Photosynthetic responses to ozone of upper and lower canopy leaves of *Fagus crenata* Blume seedlings grown under different soil nutrient conditions

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**Abstract**  
We aimed to clarify the effects of ozone (O\(_3\)) on photosynthetic ability of upper and lower canopy leaves of *Fagus crenata* Blume seedlings grown under different soil nutrient conditions. To accomplish this objective, we analyzed the response of photosynthetic parameters such as maximum carboxylation rate (\(V_{\text{cmax}}\)) to cumulative stomatal O\(_3\) uptake (\(S_{\text{Fst}}\)) and reduction rate of \(V_{\text{cmax}}\) per unit \(S_{\text{Fst}}\) as an index of detoxification capacity for O\(_3\). The seedlings of *Fagus crenata* were grown for two growing seasons (2014–2015) in nine treatments comprised of a combination of three levels of gas treatments (charcoal-filtered air or 1.0- or 1.5-times ambient O\(_3\) concentration) and three levels of soil nutrient treatments (non-fertilized or a supply of relatively low or high concentrations of compound fertilizer). The nutrient supply significantly increased the degree of O\(_3\)-induced reduction in \(V_{\text{cmax}}\) in September. However, nutrient supply did not significantly increase \(S_{\text{Fst}}\) and reduce the detoxification capacity for O\(_3\). On the other hand, the degree of O\(_3\)-induced reduction in \(V_{\text{cmax}}\) of upper canopy leaves was higher as compared with that of lower canopy leaves in August due to the higher \(S_{\text{Fst}}\). However, the reduction rate of \(V_{\text{cmax}}\) per unit \(S_{\text{Fst}}\) in lower canopy leaves was higher than that in upper canopy leaves, indicating lower detoxification capacity for O\(_3\) in lower canopy leaves. Reduction rate of \(V_{\text{cmax}}\) per unit \(S_{\text{Fst}}\) over the threshold, which is assumed to be proportional to gross photosynthetic rate, was similar between upper and lower canopy leaves. Therefore, capacity of photosynthetic CO\(_2\) assimilation is likely to be associated with detoxification capacity for O\(_3\) in upper and lower canopy leaves of *F. crenata* seedlings grown under different soil nutrient conditions.

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1. Introduction

Tropospheric ozone (O\(_3\)) concentrations have increased globally as a result of increase in emission of its precursors since the industrial revolution, and this trend is expected to continue in the future (Young et al., 2013). Ozone is a greenhouse gas and is also recognized as a phytotoxic air pollutant (Bytnerowicz et al., 2007). Photosynthetic CO\(_2\) fixation is reduced by exposure to O\(_3\) (Dizengremel, 2001; Wittig et al., 2007, 2009). Sitch et al. (2007) suggested that O\(_3\)-induced reduction in photosynthetic CO\(_2\) fixation, i.e. increase in indirect radiative forcing, contributes to global warming. Therefore, it is important to quantify the O\(_3\)-induced reduction in photosynthetic CO\(_2\) fixation of forest tree species to predict the future capacity of the forest ecosystem as a carbon sink.

Soil nutrient conditions vary across Japanese forested areas (Kawada, 1977; Kayama et al., 2002; Inagaki et al., 2004). The nutrient conditions may affect the sensitivity of trees to O\(_3\) (Matyssek and Sandermann, 2003). Maurer et al. (1997) indicated that O\(_3\)-induced reduction in photosynthetic capacity such as apparent carboxylation efficiency of high-fertilized *Betula pendula* seedlings occurred earlier than that of the low-fertilized seedlings. Furthermore, it has been suggested that the sensitivities to O\(_3\) of the

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leaves within a canopy are not uniform (Kitao et al., 2009; Watanabe et al., 2014a). Watanabe et al. (2014a) demonstrated that the degree of O₃-induced reduction in net photosynthetic rate ($A_o$) of upper canopy leaves was higher as compared with that of lower canopy leaves in Fagus crenata saplings. In contrast, the degree of O₃-induced reduction in $A_o$ of shade leaves was higher than that of sun leaves in mature Fagus sylvatica (Kitao et al., 2009). However, interactive effects of soil nutrient condition and canopy leaf position on the sensitivity of photosynthetic capacity to O₃ has not yet been clarified. Such difference in the sensitivity to O₃ depending on the nutrient conditions and/or canopy leaf positions should be taken into account for the evaluation of the effects of O₃ on photosynthetic CO₂ fixation of forest tree species.

The degree of O₃-induced reduction in photosynthetic capacity is expressed as a product of cumulative stomatal O₃ uptake ($\Sigma F_{st}$) and reduction rate of photosynthetic capacity per unit $\Sigma F_{st}$. Reduction rate of photosynthetic capacity per unit $\Sigma F_{st}$ can be used as an index of detoxification capacity for O₃ (Izuta et al., 1999). Previous studies have clarified the effects of nutrient supply to soil and canopy leaf position on $\Sigma F_{st}$ (Maurer et al., 1997; Nunn et al., 2005; Warren et al., 2007; Hoshika et al., 2015). Maurer et al. (1997) reported that stomatal O₃ uptake in the leaves of lower-crowned trees of Prunus serotina was similar to that of high-crowned fertilized seedlings. According to Hoshika et al. (2015), $\Sigma F_{st}$ of upper canopy leaves was much higher as compared with that of lower canopy leaves in F. crenata saplings. However, the effects of nutrient supply on the reduction rate of photosynthetic capacity per unit $\Sigma F_{st}$ and its difference between upper and lower canopy leaves have not yet been clarified.

