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Morphological and physiological response of Siebold's beech (*Fagus crenata*) seedlings grown under CO₂ concentration ranging from pre-industrial to expected future levels

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Abstract

Siebold's beech (*Fagus crenata*) is a common species in the cool temperate forests of Japan. As the natural regeneration of beech forests is expected to contribute to forest conservation in future, we investigated the effects of different CO₂ concentration ([CO₂]) on the growth of beech seedlings in relation to morphology and physiological changes. Acorns collected from beech forest in Minakami, central Japan were germinated and grown during a first growing season of six months under four [CO₂] levels (200, 350, 550, and 750 μL L⁻¹). Stem mass increased with increasing [CO₂]; however, root mass did not change significantly among the treatments. With increasing [CO₂], net photosynthetic rate (P_n) and leaf area increased, whereas transpiration (Tr), stomatal conductance, leaf chlorophyll content, and leaf longevity decreased. Although water use efficiency (WUE; i.e., P_n/Tr) improved with increasing [CO₂], the density of stomata did not significantly change. Increases in the number of bud and terminal bud length with increasing [CO₂] indicated accelerated formation of additional branches and leaves in the next season. At high [CO₂] environment in future, improved WUE may be nevertheless advantageous for the survival of beech seedlings preferring mesic conditions.

Keywords Regeneration, Photosynthesis, CO₂ levels, Gas exchange, Water use efficiency (WUE)

Introduction

Siebold's beech (*Fagus crenata*) is a representative deciduous broad-leaved tree species in the cool temperate forests of Japan and is often planted for monumental conservation area. Natural regeneration of beech forests is expected to contribute to forest conservation (e.g. Kataoka 1982; Maeda 1988; Nakashizuka 2004). Based on their review works, *F. crenata* has high shade tolerance capacity; however, the survival of regenerated seedlings is predicted to face a risk under increasing atmospheric CO₂ concentration ([CO₂]) (Oikawa 1986). For instance, incident light flux decreased sharply from the top layer of forest to forest floor, and is predicted to be deficient for seedling survival due to accelerated leaf production in the top layer at [CO₂] above 550 μL L⁻¹ (Oikawa 1986; Ainsworth and Long 2005). In fact, shoot development accelerated at elevated [CO₂] for spruce (Hättenschwiler and Körner 1996), willow (*Salix petosus-su*, *S. miyabeana*), alder (*Alnus hirsuta*) (Koike 1995; Koike et al. 1995), Monarch birch (*Betula maximowicziana*), oak (*Quercus mongolica* var. *crispula*) (Lei and Koike 2005) and white birch (*B. platyphylla* var. *japonica*) (Eguchi et al. 2008).

Increasing atmospheric [CO₂] also influences the daily variation in [CO₂] at forest floor (Bazzaz and Miao 1993; Bazzaz 1996; Koike et al. 2001), thus future natural regeneration of beech will occur at a higher [CO₂]. As [CO₂] increases at forest floor, plants at the forest floor will change in growth pattern and photosynthetic properties (Küppers and Schneider 1993; Osborne et al. 1997). For example, [CO₂] could affect shade tolerance of tree seedlings, which is tightly connected with the functional arrangement of leaves and light compensation point (*I_c*) of a leaf (Larcher 2003; Lei and Koike 2005). *I_c* may decrease at high [CO₂] (Farquhar and Sharkey 1982; Larcher 2003, Hida et al. 2007) and shade tolerance can be improved as dark respiration will not increase. We should know such changes in shade tolerance of *F. crenata* seedlings

through its shoot dynamics and physiological acclimation as found in European beech (*Fagus sylvatica*) (Küppers and Schneider 1993) and in *F. crenata* (Uemura et al. 2000).

Plant response at enriched [CO₂] of the nearly doubled current atmospheric level differs among tree species (Ceulemans and Mousseau 1994; Saxe et al. 1998; Hättenschwiler 2001). The response of *F. sylvatica* was evaluated through a number of experiments conducted from organ level to stand level (Ceulemans and Mousseau 1994; Saxe et al. 1998). Changes in leaf level were observed: an increase in P_n, a decrease in T_r, a decrease in leaf nitrogen content, and an improvement in WUE (Overdieck 1993; Overdieck and Forstreuter 1994; Egli et al. 1998; Dufrêne et al. 1993; Mousseau et al. 1996; Heath and Kerstiens 1997; Körner 2000; Liozon et al. 2000; Bucher-Wallin et al. 2000; Hagedorn et al. 2002). At individual level, increases in leaf area and total leaf mass (Overdieck 1993; Dyckmans and Flessa 2002; Spinnler et al. 2003; Mousseau et al. 1996; Liozon et al. 2000), and acceleration of leaf senescence (Epron et al. 1996) were observed. With respect to *F. crenata*, a yellowing of leaves, an increase in branching, and an increase in biomass (Koike et al. 1996; Matsumura et al. 2005) under elevated [CO₂] have been reported.

