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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 nmol mol^{-1} 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; Matyssek 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 O_3 uptake by O_3-induced stomatal closure in sun and shade leaves of Siebold's beech. We hypothesized that O_3-induced stomatal closure may be gradually lost after O_3 exposure throughout the growing season in Siebold's beech, and thus conducted the analysis of O_3-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 O_3 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℃, 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 O_3 exposure experiment (for details of the system; see Watanabe *et al.* 2013). We set up two plots, one for ambient O_3 and another for elevated O_3. Size of each plot is 5.5 m × 7.2 m. The distance between the O_3-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 O_3 began in 2011.

Exposure to O_3 is based on the system used at Kranzberg Forest in Germany (Werner and Fabian, 2002). The target O_3 concentration was 60 nmol mol^{-1} during daylight hours. This elevated daytime O_3 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 O_3 monitor (Mod. 202, 2B Technologies, Boulder CO, U.S.A.). The daytime hourly mean O_3 concentration in elevated O_3 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 O_3 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 O_3 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℃ 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 H_2O 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, ℃), 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:

for $A_{start} \leq DOY < (A_{start}+f_{phen_a})$,

$$f_{phen} = (1 - f_{phen_c}) \cdot ((DOY - A_{start}) / f_{phen_a}) + f_{phen_c}$$

for $(A_{start}+f_{phen_a}) \leq DOY \leq (A_{end}-f_{phen_b})$,

$$f_{phen} = 1;$$

for $(A_{end}-f_{phen_b}) < DOY \leq A_{end}$,

$$f_{phen} = (1 - f_{phen_d}) \cdot ((A_{end} - DOY) / f_{phen_b}) + f_{phen_d} \quad (2),$$

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:

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

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

The parameter for of air temperature (T, ℃) 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 (℃) for stomatal conductance.

The response of stomatal conductance to the vapor pressure

deficit (VPD, kPa) is given by:

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

where $\mathrm{VPD}_{\min}$ and $\mathrm{VPD}_{\max}$ denote the threshold of VPD (kPa) for attaining minimum and full stomatal opening, respectively. If VPD exceeds $\mathrm{VPD}_{\min}$ then f_{VPD} is set to $f_{\min}$. If VPD is lower than $\mathrm{VPD}_{\max}$ 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 μmol photons m^{-2} s^{-1} for PPFD (when PPFD values were less than 200 μmol photons m^{-2} s^{-1}, PPFD classes at 50 or 5 μmol photons m^{-2} s^{-1} steps were adopted for sun leaves and shade leaves, respectively), 2℃ 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:

$$g_{\mathrm{sw}} = g_{\max} \cdot f_{\mathrm{phen}} \cdot f_{\mathrm{O3}} \cdot f_{\mathrm{light}} \cdot \max\{f_{\min}, (f_{\mathrm{temp}} \cdot f_{\mathrm{VPD}})\} \quad (6),$$

where the new parameter f_{O3} 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_{O3} can be shown as:

$$f_{\mathrm{O3}} = 1 - \mathrm{b} \cdot \left(\frac{[\tilde{O}_3]}{27.5} - 1 \right) \cdot (1 - c \cdot [\text{Leaf age}]) \quad (7),$$

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 μmol photons m^{-2} 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.*, $f_{\mathrm{O3}} = 1$ (cf. Löw *et al.*, 2007; Hoshika *et al.*, 2012b).

