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Effects of soil nutrient availability and ozone on container-grown Japanese larch seedlings and role of soil microbes

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Abstract The interactive effects of ozone, soil nutrient availability and root microorganisms on physiological, growth, and productivity traits were studied for the first time for Japanese larch (*Larix kaempferi*) seedlings grown in containers over a growing season, using a free air ozone-concentration enrichment exposure system. High nutrient availability altered leaf and root nutrient dynamics and enhanced plant growth; however, it also enhanced seedling susceptibility to damping-off disease compared to low nutrient availability. Negative effects of elevated ozone, as compared with ambient ozone, on leaf gas exchange and plant stem form were neither offset nor exacerbated by soil nutrient availability and root colonizers. Such negative effects suggest that elevated ozone may have implications for ecological health even when plant vigor is limited by factors other than ozone. Inoculation of roots with ectomycorrhizae had negligible influence on the effects of either soil nutrient availability or ozone. However, this lack of effect may be upon impeded formation of complete mycorrhizal root tips due to factors other than the manipulated variables. B and Na appeared to have an important role in stress responses, so further studies to examine their link with physiological mechanisms as a function of time. This study provides an important perspective for designing forestry practices to enhance seedling health.

Keywords Ectomycorrhizae · Fertilizer · Oxidative stress · Plant–fungi interaction

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Introduction

The degraded quality of the atmospheric environment seen over the last decades is in part due to the increases in the levels of ground-level ozone (O_3) (Akimoto 2003; Akimoto et al. 2015; Lefohn et al. 2018; Nair et al. 2018). Local and regional regulations has helped decrease O_3 levels in parts of the world (Sicard et al. 2013) and the level of air pollutants that interplay in O_3 formation and thus may decrease

or increase O_3 levels (Sicard et al. 2013; Nopmongcol et al. 2017; Li et al. 2019a). Nonetheless, O_3 remains at potentially phytotoxic levels throughout the world, especially in wide O_3-polluted hotspots in Asia (P. Nagashima et al. 2017; Sicard et al. 2017; Li et al. 2018a, 2019a).

Some of the O_3 near plants reacts with volatile organic compounds emitted by plants or is eliminated by leaf trichomes (Li et al. 2016; Oksanen 2018). Other O_3 molecules enter the leaf through stomata during gas exchange processes (Paoletti and Grulke 2005). Elevated O_3 uptake by stomata can inhibit leaf area growth, decrease carbon uptake and Rubisco and chlorophyll content, alter growth, and suppress productivity of forest plants (Paoletti and Grulke 2005; Wittig et al. 2009; Jolivet et al. 2016; Li et al. 2017a, b; Cailleret et al. 2018). Because of the negative O_3 effects on forest plants and associated ecological processes (Karnosky et al. 2005; Ainsworth et al. 2012; Blande et al. 2014; Agathokleous et al. 2016b; Chappelka and Grulke 2016; Braun et al. 2017), we must understand the biology of O_3-induced stress and devise innovative methods to mitigate the deleterious effects of O_3.

From ancient days, plants have been fertilized using natural sources such as manure. In the modern world, soils have deteriorated and their quality is likely to worsen in the 21st century, requiring greater dependence on synthetic fertilizers as is the case for reforestation seedlings, which commonly receive synthetic fertilizers (Yang et al. 2016). Soil fertility significantly affects plant responses to atmospheric gases (Körner 2009; Bielenberg et al. 2001; Terrer et al. 2016; Koike et al. 2018), soil biota and their interactions with plants, including those between mycorrhiza and plants (Jiang-shan et al. 2005; Terrer et al. 2016; Wang et al. 2018). How soil fertility affects tree–mycorrhizal symbioses under elevated O_3, and plant response to O_3 overall, has received little attention and remains poorly understood.

Plant-associated soil biota can enhance the tolerance of plants to environmental stress and protect ecosystem stability (Futai et al. 2008; Yang et al. 2018). Mycorrhizae are fungi present in the soil at large scales, and with the rest of the soil microbial community, can drive plant and ecosystem functions (Bolan 1991; van der Heijden et al. 1998). Mycorrhizae enable plants to absorb more nutrients, especially phosphorus (P), and water in exchange for photosynthates (Bolan 1991). Ectomycorrhizae (ECM), which colonize roots without penetrating them, form mutualistic associations with a large proportion of tree species (Futai et al. 2008; Cui and Mu 2016; Wang et al. 2018). Such symbioses can promote plant tolerance to stress (Yin et al. 2016). Preliminary experimentation suggests that fertilization and elevated O_3 can alter the diversity of the ECM community and that ECM can enhance the tolerance of associated larch trees to air pollution (Wang et al. 2015, 2018), indicating that ECM are economically important for forestry practice

and ecosystem stability. Such research highlights an important area for further studies on soil fertility–ECM–O_3 interaction with potential applications in forestry practice.

Japanese larch [*Larix kaempferi* (Lamb.) Carr] is an ectomycorrhizal coniferous tree species widely distributed in Northeast Asia and planted in wide forested areas of Japan (Fuchen et al. 1998; Ryu et al. 2009). However, this fast-growing species is also susceptible to diseases (Ryu et al. 2009) and sensitive to O_3 (Wang et al. 2015; Agathokleous et al. 2017; Sugai et al. 2018;) and other stresses (Li et al. 2018b; Wang et al. 2018). Soil fertilization also changes the symbiosis between Japanese larch roots and ECM (Wang et al. 2018) as does elevated O_3 for a hybrid larch (*L. gmelinii* var. *japonica* × *L. kaempferi*), suggesting that Japanese larch may also be similarly affected because it shares half of its genome with this hybrid (Wang et al. 2015). These studies highlight the need for more studies on the Japanese larch association with ECM under O_3 and on how the performance of O_3-stressed plants may change as soil fertility changes.

The purpose of this study was to assess the effect of ECM on container-grown Japanese larch seedlings, and the influence of soil nutrients on their interaction under ambient and elevated O_3 levels, in a free air O_3-concentration enrichment (FACE) system. We hypothesized that ECM can enhance O_3 tolerance of plants, which would suppress physiology and inhibit growth of plants due to lower photosynthetic efficiency. However, we hypothesized that the degree of protection will depend on the fertility of the soil because plants may form fewer ectomycorrhizal root tips when nutrients are abundant and directly available.

Materials and methods

Experimental area

This experiment was done at the Sapporo Experimental Forest of Hokkaido University, Sapporo, Japan (43°04′N, 141°20′E, 15 m a.s.l.) in 2017. Meteorological conditions for this area are presented in (Supplementary Material Table S1).

Plant materials and treatments

In 2016, seeds of Japanese larch (*L. kaempferi*) were collected from mother trees naturally grown in Hokkaido, Japan, and stored at low temperature in an incubator. In spring 2017, the seeds were disinfected, then placed on wet paper filter in Petri dishes at room temperature to germinate. Seeds germinated after 10–12 days (67% germination). On May 30, among 400 seedlings, 180 healthy-looking seedlings were transplanted into 180 pots (5.5 cm diameter, 13 cm height, 0.2-L volume) filled with coco peat

(topcocopeat, Top, Osaka, Japan) and irrigated until soil run-off. The pots were placed onto plastic flats that had multiple slots to hold the small pots according to recent reforestation practices in Japan because container-grown seedlings have higher survival rates than bare-root seedlings (Masaki et al. 2017). From this point, the soil substrate was kept continuously moist using tap water, until plants were moved to the field. Plants were kept on tables in a glasshouse of Hokkaido University (43°04′N, 141°20′E, 15 m a.s.l.), with open windows and uncontrolled environment.

The day after transplanting, each plant was treated with 1 g (low fertilization, hereafter LF) or 2 g (high fertilization, hereafter HF) slow-release fertilizer (Osmocote Exact Standard 8-9M, 15-9-11 + 2MgO + TE; Hyponex Japan, Osaka, Japan).

On July 8, plants were inoculated with ECM. Briefly, inoculum was obtained from freshly excavated root systems of 5-year-old Japanese larch saplings maintained in the Sapporo Experimental Forest for this purpose. Tap water (5 L) in two buckets was infested with ECM by agitating ECM-infected roots for 10 min. The water of the one bucket was subsequently sterilized by boiling for 40 min. After sterilization, 5 L of cold tap water was added to each bucket. The glasshouse was separated into two similar sides by closing a glass door in the middle. Half of the plants from each fertilization treatment were moved to the other half of the glasshouse. Plants in the one half of the glasshouse received 100 mL of sterilized water each (hereafter, non-ECM). Plants in the other half received 100 mL of non-sterilized water each (ECM).

On July 21, damping off symptoms were observed on some seedlings of both ECM treatments. Seedlings with such symptoms were moved to a field with no nearby vegetation, and, all the remaining non-ECM plants were treated (a dilution of 1000) with a fungicide (Sankyo Thiuram 80, HOKUSAN, Kitahiroshima, Hokkaido, Japan). ECM plants were treated with only water. All plants were treated at night when stomata were closed. This treatment was repeated 9 days later. With the same methods, on July 25, non-ECM plants were sprayed (1:000) again with fungicide (Tachigaren, MITSUI CHEMICALS AGRO, Tokyo, Japan). This treatment was repeated on August 1.

The experimental design, for the duration plants remained in the glasshouse, was fully randomized, and the position of the plants was rotated on a weekly basis.

