

Evaluation of O₃ effect on net photosynthetic rate in flag leaves of rice (*Oryza sativa* L.) by stomatal O₃ flux and radical scavenging enzyme activities

Masahiro YAMAGUCHI^{a, c}, Daiki HOSHINO^a, Taisuke KONDO^a, Ryohei SATOH^a, Hidetoshi INADA^a and Takeshi IZUTA^{b, †}

^a Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

^b Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

^c Current address: Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki, Nagasaki 852-8521, Japan

Abstract

To evaluate the effects of O₃ on the net photosynthetic rate in flag leaves of rice (*Oryza sativa* L.) based on stomatal O₃ flux and reactive oxygen species (ROS) scavenging enzyme activities, the Japanese rice cultivar Koshihikari was exposed to O₃ for 1 month after heading in O₃-exposure chambers. During the exposure period, rice plants were exposed to charcoal-filtered air or O₃ at 60 or 100 nl l⁻¹ (ppb) daily (10:00–17:00). Light-saturated net photosynthetic rate (A_{sat}) and ROS scavenging enzyme activities in the flag leaves were periodically measured during the exposure period. The O₃ exposure significantly reduced A_{sat} on the 20th and 31st days after the beginning of O₃ exposure (DAE). The degree of O₃-induced reduction in A_{sat} was explained by the cumulative flux of O₃ compared with concentration-based O₃ indices. The activities of ROS scavenging enzymes such as ascorbate peroxidase and catalase at 32 DAE were lower than at 1 DAE. During the latter half of the O₃ exposure period (21–31 DAE), the daily average stomatal O₃ flux was also lower than that during the first half of the period (1–20 DAE), which was mainly caused by lower photosynthetic photon flux density, lower air temperature and leaf senescence. These results suggest that the balance between stomatal O₃ flux and leaf cellular detoxification capacity in the flag leaves of rice, which determines the degree of O₃ damage, might have been similar between the first half and latter half of the O₃ exposure period.

Key words: Net photosynthetic rate, Ozone, Radical scavenging enzymes, Rice, Stomatal ozone flux.

1. Introduction

Ozone (O₃) in the troposphere has detrimental effects not only on human health but also on vegetation (Fuhrer, 2009; Emberson *et al.*, 2013). It has been demonstrated that exposure to O₃ reduces the growth and yield of crop plants (Fuhrer, 2009). The tropospheric O₃ concentration in Asia has been increasing and this trend is predicted to continue in the future (Ohara *et al.*, 2007; Yamaji *et al.*, 2008). Therefore, it is necessary to evaluate and predict current and near-future effects of O₃ on Asian crops.

The O₃ enters the leaf through the stomata and then damages cellular components (Nouchi, 2002). Because the real impacts of O₃ mainly depend on the amount of O₃ reaching the sites of damage within the leaf, the cumulative flux of O₃ through the stomata is a suitable index to evaluate the effects of O₃ on plants (Mills *et al.*, 2010). Therefore, O₃ flux-based critical levels and associated response functions have been reported for agricultural crops using O₃ index of Phytotoxic Ozone Dose (*i.e.*, the accumulated stomatal flux of O₃) above a flux threshold of Y nmol O₃ m⁻² PLA s⁻¹ (POD_Y) (*e.g.*, Mills *et al.*, 2010). However, it has been suggested that the degree of O₃ damage is determined by the balance between stomatal O₃ flux and leaf cellular detoxification (Musselman *et al.*, 2006; Matyssek *et al.*, 2008; Dizengremel *et*

al., 2009). Therefore, it is necessary to evaluate O₃ effects on plants based on not only cumulative flux of O₃ but also leaf cellular detoxification capacity.

In our previous report, the O₃-induced reduction in the yield of Japanese rice was evaluated based on cumulative stomatal O₃ flux (Yamaguchi *et al.*, 2014). However, leaf detoxification capacity was not considered in our evaluation. Because rice is a primary agricultural crop in Japan, it is necessary to evaluate the adverse effects of O₃ on rice yield more mechanistically. Therefore, the objective of the present study was to evaluate O₃ effects on net photosynthesis based on not only cumulative O₃ flux but also leaf cellular detoxification capacity in the flag leaf of rice. To achieve this objective, it was necessary to clarify the O₃-induced and senescence-related change of leaf detoxification capacity such as the activity of radical oxygen species (ROS)-scavenging enzymes. In the present study, therefore, a Japanese rice cultivar, Koshihikari, was exposed to O₃ for 1 month after heading in O₃-exposure chambers, and the light-saturated net photosynthetic rate (A_{sat}) and activities of ROS scavenging enzymes in the flag leaves were periodically measured during the exposure.

2. Materials and methods

2.1 Plant material

A Japanese rice cultivar, *Oryza sativa* L. 'Koshihikari', was used as the plant material. On 19 June 2008, Koshihikari seedlings were planted in pots (22.5 cm diameter and 27.0 cm depth) filled with flooded Andisol (7 l). Fertilizer (N-P-K = 14:14:14) was applied at 5.4 g per pot (*i.e.*, 1.9 kg N a⁻¹) before planting. There

Received; June 3, 2014.

Accepted; April 1, 2015.

