Relation between O₃-Inhibition of Photosynthesis and Ethylene in Paddy Rice Grown under Different CO₂ Concentrations

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Abstract: This study was conducted to evaluate the role of ethylene in acute ozone (O₃: 0, 0.1, and 0.3 cm⁻³ m⁻³; O₀, O₀.1, and O₀.3, respectively)-induced photosynthetic inhibition of paddy rice leaves grown under different atmospheric carbon dioxide concentrations (CO₂: 400 and 800 cm⁻³ m⁻³; C₄₀₀ and C₈₀₀, respectively). Ethephon and silver thiosulfate complex (STS) were applied one day before exposure to O₃. Gas exchange, chlorophyll fluorescence, and ascorbic acid were measured immediately before (BE), immediately after (AE-0), and 1 d and 3 d after (AE-1, AE-3) the start of the exposure to O₃. In the plants exposed to O₃, visible leaf symptoms on the adaxial leaf surface appeared at AE-3. O₃ decreased photosynthesis-related parameters, total ascorbic acid content, and redox state of ascorbic acid (RDS), and C₈₀₀ ameliorated O₃-induced damage. STS ameliorated the O₃-induced visible leaf symptoms and O₃-inhibition of photosynthesis but ethephon worsened slightly or did not affect them. Additionally, we evaluated the effects of O₃ and CO₂ on ethylene production in rice leaves. Although elevated CO₂ did not affect ethylene production, exposure to O₃ greatly increased ethylene production at AE-0 and rapidly reduced it at AE-1. These results indicate that ethylene is an important component of signal transduction for the extension of O₃ injury in paddy rice.

Key words: Ascorbic acid, Chlorophyll fluorescence, Ethylene, Gas exchange, O₃, Oryza sativa, Quantum efficiency.

During windless, warm and sunny daytime periods, photochemical oxidants are generated when nitrogen oxides and hydrocarbons emitted from automobiles and factories are exposed to ultraviolet rays. Growing concern has arisen because long-distance transport of air pollution increases the background O₃ concentration (Ohara, 2011). In Japan’s Kanto region, 10 – 20 photochemical oxidant warnings are issued every rice-growing season. Hourly peak values of photochemical oxidants sometimes approach 0.2 cm⁻³ m⁻³ (Environ. Improv. Div., Tokyo Metro. Bureau Environ., 2005). Of photochemical oxidant components, 90% or more are O₃ (Cabrera et al., 1988; Nouchi, 2001). When exposed to O₃, rice plants suffer damage: inhibition of net photosynthetic rate (Pₙ), stomatal conductance (gₛ) and PSII (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a, 2012a), decreased ribulose 1,5-bisphosphate carboxylase/oxygenase (Ishioh and Imai, 2005), chlorophyll and carotenoid (Rai and Agrawal, 2008) contents and nitrite reductase activity (Kobayakawa and Imai, 2011b), in addition to visible leaf-related symptoms (Imai and Kobori, 2008) and breakdown of the cellular ultrastructure (Toyama et al., 1989). By using a passive light microscope, the 3-D chlorophyll fluorescence imaging suggested that O₃ injury began just under the epidermal cells (Endo and Omasa, 2007). Moreover, O₃ suppresses growth (Imai and Ookoshi, 2011), alters photoassimilate partitioning (Nouchi et al., 1995), and decreases grain yields (Reid and Fiscus, 2008; Imai and Ookoshi, 2011).

The atmospheric CO₂ concentration has increased worldwide from a pre-industrial level of about 280 cm⁻³ m⁻³ to the current level of 391 cm⁻³ m⁻³. This trend is expected to continue unless human activities are curtailed substantially (WMO WDCGG, 2013). Under elevated CO₂, the gₛ of plants generally decreases. Consequently, O₃-inhibition decreases because of the limited inflow of O₃ through stomata (Booker and Fiscus, 2005; Imai and Kobori, 2008). In rice plants, O₃-inhibition of photosynthesis is ameliorated by elevated CO₂ (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a, 2012a). Similar responses have been reported for wheat (McKee et al., 1997) and soybean (Booker and Fiscus, 2005).
Recently, plant hormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene have been recognized as important signals in O₃ stress (Rao and Davis, 2001; Kangasjärvi et al., 2005). In paddy rice, Kobayakawa and Imai (2012b, 2013b) reported that methyl jasmonate (MeJA) and SA application ameliorated the O₃-inhibition of Pₚ₅. In addition, jasmonates (JA and MeJA) and SA contents in rice leaves are increased by O₃ (Kobayakawa and Imai, 2012b, 2013c). However, the amelioration of O₃-inhibition by SA application and the increase of endogenous SA in rice leaves (Kobayakawa and Imai, 2012b) are less than in other plants such as Arabidopsis (Sharme et al., 1996), tobacco (Váñpani et al., 1994) and soybean (Zhao et al., 2010). Moreover, elevated CO₂ induces SA production and suppresses JA production in leaves such as tomato, soybean, and ginger (DeLucia et al., 2012). In paddy rice, Kobayakawa and Imai (2013c) found that endogenous JA and MeJA contents in leaves were decreased slightly by elevated CO₂ (800 cm⁻³ m⁻³). Therefore, the roles of plant hormones on O₃-inhibition might differ among plant species, and might change under future CO₂ concentrations.