On August 2, all plants were moved outside to moderate shade to acclimate to field conditions, then transferred to the FACE system on August 11. Plants were not irrigated manually for the duration of the O_3 treatments.

The O_3 treatments in the FACE system of Hokkaido University, Sapporo, Japan (Agathokleous et al. 2017) included ambient O_3 (AOZ) and elevated O_3 (EOZ). For EOZ, O_3-enrichment was performed on a daily basis during the daytime, from August 18 to October 28. To increase the experimental robustness by accounting for environmental variability, two subplots were created in each FACE plot by placing 3–4 pots/plants per fertilizer and ECM treatments in a multiwall plastic flat (completely randomized); one flat was placed on the south side of each plot, and the other on the north side of each plot for a total of 18 plants on average per fertilizer, ECM, and O_3 treatments.

The plants in each O_3 treatment were rotated among the experimental units approximately every 10 days. Ambient O_3 levels were recorded continuously (1-min intervals) using an ultraviolet absorption O_3 analyzer (TUV-1100; Tokyo Industries, Tokyo, Japan). The average daily 10-h (08:00–18:00 h Japan Standard Time, JST) ambient O_3 level over the treatment period was 39.1 ($\pm$ 6.0 SD) nmol mol^{-1}. In the three EOZ plots, the average daily 10-h (07:00–17:00 h JST) O_3 level over the treatment period was 65.2 ($\pm$ 4.3 SD) nmol mol^{-1}, i.e. ~ 1.7 times the ambient O_3 level.

Data collection

Gas exchange

Gas exchange was measured in situ using a LiCor gas exchange systems (LI-6400, Li-Cor, Lincoln, NE, USA) from 8 to 10 October. Stomatal conductance (Gs_{390}), photosynthetic rate (A_{390}) and transpiration rate (E_{390}) were determined at 390 µmol mol^{-1} CO_2, 60 $\pm$ 5% relative air humidity, and 1500 µmol m^{-2} s^{-1} light. Leaf temperature was maintained at 25 °C. After recording Gs_{390} and A_{390}, the light was set to 100 µmol m^{-2} s^{-1}, and Gs_{390} and A_{390} were recorded again 15 min later. The response of Gs_{390} and A_{390} to light was calculated as the percentage of change in the values of Gs_{390} and A_{390} recorded at 100 µmol m^{-2} s^{-1} (Gs_{100} and A_{100}) relative to those recorded at 1500 µmol m^{-2} s^{-1} (Gs_{1500} and A_{1500}). Gas exchange was measured in randomly selected sunlit mature needles, and the projected area of the measured needles was estimated (software LAI-32 v. 0.377e, Dr. Yamamoto K, Graduate School of Bioagricultural Sciences, Nagoya University, Japan) after scanning the needles (Canon LIDE 40, Tokyo, Japan). Gas exchange was measured in the morning and completed by 11:00 h JST.

Growth

Growth traits were measured on each plant on November 2. The stem height was measured as the distance from the soil surface to the top of the stem. The plant height was measured as the distance from the soil surface to the top of the crown. The height at which the crown starts (from the soil surface) was also measured, and the depth of the crown was estimated by subtracting the height at which the crown starts from the plant height. The crown span was measured by

taking two crosswise measurements (the two widest points of the crown) using a measuring tape with 1-mm scaling. The stem basal diameter was also measured by taking two cross measurements per plant using a digital vernier caliper with 0.001 accuracy.

Productivity

The seedlings were harvested on December 7 and separated into leaves, shoots and stem. Roots were washed gently to remove all soil particles, and their length was measured. Samples were air-dried at 75 °C to constant mass. The buds on each plant were counted. Stems (with branches) and foliage were weighed for each plan. Shoot dry matter was calculated by summing the foliage dry matter and stem dry matter per plant.

Root ECM traits

At the end of the experiment, roots were analyzed only for seedlings grown in the presence of LF to confirm the effectiveness of the ECM treatment. Therefore, three plants were analyzed for each O_3 treatment for the ECM-treated plants and four for each O_3 treatment for the non-ECM-treated plants. The plants of each treatment had similar heights, and ECM ($F = 0.20$, $P = 0.664$), $O_3 \times$ ECM ($F < 0.01$, $P = 0.981$) and ECM $\times O_3$ ($F = 1.92$, $P = 0.197$) had no statistically significant effect. The root traits analyzed were (1) total number of root tips with fully formed mycorrhizae (with a perfect mantle, permitting categorization into morphological types), (2) total number of nonmycorrhizal roots, (3) total number of root tips with mycorrhiza forming (i.e., incomplete mycorrhizae with no mantle), and (4) total number of roots with no root tip or aerial cut of root tip. The analyses were conducted using an electronic stereoscope connected to a computer. The total number of roots was calculated as the sum of the four measured root traits. The total number of root tips with fully formed mycorrhizae was 0 for all the measurements.

DNA of the ITS1–ITS2 regions (5.8S rDNA) was analyzed. The basic local alignment search tool (BLAST) for the GenBank database of NCBI was used to identify the symbiotic microorganisms as described previously (Wang et al. 2015).

Survival from damping off

By the end of the experiment (November), seedlings with damping off in August were dead. These dead plants were counted, and the survival rate of plants in each fertilization treatment was calculated as $(N_{\text{alive}}/N_{\text{total}}) \times 100$ where N_{alive} is the number of alive/healthy plants and N_{total} is the initial total number of healthy plants before damping off.

Leaf and root chemical composition

After the harvest, the dried foliage from one or two plants was pooled to obtain a composite sample for analysis, then ground to a fine powder. The same was done for the roots. These samples were digested in HNO_3 using 100 mL PP tubes (DigiTUBES, SCP Science, New York, USA) and a thermal unit (DigiPREP, SCP Science, New York, USA) at 105 °C. The digested residue was diluted with 2% (v/v) HNO_3 and filtered through a 0.45 μm Teflon membrane filter (DigiFILTER, SCP Science). The Al, B, Ca, Fe, K, Mg, Mn, Na, P, and Zn concentrations in the samples were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700a, Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis of data

When more than one measurement was taken per plant, the data were averaged per plant. Because all the plants were rotated among the experimental units of each O_3 treatment, for each response variable, the plant-level data ($n = 8$–20 per experimental condition) were allocated to three groups using sampling without replacement. The data for each group ($n = 2$–7) were then averaged to give one robust estimate per virtual experimental unit (i.e., statistical unit), hence, resulting in 3 values per experimental condition ($n = 3$) for further statistical testing. This process was followed for all the response variables except seedling survival.

A level of statistical significance of $\alpha = 0.05$ was chosen a priori. Data were tested for normality with a χ^2 test. Data for survival rate, stem height, crown depth and crown width in November, growth (except stem diameter), production and nutrient data, Gs_{100} and A_{100}, and root tip traits deviated significantly ($P < 0.05$) from normal distribution and thus were transformed using a Box–Cox transformation (Box and Cox 1964), as described previously (Agathokleous et al. 2016a).

Survival rate data were matched to a sigma-restricted type III generalized linear model (GLM) with Fertilizer as fixed factor. Data for gas exchange, light response, growth and production were subjected to a sigma-restricted type III GLM with fertilizer, ECM and O_3 as fixed factors and plot as the random factor. Data for root tip traits were subjected to a GLM with a sigma-restricted type VI parameterization; ECM and O_3 were fixed factors. When GLMs revealed a significant interaction, Bonferroni test followed for multiple comparisons among the experimental groups. The results are reported as mean $\pm$ SE. EXCEL 2010 (Microsoft, Redmond, CA, USA) and STATISTICA v.10 (StatSoft, Tulsa, OK, USA) software was used for all data processing and statistics.

