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Author(s)	Sugai, Tetsuto; Watanabe, Toshihiro; Kita, Kazuhito; Koike, Takayoshi
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Title: Nitrogen loading increases the ozone sensitivity of larch seedlings with higher sensitivity to nitrogen loading

Author names and affiliations: Tetsuto Sugai[1]・Toshihiro Watanabe[2*]・Kazuhito Kita[3]・Takayoshi Koike[2]

[1] Graduate School of Agriculture, Hokkaido University, Sapporo 060-8689, Japan

[2] Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8689, Japan

[3] Hokkaido Forest Research Institute, HRO, Bibai, Hokkaido 079-0198, Japan

[*]Corresponding authors (Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8689, Japan, E-mail: nabe@chem.agr.hokudai.ac.jp; Tel: +81-11-706-2498)

1. Abstract

Larch (*Larix* sp.) tree is a critical species for the future afforestation in Northeast Asia. The impacts of elevated concentrations of ground-level ozone (O_3) and nitrogen (N) deposition are raising concerns. However, knowledge of the combined effects of elevated O_3 and N loading are still limited. We investigated whether nitrogen loading mitigates the negative impacts of ozone on two larch species: the Japanese larch (*L. kaempferi*) and its hybrid larch F_1 (*L. gmelinii* var. *japonica* × *L. kaempferi*) or not. We used open-top cambers and compared responses of the larch seedlings. Results showed the N loading mitigated the negative effects of O_3 on Japanese larch. However, in hybrid larch F_1, N loading did not mitigate O_3-induced inhibition of growth and photosynthetic capacity. Mitigation effect of N loading on negative O_3 impacts may vary between the two *Larix* spp. Hybrid larch F_1 could be more affected by the combined effects of O_3 and N loading due to its higher growth response to N loading. Elevated O_3 also reduced leaf nitrogen/phosphorus (N/P) ratio by elevated O_3, with significant effects in hybrid larch F_1, particularly under N loading. In the present study, leaf N/P ratio was utilized to validate the hypothesis that a positive effect of N loading may be observed if O_3 does not induce P limitation in *Larix* spp. We demonstrated a potential leaf N/P ratio function, which could reflect responses to O_3 and N loading in hybrid larch F_1.

Keywords:

Ozone sensitivity; nitrogen loading; interspecific difference; hybrid larch F_1; *Larix kaempferi*; nitrogen phosphorus ratio;

Abbreviations

Ozone (O_3), Nitrogen (N), Phosphorus (P), Nitrogen-phosphorus ratio (N/P), Leaf mass area (LMA), Net photosynthetic rate at 380 μmol CO_2 mol^{-1} and light saturation per leaf area (A_{380}), Stomatal conductance (Gs_{380}), Photosynthetic nitrogen use efficiency (NUE), Photosynthetic phosphorus use efficiency (PUE), Maximum rate of carboxylation (Vc_{max}), Maximum electron transport rate (J_{max}), Net photosynthetic rate at 1600 μmol

CO_2 mol^{-1} (A_{max}), Net photosynthetic rate at light saturation per leaf mass (A_{mass}).

2. Introduction

Larch (*Larix* sp.) species are widely distributed in Northeast Asia and have been planted for timber production and ecosystem rehabilitation (Abaimov et al. 2000; Zhang et al. 2000). In addition to the native Japanese larch (*Larix kaempferi*), a hybrid larch F_1 (*L. gmelinii* var. *japonica* x *L. kaempferi*), which is more tolerant to stress and exhibits higher photosynthetic performance, has also been developed (Kita et al. 2009). However, increasing concentrations of ground-level ozone (O_3) (Akimoto 2017) and nitrogen (N) deposition (Galloway et al. 2004; Liu et al. 2013; Yamaguchi et al. 2014) are raising concern, particularly in Northeast Asian forests (Yamaji et al. 2008; Koike et al. 2013). Concentrations of such air pollutants are projected to continue increasing in future (Nagashima et al. 2010; Ainsworth et al. 2012; Izuta 2017). Therefore, there is a need to evaluate the responses of the *Larix* spp. to elevated O_3 and N deposition (Ryu et al. 2009; Koike et al. 2012).

O_3 is a toxic pollutant that inhibits numerous physiological functions such as photosynthesis and it eventually causes a decrease in growth, particularly belowground growth (Li et al. 2016; Shi et al. 2016; Wang et al. 2016; Agathokleous et al. 2016; Sugai et al. 2018). Conversely, N loading may promote the growth of trees when soil N limits growth, such as in a boreal forest (Vitousek et al. 2002). However, excess N loading could also have negative effects on tree growth (Aber et al. 1989; Ryu et al. 2009; Wang et al. 2018). Although numerous studies have reported the combined effects of elevated O_3 and N deposition, they have primarily concluded that N loading might not affect or increase the sensitivity of woody species to O_3 (e.g., Nakaji and Izuta 2001; Yamaguchi et al. 2011; Izuta et al. 2017). Regarding larch species, contrary to previous reports, Watanabe et al. (2006) reported that N loading could mitigate the negative impacts of O_3 on Japanese larch seedlings. However, a recent study reported that N loading could not mitigate the negative impacts of O_3 on hybrid larch F_1 (Dong-Gyu et al. 2015). Consequently, researchers' views regarding the combined effects of N loading and O_3 on larch species are inconsistent. In addition, the physiological mechanisms of the effects on combined N loading and O_3 deposition remain unclear (e.g., Mills et al. 2016; Izuta 2017).

The effects of N loading vary depending on the phosphorus (P) conditions in plants (e.g., Güsewell 2004; Elser et al. 2010). For instance, N loading promoted the growth of hybrid larch F_1 under high P conditions (Fujita et al. 2018). Indeed, the leaf N/P ratio could be a critical indicator of N or P limitation (Güsewell 2004) since N loading increases the N/P ratio in plants (e.g., Reich and Oleksyn 2004; Güsewell 2004; Elser et al. 2010). Conversely, one of the effects of O_3 is accelerated leaf senescence, resulting in imbalances in leaf nutrients (Uddling et al. 2006; Shi et al. 2016; Shi et al. 2017). However, few studies have reported leaf N and P reductions in larch species under elevated O_3 (Koike et al. 2012; Wang et al. 2015). The relationship between leaf N/P ratio and physiological activities under elevated O_3 and/or N loading remains relatively poorly understood (Beyears et al. 1992; Kainulainen et al. 2000; Utriainen and Holopainen 2001; Waillin et al. 2002; Wang et al. 2015). Considering that the senescence effect of O_3 does not induce reductions in leaf P concentrations (Wang et al. 2015), a positive effect of N loading on hybrid larch F_1 and lower leaf N/P ratio may be observed (Fujita et al. 2018). Here we expected that leaf N/P ratio could be a useful indicator of N and P limitation in the larches and facilitate comparison of their responses to N loading under elevated O_3.

