Ectomycorrhizal colonization and growth of the hybrid larch F_1 under elevated CO_2 and O_3

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Abstract

We studied the species abundance and the amount of ectomycorrhizal fungi colonizing a hybrid larch (F_1) under elevated CO_2 and O_3. Two-year-old larch seedlings were planted in an Open-Top-Chamber system under four gas treatments: Control (O_3<6 nmol/mol), O_3 (60 nmol/mol), CO_2 (600 μmol/mol), and CO_2+O_3. After two growing seasons the ectomycorrhiza (ECM) colonization and root biomass were found to be increased by elevated CO_2. Ozone impaired the ECM colonization and species richness, and reduced the stem biomass. There was no clear inhibition of photosynthetic capacity by O_3, however. The concentrations of Al, Fe, Mo and P in needles were reduced by O_3, but K and Mg in roots increased. This might explain the differences observed in the ECM colonization rate and diversity. No effects of combined fumigation were found for any measured parameter except the P concentration in needles. The tolerance of F_1 to O_3, as documented, might be a factor in shifts in the ECM community structure.

Key words: Ectomycorrhiza, Elevated ozone, Elevated CO_2, Hybrid larch F_1, Species richness

Capsule

Elevated CO_2 moderated the negative effects of O_3 on growth of the hybrid larch F_1, because element uptake by ectomycorrhiza led to a shift in the ectomycorrhizal community structure.

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 their increase in the coming decades, because of the continued burning of fossil fuels and deforestation (e.g. Cubasch et al., 2001; Tans, 2008; Koike et al., 2013). This rise in atmospheric CO_2 and tropospheric O_3 may affect the above-ground and below-ground growth and development of trees, and therefore impact

the CO_2 sink provided by forest ecosystems (e.g. Larcher, 2003; Qu et al., 2010).

The majority of below-ground root systems in boreal forests are colonized symbiotically by ectomycorrhizal fungi (ECMF) (Taylor et al., 2000). Larch trees (*Larix* sp.) are typical species colonized by ectomycorrhiza (ECM) (Smith and Read, 1997; Qu et al., 2004); they are widely planted in the northeast of Eurasia (Koike et al., 2000; Qu et al., 2010) and part of Europe (Matyssek and Schulze, 1987) as a dominant tree species for afforestation. A hybrid larch F_1 (*Larix gmelinii* var. *japonica* × *L. kaempferi*; here after F_1) has recently been developed as a promising species for afforestation; it has much better tolerance to cold climate, to grazing damage by the red-back vole (*Clethrionomys rutilus*) and to shoot blight disease, as well as tolerance to strong wind (Ryu et al., 2009). The growth of F_1 is closely related to its invariable association with ECM (Qu et al., 2004), although details of this symbiosis are still limited (Qu et al., 2003; 2010). It is estimated that up to 30% of total photo-assimilation products can be used in growth and maintenance of ECM (Hampp and Nehls, 2001). ECM usually act as an efficient resource supplying the root system of the host, by absorbing water and essential nutrients, particularly phosphorus (P) and sometimes nitrogen (N) (e.g. Quoreshi et al., 2003; Cairney, 2011).

Many studies have found that the net primary production and growth of trees are enhanced by elevated CO_2, in which there is also an increase in carbon allocation to below-ground parts (Nowak et al., 2004; Qu et al., 2004; Choi et al., 2005; McElrone et al., 2005). Choi et al. (2005) reported that symbiosis with ECMF increased the growth of seedlings of the Japanese red pine *(Pinus densiflora*) under elevated CO_2, because the photosynthetic activities of the host plants were enhanced by an increase in the root surface area via widely ramified ECM hyphae. These authors also found an improvement in water use efficiency (WUE), and suggested that colonization of pine with ECM leads to greater photosynthate allocation to roots under conditions of elevated CO_2. Colonization by ECM increased the growth of the Japanese larch (*Larix. kaempferi*) and its hybrid larch F_1 by a factor of 1.5-2.0 in nutrient-poor soil in northern Japan and eastern Russia (Qu et al., 2004; 2010).

Buscot et al. (2000) emphasized that a greater species richness in ECM communities improves 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 than by a single species (e.g. Quoreshi, 2003; Qu et al., 2004; Choi, 2008). Greater ECM species richness may therefore improve 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. It impairs the physiological and biochemical processes in leaves, and accelerates leaf senescence (Zhang et al., 2002; Matyssek and Sandermann, 2003; Watanabe et al., 2010a; Agathokleous et al., 2014). These negative effects have consequences below ground (Blum and Tingey, 1977; Agathokleous et al., 2014). Carbon assimilation is reduced by O_3, limiting the below-ground allocation (Grantz and Farrar, 2000; King et al., 2005) and reducing the standing fine-root mass (Kasurinen et al., 2005). These changes are expected to decrease ECM colonization and affect the species-host compatibility.

A study of the silver birch (*Betula pendula* Roth) in Open-Top-Chambers (OTCs) found that O_3 at double the ambient concentration clearly decreased the proportions of black and liver-brown mycorrhizae after three growing seasons (Kasurinen et al., 2005). Haberer et al. (2007) used radioactive isotopes (delta N-15) to measure N uptake and symbiosis of ECM with adult beech trees (*Fagus sylvatica*). They found that the number of fine roots – which were all mycorrhizal – increased markedly with long-term O_3 fumigation. Other studies, of a 70-year old mixed spruce-beech forest stand, found that the number of vital ECM root tips increased, and the ECMF community was significantly different after two-year fumigation with O_3 (Grebenc et al., 2007). This is not always the situation, however. Zeleznik et al. (2007) reported ambiguous results for two-year

old beech seedlings exposed to elevated O_3 for two years; there was very little colonization of seedlings by mycorrhizae, and the ECM types were lower ***FEWER SPECIES OR LESS OF EACH SPECIES OR BOTH? CLARIFY – EDITOR*** under O_3 fumigation than in control plants. In particular, the number of vital mycorrhizal root tips was reduced by the O_3 treatment (Zeleznik et al., 2007).

