Down-regulation of photosynthesis and its relation to changes in leaf N allocation and N availability under elevated CO_2 after long-term exposure

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Abstract

Down-regulation of photosynthesis in elevated CO_2 (eCO_2), could be attributed to depletions in nitrogen (N) availability after long term exposure to eCO_2 (progressive nitrogen limitation, PNL) or N dilution due to excessive carbon accumulation of nonstructural carbohydrates. To investigate this, we examined N availability, photosynthetic characteristics, and leaf N allocation in *Pinus densiflora*, *Fraxinus rhynchophylla*, and *Sorbus alnifolia*, grown under three different CO_2 concentrations, ambient CO_2 (aCO_2), aCO_2 × 1.4 ppm (e$CO_2$1.4), and aCO_2 × 1.8 ppm (e$CO_2$1.8), for 9 years. N availability increased under e$CO_2$1.8, and its allocation to chlorophyll (NF_{chl}) and photosynthetic N use efficiency also increased under eCO_2. The maximum carboxylation rate, leaf N per mass (N_{mass}), and N allocation to Rubisco (NF_{Rub}), however, were all lower under e$CO_2$1.8. There were interactions in NF_{chl} between canopy × species × CO_2 concentrations. The greatest changes in N allocation under eCO_2 were in the lower canopy of *S. alnifolia*, the most shade-tolerant species and this species have relatively high flexibility in N operations compared to shade-intolerant species. Based on the reduction in N_{mass} that was diluted by increased nonstructural carbohydrates and increased N availability, down-regulation of photosynthesis was found to be caused by the dilution and change in N allocations, rather than PNL.

Introduction

Increases in atmospheric CO_2 concentrations are expected to enhance photosynthetic rates in C3 plants (Drake, GonzalezMeler, & Long, 1997). Photosynthesis stimulation has previously been widely reported (Ainsworth & Long, 2005; Leakey et al., 2009), but there is uncertainty regarding the sustainability of enhanced productivity and carbon (C) storage under elevated CO_2(eCO_2) levels (Terrer et al., 2018). The uncertainty regarding the CO_2 "fertilization effect" has been partially attributed to the role of nitrogen (N) availability over long-term time scales (Hungate, Dukes, Shaw, Luo, & Field, 2003). For example, the enhancement of net primary production was mainly determined from the N availability at the eCO_2 Duke Free-air CO_2 enrichment (FACE) site (McCarthy et al., 2010). At the Oak Ridge FACE site, the enhancement of productivity diminished with time as N availability decreased (Norby, Warren, Iversen, Medlyn, & McMurtrie, 2010). A suboptimal N supply could lead to down-regulation of photosynthesis (Luo et al., 2004).

Nitrogen is among the most important limiting factors in ecosystem production. Leaf N is invested in approximately 75% of photosynthetic apparatus, with an average of 20% being invested in ribulose-1.5-

bisphosphate carboxylase/oxygenase (Rubisco). However, leaf N allocation to Rubisco can be changed to optimize productivity by various factors, including canopy position (Sharwood, Crous, Whitney, Ellsworth, & Ghannoum, 2017). Upper canopy leaves allocate a higher fraction of N to Rubisco and less to thylakoid complexes, because carboxylation limits photosynthesis more than electron transport or light harvesting in the upper canopy, where irradiance is high (Boardman, 1977). Under elevated CO_2 condition, the lower canopy leaves of *Eucalyptus globulus* have previously shown weaker responses to elevated CO_2, compared with the upper canopy, due to light differences within the canopy (Sharwood et al., 2017). Similarly, at the Duke FACE site, a reduction in Rubisco content in *Pinus taeda* was found in overwintering needles in the upper canopy under eCO_2, and these one-year-old needles later reallocated N to the current years' young growing needles (D. S. Ellsworth et al., 2012; Rogers & Ellsworth, 2002). However, only a few studies have investigated the changes in N allocation within the different canopy positions under eCO_2. A change in N allocation could provide an opportunity to increase photosynthetic N use efficiency (PNUE), which is defined as the net C assimilation rate per unit leaf N_{area} (Leakey et al., 2009). The PNUE showed interspecific variation in photosynthetic capacity among species. The PNUE was lower in evergreen than deciduous species, due to a smaller fraction of N being allocated to the Rubisco (Takashima, Hikosaka, & Hirose, 2004). However, a decrease in leaf N investment into Rubisco leads to an increased PNUE and enhanced efficiency of Rubisco under eCO_2 (Gifford, Barrett, & Lutze, 2000).

Down-regulation of photosynthesis, which involves a decrease in total N concentration (Ainsworth & Long, 2005) and amount of Rubisco (Rogers & Ellsworth, 2002), has been explained by several hypotheses, including progressive nitrogen limitation (PNL) (Luo et al., 2004), the dilution effect, a decrease in N demand and uptake, and N loss (Taub & Wang, 2008). In the case of PNL, enhanced photosynthesis via elevated CO_2, results in additional plant biomass growth in the ecosystem, which progressively decreases N availability in the soil without an increase in N input or reduction in N losses (Luo et al., 2004). The deficiency in N availability leads to a decrease, not only in N per unit mass (N_{mass}, g[?]g^{-1}) but also N per unit area (N_{area}, g[?]m^{-2}). Without sufficient N, plants cannot maintain their initial enhanced C uptake, due to the N-driven down-regulation of photosynthesis after long-term CO_2 exposure (Norby et al., 2010). Alternatively, the dilution effect caused by the excessive accumulation of nonstructural carbohydrates (NSC), which results in reduced N_{mass} under CO_2 enrichment conditions, is another commonly considered hypothesis (Kitaoka et al., 2016; Rogers & Ellsworth, 2002). However, we propose that the dilution effect should not be confused with a reduction in N_{area} because a decrease in N_{area} under eCO_2 is commonly attributed to a reduction in the rate of N uptake (McDonald, Erickson, & Kruger, 2002) or the availability of N (Norby et al., 2010). When an NSC (e.g., glucose and starch), excessively accumulates under elevated CO_2 conditions, sugar suppresses the gene expression of Rubisco (Moore, Cheng, Sims, & Seemann, 1999). The NSC accumulation is accelerated under eCO_2 and low N availability, due to imbalances between the sink (biomass accumulation) and source organs (mature photosynthetic leaves), but not under high N availability (Sugiura, Watanabe, Betsuyaku, & Terashima, 2017). However, this hypothesis has not been described in detail (Taub & Wang, 2008) and carbohydrate accumulation has not been shown to directly affect the gene expression of the photosynthetic apparatus in previous studies (Araya, Noguchi, & Terashima, 2010).