There is a threshold of stomatal O₃ uptake below which the adverse effects of O₃ on plants do not occur. Based on this concept, it has been suggested that the degree of O₃ injury to plants is proportional to the cumulative value of stomatal O₃ uptake ($F_{st}$) over the threshold ($D_t$, i.e. $\Sigma F_{st} - D_t$) (Musselman and Massman, 1999; Massman et al., 2000; Mills et al., 2010). There is a broad consensus on this theory, and it is therefore widely used to quantify the O₃ injury to plants and to establish critical levels with respect to O₃ injury (Pleijel et al., 2002; Karlsson et al., 2004, 2007; Löw et al., 2007; Bagard et al., 2008, 2015; Mills et al., 2010; Watanabe et al., 2014b; Büker et al., 2015). In Europe, $D_t$ has been set as a constant value (Pleijel et al., 2002; Karlsson et al., 2004; Mills et al., 2010). However, it has been suggested that $D_t$ is not a constant value (e.g. Massman, 2004). Massman (2004) assumed that $D_t$ is proportional to the gross photosynthetic rate ($A_g$) because a photosynthetic product is needed for the detoxification of O₃ in the leaves (Musselman and Massman, 1999). The $F_{st}/A_g$ value of shade leaves (i.e. ratio of stomatal O₃ uptake to detoxification capacity to O₃) was relatively high, and actually the extent of O₃-induced visible foliar injury in shade leaves was greater than that in sun leaves of seedlings, saplings and mature trees of Picea abies (Matyssek et al., 2007). The significant negative correlation may be due to the fact that leaves with lower SLA have thick layers of parenchyma cells with antioxidant capacity (Matyssek et al., 2007, 2008). Li et al. (2016) showed a significant negative correlation between leaf mass per area (LMA, inverse of SLA) and the degree of O₃-induced reduction in $A_o$ of 29 deciduous and evergreen tree species. Therefore, the photosynthetic productivity and LMA may be related to detoxification capacity for O₃ and $D_t$. However, it has not yet been clarified whether these indices can explain the differences in detoxification capacity for O₃ between upper and lower canopy leaves under different soil nutrient conditions.

Photosynthetic CO₂ fixation of trees has been estimated using the photosynthetic model proposed by Farquhar et al. (1980) coupled with the $g_s$ model proposed by Ball et al. (1986) (Collatz et al., 1991; Sitch et al., 2003). Photosynthetic parameters such as maximum carboxylation rate ($V_{c_{max}}$) and maximum electron transport rate ($J_{max}$) are needed to estimate photosynthetic CO₂ fixation rates using these models. Therefore, it is important to clarify the effects of O₃ on these photosynthetic parameters for evaluating the impacts of O₃ on photosynthetic CO₂ fixation of forest tree species. In the present study, we aimed to clarify the effects of O₃ on photosynthetic parameters such as $V_{c_{max}}$ and $J_{max}$ in upper and lower canopy leaves of the seedlings of F. crenata, representative Japanese deciduous trees, grown under different soil nutrient conditions. To accomplish this objective, we evaluated $\Sigma F_{st}$ and analyzed the relationships between $\Sigma F_{st}$ and photosynthetic parameters using several indices of $D_t$.

2. Materials and methods

2.1. Plant material

From the A-horizon at a forest floor of deciduous trees in the Field Museum Mt. Karasawa of Tokyo University of Agriculture and Technology (Sano, Tochigi, Japan), brown forest soil was collected and passed through a sieve with a mesh size of 5 mm. On 7 May 2014, seedlings of F. crenata Blume (2-year-old) were individually planted in 1/2000 a Wagner pots (bulk: 12 l, width: 228–240 mm, depth: 259 mm) filled with the soil. The seedlings were grown for two growing seasons from 15 May 2014 to 26 October 2015 in nine O₃-exposure chambers located at the Field Museum Tamakuyuro of Tokyo University of Agriculture and Technology (Hachioji, Tokyo, Japan) (latitude: 35°38′N; longitude: 139°22′E; above sea level: 144.1 m). The mean values of plant height and stem diameter of the seedlings on 15 May 2014 were 49 cm and 6.3 mm, respectively. Gas treatments (described in the next section) were conducted when leaves were attached (from 15 May to 30 November 2014 and from 21 April to 26 October 2015). Air temperature and relative air humidity were continuously measured at 10-min intervals in three of the nine chambers using a TR-72U Thermo Recorder (T&D Corporation, Matsumoto, Nagano, Japan). Daily mean air temperature and relative air humidity inside the chambers during the gas treatment period of the first growing season were 5.2–29.4 °C and 52.5–99.4%, respectively, and those of the second growing season were 11.7–29.6 °C and 49.4–99.0%, respectively. Illuminance was also measured in the three chambers at 5-min intervals using three HOBO Pendant temperature/light dataloggers (UA-002-64; Onset Computer Co., Bourne, MA, USA), which were calibrated against a quantum sensor (LI-190SA; Li-Cor Inc., Lincoln, NE, USA). Photosynthetic photon flux density (PPFD) outside the chambers was measured at 1-min intervals using the quantum sensor. The mean light transmissibility of the chambers during the gas treatment period was approximately 80%. Average cumulative solar radiation per day above the canopies during the gas treatment period of the first growing season was 8.1 Mj m⁻² day⁻¹, and that of the second growing season was 8.5 Mj m⁻² day⁻¹. The seedlings were well irrigated to avoid drought stress.

2.2. Ozone exposure and nutrient supply

This study had a split-plot factorial design. The whole-plot treatment consisted of three levels of O₃ with three chamber replications (a total of nine O₃-exposure chambers). The sub-plot treatment comprised of three levels of soil nutrient treatments in
each chamber. We assigned a total of 30 *F. crenata* seedlings to each treatment (10 seedlings per treatment in each chamber; three chamber replicates).