Based on the former studies, we expected acceleration in growth of *F. crenata* seedlings through the improvements in P_n and WUE with increasing growth [CO₂]. In addition, certain morphological modifications are expected (e.g., changes in leaf area, leaf thickness, and number of winter buds; i.e., branch development) as well as a leaf phenological change. To test these predictions, we cultivated *F. crenata* seedlings under different [CO₂]. Based on the results, we discuss the plausible understanding of adjustment and improvement in growth and gas exchange traits in the seedlings at

different [CO₂].

Materials and Methods

Plant materials

The acorns used in this study were collected from a pure stand of Siebold's beech (*F. crenata* Blume) located in the Minakami region (at 36°51'N, 139°10'E) in central Japan. The stand represents the population in this region of heavy snow (Koike and Maruyama 1998; Koike et al. 1998). The acorns were prechilled for 3 months under 4°C, and then sowed in containers filled with moist soil. Soil, temperature, and humidity conditions for germination were the same as those under which the seedlings were grown. Two weeks after sowing, each single seedling with fully expanded cotyledons was transplanted into one 20-liter pot.

Experimental design and structure

Seedlings were grown in four environment-controlled cabinets (S-152A, Koito Kogyo, Yokohama, Japan), illuminated by metal haloid lamps (MF1000, Toshiba, Tokyo, Japan). Photosynthetic photon flux (PPF) was controlled at 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which represents the light condition at the forest floor under a developed canopy in a mixed deciduous broadleaf forest (Lei et al. 1998). Total daily PPF was set at 15 $\text{mol m}^{-2} \text{day}^{-1}$ by 12-h continuous illumination. These conditions are suitable for avoiding photoinhibition because the photosynthesis of *F. crenata* reaches saturation at 300~400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Koike and Maruyama 1998). Variance of PPF among the cabinets and inside each cabinet was adjusted by partial shading.

The [CO₂] in each cabinet was controlled at 200, 350, 550, and 750 μL L⁻¹, respectively. The cabinet setting at 200 μL L⁻¹ was chosen to reflect ancient conditions, as forests of Siebold's beech were originally formed under atmospheric [CO₂] of approximately 270 μL L⁻¹ (Tsukada 1982). The cabinet setting at 350 μL L⁻¹ represented a case in which [CO₂] at forest floor was nearly equal to the present atmospheric [CO₂]. The cabinet settings at 550 and 750 μL L⁻¹ were assumed to reflect future conditions, around 2050 and 2080, respectively, in which atmospheric [CO₂] is expected to be greater than the current level (IPCC 2007). Temperature in the cabinets was maintained at 20°C during the illuminated period and at 10°C during the dark period. Relative humidity in the cabinets was kept at a constant 80%.

To reduce both of position effect and chamber effect and enable statistical analysis (Koike 1995), each treatment was rotated by turns among four cabinets, 120 and 240 days after sowing. Pots were also rotated randomly in each cabinet once per month simultaneously with the sampling for measurement, as described later. PPF, [CO₂], temperature, and humidity in each cabinet were recorded throughout the experiment. Among all the cabinets, the standard deviation of PPF, [CO₂], temperature, and relative humidity around the set value was 4.2 μmol m⁻² s⁻¹, 10 μL L⁻¹, 0.31°C, and 2.7%, respectively.