To verify the PNL and dilution effects hypotheses, we investigated N availability, leaf N concentrations, and N allocations, under different canopy positions, with three different temperate species, grown under three different CO_2 concentrations (ambient, ambient × 1.4 ppm, and ambient × 1.8 ppm), for 9 years. The objectives of our study were to: (i) verify the occurrence of the down-regulation of photosynthesis at this site; (ii) determine whether the PNL or dilution effects hypothesis best explained photosynthesis down-regulation (iii) quantify the variations in the responses to the CO_2effects according to the species and different canopy positions, and determine which species showed flexibility under elevated CO_2 conditions.

Materials and Methods

Study Site

This investigation was conducted at the open top chamber (OTC) site at the National Institute of Forest Science (37°15'04"N, 136°57'59"E) in South Korea. The annual precipitation at the site is 1328.6 mm[?]yr^{-1}

and rainfall is highly seasonal with 78% occurring between July and August. Data for this experiment were collected over a 9-year period; the annual rainfall varied from 751.16 mm yr^{-1} to 1975.96 mm[?]yr^{-1}. The mean annual temperature was 12.6 °C, and the monthly mean temperature was 26.5 °C in July, which was the highest temperature recorded in 2017. The OTC experiment consisted of 3 decagonal chambers (10 m in diameter by 7 m in height), with different atmospheric CO_2concentrations: ambient (aCO_2), ambient × 1.4 ppm (e$CO_2$1.4), and ambient × 1.8 ppm (e$CO_2$1.8) (one OTC per treatment). The seedlings of three temperate species, Korean red pine, an evergreen and shade-intolerant species (*Pinus densiflora* Siebold & Zucc.)*,* Korean ash, a deciduous and shade-tolerant species (*Fraxinus rhynchophylla* HANCE), and Korean whitebeam, a deciduous and the most shade-tolerant species tested (*Sorbus alnifolia*(Siebold & Zucc.) K.koch), were planted in September 2009 and there were three replicate trees in each chamber. In April 2009, the soil was excavated to a depth of 1 m and replaced with near forest soil prior to the planting (Lee, Kim, Kim, Kim, & Han, 2012). The CO_2 concentrations in the chambers were measured using an infrared gas analyzer (ZRH type, Fuji Electric System Co. Ltd, Japan), and the CO_2concentrations inside were controlled by mixing pure liquefied CO_2 and atmospheric air. The CO_2enrichment was conducted for 9 years from 8:00 am to 6:00 pm.

Measurements

Light response curves were generated and$A$ /C i curve measurements were taken for the same sunlight exposure for each tree (3 replicates per species × 3 species in each treatment, for a total of 9 leaves per treatment) from June to August in 2017 using a portable open gas exchange system LI-6400 (Li-Cor Inc., Lincoln). The temperature and relative humidity were set at 25 and 55%–60%, respectively. The light response curve was generated by changing the irradiance in the following order: 1400, 1200, 1000, 800, 600, 400, 200, 100, 75, 50, 25, 0, and 1200 µmol $m^{-2}s^{-1}$. The CO_2samples were set at 400, 560, and 720 µmol $m^{-2}s^{-1}$ for aCO_2, e$CO_2$1.4, and e$CO_2$1.8, respectively. The maximum photosyntic rate (A max) was estimated by selecting the photosynthesis value at the light saturation point from the light response curve. The maximum carboxylation rate (V Cmax) and the maximum electron transfer rate (J max) were measured by plotting$A$ /C_i curves under high irradiance (1200 µmol m^{-2} s^{-1}). The change in the reference CO_2 concentrations for each chamber were in the following order: aCO_2 : 400, 300, 200, 100, 75, 50, 25, 0, 400, 400, 600, 800, 1000, and 1200 µmol $m^{-2}s^{-1}$; eCO_2 1.4: 560, 400, 300, 200, 100, 75, 50, 25, 0, 560, 560, 800, 1000, and 1200 µmol m^{-2} s^{-1}; and e$CO_2$1.8: 720, 600, 400, 300, 200, 100, 75, 50, 25, 0, 720, 720, 100, and 1200 µmol m^{-2} s^{-1}. The$V$ cmax and J max were estimated using the curve fitting model of version 2.0 developed by (Sharkey, 2016).

Leaf size, leaf mass per area, carbon and nitrogen contents, and PNUE

The "upper canopy" was defined as the leaves in the upper 10% of the tree crown, the "middle canopy" for leaves of 50% of the tree crown, and the "lower canopy" was leaves at the lowest part of the crown (D. S. Ellsworth et al., 2012). Leaves from the three canopy positions (upper, middle, and lower) were collected from June to August in 2017 (3 canopy positions × 2 leaves × 3 replicates × 3 species in each treatment × 2 months (July and August) and 2 leaves × 3 replicates × 3 species × 1 month for a total of 72 leaves per treatment). Leaf discs with 1 cm diameters were punched out from the leaves excluding the midrib and stored in a liquid nitrogen tank (-196). Five discs were dried for 72 h at 70 in a dry oven, and the leaf mass per area (LMA, g[?]m^{-2}) was calculated by dividing the leaf area by its dry mass. Dried samples were powdered using a homogenizer (FastPrep-24, MP Biomedicals, Solon, OH), and the N and C contents (g[?]g^{-1}, %) were determined using a CHNS-Analyzer Flash EA 1112 (Thermo Electron Corporation, USA) at the NICEM, Seoul National University. PNUE was calculated as the ratio of$A$ max to N_{area}.

