

Effects of ozone-induced stomatal closure on ozone uptake and its changes due to leaf age in sun and shade leaves of Siebold's beech

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Abstract

An estimation of stomatal ozone uptake for the assessment of ozone risks in forest trees can be modified by ozone-induced stomatal closure. We thus examined a seasonal course of stomatal conductance in sun and shade leaves of Siebold's beech native to northern Japan (*Fagus crenata*) grown under free-air ozone exposure. A performance of multiplicative stomatal conductance model was also tested, when considering ozone-induced stomatal closure into the model. Ozone caused stomatal closure in both sun and shade leaves (20% and 30–40% reduction of stomatal conductance in sun and shade leaves, respectively) during early summer. However, in autumn, stomatal closure was diminished regardless of canopy positions (approximately 7% and 6% reduction of stomatal conductance in sun and shade leaves, respectively). When observed seasonal course of stomatal closure was taken into account in stomatal conductance model, the model provided a good agreement with measurements even under conditions of elevated ozone. As a result, ozone-induced stomatal closure limited stomatal ozone uptake by 11% and 17% in sun and shade leaves of Siebold's beech, respectively. In addition, stomatal ozone uptake in shade leaves under elevated ozone was much less than that in sun leaves (35% of the value in sun leaves), indicating better avoidance of ozone stress in shade leaves. Our results suggest that a loss of ozone-induced stomatal closure after ozone exposure in the late growing season should be considered in modeling stomatal ozone uptake for the assessment of ozone impacts on Siebold's beech.

Key words: Free-air ozone exposure, Siebold's beech, Stomatal conductance model, Stomatal ozone uptake, Sun and shade leaves.

1. Introduction

Tropospheric ozone (O_3) is recognized as a widespread phytotoxic air pollutant and its concentration has been increasing in the northern hemisphere since the pre-industrial times (e.g., Akimoto, 2003). Ohara (2011) reported that the annual average concentrations of photochemical oxidant, mainly O_3 , increased in Japan at the large rate of $0.25 \text{ nmol mol}^{-1} \text{ year}^{-1}$ from 1985 to 2007, and this trend is expected to continue (Yamaji *et al.*, 2008). The phytotoxic nature of O_3 may cause adverse effects on physiological and biochemical processes in forest trees (Karnosky *et al.*, 2003; Matussek and Sandermann, 2003). The present O_3 concentration in Japan may have a negative effect on the growth of forest tree species (e.g., Watanabe *et al.*, 2010, 2012).

Recent O_3 risk assessments for forest trees are focused on a stomatal O_3 uptake basis (Omasa *et al.*, 2002; Mills *et al.*, 2010), because stomata are the principal interface for entry of O_3 into a leaf (Omasa *et al.*, 2002). Hence, accurate parameterization of stomatal conductance model is essential to develop a stomatal flux-based approach for assessments of O_3 impact on forest trees (Emberson *et al.*, 2000; Mills *et al.*, 2010).

Ozone is known to induce stomatal closure, and may therefore

limit stomatal O_3 uptake (e.g., Wittig *et al.*, 2007). Hoshika *et al.* (2012b) tried to parameterize O_3 -induced stomatal closure based on stomatal conductance data during 3-month exposure to O_3 in 2011. They reported that stomatal conductance was decreased with increasing cumulative O_3 exposure or uptake. However, O_3 -induced stomatal closure may be lost in autumn after O_3 exposure throughout the growing season in forest trees (Oksanen, 2003; Yamaguchi *et al.*, 2007; Onandia *et al.*, 2011; Hoshika *et al.*, 2015). We therefore needed to analyze the stomatal conductance data under O_3 exposure throughout the growing season.

Siebold's beech (*Fagus crenata*) is widely distributed representative deciduous trees in cool-temperate region in Japan, and classified as late successional species (Koike, 1988). Experimental studies reported the susceptibility of growth response to O_3 in Siebold's beech as sensitive (Kohno *et al.*, 2005; Watanabe *et al.*, 2013).

Within forest tree canopies, different responses of individual leaves to O_3 in relation to a gradient of light conditions were found (Kitao *et al.*, 2009; Watanabe *et al.*, 2014a). Watanabe *et al.* (2014a) reported that photosynthesis and respiration were more susceptible to O_3 in the leaves of the upper canopy (sun leaves) than the lower canopy (shade leaves) of Siebold's beech. Therefore, we can hypothesize that O_3 may also affect stomata in sun leaves rather than shade leaves. Greater O_3 -induced stomatal closure may thus occur in sun leaves compared to shade leaves, and limits stomatal O_3 uptake.

From these, the aim of the present study was (1) to clarify O_3

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effects on stomatal conductance throughout the growing season in sun and shade leaves of Siebold's beech, which is representative deciduous tree species in Japan, and (2) to investigate how much is the limitation of stomatal O₃ uptake by O₃-induced stomatal closure in sun and shade leaves of Siebold's beech. We hypothesized that O₃-induced stomatal closure may be gradually lost after O₃ exposure throughout the growing season in Siebold's beech, and thus conducted the analysis of O₃-induced stomatal closure in sun and shade leaves with leaf age (cf. Reich and Lassoie, 1984). According to the observations, we discussed further development of the stomatal flux-based approach for assessment of O₃ impacts on forest trees.

2. MATERIALS AND METHODS

2.1 Experimental site

The experimental site was located in Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l., annual mean temperature: 9.3°C, total precipitation: 1279 mm in 2012). The snow-free period is usually from early-May to late December. The soil is brown forest soil. Measurements were carried out in a free-air O₃ exposure experiment (for details of the system; see Watanabe *et al.* 2013). We set up two plots, one for ambient O₃ and another for elevated O₃. Size of each plot is 5.5 m × 7.2 m. The distance between the O₃-elevated plot and the ambient plot was about 20 m. In the present study, ten seedlings of two-year-old Siebold's beech were planted in each plot in May 2003, and were grown under ambient conditions from 2003 to 2010. They were therefore ten years old when the fumigation with O₃ began in 2011.

Exposure to O₃ is based on the system used at Kranzberg Forest in Germany (Werner and Fabian, 2002). The target O₃ concentration was 60 nmol mol⁻¹ during daylight hours. This elevated daytime O₃ treatment was applied to target trees from August to November 2011, and from May to November 2012. Ozone concentrations at canopy height were recorded continuously by an O₃ monitor (Mod. 202, 2B Technologies, Boulder CO, U.S.A.). The daytime hourly mean O₃ concentration in elevated O₃ in 2012 was 61.5 ± 13.0 nmol mol⁻¹ (at upper canopy where sun leaves developed) and 51.2 ± 12.2 nmol mol⁻¹ (at lower canopy where shade leaves developed); that in the ambient was 27.5 ± 11.6 nmol mol⁻¹. The mean tree height was 3.4 ± 0.4 m, and the mean stem diameter at breast height was 33.2 ± 7.1 mm in 2012. The light intensity at upper and lower canopy was continuously monitored in both ambient and elevated O₃ using a HOBO Pendant temperature/light data logger (UA-002-64, Onset Computer, Co., MA, USA) during the experimental period in 2012. The soil moisture was measured in the root layer (at depth 20 cm) by 10HS sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, U.S.A.).

