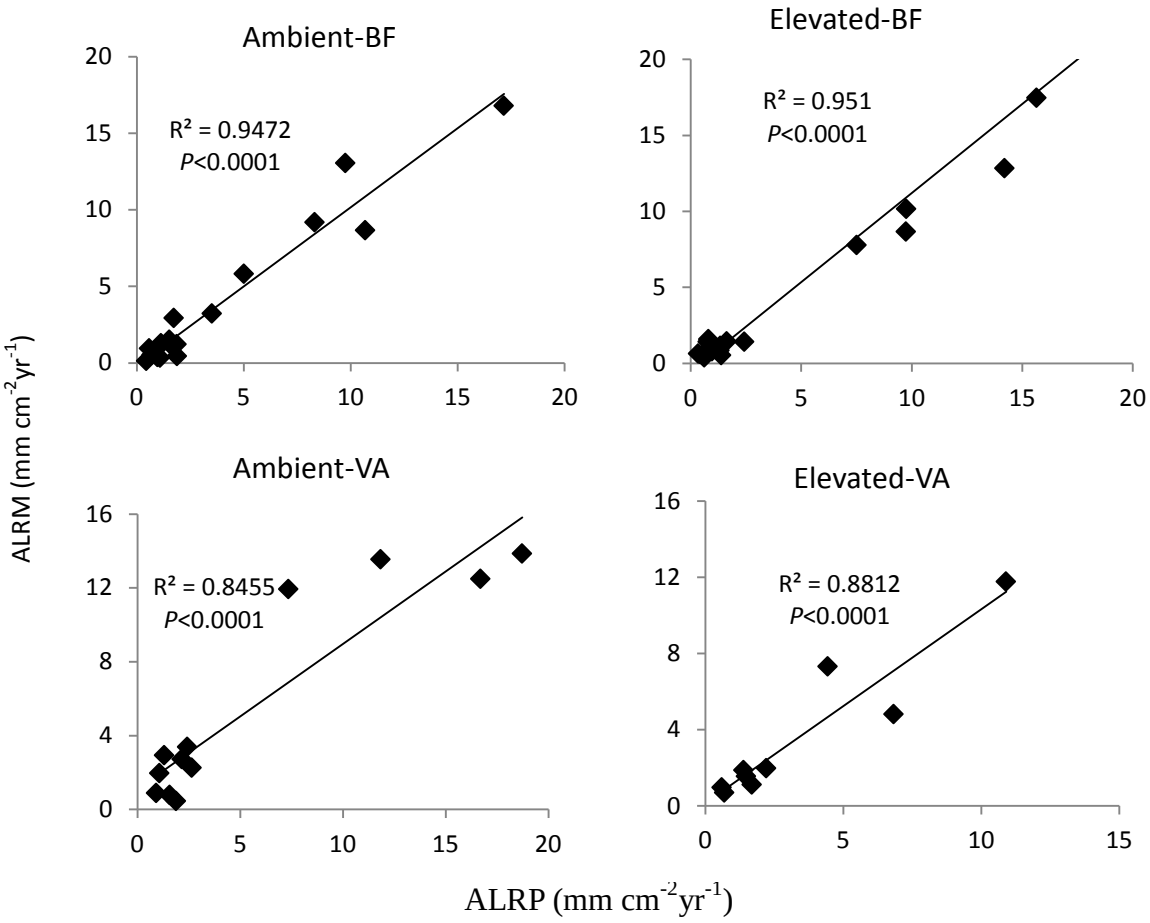


Figure 2.6 Relationship between annual length-based root production (ALRP) and annual length-based root mortality (ALRM) in each tube and treatment. Pearson correlation test showed $P < 0.0001$.

Table 2.1 Soil C and N concentration in ambient and elevated CO_2 during 2011 and 2012.

Year	Nutrient	BF soil		VA soil		*p*		
		Ambient	Elevated	Ambient	Elevated	Soil	CO_2	Soil×CO_2
2011	C (mg 100mg^{-1})	2.93 ± 0.18	3.24 ± 0.26	2.24 ± 0.16	2.24 ± 0.15	**	n.s.	n.s.
	N (mg 100mg^{-1})	0.25 ± 0.01	0.27 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	***	n.s.	n.s.
	C / N	11.69 ± 0.23	11.96 ± 0.41	12.23 ± 0.59	12.17 ± 0.35	n.s.	n.s.	n.s.
2012	C (mg 100mg^{-1})	2.78 ± 0.21	3.52 ± 0.29	1.75 ± 0.14	2.09 ± 0.15	**	n.s.	n.s.
	N (mg 100mg^{-1})	0.24 ± 0.02	0.28 ± 0.02	0.15 ± 0.01	0.17 ± 0.01	***	n.s.	n.s.
	C / N	11.50 ± 0.19	12.43 ± 0.48	11.92 ± 0.18	12.20 ± 0.28	n.s.	n.s.	•

Eatch value is the average ± SD of three replications (statistic unit is the FACE system) ANOVA: ** P<0.01,*** P<0.001, ns denotes not significant.

Table 2.2 Turnover of fine root production and mortality of each growing season (yr^{-1})

			Production		Mortality	
Year	Soil	CO_2	ALRP/ARLmax	ALRP/ARLmean	ALRP/ARLmax	ALRP/ARLmean
2011	B	Ambient	1.04(0.15) a	1.54(0.19)	1.14(0.17)	1.68(0.22)
		Elevated	0.93(0.06) a	1.35(0.12)	0.98(0.07)	1.43(0.14)
	V	Ambient	1.00(0.21)a	1.23(0.22)	1.07(0.08)	1.33(0.11)
		Elevated	0.84(0.14)a	1.06(0.12)	0.95(0.23)	1.22(0.30)
2012	B	Ambient	1.17(0.15)a	1.68(0.25)a	1.19(0.11)a	1.69(0.12)a
		Elevated	1.07(0.03)ab	1.43(0.09)ab	0.93(0.08)a	1.23(0.09)ab
	V	Ambient	0.70(0.08)b	0.96(0.12)ab	0.96(0.14)a	1.33(0.20)ab
		Elevated	0.71(0.04)b	0.92(0.06)b	0.78(0.06)a	1.01(0.12)b
2013	B	Ambient	0.64(0.09)	0.88(0.17)	0.25(0.05)a	0.33(0.06)
		Elevated	0.88(0.13)	1.04(0.14)	0.76(0.19)a	0.90(0.22)
	V	Ambient	0.71(0.06)	0.93(0.08)	0.56(0.17)a	0.74(0.23)
		Elevated	0.78(0.16)	1.00(0.25)	0.78(0.10)a	0.97(0.08)
CO_2			ns	ns	ns	ns
Soil			*	**		
Year			*	**	***	***
CO_2×Year			ns	ns	**	**
Soil×Year			**	**	ns	ns
CO_2×Soil			ns	ns	ns	ns
CO_2×Soil×Year			ns	ns	ns	ns

Each value is the average (SE) of 6 replications and statistical analysis unit is FACE site. ANOVA: * $P< 0.05$, ** $P<0.01$, *** $P<0.001$, ns

Table 2.3 Fine **r**oot lifespan of birch seedlings growing in elevated and ambient [CO_2] on volcanic ash (VA) and brown forest (BF) soil.

Year		Ambient-BF	Elevated-BF	Ambient-VA	Elevated-VA	*P*-value
2011	Median	15(0.29)c	18(0.44)b	18(0.32)b	44(2.87)a	***
	Mean	23(0.42)	26(0.35)	25(0.38)	32(0.53)	***
	N	1364	2419	1643	680	
2012	Median	18 (1.35)c	18 (2.31)b	18 (1.08)c	24 (3.74)a	**
	Mean	24 (1.12)	27(0.97)	25(0.78)	29(1.09)	**
	N	284	414	574	314	
2013	Median	21(2.92)a	18(2.44)a	12(0.72)d	15(1.62)c	***
	Mean	32(1.27)	33(1.04)	22(0.82)	25(1.33)	***
	N	672	842	886	399	

Each value is the root lifespan of six replications, statistical analysis unit is three FACE site. Significant was tested by Log Rank (Mantel-Cox): * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 2.4 Lifespan of different root diameter classes of birch seedlings growing in elevated and ambient [CO_2] on volcanic ash (VA) and brown forest (BF) soil.

Year	D (mm)	Ambient-BF	Elevated-BF	Ambient-VA	Elevated-VA	*P* value
2011	D<0.2	12(2.76)	12(0.69)	15(1.08)	15(3.93)	ns
	D 0.2-.03	15(0.29)	18(0.45)	18(0.34)	44(2.91)	***
	D 0.3-0.4	18(1.14)	29(3.61)	15(0.70)	18(1.95)	***
	D 0.4-0.5	15(0.80)	24(2.88)	15(0.43)	50(4.24)	***
	D>0.5	18(1.33)	21(3.49)	15(0.80)	18(2.88)	***
2012	D<0.2	12(3.97)	9(1.54)	12(6.00)	39(9.80)	ns
	D 0.2-.03	18(1.16)	21(4.14)	18(1.11)	24(3.51)	***
	D 0.3-0.4	15(4.06)	36(3.81)	18(4.90)	48(2.49)	***
	D 0.4-0.5	21(2.41)	42(2.60)	15(4.32)	27(10.51)	ns
	D>0.5	27(20.17)	----	42(13.35)	----	***
2013	D<0.2	9(1.49)	12(0.75)	9(1.97)	3(1.96)	**
	D 0.2-.03	30(4.86)	21(3.71)	12(0.73)	18(1.43)	**
	D 0.3-0.4	18(3.62)	48(5.55)	15(4.15)	48(7.76)	**
	D 0.4-0.5	24(10.64)	51(4.51)	24(10.22)	33(9.26)	ns
	D>0.5	27(13.98)	57(7.90)	45(5.23)	63(3.16)	ns

Each value is the root lifespan of six replications, statistical analysis unit is three FACE site. Significance was tested by Log Rank (Mantel-Cox): * $P<0.05$, ** $P<0.01$, *** $P< 0.001$, ns denotes not significant.

Chapter 3

ECTOMYCORRHIZAL COLONIZATION AND GROWTH OF HYBRID LARCH F_1 UNDER ELEVATED CO_2 AND O_3

3.1 INTRODUCTION

Concentrations of atmospheric CO_2 and tropospheric or ground surface ozone (O_3) have been increasing sharply since the Industrial Revolution. Both are predicted to continue rising during the next decades because of the over-burning of fossil fuels and deforestation (e.g., Cubasch et al. 2001; Koike et al. 2013a; Tans 2008). These changes in atmospheric air may alter the aboveground and belowground growth and development of trees, and therefore impact the CO_2 sink of forest ecosystems (e.g., Larcher 2003; Qu et al. 2010).

The majority of belowground root systems in boreal forests are symbiotically colonized by ectomycorrhizal fungi (ECMF) (Taylor et al. 2000). In fact, larch (*Larix* sp.) is a typical ectomycorrhizal (ECM) species (Smith and Read 1997; Qu et al. 2004) that is widely planted as a dominant tree species for afforestation in the northeastern part of Eurasia (Koike et al. 2000; Qu et al. 2010) and parts of Europe (Matyssek and Schulze 1987). Recently, a new hybrid larch F_1 (*L. gmelinii* var. *japonica* × *L. kaempferi*; hereafter F_1) was developed as a promising species. This hybrid has much better tolerance to cold climates, damage by red-back voles (*Clethrionomys rutilus*) grazing, shoot blight disease (*Physalospora laricina*), and strong winds (Ryu et al. 2009). It is already known that the growth of F_1 is closely related to ubiquitous ECM association (Qu et al. 2004). However, studies of the relationship between F_1 and ECM symbiosis remain limited (Qu et al. 2003 ; 2010). Up to 30% of total photo-assimilation products can be used in the growth and maintenance of ECM (Hampp and Nehls 2001). In turn, ECM usually acts as an efficient patronage to the root system of the host by absorbing water and essential nutrients such as phosphorous (P) and sometimes nitrogen (N) (e.g., Cairney 2011; Quoreshi et al.

2003).

The net primary production and the growth of trees are enhanced by elevated CO_2, with a simultaneous increase in the carbon allocation to the belowground parts (Choi et al. 2005; McElrone et al. 2005; Nowak et al. 2004; Qu et al. 2004). For instance, Choi et al. (2005) reported that symbiosis with ECMF increased the growth of Japanese red pine (*Pinus densiflora*) seedlings under elevated CO_2, because the photosynthetic activities of host plants were enhanced by an increase in root surface via widely ramified ECM hyphae. They also found an improvement in water use efficiency (WUE), and suggested that the colonization of pines with ECM leads to the allocation of more photosynthates to roots under elevated CO_2 conditions. In larch species, colonization with ECM increased the growth of Japanese larch (*L. kaempferi*) and its hybrid F_1 by a factor of 1.5–2.0 relative to uninfected larches in nutrient-poor soil in northern Japan and east Russia (Qu et al. 2004; 2010). Moreover, Buscot et al. (2000) emphasized that a greater species richness of ECM communities assists the cycling of P from heterogeneous sources in forest soil ecosystems. Host spruce, larch, and pine trees grow more rapidly when they are infected by multiple ECM species rather than by a single species (e.g., Choi 2008; Qu et al. 2004; Qu et al. 2003). Therefore, greater ECM species richness may increase the effectiveness of nutrient acquisition from different locations and/or soil substrates (Jonsson et al. 2001; Leake 2001).

In contrast, O_3 usually has negative effects on tree growth. For instance, it impairs the physiological and biochemical processes in leaves and accelerates leaf senescence (Agathokleous et al. 2015; Matyssek and Sandermann 2003; Watanabe et al. 2010a; Zhang et al. 2002). These negative effects lead to belowground responses (Agathokleous et al. 2015; Blum and Tingey 1977). As a result of these negative

effects on aboveground, carbon assimilation is reduced by O_3, limiting the allocation to belowground (Grantz and Farrar 2000; King et al. 2005) and reducing standing fine-root mass (Kasurinen et al. 2005). These effects are expected to decrease ECM colonization and to affect species-host compatibility.

A study of silver birch (*Betula pendula*) in Open-Top-Chambers (OTCs) revealed that double ambient O_3 clearly decreased the proportion of black and liver-brown mycorrhizae after three growing seasons (Kasurinen et al. 2005). Haberer et al. (2007) measured N uptake and symbioses of ECM in adult beech trees (*Fagus sylvatica*) using radioactive isotopes (delta ^{15}N). They found that the number of fine roots, which were all mycorrhizal, increased markedly with long-term O_3 fumigation. Additional research on a 70-year-old mixed spruce-beech forest stand revealed that after two-year of O_3 fumigation, the number of vital ECM root tips increased, and the ECMF community changed significantly (Grebenc and Kraigher 2007). However, this result was not always found, and Zeleznik et al. (2007) reported ambiguous results for two-year-old beech seedlings exposed to elevated O_3 for two years. In this specific case, the mycorrhization of seedlings was very low, and the ECM types were lower in O_3 fumigated plants than in control plants. Predominantly, the number of vital mycorrhizal root tips decreased with O_3 treatment (Zeleznik et al. 2007).

