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Title	Photosynthetic traits of Siebold's beech and oak saplings grown under free air ozone exposure in northern Japan
Author(s)	Watanabe, Makoto; Hoshika, Yasutomo; Inada, Naoki et al.
Citation	Environmental Pollution, 174, 50-56 https://doi.org/10.1016/j.envpol.2012.11.006
Issue Date	2013-03
Doc URL	https://hdl.handle.net/2115/51003
Type	journal article
File Information	ENVPOL2012_for_HUSCAP(Watanabe).pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Title

Photosynthetic traits of Siebold's beech and oak saplings grown under free air ozone exposure in northern Japan

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Abstract

We set up a free-air ozone (O_3) exposure system for determining the photosynthetic responses of Siebold’s beech (*Fagus crenata*) and oak (*Quercus mongolica* var. *crispula*) to O_3 under field conditions. Ten-year-old saplings of beech and oak were exposed to an elevated O_3 concentration (60 nmol mol^{-1}) during daytime from 6 August to 11 November 2011. Ozone significantly reduced the net photosynthetic rate in leaves of both species in October, by 46% for beech and 15% for oak. In beech there were significant decreases in maximum rate of carboxylation, maximum rate of electron transport in photosynthesis, nitrogen content and photosynthetic nitrogen use efficiency, but not in oak. Stomatal limitation of photosynthesis was unaffected by O_3. We therefore concluded photosynthesis in beech is more sensitive to O_3 than that in oak, and the O_3-induced reduction of photosynthetic activity in beech was due not to stomatal closure, but to biochemical limitation.

Capsule:

Photosynthesis of beech is more sensitive to free air ozone exposure than that of oak

Key words:

Free air ozone exposure; Photosynthesis; Nitrogen allocation; Beech; Oak

1. Introduction

There is growing concern that the increasing concentration of ozone (O_3) in the troposphere may lead to a high risk of injury and productive decline in vegetation (Matyssek and Sandermann, 2003; Bytnerowicz et al., 2007; Sitch et al., 2007; Wittig et al., 2007, 2009). Since the Industrial Revolution the concentration of O_3 has increased, and this increase is continuing (Akimoto, 2003; Stevenson et al., 2006; Paoletti, 2007). A significant increase in O_3 concentrations in East Asia is predicted in the near future because of rapid increases in emissions of the main O_3 precursors, such as nitrogen oxides and volatile organic compounds (Naja and Akimoto, 2004; Ohara et al., 2007; Yamaji et al., 2008). This makes it important to assess the effect of increasing O_3 on forest trees in East Asia (Kohno et al., 2005; Watanabe et al., 2010; Watanabe et al., 2012).

There are big variations of sensitivity to O_3 between tree species. Many experiments have reported the sensitivities to O_3 of various tree species grown in Japan (e.g. Kohno et al., 2005; Yamaguchi et al., 2011). These previous studies applied chamber experiments to study the effects of O_3. Although chamber experiments offer an advantage in mechanism study owing to their controllability for O_3 concentration, artifacts may arise in the environmental conditions as a result of the difference in micro metrological conditions and the absence of biotic stresses such as herbivores and diseases. Accurate simulation of forest conditions is therefore questionable (Kolb and Matyssek, 2001).

Free-air O_3 fumigation of field-grown trees is a novel technique in assessing the effects of O_3 in field conditions. Studies employing this technology have been conducted in Europe and the USA (e.g. Karnosky et al., 2007; Matyssek et al., 2007;

Oksanen et al., 2007; Díaz-de-Quijano et al., 2012) However, no study has been reported in Asian region for forest tree species although the concerns for O_3 in this region are acute and important. Therefore, we considered experiment under realistic field condition is now needed for the adequate evaluation of O_3 effects.

Siebold's beech (*Fagus crenata*) and oak (*Quercus mongolica* var. *crispula*) are representative forest tree species native to northern Japan. Both of the species distribute cool-temperate region and have similar growth traits, which is late successional with shade tolerance although oak prefers relatively lighter condition than beech (Hokkaido forest tree breeding association, 2008). Kohno et al. (2005) reported that beech is more sensitive to O_3 and oak is less sensitive based on growth responses to O_3 exposure in their experimental study. This difference of O_3 sensitivities between beech and oak in Japan is similar to that between European beech and oak species (Karlsson et al., 2007).

A reduction in tree growth by O_3 is closely related to decline of photosynthetic activity in leaves. Leaf biochemical limitation especially decrease of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity is considered as a main factor of O_3-induced reduction in photosynthetic activity of Siebold's beech (Yonekura et al., 2001; Watanabe et al., 2005; Yamaguchi et al., 2007), whereas Kitao et al. (2009) reported stomatal closure restricted net photosynthetic rate in the leaves of mature European beech tree exposed to free air O_3 enrichment. According to Yamaguchi et al. (2007), O_3-induced negative effects on photosynthesis of Siebold's beech seedlings was not caused by reduction in nitrogen (N) content in leaves but by reduction in N allocation to Rubisco and thereby photosynthetic N use efficiency (PNUE).