The aim of the present study is to investigate the effect of N loading on the responses of Japanese larch and hybrid larch F_1 seedlings to O_3. Our hypothesis is N loading could mitigate the negative impacts of O_3 on larch seedlings. To assess the hypothesis, we investigated the relationship between physiological and nutritional responses based on correlation tests.

3. Materials and methods

3. 1 Experimental design

The experiments were conducted at Sapporo Experimental Forest, Hokkaido University, Japan (43°04'N, 141°20'E; 15 m above sea level). Weather data from 2015 to 2016 were recorded at a meteorological station in Sapporo (Japan Meteorological Agency, 2016). The monthly average values (± s.e.) of metrological data from May 2015 to September 2016 were: mean monthly average air temperature = 12.31±2.18°C; maximum temperature = 23.53±2.27°C; minimum temperature = 3.41±2.21°C; total precipitation = 106.8±14.6 mm; and total sunshine duration = 162.1±12.0 h, respectively. Japanese larch (*Larix kaempferi*) is tolerant to harsh conditions such as low temperature and infertile soils although it is sensitive to grazing damage from red-backed voles (*Myodes rufocanus bedfordiae*). To overcome such limitations, hybrid larch F_1 (*L. gmelinii* var. *japonica* x *L. kaempferi*) was developed (Kita et al. 2009). These two species of larch seedlings were grown for one year from seeds at a nursery at Hokkaido Research Organization, Forestry Research Institute, Bibai. On May 12, 2015, all larch seedlings were transplanted to 7 L pots before leaf formation. The initial stem diameter and height were 2.21±0.44 mm (mean ± se) and 16.56±2.93 cm, respectively, for Japanese larch, and 2.45±0.17 mm and 20.44±1.39 cm, respectively, for hybrid larch F_1. Soil in the pots was chosen so as to mimic immature volcanic ash soil (Kanuma soil and Akadama soil, volume ratio 1:1). To prevent soil desiccation and nutrient imbalances, we applied 100 mL of commercial liquid fertilizer (HYPONEX; Japan, N = 72.4 mg l^{-1}, P = 284 mg l^{-1}, K = 94.2 mg l^{-1}) as a basal dressing after complete rooting. There, all pots initially received 5.5 kgN $ha^{-1}yr^{-1}$.

N loading treatments were applied at two-week-intervals using $(NH_4)_2SO_4$ dissolved in 500 ml tap water to simulate recent N deposition such as $PM_{2.5}$ (Particulate matter less than 2.5 μm in aerodynamic diameter; Hatakeyama 2011). N loading was set to 50 kg N ha^{-1} yr^{-1}, which are the estimated levels in 2050 (Galloway et al. 2004; Liu et al. 2013). The N amount included the initial liquid fertilization. N loading was performed on June 19, July 15, August 5 and 17 in 2015, and June 27, July 22, and August 1 and 17 in 2016, while avoiding rain days. To avoid drought conditions, control seedlings that were not N treated were irrigated with 500 ml tap water over a period similar to that of N loading. For pH and inorganic N concentration measurements, surface soil (<5 cm) was sampled from each pot on 29 August 2016.

To measure soil pH, 10 g of fresh soil was sampled and mixed with 25 ml of distilled water. The samples were shaken for over one hour before measurement using a portable pH sensor (RM-30P, TOA DKK, Tokyo, Japan). To measure soil inorganic N contents, 10 g of sampled fresh soil was mixed with 100 ml of 2 M potassium chloride solution. The samples were the shaken for over one hour. For colorimetric measurement of inorganic N (NH_4^+-N and NO_3^--N) using a flow injection analyzer (AQLA-700, Aqualab Co., Ltd., Tokyo, Japan), the samples were filtered through a 1-μm filter (Toyo Roshi, No.5C filter paper, Tokyo, Japan). Soil analyses were performed based on the methods of Miura (1997).

To expose seedlings to ambient or elevated O_3, open-top cambers (OTCs; 1.2 x 1.2 x 1.2 m) framed using steel were used following complete rooting. A polyvinyl chloride film (Noh-bi Co Ltd., Sapporo, Japan), which transmits 88% sunlight (blocking only UV-B and UV-C), was used to cover the OTCs. Two O_3 concentration treatments were applied. The first consisted of a non-filtered air treatment representing low O_3 concentration, which occurs at ambient conditions, while the second was a non-filtered O_3-enriched air treatment. The target of the enriched O_3 concentration was 60 nmol

mol^{-1}, which is the value for the environmental standard for photochemical oxidants in Japan (Japanese Ministry of the Environment 1996). The enriched O_3 concentration target was also set to assume predicted future O_3 concentrations in Asian countries (Yamaji et al. 2008; Ainsworth et al. 2012). O_3 treatments were carried out from June 19, 2015 to September 18, 2015 and from May 18, 2016 until August 29, 2016. O_3 was supplied using air pumps (APN-110K, IWAKI, Japan) from an electrical discharge O_3 generator (PZ-1B, Kofloc-Kojima, Japan) into the OTCs only during daylight hours (06:00–18:00, JST). Ambient air inside the OTCs was constantly driven to the atmosphere using over 24 hours and wind speeds at the openings to the surfaces of the OTCs were set to 0.1 m^3 per second. During the exposure periods, O_3 concentrations were continuously monitored (1 minute interval) with UV absorption O_3 analyzer (Model 202-EPA, 2B technologies, Boulder, USA) and an O_3 monitoring system (EG-3000F, Ebara, Japan). An algorithm of proportional integrative differential system was applied to maintain the target O_3 concentrations. Table 1 presents the achieved O_3 concentration values and the value of accumulated exposure over the threshold of 40 nmol mol^{-1} (AOT40). During the non-exposure periods, O_3 generators were switched off and all the seedlings were maintained in the chamber except for the heavy snow seasons.

In total, there were 4 treatment: tap water + ambient O_3 (Control); tap water + elevated O_3 (O_3); N loading + ambient O_3 (N); and elevated O_3 x N loading (O_3 x N). All the treatments had four replicates, yielding 16 OTCs. OTC positions were set based on a completely randomized split-plot design. Four larch seedlings of each species were set in each chamber (64 seedlings of each species, 128 seedlings in total). All the seedlings were utilized throughout the experiments and the positions of seedlings within each OTC were regularly changed to prevent biased edge effects.