Previous researchers found that ECM colonization under O_3 might decrease (Adams and Oneill, 1991; Edwards and Kelly, 1992) or increase (Gorissen et al. 1991; Kasurinen et al. 1999; Wollmer and Kottke 1990). Therefore, the effects of O_3 on ECM symbiosis are inconsistent, and the outcome depends on the age of the material plants and the length of fumigation.

External stressors, such as drought and high O_3, may regulate stomatal conductance. Water-soluble elements potentially are selectively absorbed by ECM as

a defense against the harmful effects of these stressors (Jourand et al. 2014). It has also been reported that aquaporin expression is enhanced in ECM seedlings (Marjanovic et al. 2005a), and this enhancement could be particularly important in conditions of water stress (Marjanovic et al. 2005b). Hence, as a defense to O_3 stress, element absorption and uptake ability might be adjusted. For example, Haberer et al. (2007) found that they were reduced in the fine-roots of European beech under enhanced O_3.

Despite the extent of ECM colonization, elevated CO_2 and/or O_3 alter the ECM community composition by affecting specific ECM species. A study of silver birch in OTCs showed that elevated CO_2 impaired light brown/orange mycorrhizae (Kasurinen et al. 2005). In a long-term exposure experiment with elevated CO_2 and/or O_3 concentrations, elevated CO_2 alone induced an increase in the proportion of *Sistotrema* spp. in the ECM community colonizing aspen/birch trees (Edwards and Zak 2011). The responses of ECM to combinations of elevated CO_2 and O_3 may not be a simple sum of the effects of each. According to Volin et al. (1998), the belowground responses to O_3 were variable, and elevated CO_2 typically mitigated the negative effects of O_3 (e.g., Karnosky et al. 2003c; Watanabe et al. 2010b). For instance, the total level of mycorrhizal colonization of silver birch clones was stimulated by enhanced CO_2 and elevated O_3 separately, but not when combined (Kasurinen et al. 2005).

The effects of combined elevated CO_2 and O_3 on ECM species richness are not known in detail (Grebenc and Kraigher 2007; Matyssek et al. 2012b). I expected the species richness of ECM to depend on the photosynthetic activity of the host plants. Photosynthesis in F_1 seedlings increased under elevated CO_2 for a short period, but was reduced under elevated O_3 (Koike et al. 2012). Therefore, I anticipate that

colonization and species richness of F_1 ECM should increase in elevated CO_2 and decrease in elevated O_3.

The present work seeks to answer the following questions regarding hybrid larch F_1 afforestation in the coming decades: (1) which ECM species colonize F_1 under elevated CO_2 and/or O_3, (2) how do the gas treatments influence the ECM species community structure, and (3) what are the effects of these gases on the growth of the F_1 plant?

3.2 MATERIALS AND METHODS

3.2.1 Experimental site and plant materials

This study was conducted at Sapporo Experimental Forest of Hokkaido University, in northern Japan (43°07′ N, 141°38′ E, 15 m a.s.l., annual mean temperature and precipitation in 2011 were 13.5°C and 1254 mm). Two-year-old seedlings of the hybrid larch F_1 (*L. gmelinii* var. *japonica* × *L. kaempferi*) were provided by the Hokkaido Research Organization, Forestry Research Institute, near Sapporo. The height and diameter of each seedling was determined before planting, and the initial mean height and mean diameter of the seedlings were 38.6 ± 0.3 cm and 5.2 ± 0.2 mm, respectively.

The ECM colonization was also determined before planting (see Table 3.2). The soil at the study site was well-homogenized brown forest soil with no previous planting of tree species. All seedlings were planted in May 2011, and they were periodically irrigated with tap water to prevent desiccation. Gas treatments began one month later when all seedlings were established in the site, and the seedlings were dug

out after two growing seasons (in late October 2012).

3.2.2 CO_2 and O_3 treatment

I set up the OTC system at the experimental forest site of Hokkaido University. The 16 chambers (volume, W × W × H = 1.2 × 1.2 × 1.5 m; H = 2.2 m after Sep. 2012) were made of steel frames with polyvinyl chloride film (Noh-bi, Sapporo, Japan) that had a transmittance of 88% of full sunlight (simply cutting UV-B). I set up four gas treatment regimes: 1) control (CO_2 = about 380 μmol/mol; O_3 < 6 nmol/mol), 2) elevated O_3 (60 nmol/mol: 7 hours, 10:00–17:00), 3) elevated CO_2 (600 μmol/mol during daytime), and 4) their combination (elevated O_3 + CO_2). Charcoal-filtered ambient air was introduced and tanked CO_2 was supplied either as ambient or elevated CO_2 treatments. The CO_2 concentration in chambers was regulated by a control unit (GMM222, Vaisala, Helsinki, Finland). Regarding the O_3 fumigation, O_3 was generated from ambient air using an electrical-discharge O_3 generator (PZ-1B, Kofloc-Kojima, Kyoto, Japan).

In the growth chambers, O_3 was continuously monitored with an ultraviolet (UV) absorption O_3 analyzer (EG-3000F, Ebara, Tokyo, Japan and model 202-EPA, 2Btechnologies, Boulder, CO, U.S.A.). The O_3 concentration was lower than 6 nmol/mol in the untreated O_3 chambers. There were four replications of each treatment, and four larch seedlings were planted in each chamber (64 seedlings in total). A proportional-integrative-differential (PID) control algorithm was applied to maintain the desired concentration of O_3. The monthly data are shown in Table 3.1.

3.2.3 ECM identification

After the two-year fumigation, I analyzed ECM on the roots of F_1. The roots extended widely after two growing seasons, trying to get accurate biomass, I removed the chambers and dug out the roots manually by shovel. After removal from the soil, all roots were covered with wet paper tissue, stored in a plastic bag, and immediately transferred to the laboratory where they were refrigerated at 4°C. Within a maximum of two days, the harvested roots were first washed until no large clods remained, and the fine roots were then gently cleaned using a small paintbrush. A microscope (Olympus szx-ILLK100, Japan) was used to observe the extent of ECM colonization (as was conducted before planting the seedlings). In total, 500 root tips were randomly counted for each replicate following the method of Shinano et al. (2007), and Fig.3.1 shows the sampling process. A classification of ECM morphological types was estimated, and final ECM identification was carried out using molecular analyses (Table 3.2).

I first extracted ribosomal DNA (rDNA) from the root tips using a DNeasy™ Plant Mini Kit (Qiagen). Then I conducted PCR amplification using primer 1F/4 to determine the sequences of the ITS-region (Bellemain et al. 2010; Gardes and Bruns 1993). Sequencing reactions were performed using the BigDye Terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, USA). Finally, ECM sequences were compared with the GenBank database at the DNA Data Bank of Japan using the basic local alignment search tool (BLAST) program as follows: (http://blast.ddbj.nig.ac.jp/blast/blastn? lang=en).

The colonization rate of ECM (*CRE*) was calculated using the following formula:

$$CREi = ER/(ER + NR) \times 100\ (\%),$$

where ER and NR denote the number of ECM and non-ECM root tips, and *i* is an

ECM type (Choi et al. 2005; Shinano et al. 2007).

The ECM diversity (*H'*) was calculated as Shannon's diversity index (Keylock, 2005) according to the following formula:

$$H' = -\sum_{i=1}^{S} \mathrm{P}i\log\mathrm{P}i,$$

where *S* denotes the total number of ECM types, and *Pi* is the proportion of the *i*th ECM type (Pielou 1966).

3.2.4 Measurement of seedling growth and nutrient concentration

The diameter and height of the seedlings were measured in July and November 2011 and in September 2012. All seedlings were harvested on October 20, 2012 to estimate the dry mass of plant organs. The seedlings were then separated into needles, branches, stems, and roots. The plant organs were dried in an oven at 80°C for one week and then weighed. The needle and root samples were crushed into powder by mills and digested with HNO_3, HCl, and H_2O_2, after which an inductively coupled plasma-atomic emission spectrometer (ICP-AES, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) was used to determine the concentration of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), aluminum (Al), iron (Fe), manganese (Mn), and molybdenum (Mo). The N concentration was determined by the combustion method using an NC analyzer (NC-900, Sumica-Shimadzu, Kyoto, Japan).

3.2.5 Measurement of the leaf gas exchange rate

The leaf gas exchange rates of seedlings were measured on September 21–25, 2011 and September 11–15, 2012 using an infrared gas analyzer system (LI-6400, Li-Cor Inc., Lincoln, NE, U.S.A.). Two seedlings in each chamber were randomly selected for measurement of their leaf gas exchange rates (eight measurements per treatment). The measurements were conducted on the same seedlings throughout the experiment. The net photosynthetic rate (A) and stomatal diffusive conductance to H_2O (G_s) were determined at 24 ± 0.1°C leaf temperature, 380 μmol/mol CO_2, 60 ± 5% relative air humidity, and a photosynthetically active photon flux (PPF) of 1500 μmol $m^{-2}s^{-1}$ per the method described by Watanabe et al. (2012). Finally, the A and the stomatal conductance values at the growth concentration [CO_2] (denoted A_{growth} and G_s) were determined.

3.2.6 Statistical analysis

Statistical analyses were undertaken using *R* and SPSS (version 16.0) software. All data were distributed normally, as verified by the Kolmogorov-Smirnov Test. The statistical unit was single OTC, two-way analysis of variance (ANOVA) was used to test the independent effects of elevated CO_2 and O_3 as well as their interaction. Tukey's HSD test was applied to identify significant differences among the four treatments. Distance based redundancy analyses (db-RDA) were performed to determine the species abundance of ECM community variation according to the gas treatment regimes.

3.3 RESTULTS

3.3.1 ECM types colonizing F_1

I found six types of ECM colonizing F_1 after CO_2 and/or O_3 treatments compared with the three types identified before the treatments. *Suillus grevillei* was the only one species existed before and after the treatments. According to mycorrhizae taxonomy, all eight ECM types belong to either the classes Basidiomycetes (Type A, C, D, F, G and H) or Ascomycetes (Type B, E). Typical morphology of each ECM species was attached as appendix. The detailed morphological description for each type of ECM and their similarly matched sequence are all listed in Table 3.2.

3.3.2 Extent of colonization and diversity of ECM

The ECM colonization rate was significantly increased by elevated CO_2, whereas it was sharply reduced by O_3 compared to the control (Fig. 3.2). However, there was no interactive effect of elevated CO_2 and O_3 on the ECM colonization rate, and the ECM colonies varied in diversity among the four treatments (Fig. 3.2). ECM diversity reduced significantly by O_3 exposure, and it also decreased under elevated $CO_2 + O_3$ treatment relative to the control. The diversity between control and elevated CO_2 did not differ significantly ($P = 0.49$).

3.3.3 Abundance of ECM by species

The six ECM types were found in different amounts under the four gas treatments. According to the integrated estimation of ECM colonization and species diversity,

types A, C, D, and F were the major ECM colonizers of F_1 (Fig. 3.3b). The ECM abundance under elevated O_3 and the mixed fumigation differed significantly from control and elevated CO_2 treatments (63.7 % of the variance was explained along the axis-1 direction, $P \leq 0.01$). Based on the distance of the four ellipses in Fig. 3.3a, the ECM abundance was similar to that of the control at elevated CO_2.

The changes of ECM community were visible under different treatments. When individual types of ECM were compared, the proportion of type D increased and the proportion of type C decreased in elevated CO_2 as compared to the control (Fig. 3.4a, b). Under O_3 exposure, type B did not colonize F_1, type D colonized at a significantly higher degree, and type A colonized at a lower degree in comparison with the control (Fig. 3.4c). Under mixed fumigation with elevated CO_2 and O_3, the proportion of type C increased relative to the control, and it became the dominant species (Fig. 3.4d).

3.3.4 Growth of seedlings and element concentrations

Ozone markedly reduced seedling growth after one growing season (Table 3.3). The height and stem diameter of seedlings were not significantly changed by elevated CO_2 compared with the control in 2011 and 2012, but they were significantly reduced by O_3 at the end of the 2011 growing season. Elevated $CO_2 + O_3$ did not have any effects on the growth of height and stem diameter during the two-year treatments, and both the diameter and height were unaffected by treatments in 2012. Elevated CO_2 increased the biomass of root, stem, and total aboveground biomass, and O_3 reduced the stem and root biomass. The elevated $CO_2 + O_3$ did not differ from the control, and there was no interaction between elevated CO_2 and O_3 for all biomass parameters

(Table 3.4). The root/shoot (needle + branch + stem) ratio (R/S) was also unaffected under all gas treatments.

No clear differences were found among gas regimes for the concentrations of N, K, Ca, and Mg in needles (Table 3.5). The concentrations of P, Al, Fe, and Mo were significantly reduced by O_3, and those of P and Mn were clearly increased by elevated CO_2. There was an interactive effect of elevated CO_2 and O_3 in the P concentration. Under the fumigation of elevated CO_2 + O_3, the P concentration in needles greatly increased compared to the control. In roots, K and Mg were increased by O_3, and there was no significant effect of the four treatments on other elements.

3.3.5 Gas exchange rate

In the elevated CO_2 treatment, A_{growth} was significantly enhanced in 2011 and 2012 (Fig. 3.5). The values of A_{growth} at elevated O_3 and control did not differ significantly, while they were increased at elevated CO_2 and CO_2 + O_3 fumigation in both 2011 and 2012. No significant influence on G_s was exerted by any individual gas treatment. However, under elevated CO_2 + O_3 treatment, the G_s value was similar to the control value, but with a tendency to be lower in 2011 ($P < 0.1$). Ozone tended to increase G_s in the second year (Fig. 3.5).

3.4 DISCUSSION

Overall, the composition of the ECM community changed greatly after the treatments. *Inocybe lacera* (G) and *Thelephoraceae* spp. (H) colonized F_1 before the treatments,

but *Suillus laricinus* (C) and other ECM species replaced them during the two-year fumigated growth period. Despite the efficiency of the symbionts, the shift of ECM species in all treatments may partly be attributed to ECM succession. Nara et al. (2003b, 2006a) found a common succession sequence of symbiotic ECMs, and for larch, *Suillus* spp. appeared in late succession on well-weathered lava flows. The same pattern was observed in Japanese larch in a mature forest (Yamakawa 2012).

In general, carbon allocation to belowground parts increased with elevated CO_2, as reported in Japanese larch (Choi et al. 2005) and other tree species (Jackson et al. 2009; Nowak et al. 2004). Carbon allocation to belowground parts stimulates symbiosis involving ECM (Lukac et al. 2003), thus a significantly increased total ECM colonization of F_1 was observed under elevated CO_2. However, the diversity did not follow the same colonization rate pattern under elevated CO_2, and this demonstrates that the ECM composition did not change with an increased total colonization rate.

On the contrary, the vital support of host photosynthates for ECM survival was reduced under O_3 due to limited carbon allocation to belowground parts (e.g., Grantz and Farrar 2000), and this resulted in a colonization rate decrease. Lower ECM diversity was also found under O_3, which indicated a shift (or even a reduction) of ECM abundance, I discussed this point below.

The proportion of *Suillus grevillei* (D) increased sharply, and was slightly greater than that of *S. laricinus* (C) in the elevated O_3 regime as compared to the control. This likely occurred because *S. grevillei* colonizes larch seedlings faster than *S. laricinus*, and larch seedlings have higher shoot biomass with *S. grevillei* colonization than with *S. laricinus* (Qu et al. 2003b). Even with reduced ECM colonization, the proportion of most represented species such as *Suillus* spp. increased under elevated O_3 within the

ECM community.