The effects of O_3 on photosynthesis are generally become clear in autumn because of higher accumulated O_3 exposure and/or relatively high sensitivity of old leaves to O_3 exposure (Kull et al., 1996; Oksanen et al., 2007; Bagard et al., 2008). As a result, active growing season would become short under relatively high O_3 condition (e.g. Yonekura et al. 2004), indicating reduction of forest productivity and carbon sink strength throughout the leafy period.

We set up a free-air O_3 exposure system in northern Japan and commenced a research of the effects of O_3 on beech and oak sapling under field conditions (Hoshika et al., 2012a, b). To compare the sensitivities of two tree species to O_3, in the present study, we investigated effects of O_3 on leaf photosynthesis in late growing season when the O_3 effects become clear as mentioned above. Our aim is to determine whether beech is also sensitive to elevated O_3 than oak even under realistic field conditions. In addition, we detail our free-air O_3 exposure system.

2. Materials and methods

2.1 Location and plant materials

This study was carried out in Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l.). The annual mean temperature and precipitation in 2011 were 13.5˚C and 1254 mm. The snow-free period is usually from mid-April to late-December. The soil type is brown forest soil. We set two plots, one for ambient plot and another for elevated O_3 plot. Those two plots were separated by about 20 m. The size of each plot was 5.5 m × 7.2 m, and the height was 5.5 m. In each plot, two-year-old seedlings of beech and oak were planted in May 2003, and grown under ambient condition. Thus, those trees were ten-year-old at the start of the present study in 2011. The mean tree height and stem diameter at breast height were 3.3 ± 0.4 m and 26.7 ± 5.9 mm for beech and 5.5 ± 0.7 m and 54.6 ± 13.9 mm for oak. There was no significant difference between the plots in the heights and stem diameters of trees. The soil moisture in the root layer (20 cm depth) was measured by 10HS sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). The average soil moisture was 28.8 ± 4.8% during these measurements. These values were nearly equal to the field capacity (32%) and there was no difference for the soil moisture between two plots.

2.2 Ozone exposure

We conducted the O_3 exposure for 98 days from 6 August to 11 November 2011. The method of O_3 exposure in our system employs the system used at Kranzberg Forest in Germany (Fig. 1, Nunn et al., 2002; Werner and Fabian, 2002). Ozone was generated from pure oxygen by an O_3 generator (Model PZ-1C, Kofloc, Kyoto, Japan). The resulting O_3 was mixed with ambient air, using a three-way valve to control the concentration. The air containing O_3 was then diluted with ambient air in a mixing tank and passed into the canopies through 48 fluorine resin tubes hanging from a fixed grid above the trees down to a height of 50 cm above the ground. Each tube has 10 holes (2 mm diameter) at 50 cm intervals. The ozone concentration at canopy height was monitored continuously by an O_3 monitor (Mod. 202, 2B Technologies, Boulder CO, USA), and the observed value was used as feedback to the three-way valve so as to regulate the O_3 concentration, using the PID algorithm. The target O_3 concentration was 60 nmol mol^{-1} during daylight hours.

2.3 Evaluation of the horizontal distribution of ozone concentration

In early November, the horizontal distribution of the O_3 concentration was determined in the exposure system. We set 12 Ogawa passive samplers for O_3 (Ogawa, Kobe, Japan) at two heights, 2.5 m and 4.0 m, for two weeks. The inverse distance weighted method was applied in estimating the O_3 concentration around the passive samplers.

2.4 Measurement of leaf gas exchange rate

The gas exchange rates of the 1st flush sun leaf were measured on 8, 9, 12 and 14 October 2011 using an open gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Five saplings were randomly selected in each gas plot (i.e. ambient and elevated O_3) for each species and one leaf per each selected tree was used for the gas exchange measurement. Although we observed 2nd flush leaves in both species, we selected the 1st flush leaves for the measurement because the number of 2nd flush leaves was small. The gas exchange rates were measured between 0800 and 1500 hours. The leaf temperature and photosynthetic photon flux density during the measurement were maintained at 25.0 ± 0.5˚C and 1500 μmol m^{-2} s^{-1}. The leaf-to-air vapor pressure deficit was 1.5 ± 0.2 kPa. To obtain the intercellular CO_2 concentration (C_i)-response curve of the net photosynthetic rate (A), i.e., the A/C_i curve, A was determined at ten CO_2 concentration levels in the chamber (C_a, 60-1700 μmol mol^{-1}). We determined A, the stomatal conductance and the ratio of C_i to C_a at C_a = 380 μmol mol^{-1} CO_2 (A_{sat}, G_s and C_i/C_a, respectively). From the A/C_i curve we calculated the stomatal limitation of photosynthesis at C_a = 380 μmol mol^{-1} CO_2, the maximum rate of carboxylation (V_{cmax}) and the maximum rate of electron transport, J_{max} (Farquhar et al., 1980; Long and Bernacchi, 2003). Values of the Rubisco Michaelis constants for CO_2 (K_c) and O_2 (K_o), and the CO_2 compensation point in the absence of dark respiration (Γ^*), for analysis of the A/C_i curve, were all as according to Bernacchi et al. (2001). After the measurement of the A/C_i curve, the leaves were kept at photosynthetic photon flux density = 0 μmol m^{-2} s^{-1} and C_a = 380 μmol mol^{-1} CO_2 for 30 min., and then, dark respiration rate (R) was determined.