Some previous research has found that ECM colonization decreases under O_3 (Adams and O'Neill, 1991; Edwards and Kelly, 1992) but other work has found an increase (Wöllmer and Kottke, 1990; Gorissen et al., 1991; Kasurinen et al., 1999). The effects of O_3 on ECM symbiosis are therefore not consistent. In fact the effects of O_3 depend on the age of the material plants and the length of fumigation.

External stressors, such as drought and high O_3 concentrations, tend to regulate stomatal conductance. Water-soluble elements are likely to be selectively absorbed by ECM as a defense against the harmful effects of such stresses (Jourand et al., 2014). According to Marjanović et al. (2005a), aquaporin expression is enhanced in ECM seedlings; this enhancement could be particularly important under conditions of water stress (Marjanović et al., 2005b). It follows that, for defense against O_3 stress, element absorption and uptake ability are likely to be adjusted. They are reduced in fine-roots of the European beech under enhanced O_3 (Haberer et al., 2007).

Elevated CO_2 and/or O_3 change the ECM community composition by affecting particular ECM species. A study of the silver birch (*Betula pendula* Roth) in OTCs found that elevated CO_2 impaired light brown/orange mycorrhizae (Kasurinen et al., 2005). In long-term exposure experiments involving CO_2 and O_3, 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 a combination 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 below-ground responses to O_3 were variable, and elevated CO_2 typically mitigated the negative effects of O_3 (e.g. Karnosky et al., 2003; Watanabe et al., 2010b). The total extent of mycorrhizal colonization of silver birch clones was stimulated by enhanced CO_2 and by elevated O_3 separately, but not when combined (Kasurinen et al., 2005).

The effects of a combination of elevated CO_2 and O_3 on ECM species richness are not known in detail (Grebenc et al., 2007; Matyssek et al., 2012). We expect 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). We therefore anticipate that colonization by ECM and the species richness in the roots of F_1 should increase under elevated CO_2 and decrease under elevated O_3.

The present work seeks to answer the following questions concerning the hybrid larch F_1 used for 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 hybrid larch F_1?

2. Materials and Methods

2.1. Experimental site and plant materials

Our study was conducted in the Sapporo Experimental Forest of Hokkaido University in northern Japan (43°07′ N, 141°38′ E, 15 m a.s.l.; the annual mean temperature and precipitation in 2011 were 13.5°C and 1254 mm). Two-year-old seedlings of the hybrid larch F_1 (*Larix gmelinii* var. *japonica* × *L. kaempferi*) were provided by the Hokkaido Research Organization Forestry Research

Institute near Sapporo. The height and diameter of these seedlings were determined before planting; the mean height of the seedlings at this time was 38.6 ± 0.3 cm, and the mean diameter was 5.2 ± 0.2 mm.

ECM colonization was also determined before planting (see Table 2). The soil at the study site was well homogenized brown forest soil in which there had not been any previous plantation of tree species. All seedlings were planted in May 2011, and were irrigated periodically with tap water to prevent desiccation. Gas treatments began one month later, after all of the seedlings were established at the site. After two growing seasons they were dug out (in late October 2012).

2.2 CO_2 and O_3 treatment

We set up the OTC system in the experimental forest site of Hokkaido University. The 16 chambers (dimensions W x W x H =1.2×1.2×1.5 m; 2.2 m high after Sep. 2012) were made of steel frame, with polyvinyl chloride film (Noh-bi, Sapporo, Japan) having a transmittance of 88 % of full sunlight cutting UV-B. We set up four gas treatment regimes: (1) control (CO_2 = about 380 μmol mol^{-1}; O_3< 6 nmol mol^{-1}), (2) elevated O_3 (60 nmol mol^{-1}: 7 hours, 10:00-17:00), (3) elevated CO_2 (600 μmol mol^{-1} during daytime) and (4) combination (elevated O_3+ elevated CO_2). Charcoal-filtered ambient air was introduced, and CO_2 was supplied from a tank in the elevated CO_2 treatments. ***WAS CO_2 SUPPLIED IN AMBIENT TREATMENTS? CONFUSING ORIGINAL WORDING – EDITOR*** The chamber CO_2 concentration was regulated by a control unit (MC-F20/S; Koito Co. Ltd., Japan). For O_3 fumigation, O_3 was generated from ambient air using an electrical-discharge O_3 generator (IO-1A5; Nippon Ozone Co., Ltd., Tokyo,Japan). In the growth chambers, O_3 was continuously monitored by an ultraviolet (UV) absorption O_3 analyzer (TUV-1100; Tokyo Industries Inc., Tokyo, Japan). The O_3 concentration was less than 6 nmol mol^{-1} in the untreated O_3 chambers. We set up 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 so as to maintain the desired concentration of O_3. The monthly data are shown in Table 1.

2.3 ECM identification

After 2 years of fumigation, we analyzed ECM on the roots of F_1. After being dug out, all roots were covered by wet paper tissue, stored in plastic bags, and transferred immediately to the laboratory where they were kept in a refrigerator at 4°C. In not more than 2 days these harvested roots were washed until no large clods remained, and then cleaned gently using a small painting-brush. A microscope (Olympus szx-ILLK100, Japan) was then used to observe the extent of ECM colonization, as had also been done prior to planting. A total of 500 root tips were counted randomly for each replicate, following the method of Shinano et al. (2007). Fig. 1 shows the sampling process. A classification of morphological types of ECM was estimated, ***HOW?*** and final identification of ECM was carried out by molecular analysis (Table 2), as follows:

First, we extracted ribosomal DNA (rDNA) from the root tips using a DNeasy™ Plant Mini Kit (QIAGEN). We then undertook PCR amplification via the polymerase chain reaction by primer 1F/4, in order to determine the sequences of the ITS-region (Gardeset al., 1993; Bellemain et al., 2010). Sequencing reactions were performed using the BigDye Terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, USA). ECM sequences were then compared with the GenBank database at the DNA Data Bank of Japan, using the basic local alignment search tool (BLAST) program (http://blast.ddbj.nig.ac.jp/blast/blastn?lang=en).

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

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

where ER and NR rrespectively denote the number of ECM and non-ECM root tips, and *i* is the ECM type index (Choi et al., 2005; Shinano et al., 2007).