Total non-structural carbohydrates

Three discs (0.785 cm^2) from each of the deciduous species and 15–20 mg leaf samples from the Korean red pine were dried at 70 for three days, and then powdered using a homogenizer (FastPrep-24, MP Biomedicals, Solon, OH). Powdered samples were used for the determinations of the soluble sugars and starch contents. The soluble sugars were extracted from the 15–20 mg ground samples with 1.5 ml 80% (v/v) ethanol. The extraction was done in a water bath at 80 . After centrifugation at 14 ,000 x g for 10 min, the concentrations

of soluble sugars were determined colorimetrically, at 490 nm using the phenol-sulfuric method (Ashwell, 1966). The starch in the pellet that remained after the extraction of the soluble sugars was hydrolyzed to glucose. Samples were incubated in 2.5 ml sodium acetate buffer (0.2 M) in a 100 water bath for 1 h. After cooling to room temperature, 2 ml of the buffer and 1 ml of amyloglucosidase (0.5% by weight, Sigma A9229-1G) were added. The solutions and sample were then incubated overnight in a 55 water bath. After at 14 ,000 x g for 10 min, the concentration of the starch was determined colorimetrically at 490 nm, using the phenol-sulfuric method . The total amount the soluble sugars and starch are referred to as the total non-structural carbohydrates (NSC, g[?]m^{-2}).

N allocations in Rubisco and chlorophyll

The Rubisco protein was extracted using the method of (Hikosaka & Shigeno, 2009). One leaf disc (0.785 cm^2) stored at -80 was powdered using a homogenizer (FastPrep-24, MP Biomedicals, Solon, OH), and samples were extracted in 1 ml of 4 extraction buffer. The samples of Korean ash and Korean whitebeam were extracted with a buffer including 80 mM Tris-HCl, pH 7.4 and the samples of Korean red pine were extracted with a buffer including 80 mM Tris-HCl, pH 8.0. Additionally, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 1.5% (v/v) glycerol, 100 mM β-mercaptoethanol, and 20 kg m^{-3}sodium dodecyl sulfate (SDS) were included in all extraction buffers. The samples were centrifuged at 15 000 × g for 30 min, at 4 °C, denatured at 90 °C for 5 min, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Rubisco nitrogen was assumed to be 16% of the total Rubisco content (Hikosaka & Shigeno, 2009).

Chlorophyll was extracted using the dimethyl sulfoxide (DMSO) method (Shinano et al., 1996). Two discs were placed in a brown bottle containing 5 ml of DMSO, and the sample was stored at 65 for 6 h, in a water bath. The samples were measured at two wavelengths, 649 and 665 nm, using a spectrophotometer (with a wavelength range of 190–1100 nm, a spectral bandpass width < 1 nm, and a wavelength accuracy < +- 0.5 nm; Optizen 2120 UV, Mecasys, Korea). The total chlorophyll content was derived using the DMSO equation of (Wellburn, 1994), as follows:

Total chlorophyll content (μg ml^{-1}) = 21.44A $_{649}$ + 5.97 A $_{665}$ (1)

N availability was determined from the freshly fallen litters collected every week from September to December, in 2017. The litters were dried at 70 for 3 days, and the ^{15}N/^{14}N ratio was determined using a stable isotope ratio mass spectrometer system with an elemental analyzer (Vision-EA, Isoprime, UK) (Norby et al., 2010).

Statistical analysis

The photosynthetic characteristics (via gas exchange) were analyzed using two-way analysis of variance (ANOVA) and three-way ANOVA was conducted for leaf size, LMA, N$_{mass}$, N$_{area}$, starch, soluble sugars, Rubisco content, chlorophyll content, NF$_{Rub}$, and NF$_{chl}$. One-way ANOVAs were conducted for N availability. When there were significant differences between the CO_2 treatments each species, a Tukey's test was used for the multiple comparisons. To examine the effects of the elevated CO_2, regression analyses were conducted using the linear and non-linear relationships between the NSC (total amount of starch and soluble sugars per unit area, g[?]m^{-2}) - LMA, and NSC - Rubisco. The regression lines for the data were compared by analysis of covariance (ANCOVA; (Zar, 1999)) to identify differences between treatments. All statistical analyses were performed using the R statistical program (ver. 3.3.2; R Core Team, 2016).

Results

Leaf morphological characteristics

In general, the leaf size increased with eCO_2, when compared to the aCO_2, but the pattern of increase between species were different depending on their canopy positions, and there was significant interaction among each species, CO_2 treatment, and canopy position (Fig. 1a,b,c). In the case of the Korean red pine, the enlargement of the leaf size was greater in the upper canopy than the lower canopy (Fig. 1a). There was a significant increase (~129.7%) in the CO_2 effects under e$CO_2$1.8 conditions in the lower canopy compared to that under aCO_2 (P = 0.032). However, in the upper canopy, leaf size significantly increased

by 216.7% under $eCO_21.8$ compared to aCO_2 ($P = 0.037$). In contrast, leaf size of the Korean ash and Korean whitebeam significantly decreased with canopy height, and the increment of leaf size with the eCO_2 was also greater in the lower canopy (Fig. 1b,c). In the Korean ash, the increment in the lower canopy under $eCO_21.8$, reached 205.0% (97.9 ± 11.8 cm^{-2}) ($P < 0.001$); however, the increment in the upper canopy was only 7.4%. In the Korean whitebeam, the leaf size increment was 103.7% in the lower canopy ($P < 0.001$) and 45.5% in the middle canopy ($P = 0.003$) with the $eCO_21.4$ than those of aCO_2 and leaf size significantly increased in upper canopy under $eCO_21.4$ (~22.8%, $P= 0.004$) and $eCO_21.8$ (~32.5%, $P= 0.020$) compared to aCO_2. Similar to leaf size, LMA showed significant interactions among the CO_2 treatments, species, and canopy positions; the LMA increased with the elevated CO_2 concentrations and increased with canopy height for all species (Fig. 1d,e,f). The LMA of Korean red pine increased under $eCO_21.4$ (~24.19%, $P = 0.009$) in the middle and under eCO_2 upper canopy (~16.8%, maximum $P = 0.029$) compared to that under aCO_2, but no significant differences were found in the lower canopy with the CO_2 treatments (Fig. 1d). However, the Korean ash was only enhanced in the middle canopy under $eCO_21.8$ ($P = 0.005$), and the Korean whitebeam was significantly enhanced with the $eCO_21.4$ at all canopy positions (maximum $P = 0.011$) (Fig. 1e,f).