2.4 Estimation of stomatal ozone uptake

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

$$F_{\mathrm{st}} = [\mathrm{O_3}] \cdot g_{\mathrm{sO3}} \cdot \frac{r_{\mathrm{c}}}{r_{\mathrm{b}} + r_{\mathrm{c}}} \quad (8),$$

where r_{b} is the leaf boundary layer resistance (s m^{-1}), and g_{sO3} is the stomatal conductance for O_3 ($g_{\mathrm{sO3}} = g_{\mathrm{sw}} \cdot 0.613$; mmol O_3 m^{-2} 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 (= $1/(g_{\mathrm{sO3}}+g_{\mathrm{ext}})$; 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):

$$r_{\mathrm{b}} = 1.3 \cdot 150 \cdot (L_{\mathrm{d}} / u)^{0.5} \quad (9),$$

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

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

$$\mathrm{POD_Y} = \sum \max(F_{\mathrm{st}} - \mathrm{Y}, 0) \quad (10),$$

where Y is a species-specific threshold of stomatal O_3 flux (nmol O_3 m^{-2} 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 ($\mathrm{POD_0}$) in the present study. $\mathrm{POD_0}$ 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 ± 13.0 g m^{-2} and 83.7 ± 10.6 g m^{-2} in ambient and in elevated O_3, respectively; shade leaves: 36.3 ± 5.3 g m^{-2} and 33.6 ± 3.6 g m^{-2} in ambient and in elevated O_3, respectively; mean ± standard deviation of 6 target trees in each treatment). The average soil moisture (volumetric soil water content) was 28.9 ± 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 mmol H_2O 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 μmol m^{-2} s^{-1} in sun leaves and above 100 μmol 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℃: 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.

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}	(℃)	27	25
	T_{min}	(℃)	2	1
	T_{max}	(℃)	36	41
f_{VPD}	VPD_{max}	(kPa)	1.3	1.3
	VPD_{min}	(kPa)	5.5	4.8
f_{O3}	*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_{O3} depend respectively on the photosynthetically relevant photon flux density at leaf surface (PPFD, μmol m^{-2} s^{-1}), temperature (T, ℃), 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.

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).

3.3 Test of f_{O3} 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_{O3} overestimated stomatal conductance (Fig. 4). When f_{O3} 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_{O3} 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_{O3} was lower than those in the model without f_{O3} (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_{O3}, 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 fO_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;

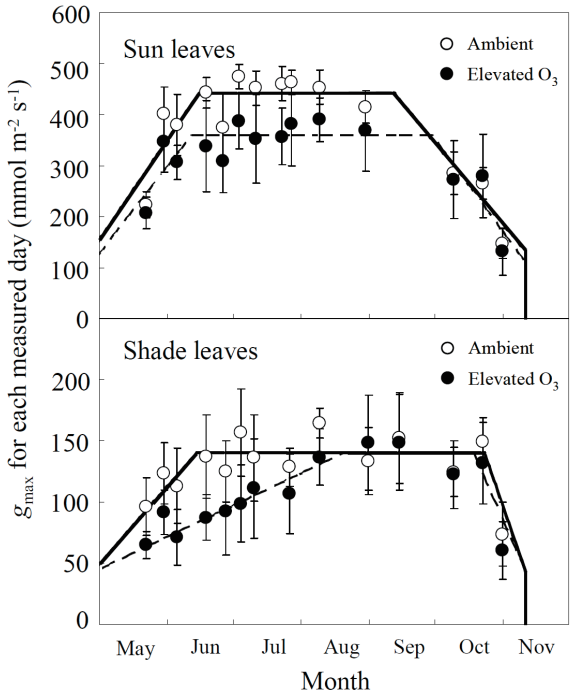

Fig. 1. Seasonal change of daily g_{max} for ambient and elevated O_3 in sun and shade leaves of Siebold's beech. Solid and dotted line denotes a regression line in ambient and elevated O_3, respectively. Daily g_{max} (obtained at 9:00 to 12:00) was identified as the daily maximum record of stomatal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

