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1 **Title:** Nitrogen loading increases the ozone sensitivity of larch seedlings with higher
2 sensitivity to nitrogen loading

3

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12

13 **1. Abstract**

14 Larch (*Larix* sp.) tree is a critical species for the future afforestation in Northeast Asia.

15 The impacts of elevated concentrations of ground-level ozone (O₃) and nitrogen (N)

16 deposition are raising concerns. However, knowledge of the combined effects of

17 elevated O₃ and N loading are still limited. We investigated whether nitrogen loading

18 mitigates the negative impacts of ozone on two larch species: the Japanese larch (*L.*

19 *kaempferi*) and its hybrid larch F₁ (*L. gmelinii* var. *japonica* × *L. kaempferi*) or not. We

20 used open-top chambers and compared responses of the larch seedlings. Results showed

21 the N loading mitigated the negative effects of O₃ on Japanese larch. However, in

22 hybrid larch F₁, N loading did not mitigate O₃-induced inhibition of growth and
23 photosynthetic capacity. Mitigation effect of N loading on negative O₃ impacts may
24 vary between the two *Larix* spp. Hybrid larch F₁ could be more affected by the
25 combined effects of O₃ and N loading due to its higher growth response to N loading.
26 Elevated O₃ also reduced leaf nitrogen/phosphorus (N/P) ratio by elevated O₃, with
27 significant effects in hybrid larch F₁, particularly under N loading. In the present study,
28 leaf N/P ratio was utilized to validate the hypothesis that a positive effect of N loading
29 may be observed if O₃ does not induce P limitation in *Larix* spp. We demonstrated a
30 potential leaf N/P ratio function, which could reflect responses to O₃ and N loading in
31 hybrid larch F₁.

32

33 **Keywords:**

34 Ozone sensitivity; nitrogen loading; interspecific difference; hybrid larch F₁; *Larix*
35 *kaempferi*; nitrogen phosphorus ratio;

36

37 **Abbreviations**

38 Ozone (O₃), Nitrogen (N), Phosphorus (P), Nitrogen-phosphorus ratio (N/P), Leaf mass
39 area (LMA), Net photosynthetic rate at 380 μmol CO₂ mol⁻¹ and light saturation per leaf
40 area (A_{380}), Stomatal conductance (G_{S380}), Photosynthetic nitrogen use efficiency (NUE),
41 Photosynthetic phosphorus use efficiency (PUE), Maximum rate of carboxylation
42 (V_{Cmax}), Maximum electron transport rate (J_{max}), Net photosynthetic rate at 1600 μmol

43 $\text{CO}_2 \text{ mol}^{-1}$ (A_{max}), Net photosynthetic rate at light saturation per leaf mass (A_{mass}).

44

45 **2. Introduction**

46 Larch (*Larix* sp.) species are widely distributed in Northeast Asia and have been planted
47 for timber production and ecosystem rehabilitation (Abaimov et al. 2000; Zhang et al.
48 2000). In addition to the native Japanese larch (*Larix kaempferi*), a hybrid larch F₁ (*L.*
49 *gmelinii* var. *japonica* x *L. kaempferi*), which is more tolerant to stress and exhibits
50 higher photosynthetic performance, has also been developed (Kita et al. 2009). However,
51 increasing concentrations of ground-level ozone (O₃) (Akimoto 2017) and nitrogen (N)
52 deposition (Galloway et al. 2004; Liu et al. 2013; Yamaguchi et al. 2014) are raising
53 concern, particularly in Northeast Asian forests (Yamaji et al. 2008; Koike et al. 2013).
54 Concentrations of such air pollutants are projected to continue increasing in future
55 (Nagashima et al. 2010; Ainsworth et al. 2012; Izuta 2017). Therefore, there is a need to
56 evaluate the responses of the *Larix* spp. to elevated O₃ and N deposition (Ryu et al.
57 2009; Koike et al. 2012).

58 O₃ is a toxic pollutant that inhibits numerous physiological functions such as
59 photosynthesis and it eventually causes a decrease in growth, particularly belowground
60 growth (Li et al. 2016; Shi et al. 2016; Wang et al. 2016; Agathokleous et al. 2016;
61 Sugai et al. 2018). Conversely, N loading may promote the growth of trees when soil N
62 limits growth, such as in a boreal forest (Vitousek et al. 2002). However, excess N

63 loading could also have negative effects on tree growth (Aber et al. 1989; Ryu et al.
64 2009; Wang et al. 2018). Although numerous studies have reported the combined
65 effects of elevated O₃ and N deposition, they have primarily concluded that N loading
66 might not affect or increase the sensitivity of woody species to O₃ (e.g., Nakaji and
67 Izuta 2001; Yamaguchi et al. 2011; Izuta et al. 2017). Regarding larch species, contrary
68 to previous reports, Watanabe et al. (2006) reported that N loading could mitigate the
69 negative impacts of O₃ on Japanese larch seedlings. However, a recent study reported
70 that N loading could not mitigate the negative impacts of O₃ on hybrid larch F₁
71 (Dong-Gyu et al. 2015). Consequently, researchers' views regarding the combined
72 effects of N loading and O₃ on larch species are inconsistent. In addition, the
73 physiological mechanisms of the effects on combined N loading and O₃ deposition
74 remain unclear (e.g., Mills et al. 2016; Izuta 2017).

