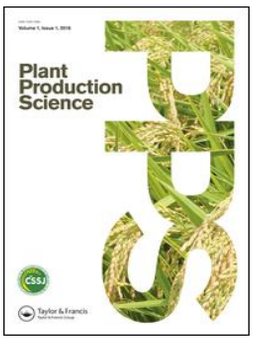

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SHORT REPORT

OPEN ACCESS

Exogenous ascorbic acid scarcely ameliorates inhibition of photosynthesis in rice leaves by O_3

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ABSTRACT

The role of ascorbic acid on acute O_3-induced inhibition of photosynthesis in solution-cultured paddy rice was evaluated. As pre-treatment, ascorbic acid (0, 5, and 10 mM) was added to the culture solution for 5 d before 5 h of O_3 exposure (0, .1, and .3 cm^3 m^{-3} O_3) during daytime. O_3 decreased photosynthesis-related parameters, total ascorbic acid content, and the redox state (RDS) of ascorbic acid. Ascorbic acid treatment enhanced the total ascorbic acid contents and its RDS level of rice leaves, but scarcely ameliorated O_3-induced inhibition of photosynthesis-related parameters. Inhibition of net photosynthetic rate (P_N) by O_3 was slightly ameliorated by exogenous ascorbic acid only at 1 d after O_3 exposure. These results indicate that ascorbic acid is a component of protection from O_3 injury but has a marginal role in the acute inhibition of P_N by O_3 in rice leaves.

Abbreviations: AA, reduced form of ascorbic acid; CO_2, carbon dioxide; DHA, dehydroascorbic acid; F_q'/F_m', operating quantum efficiency; F_v/F_m, maximum quantum efficiency; g_s, stomatal conductance; O_3, ozone; P_N, net photosynthetic rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; RDS, redox state; VPD, vapor pressure deficit

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CLASSIFICATION
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Introduction

In the Kanto region of Japan, where rice is cultivated as a staple summer crop, 10–20 warnings regarding high photochemical oxidant levels are received every growing season, and the hourly peak values of these substances are sometimes close to .2 cm^3 m^{-3} (Environmental Improvement Division, Tokyo Metropolitan Government Bureau of Environment, 2005). Of the photochemical oxidant components, 90% or more are O_3 (Cabrera et al., 1988). When exposed to O_3, paddy rice plants suffer damage: such as inhibition of the net photosynthetic rate (P_N), stomatal conductance (g_s), and PSII (Imai & Kobori, 2008; Kobayakawa & Imai, 2011a) as well as decreased nitrite reductase activity (Kobayakawa & Imai, 2011b), ribulose 1,5-bisphosphate carboxylase/oxygenase, and chlorophyll contents (Ishioh & Imai, 2005) in addition to visible leaf-related symptoms (Imai & Kobori, 2008). Moreover, O_3 decreases growth and grain yield of paddy rice (Imai & Ookoshi, 2011).

Although the production of reactive oxygen species (ROS) such as superoxide, singlet oxygen, and hydrogen peroxide is low under normal conditions, many abiotic stresses including drought stress, salt stress, heat shock, high light stress, heavy metals, ultraviolet radiation, and air pollutants such as O_3 and SO_2 enhance ROS production. ROS induces oxidative damage and cell death. However, higher plants have antioxidant metabolisms to scavenge excess ROS (Van Breusegem & Dat, 2006). Therefore, antioxidant metabolism is an important determination factor for scavenging O_3 injury of plants (Wilkinson et al., 2011). Ascorbic acid is an important non-enzymatic antioxidant in plant tissue that is synthesized by way of sugar metabolism (Lisko et al., 2013). In rice leaves, total ascorbic acid content is increased by chronic O_3 exposure (Nouchi, 1993) and decreased by acute O_3 exposure (Kobayakawa & Imai, 2011a). However, the details of the role and action mechanism of ascorbic acid in acute O_3-exposed rice leaves remain unclear. Therefore, we examined the effect of exogenous ascorbic acid on acute O_3 inhibition of photosynthesis to clarify the relationship between O_3 inhibition of photosynthesis and antioxidant levels of paddy rice leaves.

