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**Mesophyll conductance in leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) seedlings grown under elevated CO<sub>2</sub> concentration and low N availability**

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## Abstract

To test the hypothesis that mesophyll conductance ( $g_m$ ) would be reduced by leaf starch accumulation in plants grown under elevated  $\text{CO}_2$  concentration  $[\text{CO}_2]$ , we investigated  $g_m$  in seedlings of Japanese white birch grown under ambient and elevated  $[\text{CO}_2]$  with an adequate and limited nitrogen supply by using simultaneous gas exchange and chlorophyll fluorescence measurements. Both elevated  $[\text{CO}_2]$  and limited nitrogen supply decreased area-based leaf N accompanied with a decrease in the maximum rate of Rubisco carboxylation ( $V_{c,\max}$ ) on a  $\text{CO}_2$  concentration at chloroplast stroma ( $C_c$ ) basis. Conversely, only seedlings grown at elevated  $[\text{CO}_2]$  under limited nitrogen supply had significantly higher leaf starch content with significantly lower  $g_m$  among the treatment combinations. Based on a leaf anatomical analysis using microscopic photographs, however, there were no significant difference in the area of chloroplast surfaces facing intercellular space per unit leaf area among treatment combinations. Thicker cell walls were suggested in plants grown under limited N by increases in leaf mass per area subtracting non-structural carbohydrates. These results suggest that starch accumulation and/or thicker cell walls in the leaves grown at elevated  $[\text{CO}_2]$  under limited N supply might hinder  $\text{CO}_2$  diffusion in chloroplasts and cell walls, which would be an additional cause of photosynthetic down-regulation as well as a reduction in Rubisco activity related to the reduced leaf N under elevated  $[\text{CO}_2]$ .

**Abbreviations:** A, net  $\text{CO}_2$  assimilation rate;  $A_c$ , net photosynthetic rate limited by Rubisco activity;  $A_j$ , net photosynthetic rate limited by RuBP regeneration;  $C_c$ ,  $\text{CO}_2$  concentration in the chloroplast stroma;  $C_i$ , intercellular  $\text{CO}_2$  concentration;  $F_s$ , chlorophyll fluorescence under actinic light;  $F_m'$ , the maximum chlorophyll fluorescence under actinic light;  $g_m$ , mesophyll conductance to  $\text{CO}_2$  supply from substomatal cavities to sites of carboxylation;  $J_{\max}$ , the maximum rate of electron transport driving RuBP regeneration;  $J_T$ , total rate of electron transport through PSII to photosynthesis and photorespiration;  $K_c$ ,  $K_o$ , Rubisco Michaelis constant for  $\text{CO}_2$  and  $\text{O}_2$ , respectively; LMA, leaf mass per area;  $N_{\text{area}}$ , area-based leaf nitrogen content; NSC, non-structural carbohydrates; PFD, photon flux density;  $R_d$ , mitochondrial respiration in the light;  $R_n$ , mitochondrial respiration in the dark;  $S_c$ , the areas of chloroplast surfaces facing intercellular space per unit leaf area;  $V_{c,\max_{C_c}}$ ,  $V_{c,\max_{C_i}}$ ,  $C_c$ - and  $C_i$ -based maximum carboxylation capacity of Rubisco, respectively;  $\Phi_{\text{CO}_2}$ , quantum yield of  $\text{CO}_2$  assimilation;  $\Phi_e$ , quantum yield of electron transport through PSII to photosynthesis and photorespiration;  $\Phi_{\text{PSII}}$ , quantum yield of PSII electron transport;  $\Gamma^*$ ,  $\text{CO}_2$  compensation point in the absence of dark respiration

## **Introduction**

Elevated atmospheric CO<sub>2</sub> concentration [CO<sub>2</sub>] temporally stimulates the photosynthetic rate, but eventually results in photosynthetic down-regulation with carbohydrate accumulation after long-term exposure, even when root growth is not limited during FACE (free-air CO<sub>2</sub> enrichment) experiments (Nie et al. 1995, Rogers and Ellsworth 2002, Bernacchi et al. 2003, 2005, Ainsworth and Long 2005, Rogers et al. 2006, Eguchi et al. 2008). Some mechanisms underlying photosynthetic down-regulation in leaves grown under elevated [CO<sub>2</sub>] have been proposed, such as a reduced Rubisco content accompanied with reduced leaf N content (Seneweera et al. 2011), and a reduced ratio of Rubisco to total N via a reduction in RNA expression induced by sugar accumulation (e.g. Sage 1994, Drake et al. 1997, Sims et al. 1998, Stitt and Krapp 1999). Aside from the amount and activity of Rubisco, a decrease in mesophyll conductance from the stomatal cavity to chloroplast stroma due to starch accumulation has been proposed as a mechanism of photosynthetic down-regulation in crop plants with large carbon sinks based on a negative correlation between the photosynthetic rate and starch accumulation (Nakano et al. 2000, Sawada et al. 2001).

The mesophyll conductance to CO<sub>2</sub> supply from substomatal cavities to sites of carboxylation ( $g_m$ ) may pose a significant limitation to photosynthesis (Harley et al. 1992, Loreto et al. 1992, Terashima et al. 2006, Warren 2008). Simultaneous measurements of gas exchange and chlorophyll fluorescence make it possible to estimate  $g_m$ , combined with measurements under non-photorespiratory conditions (Harley et al. 1992, Epron et al. 1995, Warren and Dreyer 2006). Recent studies have reported that  $g_m$  can respond to environmental conditions, such as growth irradiance (Hanba et al. 2002), nutrient supply (Warren 2004), and short-term variations in temperature (Bernacchi et al. 2002, Warren and Dreyer 2006), and ambient CO<sub>2</sub> concentration (Centritto et al. 2003, Düring 2003, Flexas et al. 2007, Vrábl et al. 2009).  $g_m$  is considered to be determined not only by intrinsic anatomical features (Oguchi et al. 2003, Eguchi et al. 2004, Terashima et al. 2006) but possibly by physiological reactions involving a carbonic anhydrase or aquaporins (Bernacchi et al. 2002, Long and Bernacchi 2003, Hanba et al. 2004, Terashima et al. 2006, Flexas et al. 2007).  $g_m$  is also known to be smaller in tree species than in herbaceous ones, which generally results in lower photosynthetic rates in tree species (Epron et al. 1995, Flexas et al. 2008, 2012).

