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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

**Species-Specific Responses of
Photosynthesis and Nitrogen Allocation
under Nitrogen Limitation
after Nine Year's CO₂ Enrichment.**

이산화탄소 폭로 처리에 따른
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Abstract

Increased atmospheric CO₂ concentration could mitigate the climate warming via enhanced forest productivity, which is substantially affected by nutrient availability, especially N, and its efficiency. Therefore, the changes of N concentration and its allocation in leaf under long term elevated CO₂ [eCO₂] exposure is important for future prediction. This study was conducted to investigate the changes of the photosynthetic characteristics and nitrogen allocation of Japanese red pine (*Pinus densiflora*), Korean ash (*Fraxinus rhynchophylla*) and Korean whitebeam (*Sorbus alnifolia*), which have been growing under three different CO₂ concentrations (ambient [aCO₂], ambient x 1.4 [eCO₂1.4] and ambient x 1.8 [eCO₂1.8]) for nine years. There was no significant difference in growth of diameter among chambers in last two years, but the morphological characteristics such as leaf size and leaf mass per area were higher at eCO₂ than aCO₂. In case of photosynthetic characteristics, the maximum photosynthetic rate (A_{max}) was higher at eCO₂ than aCO₂, especially in Korean ash. On the other hand, the maximum carboxylation rate (V_{Cmax}) and the maximum electron transfer rate (J_{max}), decreased significantly at eCO₂1.8. Photosynthetic down-regulation was not caused by the decrease of leaf nitrogen per unit area (N_{area}), but it was rather caused by the changes in N allocation. The N allocation to Rubisco (NF_{Rub}) and cell wall (NF_{cw}) did not change among chambers, while the nitrogen fraction to chlorophyll (NF_{chl}) increased at eCO₂ than aCO₂. In addition, the changes of N allocation were species- and position-specific. The reduction of NF_{Rub} and the enhancement of NF_{chl} were the most pronounced in Korean whitebeam. NF_{cw} decreased significantly only in Korean ash. The decrease of NF_{Rub} in eCO₂ was greater in upper canopy than in lower canopy, while the enhancement of NF_{chl} was not different among canopy positions. The increment of NF_{chl} increased PNUe and increased the amount of photosynthesis and maintained biomass production despite of photosynthetic

capacity reduction. Our result implied the effect of elevated CO₂ could last longer even with the *N* limitation due to the enhancement of PNUE caused by change of *N* allocation.

keywords : photosynthesis, PNUE, nitrogen allocation, nitrogen limitation, climate change, elevated CO₂

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1. Introduction

Elevated CO₂ concentration increased the photosynthetic rate, the biomass of leaf and stem and reduced stomatal conductance (Veteli *et al.*, 2002; Ainsworth & Long, 2004; Warren *et al.*, 2011; Kirschbaum, 2011). Net primary production (NPP) is also generally expected to increase due to elevated CO₂ concentrations, and FACE studies have shown that the average of net primary production increased by 23% compared to presently exposed atmospheric CO₂ (Nordy *et al.*, 2005). However, despite the increase of CO₂ concentration, there was no increase in net primary production when soil N availability was low (Terrer *et al.*, 2018 ; Feng *et al.*, 2015; Oren *et al.*, 2001; Menge and Field, 2007) and the increase of biomass was not significant in nitrogen limited forests condition (Oren, *et al.* 2001). The reduction of productivity in previous study was directly caused by down-regulation of photosynthesis (Darbah, *et al.* 2010).

Plants that grown under elevated CO₂ could not maintain initial CO₂ uptake due to growth acclimation (Reich *et al.*, 2018), which was caused by reduced carboxylation capacity of Rubisco (Rogers *et al.*, 2001 ; Rogers & Humphries 2000) and the decrease in Rubisco content (Tingey *et al.*, 2003; Sharwood *et al.*, 2017; Gutiérrez *et al.*, 2013). These results were caused by high CO₂ and low soil N availability, which resulted in the reduction of leaf N (Norby *et al.*, 2010; Sanz-Saez *et al.*, 2010). Several land models have the potential to reduce the photosynthesis due to nitrogen limitation (Gerber *et al.*, 2010). The deficiency of nitrogen in the soil predicted a progressive nitrogen limitation (PNL) hypothesis in which nitrogen availability is reduced under elevated CO₂ and declined in productivity (Luo *et al.*, 2004).

Nitrogen as a limiting factor in plant growth, the efficient use of nitrogen is an essential element for plant growth survival. The photosynthetic capacity is affected by the leaf nitrogen concentration (Madhu & Hatfield, 2014), because 50% of the leaf nitrogen is distributed to the photosynthetic organs. Therefore, the photosynthetic capacity and nitrogen concentration have a strong correlation (Hikosaka & Shigeno, 2009). The maximum rate of Rubisco carboxylation (V_{cmax}), photosynthetic capacity, is the most crucial parameter for global forest productivity and climate change prediction (Rogers, 2014). The fraction of nitrogen invested in the maximum carboxylation rate is also used as evaluation factors of carbon model (Friend, 2010).

Rubisco, which is involved in the maximum carboxylation rate, is a major photosynthetic enzyme with an investment of 15 to 35% of nitrogen. Rubisco content is considered to be a limiting factor in the rate of light-saturation point in the present CO₂ concentration (Parry *et al.*, 2008). However, Rubisco is not always a limiting factor in photosynthesis, and it may be limited by the use of Pi for ATP synthesis or the transport capacity of CO₂ in an environment with increased CO₂ level (Farquhar *et al.*, 1980; Sharkey, 1985). Previous studies have reported decreased activity of Rubisco protein because decreased leaf nitrogen under increased carbon dioxide (Choi *et al.*, 2017).

The reduction of leaf N content caused decline of V_{cmax} and RuBP regeneration (the maximum electron transport rate, J_{max}) ; photosynthetic down-regulation (Sage, 1994; Long *et al.*, 2004; Ainsworth and Rogers, 2007; Norby and Zak, 2011). The down-regulation of photosynthesis has the potential to efficiently uptake C by redistribution of nitrogen, and to increase photosynthetic nitrogen use efficiency (PNUE) in an

condition with elevated CO₂ (Leakey *et al.*, 2009; Zong & Shangguan, 2014).

A plant allocate nitrogen to enzymes and pigments involved in photosynthesis to absorb light and electron transport in photosystem I and II and protein is used as enzyme in the Calvin cycle (Evans & Poorter, 2001). The characteristics of leaf nitrogen allocation vary depending on species and growth environment, and these variations affect the photosynthetic nitrogen use efficiency of plants (PNUE) (Feng *et al.*, 2007). Therefore, the research at the molecular level as well as an ecological point of view is needed.

However, only a few studies on the changes of leaf nitrogen allocation under the elevated CO₂ environment have been reported ; black locust (Choi *et al.*, 2017), Eucalyptus (Sharwood *et al.*, 2017). This study investigated the correlation of increased PNUE by quantifying the changes of leaf nitrogen allocation under limited nitrogen environment when exposed to elevated CO₂ using open top chamber (OTC) for nine years.

The purposes of this study are 1) to investigate the morphological and the productivity changes of three native temperate species, *Pinus densiflora*, *Fraxinus rhynchophylla* and *Sorbus alnifolia*, grown under elevated CO₂ concentration for nine years, 2) to examine the occurrence of photosynthetic acclimation under nitrogen limitation, 3) to examine the changes of nitrogen allocation within cell for different species and canopy positions and 4) to verify their effects on nitrogen use efficiency.

2. Materials and Methods

2.1. Study site

The open top chamber (OTC) site is located at National Institute of Forest Science (37° 15' 04"N, 136° 57' 59"E) in Korea. The experiment OTC consists of 3 decagon chambers (diameter 10m by height 7m) with different atmospheric CO₂ concentration (ambient [aCO₂], ambient x 1.4 [eCO₂1.4] and ambient x 1.8 [eCO₂1.8]). The seedlings of three Korean temperate species, Japanese red pine (*Pinus densiflora*), Korean ash (*Fraxinus rhynchophylla*) and Korean whitebeam (*Sorbus alnifolia*), were planted in the chambers in September, 2009. In April 2009, prior to the plantation, the soil was excavated to 1m depth from the ground and replaced with forest soil in the same area to experiment under the same soil characteristics (Lee *et al.*, 2012). The CO₂ concentration of the treatment was measured by the infrared gas analyzer (ZRH type, Fuji Electric System Co. Ltd, Japan), and the concentration of CO₂ inside was controlled by mixing pure liquefied carbon dioxide and atmospheric air. The CO₂ enrichment was conducted throughout years from 8:00 am to 6:00 pm, when the plant's transpiration occurred.

