Species-specific morphological and physiological characteristics and progressive nitrogen limitation under elevated CO_2 concentration

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Elevated atmospheric CO_2 (eCO_2) concentration initially enhances photosynthesis, growth and ecosystem productivity, but the excessive use of nitrogen due to the increased productivity causes uncertainty in long-term ecosystem responses. We exposed Korean red pine, Chinese ash, and Korean mountain ash to current atmospheric CO_2 concentration (aCO_2), 1.4 times higher CO_2 concentration (e$CO_2$1.4), and 1.8 times higher CO_2 concentration (e$CO_2$1.8) in an Open-Top Chamber (OTC) experiment for eight years (2010-2017) to investigate the effect on the morphological and physiological properties of trees. We also assessed whether nitrogen limitation occurred with time by comparing leaf and soil nitrogen concentration. CO_2 fertilization effect was observed on tree growth for the first two years ($p < 0.05$), but there was no difference thereafter. For photosynthetic properties, CO_2 effects were species-specific; no effects on Korean red pine and Chinese ash *vs.* significant effect on Korean mountain ash. However, maximum photosynthetic and carboxylation rates significantly decreased by 24.3% and 31.3% from 2013 to 2017, respectively. Leaf nitrogen significantly decreased by 21.0 % at e$CO_2$1.4 and 18.5 % at e$CO_2$1.8 compared with aCO_2 treatment. This study showed the decline of leaf nitrogen and species-specific responses to long-term high CO_2 concentration, which will effect on species competition and ecosystem succession.

Keywords: Elevated CO_2, Photosynthetic Properties, Down-regulation, Progressive Nitrogen Limitation, Carbon dioxide

Introduction

Elevated CO_2 concentration has become a common phenomenon in the Earth's atmosphere over the last half-century (IPCC 2013). The indiscreet use of fossil fuels and deforestation has raised the atmospheric CO_2 concentration, thus promoting photosynthesis and growth (Drake et al. 1997, Ainsworth & Long 2005, Sang et al. 2019) and affecting the productivity of plants (McCarthy et al. 2010, Norby et al. 2010), which is referred to as the CO_2 fertilization effect (Norby et al. 2010). However, there are conflicting opinions on whether such an effect will continue with increasing CO_2 concentration (Hungate et al. 2003). In general, the productivity of terrestrial ecosystems is affected by nutrient availability in the soil (Terrer et al. 2018). It should be taken into account that, if CO_2 fertilization promotes plant growth, the excessive nutrient use will result in insufficient nutrients in the soil, failing to meet the nutrient requirements needed for plant growth (Hungate et al. 2003, Wang & Houlton 2009) and decreasing the CO_2 fertilization effect. Therefore, the expected increase of ecosystem productivity and carbon storage due to CO_2 fertilization is still uncertain (Terrer et al. 2018).

Early studies using pots and growth chambers hardly reflected the real forest ecosystem conditions (Curtis & Wang 1998, Norby et al. 2010), therefore experiments with Open-Top chamber (OTC) and Free Air CO_2 Enrichment (FACE) techniques have been widely employed (Norby et al. 2010). Many OTC and FACE experiments reported an increased growth and photosynthesis under elevated CO_2 (Hungate et al. 2013, Talhelm et al. 2014). However, whether these effects would last for a long time is questioned. In particular, experiments on elevated CO_2 and soil nitrogen fertilization (Finzi et al. 2007), which was conducted at the Duke-FACE and Oak Ridge-FACE, confirmed that nitrogen can directly limit the increase in forest productivity due to the carbon fertilization response, highlighting the relevance of nitrogen on forest productivity.

Nitrogen is an important component of plant photosynthetic organs and of functional and structural proteins (Kwon et al. 2019). In particular, nitrogen in the leaves constitutes the photosynthetic enzymes, such as chlorophyll and rubisco (ribulose-1,5-biphosphate carboxylase/oxygenase), which determines the maximum carboxyla-

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tion rate (V_{Cmax} – Makino & Osmond 1991, Hikosaka 2004), with a decisive influence on forest productivity (Harley et al. 1992, Harrison et al. 2009). However, owing to increased productivity by long-term exposure to a high CO_2 concentrations, the consumption of nutrients may overcome the nutrients input, leading to a reduction in effective nutrients in the soil, and consequently to a reduction of plants' photosynthesis ability. In particular, as the nitrogen concentration absorbed by plants decreases, its concentration in the leaves is lowered, thus decreasing photosynthesis. This in turn results in a progressive nitrogen limitation (PNL), which offsets the CO_2 fertilization effect (Drake et al. 1997, Luo et al. 2004, Cai et al. 2018). This phenomenon is more common under nitrogen deficiency in the soil (Pettersson & McDonald 1994, McCarthy et al. 2010).

Under PNL, an increase in productivity due to the CO_2 fertilization effect is a short-term phenomenon, whereas in the long term, the ecosystem productivity might decrease again and return to its original state (Luo et al. 2004, Finzi et al. 2007). This suggests that increased forest productivity due to a high atmospheric concentration of CO_2 might be inhibited by soil nitrogen deficiency (Feng et al. 2015). Reich et al. (2006) and Feng et al. (2015) confirmed that the response of plants to CO_2 fertilization was limited by nitrogen deficiency. Moreover, PNL is expected to be more prominent when the high CO_2 concentration environment is maintained for a long time (Rütting 2017).

The main objective of this study was to determine the occurrence of nitrogen limitation under long-term CO_2 fertilization on three major species of the Korean temperate region: Korean red pine (*Pinus densiflora*), Chinese ash (*Fraxinus rhynchophylla*), and Korean mountain ash (*Sorbus alnifolia*). The effect of the increase in atmospheric CO_2 concentration on seedling diameter and height, as well as photosynthetic properties, such as maximum photosynthetic rate, maximum carboxylation rate, and maximum electron transfer rate, was evaluated over a period of 8 years. Moreover, we compared the nitrogen concentration in the leaves and soils to investigate whether the increased productivity due to CO_2 fertilization is a short-term effect owing to a progressive nitrogen limitation (PNL) in the soil.

Materials and methods

Experimental system and design

This study was carried out in an Open Top Chamber (OTC) of the Forest Biotechnology Division at National Institute of Forest Science in Suwon-si, Gyeonggi-do, Republic of Korea (37° 15′ 04″ N, 126° 57′ 29″ E). The OTC was made of a decagon structure of diameter 10 m and height 7 m (see Fig. S1 in Supplementary material). The angle of the roof was 45° in order to maintain an open-

ing ratio of 75% or higher. A polyolefin film of thickness 0.15 mm with a light transmittance of approximately 88%, low specific gravity, and excellent chemical and water resistance was used as an external covering material (Lee et al. 2012).