The effects of long-term elevated [CO<sub>2</sub>] on  $g_m$  have been reported as species- and experiment dependent (Singsaas et al. 2003, Bernacchi et al. 2005). Bernacchi et al. (2005) reported

that  $g_m$  is unaffected in soybean grown at elevated  $[\text{CO}_2]$  though the effect of starch accumulation on  $g_m$  was not assessed. It is proposed that starch accumulation in the chloroplast hinders  $\text{CO}_2$  diffusion through an increase in the length of the diffusion pathway around large starch grains (Krieg 1986). To our knowledge, there has been little study on  $g_m$  in plants grown under elevated  $\text{CO}_2$  in relation to starch accumulation. In the previous studies, limited N supply was found to be capable of inducing starch accumulation accompanied by the photosynthetic down-regulation in Japanese white birch (*Betula platyphylla* var. *japonica*) grown under elevated  $[\text{CO}_2]$  (Kitao et al. 2005, 2007).

We hypothesized that leaf starch accumulation would decrease  $g_m$ , which could be a cause of apparent photosynthetic down-regulation under elevated  $[\text{CO}_2]$  as well as a reduction in Rubisco activity in Japanese white birch. To test this hypothesis, we investigated the effects of elevated  $\text{CO}_2$  on  $g_m$  in its leaves, which are feasible to induce starch accumulation grown at elevated  $\text{CO}_2$  while limiting N supply, via simultaneous measurements of gas exchange and chlorophyll fluorescence (Harley et al. 1992).

## **Materials and methods**

### **Plants and growing conditions**

Two-year-old seedlings of Japanese white birch (*Betula platyphylla* Sukatchev var. *japonica* Hara) obtained from a commercial nursery (Oji Forestry & Landscaping Co., Ltd., Sapporo, Japan) were transplanted in free-draining plastic pots (diameter: 18 cm, depth: 15 cm, volume: approx. 3 l) filled with clay loam soil mixed with Kanuma pumice soil (1:1 in volume) to simulate typical of immature volcanic ash characterized by Japanese land. Pots were placed on the trays to prevent nutrient drainage. The seedling height was 15 to 20 cm. The seedlings were placed in natural daylight (approx. 90% of full sunlight) phytotron (26/16 °C, day/night) for about 45 days from the end of May to the beginning of July. One chamber was used for each  $\text{CO}_2$  treatment: 360  $\mu\text{mol mol}^{-1}$  (ambient  $\text{CO}_2$  treatment, Ambient); and 720  $\mu\text{mol mol}^{-1}$  (elevated  $\text{CO}_2$  treatment, Elevated). To reduce the chamber effects, the treatments and seedlings were switched between the chambers about every 10 days. Details of the  $\text{CO}_2$  regulation are as described previously (Koike 1995, Koike et al. 1996). Two nitrogen levels were applied: 700 mg per plant (high nitrogen, +N), or 100 mg per plant (low nitrogen, -N).

### Measurements of CO<sub>2</sub> assimilation rate and chlorophyll fluorescence

Plants grown in the growth chambers were transferred to the laboratory and supplied with adequate water in the evening prior to measurement. The CO<sub>2</sub> assimilation rate and chlorophyll fluorescence were measured simultaneously on fully expanded mature leaves, which were developed after treatments began (approx. 30 days old) with a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany) combined with a portable photosynthesis system (LI-6400, Li-Cor, Nebraska, USA) using a PAM-2000 adapter chamber (Li 6400-06, Li-Cor). As we observed small differences in the number of newly-developed leaves in the main shoots of seedlings (from 8 to 11) 45 days after treatment among combinations of CO<sub>2</sub> and N, the 30-day-old leaves used in the present study could be considered to be at virtually the same developmental stage. Firstly, we measured the CO<sub>2</sub> assimilation rate and chlorophyll fluorescence under various light intensities and non-photorespiratory conditions (at 1 % O<sub>2</sub>) in order to derive the relationship between the quantum yield of CO<sub>2</sub> assimilation ( $\Phi_{\text{CO}_2}$ ) and that of electron transport ( $\Phi_{\text{PSII}}$ ). The leaf temperature was maintained at 30 °C and relative humidity at the inlet of the chamber between 70 and 80%.  $\Phi_{\text{CO}_2}$  is calculated as:  $\Phi_{\text{CO}_2} = (A + R_d)/\text{PFD}$  (Epron et al. 1995, Valentini et al. 1995), where A is the net CO<sub>2</sub> assimilation rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $R_d$  the mitochondrial respiration in the light ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and PFD the photon flux density ( $\mu\text{mol m}^{-2} \text{s}^{-2}$ ). The ratio of  $R_d$  to mitochondrial respiration in the dark ( $R_n$ ) is species-specific, but dependent on leaf age and leaf temperature (Villar et al. 1995). In a preliminary study, we derived the  $R_d:R_n$  ratio at a leaf temperature of 30°C for approximately 30-day-old leaves of *B. platyphylla* seedlings grown under ambient and elevated CO<sub>2</sub> using the Laisk method (1977). There was no significant difference in the  $R_d:R_n$  ratio between the CO<sub>2</sub> treatments (n = 4 for each treatment), and the mean value of the pooled data (n = 8) was  $0.59 \pm 0.03$  (mean  $\pm$  SE). In the present study,  $R_d$  was estimated from  $R_n$  assuming that the  $R_d:R_n$  ratio was constant across the treatment combinations as follows:

$$R_d = 0.59 R_n \quad (r^2 = 0.998, p < 0.001, n = 8) \quad (1).$$

The fluorescence under actinic light,  $F_s$ , and the maximum fluorescence,  $F_m'$  (determined under saturating light of  $8000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PFD for 1 s), were used to calculate the quantum yield of PSII electron transport,  $\Phi_{\text{PSII}} (= (F_m' - F_s)/F_m')$  (Genty et al. 1989). Subsequently, we measured the CO<sub>2</sub> assimilation rate and chlorophyll fluorescence under various ambient CO<sub>2</sub> concentrations ( $C_a$ ) (2000, 720, 360, 200, and  $100 \mu\text{mol mol}^{-1}$ ), at 21% O<sub>2</sub> and saturating light ( $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$  PFD). We

chose a light intensity ( $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), which was sufficient to saturate the net photosynthetic rate but not strong enough to induce a light-dependent decrease in the photosynthetic rate based on a preliminary study. The  $C_i$ -based maximum carboxylation capacity of Rubisco ( $V_{c,\text{max}_C_i}$ ) was estimated from the following equation (Farquhar et al. 1980, Long and Bernacchi 2003):

$$A_c = (C_i - \Gamma^*) / (C_i + K_c(1 + (O/K_o))) V_{c,\text{max}_C_i} - R_d \quad (2),$$

where  $A_c$  is the net photosynthetic rate limited by Rubisco activity, and the  $\text{CO}_2$  photo-compensation point ( $\Gamma^*$ ), the Michaelis-Menten constants for  $\text{CO}_2$  ( $K_c$ ) and  $\text{O}_2$  ( $K_o$ ) on the  $C_i$  basis were estimated after Bernacchi et al. (2001) and Long and Bernacchi (2003) assuming  $g_m$  is infinite.

