The photosynthetic response of four seral deciduous broad-leaved tree seedlings grown under elevated CO₂ concentrations

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Abstract
To test the relationship between successional traits and photosynthetic response to elevated CO₂, we evaluated the photosynthetic and biochemical traits in the leaves of four deciduous broad-leaved tree species with different successional traits grown under ambient (370 µmol mol⁻¹) and elevated (720 µmol mol⁻¹) CO₂ concentrations (CO₂). All four seedlings showed photosynthetic downward regulation, which is characterized by successional type-specific differences. In the mid-successional species, Magnolia hyporeuca Siebold et Zucc., both stomatal factors; stomatal conductance (gs) and stomatal limitation (ls) and non-stomatal factors; maximum carboxylation rate of RuBP (V max), leaf nitrogen content (N leaf), leaf mass per area (LMA) are affected, but in the mid-successional tree species, Quercus mongolica Fischer ex Ledeb var. crispula (Blume) Ohashi, and the two late successional tree species, Prunus ssiori F. Schmidt and Carpinus cordata Blume, non-stomatal factors are affected by the photosynthetic rates. The decrement of N leaf and increment of LMA imply the accumulation of photosynthates in the leaves and the dilution of nitrogen. The accumulated photosynthates and the dilution of nitrogen affected the photosynthetic response of the studied species to elevated CO₂. The results suggested that tree species with different successional traits show different stomatal and non-stomatal responses to elevated CO₂.

Key words: CO₂ enrichment, Photosynthesis, Stomatal conductance.

I. Introduction
Atmospheric CO₂ concentration increases year by year and in 2014 the annual atmospheric CO₂ concentration exceeded 400 µmol mol⁻¹ at Mauna Loa (Keeling et al. 2014). Global atmospheric model simulations estimated this incremental trend will continue (IPCC 2013). Thus, the evaluation of the responses of tree species to elevated CO₂ is an important subject for the future prediction of ecosystem response to elevated CO₂ and for sustainable forestry.

Initially, elevated CO₂ increases photosynthetic CO₂ assimilation rates. However, over time, photosynthetic traits decreased due to a biochemical and morphological acclimation and environmental constraints, such as water and nutrient supply (Leakey et al.2009; Watanabe et al. 2011; Warren et al. 2014). The photosynthetic decrement (photosynthetic down regulation) are commonly observed as a decrease in the maximum carboxylation rate of Rubisco (V max) and the electron transport rate, which lead to RuBP regeneration (J max) (Leakey et al. 2009). Those changes are associated with decreases in the nitrogen concentrations and the increase of leaf mass per area (LMA) due to the assimilation of photosynthetic products (Leakey et al. 2009).

Nitrogen has an essential role in plant carbon gain; most nitrogen in the plant body is allocated to the leaves, and more than 40-60% of leaf nitrogen is allocated to photosynthetic protein (Evans, 1989; Evans and Seemann, 1989). Nitrogen is often a limited resource in forest ecosystems (Poorter, 1998; Bauer et al., 2001). Plants are therefore expected to make an optimal nitrogen allocation into leaf photosynthetic proteins to achieve the maximum carbon gain in their own light environment (Evans, 1989; Evans and Seemann, 1989; Hikosaka and Terashima, 1995; Franklin and Whitelam, 2005). The photosynthetic nitrogen allocation patterns are strongly related to the leaf biochemical traits (Niinemets and Tenhunen, 1997); for example, nitrogen allocation to Rubisco is related to V max and nitrogen allocation to proteins for electron transport is related to J max. The photosynthetic nitrogen use traits of each species show a specific pattern that corresponds to the successional traits and surrounding environment of the species (Kitaoka and Koike, 2004). For example, Quercus mongolica Fisch. ex Ledeb. var. crisipula (Blume) Ohashi (Q. mongolica) and three other different successional broad-leaved tree seedlings, Magnolia hyporeuca Siebold et Zucc. (M. hyporeuca), Prunus ssiori F. Schmidt, and Carpinus cordata Blume, which co-existed with the understory on unmanaged larch (Larix kaemprifii (Lamb.) Carrière) plantation, showed unique nitrogen use traits and allocation patterns. M. hyporeuca and Q. mongolica would have higher nitrogen demand;
they slightly responded to nitrogen fertilization, and achieved higher photosynthetic rates with developing higher leaf nitrogen contents leaves than P. ssiori and C. cordata (Kitaoka et al. 2009a). During the leafless period of the larch canopy, regenerated C. cordata seedlings allocated more nitrogen to Rubisco for maximizing photosynthetic rates under high light intensity. However, nitrogen in leaves is preferentially allocated to light-harvesting proteins during the leafy period of larch under low light condition (Kitaoka and Koike, 2004).