The atmospheric CO_2 concentrations kept in the chambers during the experiment were: (i) the current atmospheric CO_2 concentration (Chamber 1, aCO_2); (ii) 1.4 times higher CO_2 concentration than the current concentration (Chamber 2, $eCO_21.4$), which is expected to be the atmospheric CO_2 concentration by 2050, according to the IPCC scenario (IPCC 2013); and (iii) 1.8 times higher CO_2 concentration than the current concentration, which is expected to be the atmospheric CO_2 concentration by 2070 (Chamber 3, $eCO_21.8$). The exposure of CO_2 were conducted for 10 hours (08:00-18:00) a day during the growing season (April-November). Pest controls were conducted every year and weed controls were conducted for first four years of the experiment. The annual average, minimum and maximum monthly temperature in chambers were 12.4 ± 0.2 °C, -1.3 ± 0.4 °C, 22.5 ± 0.2 °C, respectively. The annual rainfall range was 751.16 mm yr^{-1} to 1975.96 mm yr^{-1} over the 9 years of the experimental period.

In each chamber, the same three clones for each species were tested. Four-year-old seedlings of Korean red pine (*Pinus densiflora*), two-year-old seedlings of Chinese ash (*Fraxinus rhynchophylla*), and two-year-old seedlings of Korean mountain ash (*Sorbus alnifolia*) were planted in September 2009. The density within the OTC was 2547 seedlings ha^{-1}.

Growth measurements

Growth measurements (height and diameter) on trees started in May 2010 and were repeated every year until 2017 at the beginning (mid-April, mean daily temperature: ~10 °C) and the end (end of October, mean daily temperature: ~5 °C) of the growing season. The annual increase in growth was obtained as the difference in these two measures. Also, the lack of differences in diameter between the last measurement of the previous year and the first of the subsequent year was verified.

Tree height was recorded using a measuring rod (A-15, SENSHIN Industry Co., LTD., Osaka, Japan), while the tree diameter was measured twice in orthogonal directions 10 cm above the root collar using a digital Vernier callipers (CD-10CPX, Mitutoyo, Kawasaki, Japan), and the average value was used. Exceptionally, since 2016, Korean red pine and Chinese ash trees were measured using a diameter tape (F10-02DM, KDS, Malaysia) because of their large diameters.

Gas exchange measurements

The photosynthetic parameters were measured on tree leaves using a portable device LI-6400 (LI-COR Inc., Lincoln, NE,

USA). From 2013 to 2017, except for 2015, the parameters were measured 1-3 times a year from June to August on 3 sunlit leaves for each species. The temperature of the leaves was set at 25 °C and the relative humidity at 55%-60%. The leaves were stabilized before measurements. The area of leaves was set at 6 cm^2 for all the species except Korean red pine. For this species the leaf area was recalculated by measuring the actual leaf area using a scanner after all measurements.

The light response curve was obtained using the photosynthetic rate recorded by sequentially varying the intensity of light irradiated on the leaves, using the following sequence in all the chambers: 1400, 1200, 1000, 800, 600, 400, 200, 100, 75, 50, 25, 0, and 1200 μmol m^{-2} s^{-1}. The reference CO_2 supplied in each LI-6400 chamber was set at the atmospheric CO_2 in each OTC. The maximum photosynthetic rate (A_{max}) was estimated as the photosynthetic rate at light saturation (1200 μmol m^{-2} s^{-1}) under the CO_2 concentration in the chambers, which was measured using the light response curve (Taiz & Zeiger 2010).

The A/C_i curve was achieved by varying the reference CO_2 concentration at light saturation point in the following order: (i) aCO_2: 400, 300, 200, 100, 75, 50, 25, 0, 400, 400, 600, 800, 1000, and 1200 μmol m^{-2} s^{-1}; (ii) $eCO_21.4$: 560, 400, 300, 200, 100, 75, 50, 25, 0, 560, 560, 800, 1000, and 1200 μmol m^{-2} s^{-1}; and (iii) $eCO_21.8$: 720, 600, 400, 300, 200, 100, 75, 50, 25, 0, 720, 720, 1000, and 1200 μmol m^{-2} s^{-1}. The parameters V_{Cmax} and J_{max} were derived using the model proposed by Sharkey (2016) and estimated in Excel™ spreadsheet version 2.0 (Microsoft, Redmond, WA, USA).
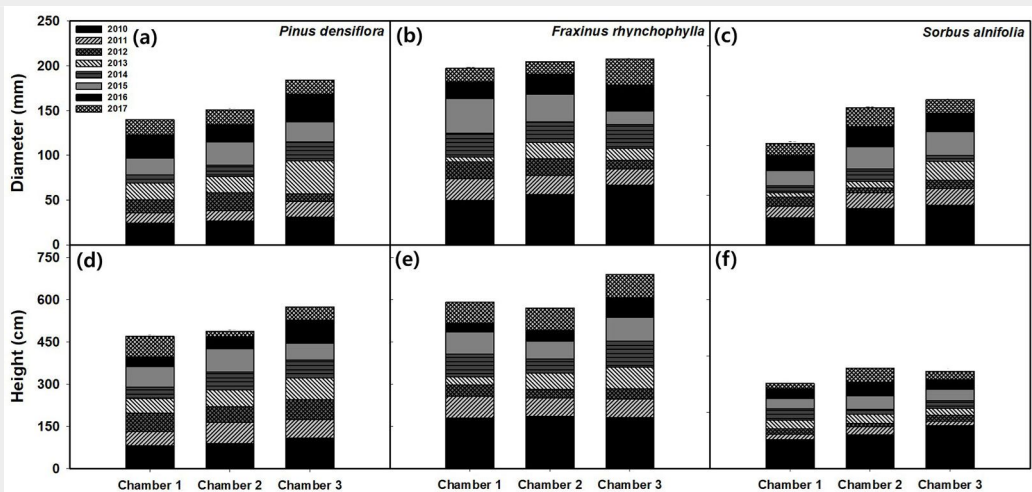
Leaf total nitrogen analysis

Three sunlit leaves were chosen on the same branch and used for photosynthetic measurements (n = 9 for each species, three repetitions for three species), and 1 cm^2 leaf disk was collected in 2017. The leaves were dried in a 70 °C in the lab for more than 72 h, then crushed using a Fast-Prep-24® crusher (MP Biomedicals, Solon, OH, USA), homogenized, and finally analysed for nitrogen content using CHNS Analyzer Flash EA 1112® (Thermo Electron Corporation, USA) at the National Instrumentation Center for Environmental Management (NICEM).