Fig. 1 Mean values ± SE ($n = 3$) for gas exchange variables in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). A_{390}, net photosynthetic rate; Gs_{390}, stomatal conductance; and E_{390}, transpiration rate. An asterisk indicates significant single effect and "ns" indicates nonsignificant single effect, within a response variable, according to a generalized linear model at $\alpha = 0.05$. The only significant interaction was Fertilizer × ECM for E_{390}; in particular, between plants treated with ECM in LF and non-ECM plants in LF (Bonferroni post hoc test)
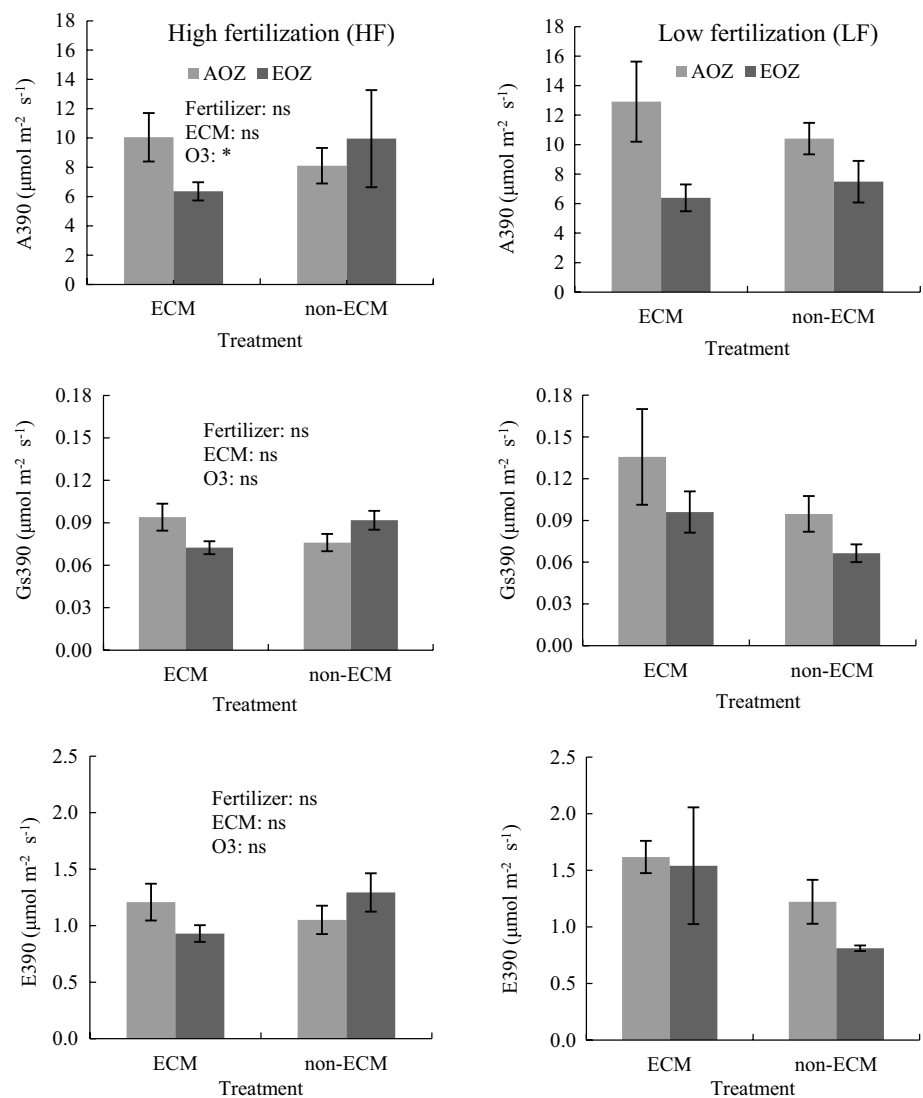
Results

Gas exchange

A_{390} was 29% lower in EOZ than in AOZ and was not significantly affected by other treatments (Fig. 1, Table 1). Although Gs_{390} was 18% lower in EOZ than in AOZ, the difference was statistically nonsignificant. Gs_{390} was not significantly affected by other treatments. However, the only factors significantly affecting E_{390} were the interactions Fertilizer × ECM and Fertilizer × ECM × O_3. For the interaction Fertilizer × ECM, the only significant difference among groups was a 60% higher E_{390} in plants treated with ECM in LF than in non-ECM plants in LF. Regarding the interaction Fertilizer × ECM × O_3, there was only one significant difference among groups, which had no biological relevance (ECM × LF × AOZ vs. non-ECM × LF × EOZ).

The response of Gs_{390} and A_{390} to light (%Gs_{390} and %A_{390}) was not significantly affected by any treatment (Fig. 2, Table 1).

Plant growth

Stem height was significantly affected by fertilizer only, with HF plants having 1.29 times higher stem height than LF plants (Fig. 3, Table 1). Roots were 20% longer in AOZ than in EOZ (Fig. 3, Table 1). Crown depth was affected only by fertilizer, with HF plants having 1.18 times greater crown depth than LF plants (Fig. 4, Table 1). Crown span was increased by HF (+17%), compared to LF, and decreased by EOZ (−8%), compared to AOZ; no other factors were significant. Stem diameter was higher in HF (+11%) than in LF, lower in EOZ (−21%) than in AOZ, and lower in ECM plants (−10%) than in non-ECM plants; interactions were nonsignificant (Fig. 3, Table 1).

Table 1 Statistical results of gas exchange, light response and growth traits

| Gas exchange	Fertilizer	ECM	O_3	Fertilizer×O_3	Fertilizer×ECM	ECM×O_3	Fertilizer×ECM×O_3
A_{390}	$F=0.27$, $P=0.614$	$F=0.08$, $P=0.788$	**$F=7.7, P<0.05$**	$F=1.57$, $P=0.228$	$F=0.04$, $P=0.848$	$F=2.55$, $P=0.130$	$F=0.22$, $P=0.644$
Gs_{390}	$F=0.63$, $P=0.441$	$F=2.50$, $P=0.134$	$F=3.45$, $P=0.082$	$F=2.29$, $P=0.150$	$F=3.29$, $P=0.088$	$F=0.75$, $P=0.401$	$F=3.02$, $P=0.102$
E_{390}	$F=0.39$, $P=0.542$	$F=2.58$, $P=0.128$	$F=3.80$, $P=0.069$	$F=2.60$, $P=0.126$	**$F=7.68$, $P<0.05$[a]**	$F=0.33$, $P=0.574$	$F=4.37$, $P=0.053$
Light response							
Gs_{100}	$F=0.19$, $P=0.669$	$F=0.01$, $P=0.934$	$F=1.13$, $P=0.304$	$F=0.69$, $P=0.418$	$F=0.57$, $P=0.462$	$F=0.16$, $P=0.695$	$F=0.15$, $P=0.709$
A_{100}	$F=1.54$, $P=0.232$	$F=0.08$, $P=0.786$	$F=2.29$, $P=0.150$	$F=0.73$, $P=0.405$	$F=0.29$, $P=0.599$	$F<0.01$, $P=0.974$	$F=0.01$, $P=0.930$
Growth							
Stem height	**$F=13.65$, $P<0.01$**	$F=2.26$, $P=0.152$	$F<0.01$, $P=0.994$	$F=0.04$, $P=0.847$	$F<0.01$, $P=0.992$	$F=0.04$, $P=0.850$	$F=1.90$, $P=0.188$
Stem diameter	**$F=7.04$, $P<0.05$**	**$F=6.86$, $P<0.05$**	**$F=35.07$, $P<0.001$**	$F=0.01$, $P=0.908$	$F=3.61$, $P=0.076$	$F=3.56$, $P=0.078$	$F=1.33$, $P=0.266$
Crown depth	**$F=7.79$, $P<0.05$**	$F=1.04$, $P=0.324$	$F=1.37$, $P=0.259$	$F=0.04$, $P=0.852$	$F=1.21$, $P=0.288$	$F=0.10$, $P=0.752$	$F=0.76$, $P=0.396$
Crown span	**$F=16.54$, $P<0.001$**	$F=4.35$, $P=0.053$	**$F=5.02$, $P<0.05$**	$F=0.40$, $P=0.536$	$F=0.88$, $P=0.362$	$F=0.10$, $P=0.762$	$F=3.01$, $P=0.102$
Root length	$F=0.03$, $P=0.877$	$F=0.01$, $P=0.911$	**$F=7.45$, $P<0.05$**	$F=0.77$, $P=0.394$	$F=0.04$, $P=0.843$	$F=0.20$, $P=0.658$	$F=0.34$, $P=0.567$

The data were tested using a generalized linear model ($\alpha=0.05$). Values in bold indicate statistical significance. Data are presented in Figs. 1, 2, 3 and 4

[a]The only significant difference among groups was between plants treated with ECM in LF and non-ECM plants in LF