As previously mentioned, lower ECM diversity caused by O_3 indicated a shift in the ECM abundance. Particularly, temporary partners could not have a symbiotic relationship with F_1, such as the species of *Peziza* spp. (B) in my case. Moreover, *Laccaria* cf. *laccata* (F) and *Tomentella* spp. (A) individuals were present in lower proportions under elevated O_3, which suggests that the assistance and function of these species to the host F_1 was weak, or that their symbiotic activity was lower than other ECM types (*Suillus* spp.). A study of long-term exposure of aspen-birch to elevated CO_2 and O_3 supports my results (Edwards and Zak 2011).

The study revealed that *Laccaria* spp. and *Tomentella* spp. declined along with decreased cello-bio-hydrolase activity in an elevated O_3 regime. Both the ECM colonization rate and diversity were reduced by elevated $CO_2 + O_3$ compared to the control, but ECM diversity was significantly higher than under the single O_3 treatment. Specifically, *Suillus* spp. colonized the same proportion of the roots as in the control. Therefore, I conclude that under the combined treatment, elevated CO_2 counteracted the reduction of diversity induced by O_3.

The F_1 growth (stem diameter and height) and ECM abundance did not accelerate significantly under elevated CO_2. However, the increased stem and root biomass proved that F_1 benefited from elevated CO_2, and the same result was also observed in seedlings of Japanese larch (Yazaki et al. 2004). Ozone decreased the stem diameter and height at the end of the first growing season, which is similar to the result of Noormets et al. (2001a) in aspen. For instance, they found that the growth of two aspen clones was reduced by O_3 (especially the stem diameter). Moreover, the inhibition of stem diameter by O_3 was also found in potted F_1 plants (Koike et al. 2012).

As symbiotic partners, ECMs surely enhance the capability for nutrient uptake of the host plant (Buscot et al. 2000). Katanic et al. (2014) found that shift of ECM community into types aided the distant nutrient acquisition when plants were treated by ethylenediurea (EDU) under O_3 treatment. In the present study, O_3 did not inhibit the growth of F_1 during the second growing season. The shift of ECM community mentioned previously led to higher nutrient uptake, which resulted in avoidance of the O_3 harmful effects on F_1 growth. Therefore, I examined the element composition of aboveground and belowground parts of F_1 plants. With higher ECM colonization rate under elevated CO_2, I found an increased concentration of P and Mn in needles. This increase might be derived from an efficient uptake due to higher ECM colonization. Phosphorus is an essential macro-element for ATP and NADPH, which are related to light reactions in photosynthesis (e.g., Reich et al. 2009). Manganese is also important for photosynthesis as a co-factor for photosynthetic oxygen evolution (Henriques 2003; Raven 1990). Higher ECM colonization stimulated the uptake of P and Mn, and thus enhanced carbon assimilation and the growth of F_1 (Cairney 2011).

The concentrations of K and Mg in F_1 roots were also increased by O_3. This change may help maintain a stable concentration of Fe in roots, since K is vital for maintaining the root iron balance (e.g., Kraemer 2004). In addition, Fe is also essential for ECM formation (e.g., Van Hees et al. 2006). As a result, I postulate that F_1 selected the most beneficial ECM partners under elevated O_3, thus the structure of ECM community changed. This is one reason why the structure of the ECM community was altered with *Peziza* spp. absence under elevated O_3 compared with control and elevated CO_2. Although the reduced concentrations of Fe and Mo by O_3 in needles may inhibit the growth of F_1 seedlings (Raven et al. 1990), macro-element concentrations of N and K did not change significantly.

Furthermore, concentrations of Ca and Mg were unaffected in the needles under elevated O_3. These two elements are important in that Ca is usually correlated with the activity of various enzymes that regulate photosynthesis, and Mg is essential for chlorophyll function (e.g., Liang et al. 2009). Therefore, this might be one reason supporting my hypothesis (slight inhibition of photosynthesis). On the other hand, under external stress, the internal allocation of nutrients is liable to be altered by various ECM species (Weigt et al. 2011). The stable concentrations of Mg and Ca in needles may be due to positive effects of ECM and the changes in species abundance, which may have indirectly led to the weak inhibition of photosynthesis by O_3 treatment. Because colonizing mycorrhizal species are regulated by their host plants according to the efficiency of symbionts, especially when carbon allocation to shoots or fine roots changes under external stress, lower carbon-requiring and effective species are selected (Hoeksema and Kummel 2003).

Further, *Suillus* spp. were reported to reduce the transfer of large quantities of metals towards the plant-fungus interface without hampering normal nutrient uptake to the host plant (Colpaert et al. 2011). In my case, the O_3-reduced concentration of Al in needles was potentially caused by the shift in ECM abundance to protect F_1 from metal toxicity. This might be beneficial to the maintenance of photosynthetic activity under elevated O_3.

The net photosynthetic rate (A_{growth}) at elevated CO_2 and elevated $CO_2 + O_3$ was markedly higher than in the control. Usually, plant growth and root systems increase under elevated CO_2, and this is accompanied by higher efficiency in plant water and nitrogen use (e.g., Koike et al. 2010; Norby and Zak, 2011; Qu et al. 2004). As a result, the stem and root biomasses were increased by elevated CO_2. I did not find any significant effects on the growth and biomass of F_1 with elevated $CO_2 + O_3$, or any

interactive effects of these two factors. The A_{growth} of seedlings in elevated CO_2 + O_3 was higher than that of seedlings in elevated O_3 alone, indicating the positive effects of elevated CO_2. The potential impact of O_3 on the growth of F_1 seedlings may be ameliorated by elevated CO_2 associated with abundant P uptake via ECM, which has been reported for beech in central Europe (Matyssek and Sandermann, 2003; Weigt et al. 2012).

Moreover, this might explain why the structure of ECM abundance under elevated CO_2 + O_3 was similar to that of the control. The G_s was not influenced by any treatment, but it was increased by O_3 in the second growing season. As it has been reported, the decrease in the hydraulic conductance of plant stems (Saliendra et al. 1995; Sperry and Pockman 1993), roots (Kavanagh et al. 1999; Meinzer and Grantz 1990), and even leaves (Salleo et al. 2000) may lead to stomatal closure. However, the water uptake of host plant was efficient with the ECM assistance, as it is supported by higher stomatal conductance occurred in the high mycorrhization (Auge et al. 2004; Ebel et al. 1997).

In the present results, although higher G_s increased the risk of high O_3 damage, the growth of F_1 was found to be unaffected. Moreover, A_{growth} was not reduced by O_3 in the second year when compared with the control. Thus, the increased stomatal conductance might be a driving factor for nutrient uptake through specific ECM species under O_3 stress. This trail requires further research.

3.5 SUMMARY

Elevated CO_2 increased the ECM colonization rate but not ECM type diversity. The

higher A_{growth} of F_1 under elevated CO_2 stimulated the biomass of belowground and stem, which increased the ECM colonization rate. Elevated O_3 negatively affected the ECM colonization rate and species abundance, with a great shift in the latter. The growth of F_1 was restricted, and the biomass was reduced by O_3. The altered concentrations of individual elements in needles and roots interactively affected the shift of ECM abundance under elevated O_3. This might contribute to the defense capability of F_1 seedlings against O_3 by selecting those ECM species capable of flourishing under enhanced O_3. However, this point needs further study with specific ECM species. Elevated CO_2 + O_3 reduced the colonization rate and diversity but increased A_{growth} with parallel unaffected growth and biomass, which suggests that elevated CO_2 diminished the influence of O_3 on photosynthetic ability. Therefore, it seems that a symbiotic partnership between host F_1 seedlings and ECM specialists such as *Suillus* spp. may be essential for the survival of F_1 seedlings. The present results provide information on ECM symbiosis with F_1 seedlings under elevated CO_2 and/or O_3 conditions, which are useful for further field inoculations of F_1 with optimal ECM species to enhance survival under changing environments.

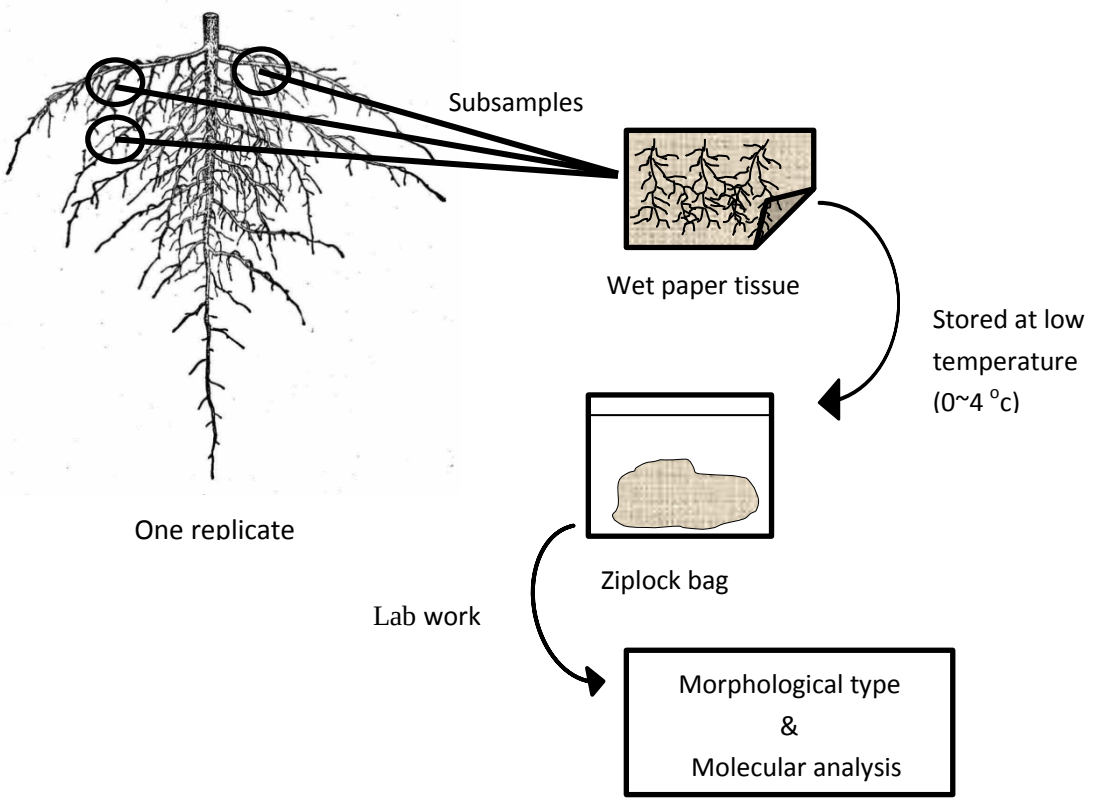


Figure 3.1 The sampling process for an individual seedling replicate. Three subsamples were randomly selected.

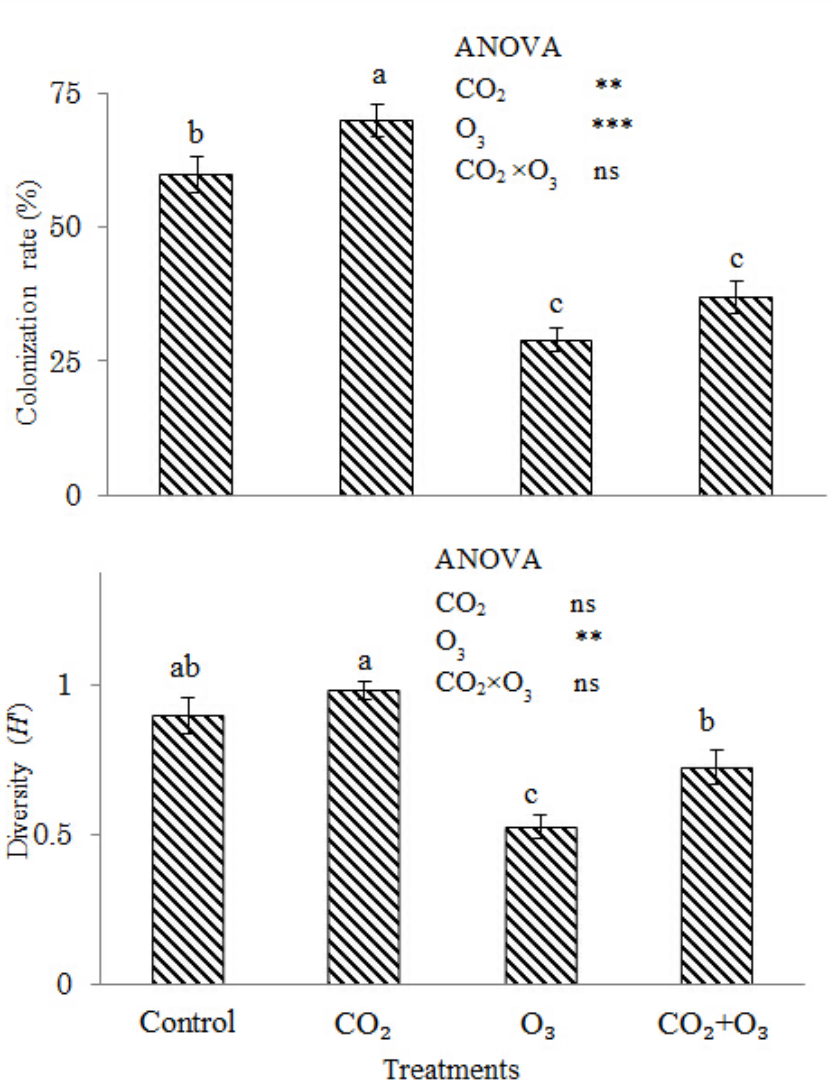


Figure 3.2 Ectomycorrhiza (ECM) colonization rate and diversity of hybrid larch F_1 under different treatments at the end of the experimental period. The ECM diversity (*H'*) was calculated as Shannon's diversity index, and each value is the average of four chamber replications. A vertical bar indicates the standard error. Different character symbols denote the degree of significance between the four treatments ($P < 0.05$). ANOVA: **$P < 0.01$, *** $P < 0.001$, "ns" means not significant.