75 The effects of N loading vary depending on the phosphorus (P) conditions in plants
76 (e.g., Güsewell 2004; Elser et al. 2010). For instance, N loading promoted the growth of
77 hybrid larch F₁ under high P conditions (Fujita et al. 2018). Indeed, the leaf N/P ratio
78 could be a critical indicator of N or P limitation (Güsewell 2004) since N loading
79 increases the N/P ratio in plants (e.g., Reich and Oleksyn 2004; Güsewell 2004; Elser et
80 al. 2010). Conversely, one of the effects of O₃ is accelerated leaf senescence, resulting
81 in imbalances in leaf nutrients (Uddling et al. 2006; Shi et al. 2016; Shi et al. 2017).
82 However, few studies have reported leaf N and P reductions in larch species under
83 elevated O₃ (Koike et al. 2012; Wang et al. 2015). The relationship between leaf N/P

84 ratio and physiological activities under elevated O₃ and/or N loading remains relatively
85 poorly understood (Beyears et al. 1992; Kainulainen et al. 2000; Utriainen and
86 Holopainen 2001; Waillin et al. 2002; Wang et al. 2015). Considering that the
87 senescence effect of O₃ does not induce reductions in leaf P concentrations (Wang et al.
88 2015), a positive effect of N loading on hybrid larch F₁ and lower leaf N/P ratio may be
89 observed (Fujita et al. 2018). Here we expected that leaf N/P ratio could be a useful
90 indicator of N and P limitation in the larches and facilitate comparison of their
91 responses to N loading under elevated O₃.

92 The aim of the present study is to investigate the effect of N loading on the
93 responses of Japanese larch and hybrid larch F₁ seedlings to O₃. Our hypothesis is N
94 loading could mitigate the negative impacts of O₃ on larch seedlings. To assess the
95 hypothesis, we investigated the relationship between physiological and nutritional
96 responses based on correlation tests.

97

98 **3. Materials and methods**

99 **3.1 Experimental design**

100 The experiments were conducted at Sapporo Experimental Forest, Hokkaido University,
101 Japan (43°04'N, 141°20'E; 15 m above sea level). Weather data from 2015 to 2016 were
102 recorded at a meteorological station in Sapporo (Japan Meteorological Agency, 2016).
103 The monthly average values (\pm s.e.) of metrological data from May 2015 to September
104 2016 were: mean monthly average air temperature = 12.31 \pm 2.18°C; maximum

105 temperature = $23.53 \pm 2.27^\circ\text{C}$; minimum temperature = $3.41 \pm 2.21^\circ\text{C}$; total precipitation
106 = 106.8 ± 14.6 mm; and total sunshine duration = 162.1 ± 12.0 h, respectively. Japanese
107 larch (*Larix kaempferi*) is tolerant to harsh conditions such as low temperature and
108 infertile soils although it is sensitive to grazing damage from red-backed voles (*Myodes*
109 *rufocanus bedfordiae*). To overcome such limitations, hybrid larch F₁ (*L. gmelinii* var.
110 *japonica* x *L. kaempferi*) was developed (Kita et al. 2009). These two species of larch
111 seedlings were grown for one year from seeds at a nursery at Hokkaido Research
112 Organization, Forestry Research Institute, Bibai. On May 12, 2015, all larch seedlings
113 were transplanted to 7 L pots before leaf formation. The initial stem diameter and height
114 were 2.21 ± 0.44 mm (mean \pm se) and 16.56 ± 2.93 cm, respectively, for Japanese larch,
115 and 2.45 ± 0.17 mm and 20.44 ± 1.39 cm, respectively, for hybrid larch F₁. Soil in the pots
116 was chosen so as to mimic immature volcanic ash soil (Kanuma soil and Akadama soil,
117 volume ratio 1:1). To prevent soil desiccation and nutrient imbalances, we applied 100
118 mL of commercial liquid fertilizer (HYPONEX; Japan, N = 72.4 mg l^{-1} , P = 284 mg l^{-1} ,
119 K = 94.2 mg l^{-1}) as a basal dressing after complete rooting. There, all pots initially
120 received $5.5 \text{ kgN ha}^{-1} \text{ yr}^{-1}$.

121 N loading treatments were applied at two-week-intervals using $(\text{NH}_4)_2\text{SO}_4$
122 dissolved in 500 ml tap water to simulate recent N deposition such as PM_{2.5} (Particulate
123 matter less than $2.5 \mu\text{m}$ in aerodynamic diameter; Hatakeyama 2011). N loading was set
124 to $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, which are the estimated levels in 2050 (Galloway et al. 2004; Liu
125 et al. 2013). The N amount included the initial liquid fertilization. N loading was

126 performed on June 19, July 15, August 5 and 17 in 2015, and June 27, July 22, and
127 August 1 and 17 in 2016, while avoiding rain days. To avoid drought conditions, control
128 seedlings that were not N treated were irrigated with 500 ml tap water over a period
129 similar to that of N loading. For pH and inorganic N concentration measurements,
130 surface soil (<5 cm) was sampled from each pot on 29 August 2016.

131 To measure soil pH, 10 g of fresh soil was sampled and mixed with 25 ml of
132 distilled water. The samples were shaken for over one hour before measurement using a
133 portable pH sensor (RM-30P, TOA DKK, Tokyo, Japan). To measure soil inorganic N
134 contents, 10 g of sampled fresh soil was mixed with 100 ml of 2 M potassium chloride
135 solution. The samples were the shaken for over one hour. For colorimetric measurement
136 of inorganic N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) using a flow injection analyzer (AQLA-700,
137 Aqualab Co., Ltd., Tokyo, Japan), the samples were filtered through a 1- μm filter (Toyo
138 Roshi, No.5C filter paper, Tokyo, Japan). Soil analyses were performed based on the
139 methods of Miura (1997).

