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Effects of elevated atmospheric carbon dioxide, soil nutrients and water conditions on photosynthetic and growth responses of *Alnus hirsuta*

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Abstract. The objective of this paper is to clarify the effects of multiple environmental conditions, elevated atmospheric CO_2 concentration ($[CO_2]$) and soil conditions on the physiological and morphological properties of *Alnus hirsuta* Turcz., an N_2-fixing species, to predict its responses to environmental changes. We examined the responses of photosynthetic properties, leaf characteristics, biomass and N allocation of *A. hirsuta* to elevated $[CO_2]$, soil N and phosphorus availability, and soil drought by using the results of two experiments. The effects of P availability were more marked than those of N availability and soil drought. The photosynthetic responses of *A. hirsuta* to elevated $[CO_2]$ under high P were considered to be 'photosynthetic acclimation', while *A. hirsuta* presented the obvious 'photosynthetic downregulation' to elevated $[CO_2]$ under low P. Soil P availability affected the growth responses to elevated $[CO_2]$ through effects on these photosynthetic properties and biomass allocation. Though elevated $[CO_2]$ caused no marked change in the allometric relationships in biomass, with some exceptions, the responses of N allocation among tissue to elevated $[CO_2]$ differed from those of biomass allocation. These results suggest that it is necessary to evaluate N mass allocation as well as biomass when we consider the N_2-fixing ability of *Alnus* under elevated $[CO_2]$.

Additional keywords: actinorhizal plants, *Frankia*, J_{max}, top to root ratio, total non-structural carbohydrate, V_{cmax}.

Introduction

Increasing atmospheric CO_2 concentration ($[CO_2]$) is an important factor in global climate change (Houghton *et al*. 2001; IPCC 2007). The role of nutrient availability is a key unresolved issue regarding plant responses to rising $[CO_2]$ (McCarthy *et al*. 2010). Because forest ecosystems are usually nitrogen-limited (Vitousek and Howarth 1991), soil N availability might become progressively deficient and limit growth under elevated $[CO_2]$. This is known as the progressive N limitation hypothesis (Luo *et al*. 2004). It is suggested that added N input from outside the forest ecosystems is needed to maintain enhanced growth in an increased $[CO_2]$ environment (Reich *et al*. 2006). For this reason, symbiotic N_2 fixation becomes an important aspect for N input in a future elevated $[CO_2]$ environment (Houghton *et al*. 2001).

In temperate to boreal zones, actinorhizal plants, including *Alnus* species, can contribute significant amounts of fixed N to forest ecosystems (Dawson 2008). However, the N_2 fixation of *Alnus* species is also influenced by other environmental factors. Soil phosphorus availability is one of the constraining factors for nodulation and N_2 fixation because a large amount of P is used for symbiotic N_2 fixation (Uliassi and Ruess 2002; Gentili and Huss-Danell 2003; Edwards *et al*. 2006; Dawson 2008). Most studies focus on N (Reich *et al*. 2006), but P limitation is also common in many terrestrial ecosystems (Nord and Lynch 2009). In addition, it has been argued that the P demand of tree species increases in an increased $[CO_2]$ environment (Kogawara *et al*. 2006; Lewis *et al*. 2010).

Furthermore, water shortages with high $[CO_2]$ may occur in the future as the global climate changes (IPCC 2007). It is also predicted that increasing temperatures cause decreased rainfall during the summer season (Calfapietra *et al*. 2010). Drought may decrease photosynthetic rates and limit growth through stomatal closure (Flexas and Medrano 2002), which may also affect nitrogenase activity and N_2 fixation of nodules (Pawlowski and Newton 2008).

Long-term nutrient limitation may affect plant responses to elevated $[CO_2]$ through effects on physiology, morphology and

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ontogeny (Lewis *et al*. 2010). In the most efficient N allocation within leaves, according to Farquhar's photosynthetic biochemical model (Farquhar *et al*. 1980), a decreased maximum carboxylation rate (V_{cmax}) and an increased maximum electron transport rate (J_{max}) are expected in elevated [CO_2] conditions, such as double [CO_2] (Long *et al*. 2004). However, tree species seldom exhibit such identical photosynthetic responses to high [CO_2], and both V_{cmax} and J_{max} often decrease under elevated [CO_2], especially in nutrient-deficient conditions (Medlyn *et al*. 1999). These photosynthetic responses (photosynthetic downregulation) are usually caused by a change in sink–source balance and in accumulation of carbohydrates within leaves (Ainsworth and Rogers 2007). In our previous two experiments, *Alnus hirsuta* Turcz. grown under high [CO_2] with adequate P supply demonstrated decreased V_{cmax} (Tobita *et al*. 2010), but the photosynthetic rate measured at growth [CO_2], which defined as each ambient [CO_2], condition ($A_{sat\text{-}growth}$) was increased under high [CO_2] (Tobita *et al*. 2005, 2010). N_2-fixing species might show photosynthetic responses close to the most efficient response because of the non-N limitation for growth and the high sink of photosynthate for their symbiont, *Frankia*, if other stressful conditions, such as P limitation, are absent (Dawson 2008).

