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**Title**

Growth and photosynthetic traits of hybrid larch F<sub>1</sub> (*Larix gmelinii* var. *japonica* × *L. kaempferi*) under elevated CO<sub>2</sub> concentration with low nutrient availability

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EFFECTS OF ELEVATED CO<sub>2</sub> ON HYBRID LARCH F<sub>1</sub>

## Summary

The hybrid larch F<sub>1</sub> (*Larix gmelinii* var. *japonica* × *Larix kaempferi*) is considered as one of the most important tree species not only for timber production but also as an afforestation material for severe conditions such as infertile soil. To predict the ability of hybrid larch F<sub>1</sub> as an afforestation material under potential climates in the future, it is important to understand the response of hybrid larch F<sub>1</sub> to elevated CO<sub>2</sub> concentration ([CO<sub>2</sub>]) under low nutrient availability. Three-year-old seedlings of hybrid larch F<sub>1</sub> were grown under two different levels of [CO<sub>2</sub>], 360 (ambient) and 720 μmol mol<sup>-1</sup> (elevated), in combination with two different levels of nitrogen (N) supply (0 and 30 kg ha<sup>-1</sup>) for one growing season. Elevated [CO<sub>2</sub>] reduced the maximum rates of carboxylation and electron transport in the needles. Net photosynthetic rates at growth [CO<sub>2</sub>] (i.e., 360 and 720 μmol mol<sup>-1</sup> for ambient and elevated treatment, respectively) did not differ between the two CO<sub>2</sub> treatments. Reductions in the N content and the N use efficiency to perform photosynthetic functions owing to the deficiency of nutrients other than N, such as P and K, and/or increase of cell wall mass were considered as the factors of photosynthetic down-regulation under elevated [CO<sub>2</sub>], whereas stomatal closure little affected the photosynthetic down-regulation. Although we observed the strong down-regulation of photosynthesis, the dry matter increase of hybrid larch F<sub>1</sub> seedlings was enhanced under elevated [CO<sub>2</sub>]. This is mainly attributable to the increase in the amount of needles with increasing the number of syllptic branches. These results suggest that elevated CO<sub>2</sub> may increase growth of hybrid larch F<sub>1</sub> even under low nutrient availability, and that this increase may be regulated by both changes in crown architecture and in

needle photosynthesis, which is mainly affected by not stomatal limitation but biochemical limitation.

### **Key words**

Acclimation to high CO<sub>2</sub>, *Larix gmelinii* var. *japonica* × *Larix kaempferi*, needle characteristics, nitrogen allocation, nutrient condition, photosynthetic adjustment

### **1. Introduction**

*Larix* species are widely distributed in cool temperate northern hemisphere regions (Gower and Richards 1990). In Japan, the Japanese larch (*Larix kaempferi*) was introduced from the central subalpine region of Japan to northern Japan as a candidate plantation species in the 1870s because it grows more rapidly and has more tolerance to cold than the other traditional silvicultural species. However, some serious disadvantages associated with the plantation of Japanese larch, such as susceptibility to diseases and grazing, raised concerns (Ito 1963, Igarashi and Takeuchi 1985, Koike et al. 2000, 2004). To overcome these difficulties, the hybrid larch, obtained by crossing *Larix gmelinii* var. *japonica* and *L. kaempferi*, was developed. The studies conducted subsequently have demonstrated that the selected clones of hybrid larch F<sub>1</sub> had improved tolerance to grazing by redback voles and deer, and to wind and snow damage. Furthermore, it showed faster growth rate and high capacity of carbon accumulation as compared to Japanese larch (Miyaki 1990, Kita et al. 2009). Because larch species can survive in infertile soil, they have been recognized as a useful material for timber production, absorption of atmospheric CO<sub>2</sub> as a carbon sink, and afforestation in

severe conditions, e.g. low nutrient availability (Zhang et al., 2000, Ryu et al. 2009, Ryu et al. 2010).

The atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) has dramatically increased since the industrial revolution, and this increase is continuing (Meehl et al. 2007). Because CO<sub>2</sub> is the primary substrate for photosynthesis, its increased concentration can act as a kind of fertilizer. However, a decrease in photosynthetic activity, called ‘down-regulation of photosynthesis’, is occasionally observed, especially under low nutrient conditions (Tissue et al. 1987, Tissue et al. 1993, Eguchi et al. 2004, Ellsworth et al. 2004, Ainsworth and Long 2005, Eguchi et al. 2008, Tissue et al. 2010). Photosynthetic down-regulation was frequently observed along with a decrease in N concentration in the leaves and whole plant, as well as an accumulation of non-structural carbohydrates in the leaves (Stitt and Krapp 1999, Nowak et al., 2004, Ellsworth et al. 2004). Under low nutrient and elevated [CO<sub>2</sub>] condition, plants will suffer the dilution of nutrients owing to the nutrient imbalance between limited supply from the soil and the stimulated nutrient demand for growth. In addition, production of new organs, which acts as a sink for carbon, is limited under low nutrient conditions, non-structural carbohydrates will accumulate in leaves under elevated [CO<sub>2</sub>], and photosynthetic activity will decrease by the feedback mechanism (Herold 1980). Therefore, there is a possibility that the benefit of elevated [CO<sub>2</sub>] is small in the case of afforestation under infertile soil conditions with low nutrient availability.

It has been reported that the growth and photosynthesis of *Larix* species were generally stimulated under elevated [CO<sub>2</sub>] (Tjoelker et al. 1998a, 1998b, Handa et al. 2005, Handa et al. 2006). However, Eguchi et al. (2004) observed

photosynthetic down-regulation of *L. kaempferi* seedlings under elevated [CO<sub>2</sub>] with relatively low nutrient conditions. Yazaki et al. (2001) observed an elevated [CO<sub>2</sub>]-induced stimulation of stem diameter growth of *Larix sibirica* under relatively high nutrient conditions, but not under low nutrient conditions. On the other hand, Yazaki et al. (2004) reported the minimal effects of [CO<sub>2</sub>] on the biomass of *L. kaempferi* seedlings that is independent of soil fertility. These studies suggest that the responses of *Larix* species to elevated [CO<sub>2</sub>] are different among different species, and are affected by environmental conditions. However, there was no information on the response of hybrid larch F<sub>1</sub> to elevated [CO<sub>2</sub>].