[†] Corresponding Author: izuta@cc.tuat.ac.jp

DOI: 10.2480/agrm.14-00014

Table 1. Air temperature and relative air humidity inside the chambers and photosynthetic photon flux density (PPFD) outside the chambers.

| Period | Air temperature (°C) | | | Relative humidity (%) | | PPFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | |
|---------------|----------------------|-------------------------|-------------------------|-----------------------|-------------------------|-----------------------------------------------|-----------------------|
| | Daily mean | Daily max. ^a | Daily min. ^b | Daily mean | Daily min. ^b | Daily mean | 7 h mean ^c |
| 27 Aug–14 Sep | 25.4 (0.3) | 36.1 (0.3) | 19.9 (0.1) | 86.8 (1.5) | 55.4 (2.2) | 241 | 586 |
| 15 Sep–26 Sep | 22.0 (0.2) | 28.0 (0.5) | 18.5 (0.1) | 90.6 (1.5) | 70.8 (3.0) | 135 | 336 |
| 27 Aug–26 Sep | 24.1 (0.2) | 33.0 (0.3) | 19.4 (0.0) | 88.3 (1.5) | 61.3 (2.5) | 200 | 490 |

Each value is the mean value of air temperature or relative air humidity in the three chambers; the standard deviation is shown in parentheses except for PPFD.

^a Mean of daily 1 h maximum value.

^b Mean of daily 1 h minimum value.

^c 7 h: 10:00–17:00.

were 3 seedlings per pot, and 30 pots were used. The seedlings were grown to heading stage in greenhouse-type chambers located at the Field Museum Tamakuryo of Tokyo University of Agriculture and Technology (Hachioji, Tokyo) from 19 June to 26 August 2008. Chamber construction details are described in Akhtar *et al.* (2010a, b). Air temperature and relative air humidity in the chambers were continuously measured at 10-min intervals using a TR-72U Thermo Recorder (T&D Corporation, Nagano, Japan). The air temperatures and relative air humidities recorded inside the chambers during the O₃-exposure period are shown in Table 1.

2.2 Gas treatments

From 19 June to the heading date of 26 August, rice plants were exposed to charcoal-filtered air (CF) daily. On 27 August, the plants were assigned to three gas treatments: CF treatment, 60 ppb O₃ treatment and 100 ppb O₃ treatment. In the 60 and 100 ppb O₃ treatments, the plants were exposed to O₃ at 60 ppb and 100 ppb daily from 10:00 to 17:00. Three chamber replications were used in each treatment, for a total of nine chambers. Three pots were assigned to each chamber, for a total of nine plants. Details of the O₃-fumigation system are described in Akhtar *et al.* (2010a, b).

The concentrations of O₃ in each chamber and in the ambient air were independently and continuously monitored at intervals of 30 min and 10 min, respectively, using ultraviolet absorption O₃ analyzers (MODEL 1210; Dylec, Ibaraki, Japan). Average concentration, the sum of the hourly average concentrations (SUM0) and accumulated exposure over a threshold of 40 ppb (AOT40) of O₃ during the O₃-exposure period were calculated based on the O₃ concentration recorded in the chambers. The phytotoxic O₃ dose (POD₀) was calculated from following equation:

$$\text{POD}_0 = \sum ([\text{O}_3]_{\text{air}} \times g_{\text{sto}})$$

where $[\text{O}_3]_{\text{air}}$ is the hourly mean atmospheric concentration of O₃ and g_{sto} is the hourly mean stomatal diffusive conductance to O₃ estimated from the Jarvis-type stomatal conductance model (Jarvis, 1976; Emberson *et al.*, 2000), parameterized for Japanese rice by Yamaguchi *et al.* (2014). In the calculation of POD₀, the O₃ concentration within intracellular spaces was zero (Laisk *et al.*, 1989). In the present study, boundary layer resistance was considered to be negligible because of the narrow leaf widths and constant and relatively high wind speed in the chamber at canopy height (e.g. Ishihara, 1995; Ryan *et al.*, 2009). The average con-

Table 2. Concentration, SUM0, AOT40 and POD₀ of O₃ in each gas treatment during the O₃ exposure period.

