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Growth and Photosynthetic Responses of Cuttings of a Hybrid Larch (*Larix gmelinii* var. *japonica* x *L. kaempferi*) to Elevated Ozone and/or Carbon Dioxide

Takayoshi Koike, Qiaozhi Mao, Naoki Inada, Korin Kawaguchi, Yasutomo Hoshika, Kazuhito Kita¹⁾ and Makoto Watanabe*

Silviculture and Forest Ecological Studies, Hokkaido University, Sapporo 060-8589, Japan

¹⁾Hokkaido Forestry Research Institute, Bibai 079-0198, Japan

*Corresponding author. Tel: +81-11-706-4180, E-mail: nab0602@for.agr.hokudai.ac.jp

ABSTRACT

We studied the effects of elevated ozone ([O₃]) and CO₂ concentrations ([CO₂]) on the growth and photosynthesis of the hybrid larch F₁ (F₁) and on its parents (the Dahurian larch and Japanese larch). F₁ is a promising species for timber production in northeast Asia. Seedlings of the three species were grown in 16 open top chambers and were exposed to two levels of O₃ (<10 ppb and 60 ppb for 7 h per day) in combination with two levels of CO₂ (ambient and 600 ppm for daytime) over an entire growing season. Ozone reduced the growth as measured by height and diameter, and reduced the needle dry mass and net photosynthetic rate of F₁, but had almost no effect on the Dahurian larch or Japanese larch. There was a significant increase in whole-plant dry mass induced by elevated [CO₂] in F₁ but not in the other two species. Photosynthetic acclimation to elevated [CO₂] was observed in all species. The net photosynthetic rate measured at the growing [CO₂] (i.e. 380 ppm for ambient treatment and 600 ppm for elevated CO₂ treatment) was nevertheless greater in the seedlings of all species grown at elevated [CO₂]. The high [CO₂] partly compensated for the reduction of stem diameter growth of F₁ at high [O₃]; no similar trend was found in the other growth and photosynthetic parameters, or in the other species.

Key words: Tropospheric ozone, High CO₂, Hybrid larch, Tree growth, Photosynthesis

1. INTRODUCTION

In the 1960s and 1970s, Japanese forests and living standards suffered from various types of environmental pollution as a result of rapid economic development after World War II (e.g. Izuta, 2006). These difficulties

were overcome with the use of desulfurization equipment and various anti-pollution initiatives. A concern today is the effect of high tropospheric ozone concentration ([O₃]) on plant production (Izuta, 2006; Omasa *et al.*, 2005). Tropospheric ozone is an air pollutant that is deeply harmful to the growth of tree species (e.g. Kume *et al.*, 2009; Izuta, 2006; Karnosky *et al.*, 2005).

In Japan, [O₃] has been increasing since the late 1980s, and global and regional (i.e. Asian) air pollution is believed to be a major reason for the increase (Ohara, 2011). Several researchers suggest that current levels of O₃ cause forest decline in several parts of Japan (Kume *et al.*, 2009; Takeda and Aihara, 2007; Yonekura *et al.*, 2001). Moreover, the emission of NO_x in East Asia has been increasing, and will continue to increase for the foreseeable future (Ohara, 2011; Ohara *et al.*, 2007), so that [O₃] in Japan will continue to increase. The atmospheric CO₂ concentration ([CO₂]) also continues to increase year by year (IPCC, 2007). It is therefore important to predict the combined effects of high [O₃] and [CO₂] on the growth and photosynthetic capabilities of representative afforestation species (Matsumura *et al.*, 2005; Paoletti and Grulke, 2005).