Soil nitrogen analysis

Before planting trees in 2009, the soil in the OTC was excavated to a depth of 1 m and replaced with forest soil to control the soil characteristics of all the treatment groups. Soil at depth < 30 cm was composed of forest soil and sand with a ratio of 1:1 (Lee et al. 2012). For all treatment groups, samples of soil from 0 to 5 cm depth were collected at five randomly selected points in September 2017, air-dried for 3 days at room temperature, and then analysed using the CHNS Analyzer Flash EA

Fig. 1 - Effects of elevated CO_2 concentration (aCO_2, Chamber 1; $eCO_2$1.4, Chamber 2; $eCO_2$1.8, Chamber 3) on average tree diameter (a, b, c) and height (d, e, f) growth per year from 2010 to 2017. There were no significant differences among CO_2 treatments ($p > 0.05$).

1112 described above at the NICEM.

Statistical analysis

All statistical analyses were carried using the software R ver. 3.3.2 (R Core Team 2016). Growth, photosynthesis variables and leaf total nitrogen over time were analyzed by repeated-measures ANOVA with CO_2 treatments as a fixed factor and the recording year as the repeated-measure. When significant CO_2 × Year interactions and CO_2 or Year effect were detected, means were compared using Tukey *post-hoc* comparisons. In addition, one-way ANOVA of individual species and Tukey *post-hoc* comparisons were conducted to assess the CO_2 effect for each year.

Results

To investigate the CO_2 fertilization effect over the period 2010-2017, diameter and height increments of trees of different treatments and species were recorded (Tab. 1, Tab. 2). The cumulative increments for height and diameter over the 8 years of the experiment are presented in Fig. 1.

Diameter growth

Tab. 1 summarizes the annual diameter in-

crease measured after CO_2 exposure. The results showed a significant increment in diameter growth under high CO_2 concentration due to the CO_2 fertilization effect (p <0.001 – Tab. 3). On average, the difference between aCO_2 and $eCO_2$1.8 was 47.7%, 25.3%, and 3.1% in Korean mountain ash (41.4 ± 6.2 *vs.* 61.1 ± 7.9 mm), Korean red pine (144.7 ± 15.1 *vs.* 181.3 ± 13.5 mm), and Chinese ash (70.9 ± 9.2 *vs.* 73.1 ± 5.5 mm), respectively.

The annual diameter increment of individual species under eCO_2 was significantly enhanced in all the species in 2010-2011 (maximum $p = 0.029$ – Tab. 3). However, since 2012, there was no significant difference in diameter increment under eCO_2. Korean red pine showed a significant difference in diameter growth under eCO_2 in 2011 in the order of $aCO_2 < eCO_2$1.4 $< eCO_2$1.8, an increase of about 100.0% (aCO_2 *vs.* $eCO_2$1.8, $p = 0.015$ – Tab. 1). Korean mountain ash showed significant difference under eCO_2 in 2010, an increase of about 57.9% (aCO_2: 5.7 ± 0.7 *vs.* $eCO_2$1.8: 9.0 ± 0.5 mm; $p = 0.02$).

Height growth

Similarly to growth in diameter, annual in-

crease in tree height was recorded each year from 2010 until 2017 (Fig. 1). For all the species, the increase in height was high under eCO_2 ($p < 0.01$ – Tab. 2). On average, the difference between aCO_2 and $eCO_2$1.8 was 22.01%, 18.3% and 14.3% in Korean red pine (470.7 ± 22.8 *vs.* 574.3 ± 72.5 cm), Chinese ash (585.3 ± 39.1 *vs.* 692.3 ± 48.1 cm) and Korean mountain ash (299.3 ± 40.5 *vs.* 342.0 ± 8.2 cm), respectively.

Tab. 2 summarizes the annual growth of tree height measured after CO_2 exposure. For all the species, results were similar to the diameter growth. Chinese ash showed a significant difference in height growth under eCO_2 in 2010 ($p = 0.029$). Differences were observed under $eCO_2$1.8 and aCO_2, which was increased about 57.9%. Korean red pine showed a significant increase under eCO_2 in 2011, an increase of about 110.7% ($p = 0.006$). However, after 2012, there was no difference in the increase in height due to CO_2 concentration for all the species.

Maximum photosynthetic rate (A_{max})

The average A_{max} under eCO_2 measured from 2013 increased in the following order: aCO_2 (12.7 ± 0.7 µmol m^{-2} s^{-1}) < $eCO_2$1.4 (13.6

Tab. 1 - Annual growth in diameter (mm, mean ± SE) of Korean red pine (*Pinus densiflora*, Pd), Chinese ash (*Fraxinus rhynchophylla*, Fr) and Korean mountain ash (*Sorbus alniflolia*, Sa) in response to aCO_2 (Chamber 1), $eCO_2$1.4 (Chamber 2) and $eCO_2$1.8 (Chamber 3) during 8 years. Different lowercase letters indicate significant ($p < 0.05$) multiple comparison results among CO_2 treatments (one-way ANOVA followed by Tukey *post-hoc* test). (ns): not significant.