Can the hybrid larch F<sub>1</sub> maintain high performance under elevated [CO<sub>2</sub>] with low nutrient availability? To answer this question, we studied the growth and photosynthetic responses of hybrid larch F<sub>1</sub> to elevated [CO<sub>2</sub>] under low nutrient availability. Because increase in N deposition is another important factor in the changing environment (Galloway et al. 2008, Ryu et al. 2009), we incorporated N supply to soil as a sub factor of the present study to test whether this treatment enhances the responses of hybrid larch F<sub>1</sub> to elevated [CO<sub>2</sub>].

## **2. Materials and methods**

### **2.1 Plant materials**

Three-year-old cutting seedlings of the hybrid larch F<sub>1</sub> clone (sp. “Gream” as a nickname) were planted in 7-L pots filled with 1:2 (v/v) mixture of Kanuma pumice soil and clay soil on 23 May 2008, and were placed in a room at about 15°C without direct sunlight but close to windows until needle emergence. These pots were set in trays to prevent nutrient drainage. On 30 May, the seedlings were

transferred into six phytotrons (Koito KG, Yokohama, Japan) at Forestry and Forest Product Research Institute in Sapporo, Japan (43°N, 141°E; 180 m a.s.l.) in natural daylight (approximately 90% of full sunlight) at 25/16°C (day/night) and were grown for 139 days until 16 October. Plants were irrigated with tap water during the experiment. This potted soil mixture does not have sufficient nutrients to grow as compared to the one in field soil such as brown forest soil, this type of soil being most widely distributed one in Japan (Koike 1995). In the present study, we applied the least amount of fertilizer to maintain low nutrient availability. On 4 July, 8 August and 4 September, we supplied liquid fertilizer (N:P:K=5:10:5, Hyponex Japan, Osaka, Japan) to all potted soils, for a total N application of 4.8 kg ha<sup>-1</sup> (15 mg N pot<sup>-1</sup>). At the end of experiment, we collected the soil and determined N concentration using an NC analyzer (NC-900, Sumika Chemical Analysis Service, Osaka, Japan). The average N concentration of all soil samples was 0.08% and there was no significant difference among the treatments.

## **2.2 Experimental design**

The present experiment had a split-plot factorial design and employed a randomized block method. The whole-plot treatment was comprised of two levels of CO<sub>2</sub> with three chamber replications, totally six chambers. The sub-plot treatment consisted of two levels of N treatment in each chamber. We measured height and diameter at ground level of all seedlings and then six seedlings (three seedlings in each N treatment) were assigned to each chamber (total 36 seedlings). The averages of stem volume, calculated as height × diameter × diameter (D<sup>2</sup>H), in each treatment were uniformed. The averages ± standard deviations of height,

diameter, and D<sup>2</sup>H of all seedlings were  $65.7 \pm 8.3$  cm,  $8.6 \pm 1.1$  mm, and  $50.6 \pm 16.8$  cm<sup>3</sup>, respectively.

The seedlings were grown at  $360 \mu\text{mol mol}^{-1}$  (ambient) or  $720 \mu\text{mol mol}^{-1}$  (elevated) CO<sub>2</sub>. The details of the CO<sub>2</sub> treatments were described previously (Koike 1995, Koike et al. 1996, Yazaki et al. 2001, 2004). We supplied an ammonium sulphate solution to the potted soil in half of the seedlings (high N) on 24 and 27 June. The total amounts of N added to the potted soil were  $30 \text{ kg N ha}^{-1}$  on the basis of the potted soil surface area ( $94 \text{ mg N pot}^{-1}$ ). The remaining seedlings were supplemented with tap water instead of ammonium sulphate solution (low N). The amount of N supply was based on the field observations of N deposition by wet deposition (bulk precipitation) and that by throughfall and stemflow ranged from  $10$  to  $25 \text{ kg ha}^{-1} \text{ year}^{-1}$  (Baba and Okazaki 1998, Baba et al. 2001, Okochi and Igawa 2001) and from  $10$  to  $40 \text{ kg ha}^{-1} \text{ year}^{-1}$  (Kobayashi et al. 1995, Baba and Okazaki 1998, Baba et al. 2001), respectively, in the forested area of Japan

### **2.3 Measurement of needle gas exchange rates**

On the 118th day after the start of CO<sub>2</sub> treatments, the gas exchange rates of fully expanded young needles were measured using an open gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Two seedlings per treatment-chamber combination were randomly selected for the measurement. The measurements of gas exchange rates were conducted between 0900 and 1500 hours. The needle temperature and photosynthetic photon flux density (PPFD) during the measurement were maintained at  $25 \pm 0.5^\circ\text{C}$  and  $1600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , respectively.



The vapor pressure deficit was kept below 1.5 kPa. To obtain the intercellular CO<sub>2</sub> concentration ( $C_i$ )-response curve of the net photosynthetic rate ( $A$ ), i.e., the  $A/C_i$  curve,  $A$  was determined at 14 steps of CO<sub>2</sub> concentration in the chamber ( $C_a$ , 50-1700  $\mu\text{mol mol}^{-1}$ ). We determined  $A$  at growth [CO<sub>2</sub>] (i.e., 360 and 720  $\mu\text{mol mol}^{-1}$  for ambient and elevated treatment, respectively,  $A_{\text{growth}}$ ) and at 1700  $\mu\text{mol mol}^{-1}$  ( $A_{\text{max}}$ ), and the stomatal conductance at growth [CO<sub>2</sub>] ( $G_s$ ). The maximum rate of carboxylation ( $V_{\text{cmax}}$ ) and the maximum rate of electron transport ( $J_{\text{max}}$ ) were calculated from the  $A/C_i$  curve (Farquhar et al. 1980, Long and Bernacchi 2003). The values of Rubisco Michaelis constants for CO<sub>2</sub> ( $K_c$ ) and O<sub>2</sub> ( $K_o$ ) and CO<sub>2</sub> compensation point in the absence of dark respiration ( $\Gamma^*$ ) for the analysis of the  $A/C_i$  curve were according to Bernacchi et al. (2001) All the gas exchange parameters were expressed on the basis of the projected needle area measured with an image scanner.