2.2. Growth of diameter at root collar (DRC)

The diameter at 5cm above the ground of each tree was measured every three to four weeks starting from April 20th to November 24th in 2017 and similarly from March 28th to November 24th in 2018. The average diameter was calculated after two measurements in perpendicular directions using digital vernier calipers (CD-10CPX, Mitutoyo, Kawasaki, Japan). At the first measurement, the line was marked on the bark of the trees to measure the same points every time.

2.3. Leaf gas exchange

Light response curve and A/C_i curve measurement were conducted on the same one sunlit from each trees (3 leaves per species \times 3 species in each treatment, total of 9 leaves per treatment) using portable open gas exchange system LI-6400 (LI-Cor, Inc., Lincoln, NE, USA). The measurement was taken in the morning between 08:00 and 12:00 to avoid measurement under water stress. Leaf temperature and relative humidity were set at 25°C and at 55~60%, respectively. Four measurement campaigns were accomplished during the growing season of 2017 and 2018. Light response curve was measured by changing the irradiance in the following order: 1400, 1200, 1000, 800, 600, 400, 200, 100, 75, 50, 25, 0, 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Sample CO_2 were set at 400, 560 and 720 $\mu\text{mol m}^{-2}\text{s}^{-1}$ in a CO_2 , e CO_2 1.4 and e CO_2 1.8, respectively. A_{max} was estimated by selecting the photosynthesis value at light saturation point from light response curve (Taiz & Zeiger, 2010). The V_{Cmax} and J_{max} were measured by plotting A/C_i curves under high irradiance (1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$). The change in the reference CO_2 concentration for each chamber was set differently, and the order is as follow. a CO_2 ; 400, 300, 200, 100, 75, 50, 25, 0, 400, 400, 600, 800, 1000, 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$, e CO_2 1.4; 560, 400, 300, 200, 100, 75, 50, 25, 0, 560, 560, 800, 1000, 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$, e CO_2 1.8 ; 720, 600, 400, 300, 200, 100, 75, 50, 25, 0, 720, 720, 100, 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The V_{cmax} and J_{max} were estimated using the curve fitting model of version 2.0 developed by Sharkey (2016).

2.4. Leaf sizes, leaf mass per area and nitrogen contents

Five leaves from three canopy positions (upper, middle and lower) were collected from each tree for twice during 2017 and 2018. Total of

90 leaves per species in each treatment and total of 810 leaves were sampled for the analysis. The leaves were scanned and the leaf size was measured with ImageJ program. After the area measurement, the leaves were dried for 72 hours in 70°C dry oven and their dry weights were measured using digital micro-scale (HS-F series, HANSUNG, 0.001g/0.1g). Leaf mass per area (LMA, gm^{-2}) was calculated by dividing leaf area with its dry mass. Dried samples were powdered using homogenizer (FastPrep-24, MP Biomedicals, Solon, OH) and then determined the total N and C with a CHNS-Analyzer Flash EA 1112 (Thermo Electron Corporation, USA) at NICEM, Seoul National University.

2.5. N allocation in Rubisco, chlorophyll and cell wall

Three to four leaves including photosynthesis measurement were collected from the same branch, and leaves were punched out to make about 10 discs of 1cm in diameter, excluding leaf midrib and stored in nitrogen liquid tank. Rubisco protein was extracted by following method of Hikosaka & Shigeno (2009). One disc (0.785cm^2) stored at -80°C was powdered using homogenizer (FastPrep-24, MP Biomedicals, Solon, OH) and samples were extracted in 1ml of 4°C extraction buffer. Samples of Korean ash and Korean whitebeam was extracted by buffer included 80Mm Tris-HCl pH 7.4, samples of Japanese red pine was extracted by buffer included 80Mm Tris-HCl pH 8.0. 1%(w/v) Polyvinylpolypyrrolidone (PVPP), 1.5%(v/v) glycerol, 100mM β -mercaptoethanol, 20kgm^{-3} Sodium Dodecyl Sulfate (SDS) were included in all extraction buffer. The samples were centrifuged at 15000 g for 30 minutes at 4°C , denatured at 90°C for 5 minutes, and then analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis SDS-PAGE. Rubisco nitrogen was assumed to be 16% of total Rubisco content

(Hikosaka & Terashima 1995).

Chlorophyll was extracted with dimethylsulphoxide (DMSO) (Baren *et al.* 1992, Shinano *et al.* 1996). Two discs were placed in a brown bottle containing 5 ml of DMSO and the sample was stored at 65°C for 6 hours in a water bath. The samples were measured at two wavelengths of 649 nm and 665 nm using a spectrophotometer (wavelength range 190 – 1100 nm, spectral band pass width < 1nm, wavelength accuracy < ± 0.5 nm; Optizen 2120 UV, Mecasys, Korea), the total chlorophyll content was derived from the DMSO equation of Wellburn (1994).

$$\text{Total chlorophyll content } (\mu\text{gml}^{-1}) = 21.44A_{649} + 5.97A_{665}$$

Cell wall protein extraction was extracted according to Hikosaka and Shigeno (2009). The sample was extracted in same way with Rubisco before denatured 90°C. After centrifuged, the sample was washed by distilled water. And then to remove chlorophylls completely, the sample was washed three times (1hour-overnight-1hour) by PAW (phenol : acetic acid : water=2:1:1). The sample was further washed three times with ethanol and dried for 72hours at 70°C. The dry mass was analyzed by cell wall N concentration at NICEM, Seoul National University.

2.6. Data analysis

For growth of diameter at root collar, one-way ANOVA was used to test the significant difference of CO₂ concentration (treatment). In photosynthetic characteristics, two-way ANOVA was used to test the significance of species and treatment. For leaf size, LMA, C:N ratio, N_{mass} , N_{area} , N_{rub} , N_{chl} , and N_{cw} , three-way ANOVA were used to test the significance of species, treatment and canopy. When significant

differences were found, Duncan post-hoc test was used for the comparison of chamber. All statistical analyzes were performed using the R statistical program (ver. 3.3.2 R Core Team, 2016).

3. Results

3.1. Growth of diameter at root collar (DRC)

The diameter growth of two years was different among species ($p = 0.002$), but there were no differences among treatments and years ($p = 0.672$ and $p = 0.071$). The two years' average diameter growth of Korean ash ($13.89 \pm 2.07 \text{ mm yr}^{-1}$) and Japanese red pine ($10.36 \pm 1.44 \text{ mm yr}^{-1}$) was higher than that of Korean whitebeam ($5.84 \pm 0.65 \text{ mm yr}^{-1}$). Even though, there were tendencies of higher growth at eCO₂ than aCO₂ in 2017, but they were not significant ($p = 0.110$), these tendencies disappeared in 2018 (Table 1).

Table 1. The average of diameter at root collar (mm) per year from 2017 to 2018.

Species	Treatments	2017	2018
Japanese red pine	aCO ₂	10.8 ± 3.0 _a	8.7 ± 1.1 _a
	eCO ₂ 1.4	15.3 ± 3.1 _a	5.7 ± 2.0 _a
	eCO ₂ 1.8	16.3 ± 2.9 _a	4.3 ± 2.3 _a
Korean ash	aCO ₂	10.2 ± 3.2 _a	13.9 ± 10.6 _a
	eCO ₂ 1.4	16.3 ± 8.1 _a	9.5 ± 2.0 _a
	eCO ₂ 1.8	18.4 ± 6.1 _a	17.6 ± 5.1 _a
Korean whitbeam	aCO ₂	5.5 ± 0.5 _a	4.6 ± 1.7 _a
	eCO ₂ 1.4	7.4 ± 2.9 _a	5.3 ± 0.7 _a
	eCO ₂ 1.8	5.4 ± 2.4 _a	6.9 ± 1.0 _a

Different letters show statistical significance among treatments at $p < 0.05$ (one-way ANOVA followed by a Duncan post-hoc test).

3.2. Leaf size, LMA

In general, leaf size increased at eCO₂ than aCO₂, but the pattern of increase among species were different depending on their canopy positions (Figure 1a). In Japanese red pine, the average leaf size of

eCO₂1.4 (6.4±0.7 cm²) and eCO₂1.8 (9.2±0.9 cm²) increased compared to aCO₂ (4.3±0.6 cm²) ($p < 0.001$). In addition, the enlargement of leaf size was greater in the upper canopy than the lower canopy. In the lower canopy, there was 32.6% (5.5±0.3 cm²) and 49.0% (7.3±1.2cm²) increase at eCO₂1.4 and eCO₂1.8 compared to aCO₂, respectively. However, in the upper canopy, leaf size increased by 53.2% and 68.4% in order of eCO₂1.4 and eCO₂1.8. In contrast, leaf size of Korean ash and Korean whitebeam decreased with canopy height and the increment at eCO₂ was also greater in the lower canopy. In Korean ash, the increment in the lower canopy reached 67.2% (97.9 ± 11.8cm²), however the increment in the upper canopy was only 6.9%. In Korean whitebeam, leaf size increment occurred in the lower canopy by 51% at eCO₂1.4, but there was no difference in the upper canopy ($p = 0.163$).