3. 2 Leaf gas-exchange measurements

Gas-exchange measurements were conducted for all the seedlings in all the treatments between the 17 and 21 August 2016. An open gas-exchange system (LI-6400, Li-Cor Inc., NE, U.S.A.) was used to measure the gas-exchange parameters. For each seedling, approximately six fresh mature needles were selected from the non-shaded position of a branch and attached to the leaf clip chamber of a LI-6400. Measurements were conducted between 05:00 and 15:00, JST, and the leaf temperature was maintained at 25±0.5°C. The water vapor deficit in the leaf chamber was approximately 1.2±0.3 kPa and the photosynthetic photon flux was approximately 1500 μmol m^{-2} s^{-1}. To plot curves of net CO_2 assimilation rate (A) versus intercellular CO_2 concentration (C_i), i.e., A/C_i curves; A was determined at ten C_i levels (C_a: 60–1600 μmol mol^{-1}). The net photosynthesis rate and stomatal conductance at 380 μmol mol^{-1} C_a (A_{380} and Gs_{380}, respectively) were determined from the A/C_i curves. In addition, using the individual leaf photosynthetic model, the curves were used to calculate the maximum net photosynthetic rate at 1600 μmol mol^{-1} C_a (A_{max}), the maximum rate of carboxylation (V_{cmax}), and the maximum rate of electron transport (J_{max}) (Farquhar et al. 1980; Long and Bernacchi 2003). Values of Rubisco Michael's constants for CO_2 (K_c), O_2 (K_o) and CO_2 compensation points in the absence of dark respiration (Γ^*) were derived following analyses of the *A/Ci* curves as described by Bernacchi et al. (2001). After photosynthetic measurements, the measured needles were scanned and needle areas calculated using Image-J software (U. S. National Institutes of Health, Bethesda, Maryland, USA; Schneider et al. 2012).

3. 3 Leaf nutrients and photosynthetic parameters

The needles used for gas-exchange measurements were used to conduct further analyses of leaf nitrogen content per mass (N) and leaf mass per area (LMA). The needles were dried at 70°C for approximately 3 days until they achieved a constant dry mass and then weighed. Nutrient contents per needle area were calculated based on the obtained LMA value. N was measured using an NC analyzer (Elemntar, VarioEL III, Japan). In addition, approximately twenty fresh mature needles were selected on 29 August 2016 from the same non-shaded position as the photosynthetic measurement as much as possible. They were digested with HNO_3 and subjected to elemental analysis using inductively coupled plasma mass spectrometry (ELAN, DRC-e; PerkinElmer, Waltham, MA, U.S.A.), based on the method of Sha et al. (2012). Photosynthetic nitrogen-use efficiency (*NUE*) was calculated as follows:

$$NUE = A_{380} / (\mathrm{LMA} \times \mathrm{N}).$$

Photosynthetic phosphorus use efficiency (*PUE*) was calculated as follows:

$$PUE = A_{380} / (\mathrm{LMA} \times \mathrm{P}).$$

Photosynthesis rate per mass (A_{mass}) was calculated as follows:

$$A_{mass} = A_{max} / \mathrm{LMA}.$$

These calculations were done based on the method of Koike et al. 2012.

3. 4 Production and allocation of dry matter

In total, 128 seedlings were harvested on 29 August 2016 and then separated into foliage, branches, stems, and roots. The roots were gently washed with tap water to remove soil particles. The samples were oven-dried at 70°C until they achieved a constant dry mass and then weighed using a digital balance. Top shoot/root ratio (T/R_{ratio}) was calculated as follows:

$$T/R_{ratio}\ (\mathrm{g\ g^{-1}}) = (\mathrm{Needle + Branch + Stem})/\mathrm{Root}.$$

Allocation in each shoot and root was calculated using each corresponding dry mass value.

3. 5 Statistical analyses

The data were averaged per experimental unit (block) resulting in four values per treatment ($n = 4$). All parameters were analyzed for each species using a general–linear mixed model (GLMM) randomized by blocks. O_3 exposure, N loading treatments, and different species constituted the predictors. For significant interaction factors with more than two levels, we applied the multiple comparison test of Tukey’s honestly significant difference (HSD). $P = 0.05$ was considered statistically significant. R software ver. 3.4.3 was used for data processing and statistics.

4. Results

4. 1. Environmental conditions

Table 1 presents O_3 concentrations, AOT40, and soil environmental conditions in each treatment. The O_3 concentrations in each year reached the target levels in the present study. Average O_3 concentrations in elevated O_3 treatments were approximately 2 times higher than those at the ambient levels. The pH (H_2O) of soil was > 6.0 in the control and the O_3 treatment, whereas it was <6.0 in the N and O_3 + N loading treatments. NH_4^+ and NO_3^- concentrations in the N and O_3 + N loading treatments were significantly higher than those in the other treatments.

4. 2. Dry mass

O_3 enrichment significantly decreased needle, branch, and stem dry mass in Japanese larch (Fig. 1*A*-*E*, $P < 0.05$) whereas significant reductions attributable to O_3 were not observed in hybrid larch F_1. In Japanese larch, although N loading did not have significant effects (Fig. 1*B, D*), needle dry mass increased whereas stem dry mass slightly decreased due to N loading. However, in hybrid larch F_1, N loading increased needle, branch, and stem dry mass (Fig. 1*A*-*E*, $P < 0.05$).

Under N loading, O_3 did not decrease any dry mass in Japanese larch (Fig. 1*A*-*E*, P

> 0.05). O_3 slightly increased branch and stem dry mass in Japanese larch although there were no significant differences (Fig. 1*C, D*). In hybrid larch F_1, O_3 significantly decrease dry mass in under N loading in all the tested parts (Fig. 1*A,B, D,* and *E*, $P < 0.05$) except in the branches (Fig. 1*C*). Notably, the branch dry mass responses to O_3 were contrasting in the two investigated species *Larix* spp. under N loading. Although there were no significant changes (Fig. 1*C*), branch dry mass in Japanese larch increased whereas that in hybrid larch F_1 decreased following O_3 enrichment.

4. 3. Dry mass allocation

O_3 decreased root proportions in the two *Larix* spp. whereas it increased the aboveground proportions (Fig. 2*D, E*). In Japanese larch, O_3 and N loading significantly increased the needle proportions (Fig. 2*A, D,* $P < 0.05$). Responses of the branches to N loading were contrasting between species although the degree was marginal (i.e., < 5%). The needle proportions under N loading were decreased by O_3 in both *Larix* spp. although these were no significant changes. Regarding the differences between the species, the needle proportions under N loading were significantly higher in Japanese larch than in hybrid larch F_1 (Fig. 2*A, D,* $P < 0.05$). The differences between the species were maintained under elevated O_3 and N loading.

4. 4. Photosynthetic activity

O_3 significantly decreased Gs_{380} and A_{max} in Japanese larch (Fig. 3*B*, *G*; $P < 0.05$). In

Japanese larch, N loading did not increase any photosynthetic parameters except A_{380}. However, in hybrid larch F_1, N loading significantly increased A_{max} and J_{max} (Fig. 3*F, G*; $P < 0.05$). In addition, O_3 did not decrease any parameters except A_{380} in hybrid larch F_1. In both *Larix* spp., O_3 decreased A_{380} under N loading (Fig. 3*A*, $P < 0.05$). The effects of O_3 on *NUE*, *PUE*, and Vc_{max} were not significant while negative effects on species were observed (Fig. 3*C-E*).