There is a correlation between the seedling response to a high CO₂ environment and the nitrogen use traits of tree species (Dyckmans and Flessa, 2005; Onoda et al., 2005; Kita et al., 2007). In oak species, the amount of available soil nutrients would strongly affect the growth and physiological responses to elevated CO₂ (Tognetti and Johnson, 1999a, 1999b).

Elevated CO₂ not only affects the leaf nitrogen content and biochemical traits but also the possibility of changes to the nitrogen allocation pattern to each photosynthetic protein (Nakano et al., 1997; Hikosaka and Hirose, 1998; Onoda et al., 2005). We hypothesized that the elevated CO₂ would change the nitrogen allocation patterns more significantly in mid- and mid-late- successional species (M. hyporeuca and Q. mongolica) than in late-successional species (P. ssiori and C. cordata), as M. hyporeuca and Q. mongolica, which have higher growth rates, demand much nitrogen than P. ssiori and C. cordata (Kitaoka et al., 2009a). To test this hypothesis, we evaluated the photosynthetic and biochemical traits in the leaves of four deciduous broad-leaved tree species with different successional traits, M. hyporeuca, Q. mongolica, P. ssiori and C. cordata grown under elevated and ambient CO₂. Based on the results, we discuss the successional type-specific pattern of photosynthetic response to elevated CO₂.

### 2. Materials and methods

#### 2.1 Plant materials

Four deciduous broad-leaved tree species were studied: mid-successional species, M. hyporeuca; mid-late species, Q. mongolica; and two late-successional species, P. ssiori and C. cordata.

Before bud break in mid-April 2001, 3-4 year-old seedlings of these species, 36 individuals of each species, were collected from the Tomakomai Experimental Forest in Hokkaido University, Japan (42.6763°N 141.5996°E 58 m above sea-level). The collected seedlings were transplanted into free-draining plastic pots (diameter 26 cm, depth 24 cm and volume of approximately 7 L) filled with clay loam soil mixed with Kanuma pumice soil (1:1 in volume) on April 24th 2001. The height of the seedlings was approximately 15 to 20 cm. Each pot was fertilized with 250 ml of a commercial balanced-nutrient solution (Hypoxon 5-10-5, Hypoxen Japan, Osaka, Japan) diluted to 1:1000 strength (N: 50 mg, P: 32 mg and K: 50 mg per pot). In early May, two weeks after transplanting, we selected four seedlings of each species and moved them to phytotron chambers (Koito Industries, Yokohama, Japan). The CO₂ was maintained at 370 μmol mol⁻¹ (ambient) and 720 μmol mol⁻¹ (elevated CO₂) with a CO₂ analyzer (FD-DF23, Fuji, Tokyo, Japan) as described previously (Yazaki et al., 2004; Kito et al., 2007). There were three chamber replicates for each CO₂ level; a total of six chambers were used for this experiment.