The growth responses of tree species to elevated [CO_2] are influenced by the responses of biomass allocation, which depend not only on the effects of tree size through increasing growth rate, but also on the effects of changing allometric relationships among tissues (Lewis *et al*. 2010). In our previous experiments, nodule biomass of *A. hirsuta* increased under high [CO_2] by the effects of tree size with no change in allometric relationships (Tobita *et al*. 2005, 2010). In previous studies for some species, such as *Pinus taeda* L., P limitation regulated growth responses to elevated [CO_2] primarily through effects on biomass allocation rather than through direct effects on photosynthetic capacity (Lewis and Strain 1996).

N concentration within each tissue often changes under elevated [CO_2]. In general, leaf N concentration decreases in elevated [CO_2] conditions (Ainsworth and Rogers 2007), but N concentration changes will not be the same among tissues within trees. Therefore, it was predicted that the N allocation pattern is different from the biomass allocation, depending on the growth environment. The impact of either elevated [CO_2] or P deficit in addition to drought stress on plant growth has been studied extensively, but their interactive effects on plant biomass and N allocation are yet inadequately understood (Xu *et al*. 2007).

In previous experiments, we examined the combined effects of elevated [CO_2] and soil N availability (Tobita *et al*. 2005), and the combined effects of elevated [CO_2], soil P deficiency and soil drought (Tobita *et al*. 2010) on the photosynthetic and growth responses of *A. hirsuta*. In this paper, we have two questions about the responses of *A. hirsuta* to elevated [CO_2] with other environmental constraints: (i) whether growth responses to elevated [CO_2] are affected by the combination of physiological (photosynthetic rate) and morphological (biomass allocation) properties, and (ii) whether the responses of N allocation to elevated [CO_2] differ from those of biomass allocation. To clarify these questions, we present new data on the photosynthetic properties, leaf characteristics and N allocation from our previous two experiments, and we analyse the extent of change in the physiological and morphological parameters of *A. hirsuta* to each growth condition by comparing the previous reported data on V_{cmax} (Tobita *et al*. 2010) and total biomass (Tobita *et al*. 2005, 2010).

Materials and methods

Plant material

Two experiments (Experiment I (Exp-I) and Experiment II (Exp-II)) were conducted in different years and detailed explanations of both experiments have been provided in the previous reports (Tobita *et al*. 2005, 2010). *Alnus hirsuta* Turcz. is a tall tree living at a relatively low elevation that shows successive leaf expansion patterns. One-year-old seedlings of *A. hirsuta* obtained from a commercial nursery (Oji Forestry and Landscaping, Sapporo, Japan) were transplanted individually into free-draining 5-L plastic pots (diameter: 21 cm) filled with 1 : 1 (v/v) Kanuma pumice and clay loam. Each pot was placed in a tray to prevent nutrient drainage. The initial height of the seedlings was 14–18 cm in both experiments. Nodule formation in the root systems was observed at the time of transplanting.

Elevated [CO_2], soil nitrogen, and phosphorus supply and soil drought treatments

In both experiments, seedlings were placed in a natural daylight phytotron (Koito Industries, Yokohama, Japan) equipped with [CO_2] controllers (DAIWA Air Co. Ltd, Sapporo, Japan) at Hokkaido Research Center, Forestry and Forest Products Research Institute, Sapporo, Japan (43°59′N, 141°23′E; 180 m above sea level). Seedlings were grown at 36 Pa [CO_2] (ambient [CO_2]) or 72 Pa (elevated [CO_2]). Air temperature was maintained at 26 : 16°C (day : night) from May to September 2001 for Exp-I and May to September in 2006 and 2007 for Exp-II. PPFD in the chamber was up to ~2200 µmol m^{-2} m^{-1} in maximum instantaneous values and ~1800 µmol m^{-2} m^{-1} in maximum 5-min average values measured using a LI-1400 sensor (Li-Cor Inc., Lincoln, NE, USA). Relative humidity in the chamber was not controlled, and ranged from ~60% in the daytime to more than 90% during the night-time, along with the change of air temperature within a day, and was measured with a remote data logger (3641; HIOKI E. E. Co., Nagano, Japan). The photoperiod was natural daylength, and changed from ~11 h to 15 h during May to October.

In Exp-I, each [CO_2] treatment was replicated in two chambers. Seedlings were supplied with N at 52.5 mg N per pot per week (high N; '+N') or 5.25 mg N per pot per week (low N: 'control') in modified 0.5× Hoagland solution (Asher and Edwards 1983) as described in Watanabe *et al*. (2008). Pots were set in trays with water in order to avoid desiccation.

In Exp-II, each [CO_2] treatment was replicated in three chambers. Twenty-four seedlings per species were grown in each chamber. Twelve seedlings were supplied with P at 7.74 mg P per pot per week (high P: 'control'), and the other 12 seedlings received 0.774 mg P per pot per week (low P: '–P') in modified 0.5× Hoagland solution as described in Tobita *et al*. (2010). In order to avoid an inhibition of nodule formation by N, only one-tenth of the N of the standard nutrient solution was

applied. This rate was the same as the low N treatment of Exp-I. Six seedlings in each P treatment received 250 mL water three times per week (well-watered conditions: 'control'); the other seedlings received no water in addition to the nutrient solution (soil drought conditions: '–water'). The water conditions in Exp-I were considered as being similar to the well-watered conditions in Exp-II, and the P supply level in Exp-I was the same as the high P level of Exp-II. Therefore, the control treatments of Exp-I ('control I') and Exp-II ('control II') were considered as having the same conditions (low N, high P and well-watered conditions). The seedlings were grown for two growing seasons in Exp-II. In accordance with the increased plant size, the amount of P and water supplied was increased 1.5 times in the second season.