4. 5. Nutrient conditions of leaves

Table 2 presents the concentration of each nutrient element per needle mass, the LMA values, and the coefficients of correlation between leaf N and leaf N/P with A_{mass} and needle dry mass. As expected, O_3 did not decrease leaf P in both species. However, N loading significantly increased leaf P in the hybrid larch F_1 (Table 2). In addition, the O_3 and N loading combined increased nutrient element concentrations in hybrid larch F_1. In addition, leaf N/P under N loading in hybrid larch F_1 was decreased by O_3. Needle dry mass of both *Larix* spp. exhibited significant positive correlations with leaf N and leaf N/P.

5. Discussion

Growth responses to the combined effect of O_3 and N loading varied between Japanese larch and hybrid larch F_1. O_3 enrichment did not result in a significant reduction in any dry mass in Japanese larch under N loading. The same type of response was also observed in the previous study (Watanabe et al. 2006; Dong-Gyu et al. 2015). Watanabe et al. (2006) suggested that reducing O_3 sensitivity in Japanese larch with N loading was not related to the amount of O_3 absorbed through the stomata. In the present study, we observed no significant combined effect on Gs_{380} in Japanese larch, although O_3 decreased Gs_{380}. Such a stomatal response suggested that N loading did not alter the amount of O_3 absorbed, which is consistent with some previous findings (Watanabe et al. 2006).

In contrast, O_3 significantly reduced the total dry mass of hybrid larch F_1 under N loading (Fig. 1*A*). We observed a reduction in photosynthetic parameters following O_3 enrichment under N loading, as well as dry mass (Fig. 3). The results are consistent with those of a previous study that reported that NH_4NO_3 loading might not mitigate the negative impacts of O_3 on hybrid larch F_1 (Dong-Gyu et al. 2015). The results suggest that the responses to the combined effects of O_3 and N loading in the two *Larix* spp. are clone-specific. In addition, the results reinforce the argument that N loading could intensify the negative effects of O_3 on hybrid larch F_1 to some extent. Previous studies have suggested that excess N loading could simulate detoxification and repair activities against O_3 stress (Watanabe et al. 2006; Izuta 2017). However, Yamaguchi et al. (2010) reported that N loading did not change anti-oxidant capacities activated by O_3 such as acidic amino acids. As described, the mechanisms of changes in O_3 sensitivity attributable to N loading remain unclear (e.g., Mills et al. 2016). Hereafter, we discuss the contrasting responses in two *Larix* spp. to consider the potential mechanisms.

Hybrid larch F_1 could be affected more by the combined effects of O_3 and N loading (Fig. 1) potentially due to its higher growth response to N loading. This hypothesis could be supported by several previous studies (Pell et al. 1995; Matyssek et al. 1997; Maurer et al. 1997; Yamaguchi et al. 2010; Kinose et al. 2017). The seasonal responses of photosynthesis rates in Siebold's beech (*Fagus crenata*) were significantly decreased by O_3 only when the levels of liquid fertilizer were relatively high (Hyponex 6-10-5, HYPONex Japan, Japan) (Kinose et al. 2017). In birth clone (*Betula pendula*) seedlings, macro- and micro nutrient supply (Hauert, Nutrient typ A, Swiss) did not mitigate the negative impacts of O_3 (Maurer et al. 1997). Conversely, the degree of physiological inhibition by O_3 was significantly increased by nutrient supply. In addition, total dry mass in *Fagus crenata* seedlings decreased due the combined effect of N loading and elevated O_3 because of a higher growth response to NH_4NO_3 loading (50 kg N ha^{-1} yr^{-1} equivalent) (Yamaguchi et al. 2007). Pell et al. (1995) also reported that the negative effect of O_3 on *Populus tremuloides* increased with increase in N supply. Based on such reports, we postulate that the responses to O_3 could be more significant when plants have higher sensitivity to N loading. In addition, we should consider the degree of change in response to O_3 and sensitivity to N loading by comparing the contrasting responses in the larch species. In the next section, we focus on role of the effect of N loading in understanding the effect of N loading on O_3-induced changes such as increments in leaves.

N loading responses were particularly varied between the between two *Larix* spp.

Soil inorganic N, particularly NO_3^- concentrations, in the N loading treatment in Japanese larch and its hybrid larch F_1 were 3.41 and 2.66 mg l^{-1}, respectively (Table 2). However, the growth responses of hybrid larch F_1 were higher under similar N treatment. Increment in total dry mass following N loading was higher in hybrid larch F_1 than in Japanese larch. Plant growth is largely regulated by the spatial distribution of leaves i.e., the quantity of leaves and photosynthetic capacity (e.g., Poorter 1989). We observed a positive effect of N loading on A_{380} in both *Larix* spp (Fig. 3*A*). However, N loading did not increase the total dry mass in Japanese larch (Fig. 1*A*). The results are consistent with the previous report in Watanabe et al. (2006). In the present study, stem dry mass was not changed in Japanese larch whereas it was significantly increased by N loading in hybrid larch F_1 (Fig. 1*D*). Notably, N loading increased only needle proportions in Japanese larch. Therefore, Japanese larch might have a relatively lower capacity for N utilization than hybrid larch F_1. Soil NO_3^- concentrations in control treatments in Japanese larch and hybrid larch F_1 species were 0.08 and 0.28 mg l^{-1}, respectively (Table 2). The growth rate in hybrid larch was higher even under similar control treatments (Relative growth amount of height (10^{-4} log cm) were 7.56±0.20 in hybrid larch F_1 and 6.76±0.63 in Japanese larch, respectively) (cf. Appendix A). The results could also suggest that the Japanese larch initially had a lower capacity for N absorption and/or utilization rate.

N loading significantly increased leaf P in hybrid larch F_1 (Table 2, $P < 0.05$). The increment in leaf P certainly decreased N/P ratio in hybrid larch F_1. The results could suggest that N loading promotes P absorption and utilization. In addition, the P increment was significant under N loading and elevated O_3 (Table 2, $P < 0.05$). The result could indicate that relative N limitation may occur following O_3 enrichment even under N loading. Such key responses were not observed in Japanese larch, suggesting that the contradictory responses in the two larch species could be associated with physiological responses to changes in leaf nutrient contents, in particular under N loading.