We also estimated the  $C_i$ -based maximum rate of electron transport driving RuBP regeneration ( $J_{\text{max}_C_i}$ ) from the following equation (Farquhar et al. 1980, Ethier and Livingston 2004):

$$A_j = (C_i - \Gamma^*) / (C_i + 2\Gamma^*) J_{\text{max}_C_i} / 4 - R_d \quad (3)$$

where  $A_j$  is the net photosynthetic rate limited by RuBP regeneration.

### **Estimation of mesophyll conductance by the variable J method**

We used ‘the variable J method’ to estimate  $g_m$  based on gas exchange and chlorophyll fluorescence measurements (Harley et al. 1992, Valentini et al. 1995, Long and Bernacchi 2003). The relationship between the quantum yield of  $\text{CO}_2$  assimilation and PSII electron transport under non-photorespiratory conditions was used to estimate the total electron transport rate ( $J_T$ ).

$\Phi_{\text{PSII}}$  is linearly related to the apparent quantum efficiency of the photosynthetic linear electron flow, which can be estimated under non-photorespiratory conditions by  $\Phi_{\text{CO}_2}$  as:

$$\Phi_{\text{PSII}} = k \Phi_{\text{CO}_2} + b \quad (4),$$

where  $k$  is the slope and  $b$  is the y-axis intercept.

The relationships between the quantum yield of  $\text{CO}_2$  assimilation under the non-photorespiratory condition ( $\Phi_{\text{CO}_2}$ ) and that of electron transport ( $\Phi_{\text{PSII}}$ ) were almost identical among the combinations

of CO<sub>2</sub> and N supply, suggesting little effects of CO<sub>2</sub> and N supply on alternative electron pathways other than photosynthesis and photorespiration (Biehler and Fock 1996, Kitao et al. 2003), allowing the use of ‘the variable J method’.

Quantum yield of electron transport through PSII to photosynthesis and photorespiration ( $\Phi_e$ ) is estimated as

$$\Phi_e = 4 \Phi_{\text{CO}_2} = 4 (\Phi_{\text{PSII}} - b) / k \quad (5),$$

where 4 is the number of electrons needed to fix one CO<sub>2</sub>.

The total rate of electron transport through photosystem II to photosynthesis and photorespiration is calculated as

$$J_T = \text{PFD } \Phi_e \quad (6),$$

$g_m$  is estimated as

$$g_m = A / (C_i - \Gamma^* (J_T + 8(A + R_d)) / (J_T - 4(A + R_d))) \quad (7),$$

where A and C<sub>i</sub> are taken from the gas exchange measurements described above, and C<sub>c</sub>-based  $\Gamma^*$  at leaf temperature of 30 °C was estimated after Bernacchi et al. (2002).

The calculated values of  $g_m$  were used to convert A-C<sub>i</sub> curves into A-C<sub>c</sub> curves using the following equation:

$$C_c = C_i - (A/g_m) \quad (8),$$

where C<sub>c</sub> is the CO<sub>2</sub> concentration in the chloroplast stroma. Because the sensitivity to errors in estimating  $g_m$  was relatively low between 100 and 300  $\mu\text{mol mol}^{-1}$  C<sub>i</sub> (Harley et al. 1992), we used  $g_m$  estimated at ambient CO<sub>2</sub> (C<sub>a</sub>) of 360  $\mu\text{mol mol}^{-1}$ , corresponding to C<sub>i</sub> 267  $\pm$  5  $\mu\text{mol mol}^{-1}$  (mean  $\pm$  SE), as a representative for each leaf.



From A-C<sub>c</sub> curves, the C<sub>c</sub>-based maximum carboxylation capacity of Rubisco (V<sub>c,max\_Cc</sub>) was calculated using the temperature dependence of the kinetic parameters of Rubisco on a C<sub>c</sub> basis (Bernacchi et al. 2002) with the following equation:

$$A_c = (C_c - \Gamma^*) / (C_c + K_c(1 + (O/K_o))) V_{c,max\_Cc} - R_d \quad (9).$$

We also estimated the C<sub>c</sub>-based maximum rate of electron transport (J<sub>max\_Cc</sub>) from the following equation (Farquhar et al. 1980, Ethier and Livingston 2004):

$$A_j = (C_c - \Gamma^*) / (C_c + 2\Gamma^*) J_{max\_Cc} / 4 - R_d \quad (10).$$

### **Estimation of mesophyll conductance by the curve-fitting method**

In addition, we estimated g<sub>m</sub> using the curve-fitting method proposed by Ethier and Livingston (2004), for a validation of g<sub>m</sub> estimated by the variable J method. Substituting Eq. 8 in Eq. 9 gives a quadratic equation (von Caemmerer 2000) whose solution is the positive root:

$$\begin{aligned} A_c &= (-b + (b^2 - 4ac)^{0.5}) / 2a, \\ a &= -1/g_m, \\ b &= (V_{c,max\_Cc} - R_d) / g_m + C_i + K_c(1 + O/K_o), \\ c &= R_d(C_i + K_c(1 + O/K_o)) - V_{c,max\_Cc} (C_i - \Gamma^*) \quad (11). \end{aligned}$$

C<sub>c</sub>-based kinetic constants of Rubisco (Γ\*, K<sub>c</sub>, and K<sub>o</sub>) were estimated after Bernacchi et al. (2002). The value of R<sub>d</sub> was estimated as described above. We set these constants as a known priori and then estimated g<sub>m</sub> and V<sub>c,max\_Cc</sub> from a non-linear least-squares fit.

### **Light microscopy**

Interveinal sections of leaf samples were fixed in an aqueous 4% solution of glutaraldehyde and post-fixed in 1% osmium tetroxide, before being dehydrated through a graded ethanol series and embedded in epoxy resin. The embedded samples were then cut into transverse, thin sections (1 μm) and stained with 1% toluidine blue. Following Evans et al. (1994), the areas of chloroplast surfaces facing intercellular space per unit leaf area (S<sub>c</sub>) were calculated from the microscopic photographs. A curvature factor (F) was determined assuming that the shape of the cells was a cylinder with hemispherical ends (Thain, 1983; Miyazawa and Terashima, 2001). The mean value

of F was 1.48 for the palisade and 1.39 for the spongy cells.