Materials and methods

Plant materials, gas exposures, and application of ascorbic acid

In April 2015, seeds of japonica rice (*Oryza sativa* L. cv. Koshihikari) were sown in 1/5000 a Wagner pots filled with

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Kimura B solution (Baba & Takahashi, 1956) with minor elements at pH 5.5. The nutrient solutions were changed every four days. Plants were grown in natural-light growth chambers (width × depth × height = 2 m × 2 m × 1.9 m: S-2003A; Koito Industries, Ltd, Yokohama, Japan) at 28/23 °C (12-h day/12-h night), 60% RH, and 400 $cm^3\ m^{-3}$ CO_2 (accuracy: within ± 1%) without O_3. The differences in environmental parameters (temperature, humidity, and light intensity) among growth chambers were within 5% at any period of time. Immediately after full expansion of the eighth leaves (ca. one month from sowing), plant roots were filled with Kimura B solution including 0 (A^0), 5 (A^5), and 10 (A^{10}) mM L(+)-ascorbic acid (Kanto Chemical Co. Inc., Tokyo, Japan). These solutions were renewed daily during five days before O_3 exposure. The concentration of ascorbic acid and period of pre-treatment were determined by reference to our preliminary experiment (data not shown) and previous report (Inoue et al., 1996). Because ascorbic acid treatment lowered the pH of nutrient solution (3.31–3.70), low pH-treated plants (L plants) were included to check the effect of low pH on photosynthesis-related parameters. Five days after ascorbic acid treatment, rice plants were exposed to 0 (<.002), .1, and .3 $cm^3\ m^{-3}$ O_3 (expressed, respectively, as O^0, $O^{0.1}$, and $O^{0.3}$, accuracy: within ± 2%) during five hours of local daytime (08:00–13:00) using three chambers under growth CO_2 concentrations. O_3 was supplied using a high-voltage O_3 generator with dry air (ED-OG-R6; Ecodesign Inc., Ogawa, Saitama, Japan), and CO_2 was supplied from cylinders containing liquid CO_2. These gases were injected into air that had been filtered through activated charcoal layers. The O_3 and CO_2 concentrations were measured and computer-controlled, respectively, using an ultraviolet absorption-type O_3 analyzer (EG-2001F; Ebara Jitsugyo Co. Ltd, Tokyo, Japan) and infrared CO_2 analyzer (ZRH; Fuji Electric Systems Co. Ltd, Tokyo, Japan).

Gas exchange measurements

In situ gas-exchange measurements (13:00–14:30, local time) of the attached, eighth leaves at the middle portion were conducted 1 d before (BE), immediately after (AE-0: .1–1.1 h after), and 1 and 3 d after (AE-1, AE-3) exposure to O_3 at 400 $cm^3\ m^{-3}$ CO_2 for four replicate plants in each treatment using a portable photosynthesis and transpiration measurements system (Li-6400XT; Li-Cor Inc., Lincoln, NE, USA). Environmental conditions within the Li-COR cuvette during measurements were set at 28 °C leaf temperature, 1.5 kPa VPD, 1500 µmol $m^{-2}\ s^{-1}$ PPFD (mixed light from red and blue LEDs), and 400 $cm^3\ m^{-3}$ CO_2.

Chlorophyll fluorescence measurements

The chlorophyll fluorescence of the eighth leaves was measured at 28 °C almost simultaneously with gas exchange measurements for four replicate plants using a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany). The chlorophyll fluorescence parameters were obtained by the respective application of .2, 7000, and 1400 µmol $m^{-2}\ s^{-1}$ of measuring light, saturation pulse (.8 s flash), and actinic light. Before measurements, the objective leaf was kept in the dark for 30 min in general. However, the leaf was kept in the dark for 10 min to avoid the effects of non-steady-state factors on the chlorophyll fluorescence parameters disappearing during this dark period (Kobayakawa & Imai, 2013; Sonoike, 2009). Next, the minimum (F_0) and maximum (F_m) fluorescence were determined by irradiating the measuring light and/or saturation pulses. Thereafter, the steady fluorescence (F') and maximum fluorescence in the steady state (F_m') were determined under actinic light irradiation. The maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies of PSII were obtained using the following equations (Baker, 2008).