Unlike leaf size, LMA increased with canopy height in all species ($p < 0.001$), however, the increment pattern was different among species (Figure 1b). In Japanese red pine, LMA increment increased with height. The increments were 14.4% and 19.5% at eCO₂ in the middle ($p = 0.03$) and the upper ($p = 0.009$) compared to aCO₂, but lower canopy had no difference among treatments. In other hand, Korean ash only showed enhancement in the middle canopy ($p = 0.004$) and Korean whitebeam showed similar enhancement in all canopy positions (maximum $p = 0.012$).

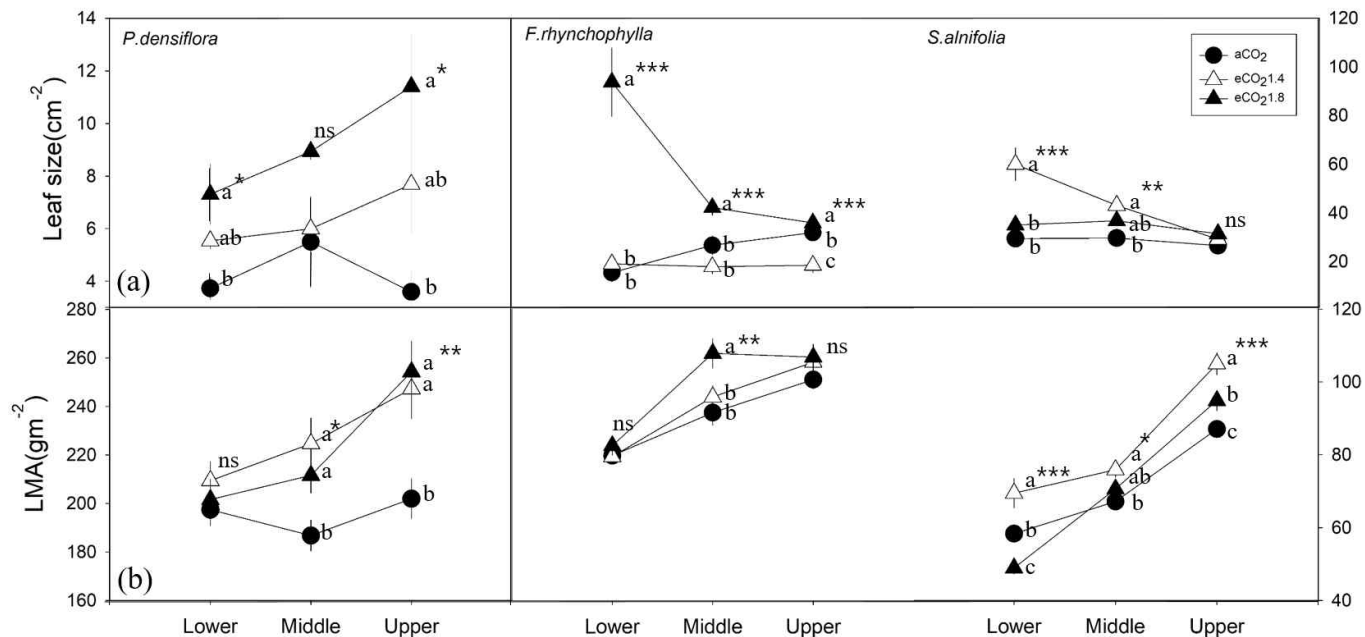


Figure 1. The average of (a) leaf size and (b) leaf mass per area in each three canopy positions. Different letters show statistical significance among treatments [filled circle (ambient), open triangle (ambient×1.4) and filled triangle (ambient×1.8)]. *, **, *** represent statistical differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ (three-way ANOVA followed by a Duncan post-hoc test), respectively.

3.3. Photosynthetic characteristics

Overall, there was no effect of elevated CO₂ on A_{\max} ($p = 0.110$), however, the effects were different among species. Korean ash showed significant enhancement by 37.9% than aCO₂ in eCO₂1.8 ($p = 0.001$, Figure 2), but A_{\max} of Japanese red pine and Korean whitebeam were not significant, even under elevated CO₂ concentration.

In contrast to A_{\max} , $V_{C_{\max}}$ reduction was observed at eCO₂1.8 ($p < 0.001$, Table 2). Japanese red pine had lower $V_{C_{\max}}$ at eCO₂1.8 ($32.0 \pm 5.6 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared to aCO₂ ($58.1 \pm 7.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and eCO₂1.4 ($68.6 \pm 12.3 \mu\text{mol m}^{-2} \text{s}^{-1}$) ($p = 0.021$, Figure 3a). Korean ash and Korean whitebeam had lower tendencies of $V_{C_{\max}}$ at eCO₂1.8 but they were not significant ($p = 0.282$, Figure 3a).

Similar to $V_{C_{\max}}$, J_{\max} of eCO₂ was significantly lower compared to aCO₂ (Table 2). In case of Japanese red pine, J_{\max} was the lowest at eCO₂1.8 ($p = 0.002$, Figure 3b). J_{\max} of Korean ash and Korean whitebeam were not statistically significant among treatments but it tended to be lower at eCO₂ ($p = 0.592$, Figure 3b). Furthermore, there was differences in J_{\max} among species ($p < 0.001$, Table 2). Japanese red pine ($91.0 \pm 4.4 \mu\text{mol m}^{-2} \text{s}^{-1}$) was highest and it was followed by Korean ash ($72.1 \pm 3.6 \mu\text{mol m}^{-2} \text{s}^{-1}$) and Korean whitebeam ($66.5 \pm 3.0 \mu\text{mol m}^{-2} \text{s}^{-1}$) without differences between two species.

Table 2. Two-way ANOVA for photosynthesis parameters.

Term	Variable			
	A_{\max}	$V_{C\max}$	J_{\max}	PNUE
Chamber	0.110	0.002	$P < 0.001$	$P < 0.001$
Species	0.697	0.093	$P < 0.001$	$P < 0.001$
Species×Chamber	0.048	0.126	0.124	0.039

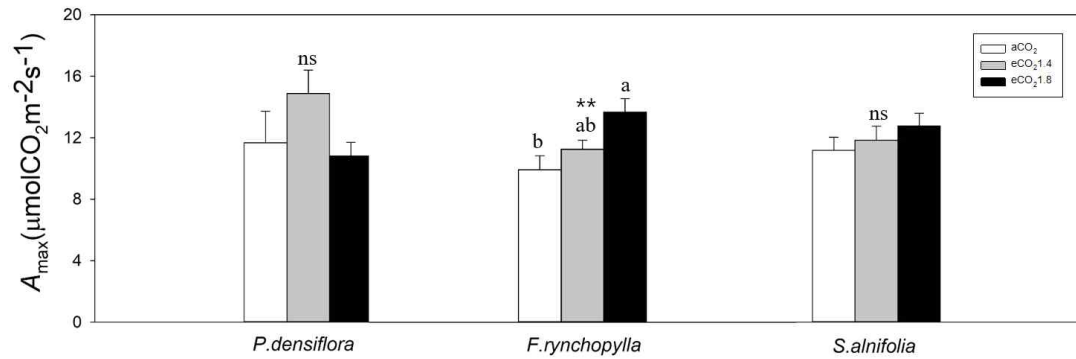


Figure 2. The average of light-saturated rates of photosynthesis ; A_{\max} . Different letters show statistical significance among treatments [open columns (ambient), gray columns (ambient×1.4) and black columns (ambient×1.8)]. *,** represent statistical differences at $p < 0.05$ and $p < 0.01$ (three-way ANOVA followed by a Duncan post-hoc test), respectively.