Fig. 2 Mean values $\pm$ SE ($n=3$) of light response variables in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). A_{390}, net photosynthetic rate; Gs_{390}, stomatal conductance. Values are percentage of change in Gs_{390} and A_{390} recorded at 100 μmol m^{-2} s^{-1} (Gs_{100} and A_{100}) from those recorded at 1500 μmol m^{-2} s^{-1} (Gs_{1500} and A_{1500}). None of the single or interactive effects were significant according to a generalized linear model at $\alpha=0.05$
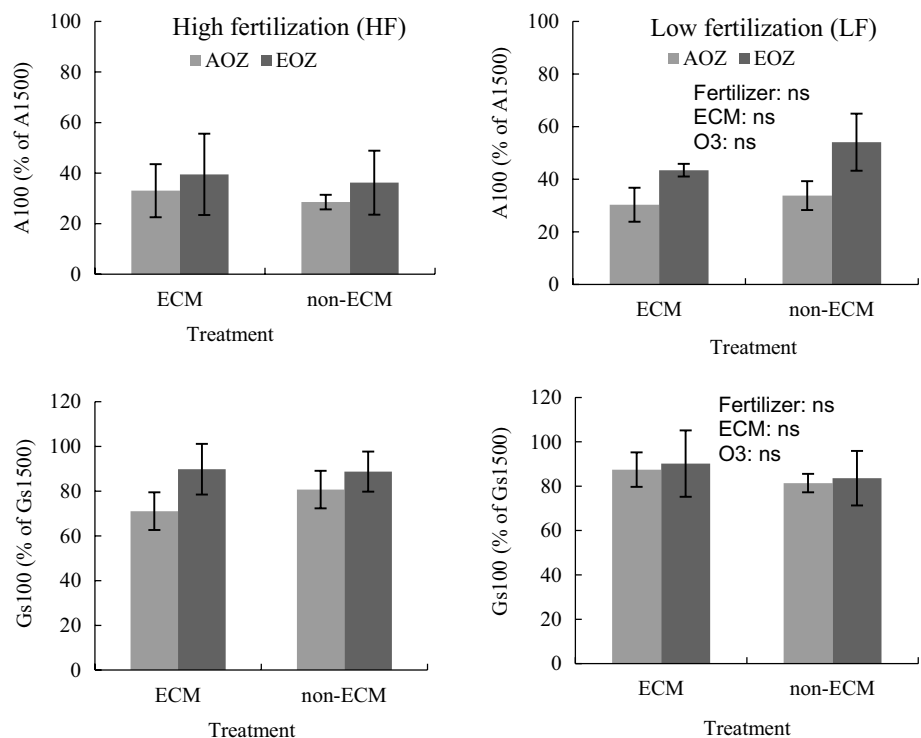
Fig. 3 Mean values ± SE
($n = 3$) of plant growth traits of
Japanese larch seedlings grown
in two soil fertilization regimes,
treated with ectomycorrhizae
and no fungicide (ECM) or
sterilized water and fungicide
(non-ECM) and exposed to
ambient (AOZ) or elevated
(EOZ) ozone (O_3). An asterisk
indicates significant single
effect and "ns" indicates non-
significant single effect, within
a response variable, according
to a generalized linear model at
$\alpha = 0.05$. No interactions were
significant
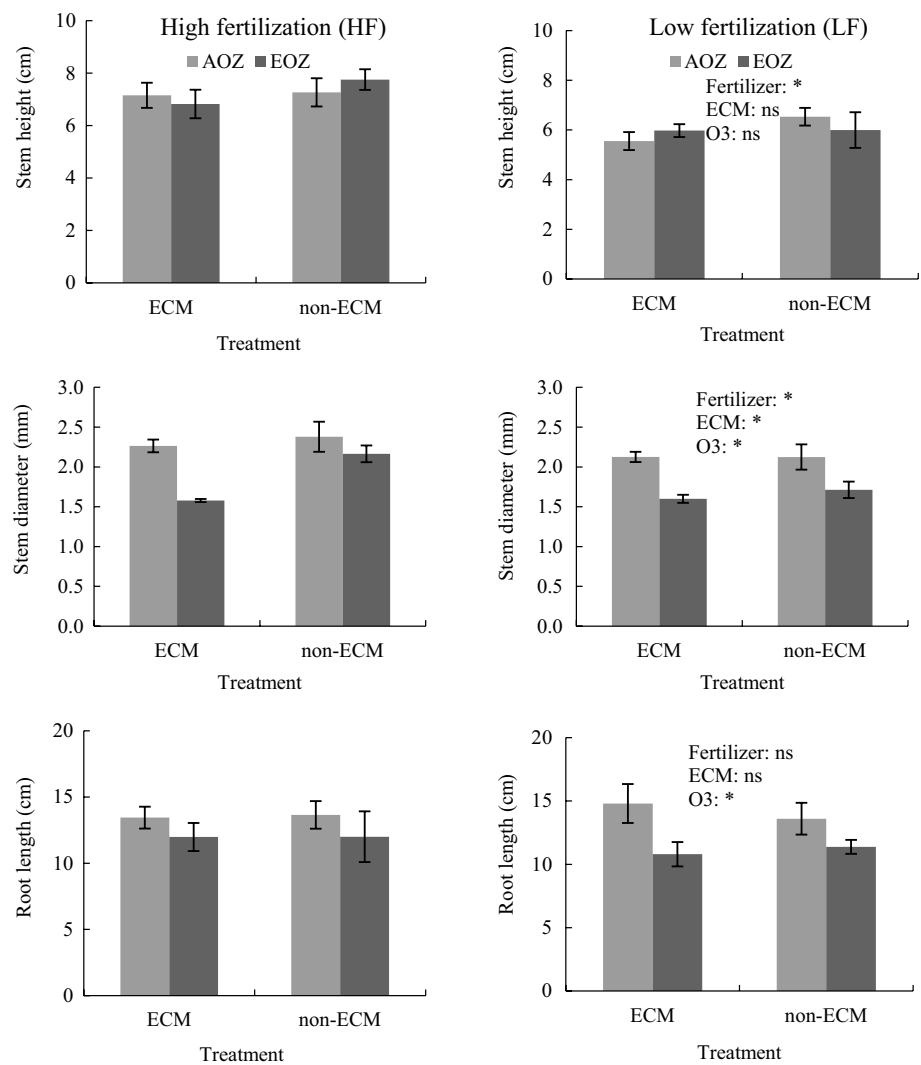
Plant productivity

Plants in HF (mean 14.9) had 55% more buds than plants in
LF (mean 9.6) (Table 2, Supplementary Materials Fig. S1).
Roots, foliage, stems and total plant dry mass were not sig-
nificantly affected by any treatment (Supplementary Mate-
rial Fig. S2), as was also the case for the ratio of root dry
mass to foliage dry mass (R/F) and root dry mass to root
dry mass (R/S) (Supplementary Material Fig. S3), number
of buds per root dry matter, and number of buds per total
plant dry mass (Table 2). While the mean dry mass was
much higher in HF than in LF, there were large variances
(Supplementary Material Fig. S2), and the results were non-
significant (Table 2).

Root-ECM traits

Analysis of root as to ECM-forming capacity (Fig. 5)
revealed that the values of non-mycorrhizal roots, forming

mycorrhizal roots, no root tip roots, and total number of
root tips of ECM-treated plants were on average 197, 183,
210, and 185% of the values of non-ECM-treated plants,
respectively (Table 3). O_3, as a single factor, was nonsig-
nificant for all traits. The ECM × O_3 interaction was signif-
icant for nonmycorrhizal roots and no-root-tip roots only.
Nonmycorrhizal roots did not differ significantly between
non-ECM-treated and ECM-treated plants in AOZ; how-
ever, in EOZ, the mean value was 7.8 times lower in non-
ECM-treated plants than in ECM-treated plants (Table 3).
Similarly, no root tip roots differed significantly between
non-ECM-treated and ECM-treated plants in AOZ; how-
ever, in EOZ, the mean value was 4.4 times lower in non-
ECM-treated plants than in ECM-treated plants.

The BLAST analysis revealed that ECM-treated seed-
lings were infected only with *Fusarium* sp. and untreated
seedlings were infected with *Suillus grevillei* and *Doth-
ideomycetes* sp.

Fig. 4 Mean values $\pm$ SE ($n = 3$) for crown growth traits of Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). An asterisk indicates significant single effect and "ns" indicates non-significant single effect, within a response variable, according to a generalized linear model at $\alpha = 0.05$. No interactions were significant
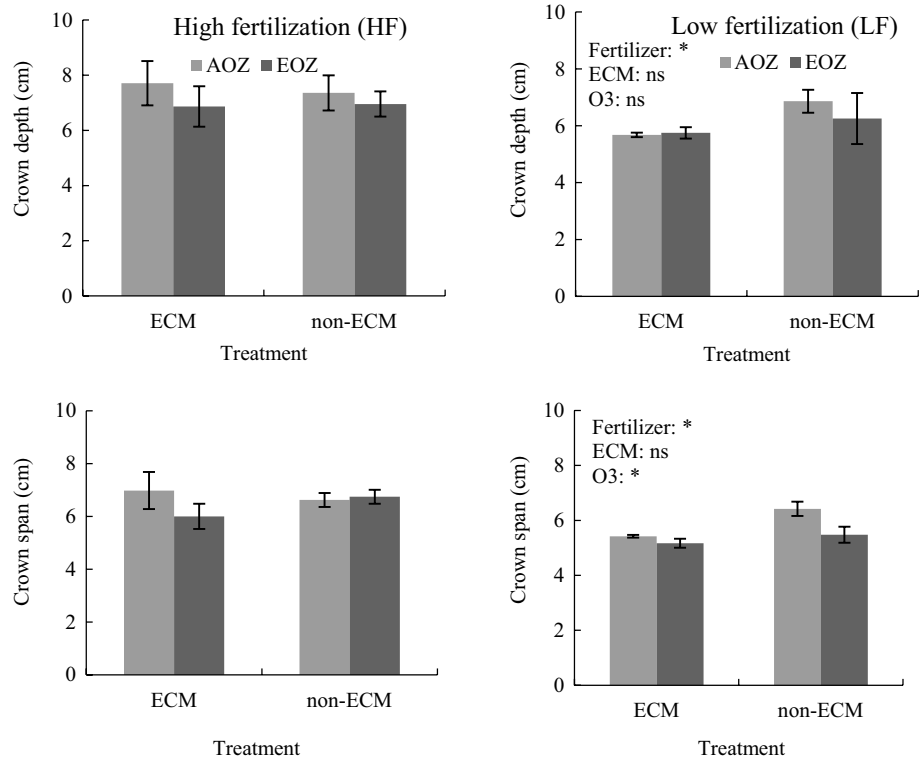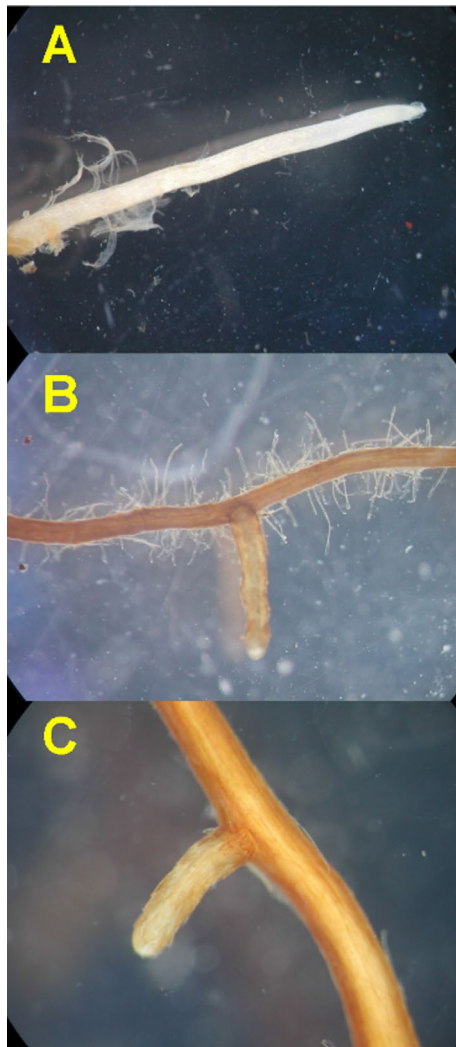