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

147 mol^{-1} , which is the value for the environmental standard for photochemical oxidants in
148 Japan (Japanese Ministry of the Environment 1996). The enriched O_3 concentration
149 target was also set to assume predicted future O_3 concentrations in Asian countries
150 (Yamaji et al. 2008; Ainsworth et al. 2012). O_3 treatments were carried out from June
151 19, 2015 to September 18, 2015 and from May 18, 2016 until August 29, 2016. O_3 was
152 supplied using air pumps (APN-110K, IWAKI, Japan) from an electrical discharge O_3
153 generator (PZ-1B, Kofloc-Kojima, Japan) into the OTCs only during daylight hours
154 (06:00–18:00, JST). Ambient air inside the OTCs was constantly driven to the
155 atmosphere using over 24 hours and wind speeds at the openings to the surfaces of the
156 OTCs were set to 0.1 m^3 per second. During the exposure periods, O_3 concentrations
157 were continuously monitored (1 minute interval) with UV absorption O_3 analyzer
158 (Model 202-EPA, 2B technologies, Boulder, USA) and an O_3 monitoring system
159 (EG-3000F, Ebara, Japan). An algorithm of proportional integrative differential system
160 was applied to maintain the target O_3 concentrations. Table 1 presents the achieved O_3
161 concentration values and the value of accumulated exposure over the threshold of 40
162 nmol mol^{-1} (AOT40). During the non-exposure periods, O_3 generators were switched
163 off and all the seedlings were maintained in the chamber except for the heavy snow
164 seasons.

165 In total, there were 4 treatment: tap water + ambient O_3 (Control); tap water +
166 elevated O_3 (O_3); N loading + ambient O_3 (N); and elevated O_3 x N loading (O_3 x N).
167 All the treatments had four replicates, yielding 16 OTCs. OTC positions were set based

168 on a completely randomized split-plot design. Four larch seedlings of each species were
169 set in each chamber (64 seedlings of each species, 128 seedlings in total). All the
170 seedlings were utilized throughout the experiments and the positions of seedlings within
171 each OTC were regularly changed to prevent biased edge effects.

172

173 **3. 2 Leaf gas-exchange measurements**

174 Gas-exchange measurements were conducted for all the seedlings in all the treatments
175 between the 17 and 21 August 2016. An open gas-exchange system (LI-6400, Li-Cor
176 Inc., NE, U.S.A.) was used to measure the gas-exchange parameters. For each seedling,
177 approximately six fresh mature needles were selected from the non-shaded position of a
178 branch and attached to the leaf clip chamber of a LI-6400. Measurements were
179 conducted between 05:00 and 15:00, JST, and the leaf temperature was maintained at
180 $25\pm 0.5^\circ\text{C}$. The water vapor deficit in the leaf chamber was approximately 1.2 ± 0.3 kPa
181 and the photosynthetic photon flux was approximately $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. To plot
182 curves of net CO_2 assimilation rate (A) versus intercellular CO_2 concentration (C_i), i.e.,
183 A/C_i curves; A was determined at ten C_i levels (C_a : 60–1600 $\mu\text{mol mol}^{-1}$). The net
184 photosynthesis rate and stomatal conductance at 380 $\mu\text{mol mol}^{-1} C_a$ (A_{380} and G_{S380} ,
185 respectively) were determined from the A/C_i curves. In addition, using the individual
186 leaf photosynthetic model, the curves were used to calculate the maximum net
187 photosynthetic rate at 1600 $\mu\text{mol mol}^{-1} C_a$ (A_{max}), the maximum rate of carboxylation
188 (V_{max}), and the maximum rate of electron transport (J_{max}) (Farquhar et al. 1980; Long

210
$$PUE = A_{380} / (LMA \times P).$$

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

212
$$A_{mass} = A_{max} / LMA.$$

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

214

215 **3. 4 Production and allocation of dry matter**

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

221
$$T/R_{ratio} \text{ (g g}^{-1}\text{)} = (\text{Needle} + \text{Branch} + \text{Stem}) / \text{Root}.$$

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

224

225 **3. 5 Statistical analyses**

226 The data were averaged per experimental unit (block) resulting in four values per
227 treatment (n = 4). All parameters were analyzed for each species using a general-linear
228 mixed model (GLMM) randomized by blocks. O₃ exposure, N loading treatments, and
229 different species constituted the predictors. For significant interaction factors with more
230 than two levels, we applied the multiple comparison test of Tukey's honestly significant

231 difference (HSD). $P = 0.05$ was considered statistically significant. R software ver. 3.4.3
232 was used for data processing and statistics.

233

234 **4. Results**

235 **4.1. Environmental conditions**

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

243

244 **4.2. Dry mass**

245 O_3 enrichment significantly decreased needle, branch, and stem dry mass in Japanese
246 larch (Fig. 1A-E, $P < 0.05$) whereas significant reductions attributable to O_3 were not
247 observed in hybrid larch F₁. In Japanese larch, although N loading did not have
248 significant effects (Fig. 1B, D), needle dry mass increased whereas stem dry mass
249 slightly decreased due to N loading. However, in hybrid larch F₁, N loading increased
250 needle, branch, and stem dry mass (Fig. 1A-E, $P < 0.05$).

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

252 > 0.05). O₃ slightly increased branch and stem dry mass in Japanese larch although
253 there were no significant differences (Fig. 1C, D). In hybrid larch F₁, O₃ significantly
254 decrease dry mass in under N loading in all the tested parts (Fig. 1A,B, D, and E, P <
255 0.05) except in the branches (Fig. 1C). Notably, the branch dry mass responses to O₃
256 were contrasting in the two investigated species *Larix* spp. under N loading. Although
257 there were no significant changes (Fig. 1C), branch dry mass in Japanese larch
258 increased whereas that in hybrid larch F₁ decreased following O₃ enrichment.