$$F_v/F_m = \left(F_m - F_0\right)/F_m$$

$$F_q'/F_m' = \left(F_m' - F'\right)/F_m'$$

Ascorbic acid measurements

For each treatment, four eighth leaves were sampled and used for measurements of ascorbic acid (reduced form, AA; oxidized form, DHA) content simultaneously with gas exchange measurements. Immediately after measurements of the fresh weight, leaves were frozen in liquid N_2 and ground with a mortar and pestle adding metaphosphoric acid to obtain leaf extracts. Next, these were determined using the hydrazine method (Fujita & Yamada, 2006) with a spectrophotometer (Ubest-30; Jasco Corp., Tokyo, Japan), with L(+)-ascorbic acid (Kanto Chemical Co. Inc., Tokyo, Japan) used as a standard reagent. This method uses a color reaction (540 nm) between 2,4-dinitrophenylhydrazine and sulfuric acid to assay DHA. When sodium 2,6-dichloroindophenol is added to the sample solution beforehand, the AA is transformed to DHA and total ascorbic acid (AA + DHA) is obtainable. The AA content was calculated by subtraction of DHA from total ascorbic acid. The RDS of ascorbic acid was calculated as AA/(AA + DHA).

Statistical analysis

All data were subjected to two-way analyses of variance (ANOVA) using software (Bell Curve for Excel; Social Survey Research Information Co. Ltd, Tokyo, Japan). Because of the lack of chamber replications, the O_3 effect was not separable from the chamber effect. Therefore, the former might be biased. However, the chamber effect did not seem large from our experience with environmental regulation. The significance among O_3 treatments or ascorbic