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

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

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

2.4 Measurement of seedling growth and nutrient concentration

The diameter and height of the seedlings were measured in July and November 2011, and again in September 2012. All seedlings were harvested on 20 October 2012 in order to estimate the dry mass of plant organs. At this point the seedlings were separated into needles, branches, stems and roots. The plant organs were dried in an oven at 80°C for one week and weighed. Needle and root samples were crushed into powder by mills and digested by HNO_3, HCl and H_2O_2. An inductively coupled plasma-atomic emission spectrometer (ICP-AES, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) was then 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).

2.5 Measurement of leaf gas exchange rate

Leaf gas exchange rates of the seedlings were measured on the 21-25th September 2011 and 11-15th September 2012 using an infrared gas analyzer system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Two seedlings in each chamber were randomly selected to undergo measurements of their leaf gas exchange rates (8 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 a leaf temperature of 24 ± 0.1°C, 380 μmol mol^{-1} CO_2, relative air humidity 60 ± 5% and a photosynthetically active photon flux (PPF) of 1500 μmol m^{-2}s^{-1}, following Watanabe et al. (2012). Finally, the value of *A* and the stomatal conductance at the growth concentration [CO_2] (denoted A_{growth} and G_s) were measured.

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. 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 between the four treatments. Distance based redundancy analysis (db-RDA) was performed to determine the varying species abundance of the ECM community across the gas treatment regimes.

3. Results

3.1 ECM types colonizing F_1

We found six types of ECM colonizing F_1 after the treatments, compared with three types before the CO_2 and/or O_3 treatments. According to mycorrhiza taxonomy, eight ECM types belong to either the class Basidiomycetes (Type A, C, D, F, G and H) or Ascomycetes (Type B, E). Table 2 sets out the morphological specification of each type of ECM and the similarity of matched sequences in their identification.

3.2 Extent of colonization and diversity of ECM

The ECM colonization extent ***NOT 'RATE' WHICH REFERS TO A SPEED*** was significantly increased by elevated CO_2, but was sharply reduced by O_3 relative to the control (Fig. 2). There was no interactive effect of elevated CO_2 and O_3 on the ECM colonization rate, however. The ECM colony varied in diversity across the four treatments (Fig. 2). ECM diversity was significantly reduced by O_3 exposure, and diversity also decreased under elevated CO_2+O_3 treatment relative to the control, whereas a greater diversit wasy found in the control and elevated CO_2 regimes. There was no significant difference between control and elevated CO_2 (P=0.49).

3.3 Abundance of ECM by species

The six ECM types were found in differing amounts in the four gas treatments. According to the integrated estimation of ECM colonization and species, the major ECM colonizers of F_1 were types A, C, D and F (Fig. 3b). The ECM abundance under elevated O_3 and the mixed fumigation differed significantly from thre control and elevated CO_2 regimes (along the axis-1 direction, 63.7% of the variance was explained, $P \leq 0.01$). At elevated CO_2 the ECM abundance was similar to that in the control, based on the details of the four ellipses in Fig. 3a.

The number of colonizing species of ECM in the control was unchanged with elevated CO_2. In a comparison of the particular types of ECM, the proportion of type D was greater and the proportion of type C was less under elevated CO_2 than in the control (Fig. 4a, b). Under exposure to O_3, type B failed to colonize F_1 whereas type D colonized to a significantly greater extent, and type A colonized less than in the control (Fig. 4c). Under mixed fumigation in which CO_2 and O_3, were both elevated, the proportion of type C increased relative to the control, and became the dominant species (Fig. 4d).

3.4 Growth of seedlings and element concentrations

Ozone markedly reduced seedling growth by the end of the first growing season (Table 3). The height and stem diameter of seedlings were not significantly changed by elevated CO_2 relative to the control in 2011 and 2012, but these parameters were significantly reduced by O_3 by the end of the 2011 growing season. Elevated CO_2+O_3 did not exert any effect on the growth of height and stem diameter during the two years of treatment. Neither the diameter nor the height was affected by any treatment in 2012. Elevated CO_2 increased the biomass of root, stem and total above-ground biomass, and O_3 reduced the biomass of stem and root. The combined elevated CO_2+O_3 did not give rise to differences from the control, and there was no interaction between elevated CO_2 and O_3 for any biomass parameter (Table 4). The root/shoot (needle + branch + stem) ratio (R/S) was also unaffected in every gas treatment.

No clear differences between gas regimes were found for the concentrations of N, K, Ca and

Mg in needles (Table 5). The concentrations of P, Al, Fe and Mo were significantly reduced by O_3, and concentrations of P and Mn were clearly increased by elevated CO_2. An interactive effect of elevated CO_2 and O_3 was found in the P concentration. Under the fumigation with elevated CO_2+O_3, the P concentration in needles increased by a large amount over the control. In roots, only K and Mg were increased by O_3, and there was no significant effect of the four treatments on the other elements measured.

3.5 Gas exchange rate

In the elevated CO_2 treatment, A_{growth} was significantly enhanced in both 2011 and 2012 (Fig. 5). Under elevated O_3 the values of A_{growth} did not differ significantly from the control, but did increase with elevated CO_2 and CO_2+O_3 fumigation in 2011 and in 2012. No significant influence was exerted upon G_s by any gas treatment. Under both elevated CO_2+O_3, G_s was similar to its control value, but with a tendency to be less in 2011 (P<0.1). Ozone tended to induce an increase in G_s in the second year (Fig. 5).

4. Discussion

Overall, the composition of the ECM community was very different by the end of the treatments. *Inocybelacera* (G) and *Thelephoraceae* spp. (H) had colonized F_1 before the treatments, but *Suillus laricinus* (C) and other ECM species replaced them during the 2 years of fumigation. In all treatments, changes in the observed ECM species may be due in part to ECM succession. Nara et al. (2003, 2006) found a common sequence of succession of symbiotic ECMs; for larches, *Suillus* spp. appeared late in the pattern on soul from well weathered lava flow. A similar pattern has been observed in the Japanese larch in a mature forest (Yamakawa, 2012).