259

260 **4. 3. Dry mass allocation**

261 O₃ decreased root proportions in the two *Larix* spp. whereas it increased the
262 aboveground proportions (Fig. 2D, E). In Japanese larch, O₃ and N loading significantly
263 increased the needle proportions (Fig. 2A, D, P < 0.05). Responses of the branches to N
264 loading were contrasting between species although the degree was marginal (i.e., < 5%).
265 The needle proportions under N loading were decreased by O₃ in both *Larix* spp.
266 although these were no significant changes. Regarding the differences between the
267 species, the needle proportions under N loading were significantly higher in Japanese
268 larch than in hybrid larch F₁ (Fig. 2A, D, P < 0.05). The differences between the species
269 were maintained under elevated O₃ and N loading.

270

271 **4. 4. Photosynthetic activity**

272 O₃ significantly decreased GS_{380} and A_{max} in Japanese larch (Fig. 3B, G; P < 0.05). In

273 Japanese larch, N loading did not increase any photosynthetic parameters except A_{380} .
274 However, in hybrid larch F₁, N loading significantly increased A_{max} and J_{max} (Fig. 3F,
275 G; $P < 0.05$). In addition, O₃ did not decrease any parameters except A_{380} in hybrid larch
276 F₁. In both *Larix* spp., O₃ decreased A_{380} under N loading (Fig. 3A, $P < 0.05$). The
277 effects of O₃ on NUE , PUE , and $V_{c_{max}}$ were not significant while negative effects on
278 species were observed (Fig. 3C-E).

279

280 **4. 5. Nutrient conditions of leaves**

281 Table 2 presents the concentration of each nutrient element per needle mass, the LMA
282 values, and the coefficients of correlation between leaf N and leaf N/P with A_{mass} and
283 needle dry mass. As expected, O₃ did not decrease leaf P in both species. However, N
284 loading significantly increased leaf P in the hybrid larch F₁ (Table 2). In addition, the O₃
285 and N loading combined increased nutrient element concentrations in hybrid larch F₁. In
286 addition, leaf N/P under N loading in hybrid larch F₁ was decreased by O₃. Needle dry
287 mass of both *Larix* spp. exhibited significant positive correlations with leaf N and leaf
288 N/P.

289

290 **5. Discussion**

291 Growth responses to the combined effect of O₃ and N loading varied between Japanese
292 larch and hybrid larch F₁. O₃ enrichment did not result in a significant reduction in any
293 dry mass in Japanese larch under N loading. The same type of response was also

294 observed in the previous study (Watanabe et al. 2006; Dong-Gyu et al. 2015). Watanabe
295 et al. (2006) suggested that reducing O₃ sensitivity in Japanese larch with N loading was
296 not related to the amount of O₃ absorbed through the stomata. In the present study, we
297 observed no significant combined effect on G_{S380} in Japanese larch, although O₃
298 decreased G_{S380} . Such a stomatal response suggested that N loading did not alter the
299 amount of O₃ absorbed, which is consistent with some previous findings (Watanabe et
300 al. 2006).

301 In contrast, O₃ significantly reduced the total dry mass of hybrid larch F₁ under N
302 loading (Fig. 1A). We observed a reduction in photosynthetic parameters following O₃
303 enrichment under N loading, as well as dry mass (Fig. 3). The results are consistent with
304 those of a previous study that reported that NH₄NO₃ loading might not mitigate the
305 negative impacts of O₃ on hybrid larch F₁ (Dong-Gyu et al. 2015). The results suggest
306 that the responses to the combined effects of O₃ and N loading in the two *Larix* spp. are
307 clone-specific. In addition, the results reinforce the argument that N loading could
308 intensify the negative effects of O₃ on hybrid larch F₁ to some extent. Previous studies
309 have suggested that excess N loading could simulate detoxification and repair activities
310 against O₃ stress (Watanabe et al. 2006; Izuta 2017). However, Yamaguchi et al. (2010)
311 reported that N loading did not change anti-oxidant capacities activated by O₃ such as
312 acidic amino acids. As described, the mechanisms of changes in O₃ sensitivity
313 attributable to N loading remain unclear (e.g., Mills et al. 2016). Hereafter, we discuss
314 the contrasting responses in two *Larix* spp. to consider the potential mechanisms.

315 Hybrid larch F₁ could be affected more by the combined effects of O₃ and N
316 loading (Fig. 1) potentially due to its higher growth response to N loading. This
317 hypothesis could be supported by several previous studies (Pell et al. 1995; Matyssek et
318 al. 1997; Maurer et al. 1997; Yamaguchi et al. 2010; Kinose et al. 2017). The seasonal
319 responses of photosynthesis rates in Siebold's beech (*Fagus crenata*) were significantly
320 decreased by O₃ only when the levels of liquid fertilizer were relatively high (Hyponex
321 6-10-5, HYPONex Japan, Japan) (Kinose et al. 2017). In birth clone (*Betula pendula*)
322 seedlings, macro- and micro nutrient supply (Hauert, Nutrient typ A, Swiss) did not
323 mitigate the negative impacts of O₃ (Maurer et al. 1997). Conversely, the degree of
324 physiological inhibition by O₃ was significantly increased by nutrient supply. In
325 addition, total dry mass in *Fagus crenata* seedlings decreased due the combined effect
326 of N loading and elevated O₃ because of a higher growth response to NH₄NO₃ loading
327 (50 kg N ha⁻¹ yr⁻¹ equivalent) (Yamaguchi et al. 2007). Pell et al. (1995) also reported
328 that the negative effect of O₃ on *Populus tremuloides* increased with increase in N
329 supply. Based on such reports, we postulate that the responses to O₃ could be more
330 significant when plants have higher sensitivity to N loading. In addition, we should
331 consider the degree of change in response to O₃ and sensitivity to N loading by
332 comparing the contrasting responses in the larch species. In the next section, we focus
333 on role of the effect of N loading in understanding the effect of N loading on
334 O₃-induced changes such as increments in leaves.

