Changes in morphology, anatomy, and photosynthetic capacity of needles of Japanese larch (*Larix kaempferi*) seedlings grown in high CO_2 concentrations

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Abstract

Photosynthetic traits of two-year-old Japanese larch seedlings (*Larix kaempferi* Carr.) grown at elevated CO_2 concentrations were studied in relation to structural changes in the needles. Seedlings were grown at two CO_2 concentrations, 360 (AC) and 720 (EC) μmol mol^{-1} at high and low nutrient supply rates, high N (HN) and low N (LN). The photosynthetic capacity fell significantly in EC+LN, but increased significantly in EC+HN. Since the mesophyll surface area exposed to intercellular space per unit leaf area (A^{mes}/A) is correlated with the photosynthetic rate, we measured A^{mes}/A for larch needles growing in EC. Changes of A^{mes}/A in both EC+HN and EC+LN were very similar to the changes in photosynthetic capacity. This suggests that the changes of A^{mes}/A in EC probably caused the changes in the photosynthetic capacity. The changes of A^{mes}/A in EC were attributed to changes in the mesophyll cell size and mesophyll cell number. The photosynthetic capacity in EC can be explained by taking morphological and structural adaptations into account as well as biochemical factors.

Additional key words: carboxylation efficiency; cell number; intercellular CO_2 concentration; mesophyll surface area; needle thickness and width; net photosynthetic rate; nitrogen content; specific leaf area; starch.

Introduction

Plants raised under high concentrations of CO_2 usually show photosynthetic depression, recognized as photosynthetic down-regulation (Cook *et al*. 1998, Koike *et al*. 2000, Ainsworth *et al*. 2003). However, the down-regulation mechanism is still uncertain, since the response differs with species (Sage *et al*. 1989), leaf age (Turnbull *et al*. 1998), and tree size (Greenep *et al*. 2003).

There are two major reasons for photosynthetic down-regulation. The first is reduction of nitrogen concentration in foliage (Coleman *et al*. 1993, Nakano *et al*. 1997). The second is reduced CO_2 diffusion within the leaves, following from reduction in stomatal conductance (Vodnik *et al*. 2002) and excessive accumulation of starch inside the chloroplasts (Makino 1994).

A further important factor affecting photosynthetic capacity is structural features within the leaves (Šesták *et al*. 1985, Tichá 1985, Terashima *et al*. 2001). Intercellular space per unit leaf area (A^{mes}/A) is a particularly important parameter that is correlated with the photosynthetic rate (Nobel *et al*. 1975, Nobel 1999, Slaton and Smith 2002). The A^{mes}/A is changed easily by the environmental influences (*e.g.* Körner and Larcher 1988, Nobel 1999).

In the higher latitudes of the northern hemisphere, larch is the dominant species, having huge biomass even in permafrost regions (Gower and Richards 1990). Since

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Abbreviations: A^{mes}/A = the mesophyll surface area exposed to intercellular space per unit leaf area; AC = ambient CO_2 concentration; C_i = internal needle CO_2 partial pressure; EC = enhanced CO_2 concentration; HN = high nutrient supply; LN = low nutrient supply; P = photosynthetic rate; P_{growth} = photosynthetic rate at growing environment; P_{max} = photosynthetic rate under CO_2 saturation; SLA = specific leaf area.
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the Japanese larch (*Larix kaempferi* Carr.) grows very fast, this species has been widely planted in Japan and Europe (Matyssek and Schulze 1987, Koike *et al*. 2000, Onaindia and Amezaga 2000). In experiments with Siberian larch (*Larix sibirica*) in high concentrations of CO_2, photosynthetic depression was accompanied by low nutrient condition (Koike *et al*. 2000); growth rates increased significantly with better nutrient condition (Yazaki *et al*. 2001). However, no information is available to infer the physiological depression as related to anatomical structure of larch species in raised [CO_2] (Koike *et al*. 2000). It is expected that down-regulation or physiological adjustment may be correlated with both physiological function and structural changes in high [CO_2].

To access this expectation, we tried to find the photosynthetic traits of Japanese larch at raised [CO_2] (EC) from the inner structural changes in the needles. Since inside structure of leaves is readily altered by environmental factors (Körner and Larcher 1988, Yáñez-Espinosa *et al*. 2003), larch seedlings were grown in a growth cabinet under precisely regulated environmental conditions in order to study the effect of EC on the morphology and anatomy of the needles. In addition, since photosynthetic traits at EC are strongly affected by soil nutrient conditions (Koike *et al*. 2000, Yazaki *et al*. 2001), our larch seedlings were raised under two nutrient regimes, known as high (HN) and low (LN) nutrient condition. We then determined the photosynthetic capacity and the factors believed to influence it (nitrogen and starch contents, and structural features inside the needles).