#### 2.2 Measurement of gas exchange rates and leaf traits

In early July, after 60 days growth under each CO₂ condition, we measured the leaf gas exchange traits of fully expanded and mature leaves (Koike, 1995; Kitaoka and Koike, 2005). The day before the measurement, 500 ml of tap water was supplied to prevent water stress, especially to circumvent stomatal patchiness (Terashima 1992). The net photosynthetic rate-CO₂ response curves (A–C) were measured using a portable gas analyzer (LI-6400, LiCor, Nebraska USA) with a CO₂ control module and a light-emitting diode (LED) light source (6400-02B, LiCor, Nebraska USA). During the measurement, leaf temperatures were maintained at 25°C and the photosynthetic photon flux density was maintained at 800 μmol m⁻² s⁻¹, which has been previously determined as the optimal intensity for these species (Koike, 1986; Kitaoka and Koike, 2004). The external CO₂ partial pressures (Cᵀ) were supplied in 12 steps. Firstly, we measured the stable photosynthetic rates at growth CO₂ concentration (i.e., 370 and 720 μmol mol⁻¹ for ambient and elevated CO₂ treatment, respectively), then we gradually decreased Cᵀ and measured net photosynthetic rate until Cᵀ of 50 μmol mol⁻¹. After that, Cᵀ was set at the growth CO₂ concentration. After we confirmed the net photosynthetic rates at growth CO₂ as original values, we gradually increased Cᵀ and measured the net photosynthetic rates until a Cᵀ of 1200 μmol mol⁻¹. For each external CO₂ concentration, we checked the stability and matched IRGAs, then logged the four measurements. We analyzed leaf biochemical parameters Vₑₘₐₓ (maximum carboxylation rate of RuBP) and Jₑₘₐₓ (maximum electron transport rates) from the A–C curves according to a specific model (Farquhar et al., 1980; Long and Bernacchi, 2003; Onoda et al., 2005). The relative stomatal limitation to photosynthesis (lₛ), an estimate of the proportion of the reduction in photosynthesis attributable to CO₂ diffusion between the atmosphere and the site of carboxylation, was calculated at 370 and 720 μmol mol⁻¹ of Cᵀ from A–C curves following the methods used in previous studies (Farquhar and Sharkey, 1982; Tissue et al., 2005).

After these measurements, we sampled the leaves and stamped out two disks (each 1 cm²) for determining the leaf mass per area (LMA), nitrogen content and leaf chlorophyll (Chl) content. The disks used to determine the LMA and leaf nitrogen content were dried at 60°C for 48 h. We then measured the dry weight to determine the LMA. The leaf nitrogen content was determined using an NC analyzer (NC-900, Shimadzu, Kyoto, Japan). The samples were again dried at 60°C for several hours before being placed in an analyzer. The nitrogen content was calibrated and checked against a known standard (acetamidile; N = 10.36%, C = 71.09%; Wako Chemical Co. Ltd., Osaka, Japan). The measurements of leaf Chl content followed the DMSO (dimethyl sulfoxide (CH₃)₂SO) method (Barnes et al., 1992; Shinano et al., 1996). The Chl absorption in the extract was determined using a double-beam spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) with a bandwidth resolution of 1 nm. We calculated the ratios of Vₑₘₐₓ, Jₑₘₐₓ and Chl to N content (Vₑₘₐₓ/N, Jₑₘₐₓ/N, and Chl/N, respectively), which are indices of nitrogen allocation to Rubisco, electron transport and light harvesting complex (LHC) protein (Warren and Adams, 2004; Watanabe et al., 2011).
2.3 Statistical analysis

All of the statistical analyses were performed using R ver. 2.8.1 software (The R Project in Statistical Computing, http://www.r-project.org/index.html). We analyzed the effects of the CO2 growth using a linear mixed model (Crawley, 2005), we defined CO2 growth and species as fixed factors, and the growth chamber.
as a random factor. When significant effects of CO2 were detected, contrast analyses were used for comparison of the effects of CO2 growth among the species.