#### **2.4 Measurement of needle traits**

After the measurement of gas exchange rate, the needles were collected to determine the leaf mass per area (LMA), amounts of chlorophyll, cell wall, starch, and nutrient element. After the measurement of the needle projected area using an image scanner, the samples for chlorophyll and cell wall were frozen and stored in a freezer at -80°C.

Chlorophyll in needles was extracted with dimethyl sulfoxide according to Barnes et al. (1992) and determined using a spectrophotometer (Gene spec III, Hitachi, Tokyo, Japan). The cell wall mass was measured according to the method described by Hikosaka et al. (2009). Frozen needles were powdered with liquid

nitrogen in a mortar with a pestle, and homogenized in a 1.5 mL of buffer containing 100 mM HEPES (pH 8.0), 5 mM EDTA, 0.7% (w/v) polyethylene glycol 20000, 2% sodium dodecyl sulfate (SDS), 1% polyvinylpyrrolidone and 24 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000 g for 10 min. The pellet was washed with the same buffer two times and then washed with deionized water to remove SDS. After this procedure, the pellet was treated with PAW (phenol:acetic acid:water = 2:1:1, w:v:v) three times (1 h, overnight, and 1 h, respectively). The pellet was then washed with ethanol until there was no smell of phenol. The dry mass of the remaining pellet was regarded as the mass of cell walls.

The other samples for determination of LMA and contents of starch and nutrient element were dried in an oven at 70°C for five days. The LMA was calculated as the ratio of dry mass to the area of the needles. Dried needles were ground to a fine powder with a sample mill. Fifty mg of powdered dry samples were used for the analyses of starch and nutrient elements. The starch content was determined according to Yonekura et al. (2004) using F-Kit Starch (Boehringer-Mannheim K.K., Japan). The N content of the needle sample was determined using an NC analyzer described above. We calculated the ratios of  $V_{\text{cmax}}$  ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $J_{\text{max}}$  ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and chlorophyll content ( $\text{g m}^{-2}$ ) to N content ( $N_{\text{area}}$ ,  $\text{g m}^{-2}$ ) ( $V_{\text{cmax}}/N$ ,  $J_{\text{max}}/N$ , and  $\text{Chl}/N$ , respectively) for evaluating N use efficiency for carboxylation, electron transport, and light harvesting in photosynthesis, respectively. To measure the concentrations of phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) in the needles, the needle sample was digested with  $\text{HNO}_3$ ,  $\text{HCl}$ , and  $\text{H}_2\text{O}_2$ . The concentration of elements

in the sample solutions was determined using a inductively coupled plasma optical emission spectrometry (IRIS, Jarrel Ash, Franklin, MA, U.S.A.).

## **2.5 Anatomical analysis**

Samples collected after the gas exchange measurement were fixed with 4% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2). After these samples were washed, they were cut into small pieces, dehydrated through a graded alcohol series, and embedded in epoxy resin (Epok 812; Oken, Japan). 1- $\mu\text{m}$ -thick transverse sections were cut using an ultramicrotome (Ultracut N; Reichert-Nissei, Germany), and were stained with toluidine blue (1% w/v) for 10 min. Toluidine blue has low specificity of staining and stains cell wall, cell nucleus, DNA, RNA, vacuole with abundant phenolic compound such as tannin, and polysaccharide with abundant carboxyl and sulphate groups. We photographed the sections using a light microscope (Axioskop 2 plus; Zeiss, Germany) equipped with a digital camera (Nikon Digital Sight; Nikon, Tokyo, Japan). The needle shape ratio, defined as the ratio of needle thickness to width, was calculated from the photographs.

## **2.6 Growth measurements**

On the 139th day after the start of CO<sub>2</sub> treatments, all the seedlings were harvested to determine the dry mass of the plant organs. We took three-week interval between the dates of measurement for needle traits and that for dry mass to reflect the evaluated photosynthetic traits in needles to the growth. The seedlings were separated into needles, branches, stems, and roots. Because

sylleptic branches were identified, we separated the current year branches into branches elongated from the previous year's bud (primary branch) and sylleptic branches (secondary branch), and counted them. The plant organs were dried at 70°C for 1 week and weighed. Although the roots reached the bottom of the pot and circled a little, we did not find intertwining roots at the end of the experiment.

### **2.7 Statistical analysis:**

Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the effects of CO<sub>2</sub> and N treatment. Hypothesis concerning CO<sub>2</sub> effects (1 d.f.) were tested against whole-plot chamber variation (4 d.f.), whereas the main effects of N and CO<sub>2</sub> × N interaction (1 d.f.) were tested against the N × chamber/CO<sub>2</sub> error term (4 d.f.). To test the carry-over effects on growth of the seedlings from prior to the experiment, analysis of covariance with initial D<sub>2</sub>H as a covariate was also applied for the growth parameter. However, the results were the same with that of ANOVA. The reduced major axis regression method was used to analyze the relationship between variable pairs.

## **3. Results**

### *CO<sub>2</sub> effects on needle gas exchange parameters and traits*

Elevated [CO<sub>2</sub>] significantly decreased  $A_{\max}$ ,  $V_{c\max}$ ,  $J_{\max}$ , and  $G_s$  in the needles of hybrid larch F<sub>1</sub> seedlings (Table 1). No significant increase was detected in  $A_{\text{growth}}$  under elevated [CO<sub>2</sub>]. Neither the N supply nor the interaction between elevated [CO<sub>2</sub>] and N supply have any significant effects on all parameters. Elevated [CO<sub>2</sub>]

resulted in a significant increase in the LMA, cell wall mass per area, and starch contents, but a decrease in chlorophyll, P, and K contents in the needles (Table 2). The N supply significantly increased Ca and Mg content in the needles. There was no significant interaction between elevated  $[\text{CO}_2]$  and N supply for any parameters.