Materials and methods

Plants: Two-year-old seedlings of Japanese larch (*Larix kaempferi* Carr.) were cultivated at Kuriyama town, near Sapporo, northern Japan (43˚N, 141˚E). The mean height and diameter of seedlings at the stem base were 12.5 cm and 3.0 mm, respectively. These seedlings were raised in four environmental growth cabinets (*Koito Industries*, Yokohama, Japan) at the Forestry and Forest Products Research Institute (FFPRI) located in Sapporo; full specifications are given by Koike (1995). In May 2003, the seedlings were transplanted to 9 500 cm^3 vinyl pots filled with a 1 : 1 (v/v) mixture of Kanuma pumice soil and clay soil. The pots were large enough to allow unrestricted root growth during the experiment. The environmental treatments commenced in May 2003 and finished in October 2003; total 160 d.

Treatments: Six seedlings per treatment were grown in two concentrations of CO_2 (360 and 720 μmol mol^{-1}) with two nutrient supply rates (HN and LN). Liquid fertilizer (*Hyponex*, 5 : 10 : 5, N : P : K, *O.M. Scott and Sons*, Marysville, OH, USA) was supplied once a week [HN: 18 g(N) m^{-3} $week^{-1}$] or just once at the start of CO_2 treatment [LN: 18 g(N) m^{-3} per five-months]. Seedlings were grown under natural daylight and photoperiod. The photosynthetic photon flux density (PPFD) in each chamber was about 80 % of full sunlight (Yazaki *et al*. 2001). The day/night temperatures were maintained at optimal values (26/16 °C) (Koike *et al*. 2000). All pots were moved monthly across the chambers, and weekly within the chambers, to minimize any effect of individual chambers or locations (Koike 1995, Yazaki *et al*. 2001).

Photosynthetic characteristics of larch needles were determined using three machines of a gas analysis system (*Li-6400*, *Li-Cor*, Lincoln, NE, USA). Four samples were used per treatment. P/C_i response curves (Farquhar and Sharkey 1982) were determined by measuring the steady-state response of photosynthetic rate (P) to varying internal needle CO_2 partial pressures (C_i). External CO_2 concentrations were supplied in seven steps, from 1 500 to 0 μmol mol^{-1}. The PPFD in the leaf chamber (measured with a *Li-6400* device; *Li-Cor*, Lincoln, NE, USA) was 1 500 μmol m^{-2} s^{-1}, which is saturation level for Japanese larch (Koike *et al*. 2000, Kitaoka *et al.* 2001). The temperature in the leaf chamber was 25 °C at all CO_2 concentrations. The water vapour deficit in the leaf chamber was about 1.5 kPa. P/C_i curves were determined on August 28th and 29th, about 90 d after treatment began and when the long shoot needles had expanded fully. After photosynthetic measurement, the needle surface areas were calculated by image processing and analysis software, *Image J* (National Institutes of Health, Maryland, USA) in order to determine P per unit area and the specific leaf area (SLA). The initial slope of the P/C_i curves (giving the carboxylation efficiency, CE) and the photosynthetic rate at growing environment (P_{growth}) and under CO_2 saturation (P_{max}) were also calculated from the P/C_i curves.

Nitrogen and starch measurements: After the photosynthetic measurements, the needles were collected and dried. The nitrogen content per unit dry needle mass was determined using a *N.C.* Analyzer *NC-900* (*Shimadzu*, Kyoto, Japan). The nitrogen content per unit needle area was calculated from the SLA and needle moisture content. Four samples were used per treatment.