Photosynthetic characteristics

To verify that photosynthesis was downregulated under elevated CO_2 treatments, we investigated the photosynthetic characteristics. Overall, there was no effect from the elevated CO_2 on the A_{max}, but there was an significant interaction between the CO_2 concentrations and the species (Table 1). The Korean ash had the largest and most significant enhancement in A_{max} with 37.9% with the $eCO_21.8$ ($P = 0.008$). In contrast to the A_{max}, the $V_{C\,max}$ and J_{max} were affected by the CO_2 (Table 1). On average, a 31.9% $V_{C\,max}$ reduction was observed under $eCO_21.8$ conditions compared with aCO_2, and the Korean red pine had a 45.0% lower V_{Cmax} under $eCO_21.8$ conditions, compared to that under aCO_2. Korean ash and Korean whitebeam had lower V_{Cmax} under $eCO_21.8$ conditions. Similar to V_{Cmax}, the J_{max} was 20.6% lower, compared to that under aCO_2. There were no significant interactions between the CO_2 concentrations and the species for the V_{Cmax} and J_{max}. The PNUE was enhanced by 36.0% under $eCO_21.8$ conditions, and there was ere also interaction between the CO_2 concentrations and species (Table 1). For the Korean ash, the PNUE was 74.6% higher with the $eCO_21.8$ ($P < 0.001$). The average of the PNUE for the Korean ash was the highest of the species investigated, while that of the Korean red pine was lower than that of the deciduous species.

Leaf total N and nonstructural carbohydrates

On average, N_{mass} significantly decreased by 14.9 % with the elevated CO_2 concentrations (1.15 ± 0.04 g[?]g^{-1},%) compared to aCO_2 (1.35 +- 0.05 g[?]g^{-1} ,%) (Fig. 2a) and there was a significant difference in the N_{mass} among the species, in the order of Korean ash (1.43 +- 0.04 g[?]g^{-1},%), Korean whitebeam (1.25 +- 0.03 g[?]g^{-1} ,%), and Korean red pine (0.95 +- 0.02 g[?]g^{-1},%). In contrast to the N_{mass}, the N_{area} was not affected by the CO_2 concentrations (Fig. 2b); however, there were significant interactions between the CO_2 concentrations x species, and the average N_{area} in the Korean ash showed a significant decrease of 21.0% under $eCO_21.4$ ($P = 0.024$). In addition, differences in the N_{area} were observed between the canopy positions (Fig. 2b). Among the three canopy positions, the lower canopy leaves (1.53 +- 0.07 g[?]m^{-2}) tended to have significantly smaller N_{area} than the middle (1.47 +- 0.07 g[?]m^{-2}) or upper (1.53 +- 0.08 g[?]m^{-2}) canopy positions.

To examine the reduction in N_{mass} due to the N dilution caused by NSC, we quantified NSC (the total amount of starch and soluble sugars) in each CO_2 treatment, species, and canopy (Fig. 2c). Starch increased under eCO_2, and starch was higher in the deciduous Korean ash (25.07 +- 0.04 g[?]m^{-2}) and Korean whitebeam (14.69 +- 0.61 g[?]m^{-2}) species, than the evergreen Korean pine (12.90 +- 0.41 g[?]m^{-2}). There were significant interaction effects between the species x CO_2 concentrations (Fig. 2c). The Korean ash showed significant increments with increasing CO_2 concentrations (maximum $P = 0.033$), and the Korean white beam was the highest under $eCO_21.4$ conditions ($P = 0.047$). While the broadleaved and shade - tolerant species increased under eCO_2 conditions, there was no clear tendency for starch in the Korean red pine. However, there was no effect of elevated CO_2 on the soluble sugars (Fig. 2d). NSC and LMA showed similar increment with CO_2 treatment except for Korean red pine (Fig. 1d,e,f and Fig. 2c,d). The NSC was negatively correlated

with the LMA in the Korean red pine (Fig 3a), whereas there were positive correlations for the Korean ash and Korean whitebeam (Fig. 3b,c).

Leaf N allocations to Rubisco and chlorophyll

The Rubisco content showed significant differences among the CO_2 treatments, species, and canopy positions (Fig. 4a). For the CO_2 treatments, the average Rubisco content was lowest with the $eCO_2$1.8 (0.96 +- 0.04 g[?]m^{-2}) compared to $eCO_2$1.4 (1.07 +- 0.04 g[?]m^{-2}) and aCO_2 (1.16 +- 0.04 g[?]m^{-2}). The Rubisco content was highest in the Korean ash (1.28 +- 0.04 g[?]m^{-2}), followed by the Korean whitebeam (1.08 +- 0.04 g[?]m^{-2}), and Korean red pine (0.75 +- 0.04 g[?]m^{-2}). The upper canopy leaves on average had a higher Rubisco content by 29.6% and 36.0% than the middle and lower canopies, respectively. Similar to the Rubisco content, there were significant difference in the NF_{Rub} with the CO_2 treatments, species, and canopy positions (Fig. 4b). The Korean red pine was the lowest in NF_{Rub} among species and in case of canopy position analysis in NF_{Rub}, upper canopy was the highest among canopy position. There were interactions between CO_2 concentrations x the species in the NF_{Rub}, and the Korean whitebeam showed significant decreases of 15.7% under $eCO_2$1.8 ($P = 0.011$), compared to the aCO_2 .

Unlike the Rubisco that was reduced under eCO_2conditions, the chlorophyll content increased under $eCO_2$1.8 conditions, despite there being no significant differences in the N_{area} between the CO_2 treatments (Fig. 4c). In the Korean red pine and Korean whitebeam, the chlorophyll content significantly increased in the $eCO_2$1.4 and $eCO_2$1.8, respectively ($P = 0.034$ and $P = 0.008$). The chlorophyll contents showed interactions between the CO_2 treatment x species x canopy positions (Fig. 4c). The NF_{chl} analysis showed that there were significant differences among the CO_2 treatments, species, and canopy positions (Fig 4d). In general, the increase in the chlorophyll content resulted in an increase in NF_{chl}under $eCO_2$1.8. The NF_{chl} in the evergreen Korean red pine, which is a shade-intolerant species, was the lowest among species. There were interactions in the CO_2treatment x species (Fig. 4d). Particularly, the Korean ash and Korean whitebeam increased by 11.2% and 22.0% under $eCO_2$1.8, respectively. On average, the NF_{chl} decreased with increasing canopy heights; lower (0.16 +- 0.01), middle (0.14 +- 0.01), and upper (0.12 +- 0.00). In addition, there were interactions in the CO_2 treatment x species x canopy positions (Fig. 4d). Increases in NF_{chl} are more noticeable in the middle (˜19.1%, $P = 0.004$) and lower canopies (˜50.0%, $P = 0.006$) than the upper canopy (not significant) in the Korean whitebeam under $eCO_2$1.8, compared to those under aCO_2. The Korean red pine also showed increments in NF_{chl} in the lower and middle canopies under $eCO_2$1.8 and $eCO_2$1.4 conditions, respectively ($P = 0.030$ and $P = 0.011$).