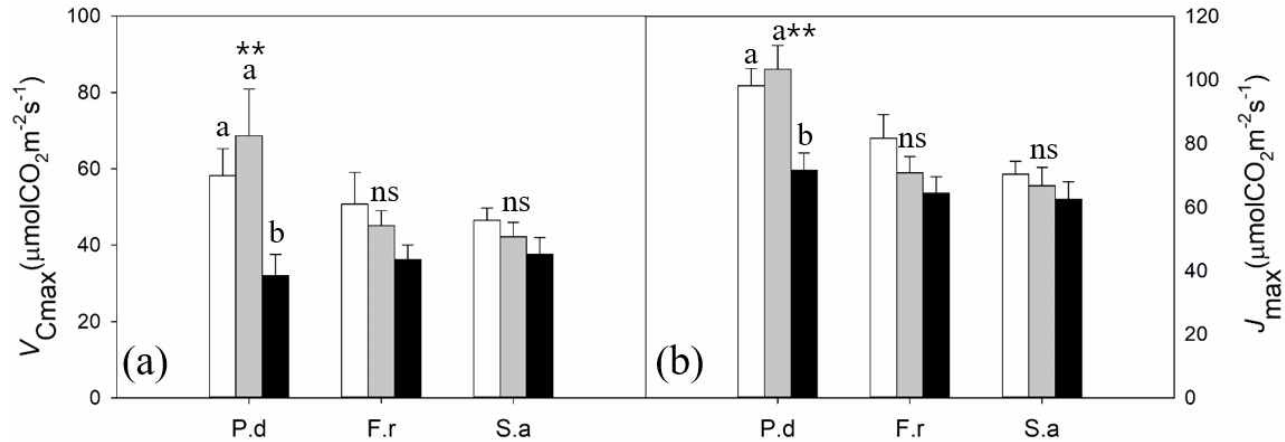


Figure 3. The average of (a) the maximum carboxylation rate ; V_{Cmax} and (b) maximum electron transport rate ; J_{max} . Different letters show statistical significance among treatments [open columns (ambient), gray columns (ambient×1.4) and black columns (ambient×1.8)]. *,** represent statistical differences at $p < 0.05$ and $p < 0.01$ (three-way ANOVA followed by a Duncan post-hoc test), respectively.

3.4. Total leaf N content and C:N ratio

Leaf N content per unit dry mass (N_{mass}) decreased under elevated CO_2 in all species ($p < 0.001$, Table 3). Especially, in Japanese red pine and Korean ash, reduction of N_{mass} occurred by 17.7% and 20.6%, respectively, at $e\text{CO}_2$ 1.8 than $a\text{CO}_2$ in the middle canopy (Table 4). N_{mass} also showed difference among species ($p < 0.001$, Table 3), in the order of Korean ash ($1.43 \pm 0.04 \text{ g g}^{-1}$), Korean whitebeam ($1.25 \pm 0.03 \text{ g g}^{-1}$) and Japanese red pine ($0.95 \pm 0.02 \text{ g g}^{-1}$).

Unlike N_{mass} , in case of total leaf N content per unit area (N_{area}), there was no significant difference among treatments but showed differences among canopy positions (Table 3). Even though there was no differences with the treatments, N_{area} of Korean ash significantly decreased by 20.9% at $e\text{CO}_2$ 1.4 compared to $a\text{CO}_2$ ($p = 0.032$). Among canopy positions, the lower canopy ($1.53 \pm 0.07 \text{ g m}^{-2}$) leaves tended to have less N_{area} in lower than other canopy; middle ($1.47 \pm 0.07 \text{ g m}^{-2}$) and the upper ($1.53 \pm 0.08 \text{ g m}^{-2}$) (Table 3). In addition, the similar to N_{mass} there was difference among species in N_{area} (Table 3).

The C:N ratio increased with canopy height and CO_2 treatment ($p < 0.001$). Overall, the $e\text{CO}_2$ 1.4 (45.3 ± 1.6) and $e\text{CO}_2$ 1.8 (43.3 ± 1.5) had higher C:N ratio than $a\text{CO}_2$ (38.2 ± 1.5) ($p = 0.003$), but there was difference among species; differences in Japanese red pine ($p = 0.008$) and Korean ash ($p = 0.001$) vs. no differences in Korean whitebeam ($p = 0.050$). The ratios at upper canopy (45.9 ± 1.8) was the highest ($p = 0.038$) and it was followed by the middle (41.0 ± 1.4) and the lower canopy (40.6 ± 1.5) without differences between them. There were differences among species as well, in the order of Japanese red pine (54.9 ± 1.1), Korea whitebeam (38.1

± 1.1) and Korean ash (33.8 ± 1.0) ($p < 0.001$, Table 3).

Table 3. Three-way ANOVA for leaf nitrogen parameters.

Term	Variable								
	N_{mass}	N_{area}	C:N ratio	Rubisco content	NF _{Rub}	chlorophyll content	NF _{chl}	cell wall content	NF _{cw}
Chamber	<i>p</i> < 0.001	0.24	<i>p</i> < 0.001	<i>p</i> < 0.001	0.016	0.011	<i>p</i> < 0.001	<i>p</i> < 0.001	0.823
Species	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001
Canopy	0.046	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	0.653	0.146
Species×Chamber	0.154	0.037	0.552	0.184	0.021	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001
Canopy×Chamber	0.940	0.291	0.048	0.184	0.317	0.050	0.579	0.447	0.388
Canopy×Species	0.018	0.037	0.869	<i>p</i> < 0.001	0.008	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	0.367
Chamber×Species×Canopy	0.853	0.982	0.567	0.302	0.523	0.002	0.003	0.251	0.036

Table 4. The average of leaf N per unit dry mass ($\text{g g}^{-1},\%$) and leaf N per unit area (g m^{-2}) in each three canopy positions.

Species	Treatments	N_{mass}			N_{area}		
		Upper	Middle	Lower	Upper	Middle	Lower
<i>Pinus densiflora</i>	aCO ₂	0.99 ± 0.07 _a	1.09 ± 0.06 _a	1.04 ± 0.11 _a	2.07 ± 0.19 _a	2.03 ± 0.13 _a	2.03 ± 0.15 _a
	eCO ₂ 1.4	0.84 ± 0.04 _a	0.89 ± 0.03 _b	1.02 ± 0.10 _a	2.12 ± 0.17 _a	2.12 ± 0.23 _a	2.23 ± 0.15 _a
	eCO ₂ 1.8	0.85 ± 0.02 _a	0.89 ± 0.03 _b	0.96 ± 0.03 _a	2.16 ± 0.14 _a	1.98 ± 0.10 _a	1.77 ± 0.09 _a
<i>Fraxinus rhynchophylla</i>	aCO ₂	1.66 ± 0.13 _a	1.66 ± 0.09 _a	1.60 ± 0.13 _a	1.59 ± 0.20 _a	1.64 ± 0.16 _a	1.21 ± 0.12 _a
	eCO ₂ 1.4	1.26 ± 0.12 _a	1.28 ± 0.09 _b	1.33 ± 0.11 _a	1.30 ± 0.11 _a	1.23 ± 0.08 _a	0.97 ± 0.06 _a
	eCO ₂ 1.8	1.39 ± 0.13 _a	1.36 ± 0.12 _b	1.36 ± 0.16 _a	1.49 ± 0.15 _a	1.51 ± 0.13 _a	0.96 ± 0.05 _a
<i>Sorbus alnifolia</i>	aCO ₂	1.18 ± 0.09 _a	1.38 ± 0.07 _a	1.47 ± 0.10 _a	0.97 ± 0.08 _a	0.91 ± 0.05 _a	0.83 ± 0.04 _a
	eCO ₂ 1.4	0.99 ± 0.07 _a	1.26 ± 0.05 _a	1.17 ± 0.15 _a	1.01 ± 0.04 _a	0.94 ± 0.04 _a	0.75 ± 0.08 _a
	eCO ₂ 1.8	1.14 ± 0.09 _a	1.29 ± 0.06 _a	1.29 ± 0.07 _a	1.10 ± 0.13 _a	0.87 ± 0.08 _a	0.61 ± 0.08 _a

Different letters show statistical significance among treatments at $p < 0.05$ (three-way ANOVA followed by a Duncan post-hoc test).

3.5. Photosynthetic nitrogen use efficiency

Overall, there was enhancement in PNUE at eCO₂1.8 (171.0 ± μmol (mol N)⁻¹ s⁻¹) than aCO₂ (123.8 ± μmol (mol N)⁻¹ s⁻¹) (Table 2). PNUE of Korean ash was higher by 42.7% at eCO₂ (201.7 ± 12.7μmol (mol N)⁻¹ s⁻¹) compared to aCO₂ (115.5 ± 9.8μmol (mol N)⁻¹ s⁻¹) ($p < 0.001$, Figure 4). In Japanese red pine and Korean whitebeam, PNUE tend to increase at eCO₂ but there were no significant ($p=0.336$, $p=0.248$). PNUE showed differences among species in order of Korean whitebeam (193.0 ± 11.4μmol (mol N)⁻¹ s⁻¹), Korean ash (163.8 ± 9.2μmol (mol N)⁻¹ s⁻¹) and Japanese red pine (89.2 ± 6.9μmol (mol N)⁻¹ s⁻¹) (Table 2).