2.5 Measurement of leaf traits

After measuring the gas exchange rate, we collected seven leaf discs (12 mm diameter) in order to determine the leaf mass per area (LMA) and the content of N, Rubisco and chlorophyll in the leaves. Four leaf discs, three for Rubisco and one for chlorophyll, were frozen in liquid N immediately after collection, and were stored in a deep freezer at -80˚C prior to analysis; the remaining leaf discs were dried in an oven for 5 days at 70˚C. The LMA was calculated from the area and dry mass of the leaves. The N content of the leaves per unit mass (N_{mass}) was determined by gas chromatography (GC-8A, Shimadzu, Kyoto, Japan) after combustion with circulating O_2 using an NC analyzer (Sumigraph NC-900, Sumika Chemical Analysis Service, Osaka, Japan). A calibration curve was generated using acetanilide (N = 10.36%, C = 71.09, Wako, Osaka, Japan). We calculated the area-based N content (N_{area}) as the product of N_{mass} and LMA, and the PNUE as A_{sat} divided by N_{area}.

For the analysis of Rubisco, frozen leaf discs were powdered with liquid N using a pestle and mortar, and were homogenized with 1 ml extraction buffer containing 100 mM HEPES (pH 8.0), 5 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone, 0.7% (w/v) polyethylene glycol 20000, 1% (v/v) Tween-80 and 24 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000 g for 10 min. at 4˚C. The Rubisco protein in the supernatant was separated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE, Laemmli, 1970). The band of Rubisco was stained by Coomassie brilliant blue and was scanned by an image scanner. The amount of Rubisco was determined using Image J software (National Institutes of Health, Bethesda, MD). Chlorophyll was extracted with dimethyl sulfoxide according to Barnes et al. (1992) and Shinano et al. (1996), and was determined using a spectrophotometer (Gene spec III, Hitachi, Tokyo, Japan).

2.6 Estimation of nitrogen allocation to photosynthetic functions

The photosynthetic apparatus was divided into three parts: Rubisco, bioenergetics (electron carriers except for photosystems, coupling factor and Calvin cycle enzymes except Rubisco), and light-harvesting complex and photosystems. The fraction of leaf N deployed in each function is denoted by N1, N2 and N3 respectively. The N concentration of Rubisco was assumed to be 16% (Takashima et al., 2004; Vogan and Sage, 2012), and N1 was accordingly estimated from the following equation:

$$\mathrm{N1} = 0.16\ \mathrm{Rubisco} / N_{\mathrm{area}}$$

The value of N2 was estimated from gas exchange characteristics according to the following equation (Kitaoka and Koike, 2004; Takashima et al., 2004):

$$N2 = J_{\mathrm{max}} / (156 \times 9.53\ N_{\mathrm{area}})$$

It was assumed that N in bioenergetics is proportional to J_{max}, where the ratio of J_{max} to the cytochrome f content is 156 mmol mol^{-1} s^{-1} (Niinemets and Tenhunen, 1997), and the value of N in bioenergetics per unit cytochrome f is 9.53 mol mmol^{-1} (Hikosaka and Terashima, 1995). N3 was estimated according to the following equation:

$$N3 = 37.1\ \mathrm{Chl} / N_{\mathrm{area}}$$

where Chl is the chlorophyll content (mol m^{-2}), and the N content per unit chlorophyll is 37.1 mol mol^{-1} (Evans and Seemann, 1989).

2.7 Statistical analysis

Statistical analyses were run using PASW Statistics v.18 (IBM, NY, USA). We used a T-test to test the effects of O_3 on each parameter. Individual trees were considered as replication. The reduced major axis regression method was applied to analyze the relationship between N_{area} and photosynthetic parameters.

3. Results

3.1 Regulation of ozone concentration in the exposure system

The average O_3 concentration in the elevated O_3 plot during daytime of O_3 exposure period was 56.7 nmol mol^{-1}, and that in the ambient plot was 25.7 nmol mol^{-1} (Table 1). Regulation of the O_3 concentration was more accurate at low wind speed (<1.0 m); accuracy decreased with increasing wind speed. The proportion of the time within 10% of the target O_3 concentration (i.e. 60 nmol mol^{-1}) in elevated O_3 plot for whole exposure period was 45% and within 20% of the target was 73%. The AOT40 (accumulated exposure over a threshold of 40 nmol mol^{-1}) in daytime during experimental period from 6 August to 11 November was 401 nmol mol^{-1} h for the ambient plot, and 13323 nmol mol^{-1} h for the O_3-enhanced plot.

Figure 2 shows horizontal distributions of the O_3 concentration at heights 2.5 m and 4.0 m. Although we found variation in the O_3 concentration in the system at each height, the average O_3 concentrations at the two heights were similar (50.7 nmol mol^{-1} at 2.5 m and 52.3 nmol mol^{-1} at 4.0 m).