The seedlings were exposed to charcoal-filtered air (CF treatments, mean removal efficiency of O$_3$: ca. 60%), O$_3$ at 1.0-time the ambient concentration (1.0 × O$_3$ treatment), i.e. the same O$_3$ concentration as ambient concentration, or O$_3$ at 1.5-times the ambient concentration (1.5 × O$_3$ treatment). The ambient concentration of O$_3$ outside the chambers was used as the standard concentration for the regulation of the concentrations of O$_3$ in the chambers of 1.0 × O$_3$ and 1.5 × O$_3$ treatments. Further details of the O$_3$-exposure and O$_3$-monitoring systems are described in Kinose et al. (2014). The 12-h mean (6:00—18:00) concentration of O$_3$ in the ambient air during the gas treatment period of the first and second growing seasons were 24.7 and 28.3 nmol mol$^{-1}$, respectively. The 12-h mean concentration of O$_3$ in the CF, 1.0 × O$_3$ and 1.5 × O$_3$ treatments during the gas treatment period of the first growing season was 9.4, 20.1 and 29.5 nmol mol$^{-1}$, respectively. Those of the second growing season were 9.8, 29.1 and 43.1 nmol mol$^{-1}$, respectively. The accumulated exposure over a threshold of 40 nmol mol$^{-1}$ of O$_3$ (AOT40) in daylight hours (solar radiation > 50 W m$^{-2}$) was calculated based on the monitored O$_3$ concentrations in the chambers. The seedlings were supplied with 500 mL of water (NF: non-fertilized treatment), 2000-fold diluted water (LF: low-fertilized treatment) or 1000-fold one (HF: high-fertilized treatment) to soil once per 2 weeks during the gas treatment period.

2.3. Measurements of soil nutrient status

On 26 October 2015, nine potted soils per treatment (three potted soil per treatment in each chamber; three chamber replicates) were collected to determine the concentrations of total nitrogen (N), available phosphorus (P) and exchangeable potassium (K) in the soil. After air-drying of the soil, the dried soil was passed through a 2 mm sieve. The concentration of N in the soil was determined with a C/N analyzer (MT-700, Yanaco, Tokyo, Japan). Phosphorus was extracted from the soil according to the Bray-2 method (Bray and Kurtz, 1945), and the concentration was determined by the molybdenum blue method (Murphy and Riley, 1962). Potassium was extracted from the soil using 1 M ammonium acetate and the concentration was determined using an inductively coupled plasma optical emission spectrometry (iCAP-6300; Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4. Model description

We estimated leaf gas exchange parameters such as $A_g$ and $g_s$ by coupling the photosynthetic model and $g_s$ model (Farquhar et al., 1980; Ball et al., 1986; Collatz et al., 1991). The $A_g$ (μmol m$^{-2}$ s$^{-1}$) and $g_s$ (μmol m$^{-2}$ s$^{-1}$) are expressed as follows:

$$A_g = \min(W_c, W_l),$$ (1)

$$A_n = \min(W_c, W_j) - R,$$ (2)

where $W_c$ is the Rubisco-limited gross photosynthetic rate (μmol m$^{-2}$ s$^{-1}$), $W_l$ is the RubP regeneration rate-limited gross photosynthetic rate (μmol m$^{-2}$ s$^{-1}$), and $R$ is the mitochondrial respiration rate (μmol m$^{-2}$ s$^{-1}$). The $W_c$ and $W_j$ are given by:

$$W_c = \frac{V_{c\text{max}}(C_i - f^*)}{C_i + K_c(T + O/K_o)},$$ (3)

$$W_j = \frac{J(C_i - f^*)}{4C_i + 8T},$$ (4)

where $C_i$ is the intercellular CO$_2$ concentration (μmol mol$^{-1}$), $j^*$ is the CO$_2$ compensation point in the absence of mitochondrial respiration (μmol mol$^{-1}$), $K_c$ is the Michaelis–Menten constant of Rubisco for CO$_2$ (μmol mol$^{-1}$), $O$ is the O$_2$ concentration (nmol mol$^{-1}$), $K_o$ is the Michaelis–Menten constant of Rubisco for O$_2$ (mmol mol$^{-1}$), and $J$ is the electron transport rate (μmol m$^{-2}$ s$^{-1}$). The $J$ is written as follows:

$$J = \frac{j \text{ PPFD} + J_{\text{max}} - \sqrt{(j \text{ PPFD} + J_{\text{max}})^2 - 4 j \text{ PPFD} j_{\text{ max}}}}{2j},$$ (5)

where $j$ is the initial slope of response curve of $J$ to PPFD (mol m$^{-2}$ s$^{-1})$, $J_{\text{max}}$ is the maximum electron transport rate (μmol m$^{-2}$ s$^{-1}$) and $\theta$ is the convexity of the response curve of $J$ to PPFD.

The day respiration rate ($R_d$, μmol m$^{-2}$ s$^{-1}$) was set as 40% of the dark respiration ($R_{d0}$, μmol m$^{-2}$ s$^{-1}$) (Villar et al., 1995). The $R$ was determined as follows:

$$R = R_{d0},$$ (6)

when PPFD = 0,

$$R = R_{d0} + 0.4 R_{d0},$$ (7)

The values of $V_{c\text{max}}, J_{\text{max}}, K_c, K_o, j^*$ and $R$ at given leaf temperature are calculated from those values at leaf temperature of 25 °C ($V_{c\text{max},25}, J_{\text{max},25}, K_c25, K_o25, j^*_{25}$ and $R_{25}$) using the following equations (Farquhar et al., 1980; Bernacchi et al., 2001; Medlyn et al., 2002):

For $V_{c\text{max}}, K_c, K_o, j^*$ and $R$ (following equation is an example for $V_{c\text{max}}$),

$$V_{c\text{max}} = V_{c\text{max},25}\exp \left[ \frac{(T_{\text{leaf}} - 25) \Delta H_s}{298 R (T_{\text{leaf}} + 273)} \right].$$ (8)

for $J_{\text{max}}$,

$$J_{\text{max}} = J_{\text{max},25}\exp \left[ \frac{(T_{\text{leaf}} - 25) \Delta H_s}{298 R (T_{\text{leaf}} + 273)} \right] \frac{1 + \exp \left( \frac{298 \Delta S - \Delta H_s}{298 R} \right)}{1 + \exp \left( \frac{298 \Delta S - \Delta H_s}{R (T_{\text{leaf}} + 273)} \right)},$$ (9)

where $T_{\text{leaf}}$ is the leaf temperature (°C), $\Delta H_s$ is activation energy (kJ mol$^{-1}$), $R'$ is the universal gas constant (kJ K$^{-1}$ mol$^{-1}$), $\Delta S$ is the entropy factor (kJ K$^{-1}$ mol$^{-1}$), and $\Delta H_d$ is the deactivation energy (kJ mol$^{-1}$). The values of these parameters are listed in Table 1. The $T_{\text{leaf}}$ at given time was estimated using energy budget model at the leaf according to Amthor (1994).