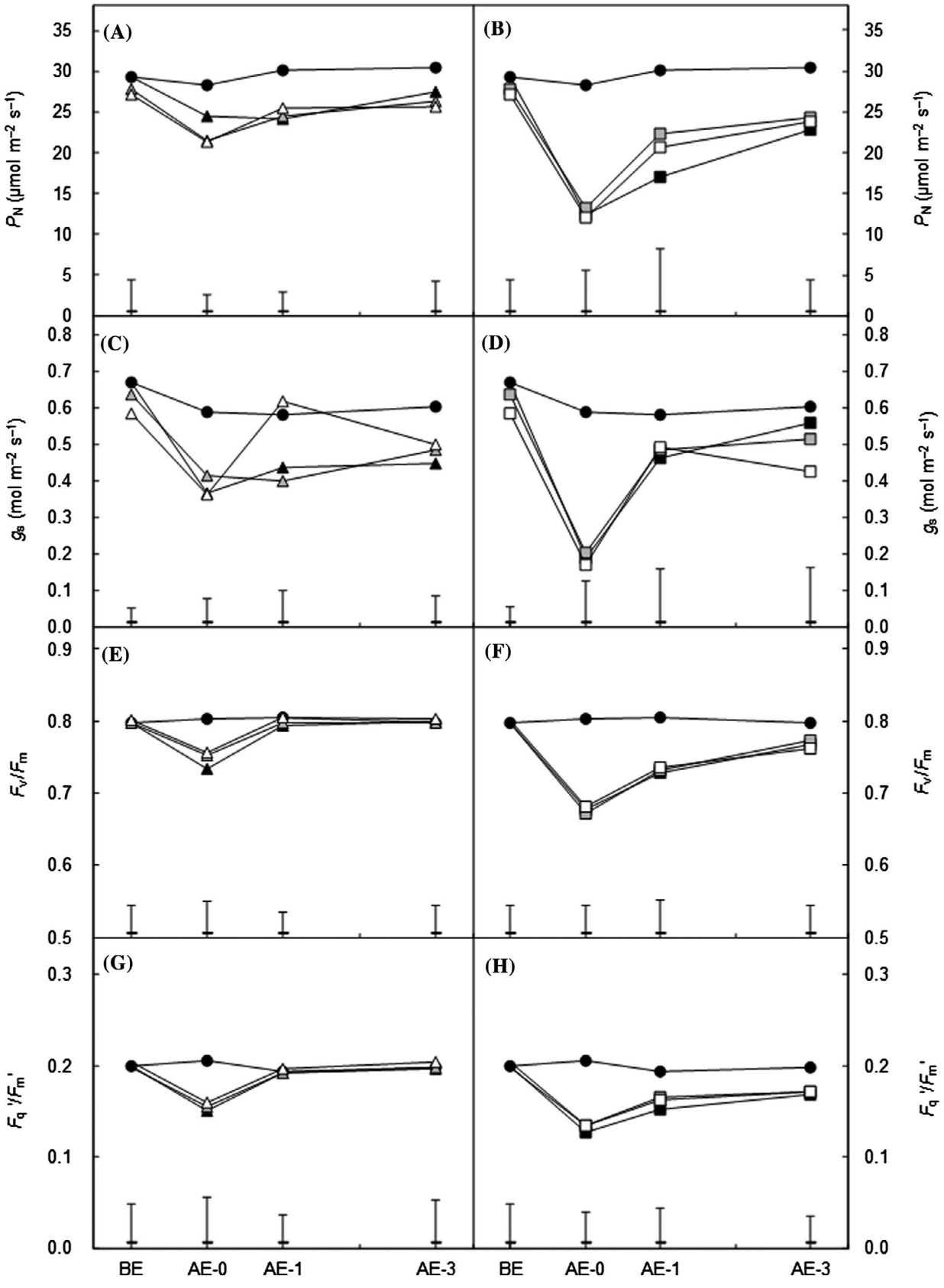

Figure 1. Effects of O_3 and ascorbic acid application on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies of photosystem II in rice leaves. Vertical bars represent Tukey's HSD at 5% on each day. BE, AE-0, AE-1, AE-3: before, immediately after, 1 and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, .1, .3 cm^3 m^{-3} O_3; A^0, A^5, A^{10}: 0, 5, 10 mM ascorbic acid. ●: $O^0 + A^0$; ▲: $O^{0.1} + A^0$; ▲: $O^{0.1} + A^5$; △: $O^{0.1} + A^{10}$; ■: $O^{0.3} + A^0$; ■: $O^{0.3} + A^5$; □: $O^{0.3} + A^{10}$.

acid applications in each interval was determined using Tukey's honestly significant difference (HSD) test ($P \leq .05$).

Results

Effect of ascorbic acid on inhibition of photosynthesis by O_3

From AE-0 to AE-3, photosynthesis-related parameters in the O^0 plants were unaffected by ascorbic acid application and by low pH treatment (data not shown). At AE-0, P_N in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was, respectively, 86% and 43% of that in $O^0 + A^0$ plants. Thereafter, the plants recovered from O_3 inhibition. At AE-3, P_N had recovered in $O^{0.1} + A^0$ plants, but inhibition persisted in the $O^{0.3} + A^0$ plants (−25% of $O^0 + A^0$ plants). At AE-0, the O_3 inhibition of P_N was scarcely affected by ascorbic acid application. Therefore, significant differences among ascorbic acid applications in each O_3 concentration were not observed. At AE-1, the O_3 inhibition of P_N in the $O^{0.3} + A^0$ plants was ameliorated slightly (not significant by Tukey's HSD test) (Figure 1(A) and (B)). Therefore, a two-way ANOVA for P_N showed interaction ($P \leq .05$) between O_3 and ascorbic acid at AE-1 (Table 1). At AE-0, g_s in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was, respectively, 62 and 32% of that in $O^0 + A^0$ plants. Thereafter, the g_s recovered from O_3 inhibition. At AE-3, significant differences among ascorbic acid applications in each O_3 concentration were not observed. At AE-0, the O_3-inhibition of g_s in the $O^{0.1} + A^0$ plants was scarcely affected by ascorbic acid application (Figure 1(C) and (D)). Two-way ANOVA for P_N and g_s showed clear negative effects of O_3 from AE-0 to AE-3 (Table 1).