In the expectation of high biomass and timber production, the Japanese larch (*Larix kaempferi*) has been planted in northern Japan from approximately the 1950s, but this larch suffers severely from biotic and abiotic stresses (Koike, 2009; Ryu *et al.*, 2009). To overcome various difficulties, a new hybrid larch F₁ has recently been developed (*Larix gmelinii* var. *japonica* x *L. kaempferi*) for afforestation (Kuromaru *et al.*, 2011; Ryu *et al.*, 2009). This hybrid larch F₁ is characterized by its high resistance to biological stresses (grazing by voles, shoot blight disease, etc.), high rate of growth and high specific gravity of the stem (Kita, 2011). Following the Kyoto protocol (IGBP, 1998), we aim for high [CO₂] fixation capacity of forest ecosystems to moderate global warming, especially via high CO₂ fixa-

tion and storage capability of the tree species selected.

The newly developed hybrid larch F_1 (its nickname is the “clean larch”, Kuromaru *et al.*, 2011) is now being planted in many parts of northern Japan. However, no information on its response to elevated $[O_3]$ with elevated $[CO_2]$ is yet available (Kuromaru *et al.*, 2011; Ryu *et al.*, 2009).

In the present study, we examined the effect of elevated $[O_3]$ together with elevated $[CO_2]$ on the growth and physiology of the hybrid larch F_1 and on its parents, the Japanese larch and Dahurian larch. High $[CO_2]$ usually induces stomatal closure so as to improve the water use efficiency of most C3 plants (Watanabe *et al.*, 2011, 2010; Körner, 2005; Schulze *et al.*, 2005; Koike, 1996). We therefore expect that high $[CO_2]$ will moderate the harmful effects of O_3 , because of the resulting reduction in stomatal O_3 uptake. Consequently, our hypothesis is that larch growth is not suppressed at high $[O_3]$ under the future high $[CO_2]$.

2. MATERIALS AND METHODS

2.1 Plant Materials

We used 2-year-old seedlings of the Japanese larch (*Larix kaempferi*) and Dahurian larch (*Larix gmelinii* var. *japonica*), and 3-year-old cuttings of the hybrid larch F_1 (hereafter F_1 , *Larix gmelinii* var. *japonica* × *L. kaempferi*). The chloroplast DNA and mitochondrial DNA of *Larix* species indicate paternity of the Japanese larch and maternity of the Dahurian larch, respectively (Szmidt *et al.*, 1987). Three-year-old specimens of F_1 were used for practical reasons relating to the Hokkaido Regional Government. The basal diameter of the stem and the height at the start of the experiment were 5.1 mm and 30 cm for the Japanese larch, 5.0 mm and 22 cm for the Dahurian larch, and 8.5 mm and 52 cm for F_1 . Prior to bud breaking, we planted these seedlings and cuttings in 7 liter pots filled with 1 : 1 (v/v) mixture of Kanuma pumice soil and clay soil. 200 mL of liquid fertilizer (N : P : K = 6 : 10 : 5, Hyponex, Ohio, USA), diluted 500-fold, was supplied to all potted soils at 2-weeks interval, for a total nitrogen (N) application of 192 mg N per pot. Watering with tap water was carried out at 3-7 day intervals to prevent desiccation.

2.2 Treatments

We ran an open-top chamber (OTC) experiment from July to October with $[O_3]$ (60 ppb: 7 hrs, 10:00-17:00), $[CO_2]$ (600 ppm: during daytime) and their combination. We targeted the long-shoot needles because most needles of seedlings are long-shoot needles and in central Hokkaido begin to develop usually in late June, and to mature from early September (Kitaoka *et al.*,

2000). Inlet air was cleaned by a charcoal filter. The target $[O_3]$ was the same concentration with environmental standard value for the photochemical oxidant in Japan, and similar or higher $[O_3]$ has often been observed in many regions, including forested areas in Japan (Izuta, 2006). The chosen $[CO_2]$ of 600 ppm is predicted to be attained around the year 2060 (IPCC, 2007). The 16 OTCs (1.2 m × 1.2 m × 1.2 m high) were set in the experimental nursery of the Field Science Center for the Northern Biosphere of Hokkaido University (43°06' N, 141°20' E, 15 m a.s.l.). The OTCs were replicated four times for each gas treatment. Each OTC had two seedlings of each larch species.