### **Leaf N, sugar and starch concentration**

Leaf N, soluble sugar and starch contents were determined for the leaves used for the gas exchange and chlorophyll fluorescence measurements. Dried leaf samples, kept at 70°C, were weighted for the calculation of leaf mass per area (LMA), then ground using a mortar. The leaf N content was determined by an NC analysis system composed of a nitrogen/carbon determination unit (Sumigraph, NC-800, Sumica Chem. Anal. Service, Osaka, Japan), a gas chromatograph (GC-8A, Shimadzu, Kyoto, Japan) and a data processor (Chromatopac, C-R6A, Shimadzu). Sugars were extracted with 80% ethanol and determined by the phenol-sulfuric acid method (Dubois et al. 1956). Starch in the residue was solubilized by potassium hydroxide and digested to glucose with amyloglucosidase (A9228, Sigma, St. Louis, MO) solution (Kabeya et al. 2003). The digested glucose was determined with Wako Autokit Glucose (439-90901, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

### **Statistical analysis**

Two-factorial ANOVA ( $N \times CO_2$ ) was used to test the differences in the treatment means of net  $CO_2$  assimilation rate ( $A$ ), stomatal conductance ( $g_s$ ), total rate of electron transport ( $J_T$ ), mitochondrial respiration rate in the dark ( $R_n$ ),  $C_i$ -based  $V_{c,max}$ ,  $C_c$ -based  $V_{c,max}$ ,  $C_i$ -based  $J_{max}$ ,  $C_c$ -based  $J_{max}$ , the ratio of  $C_c$ -based  $J_{max}$  to  $V_{c,max}$  ( $J_{max,Cc}:V_{c,max,Cc}$ ), area-based leaf N, leaf starch and sugar content,  $g_m$  estimated at a  $C_a$  of  $360 \mu\text{mol mol}^{-1}$  ( $g_{m,360}$ ),  $S_c$ , leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC) by the `aov` function in R (R Development Core Team, 2014). Furthermore, as the interaction term between N and  $CO_2$  was significant for  $J_{max,Cc}:V_{c,max,Cc}$ , leaf starch content and  $g_{m,360}$ , significant differences in their means among the combinations of  $N \times CO_2$  were re-tested using one-factorial ANOVA and the Holm pairwise comparisons. One-factorial analysis of covariance (ANCOVA) was used to test the effect of N supply on  $C_i$  and  $C_c$ -based  $V_{c,max}$  taking area-based leaf N ( $N_{area}$ ) as a covariate (`glm` function in R; R Development Core Team 2014).

## **Results**

Photosynthetic traits, including the net CO<sub>2</sub> assimilation rate (A), stomatal conductance (g<sub>s</sub>), total rate of electron transport (J<sub>T</sub>), dark respiration rate (R<sub>n</sub>), C<sub>i</sub>- and C<sub>c</sub>-based maximum carboxylation capacity of Rubisco (V<sub>c,max\_Ci</sub> and V<sub>c,max\_Cc</sub>, respectively), C<sub>i</sub>- and C<sub>c</sub>-based maximum rate of electron transport (J<sub>max\_Ci</sub> and J<sub>max\_Cc</sub>, respectively), and the ratio of C<sub>c</sub>-based J<sub>max</sub> to V<sub>c,max</sub> (J<sub>max\_Cc</sub>:V<sub>c,max\_Cc</sub>), in the seedlings of *B. platyphylla* grown under ambient and elevated [CO<sub>2</sub>], with adequate and limited nitrogen supply are shown in Table 1. Both elevated [CO<sub>2</sub>] and limited N supply significantly decreased A and J<sub>T</sub> measured at the same C<sub>a</sub> (360 μmol mol<sup>-1</sup>), whereas g<sub>s</sub> was only affected by the limited N supply. The limited N supply also decreased R<sub>n</sub>, but no significant effect of elevated [CO<sub>2</sub>] was observed. The C<sub>c</sub>-based V<sub>c,max</sub> was generally higher than the C<sub>i</sub>-based V<sub>c,max</sub>. Both C<sub>i</sub>- and C<sub>c</sub>-based V<sub>c,max</sub> were significantly decreased by elevated [CO<sub>2</sub>] and limited N supply. The C<sub>i</sub>- and C<sub>c</sub>-based J<sub>max</sub> were almost identical and decreased by elevated [CO<sub>2</sub>] and limited N supply as well as A, J<sub>T</sub> and V<sub>c,max</sub>. A significant (p < 0.05) effect of the interaction between CO<sub>2</sub> and N supply on J<sub>max\_Cc</sub>:V<sub>c,max\_Cc</sub> was observed (Table 1). Significant differences among the combinations were re-tested by one-way ANOVA and the Holm pairwise comparisons. The ranking of J<sub>max\_Cc</sub>:V<sub>c,max\_Cc</sub> was Elevated – N:1.41<sup>a</sup> > Elevated + N:1.12<sup>b</sup> > Ambient – N:0.98<sup>b</sup> > Ambient + N:0.94<sup>b</sup> (different letters indicate significant differences at p < 0.05). The significantly higher J<sub>max\_Cc</sub>:V<sub>c,max\_Cc</sub> observed in the plants grown at elevated [CO<sub>2</sub>] under limited N supply was attributed to the severer decrease in V<sub>c,max\_Cc</sub> than that in J<sub>max\_Cc</sub> (by 70% and 50% relative to the plants grown at ambient [CO<sub>2</sub>] under adequate N supply, respectively).

We estimated g<sub>m</sub> using two different methods: the variable J method (Harley et al. 1992, Valentini et al. 1995, Long and Bernacchi 2003) and the curve-fitting method (Ethier and Livingston 2004). As shown in Figure 1, a positive and significant correlation (r<sup>2</sup> = 0.49, p < 0.001) was observed in g<sub>m</sub> estimated using the two different methods, which can support the validity of the variable J method used in the present study.

Based on the response of g<sub>m</sub> to sub-stomatal CO<sub>2</sub> concentration (C<sub>i</sub>), g<sub>m</sub> showed generally decreasing trends with higher C<sub>i</sub> above 470 and below 100 μmol mol<sup>-1</sup> in all treatment combinations (Fig. 2). Because the sensitivity to errors in estimating g<sub>m</sub> was relatively low between 100 and 300 μmol mol<sup>-1</sup> C<sub>i</sub> (Harley et al. 1992), we used g<sub>m</sub> estimated at ambient CO<sub>2</sub> (C<sub>a</sub>) of 360 μmol mol<sup>-1</sup>, corresponding to C<sub>i</sub> 267 ± 5 μmol mol<sup>-1</sup> (mean ± SE), as a representative for each leaf. As a significant (p < 0.05) effect of the interaction of CO<sub>2</sub> and N supply on g<sub>m</sub> at 360 μmol mol<sup>-1</sup> C<sub>a</sub> (g<sub>m,360</sub>) was observed (Table 2), significant differences among the combinations were re-tested by one-way ANOVA and the Holm pairwise comparisons. The ranking of g<sub>m,360</sub> was

Ambient + N:  $0.16^a \pm 0.01$  > Elevated + N:  $0.13^a \pm 0.008$  > Ambient - N:  $0.12^a \pm 0.01$  > Elevated - N:  $0.06^b \pm 0.003$  mol m<sup>-2</sup> s<sup>-1</sup> (means  $\pm$  SE, different letters indicating statistically significant differences at  $p < 0.05$ , Fig. 3). The leaf starch content also exhibited the significant effect of the interaction of CO<sub>2</sub> and N supply (Table 2). Based on the Holm pairwise comparisons, the ranking of starch was Elevated - N:  $4.38^a \pm 0.80$  > Ambient - N:  $0.61^b \pm 0.12$  > Elevated + N:  $0.27^b \pm 0.07$  > Ambient + N:  $0.24^b \pm 0.09$  g m<sup>-2</sup> (Fig. 3A). The leaf sugar concentration significantly increased under limited N supply (Table 2, Fig. 3B). A stronger negative correlation was observed between leaf starch and  $g_{m_{360}}$  than between leaf sugar and  $g_{m_{360}}$  (Fig. 3A,B).