At AE-0, F_v/F_m in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was, respectively, 91 and 84% of that in $O^0 + A^0$ plants. Thereafter, the plants recovered from O_3 inhibition. At AE-1, F_v/F_m had recovered in $O^{0.1} + A^0$ plants but inhibition persisted in the $O^{0.3} + A^0$ plants (−10% of $O^0 + A^0$ plants). At AE-3, all plants had recovered from O_3-inhibition. Therefore, there were no significant differences among all plants by Tukey's HSD test. O_3 inhibition of F_v/F_m in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was scarcely affected by ascorbic acid treatment from AE-0 to AE-3 (Figure 1(E) and (F)). At AE-0, F_q'/F_m' in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was, respectively, 74 and 62% of those in $O^0 + A^0$ plants. Thereafter, the plants recovered from O_3 inhibition. At AE-3, there were no significant differences among all plants by Tukey's HSD test ($P \leq .05$). O_3 inhibition of F_q'/F_m' in the $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants was scarcely affected by ascorbic acid treatment from AE-0 to AE-3 (Figure 1(G) and (H)). A two-way ANOVA for F_v/F_m and F_q'/F_m' showed clear negative effects of O_3 from AE-0 to AE-3. Because the O_3 inhibition of chlorophyll fluorescence parameters was not ameliorated by ascorbic acid application, there were no interaction between O_3 and ascorbic acid from AE-0 to AE-3 (Table 1).

Effects of O_3 and exogenous ascorbic acid on ascorbic acid contents

Total ascorbic acid (AA + DHA) and RDS of ascorbic acid in the O^0 plants were unaffected by low pH treatment (data not shown). At AE-0, total ascorbic acid was decreased by O_3 in $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants: the contents of $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants were 80% and 81% of that in $O^0 + A^0$ plants. At AE-1, the contents had decreased further by O_3: the contents of $O^{0.1} + A^0$ and $O^{0.3} + A^0$ plants were 64 and 68% of that in $O^0 + A^0$ plants. At AE-3, the contents recovered from O_3-induced decrease (Figure 2(A) and (B)). On the other hand, the total ascorbic acid content was increased by the addition of ascorbic acid into culture solution: the contents of $O^0 + A^5$ and $O^0 + A^{10}$ plants were 116% and 123% of that in $O^0 + A^0$ plants at BE. These were maintained from BE to AE-3. Therefore, a two-way ANOVA for total ascorbic acid content showed clear positive effects of ascorbic acid treatment at AE-1 and AE-3 ($P \leq .001$, Table 1). In addition, the decrease in total ascorbic acid content by O_3 was ameliorated by ascorbic acid treatment. Therefore, there were no significant differences ($P \leq .05$, Tukey's HSD test)

Table 1. Results of statistical analyses of the effects of O_3 and ascorbic acid application on photosynthesis-related parameters and ascorbic acid contents per unit of fresh weight in rice leaves shown in Figures 1 and 2. AE-0, AE-1, and AE-3, respectively, denote values obtained immediately after, and 1 and 3 d after gas exposure.

Time	Factor	P_N	g_s	F_v/F_m	F_q'/F_m'	AA + DHA	RDS
BE	AsA	n.s.	n.s.	n.s.	n.s.	*	n.s.
AE-0	O_3	***	***	***	***	*	**
	AsA	n.s.	n.s.	n.s.	n.s.	***	n.s.
	$O_3 \times$ AsA	*	***	n.s.	n.s.	n.s.	n.s.
AE-1	O_3	***	*	***	***	***	*
	AsA	n.s.	*	n.s.	n.s.	***	***
	$O_3 \times$ AsA	*	**	n.s.	n.s.	**	**
AE-3	O_3	***	**	***	**	**	n.s.
	AsA	n.s.	n.s.	n.s.	n.s.	***	n.s.
	$O_3 \times$ AsA	n.s.	*	n.s.	n.s.	n.s.	n.s.

n.s.–not significant, by one-way or two-way ANOVA.
*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.