2.3 Growth Measurements

The height and diameter of the seedlings were measured at the beginning and end (mid October) of the experiment, to determine growth. At the end of the experiment, all seedlings were harvested and were separated into each organ (i.e. stem, needle and root). The samples were oven-dried at 70°C for at least one week and then weighed. The T/R (Top/ Root) ratio was calculated as the dry mass ratio of above-ground to root.

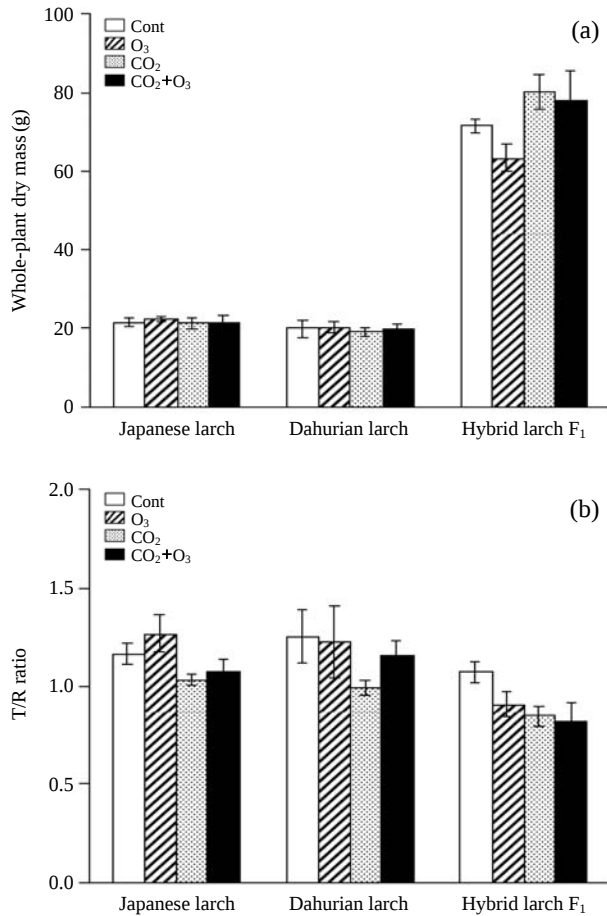
2.4 Gas Exchange Measurements

In the middle of September, the gas exchange rates of mature needles were measured using an open gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA), as specified in Watanabe *et al.* (2011). One or two seedlings per treatment-chamber combination were selected randomly for measurement. We determined the net photosynthetic rate (A_{growth}) and the stomatal conductance (G_s) for water vapor at the chosen values of $[CO_2]$, i.e., 380 ppm for the ambient treatment and 600 ppm for the elevated treatment. We calculated the net photosynthetic rate at 1700 $\mu\text{mol mol}^{-1}$ (A_{max} , indicating maximum rate of ribulose-1,5-bisphosphate regeneration), the maximum rate of carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) from the intercellular CO_2 concentration-response curve of the net photosynthetic rate (Long and Bernacchi, 2003; Farquhar *et al.*, 1980). After measurement of the gas exchange rate, the needles were collected to determine the projected needle area as measured by an image scanner. The collected needles were dried in an oven at 70°C for at least one week and then weighed. The leaf mass per unit area (LMA) of needles was calculated as the ratio of dry mass to the area of the needles. Needle N and C concentrations were determined by a NC analyzer (Sumica-Shimadzu NC-900, Kyoto, Japan). We calculated the ratio of A_{growth} to the N content as the photosynthetic N use efficiency (PNUE).

2.5 Statistics

Statistical analyses were performed using R software,

version 2.8.1. Two-way analysis of variance (ANOVA) was used to test the effects of O_3 and elevated $[CO_2]$. In each analysis, a chamber was nested within each gas treatment and added as a random effect to the model. The reduced major axis regression method was used to analyze the relationship between pairs of variables.