Ethylene is the simplest olefin synthesized from methionine through intermediates S-adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). In higher plants, ethylene production increases during leaf abscission, flower senescence, and fruit ripening. In addition, ethylene biosynthesis increases under stress conditions such as drought, flooding, chilling and mechanical wounding (Taiz and Zeiger, 2010). The O₃-exposure increases ethylene production in leaves of Arabidopsis (Rao et al., 2002), pea (Mehlhorn et al., 1991) and tobacco (Mehlhorn et al., 1991; Ogawa et al., 2005). Likewise, Rao et al. (2002) reported that ethylene-overproducer mutants (eto1, eto3) were more sensitive to O₃ than wild type. Overmyer et al. (2000) showed that ethylene-insensitive mutant (ein2) was more insensitive to O₃ than wild type, and O₃-sensitive mutant (rdl) produced ethylene more than wild type, and was therefore more sensitive to O₃. Furthermore, the O₃-induced visible leaf symptoms are ameliorated by ethylene biosynthesis inhibitor, aminooxethoxyvinylglycine (AVG), application in pinto bean and tobacco (Mehlhorn et al., 1991). However, there were few studies on the relation between O₃-inhibition of photosynthesis and ethylene in paddy rice, although O₃ exposure increased ethylene production in rice leaves (Ohki et al., 1999).

The objective of current study is to clarify the role of ethylene on O₃-inhibition in rice leaves under different CO₂ concentrations. The level or function of ethylene can be manipulated in different ways. In this study, we used the following two methods to determine possible associations of ethylene in the O₃-inhibition of photosynthesis under different CO₂ concentrations (Exp. 1); application of ethylene-generating agent (ethephon) or ethylene-action inhibitor (STS). The former is intended to increase the level of ethylene and the latter is to suppress ethylene functions even in the presence of it. We also examined the endogenous ethylene level under different CO₂ concentrations (Exp. 2). Our hypothesis is that elevated ethylene promotes and deficient ethylene suppresses the O₃-inhibition in rice leaves. However, this situation may change under elevated CO₂.

### Materials and Methods

1. **Plant materials, exposure to gas, and application of ethylene-related plant growth regulators**

In April (Exp. 1) and July (Exp. 2) of 2013, seeds of a japonica rice (Oryza sativa L. cv. Koshihikari) were sown in 1/5000 a Wagner pots filled with 2.5 kg of dry soil and 12.5 g of compound fertilizer (N, P₂O₅, K₂O = 8, 8, 8%). 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-hr day / 12-hr night), 60% RH and 400 or 800 cm⁻³ m⁻³ CO₂ (C₄₀ or C₈₀₀, variation: within ±2%) without O₃. The average temperature, relative humidity, and relative light intensity among growth chambers were 27.7 / 22.8°C, 60.6%, and 51.7% (relative value to outside of green house), respectively. These were within 3% of target values. In addition, the differences in environmental parameters (temperature, humidity, and light intensity) among growth chambers were within 3% in average.

In Exp. 1, immediately after full expansion of the eighth leaves (ca. one month from sowing), a solution of 0.1% ethephon (Nissan Ethrel 10; Nissan Chemical Industries Ltd., Tokyo, Japan) or 2 mM STS (K-20C; Chrysal Japan Ltd., Oosaka, Japan) was applied to the plants. Ethephon was sprayed to the shoots using a mister, and STS was applied to the soil for absorption from the plant roots. One day after ethephon or STS application (Exp. 1), or immediately after full expansion of the eighth leaves (Exp. 2), rice plants were exposed to 0 (< 0.002), 0.1, and 0.3 cm⁻³ m⁻³ O₃ (expressed respectively as O₃, O₃¹, and O₃³) during 5-hr local daytime (0800 – 1300) using three chambers under growth CO₂ concentrations. Ozone was supplied using a high-voltage O₃ generator with dry air (EDOG-R6; Ecodesign Inc., Ogawa, Saitama, Japan), and CO₂ was supplied from cylinders containing liquid CO₂. These gases were injected into air that had been filtered through activated charcoal layers. The O₃ and CO₂ concentrations were measured every 1 min and computer-controlled using an ultraviolet absorption-type O₃ analyzer (EG-2001F; Ebara Jitsugyo Co. Ltd., Tokyo, Japan) and infrared CO₂ analyzer (ZRH; Fuji Electric Systems Co. Ltd., Tokyo, Japan), respectively. The average concentrations of O₃ in the O₃¹ and O₃³ treatments were 0.106 and 0.307 cm⁻³ m⁻³, respectively, and those of CO₂ in the C₄₀₀ and C₈₀₀
treatments were 396 and 792 cm\(^{-3}\) m\(^{-2}\), respectively. These were within 3% of target values.