2.2 Parameterization of the stomatal conductance model

Diurnal courses of the stomatal conductance were measured using a steady-state diffusion porometer (Model LI-1600, Li-Cor instruments, Lincoln, NE, U.S.A.), from May to October 2012. All measurements were conducted on fully expanded sun and shade leaves in the canopy of 6 trees in each ambient and elevated O₃ treatment. The measurement time interval was 2 to 3 hours from morning to evening (the earliest and latest measurements

were made at 5:30 and 20:00, respectively) for all target trees. Pooled data (736 and 622 measurements in sun and shade leaves of Siebold's beech, respectively) were used to estimate the parameters of the stomatal conductance model. In August, after measurement of the gas exchange rate, three leaf discs (12 mm diameter) were collected for determination of the leaf mass per unit area (LMA). These leaf discs were dried in an oven at 70°C for 1 week and were then weighted. The LMA was calculated as the ratio of the dry mass to the area of the leaves.

Our stomatal conductance model was based on the multiplicative algorithm described by Jarvis (1976) and modified by Emberson *et al.* (2000), as follows:

$$g_{sw} = g_{max} \cdot f_{phen} \cdot f_{light} \cdot \max\{f_{min}, (f_{temp} \cdot f_{VPD} \cdot f_{SWP})\} \quad (1),$$

where g_{max} is the maximum stomatal conductance (mmol H₂O m⁻² Projected Leaf Area (PLA) s⁻¹). The other functions are limiting factors of g_{max} and are scaled from 0 to 1. Here, f_{min} is the minimum stomatal conductance, f_{phen} is the variation in stomatal conductance with leaf age, and f_{light} , f_{temp} , f_{VPD} , and f_{SWP} depend respectively on the photosynthetic photon flux density at the leaf surface (PPFD, μmol photons m⁻² s⁻¹), the temperature (T, °C), the vapor pressure deficit (VPD, kPa), and the soil water potential (MPa).

The response of stomatal conductance to phenology (f_{phen}) during the experiments is described as follows:

$$\begin{aligned} & \text{for } A_{start} \leq \text{DOY} < (A_{start} + f_{phen_a}), \\ & f_{phen} = (1 - f_{phen_c}) \cdot ((\text{DOY} - A_{start}) / f_{phen_a}) + f_{phen_c} \\ & \text{for } (A_{start} + f_{phen_a}) \leq \text{DOY} \leq (A_{end} - f_{phen_b}), \\ & f_{phen} = 1; \\ & \text{for } (A_{end} - f_{phen_b}) < \text{DOY} \leq A_{end}, \\ & f_{phen} = (1 - f_{phen_d}) \cdot ((A_{end} - \text{DOY}) / f_{phen_d}) + f_{phen_d} \end{aligned} \quad (2),$$

where DOY is the day of the year. Here A_{start} and A_{end} are the year days for the leaf onset and fall, respectively. The parameter f_{phen_a} and f_{phen_b} represent the number of days of f_{phen} to reach its maximum and the number of days during the decline of f_{phen} to the minimum value. The parameter f_{phen_c} and f_{phen_d} represent maximum fraction of f_{phen} at A_{start} and A_{end} , respectively.

The response of stomatal conductance to PPFD, *i.e.*, f_{light} , is specified as:

$$f_{light} = 1 - \exp(-a \cdot \text{PPFD}) \quad (3),$$

where a is a species-specific parameter defining the shape of the exponential relationship.

The parameter for of air temperature (T, °C) is expressed as:

$$f_{temp} = \left(\frac{T - T_{min}}{T_{opt} - T_{min}} \right) \left\{ \left(\frac{T_{max} - T}{T_{max} - T_{opt}} \right) \left(\frac{T_{max} - T_{opt}}{T_{opt} - T_{min}} \right) \right\} \quad (4),$$

where T_{opt} , T_{min} , and T_{max} respectively denote the optimum, minimum, and maximum temperature (°C) for stomatal conductance.

The response of stomatal conductance to the vapor pressure

deficit (VPD, kPa) is given by:

$$f_{VPD} = \frac{(1 - f_{min}) \cdot (VPD_{min} - VPD)}{VPD_{min} - VPD_{max}} + f_{min} \quad (5),$$

where VPD_{min} and VPD_{max} denote the threshold of VPD (kPa) for attaining minimum and full stomatal opening, respectively. If VPD exceeds VPD_{min} then f_{VPD} is set to f_{min} . If VPD is lower than VPD_{max} then f_{VPD} is 1.

Terms describing modification of stomatal conductance by the soil moisture (*i.e.*, f_{SWP}) were not used in this study. As mentioned above, the soil moisture approached to the field capacity, and no reductions in stomatal conductance due to soil water content were recorded (data not shown).

Parameter estimation was carried out using a boundary line analysis (*e.g.*, Alonso *et al.*, 2008; Hoshika *et al.*, 2012b). First, the g_{sw} data were divided into classes with the following step-wise increases for each variable: 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for PPFD (when PPFD values were less than 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, PPFD classes at 50 or 5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ steps were adopted for sun leaves and shade leaves, respectively), 2°C for T and 0.2 kPa for VPD. A function was fitted against each model variable based on 98th percentile values per each class of environmental factors. The g_{max} value corresponds to an averaged value over 97th percentile value (Alonso *et al.*, 2008). We took f_{min} as mean value less than the 5th percentile values of conductance.

2.3 Introducing a function of ozone effect on stomatal conductance model

In the present study, lower stomatal conductance was found in the elevated O_3 treatment than in controls. A further parameter was tested in the model:

$$g_{sw} = g_{max} \cdot f_{phen} \cdot f_{O_3} \cdot f_{light} \cdot \max\{f_{min}, (f_{temp} \cdot f_{VPD})\} \quad (6),$$

where the new parameter f_{O_3} represents a response of stomatal conductance to O_3 .