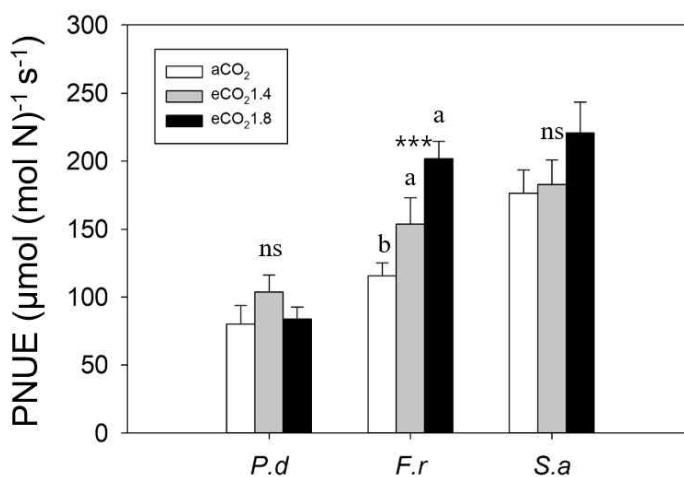


Figure 4. The average of photosynthetic nitrogen use efficiency (PNUE). Different letters show statistical significance among treatments [open columns (ambient), gray columns (ambient×1.4) and black columns (ambient×1.8)]. *, **, *** represent statistical differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ (three-way ANOVA followed by a Duncan post-hoc test), respectively.

3.6. Leaf N allocation

Nitrogen in Rubisco

Similar to leaf *N*, Rubisco content showed differences among species, treatment and canopy positions (Table 3). Rubisco content was highest in Korean ash ($1.28 \pm 0.04 \text{ g m}^{-2}$) and followed by Korean whitebeam ($1.08 \pm 0.04 \text{ g m}^{-2}$) and Japanese red pine ($0.75 \pm 0.04 \text{ g m}^{-2}$) ($p < 0.001$). For CO_2 treatment, Rubisco contents were lower at $\text{eCO}_2 1.8$ ($p < 0.001$, Table 3) but showed different tendency among species. Only in Korean whitebeam, there was significant difference among treatments in Rubisco content ($p = 0.002$). In case of canopy positions, upper canopy leaves had more Rubisco content by 29.6% and 36.0% than the middle and the lower canopy, respectively ($p < 0.001$). Furthermore, reduction in Rubisco content was higher in the middle canopy (22.1%) at $\text{eCO}_2 1.8$ compared to aCO_2 ($p = 0.028$) and followed by the lower (19.4%) and the upper canopy (14.8%). Especially, Korean whitebeam showed pronounced reduction in Rubisco in middle canopy ($p = 0.002$, Table 5), but Japanese red pine and Korean ash had similar tendencies without significance.

Like Rubisco content, fraction of Rubisco content showed the differences among species, treatments and canopy positions (Table 3). Fraction of Rubisco content was highest in Korean whitebeam (0.20 ± 0.06) and followed by Korean ash (0.17 ± 0.05) and Japanese red pine (0.06 ± 0.03) ($p < 0.001$). The fraction of Rubisco nitrogen (NF_{Rub}) decreased at $\text{eCO}_2 1.8$ ($p < 0.001$), especially in Korean whitebeam, the reduction was 15.7% at $\text{eCO}_2 1.8$ (0.18 ± 0.01) compared to aCO_2 (0.22 ± 0.01) ($p = 0.011$). Rubisco fraction decreased with crown depth ($p < 0.001$). Furthermore, there was an interaction effect between species and canopy positions ($p = 0.008$). Therefore, in Korean whitebeam, upper and lower canopy of NF_{Rub} significantly decreased at $\text{eCO}_2 1.4$ and $\text{eCO}_2 1.8$ than ambient in Korean

whitebeam (maximum $p = 0.027$). However, there were no differences among treatments in Japanese red pine and Korean ash in all canopy (Figure 5a).

Table 5. The average of total Rubisco content per unit area (g m^{-2}) and chlorophyll content per unit area (g m^{-2}) in each three canopy positions.

Species	Treatments	Rubisco content			chlorophyll content		
		Upper	Middle	Lower	Upper	Middle	Lower
<i>Pinus densiflora</i>	aCO ₂	1.19 ± 0.12 _a	0.57 ± 0.07 _a	0.63 ± 0.08 _a	0.10 ± 0.01 _a	0.12 ± 0.01 _b	0.06 ± 0.00 _b
	eCO ₂ 1.4	1.41 ± 0.17 _a	0.53 ± 0.05 _a	0.63 ± 0.09 _a	0.09 ± 0.01 _{ab}	0.14 ± 0.01 _a	0.07 ± 0.00 _{ab}
	eCO ₂ 1.8	1.09 ± 0.06 _a	0.47 ± 0.04 _a	0.43 ± 0.06 _a	0.08 ± 0.01 _b	0.12 ± 0.00 _b	0.08 ± 0.01 _a
<i>Fraxinus rhynchophylla</i>	aCO ₂	2.06 ± 0.15 _a	1.48 ± 0.12 _a	1.06 ± 0.09 _a	0.22 ± 0.01 _{ab}	0.20 ± 0.01 _b	0.20 ± 0.00 _a
	eCO ₂ 1.4	1.59 ± 0.17 _a	1.21 ± 0.08 _a	1.17 ± 0.09 _a	0.19 ± 0.01 _b	0.19 ± 0.01 _b	0.17 ± 0.01 _b
	eCO ₂ 1.8	1.73 ± 0.12 _a	1.31 ± 0.10 _a	1.01 ± 0.06 _a	0.25 ± 0.02 _a	0.24 ± 0.02 _a	0.18 ± 0.00 _b
<i>Sorbus alnifolia</i>	aCO ₂	1.38 ± 0.09 _a	1.31 ± 0.11 _a	0.93 ± 0.05 _a	0.14 ± 0.00 _b	0.18 ± 0.00 _b	0.14 ± 0.01 _a
	eCO ₂ 1.4	1.12 ± 0.09 _a	1.09 ± 0.09 _{ab}	0.96 ± 0.09 _a	0.16 ± 0.01 _{ab}	0.18 ± 0.01 _b	0.14 ± 0.01 _a
	eCO ₂ 1.8	1.24 ± 0.13 _a	0.99 ± 0.09 _b	0.56 ± 0.09 _b	0.17 ± 0.01 _a	0.21 ± 0.01 _a	0.15 ± 0.01 _a

Different letters show statistical significance among treatments at $p < 0.05$ (three-way ANOVA followed by a Duncan post-hoc test).

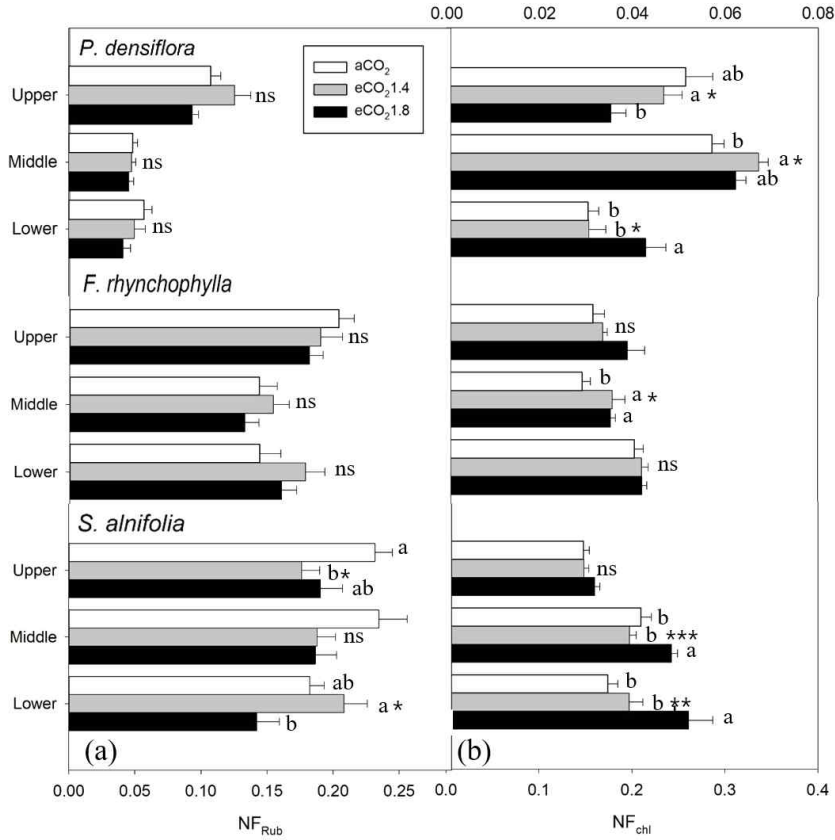


Figure 5. The average of (a) NF_{Rub} and (b) NF_{chl} in each three canopy positions. Different letters show statistical significance among treatments [open columns (ambient), gray columns (ambient \times 1.4) and black columns (ambient \times 1.8)]. *, **, *** represent statistical differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ (three-way ANOVA followed by a Duncan post-hoc test), respectively.