Knowledge regarding leaf P content responses to O_3 remains relatively limited (e.g., Beyears et al. 1992; Kainulainen et al. 2000; Shi et al. 2017). Previous studies have reported no significant reductions, rather, slight increments of leaf P contents under elevated O_3 (e.g., Beyears et al. 1992; Kainulainen et al. 2000; Utriainen and Holopainen 2001; Waillin et al. 2002; Wang et al. 2015). In the present study, we also observed a slight increment in leaf P content under elevated O_3 in both *Larix* spp. The increment in P in the present study was significant in hybrid larch F_1 under N loading, particularly with elevated O_3 (Table 2, $P < 0.05$). Further studies are required to evaluate why leaf P increased in hybrid larch F_1. Potential mechanisms would be related to ectomycorrhizal fungi (ECM) infection ratio and species diversity of under elevated O_3 conditions (Wang et al. 2015). It is also critical consider the role of ECM symbioses since symbiotic ECM enhance nutrient conditions for the host plants as long as the host plants have the capacity to photosynthesize efficiently.

Demand for P could vary depending on N conditions. In Japanese larch, P demand is much greater than N demand (Li et al. 2016). Therefore, to investigate the leaf N and P balances, we evaluated leaf N/P ratio under elevated O_3 and N loading. In the present study, all leaf N/P values in in Japanese larch were >20 irrespective of treatments (Table 2). A leaf N/P ratio >20 is indicative of P limited biomass production (Güsewell 2004). Therefore, the results indicate that P could limit biomass production in Japanese larch. In addition, higher N/P, i.e., relative P deficiency, accelerates leaf senescence, which becomes a physiological limiting factor (Güsewell 2004; Killingbeck 2004). However, in the present study, N loading decreased *NUE* in hybrid larch F_1 (Fig. 3*C*, 6.40±0.39 to 4.71±0.56 μmol mgN^{-1} s^{-1} in the control and N loading treatment, respectively). Translocation of photosynthates in chloroplasts is inhibited by relative deficiency in inorganic phosphorous, i.e., higher leaf N/P constrains the carboxylation cycle (e.g., Sharkey 1985). Although we did not observe any significant inhibition of *PUE* in both species (Fig. 3*D*), a plausible explanation for the observed phenomenon could be an increase in sucrose synthesis via the Calvin cycle due to N loading. It would induce P limitations at the early stages of photosynthate translocation, which would also reduce *NUE* (Luo et al. 2014; Li et al. 2016).

In conclusion, growth and physiological responses of Japanese larch (*Larix kaempferi*) and a hybrid larch F_1 (*L. gmelinii* var. *japonica* x *L. kaempferi*) varied based on the combined effects of N loading and elevated O_3. N loading mitigated the negative effects of O_3 on the total dry mass of Japanese larch. This response was partly consistent with that of the previous results in Japanese larch with increasing N loading under elevated O_3 (Watanabe et al. 2006; Koike et al. 2013). Conversely, N loading increased O_3 sensitivity in hybrid larch F_1 to some extent. The contradictory responses in the two *Larix* spp. could be associated with physiological reactions that influence nutrient contents. To further enhance our understanding of the nutrient dynamics, the synergistic relationship with ECM should be evaluated under elevated O_3 and N loading.

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8. Appendix A. Supplementary data

Seasonal growth responses of stem diameter (mm) and height (cm) in two larch species (black lines and symbols: Japanese larch; gray lines and symbols: Hybrid larch F_1) of each treatment (control: full line, elevated ozone: dotted line, nitrogen loading: bold line, ozone and nitrogen loading: bold dotted line). The values were measured on May 13 and September 11 in 2015, and June 11, July 15, and August 27 in 2016 using measuring tapes (1 mm gradient) and the calipers (Mitsutoyo, Japan). The diameter values were calculated as the means of two crosswise measurements at the base of stem. Each point + error bar is the mean value and standard errors (n=4).

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10. Captions

Fig. 1

The total dry mass (g), needle dry mass (g), branch dry mass (g), stem dry mass (g), and root biomass (g) of two larch species (white bar: Japanese larch; gray bar: Hybrid larch F_1) in each treatment (control: C, elevated ozone: O_3, nitrogen loading: N, ozone and nitrogen loading: O_3 x N). Each bar + error bar is the mean value and standard errors. GLMM analysis: ozone (O_3), N loading (N), species difference (Sp.), and these interactions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s. denotes non-significant values; Different lower case letters denote significant differences between species and treatments (Tukey HSD test; $P < 0.05$), and different capital letters denote significant differences between treatments (species pooled).

Fig. 2

Dry mass allocation (%) to needle, branch, stem, and root and top shoot/root ratio (T/R_{ratio}, g g^{-1}) in two larch species (white bar: Japanese larch; gray bar: Hybrid larch F_1) for each treatment (control: C, elevated ozone: O_3, nitrogen loading: N, ozone and nitrogen loading: O_3 x N).

Fig. 3

The net photosynthesis rate at a CO_2 concentration of 380 ppm (A_{380}, µmol m^{-2} s^{-1}), stomatal conductance (Gs_{380}, mol m^{-2} s^{-1}), photosynthetic nitrogen-use efficiency (*NUE*, µmol mgN^{-1} s^{-1}), photosynthetic phosphorus use efficiency (*PUE*, µmol mgP^{-1} s^{-1}), maximum carboxylation rate (Vc_{max}, µmol m^{-2} s^{-1}), maximum electronic transport rate (J_{max}, µmol m^{-2} s^{-1}), and maximum photosynthetic rate (A_{max}, µmol m^{-2} s^{-1}) in two larch species (white bar: Japanese larch; gray bar: Hybrid larch F_1) for each treatment (control: C, elevated ozone: O_3, nitrogen loading: N, ozone and nitrogen loading: O_3 x N).