If O_3 -induced stomatal closure may be gradually lost with increasing leaf age (*cf.* Reich and Lassoie, 1984), the parameter of f_{O_3} can be shown as:

$$f_{O_3} = 1 - b \cdot \left(\frac{[\tilde{O}_3]}{27.5} - 1 \right) \cdot (1 - c \cdot [\text{Leaf age}]) \quad (7),$$

where b is the parameter showing a fractional reduction of stomatal conductance by doubled ambient O_3 concentration (*i.e.*, 55 nmol mol^{-1} in the present study) without any effects of leaf age, c is a empirical constant relating to leaf age. $[\tilde{O}_3]$ is the long-term mean O_3 concentration (nmol mol^{-1}) under elevated O_3 . $[\tilde{O}_3]$ can be taken as 61.5 and 51.2 (nmol mol^{-1}) at upper and lower canopy which are the daytime mean value during the experiment. The value of 27.5 is the daytime mean O_3 concentration (nmol mol^{-1}) at ambient condition. In the present study, [Leaf age] was denoted as days after full expansion of leaves. The parameters of b and c were estimated by a regression analysis using the ratio of daily g_{max} to the control value. Daily g_{max} (obtained at 9:00 to 12:00) was identified as the daily maximum record of stomatal conductance of the measured trees when PPFD > 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and VPD < 1.5 kPa for sun leaves, and when VPD < 1.5 kPa for shade leaves. When applying the model in control conditions,

there was taken to be no effect of O_3 on stomatal conductance, *i.e.*, $f_{O_3}=1$ (*cf.* Löw *et al.*, 2007; Hoshika *et al.*, 2012b).

2.4 Estimation of stomatal ozone uptake

Stomatal O_3 uptake (F_{st} ; $\text{nmol O}_3 \text{ m}^{-2} \text{s}^{-1}$) was calculated as:

$$F_{st} = [O_3] \cdot g_{sO_3} \cdot \frac{r_c}{r_b + r_c} \quad (8),$$

where r_b is the leaf boundary layer resistance (s m^{-1}), and g_{sO_3} is the stomatal conductance for O_3 ($g_{sO_3} = g_{sw} \cdot 0.613$; $\text{mmol O}_3 \text{ m}^{-2} \text{ PLA s}^{-1}$); the factor of 0.613 is the ratio of diffusivities between O_3 and water vapor. Here, r_c is the leaf surface resistance ($= 1/(g_{sO_3} + g_{ext})$; s m^{-1}), and g_{ext} is the external leaf or cuticular conductance (s m^{-1}), chosen as 0.0004 m s^{-1} (Mills *et al.*, 2010).

Leaf boundary layer resistance (r_b) was calculated from the wind speed, u (m s^{-1}), and the cross-wind leaf dimension, L_d (0.05 m for Siebold's beech, see Hoshika *et al.*, 2012b) (Mills *et al.*, 2010):

$$r_b = 1.3 \cdot 150 \cdot (L_d/u)^{0.5} \quad (9),$$

where the factor 1.3 accounts for differences in diffusivity between heat and O_3 , and 150 is the empirical constant ($\text{s}^{1/2} \text{ m}^{-1}$) (McNaughton and van den Hurk, 1995).

In Europe, phytotoxic O_3 dose above a flux threshold of Y (POD_Y) was recommended to assess O_3 risk for forest species (Mills *et al.* 2010). It is given by:

$$\text{POD}_Y = \sum \max(F_{st} - Y, 0) \quad (10),$$

where Y is a species-specific threshold of stomatal O_3 flux ($\text{nmol O}_3 \text{ m}^{-2} \text{ s}^{-1}$). As it was not clear which threshold Y can be applied for the species in Japan, we did not set a threshold for the F_{st} value (POD_0) in the present study. POD_0 was then estimated using hourly meteorological data and O_3 concentration data obtained in the experimental site.

2.5 Data analysis

Simple correlation analysis was used to test the relationship between measured and estimated stomatal conductance. And the relationship between [Leaf age] and the ratio of daily g_{max} to control was assessed by regression of linear function. The analysis was performed using SPSS software (SPSS, Chicago, USA).

3. Results

3.1 Relative light intensity and LMA at upper and lower canopy, and average soil moisture

The relative light intensity of lower canopy was 10–15% as compared to that of upper canopy (*cf.* Watanabe *et al.*, 2014a). Different light environment made a difference of LMA between sun leaves at upper canopy and shade leaves at lower canopy (sun leaves: $87.6 \pm 13.0 \text{ g m}^{-2}$ and $83.7 \pm 10.6 \text{ g m}^{-2}$ in ambient and in elevated O_3 , respectively; shade leaves: $36.3 \pm 5.3 \text{ g m}^{-2}$ and $33.6 \pm 3.6 \text{ g m}^{-2}$ in ambient and in elevated O_3 , respectively; mean \pm standard deviation of 6 target trees in each treatment). The average soil moisture (volumetric soil water content) was $28.9 \pm 2.8\%$ 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.

3.2 Parameterization of the stomatal conductance model

The g_{max} values were set to around 485 and 175 $\text{mmol H}_2\text{O m}^{-2}$

PLA s^{-1} in sun and shade leaves of Siebold's beech, respectively (Table 1). Parameters of limiting functions of stomatal conductance (*i.e.* f_{light} , f_{temp} , f_{VPD} and f_{phen}) were listed in Table 1. The response of stomatal conductance to PPFD (f_{light}) followed a typical light-response curve, with a light saturation point above 500 $\mu mol m^{-2} s^{-1}$ in sun leaves and above 100 $\mu mol m^{-2} s^{-1}$ in shade leaves. The response of stomatal conductance to air temperature (f_{temp}) indicated an optimal temperature for stomatal opening did not differ between canopy position (around 25°C: Table 1). More than around 1.0 kPa of VPD induced stomatal closure in both sun

and shade leaves. Stomatal conductance decreased to 40–50% of g_{max} at 3.0 kPa of VPD.

Lower stomatal conductance in sun and shade leaves under elevated O_3 conditions was found compared to those under ambient conditions (Fig. 1). In early summer, O_3 caused 30–40% reduction of stomatal conductance in shade leaves, while sun leaves showed 20% decline of stomatal conductance in elevated O_3 treatment compared to in ambient conditions. Observed decrease in stomatal conductance was, however, diminished with increasing leaf age (Fig. 2). In autumn, we found approximately 7% and 6% reduction of stomatal conductance by elevated O_3 in sun and shade leaves, respectively (Fig. 2).

Table 1. Summary of model parameters in stomatal conductance of sun and shade leaves in Siebold's beech obtained from measured data in 2012.