Nitrogen in Chlorophyll

Similar to Rubisco, chlorophyll content and its fraction were significantly different among species, treatment and canopy positions (Table 3). Korean ash ($0.20 \pm 0.00 \text{ g m}^{-2}$) had the highest chlorophyll content followed by Korean whitebeam ($0.17 \pm 0.00 \text{ g m}^{-2}$) and Japanese red pine ($0.10 \pm 0.00 \text{ g m}^{-2}$) ($p < 0.001$). However, unlike Rubisco, which showed reduction in content and fraction, chlorophyll content increased at $e\text{CO}_2 1.8$ in all species (maximum $p = 0.008$). Within canopy, chlorophyll content decreased with depth ($p < 0.001$). In addition, there were several interaction effects (Table 3). In Japanese red pine, chlorophyll content increased by 24.4% and 13.3% under $e\text{CO}_2$ respectively than $a\text{CO}_2$ in middle canopy and lower canopy (maximum $p = 0.04$). In upper canopy, chlorophyll content was highest at $a\text{CO}_2$ but it was not statistically significant ($p = 0.057$). Chlorophyll content of Korean ash in middle and upper canopy also increased at $e\text{CO}_2 1.8$, similar to Japanese red pine (maximum $p = 0.031$ and Table 5). In contrast, chlorophyll content enhanced by 11.3% at $a\text{CO}_2$ compared to $e\text{CO}_2$ in lower canopy ($p < 0.001$, Table 5). In case of Korean whitebeam, increase of chlorophyll content showed in all canopy, but there was no significant difference in lower canopy. In middle and upper canopy, chlorophyll content increased by 12.5% and 18.1% at $e\text{CO}_2 1.8$ than $a\text{CO}_2$, respectively (maximum $p = 0.042$) (Table 5).

The increase of chlorophyll content resulted in the increase of NF_{chl} ($p < 0.001$, Table 3) and showed similar tendency in species and canopy. In the middle and lower canopy, enhancement of NF_{chl} was observed by 16.2% and 16.0% but in the upper canopy was only 6.5%. In Japanese red pine increased showed increment by 28.9% and 15.0% at $e\text{CO}_2$ compared to $a\text{CO}_2$ in lower and middle canopy, respectively. ($p = 0.030$). On the other hand, NF_{chl} was improved by 31.6% at $a\text{CO}_2$ (0.52 ± 0.05) than $e\text{CO}_2 1.8$ (0.35 ± 0.03) in upper canopy ($p = 0.042$). Korean ash showed increment of NF_{chl} at

eCO₂1.8 compared to aCO₂, but there was significant difference among treatments only in middle canopy ($p = 0.049$). Korean whitebeam also tended to be higher by 13.4% and 33.3% at eCO₂1.8 than aCO₂ in middle canopy ($p < 0.001$) and lower canopy ($p = 0.006$, Figure 5b).

Nitrogen in Cell wall

Cell wall nitrogen content decreased by 33.4% under eCO₂ compared to aCO₂ especially in Korean ash ($p < 0.001$). Cell wall nitrogen content of Korean ash at eCO₂1.8 was lower by 36.1%, 39.6% and 57.8% than aCO₂ in the lower, middle and upper canopy, respectively (maximum $p = 0.023$ and Table 6). In Japanese red pine and Korean whitebeam, the average of cell wall nitrogen content tended to be higher at eCO₂ but were not significant. There was significant difference among species ; Korean ash ($0.56 \pm 0.98 \text{ g m}^{-2}$), Korean whitebeam ($0.44 \pm 0.72 \text{ g m}^{-2}$) and Japanese red pine ($0.25 \pm 0.01 \text{ g m}^{-2}$)

Decrease of cell wall nitrogen fraction (NF_{cw}) also occurred at eCO₂ (0.48 ± 0.35) than aCO₂ (0.62 ± 0.24) like cell wall nitrogen content in Korean ash ($p < 0.001$) In all canopy, NF_{cw} of Korean ash tended to be lower at eCO₂1.8 , but there were no difference among treatments in all canopy (Table 6). Japanese red pine in middle canopy decreased in NF_{cw} at eCO₂1.8. In Korean whitebeam, NF_{cw} tended to be higher at eCO₂1.8 at all canopy, but there was no difference.

Table 6. The average of cell wall nitrogen content (g m^{-2}), cell wall nitrogen fraction per canopy position.

Species	Treatments	Cell wall nitrogen content			Cell wall nitrogen fraction (NF_{cw})		
		Upper	Middle	Lower	Upper	Middle	Lower
<i>Pinus densiflora</i>	aCO ₂	0.63 ± 0.07 _a	0.62 ± 0.12 _a	0.99 _a ± 0.09 _a	0.32 ± 0.04 _a	0.25 ± 0.04 _a	0.50 ± 0.06 _a
	eCO ₂ 1.4	0.75 ± 0.06 _a	0.88 ± 0.15 _a	0.79 _a ± 0.04 _a	0.36 ± 0.01 _a	0.43 ± 0.05 _a	0.39 ± 0.05 _a
	eCO ₂ 1.8	0.71 ± 0.03 _a	0.96 ± 0.21 _a	0.74 _a ± 0.07 _a	0.33 ± 0.02 _a	0.40 ± 0.03 _b	0.38 ± 0.05 _a
<i>Fraxinus rhynchophylla</i>	aCO ₂	1.35 ± 0.11 _a	0.94 ± 0.13 _a	0.79 ± 0.09 _a	0.64 ± 0.04 _a	0.58 ± 0.05 _a	0.65 ± 0.03 _a
	eCO ₂ 1.4	0.67 ± 0.19 _b	0.59 ± 0.05 _b	0.53 ± 0.04 _b	0.45 ± 0.07 _a	0.49 ± 0.06 _a	0.55 ± 0.03 _a
	eCO ₂ 1.8	0.57 ± 0.05 _b	0.57 ± 0.09 _b	0.50 ± 0.08 _b	0.41 ± 0.05 _a	0.39 ± 0.04 _a	0.50 ± 0.07 _a
<i>Sorbus alnifolia</i>	aCO ₂	0.52 ± 0.09 _a	0.40 ± 0.05 _a	0.33 ± 0.05 _a	0.55 ± 0.10 _a	0.43 ± 0.04 _a	0.40 ± 0.05 _a
	eCO ₂ 1.4	0.56 ± 0.04 _a	0.44 ± 0.05 _a	0.37 ± 0.08 _a	0.53 ± 0.02 _a	0.48 ± 0.06 _a	0.46 ± 0.06 _a
	eCO ₂ 1.8	0.59 ± 0.07 _a	0.51 ± 0.07 _a	0.32 ± 0.05 _a	0.55 ± 0.04 _a	0.53 ± 0.05 _a	0.53 ± 0.05 _a

Different letters show statistical significance among treatments at $p < 0.05$ (three-way ANOVA followed by a Duncan post-hoc test).

4. Discussion

4.1. Changes in leaf morphological characteristics and relationship with nitrogen concentration under elevated CO₂

Increasing leaf size (Ainsworth & Long 2005), LMA (Poorter *et al.*, 2009) cell size (Riikonen, Percy *et al.* 2010) under elevated CO₂ have been reported. In this study, leaf size increased under elevated CO₂ in all species but reacted differently depending on the CO₂ concentration. Korean ash and Japanese red pine showed the highest leaf size at eCO₂1.8, but leaf size in Korean whitebeam, increased at eCO₂1.4 (Figure 1). The CO₂ effect not only caused these morphological changes but also chemical changes such as reduction of leaf nitrogen per unit mass (N_{mass}) (Yin, 2002; Kovenock & Swann, 2018). N_{mass} at elevated CO₂ decreased significantly in all species while C:N ratio increased. These results are in agreement with increasing LMA represented by the carbon cost of constructing the leaf structure. In previous studies, the decrease of N_{mass} was related leaf thickness and density (Tricker, Calfapietra *et al.* 2004) and accumulation of nonstructural carbohydrates such as starch and soluble sugar concentration was reported to be associated with photosynthesis acclimation (Nowak *et al.*, 2004 ; Ibrahim *et al.*, 2010).