Species	Treatments	Diameter growth (mm)							
		2010	2011	2012	2013	2014	2015	2016	2017
Pd	aCO_2	11.2 ± 1.4 [ns]	8.5 ± 1.4 [b]	11.6 ± 10.1 [ns]	19.0 ± 3.5 [ns]	9.3 ± 3.3 [ns]	18.8 ± 7.7 [ns]	25.8 ± 7.7 [ns]	16.7 ± 5.2 [ns]
	$eCO_2$1.4	11.9 ± 0.2 [ns]	11.4 ± 1.1 [ab]	20.3 ± 4.2 [ns]	18.0 ± 6.7 [ns]	12.3 ± 3.8 [ns]	26.3 ± 6.3 [ns]	19.3 ± 9.1 [ns]	16.5 ± 3.8 [ns]
	$CeCO_2$1.8	12.5 ± 2.9 [ns]	17.0 ± 1.7 [a]	8.9 ± 3.4 [ns]	40.0 ± 24.1 [ns]	20.1 ± 4.8 [ns]	25.2 ± 0.9 [ns]	31.0 ± 5.4 [ns]	19.2 ± 3.0 [ns]
Fr	aCO_2	11.7 ± 0.8 [ns]	8.6 ± 2.3 [ns]	8.8 ± 3.4 [ns]	2.0 ± 1.2 [ns]	12.6 ± 5.6 [ns]	13.8 ± 1.1 [ns]	6.8 ± 1.7 [ns]	5.5 ± 1.0 [ns]
	$eCO_2$1.4	14.4 ± 2.0 [ns]	7.6 ± 1.4 [ns]	4.7 ± 6.0 [ns]	3.0 ± 1.7 [ns]	8.4 ± 4.6 [ns]	11.0 ± 1.9 [ns]	8.2 ± 2.0 [ns]	5.0 ± 2.2 [ns]
	$CeCO_2$1.8	18.5 ± 2.5 [ns]	5.3 ± 2.9 [ns]	3.4 ± 6.0 [ns]	5.0 ± 3.1 [ns]	7.0 ± 0.7 [ns]	5.5 ± 0.1 [ns]	10.5 ± 1.9 [ns]	10.6 ± 2.7 [ns]
Sa	aCO_2	5.7 ± 0.7 [b]	4.5 ± 0.5 [ns]	3.8 ± 4.9 [ns]	1.7 ± 0.7 [ns]	3.0 ± 0.6 [ns]	6.1 ± 2.5 [ns]	6.0 ± 3.3 [ns]	4.9 ± 0.9 [ns]
	$CeCO_2$1.4	7.0 ± 0.5 [ab]	6.3 ± 0.2 [ns]	5.1 ± 27 [ns]	2.7 ± 0.8 [ns]	4.7 ± 0.9 [ns]	9.0 ± 1.9 [ns]	8.0 ± 0.9 [ns]	7.6 ± 2.9 [ns]
	$eCO_2$1.8	9.0 ± 0.5 [a]	6.9 ± 0.6 [ns]	3.2 ± 2.6 [ns]	7.7 ± 4.7 [ns]	2.3 ± 2.0 [ns]	9.6 ± 1.2 [ns]	7.4 ± 1.7 [ns]	3.5 ± 2.3 [ns]

Tab. 2 - Annual growth in height (cm, mean ± SE) of Korean red pine (*Pinus densiflora*, Pd), Chinese ash (*Fraxinus rhynchophylla*, Fr) and Korean mountain ash (*Sorbus alniflolia*, Sa) in response to aCO_2 (Chamber 1), $eCO_21.4$ (Chamber 2) and $eCO_21.8$ (Chamber 3) during 8 years. Different lowercase letters indicate significant ($p < 0.05$) multiple comparison results among CO_2 treatments (one-way ANOVA followed by Tukey *post-hoc* test). (ns): not significant.

Species	Treatment	Height growth (cm)							
		2010	2011	2012	2013	2014	2015	2016	2017
Pd	aCO_2	10.5 ± 1.6 [ns]	27.0 ± 0.9 [b]	65.2 ± 6.6 [ns]	52.3 ± 13.0 [ns]	41.3 ± 18.3 [ns]	72.0 ± 5.1 [ns]	35.3 ± 2.7 [ns]	73.0 ± 22.1 [ns]
	$eCO_21.4$	11.5 ± 1.5 [ns]	11.4 ± 1.1 [b]	57.6 ± 19.1 [ns]	59.3 ± 9.5 [ns]	65.7 ± 8.7 [ns]	94.7 ± 49.6 [ns]	50.0 ± 46.0 [ns]	17.7 ± 24.6 [ns]
	$eCO_21.8$	6.3 ± 1.6 [ns]	56.9 ± 7.5 [a]	70.5 ± 34.6 [ns]	77.0 ± 5.3 [ns]	63.0 ± 6.1 [ns]	60.0 ± 20.0 [ns]	99.0 ± 21.7 [ns]	48.0 ± 17.1 [ns]
Fr	aCO_2	136.2 ± 23.3 [b]	86.5 ± 11.9 [ns]	29.0 ± 16.0 [ns]	13.0 ± 2.1 [ns]	90.7 ± 34.7 [ns]	89.3 ± 44.9 [ns]	30.2 ± 22.3 [ns]	78.3 ± 2.6 [ns]
	$eCO_21.4$	145.4 ± 14.8 [ab]	7.6 ± 1.3 [ns]	22.9 ± 3.3 [ns]	27.7 ± 12.8 [ns]	60.3 ± 29.5 [ns]	73.7 ± 18.2 [ns]	29.3 ± 20.1 [ns]	86.7 ± 0.9 [ns]
	$eCO_21.8$	215.0 ± 8.3 [a]	84.4 ± 18.4 [ns]	9.0 ± 3.3 [ns]	75.7 ± 33.1 [ns]	92.0 ± 40.8 [ns]	81.7 ± 10.9 [ns]	70.3 ± 19.7 [ns]	82.7 ± 39.8 [ns]
Sa	aCO_2	39.1 ± 7.0 [ns]	27.0 ± 4.3 [ns]	18.2 ± 12.8 [ns]	51.0 ± 16.8 [ns]	42.3 ± 20.3 [ns]	38.0 ± 3.21 [ns]	43.0 ± 2.1 [ns]	18.7 ± 9.8 [ns]
	$eCO_21.4$	55.7 ± 15.9 [ns]	33.4 ± 9.7 [ns]	10.6 ± 5.5 [ns]	53.7 ± 33.7 [ns]	17.7 ± 7.2 [ns]	46.6 ± 13.1 [ns]	59.0 ± 11.5 [ns]	50.0 ± 8.0 [ns]
	$eCO_21.8$	85.8 ± 11.4 [ns]	17.3 ± 6.5 [ns]	22.3 ± 15.1 [ns]	16.0 ± 0.7 [ns]	29.3 ± 14.4 [ns]	45.3 ± 7.8 [ns]	35.7 ± 6.0 [ns]	36.0 ± 18.6 [ns]

Tab. 3 - Results statistics for diameter, height, A_{max}, Vc_{max}, J_{max} and leaf total N of all species with F statistics and p-values from two-way repeated-measure ANOVA.