2. Gas exchange measurements

In Exp. 1, in situ gas-exchange measurements (1300 – 1430, local time) of the attached, eighth leaves at the middle portion were conducted immediately before (BE: 1 – 0 hr before), immediately after (AE-0: 0.1 – 1.1 hr after), and 1 and 3 d after (AE-1, AE-3) exposure to O\(_3\) at C\(^{400}\) or C\(^{800}\) 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, and 1500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PPFD (mixed light from red and blue LEDs) under growth CO\(_2\) concentrations.

3. Chlorophyll fluorescence measurements

In both Exp. 1 and Exp. 2, chlorophyll fluorescence of the eighth leaves were measured at 28°C right after (within about 1 min) the 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 applications of 0.2, 7000, and 1400 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) of measuring light, saturation pulse (0.8 s flash), and actinic light. Before measurements, the leaf was kept in the dark for 10 min because the effect of O\(_3\) remained maximal, although the effects of other non-steady-state factors on chlorophyll fluorescence parameters disappeared during this period (Kobayakawa and Imai, 2013a). Then the minimum (\(F_0\)) and maximum (\(F_m\)) fluorescence were determined, respectively, by irradiating the measuring light and saturation pulses. Thereafter, the steady fluorescence (\(F'\)) and maximum fluorescence in the steady state (\(F_m'\)) were determined under actinic light irradiation. The maximum (\(F'/F_m\)) and operating (\(F'_o/F_m'\)) quantum efficiencies of PSII were obtained using the following equations (Baker, 2008; Sonoike, 2009).

\[
\frac{F_o}{F_m} = \frac{(F_m - F_o)}{F_m} \\
\frac{F'_o}{F'_m} = \frac{(F'_m - F'_o)}{F'_m}
\]

4. Ascorbic acid measurements

In Exp. 1, four eighth leaves for each treatment were sampled and used for the measurements of ascorbic acid (reduced form, AA; oxidized form, DHA) contents right after (within about 5 min) the chlorophyll fluorescence measurements. Immediately after measurements of the FW, leaves were sealed in a test tube with 4 mL water. They were incubated at 28°C for 4 hr using an incubator (LTI-700; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Thereafter, the gas sample in a test tube was injected into a gas chromatograph (GC-2010, Shimadzu Corp., Kyoto, Japan) with a flame ionization detector (FID-2010Plus; Shimadzu Corp., Kyoto, Japan) using a gas-tight syringe (MS-GANX00; Ito Corp., Shizuoka, Japan). Separation of ethylene was conducted using a capillary column (30 m x 0.25 mm x 0.25 \(\mu\)m; Rxi®-1 ms; Restek Corp., Pennsylvania, U.S.) with He as a carrier gas. Temperatures of the injection port and detector were maintained, respectively, at 250°C and 280°C. The column temperature was maintained at 170°C for 5 min. The ethylene retention time was 1.392 min.

5. Ethylene production measurements

In Exp. 2, four eighth leaves for each treatment were sampled and used for measurements of ethylene production almost simultaneously with chlorophyll fluorescence measurements. Immediately after measurements of the FW, leaves were sealed in a test tube with 4 mL water. They were incubated at 28°C for 4 hr using an incubator (LTI-700; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Thereafter, the gas sample in a test tube was injected into a gas chromatograph (GC-2010, Shimadzu Corp., Kyoto, Japan) with a flame ionization detector (FID-2010Plus; Shimadzu Corp., Kyoto, Japan) using a gas-tight syringe (MS-GANX00; Ito Corp., Shizuoka, Japan). Separation of ethylene was conducted using a capillary column (30 m x 0.25 mm x 0.25 \(\mu\)m; Rxi®-1 ms; Restek Corp., Pennsylvania, U.S.) with He as a carrier gas. Temperatures of the injection port and detector were maintained, respectively, at 250°C and 280°C. The column temperature was maintained at 170°C for 5 min. The ethylene retention time was 1.392 min.

6. Statistical analysis

All data were subjected to three-way (Exp. 1) or two-way (Exp. 2) analysis of variance (ANOVA) using software (Excel Statistics 2010 for Windows; 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 treatments in each interval was determined using Tukey’s honestly significant difference (HSD) test (P ≤ 0.05).