Figures 1-4 show the relationships between  $A_{\text{max}}$ , and  $N_{\text{area}}$  and starch content (Fig. 1),  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$ , and LMA (Fig. 2),  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$ , and P and K content (Fig. 3), and LMA and cell wall mass per area (Fig. 4). A significant correlation was detected between  $N_{\text{area}}$  and  $A_{\text{max}}$  ( $P < 0.001$ );  $A_{\text{max}}$  decreased with decreasing  $N_{\text{area}}$  (Fig. 1). A similar trend was also observed between  $N_{\text{area}}$  and  $A$  at 360 and 720  $\mu\text{mol mol}^{-1} \text{CO}_2$ ,  $V_{\text{cmax}}$ , and  $J_{\text{max}}$  (data not shown). There was no correlation between starch content and  $A_{\text{max}}$ . As shown in Figure 2,  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$  were negatively correlated with LMA ( $P < 0.01$ , in all parameters). We found positive correlations of  $J_{\text{max}}/\text{N}$  and  $\text{Chl}/\text{N}$  with P content ( $P < 0.05$  and  $P = 0.094$ , respectively) (Fig. 3).  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$  showed significant positive correlation with K content. LMA increased with cell wall mass per area ( $P < 0.01$ ), and the slope of the regression line was 1.0 (Fig. 4).

The micrographs of needle cross sections of hybrid larch  $F_1$  seedlings are shown in Figure 5. We did not observe any change in the thickness of needles and mesophyll cell layers in the adaxial side in response to the elevated  $[\text{CO}_2]$  and N supply. Cells of the needles under elevated  $[\text{CO}_2]$  were strongly stained by toluidine blue compared to those under ambient  $[\text{CO}_2]$ , irrespective of nitrogen treatment, indicating phenolic compounds such as tannin to accumulate in the central vacuole of mesophyll cells.

### *CO<sub>2</sub> Effects on growth and branch number*

A significant increase in needle, branch, root, and whole-plant dry mass under elevated [CO<sub>2</sub>] was observed (Table 3). Needle, root, and whole-plant dry mass were significantly increased by N supply. Elevated [CO<sub>2</sub>] significantly increased the secondary branch number, whereas N supply had no effect. There was no significant interaction between elevated [CO<sub>2</sub>] and N supply for any parameters related to growth and branch number.

## **4. Discussion**

### *Down-regulation of photosynthesis under elevated [CO<sub>2</sub>]*

We found a strong down-regulation of photosynthesis in the needle of hybrid larch F<sub>1</sub> seedlings under elevated [CO<sub>2</sub>] (Table 1). As a result,  $A_{\text{growth}}$  in elevated [CO<sub>2</sub>] was not significantly different from that in ambient [CO<sub>2</sub>]. We also found a decrease in  $G_s$  under elevated [CO<sub>2</sub>]. The reducing photosynthetic rate may be due to elevated [CO<sub>2</sub>]-induced stomatal closure, a frequently reported observation (Ainsworth and Rogers 2007). However, the ratio of  $C_i$  to  $C_a$  of the needles in the seedlings grown under elevated [CO<sub>2</sub>] was significantly higher than under ambient [CO<sub>2</sub>] (data not shown), indicating that the main factor for photosynthetic down-regulation is not stomatal closure but a reduction in the assimilation capacity in the needles.

Photosynthetic down-regulation was frequently observed along with an accumulation of non-structural carbohydrates in the leaf and a decrease in N concentration in the leaves and whole plant (Stitt and Krapp 1999, Nowak et al. 2004, Ellsworth et al. 2004). In the present study, we found a significant increase

in starch content in the needles under elevated  $[\text{CO}_2]$  (Table 2). However, the extent of starch accumulation was not pronounced as compared to the other studies (Tissue et al. 1997, Tissue et al. 2010), and there was no significant correlation between  $A_{\text{max}}$  and starch content (Fig 1). On the other hand,  $A_{\text{max}}$  decreased linearly with  $N_{\text{area}}$ . The elevated  $[\text{CO}_2]$ -induced decrease of  $N_{\text{area}}$  would closely relate to the dilution of N with increasing plant body size (Table 3), as observed by Coleman et al. (1993). Therefore, we consider that the reduction of N content in needles with increasing amount of whole-plant needle is one of the reasons for the photosynthetic down-regulation of hybrid larch  $F_1$  seedlings under elevated  $[\text{CO}_2]$ .

The slope of the regression line between  $A_{\text{max}}$  and  $N_{\text{area}}$  was steep and had a positive value of x-axis intercept (Fig. 1), indicating a decrease in photosynthetic N use efficiency (PNUE) under elevated  $[\text{CO}_2]$ . We found a marginal decrease in  $N_{\text{area}}$  under elevated  $[\text{CO}_2]$  ( $P = 0.068$ ), because the significant decrease in mass-based N content (data not shown) was outweighed by a significant increase in LMA (Table 2). Similar results were reported in the needles of *Pinus ponderosa* showing the photosynthetic down-regulation under elevated  $[\text{CO}_2]$  (Tissue et al. 1999). We consider that N utilizations in the needles of hybrid larch  $F_1$  seedlings grown under elevated  $[\text{CO}_2]$  were different as compared to those under ambient  $[\text{CO}_2]$  owing to the elevated  $[\text{CO}_2]$ -induced increase in LMA even though  $N_{\text{area}}$  is similar. In fact, decreases in  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$  with increasing LMA were observed, as shown in Figure 2. These results indicate a decrease in N use efficiency with respect to photosynthetic function under elevated  $[\text{CO}_2]$ . Therefore, we consider that the decrease in N

content in the needles and the decrease in N use efficiency to perform photosynthetic functions contributed to the down-regulation of photosynthesis under elevated  $[\text{CO}_2]$ . We discuss the decrease in N use efficiency to perform photosynthetic functions from the viewpoints of nutrient condition other than N and needle structure.