The starch content in the needles was determined by the anthron-sulphuric acid technique (Koehler 1952). Four samples were used per treatment. Needles were collected and dried after photosynthetic measurement. 50 mg samples of powdered needles were used, and each sample was washed three times in hot 80 % ethanol to remove soluble sugar and protein. The beaker containing the residue was then placed in boiling water for 10 min. Next, 6.5 cm^3 of 52 % $HClO_4$ was added to hydrolyze starch into glucose. The mixture was stirred for 10 min and left

for 24 h. After filtration, the filtrate was made up to 50 cm^3. 1 cm^3 of this extracted solution was mixed with 2 cm^3 of anthron-sulphuric acid in a test tube on ice. The test tube was then placed in boiling water for 7.5 min. After cooling, the absorption at 630 nm was measured using a spectrophotometer (*Ultraspec 3000 pro*, *Amersham Biosciences*, Tokyo, Japan). This value corresponds to the sucrose concentration deriving from starch, so that the starch concentration was given by multiplying the glucose concentration by 0.9. Then, the starch content per dry mass was calculated.

Structural features inside the needles: Four samples were used per treatment. After photosynthetic measurement, the needles were fixed overnight in 4 % glutaraldehyde, and then dehydrated through a graded ethanol series and embedded in epoxy resin (Yazaki *et al*. 2001). Transverse sections of 1 μm thickness were cut with an ultra-microtome (*EM-Ultracut-J*, *JEOL Co.*, Tokyo, Japan) and double-stained with a solution of 1 % safranin and 1 % gentian violet in water (Kitin *et al*. 1999). Digital images (512×512 pixels, 256 level on a gray scale) were obtained with a confocal laser scanning microscope (*LSM-310*, *Carl Zeiss*, Oberkochen, Germany) operating in transmission mode (Funada *et al*. 1997). The images were analyzed with *Image J* (National Institutes of Health, Maryland, USA).

Thickness and width of needles were measured from the observed images. The calculation methods of the surface area of mesophyll cells exposed to intercellular space per unit needle area (A^{mes}/A) have been suggested by many researchers (Thain 1983, James *et al*. 1999, Nobel 1999, Michèle *et al*. 2002, Oguchi *et al*. 2003). James *et al*. (1999) and Michèle *et al*. (2002) provided the methods using the oblique-paradermal section, which could indicate more accurate A^{mes}/A data. In our study, however, we determined relative value of A^{mes}/A in the needles with use of the transversal sections for comparison between two CO_2 conditions. We applied transversal sections to calculate A^{mes}/A following the methods of Oguchi *et al*. (2003) and Thain (1983), which assume that mesophyll cells are cylinders having flat ends or are spheroid. Images of the transversal sections were examined with use of this method to investigate the anatomical traits within the needles of pine and spruce (Koike *et al*. 1994). The corresponding curvature factors (F) were 1.34–1.41 and 1.36–1.43, respectively (Thain 1983).

Statistical analysis: The effect of CO_2 was evaluated by one-way analysis of variance (ANOVA) for each nutrient treatment, using the *Statview* software package (*SAS Institute*, Cary, NC, USA). Differences were considered significant at $p<0.05$.

Results

Photosynthetic characteristics: Differences were visible in P/C_i curves between EC and AC with both LN and HN treatments (Fig. 1*A,B*). The carboxylation efficiency was significantly reduced in EC+LN, but did not differ significantly between the CO_2 concentrations in HN (Table 1). P_{growth} did not differ significantly between CO_2 concentrations in LN, but increased significantly in EC+HN (Table 1). P_{max} was significantly reduced in EC+LN, but increased significantly in EC+HN (Table 1).

Nitrogen and starch measurements: Nitrogen contents per needle dry mass and needle area did not differ significantly between the CO_2 concentration treatments at either LN or HN (Table 1). Needle starch content increased significantly in EC with both LN and HN (Table 1).

Structural features inside needles: Fig. 2 shows light micrographs of transverse sections of needles from each

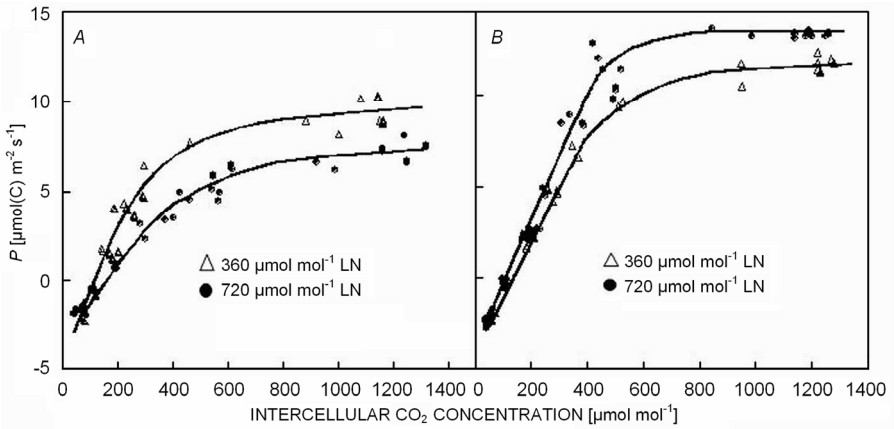

Fig. 1. Response of photosynthetic rate (P) to intercellular CO_2 concentration (C_i) ($n = 4$). *A*: Low nutrient supply (LN). *B*: High nutrient supply (HN).