Table. 1

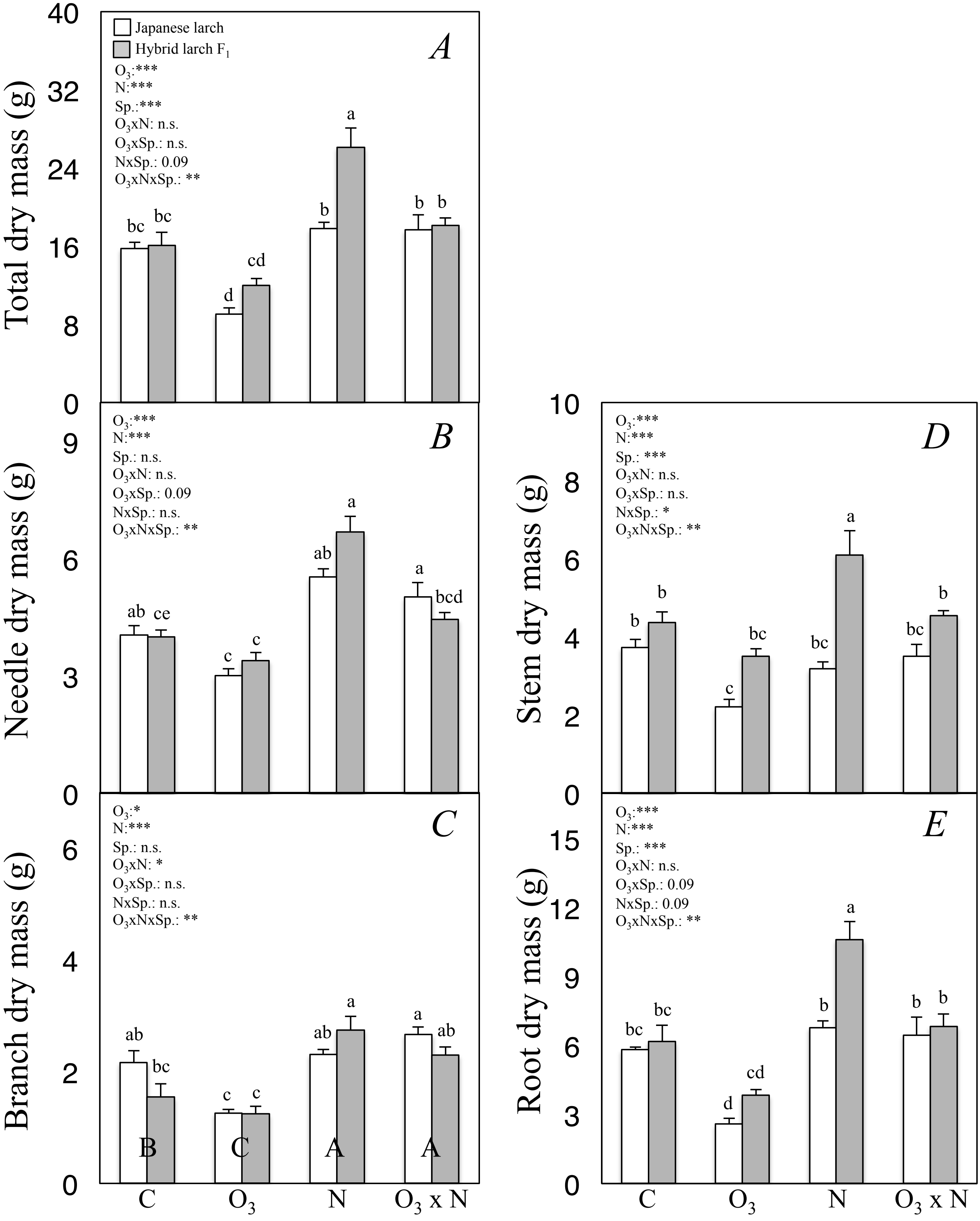
The mean value (standard error; SE, n=4) of ozone concentration, AOT40 in each month of the years 2015 and 2016 (nmol O_3 mol^{-1}), inorganic nitrogen concentrations in surface soil (NH_4^+, NO_3^-, mg l^{-1}), and pH (H_2O) in two larch species (JL: Japanese larch; HL: Hybrid larch F_1) for each treatment (Control: control, O_3: elevated O_3, N: nitrogen loading, O_3 x N: ozone and nitrogen loading).

Table. 2

Summary of needle element concentrations (N, P, mg g^{-1}), N/P and leaf mass area (LMA, g m^{-2}) in the Japanese larch and hybrid larch F_1 in each treatment (control: control, O_3: elevated ozone, N: nitrogen loading, O_3 x N: ozone and nitrogen loading), n = 4. Data indicate mean values (SE); GLMM analysis: ozone (O_3), N loading (N), species difference (Sp.), and these interactions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s. denotes non-significant values; Different letters indicate significant differences (Tukey HSD test).

Correlation coefficients between nutrients, photosynthesis, and needle dry mass were calculated using Pearson's correlation test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. No sign denotes non-significant values.

Fig.1



Japanese larch
Hybrid larch F_1
A
O_3:***
N:***
Sp.:***
O_3xN: n.s.
O_3xSp.: n.s.
NxSp.: 0.09
O_3xNxSp.: **
Total dry mass (g)
0
8
16
24
32
40
bc
bc
d
cd
b
a
b
b
B
O_3:***
N:***
Sp.: n.s.
O_3xN: n.s.
O_3xSp.: 0.09
NxSp.: n.s.
O_3xNxSp.: **
Needle dry mass (g)
0
3
6
9
ab
ce
c
c
ab
a
a
bcd
C
O_3:*
N:***
Sp.: n.s.
O_3xN: *
O_3xSp.: n.s.
NxSp.: n.s.
O_3xNxSp.: **
Branch dry mass (g)
0
2
4
6
ab
bc
c
c
ab
a
a
ab
B
C
A
A
C
O_3
N
O_3 x N
D
O_3:***
N:***
Sp.: ***
O_3xN: n.s.
O_3xSp.: n.s.
NxSp.: *
O_3xNxSp.: **
Stem dry mass (g)
0
2
4
6
8
10
b
b
c
bc
bc
a
bc
b
E
O_3:***
N:***
Sp.: ***
O_3xN: n.s.
O_3xSp.: 0.09
NxSp.: 0.09
O_3xNxSp.: **
Root dry mass (g)
0
3
6
9
12
15
bc
bc
d
cd
b
a
b
b
C
O_3
N
O_3 x N