The $g_s$ (μmol m$^{-2}$ s$^{-1}$) was estimated using the model of Iorio et al. (2009), originally proposed by Ball et al. (1986), as follows:
g_s = \frac{A_r}{C_s - \Gamma_s} \sqrt{D} + g_0. \tag{10}

where $C_s$ is CO$_2$ concentration at the leaf surface (µmol mol$^{-1}$), $D$ is the vapor pressure deficit from leaf to air (kPa), $g_s$ is a constant, and $g_0$ is $g_s$ when $A_r$ is equal to 0 (µmol m$^{-2}$ s$^{-1}$). The results of the parameterization of the $g_s$ models are given in the supplementary information (Fig. S4; Table S2).

The $F_{st}$ (µmol m$^{-2}$ s$^{-1}$) is expressed as follows:

$$F_{st} = \frac{[O_3]}{r_{s,03} + R_{o,03}}. \tag{11}$$

where $[O_3]$ is the O$_3$ concentration in the air (µmol mol$^{-1}$), $r_{s,03}$ is the stomatal resistance to O$_3$ (m$^2$ s µmol$^{-1}$), and $R_{o,03}$ is the leaf boundary layer resistance to O$_3$ (µmol m$^{-2}$ s µmol$^{-1}$).

The $r_{s,03}$ is inverse of stomatal conductance to O$_3$ ($g_{s,03}$), which is calculated by multiplying $g_s$ by a conversion factor of 0.663 to account for the difference in molecular diffusivity between water vapor and O$_3$ (Massman, 1998; Mills et al., 2010):

$$r_{s,03} = \frac{1}{0.663g_s}. \tag{12}$$

The $R_{o,03}$ was calculated from wind speed ($u$, m s$^{-1}$) and crosswind leaf dimension ($L_d$, m) as follows (Mills et al., 2010):

$$R_{o,03} = 1.3 \cdot 150 \cdot \sqrt{\frac{L_d}{u}}. \tag{13}$$

where the factor 1.3 accounts for the difference in diffusivity between heat and O$_3$.

The tree height of F. crenata seedlings after the leaf emergence of the second growing season was 89 ± 16 cm. During the second growing season, the canopy was vertically separated into five layers from the surface of the potted soil at 30 cm intervals (Layer 1: 0–30 cm; Layer 2: 30–60 cm; Layer 3: 60–90 cm; Layer 4: 90–120 cm; and Layer 5: >120 cm). Diffuse PPFD in the canopy was measured on a cloudy day and wind velocity in the canopy was also measured using an anemometer (WS-03SD, Custom Corp., Chiyoda, Tokyo, Japan) in July of the second growing season. The PPFD and wind velocity were different depending on vertical locations in the canopy (described in the next section), although there were no differences in atmospheric concentrations of O$_3$, air temperature and relative air humidity among the layers. Based on the result of diffuse PPFD in the canopy, diffuse PPFD and the sum of direct and diffuse PPFD at the leaf surface were estimated in each layer according to Anten (1997). The ratio between the leaf area receiving only diffuse PPFD and that receiving both direct and diffuse PPFD were calculated in each layer by measurement of leaf angle. Based on these environmental data and photosynthetic parameters such as $V_{max}$ and $J_{max}$ in each layer, $A_r$, $g_s$ and $F_{st}$ were calculated using the coupled model of photosynthesis and $g_s$ according to the method in Collatz et al. (1991).

2.5. Measurements of leaf gas exchange rates and leaf traits

The $A$–$C_i$ and $A$–$light$ curves were determined for the first-flush leaves located in Layer 3 (upper canopy leaves) and Layer 1 (lower canopy leaves). We selected these layers because most of the leaves were within Layers 1–3 (ca. 85% of the total leaf area per seedling). The diffuse PPFD in Layer 1 was approximately 30% of that in Layer 3. Wind velocities in Layers 1 and 3 were 0.11 and 0.51 m s$^{-1}$, respectively. The measurement of leaf gas exchange rates was conducted once per month from May to September during the second growing season using an infrared gas analyzer system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). The nine seedlings per treatment (three seedlings per treatment in each chamber; three chamber replicates) were randomly selected for the measurement. In the measurement, $T_{leaf}$ values were set as 24, 25, 29, 30 and 23 ± 0.5 °C in May, June, July, August and September, respectively, and $D$ was set as 1.3 ± 0.1 kPa in all the measurement months. The $T_{leaf}$ value during the measurements of leaf gas exchange rates was adjusted to coincide with the mean daytime air temperature (6:00–18:00) during the 10 days before the measurement period. The $A$–$C_i$ curve was determined at nine levels of CO$_2$ concentration (50, 150, 300, 400, 500, 800, 1,200, 1,500, 1800 ppm) and at PPFD of 1500 µmol m$^{-2}$ s$^{-1}$. The $A$–$light$ curve was determined at seven levels of PPFD (25, 75, 100, 300, 800 and 1500 µmol m$^2$ s$^{-1}$) and at 1800 ppm of CO$_2$ concentration. The $V_{max}$ $J_{max}$ $\varphi$ and $\theta$ were calculated using simultaneous fitting of Eqs. (2)–(6) to A–C$_i$ and $A$–$light$ curves, and $V_{max}$ $J_{max}$ were calculated using temperature dependencies of $V_{max}$ and $J_{max}$ (Eqs. (8) and (9)). To obtain $g_s$ and $g_0$, $A_r$ and $g_s$ were simultaneously measured under the growth conditions inside the chambers during the daytime in May, June, July, August and September of the second growing season. The nine seedlings grown in CF treatment were selected and the temperature dependency of $R_e$ was determined at 17–35 °C of $T_{leaf}$ in July of the first growing season. The $\Delta H_4$ for $R$ was calculated as 46.345 kJ µmol$^{-1}$. The $R_e$ at 25 °C of $T_{leaf}$ ($R_{e,25}$) was determined.
based on the measurement result of $R_n$ in our previous study (Kinose et al., 2016).