In general, carbon allocation to below-ground organs increased with elevated CO_2, in the Japanese larch (Choi et al., 2005) and also other tree species (Nowak et al., 2004; Jackson et al., 2009). Carbon allocation to below-ground 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. The diversity was different from the colonization rate pattern under elevated CO_2, however. This shows that the ECM composition did not change with an increased total colonization rate. In fact, the vital support of photosynthates for ECM survival from the host was reduced under O_3 as a result of the limited carbon allocation to below-ground (e.g. Grantz and Farrar, 2000). This slowed the rate of colonization. Lower ECM diversity was also found under O_3; we discuss this point later.

The proportion of *Suillus grevillei* (D) increased sharply, and was slightly larger than that of *S. laricinus* (C) in the elevated O_3 regime relative to the control. This is probably because *S. grevillei* colonizes larch seedlings more rapidly than *S. laricinus*, and larch seedlings have higher shoot biomass when colonized by *S. grevillei* than by *S. laricinus* (Qu et al., 2003). Even with reduced ECM colonization, the proportion of favored species increased within the ECM community under elevated O_3 conditions, exactly as *Suillus* spp. did here.

The lower ECM diversity due to O_3 gives rise to a change in the abundance of ECM. ***CLARIFY WHETHER ‘ABUNDANCE’ MEANS NUMBER OF DISTINCT SPECIES OR THE TOTAL AMOUNT OF ECM*** ***In particular, temporary partners could not have a symbiotic relationship with F_1—as the species of *Peziza* spp. (B) did in this work. ***CONFUSING; A TEMPORARY PARTNER DOES HAVE A SYMBIOTIC RELATIONSHIP DURING THE TIME IT IS A PARTNER*** Also the individuals of *Laccaria cf. laccata* (F) and

Tomentella spp. (A) were present in smaller proportions under elevated O_3, suggesting that the assistance and function of these species to the host F_1 was weak, or that their symbiotic activity was lower than that of the other ECM types (*Suillus* spp.). A study of long-term exposure of aspen-birch to elevated CO_2 and O_3 supports our results (Edwards and Zak, 2011). This study found that *Laccaria* spp. and *Tomentella* spp. declined together with decreased cello-bio-hydrolase activity in an elevated O_3 regime. The ECM colonization rate and diversity were both reduced by elevated CO_2+O_3 relative to the control, but ECM diversity was significantly higher under this combination than under O_3 alone. *Suillus* spp. colonized the same proportion of roots as in the control. We conclude that, under elevated CO_2+O_3, the lower diversity induced by O_3 was compensated for by the effect of CO_2.

The growth (stem diameter and height) and the ECM abundance of F_1 were not accelerated significantly under elevated CO_2. Nevertheless the increased biomass of stem and root demonstrate that F_1 benefited from elevated CO_2. The same result has been observed in seedlings of the Japanese larch (Yazaki et al., 2004); O_3 reduced the stem diameter and height at the end of the first growing season. This is a similar result to Noormets et al. (2001), studying aspen; the growth of two aspen clones was reduced by O_3 (particularly the stem diameter). The tendency of O_3 to inhibit growth of the stem diameter also occurred with potted F_1 plants (Koike et al., 2012). O_3 did not inhibit the growth of F_1 during the second growing season. It appears that F_1 benefited visibly from uptake of nutrient elements by ECM in the second year.

As a symbiotic partners ECM enhances the host plant capability for nutrient uptake of ***WHAT?*** (Buscot et al., 2000). We therefore examined the element composition of above- and below-ground parts of F_1 plants. With the greater ECM colonization under elevated CO_2, we found increased concentrations of P and Mn in needles. This increase might be due to increased uptake as a result of the greater ECM colonization. Phosphorus is an essential macro-element for ATP and NADPH, and is 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 (Raven, 1990; Henriques, 2003). The uptake of P and Mn enhanced the growth of F_1 and strengthened the symbiosis by presenting a greater opportunity for ECM colonization (Cairney, 2011).

The concentrations of K and Mg in the roots of F_1 were increased by O_3. This change may assist in maintaining a stable concentration of Fe in roots, since K is vital to maintenance of the iron balance in roots (e.g. Kraemer, 2004). Fe is also essential in the formation of ECM (e.g. Van Hees et al., 2006). We are led to postulate that beneficial and functional ECM partners were previously selected by host F_1 under elevated O_3. This may explain why the proportions of distinct ECM types in the ECM community were different under elevated O_3 than in the other gas regimes. On the other hand, concentrations of elements in needles – such as Fe and Mo – were reduced by O_3, and this effect might have inhibited the growth of F_1 seedlings. Similar trends have been found by Norby et al. (1986) for oak and by Alina (2001) for many species. The concentrations of N and K did not change significantly, however. Furthermore, the concentrations of Ca and Mg were unaffected, and remained stable in needles of F_1 under elevated O_3. These two elements are important: 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). This might support our hypothesis, ***REMIND READERS BRIEFLY BY STATING HYPOTHESIS IN A SHORT PHRASE*** in that there was little inhibition of photosynthesis. On the other hand, under external stress the allocation of nutrients within the plant 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. This could be the cause of the weak inhibition of photosynthesis in the O_3 treatment. Since the 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, species with lower carbon requirement and/or which utilize carbon effectively are favored (Hoeksema and Kummel, 2003). Further, *Suillus* spp. reportedly reduces the transfer of large quantities of metals towards the plant-fungus interface without impairing normal nutrient uptake to the host plant (Colpaert et al., 2011). In our experiment, the reduction of Al in needles under O_3 was possibly caused by the change in ECM abundance, protecting F_1 from metal toxicity.