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

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

355 N loading significantly increased leaf P in hybrid larch F_1 (Table 2, $P < 0.05$).
356 The increment in leaf P certainly decreased N/P ratio in hybrid larch F_1 . The results

357 could suggest that N loading promotes P absorption and utilization. In addition, the P
358 increment was significant under N loading and elevated O₃ (Table 2, P < 0.05). The
359 result could indicate that relative N limitation may occur following O₃ enrichment even
360 under N loading. Such key responses were not observed in Japanese larch, suggesting
361 that the contradictory responses in the two larch species could be associated with
362 physiological responses to changes in leaf nutrient contents, in particular under N
363 loading.

364 Knowledge regarding leaf P content responses to O₃ remains relatively limited
365 (e.g., Beyears et al. 1992; Kainulainen et al. 2000; Shi et al. 2017). Previous studies
366 have reported no significant reductions, rather, slight increments of leaf P contents
367 under elevated O₃ (e.g., Beyears et al. 1992; Kainulainen et al. 2000; Utriainen and
368 Holopainen 2001; Waillin et al. 2002; Wang et al. 2015). In the present study, we also
369 observed a slight increment in leaf P content under elevated O₃ in both *Larix* spp. The
370 increment in P in the present study was significant in hybrid larch F₁ under N loading,
371 particularly with elevated O₃ (Table 2, P < 0.05). Further studies are required to
372 evaluate why leaf P increased in hybrid larch F₁. Potential mechanisms would be related
373 to ectomycorrhizal fungi (ECM) infection ratio and species diversity of under elevated
374 O₃ conditions (Wang et al. 2015). It is also critical consider the role of ECM symbioses
375 since symbiotic ECM enhance nutrient conditions for the host plants as long as the host
376 plants have the capacity to photosynthesize efficiently.

377 Demand for P could vary depending on N conditions. In Japanese larch, P demand

378 is much greater than N demand (Li et al. 2016). Therefore, to investigate the leaf N and
379 P balances, we evaluated leaf N/P ratio under elevated O₃ and N loading. In the present
380 study, all leaf N/P values in Japanese larch were >20 irrespective of treatments (Table
381 2). A leaf N/P ratio >20 is indicative of P limited biomass production (Güsewell 2004).
382 Therefore, the results indicate that P could limit biomass production in Japanese larch.
383 In addition, higher N/P, i.e., relative P deficiency, accelerates leaf senescence, which
384 becomes a physiological limiting factor (Güsewell 2004; Killingbeck 2004). However,
385 in the present study, N loading decreased *NUE* in hybrid larch F₁ (Fig. 3C, 6.40±0.39 to
386 4.71±0.56 μmol mgN⁻¹ s⁻¹ in the control and N loading treatment, respectively).
387 Translocation of photosynthates in chloroplasts is inhibited by relative deficiency in
388 inorganic phosphorous, i.e., higher leaf N/P constrains the carboxylation cycle (e.g.,
389 Sharkey 1985). Although we did not observe any significant inhibition of *PUE* in both
390 species (Fig. 3D), a plausible explanation for the observed phenomenon could be an
391 increase in sucrose synthesis via the Calvin cycle due to N loading. It would induce P
392 limitations at the early stages of photosynthate translocation, which would also reduce
393 *NUE* (Luo et al. 2014; Li et al. 2016).

394 In conclusion, growth and physiological responses of Japanese larch (*Larix*
395 *kaempferi*) and a hybrid larch F₁ (*L. gmelinii* var. *japonica* x *L. kaempferi*) varied based
396 on the combined effects of N loading and elevated O₃. N loading mitigated the negative
397 effects of O₃ on the total dry mass of Japanese larch. This response was partly
398 consistent with that of the previous results in Japanese larch with increasing N loading

399 under elevated O₃ (Watanabe et al. 2006; Koike et al. 2013). Conversely, N loading
400 increased O₃ sensitivity in hybrid larch F₁ to some extent. The contradictory responses
401 in the two *Larix* spp. could be associated with physiological reactions that influence
402 nutrient contents. To further enhance our understanding of the nutrient dynamics, the
403 synergistic relationship with ECM should be evaluated under elevated O₃ and N
404 loading.

405

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413

414 **8. Appendix A. Supplementary data**

415 Seasonal growth responses of stem diameter (mm) and height (cm) in two larch species
416 (black lines and symbols: Japanese larch; gray lines and symbols: Hybrid larch F₁) of
417 each treatment (control: full line, elevated ozone: dotted line, nitrogen loading: bold line,
418 ozone and nitrogen loading: bold dotted line). The values were measured on May 13
419 and September 11 in 2015, and June 11, July 15, and August 27 in 2016 using

420 measuring tapes (1 mm gradient) and the calipers (Mitsutoyo, Japan). The diameter
421 values were calculated as the means of two crosswise measurements at the base of stem.
422 Each point + error bar is the mean value and standard errors (n=4).

423

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608

609 **10. Captions**

610 Fig. 1

611 The total dry mass (g), needle dry mass (g), branch dry mass (g), stem dry mass (g), and
612 root biomass (g) of two larch species (white bar: Japanese larch; gray bar: Hybrid larch
613 F₁) in each treatment (control: C, elevated ozone: O₃, nitrogen loading: N, ozone and
614 nitrogen loading: O₃ x N). Each bar + error bar is the mean value and standard errors.
615 GLMM analysis: ozone (O₃), N loading (N), species difference (Sp.), and these
616 interactions, *P < 0.05, **P < 0.01, ***P < 0.001, n.s. denotes non-significant values;
617 Different lower case letters denote significant differences between species and
618 treatments (Tukey HSD test; P < 0.05), and different capital letters denote significant
619 differences between treatments (species pooled).