P value for two-way ANOVA

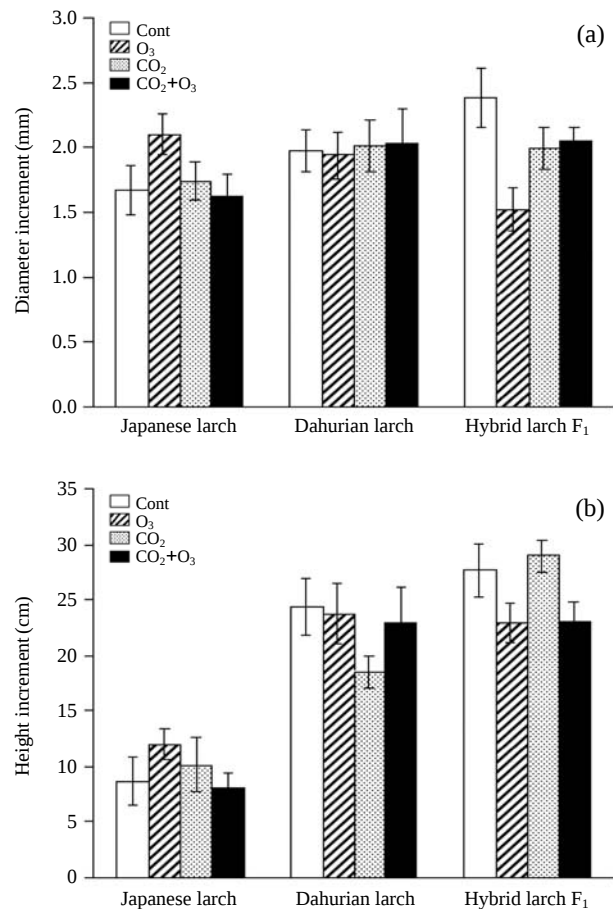
	O_3	CO_2	$CO_2 \times O_3$
Whole-plant dry mass			
Japanese larch	0.753	0.651	0.770
Dahurian larch	0.806	0.772	0.910
Hybrid larch F_1	0.277	0.027	0.555
T/R ratio			
Japanese larch	0.184	0.006	0.531
Dahurian larch	0.555	0.155	0.397
Hybrid larch F_1	0.177	0.038	0.306

Fig. 1. Effects of elevated $[O_3]$ and/or $[CO_2]$ on the whole-plant dry mass and Top-root dry mass ratio (T/R ratio) of three larch species. Each value is the mean of 8 determinations; the error bar shows the standard error. Cont: O_3 free ambient air, O_3 : 60 ppb O_3 , CO_2 : 600 ppm CO_2 . The explanation is the same for the following figures.

3. RESULTS

3.1 Biomass and Allocation (T/R)

No significant effect of elevated $[O_3]$ and/or elevated $[CO_2]$ was found for the whole-plant dry mass of Japanese and Dahurian larch seedlings (Fig. 1a). Elevated $[O_3]$ tended to reduce the whole-plant dry mass of F_1 . On the other hand, whole-plant dry mass of F_1 grown at high $[CO_2]$ was significantly larger than at ambient



P value for two-way ANOVA

	O_3	CO_2	$CO_2 \times O_3$
Diameter increments			
Japanese larch	0.322	0.189	0.086
Dahurian larch	0.686	0.853	0.609
Hybrid larch F_1	0.013	0.637	0.006
Height increment			
Japanese larch	0.676	0.473	0.116
Dahurian larch	0.259	0.098	0.188
Hybrid larch F_1	0.012	0.696	0.769

Fig. 2. Stem basal diameter and height increments of three kinds of larch under elevated $[O_3]$ and/or $[CO_2]$. See legend for Fig. 1.