After the measurement of leaf gas exchange rates, the leaves of nine seedlings per treatment (three seedlings per treatment in each chamber; three chamber replicates) were collected to determine LMA. The LMA was measured in the first-flush leaves located in upper and lower canopies. The projected area of the leaves was analyzed using the ImageJ software. The leaves were dried at 80°C in the oven for 1 week and weighed. The LMA was calculated as the ratio of leaf dry mass to projected leaf area.

2.6. Validation of model performance

To validate the estimation accuracy of $A_m$, $g_s$, and $T_{leaf}$, the dataset of leaf gas exchange measured under the growth conditions inside the chambers during the daytime were randomly divided into two groups: one for parameterization of the $g_s$ model and another for validation of the estimation accuracy. Mean absolute error (MAE) and mean bias error (MBE) of $A_m$, $g_s$, and $T_{leaf}$ were calculated (Willmott, 1982). Results of the MAE and MBE in $A_m$, $g_s$, and $T_{leaf}$ are provided in the supplementary information (Fig. S5).

2.7. Relationships between $\Sigma(F_{st} - D_f)$ and photosynthetic parameters

Previous studies proposed that $D_f$ is a constant (Pleijel et al., 2002; Karlsson et al., 2004; Mills et al., 2010), or is proportional to $A_g$ (Massman, 2004). On the other hand, we applied that $D_f$ is proportional to LMA because it has been suggested that LMA is a useful index of detoxification capacity for $O_3$ (Matyssek et al., 2007). In the present study, three functions of $D_f$ were defined as follows:

\[
D_t = \alpha, \\
D_t = \beta \times LMA, \\
D_t = \gamma \times A_g.
\]

The constant values ($\alpha$, $\beta$ and $\gamma$) were determined as the values when $R^2$ in the linear relationships between $\Sigma(F_{st} - \alpha)$, $\Sigma(F_{st} - \beta \times LMA)$ or $\Sigma(F_{st} - \gamma \times A_g)$ and photosynthetic parameters were highest. Annual mean LMA was used as $D_f$ in the present study and $A_g$ was calculated using the coupled model of photosynthesis and $g_s$ (Section 2.4).

2.8. Statistical analyses

Three-way analysis of variance (ANOVA) was performed to test the effects of $O_3$, nutrient supply to soil and leaf position in the canopy (i.e. upper and lower canopies) on photosynthetic parameters and $\Sigma F_{st}$. Two-way ANOVA was performed to test the effects of $O_3$ and nutrient supply to soil on soil nutrient conditions. Analysis of covariance (ANCOVA) was performed to test the effects of $O_3$, nutrient supply to soil and canopy leaf position on slope ($g_{sl}$) and intercept ($g_{si}$) in the linear relationships between $A_m(\frac{C_s - T^*}{D})$ and $g_s$. ANCOVA was also performed to test the effects of nutrient supply to soil and canopy leaf position on the slopes of linear relationships between $AOT40$, $\Sigma F_{st}$, $\Sigma(F_{st} - \alpha)$, $\Sigma(F_{st} - \beta \times LMA)$ or $\Sigma(F_{st} - \gamma \times A_g)$ and photosynthetic parameters. Statistical analyses were run using R 3.1.3 software (R Development Core Team, 2014).

3. Results

Table 2 shows nutrient concentrations in the potted soil on 26 October of the second growing season. Exposure to $O_3$ did not significantly affect soil nutrient concentrations. Conversely, nutrient supply to soil significantly increased N, P and K concentrations in the soil. There were no interactive effects of $O_3$ and nutrient supply on soil nutrient concentrations. On the other hand, nutrient concentrations in the leaves of non-, low- and high-fertilized seedlings averaged across the $O_3$ treatments and canopy leaf positions were 1.4, 1.5 and 1.6 g m$^{-2}$ for N, 49, 58 and 61 mg kg$^{-1}$ for P, and 268, 268 and 285 mg m$^{-2}$ for K, respectively (Kinose et al., 2016).

Table 3 indicates annual mean LMA of upper and lower canopy leaves during the second growing season. There was no significant

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Gas</th>
<th>$N$ (g kg$^{-1}$)</th>
<th>$P$ (mg kg$^{-1}$)</th>
<th>K (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>CF</td>
<td>1.83 (0.03)</td>
<td>5.1 (0.5)</td>
<td>51 (2)</td>
</tr>
<tr>
<td>1.0 × $O_3$</td>
<td></td>
<td>1.90 (0.10)</td>
<td>5.8 (0.6)</td>
<td>55 (2)</td>
</tr>
<tr>
<td>1.5 × $O_3$</td>
<td></td>
<td>1.78 (0.12)</td>
<td>5.0 (0.1)</td>
<td>55 (1)</td>
</tr>
<tr>
<td>LF</td>
<td>CF</td>
<td>1.94 (0.08)</td>
<td>10.9 (1.3)</td>
<td>59 (5)</td>
</tr>
<tr>
<td>1.0 × $O_3$</td>
<td></td>
<td>2.03 (0.06)</td>
<td>10.7 (1.3)</td>
<td>60 (4)</td>
</tr>
<tr>
<td>1.5 × $O_3$</td>
<td></td>
<td>1.86 (0.06)</td>
<td>11.4 (2.6)</td>
<td>59 (2)</td>
</tr>
<tr>
<td>HF</td>
<td>CF</td>
<td>1.97 (0.08)</td>
<td>18.6 (3.0)</td>
<td>69 (4)</td>
</tr>
<tr>
<td>1.0 × $O_3$</td>
<td></td>
<td>1.95 (0.10)</td>
<td>16.1 (4.4)</td>
<td>67 (3)</td>
</tr>
<tr>
<td>1.5 × $O_3$</td>
<td></td>
<td>1.91 (0.09)</td>
<td>14.6 (1.1)</td>
<td>68 (3)</td>
</tr>
</tbody>
</table>

The levels of nutrient treatments are indicated in parenthesis. Statistical significance by three-way ANOVA: *; p < 0.05, **; p < 0.001, n.s.; not significant.