The net photosynthetic rate (A_{growth}) at elevated CO_2 and elevated CO_2+O_3 was markedly higher than in the control. Plant growth and root system are usually enhanced under elevated CO_2, and there is accompanying higher water and nitrogen use efficiency of the plant (e.g. Qu et al., 2004; Koike et al., 2010; Norby and Zak, 2011). As a result the biomasses of stem and root were increased by elevated CO_2. We did not find any significant effects on growth and biomass of F_1 of elevated CO_2+O_3, or any interaction effects of the two gases. The value of A_{growth} for seedlings under elevated CO_2 + O_3 was higher than that for seedlings under elevated O_3 alone, indicating the positive effects of elevated CO_2. The impact of O_3 on the growth of F_1 seedlings may be mitigated by elevated CO_2 in association with abundant P uptake by ECM, as reported for beech in central Europe (Matyssek and Sandermann, 2003; Weigt et al., 2012). This could explain why the structure of ECM abundance ***CLARIFY 'STRUCTURE OF ECM ABUNDANCE – DO YOU MEAN SPECIES IN SAME PROPORTION, OR SAME AMOUNT OF COLONIZATION, OR BOTH?*** under elevated CO_2+O_3 was similar to that of the control. The value of G_s was not influenced by any gas treatment, IN THE FIRST YEAR? but it was increased by O_3 in the second growing season. This increased the risk of damage due to O_3; however, the growth of F_1 was found to be unaffected, and A_{growth} was not reduced by O_3 relative to the control in the second year. It is possible that the increased G_s is a driving factor for nutrient uptake by the favoring of particular ECM species under O_3 stress.

5. Conclusion

Elevated CO_2 increased the extent of ECM colonization but not the diversity. The higher value of A_{growth} for F_1 under elevated CO_2 led to increased biomass of below-ground parts and stem, increasing the ECM colonization rate. Elevated O_3 impaired the extent of ECM colonization and number of species abundance; the latter was very different. ***DO NOT USE 'ABUNDANCE' AS SHORT FOR 'SPECIES ABUNDANCE' ANYWHERE!*** Growth of F_1 was reduced by O_3, and the biomass was reduced. The higher value of G_s observed under high O_3 did not cause severe inhibition of photosynthesis, however, due to the uptake of vital elements by specific ECMs. The changed concentrations of individual elements in needles and roots is related to the change in ECM abundance under elevated O_3. This may have contributed to the defense capability of F_1 seedlings against O_3 by selecting those ECM species capable of flourishing under enhanced O_3. This point warrants further study with specific ECM species. Elevated CO_2+O_3 together reduced the extent of colonization and diversity but increased A_{growth}, leading to no overall effect on growth or biomass; this suggests that elevated CO_2 mitigates the harm done by O_3 to photosynthetic capability. 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. Our present results provide information on ECM symbiosis with F_1 seedlings under elevated CO_2 and/or O_3; these results inform us which ar the best ECM species for field inoculation of F_1 to improve plant survival under harsh conditions.

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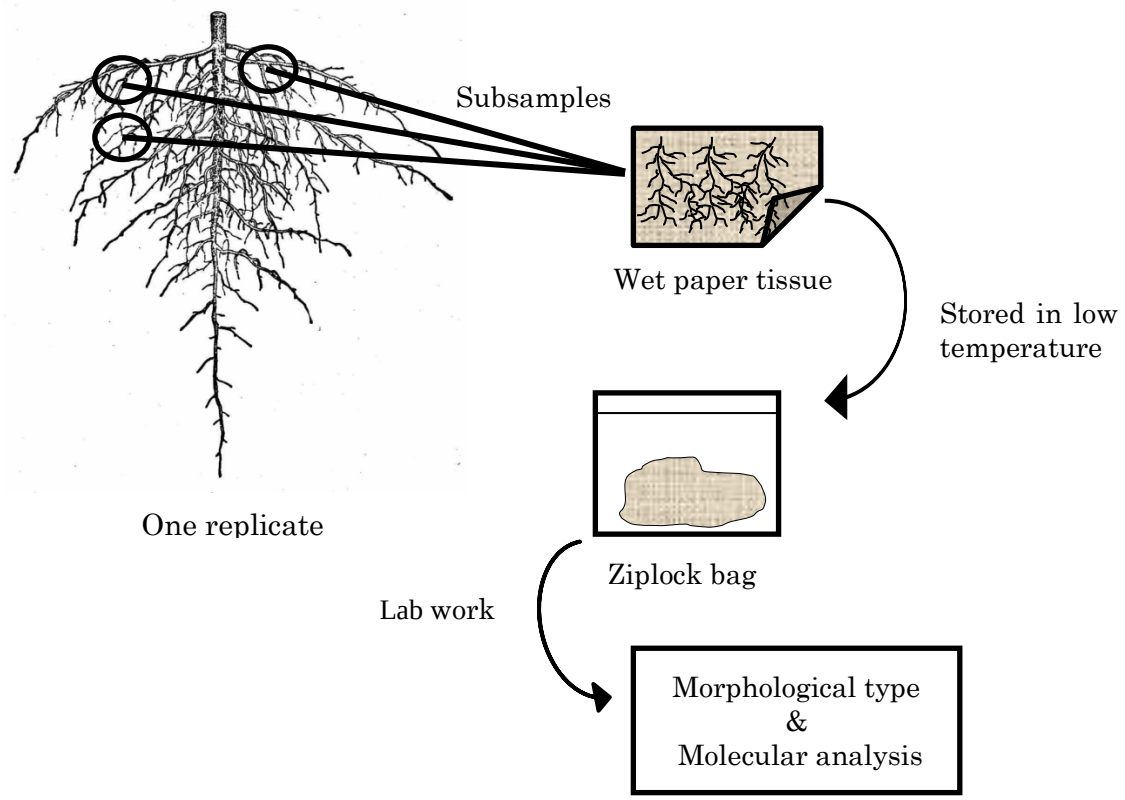
640 **Figures and Tables**



641
642 **Fig. 1.** The sampling process for a single replicate seedling; three subsamples were
643 selected at random.