Parameter	Factor	F	*p*
Diameter	CO_2	58.03	< 0.001
	Year	19.08	< 0.001
	$CO_2 \times$ Year	1.04	0.982
Height	CO_2	17.727	< 0.001
	Year	9.201	< 0.001
	$CO_2 \times$ Year	0.367	0.416
A_{max}	CO_2	2.942	0.06
	Year	13.474	< 0.001
	$CO_2 \times$ Year	0.771	0.594
Vc_{max}	CO_2	1.662	0.194
	Year	2.791	0.030
	$CO_2 \times$ Year	0.545	0.819
J_{max}	CO_2	0.192	0.825
	Year	0.628	0.644
	$CO_2 \times$ Year	0.251	0.979
Leaf Total N	CO_2	3.701	0.026
	Year	5.295	0.002
	$CO_2 \times$ Year	0.945	0.464

age A_{max} (minimum $p = 0.094$ – Tab. 4), but Korean mountain ash showed significant CO_2 effect. Korean red pine in all the years under eCO_2 showed no specific tendency. The A_{max} of Korean mountain ash was 14.0 ± 1.3 µmol m^{-2} s^{-1} under $eCO_21.8$, which showed an enhancement from 10.7 ± 0.7 µmol m^{-2} s^{-1} under aCO_2 ($p= 0.014$). The difference in the A_{max} due to CO_2 exposure in each year did not show significant differences (Fig. 2c). The A_{max} of Chinese ash decreased in the following order: aCO_2 (12.4 ± 1.2 µmol m^{-2} s^{-1}) < $eCO_21.4$ (15.0 ± 1.7 µmol m^{-2} s^{-1}) < $eCO_21.8$ (17.4 ± 1.6 µmol m^{-2} s^{-1}), and also decreased steadily from 2013 (19.4 ± 1.1 µmol m^{-2} s^{-1}) to 2017 (11.7 ± 0.8 µmol m^{-2} s^{-1}) by about 39.7%, though not significantly ($p=0.094$, $p=0.095$, respectively – Fig. 2b). However, the enhancement of A_{max} due to elevated CO_2 was 27.7% at $eCO_21.8$, and the increase was constantly maintained at 28.6% under $eCO_21.8$ in 2013 and 27.5% in 2017 relative to the rate under aCO_2.

## Maximum rate of carboxylation (V_{Cmax}) and electron transport (J_{max})

Unlike the maximum photosynthetic rate, the V_{Cmax} was decreased of about 13.6% from $eCO_21.8$ (49.6 ± 3.4 µmol m^{-2} s^{-1}) to aCO_2 (57.4 ± 3.5 µmol m^{-2} s^{-1}) but did not dif-

± 0.8 µmol m^{-2} s^{-1}) < $eCO_21.8$ (14.9 ± 0.8 µmol m^{-2} s^{-1}) but did not differ significantly ($p = 0.06$ – Tab. 3). In terms of response by year, the average A_{max} decreased by about 28.9%, from 15.9 ± 0.8 µmol m^{-2} s^{-1} in 2013 to 11.3 ± 0.9 µmol m^{-2} s^{-1} in 2016 ($p < 0.001$). However, these results showed differences among species and year (Fig. 2, Tab. 4).

Korean red pine and Chinese ash did not show a significant difference in the aver-

Tab. 4 - Results statistics of diameter, height, A_{max}, Vc_{max}, J_{max} and leaf total N of the studied species with F statistics and p-values from two-way repeated measured ANOVA. (Pd): Korean red pine (*Pinus densiflora*); (Fr): Chinese ash (*Fraxinus rhyncophylla*); (Sa): Korean mountain ash (*Sorbus alnifolia*).

Species	Factors	Diameter		Height		A_{max}		Vc_{max}		J_{max}		Leaf Total N	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Pd	CO_2	0.632	0.540	14.449	< 0.001	0.266	0.771	0.618	0.554	0.719	0.506	1.459	0.247
	Year	5.827	< 0.001	9.673	< 0.001	0.840	0.498	0.979	0.452	1.957	0.161	7.837	< 0.001
	$CO_2 \times$ Year	0.249	0.995	1.250	0.306	1.189	0.374	2.817	0.051	0.511	0.810	3.793	0.006
Fr	CO_2	30.737	< 0.001	5.527	0.010	2.891	0.094	1.594	0.238	0.486	0.625	4.025	0.021
	Year	10.920	< 0.001	0.403	0.890	2.661	0.095	2.232	0.118	2.364	0.103	13.145	< 0.001
	$CO_2 \times$ Year	2.537	0.021	0.456	0.935	0.545	0.765	1.368	0.292	0.507	0.815	2.646	0.021
Sa	CO_2	11.368	< 0.001	13.344	< 0.001	6.983	0.014	2.890	0.094	1.782	0.210	0.178	0.838
	Year	0.179	0.987	0.583	0.762	0.918	0.433	5.243	0.015	2.931	0.0768	0.765	0.522
	$CO_2 \times$ Year	2.611	0.018	1.809	0.097	1.123	0.404	0.632	0.702	0.708	0.649	1.288	0.289

Fig. 2 - Effects of CO_2 concentration (aCO_2, $eCO_21.4$, $eCO_21.8$) on maximum photosynthetic rate (A_{max}), maximum rate of carboxylation (Vc_{max}), and maximum rate of electron transport (J_{max}) in: (a, d, g) Korean red pine (*Pinus densiflora*); (b, e, h) Chinese ash (*Fraxinus rhynchophylla*); and (c, f, i) Korean mountain ash (*Sorbus alnifolia*). The values shown are mean ± SE. Different lowercase letters above the bars indicate significant ($p < 0.05$) differences from multiple comparison among CO_2 treatments. (ns): not significant.
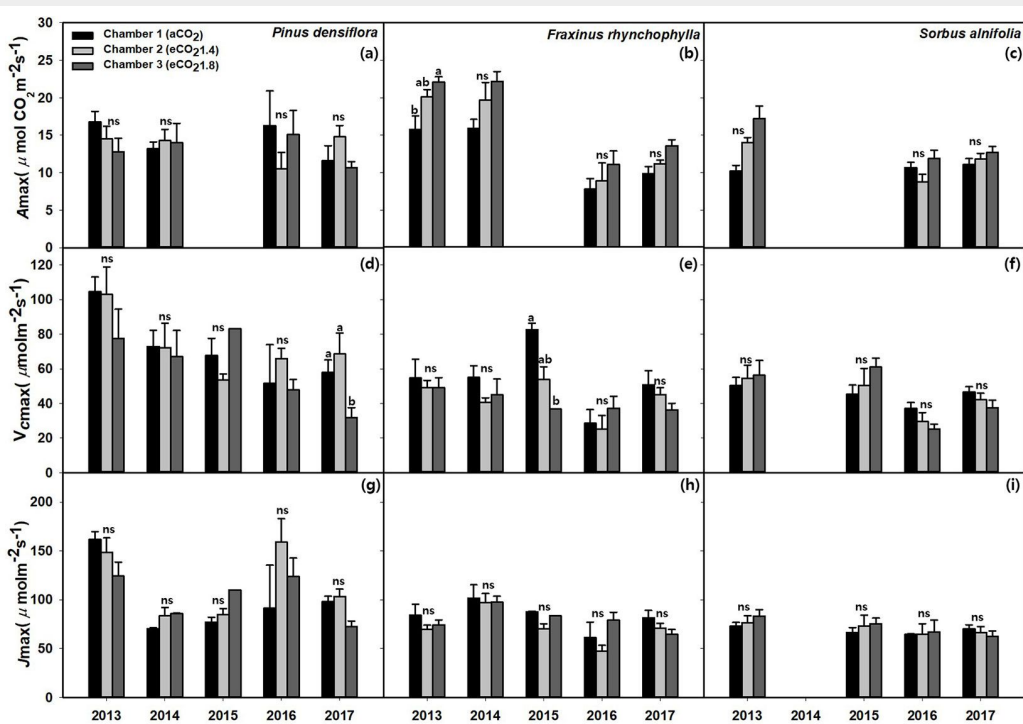
fer significantly ($p = 0.194$ – Tab. 3). Similar to the A_{max}, the Vc_{max} value by year was 68.0 ± 5.2 μmol m^{-2} s^{-1} in 2013 and it significantly decreased to 46.7 ± 2.6 μmol m^{-2} s^{-1} in 2017 ($p = 0.03$).