Parameter		Siebold's beech		
		Sun leaves	Shade leaves	
g_{max}	($mmol H_2O m^{-2} PLA s^{-1}$)	485	175	
f_{min}	(fraction)	0.08	0.09	
f_{phen}	A_{start}	(day of year)	117	117
	A_{end}	(day of year)	315	315
	f_{phen_a}	(days)	48	49
	f_{phen_b}	(days)	45	23
	f_{phen_c}	(fraction)	0.3	0.3
f_{phen_d}	(fraction)	0.3	0.3	
f_{light}	a	(constant)	-0.0071	-0.10
f_{temp}	T_{opt}	(°C)	27	25
	T_{min}	(°C)	2	1
	T_{max}	(°C)	36	41
f_{VPD}	VPD_{max}	(kPa)	1.3	1.3
	VPD_{min}	(kPa)	5.5	4.8
f_{O_3}	b	(constant)	0.17	0.40
	c	(constant)	0.0042	0.0055

g_{max} , maximum stomatal conductance; f_{min} , minimum stomatal conductance;

f_{phen} ; the variation in stomatal conductance with leaf age;

f_{light} , f_{temp} , f_{VPD} and f_{O_3} depend respectively on the photosynthetically relevant photon flux density at leaf surface (PPFD, $\mu mol m^{-2} s^{-1}$), temperature (T, °C), vapor pressure deficit (VPD, kPa), and daytime mean ozone concentration during the experiment ($nmol mol^{-1}$);

A_{start} and A_{end} are the year days for the leaf onset and fall, respectively.

b denotes a fractional reduction of stomatal conductance by doubled ambient O_3 concentration (*i.e.*, 55 $nmol mol^{-1}$ in the present study) without any effects of leaf age, c is a empirical constant relating to leaf age.

3.3 Test of f_{O_3} function

Predicted stomatal conductance by the model fitted well with the measured values under ambient conditions in both sun and shade leaves (Fig. 3). In the elevated O_3 treatment, however, the model without f_{O_3} overestimated stomatal conductance (Fig. 4). When f_{O_3} was included into the model, the error of the model estimation under elevated O_3 decreased (for sun leaves, from 113.5 to 82.7 $mmol m^{-2} s^{-1}$ of RMSE (-27%), respectively; for shade leaves, from 43.2 to 28.2 $mmol m^{-2} s^{-1}$ of RMSE (-35%), respectively. Fig. 4, 5). These RMSE of the model estimations under elevated O_3 were similar to that under ambient conditions (Fig. 3, 5).

Table 2 shows POD_0 estimated by the model with and without f_{O_3} in each O_3 treatment. POD_0 in sun and shade leaves of Siebold's beech were greater under elevated O_3 than those under ambient conditions. POD_0 in the model with f_{O_3} was lower than those in the model without f_{O_3} (11% and 17% in sun and shade leaves, respectively), indicating a limitation of stomatal O_3 uptake by stomatal closure.

4. Discussion

Ozone-induced reduction in stomatal conductance occurred in both sun and shade leaves of Siebold's beech (Fig. 1, 2). The parameter of b in the empirical function of f_{O_3} , which shows a fractional reduction of stomatal conductance by doubled ambient O_3 concentration (*i.e.*, 55 $nmol mol^{-1}$ in the present study), was 0.17 and 0.40 in sun and shade leaves, respectively (Table 1). Matyssek *et al.* (2015) reported that decline of stomatal conductance by elevated O_3 could reach up to 30% at the whole-tree foliage level for European beech at Kranzberg Forest (36 $nmol mol^{-1}$ at ambient, 66 $nmol mol^{-1}$ at elevated O_3 as daytime mean O_3 concentration). Their result of O_3 -induced reduction of stomatal conductance for European beech could be translated into $b = 0.26$ in the empirical equation of f_{O_3} (Eq. 7). Interestingly the result suggests that similar extents of limitation in stomatal conductance caused by elevated O_3 were found between both experiments for beeches (Hoshika *et al.*, 2015).

Contrary to expectations, the reduction of stomatal conductance was greater in shade leaves compared to in sun leaves as indicated by higher b in shade leaves (Fig. 2; Table 1), although shade leaves of Siebold's beech did not show O_3 -induced decline of photosynthetic capacity in the same experimental site (Watanabe *et al.*, 2014a). Ozone-induced stomatal closure may occur by both photosynthesis-dependent and -independent mechanisms (Kangasjärvi *et al.*, 2005; Kitao *et al.*, 2009; Matyssek *et al.*, 2010;

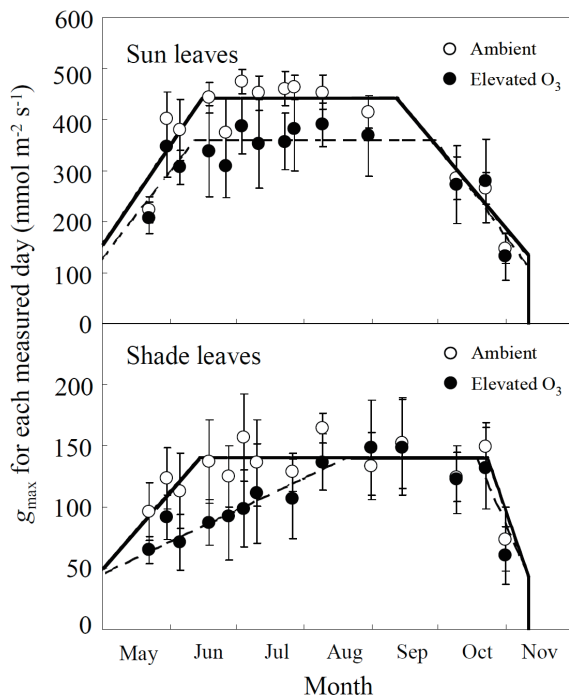


Fig. 1. Seasonal change of daily g_{\max} for ambient and elevated O_3 in sun and shade leaves of Siebold's beech. Solid and dotted line denotes a regression line in ambient and elevated O_3 , respectively. Daily g_{\max} (obtained at 9:00 to 12:00) was identified as the daily maximum record of stomatal conductance of the measured trees when PPFD > 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and VPD < 1.5 kPa for sun leaves, and when VPD < 1.5 kPa for shade leaves. Error bars mean \pm S.D of 6 trees in each plot.