Differences in the N availability between the two CO_2 treatments

N availability was quantified by determining the ratio of^{14}N/^{15}N using fresh fallen litter after 9 years of eCO_2 exposure to examine the occurrence of PNL at the study site. However, unexpectedly, N availability was significantly higher by 32.5% under $eCO_2$1.8 (-1.38 +- 0.14($P < 0.001$).

Discussion

Changes in leaf morphological characteristics and relationships with nitrogen concentration under elevated CO_2

Similar to the results of previous investigations, Fig 1 shows the changes in the morphological parameters such as leaf size (Riikonen et al., 2010) and LMA (Aspinwall et al., 2017) under elevated CO_2. The leaf size increases were caused by enhanced cell production and expansion, which could be attributed to increases in xyloglucan endotransglycosylase, a cell-wall-loosening enzyme (Riikonen et al., 2010). LMA was coupled to the increased accumulation of NSCs, such as starch and soluble sugars concentrations, with elevated CO_2 (Aspinwall et al., 2017). Similarly, our results also showed that the LMA was positively correlated with the NSC in the deciduous Korean ash and Korean whitebeam ($P < 0.001$ and $P < 0.001$, respectively, Fig. 3b,c), but there was a negative correlation with the Korean red pine ($P < 0.001$, Fig. 3a). In general, increased NSC led to increased LMA; however, in high-LMA species such as the Korean red pine, increase in LMA was mainly driven by the increase in the constituent compounds, such as structural carbohydrates

and lignin, while NSC and minerals only increased marginally. Therefore, no significant differences in the NSC under elevated CO_2 in the Korean red pine and the large increments in LMA in the middle and upper canopies resulted in a decrease in NSC with an increase in LMA (Fig. 1d, Fig. 2 and Fig. 3a) (Poorter, Niinemets, Poorter, Wright, & Villar, 2009).

Dilution effects and down-regulation of photosynthesis

During long-term CO_2 enrichment experiments, most plants are unable to maintain their initial growth increments due to their acclimation to elevated CO_2 (Reich, Hobbie, Lee, & Pastore, 2018), as it results in the down-regulation of photosynthesis, a decrease in V_{Cmax}, J_{max}, and Rubisco protein content (Rogers & Ellsworth, 2002). There was a reduction in the V_{Cmax} and J_{max} under $eCO_2 1.8$ conditions (Table 1). The average Rubisco content decreased under $eCO_2 1.8$ (Fig. 4a). Furthermore, the NF_{Rub} were also affected by elevated CO_2 and decreased under elevated CO_2 in the Korean whitebeam, while showing a decreasing tendency in the other species (Fig. 4b). Several previous studies have suggested that down-regulation of photosynthesis is caused by a dilution effect, termed a sugar-mediated mechanism, and that the high sugar concentration at high LMA can repress Rubisco gene expression under elevated CO_2. This hypothesis demonstrated that the photosynthate sink–source balance was controlled by the mediation of sugar signaling (Gutierrez, Morcuende, Del Pozo, Martinez-Carrasco, & Perez, 2013). In the present study, the LMA increased under elevated CO_2 (Fig. 1d,e,f) and starch, and the photosynthate-reservoir also increased under elevated CO_2 conditions in the Korean ash and Korean whitebeam, but not the Korean red pine (Fig. 2c), indicating that large amounts of starch diluted the leaf N (Gifford et al., 2000), specifically for hardwood rather than conifer species.

Some studies that reported a decrease in the N_{area} have claimed that down-regulation of photosynthesis was due to the dilution effect (Gutierrez et al., 2013; Kitaoka et al., 2016), but, as defined in the introduction of the present study, it should be attributed to N limitation rather than dilution. In the present study, there was a decrease in N_{mass} with no differences in the N_{area} among treatments (Fig. 2a,b), which implied that dilution occurred, and not N limitation. Furthermore, NF_{Rub} was decreased under elevated CO_2 conditions in the Korean whitebeam (Fig. 4b), which supports the hypothesis that the reduced Rubisco gene expression was due to the accumulated NSC (Moore et al., 1999). In the case of the Korean ash, even though there were no differences in the NF_{Rub}, the starch content significantly increased under eCO_2 (Fig. 2c). In addition, the relationship between the NSC and Rubisco content showed smaller slopes under $eCO_2 1.8$, which implied that the Rubisco content of $eCO_2 1.8$ was lower than those of aCO_2 and eCO_2 at the same NSC ($P = 0.055$, $P = 0.006$ and Fig. 5b). Similarly, the Rubisco content of the Korean whitebeam was also lower at the same NSC in the $eCO_2 1.8$ than in the aCO_2, even though the relationship with aCO_2 was not significant (Fig. 5c). Although the Korean red pine had the same negative relationships between the NSC and Rubisco for the CO_2 treatments ($P = 0.021$, Fig. 5a), the reduction in N_{mass} (but not in N_{area}) in the Korean red pine would imply the dilution of N by increments in structural components, and not NSC, as shown in Fig. 1d, Fig. 2 and Fig. 3a.

Changes in leaf N allocations and increments in PNUE under eCO_2

Unlike Rubisco, chlorophyll per unit area and NF_{chl} were significantly increased under elevated CO_2 (Fig. 4c,d), and the PNUE was also increased (Table 1), which implied changes to the N allocation from Rubisco to chlorophyll, and increments in the N use efficiency that were associated with no reductions in A_{max} under $eCO_2 1.8$ (Table 1). Similar to our findings, chlorophyll content/total N in *Robinia pseudoacacia L* increased by 22% after 4 years, in the Hokkaido FACE experiment (Choi et al., 2017), and a small decrease in Rubisco content via individual suppression of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RBCS) genes led to increased chlorophyll (Kanno, Suzuki, & Makino, 2017). Unlike under ambient CO_2 conditions, the Rubisco content was not the limiting factor in the elevated CO_2 concentrations (Parry, Keys, Madgwick, Carmo-Silva, & Andralojc, 2008); however, the use of Pi for ATP synthesis or the transport capacity of CO_2 might limit photosynthesis under elevated CO_2 conditions. Therefore, to enhance the use efficiency, more nitrogen must be invested in chlorophyll than the Rubisco to improve the light harvesting, as sufficient CO_2 can be used for carbon fixing under high CO_2 concentrations to maximize C gains (Choi

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et al., 2017; Kanno et al., 2017; Leakey et al., 2009).