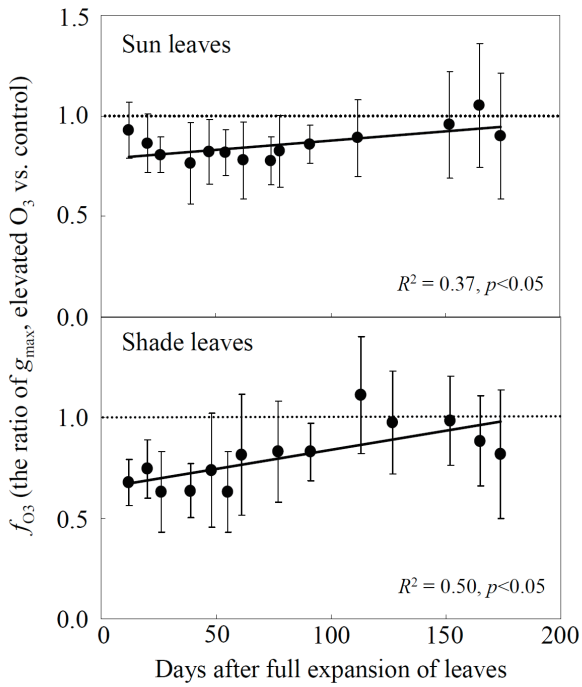

Fig. 2. Ratio of daily g_{max} in elevated O_3 to those in ambient conditions in sun and shade leaves of Siebold's beech, as a linear function of leaf age (days after full expansion of leaves). The regression line was obtained as y=0.0009x+0.79 and y=0.0019x+0.65 in sun and shade leaves, respectively. 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 μmol photons m^{-2} s^{-1} and VPD<1.5 kPa for sun leaves, and when VPD<1.5 kPa for shade leaves. Error bars mean ± 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_{O3} (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.

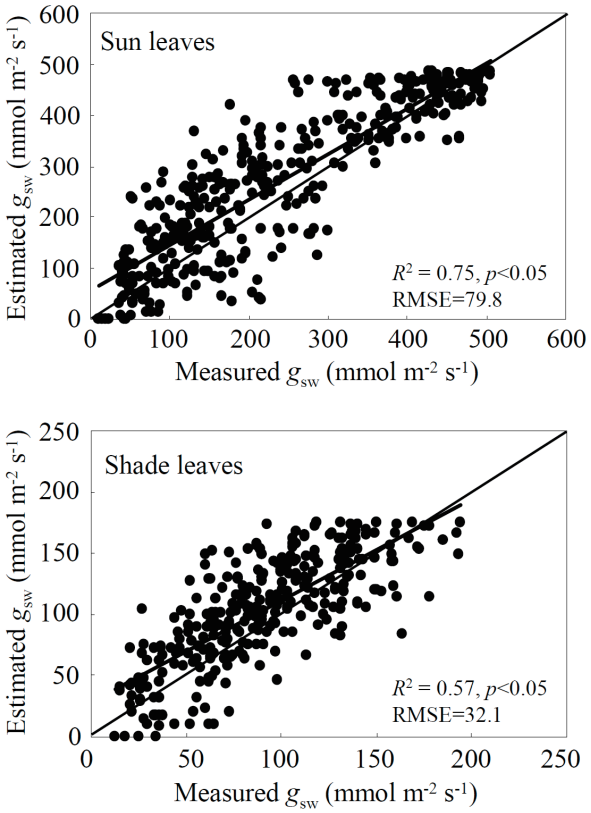

Fig. 3. A comparison between measured and estimated g_{sw} under ambient conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as y=0.89x+58.5 and y=0.83x+28.3 in sun and shade leaves, respectively.