Photosynthetic properties

In both experiments (Exp-I and Exp-II), leaf gas exchange measurements were taken on six attached mature leaves of each treatment by using an open gas exchange system (LI-6400; Li-Cor Inc.). The age of the leaves was ~30 days in Exp-I (Tobita *et al*. 2005) and 38–56 days in Exp-II (Tobita *et al*. 2010). The sixth to eighth leaves, counted from the tip of the leader shoots, were used. The full light-saturated net photosynthetic rates per leaf area (A_{sat}) versus internal [CO_2] (C_i) curves were determined. Saturating PPFD at the upper leaf surface was 1200 µmol m^{-2} s^{-1}, as determined based on light response curves of photosynthetic rates (data not shown). Each curve consisted of 7 steps (Exp-I) to 10 steps (Exp-II), starting from ambient [CO_2] (360 µmol CO_2 mol^{-1}) and decreasing to 200, 100 and 50 µmol CO_2 mol^{-1}, before increasing to 360, 500, 720, 1000, 1500 and 1800 µmol CO_2 mol^{-1} (except for 500 and 1000 µmol CO_2 mol^{-1} in Exp-I). Leaf temperature was maintained at 25°C and leaf to air vapour pressure saturation deficit (VPD) was kept at less than 1.2 kPa. The maximum rate of carboxylation (V_{cmax}) and the maximum electron transport rate (J_{max}) were estimated using the equations of the Farquhar-type model (Farquhar *et al*. 1980):

$$A_{sat} = \frac{V_{cmax} \times (C_c - \Gamma^*)}{\left\{C_c + K_c \times \left(1 + \frac{O_i}{K_o}\right)\right\}} - R_d. \quad (1)$$

$$A_{sat} = \frac{J \times (C_c - \Gamma^*)}{(4C_c + 8\Gamma^*)} - R_d \quad (2)$$

where C_c is the [CO_2] in the chloroplasts, Γ* is the CO_2 compensation point, K_c and K_o are the Michaelis–Menten constants for carboxylation and oxygenation, O_i is the intercellular O_2 concentration, and R_d is the rate of daytime respiration. In this study, we ignored possible CO_2 diffusion limitation within the leaf, and C_i was used as C_c. For the coefficients K_c, K_o and Γ*, we used 404.9 µmol mol^{-1}, 278.4 mmol mol^{-1} and 42.75 µmol mol^{-1}, respectively, at 25°C (Bernacchi *et al*. 2001). For leaves in which full $A : C_i$ curves were measured, V_{cmax} was calculated by fitting Eqn (1) to the initial slope of the $A : C_i$ curve ($C_i < 300$; Tobita *et al*. 2010) and J_{max} was calculated by fitting Eqn (2) to the near-plateau of the $A : C_i$ curve ($C_i > 600$). Stomata conductance at growth [CO_2] ($g_{sw\text{-}growth}$) was determined as the value measured at each [CO_2] condition (36 Pa or 72 Pa [CO_2]).

Carbohydrate analysis

We measured soluble sugar and starch contents in the leaves that were used for the gas exchange measurements in each experiment. Leaf samples were ground after drying at 80°C for 48 h. Leaf soluble sugars were extracted with 80% ethanol, and then determined by the phenol–sulfuric acid method (Kabeya *et al*. 2003). Leaf starch in the residue was solubilised by potassium hydroxide, and then digested to glucose with amyloglucosidase (A9228, Sigma, St Louis, MO, USA) solution. The digested glucose was determined with the Wako Auto Kit Glucose (439–90901, Wako Pure Chemical Industries Ltd, Osaka, Japan). The total nonstructural carbohydrate (TNC) was calculated as the sum of the soluble sugar and the starch.

Growth and biomass allocation

After 100 days (late August) in Exp-I (Tobita *et al*. 2005) and in August of the second growing season in Exp-II (Tobita *et al*. 2010), six seedlings of each treatment were harvested in each experiment. Dry masses of seedling leaves, shoots, stems, coarse roots (>2 mm), fine roots (<2 mm) and nodules were determined after drying at 80°C, and the total leaf area (LA) was measured. The top to root ratio (T : R ratio; aboveground dry mass per root dry mass) of each seedling was calculated.

N content and N allocation

The N content of each organ of the cultivated seedlings in each experiment was determined by combustion using an NC-analyser (Sumigraph NC-800; Sumika Chemical Analysis Service, Osaka, Japan, for Exp-I; Vario Max CN; Elementar Analysensysteme GmbH, Hanau, Germany, for Exp-II) after drying at 80°C for 48 h. The top to root ratio for N content (T : R (N) ratio; aboveground N mass per root N mass) of each seedling was calculated. Leaf mass per unit leaf area (LMA) and the N content of the leaves that were used for the gas exchange measurements in each experiment were also determined and the leaf N content per unit leaf area (N_{area}) was calculated.

Statistical analysis

Two-way ANOVA for split-plot designs was used to evaluate the effects of [CO_2] and N-treatment in Exp-I, and in Exp-II, three-way ANOVA for split-plot designs was used to evaluate the effects of [CO_2], P and soil water treatment on physiological properties (J_{max} and $g_{sw\text{-}growth}$), leaf characteristics (LMA, N_{area}, soluble sugar, starch and TNC), growth properties (T : R ratio), and N status (T : R (N) ratio and total N mass) at the probability level $P < 0.05$ using JMP software (SAS Institute 2003). The probability level $P < 0.15$ was considered to indicate a trend. Differences among treatments in the biomass and N content of each organ were analysed as dependent factors with an analysis of covariance, including treatments as independent factors as described in Tobita *et al*. (2010).