Fig. 2

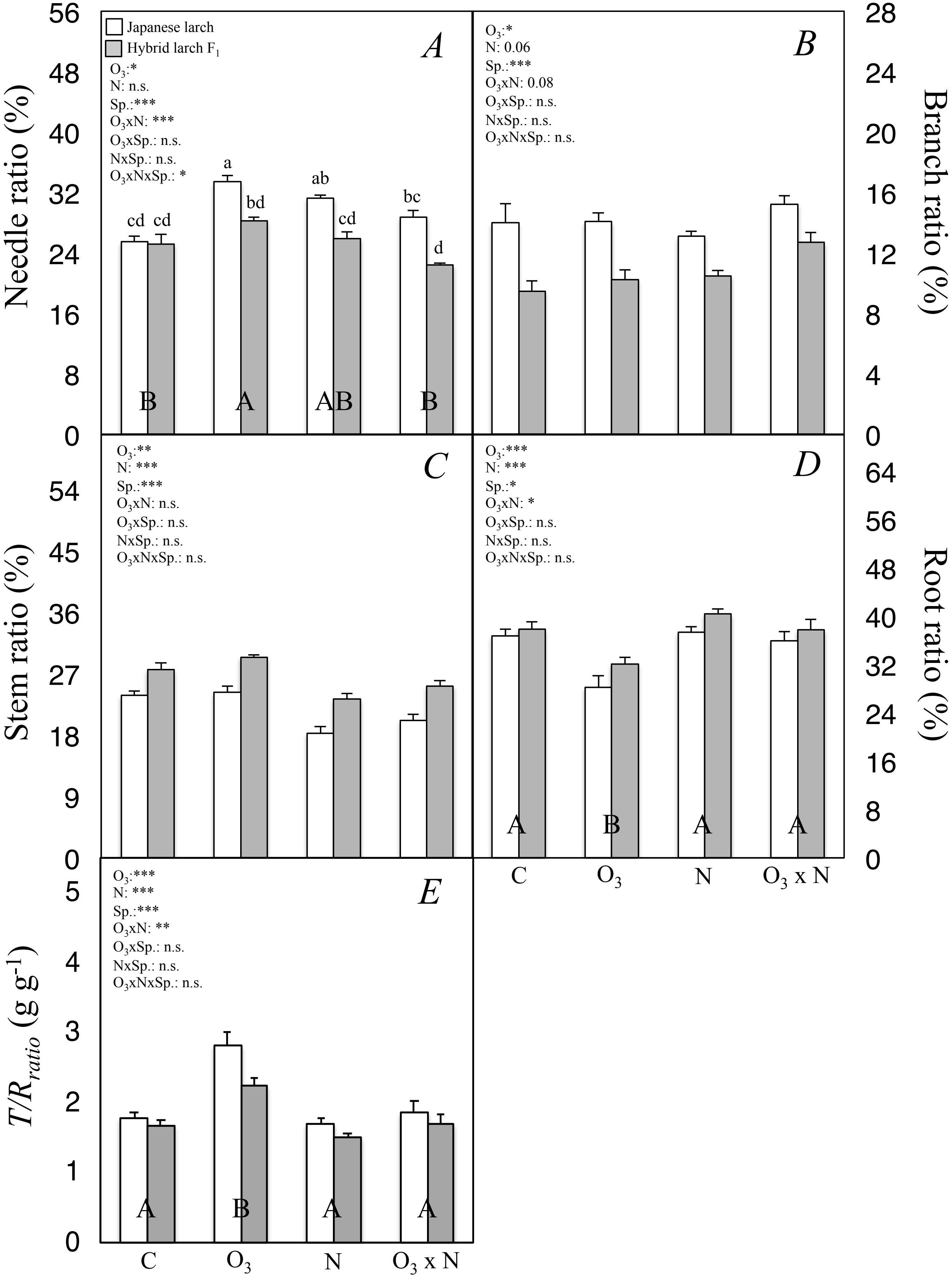
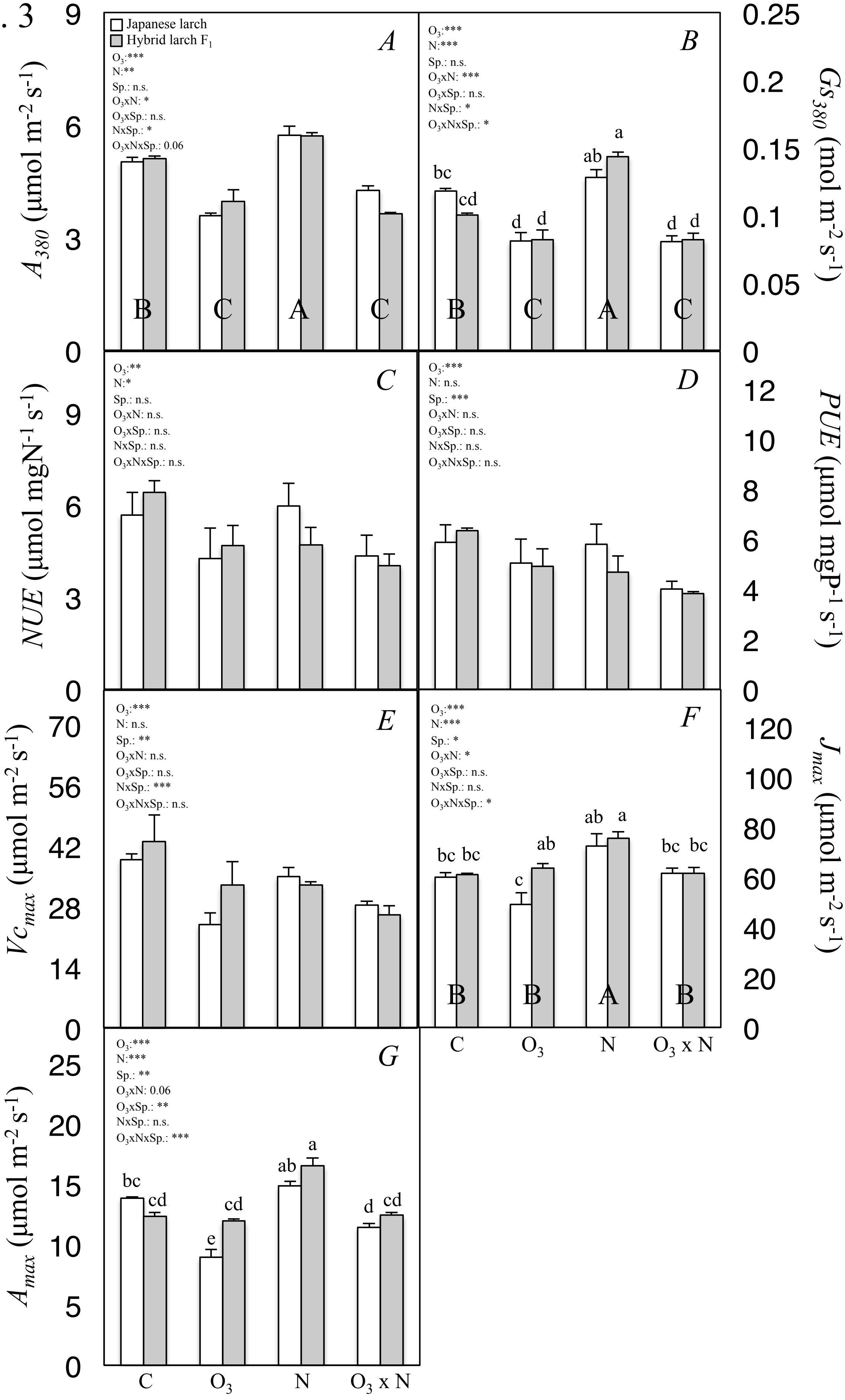