3.2 Leaf traits

For beech, we observed significant O_3-induced reductions in A_{sat}, V_{cmax}, J_{max}, PNUE, N_{area} and the content of Rubisco and chlorophyll, but C_i/C_a and R increased significantly with O_3 (Table 2). The decrease in N_{mass} with exposure to O_3 was marginal. In contrast, the effects of O_3 on leaf traits of oak was small; we found a significant reduction in A_{sat}, a trend of reduction in PNUE and significant increase in R for oak in elevated O_3. The reduction in A_{sat} of beech (46%) was greater than for oak (15%). There was no significant effect of O_3 on G_s or stomatal limitation for either species although G_s of both species in elevated O_3 plot were approximately 18% lower than that in control.

Figure 3 shows the relations between N_{area} and A_{sat}, V_{cmax}, J_{max}, Rubisco content and chlorophyll content for beech saplings. We found significant positive correlations in all relations, and joint decreases in photosynthetic parameters and N_{area} under elevated O_3. The ratio of N allocation to photosynthetic functions tended to be less under elevated O_3 ($P = 0.098$, Fig. 4), as a result of reduced N allocation to N1 and N3.

4. Discussion

The ambient O_3 concentration in this study site was low and constant. Concentrations of O_3 above 50 nmol mol^{-1} were observed for only 6 hours, and the maximum concentration was 57 nmol mol^{-1}. We therefore believe that the ambient plot in the present study was an adequate control although there might be an effect of O_3 on the physiology of the leaves even by this low level of O_3 concentration. The O_3 concentration in the elevated O_3 plot was satisfactorily regulated, especially at relatively low wind speeds, although the average O_3 concentration during the fumigation period was low relative to the target concentration (Table 1). There was no sharp spike of high O_3 concentration, above 90 nmol mol^{-1}, and we therefore consider that our system successfully produced a stably low O_3 concentration.

There were horizontal variations in the concentration of O_3 in the elevated O_3 plot (Fig. 2). Although we did not find any correlation between O_3 concentrations at individual tree and their photosynthetic parameters (data not shown), there is a possibility that horizontal difference of O_3 concentration induces a variation to tree performance after several growing seasons. In subsequent growing seasons we shall adjust for differences in O_3 concentration between positions by opening or closing holes in the fluorine resin tubes carrying the O_3-enhanced air.

Beech showed greater photosynthetic sensitivity to O_3 than oak (Table 2). There was a clear decrease in photosynthetic activity in beech, but not in oak. This trend is consistent with the results of a previous chamber experiment (Kohno et al., 2005). Difference of O_3 uptake in leaves is important factor explaining different O_3 sensitivity between tree species (Karlsson et al., 2007). Unfortunately, we do not have information on the model parameters for estimating O_3 uptake of oak, while that of beech was reported by Hoshika et al. (2012b). According to Table 2, on the other hand, G_s of oak was higher than that of beech, indicating higher O_3 uptake in oak. In that case, the differing sensitivities of beech and oak are due mainly to the detoxification capacity for O_3 and/or reactive oxygen species derived from O_3, but not the actual amount of O_3 taken up (Musselman et al., 2006; Tausz et al., 2007).

We determined photosynthetic activity in autumn to evaluate the effects of O_3 on photosynthesis. Generally, older senescent leaves are more sensitive to elevated O_3 (Kull et al., 1996; Oksanen et al., 2007; Bagard et al., 2008). Following possibility could be raised: the strong effects of O_3 on beech than oak were because of earlier leaf senescence in beech. Lower A_{sat} of beech than that of oak under ambient condition seems to support this possibility (Table 2). However, this trend was also observed in July (before the initiation of O_3 exposure), A_{sat} of beech and oak were 11.8 and 15.3 μmol m^{-2} s^{-1}, respectively (Hoshika et al., 2012a for the A_{sat} of beech). In addition, timing of yellowing and leaf fall of beech and oak were the same under ambient condition in this experimental year 2011 as well as the previous year

2010 (before experiment). Furthermore, O_3-induced decrease of A_{sat} in beech was started from mid-August when the photosynthesis in beech was still active (Hoshika et al., 2012a). Therefore, we consider the timing of leaf senescence was the same between two species and the different photosynthetic responses of the two species to O_3 were due to the difference of the sensitivities to O_3.