Results

Photosynthetic properties

J_{max} increased under high N compared with low N, irrespective of the [CO_2] treatment in Exp-I, and J_{max} decreased under low P compared with high P, irrespective of the [CO_2] treatment in

Exp-II (Fig. 1*a*). In general, the percent change of J_{max} compared to the control (the ambient $[CO_2]$ of the control in each experiment) was smaller than the percent change in V_{cmax} (Fig. 2). In addition, the extent of decline in J_{max} under elevated $[CO_2]$ from J_{max} under ambient $[CO_2]$ in each treatment was smaller than V_{cmax} (Fig. 2). Irrespective of $[CO_2]$ treatments, the variation of $g_{sw\text{-}growth}$ from the control was larger in soil drought conditions than that in well-watered conditions (Fig. 1*b*). In well-watered conditions, the variation of $g_{sw\text{-}growth}$ from the control was larger under elevated $[CO_2]$ than the variation under ambient $[CO_2]$. As a result, $g_{sw\text{-}growth}$ decreased under elevated $[CO_2]$ in well-watered conditions (20–35% lower compared to ambient $[CO_2]$), but showing little or no decrease under increased $[CO_2]$ in soil drought conditions (1–11% lower compared to ambient $[CO_2]$).

Leaf characteristics

LMA increased under elevated $[CO_2]$, especially in Exp-II (Fig. 3*a*; $P = 0.003$). N_{area} decreased in low P irrespective of $[CO_2]$ treatment in Exp-II ($P = 0.02$), and showed no significant decline under elevated $[CO_2]$ compared with ambient $[CO_2]$ (Fig. 3*b*). The percent change of N_{area} compared to the control (ambient $[CO_2]$ of the control in each experiment) was smaller than that of V_{cmax} (Fig. 4), and the extent of decline in N_{area} under elevated $[CO_2]$ compared to ambient $[CO_2]$ in each treatment was smaller than that of V_{cmax} (Fig. 4). TNC content in Exp-II showed interactive effects between $[CO_2]$ and P treatments (Fig. 3*c*; $P = 0.01$). Starch content in Exp-II also exhibited interactive effects between $[CO_2]$ and P treatments (Fig. 3*d*; $P = 0.002$), and increased under elevated $[CO_2]$ in high P conditions. Soluble sugar content increased clearly at low P (Fig. 3*e*; $P = 0.002$). TNC content also demonstrated interactive effects between $[CO_2]$ and soil water treatments (Fig. 3*c*; $P = 0.004$), and decreased in soil drought conditions at ambient $[CO_2]$.

Biomass allocation

In general, there were no marked effects of elevated $[CO_2]$ on biomass allocation. There were always interactive effects between P and soil water treatments in the parameters of biomass allocation. T : R ratio, as well as leaf area ratio (LAR), as previously reported in Tobita *et al*. (2010), markedly decreased under low P compared with high P, and increased in soil drought conditions in high P under ambient $[CO_2]$ compared with the ambient $[CO_2]$ of the control in Exp-II (Fig. 5*a*). Leaf weight ratio (leaf biomass per total biomass) also decreased under low P ($P < 0.0001$, data not shown).

Whole-plant N content

The effects of elevated $[CO_2]$ on the total N mass of *A. hirsuta* were also dependent on P treatments (Fig. 5*c*). In high P, total N mass increased under elevated $[CO_2]$ irrespective of N treatment (13–17% of total N mass under ambient $[CO_2]$ in Exp-I; not significant) or soil water treatments (15–17% of total N mass under ambient $[CO_2]$ in Exp-II), but exhibited no enhancement of total N mass under low P.

N allocation within tree

In both experiments, there were no marked effects of elevated $[CO_2]$ on N allocation to each tissue. In Exp-II, there were marked effects of P treatments on N allocation to each tissue, and the leaf N mass to total N mass ratio (LNR) decreased under low P. Contrary to expectations, root N mass to total N mass ratio (RNR) increased under low P. The T : R (N) ratio decreased under low P (Fig. 5*b*).

Allometric relationships for biomass

Elevated $[CO_2]$ did not alter the allometric relationships between total biomass and the biomass of each tissue, with some exceptions. As previously reported, only nodule biomass