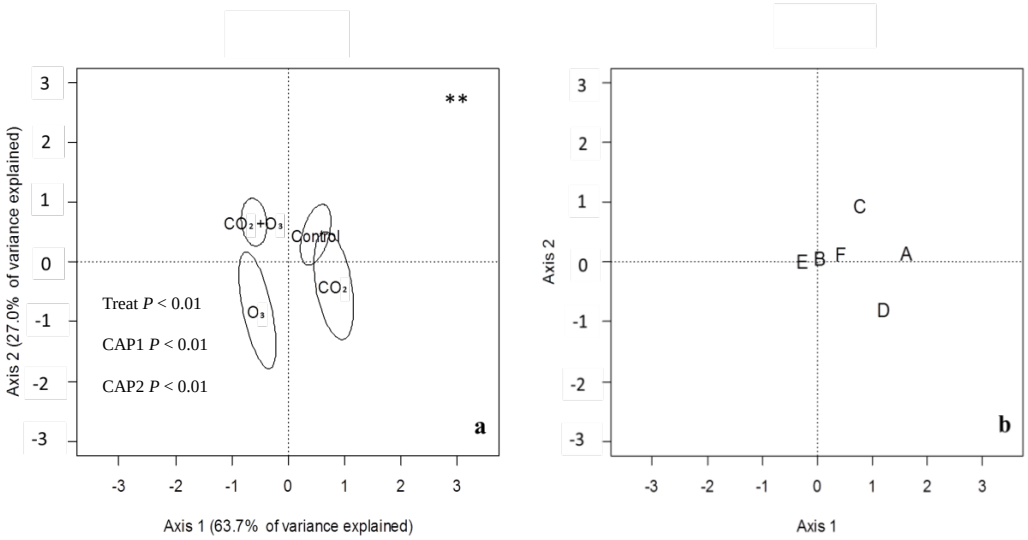


Figure 3.3 Abundance of colonized ECM species in response to four fumigation treatments at the end of the experimental period. The colonization rate, together with diversity of different ECM species, was estimated using a distance-based redundancy analysis (db-RDA) among the four gas treatments. The db-RDA depends on the matrix created using ECM species and the four treatments, and it calculates the eigenvalues and their contribution to the squared Bray distance. The results of the ANOVA use permutation tests for capscale under a reduced model (CAP1, CAP2, and CAP3 are shown). Independent variables are identified as factors (Treat.) CAP1 and CAP2 refer to axis 1 and axis 2 information, respectively, and they represent the proportion explained by the difference between each treatment. From the R results, we determined that the directions of both axis 1 (P = 0.005) and axis 2 (P = 0.005) were significantly different. This result indicates that the abundance of colonized ECM species from elevated O_3 and mixed conditions was largely different from that of control and elevated CO_2 conditions (axis 1 direction). Between elevated O_3 and mixed conditions, the abundance of ECM species was also markedly changed (explained by axis 2). An asterisk refers to significant codes: $^{**}P < 0.01$.

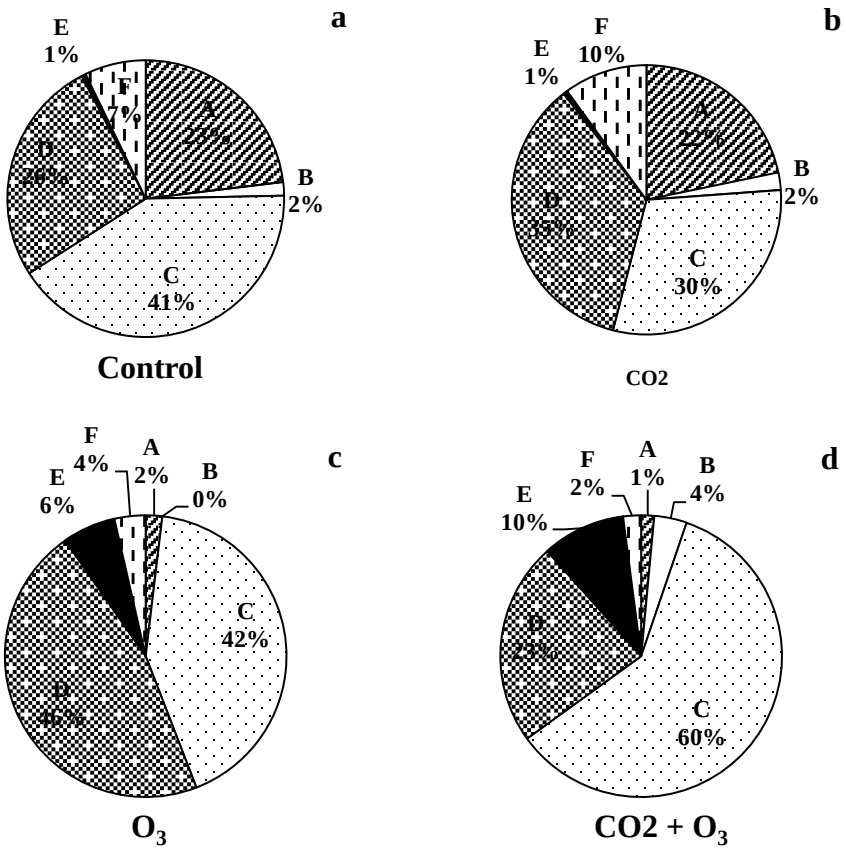


Figure 3.4 Changes in the abundance of the ECM community of the four treatments. Each value is the proportion of each type of ECM identified with the hybrid larch F_1 under the four types of fumigation (a, b, c, d). a: *Tomentella* sp. B: *Peziza* sp. C: *Suillus laricinus* D: *S. grevillei* E: *Cadophora finlandia* F: *Laccaria cf. laccata*

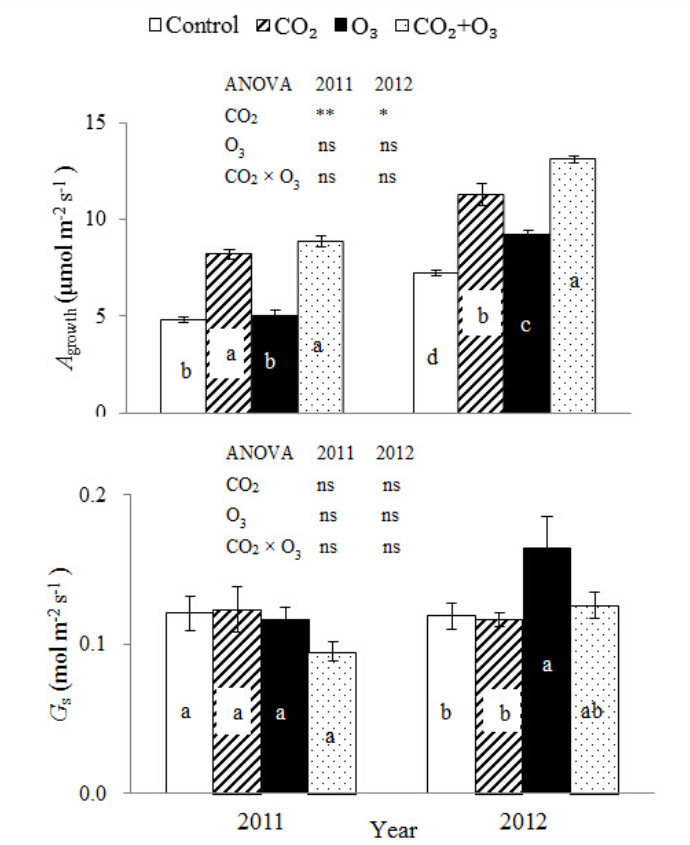


Figure 3.5 Net photosynthetic rate of growth under [CO_2] concentrations (A_{growth}) and stomatal conductance (G_s). Each value is the mean of four chamber replications, and the vertical bar indicates the standard error. Different character symbol denote significant differences between the four treatments ($P < 0.05$). ANOVA: *$P < 0.05$, **$P < 0.01$, "ns" means not significant.

Table 3.1 Daily concentration (average/peak) of CO_2 (ppm) and O_3 (ppb) for each treatment.

Treatment	2011.7	2011.8	2011.9	2012.5	2012.6	2012.7	2012.8	2012.9
Control	383.3/418.9	375.1/417.8	392.7/419.7	398.8/419.1	390.3/419.9	386.4/419.7	390.9/419.6	385.5/416.7
CO_2	573.7/582.1	570.0/581.7	571.1/581.6	639.5/660.7	624.5/663.2	610.4/622.6	609.4/620.7	603.1/621.7
O_3	—	—	—	—	—	—	—	—
$CO_2 + O_3$	594.0/608.4	592.5/608.7	593.7/606.7	624.2/643.9	615.8/647.4	604.8/619.2	603.8/619.7	595.4/615.7
Control	9.5/19.7	9.0/18.9	8.9/47.3	27.2/38.5	13.0/27.3	9.6/20.1	9.1/18.9	8.1/20.9
CO_2	—	—	—	—	—	—	—	—
O_3	52.3/114.2	57.3/125.6	51.0/ 97.0	80.7/104.2	67.7/115.8	55.3/88.6	27.3/84.8	39.7/76.0
$CO_2 + O_3$	48.9/123.5	53.1/158.5	103.4/163.8	61.3/74.8	62.6/102.6	55.2/78.7	45.3/93.2	45.7/62.9

Each value is the average of four chamber replications.

Table 3.2 Morphotype and genetic identification of ECM species colonized with hybrid larch F_1 seedlings before and after treatment.

Treatment	ECM ID	Color	Ramification[a]	Tip shape[b]	Mantle-surface[c]	Emanating hyphae	Accession number	Query cover	ECM Species
After	A	Brown	1	b	10	-	AB971275	99%	*Tomentella* sp.
	B	Brown-whitish	5	b	12	++	AB971274	98%	*Peziza* sp.
	C	Dark brown	2	d	11	-	AB971277	98%	*Suillus laricinus*
	D	Brown-whitish	4	d	13	+++	AB971278	100%	*Suillus grevillei*
	E	Black-brown	5	c	11	+	EU557316.1	100%	*Cadophora finlandia*
	F	Brown	4	a	10	++	AB971276	100%	*Laccaria cf. laccata*
Before	D	Dark brown	2	d	11	-	AB971277	98%	*Suillus laricinus*
	G	Light brown	4	a	12	-	AB971280	99%	*Inocybe lacera*
	H	Orange brown	5	b	10	-	AB971281	99%	*Thelephoraceae sp.*

Note: The symbol "+" indicates the presence of emanating hyphae, the number of "+" describes the frequency. The symbol "-" indicates absence.

[a] See Agerer, pp. 9i-11i

[b] See Agerer, pp. 12i

[c] See Agerer, pp. 13i

Table 3.3 Growth of height and stem diameter of hybrid larch F_1 seedlings for two growing seasons.

		2011.07		2011.11		2012.09	
		Diameter (mm)	Height (cm)	Diameter (mm)	Height (cm)	Diameter (cm)	Height (cm)
Treatment	Control	5.51 (0.20) a	38.65 (1.42) a	9.60 (0.39) a	72.98 (4.37) a	16.69 (1.13) a	202.69 (14.02) a
	CO_2	5.00 (0.21) a	38.68 (1.72) a	9.53 (0.52 a	73.05 (5.93) a	17.28 (1.29) a	198.39 (18.56) a
	O_3	5.08 (0.12) a	38.78 (1.19) a	7.37 (0.36) b	55.74 (3.11) b	14.89 (1.16) a	173.44 (14.43) a
	$CO_2 + O_3$	5.00 (0.20) a	38.24 (1.57) a	8.80 (0.41) ab	67.86 (3.63) ab	16.54 (0.84) a	203.75 (8.84) a
ANOVA	CO_2	ns	ns	ns	ns	ns	ns
	O_3	ns	ns	**	*	ns	ns
	$CO_2 \times O_3$	ns	ns	ns	ns	ns	ns

Each value is the average (SE) of four chamber replications. Different character symbol denote significant differences between the four treatments ($P < 0.05$). ANOVA: *$P < 0.05$, **$P < 0.01$, “ns” means not significant.

Table 3.4 Dry mass of plant organs and the ratio of root biomass to shoot biomass (R/S) of hybrid larch F_1 seedlings at the end of the experimental period.

		Needle	Branch	Stem	Root	Aboveground	R/S
Treat.	Control	37.57 (3.61) a	34.91 (3.75) a	44.66 (5.38) ab	36.88 (4.02) ab	116.23 (10.97) ab	0.32 (0.02) a
	CO_2	48.22 (7.59) a	41.12 (8.88) a	51.67 (6.50) a	49.29 (6.07) a	144.76 (22.43) a	0.36 (0.04) a
	O_3	32.92 (5.53) a	20.82 (3.63) a	28.64 (4.87) b	25.57 (4.17) b	082.38 (13.70) b	0.33 (0.04) a
	$CO_2 + O_3$	47.07 (6.68) a	37.50 (5.54) a	45.21 (4.29) ab	40.13 (3.86) ab	129.78 (15.08) ab	0.32 (0.02) a
ANOVA	CO_2	ns	ns	*	**	*	ns
	O_3	ns	ns	*	*	ns	ns
	$CO_2 \times O_3$	ns	ns	ns	ns	ns	ns

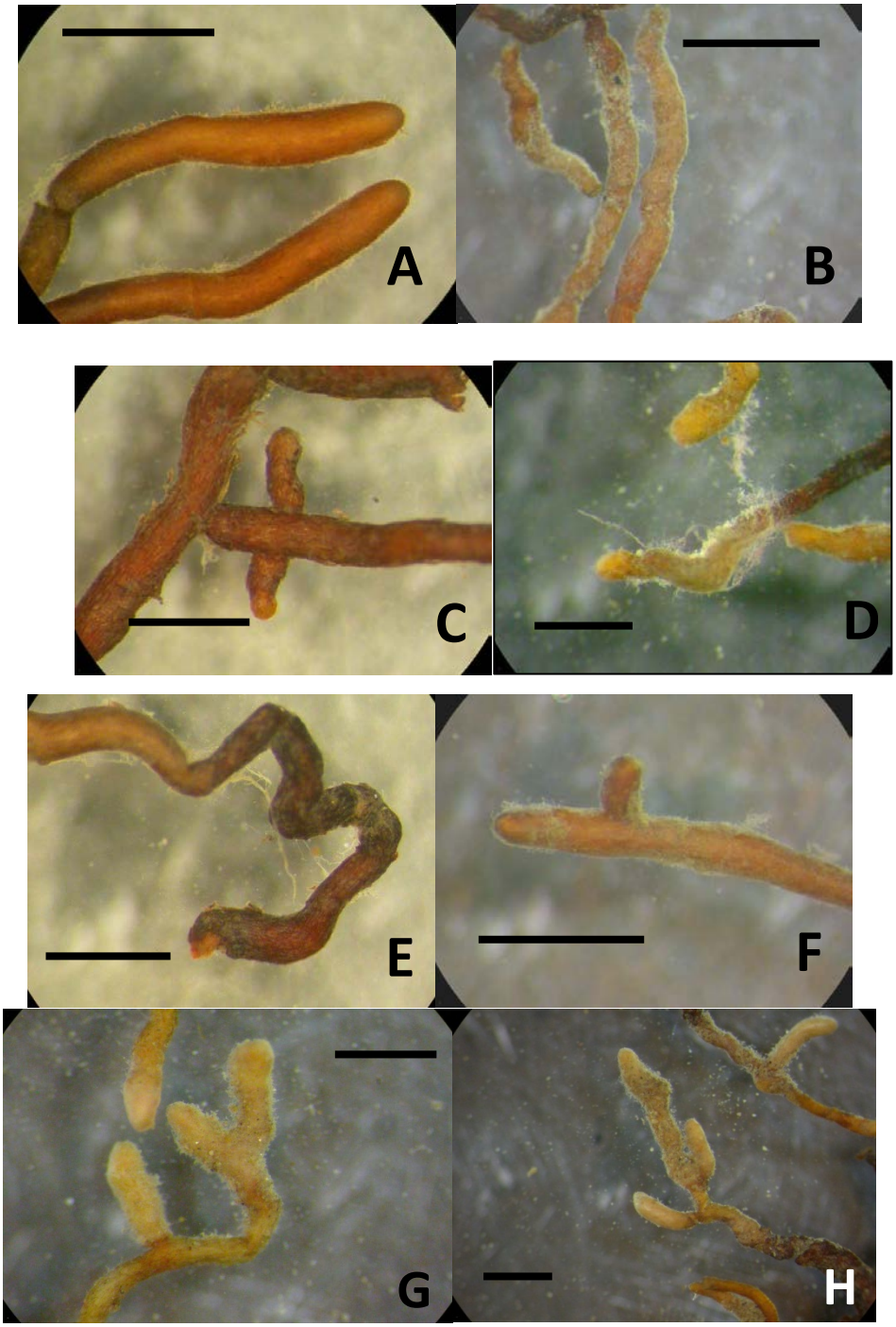
Each value is the average (SE) of four chamber replications. R/S: Root biomass to shoot biomass (needle + branch + stem). Different character symbol denote significant differences between the four treatments ($P < 0.05$). ANOVA: *P<0.05, **P<0.01, “ns” means not significant.