We compared the quantitative decrease of light saturated net photosynthetic rate (A_{sat}) in beech against AOT40 found in the present study and previous studies (Fig. 5, Yonekura et al., 2001; Watanabe et al., 2005; Yamaguchi et al., 2007). It should be noted that the O_3 treatments in the previous studies were started from spring (Yonekura et al., 2001; Watanabe et al., 2005) or previous year (Yamaguchi et al., 2007), while that of the present study was from August. The regimes of O_3 exposure also differ, constant 60 ppb for 7 h (10:00-17:00) in Yonekura et al. (2001) and Watanabe et al. (2005), and proportional regime to ambient O_3 concentration for 24 h (1.0, 1.5 and 2.0 times ambient concentration) in Yamaguchi et al. (2007). All the three previous studies applied charcoal-filtered air as a control. The decrease of A_{sat} against AOT40 was greater in the present study than in previous studies. As the maximum G_s was also greater in the present study (Hoshika et al., 2012a, b), it is possible that the difference in stomatal O_3 uptake explains the high sensitivity of beech found here. On the other hand, O_3 exposure was started after development of 1st flush leaves in our study, Yonekura et al. (2001) and Watanabe et al. (2005), while continuous O_3 exposure from previous year was conducted in the experiment of Yamaguchi et al. (2007). Some extent of O_3 exposure during leaf development may confer tolerance to O_3 in beech seedlings of Yamaguchi et al. (2007). To clarify the difference of sensitivity to O_3 between three studies adequately, analysis based on stomatal O_3 uptake is needed as a next step.

Approximately 18% reduction in stomatal conductance was observed in beech grown in elevated O_3 plot although the reduction was not significant. Hoshika et al. (2012a) reported significant reduction in G_s of beech in the same study site. In general, stomatal conductance of angiosperm tree tends to decrease by the exposure to O_3 (Witting et al., 2007). We consider relatively low number of replication for the gas exchange measurement in the present study was a factor of no significance of O_3-induced negative effect on G_s. However, we found no increase of L_s and significant increase of C_i/C_a, indicating O_3-induced reduction in A_{sat} in beech was mainly due not to the stomatal closure but to the reduction in photosynthetic activity in chloroplasts (Table 2).

Most leaf N is used in relation to photosynthesis, and N is considered to be the main factor regulating photosynthetic activity (e.g Lambers et al., 2008). We found joint decreases in photosynthetic parameters and N_{area} under elevated O_3 (Fig. 3). We therefore believe that the decrease in photosynthetic activities under elevated O_3 was due mainly to the decrease in N content of the leaves. Furthermore, we found an O_3-induced decrease in PNUE (Table 2). Yamaguchi et al. (2007; 2010) reported a reduction in PNUE in Siebold’s beech seedlings under elevated O_3, and inferred an O_3-induced decrease in efficiency of N use for soluble protein (i.e., the ratio of soluble protein to N) under relatively high soil N loading. A declining trend in N allocation to photosynthetic function was also observed in the present study under elevated O_3 (Fig. 4); we believe that this is the main reason for the reduction in PNUE with O_3.

Early senescence-like symptoms are frequently observed in the leaves of plants exposed to O_3 (e.g. Yonekura et al., 2004). Degradation of Rubisco and chlorophyll, and reabsorption of N from leaves, would take place during this process, as well as normal senescence (Pell et al., 1999; Matyssek and Sandermann, 2003; Oksanen, 2005). In fact, we observed O_3-induced acceleration of leaf abscission in the following November. The O_3-induced decreases in N_{area} and PNUE found in beech in the present study may therefore be explained in part as senescence-like symptoms induced by O_3.

Exposure to O_3 significantly increased the value of R in both species (Table 2). Similar increases in R have been reported for mature European beech exposed to O_3 with free air fumigation (Kitao et al., 2009), but the meta-analysis by Wittig et al. (2009) indicated that R in woody plants generally decreases by O_3 exposure. We believe that the increase in R was partly responsible for the decline in A_{sat} in both species. The increase in R under elevated O_3 may be due to O_3-related detoxification involving O_3-derived reactive oxygen species, and/or repair of damaged tissues (Landolt et al., 1997; Matyssek and Sandermann, 2003).

5. Conclusion

In this paper, we reported photosynthetic responses of two representative deciduous broad-leaved tree species native to northern Japan, Siebold’s beech and oak, to free air O_3 exposure. The results indicate photosynthetic activity of beech was more sensitive to O_3 than that of oak under O_3 fumigation into free air, and the O_3-induced reduction in light-saturated photosynthetic rate observed in beech was not due to stomatal closure, but rather to biochemical limitation. These qualitative trends in sensitivity are consistent with previous studies using chambers. The amount of reduction of A_{sat} for given AOT40 was different from previous studies, however. We propose to investigate next whether this difference in sensitivity between experiments is due to accumulative stomatal O_3 uptake.

Acknowledgments

This study was supported partly by the Environment Research and Technology Development Fund (B-1105) of the Ministry of the Environment, Japan (grant to T. Koike), and a Grant–in–Aid from the Japan Society for the Promotion of Science through its Type B program (to T. Koike, 23380078) and Young Scientists B (to M. Watanabe, 24710027 and to Y. Hoshika, 24780239).

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Table 1 Frequency, average concentration of ozone and proportion of time within 10% and 20% of the target concentration of ozone (54-66 and 48-72 nmol mol^{-1}, respectively).

		Average concentration	Proportion of time (%)	
Wind speed (m s^{-1})	Frequency (%)	(nmol mol^{-1})	±10%	±20%
0-0.5	38.6	58.7	49.5	80.7
0.5-1.0	35.6	56.7	54.8	82.9
1.0-1.5	17.2	55.8	36.2	59.1
1.5-	8.7	51.8	6.3	26.6
All	100.0	56.7	45.3	73.1

Table 2 Leaf traits of Siebold’s beech and oak saplings grown under ambient and elevated concentrations of O_3.