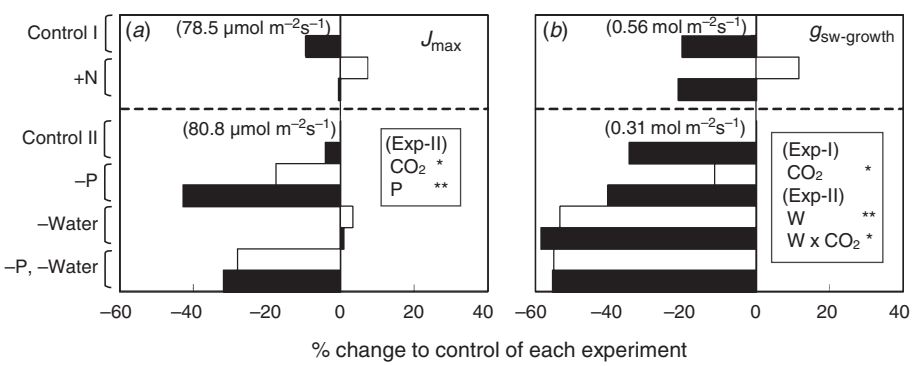

Fig. 1. The percent change in (*a*) maximum electron transport rate (J_{max}) and (*b*) stomata conductance under each ambient CO_2 level ($g_{sw\text{-}growth}$) compared to the control of each experiment in *Alnus hirsuta*. Values shown are (100 × ((means of each treatment) ÷ (mean of ambient $[CO_2]$ of control in each experiment) – 1)). Open column, ambient $[CO_2]$; closed column, elevated $[CO_2]$. The upper four columns show the results of Experiment I (Exp-I), and the lower eight columns show the results of Experiment II (Exp-II). Control I, low N; +N, high N; Control II, high P and well-watered conditions; –P, low P; –water, soil drought conditions; '–P, –water', low P and soil drought conditions. The values in parentheses on each control column show the mean ambient $[CO_2]$ of each control treatment. ANOVA results are also shown when $P < 0.05$: *, significant at $P < 0.05$; **, significant at $P < 0.01$. W, water treatment.

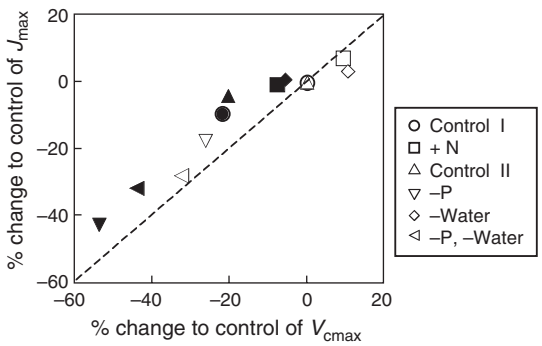

Fig. 2. Comparison of the percent change in V_{cmax} compared to the control and the percent change in J_{max} compared to the control. Values shown are (100 × ((means of each treatment) ÷ (mean of ambient [CO_2] of control in each experiment) – 1)). The labels of each treatment are the same as in Fig. 1. Open symbols, ambient [CO_2]; closed symbols, elevated [CO_2]. The dotted line represents the same variation from the control in both the *x*-axis and *y*-axis.

showed a significant alteration in the allometric relationships with total biomass by N treatment in Exp-I (Tobita *et al*. 2005) and by soil water treatment in Exp-II (Tobita *et al*. 2010). In Exp-I, the allometric relationship with total biomass was affected by [CO_2] treatment in fine root biomass ($P = 0.01$; ambient [CO_2] > elevated [CO_2]). In Exp-II, there were interactive effects between soil water treatment and total biomass on the biomass of coarse roots ($P = 0.01$). There were some insignificant effects on leaf biomass by N treatment ($P = 0.06$; high N > low N), and on shoot biomass by [CO_2] treatment ($P = 0.09$; elevated [CO_2] > ambient [CO_2]) and by N treatment ($P = 0.13$; low N > high N) in Exp-I.

Allometric relationships for N content

In Exp-I, the relationships between total biomass and both total N mass and leaf N mass were affected by N treatment (Table 1; $P = 0.005$ and 0.03, respectively; high N > low N). In Exp-II, the relationships between total biomass and both total N mass and leaf