| Period | Treatment | Average concentration (nl l ⁻¹) | | SUM0 ^a ($\mu\text{l l}^{-1} \text{h}$) | | AOT40 ^b ($\mu\text{l l}^{-1} \text{h}$) | | POD ₀ ^c (mmol m ⁻²) | | Daily POD ₀ ^c ($\mu\text{mol m}^{-2} \text{day}^{-1}$) | |
|-----------|------------------------|---------------------------------------------|------------------|-----------------------------------------------------|------------------|------------------------------------------------------|------------------|-------------------------------------------------------|------------------|--------------------------------------------------------------------------------|------------------|
| | | 24 h | 7 h ^d | 24 h | 7 h ^d | 24 h | 7 h ^d | 24 h | 7 h ^d | 24 h | 7 h ^d |
| 27 Aug | CF | 5.2 (0.5) | 9.0 (0.9) | 2.06 (0.20) | 1.13 (0.11) | 0.00 (0.00) | 0.00 (0.00) | 1.02 | 0.87 | 53.5 | 45.7 |
| –14 Sep | 60 ppb O ₃ | 18.5 (0.5) | 44.3 (1.6) | 7.66 (0.22) | 5.58 (0.20) | 1.86 (0.17) | 1.85 (0.17) | 3.62 | 3.30 | 190.5 | 173.8 |
| (19 days) | 100 ppb O ₃ | 23.8 (0.3) | 67.5 (1.7) | 9.87 (0.12) | 8.50 (0.22) | 4.93 (0.22) | 4.90 (0.22) | 3.80 | 3.59 | 200.0 | 188.7 |
| 15 Sep | CF | 5.3 (0.8) | 8.5 (1.4) | 1.52 (0.23) | 0.71 (0.12) | 0.00 (0.00) | 0.00 (0.00) | 0.32 | 0.27 | 26.3 | 22.8 |
| –26 Sep | 60 ppb O ₃ | 23.4 (0.7) | 64.8 (1.7) | 6.75 (0.21) | 5.44 (0.14) | 2.08 (0.14) | 2.08 (0.14) | 1.44 | 1.37 | 119.9 | 114.4 |
| (12 days) | 100 ppb O ₃ | 33.3 (0.2) | 101.2 (1.7) | 9.58 (0.05) | 8.50 (0.15) | 5.14 (0.14) | 5.14 (0.15) | 1.56 | 1.50 | 129.8 | 125.1 |
| 27 Aug | CF | 5.2 (0.6) | 8.8 (1.1) | 3.58 (0.42) | 1.84 (0.23) | 0.00 (0.00) | 0.00 (0.00) | 1.33 | 1.14 | 43.0 | 36.8 |
| –26 Sep | 60 ppb O ₃ | 20.6 (0.2) | 52.5 (0.6) | 14.41 (0.15) | 11.02 (0.13) | 3.94 (0.10) | 3.94 (0.10) | 5.06 | 4.68 | 163.2 | 150.8 |
| (31 days) | 100 ppb O ₃ | 27.9 (0.2) | 81.0 (1.7) | 19.45 (0.14) | 17.00 (0.36) | 10.07 (0.36) | 10.04 (0.36) | 5.36 | 5.09 | 172.8 | 164.1 |

Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses, except for POD₀.

CF: Charcoal-filtered air; 60 ppb O₃: O₃ at 60 ppb (10:00–17:00); 100 ppb O₃: O₃ at 100 ppb (10:00–17:00).

^a SUM0: Sum of all hourly average concentrations.

^b AOT40: Accumulated exposure over a threshold of 40 nl l⁻¹.

^c POD₀: Phytotoxic O₃ dose without flux threshold.

^d 7 h: 10:00–17:00.

centration, SUM0, AOT40 and POD₀ of O₃ are shown in Table 2.

2.3 Measurement of leaf gas exchange rates

Light-saturated net photosynthetic rate (A_{sat}) and stomatal diffusive conductance to H₂O (G_s) of the flag leaves were measured on 14–15 September and 25–26 September 2008 using an infrared gas analyzer system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Three plants per treatment–chamber combination were randomly selected for A_{sat} and G_s measurements (nine measurements per treatment). During the measurements, the atmospheric CO₂ concentration, air temperature, relative air humidity and photosynthetic photon flux density (PPFD) in the leaf chamber were maintained at 380 $\mu\text{mol mol}^{-1}$, $25 \pm 0.1^\circ\text{C}$, $70 \pm 5^\circ\text{C}$ and 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. To avoid the effects of environmental conditions on the gas exchange rate at a measurement time (*e.g.*, mid-day depression), we carried out the measurement in an experimental room. Before sunrise on the day of gas exchange rate measurement, the pots were moved from the chambers into an experimental room and placed under dark conditions until the measurement was performed.

2.4 Concentrations of Rubisco and chlorophyll

On 27 September 2008, flag leaves of rice were harvested from 5:00 to 8:00 to determine the concentrations of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll. The harvested leaves were washed with deionized water and the area of the leaves (100 mg fresh weight [FW]) was measured with an area meter. Immediately after the measurement of leaf area (LA), the leaf samples were frozen in liquid nitrogen and stored at -80°C . Two plants per treatment–chamber combination were randomly selected for the analyses (six determinations per treatment).

Stored leaf samples were homogenized to a fine powder in liquid nitrogen using a mortar and pestle with 20 mg of PVPP and a small amount of quartz sand. Subsequently, Rubisco and chlorophyll were extracted by grinding the fine powder in 1.0 ml of extraction buffer containing 100 mM HEPES-KOH (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol and 1mM PMSF. The crude homogenate was used for determination of chlorophyll concentration according to Barnes *et al.* (1992). To determine the Rubisco concentration, the crude homogenate was centrifuged at $16,000 \times g$ for 10 min at 4°C . The supernatant of the sample was used for determination of Rubisco concentration. Details of the measurement of Rubisco and chlorophyll concentrations are described in our previous report (Inada *et al.*, 2008).