Based on the anatomical analysis, there were no effects of CO<sub>2</sub> and N on the cumulated chloroplast surface area that faces the intercellular spaces on a leaf area basis ( $S_c$ ) (Table 2), and a low correlation between  $S_c$  and  $g_{m_{360}}$  was observed (Fig. 4).

The area-based leaf N content ( $N_{area}$ ) decreased significantly both by elevated [CO<sub>2</sub>] and limited N supply (Fig. 5, Table 2). Concomitantly, C<sub>i</sub>- and C<sub>c</sub>-based  $V_{c,max}$  decreased both by elevated [CO<sub>2</sub>] and limited N (Figs. 5A and B, Table 1). One-factorial ANCOVA with  $N_{area}$  as a covariate provides that the slope of C<sub>i</sub>-based  $V_{c,max}$  to  $N_{area}$  in plants grown under adequate N supply at both ambient and elevated [CO<sub>2</sub>] was significantly lower than that under limited N supply ( $F_{1,15} = 4.6$ ,  $p = 0.049$ ) (Fig. 5A). This result also indicates a steeper decline in C<sub>i</sub>-based  $V_{c,max}$  with decreasing  $N_{area}$  caused by elevated [CO<sub>2</sub>] in plants grown under limited N supply. Conversely, the slope of C<sub>c</sub>-based  $V_{c,max}$  to  $N_{area}$  in plants grown under adequate N supply with different CO<sub>2</sub> concentrations was not significantly different from that in plants grown under limited N supply ( $F_{1,15} = 1.5$ ,  $p = 0.24$ ); the intercepts were not significantly different ( $F_{1,15} = 0.46$ ,  $p = 0.51$ ) (Fig. 5B).

To assess leaf structural change, we used corrected values of LMA, which is subtracting total mass of nonstructural carbon (NSC = soluble sugar + starch) from the measured LMA. Limited N significantly increased LMA-NSC, whereas elevated [CO<sub>2</sub>] had no significant effect on LMA-NSC (Table 2, Fig. 6). Conversely, no difference was observed in leaf thickness among treatment combinations (data not shown).

## Discussion

In the present study, elevated [CO<sub>2</sub>] and limited N supply were found to be capable of inducing leaf starch accumulation in the Japanese white birch (Fig. 3A), accompanied with photosynthetic down-regulation indicated by a decrease in  $V_{c,max}$  both on C<sub>i</sub> and C<sub>c</sub> basis (Table 1, Figs. 5) (Kitao et al.

2005, 2007). Conversely, photosynthetic down-regulation, i.e., a decrease in  $V_{c,max}$ , was also observed in plants with adequate N supply under elevated  $[CO_2]$  (Table 1, Fig 5). Decreases in  $A$  and  $J_T$  measured at a  $C_a$  of  $360 \mu\text{mol mol}^{-1}$  by elevated  $[CO_2]$  also suggests photosynthetic down-regulation (Table 1, Ainsworth and Long 2005, Rogers et al. 2006). Limited N supply reduced  $R_n$ , whereas elevated  $[CO_2]$  had no significant effect on  $R_n$  in Japanese white birch, as has been reported (Gonzalez-Meler et al. 2004).  $J_{max}$  on the basis of both  $C_i$  and  $C_c$  was decreased by elevated  $[CO_2]$  and limited N supply (Table 1). Although both  $C_c$ -based  $V_{c,max}$  and  $C_c$ -based  $J_{max}$  decreased with elevated  $CO_2$  and limited N supply, the extent of decrease in  $V_{c,max}$  was greater than that in  $J_{max}$  (Table 1). Consequently, an increase in  $J_{max}:V_{c,max}$  was observed in plants grown under the combination of elevated  $[CO_2]$  and limited N supply on a  $C_c$  basis, even after considering mesophyll conductance (von Caemmerer 2000, Leakey et al. 2009). This suggests that the limited N supply had a severe effect on  $V_{c,max}$  relative to  $J_{max}$  in plants grown under elevated  $[CO_2]$  (Ainsworth and Long 2005, Rogers et al. 2006, Leakey et al. 2009).

$g_m$  is considered to respond in terms of matching the availability of  $CO_2$  and photosynthetic capacity (Long and Bernacchi 2003), where  $g_m$  declined at limiting PFD or high  $C_i$  (Düring 2003, Flexas et al. 2007, Vrábl et al. 2009). In the present study, we also observed declines in  $g_m$  at lower and higher  $C_i$  than ambient levels in all treatment combinations (Fig. 2). Although responses of  $g_m$  to  $C_i$  are reportedly species-dependent such that some species have constant  $g_m$  at a certain range of  $C_i$  (von Caemmerer and Evans 1991, Loreto et al. 1992, Tazoe et al. 2009), Japanese white birch is suggested to be a species changing  $g_m$  to match the availability of  $CO_2$  (cf. Düring, 2003, Flexas et al. 2007, Vrábl et al. 2009). Recently, Tholen et al. (2012) reported that an increase in photorespiration would result in an apparent decrease in  $g_m$  at  $C_i$  below ambient levels even when no intrinsic change in diffusion properties of the mesophyll occurs. Furthermore, a possibility has also been proposed that the  $C_i$ -dependent  $g_m$  response is a methodological artifact at both low and high  $C_i$  regions (Gu and Sun 2014). However, as we compared  $g_m$  at the same  $C_i$  and the same irradiance,  $g_{m,360}$  used in the present study estimated by the variable  $J$  method could reflect the difference in  $g_m$  among the treatment combinations, which is also validated by the correlation with the curve-fitting method (Fig. 1, Ethier and Livingston 2004).

$g_{m,360}$  is unaffected in plants grown under elevated  $CO_2$  with adequate N supply (Fig. 3, Table 2), as has been reported in a N-fixing plant, soybean at FACE experiments (Bernacchi et al. 2005). However, seedlings grown at elevated  $[CO_2]$  under limited N supply had significantly lower  $g_{m,360}$  accompanied with higher leaf starch content among the treatment combinations (Fig. 3).