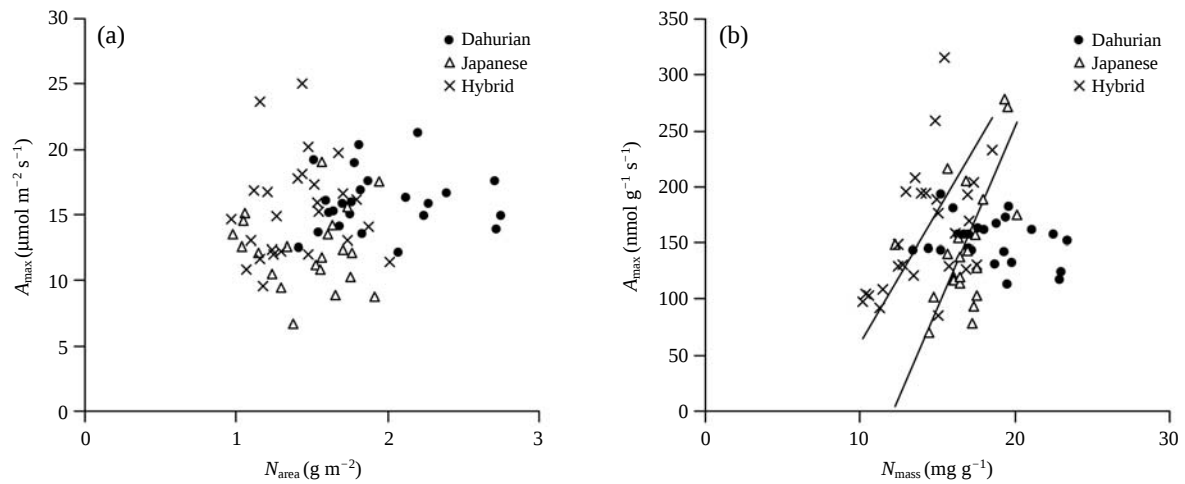


Fig. 3. Relationship between area-based (left) and mass-based (right) $A_{\max}$ and N in three larch species. $A_{\max}$: net photosynthetic rate at CO_2 and light saturation. The regression lines are obtained using the reduced major axis regression method ($y=32.2x-390.0$, $R^2=0.24^*$, for Japanese larch; $y=23.6x-174.8$, $R^2=0.24^*$, for F_1). There was no significance in the regression line for dahurian larch ($R^2=0.09$).

Table 1. Needle photosynthetic parameters of three kinds of larch treated with elevated $[\text{O}_3]$ and/or $[\text{CO}_2]$.