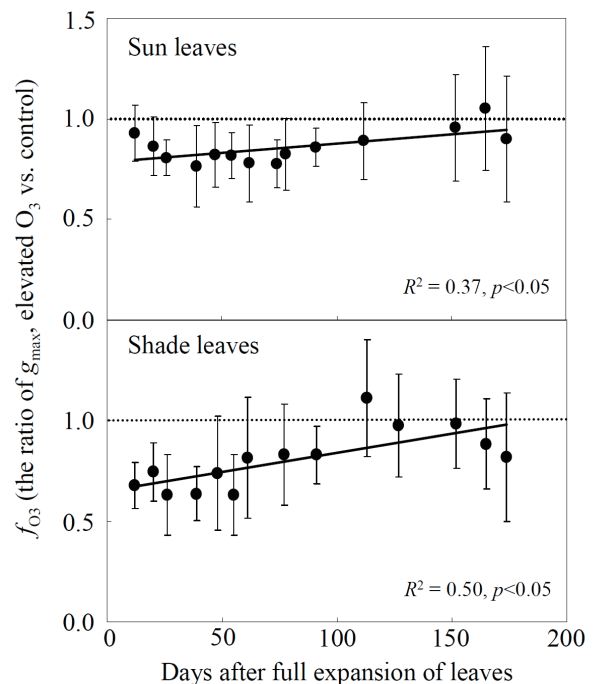


Fig. 2. Ratio of daily g_{\max} in elevated O_3 to those in ambient conditions in sun and shade leaves of Siebold's beech, as a linear function of leaf age (days after full expansion of leaves). The regression line was obtained as $y=0.0009x+0.79$ and $y=0.0019x+0.65$ in sun and shade leaves, respectively. Daily g_{\max} (obtained at 9:00 to 12:00) was identified as the daily maximum record of stomatal conductance of the measured trees when PPFD > 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and VPD < 1.5 kPa for sun leaves, and when VPD < 1.5 kPa for shade leaves. Error bars mean \pm S.D of 6 trees in each plot.

Hoshika *et al.*, 2013, 2015). Ozone-induced decline of photosynthetic capacity may cause stomatal closure (Reich, 1987; Farage and Long, 1995), because stomatal conductance is generally regulated so as to maintain the ratio of leaf internal (C_i) to ambient CO_2 concentration (C_a ; Lambers *et al.*, 2008). In addition, O_3 induces stomatal closure as a direct action of O_3 or O_3 -induced reactive oxygen species (ROS) (Kangasjärvi *et al.*, 2005, Vahisalu *et al.*, 2010), and the involvement of abscisic acid (ABA) signaling (Ahlfors *et al.*, 2004; Matyssek *et al.*, 2010). Ozone therefore caused a reduction of stomatal conductance in Siebold's beech regardless of the canopy position. The large reduction of stomatal conductance in shade leaves suggests greater avoidance of O_3 uptake. Hoshika *et al.* (2013) suggested that stomatal conductance for *Fagus crenata* under elevated O_3 was optimized for maximizing carbon gain while minimizing accompanying water loss and O_3 uptake, especially in early summer. A contribution of carbon assimilation of shade leaves in entire canopy is generally low (cf. Kitao *et al.*, 2012; Watanabe *et al.*, 2014a). And the detoxification capacity may be lower in shade leaves than in sun leaves (Haberer *et al.*, 2007). Shade leaves thus may have shown greater stomatal closure to avoid O_3 stress with some small loss of carbon assimilation.

Our previous study suggested that such a reduction of stomatal conductance was observed with increasing cumulative O_3 exposure or uptake during 3-month O_3 exposure to Siebold's beech (Hoshika *et al.*, 2012b). However, in the present study, O_3 -induced reduction of stomatal conductance of Siebold's beech was diminished in autumn after O_3 exposure throughout the growing season (Fig. 2). And we then tried to apply an empirical function of leaf age regarding O_3 -induced stomatal closure as f_{O_3} (eq. 7, Fig. 2). The both results may indicate following possible mechanisms: 1) there may be a difference of stomatal response to O_3 with leaf age during O_3 exposure (cf. Reich and Lassoie, 1984), and 2) further O_3 exposure or uptake may cause a change of stomatal response to O_3 . Ozone is known to cause not only stomatal closure, but also a weaker ability to close stomata, i.e., increasing loss of stomatal regulation, which is called as " O_3 -induced stomatal sluggishness" (Paoletti, 2005; Paoletti and Grulke, 2005; Mills *et al.*, 2009; Hoshika *et al.*, 2012a, 2013). However, it is hard to identify which mechanism involved in the loss of O_3 -induced stomatal closure in this study because cumulative O_3 exposure or uptake increased with increasing leaf age at the same time. Further studies are needed to understand the underlying mechanisms of such a loss of O_3 -induced stomatal closure.

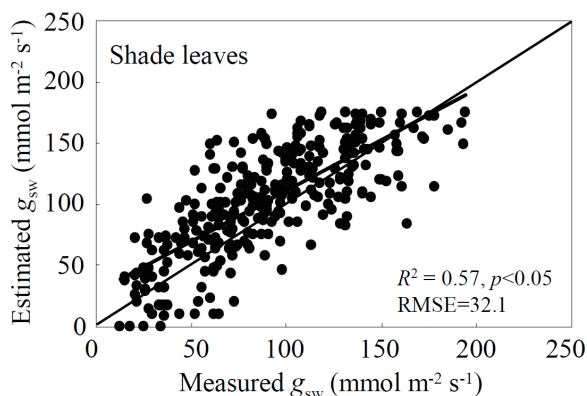
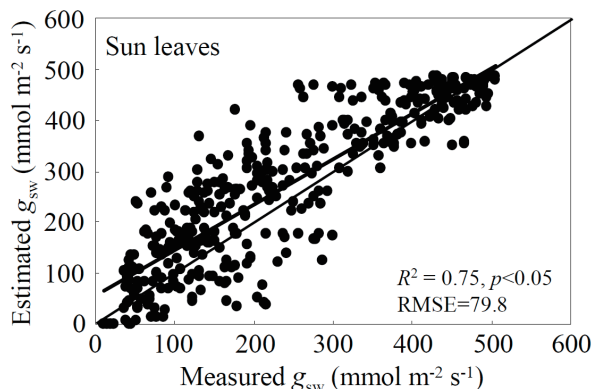


Fig. 3. A comparison between measured and estimated g_{sw} under ambient conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as $y=0.89x+58.5$ and $y=0.83x+28.3$ in sun and shade leaves, respectively.

In previous modeling studies with crops, a development of f_{O_3} was successfully achieved for potato (Pleijel *et al.*, 2002), rice (Oue *et al.*, 2008) and wheat (Grüters *et al.*, 1995; Danielsson *et al.*, 2003; Oue *et al.*, 2009), describing a monotonic reduction in stomatal conductance with increasing cumulative O_3 exposure or uptake. However, the result presented here (Fig. 2) suggests that a development of f_{O_3} function as reported in crops (*i.e.*, a monotonic reduction in stomatal conductance with increasing cumulative O_3 exposure or uptake) could not be applied for forest trees (Elvira *et al.*, 2007). The absence of O_3 effects on stomatal conductance in autumn has been reported after O_3 exposure throughout the growing season in several forest trees species (Oksanen, 2003; Löw *et al.*, 2007; Yamaguchi *et al.*, 2007). Also the studies of sapflow measurement in free-air O_3 exposure experiment reported that O_3 -induced stomatal closure was gradually lost during the growing season as reported in Aspen-birch communities (*Betula papyrifera* and *Populus tremuloides*, Onandia *et al.*, 2011) and European beech (*Fagus sylvatica*, Matyssek *et al.*, 2015).