	Beech			Oak		
	Ambient	Ozone	T-test	Ambient	Ozone	T-test
A_{sat} (μmol m^{-2} s^{-1})	8.8(0.3)	4.7(0.4)	***	12.0(0.5)	10.2(0.4)	*
G_{s} (mol m^{-2} s^{-1})	0.17(0.02)	0.14(0.01)	n.s.	0.23(0.02)	0.19(0.01)	n.s.
$C_{\mathrm{i}}/C_{\mathrm{a}}$	0.75(0.02)	0.83(0.01)	**	0.71(0.02)	0.75(0.02)	n.s.
L_{s}	0.37(0.03)	0.32(0.04)	n.s.	0.28(0.03)	0.32(0.02)	n.s.
V_{cmzx} (μmol m^{-2} s^{-1})	55.8(3.0)	31.3(2.1)	***	63.4(6.7)	55.3(1.6)	n.s.
J_{mzx} (μmol m^{-2} s^{-1})	118.6(6.1)	85.2(5.3)	**	126.4(9.1)	126.3(3.7)	n.s.
R (μmol m^{-2} s^{-1})	1.1(0.2)	1.8(0.1)	*	0.8(0.1)	1.6(0.2)	**
PNUE (μmol mol^{-1} s^{-1})	94.1(2.9)	64.9(7.2)	**	105.9(5.1)	87.0(7.5)	0.07
LMA (g m^{-2})	64.6(3.5)	61.9(5.3)	n.s.	74.2(5.0)	76.1(5.9)	n.s.
N_{mass} (%)	2.1(0.2)	1.7(0.1)	0.10	2.2(0.1)	2.2(0.1)	n.s.
N_{area} (g m^{-2})	1.3(0.1)	1.0(0.1)	*	1.6(0.1)	1.7(0.1)	n.s.
Rubisco (g m^{-2})	1.7(0.2)	1.1(0.1)	*	2.6(0.2)	3.2(0.3)	n.s.
Chlorophyll (g m^{-2})	0.51(0.03)	0.32(0.07)	*	0.88(0.06)	0.87(0.07)	n.s.

A_{sat}, light-saturated net photosynthetic rate at 380 μmol mol^{-1} CO_2; G_{s}, stomatal conductance to water vapor; $C_{\mathrm{i}}/C_{\mathrm{a}}$, ratio of intercellular CO_2 concentration to ambient CO_2 concentration; L_{s}, stomatal limitation of photosynthesis; V_{cmax}, maximum rate of carboxylation; J_{max}, maximum rate of electron transport; R, dark respiration; PNUE, photosynthetic nitrogen use efficiency; LMA, leaf mass per area; N_{mass}, mass-based nitrogen content; N_{area}, area-based nitrogen content.

Each value is the mean of five measurements; standard error is shown in parentheses.

T-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant. The actual P value is shown if $0.05 < P < 0.10$.

Figure captions

Fig. 1. Overview of free air ozone exposure system located in northern Japan.

Fig. 2. Horizontal distribution of ozone concentration at two heights (2.5 and 4.0 m) in the free air ozone exposure system.

Fig. 3. Relation between area-based N content (N_{area}) and light-saturated net photosynthetic rate at 380 μmol mol^{-1} CO_2 (A_{sat}), maximum rate of carboxylation (V_{cmax}), maximum rate of electron transport (J_{max}), Rubisco content and chlorophyll content of Siebold's beech saplings. Y = -6.29 + 11.1 X, $R^2 = 0.53^{**}$ for N_{area} vs. A_{sat}, Y = -36.4 + 67.9 X, $R^2 = 0.56^{**}$ for N_{area} vs. V_{cmax}, Y = -19.7 + 103.2 X, $R^2 = 0.60^{**}$ for N_{area} vs. J_{max}, Y = -1.8 + 2.7 X, $R^2 = 0.48^{*}$ for N_{area} vs. Rubisco content and Y = -0.38 + 0.61 X, $R^2 = 0.48^{*}$ for N_{area} vs. chlorophyll content.

Fig. 4. Nitrogen allocation to photosynthetic functions in leaves of Siebold's beech saplings. N1, nitrogen allocated to Rubisco; N2, nitrogen allocated to electron carriers except for photosystems, coupling factor and Calvin cycle enzymes apart from Rubisco; N3, nitrogen allocated to light-harvesting complex and photosystems.

Fig. 5. Comparison of relationships between AOT40 during daylight hours and light-saturated net photosynthetic rate (A_{sat}) of Siebold's beeches, between the present study and previous studies. Dates of measurement for A_{sat} and period of AOT40 accumulation were 2 October 1999 and 146 days (from 10 May to 2 October 1999) for Yonekura et al. (2001), 8 October

2000 and 151 days (from 10 May to 8 October 2000) for Watanabe et al. (2005), 21-23 September 2005 and 183 days (from 1 April to 30 September 2005) for Yamaguchi et al. (2007), and 8-14 October 2011 and 63 days (from 6 August to 8 October 2011) for the present study. A_{sat} was determined at 350 μmol mol^{-1} CO_2 for Yonekura et al. (2001) and Watanabe et al. (2005), and 380 μmol mol^{-1} CO_2 for Yamaguchi et al. (2007) and present study.