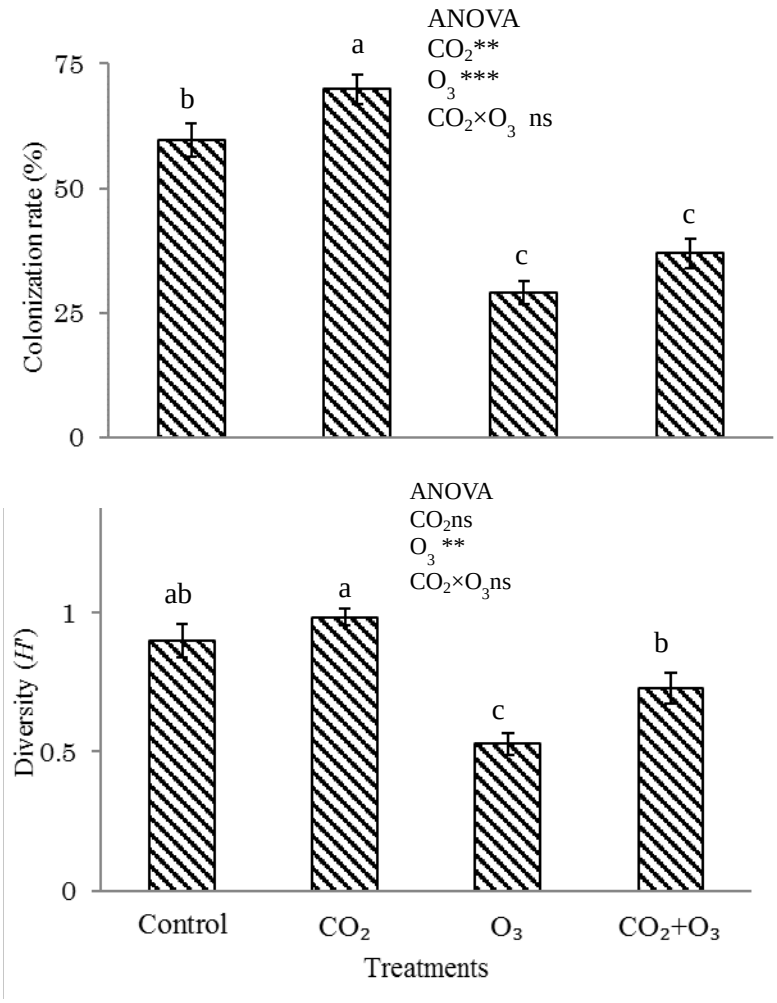


Fig. 2. The ECM colonization rate and diversity on the hybrid larch F_1 with differing gas treatments at the end of the experimental period. The ECM diversity (*H'*) is calculated as Shannon's diversity index; each value is the average of four chamber replications. A vertical bar indicates the standard error. Different character symbols denote a significant difference between the four treatments; $P<0.05$. ANOVA: $^{**}P<0.01$, $^{***}P<0.001$, ns denotes not significant.

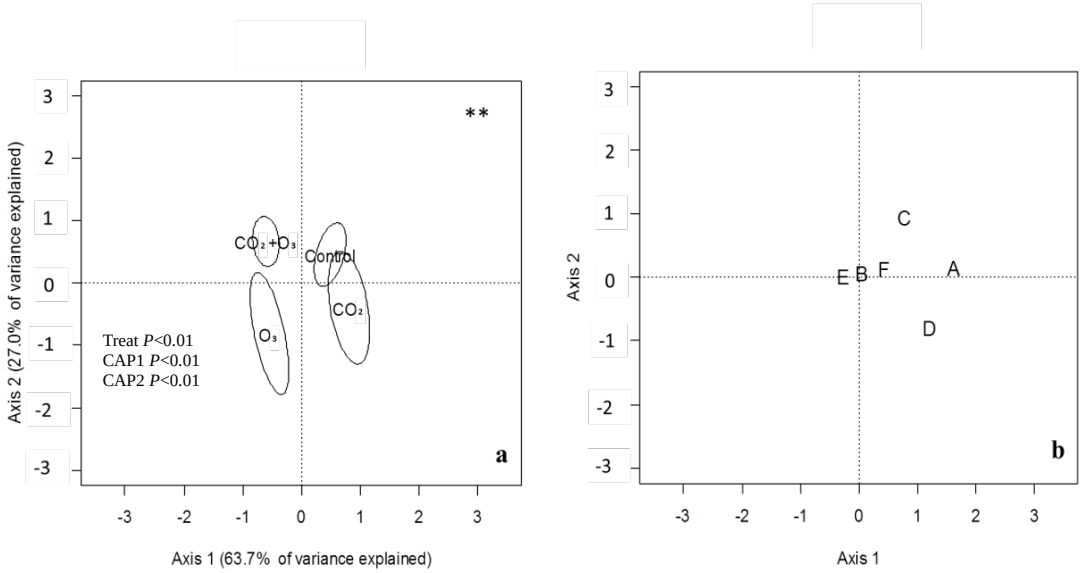


Fig. 3. Abundance of infecting ECM species in response to four fumigation treatments. The colonization rate ***EXTENT?*** involving different ECM species was estimated by distance-based redundancy analysis among the four gas treatments. Distance-based redundancy analysis depends on the matrix generated from the ECM species and the 4 treatments, and derives the eigenvalues and their contribution to the squared Bray distance. ***IF THERE ARE MORE THAN 4 ECM SPECIES THEN THIS MATRIX IS NOT SQUARE AND IT MAKES NO SENSE TO REFER TO TIS EIGENVALUES?*** The results of ANOVA use a permutation test for the cap scale under a reduced model (CAP1, CAP2 and CAP3 are shown). Independent variables were identified as factors ***MEANING UNCLEAR*** (Treat.) CAP1 means axis-1 information, and CAP 2 likewise means axis-2. They are the proportion of the results explained by the difference between each treatment. From the R result we obtain both the direction of axis-1 (P=0.005) and axis-2 (P=0.005), which differ significantly. This results shows that the abundance of colonized ECM species under elevated O_3 and both gases different widely from the control and elevated CO_2 (axis-1 direction). The abundance of ECM species also differs markedly between elevated O_3 and both gases (explained via axi- 2). An asterisk denotes significance: **P<0.01.