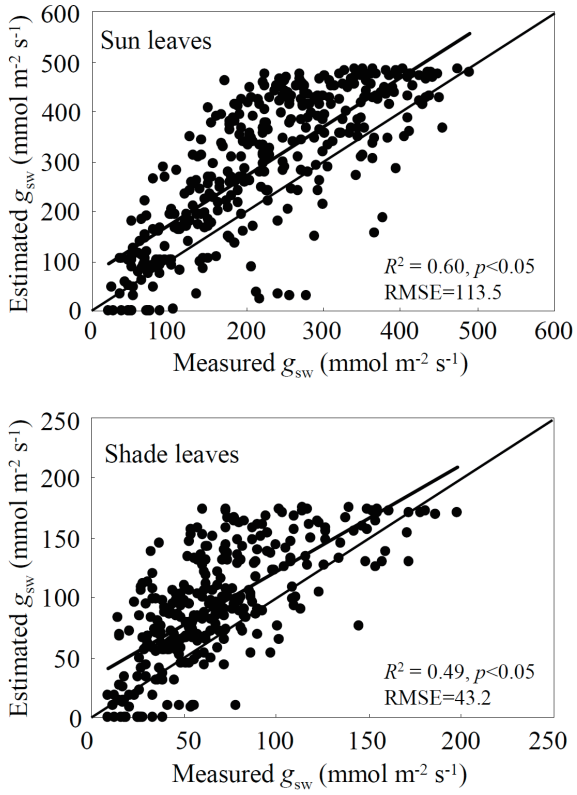

Fig. 4. A comparison between measured and estimated g_{sw} using the model without f_{O3} (neglecting O_3-induced stomatal closure) under elevated O_3 conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as y=0.98x+73.3 and y=0.88x+33.7 in sun and shade leaves, respectively.

In previous modeling studies with crops, a development of f_{O3} 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_{O3} 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_{O3} 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 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_{O3} 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 O_3 uptake (POD_0) under ambient and elevated O_3 using stomatal conductance model neglecting or considering O_3-induced stomatal closure (f_{O3}) 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_{O3})	Elevated O_3 (model with f_{O3})	
Siebold's beech				
Sun leaves	25.6	53.0	47.4	11%
Shade leaves	11.8	19.9	16.5	17%

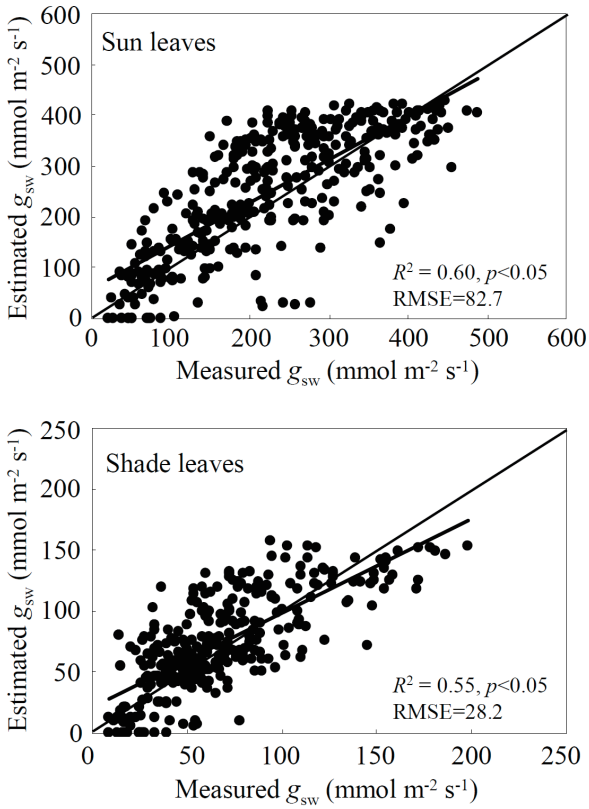

Fig. 5. A comparison between measured and estimated g_{sw} using the model with f_{O3} (considering O_3-induced stomatal closure) under elevated O_3 conditions in sun leaves (above figure) and shade leaves (bottom figure) of Siebold's beech. The regression line was obtained as y=0.84x+60.5 and y=0.78x+20.5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

This may result in lower decline of photosynthetic traits with exposure to O_3 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 O_3-induced stomatal closure should be considered in f_{O3} 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 O_3 for further development of modeling for the assessment of O_3 impacts on forest trees (cf. Watanabe *et al.*, 2014b).

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