	Cont	O ₃	CO ₂	CO ₂ +O ₃	P value for Two-way ANOVA		
					O ₃	CO ₂	CO ₂ × O ₃
Japanese larch							
A _{grwoth} (μmol m ⁻² s ⁻¹)	4.9 (0.7)	5.6 (1.2)	6.3 (0.5)	7.6 (1.2)	0.373	0.146	0.782
A _{max} (μmol m ⁻² s ⁻¹)	13.4 (1.0)	13.1 (2.0)	11.3 (1.2)	11.8 (0.9)	0.966	0.177	0.749
G _s (μmol m ⁻² s ⁻¹)	0.09 (0.02)	0.09 (0.02)	0.06 (0.01)	0.07 (0.00)	0.844	0.126	0.625
V _{cmax} (μmol m ⁻² s ⁻¹)	35.2 (2.9)	39.0 (4.4)	21.9 (3.5)	26.4 (4.8)	0.305	0.016	0.935
J _{max} (μmol m ⁻² s ⁻¹)	74.0 (2.5)	75.5 (11.7)	58.3 (10.5)	54.9 (3.9)	0.888	0.031	0.706
LMA	77.3 (8.5)	88.6 (10.6)	97.7 (6.9)	93.1 (5.4)	0.630	0.113	0.276
N _{area} (g m ⁻²)	1.3 (0.2)	1.4 (0.1)	1.6 (0.1)	1.6 (0.1)	0.798	0.033	0.357
N _{mass} (mg g ⁻¹)	17.4 (1.1)	16.4 (0.9)	16.5 (0.4)	16.6 (0.3)	0.701	0.773	0.609
PNUE (μmol mol ⁻¹ s ⁻¹)	55.6 (12.6)	57.2 (14.0)	56.6 (7.5)	73.4 (16.8)	0.575	0.598	0.643
Dahurian larch							
A _{grwoth} (μmol m ⁻² s ⁻¹)	9.5 (0.5)	8.1 (1.0)	11.2 (0.8)	11.3 (1.4)	0.455	0.016	0.370
A _{max} (μmol m ⁻² s ⁻¹)	16.2 (1.3)	15.9 (1.0)	15.8 (0.8)	15.8 (0.8)	0.878	0.816	0.863
G _s (μmol m ⁻² s ⁻¹)	0.11 (0.02)	0.09 (0.01)	0.10 (0.01)	0.10 (0.02)	0.448	0.885	0.521
V _{cmax} (μmol m ⁻² s ⁻¹)	51.8 (5.7)	49.2 (4.0)	39.4 (2.2)	35.8 (2.2)	0.565	0.034	0.921
J _{max} (μmol m ⁻² s ⁻¹)	77.6 (3.4)	80.4 (1.1)	77.4 (2.2)	71.3 (2.8)	0.569	0.132	0.142
LMA	112.6 (9.3)	98.0 (4.1)	113.1 (4.6)	100.7 (3.9)	0.029	0.758	0.842
N _{area} (g m ⁻²)	2.0 (0.2)	1.8 (0.1)	2.0 (0.2)	2.0 (0.2)	0.375	0.513	0.581
N _{mass} (mg g ⁻¹)	18.2 (1.4)	18.0 (0.7)	18.0 (1.3)	19.5 (1.2)	0.668	0.679	0.610
PNUE (μmol mol ⁻¹ s ⁻¹)	69.1 (8.3)	64.2 (6.9)	80.5 (10.7)	85.3 (16.5)	0.997	0.223	0.703
Hybrid larch F ₁							
A _{grwoth} (μmol m ⁻² s ⁻¹)	8.3 (0.9)	6.9 (0.4)	10.0 (1.1)	9.0 (0.6)	0.068	0.009	0.762
A _{max} (μmol m ⁻² s ⁻¹)	17.8 (2.0)	16.0 (0.8)	12.6 (1.1)	14.5 (1.3)	0.946	0.045	0.213
G _s (μmol m ⁻² s ⁻¹)	0.09 (0.02)	0.08 (0.01)	0.08 (0.01)	0.07 (0.01)	0.372	0.532	0.777
V _{cmax} (μmol m ⁻² s ⁻¹)	51.8 (5.8)	52.8 (4.8)	37.5 (5.3)	36.8 (6.1)	0.970	0.036	0.890
J _{max} (μmol m ⁻² s ⁻¹)	82.7 (9.1)	86.8 (6.7)	66.4 (7.6)	71.8 (7.6)	0.563	0.080	0.940
LMA	98.9 (7.9)	88.8 (2.4)	105.5 (9.4)	109.4 (11.2)	0.687	0.149	0.458
N _{area} (g m ⁻²)	1.5 (0.1)	1.4 (0.0)	1.3 (0.2)	1.3 (0.1)	0.228	0.026	0.204
N _{mass} (mg g ⁻¹)	15.6 (0.6)	15.9 (0.6)	12.5 (0.8)	12.5 (0.9)	0.853	0.005	0.877
PNUE (μmol mol ⁻¹ s ⁻¹)	79.6 (11.9)	68.8 (3.5)	114.1 (20.4)	96.6 (6.9)	0.042	0.000	0.577

A_{growth} , net photosynthetic rate at growth CO_2 concentration; $A_{\max}$, net photosynthetic rate at CO_2 saturation; G_s , stomatal conductance to water vapor; V_{cmax} , maximum rate of carboxylation; $J_{\max}$, maximum rate of electron transport; LMA, leaf mass per area; N_{area} , nitrogen content per unit needle area; PNUE, photosynthetic nitrogen use efficiency. Each value is the mean of 8 measurements; the standard error is shown in parentheses.