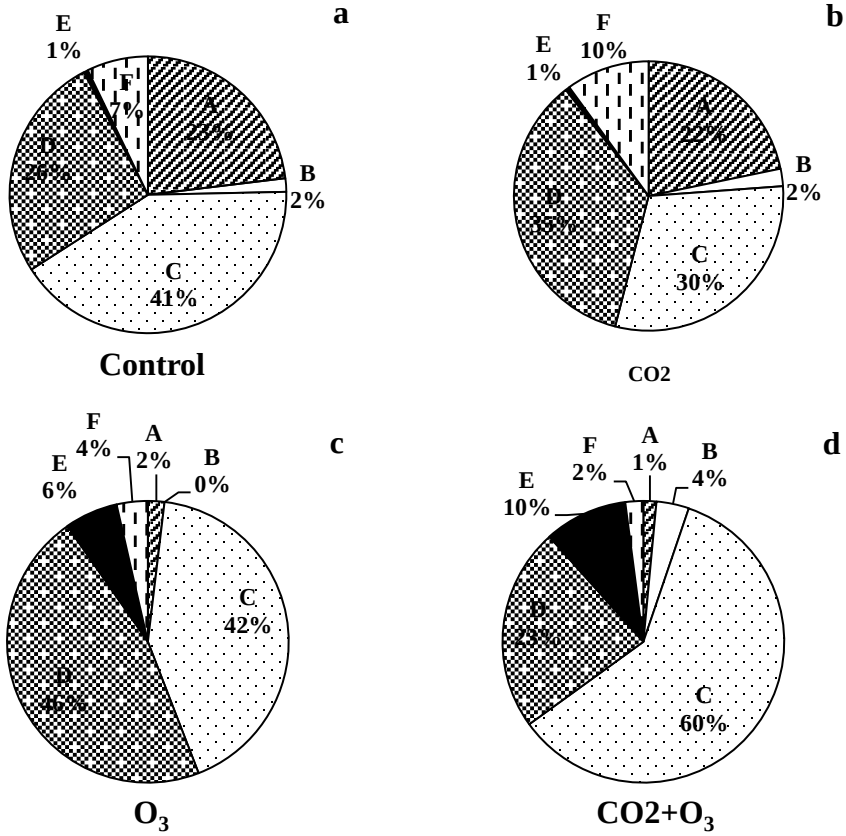


Fig. 4. ECM community: changes of abundance in the four treatments. Each value is the proportion of all types of ECM identified colonizing the hybrid larch F_1 under 4 types of fumigation (**a, b, c, d**). A: *Tomentella* sp. B: *Peziza* sp. C: *Suillus laricinus* D: *Suillus grevillei* E: *Cadophora finlandica* F: *Laccaria cf. Laccata.*

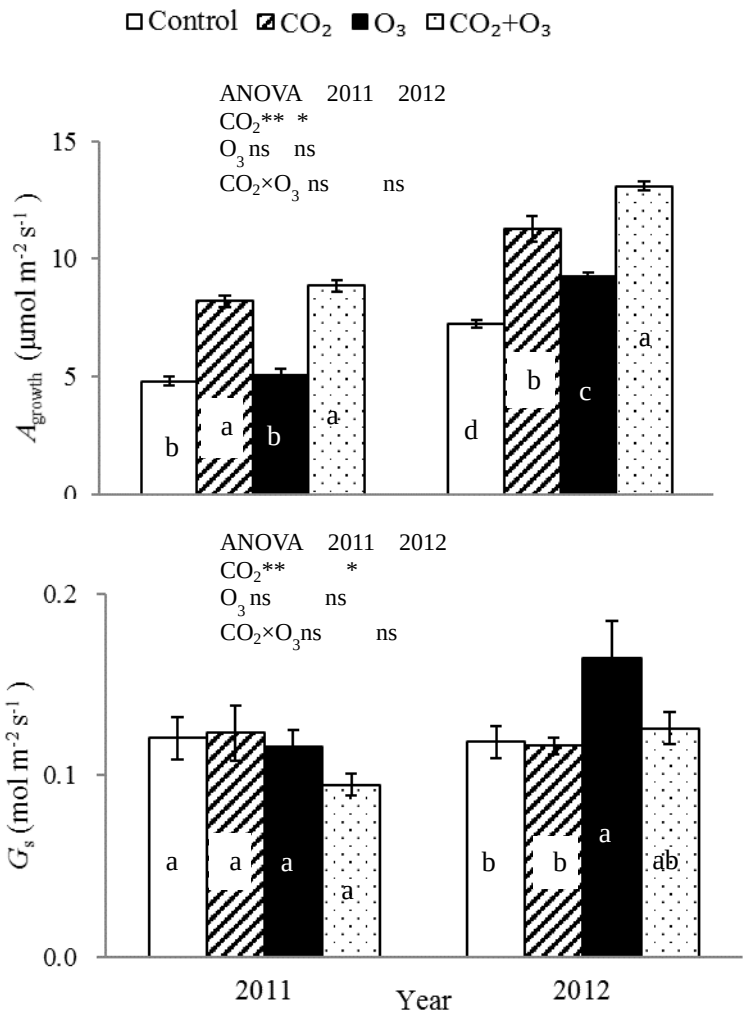


Fig. 5. The net photosynthetic rate at growing [CO_2] concentrations (A_{growth}), and stomatal conductance (G_s). Each value is the mean of four chamber replications, and the vertical bar indicates the standard error. ANOVA: *P < 0.05, **P < 0.01, no asterisk means not significant. Different character symbols denote a significant difference between the four treatments; P < 0.05.

Table 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 2

Morpho-type and genetic identification of ECM species colonizing hybrid larch F_1 seedlings before and after the gas treatments.

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 finlandica*
	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%	*Inocybelacera*
	H	Orange brown	5	b	10	-	AB971281	99%	*Thelephoraceae sp.*

Note: A symbol '+' indicates the presence of emanating hyphae; the number of '+' symbols increases with their number. The symbol '-' means no hyphae.

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

[b] See Agerer, pp. 12i

[c] See Agerer, pp. 13i

Table 3

Growth in height and stem diameter of hybrid larch F_1 seedlings in 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(0.52a	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. ANOVA: *P<0.05, **P<0.01, ns not significant. Different character symbols denotes a significant difference between the four treatments; P< 0.05.