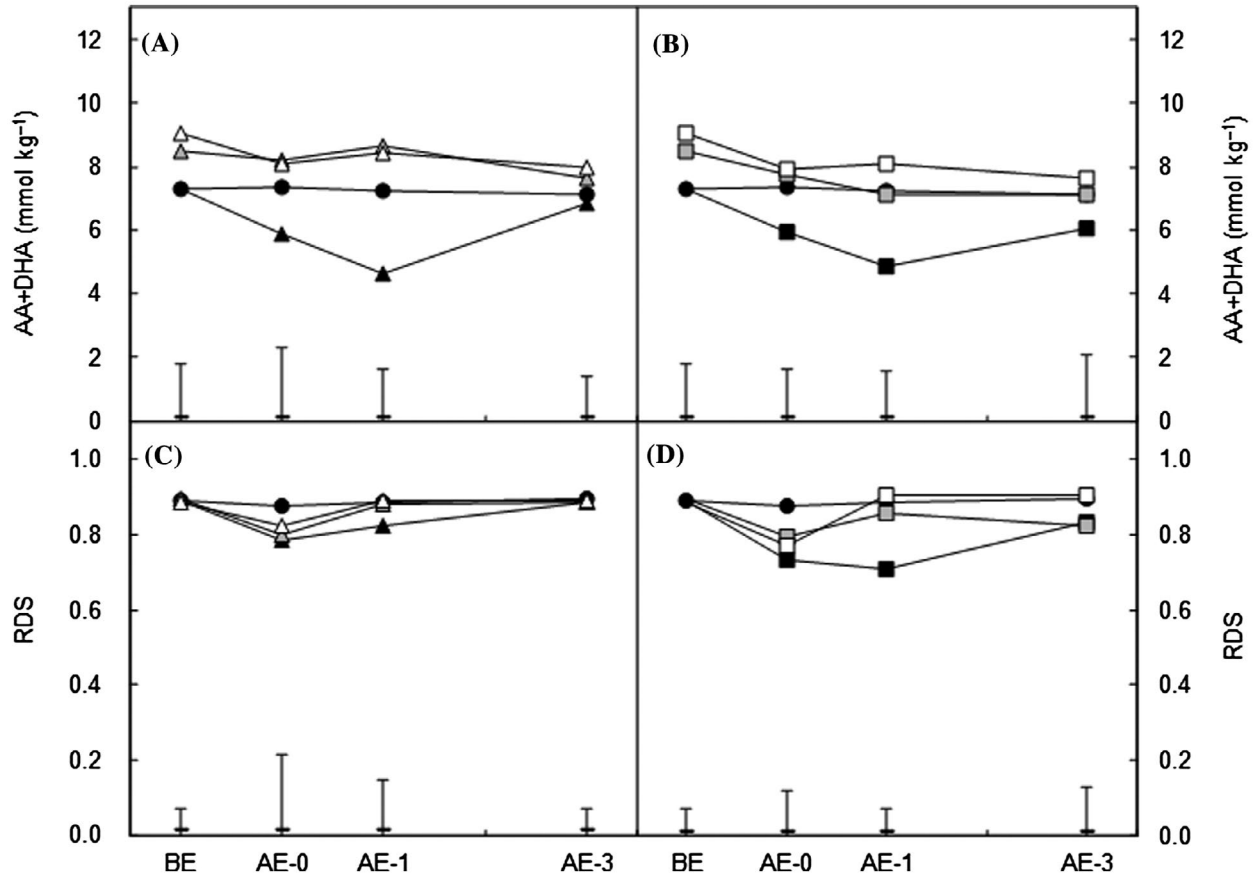

Figure 2. Effects of O_3 and ascorbic acid application on total ascorbic acid and redox state of ascorbic acid (RDS) in rice leaves. Vertical bars represent Tukey's HSD at 5% on each day. AA: reduced form of ascorbic acid; DHA: dehydroascorbic acid (oxidized form of ascorbic acid), RDS: redox state of ascorbic acid [AA/(AA + DHA)]. BE, AE-0, AE-1, AE-3: before, immediately after, 1 and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, .1, .3 cm^3 m^{-3} O_3; A^0, A^5, A^{10}: 0, 5, 10 mM ascorbic acid. ●: $O^0 + A^0$; ▲$O^{0.1} + A^0$; ▲: $O^{0.1} + A^5$; △: $O^{0.1} + A^{10}$; ■: $O^{0.3} + A^0$; ■: $O^{0.3} + A^5$; □: $O^{0.3} + A^{10}$.