3. Results

3.1 Photosynthetic traits (Photosynthetic rates, gs, Vcmax, Jmax, Is), leaf mass per area (LMA) and leaf nitrogen content (Narea)

The light-saturated photosynthetic rates at 370 μmol mol⁻¹ (P370) and 720 μmol mol⁻¹ (P720) CO2 showed similar patterns in all four studied tree species. Statistically significant decrements of P370 and P720 were observed in the elevated CO2 (Fig. 1a, Table 1).

In M. hyporeuca, the stomatal conductance (gs) at 370 μmol mol⁻¹ (gs370) and 720 μmol mol⁻¹ (gs720) was significantly decreased by the elevated CO2 (Table 1). However, in Q. mongolica, P. ssiorei and C. cordata, there were no significant differences of gs370 and gs720 between the ambient and elevated CO2 concentrations (Fig. 1b).

The both Is370 and Is720 showed specific response to elevated CO2 in M. hyporeuca, in which Is significantly increased in the presence of elevated CO2 (Fig. 1c). However, in Q. mongolica, P. ssiorei and C. cordata, there were no significant differences of Is between the ambient and elevated CO2 concentrations (Fig. 1c).

The leaf biochemical characters: Vcmax and Jmax showed different responses to elevated CO2. In all species, Vcmax at the ambient CO2 was higher than that at the elevated CO2, while no significant effects of elevated CO2 on Jmax were observed (Fig. 1g, h, Table 1).

Elevated CO2 also significantly affected the area-based leaf nitrogen content (Narea) in all studied species (Table 1, Fig. 1i); Narea was lower in the leaves with elevated CO2 than for those with ambient CO2 levels. In all four studied tree species, there were no marked differences in Chl content (Fig. 1j). The two late-successional tree species (P. ssiorei and C. cordata) had lower LMA leaves than the other two tree species, M. hyporeuca and the mid to late-successional tree species, Q. mongolica (Fig. 1k). All species had significantly higher LMA at elevated CO2 than at ambient CO2 (P < 0.01 in M. hyporeuca and Q. mongolica, P < 0.05 in P. ssiorei and C. cordata).

3.2 Nitrogen use traits of leaf photosynthesis: Vcmax/N ratio, Jmax/N ratio and Chl/N ratio

In M. hyporeuca and Q. mongolica, Vcmax/N significantly decreased at elevated CO2. However, no significant difference was observed in the other two late-successional species, P. ssiorei and C. cordata. On the other hand, no significant effects of CO2 on Jmax/N were detected (Table 1). Chl/N ranged from 0.2 to 0.5 in all of the studied tree species. Chl/N was higher at elevated CO2 treatments in the late-successional tree species, P. ssiorei and C. cordata.

4. Discussion

Photosynthesis is regulated by both stomatal (gs, Is) and biochemical (Vcmax, Jmax) factors (Lenz et al. 2010). Previous studies suggested that the stomatal response is one of the most relevant factors in response to elevated CO2 (Rey and Jarvis, 1998;

Table 1. Results of statistical analysis of data related to leaf gas exchanges, biochemical and functional traits of four seral deciduous broad-leaved tree species. Significance of main effects and interaction effects are shown as symbols (* p<0.05, ** p<0.01), or as non-significant (n.s.).
Körner et al., 2000; Urban, 2003; Keel et al., 2007). In this study, significant changes of stomatal factors were observed only in *M. hyporeuca* between CO₂ treatments (Fig. 1b). The gs decrement and the increment of *ls* suggest that CO₂ diffusion was hindered by stomatal closure under elevated CO₂. Furthermore, when compared at the growth CO₂ concentrations (i.e. gs370 vs gs210, ls370 vs ls210), significantly greater stomatal limitation was observed under elevated CO₂ in *M. hyporeuca* and *Q. mongolica* (Fig. 1f). Therefore, stomatal factors are one of the drivers of the photosynthetic acclimation in the mid- and mid-late successional species, *M. hyporeuca* and *Q. mongolica*.