Depending on the size of plant materials, down-regulation due to root restriction is generally observed under conditions in which the pot sizes are below 3.5-liter or 0.5-liter (Arp 1991; Curtis and Wang 1998). In this study, 20-liter pots (W200×D200×H500 mm) were used to avoid down-regulation through the restriction of root growth. The pot shape was considered suitable for beech, which is a deep-rooted species (Mori 1991). Indeed, growth of plant materials depends more on soil nutrients

but not so much on pot size and shape (Berntson et al. 1993; McConnaughay et al. 1993b; McConnaughay et al. 1996). However, it is difficult to control soil nutrients (Pettersson and McDonald 1994). As nitrogen (N) and phosphate (P) are consistently insufficient in most terrestrial ecosystems (Aerts and Chapin 2000), we used a 2/1 (volume/volume) mixture of clay loam and the Kanuma pumice containing little nutrients. Nutrients were supplied three times during the experiment (i.e., 30, 60, and 120 days after sowing). At each time, 25 mg NO₃-N, 50 mg PO₄-P and 25 mg potassium per pot were supplied as a diluted solution (Hyponex 5-10-5, Hyponex, Osaka, Japan). To avoid a soil water deficit, the seedlings were watered twice a day at the start and at the end of the illuminated period.

Growth analysis

After 31, 59, 93, 118, 154, and 201 days following sowing (hereafter referred to as from 1 to 6 months after sowing), ten seedlings per each treatment were randomly harvested. Stem height, stem diameter at the boundary of a stem and its roots, leaf area, and the length of terminal bud were measured before drying the samples. Then, leaves, stems, taproots, and fine roots were separated and dry mass was determined following 72-hours oven-drying at 70°C. Leaf thickness was measured by optical microscopic observation (Vannox S, Olympus, Tokyo, Japan) at a cross section of sampled raw leaves at 2 months after sowing. At the same time, stomatal density at the abaxial surface was also counted by electron microscopic observation (JSM-5410, JOEL, Tokyo, Japan).

Formation of a winter bud was determined by the observable formation of bud scale. Growth of buds was estimated as the growth in length of terminal buds in the

latter 3 months of the growing season. Total root mass was defined as the total of taproot mass and fine root mass. The following indices were used for describing morphological features:

Specific leaf area (SLA; $\text{cm}^2 \text{g}^{-1}$) = leaf area (cm^2) / leaf mass (g).

Stem-root ratio (g g^{-1}) = stem mass (g) / total root mass (g).

Fine root-taproot ratio (g g^{-1}) = fine root mass (g) / taproot mass (g).

Physiological measurements

Relative chlorophyll content of leaves was measured by a chlorophyll meter (SPAD-502, Minolta, Tokyo, Japan) and represented as SPAD value (Masuda and Ishii 1985). Nitrogen content of leaves was determined by using a NC analyzer (NC-800, Sumica-Shimadzu, Kyoto, Japan).

Two months after sowing, when the SPAD values were almost at the maximum, ten fully expanded leaves were randomly selected from each treatment for the measurement of gas exchange. Gas exchange of CO_2 and H_2O were measured *in situ* at the center of each leaf placed in the measuring cuvette of a photosynthesis analysis system (CIRAS $\text{CO}_2/\text{H}_2\text{O}$ analyzer, PP Systems, Massachusetts, U.S.A. through Koito Kogyo Co.Ltd., Yokohama, Japan). $[\text{CO}_2]$ in the air flowing into the cuvette was regulated to the same level as that for the growing condition for each leaf. In all measurements, PPF, temperature, and relative humidity on the surface of the leaves was kept at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$, 25°C , and 60%, respectively. The system automatically calculated P_n , T_r , and stomatal conductance on an area basis. T_r divided by P_n was defined as being equal to WUE.

Statistical analysis

Statistic analyses were carried out using a software package (SPSS 12.0.1J, SPSS Japan, Tokyo, Japan). To discuss significant differences among the treatments, parametric one-way ANOVA or non-parametric testing of Kruskal Wallis accompanied by multiple comparison tests with the Bonferroni correction was carried out according to the results of tests for normality and equality of variance. Chamber and position effects could be canceled by switching the chambers and rotating pots, respectively.

Results

The acorns germinated 5 to 15 days after sowing. CO₂ concentration had no effect on the speed and the rate of germination (data not shown, the significance level (P) > 0.05). The lack of effect of [CO₂] on the speed might have resulted from a dependency of germination on the reserved substrates in the acorns. Stem height had reached a constant level with the first shoots; i.e., the height did not change significantly throughout the growing season, with no second shoots (data not shown, P > 0.05). Significantly higher stem height was observed with increasing treatment [CO₂] (Table 1). In contrast to stem height, stem diameter continuously increased throughout the season, which resulted in a continuous increase in stem mass (Fig. 1).