between control ($O^0 + A^0$) plants and $O^{0.1 \text{ and } 0.3} + A^{5 \text{ and } 10}$ plants from AE-0 to AE-3 (Figure 2(A) and (B)). At AE-0, the RDS of ascorbic acid was unaffected by O_3 and ascorbic acid treatment. At AE-1, the RDS of ascorbic acid in the $O^{0.3} + A^0$ plants decreased to 80% of that in $O^0 + A^0$ plants. At AE-3, it had recovered in $O^{0.3} + A^0$ plants. The decrease of RDS in the $O^{0.3} + A^0$ plants disappeared by ascorbic acid treatment (Figure 2(D)).

Discussion

The present study was conducted to clarify the role of ascorbic acid in acute O_3-exposed rice leaves. Coincident with previous studies of rice (Imai & Kobori, 2008; Kobayakawa & Imai, 2011a), the photosynthesis-related parameters and content of ascorbic acid and its RDS were inhibited by acute O_3 exposure (Figures 1 and 2). Ascorbic acid is an important antioxidant in plant tissues that has been shown to have an essential role in several physiological processes in plants, including growth, differentiation, and metabolism. It protects cells and organelles from oxidative damage by scavenging ROS. Further, it acts as a substance for the synthesis of important organic acids, as well as a cofactor for enzymes involved in multiple processes including flavonoid and phytohormone biosynthesis, and the xanthophyll cycle (Lisko et al., 2013). Several studies reported that exogenous ascorbic acid ameliorated stresses such as salt (Hasanuzzaman et al., 2013), high temperature (Shah et al., 2011), and drought (Malik & Ashraf, 2012). Lisko et al. (2013) showed that an *Arabidopsis* line overexpressing ascorbic acid biosynthesis enzyme was more tolerant to abiotic stress. In our study, the O_3 inhibition of rice photosynthesis was scarcely ameliorated by ascorbic acid treatment (Figure 1 and Table 1), although the exogenous ascorbic acid increased ascorbic acid levels at AE-0 (Figure 2). In the case of *Plantago major*, exogenous ascorbic acid increased leaf ascorbic acid content and prevented O_3-induced visible injury with a relatively small decline (−26%) of P_N than in plants without exogenous ascorbic acid (−57%) (Zheng et al., 2000). Frei et al. (2012) showed that rice mutant ND6172 had around 20–30% lower ascorbic acid level than its wild type (cv. Nipponbare)

and exhibited a higher level of visible leaf symptoms due to O_3 exposure, but the sensitivity of P_N to O_3 was not different. On the other hand, Chen and Gallie (2005) reported that tobacco plants with larger and smaller ascorbic acid pool sizes, respectively, exhibited more and less tolerance of P_N to acute and chronic O_3 exposure than plants with a normal pool size. Furthermore, Xie et al. (2009) reported that exogenous ascorbic acid application ameliorated the chronic inhibition of photosynthesis-related parameters (P_N, chlorophyll contents, and net assimilation rate) by O_3 in rice. From these reports and our present results, we can conclude that ascorbic acid is one of the determinants for O_3 injury in rice plants, but its role is dependent on the mode of O_3 exposure (such as concentration and duration) and examined plant traits (such as visible leaf symptoms, photosynthesis-related parameters and yield). This is not conflicting with the report on rice by Sawada and Kohno (2009) that mechanisms inducing acute leaf injury do not relate to chronic O_3 toxicity that reduces yield. In addition, the effects of ascorbic acid application may differ depending on application methods and concentrations. Thus, the role of ascorbic acid in acute O_3 inhibition of photosynthesis in rice leaves seems not important.

Disclosure statement

No potential conflict of interest was reported by the authors.

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