Table 4

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

		Needle	Branch	Stem	Root	Above ground	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× O_3	ns	ns	ns	ns	ns	ns

Each value is the average (SE) of four chamber replications. R/S: Ratio of root biomass to shoot biomass (needle+branch+stem). ANOVA: *P<0.05,**P<0.01. Different character symbols denote a significant difference between the four treatments; P< 0.05.

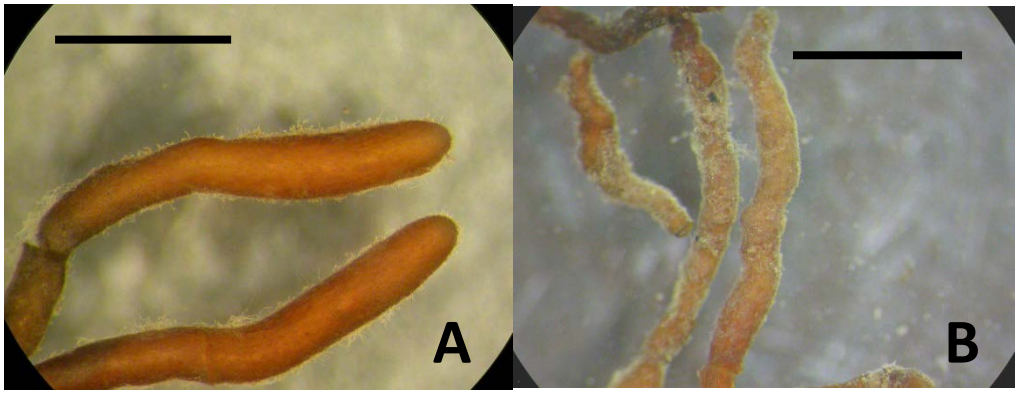
Table 5

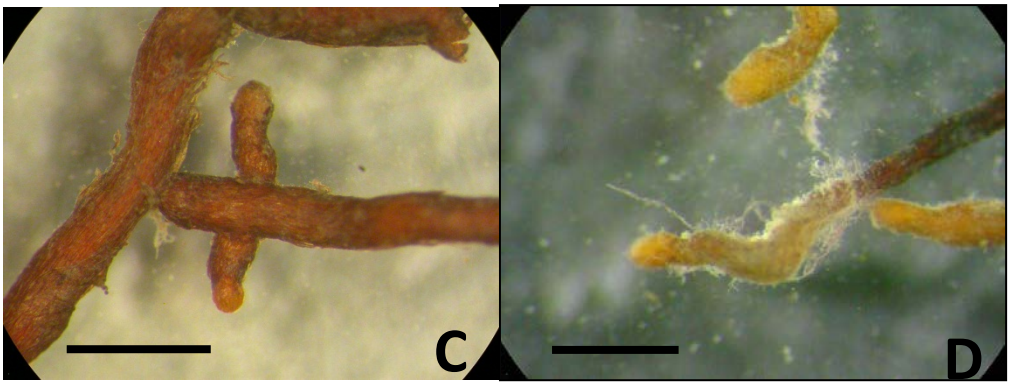
Effect of elevated CO_2 and O_3 on nutrient concentration in needles and roots of seedlings.

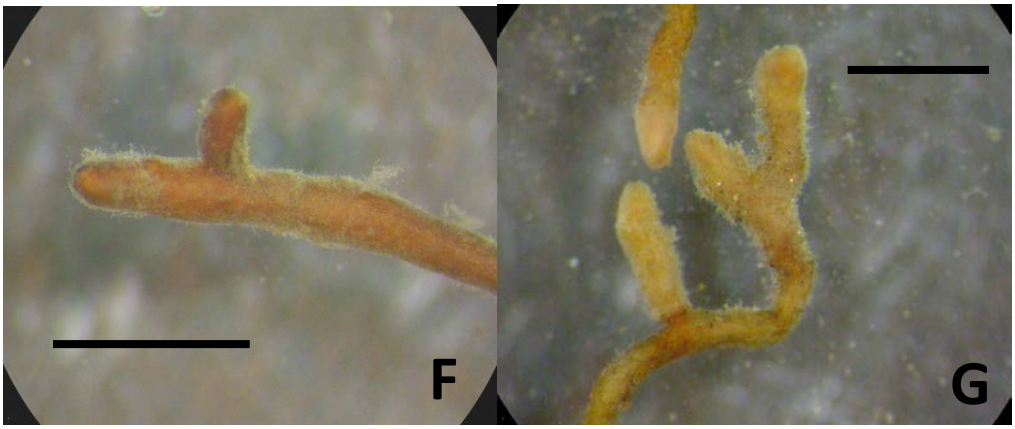
Organs	Treatment	Nutrient concentration (mg/g)								
		N	P	K	Ca	Mg	Al	Fe	Mn	Mo
Needle	Control	20.42(6.16)a	1.62(0.18)b	10.21(0.63)a	7.67(1.76)a	1.25(0.20)a	0.12(0.02)ab	0.14(0.02)a	0.04(0.03)a	0.03(.002)a
	CO_2	19.10(6.37)a	2.09(0.26)b	11.44(1.03)a	5.20(0.78)a	0.96(0.12)a	0.12(0.01)a	0.13(0.03)a	0.09(0.06)a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

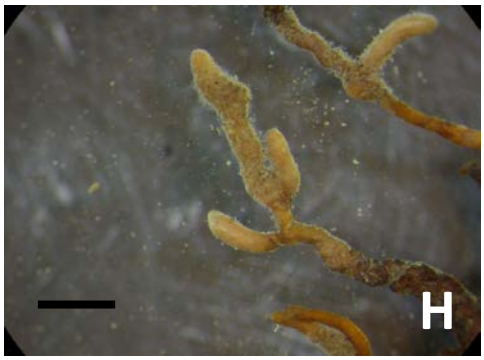
Each value is the average (SE) of four chamber replications. ANOVA: *P<0.05,**P<0.01,***P<0.001, ns not significant. Different character symbols denotes a significant difference between the four treatments; P< 0.05.

Appendix









Note: Each photo shows typical morphology of the different ECM species; black bar=1 mm.

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