Two months after sowing, foliage leaves had completely expanded. Leaf area and leaf mass reached constant values under all treatments; i.e., these values did not increase significantly after that (data not shown, P > 0.05). At the same time, SPAD value (i.e., relative chlorophyll content) also reached constant values, signifying the maturation of leaves (Fig. 2, P < 0.05). Various features of the leaves were estimated as follows: Leaf area, leaf mass, and leaf thickness increased with the increasing treatment level; on the

other hand, relative chlorophyll content decreased with the increasing treatment level (Table 2). Such a decrease in relative chlorophyll content was observed throughout the growing season (Fig. 2). Although nitrogen content and stomatal density of the leaves also seemed negatively to correlate with treatment [CO_2], the differences were not significant (Table 2).

Measuring the gas exchange of leaves, higher CO_2 treatment resulted in higher P_n (Table 2). In contrast, higher CO_2 treatment resulted in lower T_r , leading to an improvement in WUE (Table 2). P_n associated with the treatment levels of 200, 550, and $750 \mu\text{L L}^{-1}$ compared with that at the treatment level of $350 \mu\text{L L}^{-1}$ was 68%, 102%, and 119%, respectively. In the same way, WUE was 50%, 131%, and 209%, respectively. Stomatal conductance showed a trend similar to that found for T_r (Table 2).

The formation of winter buds almost kept in step with the maturation of leaves. It was observed 2 months after sowing at the treatment levels of 200, 350, and $550 \mu\text{L L}^{-1}$. On the other hand, it was observed 3 months after sowing at $750 \mu\text{L L}^{-1}$. The number of winter buds did not subsequently increase. A histogram of the number showed that most individuals had 1, 3, or 5 buds; i.e., one terminal bud plus even-numbered lateral buds (Data not shown). Both the significantly largest number of winter buds and the maximum growth in the length of terminal bud were observed at the treatment level of $750 \mu\text{L L}^{-1}$ among the treatments (Table 1). Terminal bud length, however, reached a maximum under the treatment level of $550 \mu\text{L L}^{-1}$ at the end of the growing season, probably as a result of the delayed formation of winter buds at $750 \mu\text{L L}^{-1}$ (Table 1).

The first growing season drew to an end with the senescence of leaves at around 5 months after sowing; the relative chlorophyll content of leaves decreased month by

month toward the end of the season (Fig. 2). Since the increase in treatment $[\text{CO}_2]$ lowered the chlorophyll content throughout the growing season, the leaves turned yellow at an early month with increasing $[\text{CO}_2]$ (Fig. 2). Six months after sowing, the remaining leaves turned brown and were almost dry. In particular, the shedding associated with the $750 \mu\text{L L}^{-1}$ treatment seemed relatively to be more advanced compared with the other treatments; i.e., at the end of the experiment, the percentage of seedlings with dry leaves still attached was 68%, 75%, 92%, and 35% at the treatment level of 200, 350, 550, and $750 \mu\text{L L}^{-1}$, respectively.

At the final stage of the first growing season, stem was significantly heavier with increasing $[\text{CO}_2]$; on the contrary, treatment $[\text{CO}_2]$ did not affect total root mass (Table 1, Fig. 3). As a result, the stem-root ratio increased significantly with increasing $[\text{CO}_2]$. This indicated a change in allocation of photosynthetic products under different growth $[\text{CO}_2]$. Compared with the case of the treatment level of $350 \mu\text{L L}^{-1}$, the increment in stem mass was 58% at the treatment level of $550 \mu\text{L L}^{-1}$, and 73% at the treatment level of $750 \mu\text{L L}^{-1}$. The ratio of fine root mass to tap root mass was not affected by $[\text{CO}_2]$ (Table 1)

Discussion

Regenerated seedlings of Siebold's beech (*F. crenata*) on forest floor disappear usually within few years after their germination (e.g. Kataoka 1982; Maeda 1988). The causes of the disappearance are thought to be insufficient light, drought stress, herbivory, fungal pathogens, and so on (Sahashi et al. 1994; Maruta 1996). Because the beech prefers mesic condition, drought stress particularly injures the seedlings (Nakashizuka

2004). The insufficient light condition on forest floor suppresses the development of root system remarkably. In addition, thick layered litter on forest floor blocks the access of the poor root system to soil moisture. Consequently, most of the current-year seedlings wither through a water deficit (Kataoka 1991). Thus the light and water availability of the seedlings may affect those survival rate.