Table 3.5 Effect of elevated CO_2 and O_3 on seedling nutrient concentration in needles and roots.

Organs	Treatmen	Nutrient concentration (mg/g)								
		N	P	K	Ca	Mg	Al	Fe	Mn	Mo
Needle	Control	20.42(6.16)	1.62(0.18)	10.21(0.63)	7.67(1.76)	1.25(0.20)	0.12(0.02)a	0.14(0.02)	0.04(0.03)	0.03(.002)
	CO_2	19.10(6.37)	2.09(0.26)	11.44(1.03)	5.20(0.78)	0.96(0.12)	0.12(0.01)a	0.13(0.03)	0.09(0.06)	0.03(.004)
	O_3	18.43(6.14)	1.91(0.18)	6.95(0.77)a	4.61(0.64)	0.98(0.22)	0.07(0.02)b	0.09(0.02)	0.04(0.01)	0.01(.002)
	$CO_2 + O_3$	18.54(5.14)	4.13(0.51)	8.83(2.25)a	4.44(0.59)	0.88(0.09)	0.07(0.01)b	0.09(0.02)	0.08(0.02)	0.01(.002)
ANOV	CO_2	ns	**	ns	ns	ns	ns	ns	**	ns
	O_3	ns	**	ns	ns	ns	**	*	ns	***
	$CO_2 \times O_3$	ns	*	ns	ns	ns	ns	ns	ns	ns
Root	Control	16.37(4.09)	2.47(0.11)	3.90(0.35)a	9.38(0.44)	2.07(0.06)	6.10(0.30)a	4.19(0.06)	0.07(0.01)	0.01(.001)
	CO_2	15.74(4.37)	2.13(0.17)	4.15(0.23)a	8.75(0.74)	1.96(0.15)	5.86(0.70)a	4.02(0.24)	0.09(0.01)	0.01(.003)
	O_3	15.93(3.98)	2.30(0.29)	5.09(0.62)a	7.76(2.00)	2.40(0.17)	6.48(0.52)a	4.39(0.13)	0.09(0.01)	0.01(.001)
	$CO_2 + O_3$	17.22(4.30)	2.58(0.24)	4.75(0.29)a	8.97(0.58)	2.30(0.17)	6.32(0.70)a	4.37(0.08)	0.10(0.01)	0.02(.000)
ANOV	CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns
	O_3	ns	ns	*	ns	*	ns	ns	ns	ns
	$CO_2 \times O_3$	ns	ns	ns	ns	ns	ns	ns	ns	ns

Each value is the average (SE) of four chamber replications. Different character symbol denote significant differences between the four treatments ($P < 0.05$). ANOVA: *P<0.05,**P<0.01,***P<0.001, “ns” means not significant

Appendix



Each photo is the typical morphology of different ECM species, and all photos were taken under stereomicroscope (black bar=1 mm).

A: *Tomentella* sp., B: *Peziza* sp., C: *Suillus laricinus*, D: *Suillus grevillei*, E: *Cadophora finlandica*, F: *Laccaria* cf. *laccata*, G: *Inocybe lacera*, H: *Thelephoraceae* sp.

Chapter 4

ECTOMYCORRHIZAL SYMBIOSIS OF THREE LARIX SPECIES WITH DIFFERENT N AND P LOADINGS

4.1 INTRODUCTION

Nitrogen (N) deposition is increasing sharply in northeast Asia, including Northwestern China (Liu et al. 2013), South Korea (Park et al. 2002), and Japan (Morino et al. 2011), as a result of rapid industrial development and overuse of N fertilizer (e.g. Galloway et al. 2008). Nitrogen is often one of the most limiting nutrients in many terrestrial ecosystems (Schulze et al. 2005; LeBauer et al. 2008), thus chronic N deposition can alter ecosystem function (Smith et al. 2009). Such altered ecosystem functions have been documented in soils (Paul 2007) where microbes play a major role in energy and nutrient cycling (Frey et al. 2004). One group of such soil microbes is mycorrhizal fungi which have symbiotic relationships with 80% of the terrestrial vascular plant species (Smith et al. 2003). Importantly, mycorrhizal fungi improve the survival in the harsh environmental conditions in forests (Smith and Read 1997; 2008; Qu et al. 2010; Agerer 2012).

In infertile soil, ectomycorrhizal fungi (ECMF) efficiently supply phosphorus (P) and water to the host plants better than the plant root (Wallander 2000; Alves et al. 2010; Qu et al. 2010). Several studies reported this effective uptake of ECM-associated plants for organic P (Po) and nitrogen (N) was due to ECM production of extracellular enzymes, compared to non-infected plants of the same species (Turnbull et al. 1996; Van Der Heijden and Sanders 2002; Qu et al. 2004; White and Hammond 2008). Moreover the formation of extrametrical mycelia (EMM), which grow from the mantle into the root of the host plant from the surrounding soil, plays a major role in the translocation of nutrients and water, and even facilitates nutrient movement between individual host plants (Agerer 2001; 2012; Anderson and Cairney 2007).

Commonly, larch (*Larix* species) is a typical coniferous species which readily establishes symbiotic relationship with ECM fungi in harsh environments (Smith and Read 1997; Qu et al. 2004; 2010). As a dominant tree species in the northeastern part of Eurasia (Koike et al. 2000; Kayama et al. 2009; Mao et al. 2010), larch has been intensively planted for afforestation especially across Northern Japan (Ryu et al. 2009; Qu et al. 2010), South Korea (Kim 2008) and Northeastern China (Hu et al. 2010; Sun et al. 2010; Zhao et al. 2011). To overcome biotic and abiotic stress, such as cold weather, wild animal graze and strong wind, etc., typical to the regions, a new hybrid larch, and known as F_1 has been developed recently (Ryu et al. 2009). Previous study has found that ECM infection increases the growth of Japanese larch and F_1 by a factor of 1.5-2.0 in nutrient-poor soil in northern Japan and central Russian forests (Qu et al. 2010). Is this relationship maintained under a changing environment, and in particular where there is increased high N deposition?

Many studies documented that N deposition changed relationship between the host plants and mycorrhizal fungi. For example, a study on a spruce forest across a stand-scale N deposition gradient (from 27 to 43 kg N $ha^{-1}yr^{-1}$) revealed that with increasing N deposition, ECM root tip abundance and mycelial production decreased five and 10-fold, and ECMF community changed and the species richness decreased (Kjoller et al. 2012). According to the study of Lilleskov et al. (2002), composition of ECM species shifted from N-uptake to N-tolerant species with N loading, and given that under high-N, low-P and acidified conditions, the ECM species changed to favor types specialized for P uptake.

Furthermore, P availability is regarded to be a limited factor to tree growth due to several mechanisms, such as P depletion, soil barriers, especially the interaction with N deposition (Vitousek et al. 2010). P exists in soil in both inorganic and organic

forms, and with low concentration in the soil solution (Hinsinger 2001), especially immature volcanic ash soil (e.g. Kayama et al. 2009). Recently, some studies have been reported that ECMF contribute significantly to weathering processes of apatite substrates (Alves et al. 2010), and uptake of P from soil where with poor soluble sources (Aquino and Plassard 2004). Japan is part of the Pacific 'Ring of Fire,' and most forest soils derive from volcanic ash soil, which is deficient in P content (e.g. Schmincke 2004). Chronic deposition of atmospheric N potentially limits the utilization of P in a broad range of forest ecosystems (Gradowski and Thomas 2006). However, as I mentioned above, ECMF assisted the P acquisition from heterogeneous soil sources in forest ecosystems (Buscot et al. 2000; Alves et al. 2010). Baxter and Dighton (2005) reported host trees having a diverse ECM fungal species are regarded as equally efficient in mediating abundant P acquisition as trees with fewer ECM species. Additionally, the growth of many host trees is enhanced when they are infected by multiple ECM species rather than a single, including larches (Qu et al. 2004; 2010), three kinds of pine species and the Japanese larch (e.g. Choi, 2008). Contrary, in some forests, the soil P status exerts a selective influence on ECM fungal community composition (Morris et al. 2008; Dickie et al. 2009). Therefore the unclear symbiotic interaction is vital to successful forest establishment in infertile soil conditions (Dahlberg 2001; Smith 2002; Koike et al. 2010).

Although larch is important as plantation species in northern Japan, few studies exist regarding the symbiotic relationship between ECM fungi and larch species with increasing N deposition (Qu et al. 2003b; Choi et al. 2005; Shinano et al. 2007). Moreover, rather limited study has focused on ECM symbiosis in immature volcanic ash soil (Leski and Rudawska 2012). Already previous studies have found some advantaged growth character of hybrid larch F_1 than their parents (Ryu et al. 2009),

there is still unclear information about the response of ECM symbiosis colonized with these three larch species under with N deposition. P as the second nutrient element after N, can further change mycorrhizal dynamics with an interaction of N deposition. I hypothesize the species richness of ECMF with larches can be changed by N loading with or without P conditioning.

In this study, I examined how three larch species responded to N and P amendments. Specifically, trying to find some clues of larch species for afforestation in early stage, I consider four questions: (1) Is there an interactive effect between P and N fertilization on the extent of colonization of ECM fungi? (2) Does an increase in N deposition affect the diversity of ECM species colonizing host larches? And (3) Does ECM communities respond to nutrients differently among the three larch species? (4) Are there variations in the community structure of ECM fungal species between host larch species? To answer these questions I conducted a model experiment using representative larch species, including the new hybrid F_1, planted in simulated immature volcanic ash soils with differing N and P levels.

4.2 MATERIALS AND METHODS

4.2.1 Plants and soil materials

This experiment was also conducted in Sapporo Experimental Forest of Hokkaido University, Japan (N43.07, E141.38, 15m a.s.l.). The snow-free period is from early May to early November. The average temperature at the experimental site during the growing period was 20.2 ^{o}C, and the relative air humidity during May to October was 74.3 %. In 2010 the monthly accumulated photosynthetic photon flux in July, August,

September and October was respectively 578.2, 625.2, 582.2 and 360.7 mol m^{-2}.

I planted 3-year-old seedlings of three larches species in 15L pots. These were Dahurian larch (DL: *Larix gmelinii* var. *japonica*), originating from the Kuril Islands; the Japanese larch (JL: *L. kaempferi*), which is native to central Japan; and their hybrid larch (F_1) (*L. gmelinii* var. *japonica* × *L. kaempferi*), which has been successfully planted in northern Japan as a reforestation tree species having fast growth and high tolerance to cold (Ryu et al. 2009). JL and DL received from the same nursery near Bibai (Hokkaido Research Organization, Forestry Research Institute). Hybrid F_1 had been cloned by the same institute and offered to us. I obtained these species after 3 years cultivation. All seedlings were kept in low temperature cabinets in a storeroom at our university before planting, to observe an initial ECM colonization and determine the initial diameter and height. The size of three larch species showed no difference. The average (±SD) value of diameter was 10.13 (± 0.35) mm, and their initial height was 30.75 (± 3.24) cm.

Soil of Kanuma-pumice and Akadama (both are well-weathered volcanic ash) were selected for this pot experiment, two kinds of soil were mixed in equal volumes. Its physical and chemical property was same condition with Eguchi et al. (2008), soil PH and NH_4^+ concentration after nutrient treatment were detected (Table 4.1). These soils have low nutrient concentrations making them ideal for a nutrient-adding experiment and as an observational substrate for mycorrhiza.

4.2.2 Nutrient treatments

The experiment was fully randomized. I set two levels of N (0 and 100 kg $ha^{-1}yr^{-1}$; zero corresponding to N control), in combination with two levels of P (0 and 50 kg

$ha^{-1}yr^{-1}$), so as to cover all four combinations. The four treatments were denoted control (P0N0), high P (P50N0), high N (P0N100) and high P×N (P50N100), each treatment had six replicates. The concentration gradient was set according to a report that the average N deposition rates worldwide now exceed 10 kg ha^{-1} yr^{-1}, and that by 2050 some regions in Asia will reach 50 kg ha^{-1} yr^{-1} (Galloway 2004; 2008).I used ammonium nitrate solution (NH_4NO_3) to simulate acid rain and potassium phosphate monobasic (KH_2PO_4) as fertilizer source, because NH_4NO_3 is a major component of recent acid rain in northern Japan. Potassium chloride (KCl) was used to equilibrate the potassium concentration. To prevent nutrient leaching from heavy rain, a matched tray was set in the bottom of each pot, and the collected water was returned to the same pot. Irrigation was applied manually avoiding desiccation, and N and P were applied with two weeks interval after one month from planting (for adapting soil condition). Treatments period started from June 2010 until October 2011 except the winter time (from November to April).

4.2.3 Colonization rate and diversity of ECM

The ECM taxa were assessed via both morphological as well as molecular analyses, before planting and after harvesting (Table 4.2, 4.3). At the end of growing season, October, 2011, the entire seedlings were harvested and roots were stored in plastic bag covering by wet paper tissue and transferred to laboratory immediately, keeping in refrigerator at 4 ^{o}C. Within 3 days, the harvested roots were first washed until no clod and then gently cleaned the root tips using a paintbrush. Sampling method referred to Wang et al. (2015a). Finally, a microscope (Olympus szx-ILLK100, Japan) was used to observe the extent of colonization of ECM. Taxa of ECM were classified based on

morphological characteristics, including color, texture, ramification, root tip shape, and emanating hyphae (Grand and Harvey, 1982; Agerer 1987-1993). The taxonomic classification based on the morphology was verified via molecular analysis.

Ribosomal DNA (rDNA) of ECM was extracted from the root tips infected with ECM using a DNeasyTM Plant Mini Kit (QIAGEN). Internal transcribed spacer (ITS) was amplified via PCR using a primer set of ITS1-F (CTTGGTCATTTAGAGGAAG TAA) and ITS4-B (CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns 1993; Bellemain et al. 2010). PCR reactions were performed using 50 µL assays: 5µL of 10 × PCR buffer, 5µL of 25 mM $MgCl_2$, 4µLof a 0.2 mM dNTP mixture containing, 0.5µL of taq DNA polymerase, 1 µL each of 10 µM forward and reverse primer, 2 µL of a genomic DNA template (40 ng μL^{-1}) and 31.5µL of PCR grade water. The PCR thermal profile included an initial denaturation and enzyme activation step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 sec, 50 °C for 40 sec and 72 °C for 30 sec. PCR products were evaluated for amplification and their lengths by agarose gel electrophoresis, and purified with the PCR purification Kit (Labopass Cosmogenetech co, Ltd). Amplicons were sequenced by a BigDye Terminator v3.1/1.1Cycle Sequencing Kit (Applied Biosystems, USA). Finally the ECM sequences were then compared with the GenBank database of NCBI via basic local alignment search tool (BLAST). All the sequences were deposited at NCBI, the alignment similarity and accession number was list in Table 4.3.