620

621

622 Fig. 2

623 Dry mass allocation (%) to needle, branch, stem, and root and top shoot/root ratio
624 (T/R_{ratio} , g g⁻¹) in two larch species (white bar: Japanese larch; gray bar: Hybrid larch
625 F₁) for each treatment (control: C, elevated ozone: O₃, nitrogen loading: N, ozone and
626 nitrogen loading: O₃ x N).

627

628

629

630 Fig. 3

631 The net photosynthesis rate at a CO₂ concentration of 380 ppm (A_{380} , $\mu\text{mol m}^{-2} \text{s}^{-1}$),
632 stomatal conductance (G_{S380} , $\text{mol m}^{-2} \text{s}^{-1}$), photosynthetic nitrogen-use efficiency (NUE ,
633 $\mu\text{mol mgN}^{-1} \text{s}^{-1}$), photosynthetic phosphorus use efficiency (PUE , $\mu\text{mol mgP}^{-1} \text{s}^{-1}$),
634 maximum carboxylation rate (V_{Cmax} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum electronic transport rate
635 (J_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), and maximum photosynthetic rate (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) in two larch
636 species (white bar: Japanese larch; gray bar: Hybrid larch F₁) for each treatment
637 (control: C, elevated ozone: O₃, nitrogen loading: N, ozone and nitrogen loading: O₃ x
638 N).

639

640 Table. 1

641 The mean value (standard error; SE, n=4) of ozone concentration, AOT40 in each
642 month of the years 2015 and 2016 ($\text{nmol O}_3 \text{mol}^{-1}$), inorganic nitrogen concentrations in
643 surface soil (NH_4^+ , NO_3^- , mg l^{-1}), and pH (H₂O) in two larch species (JL: Japanese
644 larch; HL: Hybrid larch F₁) for each treatment (Control: control, O₃: elevated O₃, N:
645 nitrogen loading, O₃ x N: ozone and nitrogen loading).

646

647

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649

650

651 Table. 2

652 Summary of needle element concentrations (N, P, mg g⁻¹), N/P and leaf mass area
653 (LMA, g m⁻²) in the Japanese larch and hybrid larch F₁ in each treatment (control:
654 control, O₃: elevated ozone, N: nitrogen loading, O₃ x N: ozone and nitrogen loading), n
655 = 4. Data indicate mean values (SE); GLMM analysis: ozone (O₃), N loading (N),
656 species difference (Sp.), and these interactions. *P < 0.05, **P < 0.01, ***P < 0.001, n.s.
657 denotes non-significant values; Different letters indicate significant differences (Tukey
658 HSD test).

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

662

Fig.1

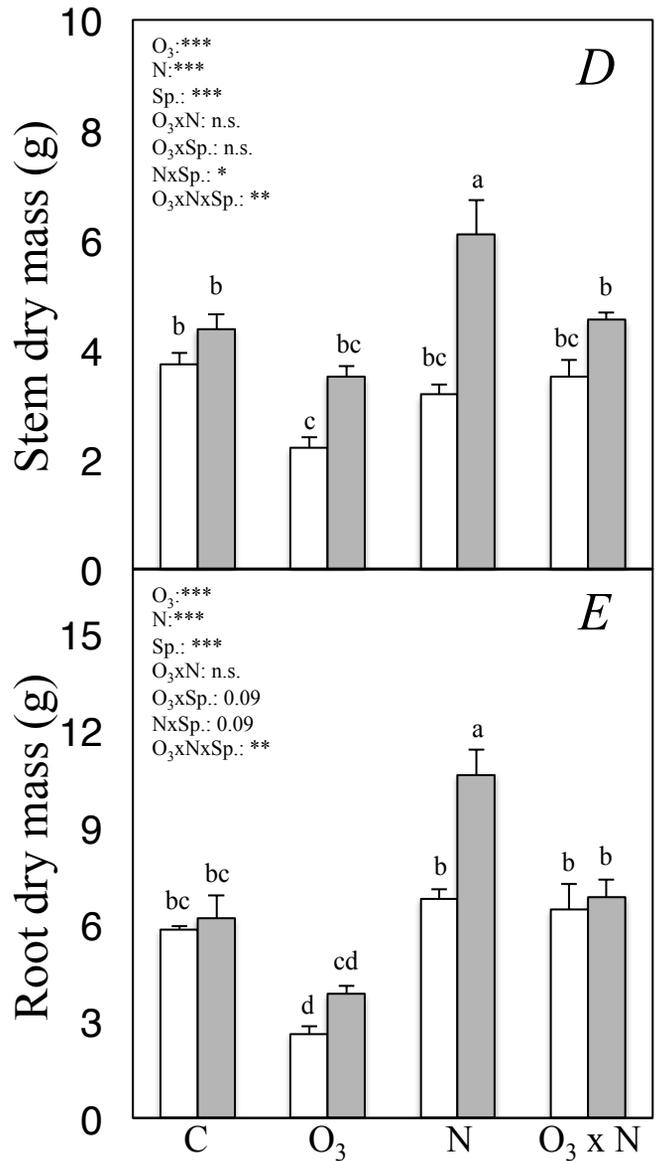
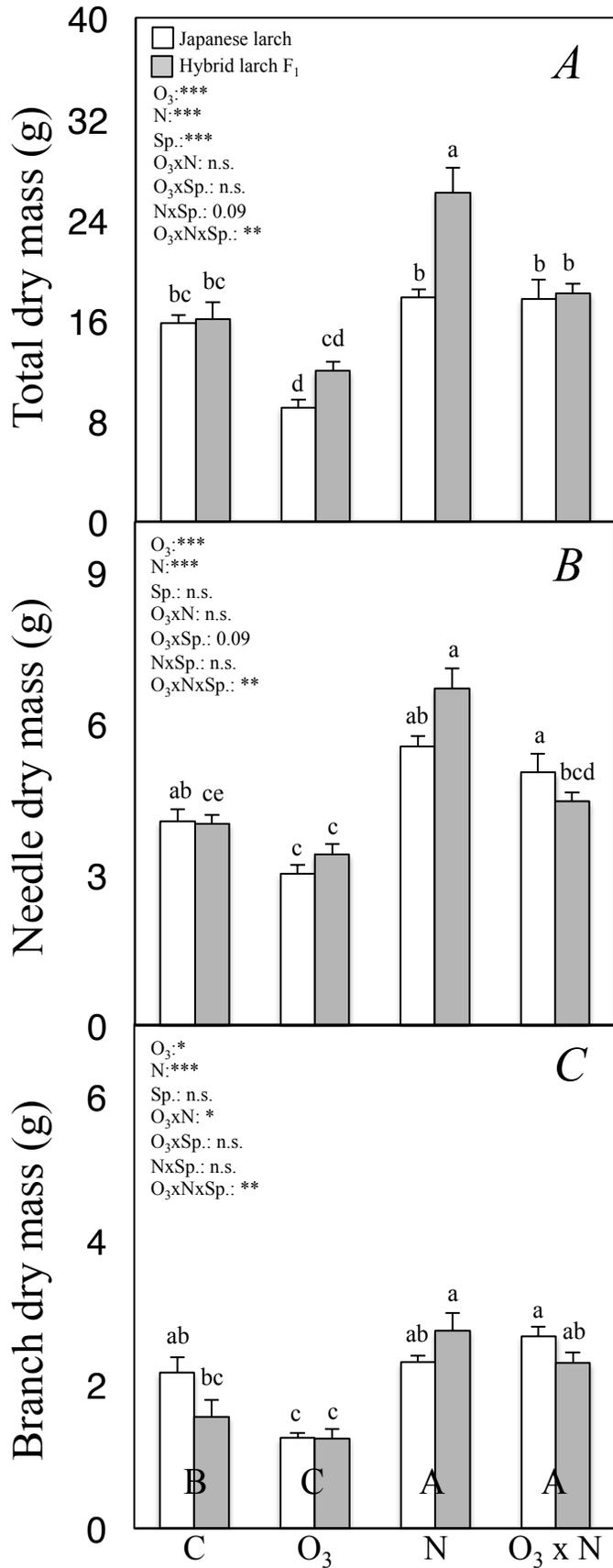