4.2. Changes of photosynthetic characteristics and nitrogen allocation characteristics under elevated CO₂

Most plants are unable to maintain their initial growth due to acclimation in an environment with increasing CO₂ (Sage 1994 ; Reich, 2018) and this results is attributed to photosynthetic acclimation; a reduction in the carboxylation of Rubisco (Ainsworth & Rogers 2007 ; Rogers & Humphries 2000). In a WTC experiment with *Eucalyptus globulus*, Rubisco content was reduced by 16% and 51%, respectively, in the upper and lower canopy under elevated CO₂ (Sharwood, Crous *et al.* 2017). In this study, Rubisco content was significantly decreased by 16.5% at eCO₂1.8 compared to aCO₂, but the degree of response to the CO₂ concentration was different depending on canopy position by species. Overall, in the middle canopy, Rubisco content decreased significantly. In case of the upper canopy and the lower canopy, Rubisco content decreased by 14.8% and 19.3% at eCO₂1.8, respectively, compared to aCO₂ but was not significant. Rubisco content of Korean whitebeam decreased significantly with increasing CO₂ concentration, and NF_{Rub} also showed a tendency to decrease. Results of in vivo experiment

(maximum carboxylation Rubisco capacity; V_{cmax} and maximum rate of electron transport ; J_{max}) also observed photosynthetic acclimation. The V_{cmax} was decreased significantly at $e\text{CO}_2$ 1.8 and J_{max} also showed similar tendency. These are associated with decreased leaf N that is largely invested in photosynthetic organs under elevated CO_2 (Hikosaka & Shigeno, 2009), resulting in a decreased V_{cmax} and J_{max} (reviewed in Long *et al.*, 2004; Nowak *et al.*, 2004; Ainsworth & Rogers, 2007). The N_{mass} decreased with increasing CO_2 concentration in all species in OTC, but leaf nitrogen per unit area (N_{area}) was no significant result among treatments. The N_{area} of Korean ash only significantly decreased at $e\text{CO}_2$ 1.8. Despite the increase in LMA under elevated CO_2 , which resulted increment thickness, the fact that there was no difference in N_{area} can be judged that the concentration of leaf nitrogen content per unit area actually decreased.

In this study, Rubisco content was decreased as decreasing N_{mass} at $e\text{CO}_2$ 1.4 and 1.8, but chlorophyll content was increased significantly under elevated CO_2 . chlorophyll content at $e\text{CO}_2$ showed higher chlorophyll content of *Korean ash* and *Korean whitebeam* than $a\text{CO}_2$. Chlorophyll nitrogen fraction (NF_{chl}) was also increased at two elevated CO_2 chamber, especially at $e\text{CO}_2$ 1.8. In previous studies, Leaf chlorophyll content declined under elevated CO_2 (Mu *et al.*, 2016), and in contrast, NF_{chl} in *Robinia pseudoacacia L* increased by 22% for 4 years in FACE experiment. This result is that more nitrogen is invested in light harvesting than Rubisco which fixes carbon to maintain the similar photosynthetic rate even at low Rubisco content under high concentration of CO_2 (Choi, Watanabe *et al.* 2017).

4.3. Changes in nitrogen allocation characteristics under elevated CO_2 and canopy position

Allocated nitrogen in Rubisco was significantly increased in upper canopy relative to middle and lower canopy and chlorophyll nitrogen fraction decreased with increasing canopy height. This result is consistent with previous studies (Weerasinghe, 2014 ; Ellsworth, 2012; Seneweera *et al.*, 2011). Compared among chambers, NF_{rub} was decreased at $e\text{CO}_2$ 1.8 in upper canopy than other canopy. This is consistent with in previous studies that the lower canopy showed weaker responses with elevated CO_2 (Sharwood *et al.*, 2017). This result appears to be due to the assumption that the canopy difference in the light environment can affect the response to CO_2 (Kubiske *et al.*, 2002)

4.4. Correlation between increase of PNUE and changes in nitrogen allocation characteristics under elevated CO₂

PNUE is defined as net amount of CO₂ assimilated per unit of leaf N. C3 plants were expected to reduce leaf N to maximize carbon uptake efficiency and FACE experiments showed improved PNUE under high concentrations of CO₂ (Leakey, Ainsworth *et al.*, 2009). On the other hand, PNUE was lower than aCO₂, but statistically not significant (Choi, Watanabe *et al.*, 2017). In this study, PNUE increased with elevated CO₂. In particular, Korean ash significantly increased at eCO₂1.8 (Figure 4). Previous studies have shown that PNUE is positively correlated with NF_{rub} but not significant (Hikosaka & Shigeno 2009) and LMA, leaf life span, and cell wall nitrogen are negatively correlated with PNUE (Onoda, Hikosaka *et al.* 2004). In contrast, It was also reported that the increase in LMA resulted in a decrease in PNUE, but this was not related to cell wall nitrogen fraction. But not with cell wall , was also reported (Harrison, Edwards *et al.* 2009).

In OTC experiment, LMA and PNUE had the similar negative correlation with the previous studies, and the strongest correlation was found at eCO₂1.8 and aCO₂ (Figure 8a). LMA at eCO₂1.4 were statistically significant but relatively weak correlations. In case of NF_{cw}, there was a weak linear relationship between NF_{cw} and PNUE at eCO₂1.8, aCO₂ and 1.4 were not statistically significant (Figure 6b). NF_{cw} was important nitrogen sink but not directly affected PNUE (Hikosaka & Shigeno 2009). As a result of photosynthesis nitrogen analysis, NF_{rub} and NF_{chl} showed a significant correlation with PNUE in all chamber (Figure 7). NF_{rub} showed the strongest correlation with PNUE at eCO₂1.8, and PNUE increased as the fraction of Rubisco nitrogen increased (Figure 7a). NF_{chl} showed the strongest linear relationship at aCO₂, and there was a strong correlation between PNUE and NF_{chl} (Figure 7b). Therefore, the increase of PNUE is considered to be due to the increase of NF_{chl}.

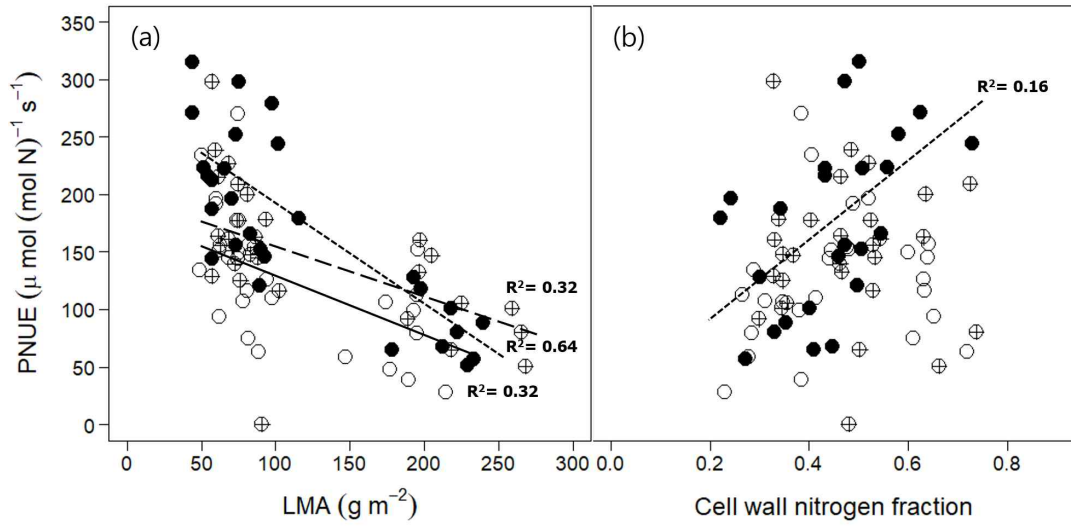


Figure 6. Leaf mass per area and cell wall nitrogen fraction (NF_{cw}) changes on PNUE at aCO₂ (unfilled circle), eCO₂1.4 (crossed circle) and eCO₂1.8 (filled circle). (a) LMA, (b) cell wall nitrogen fraction. Trend lines represent LMA and NF_{cw} -PNUE linear relationships (aCO₂ ; solid line (a) $R^2=0.32$ $p < 0.001$, eCO₂1.4 ; long-dashed line (a) $R^2=0.32$ $p < 0.001$, Ch1.8 ; dotted line (a) $R^2=0.64$ $p < 0.001$ (b) $R^2=0.16$ $p = 0.027$).