The colonization rate of ECM (CRE) was determined based on the formula: CRE*i* (%) = ER/(ER+NR) × 100, where ER and NR denote the number of ectomycorrhizal and non-ectomycorrhizal root tips, *i* is an ECM type (Choi et al. 2005; Shinano et al. 2007). The diversity of ECM was calculated as Shannon's diversity

index (Keylock 2005): $H' = -\sum^{S}_{i=1} Pi \log Pi$, where S denotes the total number of types of ECM, and *Pi* is the proportion of the *i*th ECM type (Pielou 1966).

4.2.4 Plant growth and concentration of P in needles

To determine the total dry mass of each organ of seedlings, all the harvested seedlings were separated into needles, branches and roots, and then put into an oven at 80 °C for 48h (root for one week). The dry mass of different organs was weighted.

P uptake capacity to distal parts of seedlings were checked, the dried needles were ground to fine powder digesting by HNO_3, HCl and H_2O_2, after which an inductively coupled plasma-atomic emission spectrometer (ICP-AES, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, USA) was used to determine the concentration of P in the needles.

4.2.5 Statistical analysis

All data were following normal distribution, two-way ANOVA was performed for the fertilizer effects on colonization rates, ECM diversity and element concentration. I ran distance-based redundancy analyses for ECM composition with R software (version 2.15.0), and each calculation carried out with six individual seedlings.

4.3 RESULTS

4.3.1 Taxonomic identification

Four ECM types *Suillus laricinus*, *S. grevillei*, *Inocybe* sp. and *Thelephora* sp. (Type A, B, D and F) were observed before nutrient treatment (Table 4.2). After two growing seasons, three more ECM species appeared with three larch species. These were *Russula* sp. (C), *Hebeloma* sp. (E) and *Tomentella* sp. (G) (Table 4.3). According to taxonomy, all seven types were Hymenomycetes of Basidiomycotina, and were divided into two orders, Agaricales and Aphyllophrales. Agaricales contained three families: Boletaceae (Type A, B), Russulaceae (Type C) and Cortinariaceae (Type D, E). Aphyllophrales involved one family, *Thelephoraceae* (Type F, G).

4.3.2 ECM colonization and species diversity

The extent of ECM colonization in response to the N and P treatments were different in the three larch species (Fig. 4.2). Individual factor of N and P did not impact the ECM colonization rate for DL, the species diversity was significantly affected with increasing trend over N and P loading (R^2 = 0.94) (Fig.4.2 a, d). No pronounced effects of N and P were found on colonization rate and species diversity of JL except an interaction effect of N and P on colonization rate (Fig.4.2 b, e). F_1 showed same response with JL under different N and P treatments, and species diversity appeared a positive trend with increased nutrient level (R^2 = 0.96) (Fig. 4.2 c, f).

With N loading, colonization rate was reduced by 19.76% for DL, and increased by 39.44% and 33.59% for JL and F_1. Phosphorous application positively affected the ECM colonization for all three larch species. Phosphorous application positively affected the ECM colonization for all three larch species.

4.3.3 Species composition of ECM

The ECM composition responded to the N and P treatments with all of the three larch species (Fig. 4.1). After two-year treatment, type B was replaced by new types (C, D, E, F and G) for the DL. For the JL, the number of ECM types increased from one to five (A, C, E, F, and G) and for F_1, type D was replaced by types A, C, E and G (Fig. 4.1). In control conditions, the JL and F_1 were dominated by ECM types A and E, and the DL was dominated by type A. Increasing N loading to the high N (P0N100) level, DL was shifted from type D to type C, whereas type C replaced types E and F for JL. As for F_1, type G out-competed type B. With high P (P50N0) and high P+N (P50N100) applications, the dominant ECM types were type G for DL and type A for JL and F_1. With increased P and N loading together (to P50N100), type F appeared on DL, and types C and E replaced type A on JL, whereas for F_1, two new ECM types appeared (C and E), and type E became the dominant species (Fig. 4.1).

4.3.4 Community structure of ECM species

The ECM community structure differed within four nutrient treatments of each larch species (Fig.4.3). For the JL, there was a significant difference in the ECM community structure among nutrient treatments (P = 0.01) (Fig. 4.3 b), but no distinct differences in the DL and F_1 (Fig. 4.3 a, c). The asterisk shows the significance along the axis 1 direction, which explains 50.5 % of the variance of the JL**,** and revealing a significant difference between P50N0 and P50N100 (P=0.02). F_1 had properties intermediate between its parents, exhibited no significant difference among the four nutrient regimes; only 69.9 % of the variance was explained along axis 1, having marginal significance (P=0.06; see Fig. 4.3 c).

Under the same nutrient treatment, the ECM community structure differed significantly of three larch species. A significant difference was found among the three larch species in the control (P0N0) and high P (P50N0) treatments (Fig. 4.4 a, b). Distinction was obvious along axis 1(explaining 74.9 % and 80.1 % of the variance, respectively), with *P* values of 0.03 and 0.02 in each case (Fig. 4.4 a, b). This implies that, in the controls (P0N0)**,** the community structure of ECM species differed significantly between the DL and other two species (Fig. 4.4 a), whereas in the high P (P50N0) regime a significant difference had opened between the DL and F_1 (Fig. 4.4 b). The community structure of ECM species did not differ significantly with high N (P0N100) and high P+N (P50N100) nutrient loading among the three larches (Fig. 4.4 c, d).

4.3.5 Biomass of seedlings

F_1 showed higher biomass (shoots and roots) in comparing with their parents (Table 4.4). P significantly affected the root biomass of DL, and JL was unaffected by any nutrient factor, F_1 showed marked effect of P and the interaction effect with N on root and shoot biomass.

4.3.6 Concentration of P in distal parts of seedlings

The concentration of P was determined in needles. No significant effect of N and P was found on F_1. However N greatly decreased the P concentration in needles of its parents. In high N (P0N100) condition, the needle P concentration was decreased by 54.4 % for DL and 35.5 % for JL comparing with control. And with high P+N

3(P50N100) treatment, 23.2 % decrease for Dahurian larch and 28.9 % decrease for Japanese larch were detected respectively (Fig. 4.5).

4.4 DISCUSSION

I estimated the ECM colonization and diversity in symbiosis with larch seedlings grown in immature volcanic ash undergoing N and P application, which had a statistically significant impact on all three larches. Especially the community structure of ECM differed among three host larch species and four nutrient regimes.

The ECM species and colonization rate changed from their initial values after two-year treatment. It has been shown that larch species are mostly associated with *Suillus* spp. under natural conditions (Zhou et al. 2000; Zhou and Hogetsu 2002).The DL was colonized with *Suillus* spp. as the dominant ECM species at the beginning. In the end of experiment, it hosted up to six species of ECM. *Thelephora* spp. was the only one ECM species colonized JL before treatment, finally the species number increased to six with JL. This greater shift indicates a higher variety of ECM community structure among different treatments of JL (Fig.4.2 b). F_1 colonized more ECM species than their parents before the treatment, while *S. grevillei* only survived symbiosis with F_1 after two growing seasons. It has been found that larch species-*Larix laricina* seedlings were colonized rapidly and extensively by *S. grevillei* with inoculum of 109 fungal isolates (Samson and Fortin, 1986). This intensive symbiosis was also reported by Qu et al. (2003b), they detected that *S. grevillei* colonizes larch seedlings faster than *S. laricinus*, especially for F_1. However, for JL, *Suillus* spp. did not appear firstly, probably due to a common sequence of succession of symbiotic ECM (Nara et al. 2003b, 2006b; Yamakawa 2012). Additionally, ECM

succession was investigated in the coniferous species-Scots pine (*Pinus sylvestris*), the results showed that *Suillus* spp. was not the earliest pioneer species but developed after *Paxillus involutus* (Shaw et al. 2003). *Thelephora* spp. has also been found in naturally regenerated European larch seedlings (*Larix decidua*), and as a less abundant species with other ECMs (*Hydnotrya tulasnei, Pseudotomentella tristis, Tomentella sublilacina* and *Russula puelaris*) (Leski and Rudawska 2012). After the treatment, *Thelephora* spp. was found in all three larch species, probably because *Thelephora* spp. is a universal type in woody plants with wide age range. Ma et al. (2010) reported seedlings of the Red Pine (*Pinus densiflora*) aged from 1 to 5 years in a naturally regenerating area of a mature forest have also been colonized by *Thelephora* spp. Thus, in my case, all three host larches were likely to be colonized by *Thelephora* spp. after two-year treatment.

The ECM colonization and diversity were significantly affected by nutrient treatment, which led to the distinct difference of ECM community structure of three larch species. The colonization enhanced with elevated N and also with elevated P, and there was an interaction effect of N and P for JL and F_1. Some studies has been indicated that the ECM colonization rate or EMM production increase with high P availability (Jentschke et al. 2001; Bakker et al. 2009). It is possible that high N and high P treatment lead to nutrient imbalance, and ECM assistance was required for nutrient uptake. Furthermore, plants grown under high nutrient level tend to have more dichotomous architecture, readily generate more root tips. It is therefore with greater root tips, potentially colonized with greater amount of ECM (Fitter and Stickland 1991; Taub and Goldberg 1996). In addition, the JL and F_1 have the same pattern of root growth (Sato, 1995), perhaps resulted in the same trend of colonization rate as well. In the deep rhizosphere, especially beyond the inorganic phosphate

(Pi)-depletion zone, their shallow root system requires greater colonization by ECM for efficient nutrient-absorption. Another possibility may be related to inheritance (Fujimoto et al. 2006; 2008), there are many similar patterns of character expression between Japanese larch and F_1. One typical character is the chloroplast DNA, it predicts the genus *Larix* exhibits paternal inheritance (Szmidt et al. 1987), thus showing the same colonization rate of ECM for JL and F_1. There was no significant effect on ECM colonization rate for the DL, the value was lower in high N condition than control. Probably because the first-order roots colonized by ECM were 17 % reduced under high N (100 kg N $ha^{-1}yr^{-1}$) condition (Sun et al. 2010). The greatly affected diversity of DL by individual N and P factor showed an increasing trend with nutrient loading, but with lower changes of community structure among four nutrient regimes. This indicated less flexibility of DL for adapting soil nutrient stress, and this probably leading to inefficient nutrient absorption, especially P uptake. Overall, with the trend of unaffected colonization, the diversity showed increasing trend for JL and F_1 (Fig. 4.2). It revealed a markedly difference of community structure under different nutrient condition (Fig. 4.3).

The difference of ECM community structure was diminished with high N loading. This revealed the trend of similar ECM composition of three larches. However, the N loading reduced P content in needles for the parents not F_1, demonstrated F_1 had a high capacity and efficient uptake of P under high N condition (Goldstein 2013). Since there was no difference of community structure for three larches under P0N100 and P50N100, the benefit for unaffected P uptake of F_1 may from some dominate ECM species, perhaps type A, B and E, particular type B (*S. grevillei*) which only colonized F_1 in P50N100 condition. Strictly, it was not comprehensive to explain the P uptake by community structure or colonization rate and diversity, the plasticity of

ECM short root can affect the P uptake significantly, such as specific root length (SRL), specific root area (SRA) and root tissue density (Lambers et al. 2006). Changes in these morphological root traits are related to species-specific impact of ECM fungi (Ostonen et al. 2009).

Additionally, P and its interaction with N affected the above- and belowground biomass of F_1, reduced biomass was found with high P loading, especially without N application (P50N0). Based on the photosynthetic parameters, the growth of F_1 was not enhanced by P application up to 50 kg $hr^{-1}yr^{-1}$ (Ryu et al. 2009; Mao et al. 2014). As I mentioned before, N was vital for keeping needle P content. Since P in leaves is an essential macro-element for ATP and NADPH, which are related to light reactions in photosynthesis (e.g., Reich et al. 2009), therefore, photolysis activity was inhibited without N loading (P50N0) which leading to a reduced aboveground biomass and fewer allocation to belowground. On the other hand, the high P condition may enhance root length of F_1 without a proportional increase in root biomass as reported by Steingrobe (2001), as another possibility, the root biomass was reduced. There was no effect on biomass of JL, perhaps because a benefited flexible shift of ECM community structure under different nutrient regimes.

4.5 SUMMARY

I have studied the distribution of different ECM types colonizing three larch species in high N condition with and without P loading. Species-specific diversity was found in the relationship between ECM and the host larches planted in volcanic ash soil. High N loading increased ECM colonization rate for JL and F_1, not for DL. Contrary, ECM diversity was affected by nutrient treatment for DL neither JL nor F_1. In general, the

JL was more sensitive to N and P treatment, with marked shift in ECM community structure to different nutrient regimes. More importantly, F_1 kept efficient P uptake in high N condition than its parents due to the stable ECM diversity, particularly the contribution of *Suillus grevillei*. Or because of the different plasticity of ECM short root, further efforts are required to detect this prediction. The present results proved P was not a limited element for F_1. It was tolerant to N deposition with stable ECM community in early age stage, promising for plantations in P efficiency region. However, this is a short term treatment with larch samplings, further studies are required to detect response of long-term or with specific ECM species under several combined changing environments.

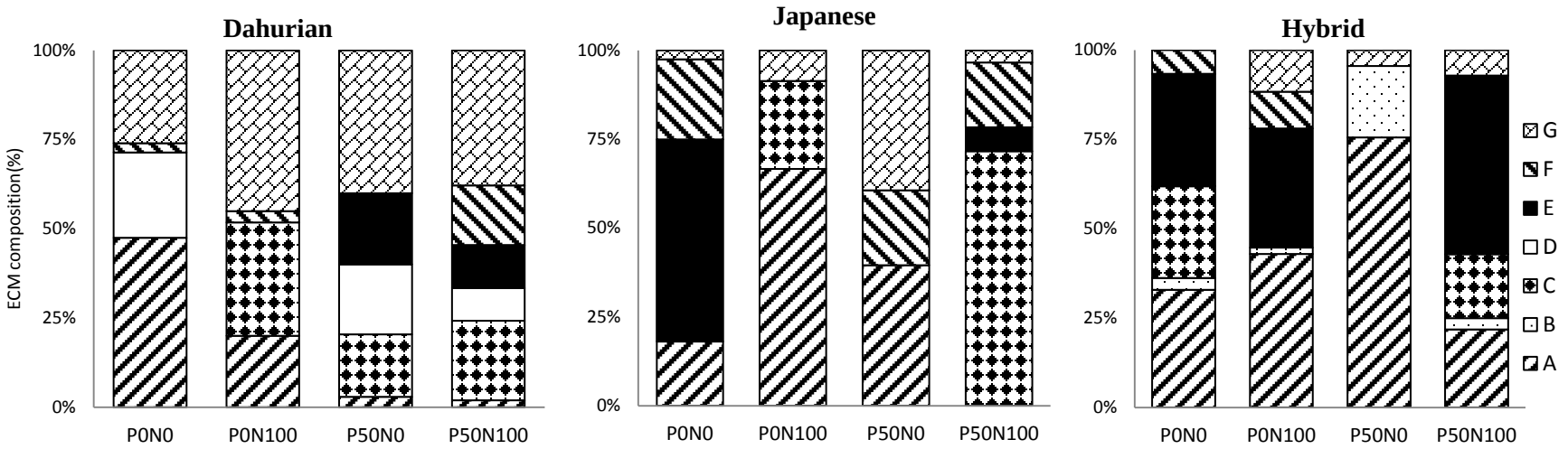


Figure 4.1 Composition of ECM fungal species of three larch seedlings with four different nutrient regimes. The different streak symbols correspond to the proportions of ECM fungal types colonizing host seedlings; values are calculated from the colonization rate of ECM fungi in different nutrient conditions.