Needle ratio (%)
56
48
40
32
24
16
8
0
Japanese larch
Hybrid larch F$_1$
A
O$_3$:*
N: n.s.
Sp.:***
O$_3$xN: ***
O$_3$xSp.: n.s.
NxSp.: n.s.
O$_3$xNxSp.: *
cd
cd
a
bd
ab
cd
bc
d
B
A
AB
B
B
O$_3$:*
N: 0.06
Sp.:***
O$_3$xN: 0.08
O$_3$xSp.: n.s.
NxSp.: n.s.
O$_3$xNxSp.: n.s.
Branch ratio (%)
28
24
20
16
12
8
4
0
C
O$_3$:**
N: ***
Sp.:***
O$_3$xN: n.s.
O$_3$xSp.: n.s.
NxSp.: n.s.
O$_3$xNxSp.: n.s.
Stem ratio (%)
54
45
36
27
18
9
0
D
O$_3$:***
N: ***
Sp.:*
O$_3$xN: *
O$_3$xSp.: n.s.
NxSp.: n.s.
O$_3$xNxSp.: n.s.
Root ratio (%)
64
56
48
40
32
24
16
8
0
A
B
A
A
C
O$_3$
N
O$_3$ x N
E
O$_3$:***
N: ***
Sp.:***
O$_3$xN: **
O$_3$xSp.: n.s.
NxSp.: n.s.
O$_3$xNxSp.: n.s.
T/R_{ratio} (g g^{-1})
5
4
3
2
1
0
A
B
A
A
C
O$_3$
N
O$_3$ x N

Fig. 3



Japanese larch
Hybrid larch F1
A
O3:***
N:**
Sp.: n.s.
O3xN: *
O3xSp.: n.s.
NxSp.: *
O3xNxSp.: 0.06
B C A C
A380 (µmol m-2 s-1)
0 3 6 9
B
O3:***
N:***
Sp.: n.s.
O3xN: ***
O3xSp.: n.s.
NxSp.: *
O3xNxSp.: *
bc cd d d ab a d d
B C A C
Gs380 (mol m-2 s-1)
0 0.05 0.1 0.15 0.2 0.25
C
O3:**
N:*
Sp.: n.s.
O3xN: n.s.
O3xSp.: n.s.
NxSp.: n.s.
O3xNxSp.: n.s.
NUE (µmol mgN-1 s-1)
0 3 6 9
D
O3:***
N: n.s.
Sp.: ***
O3xN: n.s.
O3xSp.: n.s.
NxSp.: n.s.
O3xNxSp.: n.s.
PUE (µmol mgP-1 s-1)
0 2 4 6 8 10 12
E
O3:***
N: n.s.
Sp.: **
O3xN: n.s.
O3xSp.: n.s.
NxSp.: ***
O3xNxSp.: n.s.
Vcmax (µmol m-2 s-1)
0 14 28 42 56 70
F
O3:***
N:***
Sp.: *
O3xN: *
O3xSp.: n.s.
NxSp.: n.s.
O3xNxSp.: *
bc bc c ab ab a bc bc
B B A B
Jmax (µmol m-2 s-1)
0 20 40 60 80 100 120
C O3 N O3 x N
G
O3:***
N:***
Sp.: **
O3xN: 0.06
O3xSp.: **
NxSp.: n.s.
O3xNxSp.: ***
bc cd e cd ab a d cd
Amax (µmol m-2 s-1)
0 5 10 15 20 25
C O3 N O3 x N

Table. 1

	O_3 concentration (nmol O_3 mol^{-1})			
	Control	O_3	N	O_3 x N
2015				
July	29.1 (1.6)	60.9 (1.4)	25.6 (1.4)	60.8 (1.4)
August	30.9 (1.3)	64.1 (1.4)	27.2 (1.2)	64.0 (1.4)
September	35.1 (1.7)	59.6 (2.2)	30.9 (1.5)	59.6 (2.1)
2016				
June	33.6 (1.5)	64.3 (2.3)	32.9 (1.4)	64.2 (2.3)
July	27.4 (1.8)	62.3 (1.7)	26.9 (1.7)	63.2 (1.6)
August	28.9 (1.5)	61.2 (1.9)	28.3 (1.5)	61.1 (1.9)
	AOT40 (μmol O_3 mol^{-1} h)			
	Control	O_3	N	O_3 x N
2015				
July	1.00 (0.5)	6.89 (0.7)	0.92 (0.4)	6.88 (0.4)
August	1.01 (0.4)	7.26 (0.6)	0.89 (0.3)	7.24 (0.5)
September	1.14 (0.5)	6.75 (0.9)	1.01 (0.4)	6.74 (0.9)
2016				
June	1.10 (0.5)	7.28 (1.1)	1.07 (0.3)	7.27 (1.0)
July	0.89 (0.6)	7.05 (0.6)	0.88 (0.6)	7.16 (0.6)
August	0.94 (0.5)	6.92 (0.7)	0.92 (0.5)	6.92 (0.8)
	Soil conditions			
	Control	O_3	N	O_3 x N
NH_4^+ (mg l^{-1})				
JL	3.42 (0.30)	3.68 (0.07)	5.23 (0.36)	5.51 (0.11)
HL	3.02 (0.30)	3.51 (0.10)	5.81 (0.43)	5.14 (0.45)
NO_3^- (mg l^{-1})				
JL	0.08 (0.04)	0.32 (0.10)	3.41 (0.68)	2.31 (0.31)
HL	0.28 (0.01)	0.27 (0.06)	2.66 (0.66)	2.42 (0.43)
pH (H_2O)				
JL	6.03 (0.02)	6.12 (0.01)	5.84 (0.01)	5.90 (0.02)
HL	6.05 (0.01)	6.16 (0.20)	5.87 (0.01)	5.88 (0.04)

Table. 2

	Japanese larch				Hybrid larch F$_1$				P value						
	C	O$_3$	N	O$_3$ x N	C	O$_3$	N	O$_3$ x N	O$_3$	N	Sp.	O$_3$ x N	O$_3$ x Sp.	N x Sp.	O$_3$ x N x Sp.
Leaf N	8.82	7.30	13.25	12.01	7.81	7.62	12.20	10.92	*	***	0.07	n.s.	n.s.	n.s.	n.s.
	(0.27)	(0.24)	(0.77)	(0.47)	(0.28)	(0.33)	(1.10)	(0.27)							
Leaf P	0.37^{bc}	0.38^{bc}	0.44^{bc}	0.54^{bc}	0.36^{c}	0.46^{bc}	0.55^{b}	0.96^{a}	***	***	***	**	**	***	*
	(0.02)	(0.02)	(0.01)	(0.01)	(0.01)	(0.02)	(0.05)	(0.09)							
N/P	24.03	19.28	29.98	22.29	21.67	16.72	23.32	11.61	***	n.s.	***	0.06	n.s.	*	n.s.
	(0.75)	(0.84)	(1.87)	(0.78)	(1.28)	(0.61)	(4.12)	(0.90)							
LMA	100	106	107	101	104	113	109	107	n.s.	0.06	n.s	n.s.	n.s.	n.s.	n.s.
	(10.1)	(14.2)	(4.6)	(1.4)	(2.0)	(10.8)	(11.6)	(1.2)							

	Nutrient factors			
	Japanese larch		Hybrid larch F$_1$	
Coefficient	Leaf N	N/P	Leaf N	N/P
Photosynthesis	0.64*	0.55	0.79**	0.65*
Needle dry mass	0.81**	0.56*	0.92***	0.84***

Supplementary data