Table 2 Statistical results of production traits

Trait	Fertilizer	ECM	O_3	Fertilizer $\times$ O_3	Fertilizer $\times$ ECM	ECM $\times$ O_3	Fertilizer $\times$ ECM $\times$ O_3
No. of buds	$F = 4.72$, $P < 0.05$	$F = 0.42$, $P = 0.529$	$F = 0.01$, $P = 0.923$	$F = 0.20$, $P = 0.662$	$F = 0.13$, $P = 0.724$	$F = 1.03$, $P = 0.325$	$F = 1.18$, $P = 0.294$
Root dry mass	$F = 2.04$, $P = 0.172$	$F = 0.14$, $P = 0.717$	$F = 1.82$, $P = 0.196$	$F = 0.03$, $P = 0.862$	$F = 0.17$, $P = 0.687$	$F = 0.47$, $P = 0.504$	$F = 0.60$, $P = 0.449$
Foliage dry mass	$F = 4.28$, $P = 0.055$	$F = 0.01$, $P = 0.919$	$F = 1.05$, $P = 0.322$	$F = 0.05$, $P = 0.833$	$F = 0.02$, $P = 0.885$	$F = 0.19$, $P = 0.666$	$F = 0.64$, $P = 0.437$
Stem dry mass	$F = 2.64$, $P = 0.124$	$F = 0.60$, $P = 0.451$	$F = 0.28$, $P = 0.607$	$F = 0.45$, $P = 0.513$	$F < 0.01$, $P = 0.965$	$F = 0.11$, $P = 0.741$	$F = 0.06$, $P = 0.817$
Plant dry mass	$F = 2.79$, $P = 0.114$	$F = 0.17$, $P = 0.686$	$F = 1.10$, $P = 0.309$	$F = 0.03$, $P = 0.864$	$F = 0.07$, $P = 0.799$	$F = 0.27$, $P = 0.612$	$F = 0.46$, $P = 0.510$
R/F ratio	$F = 0.76$, $P = 0.395$	$F = 0.28$, $P = 0.603$	$F = 1.86$, $P = 0.191$	$F = 1.13$, $P = 0.304$	$F = 0.59$, $P = 0.453$	$F = 0.63$, $P = 0.440$	$F = 0.09$, $P = 0.763$
R/S ratio	$F = 0.47$, $P = 0.503$	$F = 0.04$, $P = 0.851$	$F = 2.95$, $P = 0.105$	$F = 2.81$, $P = 0.113$	$F = 1.19$, $P = 0.291$	$F = 1.09$, $P = 0.312$	$F = 0.73$, $P = 0.406$
Buds/root dry mass	$F = 0.75$, $P = 0.399$	$F < 0.01$, $P = 0.968$	$F = 2.00$, $P = 0.176$	$F = 0.13$, $P = 0.720$	$F = 0.81$, $P = 0.383$	$F = 0.08$, $P = 0.776$	$F < 0.01$, $P = 0.949$
Buds/total dry mass	$F = 0.01$, $P = 0.924$	$F = 0.01$, $P = 0.919$	$F = 2.98$, $P = 0.104$	$F = 0.67$, $P = 0.425$	$F = 1.17$, $P = 0.296$	$F = 0.23$, $P = 0.637$	$F = 0.01$, $P = 0.932$

The data were tested using a generalized linear model at $\alpha = 0.05$. Values in bold indicate statistical significance. R/F, ratio of root dry mass to foliage dry mass;, R/S, ratio of root dry mass to root dry mass; buds/root mass, number of buds per root dry mass; buds/total dry mass, number of buds per total plant dry mass. Data are presented in Supplementary Material S1–S3

Leaf and root nutrient content

Fertilizer was a significant factor for Fe, K, Mn, Na and Zn content in the leaves and for K, Mn and P content in the roots (Table 4). Leaves of seedlings grown in LF had 42.7% higher content of Na but 17.8%, 30.5%, 38.3%, and 27.3% lower content of Fe, K, Mn and Zn, respectively, than leaves of seedlings grown in the presence of HF (Figs. 6, 7). Roots of seedlings grown in LF had 29.5%, 40.2%, and 20.5% lower K, Mn, and P content, respectively, than in roots of seedlings grown in the presence of HF (Fig. 8).

ECM was a significant factor for B content in the leaves (Table 4). Leaves of ECM-treated plants were 21.7% richer in B content than leaves of non-ECM-treated plants (Fig. 7). Ozone was a nonsignificant factor for all the nutrients (Table 4).

Fertilizer×O_3 interaction was nonsignificant for all leaf nutrients but significant for root Al and Na content; but there were no statistically significant differences when the means were tested post hoc with an α level Bonferroni correction (Table 4). This result suggests that any differences among means were from random variation at the tested level of significance (Fig. 9).

Fertilizer×ECM interaction was significant only for leaf B content due to 49.0% greater B content in leaves of ECM-treated plants than in leaves of non-ECM-treated plants grown in LF (Fig. 7). ECM×O_3 interaction was significant only for leaf Na content (Table 4), which was 50.7% lower for ECM-treated plants in EOZ than for ECM-treated plants in AOZ (Fig. 7). Fertilizer×ECM×O_3 interaction was nonsignificant for all nutrients in roots and leaves (Table 4). Al, Co and P content in the leaves and of B, Fe and Zn content in the root did not differ significantly among treatments (Supplementary Materials, Figs. S4, S5).

Fig. 5 Representative root with no mycorrhizae (**a**), root forming mycorrhizae with root hair (**b**) and root forming mycorrhizae without root hair (**c**)

Table 3 Means ± SE of production traits of Japanese larch seedlings grown in low fertilization (LF) and treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone

Trait	ECM×AOZ	ECM×EOZ	Non-ECM×AOZ	Non-ECM×EOZ
Nonmycorrhizal	$47.0^a \pm 4.6$	$80.3^a \pm 26.0$	$54.5^a \pm 15.6$	$10.3^b \pm 3.8$
Forming mycorrhiza	1315.7 ± 55.1	1036.0 ± 87.4	763.0 ± 153.5	523.0 ± 181.2
No root tips	$49.0^{ab} \pm 7.5$	$90.0^a \pm 18.7$	$46.0^{bc} \pm 11.4$	$20.3^c \pm 5.3$
Total root tips	1411.7 ± 67.3	1206.3 ± 109.9	863.5 ± 177.8	553.5 ± 187.5
Results of GLM				
	Nonmycorrhizal	Forming mycorrhiza	No root tips	Total root tips
ECM	$\boldsymbol{F=6.19, P<0.05}$	$\boldsymbol{F=13.68, P<0.01}$	$\boldsymbol{F=10.79, P<0.01}$	$\boldsymbol{F=14.5, P<0.01}$
O_3	$F=1.53, P=0.245$	$F=3.28, P=0.100$	$F<0.01, P=0.926$	$F=2.64, P=0.135$
ECM×O_3	$\boldsymbol{F=7.01, P<0.05}$	$F=0.05, P=0.826$	$\boldsymbol{F=7.94, P<0.05}$	$F=0.05, P=0.831$

Traits assessed: total number of nonmycorrhizal roots (nonmycorrhizal), total number of root tips with forming mycorrhiza (forming mycorrhiza), total number of roots with no root tip or aerial cut of root tip (no root tips) and total number of root tips. Different letters after means indicate statistically significant differences according to Bonferroni test. Results in bold indicate statistical significance according to generalized linear model (GLM) testing

Table 4 Statistical results for comparison of nutrient levels in leaves and roots