We found several significant correlations between the ratios of  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$ , and  $\text{Chl}/\text{N}$ , and the contents of P and K in the needles, which showed a significant decrease under elevated  $[\text{CO}_2]$  (Fig. 3; Table 2). The deficiency of P and K under elevated  $[\text{CO}_2]$  may lead to a decrease in N use efficiency to perform photosynthetic functions. Triose phosphate utilization (TPU) in the chloroplast has been reported as one of the important biochemical limitations in photosynthesis (e.g. Sharkey 1985). The TPU limitation will become serious with decreasing P concentration in leaves. K has been suggested to be an important regulatory cation in maintaining the pH of cytoplasm and stroma (Kurkdjian and Guern 1989, Wu and Berkowitz 1992). The K deficiency induces a decrease in stromal pH, resulting in decrease in photosynthetic activity (Werdan et al. 1975, Marschner 1995). The effects of P and K deficiencies on N use efficiency to perform photosynthetic functions would be the results of feedback effect to balance the reduction in photosynthetic activity by direct effects of P and K limitations. However, it should be noted that because we did not control soil P and K availabilities, it is hard to conclude whether P and/or K contents drove the relationships between needle physiology and N content, or were simply correlated with these relationships.

An increase in the cell wall mass per area with the increase of LMA is



illustrated in Fig. 4. This result may suggest that an increase in N allocation to cell wall led to the decrease in N allocation to photosynthetic functions (Takashima et al. 2004, Feng et al. 2009). According to Hikosaka et al. (2009), the concentration of N in cell wall of deciduous tree species is 0.19–0.37%. Applying these values to our case, the increase of 11.7 g m<sup>-2</sup> cell wall mass per area under elevated [CO<sub>2</sub>] in the present study (averaged in two N treatments, Table 2) results in an increase of 0.02–0.04 g N m<sup>-2</sup> allocated to cell wall. We then calculated the contents of N allocated to photosynthesis in the needle based on the values of  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ , and chlorophyll content (Niinemets and Tenhunen 1997, Kitaoka and Koike 2004, Tissue and Lewis 2010). The average values of two N treatments at ambient and elevated [CO<sub>2</sub>] were 0.46 and 0.31 g N m<sup>-2</sup>, respectively. The increase of N allocated to cell wall (0.02–0.04 g N m<sup>-2</sup>) seems a significant decrease in the N allocated to photosynthesis under elevated [CO<sub>2</sub>] (0.15 g N m<sup>-2</sup>). Therefore, the hypothesis that a trade-off exists between N allocation to photosynthesis and cell wall might partly explain the decreased N use efficiency to perform photosynthetic functions under elevated [CO<sub>2</sub>].

Because we did not observe any increase in the thickness of needles and mesophyll cell layers in the adaxial side under elevated [CO<sub>2</sub>] (Table 2; Fig. 5), the increase in cell wall mass per area is not due to the change in the structure of the needles, but due to the increase in density and/or thickness of cell wall itself. An alternative possibility of the decreased N use efficiency to perform photosynthetic functions under elevated [CO<sub>2</sub>] could be that the diffusion of CO<sub>2</sub> from the intercellular space to chloroplast stroma is inhibited by dense and/or thick cell wall under elevated [CO<sub>2</sub>], although the main location for resistance of

CO<sub>2</sub> diffusion is considered to be plasma membranes including aquaporins (Terashima and Ono 2001, Hanba et al. 2004).

Consequently, we attribute the reduction in the PNUE of hybrid larch F<sub>1</sub> seedlings under elevated [CO<sub>2</sub>] in the present study to one or more of the following factors: (1) the deficiency of nutrients other than N, such as P and K; (2) the increase in N allocation to cell wall; and (3) the increase of resistance in CO<sub>2</sub> diffusion from intercellular space to chloroplast stroma.

#### *Growth response to elevated [CO<sub>2</sub>]*

Although we found a strong down-regulation of photosynthesis, dry matter growth of hybrid larch F<sub>1</sub> seedlings was stimulated under elevated [CO<sub>2</sub>] (Tables 1 and 3). This is due to an increase in the amount of needles. We expect that  $A_{\text{growth}}$  under elevated [CO<sub>2</sub>] would be higher than that under ambient [CO<sub>2</sub>] during the early period of the experiment (short-term response to elevated [CO<sub>2</sub>]), in turn causing the seedlings under elevated [CO<sub>2</sub>] to use more carbohydrates to produce needles. This may lead to an increase in the assimilation of the whole-plant scale even after down-regulation of photosynthesis. An increase in the total needle amount under elevated [CO<sub>2</sub>] resulted in an increase in the secondary (syllaptic) branch number (Table 3). Such increase under elevated [CO<sub>2</sub>] was also observed in *Salix* species (Koike et al. 1995). Furthermore, Tissue et al. (1987) reported that an increased production of new tiller of *Eriophorum vaginatum* under elevated [CO<sub>2</sub>]. These results indicate that ordinal emergence of new leaves from the top of the branch may not be sufficient to act as the sink for increased carbohydrates under elevated [CO<sub>2</sub>]. Although we can expect the stimulation of the growth

performance of hybrid larch F<sub>1</sub> under elevated [CO<sub>2</sub>] in the future even in low nutrient availability, the crown structure in the future may change as compared to the current form. Crown structure is highly regulated to effectively obtain the light resource (Sterck 2005). The elevated [CO<sub>2</sub>]-induced change of crown structure may reduce the efficiency of light capture (e.g. self-shading) and thereby gradually down-regulate the growth during long term.

We found an accumulation of phenolic compounds in the central vacuole of mesophyll cells (Fig. 5). This accumulation may be provided by extra carbon under elevated [CO<sub>2</sub>] in agreement with other studies (Pritchard et al. 1997, Knepp et al. 2005, Peltonen et al. 2005). We suggest the accumulation of phenolic compounds may increase resistance against herbivores and thereby prevent the growth reduction by herbivory under actual field condition.

#### *Effects of N supply*

N supply on the soil did not affect the response of hybrid larch F<sub>1</sub> seedlings to elevated [CO<sub>2</sub>]. A similar result was observed in *P. ponderosa* to elevated [CO<sub>2</sub>] with N supply at 20 kg N ha<sup>-1</sup> year<sup>-1</sup> (Tissue et al 1999). In the present study, N supply stimulated the growth of the seedlings (Table 3). The additional N was used for the production of new needles as it did not increase the N concentration in the needles (Tables 2 and 3). We also found that the area of individual needles marginally increased with the N supply ( $P = 0.064$ , data not shown). The change in leaf size may be a result of improvement in the nutrient status in the seedlings (e.g. Westoby et al. 2002); this may partly contribute to the needle amount of the whole plant.