As for biochemical factors, all four studied tree species (*M. hyporeuca*, *Q. mongolica*, *P. ssiori*, *C. cordata*) showed a decrement of *V_{cmax}* at elevated CO₂, suggesting that photosynthetic down-regulation occurred in all species (Tissue et al., 1993, 2001; Eguchi et al., 2004; Kitao et al., 2007). A reason for this decrement would be a dilution of the leaf nitrogen indicated by the decrease in *N*_{area} under elevated CO₂ (Fig. 1i, Tabub and Wang, 2008).

As leaf Rubisco contents are strongly connect with the light-saturated photosynthetic rates and *V_{cmax}* (Evans, 1989; Makino et al., 1994; Hikosaka and Terashima, 1995; Poorter and Evans, 1998), *V_{cmax}/N* represents the leaf nitrogen allocation to Rubisco.

In this study, *V_{cmax}* and *N*_{area} of all of the studied tree species significantly decreased at elevated CO₂ (Fig. 1c, f). Conversely, *V_{cmax}/N*, the measure of nitrogen allocation into Rubisco (Warren and Adams, 2004; Watanabe et al., 2011) in *M. hyporeuca* and *Q. mongolica* significantly decreased, but *P. ssiori* and *C. cordata* showed no marked decrease of *V_{cmax}/N* (Fig. 2a). The decrements in *V_{cmax}/N* in *M. hyporeuca* and *Q. mongolica* at elevated CO₂ might exacerbate photosynthetic down-regulation because of the reduction of nitrogen allocation to Rubisco in addition to the decreases in *N*_{area} per se (Tissue et al., 1993; Nakano et al., 1997).

The observed increment of LMA was a typical acclimation response to elevated CO₂, which are partly explained by the accumulation of non-structural carbohydrate including sucrose and starch in leaves (Rey and Jarvis, 1998; Urban, 2003). In general, elevated CO₂ initially increases the photosynthetic carbon fixation, however, rapidly enhanced growth induces a dilution of nitrogen in plant body (Taub and Wang 2008). The propensity of excess carbohydrates in leaves, particularly observed in *M. hyporeuca* indicated by greater extent of increase in LMA (41% other three species ranged from 32 to 36%), suggests a sink limitation under elevated CO₂ (Paul and Driscoll, 1997; Leakey et al., 2009).

As mid- and mid-late successional species, *M. hyporeuca* and *Q. mongolica* had higher nitrogen demand with higher growth rates than late successional species, *P. ssiori* and *C. cordata* than (Kitaoka et al., 2009a, b), the former two species might cause more severe nitrogen deficiency at the whole plant level accomplished with an enhanced growth under elevated CO₂ (Taub and Wang 2008). Actually, the decreases in *N*_{area} by elevated CO₂ were greater in *M. hyporeuca* and *Q. mongolica* (~31% and ~34%) than in *P. ssiori* and *C. cordata* (~26 and ~25%, respectively, Fig. 1j). This might, in turn, cause a sink limitation owing to growth retardation, which consequently decreased nitrogen allocation into Rubisco via feedback regulation particularly in the mid-and mid-late successional species (Field et al., 1992; Bazzaz and Miao, 1993). In addition, the two late-successional species may intensively allocate photosynthates into storage organs, such as roots and stems because of their determinate growth traits (Chapin et al., 1990; Field et al., 1992; Bazzaz and Miao, 1993; Bazzaz 1996), which may lead to less down-regulation in photosynthesis. Thus, the resource use traits for each species would affect the acclimation responses to elevated CO₂ (Field et al., 1992; Bazzaz and Miao 1993).

In conclusion, all four different successional deciduous broad-leaved tree seedlings showed photosynthetic downward regulation. In the mid- and mid-late successional tree species, *M. hyporeuca*...
and Q. mongolica, both stomatal (gs and ls) and non-stomatal factors ($V_{\text{max}}$, $N_e$, and LMA) were involved in the photosynthetic down-regulation induced by only non-stomatal factors were involved. Differences in resource use traits among tree species would influence the photosynthetic down-regulation, particularly related to the nitrogen allocation into Rubisco.

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