Table 3

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Gas</th>
<th>$N$ (g kg$^{-1}$)</th>
<th>$P$ (mg kg$^{-1}$)</th>
<th>K (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>CF</td>
<td>1.94 (0.08)</td>
<td>10.9 (1.3)</td>
<td>59 (5)</td>
</tr>
<tr>
<td>1.0 × $O_3$</td>
<td></td>
<td>2.03 (0.06)</td>
<td>10.7 (1.3)</td>
<td>60 (4)</td>
</tr>
<tr>
<td>1.5 × $O_3$</td>
<td></td>
<td>1.86 (0.06)</td>
<td>11.4 (2.6)</td>
<td>59 (2)</td>
</tr>
<tr>
<td>HF</td>
<td>CF</td>
<td>1.97 (0.08)</td>
<td>18.6 (3.0)</td>
<td>69 (4)</td>
</tr>
<tr>
<td>1.0 × $O_3$</td>
<td></td>
<td>1.95 (0.10)</td>
<td>16.1 (4.4)</td>
<td>67 (3)</td>
</tr>
<tr>
<td>1.5 × $O_3$</td>
<td></td>
<td>1.91 (0.09)</td>
<td>14.6 (1.1)</td>
<td>68 (3)</td>
</tr>
</tbody>
</table>

See legend to Table 2 for treatment description.
single effect of O₃ or nutrient supply to soil on LMA. Conversely, LMA of upper canopy leaves was significantly higher as compared with that of lower canopy leaves. Significant interactive effect of O₃, nutrient supply and canopy leaf position on LMA was not detected.

Figs. 1 and 2 show $V_{\text{cmax},25}$ and $J_{\text{max},25}$ of upper and lower canopy leaves. Table 4 shows the results of three-way ANOVA for the effects of O₃, nutrient supply and leaf position on $V_{\text{cmax},25}$ and $J_{\text{max},25}$. Exposure to O₃ significantly decreased $V_{\text{cmax},25}$ in May, July, August and September, and $J_{\text{max},25}$ in August and September. Conversely, nutrient supply to soil increased $V_{\text{cmax},25}$ and $J_{\text{max},25}$ in all the measurement months. $V_{\text{cmax},25}$ and $J_{\text{max},25}$ of upper canopy leaves were higher as compared with those of lower canopy leaves in all the measurement months. Significant interactive effects of O₃ and nutrient supply on $V_{\text{cmax},25}$ and $J_{\text{max},25}$ were detected in September.

The degrees of O₃-induced reductions in $V_{\text{cmax}}$ and $J_{\text{max}}$ were higher in low- and high-fertilized seedlings than in non-fertilized seedlings. Significant interactive effects of O₃ and canopy leaf position were detected on $V_{\text{cmax},25}$ in August and $J_{\text{max},25}$ in June. The degrees of O₃-induced reductions in $V_{\text{cmax}}$ and $J_{\text{max}}$ of upper canopy leaves were higher as compared with those of lower canopy leaves.

![Fig. 1](image1.png)

**Fig. 1.** Maximum carboxylation rate at leaf temperature of 25 °C ($V_{\text{cmax},25}$) of upper and lower canopy leaves of Fagus crenata seedlings during the second growing season. The seedlings were grown in nine treatments comprised of a combination of three levels of gas treatments (CF: charcoal-filtered air, 1.0 × O₃: 1.0 time ambient O₃ concentration, 1.5 × O₃: 1.5 times ambient O₃ concentration) with three levels of soil nutrient treatments. The seedlings were supplied with 500 mL of water (NF: non-fertilized), 2000-fold diluted liquid fertilizer (N:P:K = 6:10:5) (LF: low-fertilized) or 1000-fold one (HF: high-fertilized) to potted soil once per two weeks. The leaves were emerged on 21 April 2015 and the $V_{\text{cmax},25}$ were measured once per month from May to September. Each value is the mean ± standard deviation of three chamber replicates. Results of three-way ANOVA are shown in Table 4.

![Fig. 2](image2.png)

**Fig. 2.** Maximum electron transport rate at leaf temperature of 25 °C ($J_{\text{max},25}$) of upper and lower canopy leaves of Fagus crenata seedlings during the second growing season. See legend to Fig. 1 for treatment and measurement description. Each value is the mean ± standard deviation of three chamber replicates. Results of three-way ANOVA are shown in Table 4.
Table 4
Results of three-way ANOVA for the effects of ozone, nutrient supply to soil and leaf position on maximum carboxylation rate at leaf temperature of 25°C ($V_{\text{max,25}}$, Fig. 1) and maximum electron transport rate at leaf temperature of 25°C ($J_{\text{max,25}}$, Fig. 2) of upper and lower canopy leaves of Fagus crenata seedlings during the second growing season.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max,25}}$</th>
<th>$J_{\text{max,25}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone ($O_3$)</td>
<td>**</td>
<td>n.s.</td>
</tr>
<tr>
<td>Nutrient (N)</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>Leaf position (1P)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>$O_3 \times N$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$N \times 1P$</td>
<td>**</td>
<td>n.s.</td>
</tr>
<tr>
<td>$O_3 \times N \times 1P$</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Statistical significance by three-way ANOVA: *; p<0.05, **; p<0.01, ***; p<0.001, n.s.; not significant.