Although  $g_m$  is generally coordinated with  $g_s$  across diverse species (Flexas et al. 2013), a significant decline in  $g_m$  was observed in plants grown under elevated  $[\text{CO}_2]$  with limited N supply (Fig. 3) despite of no significant effect of elevated  $[\text{CO}_2]$  on  $g_s$  (Table 1). This suggests some factor existing to interfere the coordination between  $g_m$  and  $g_s$  in these plants (see discussion below). The lower  $g_{m_{360}}$  might lead to an apparently greater decrease in the  $C_i$ -based  $V_{c,\text{max}}$  relative to the  $C_c$ -based  $V_{c,\text{max}}$  in the seedlings grown under elevated  $[\text{CO}_2]$  with limited N supply (Fig. 5).

As the cumulated chloroplast surface area that faces the intercellular spaces on a leaf area basis ( $S_c$ ) represents the area for  $\text{CO}_2$  dissolution, an increase in  $S_c$  may contribute to increase  $g_m$  (Oguchi et al. 2003, Terashima et al. 2006). In the present study, there were no significant effects of both  $\text{CO}_2$  and N on  $S_c$ , suggesting that leaf anatomical traits related to  $g_m$  might not be affected by elevated  $[\text{CO}_2]$ .

Thicker cell walls accompanied with higher cellulose contents have been reported in *Arabidopsis thaliana* grown under elevated  $\text{CO}_2$  (Teng et al. 2006). In the present study, thicker cell walls, indicated by the higher LMA-NSC (Osborne and Beerling 2003), might be a cause of the decreased  $g_m$  in plants grown under elevated  $[\text{CO}_2]$  and limited N supply (Terashima et al. 2006). However, because plants grown under ambient  $[\text{CO}_2]$  and limited N, which also had higher LMA-NSC, showed no significant difference in  $g_m$  from Ambient + N and Elevated + N plants, there is a high probability that starch accumulation in Elevated – N plants hindered  $\text{CO}_2$  diffusion in chloroplasts, leading to the decrease in  $g_m$  (Nakano et al. 2000, Sawada et al. 2001).

Sawada et al. (2000) suggested that the suppression of photosynthesis in single-rooted soybean leaves during the acclimation to elevated  $\text{CO}_2$  was largely caused by the hindrance of  $\text{CO}_2$  diffusion due to starch accumulation in the chloroplast. In the present study, however,  $C_c$ -based  $V_{c,\text{max}}$ , which represents photosynthetic activity taking differences in  $g_m$  into account, still decreased under elevated  $[\text{CO}_2]$  accompanied with reduced  $N_{\text{area}}$ , maybe due to N dilution by the enhanced growth as a whole plant (Kitao et al. 2005, 2007, Seneweera et al. 2011).

Consequently, leaf starch accumulation and/or thicker cell wall at elevated  $[\text{CO}_2]$  under limited N supply declined  $g_m$ , which could be an additional cause of photosynthetic down-regulation as well as a reduction in Rubisco activity due to the reduced area-based leaf N content.

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Table 1. Photosynthetic traits, including the net CO<sub>2</sub> assimilation rate (A), stomatal conductance (g<sub>s</sub>), total rate of electron transport (J<sub>T</sub>), mitochondrial respiration rate in the dark (R<sub>n</sub>), C<sub>i</sub>-based maximum carboxylation capacity of Rubisco (V<sub>c,max\_Ci</sub>), C<sub>c</sub>-based maximum carboxylation capacity of Rubisco (V<sub>c,max\_Cc</sub>), C<sub>i</sub>-based maximum rate of electron transport (J<sub>max\_Ci</sub>), C<sub>c</sub>-based maximum rate of electron transport (J<sub>max\_Cc</sub>), and ratio of C<sub>c</sub>-based J<sub>max</sub> to V<sub>c,max</sub> (J<sub>max\_Cc</sub>:V<sub>c,max\_Cc</sub>), in the seedlings of Japanese white birch grown under ambient and elevated CO<sub>2</sub> with adequate and limited N supply. A, g<sub>s</sub>, and J<sub>T</sub> were measured at saturating light intensity (1200 μmol m<sup>-2</sup> s<sup>-1</sup>), a leaf temperature of 30°C and 360 μmol mol<sup>-1</sup> CO<sub>2</sub>. R<sub>n</sub> was measured in the darkness at a leaf temperature of 30°C and 360 μmol mol<sup>-1</sup> CO<sub>2</sub>. Values are mean ± SE (n = 4–6). The summary of the two-way ANOVA to test the effects of the CO<sub>2</sub> treatment (F<sub>1,15</sub>), N supply (F<sub>1,15</sub>), and CO<sub>2</sub> treatment × N supply (F<sub>1,15</sub>) is shown. \* p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001.

	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		F statistics		
	+N	-N	+N	-N	CO <sub>2</sub>	N	CO <sub>2</sub> × N
A (μmol m <sup>-2</sup> s <sup>-1</sup> )	13.7 ± 0.4	9.4 ± 0.3	11.4 ± 0.2	5.7 ± 0.4	69 ***	154 ***	2.8
g <sub>s</sub> (mol m <sup>-2</sup> s <sup>-1</sup> )	0.30 ± 0.02	0.24 ± 0.02	0.30 ± 0.02	0.22 ± 0.04	0.18	4.7 *	0.03
J <sub>T</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	126.9 ± 1.4	77.4 ± 3.2	98.4 ± 2.9	47.0 ± 3.2	92 ***	226 ***	0.08
R <sub>n</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	1.35 ± 0.04	0.97 ± 0.03	1.23 ± 0.10	0.86 ± 0.04	4.5	38 ***	0.01
V <sub>c,max_Ci</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	102.9 ± 2.3	65.1 ± 3.1	81.5 ± 4.0	36.3 ± 2.2	70 ***	157 ***	1.3
V <sub>c,max_Cc</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	142.5 ± 1.9	79.4 ± 4.1	104.5 ± 5.1	46.5 ± 3.3	77 ***	195 ***	0.35
J <sub>max_Ci</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	131.7 ± 3.9	77.9 ± 4.6	115.5 ± 2.0	63.6 ± 2.0	22 ***	199 ***	0.65
J <sub>max_Cc</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	133.8 ± 4.8	78.1 ± 4.7	116.6 ± 1.9	64.4 ± 2.0	20 ***	180 ***	0.19
J <sub>max_Cc</sub> :V <sub>c,max_Cc</sub>	0.94 ± 0.03	0.98 ± 0.02	1.12 ± 0.04	1.41 ± 0.07	39 ***	10 **	5.1 *

Table 2. Summary of the two-factorial ANOVA to test for the effects of growth [CO<sub>2</sub>] (F<sub>1,15</sub>), N supply (F<sub>1,15</sub>) and their interaction (F<sub>1,15</sub>) on area-based leaf N (N<sub>area</sub>), leaf starch and sugar content, g<sub>m</sub> estimated at a C<sub>a</sub> of 360 μmol mol<sup>-1</sup> (g<sub>m\_360</sub>), the areas of chloroplast surfaces facing intercellular space per unit leaf area (S<sub>c</sub>), and leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC). \* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.001.