Table 1. Carboxylation efficiency, photosynthetic rate at growing environment (P_{growth}) and under CO_2 saturation (P_{max}), calculated from P/C_i curves (Fig. 1) and nitrogen content of needles per unit dry mass and per unit area, and starch content of needles per unit dry mass. LN = low nutrients, HN = high nutrients. Means±S.E., $n = 4$. $^{*}p<0.05$, $^{**}p<0.01$, NS = non-significant.

Treatment	LN			HN		
Parameter	360 μmol mol^{-1}	720 μmol mol^{-1}	*p*	360 μmol mol^{-1}	720μmol mol^{-1}	*P*
Carboxylation efficiency	0.033±0.004	0.022±0.001	**	0.045±0.001	0.044±0.001	NS
P_{growth} [μmol(C) m^{-2} s^{-1}]	4.14±0.24	5.13±0.35	NS	4.86±0.06	10.46±0.35	**
P_{max} [μmol(C) m^{-2} s^{-1}]	9.28±0.31	6.79±0.18	**	11.81±0.23	13.30±0.07	**
Nitrogen in needle [g kg^{-1}]	11.5±0.6	10.0±0.9	NS	17.7±0.4	17.4±0.7	NS
Nitrogen in needle [g m^{-2}]	13.5±0.5	12.7±0.9	NS	22.9±1.6	22.1±0.8	NS
Starch in needle [g kg^{-1}]	50.4±3.6	65.0±2.6	*	26.4±1.4	35.3±2.1	*

Table 2. Architectural features calculated from light micrographs (Fig. 3). Means±S.E., $n = 4$. $^{*}p<0.05$, $^{**}p<0.01$, NS = non-significant. SLA = specific leaf area. A^{mes}/A = surface area of mesophyll cells exposed to intercellular space per unit leaf area.

Treatment	LN			HN		
Parameter	360 μmol mol^{-1}	720 μmol mol^{-1}	*p*	360 μmol mol^{-1}	720 μmol mol^{-1}	*p*
SLA [cm^2 g^{-1}]	50.50±1.90	47.20±2.20	NS	46.70±2.30	47.10±0.90	NS
Needle thickness [mm]	0.37±0.20	0.34±0.09	NS	0.52±0.31	0.48±0.43	NS
Needle width [mm]	0.91±0.68	0.93±0.23	NS	1.31±0.59	1.03±0.65	*
A^{mes}/A [m^2 m^{-2}]	11.70±0.38	9.60±0.35	**	14.20±0.37	15.60±0.30	*
Mesophyll cell height [μm]	38.50±1.51	48.10±4.66	NS	60.80±2.67	47.90±3.35	*
Mesophyll cell diameter [μm]	21.00±1.31	33.00±1.08	*	31.20±2.18	28.60±1.51	**
Mesophyll cell number per μm^{-2}	0.12±0.01	0.09±0.01	**	0.12±0.01	0.15±0.01	*

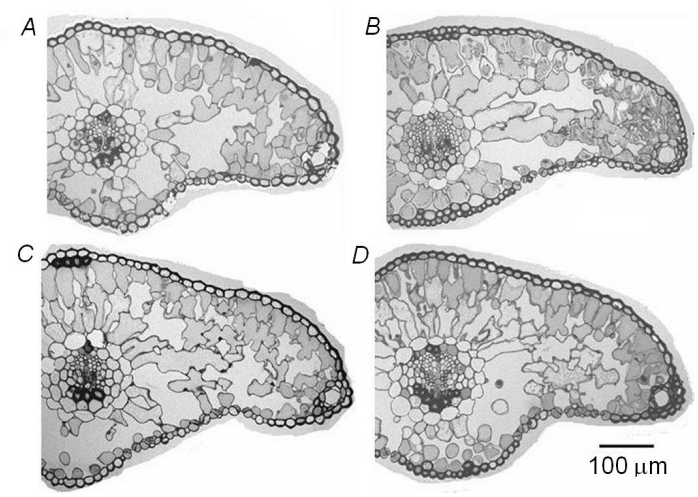

Fig. 2. Light micrographs of transverse sections of needles grown (*A*) at 360 μmol mol^{-1} CO_2 (AC) with low nutrients (LN), (*B*) at 720 μmol mol^{-1} (EC) with LN, (*C*) at AC with high nutrients (HN), or (*D*) at EC with HN. *The bar* is 100 μm.