[CO₂] on forest floor sometime exceeds atmospheric [CO₂] during after sunset and before dawn, but the former roughly closes to the later (Norisada 1997; Koike et al. 2001). It means that [CO₂] in forest and on forest floor might have been increasing from nearly pre-industrial level to future level. In this study, current-year seedlings of *F. crenata* showed significant responses to four levels of treatment [CO₂], which represent past, near present, and future conditions. Namely, with increasing treatment [CO₂], stem mass, leaf area, the number of winter buds, P_n, and WUE tended to increase, whereas T_r and chlorophyll content of leaves decreased (Table 1, Table 2). In response to a stepwise set-up of treatment CO₂ level, indexes of physiological features such as P_n and T_r showed stepwise increase or decrease (Table 2). In contrast, indexes of morphological features such as stem height, stem mass, and leaf area did not differ significantly in response to a change in the treatment CO₂ level from 200 μL L⁻¹ to 350 μL L⁻¹; on the other hand, the indexes increased significantly in response to that from 350 μL L⁻¹ to 750 μL L⁻¹ (Table 1, Table 2). It could be suggested that the beech seedlings might alter their morphology from the present days to the future under a rapid and larger increase in [CO₂] level, in contrast to the insensitiveness under a comparatively slow and small increase in [CO₂] level from the pre-industrial days to the present. Such a difference between sensitiveness and insensitiveness was found in the density of stomata in many plant species; for instance, the density increased under the

elevation of [CO₂] from the pre-industrial level to the current atmospheric level, whereas it did not respond to further elevation of [CO₂] (Woodward 1987; Woodward and Bazzaz 1988). Both of morphological and physiological changes in the coming generations of the beech seedlings would affect its survival rate on future forest floor.

The improved WUE with increasing CO₂ might be profitable for the survival of the seedlings (Table 2). Stomatal conductance was significantly reduced with increasing [CO₂] without any change in the density of stomata (Table 2). This meant that the conductance was controlled by the partial closure of stomata (Morison 1985; Mansfield et al. 1990; Terashima 1992). By contrast, stomatal conductance of *F. sylvatica* is insensitive to elevated [CO₂] (Heath and Kerstiens 1997). The insensitivity accompanied with the increase in leaf area may decrease water availability of whole seedlings of *F. sylvatica*; and the drought tolerance may not improve under elevated [CO₂] (Küppers and Schneider 1993; Körner 2000). In this study of *F. crenata*, leaf area increased 1.1 times larger, on the other side, WUE on an area basis improved 2.1 times larger with an increase in [CO₂] from 350 μL L⁻¹ to 750 μL L⁻¹ (Table 2). Thus the improvement in WUE exceeded the increase in leaf area; consequently, water availability of whole seedlings, i.e., drought tolerance might improve in current -year seedlings of *F. crenata* in future.

The enhanced P_n accompanied with the increased leaf area improved the net photosynthetic gain of whole seedlings under the increased [CO₂] (Table 2). It is plausible that the improved gain makes it possible to enhance the growth in the next season; that means, increases in the number or in the size of winter buds predicts acceleration in leaf production in the next season (Table 1) (Maruyama 1983). Such acceleration is advantageous to seedlings for interception of light (Küppers 1985).

However, accelerated leaf production in the top layer of forest under elevated $[\text{CO}_2]$ could aggravate the photosynthesis of seedlings at the same time (Oikawa 1986). If the chlorophyll content in the leaves of top layer decreases under elevated $[\text{CO}_2]$ same as the case of current-year seedlings, it would become profitable to the seedlings. Filtering effect of layered leaves above seedlings particularly diminishes the light intensity around the absorption spectrum of chlorophyll as found in detail by Lei et al. (1998). Such a change in light quality disturbs the survival of seedlings (Kataoka 1991; Lei et al. 1998). Decreased chlorophyll in the leaves of top layer could reduce deterioration of light quality, and it might compensate a decrease in light quantity on future forest floor. Our data showed only the responses of current-year seedlings with cotyledon that store large amount of starch and other storage compounds. Therefore, we should conclude the survival of the seedlings based on the results of multiply years but we unfortunately could not infer this point.