	F-statistics		
	CO <sub>2</sub>	N	Interaction
N <sub>area</sub>	18 ***	120 ***	0.89
Leaf starch content	16 **	16 **	10 **
Leaf sugar content	0.17	23 ***	0.88
g <sub>m_360</sub>	30 ***	32 *	5.6 **
S <sub>c</sub>	1.7	2.7	1.0
LMA-NSC	2.1	10 *	1.9

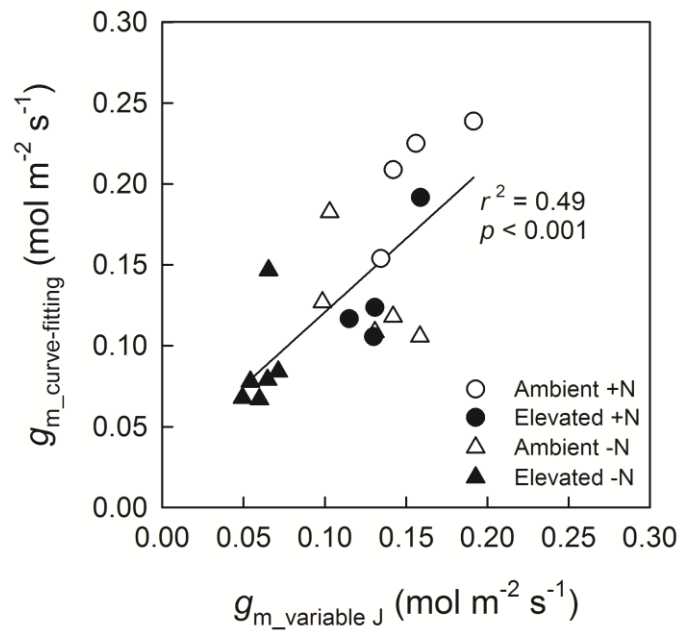


Figure 1. The relationship between mesophyll conductance ( $g_m$ ) estimated by the variable J method and the curve-fitting method in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO<sub>2</sub>] with high (circles) and low (triangles) nitrogen supplies. The data were fitted by linear regression.



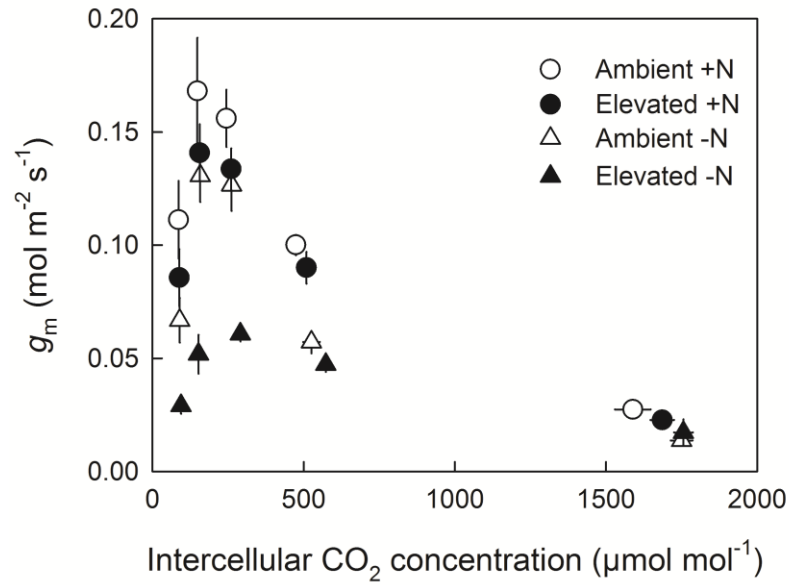


Figure 2. Mesophyll conductance ( $g_m$ ) as a function of intercellular CO<sub>2</sub> concentration ( $C_i$ ) in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO<sub>2</sub>] with high (circles) and low (triangles) nitrogen supplies. Values are mean ( $n = 4-6$ )  $\pm$  SE.

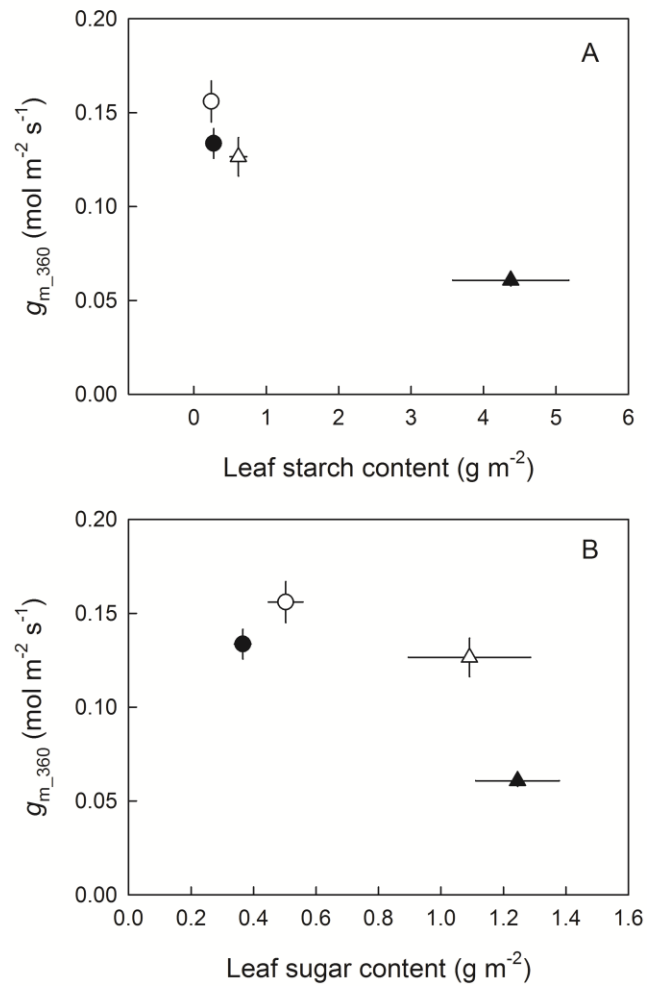


Figure 3. The relationship between mesophyll conductance ( $g_{m,360}$ ) at ambient  $\text{CO}_2$  of  $360 \mu\text{mol mol}^{-1}$  and leaf starch content (A) and leaf sugar concentration (B) in leaves of Japanese white birch grown under ambient (open) and elevated (closed)  $[\text{CO}_2]$  with high (circles) and low (triangles) nitrogen supplies. Values are mean ( $n = 4-6$ )  $\pm$  SE.