In terms of response by species and year, Korean red pine showed no significant differences with CO_2 and year ($p = 0.554$ and $p = 0.452$, respectively – Tab. 4). Contrastingly, Vc_{max} showed a decreasing tendency of about 46.4% from 2013 (98.1 ± 8.1 μmol m^{-2} s^{-1}) to 2017 (52.6 ± 5.9 μmol m^{-2} s^{-1}). Depending on the CO_2 concentration, it also showed same decreasing trend by about 14.8% from aCO_2 (70.9 ± 7.1 μmol m^{-2} s^{-1}) to $eCO_21.8$ (60.4 ± 7.5 μmol m^{-2} s^{-1}) and the difference between $eCO_21.8$ and aCO_2 was the highest and significantly different in 2017 ($p = 0.002$ – Fig. 2d). The annual Vc_{max} of Korean mountain ash decreased significantly by 23.5% from 55.0 ± 4.5 μmol m^{-2} s^{-1} in 2013 to 42.1 ± 2.0 μmol m^{-2} s^{-1} in 2017 ($p=0.015$).

For Chinese ash, no statistical differences were found. However, its annual Vc_{max} decreased under $eCO_21.8$ by 55.7% compared with that under aCO_2 in 2015 ($p = 0.03$, Fig. 2e), but there was no difference thereafter. All species showed no significant differences in Vc_{max} due to CO_2 enhancement (minimum $p = 0.094$).

The J_{max} showed no significant differences under eCO_2 and year (Tab. 3, Tab. 4). However, it had a decreasing tendency of about 23.4% from 2013 (100.2 ± 7.2 μmol m^{-2} s^{-1}) to 2017 (76.8 ± 3.1 μmol m^{-2} s^{-1}) with no significance. Depending on the CO_2 concentration, there was no significant differences and tendency (minimum $p = 0.210$). In terms of response by species, all species showed no statistical differences with CO_2 and year (Fig. 2g, Fig. 2h, Fig. 2i).

Leaf and soil N

The leaf total nitrogen was significantly

different under eCO_2 and year (maximum $p = 0.026$ – Tab. 3). The leaf total N exposed to CO_2 significantly ($p = 0.026$) decreased in the following order: aCO_2 (1.57 ± 0.05 %) > $eCO_21.8$ (1.28 ± 0.04 %) > $eCO_21.4$ (1.24 ± 0.03 %).

Korean red pine showed significant differences in leaf total N under CO_2 exposure ($p < 0.001$ – Tab. 4). The leaf total N decreased significantly by about 24.4% from 2013 (1.31 ± 0.08 %) to 2017 (0.99 ± 0.03 %). The leaf total N under eCO_2 decreased in the following order: aCO_2 (1.45 ± 0.15 %) > $eCO_21.8$ (1.21 ± 0.09 %) > $eCO_21.4$ (1.07 ± 0.04 %) but it was not significant ($p = 0.247$). The $eCO_21.4$ treatment showed the lowest leaf total N and the difference between aCO_2 and $eCO_21.4$ were 31.0% in 2013, 27.9% in 2015, and 10% in 2017 (Fig. 3a).

Chinese ash showed a significant decrease in leaf total N under eCO_2 and year ($p = 0.021$ and $p < 0.001$, respectively). The
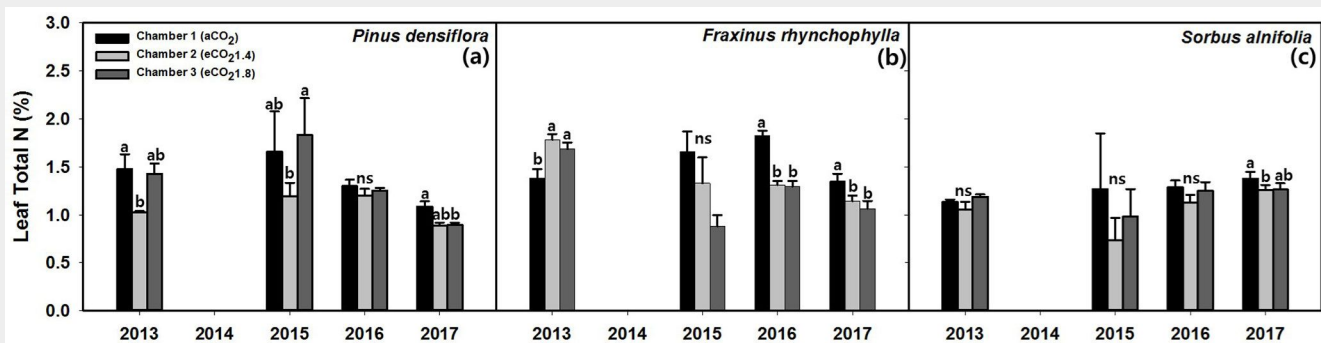
Fig. 3 - Effects of CO_2 concentration (aCO_2, $eCO_21.4$, $eCO_21.8$) on leaf total nitrogen content (%) in (a) Korean red pine (*Pinus densiflora*), (b) Chinese ash (*Fraxinus rhynchophylla*), (c) Korean mountain ash (*Sorbus alnifolia*). The values shown are mean ± SE. Different lowercase letters above the bars indicate significant ($p < 0.05$) differences from multiple comparison among CO_2 treatments. (ns): not significant.