The average increments in stem mass in this study were 58% and 73% under the future levels of $[\text{CO}_2]$. The corresponding increment is 32% or 49%, where broad-leaved trees are exposed to long-term elevated $[\text{CO}_2]$ of around $700 \mu\text{L L}^{-1}$ without environmental stress, as suggested by Curtis and Wang (1998) and Saxe et al. (1998). In a case of 2-year-old *F. sylvatica* grown under $[\text{CO}_2]$ of $700 \mu\text{L L}^{-1}$ on garden soil of medium fertility, the increment in stem mass was 34% compared with that at the treatment level of $350 \mu\text{L L}^{-1}$ (Overdieck 1993). The effect of elevated $[\text{CO}_2]$ on biomass increment is most intense when the test plants are young (Saxe et al. 1998). On the other hand, the biomass increment thought to be suppressed by following factors. One is the stress resulting from a low level of fertilization with low irradiance (Curtis and Wang 1998; Ainsworth and Long 2005). A low level fertilization causes a shortage

of phosphoric acid in plant bodies, which restricts translocation of photosynthates and biomass increment (Issop et al. 2000). Another factor is an earlier senescence under elevated $[\text{CO}_2]$. Such an earlier senescence is reported in cases of *Salix phylicifolia* (Silvola and Ahlholm 1993), two willows (Koike et al. 1995), and *F. sylvatica* (Epron et al. 1996). In a study on annuals, a relatively earlier senescence shortened the growth period, which did not lead to an increase in standing biomass under elevated $[\text{CO}_2]$ (McConnaughay et al. 1993a). Promoted aging under elevated $[\text{CO}_2]$ is particular under nitrogen limitation (Stitt and Krapp 1999), which usually reveals in decreased chlorophyll content (Ainsworth and Long 2005) as was observed in this study. The other factors are low temperature (Ainsworth and Long 2005) and exposure to elevated $[\text{CO}_2]$ from the time of germination avoiding response to a step elevation in growth $[\text{CO}_2]$ (Luo and Reynolds 1999). Though all the suppressing factors might be found in the experimental design of this study, the use of very juvenile material with the rich reserves in the seeds may give current-year seedlings of *F. crenata* a larger increment in stem mass compared with the average of broad-leaved species.

Seedlings suppressed under dense canopy need to survive until disturbances such as a gap formation, which brings an opportunity of regeneration (e.g. Kataoka 1982; Nakashizuka 2004). Even though beech seeds carry sufficient amount reserves in the acorns, which may give them tolerance to environmental stresses in their first growing season, it is actually important for the current-year beech (*F. crenata*) seedlings to get over serious dry stresses. In conclusion, the consistent improvement in WUE under increasing $[\text{CO}_2]$ would be able to give the current-year seedlings for the possibility of survival on forest floor.

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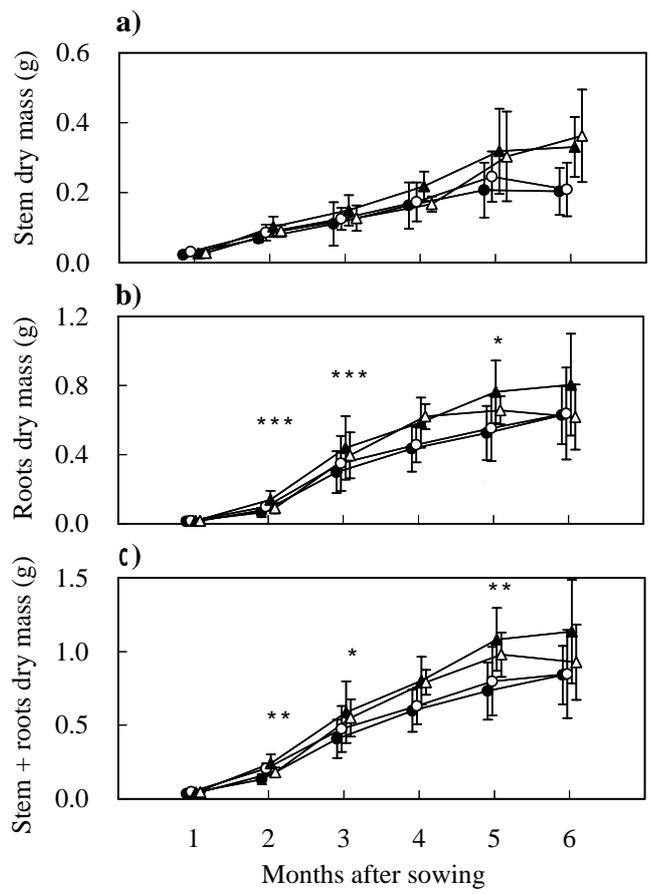
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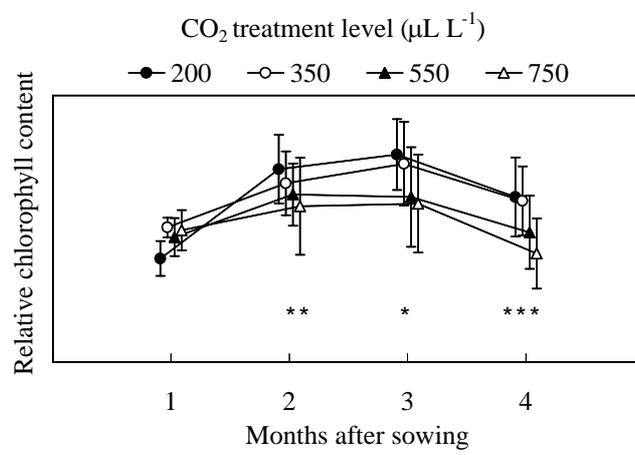
Fig. 1 Effect of CO₂ treatment on biomass productivity. **a** Stem. **b** Roots. **c** Stem + roots. Mean ± SD. n=10. Asterisks show probabilities (P) associated with ANOVA; *P<0.05, **P<0.01, ***P<0.001.