Fig. 2

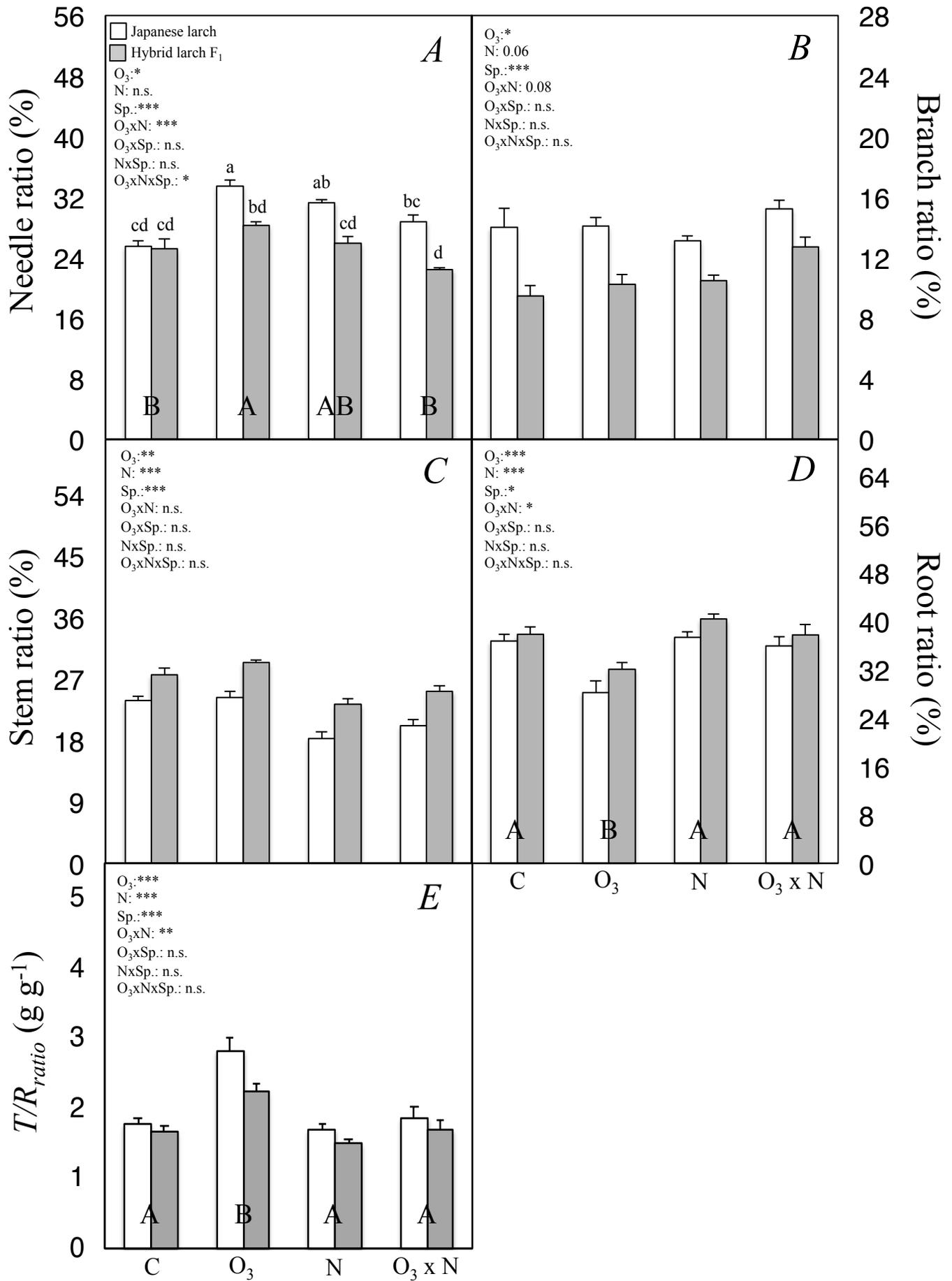


Fig. 3

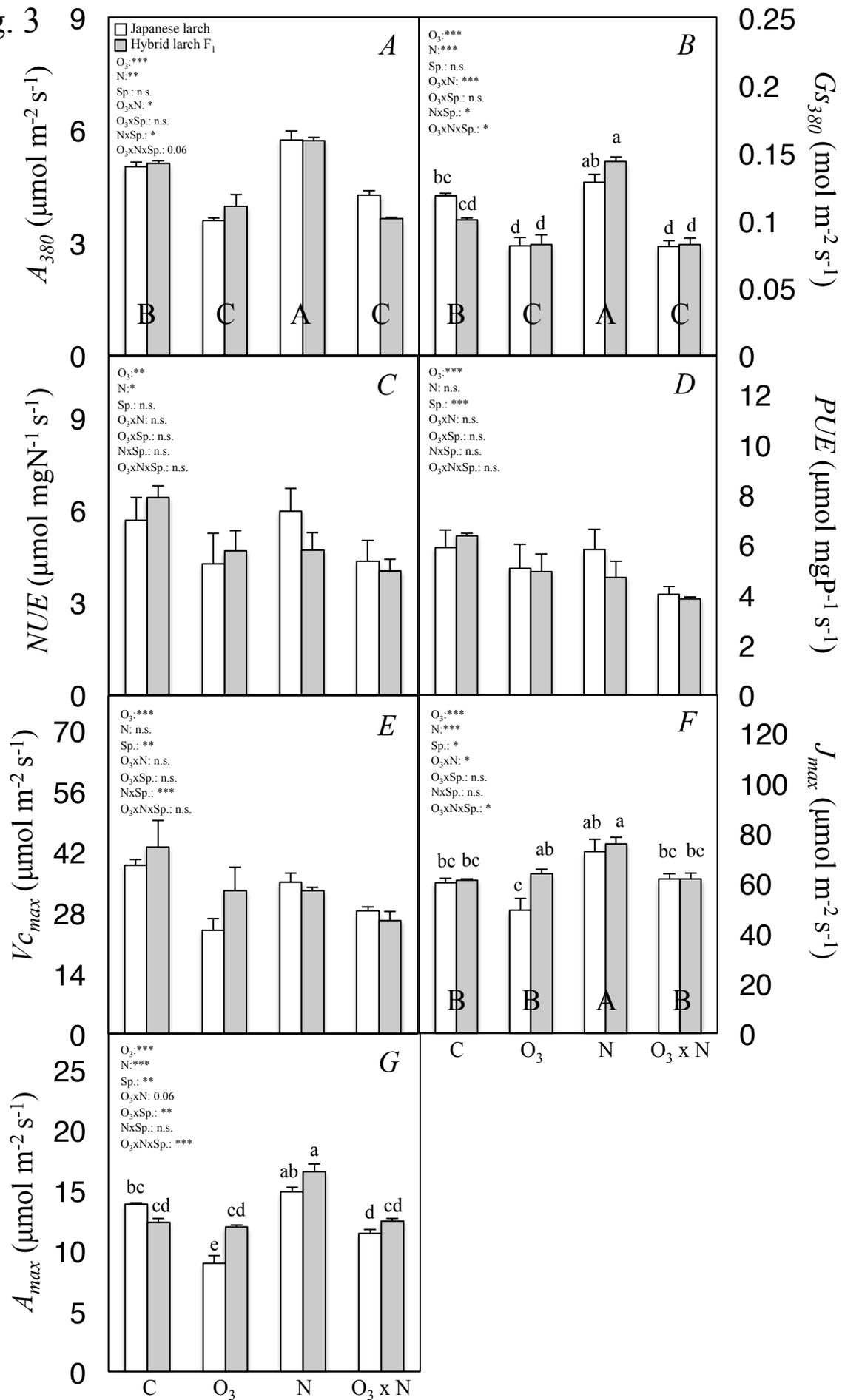


Table. 1

| | 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 ₂ O) | | | | |
| 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 (0.27) | 7.30 (0.24) | 13.25 (0.77) | 12.01 (0.47) | 7.81 (0.28) | 7.62 (0.33) | 12.20 (1.10) | 10.92 (0.27) | * | *** | 0.07 | n.s. | n.s. | n.s. | n.s. | |
| Leaf P | 0.37 ^{bc} (0.02) | 0.38 ^{bc} (0.02) | 0.44 ^{bc} (0.01) | 0.54 ^{bc} (0.01) | 0.36 ^c (0.01) | 0.46 ^{bc} (0.02) | 0.55 ^b (0.05) | 0.96 ^a (0.09) | *** | *** | *** | ** | ** | *** | * | |
| N/P | 24.03 (0.75) | 19.28 (0.84) | 29.98 (1.87) | 22.29 (0.78) | 21.67 (1.28) | 16.72 (0.61) | 23.32 (4.12) | 11.61 (0.90) | *** | n.s. | *** | 0.06 | n.s. | * | n.s. | |
| LMA | 100 (10.1) | 106 (14.2) | 107 (4.6) | 101 (1.4) | 104 (2.0) | 113 (10.8) | 109 (11.6) | 107 (1.2) | n.s. | 0.06 | n.s. | n.s. | n.s. | n.s. | n.s. | |
| 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