treatment. The specific leaf area (SLA) tended to decrease in EC+LN, but the difference between the CO_2 concentration treatments was not significant with HN (Table 2). Needle thickness did not differ significantly between CO_2 concentrations with either LN or HN (Table 2). Needle width did not differ significantly between CO_2 concentrations with LN, but decreased significantly in EC+HN (Table 2). A^{mes}/A decreased significantly in EC+LN, but increased significantly in EC+HN (Table 2). Mesophyll cell height tended to increase in EC+LN, but decreased significantly in EC+HN (Table 2). Mesophyll cell diameter increased in EC+LN, but decreased significantly in EC+HN (Table 2). Mesophyll cell number per unit area decreased in EC+LN, but increased significantly in EC+HN (Table 2).

Discussion

Down-regulation of photosynthesis of larch seedlings grown in EC+LN (Fig. 1*A* and Table 1) was observed. However, the photosynthetic capacity increased in EC+HN (Fig. 1*B* and Table 1). To explain the photosynthetic changes observed in larch seedlings in EC, we focused on the nitrogen and starch contents, and the morphological and anatomical traits in the needles, all of which affect the photosynthetic rate (Šesták *et al*. 1985, Coleman *et al*. 1993, Makino 1994).

The nitrogen content in the needles tended to decrease in EC with both LN and HN, though the difference was not significant (Table 1). This might explain why down-regulation of photosynthesis was observed in EC+LN, but cannot explain the increase in photosynthetic capacity in EC+HN.

The starch content in the needles increased significantly in EC with both LN and HN, more markedly with LN (Table 1). In general, sink-source balance of photosynthate is lost in EC (Issop *et al*. 2000) that probably induces excess accumulation of starch in chloroplasts. This reduces CO_2 diffusion in chloroplasts (Makino 1994) and is likely to be related to down-regulation. Usuda and Shimogawara (1998) reported that plants with high sink activity (for example, radish) were not down-regulated in EC because excess photosynthate did not accumulate in the leaves. In the present study, starch accumulation in EC+LN was probably one reason why photosynthetic down-regulation was observed, as a consequence of high diffusion resistance in chloroplasts (Makino 1994). However, this cannot explain the increase in photosynthetic capacity in EC+HN.

The morphological and anatomical traits of the needles also affect the photosynthetic capacity (Šesták *et al*. 1985, Tichá 1985, Terashima *et al*. 2001). In particular, A^{mes}/A is a key parameter that is correlated with the photosynthetic rate (Nobel *et al*. 1975, Nobel 1999, Slaton and Smith 2002). In our study, A^{mes}/A decreased significantly in EC+LN and increased significantly in EC+HN (Table 2). These changes of A^{mes}/A in EC with both LN and HN were very similar to the changes of P/C_i curve, or the photosynthetic capacity (Table 1). Consequently, the changes of A^{mes}/A in EC probably explain the down-regulation of photosynthesis observed in EC+LN, and the increase in photosynthetic capacity in EC+HN.

It remains to explain why A^{mes}/A changed in EC. This depends on the mesophyll cell size and mesophyll cell number inside the needles. With LN, the mesophyll cell size increased but the mesophyll cell number decreased in EC (Table 2). This probably caused the decrease in total mesophyll surface area, reducing A^{mes}/A. In the HN condition, by contrast, the mesophyll cell size decreased but the mesophyll cell number increased in EC (Table 2). This probably caused the increase in total mesophyll surface area, increasing A^{mes}/A.

In conclusion, morphological and anatomical traits inside the needles significantly influence the photosynthetic capacity of Japanese larch seedlings grown in EC. In particular, A^{mes}/A is probably responsible for changes in the photosynthetic capacity in EC. Changes of A^{mes}/A in EC are attributed to changes in mesophyll cell size and mesophyll cell number. The photosynthetic capacity in EC can be explained by taking into account morphological and structural adaptations as well as biochemical factors.

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