Fig. 2 Effect of CO₂ treatment on chlorophyll content of leaves. SPAD values indicate the content. Mean ± SD. n=10. Asterisks show probabilities (P) associated with ANOVA; *P<0.05, **P<0.01, ***P<0.001.

Fig. 3 Effect of CO₂ treatment on biomass allocation at the end of the first growing season. Mean ± SD. n=10. Capital letters above and under the bars represent significant differences of stem mass and roots (tap root + fine roots) mass, respectively (the significance level<0.05).

CO₂ treatment level (μL L⁻¹)
● 200 ○ 350 ▲ 550 △ 750





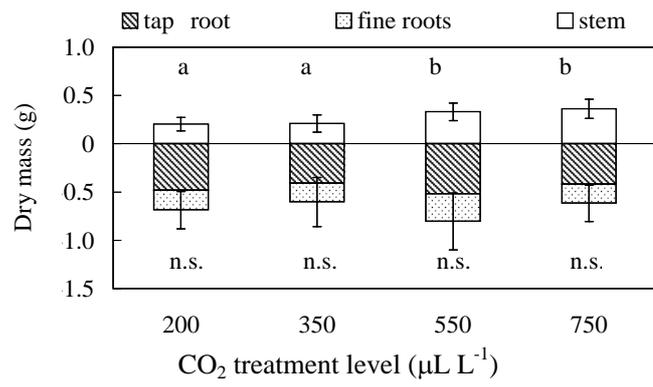


Table 1 Effect of CO₂ treatment on tree shape, biomass, and winter buds

	200 $\mu\text{L L}^{-1}$ CO ₂	350 $\mu\text{L L}^{-1}$ CO ₂	550 $\mu\text{L L}^{-1}$ CO ₂	750 $\mu\text{L L}^{-1}$ CO ₂
Stem height (cm) ^a	8.8 ± 2.0 ^{AB}	7.9 ± 1.0 ^A	10.0 ± 1.0 ^{BC}	10.4 ± 1.3 ^C
Stem diameter (mm) ^a	3.3 ± 0.6 ^A	3.3 ± 1.0 ^A	4.4 ± 0.4 ^B	4.1 ± 1.3 ^{AB}
Stem mass (g) ^a	0.20 ± 0.07 ^A	0.21 ± 0.09 ^A	0.33 ± 0.09 ^B	0.36 ± 0.10 ^B
Roots mass (g) ^a	0.68 ± 0.19 ^{n.s.}	0.60 ± 0.25 ^{n.s.}	0.81 ± 0.30 ^{n.s.}	0.62 ± 0.19 ^{n.s.}
Stem + roots mass (g) ^a	0.89 ± 0.20 ^{n.s.}	0.81 ± 0.29 ^{n.s.}	1.14 ± 0.35 ^{n.s.}	0.98 ± 0.26 ^{n.s.}
Stem-roots ratio (g g ⁻¹) ^a	0.32 ± 0.14 ^A	0.39 ± 0.24 ^A	0.44 ± 0.14 ^{AB}	0.62 ± 0.16 ^B
Fine-tap roots ratio (g g ⁻¹) ^a	0.43 ± 0.72 ^{n.s.}	0.48 ± 0.38 ^{n.s.}	0.55 ± 0.32 ^{n.s.}	0.47 ± 0.19 ^{n.s.}
Number of winter buds ^b	2.3 ± 1.4 ^A	2.1 ± 1.2 ^A	2.2 ± 1.1 ^A	2.7 ± 1.4 ^B
Terminal bud length (mm) ^b	6.0 ± 0.9 ^A	6.6 ± 1.3 ^A	7.2 ± 0.5 ^A	4.4 ± 1.3 ^B
Terminal bud length (mm) ^a	8.5 ± 1.4 ^A	9.0 ± 1.4 ^A	10.3 ± 1.3 ^B	8.7 ± 1.2 ^A
Growth of terminal bud (mm) ^c	2.5	2.4	3.1	4.3