Leaves	Fertilizer	ECM	O_3	Fertilizer×O_3	Fertilizer×ECM	ECM×O_3	Fertilizer×ECM×O_3
Al	$F=0.01$, $P=0.912$	$F=1.33$, $P=0.266$	$F=0.74$, $P=0.401$	$F=1.33$, $P=0.265$	$F=0.84$, $P=0.372$	$F=0.01$, $P=0.928$	$F=0.37$, $P=0.554$
B	$F=0.02$, $P=0.832$	**$F=6.86$, $P<0.05$**	$F=0.40$, $P=0.535$	$F=1.09$, $P=0.311$	**$F=6.48$, $P<0.05$[a]**	$F=2.16$, $P=0.161$	$F=1.84$, $P=0.194$
Ca	$F=1.45$, $P=0.246$	$F=1.09$, $P=0.312$	$F=0.02$, $P=0.892$	$F=0.16$, $P=0.695$	$F=0.06$, $P=0.817$	$F=0.14$, $P=0.713$	$F=0.41$, $P=0.533$
Fe	**$F=4.84$, $P<0.05$**	$F=1.15$, $P=0.299$	$F=0.21$, $P=0.650$	$F=0.47$, $P=0.501$	$F=1.50$, $P=0.239$	$F=0.49$, $P=0.494$	$F=0.25$, $P=0.621$
K	**$F=4.41$, $P=0.05$**	$F=1.90$, $P=0.187$	$F=1.94$, $P=0.183$	$F=0.50$, $P=0.491$	$F=0.55$, $P=0.471$	$F=0.01$, $P=0.910$	$F=0.02$, $P=0.894$
Mg	$F<0.01$, $P=0.983$	$F=0.09$, $P=0.772$	$F=0.01$, $P=0.925$	$F=0.16$, $P=0.698$	$F=0.03$, $P=0.877$	$F=0.07$, $P=0.794$	$F=1.78$, $P=0.201$
Mn	**$F=8.71$, $P<0.01$**	$F=2.50$, $P=0.134$	$F=0.48$, $P=0.499$	$F<0.01$, $P=0.970$	$F=0.11$, $P=0.746$	$F=3.14$, $P=0.096$	$F=0.24$, $P=0.631$
Na	**$F=4.40$, $P=0.05$**	$F=1.00$, $P=0.333$	$F=4.05$, $P=0.061$	$F=1.77$, $P=0.202$	$F=0.37$, $P=0.551$	**$F=5.02$, $P<0.05$[b]**	$F=0.44$, $P=0.519$
P	$F=0.43$, $P=0.522$	$F=0.03$, $P=0.891$	$F=2.33$, $P=0.146$	$F=2.34$, $P=0.145$	$F=0.26$, $P=0.615$	$F=0.07$, $P=0.793$	$F=0.19$, $P=0.672$
Zn	**$F=5.40$, $P<0.05$**	$F=1.10$, $P=0.310$	$F=0.88$, $P=0.363$	$F<0.01$, $P=0.993$	$F=0.18$, $P=0.682$	$F=0.72$, $P=0.408$	$F<0.01$, $P=0.985$
Roots							
Al	$F=1.36$, $P=0.262$	$F=0.77$, $P=0.393$	$F=0.29$, $P=0.598$	**$F=8.23$, $P<0.05$[c]**	$F=0.04$, $P=0.847$	$F=0.05$, $P=0.819$	$F=0.24$, $P=0.631$
B	$F=1.13$, $P=0.304$	$F=0.05$, $P=0.831$	$F=1.63$, $P=0.220$	$F=2.32$, $P=0.147$	$F=0.14$, $P=0.715$	$F=0.42$, $P=0.526$	$F=0.14$, $P=0.718$
Ca	$F=0.34$, $P=0.570$	$F=0.53$, $P=0.478$	$F=0.03$, $P=0.862$	$F=1.19$, $P=0.291$	$F=0.09$, $P=0.764$	$F=0.03$, $P=0.871$	$F=0.82$, $P=0.379$
Fe	$F=0.32$, $P=0.578$	$F=0.25$, $P=0.622$	$F=3.17$, $P=0.094$	$F=2.31$, $P=0.148$	$F=0.13$, $P=0.725$	$F=0.52$, $P=0.235$	$F=3.59$, $P=0.077$
K	**$F=7.18$, $P<0.05$**	$F=0.92$, $P=0.351$	$F=0.97$, $P=0.340$	$F=1.62$, $P=0.221$	$F=0.39$, $P=0.541$	$F=0.39$, $P=0.539$	$F<0.01$, $P=0.221$
Mg	$F=0.84$, $P=0.373$	$F=0.11$, $P=0.750$	$F=1.83$, $P=0.195$	$F=1.15$, $P=0.299$	$F=0.05$, $P=0.832$	$F=0.25$, $P=0.626$	$F=0.48$, $P=0.498$
Mn	**$F=10.80$, $P<0.01$**	$F=1.97$, $P=0.180$	$F=0.97$, $P=0.339$	$F=1.74$, $P=0.206$	$F=1.90$, $P=0.187$	$F<0.01$, $P=0.927$	$F=0.04$, $P=0.850$
Na	$F=1.36$, $P=0.260$	$F=0.59$, $P=0.454$	$F=0.18$, $P=0.680$	**$F=7.36$, $P<0.05$[c]**	$F<0.01$, $P=0.958$	$F=4.16$, $P=0.058$	$F<0.01$, $P=0.932$
P	**$F=4.65$, $P<0.05$**	$F=1.50$, $P=0.238$	$F=0.84$, $P=0.375$	$F=0.17$, $P=0.682$	$F=0.20$, $P=0.659$	$F=0.30$, $P=0.590$	$F=0.12$, $P=0.732$
Zn	$F=1.58$, $P=0.226$	$F=0.03$, $P=0.870$	$F=0.98$, $P=0.337$	$F=0.29$, $P=0.598$	$F=0.33$, $P=0.576$	$F=0.04$, $P=0.852$	$F=3.14$, $P=0.095$

Data were tested using a generalized linear model at $\alpha=0.05$. Values in bold indicate statistical significance. Data are presented in Figs. 6, 7, 8 and 9 or in Supplementary Materials (for those with no statistical significance)

[a]The only significance difference was between non-ECM-treated and ECM-treated plants grown in LF (Bonferroni)

[b]The only significance difference was between ECM-treated plants in AOZ and ECM-treated plants in EOZ (Bonferroni). [c]There was no statistically significant difference when the means were tested post hoc with a level Bonferroni correction

Survival rate

For survival rate of seedlings, damping off symptoms affected $52.4\pm4.1\%$ of HF plants and $88.1\pm3.0\%$ of LF plants, representing a significant difference ($F=51.7$, $P<0.001$).

Discussion

Fertilization

Although fertilizer treatment did not affect gas exchange, plants grown in the presence of HF were taller and had

Fig. 6 Means ± SE ($n = 3$) for leaf nutrient content in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). An asterisk indicates significant single effect and "ns" indicates nonsignificant single effect, within a response variable, according to a generalized linear model at $\alpha = 0.05$. No interactions were significant
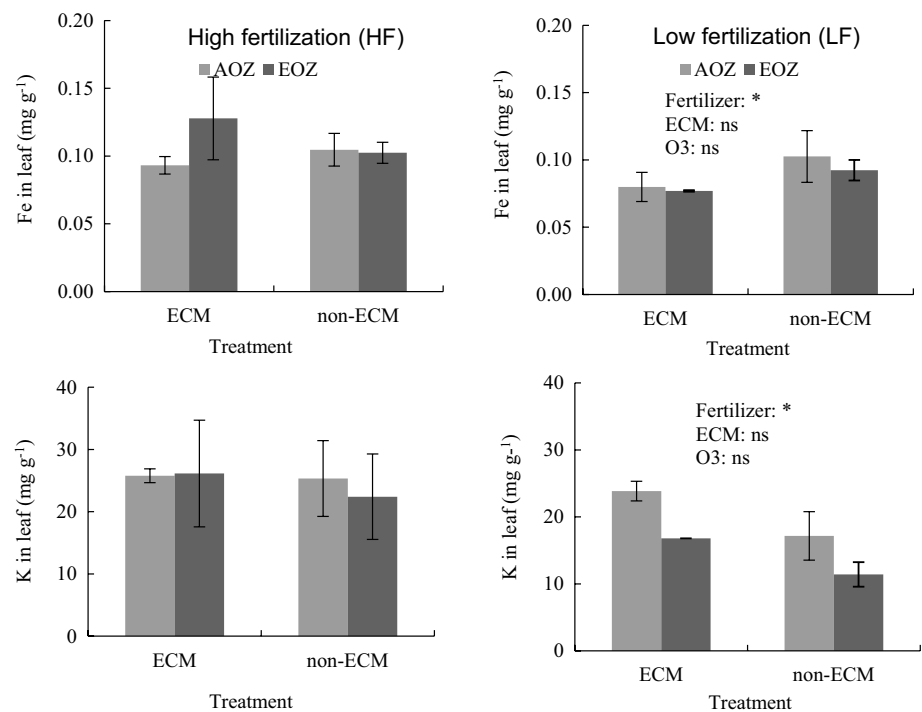
thicker stems and deeper, wider crowns than plants grown in LF. This growth enhancement did not translate to a significant gain in biomass production (despite the large arithmetic differences), because of the high variance that was likely generated by the limiting growing conditions (see next section). However, plants grown in the presence of HF had a significant gain in terms of reproductive capacity as indicated by more buds compared to plants grown in LF. The ratios of buds to root dry matter and f buds to total dry matter were not affected by HF, so there was no imbalance between reproduction and biomass productivity.

Fertilizer treatment drove nutrient dynamics in leaves and roots. Leaves of seedlings grown in LF had higher Na content than in those grown in the presence of HF, even if the content in the roots was not significantly different. The harsh conditions for the plants grown in LF may induce oxidative stress due to nutrient deficiency. The increase in Na content may be attributed to K deficiency and a need of plants grown in LF to account for K^+ deficiency by increasing Na^+, as both elements have similar efficacy in several functions (Maathuis 2014). The substitution of K by Na is well known and understood, and can occur in a wide array of plants. However, growth stimulation by Na, mainly via water balance and cell expansion, is also possible, as Na has a better osmotic adjustment and may contribute better to the solute potential in the vacuoles than K.