Comparisons of the long-term experiments of elevated CO_2 and down-regulation of photosynthesis by N availability

The long-term exposure to CO_2 enrichment in the Oakridge FACE experiments showed that the NPP enhancement decreased from 24% to 9% due to the decrease in the linearly declined $\delta^{15}N$ of the leaf litter, an indicator of N availability, from 1998 and 2005 (Norby et al., 2010). N availability based on the $\delta^{15}N$ of the leaf litter declined in both under aCO_2 and eCO_2 conditions, but increased under eCO_2 in the ORNL FACE experiment (Garten Jr, Iversen, & Norby, 2011). These results suggest a progressive nitrogen limitation (PNL) hypothesis and a reduction in photosynthesis stimulation caused by a reduction in leaf N in eCO_2 (Norby et al., 2010). Under N-deficient conditions, N is translocated within plants; this indirectly resulted in the down-regulation of photosynthesis (Rogers & Ellsworth, 2002), and NSC accumulation in photosynthesis was accelerated (Araya et al., 2010). However, in the present study, N availability significantly increased under $eCO_2$1.8 compared to that under aCO_2 after 9 years of eCO_2exposure and starch also increased under eCO_2 (Fig. 2c). Declining N availability over time is a key process in the occurrence of PNL; however, soil N availability is a difficult parameter to measure , and conventional measures of soil N availability do not assess plant-available N (Franklin et al., 2009). To quantify N availability, we looked for stable N isotopes from the leaf litterfall, as they are an accurate indicator of soil N availability (Garten Jr et al., 2011). The increased $\delta^{15}N$ in the leaves showed that the available N pool was isotopically heavier. These results also showed that there was more available N because nitrification increased compared to the immobilization under eCO_2 (Craine et al., 2009). Down-regulation of photosynthesis would decrease the N demand of a plant due to increased PNUE (Table 1), despite the increased N availability under $eCO_2$1.8, or the reduced ability of the soil-root system to supply N (Taub & Wang, 2008). As a result, down-regulation of photosynthesis was not caused by a deficiency in N availability, indicating that PNL does not occur at this site, despite the long-term elevated CO_2 exposure.

Changes in the leaf characteristics under elevated CO_2 and by canopy positions

The change in canopy position resulted in a change in leaf size, and a variation in response to elevated CO_2 occurred for specific species. The leaf size of the Korean ash and Korean white beam in the lower canopy increased compared to the upper canopy under elevated CO_2 (Fig. 1b,c). The upper canopy of the small leaves reduced the water loss from sun exposure forming a small leaf volume layer that transmitted radiation through the canopy (Hagemeier & Leuschner, 2019). In addition, cell-wall-loosening enzyme activity increased not only under elevated CO_2 conditions but also under low light conditions (Sasidharan, Chinnappa, Voesenek, & Pierik, 2008). In the canopy analysis, the leaf size of the Korean red pine, which is a shade-intolerant species compared to the other two species, increased with the canopy height and CO_2 concentration, but it was also affected by the elevated CO_2 in all of its canopy positions (Fig. 1a). The LMA of all species in the upper canopy tended to be higher than those in the lower canopy under elevated CO_2 (Fig. 1d,e,f). In general, leaves under a higher light intensity were thicker, because of the thicker palisade mesophyll cell layer with increasing canopy height (Ishii & Ohsugi, 2011)Thicker leaves that contained additional mesophyll have more total Rubisco and N_{area} (D. Ellsworth & Reich, 1993). In the present study, the leaves in the upper canopy had higher Rubisco content (Fig. 4a), and the NF_{Rub} was increased in the upper canopy, compared to that in the lower canopy (Fig. 4b). Conversely, the average of NF_{chl} decreased with increasing canopy height (Fig. 4d). In response to the eCO_2, the lower canopy had weaker responses but there were no significant canopy $\times$ CO_2 treatment interactions in the photosynthetic N allocations, such as with Rubisco (Sharwood et al., 2017). In the present study, there were also no interaction effects in the NF_{Rub} between the CO_2 concentrations and canopy positions ($P = 0.317$). However, the NF_{chl}showed interactions between the CO_2 concentrations $\times$ canopy $\times$ species (Fig. 4d). Among the species examined, the Korean whitebeam showed significant increases in the NF_{chl}under $eCO_2$1.8 in the lower and middle canopy, and the Korean red pine also showed similar responses in the lower and middle canopy. A high light intensity caused a greater increase in leaf N than increased CO_2 (Pan et al., 2020) (Fig. 2b). Therefore, in the upper canopy under high light conditions, the NF_{chl} showed no significant increases under eCO_2 with the increase

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in total N (Fig. 4d).

Changes in leaf characteristics under elevated CO_2 and by species

There was an interaction between the eCO_2 treatments and the species in terms of the PNUE (Table 1). Improvement in the PNUE occurred under eCO_2 in the deciduous species, especially the Korean ash compared to the Korean red pine. An effect of the eCO_2, was that the decrease in NF_{Rub} and the increase in NF_{chl} was the greatest in the most shade-tolerant species (Korean whitebeam) tested (Fig. 4b,d). Previously in the Duke FACE experiment, *Liquidambar styraciflua* showed greater photosynthetic enhancements than *Pinus taeda* . Furthermore, among the six understory tree species, the most shade-tolerant species (*C. florida*) showed the highest increments in A_{max} with light saturation under eCO_2, whereas the least shade-tolerant species showed the lowest increment under eCO_2 (D. S. Ellsworth et al., 2012). Shade-intolerant species provide more sufficient carbon sinks under high light intensities than shade-tolerant species, indicating that down-regulation of photosynthesis occurs more under high light intensities than low light intensities due to a reduction in N allocation to Rubisco (Springer & Thomas, 2007). Furthermore, this result implies that shade-tolerant species have a competitive advantage and utilize different light niches to profit under eCO_2, and for understory vegetation; this could be more important in carbon sequestration due to other ecosystem functions, such as less down-regulation of photosynthesis and high plasticity to changing light conditions (Holub, Klem, Linder, & Urban, 2019).