Data are means for 10 samples (\pm SD). Values with a different capital letter within a given row indicate statistical significance at the significance level < 0.05 ; n.s. means no significance. ^a Measured or calculated at 6 months after sowing; ^b Measured or counted at 3 months after sowing; ^c Growth in length of terminal buds from 3 to 6 months after sowing.

Table 2 Effect of CO₂ treatment on fully expanded leaves estimated 2 months after sowing

	200 $\mu\text{L L}^{-1}$ CO ₂	350 $\mu\text{L L}^{-1}$ CO ₂	550 $\mu\text{L L}^{-1}$ CO ₂	750 $\mu\text{L L}^{-1}$ CO ₂
Leaf mass (g)	0.16 \pm 0.04 ^A	0.21 \pm 0.04 ^{AB}	0.25 \pm 0.05 ^B	0.22 \pm 0.04 ^B
Leaf area (cm ²)	41.7 \pm 8.8 ^A	48.0 \pm 7.7 ^A	55.0 \pm 8.9 ^{AB}	53.0 \pm 7.4 ^B
Leaf thickness (μm)	85 \pm 10 ^A	90 \pm 7 ^{AB}	93 \pm 11 ^B	101 \pm 9 ^C
Specific leaf area (cm ² g ⁻¹)	270 \pm 53 ^A	235 \pm 25 ^{AB}	228 \pm 32 ^B	233 \pm 12 ^{AB}
Density of stomata (10 ³ x number cm ⁻²)	11.2 \pm 1.5 ^{n.s.}	11.0 \pm 0.9 ^{n.s.}	10.0 \pm 1.1 ^{n.s.}	9.8 \pm 1.6 ^{n.s.}
Nitrogen content (%)	1.65 \pm 0.41 ^{n.s.}	1.60 \pm 0.42 ^{n.s.}	1.18 \pm 0.15 ^{n.s.}	1.01 \pm 0.21 ^{n.s.}
SPAD value ^a	28.4 \pm 4.0 ^A	26.9 \pm 4.9 ^{AB}	25.2 \pm 3.5 ^{BC}	23.6 \pm 5.3 ^C
Net photosynthetic rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	2.9 \pm 0.3 ^A	4.3 \pm 0.4 ^B	4.3 \pm 0.7 ^B	5.1 \pm 0.7 ^C
Transpiration (mmol m ⁻² s ⁻¹)	1.62 \pm 0.19 ^A	1.13 \pm 0.21 ^B	0.87 \pm 0.16 ^C	0.72 \pm 0.15 ^C
Stomatal conductance (mmol m ⁻² s ⁻¹)	120 \pm 17 ^A	87 \pm 17 ^B	59 \pm 11 ^C	46 \pm 11 ^C
Water use efficiency ^b	1.8 \pm 0.20 ^A	3.6 \pm 0.41 ^B	4.7 \pm 0.84 ^C	7.5 \pm 1.83 ^D

Data are means for 10 leaves (\pm SD). Values with a different capital letter within a given row indicate statistical significance at the significance level < 0.05 ; n.s. means no significance. ^a SPAD value corresponds to chlorophyll content of leaves. ^b Water use efficiency was define as a ratio of net photosynthetic rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$) to transpiration (mmol m⁻² s⁻¹).