Stomatal conductance model without f_{O_3} overestimated stomatal conductance under elevated O_3 condition (Fig. 4), although it showed good performance under ambient condition with low O_3 concentration (Fig. 3). When O_3 -induced stomatal closure was

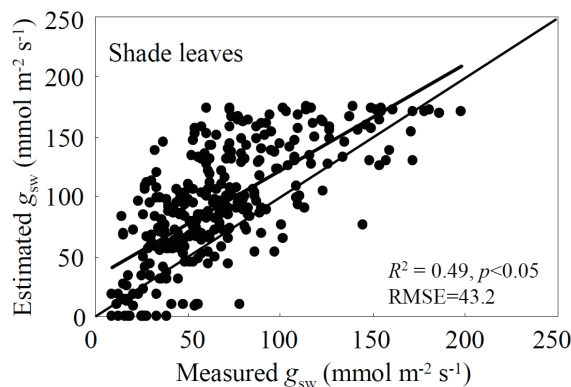
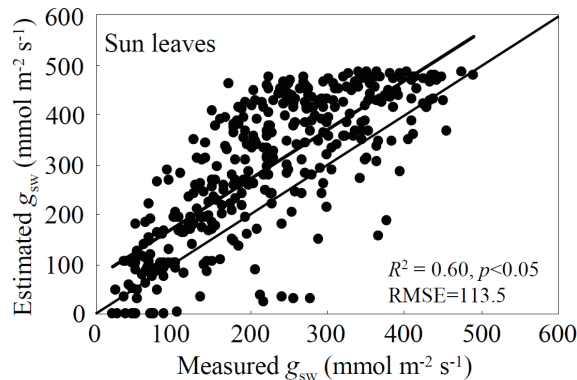


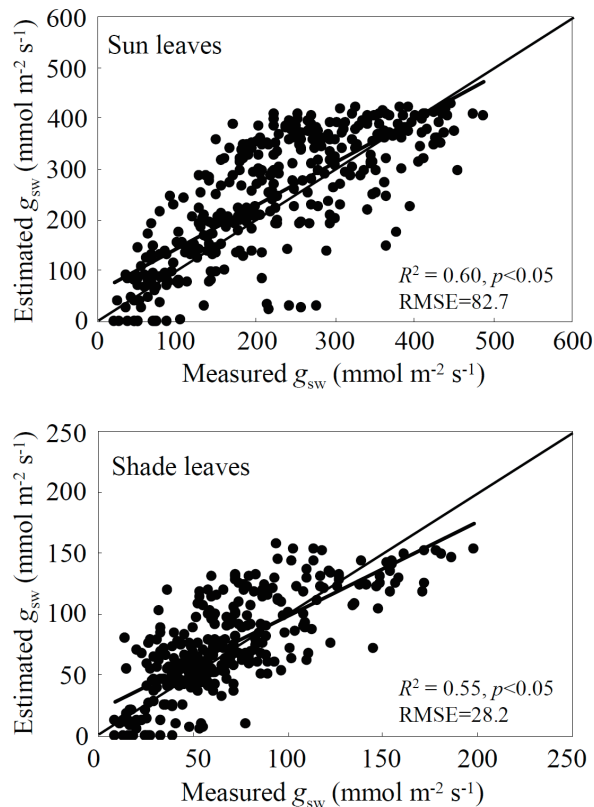
Fig. 4. A comparison between measured and estimated g_{sw} using the model without f_{O_3} (neglecting O_3 -induced stomatal closure) under elevated O_3 conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as $y=0.98x+73.3$ and $y=0.88x+33.7$ in sun and shade leaves, respectively.

considered into the model, the error of the model estimations under elevated O_3 decreased and became similar to that under ambient conditions (Fig. 3-5). The decline in daily maximum stomatal conductance results in limiting stomatal O_3 uptake (Table 2). As a result, the model without f_{O_3} may overestimate POD_0 by 11–17% under elevated O_3 conditions (Table 2). POD_0 in shade leaves under elevated O_3 was much less than that in sun leaves (Table 2, 35% of POD_0 compared to the value in sun leaves). Watanabe *et al.* (2014a) reported that larger decline in light-saturated net photosynthetic rate and increase in respiration with exposure to O_3 was found in sun leaves rather than shade leaves of Siebold's beech. Higher stomatal O_3 uptake in sun leaves may result in greater O_3 damage to photosynthetic system observed in Watanabe *et al.* (2014a), although high light intensity and high leaf temperature in sun leaves may also relate to the production of ROS from the water-water cycle in chloroplasts, inducing oxidative stress (Asada, 1996; Apel and Hirt, 2004).

In conclusion, O_3 caused stomatal closure in both sun and shade leaves of Siebold's beech, which limited cumulative O_3 uptake by 11% and 17% in sun and shade leaves, respectively. In addition, cumulative O_3 uptake in shade leaves under elevated O_3 was much lower than that in sun leaves (35% of the value in sun leaves).

Table 2. Cumulative O₃ uptake (POD₀) under ambient and elevated O₃ using stomatal conductance model neglecting or considering O₃-induced stomatal closure (f_{O_3}) in sun and shade leaves of Siebold's beech in 2012.

Species and leaf type	Cumulative O ₃ uptake (POD ₀ , mmol m ⁻²)			Limitation of O ₃ influx by O ₃ -induced stomatal closure
	Ambient	Elevated O ₃ (model without f_{O_3})	Elevated O ₃ (model with f_{O_3})	
Siebold's beech				
Sun leaves	25.6	53.0	47.4	11%
Shade leaves	11.8	19.9	16.5	17%

**Fig. 5.** A comparison between measured and estimated g_{sw} using the model with f_{O_3} (considering O₃-induced stomatal closure) under elevated O₃ conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as $y=0.84x+60.5$ and $y=0.78x+20.5$ in sun and shade leaves, respectively.

This may result in lower decline of photosynthetic traits with exposure to O₃ in shade leaves than in sun leaves of Siebold's beech as found in our previous report (Watanabe *et al.*, 2014a). Also we should note that the stomatal closure diminished in autumn, suggesting a loss of closing response of stomata with seasonal senescence. Such a loss of O₃-induced stomatal closure should be considered in f_{O_3} functions of stomatal conductance model of Siebold's beech. Ozone-induced stomatal closure may occur by both photosynthesis-dependent and -independent mechanisms (e.g., Matyssek *et al.*, 2010). Therefore, we should consider the photosynthesis-stomatal conductance relationship, and its seasonal change under elevated O₃ for further development of modeling for the assessment of O₃ impacts on forest trees (cf. Watanabe *et al.*, 2014b).