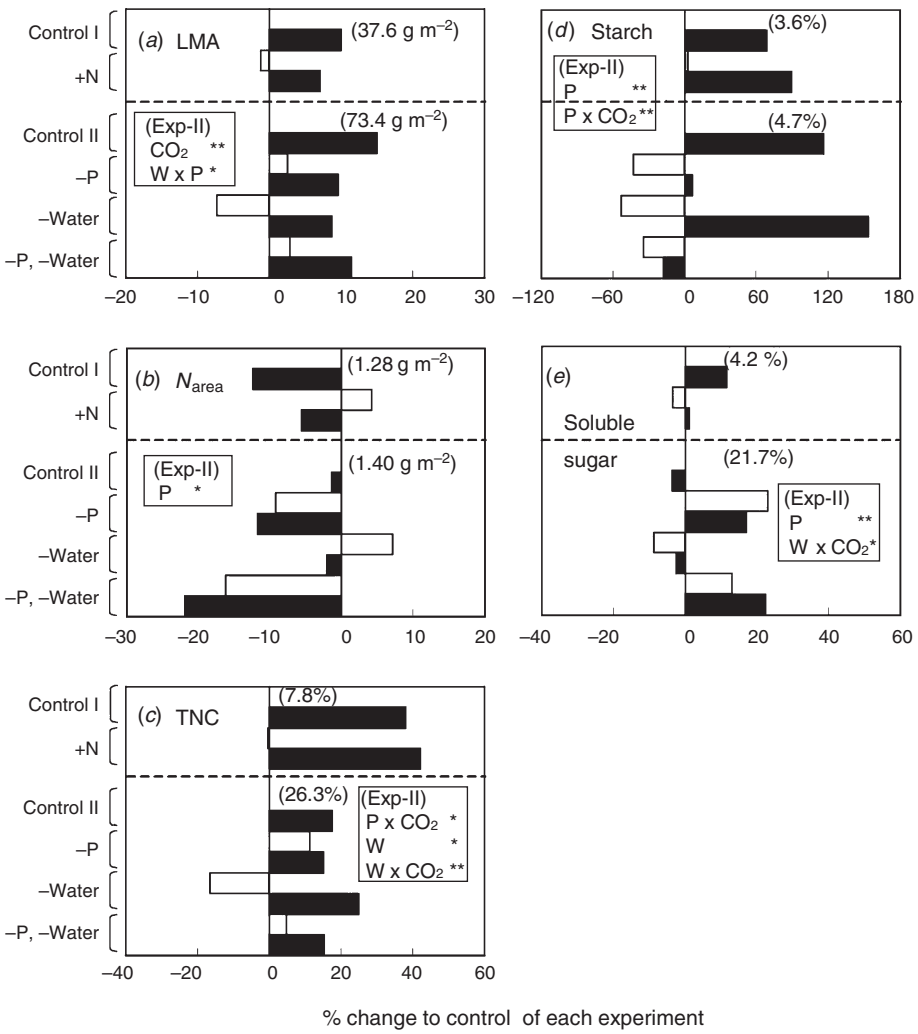

Fig. 3. The percent change in (*a*) leaf mass per area (LMA), (*b*) N content per unit leaf area (N_{area}), (*c*) total non-structural carbohydrates (TNC) content, (*d*) starch content and (*e*) soluble sugar content compared to the control of each experiment in *Alnus hirsuta*. The format of these figures is the same as in Fig. 1.

N mass were affected by [CO_2] treatment (Table 1; $P = 0.04$ and 0.002, respectively; ambient [CO_2] > elevated [CO_2]), and showed the interactive effects of soil water treatment and total biomass ($P = 0.04$ and 0.01, respectively; soil drought conditions > well-watered conditions). The percent change of total N mass and leaf N mass compared to the control (ambient [CO_2] of the control in each experiment) was not always the same as that of total biomass, for example, under the ambient [CO_2] of the '+N' and '–water' treatments (Fig. 6*a*, *b*). As the results show, the variation in total N mass and leaf N mass under elevated [CO_2] compared to the ambient [CO_2] of each treatment was smaller than the variation in total biomass (Fig. 6*a*, *b*).

In Exp-I, there were significant effects on the relationships with total biomass in root N mass by N treatment ($P < 0.0001$;

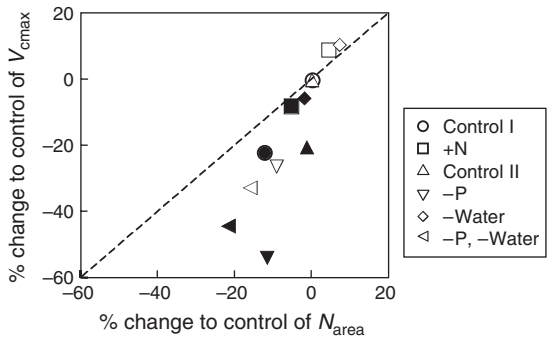

Fig. 4. Comparison of the percent change in N_{area} compared to the control and the percent change in V_{cmax} compared to the control. The format of this figure is the same as in Fig. 2.

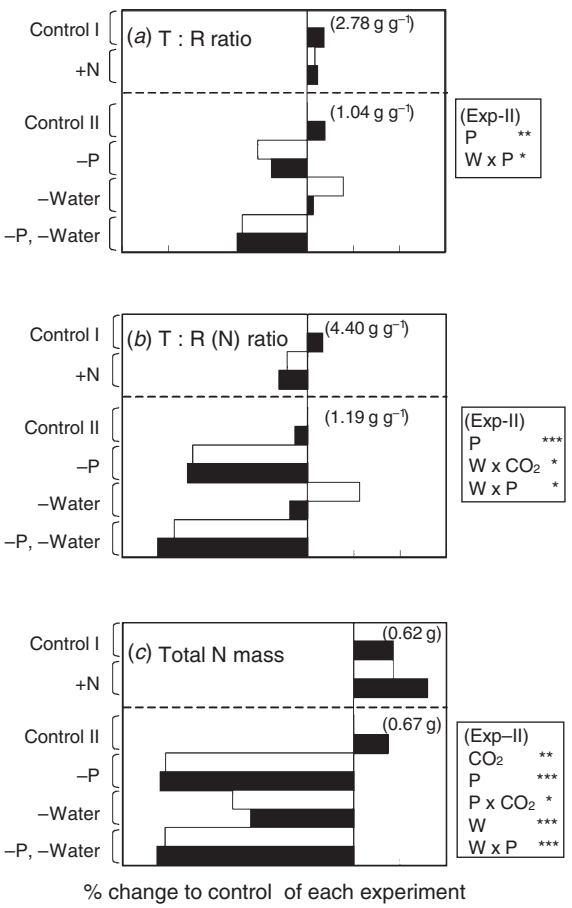

Fig. 5. The percent change in (*a*) top to root ratio of biomass (T : R ratio), (*b*) top to root ratio of N content (T : R (N) ratio) and (*c*) total N mass compared to the control of each experiment in *Alnus hirsuta*. The format of these figures is the same as in Fig. 1. ***, significant at $P < 0.001$.