Figure 1

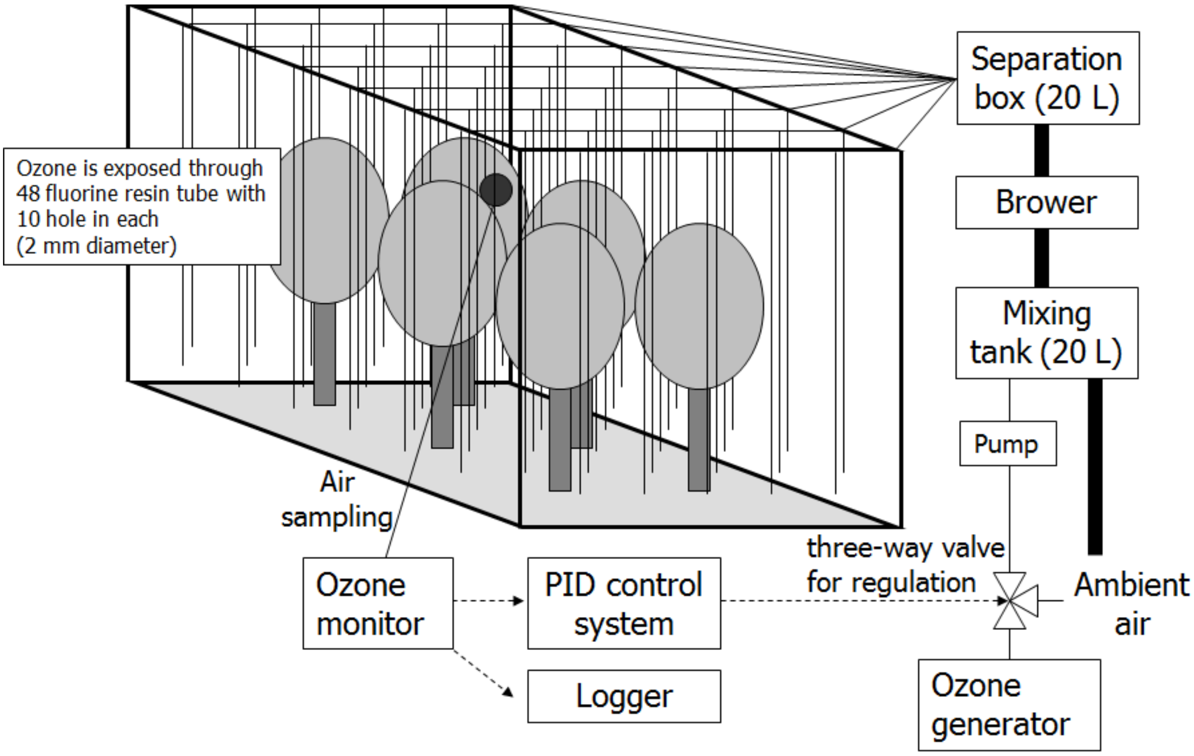

Separation box (20 L)
Brower
Mixing tank (20 L)
Pump
Ozone is exposed through 48 fluorine resin tube with 10 hole in each (2 mm diameter)
Air sampling
three-way valve for regulation
Ambient air
Ozone monitor
PID control system
Logger
Ozone generator

Figure 2

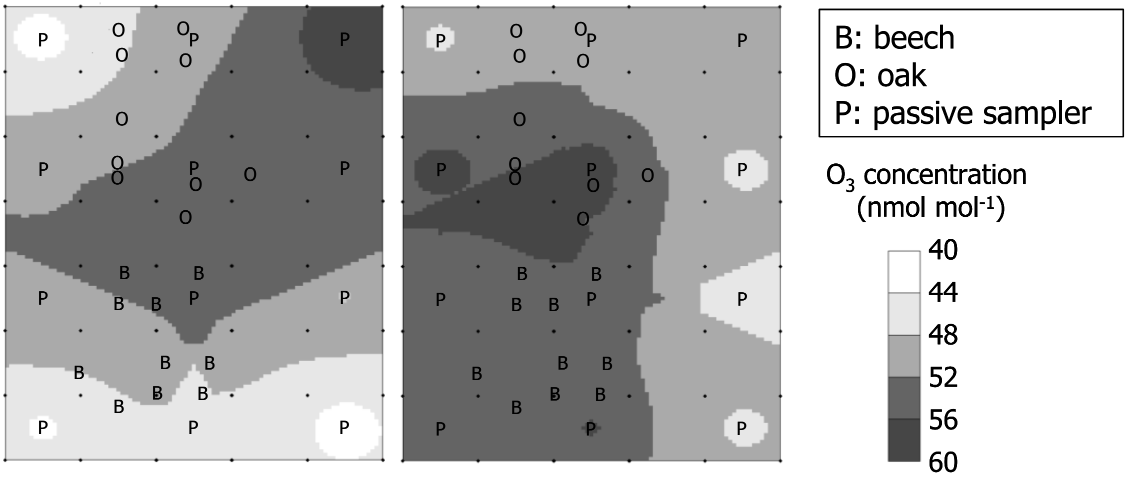

B: beech
O: oak
P: passive sampler
O3 concentration (nmol mol-1)
40
44
48
52
56
60