## **Conclusion**

Although a strong down-regulation of photosynthesis was induced, we can conclude that the growth of hybrid larch F<sub>1</sub> may be stimulated under future elevated [CO<sub>2</sub>] even in soil with low nutrient availability. A reduction in the N content and the N use efficiency to perform photosynthetic functions were the main factor of photosynthetic down-regulation, whereas stomatal limitation little affected it. The increase in N allocations to the cell wall and the increase in the resistance of CO<sub>2</sub> diffusion from the intercellular space to the chloroplast stroma are new possibilities of photosynthetic down-regulation under elevated [CO<sub>2</sub>] in infertile soil conditions. We also highlight the possibility of other nutrients deficiency such as P and K with regard to N use efficiency in the down-regulation of photosynthesis under elevated [CO<sub>2</sub>]. These possibilities should be confirmed in the future studies.

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Table 1 Gas exchange traits in the needles of hybrid larch F<sub>1</sub> seedlings grown under CO<sub>2</sub> concentration at 360 and 720 μmol mol<sup>-1</sup> in combination with 0 (low N) and 30 kg N ha<sup>-1</sup> (High N).

	360 μmol mol <sup>-1</sup> CO <sub>2</sub>		720 μmol mol <sup>-1</sup> CO <sub>2</sub>		ANOVA		
	Low N	High N	Low N	High N	CO <sub>2</sub>	N	CO <sub>2</sub> ×N
$A_{\text{growth}}$ (μmol m <sup>-2</sup> s <sup>-1</sup> )	5.7 (1.3)	6.1 (0.2)	6.2 (2.1)	5.2 (0.1)	n.s.	n.s.	n.s.
$A_{\text{max}}$ (μmol m <sup>-2</sup> s <sup>-1</sup> )	13.7 (1.6)	15.4 (2.6)	9.9 (2.4)	9.2 (1.5)	***	n.s.	n.s.
$V_{\text{cmax}}$ (μmol m <sup>-2</sup> s <sup>-1</sup> )	33.5 (1.5)	33.7 (4.5)	24.5 (7.4)	21.5 (3.6)	*	n.s.	n.s.
$J_{\text{max}}$ (μmol m <sup>-2</sup> s <sup>-1</sup> )	62.2 (8.1)	72.6 (15.8)	45.9 (3.9)	42.6 (7.0)	**	n.s.	n.s.
$G_s$ (mmol m <sup>-2</sup> s <sup>-1</sup> )	95.2 (25.2)	110.2 (30.3)	70.1 (8.4)	65.8 (17.0)	*	n.s.	n.s.

$A_{\text{growth}}$ , net photosynthetic rate at growing CO<sub>2</sub> concentration;  $A_{\text{max}}$ , net photosynthetic rate at 1700 μmol mol<sup>-1</sup> CO<sub>2</sub>;  $V_{\text{cmax}}$ , maximum rate of carboxylation;  $J_{\text{max}}$ , maximum rate of electron transport;  $G_s$ , stomatal conductance to water vapor

Each value is the mean of three replications, and the standard deviation is shown in parenthesis

ANOVA: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; n.s. not significant

Table 2 Needle traits of hybrid larch F<sub>1</sub> seedlings grown under CO<sub>2</sub> concentration at 360 and 720  $\mu\text{mol mol}^{-1}$  in combination with 0 (low N) and 30 kg N ha<sup>-1</sup> (High N).

	360 $\mu\text{mol mol}^{-1}$ CO <sub>2</sub>		720 $\mu\text{mol mol}^{-1}$ CO <sub>2</sub>		ANOVA		
	Low N	High N	Low N	High N	CO <sub>2</sub>	N	CO <sub>2</sub> ×N
LMA(g m <sup>-2</sup> )	92.8 (5.5)	93.8 (4.2)	103.9 (3.9)	104.6 (6.3)	*	n.s.	n.s.
CWMA (g m <sup>-2</sup> )	53.5 (1.0)	49.0 (1.8)	61.7 (4.6)	64.2 (4.7)	***	n.s.	n.s.
Needle shape ratio	0.42 (0.01)	0.44 (0.03)	0.43 (0.02)	0.43 (0.01)	n.s.	n.s.	n.s.
Starch (g m <sup>-2</sup> )	7.32 (2.75)	7.46 (1.47)	9.79 (1.07)	10.70 (1.76)	**	n.s.	n.s.
Chlorophyll (g m <sup>-2</sup> )	0.23 (0.03)	0.23 (0.03)	0.14 (0.01)	0.16 (0.02)	**	n.s.	n.s.
N (g m <sup>-2</sup> )	1.05 (0.02)	1.20 (0.20)	0.98 (0.08)	0.97 (0.06)	n.s.	n.s.	n.s.
P (mg m <sup>-2</sup> )	53.5 (6.1)	56.4 (6.2)	41.9 (4.7)	47.7 (5.5)	***	n.s.	n.s.
K (mg m <sup>-2</sup> )	647.6 (99.2)	637.2 (86.1)	474.3 (63.7)	511.8 (112.6)	*	n.s.	n.s.
Ca (mg m <sup>-2</sup> )	264.6 (36.2)	318.5 (26.3)	309.2 (57.7)	386.0 (45.5)	n.s.	*	n.s.
Mg (mg m <sup>-2</sup> )	104.2 (8.5)	120.5 (9.8)	107.1 (1.2)	136.7 (5.6)	n.s.	**	n.s.

LMA, leaf mass per area; CWMA, cell wall mass per area; Needle shape ratio, the ratio of needle thickness to width

Each value is the mean of three replications, and the standard deviation is shown in parenthesis

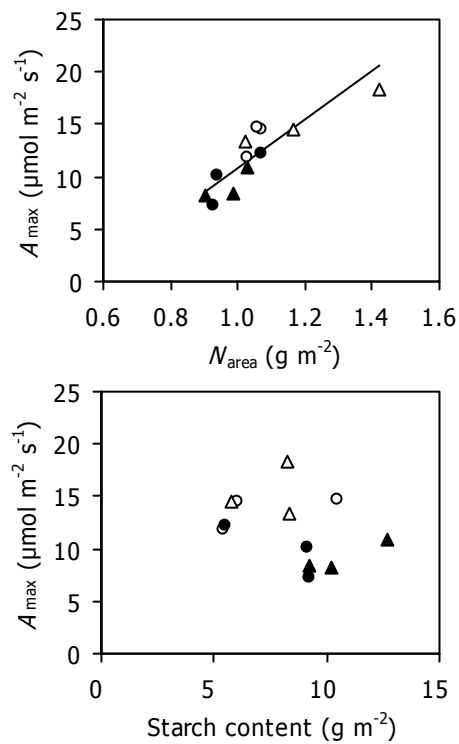
ANOVA: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; n.s. not significant

Table 3 Dry mass of plant organs and branch numbers of hybrid larch F<sub>1</sub> seedlings grown under CO<sub>2</sub> concentration at 360 and 720  $\mu\text{mol mol}^{-1}$  in combination with 0 (low N) and 30 kg N ha<sup>-1</sup> (High N).