2.5 Activities of ROS scavenging enzymes

On 27 August and 27 September 2008, flag leaves of rice were harvested after O₃ exposure (17:00–20:00) for ROS scavenging enzymes activity measurement. For each sampling date, two plants per treatment–chamber combination were randomly selected for the analysis (six determinations per treatment). The harvested leaves were washed with deionized water and their FW and LA were measured using a balance and area meter, respectively. Immediately after the measurement of FW and LA, the leaf samples were frozen in liquid nitrogen and stored at -80°C until the enzyme activity measurement, excluding the sample for measurement of ascorbate peroxidase (APX) activity.

For APX activity measurement, immediately after harvesting the flag leaves, the leaf samples (50 mg FW) were homogenized

to a fine powder in liquid nitrogen using a mortar and pestle with 10 mg of PVPP and a small amount of quartz sand. Subsequently, APX was extracted by grinding the fine powder in 0.5 ml of extraction buffer containing 50 mM potassium phosphate (pH 7.0), 1 mM Na-EDTA and 5 mM L-ascorbic acid. The crude homogenate was centrifuged at $16,000 \times g$ at 4°C for 10 min to obtain the supernatant for APX activity measurement. The APX activity was measured according to the method of Nakano and Asada (1981), and was defined as the oxidation rate of ascorbic acid by H₂O₂. Details of the measurement of APX activity are described in our previous report (Inada *et al.*, 2008).

To measure the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), stored leaf samples (100 mg FW) were homogenized to a fine powder in liquid nitrogen using a mortar and pestle with 20 mg of PVPP and a small amount of quartz sand. Subsequently, SOD, CAT and GR were extracted by grinding the fine powder in 1.0 ml of extraction buffer containing 50 mM potassium phosphate (pH 7.8) and 1 mM Na-EDTA. The crude homogenate was centrifuged at $16,000 \times g$ at 4°C for 10 min. The supernatant of the sample was used to determine the activities of SOD, CAT and GR. The SOD activity was measured according to Asada *et al.* (1973), and 1 unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the O₂⁻-induced reduction rate of oxidized cytochrome c by 50%. The CAT activity was measured according Aebi (1983), and was defined as the elimination rate of H₂O₂. The GR activity was measured according to Foyer and Halliwell (1976), and was defined as the consumption rate of NADPH for the reduction of oxidized glutathione. Details of the measurement of SOD, CAT and GR activities are described in our previous report (Inada *et al.*, 2008).

For the measurement of monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) activities, stored leaf samples (50 mg FW) were homogenized to a fine powder in liquid nitrogen using a mortar and pestle with 10 mg of PVPP and a small amount of quartz sand. Subsequently, MDAR and DHAR were extracted by grinding the fine powder in 0.5 ml of extraction buffer containing 50 mM Tris-HCl (pH 7.0), 1 mM L-ascorbic acid, 1 mM dithiothreitol, 1 mM Na-EDTA, 1 mM reduced glutathione and 5 mM MgCl₂. The crude homogenate was centrifuged at $12,000 \times g$ for 6 min at 4°C , and then centrifuged at $20,000 \times g$ for 16 min at 4°C . The supernatant of the sample was used to determine the activities of MDAR and DHAR. The MDAR activity was measured according to Hossain *et al.* (1984), and was defined as the consumption rate of NADPH for the reduction of monodehydroascorbate. The DHAR activity was measured according to Hossain and Asada (1984), and was defined as the reduction rate of dehydroascorbate. Details of the measurement of MDAR and DHAR activities are described in our previous report (Inada *et al.*, 2008).

2.6 Statistical analysis

All statistical analyses were performed using the IBM® SPSS® Advanced Statistics 19 software system. Analysis of variance (ANOVA) was used to test the effects of O₃ and the date of measurement. When there was a significant interaction between O₃ and the date of measurement for activities of ROS scavenging enzymes, one-way ANOVA was used to determine the effects of

Table 3. Effects of O₃ on stomatal conductance to water vapor (G_s) on 26 September and concentrations of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), chlorophyll a and chlorophyll b on 27 September 2008.

| Treatment | G_s (mol m ⁻² s ⁻¹) | Rubisco conc. (g m ⁻²) | Chlorophyll a conc. (mg m ⁻²) | Chlorophyll b conc. (mg m ⁻²) |
|------------------------|----------------------------------------------|------------------------------------|-------------------------------------------|-------------------------------------------|
| CF | 0.369 (0.028) | 1.15 (0.03) | 135.4 (10.6) | 51.9 (7.8) |
| 60 ppb O ₃ | 0.234 (0.061) | 0.88 (0.06) | 115.4 (14.0) | 36.4 (7.3) |
| 100 ppb O ₃ | 0.244 (0.017) | 0.81 (0.10) | 109.5 (4.8) | 32.4 (4.1) |
| One-way ANOVA | *** | *** | *** | *** |

Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses.

CF: Charcoal-filtered air; 60 ppb O₃: O₃ at 60 ppb (10:00–17:00); 100 ppb O₃: O₃ at 100 ppb (10:00–17:00).

Significance of one-way ANOVA: *** $p < 0.001$

O₃ on each date.