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References

- Akimoto, H., 2003: Global air quality and pollution. *Science*, **302**, 1716–1719.
 Ahlfors, R., Lång, S., Overmyer, K., Jaspers, P., Brosché, M.,

- Tauriainen, A., Kollist, H., Tuominen, H., Balles-Boix, E., Piippo, M., Inzé, D., Palva, E. T. and Kangasjärvi, J., 2004: Arabidopsis Radical-induced cell death1 belongs to WWE protein-protein interaction domain protein family and modulates abscisic acid, ethylene, and methyl jasmonate responses. *The Plant Cell*, **16**, 1925–1937.
- Alonso, R., Elvira, S., Sanz, M. J., Gerosa, G., Emberson, L. D., Bermejo, B. and Gimeno, B. S., 2008: Sensitivity analysis of a parameterization of the stomatal component of the DO₃SE model for *Quercus ilex* to estimate ozone fluxes. *Environmental Pollution*, **155**, 473–480.
- Apel, K. and Hirt, H., 2004: Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, **55**, 373–399.
- Asada, K., 1996: Radical production and scavenging in the chloroplasts. In: *Photosynthesis and Environment* (ed. by Baker, N. R.). Kluwer Academic Publishers, Dordrecht, Netherlands, pp.123–150.
- Danielsson, H., Karlsson, G. H., Karlsson, P. E. and Pleijel, H., 2003: Ozone uptake modeling and flux-response relationships – an assessment of ozone-induced yield loss in spring wheat. *Atmospheric Environment*, **37**, 475–485.
- Elvira, S., Alonso, R. and Gimeno, B. S., 2007: Simulation of stomatal conductance for Aleppo pine to estimate its ozone uptake. *Environmental Pollution*, **146**, 617–623.
- Emberson, L. D., Ashmore, M. R., Cambridge, H. M., Simpson, D. and Tuovinen, J. P., 2000: Modelling stomatal ozone flux across Europe. *Environmental Pollution*, **109**, 403–413.
- Farage, P. K. and Long, S. P., 1995: An *in vivo* analysis of photosynthesis during short-term O₃ exposure in three contrasting species. *Photosynthesis Research*, **43**, 11–18.
- Grüters, U., Fangmeier, A. and Jäger, H.-J., 1995: Modelling stomatal responses of spring wheat (*Triticum aestivum* L. cv. Turbo) to ozone and different levels of water supply. *Environmental Pollution*, **87**, 141–149.
- Haberer, K., Herbinger, K., Alexou, M., Tausz, M., and Rennenberg, H., 2007: Antioxidative defence of old growth beech (*Fagus sylvatica*) under double ambient O₃ concentrations in a free-air exposure system. *Plant Biology*, **9**, 215–226.
- Hoshika, Y., Watanabe, M., Inada, N. and Koike, T., 2012a: Ozone-induced stomatal sluggishness develops progressively in Siebold's beech (*Fagus crenata*). *Environmental Pollution*, **166**, 152–156.
- Hoshika, Y., Watanabe, M., Inada, N. and Koike, T., 2012b: Modeling of stomatal conductance for estimating ozone uptake of *Fagus crenata* under experimentally enhanced free-air ozone exposure. *Water, Air, and Soil Pollution*, **223**, 3893–3901.
- Hoshika, Y., Watanabe, M., Inada, N. and Koike, T., 2013: Model-based analysis of avoidance of ozone stress by stomatal closure in Siebold's beech (*Fagus crenata*). *Annals of Botany*, **112**, 1149–1158.
- Hoshika, Y., Watanabe, M., Kitao, M., Haberle, K.-H., Grams, T. E. E., Koike, T. and Matyssek, R., 2015: Ozone induces stomatal narrowing in European and Siebold's beeches: a comparison between two experiments of free-air ozone exposure. *Environmental Pollution*, **196**, 527–533.
- Jarvis, P. G., 1976: Interpretation of variations in leaf water potential and stomatal conductance found in canopies in field. *Philosophical Transactions of the Royal Society, London B*, **273**, 593–610.
- Kangasjärvi, J., Jaspers, P. and Kollist, H., 2005: Signalling and cell death in ozone-exposed plants. *Plant, Cell and Environment*, **28**, 1021–1036.
- Karnosky, D., Percy, K. E., Chappelka, A. H., Simpson, C. and Pikkariainen, J., 2003: *Air pollution, global change and forests in the new millennium*. Elsevier, Oxford, UK.
- Kitao, M., Löw, M., Heerdt, C., Grams, T. E. E., Häberle, K.-H., and Matyssek, R., 2009: Effects of chronic elevated ozone exposure on gas exchange responses of adult beech trees (*Fagus sylvatica*) as related to the within-canopy light gradient. *Environmental Pollution*, **157**, 537–544.
- Kitao, M., Winkler, J. B., Löw, M., Nunn, A. J., Kuptz, D., Häberle, K.-H., Reiter, I. M., and Matyssek, R., 2012: How closely does stem growth of adult beech (*Fagus sylvatica*) relate to net carbon gain under experimentally enhanced ozone stress? *Environmental Pollution*, **166**, 108–115.
- Kohno, Y., Matsumura, H., Ishii, T. and Izuta, T., 2005: Establishing critical levels of air pollutants for protecting East Asian vegetation – a challenge. In: *Plant Responses to Air Pollution and Global Change* (ed. by Omasa, K., Nouchi, I. and De Kok, L. J.). Springer, Tokyo, pp.243–250.
- Koike, T., 1988: Leaf structure and photosynthetic performance as related to the forest succession of deciduous broad-leaved trees. *Plant Species Biology*, **3**, 77–87.
- Lambers, H., Chapin, F. S. (III) and Pons, T. L., 2008: *Plant physiological ecology*, 2nd-edition. Springer-Verlag, New York. 640pp.
- Löw, M., Häberle, K. H., Warren, C. R. and Matyssek, R., 2007: O₃ flux-related responsiveness of photosynthesis, respiration, and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O₃ exposure. *Plant Biology*, **9**, 197–206.
- Matyssek, R. and Sandermann, H., 2003: Impact of ozone on trees: an ecophysiological perspective. In: *An Ecophysiological Perspective. Progress in Botany vol.64* (ed. by Esser, K., Lüttge, U., Beyschlag, W. and Hellwig, F.). Springer-Verlag, Berlin, Heidelberg, pp.349–402.
- Matyssek, R., Wieser, G., Ceulemans, R., Rennenberg, H., Pretzch, H., Haberer, K., Löw, M., Nunn, A. J., Werner, H., Wipfler, P., Oßwald, W., Nikolova, P., Hanke, D. E., Kraigher, H., Tausz, M., Bahnweg, G., Kitao, M., Dieler, J., Sandermann, H., Herbinger, K., Grebenc, T., Blumenröther, M., Deckmyn, G., Grams, T. E. E., Heerdt, C., Lauchner, M., Fabian, P. and Häberle, K.-H., 2010: Enhanced ozone strongly reduces carbon sink strength of adult beech (*Fagus sylvatica*) – Resume from the free-air fumigation study at Kranzberg forest. *Environmental Pollution*, **158**, 2527–2532.
- Matyssek, R., Baumgarten, M., Hummel, U., Häberle, K.-H., Kitao, M. and Wieser, G., 2015: Canopy-level stomatal narrowing in adult *Fagus sylvatica* under O₃ stress – Means of preventing enhanced O₃ uptake under high O₃ exposure?. *Environmental Pollution*, **196**, 518–526.
- McNaughton, K. G. and van den Hurk, B. J. J. M., 1995: A 'lagrangian' revision of the resistors in the two-layer model for calculating the energy budget of a plant canopy. *Boundary-Layer Meteorology*, **74**, 261–288.
- Mills, G., Hayes, F., Wilkinson, S. and Davies, W. J., 2009: Chronic exposure to increasing background ozone impairs stomatal functioning in grassland species. *Global Change Biology*, **15**, 1522–1533.
- Mills, G., Pleijel, H., Büker, P., Braun, S., Emberson, L. D., Harmens, H., Hayes, F., Simpson, D., Grünhage, L., Karlsson, P. E., Danielsson, H., Bermejo, V. and Gonzalez Fernandez, I., 2010:

- Mapping Critical Levels for Vegetation. Revision undertaken in Summer 2010 to include new flux-based critical levels and response functions for ozone, In: *Mapping Manual 2004*. International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops.
- Ohara, T., 2011: Why is the increase of tropospheric ozone concentration in mountain and island regions in Japan? *Japanese Journal of Ecology*, **60**, 77–81. (In Japanese)
- Oksanen, E., 2003: Responses of selected birch (*Betula pendula* Roth) clones to ozone change over time. *Plant, Cell and Environment*, **26**, 875–886.
- Omasa, K., Saji, H., Youssefian, S. and Kondo, K., 2002: *Air Pollution and Plant Biotechnology*. Springer-Verlag, Tokyo. 455pp.
- Onandia, G., Olsson, A.-K., Barth, S., King, J. S. and Uddling, J., 2011: Exposure to moderate concentrations of tropospheric ozone impairs tree stomatal response to carbon dioxide. *Environmental Pollution*, **159**, 2350–2354.
- Oue, H., Motohiro, S., Inada, K., Miyata, A., Mano, M., Kobayashi, K. and Zhu, J., 2008: Evaluation of ozone uptake by the rice canopy with the multi-layer model. *Journal of Agricultural Meteorology*, **64**, 223–232.
- Oue, H., Feng, Z., Pang, J., Miyata, A., Mano, M., Kobayashi, K. and Zhu, J., 2009: Modeling the stomatal conductance and photosynthesis of a flag leaf of wheat under elevated O₃ concentration. *Journal of Agricultural Meteorology*, **65**, 239–248.
- Paoletti, E., 2005: Ozone slows stomatal response to light and leaf wounding in a Mediterranean evergreen broadleaf, *Arbutus unedo*. *Environmental Pollution*, **134**, 439–445.
- Paoletti, E. and Grulke, N. E., 2005: Does living in elevated CO₂ ameliorate tree response to ozone? A review on stomatal responses. *Environmental Pollution*, **137**, 483–493.
- Pleijel, H., Danielsson, H., Vandermeiren, K., Blum, C., Colls, J. and Ojanperä, K., 2002: Stomatal conductance and ozone exposure in relation to potato tuber yield – results from the European CHIP programme. *European Journal of Agronomy*, **17**, 303–317.
- Reich, P. B., 1987: Quantifying plant response to ozone: a unifying theory. *Tree Physiology*, **3**, 63–91.
- Reich, P. B. and Lassoie, J. P., 1984: Effects of low level O₃ exposure on leaf diffusive conductance and water-use efficiency in hybrid poplar. *Plant, Cell and Environment*, **7**, 661–668.
- Vahisalu, T., Puzorjova, I., Brosche, M., Valk, E., Lepiku, M., Moldau, H., Pechter, P., Wang, Y.-S., Lindgren, O., Salojarvi, J., Loog, M., Kangasjarvi, J. and Kollist, H., 2010: Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. *The Plant Journal*, **62**, 442–453.
- Watanabe, M., Hoshika, Y., Inada, N. and Koike, T., 2014a: Canopy carbon budget of Siebold's beech (*Fagus crenata*) sapling under free air ozone exposure. *Environmental Pollution*, **184**, 682–689.
- Watanabe, M., Hoshika, Y., Inada, N., Wang, X., Mao, Q. and Koike, T., 2013: Photosynthetic traits of Siebold's beech and oak saplings grown under free air ozone exposure. *Environmental Pollution*, **174**, 50–56.
- Watanabe, M., Hoshika, Y. and Koike, T., 2014b: Photosynthetic responses of Monarch birch seedlings to differing timings of free air ozone fumigation. *Journal of Plant Research*, **127**, 339–345.
- Watanabe, M., Matsuo, N., Yamaguchi, M., Matsumura, H., Kohno, Y. and Izuta, T., 2010: Risk assessment of ozone impact on the carbon absorption of Japanese representative conifers. *European Journal of Forest Research*, **129**, 421–430.
- Watanabe, M., Yamaguchi, M., Matsumura, H., Kohno, Y. and Izuta, T., 2012: Risk assessment of ozone impact on *Fagus crenata* in Japan: consideration of atmospheric nitrogen deposition. *European Journal of Forest Research*, **131**, 475–484.
- Werner, H. and Fabian, P., 2002: Free-air fumigation of mature trees: a novel system for controlled ozone enrichment in grown-up beech and spruce canopies. *Environmental Science and Pollution Research*, **9**, 117–121.
- Wittig, V. E., Ainsworth, E. A. and Long, S. P., 2007: To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. *Plant, Cell and Environment*, **30**, 1150–1162.
- Yamaguchi, M., Watanabe, M., Iwasaki, M., Tabe, C., Matsumura, H., Kohno, Y. and Izuta, T., 2007: Growth and photosynthetic responses of *Fagus crenata* seedlings to O₃ under different nitrogen loads. *Trees*, **21**, 707–718.
- Yamaji, K., Ohara, T., Uno, I., Kurokawa, J., Pochanart, P. and Akimoto, H., 2008: Future prediction of surface ozone over east Asia using models-3 community multiscale air quality modeling system and regional emission inventory in Asia. *Journal of Geophysical Research*, **113**, D08306.