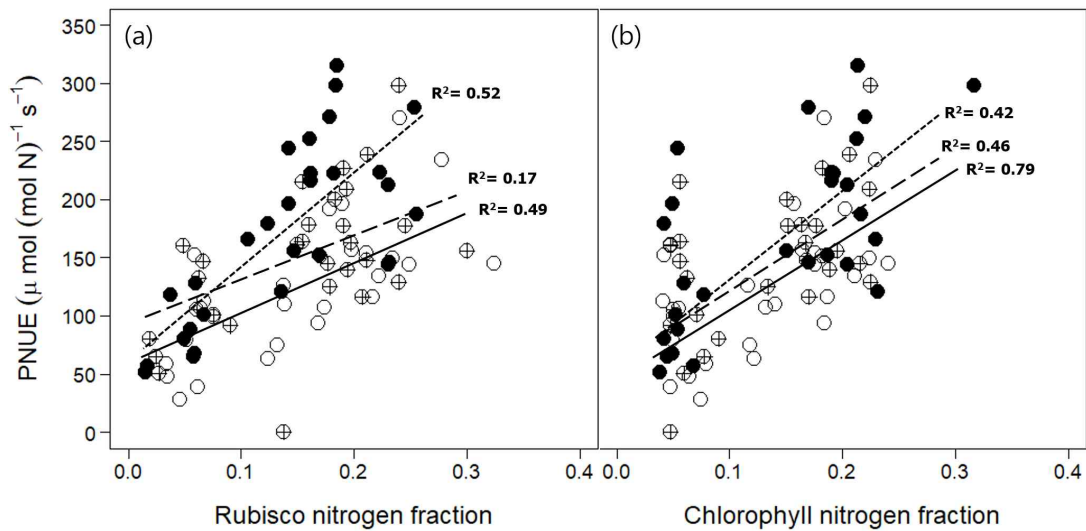


Figure 7. (a) Rubisco nitrogen fraction (NF_{Rub}) and (b) chlorophyll nitrogen fraction (NF_{chl}) changes on PNUE at aCO₂ (unfilled circle), eCO₂1.4 (crossed circle) and eCO₂1.8 (filled circle). Trend lines represent Rubisco nitrogen fraction and chlorophyll-PNUE linear relationships (aCO₂ ; solid line (a) $R^2=0.49$ $p < 0.001$, (b) $R^2=0.79$ $p < 0.001$ eCO₂1.4 ; long-dashed line (a) $R^2=0.17$ $p = 0.017$, (b) $R^2=0.46$ $p = 0.01$ and eCO₂1.8 ; dotted line (a) $R^2=0.52$ $p < 0.001$ (b) $R^2=0.42$ $p < 0.001$).

4.5. Progressive nitrogen limitation and sustainability of forest productivity through nitrogen allocation characteristics

In FACE experiments of exposure to CO₂ enrichment for a long term, sweetgum decreased leaf nitrogen concentration due to the decrease of soil inorganic nitrogen (Dijkstra *et al.*, 2010), and enhancement of NPP decreased from 24% to 9%. In addition to decrease in soil N availability, these results suggest a progressive nitrogen limitation (PNL) hypothesis (Norby, Warren *et al.*, 2010). However, the Duke FACE experiment with loblolly pine did not show a decrease in productivity from 1996 to 2004 (McCarthy, Oren *et al.* 2010; Zak *et al.*, 2011) and nitrogen redistribution may improve productivity under high CO₂ (Kant *et al.*, 2012) which makes it difficult to predict forest productivity in the future climate environment. In this study, there was no significant difference in diameter among chamber in the last 2 years (Table 1).

Total Rubisco content decreased under high concentration of carbon dioxide due to decrease of leaf N_{mass}, V_{cmax} and J_{max} decreased. Although leaf N_{mass} decreased under elevated CO₂ condition, total chlorophyll content and NF_{chl} was higher at eCO₂1.8 compared to aCO₂. NF_{chl} showed a strong positive correlation with PNUE at all chambers, and PNUE increased under elevated due to increased NF_{chl}. Thus, productivity is likely to be increased or maintained at a limited amount of nitrogen by increased PNUE. It is considered only nitrogen so we need to conduct additional experiments over a long period of time.

5. Conclusion

After nine years of CO₂ enrichment, symptoms of nitrogen limitation were rampant in the study site. For examples, there were reductions in $V_{C_{max}}$ and J_{max} , and these reductions were attributable to the reduction of leaf N . In addition, there were substantial changes in morphological such as increase of leaf size and thickness. On the other hand, despite the decrease of leaf nitrogen, the amount of nitrogen invested in light harvesting chlorophyll increased under elevated CO₂ and it showed a strong correlation with PNUE. Therefore, the increase of leaf biomass is considered to be due to the increase of PNUE due to increased chlorophyll.

In conclusion, plants are supposed to supplement the amount of nitrogen that is reduced by effective use of light under the future climate, which is likely to result in an increase or maintenance of productivity. However, this is what happens in the process of reducing nitrogen, and further research is needed for longer-term changes.

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Abstract in Korean

이산화탄소 폭로 처리에 따른 질소 제한 환경 하에서 임목의 광합성과 질소 분배 반응

증가된 대기 중 이산화탄소는 생리적 특성인 광합성률, 기공전도도 변화 뿐 아니라 잎과 줄기의 바이오매스 증가와 같은 형태적 특성 또한 변화시킨다. 고농도의 이산화탄소하에서 순일차생산량이 증가할 것으로 예상되었으나 질소가 제한된 환경에서는 광합성 저감으로 인해 초기생장을 유지하지 못하게 되는 결과를 가져오게 된다. 질소는 식물 생장의 제한요소로서 잎 질소의 대부분은 광합성 및 기능적 기관에 투자되어 있으며 이러한 질소분배는 수관, 수중, 환경에 따라 변화하며 광합성 질소이용효율에 영향을 끼치게 된다. 본 연구는 장기간 상부개방형 온실을 이용하여 이산화탄소 농도 변화로 인한 제한된 질소 환경에서 소나무(*Pinus densiflora*), 물푸레나무(*Fraxinus rhynchophylla*), 팔배나무(*Sorbus alnifolia*)의 광합성 특성 및 질소분배 특성 변화를 알아보고자하였다. 최근 2년간 온실 간 직경성장량 차이가 나타나지 않았으나 잎 크기와 엽중량비(leaf mass per area) C:N비와 같은 형태적 인자는 대조구에 비해 고농도의 이산화탄소 온실인 챔버1.4와 1.8에서 높았다. 광합성 인자의 경우, 최대광합성 속도는 챔버1.4와 1.8에서 대조구에 비해 증가하였으며, 특히 물푸레나무에서 유의하게 증가하였다. 반면 광합성 능력인 최대카르복실화 속도(V_{Cmax})와 최대전자전달속도(J_{max})는 챔버1.8에서 감소하였다. 토양질소 분석 결과, 토양 전질소 함량은 챔버 간 차이가 발생하지 않았으며, 토양 무기태 질소 또한 별다른 차이를 보이지 않았다. 광합성 능력의 감소는 이산화탄소 증가에 따른 엽면적의 증가로 인한 단위질량당 잎 질소 감소에 의해 발생하며 본 연구결과에서도 모든 수중에서 대조구에 비해 챔버1.4와 1.8에서 유의하게 감소하였다. 반면 단위면적 당 잎 질소함량은 처리구간 차이가 나타나지 않았다. 단위 질량당 잎 질소함량의 감소로 인하여 루비스코 함량 또한 챔버1.8에서 유의하게 감소하였으나 엽록소 질소 함량은 이산화탄소 농도가 증가할수록 증가하였으며 엽록소에 투자된 질소비율 또한 유의하게 증가하였다. 수관별 분석결과에서는 수관하부에 비해 수관상부의 루비스코 질소비율이 고농도의 이산화탄소에서 유의하게 감소하였으나 엽록소 질소비율은 모든 수관에서 이산화탄소 농도에 따른 차이가 발생하지 않았다. 본 연구결과에서 나타난 챔버1.8에서의 광합성 질소이용효율의 증가는 질소분배 특성인 세포벽 질소비율, 루비스코 질소비율과 엽록소 질소비율 중 광합성 인자인 루비스코 질소비율과 엽록소질소비율과 강한 상관관계를 보였으며 세포벽 질소비율과는 상관관계를 보이지 않았다. 따라서 고농도 이산화탄소 하에서의 엽록소 질소비율 증가로 인해 광합성 질소이용효율 증가가 나타나고, 이로 인해 광합성 특성의 감소에도 불구하고 광합성의 양의 증가와 바이오매스 생산량의 유지가 일어난다고 판단된다.

keywords : 광합성, 광합성 질소이용효율, 질소분배, 질소제한, 기후변화, 고농도의 이산화탄소

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