3. Results and Discussion

3.1 Effects of O₃ on net photosynthetic rate

The effects of O₃ on light-saturated net photosynthetic rate (A_{sat}) in the flag leaves of rice on 15 and 26 September 2008 (*i.e.*, the 20th and 31st days after the beginning of O₃ exposure (DAE), respectively) are shown in Fig. 1. On both dates, A_{sat} was significantly reduced by the exposure to O₃. Table 3 shows the effects of O₃ on stomatal conductance to water vapor (G_s) on 26 September and the concentrations of Rubisco and chlorophyll on 27 September. Exposure to O₃ significantly reduced the concentrations of Rubisco and chlorophyll a and b. The G_s was significantly reduced by exposure to O₃. These results indicate that exposure to O₃ reduced the carbon fixation capacity, light energy absorption capacity and CO₂ diffusivity, resulting in a significant reduction in net photosynthesis in the flag leaves of rice.

Because there was no significant interaction between O₃ and the date of measurement for A_{sat} (Fig. 1), we conducted regression analysis of the relationships between the relative value of A_{sat} and O₃ indices during the O₃-exposure period using six plots (Fig. 2).

There was no significant correlation between the relative value of A_{sat} and AOT40 (Fig. 2). However, there were significant negative correlations between the relative value of A_{sat} and average O₃ concentration, SUM0 and POD₀ (Fig. 2). The R^2 value obtained from linear regression analysis of the relationship between relative A_{sat} and POD₀ was higher than those from the relationships between relative A_{sat} and average O₃ concentration and SUM0. This result indicates that, compared with concentration-based O₃ indices, the cumulative flux of O₃ is more suitable for explaining the degree of O₃-induced reduction in the net photosynthetic rate of rice.

3.2 Changes in ROS scavenging enzyme activity

It has been suggested that the degree of O₃ damage is determined by the balance between stomatal flux and leaf cellular detoxification (Musselman *et al.*, 2006; Matyssek *et al.*, 2008; Dizengremel *et al.*, 2009). To clarify the O₃-induced and senescence-related changes of the detoxification capacity in the flag leaf of rice, we measured the activities of enzymes participating in the ROS scavenging system after O₃ exposure at 1 and 32 DAE (Table 4).

At 1 DAE, the O₃ exposure significantly increased GR activity.

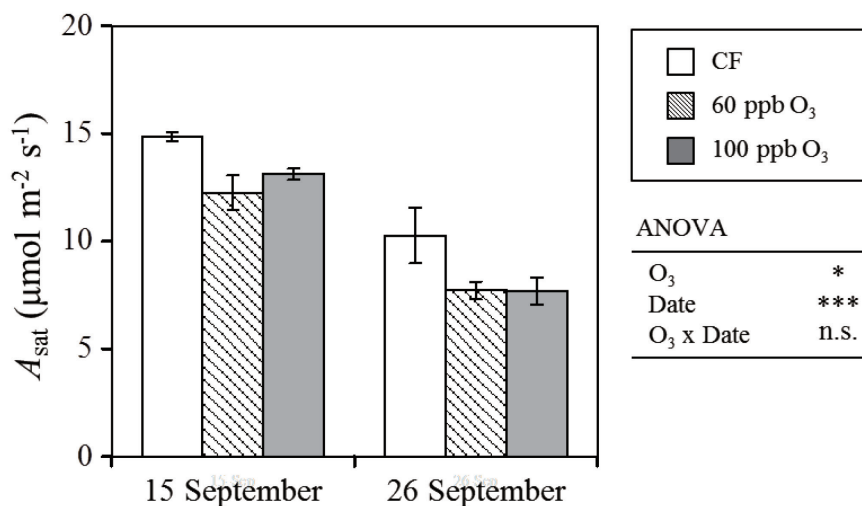


Fig. 1. Effects of O₃ on the light-saturated net photosynthetic rate (A_{sat}) in the flag leaf of rice (*Oryza sativa* L.) on 15 and 26 September 2008. Each value represents the mean of three chamber replications; vertical bars show the standard deviation (SD). Two-way ANOVA: * $p < 0.05$, *** $p < 0.001$, n.s. = not significant. The A_{sat} before the O₃ exposure period on 26 August 2008 was $21.0 \pm 1.17 \mu\text{mol m}^{-2} \text{s}^{-1}$ ($n = 6$).