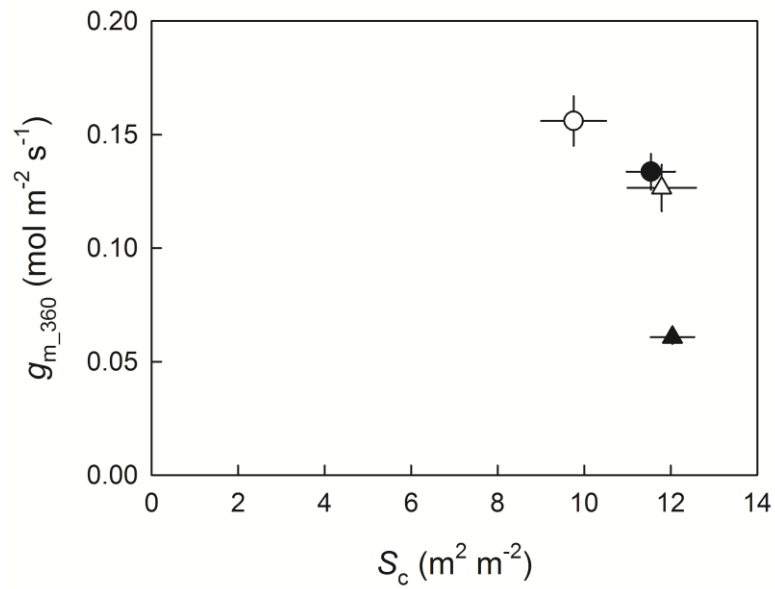


Figure 4. The relationship between mesophyll conductance ( $g_{m_{360}}$ ) at ambient  $CO_2$  of  $360 \mu mol mol^{-1}$  and the areas of chloroplast surfaces facing intercellular space per unit leaf area ( $S_c$ ) in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [ $CO_2$ ] with high (circles) and low (triangles) nitrogen supplies. Values are mean ( $n = 4-6$ )  $\pm$  SE.

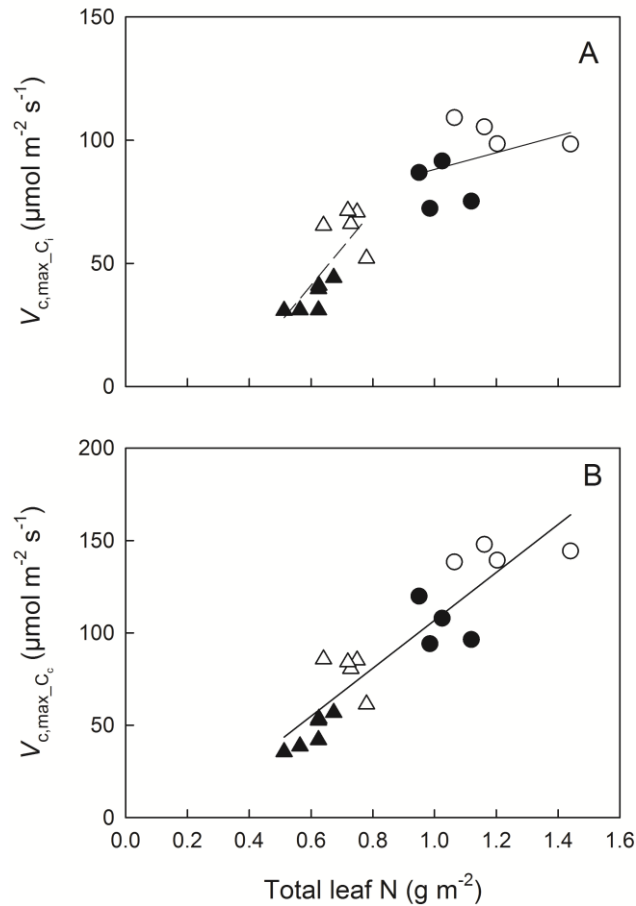


Figure 5.  $C_i$ - ( $V_{c,max,C_i}$ , A) and  $C_c$ -based maximum carboxylation capacity of Rubisco ( $V_{c,max,C_c}$ , B) as a function of total leaf nitrogen in leaves of Japanese white birch grown under ambient (open) and elevated (closed)  $[CO_2]$  with high (circles) and low (triangles) nitrogen supply. In the upper panel (A), the linear regressions for the adequate and limited N supplies are as follows:  $y = 33.9x + 54.3$  ( $r^2 = 0.16$ ,  $p = 0.33$ ) (solid line) and  $y = 153.4x - 51.5$  ( $r^2 = 0.57$  and  $p < 0.01$ ) (dashed line), respectively. A single regression line is presented in the lower panel (B) because the regressions generated for each N supply were not statistically different. The linear regression for the combined data is  $y = 129.8x - 23.0$  ( $r^2 = 0.81$  and  $p < 0.001$ ).

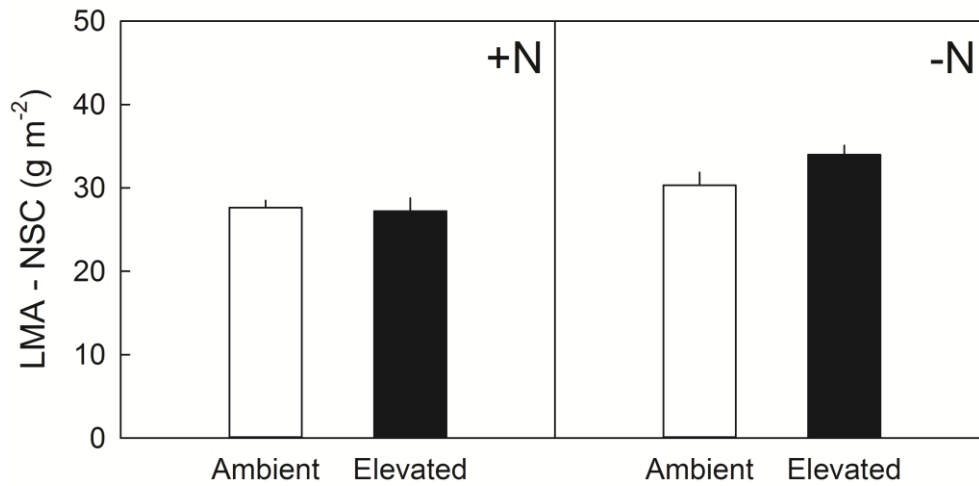


Figure 6. Leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC) in white birch seedlings grown under ambient (open) and elevated (closed bars) [CO<sub>2</sub>] with high (left) and low (right panel) nitrogen supplies. Values are mean (n = 4–6) + SE.