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Fig. 4 - Effects of elevated CO_2 concentration (aCO_2, $eCO_21.4$, $eCO_21.8$) on the soil total nitrogen (%) in 2017. Values are mean ± SE. There were no significant differences among CO_2 treatments ($p > 0.05$).
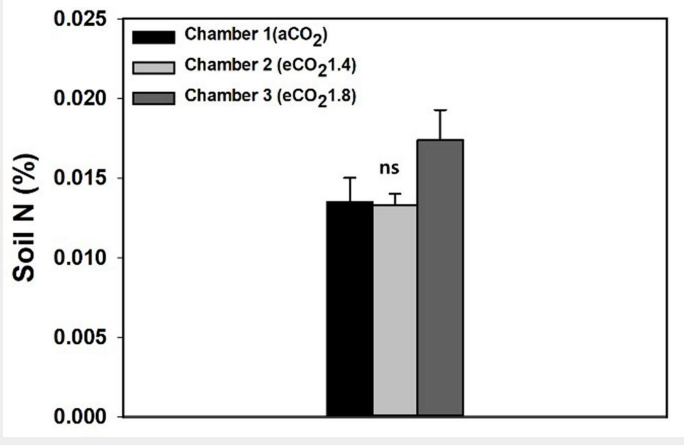
leaf total N under eCO_2 decreased significantly ($p = 0.021$) in the following order: aCO_2 (1.78 ± 0.04 %) > $eCO_21.4$ (1.41 ± 0.04 %) > $eCO_21.8$ (1.36 ± 0.05 %). In particular, the leaf total N was higher under $eCO_21.4$ and $eCO_21.8$ than under aCO_2 in 2013 ($p < 0.001$), but it became significantly lower under $eCO_21.4$ and $eCO_21.8$ than under aCO_2 over time (Fig. 3b). The difference between aCO_2 and $eCO_21.8$ was 27.8% in 2016, and 18.8% in 2017 (maximum $p = 0.04$).

Korean mountain ash showed no significant differences under eCO_2 and year (minimum $p = 0.522$). But it decreased significantly by 15.4% under $eCO_21.4$ compared with aCO_2 only in 2017 ($p = 0.03$ – Fig. 3c).

There was no significant difference in soil total N under eCO_2 in 2017 ($p = 0.125$). The absolute concentration of soil total N was very low, and therefore, the variation depending on CO_2 concentration was negligible (Fig. 4).

Discussion

Relationship between the CO_2 fertilization effect and growth

Several studies have reported that elevated CO_2 concentration due to climate change directly affects photosynthesis, suggesting that eCO_2 promotes ecosystem production and biomass accumulation (McCarthy et al. 2010, Norby et al. 2010, Reich et al. 2018). For instances, the net primary production (NPP) increased by 22-32% in the Duke-FACE experiment, which conducted the CO_2 exposure for more than 10 years. In the Pop-FACE experiment, the diameter increased about 5% and the 12 species leaf area increased about 8% in seven FACE (Ainsworth & Long 2005, Finzi et al. 2007, McCarthy et al. 2010). In this study, the annual growth of the three speices was analysed, confirming a significant enhancement in height and diameter under eCO_2 for the first two years. However, the CO_2 fertilization effect was not sustained thereafter (Tab. 1, Tab. 2, Fig. 1). Temporary CO_2 fertilization effect was also reported by other studies. In the Oak-Ridge FACE and BioCON experiments, the reduction of CO_2 effect was offset after 6 and 3

years, respectively, and nitrogen fertilization showed an immediate increase in NPP (Reich et al. 2006, Norby et al. 2010). In addition, there was no CO_2 effect in *Picea abies* growing on a very low-nutrient soil (Ward et al. 2008). Moreover, in the Duke FACE experiment, where the CO_2 fertilization effect remained for more than 10 years, soil N positively correlated with productivity increment under eCO_2 (McCarthy et al. 2010). Thus, soil nutrients and CO_2 fertilization effect appeared to be closely related. In particular, in our study site, the soil total N was very low because the nearby forest soil was mixed with sand and leaf total N decreased over time. For this reason, we argue that growth promotion through the CO_2 fertilization effect was not sustained.

Changes in photosynthetic properties under elevated CO_2 concentration

Elevated atmospheric CO_2 concentration increases the difference in the partial pressure of CO_2 between the atmosphere and leaf mesophyll tissues, increasing the maximum photosynthetic rate (Curtis & Wang 1998, Ainsworth & Rogers 2007). Twelve FACE experiments showed that the average A_{max} of all the tested species increased by about 31%. Depending on species, it was increased by about 37% in *Pinus australis* (Mozdzer & Caplan 2018), 67% in *P. taeda*, and 62% in *Liquidambar styraciflua* (Ellsworth et al. 2012). However, over time, the increase of A_{max} under eCO_2 was reduced by the acclimation of photosynthetic properties (Ainsworth & Rogers 2007). In our study, the reduction of A_{max} increase under eCO_2 varied depending on the species (Fig. 2). Korean red pine and Korean mountain ash did not show a significant difference since 2013, which was only three years after eCO_2 exposure. Chinese ash showed the enhancement in 2013, but the increase in A_{max} was reduced over time. A_{max} of Korean mountain ash under aCO_2 was maintained over time, but it was decreased in Chinese ash, which might have been due to the low soil nutrient (Fig. 2, Fig. 4). This decrease in the A_{max} resulted in the down-regulation of the Vc_{max} and J_{max}. In other words, the A_{max}