Conclusion

Our study showed that morphological characteristics, such as LMA and leaf size, increased under elevated CO_2 conditions, particularly in the lower canopies of shade-tolerant species. After nine years of CO_2 enrichment, the down-regulation of photosynthesis was evident. We discuss the hypothesis regarding dilutions derived from the excess accumulation of photosynthate, and that regarding the influence of N availability for long-term eCO_2 exposure, termed PNL. On the basis of our results, down-regulation of photosynthesis was found to be caused by the dilution effect and a change in N allocation. Our study showed a shift in N allocation from Rubisco and electron transport to the light-harvesting system under eCO_2, which caused increased PNUE. Increased N efficiency reduces N demand, which could be another cause of the down-regulation of photosynthesis, although the N availability increased under eCO_2 at the site.

We conclude that the mechanisms of the down-regulation of photosynthesis are dilution and a decrease in N demand. Shade-tolerant broadleaved species have relatively high flexibility in leaf N operations compared to shade-intolerant conifer species. However, leaf N cannot indicate the N status of the entire plant under eCO_2; thus, further research regarding other sink organ changes is required.

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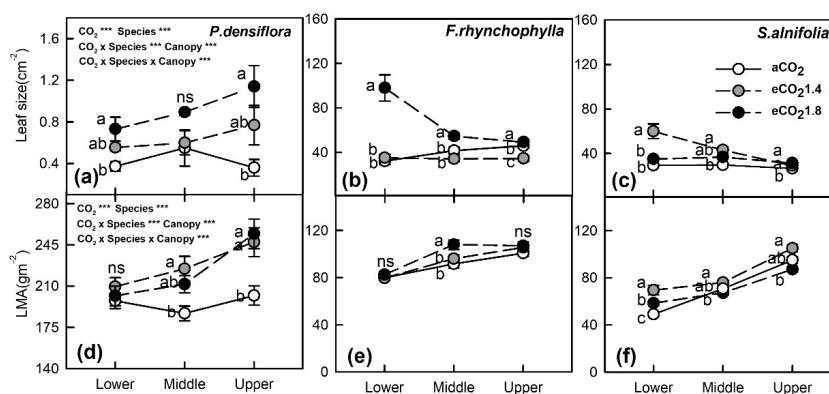
Figures

Figure 1. The average of leaf size (a). Korean red pine (*Pinus densiflora*), (b). Korean ash, *Fraxinus rhynchophylla* , (c). Korean whitebeam, *Sorbus alnifolia* and the average of LMA (d). Korean red pine (*Pinus densiflora*) (e). Korean ash, *Fraxinus rhynchophylla* , (f). Korean whitebeam,*Sorbus alnifolia.* Results are presented as mean + SE [3 species × 3 canopy × 3 replicate × 5 leaf per chambers], aCO_2(white circe), $eCO_2 1.4$ (grey circle) and $eCO_2 1.8$ (black circle). The X axis represents canopy position: lower, middle and upper. When there was interaction between CO_2 × Species × Canopy, differences between CO_2 treatments are indicated by different lower case letters by species and canopy (Tukey's test, $P < 0.05$). Leaf size and LMA showed interactions in in CO_2 × Species × Canopy ($P < 0.001$ and $P < 0.001$).
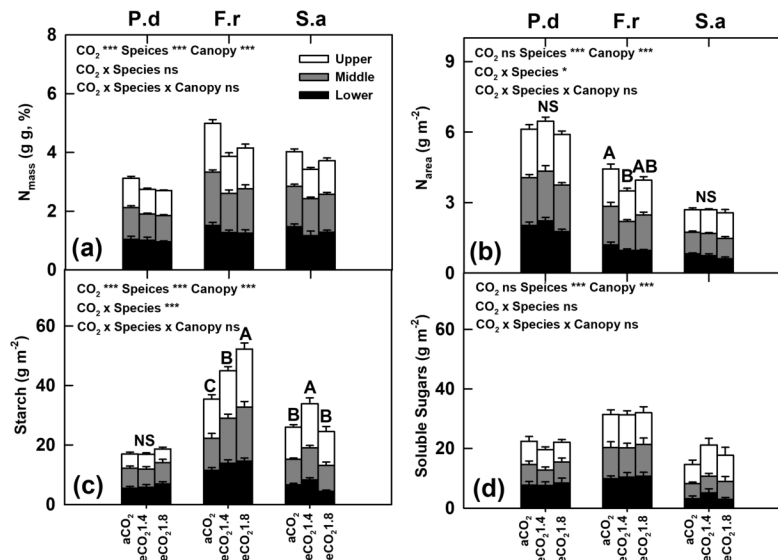
Figure 2. Results are presented as mean + SE [3 species $\times$ 3 replicates $\times$ 3 canopy per chamber]. Leaf parameters measured in Korean red pine (*P. densiflora*, P.d), Korean ash (*F. rhynchophylla*, F.r), and Korean whitebeam (*S. alnifolia,* S.a) grown at three different CO_2 chambers (ambient; ambient$\times$1.4, e $CO_2$1.4; ambient$\times$1.8, e$CO_2$1.8). (a) N_{mass} : leaf nitrogen per unit mass, (b) N_{area} : leaf nitrogen per unit area, (c) Starch content per unit area, (d) Soluble sugars content per unit area. Differences between CO_2 treatments are indicated by different upper case letters, regardless canopy. When there was interaction between CO_2 $\times$ Species $\times$ Canopy, differences between CO_2 treatments are indicated by different lower case letters by species (Tukey's test, $P < 0.05$). N_{area} and starch showed interaction in CO_2 $\times$ Species ($P = 0.037$ and $P < 0.001$).
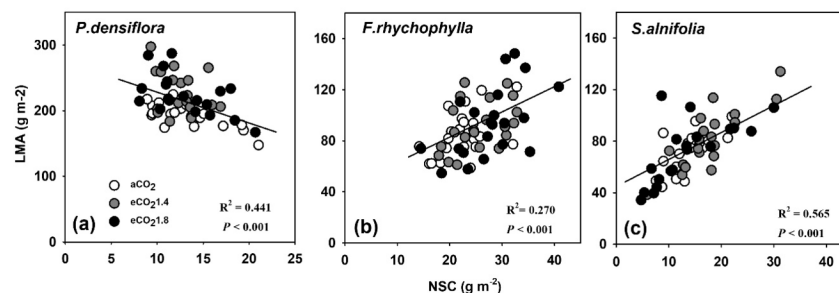