Table 1. Analysis of covariance of total N mass (mg) and leaf N mass (mg) related to [CO_2] treatments (ambient and elevated [CO_2]) at two N treatments (high N and low N) in Experiment I (Exp-I) and at two soil water treatments (well-watered and soil drought conditions) in Experiment II (Exp-II)

Total biomass was tested as a covariate in the model. [CO_2] treatment, N treatment and soil water treatment were tested as independent factors

Source of variance	Total N (mg)		Leaf N (mg)	
Exp-I	*F*	*P* > *F*	*F*	*P* > *F*
Total biomass	150	<0.0001	56	<0.0001
CO_2	0.9	0.4	0.5	0.5
N	10	0.005	6	0.03
Exp-II				
Total biomass	349	<0.0001	215	<0.0001
CO_2	5	0.04	11	0.002
Water	6	0.02	8	0.006
Water × total biomass	5	0.04	7	0.01

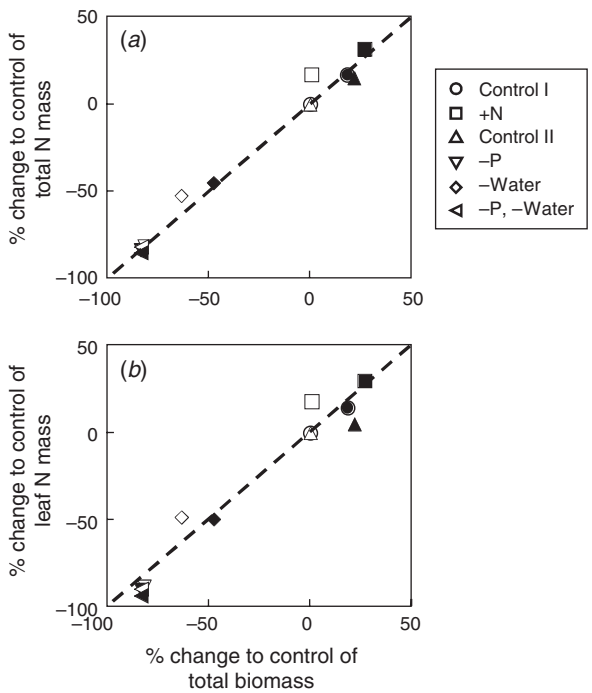

Fig. 6. Comparison of the percent change in total biomass compared to the control and the percent change in (*a*) total N mass and (*b*) leaf N mass compared to the control. The format of these figures is the same as in Fig. 2.

high N > low N) as well as by [CO_2] treatment ($P = 0.03$; ambient [CO_2] > elevated [CO_2]), and also in nodule N mass by N treatment ($P < 0.0001$; low N > high N). In Exp-II, there were also soil water treatment effects on the relationship between total biomass and fine root N mass, although they were not significant ($P = 0.14$; soil drought conditions > well-watered conditions).

Discussion

In our study, *A. hirsuta* demonstrated that J_{max} decreased to a smaller extent than V_{cmax}, which was demonstrated in Tobita *et al*. (2010), under elevated [CO_2] with sufficient P availability (Figs 1*a*, 2). These results for V_{cmax} and J_{max} lead to an increase in the $J_{max} : V_{cmax}$ ratio under elevated [CO_2] conditions. Moore *et al*. (1999) proposed a model for photosynthetic responses to elevated [CO_2] that makes a convincing case for the molecular control of Rubisco content at elevated [CO_2] via sucrose cycling. In this model, a selective loss of Rubisco is provided by an increase in the $J_{max} : V_{cmax}$ ratio and does not cause a decrease in photosynthesis (Long *et al*. 2004). In our experiments, although V_{cmax} decreased under elevated [CO_2], it was confirmed that the photosynthetic rate at growth [CO_2] condition ($A_{sat\text{-}growth}$) increased under elevated [CO_2] (Tobita *et al*. 2005, 2010). For these reasons, we could consider these photosynthetic responses of *A. hirsuta* to elevated [CO_2] under high P to be 'photosynthetic acclimation' rather than 'photosynthetic downregulation' in response to elevated [CO_2], as indicated by Long *et al*. (2004). Similar responses of *A. hirsuta* to elevated [CO_2] were also observed in a field experiment (Eguchi *et al*. 2008), although the soil nutrient status was different from our study. These phenomena might be species-specific responses to elevated [CO_2] of the N_2-fixing *A. hirsuta*, because other tree species seldom exhibit such photosynthetic responses to elevated [CO_2].

N_2-fixing plants have a relatively high demand for P over non-fixers (Ingestad 1981) because of the high energy demands of N fixation (Uliassi and Ruess 2002; Dawson 2008), and respond strongly to P fertilisation (Uliassi *et al*. 2000). Under low P, especially in well-watered conditions, J_{max} as well as V_{cmax} decreased under elevated [CO_2] in *A. hirsuta*, and no increase in $A_{sat\text{-}growth}$ was observed (Figs 1*a*, 2) (Tobita *et al*. 2010). These responses indicate the obvious 'photosynthetic downregulation' in response to elevated [CO_2]. A reduced or insufficient sink capacity may be the result of many potentially limiting processes such as N supply or developmental changes (Rogers and Ainthworth 2006), and can cause the appearance of a carbohydrate-derived signal that can lead the subsequent downregulation of photosynthetic machinery, principally Rubisco (Long *et al*. 2004; Ainsworth and Rogers 2007). Leaf TNC, especially soluble sugar, accumulated under low P even under ambient [CO_2] (Fig. 3*c*, *e*) and N_{area} also decreased compared with control treatment under ambient [CO_2] (Fig. 3*b*). These results indicate that the sink–source balance had already changed even at ambient [CO_2] under low P. It is suggested that the decline in photosynthetic capacity associated with the changes in leaf characteristics described above, and morphological changes such as the decrease in the T : R ratio (Fig. 5*a*) and LAR (Tobita *et al*. 2010) caused *A. hirsuta* to show no enhancement in growth under elevated [CO_2] with low P (Fig. 6). Previous studies on *P. taeda* suggest that long-term P limitation regulates growth responses to elevated [CO_2] primarily through its effect on biomass allocation rather than through its direct effect on photosynthetic capacity (Lewis and Strain 1996). Our study suggested that the photosynthetic responses as well as biomass allocation affected the growth responses of *A. hirsuta* to P deficiency.