Note: A *Suillus laricinus*, B *Suillus grevillei*, C *Russula* sp., D *Inocybe* sp., E *Hebeloma* sp., F *Thelephora* sp., G *Tomentella* sp.

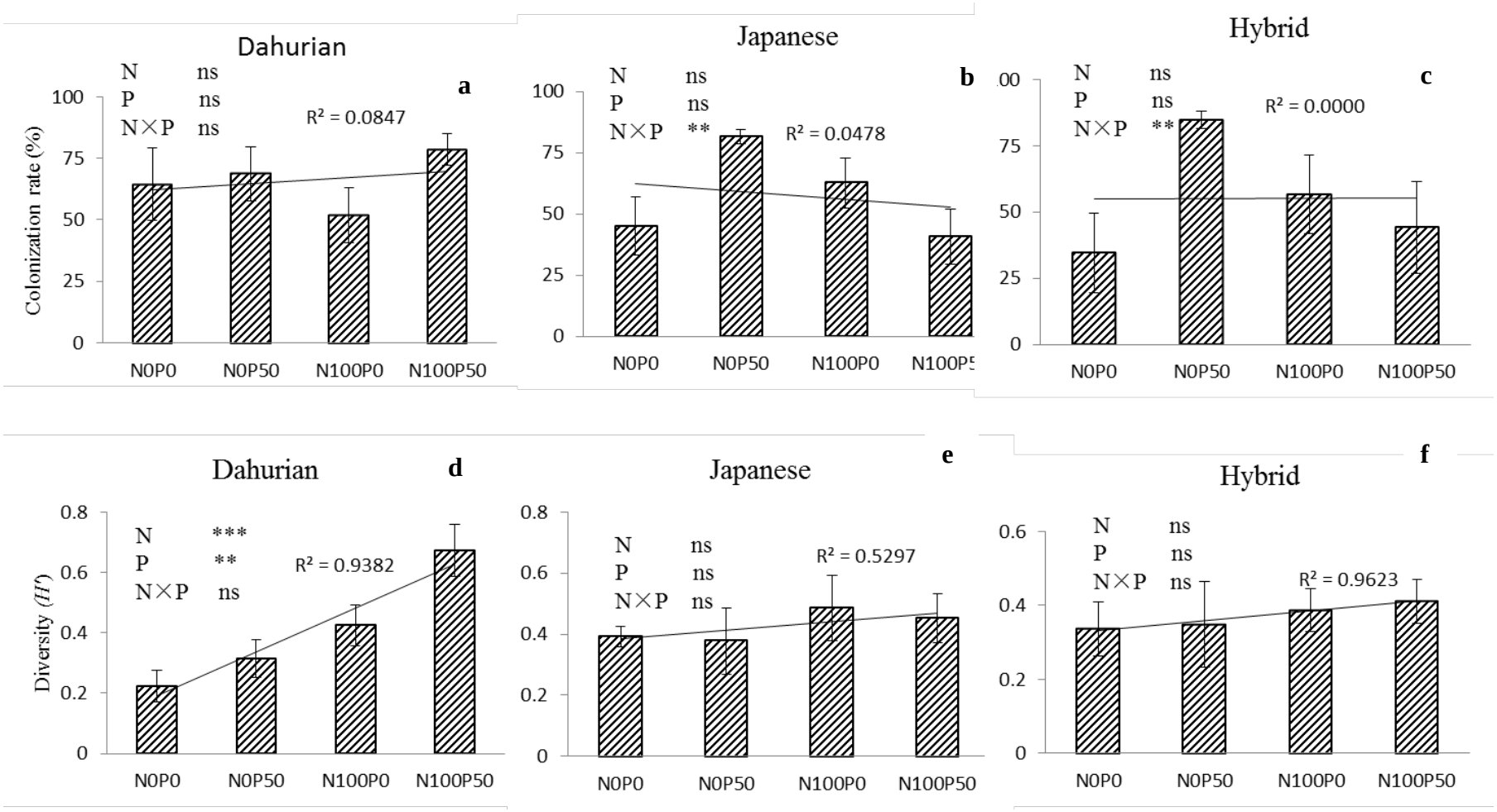


Figure 4.2 ECM colonization rate and species diversity of three larch seedlings under different N and P treatment. Each value is the average of six replications, and the error bar denotes the SE. ANOVA: *** $P < 0.001$, ** $P < 0.01$, ns: not significant.

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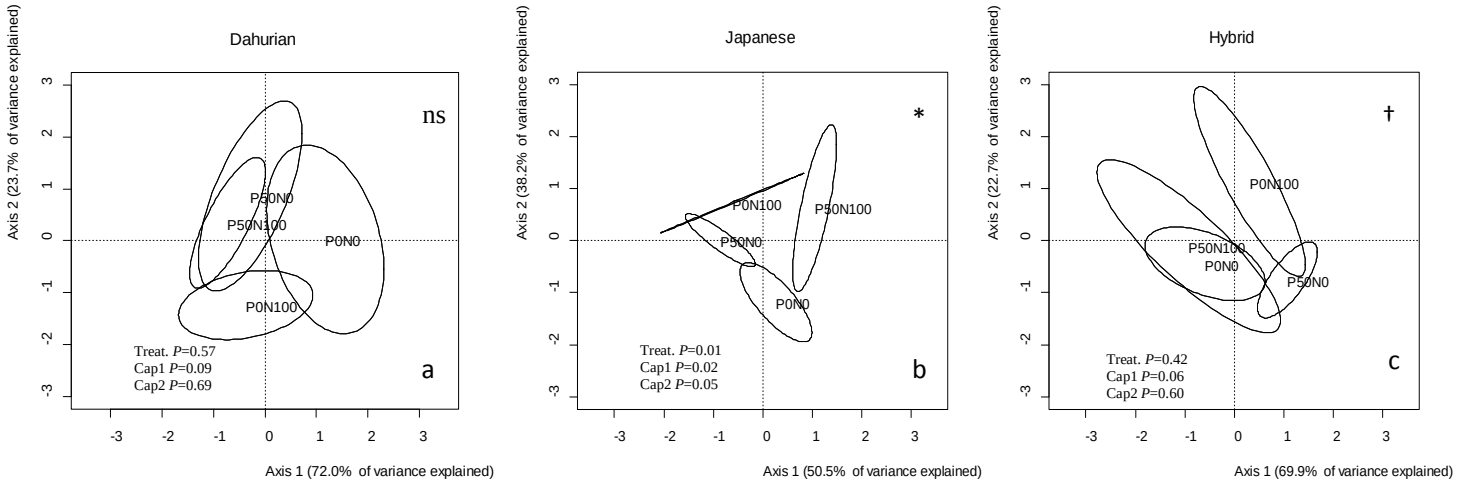

Figure 4.3 Community structure of ECM species with nutrient treatments as factors among the three *Larix* species. Results of distance-based redundancy analysis, with symbols indicating the results of ANOVA: †, $P \leq 0.10$; *, $P \leq 0.05$; ns, not significant.

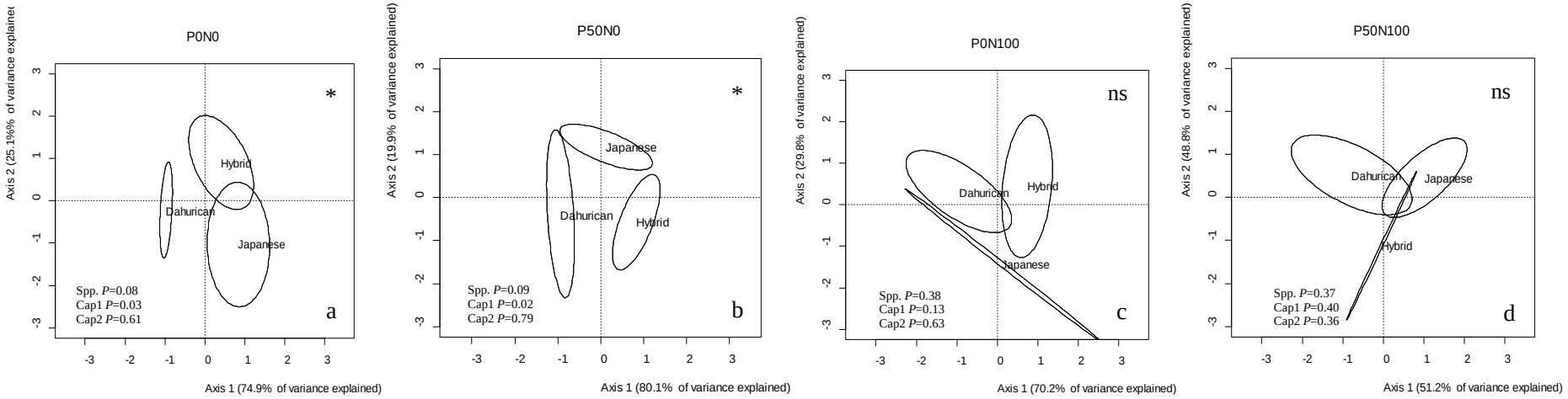

Figure 4.4 Community structure of ECM species with the three *Larix* species as factors among the different nutrient regimes. Results of distance-based redundancy analysis, with symbols indicating the results of ANOVA: *, $P \leq 0.05$; ns, not significant.

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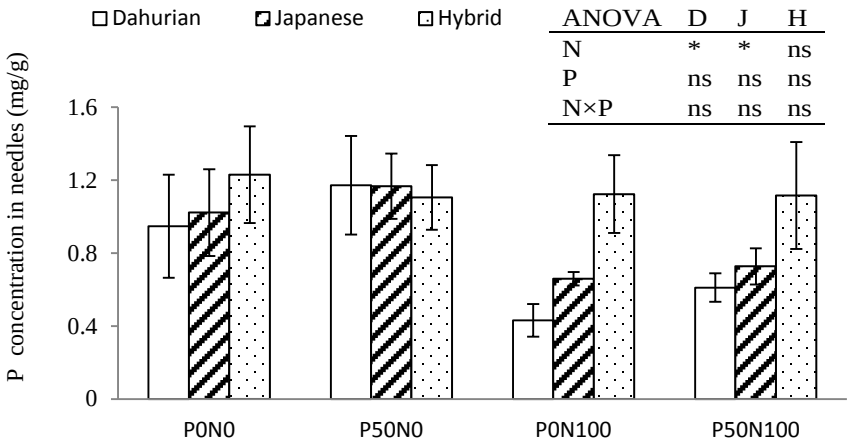


Figure 4.5 Concentration of P in needles of three larch species under the different nutrient treatments. Each value is the mean of six replications, and the error bar shows SE. ANOVA: *, $P < 0.05$; ns, not significant. D, J and H are the abbreviations for three larch species.

Table 4.1 Soil properties at the end of experimental period after the nutrient treatments.

		P0N0	P50N0	P0N100	P50N100	N	P	N+P
Dahurian	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.
Japanese	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.
Hybrid	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 4.2 ECM taxa colonizing three larches before nutrient treatment

ECM Type	Colonization rate (%)		
	Dahurian larch	Japanese larch	Hybrid larch
Suillus laricinus	38	-	-
Suillus grevillei	50	-	32
Inocybe lacera	-	-	25
Thelephora sp.	-	67	40

-: not colonized, because the samples were too small to identify.

Table 4.3 Molecular identification of ECM species with three larch seedlings after nutrient treatment

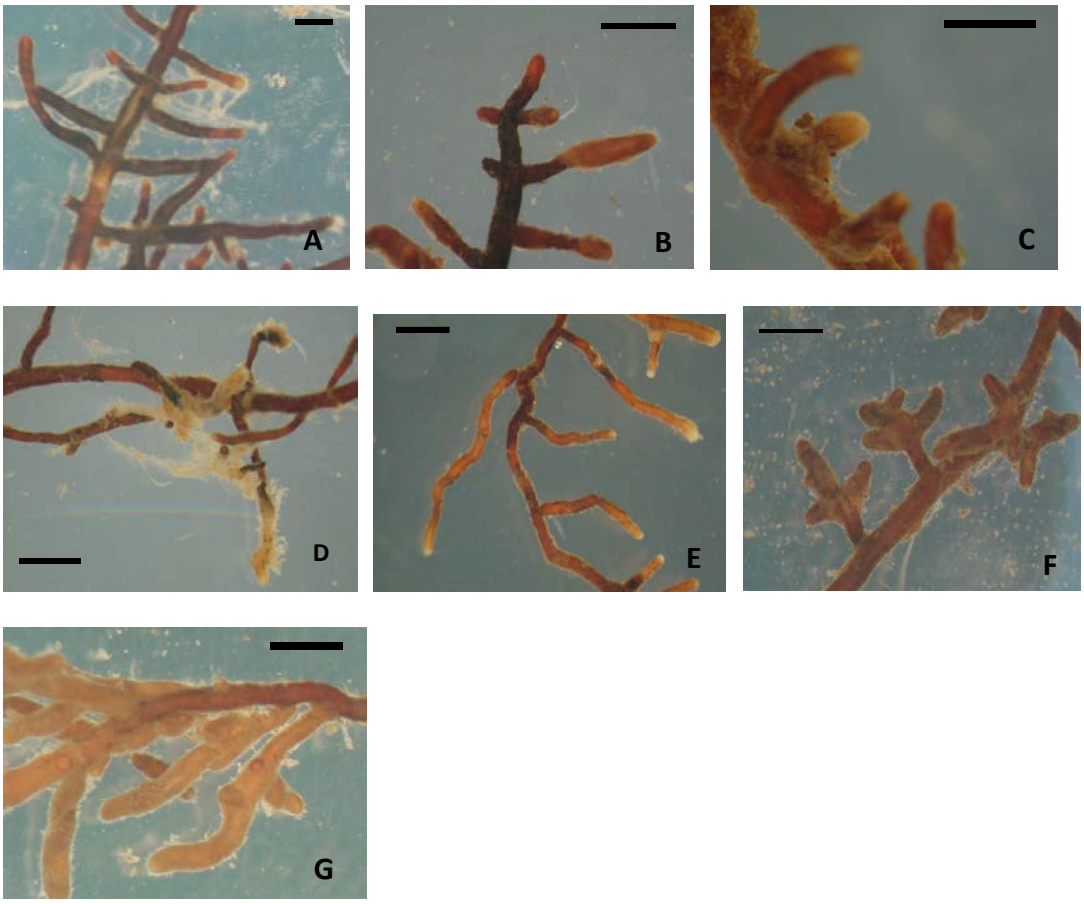
Type ID	Fungal taxon	Accession	Length (bp)	Closest match description	Query cover	E-value
A	*Suillus laricinus*	L54102.1	811	*Suillus laricinus* nuclear ribosomal RNA (rRNA) gene	79%	2E-128
B	*Suillus grevillei*	HM347659.1	926	*Suillus grevillei* voucher UF1336 18S ribosomal RNA gene	100%	9E-112
C	*Russula* sp.	FN565337.1	539	Uncultured *Russula* 18S rRNA gene (partial)	95%	3E-94
D	*Inocybe* sp.	GQ267473.1	620	*Inocybe lacera* voucher K04S3 18S ribosomal RNA gene	99%	4E-137
E	*Hebeloma* sp.	EF564171.1	848	*Hebeloma* sp. F-NB01 18S ribosomal RNA gene	97%	5E-77
F	*Thelephora* sp.	JX630835.1	591	Uncultured *Thelephoraceae* clone AR1727 internal transcribed spacer 1	99%	2E-103
G	*Tomentella* sp.	FJ013069.1	634	Uncultured *Tomentella* clone PR-67 18S ribosomal RNA gene	99%	4E-139

Table 4.4 Shoot and root biomass of three larch species in the end of experiment (g).