O_3

Gas exchange results suggested than EOZ induced uncoupling between A_{390} and Gs_{390}. Such an O_3-induced photosynthesis-conductance uncoupling was found in previous experiments with trees as well as crop plants (Tjoelker et al. 1995;Calatayud et al. 2007; Francini et al. 2007; Singh et al. 2009) and may indicate that the reduction in A_{390} was due to some extent to factors other than a reduced Gs_{390}. Although A_{390} was affected by EOZ, the response to light was not significantly affected, suggesting that EOZ-treated plants could reduce Gs_{390} and A_{390} to the same extend with AOZ plants within a time window of 15 min. While elevated O_3 can induce stomatal sluggishness and decelerate the response to light (Paoletti 2005; Hoshika et al. 2013), our results suggest that EOZ did not cause a permanent impairment in stomata/photosynthesis response to light (delayed or sluggish response was not studied in this experiment). This may be attributed to no EOZ-induced inhibition of guard cell K^+ channels that control stomata (Torsethaugen et al. 1999); EOZ did not alter K content in the leaves. The reduction in A_{390} may relate to the smaller crown span and stem diameter that were caused by EOZ. Elevated O_3 can alter nutrient composition, affect allocation to different organs, and have impacts in ecological stoichiometry of trees (Li et al. 2017a; Shang et al. 2018, 2019). However, in this study, the only significant effect of O_3 on leaf nutrients content was for Na content in ECM-treated plants grown in EOZ, which was approximately half of that in ECM-treated plants grown in

Fig. 7 Mean±SE ($n=3$) leaf nutrient content in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). An asterisk indicates significant single effect and "ns" indicates nonsignificant single effect, within a response variable, according to a generalized linear model at $\alpha=0.05$. The only significant (Bonferroni post hoc test) interactions were ECM×O_3 for Na (only between ECM-treated plants in AOZ and ECM-treated plants in EOZ) and Fertilizer×ECM for B (only between non-ECM-treated and ECM-treated plants grown in LF)
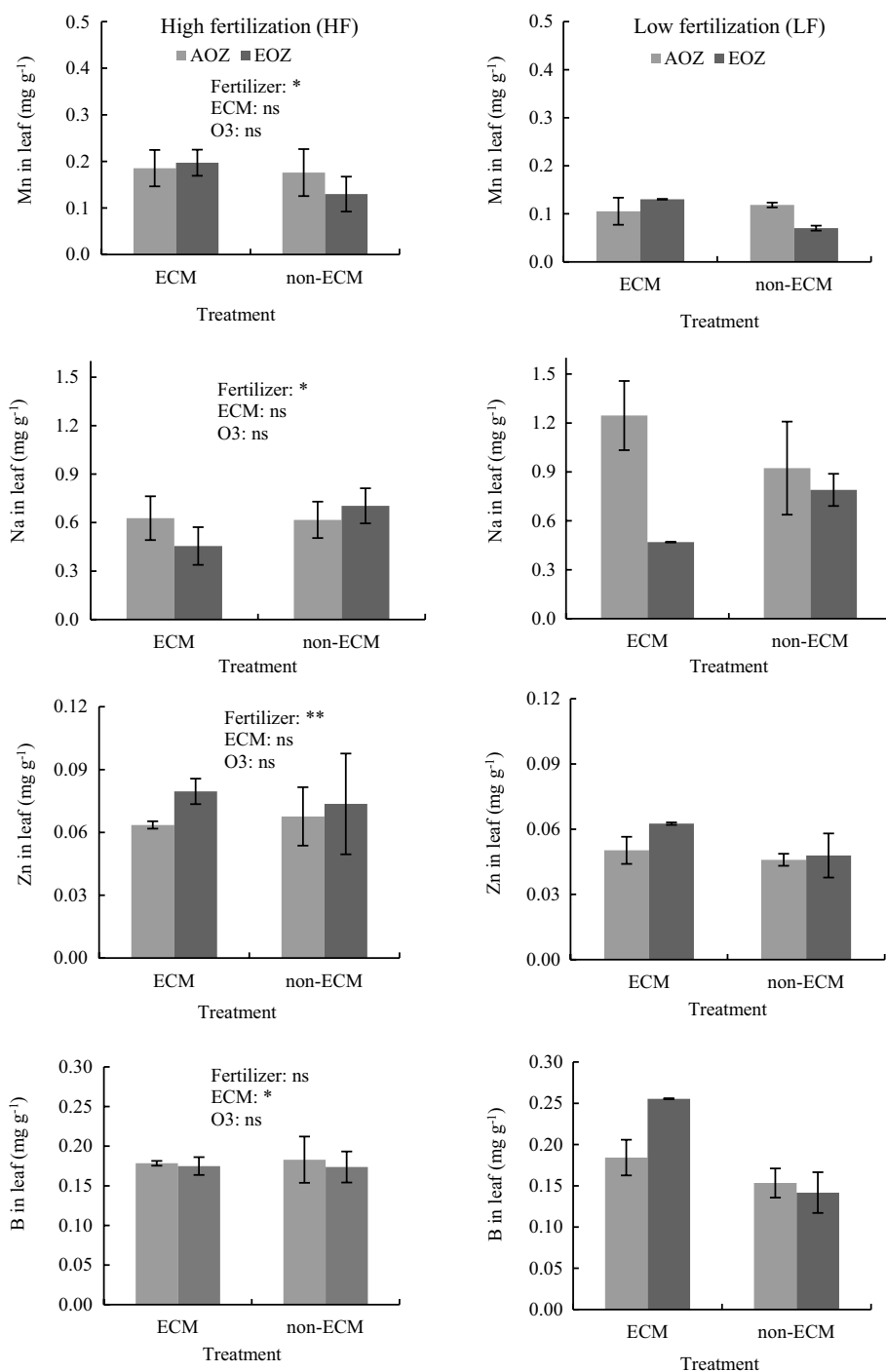

AOZ. These suggest that Na had a major role (among all leaf nutrients analyzed) in the plant response to the combined effect of EOZ and root colonizers. However, the change in Na could not be linked with any of the physiological traits analyzed.

The fact that EOZ decreased stem diameter but did not reduce stem height may indicate an altered stem shape with potentially higher susceptibility of larches to other stressors such as strong winds (Agathokleous et al. 2017; Watanabe

et al. 2013). Interestingly, EOZ increased the root length in spite of lower A_{390}, an observation that may indicate that EOZ-induced stress was modest rather than adverse (Agathokleous et al. 2019).

The overly small size of plants across experimental conditions (treatments) suggests that all plants were stressed by factors other than those experimentally manipulated. In this experiment, plants were grown in cocopeat substrate whose physical properties drive air capacity and water

Fig. 8 Mean ± SE (*n* = 3) root nutrient content in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). An asterisk indicates significant single effect and "ns" indicates nonsignificant single effect, within a response variable, according to a generalized linear model at α = 0.05. No interactions were significant (Bonferroni post hoc test)
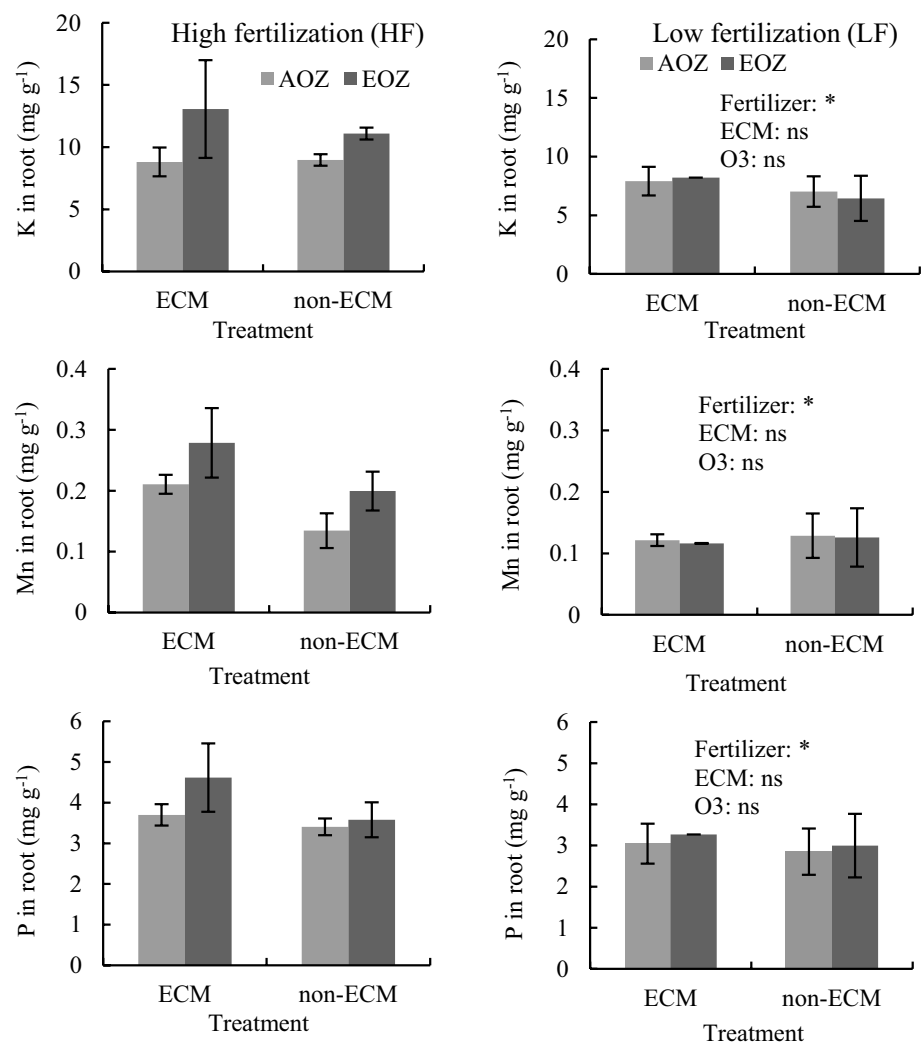