Table 5
Results of three-way ANOVA for the effects of ozone, nutrient supply to soil and canopy leaf position on cumulative stomatal O$_3$ uptake (Fig. 3) of Fagus crenata seedlings during the second growing season.

<table>
<thead>
<tr>
<th></th>
<th>$\Sigma F_i$ (mmol m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper</td>
</tr>
<tr>
<td></td>
<td>NF</td>
</tr>
<tr>
<td>Ozone ($O_3$)</td>
<td>0.0</td>
</tr>
<tr>
<td>Nutrient (N)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leaf position (1P)</td>
<td>***</td>
</tr>
<tr>
<td>$O_3 \times N$</td>
<td>n.s.</td>
</tr>
<tr>
<td>$N \times 1P$</td>
<td>**</td>
</tr>
<tr>
<td>$O_3 \times N \times 1P$</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Statistical significance by three-way ANOVA: ***; p<0.001, n.s.; not significant.

Fig. 3. Cumulative stomatal O$_3$ uptake ($\Sigma F_i$) of upper and lower canopy leaves of Fagus crenata seedlings from leaf emergence day to 26 September in the second growing season. See legend to Fig. 1 for treatment description. Each value is the mean ± standard deviation of three chamber replicates. Statistical significance by three-way ANOVA was shown in Table 5.

Fig. 4. Relationships between $\alpha$, $\beta$ or $\gamma$ and coefficient of determination ($R^2$) of linear relationships between cumulative stomatal O$_3$ uptake over thresholds [$\Sigma (F_i - \alpha)$, $\Sigma (F_i - \beta \times \text{LMA})$ or $\Sigma (F_i - \gamma \times A_g)$] and relative maximum carboxylation rate ($V_{\text{max}}$) or maximum electron transport rate ($J_{\text{max}}$) shown in Fig. 5. Mean: average values of $R^2$ in the given relationships) were highest when $\alpha$ and $\beta$ were 0, indicating no improvement of mean $R^2$ values using $\alpha$ or $\beta \times \text{LMA}$ as $D_i$. Conversely, usage of $\gamma \times A_g$ as $D_i$ improved mean $R^2$ value and the highest value was found when $\gamma$ was 1.04.

Fig. 5 indicates the relationships between AOT40, $\Sigma F_{\text{40}}$ or $\Sigma (F_{\text{40}}-1.04 \times A_g)$ and relative $V_{\text{max}}$, $\text{LMA}$, $\phi$, $\gamma$ or $R_o$ of upper and lower canopy leaves. The $R^2$ in the linear relationships between AOT40, $\Sigma F_{\text{40}}$ or $\Sigma (F_{\text{40}}-1.04 \times A_g)$ and relative $V_{\text{max}}$, were 0.360, 0.501 and 0.650, respectively. Nutrient supply did not significantly affect the absolute values of the slope of linear relationships between $\Sigma F_{\text{40}}$ or
4. Discussion

We found that nutrient supply increased the degrees of O₃-induced reduction in $V_{\text{max,25}}$ and $J_{\text{max,25}}$ (Figs. 1 and 2; Table 4). Similarly, Maurer et al. (1997) showed a higher sensitivity to O₃ of photosynthetic capacity such as carboxylation efficiency in high-fertilized *B. pendula* seedlings than low-fertilized seedlings. On the other hand, there were no significant nutrient supply-induced increase in cumulative stomatal O₃ uptake ($\Sigma^{\text{st}}$) and reduction in $V_{\text{max}}$ per unit $\Sigma^{\text{st}}$ (Figs. 3 and 5; Table 5). Nutrient supply-induced tendencies of increase in cumulative stomatal O₃ uptake and reduction in detoxification capacity for O₃ might additively result in higher degrees of O₃-induced reduction in photosynthetic parameters of low- and high-fertilized *F. crenata* seedlings.

Relatively high sensitivity to O₃ of photosynthetic traits in upper canopy leaves as compared with lower canopy leaves was also reported in *F. crenata* saplings (Watanabe et al., 2014a). Hoshika et al. (2015) indicated that this was mainly due to higher $\Sigma^{\text{st}}$ of upper canopy leaves. In general, content of proteins relating to carboxylation, light absorption and electron transport of upper canopy leaves are higher as compared with those of lower canopy leaves in tree species (Lambers et al., 2008). Thus, $V_{\text{max,25}}$ and $J_{\text{max,25}}$ of upper canopy leaves were higher than those of lower canopy leaves (Figs. 1 and 2; Table 4). Furthermore, incident PPFD on upper canopy leaves was approximately three times higher than that on lower canopy leaves. As a result, $g_{s}$ of upper canopy leaves were higher as compared with those of lower canopy leaves, which resulted in relatively high $\Sigma^{\text{st}}$ in upper canopy leaves (Fig. 3 and Table 5). Therefore, higher sensitivity of photosynthetic parameters to O₃ in upper canopy leaves is likely to be mainly due to the higher cumulative stomatal O₃ uptake of *F. crenata* seedlings.