[CO₂] ($P < 0.05$). There was no significant interaction effect of elevated [O₃] and [CO₂] on the whole-plant dry mass of F₁. The T/R of the Japanese larch and F₁ was significantly reduced at elevated [CO₂] relative to ambient [CO₂] (Fig. 1b). No increase of T/R was found in seedlings of any species treated with [O₃]; T/R for F₁ tended to be small at high [O₃] ($P = 0.177$). The needle biomass of Dahurian and Japanese larches was fairly similar in all treatments, but for F₁ it was significantly lower at high [O₃] (data not shown).

The height increment, and the diameter increment at the bottom of the stem of F₁ was significantly less under elevated [O₃], whereas no significant effect of O₃ was found in the Japanese or Dahurian larches (Fig. 2). We found a significant interaction between elevated [O₃] and [CO₂] influencing the diameter increment. Although elevated [O₃] clearly impairs the stem diameter increment under ambient [CO₂], no such effect was observed under high [CO₂].

3.2 Photosynthetic Parameters

The exposure to O₃ significantly reduced the LMA of the Dahurian larch and the PNUE of F₁, and marginally decreased A_{growth} for F₁ (Table 1). Elevated [CO₂]-induced significant increases were found in N_{area} of the Japanese larch, A_{growth} of the Dahurian larch and A_{growth} and PNUE of F₁. Significant decreases were detected in V_{max} of all species, and in A_{max} , N_{area} and N_{mass} of F₁, and marginally in J_{max} of F₁. There was no significant interaction between elevated [O₃] and [CO₂] influencing the value of any photosynthetic parameters.

No significant correlation was found between area-based A_{max} and N_{area} in needles (Fig. 3a). On the other hand, there was a positive correlation between the mass-based A_{max} and N_{mass} in needles of the Japanese larch and F₁ (Fig. 3b). No clear trend was found in the Dahurian larch, however.

4. DISCUSSION

No significant effect of elevated [O₃] and/or elevated [CO₂] was found for the whole-plant dry mass of Japanese and Dahurian larch seedlings, while F₁ treated with high [CO₂] had high dry mass (Fig. 1). The needle biomass of F₁ was lower at high [O₃] than at low [O₃]. The trend of depression in the whole-plant biomass of F₁ at high [O₃] may be due to a decrease in the needle biomass. Furthermore, the stem diameter increment and height increment of F₁ were significantly impaired by elevated [O₃] (Fig. 2). The O₃-induced growth reduction found in F₁ might be due to the lower A_{growth} , although it was marginally significant (Table 1). The extent of the O₃ effects on plant greatly depends on

amounts of O₃ uptake through stomata and detoxification for O₃ and reactive oxygen species (ROS) derived from O₃ within foliage (Fuhrer and Booker, 2003). We considered the uptakes of O₃ were similar among the three species because of no difference of G_s (Table 1). There is a possibility of lower detoxification capacity for O₃ and/or ROS in F₁. We may be able to conclude this as a weakness in F₁ against O₃.

We did not observe a significant decrease in N content in the needles of larches exposed to O₃. Yamaguchi *et al.* (2007) reported a reduction in the net photosynthetic rate of leaves of the Siebold's beech (*Fagus crenata*) due to an O₃-induced reduction in PNUE. Furthermore, their subsequent study revealed a reduction in the concentration ratio of total soluble protein (of which the main component is Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase, a key enzyme for photosynthesis), to the total N in leaves exposed to O₃. In the present study (Table 1), the PNUE under high [O₃] was significantly lower than that under low [O₃]. We therefore believe that the decrease in photosynthetic N utilization leads to the decrease in A_{growth} of F₁.