retention (Abad et al. 2002). Cocopeat has a high water-holding capacity and poor aeration, which affects O_2 diffusion to the roots (Awang et al. 2009). These characteristics of the soil substrate used may explain why plant growth was restricted even for plants grown in HF. Based on findings on the effects of CO_2 on plants, plant–soil coupling deserves more attention than plant–atmosphere coupling (Körner 2006); that is, the control over plants is far stronger for soil than for atmospheric conditions. Experiments studying the effects of O_3 on plants have been principally done with healthy/vigorous plants grown under optimum conditions, yet plants will rarely, if ever, grow under such conditions in the real world. Thus, O_3 effects on plants may be overestimated (Körner 2006). Previous experiments with vigorous plants grown in optimum or near-optimum conditions in the same experimental forest suggested significant effects of O_3 (concentrations similar to those used here) on growth and biomass production of Japanese larch seedlings and tall saplings (Agathokleous et al. 2017; Sugai et al. 2019). Conversely, the present results, from an experiment

with plants with limited vigor, revealed only limited effects on gas exchange and nearly no effects on leaf and root nutrient content, growth and biomass production. A recent study with poplars also found that the decrease in plant biomass induced by elevated O_3 was limited when water and nutrients in the soil were limiting factors (Li et al. 2019b).

ECM treatment

The smaller diameter unaccompanied by a smaller height in ECM-treated plants, compared to non-ECM-treated plants, also suggests an altered stem shape, indicative of stress, as it was observed with O_3. There were no significant differences between ECM-treated and non-ECM-treated plants as to gas exchange physiology, growth, and productivity, except a higher E_{390} in ECM-treated plants than in non-ECM plants when grown in LF. The higher E_{390} may be due to more acquisition of water in ECM-treated plants in LF (Birhane et al. 2012). Transpiration was shown before to increase in plants highly infected with root microbes (Drüge

Fig. 9 Mean ± SE ($n = 3$) root nutrient levels in Japanese larch seedlings grown in two soil fertilization regimes, treated with ectomycorrhizae and no fungicide (ECM) or sterilized water and fungicide (non-ECM) and exposed to ambient (AOZ) or elevated (EOZ) ozone (O_3). "ns" indicates nonsignificant single effect, within a response variable, according to a generalized linear model at $\alpha = 0.05$. There was no significant difference in multiple comparisons for the interactions (Bonferroni post hoc test; Table 4)
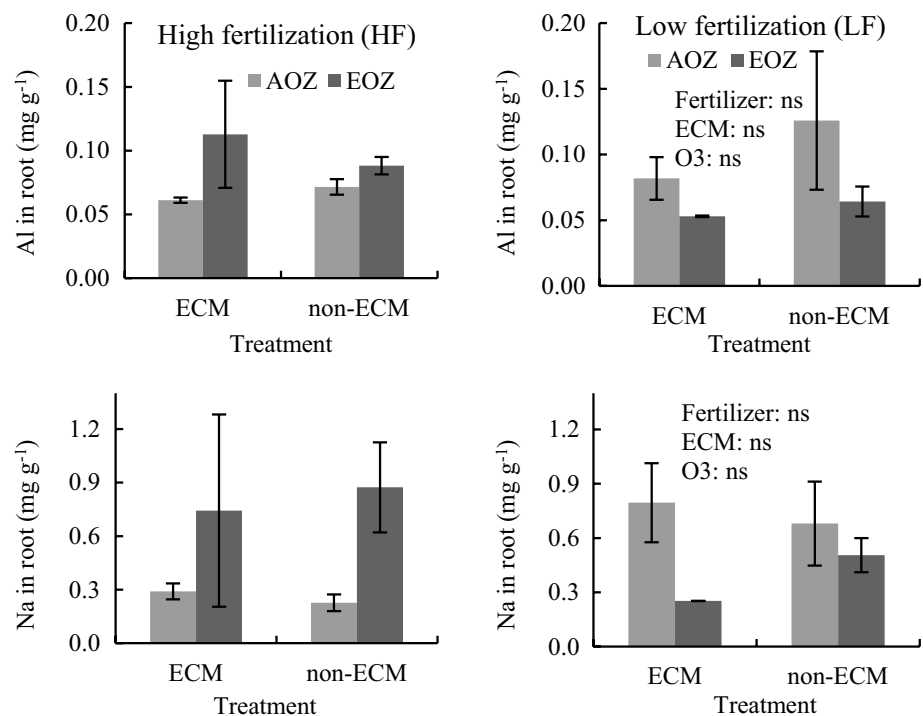
and Schonbeck 1993), an effect which likely depends on the soil water potential (Bingyun and Nioh 1997).

A following analysis of root traits in plants grown in LF revealed that inoculation of plants with ECM (ECM-treated) was successful in inducing an increase in the mycorrhizal roots. However, it is noteworthy that there were only incomplete mycorrhizal roots with no mantle and no complete mycorrhizal roots, suggesting that the development of mycorrhizal roots initiated but for some reason could not be completed. The reason cannot be confirmed, but we can speculate that the seedlings were too young or small for complete formation of mycorrhizal roots. Further DNA analyses revealed that ECM-treated plants were infected with only *Fusarium* sp. and non-treated ones were infected with *Suillus grevillei* (larch-specific ECM; Wang et al. 2015) and *Dothideomycetes* sp. (endophytic mycorrhiza).These results suggest that non-ECM-treated plants were infected normally with (ecto)mycorrhizae that can be transferred through aerial spores and that the control of damping-off was effective. On the other hand, surprisingly, no mycorrhizae could be identified in roots of ECM-treated plants. Furthermore, *Fusarium* sp. was identified, which may be responsible for the damping-off (Seseni et al. 2015), a worldwide disease caused by soil-borne pathogens that rots seedlings (Lamichhane et al. 2017). The conditions in the cocopeat substrate (Abad et al. 2002; Awang et al. 2009; see also section 4.2), might have contributed to the development of damping off. Since seeds were treated against potential infection, the entry of pathogens into the system was not via chamydospores in the seeds but likely via aerial transfer. Considering that ECM-treated

plants were not treated against damping off, the presence of only *Fusarium* sp. and the existence of a higher number of mycorrhizal roots (compared to non-ECM-treated plants) may indicate that *Fusarium* sp. replaced (ecto) mycorrhizae. The results also showed that 9 of 10 seedlings survived damping-off when grown in LF; however, only 5 of 10 seedlings survived when grown in the presence of HF, suggesting that low nutrient availability may mitigate the disease at some extend. These findings offer an important perspective for management practices.

In this experiment with plants stressed by factors other than root colonizers, root colonizers had no effect on leaf nutrient content, except more B in leaves of ECM-treated plants than in leaves of non-ECM-treated plants grown in LF. This increase in B content may be part of a defense mechanism of plants against *Fusarium* sp. for reducing or controlling disease severity (Dong et al. 2016; Jiang et al. 2016) and suggests a potentially important role for B in reducing disease development (Chappelka and Grulke 2016). Further studies should assess the role of B in stress and disease biology over time and for different experimental setups to confirm that its role in a stress-defense mechanism.

Conclusions

The interactive effects of O_3, soil substrate fertilization and root colonizers were investigated for the first time in Japanese container-grown seedlings. Soil fertilization status drove plant nutrient dynamics, as reflected in their contents

in leaves and roots, and enhanced seedling growth. Low nutrient availability protected seedlings against damping off by increasing their survival rate.

EOZ negatively affected gas exchange but had no effects on growth (except an increase in root length) and biomass production. O_3 was a minor stressor in this experiment where seedling growth and productivity was restricted by factors other than O_3 (soil and microorganisms). Nonetheless, the results suggest potential ecological risks due to elevated O_3-induced enhanced susceptibility to other stressors via changes in the form of plant stem, even under conditions where factors other than O_3 dominate plant health/stress.

In contrast to our expectations, ECM treatment did not enhance the tolerance of plants against elevated O_3, albeit it effectively increased the number of (mycorrhiza-forming) root tips. However, this is due to practical limitations arising from the effects of disease and other factors that might have affected the establishment of plant–microbe symbioses and the formation of complete mycorrhizal root tips (e.g., small plant size). Nonetheless, this research provides an important basis for designing relevant studies in the future.

Fungal root colonizers and fertilization had no significant interactive effects with O_3 in this experiment. B and Na, among 10 nutrients analyzed, seem to have an important role in stress responses. Further studies are needed to reveal potential physiological mechanisms linked to the regulation of B and Na. We conclude that elevated O_3 can have negative ecological effects on Japanese larch even if plants are grown in a harsh environment where factors other than O_3 may strongly limit plant growth and productivity.

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