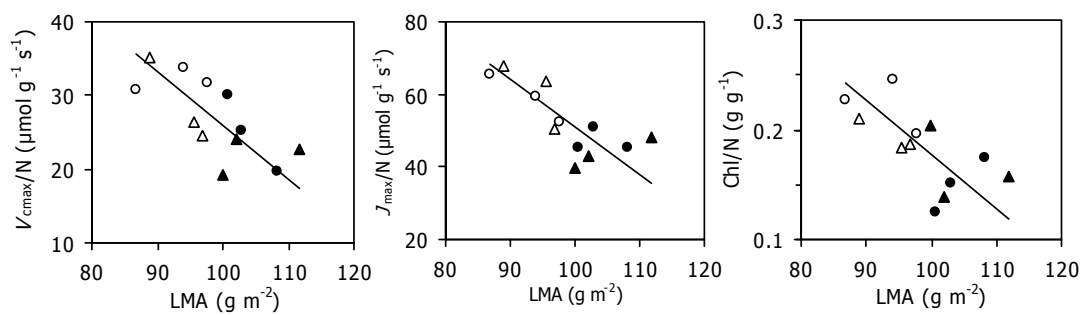
	360 $\mu\text{mol mol}^{-1}$ CO <sub>2</sub>		720 $\mu\text{mol mol}^{-1}$ CO <sub>2</sub>		ANOVA		
	Low N	High N	Low N	High N	CO <sub>2</sub>	N	CO <sub>2</sub> ×N
Dry mass (g)							
Needle	10.3 (2.9)	14.1 (1.0)	17.8 (1.6)	19.5 (1.9)	*	*	n.s.
Branch	5.2 (1.3)	7.4 (0.9)	9.3 (0.2)	8.9 (1.7)	**	n.s.	n.s.
Stem	13.8 (4.2)	17.8 (1.0)	17.1 (3.6)	18.6 (3.1)	n.s.	n.s.	n.s.
Root	25.9 (8.9)	29.8 (1.8)	35.3 (3.1)	46.1 (3.1)	*	*	n.s.
Whole-plant	55.2 (15.7)	69.0 (2.2)	79.5 (5.9)	93.0 (8.5)	*	*	n.s.
Branch number							
Primary branch	15.0 (5.8)	16.2 (3.3)	15.0 (2.7)	16.6 (4.5)	n.s.	n.s.	n.s.
Secondary branch	22.1 (11.3)	37.4 (14.3)	69.1 (14.6)	56.0 (15.9)	*	n.s.	n.s.

Each value is the mean of three replications, and the standard deviation is shown in parenthesis  
ANOVA: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; n.s. not significant

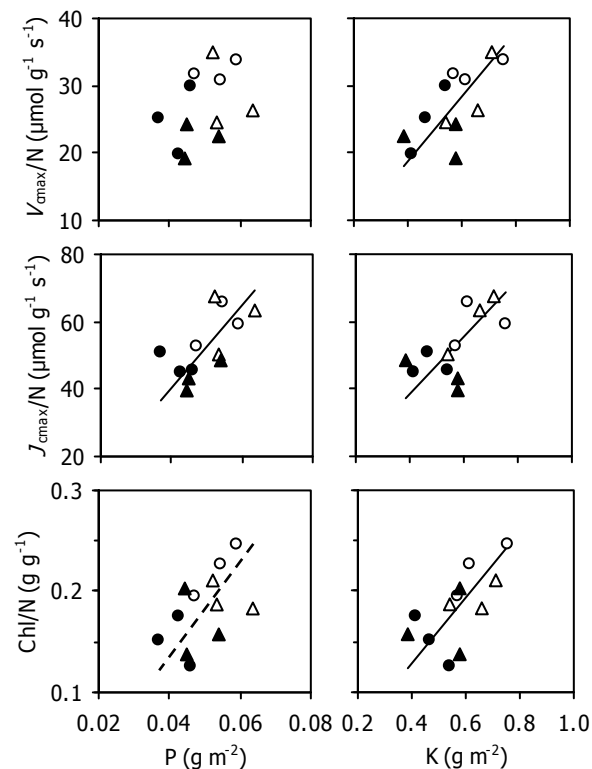




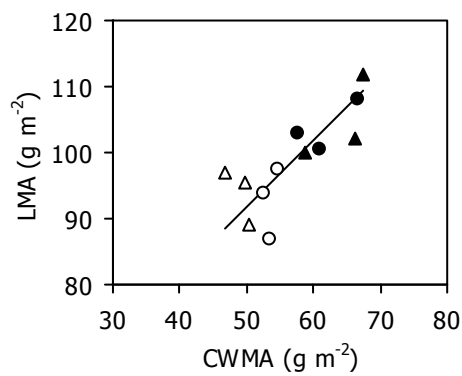
**Figure 1** Relationships between light and  $\text{CO}_2$  saturated net photosynthetic rate ( $A_{\max}$ ) and (top) N content per area ( $N_{\text{area}}$ ) and (bottom) starch content in the needles of hybrid larch  $F_1$  seedlings grown under  $\text{CO}_2$  concentration at 360 (open) and 720  $\mu\text{mol mol}^{-1}$  (solid) in combination with N supply at 0 (circle) and 30  $\text{kg N ha}^{-1}$  (triangle). Each plot indicates the value in each replication. Regression line for all treatments was obtained using reduced major axis regression method ( $Y = -12.7 + 23.5 X$ ,  $R^2 = 0.76^{***}$  for  $N_{\text{area}}$  vs.  $A_{\max}$ )