Figure 3

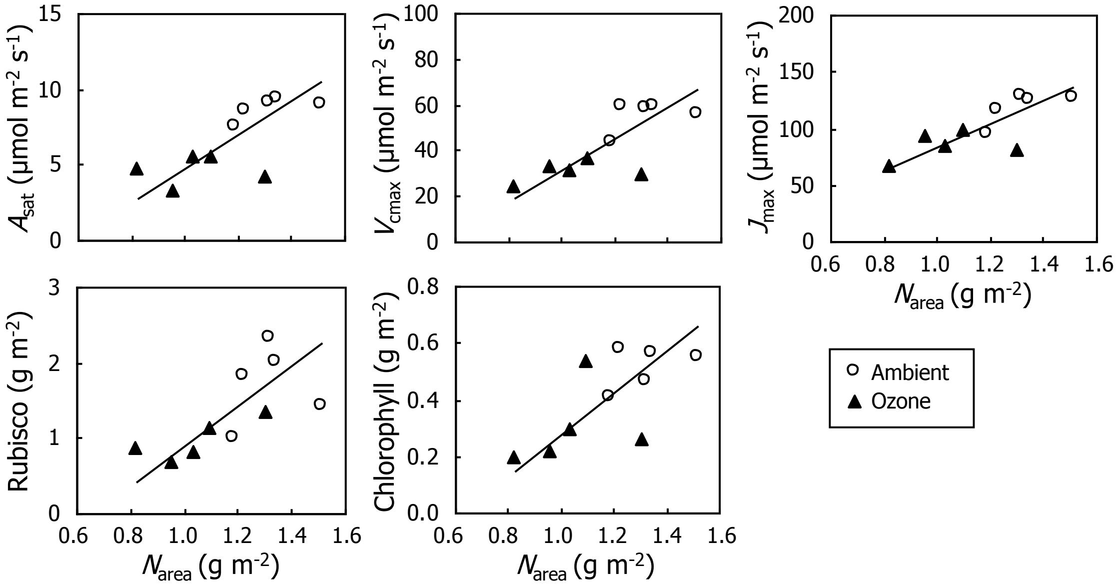

A_{sat} (µmol m^{-2} s^{-1})
0
5
10
15
V_{cmax} (µmol m^{-2} s^{-1})
0
20
40
60
80
100
J_{max} (µmol m^{-2} s^{-1})
0
50
100
150
200
Rubisco (g m^{-2})
0
1
2
3
Chlorophyll (g m^{-2})
0.0
0.2
0.4
0.6
0.8
0.6
0.8
1.0
1.2
1.4
1.6
N_{area} (g m^{-2})
○ Ambient
▲ Ozone

Figure 4

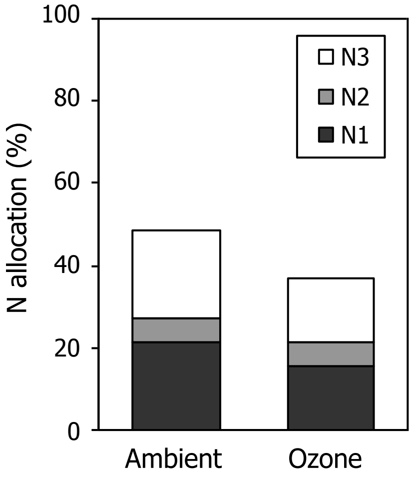
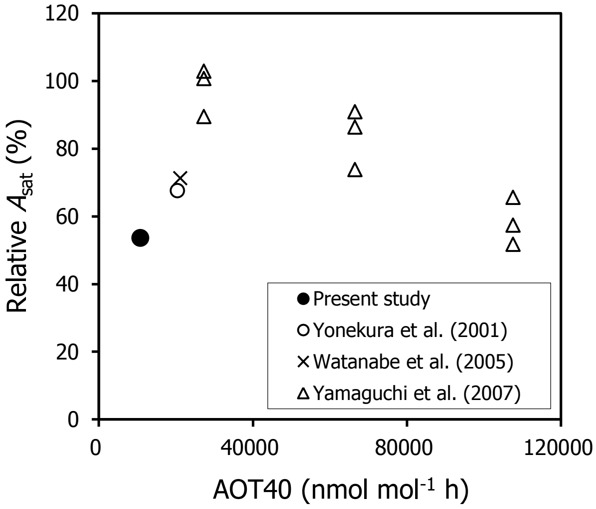


Figure 5



120
100
80
60
40
20
0
Relative A_{sat} (%)
0
40000
80000
120000
AOT40 (nmol mol^{-1} h)
● Present study
○ Yonekura et al. (2001)
× Watanabe et al. (2005)
△ Yamaguchi et al. (2007)