The growth responses of tree species to elevated [CO_2] occur via their effects on growth rates or via altering the patterns of allocation independent of the effects on growth rate (Lewis *et al*. 2010). The elevated [CO_2] caused no marked change in the allometric relationship between the biomass of each tissue and total biomass of *A. hirsuta*, except for the fine root biomass in Exp-I ($P = 0.01$; ambient [CO_2] > elevated [CO_2]). These results suggest that the differences in biomass allocation to each tissue among [CO_2] treatments were mainly attributable to differences in total plant size (growth rate) among treatments. Unlike the small change seen here in the allometric relationships of *A. hirsuta* under elevated [CO_2], Lewis *et al*. (2010) demonstrated a change in allometric relationships between total biomass and the biomass of each tissue under elevated [CO_2] for *Populus deltoides* Marsh. It was suggested that there are species-specific responses in biomass allocation to increasing [CO_2].

In general, the increased root biomass allocation, which means a decreased T : R ratio, has often been reported in relation to the responses to elevated [CO_2] as well as to soil drought conditions (Edwards *et al*. 2006; Xu *et al*. 2007). However, in our experiments, *A. hirsuta* showed a relatively high T : R ratio (Fig. 5*a*) and a high LAR under soil drought with high P at ambient [CO_2] (Tobita *et al*. 2010). In addition, these responses to soil drought conditions were not shown under low P. These results suggest that for *A. hirsuta* seedlings, P deficiency conditions had a larger effect on biomass allocation, such as T : R ratio, than soil drought conditions.

The leaf N : P ratio of *A. hirsuta* was the lowest in the soil drought × high P × ambient [CO_2] conditions (~12) out of all the treatments (Tobita *et al*. 2010). Nutrient ratios have been extensively used to examine optimum nutrition and to explain particular nutrient limitations (Gentili and Huss-Danell 2003; Bown *et al*. 2007). A lower leaf N : P ratio indicates relatively lower P limitation compared with other treatments. In the case of high P availability, in general, biomass allocation to leaves increases compared with root biomass allocation. These phenomena will lead to an increase in T : R ratio (Fig. 5*a*) and LAR (Tobita *et al*. 2010).

Nitrogen allocation within trees reflects changes in the N concentration of each tissue in addition to the change of biomass allocation. The variation from control conditions (ambient [CO_2] of the control in each experiment) was different between N allocation and biomass allocation (Figs 5, 6). For example, there were larger variations in the T : R (N) ratio among treatments compared to the T : R ratio, especially in low P conditions(Fig. 5*a*, *b*). These results reflected that the responses in N concentration differed among tissues, such as a decrease in total N concentration (ANOVA; $P = 0.006$) and leaf N concentration (ANOVA; $P = 0.01$), and no change in root N concentration under elevated [CO_2]. Xu *et al*. (2007) demonstrated for *Caragana intermedia* Kuanget H.c.Fu (a desert shrub) that elevated [CO_2] significantly decreased the partitioning of N into leaves, but increased the partitioning of

N into roots, especially in soil drought conditions. However, the variation of total N mass and leaf N mass from the control conditions was not always the same as that of total biomass (Fig. 6*a*, *b*). In particular, total N mass and leaf N mass tended to decline under elevated [CO_2] compared with ambient [CO_2] in Exp-II (Table 1). As a result, the increase of total plant N mass under elevated [CO_2] compared with ambient [CO_2] was smaller than that predicted by the responses of total biomass to elevated [CO_2] (Tobita *et al*. 2005, 2010), as shown in Fig. 6. In temperate to boreal zones, actinorhizal plants including *Alnus* species are expected to contribute significant amounts of fixed N to forest ecosystems (Dawson 2008) in elevated [CO_2] conditions. These results suggest that it is necessary to evaluate not only biomass accumulation but also N mass allocation when we consider the N_2-fixing ability of *Alnus* species under elevated [CO_2].

Conclusion

The effects of soil nutrients (N or P availability) and soil drought on the physiological and morphological responses of *A. hirsuta* to elevated [CO_2] were evaluated comprehensively by using the results of two previous experiments (Tobita *et al*. 2005, 2010). Soil P availability affected the growth responses of *A. hirsuta* to elevated [CO_2] through direct effects on photosynthetic properties and through the effects on biomass allocation. The elevated [CO_2] caused no marked change in the allometric relationships of *A. hirsuta*, with some exceptions. These results suggest that the difference among treatments in biomass allocation to each tissue were mainly attributable to differences in growth rates among the treatments. The responses of N allocation among tissues to elevated [CO_2] differed from those of biomass allocation. These results suggest that it is necessary to evaluate not only biomass accumulation but also N mass allocation when we consider the N_2-fixing ability of *Alnus* species under elevated [CO_2].

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