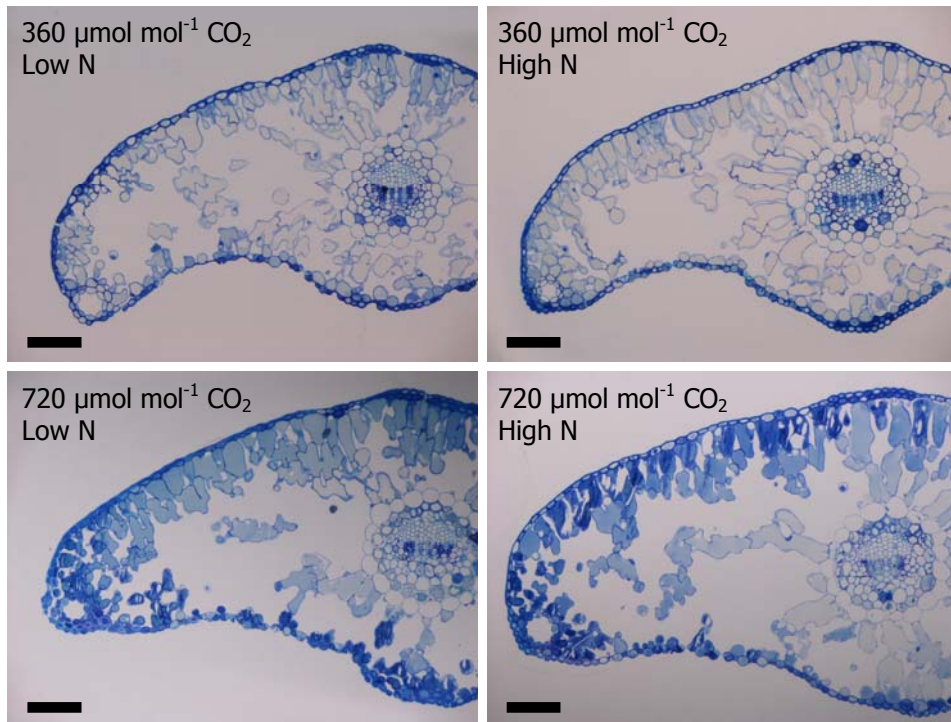
**Figure 2** Ratios of maximum rate of carboxylation ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), maximum rate of electron transport ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and chlorophyll content ( $\text{g m}^{-2}$ ) to N content ( $\text{g m}^{-2}$ ) ( $V_{\text{cmax}}/\text{N}$  (left),  $J_{\text{max}}/\text{N}$  (middle), and  $\text{Chl}/\text{N}$  (right), respectively) as a function of leaf mass per area (LMA) in the needles of hybrid larch  $F_1$  seedlings grown under  $\text{CO}_2$  concentration at 360 (open) and 720  $\mu\text{mol mol}^{-1}$  (solid) in combination with N supply at 0 (circle) and 30  $\text{kg N ha}^{-1}$  (triangle). Each plot indicates the value in each replication. Regression line for all treatments was obtained using the reduced major axis regression method ( $Y = -0.73 + 99.5 X$ ,  $R^2 = 0.52^{**}$  for LMA vs.  $V_{\text{cmax}}/\text{N}$ ;  $Y = -1.30 + 181.0 X$ ,  $R^2 = 0.57^{**}$  for LMA vs.  $J_{\text{max}}/\text{N}$ ; and  $Y = -.0050 + 0.6783 X$ ,  $R^2 = 0.44^*$  for LMA vs.  $\text{Chl}/\text{N}$ )



**Figure 3** Ratios of maximum rate of carboxylation ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), maximum rate of electron transport ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and chlorophyll content ( $\text{g m}^{-2}$ ) to N content ( $\text{g m}^{-2}$ ) ( $V_{\text{cmax}}/N$  (upper),  $J_{\text{max}}/N$  (middle), and  $\text{Chl}/N$  (lower), respectively) as a function of P (left) and K (right) contents in the needles of hybrid larch  $F_1$  seedlings grown under  $\text{CO}_2$  concentration at 360 (open) and 720  $\mu\text{mol mol}^{-1}$  (solid) in combination with N supply at 0 (circle) and 30  $\text{kg N ha}^{-1}$  (triangle). Each plot indicates the value in each replication. Regression line for all treatments was obtained using the reduced major axis regression method [ $Y = -9.1 + 1237.1 X$ ,  $R^2 = 0.43^*$  and  $Y = -0.05 + 4.77 X$ ,  $R^2 = 0.26$  ( $P = 0.094$ ) for P vs.  $J_{\text{max}}/N$  and  $\text{Chl}/N$ , respectively, and  $Y = 0.06 + 47.31 X$ ,  $R^2 = 0.49^*$ ,  $Y = 5.1 + 83.7 X$ ,  $R^2 = 0.39^{**}$ , and  $Y = 0.0001 + 0.3227 X$ ,  $R^2 = 0.41^{**}$  for K vs.  $V_{\text{cmax}}/N$ ,  $J_{\text{max}}/N$ , and  $\text{Chl}/N$ , respectively]



**Figure 4** Leaf mass per area (LMA) as a function of cell wall mass per area (CWMA) in the needles of hybrid larch F<sub>1</sub> seedlings grown under CO<sub>2</sub> concentration at 360 (open) and 720 μmol mol<sup>-1</sup> (solid) in combination with N supply at 0 (circle) and 30 kg N ha<sup>-1</sup> (triangle). Each plot indicates the value in each replication. Regression line for all treatments was obtained using the reduced major axis regression method ( $Y = 1.02 + 40.48 X$ ,  $R^2 = 0.65^{**}$ )



**Figure 5** Transverse section of the needles of hybrid larch  $F_1$  seedlings grown under  $\text{CO}_2$  concentration at 360 (upper) and 720  $\mu\text{mol mol}^{-1}$  (lower) in combination with N supply at 0 (low N, left) and 30  $\text{kg N ha}^{-1}$  (High N, right). Scale bars: 100  $\mu\text{m}$ .