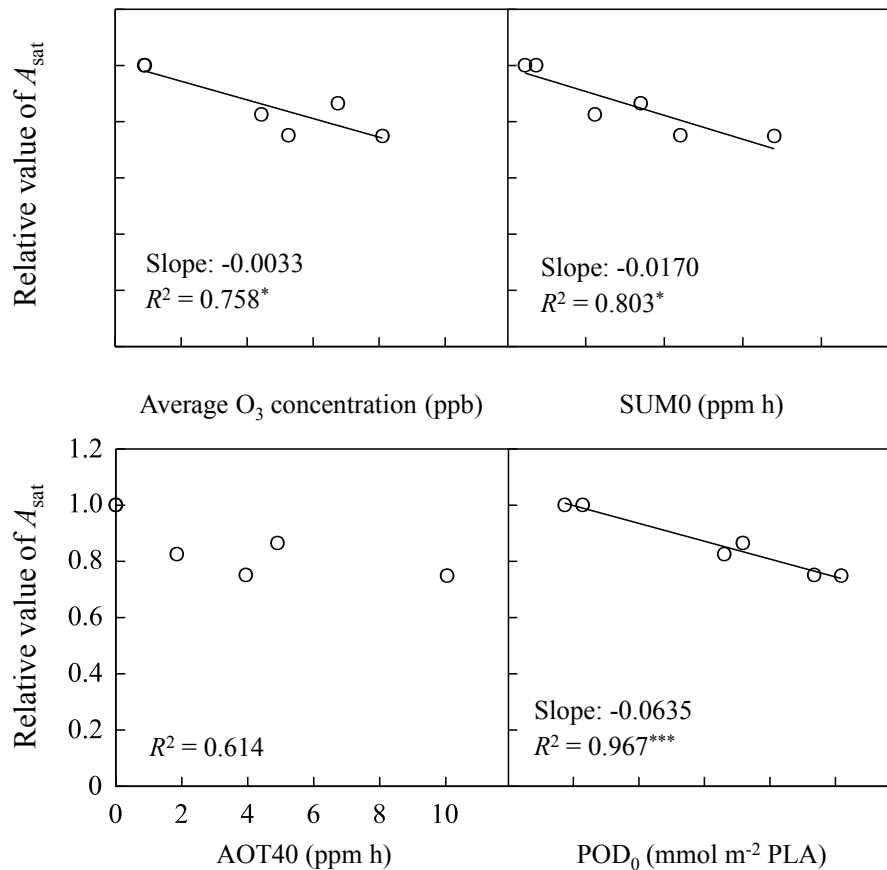


Fig. 2. The relationships between the relative value of light-saturated net photosynthetic rate (A_{sat}) and average O₃ concentration, sum of all hourly average concentrations (SUM0), accumulated exposure over a threshold of 40 nl l⁻¹ (AOT40) and phytotoxic O₃ dose without flux threshold (POD₀) during O₃ exposure time (10:00–17:00). For each A_{sat} measurement date, A_{sat} in the CF treatment was used as a reference (1.0) to calculate the relative value of A_{sat} for each O₃ treatment. The coefficient of determination (R^2) obtained from linear regression analysis and the slope of the regression line are indicated in the figures. Statistical significance for R^2 is shown: * $p < 0.05$, *** $p < 0.001$.

No significant effects of O₃ were observed on activities of APX, CAT and MDAR. There were significant interactions between O₃ and the date of measurement for the activities of SOD and DHAR. At 1 DAE, O₃ exposure significantly decreased SOD activity and significantly increased DHAR activity. Using reduced glutathione (GSH), DHAR catalyzes the regeneration of reduced ascorbate (AsA) from dehydroascorbate (DHA) originated from the elimination of H₂O₂ by the action of APX (Asada, 2006). The GR catalyzes the regeneration of GSH from oxidized glutathione (GSSG) produced by the reduction reaction of DHA (Asada, 2006). Therefore, the higher activities of DHAR and GR induced by 1 day of O₃ exposure suggest a higher demand for AsA to eliminate H₂O₂ in rice leaves.

On 27 September, the activities of APX and CAT were significantly lower, and the activities of MDAR and GR were significantly higher than those on 27 August (Table 4). Because there were significant interactions between O₃ and the date of measurement for DHAR and SOD activities, a Student's *t*-test was performed to identify significant differences between the two time points in the CF treatment. In the CF treatment, although there was no significant difference in DHAR activity between the two days, lower activities of SOD was observed on 27 September

compared with those on 27 August ($p < 0.05$) (Table 4). The SOD and CAT catalyze disproportionation reactions of O₂⁻ and H₂O₂, respectively (Aebi, 1984; Asada, 2006). Hydrogen peroxide is reduced to water by the oxidation of AsA catalyzed by APX (Asada, 2006). The MDAR catalyzes the reduction reaction of monodehydroascorbate (MDA) produced from the elimination of H₂O₂ by the action of APX (Asada, 2006). These results suggest that the senescence-related changes of detoxification capacity in rice flag leaves were a reduction in ROS elimination capacity and an increase in antioxidant regeneration capacity, although it is difficult to identify senescence-induced changes because there was no measurement time point before the O₃ exposure period.

At 32 DAE, O₃ exposure significantly increased the activities of SOD and GR (Table 4). Despite the senescence-related reductions in APX and CAT activities, SOD activity was enhanced by long-term O₃ exposure, which might cause susceptibility to H₂O₂ accumulation. This result suggests that the detoxification capacity in the flag leaves of rice declines during leaf senescence and/or long-term O₃ exposure.