We found that reduction rate of $V_{\text{max}}$ per unit $\Sigma^{\text{st}}$ of lower canopy leaves was higher than that of upper canopy leaves (Fig. 5). Kitao et al. (2009) indicated that although $\Sigma^{\text{st}}$ of shade leaves was lower than that of sun leaves, the sensitivity of $A_{g}$ to O₃ in shade leaves was relatively higher in mature *F. sylvatica* (Nunn et al., 2005; Warren et al., 2007). These studies suggest that detoxification capacity for O₃ of lower canopy leaves is lower as compared with that of upper canopy leaves. In the present study, we observed that $R^{2}$ of the relationship between $\Sigma^{\text{st}}$ and $V_{\text{max}}$ was much higher than that between $\Sigma^{\text{st}}$ and $V_{\text{max}}$ (Fig. 5). Mean daytime $A_{g}$ (6:00–18:00) of lower canopy leaves averaged across the gas and nutrient treatments during the second growing season was 1.3 μmol m⁻² s⁻¹, which was much lower than that of upper canopy leaves (4.3 μmol m⁻² s⁻¹). Thus, the relatively low detoxification capacity for O₃ of lower canopy leaves may be associated with the lower $A_{g}$ than upper canopy leaves. Photosynthetic products are needed for biosynthesis and regeneration of Asc (Smirnoff, 1996; Smirnoff and Pallanca, 1996; Noctor and Foyer, 1998), which is recognized as the most important antioxidant to detoxify O₃ because it is most abundant antioxidant in plants (Smirnoff, 2000). Therefore, the lower detoxification capacity for O₃ of lower canopy leaves may be associated with the lower Aₙ than upper canopy leaves. Photosynthetic products are needed for biosynthesis and regeneration of Asc depending on the capacity of photosynthetic CO₂ assimilation.

In contrast to $V_{\text{max}}$, significant regression lines were not obtained in the relationships between $\Sigma^{\text{st}}$ and $\Sigma^{\text{st}}$ or $\Sigma^{\text{st}}$ and $V_{\text{max}}$ (Fig. 5). This is possibly due to lower degree of O₃-induced reduction in $\phi$ as compared with that in $V_{\text{max}}$ (Figs. 1 and 5 and Fig. S1). Similarly, previous studies reported O₃-induced reductions in light saturated $A_{g}$, carboxylation efficiency and Rubisco concentration, while the exposure to O₃ did not significantly affect initial slope of $A$-light curve of *F. crenata* seedlings and saplings (Yonekura et al., 2001; Watanabe et al., 2014a). Furthermore, Bagard et al. (2008) suggested that the exposure to O₃ reduced the
capacity of carboxylation catalyzed by Rubisco, and subsequently downregulated the capacity of electron transport to avoid photo-inhibition due to excess light energy in the chloroplasts caused by O₃-induced impairment of carboxylation. These results indicate that Rubisco in chloroplast stroma is more preferentially affected by O₃ than the other proteins relating to light absorption and electron transport in chloroplast thylakoids.

Contrary to our results (Fig. 4), previous studies indicated that R² of the relationships between Σ(F₇₅ - α) and growth or photosynthetic parameters were higher than those of the relationships between Σ(F₆₅) and growth or photosynthetic parameters (Bagard et al., 2015; Büker et al., 2015). In the previous studies, single effects of O₃ on trees were investigated, and relationships between Σ(F₇₅ - α) and growth or photosynthetic parameters were established. In the present study, we investigated combined effects of O₃ and soil nutrient conditions, and those of O₃ and canopy leaf position on photosynthetic parameters. We found that the detoxification capacity for O₃ was significantly different especially between upper and lower canopy leaves (Fig. 5). As shown in Fig. 4, the usage of a constant value as D₁(α) did not improve R². Therefore, an index relating to detoxification capacity for O₃ of upper and lower canopy leaves is appropriate for D₃ to accurately evaluate the effects of O₃ on photosynthetic parameters of F. crenata seedlings.

The difference in LMA as an index of D₃ did not explain that in the reduction rates of V₃max per unit ΣF₇₅ between upper and lower canopy leaves of F. crenata seedlings (Figs. 4 and 5). However, Matyssek et al. (2007) suggested that LMA is useful as an index of detoxification capacity for O₃ because it is related to the thickness of the parenchyma cell layer with antioxidant capacity. Leaf anatomy such as leaf cell number and thickness of leaf cell layer do not usually change after leaf expansion (Sims and Pearcy, 1992). Conversely, several studies have demonstrated diurnal and seasonal changes in detoxification capacity for O₃ (Heath et al., 2009; Fares et al., 2010). The LMA probably cannot explain the diurnal and seasonal changes of detoxification capacity for O₃. Therefore, β × LMA is not adequate as D₃ to evaluate the degree of O₃-induced reduction in photosynthetic parameters of upper and lower canopy leaves of F. crenata seedlings.

Massman (2004) proposed γ × A₃ as D₃, but its suitability for evaluating the effects of O₃ on photosynthetic parameters has not been clarified to date. This is probably because A₃ has not been estimated in the previous studies since F₄₅ was estimated using the gs model proposed by Jarvis (1976) (Emerson et al., 2000; Kinose et al., 2014). Büker et al. (2007) and Op de Beeck et al. (2010) indicated that estimation accuracy of gs and F₄₅ estimated using the Jarvis gs model and that estimated using the coupled model of photosynthesis and gs were almost the same. However, we propose the usage of the coupled model to estimate F₄₅ and A₃ because Σ(F₇₅ - γ × A₃) was identified as the most appropriate index to accurately quantify the effects of O₃ on photosynthetic parameters of upper and lower canopy leaves of F. crenata seedlings grown under different soil nutrient conditions (Figs. 4 and 5).

5. Conclusions

We found that the degrees of O₃-induced reduction in V₃max and J₃max of low- or high-fertilized seedlings are higher as compared with those of non-fertilized seedlings. On the other hand, we observed that the sensitivity to O₃ of photosynthetic parameters of upper canopy leaves is higher than that of lower canopy leaves, mainly as a result of the higher cumulative stomatal O₃ uptake of upper canopy leaves. However, the detoxification capacity for O₃ of lower canopy leaves is lower than that of upper canopy leaves. Difference in the capacity of photosynthetic CO₂ assimilation between upper and lower canopy leaves is considered to be related to the difference in the detoxification capacity for O₃ between these leaves. Therefore, linear relationships between Σ(F₇₅ - γ × A₃) and photosynthetic parameters such as V₃max and J₃max are more useful than the other relationships tested in the present study (e.g. relationships between ΣF₇₅ and photosynthetic parameters) to quantify the O₃-induced reduction in photosynthetic CO₂ fixation of F. crenata.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.envpol.2017.01.014.

References