	Dahurian		Japanese		Hybrid	
Treatment	Root	Shoot	Root	Shoot	Root	Shoot
N0P0	50.80 (5.72)	44.61(7.99)	23.76(8.31)	79.86(25.75)	161.15(23.46)	126.85(18.37)
N0P50	25.61 (2.06)	24.22(2.11)	22.12(5.51)	73.42(15.08)	47.11(8.20)	47.74(7.91)
N100P0	31.13(3.64)	28.56(3.75)	12.67(4.46)	55.27(20.08)	104.89(9.51)	111.90(15.54)
N100P50	28.03(6.85)	25.03(5.44)	20.47(5.34)	68.77(16.74)	61.29(3.46)	101.63(7.35)
N	ns	ns	ns	ns	ns	ns
P	**	ns	ns	ns	***	***
N×P	ns	ns	ns	ns	**	**

Each value is the mean (SE) of six replications. ANOVA: ***, $P < 0.001$; **, $P < 0.01$; ns, not significant.

Appendix



Each photo is the different types of ECM identified during two-year treatment with N and P loadings. by microscope and molecular skill comprehensively. (black bar=1mm)
Note: A *Suillus laricinus,* B *Suillus grevillei,* C *Russula* sp., D *Inocybe* sp., E *Hebeloma* sp., F *Thelephora* sp., G *Tomentella* sp.

Chapter 5

GENERAL DISCUSSION

5. General discussion

Root is hidden half of the plant (Eshel and Beeckman, 2013) which suggests us to know more about plant function from viewpoints of shoot-root communication (e.g. Koike et al. 2003). Many trials for root research have been developed and examined their own characteristics in many instruments (e.g. Satomura et al. 2007). Among them, I employed the mini-Rhizotron method modified by Nakaji et al. (2008). During monitoring experiments for birth and death of the fine roots of white birch grown under elevated CO_2 in FACE, I also recognized real essential role of symbiotic micro-organisms in the rhizosphere, especially ectomycorrhizae (ECM). I further studied the different types of root tips colonized with several ECM of larches grown under different environmental conditions, such as elevated CO_2, O_3 and combined loading with N and P (as shown in Fig. 1.3 and Fig. 1.4).

Fortunately, number of ECM species colonized with larch have revealed to be limited, even though number of species of ECM increased with increasing of age (or size) of the host larch (Yamakawa 2012). Based on DNA analysis of the colonized ECM on larch seedlings and saplings grown under several environmental conditions, I examined essential role of ECM symbiosis with larch species. Here, I discussed first birth and death of the fine roots, and considered changes in nutrient condition of the rhizosphere caused by dead fine roots as resources (such as carbon [C] and nitrogen [N]). Second, I discussed abundance and composition of the ECM colonized with larch species, and consider about critical roles of the ECM on larch species.

5.1 Root dynamic under elevated CO_2

Fine roots were operationally classified as ≤ 2 mm in diameter based on the definition proposed by Pregitzer et al. (2002) in Aspen FACE, and an assessment of roots order and function in longleaf pine (*Pinus palustris*) forests by Guo et al. (2004). Their

rapid birth and death significantly influence the C and N cycling in a forest ecosystem. About 33% of global net primary production (NPP) is used in fine roots production and their functions (Jackson et al. 1997).

Previous studies have shown that NPP allocated to belowground is often greater than aboveground parts, and annual C and nutrients inputs to soil from fine roots frequently equal or exceed those from foliage (Norby and Jackson 2000). Because fine roots have a much shorter lifespan than coarse roots, as a consequence, their biomass varies both seasonality and environmental conditions. Therefore, effect of CO_2 enrichment on roots survivorship is important because of the implications to potential increases in carbon inputs to the soil from dead roots.

Elevated CO_2 usually increase production and turnover of fine roots (Wang et al. 2015). However, this is not consistent with the results of former studies (e.g. Pregizter et al. 2002; Norby and Zak 2011; Pregizter and Talhelm 2013). In this study, therefore, I investigated the fine roots dynamics under elevated CO_2 for three years. Length of live fine root was suppressed by elevated CO_2 in VA soil. It indicated that a lower soil nutrient level restricted the roots growth or prior supported for aboveground growth, which was found in evergreen (*Larrea tridentata*), drought-deciduous (*Ambrosia dumosa*), and winter-deciduous shrubs (*Krameria erecta*) (Housman et al. 2005). Elevated CO_2 increased length of live fine root in BF soil the first year, which was also found in previous study that root growth or biomass was increased under elevated CO_2 (Lukac et al. 2003). However, a higher root production does not necessarily result in a high root turnover.

To the contrary, FRP and its turnover rate were decreased by elevated CO_2 (Table 2.2), due to the positive correlation of ALRM and ALRP, I concluded a longer root lifespan occurred under elevated CO_2. This indication was proved by results of the root longevity (Table 2.3). One possibility is that the nutrient limitation resulted in a lower turnover of FRP and FRM, because the root longevity might be inversely related to the duration of resource supply (Fitter and Hay 1999; Pregitzer et al. 1993;

Pregizter and Talhelm 2013). Another reason is that plants are preferentially to enhance the growth of aboveground as I discussed above, which may readily occur in nutrient limited condition, because root lifespan would be increased if construction costs would be higher than maintenance costs, or if the nutrient availability is low (Eissenstat et al. 2000).

Roots (including coarse roots) of different diameter class response to the treatments were different (see Table 2.4). This difference may be caused by the mycorrhizal symbiosis. It has been widely demonstrated that plants usually increases mycorrhizal colonization and/or decreases root N concentration under elevated CO_2 (Pritchard and Rogers 2000; Tingey et al. 2000). Bidartondo et al. (2001) also found ECM colonizing the roots (D = 0.3~0.6mm) of Bishop pine (*Pinus muricata*) prolonged the root life. In my case, the shorter longevity of fine root was likely derived from the decreased ECM colonization during the third year.

Overall, fine root longevity can be influenced by several abiotic and biotic factors, such as climate, seasonality, soil conditions, diameter, root age and importantly the mycorrhizal colonization (see Eissenstat et al. 2000; Ruess et al. 2003). Therefore, to understand the fine root dynamics, I should deeply understand the response of mycorrhiza with various environmental factors.

5.2 ECM symbiosis under changing environment

The majority of belowground root systems in boreal forests are symbiotically colonized by ectomycorrhizal fungi (ECMF) (Taylor et al. 2000). Particularly, larch (*Larix* sp.) is a typical ectomycorrhizal (ECM) species (Smith and Read, 1997; Qu et al. 2004, 2010). Hybrid larch F_1 was developed as a promising afforestation species in northeastern part of Asia (Kuromaru 2008; Koike 2008; Ryu et al. 2009). Its character has been reported by Ryu et al. (2009) and the ECM symbiosis capacity was estimated by Qu et al. (2004; 2009). However, studies of the relationship between F_1 and ECM

symbiosis are still limited, especially with the effect of changing environment, such as elevated CO_2/O_3 and N deposition on it (Watanabe et al. 2013: Mao et al. 2014).

In this study with elevated CO_2 and O_3 treatments, a significant increase for total number of species of ECM colonization in F_1 under elevated $CO_2$was observed. However, the diversity did not follow the ECM colonization rate with F_1 under elevated CO_2. This result demonstrated that the ECM composition for F_1 did not change with an increased total colonization rate. Additionally, the vital support of host photosynthates for ECM survival was reduced under O_3 due to limited carbon allocation to belowground (e.g., Grantz and Farrar 2000), and this resulted in a decrease of ECM colonization rate. Importantly, lower ECM diversity caused by O_3 indicated a shift in the ECM abundance. Species of *Peziza* spp. in this study could not have a symbiotic relationship with F_1 under high O_3, but *Suillus grevillei* took great proportion than other species. This proved there may be specific symbiotic relationship of ECM and F_1 under O_3 stress.

On the other hand, for hybrid larch F_1, increased P content in needles, and K, Mg in roots were also proved that specific ECM was infected under this condition. Under external stress, the internal allocation of nutrients is liable to be altered by various ECM species (Weigt et al. 2011). According to the unaffected photosynthesis of F_1 under elevated O_3, the species of *Suillus grevillei* contributed significantly. Furthermore, elevated CO_2 diminished the harmful effects of O_3 by stimulating higher ECM colonization. Then, how about other environmental factors?

Nitrogen (N) deposition is increasing sharply in northeastern Asia which has become a severe environmental issue (e.g. Galloway et al. 2008). Even N is often one of the most limiting nutrients in many terrestrial ecosystems (e.g. Schulze et al. 2005; LeBauer et al. 2008), chronic N deposition can alter ecosystem functions, especially soil nutrient conditions. This changes influence the mycorrhizal symbiosis with the host plant (e.g. Choi 2008). Commonly, mycorrhizal fungi improve the survival in the harsh environmental conditions in forests. Several studies reported ECM-associated

Japanese larch and F_1 have more efficient uptake of organic P and N than non-ECM ones (Qu et al. 2004; 2010).

To detect the influence of N deposition on ECM symbiosis with typical ECM species, I conducted the potted experiment (Wang et al. 2013). In this study, three larch species, F_1 and its parents were planted in simulated volcanic ash soil with different N and P loading. The ECM species and colonization changed from their initial status after two-year treatment. This greater shift indicates a higher community structure of ECM among different treatments (Fig.4.2 b). The extent of colonization by ECM was enhanced by increasing N loading with elevated P (for Japanese larch and F_1). This opposite result with previous studies (Parrent and Vilgalys, 2007; Sun et al. 2010) indicates high N and P treatment lead to nutrient imbalance, ECM assistance was required for nutrients uptake. Distinctly, ECM colonization rate or EMM production will increase with high P availability (Jentschke et al. 2001; Bakker et al. 2009). In addition, several similarities were found in ECM colonization rate of Japanese larch and F_1 under different nutrient conditions. This may be due to their same pattern of root growth (Sato, 1995), which resulted in the same trend of colonization rate as well. In the deep part of rhizosphere, especially beyond the inorganic phosphate (Pi)-depletion zone, their shallow root system requires greater colonization by ECM for efficiently absorbing nutrients.

Another possibility may be related to inheritance (Fujimoto et al. 2006, 2008), there are many similar expression patterns of characters between Japanese larch and F_1.The more stable diversity of F_1 than its parents indicated some dominated species in ECM community. *S. grevillei* was only one species survived, which made symbiosis with F_1 after two growing seasons with high loading of N and P. This finding revealed that F_1 undergoes symbiosis to a greater degree with *S. grevillei* than either of its parent larches (Zhou et al. 2002; Qu et al. 2003, 2004). These results proved a specific species of ECM with F_1 for survival in high N conditions.

There was no distinct effect on biomass of aboveground by N and P treatment,

but the root biomass was reduced for F_1 by high P loading. However, there was no impact on P uptake in imbalance nutrient condition (e.g. high N loading). The high P concentration in needles of F_1 with N loading proved its high capacity and efficient uptake of P (Goldstein 2013). All of these owed to its benefit relationship of ECM symbiosis, contributing to a relative greater biomass than its parents.

Additionally, the different level of specialization to host plants is well known, with some ECM fungal species associated with a phylogenetically wide range of host plants while others are more specific to a narrow host range (den Bakker et al. 2004). Many ECM fungi have multiple hosts while most host plants can also associate with many unrelated ECM fungi (Bruns et al. 2002). These ECM have been called as "generalists." Generalist fungi might be more successful than specialists for long-distance dispersal in a spatially variable environment (Roy et al. 2013) as there is less host plant restriction. For example, among ECM fungi, a super-generalist *Cenococcum geophilum,* associates with virtually all ECM plant species (LoBuglio 1999) in many contrasting habitats world-wide. Contrarily, Suilloid group which is composed of *Suillus*, *Rhizopogon*, *Truncocolumella*, *Gomphidius*, and *Chroogomphus*, is the largest group of ECM fungi that exhibits this degree of host specificity, that is almost entirely restricted to hosts in the Pinaceae (Bruns et al. 2002).

The advantages of being specialists are less obvious, but if specialization provides greater physiological compatibility with the targeted host plant, it could result in greater competitive ability for the host or greater access to the host's resources (Bruns et al. 2002). Therefore, I study ECM fungi with investigation of generalist and specialist species

5.3 Perspective

In this study, root dynamics and ECM symbiosis were intensively investigated under elevated CO_2/or O_3 and N deposition. After the investigation of root dynamics and

ECM symbiosis in chapter 2-4, I obtained some important ecological information for afforestation with birch and larch species. However, one thing must be sure that, all the influences cannot be easily attributed to the individual environmental factor. The interactions of these factors are encouraged to detect for deep understanding of forest ecosystem under changing environment. There are still several aspects needed to be conducted in further study:

1) The understanding of fine root dynamics various greatly depending on the root diameter or a root position in branching system. It is important to classify the different root orders and find their different behaviors at each location of the root systems.

2) The characteristics of root differ with its function, some pioneer roots and other roots are functional contributor for nutrient cycling in forest ecosystem. But how much difference of their contributions, and when and how their functions change, these are the essential parts that should be revealed in relation to changing environment, such as elevated CO_2, O_3 and high N loading.

3) The ECM symbiosis with a host plant has been evaluated by several studies. This special relationship may be changed by ways of plasticity of short root, and further result in the function variation. To understand the morphology type of short root with ECM is highlighted in the near future.

Undoubtedly, it is still hard to conduct a root research because root is a hidden half of plants (e.g. Eshel and Beeckman 2013). Root research has lots of difficulties may be due to its unique trait, therefore it is challengeable and frontier for us like soil scientist as Olson suggested “Soil-The Final Frontier” (Special issue of Science 204, 11 June 2004). Most significantly, it is valuable and meaningful for any tiny contribution or further step on this research filed.

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