Plants deploy defense and repair processes against O₃ damage which require photosynthetic assimilates (Fuhrer and Booker, 2003). This diversion of assimilates to defense and/or repair processes may result in the growth reduction of needles of F₁. The O₃-induced increase of antioxidant and phenolic compounds were reported from several researches (Omasa *et al.*, 2005; Watanabe *et al.*, 2005; Wiser *et al.*, 1993). Or O₃ may simply reduce needle biomass via reduction of photosynthetic function (e.g. Matyssek *et al.*, 1993).

A positive correlation between foliar N and A_{max} is observed in many species (e.g. Schulze *et al.*, 2005; Larcher, 2003) because large part of foliar N is used for photosynthetic function such as Rubisco and chlorophyll (Kitaoka and Koike, 2004). In Japanese larch and F₁, in fact, we found positive correlation between mass-basis N and A_{max} (Fig. 3). However no such correlation was found for the Dahurian larch. This may be related to the small LMA value at elevated [O₃], suggesting that biomass production at high [O₃] was restricted.

Photosynthetic acclimation to high [CO₂] took place, as indicated by the reduction of V_{cmax} , J_{max} and/or A_{max} (Table 1). This homeostatic adjustment occurs in many species (Watanabe *et al.*, 2011; Koike *et al.*, 1996; Tissue and Oechel, 1987). Biomass production was initially accelerated by high [CO₂], but the effect usually ceased with deficiency of nutrients, dilution of N with rapid growth and depression of Rubisco activity (Watanabe *et al.*, 2011; Larcher, 2003). For fast growing species, including larch, the pot size may restrict

the growth of below-ground biomass at elevated $[\text{CO}_2]$, independent of $[\text{O}_3]$ (Koike *et al.*, 1996). In setting up new plantations with F_1 we should pay attention to the source-sink relation, i.e. the T/R ratio, as for an indicator of survival of plants under increasing $[\text{O}_3]$.

In the present study, the effect of elevated $[\text{CO}_2]$ in compensating for the O_3 -induced reduction in growth of the stem basal-diameter of F_1 was clear, but there was no similar trend in the other growth and photosynthetic parameters, and the other species. The effect of elevated $[\text{CO}_2]$ on the height-growth reduction at high $[\text{O}_3]$ was not the same for aspen and birch (Karnosky *et al.*, 2007). Moreover, trees have different sensitivity to $[\text{O}_3]$ and the sensitivity may change with growth stage (Kolb and Matyssek, 2001). In seedlings of the Siebold's beech, growth at high $[\text{O}_3]$ was dramatically accelerated with elevated $[\text{CO}_2]$ via the production of extra secondary flush leaves (Watanabe *et al.*, 2010). Although down-regulation by elevated $[\text{CO}_2]$ was found in our three larches, the net photosynthetic rate measured at the cultivated $[\text{CO}_2]$ was greater in seedlings of the Dahurian larch and F_1 grown at elevated $[\text{CO}_2]$. In F_1 , the surplus photosynthates at high $[\text{CO}_2]$ may efficiently be allocated to defense against O_3 or to repair parts of plant organs damaged by O_3 (e.g., Dizengremel *et al.*, 2008; Fuhrer and Booker, 2003).

In conclusion, a negative effect of elevated $[\text{O}_3]$ was observed mainly in F_1 . This is a concern for future afforestation with F_1 . The effect of high $[\text{CO}_2]$ in compensating for the reduction of stem diameter growth of F_1 at high $[\text{O}_3]$ is an important insight. Further detailed study of the mechanisms of these effects in the hybrid larch F_1 should clarify its suitability for future afforestation projects in an atmosphere containing elevated $[\text{O}_3]$ and $[\text{CO}_2]$.

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