3.3 Balance between stomatal O₃ flux and the activity of ROS scavenging enzymes

The degree of O₃-induced reduction in the net photosynthetic

Table 4. Effects of O₃ on the activities of SOD, APX, CAT, MDAR, DHAR and GR in the flag leaf of rice (*Oryza sativa* L.).

| Date | Treatment | SOD (U cm ⁻²) | APX (μkat m ⁻²) | CAT (mkat m ⁻²) | MDAR (μkat m ⁻²) | DHAR (μkat m ⁻²) | GR (μkat m ⁻²) |
|-----------------------|------------------------|------------------------------|--------------------------------|--------------------------------|---------------------------------|---------------------------------|-------------------------------|
| 27 Aug | CF | 5.93 (0.35) | 14.8 (1.0) | 5.65 (0.29) | 2.01 (0.13) | 10.7 (0.3) | 1.58 (0.06) |
| | 60 ppb O ₃ | 4.93 (0.19) | 14.7 (1.1) | 6.08 (0.45) | 1.95 (0.15) | 10.4 (1.3) | 1.77 (0.23) |
| | 100 ppb O ₃ | 4.42 (0.33) ** | 15.8 (1.1) | 5.24 (0.25) | 2.06 (0.26) | 13.8 (0.8) ** | 1.85 (0.10) |
| 27 Sep | CF | 4.24 (0.22) | 11.6 (0.3) | 4.72 (0.43) | 2.94 (0.38) | 11.7 (0.7) | 2.33 (0.12) |
| | 60 ppb O ₃ | 4.79 (0.49) | 10.0 (0.8) | 4.22 (0.86) | 2.93 (0.17) | 10.3 (2.2) | 2.38 (0.04) |
| | 100 ppb O ₃ | 5.27 (0.32) * | 9.8 (0.5) | 3.76 (0.60) | 3.18 (0.51) | 8.9 (1.9) n.s. | 2.54 (0.19) |
| Two-way ANOVA | | | | | | | |
| O ₃ | | n.s. | n.s. | n.s. | n.s. | n.s. | * |
| Date | | n.s. | *** | ** | *** | n.s. | *** |
| O ₃ × Date | | *** | n.s. | n.s. | n.s. | * | n.s. |

Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses.

CF: Charcoal-filtered air; 60 ppb O₃: O₃ at 60 ppb (10:00–17:00); 100 ppb O₃: O₃ at 100 ppb (10:00–17:00).

SOD: Superoxide dismutase; APX: Ascorbate peroxidase; CAT: Catalase; MDAR: Monodehydroascorbate reductase; DHAR: Dehydroascorbate reductase; GR: Glutathione reductase.

When there was a significant interaction between O₃ and date, one-way ANOVA was used to determine the effects of O₃ on each date. The levels of significance of the one-way ANOVA were shown in each row. Significance of ANOVA: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, n.s. = not significant.

rate per unit POD₀ was the same between 20 and 31 DAE (Fig. 2). Although the degree of O₃ damage is determined by the balance between stomatal O₃ flux and leaf cellular detoxification (Musselman *et al.*, 2006; Matyssek *et al.*, 2008; Dizengremel *et al.*, 2009), the leaf cellular detoxification capacity, such as the activities of APX and CAT, in the flag leaves of rice declines during leaf senescence. This discrepancy might be explained by the lower daily POD₀ (*i.e.*, daily average stomatal O₃ flux) from 15 to 26 September than that from 27 August to 14 September (Table 2), which was mainly caused by lower PPFD, lower air temperature and leaf senescence during latter time period (data not shown). Therefore, the balance between stomatal O₃ flux and leaf cellular detoxification capacity in the flag leaves of rice, which determines the degree of O₃ damage, might have been similar between the first half (1–20 DAE) and the latter half (21–31 DAE) of the O₃ exposure period.

Environmental conditions such as PPFD were quite different between the two time periods (Table 1). Both stomatal O₃ flux and leaf detoxification capacity can vary in response to changes in environmental conditions (Musselman *et al.*, 2006). Although a stomatal conductance model for estimating stomatal O₃ flux has been established for some important agricultural crops (*e.g.*, Mills, 2010), a model for evaluating the leaf detoxification capacity has not been established. Therefore, further study is required to quantify the leaf cellular detoxification capacity for the final purpose of evaluating the adverse effects of O₃ based on biologically relevant plant response relationships (Musselman *et al.*, 2006).

Acknowledgements

The authors acknowledge Prof. Kazuhiko Kobayashi, Dr. Isamu Nouchi and Dr. Haruto Sasaki (The University of Tokyo) for their valuable suggestions and technical support. The authors are greatly indebted to Prof. Hiroshi Hara (Tokyo University of Agriculture and Technology) for his comprehensive guidance. The

authors also acknowledge Mr. Masayoshi Uchida (Tokyo University of Agriculture and Technology) for his technical support. This study was financially supported by the program of the Global Environment Research Fund (C-062) from the Ministry of the Environment, Japan.