is usually measured under the CO_2 concentration of each treatment, therefore no difference or a decrease indicates that photosynthesis enhancement is not maintained, rather lowered over time. Similar to previous studies (Drake et al. 1997, Martínez-Carrasco et al. 2005), we observed a reduction in the Vc_{max} and J_{max} under eCO_2. This reduction usually occurs after long term CO_2 exposure (Ainsworth & Rogers 2007, Norby et al. 2010). In *P. ponderosa* exposed to eCO_2 for 6 years, the V_{Cmax} and J_{max} were decreased by about 36% and 21%, respectively (Tissue et al. 1999), and the V_{Cmax} was decreased by about 19% in *P. abies* (Uddling & Wallin 2012). However, in some cases, the reduction of photosynthesic ability under eCO_2 did not occur even in the long term (more than 6 years – Bader et al. 2010, Darbah et al. 2010, Warren et al. 2015). In an experiment on *Fagus sylvatica, Quercus petraea, Carpinus betulus, Acer campestre*, and *Tilia platyphyllos* in mature deciduous forests, the increase in the A_{max} was maintained even after 8 years of CO_2 treatment, and the down-regulation of V_{Cmax} and J_{max} did not occur. Similarly, in the Aspen FACE experiment, the increases in V_{Cmax} (48%-85%) and J_{max} (23%-34%) were sustained for 11 years, and no reduction occurred. Warren et al. (2015) also showed that the increase in photosynthesis was maintained during the first 8 years when the leaf N was 0.2 mg cm^{-2} or more, but after a decrease of leaf N, the CO_2 fertilization effect disappeared. Thus, the increase in photosynthesis and the decrease in photosynthesic ability are largely affected by the nitrogen available in the environment. These studies were conducted in well-developed organic layer of forest floor, such as mature stands (Bader et al. 2010), or soil N of 3% or more (Darbah et al. 2010), *i.e.*, environments with considerably higher N than normal forest soil. On the contrary, the long-term exposure under eCO_2 leads to nitrogen limitation in common forest soil, which results in PNL that decreases photosynthesis and growth increment (Reich et al. 2006, Norby et al. 2010, Feng et al. 2015, Rütting 2017).

Relationship between photosynthetic properties (Vc_{max} and J_{max}) and leaf nitrogen

In general, the leaf total N is positively correlated with the photosynthesis ability (Evans & Seemann 1989). Several studies have shown that the leaf N decreased in response to long-term eCO_2 condition (Ellsworth et al. 2012). The elevated CO_2 concentration increases the capacity of the rubisco enzyme to adsorb carbon dioxide, thereby causing carboxylation and a decrease in the demand of nitrogen for rubisco (Nowak et al. 2004, Liu et al. 2012). Similarly, in this study, the leaf N was significantly lowered under eCO_2 over time (Fig. 3). In addition, studies have shown that the Vc_{max} and photosynthesis ability, decreased as the leaf N decreased (Ainsworth & Long

2005, Warren et al. 2015). Thus, fertilization at the time of nitrogen limitation increased the CO_2 fertilization effect again or the decreasing rate was reduced (Liberloo et al. 2007, Crous et al. 2008, Terrer et al. 2018).

In general, there is a strong correlation between V_{Cmax} and J_{max}, with J_{max}/V_{Cmax} = 1.5-2.0 at 25 °C (Medlyn et al. 2002, Walker et al. 2014, Cho et al. 2019). Similarly, V_{Cmax}, J_{max}, and leaf N showed correlation under eCO_2 (Crous et al. 2008, Warren et al. 2015). Therefore, when the leaf N and V_{Cmax} were decreased after long-term eCO_2 treatment, the J_{max} also decreased in some cases (Herrick & Thomas 2001, Medlyn et al. 2002, Sholtis et al. 2004, Crous et al. 2008, Bader et al. 2010). As in our study, the decrease in J_{max} (Tab. 4) was relatively lower than that of Vc_{max} in some cases (Liberloo et al. 2007, Warren et al. 2015). Moreover, these changes are different depending on species. Chinese ash and Korean red pine showed a decrease only in the V_{Cmax}, while Korean mountain ash did not show any decrease in both the above photosynthetic parameters (Fig. 2). These differences due to the change in intracellular nitrogen distribution under eCO_2 are dependent not only on the environment but also on the species. Further studies are needed to examine how species change the intercellular nitrogen distribution under elevated CO_2 (Evans & Seemann 1989, Hikosaka 2004).

CO_2 fertilization effect and PNL

The enhanced productivity of forest ecosystem due to the CO_2 fertilization is heavily influenced by the nutrients available in the soil (Finzi et al. 2007). PNL is a hypothesis that the increased forest productivity due to eCO_2 decreases over time because of the increased N accumulation in the biomass, resulting in a decrease of soil N availability, an increase of N immobilization and a decrease of N mineralization (Luo et al. 2004). In the long-term eCO_2 at Duke-FACE, Oak-Ridge FACE, and BioCON experiments, N fertilization led to an immediate increase in NPP. Therefore, there seemed to be an interaction between eCO_2 and N in terms of increased productivity (Reich et al. 2006, McCarthy et al. 2010, Norby et al. 2010). In particular, the soil N is important and N deficiency leads to a decline in production due to PNL even under eCO_2 (Norby et al. 2016). Such N deficiency changes the N distribution in the plants' organs for effective nitrogen utilization, resulting in a decrease of the N used for above-ground photosynthesis (Pettersson & McDonald 1994). In addition, N distribution in photosynthetic apparatus such as Rubisco is greatly reduced. Therefore, nitrogen-use-efficiency is increased generally (Vicente et al. 2016, Sharwood et al. 2017), but the persistence of such an increase is controversial (Ainsworth & Rogers 2007, Leakey et al. 2009). There is limited information about N distribution in the above- and below-ground under eCO_2. Therefore, it is necessary to determine and quantify the distribution of N in the above- and below-ground through follow-up studies.

Conclusions

In this study, we investigated the physiological and morphological characteristics of Korean red pine, Chinese ash, and Korean mountain ash, which are native tree species in Korea, under eCO_2 over a period of 8 years. We also examined the longevity of CO_2 fertilization effect in our study sites.

The CO_2 fertilization effect caused by eCO_2 led to an increase in growth in the early stage of exposure, but there was no significant difference thereafter. Photosynthetic properties showed a decreasing tendency in all species and a down-regulation of photosynthetic capacity increase with time, especially in Korean red pine and Korean mountain ash. The analysis of leaf and soil N to identify the cause revealed a significant decrease of leaf N under eCO_2. We argue that the enhancement of productivity might have decreased due to low soil N.

In conclusion, progressive nitrogen limitation (PNL) caused by the N reduction might have already started or is about to start in our study sites. Further investigation is needed to clarify N use efficiency and nitrogen distribution according to species.

List of abbreviations

(OTC): Open Top Chamber; (aCO_2): current atmospheric CO_2 concentration ; (e$CO_2$1.4): 1.4 times higher CO_2 concentration than the current concentration (Chamber 2); (e$CO_2$1.8): 1.8 times higher CO_2 concentration than the current concentration (Chamber 3); (A_{max}): maximum photosynthetic rate; (V_{Cmax}): maximum rate of carboxylation; (J_{max}): maximum electron transport rate.

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Supplementary Material

Fig. S1 – The Open Top Chamber (OTC) user in the experiment.

Link: Song_3288@suppl001.pdf