Figure 3. Relationships between leaf mass per area (LMA) and NSC (nonstructural carbohydrate per unit area, total amount of starch and soluble sugars) from *P. densiflora* , (Korean red pine) (b).*F. rhynchophylla* (Korean ash), and (c). *S. anlifolia*(Korean whitebeam). Open circle, grey circle and filled circle represent values for aCO_2, e$CO_2$1.4 and e$CO_2$1.8, respectively. Solid lines represent the significant LMA-NSC relationships based on regression analyses. (a) y = -4.35 x + 251.11 (b) y = 2.08 x + 38.45 (c) y = 2.72 x + 34.32.
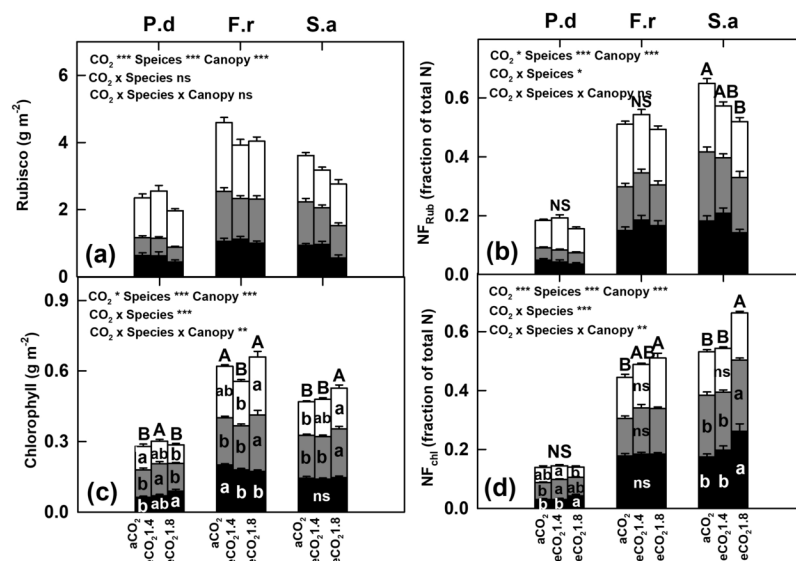
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Figure 4. Results are presented as mean + SE [3 species $\times$ 3 replicates $\times$ 3 canopy per chamber]. Leaf parameters measured in Korean red pine (*P. densiflora* , P.d), Korean ash (*F. rhynchophylla* , F.r), and Korean whitebeam (*S. alnifolia,* S.a) grown at three different CO_2 chambers (ambient; ambient$\times$1.4, e $CO_2$1.4; ambient$\times$1.8, e$CO_2$1.8). The average of the (a) Rubisco : Rubisco content per unit area, (b) N fraction of Rubisco (NF$_{Rub}$, Rubisco N/N$_{area}$), (c) chlorophyll : chlorophyll per unit area and (d) N fraction of chlorophyll (NF$_{chl}$, chloropyll N/N$_{area}$). Results are presented as mean + SE [3 species $\times$ 3 replicates $\times$ 3 canopy per chamber], upper (white bars), middle (grey bars) and lower (black). Differences between CO_2 treatments are indicated by different upper case letters by species, regardless canopy. When there was interaction between $CO_2 \times$ Species $\times$ Canopy, differences between CO_2 treatments are indicated by different lower case letters by species and canopy (Tukey's test, $P < 0.05$). Rubisco, chlorophyll, NF$_{Rub}$ and NF$_{chl}$showed interaction in $CO_2 \times$ Species ($P< 0.001$, $P = 0.011$, $P = 0.021$ and$P < 0.001$, respectively). Chlorophyll content and NF$_{chl}$ showed interaction in $CO_2 \times$ Species $\times$ Canopy ($P = 0.002$ and $P = 0.003$).
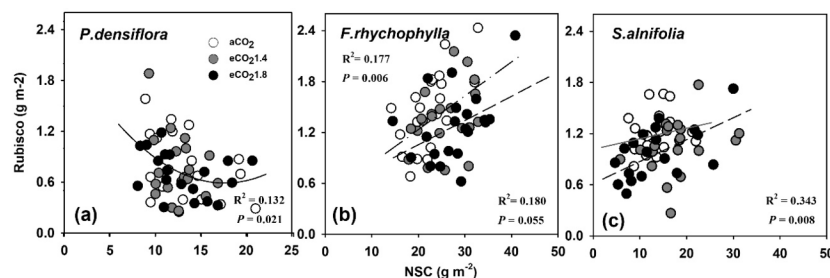

Figure 5. Relationships between Rubisco content per area and NSC (nonstructural carbohydrate per unit area, sum of starch and soluble sugars) from *P. densiflora* (Korean red pine) (b). *F. rhynchophylla* (Korean ash), and (c). *S. anlifolia* (Korean whitebeam). Open circle, grey circle and filled circle represent values for a CO_2, e$CO_2$1.4 and e$CO_2$1.8, respectively. Black lines represent the significant Rubisco-NSC relationships based on regression analyses. (a) The solid line is fitted regressions of Rubisco-NSC in Korean red pine (y = $0.0053 x^2 - 0.184 x + 2.2005$) (b) The dash-dotted line is fitted regressions of Rubisco-NSC in Korean ash under aCO_2 and e$CO_2$1.4 (y = $0.036 x + 0.532$). The dashed line is fitted regressions of Rubisco–NSC under e$CO_2$1.8 (y = $0.029 x + 0.484$). (c) Grey line represent the non-significant relationship between Rubisco-NSC under aCO_2 and the dashed line is fitted regressions of Rubisco-NSC under e$CO_2$1.8 (y = $0.025 x + 0.645$).

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