References

- Aebi, H., 1984: Catalase in vitro. *Methods in Enzymology*, **105**, 121–126.
- Akhtar, N., Yamaguchi, M., Inada, H., Hoshino, D., Kondo, T. and Izuta, T., 2010a: Effects of ozone on growth, yield and leaf gas exchange rates of two Bangladeshi cultivars of wheat (*Triticum aestivum* L.). *Environmental Pollution*, **158**, 1763–1767.
- Akhtar, N., Yamaguchi, M., Inada, H., Hoshino, D., Kondo, T., Fukami, M., Funada, R. and Izuta, T., 2010b: Effects of ozone on growth, yield and leaf gas exchange rates of four Bangladeshi cultivars of rice (*Oryza sativa* L.). *Environmental Pollution*, **158**, 2970–2976.
- Asada, K., 2006: Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology*, **141**, 391–396.
- Asada, K., Urano, M. and Takahashi, M., 1973: Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. *European Journal of Biochemistry*, **36**, 257–266.
- Barnes, J. D., Balaguer, L., Manrique, E., Elvira, S. and Davison, A. W., 1992: A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. *Environmental and Experimental Botany*, **32**, 85–100.
- Dizengremel, P., Thiec, D. L., Hasenfratz-Sauder, M.-P., Vaultier, M.-N., Bagard, M. and Jolivet, Y., 2009: Metabolic-dependent changes in plant cell redox power after ozone exposure. *Plant Biology*, **11**, 35–42.
- Emberson, L. D., Wieser, G., Ashmore, M. R., 2000: Modelling of stomatal conductance and ozone flux of Norway spruce:

- comparison with field data. *Environmental Pollution*, **109**, 393–402.
- Emberson, L. D., Kitwiroon, N., Beevers, S., B ker, P. and Cindery, S., 2013: Scorched Earth: how will changes in the strength of the vegetation sink to ozone deposition affect human health and ecosystems? *Atmospheric Chemistry and Physics*, **13**, 6741–6755.
- Foyer, C. H. and Halliwell, B., 1976: The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta*, **133**, 21–25.
- Fuhrer, J., 2009: Ozone risk for crops and pastures in present and future climates. *Naturwissenschaften*, **96**, 173–194.
- Hossain, M. A. and Asada, K., 1984: Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. *Plant Cell and Physiology*, **25**, 85–92.
- Hossain, M. A., Nakano, Y. and Asada, K., 1984: Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant and Cell Physiology*, **25**, 385–395.
- Inada, H., Yamaguchi, M., Satoh, R., Hoshino, D., Nagasawa, A., Negishi, Y., Nouchi, I., Kobayashi, K. and Izuta, T., 2008: Effects of ozone on photosynthetic components and radical scavenging system in leaves of rice (*Oryza sativa* L.). *Journal of Agricultural Meteorology*, **64**, 243–255.
- Ishihara, K., 1995: Photosynthesis and respiration in a single leaf. 2. Leaf structure and photosynthesis. In *Science of the rice plant. vol. 2. Physiology* (ed. by Matsuo, T., Kumazawa, K., Ishii, R., Ishihara, K., Hirata, H.), Food and Agriculture Policy Research Center, Tokyo, Japan, pp.491–507.
- Jarvis, P. G., 1976: The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. *Philosophical Transactions of the Royal Society of London Series B—Biological Sciences*, **273**, 593–610.
- Laisk, A., Kull, O. and Moldau, H., 1989: Ozone concentration in leaf intercellular air spaces is close to zero. *Plant Physiology*, **90**, 1163–1167.
- McCord, J. M. and Fridovich, I., 1969: Superoxide dismutase. *The Journal of Biological Chemistry*, **244**, 6049–6055.
- Matyssek, R., Sandermann, H., Wieser, G., Booker, F., Cieslik, S., Musselman, R. and Ernst, D., 2008: The challenge of making ozone risk assessment for forest trees more mechanistic. *Environmental Pollution*, **156**, 567–582.
- Mills, G., Pleijel, H., B ker, P., Braun, S., Emberson, L., Harmens, H., Hayes, F., Simpson, D., Gr nhage, L., Karlsson, P.-E., Danielsson, H., Bermejo, V. and Fernandez, I. G., 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 (ed. by Spranger, T.), UNECE Convention on Long-range Transboundary Air Pollution.
- Musselman, R. C., Lefohn, A. S., Massman, W. J. and Heath, R. L., 2006: A critical review and analysis of the use of exposure- and flux-based ozone indices for predicting vegetation effects. *Atmospheric Environment*, **40**, 1869–1888.
- Nakano, Y. and Asada, K., 1981: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, **22**, 867–880.
- Nouchi, I., 2002: Responses of whole plants to air pollutants. In *Air pollution and plant biotechnology – Prospects for phytomonitoring and phytoremediation* (ed. by Omasa, K., Saji, H., Youssefian, S., Kondo, N.), Springer-Verlag Tokyo, Tokyo, Japan, pp.3–39.
- Ohara, T., Akimoto, H., Kurokawa, J., Horii, N., Yamaji, K., Yan, X. and Hayasaka, T., 2007: An Asian emission inventory of anthropogenic emission sources for the period 1980–2020. *Atmospheric Chemistry and Physics*, **7**, 4419–4444.
- Ryan, A., Cojocariu, C., Possell, M., Davies, W. J. and Hewitt, C. N., 2009: Defining hybrid poplar (*Populus deltoids* × *Populus trichocarpa*) tolerance to ozone: identifying key parameters. *Plant, Cell and Environment*, **32**, 31–45.
- Yamaguchi, M., Hoshino, D., Inada, H., Akhtar, N., Sumioka, C., Takeda, K. and Izuta, T., 2014: Evaluation of the effects of ozone on yield of Japanese rice (*Oryza sativa* L.) based on stomatal ozone uptake. *Environmental Pollution*, **184**, 472–480.