Results

1. O\(_3\)-induced visible symptoms on adaxial leaf surface (Exp. 1)

At AE-3, O\(_3\)-induced visible symptoms appeared in the O\(^{0.1} + C^{400}\), O\(^{0.3} + C^{400}\), and O\(^{0.3} + C^{800}\) plants. Elevated CO\(_2\) (C\(^{400}\)) and STS application ameliorated but ethephon application slightly worsened the leaf symptoms. Figure 1 shows O\(_3\)-induced visible leaf symptoms in the O\(^{0} + C^{400}\) (control), O\(^{0.1} + C^{400}\), O\(^{0.1} + C^{400} + S\), and O\(^{0.1} + C^{400} + E\)
plants. The O₃-induced symptom in the O₃₀₁ + C₄₀₀₀ and O₃₀₁ + C₄₀₀₀ + E plants look similar, but injured areas of leaf between these two were different. The symptom appeared only on the edge of leaf in the O₃₀₁ + C₄₀₀₀ plants, but it spread from the edge to middle portion (around midrib) in the O₃₀₁ + C₄₀₀₀ + E plants.

2. Effect of ethylene on O₃-inhibition of photosynthesis under ambient CO₂ (Exp. 1)

Photosynthesis-related parameters in the O₃ plants were unaffected by ethephon or STS application during AE-0 to AE-3 under both C₄₀₀₀ and C₄₀₀₀ (Figs. 2 and 3). At AE-0, Pₖ in the O₃₀₁ + C₄₀₀₀ (▲) and O₃₀₅ + C₄₀₀₀ (■) plants were 52% and 24%, respectively, of those at BE. Thereafter, the decreases persisted: At AE-3, the former and the latter were, respectively, 72% and 82% of those at BE. The O₃-inhibition of Pₖ in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants slightly ameliorated by STS: At AE-0, Pₖ in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants was unaffected by ethephon or STS during AE-0 to AE-3, but the O₃₀₅ + C₄₀₀₀ plants were ameliorated slightly by STS at AE-1 and AE-3. The Fₖ/Fₘ values in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants were recovered fully, but the O₃₀₅ + C₄₀₀₀ plants was ameliorated slightly by ethylene or STS at AE-0 (Fig. 2b).

At AE-0, Fₖ/Fₘ in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants were, respectively, 78% and 66% of those at BE. Thereafter, these plants recovered from O₃-inhibition. At AE-3, Fₖ/Fₘ values in the former and the latter were, respectively, 93% and 72% of those at BE. The O₃-inhibition of Fₖ/Fₘ in O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants was unaffected by ethylene or STS at AE-0, but the O₃₀₅ + C₄₀₀₀ plants were ameliorated by STS at AE-1 and AE-3. The Fₖ/Fₘ values in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants were, respectively, 76% and 88% of those at BE (Fig. 2c). At AE-0, Fₖ/Fₘ values in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants were, respectively, 73% and 52% of those at BE. Thereafter, the O₃₀₁ + C₄₀₀₀ plants recovered from O₃-inhibition, but the O₃₀₅ + C₄₀₀₀ plants did not. At AE-3, the O₃₀₁ + C₄₀₀₀ plants recovered fully, but the O₃₀₅ + C₄₀₀₀ plants remained at 55% of BE. The O₃-inhibition of Fₖ/Fₘ in the O₃₀₁ + C₄₀₀₀ and O₃₀₅ + C₄₀₀₀ plants was ameliorated slightly by STS. At AE-3, the Fₖ/Fₘ values in the O₃₀₁ + C₄₀₀₀ plants was 75% of that at BE (Fig. 2d). Three-way ANOVA for Fₖ/Fₘ and Fₖ/Fₘ indicated strong effects of O₃ from AE-0 to AE-3 (P ≤ 0.001, Table 1).

3. Effect of ethylene on O₃-inhibition of photosynthesis under elevated CO₂ (Exp. 1)

The O₃-inhibition of all photosynthesis-related parameters was ameliorated by elevated CO₂ (Figs. 2 and 3). At AE-0, Pₖ values in the O₃₀₁ + C₄₀₀₀ (▲) and O₃₀₅ + C₄₀₀₀ (■) plants were, respectively, 85% and 43% of those at BE.
At AE-3, $P_N$ recovered fully in the $O^{0.1} + C^{800}$ plants, but remained at 68% of BE in $O^{0.3} + C^{800}$ plants. The $O_3$-inhibition of $P_N$ in the $O^{0.3} + C^{800}$ plants was worsened by ethephon but was ameliorated by STS. At AE-3, $P_N$ values in the $O^{0.3} + C^{800} + E$ (□) and $O^{0.5} + C^{800} + S$ (□) plants were, respectively, 44% and 81% of those at BE (Fig. 3a).

At AE-0, $g_s$ values in the $O^{0.1} + C^{800}$ and $O^{0.3} + C^{800}$ plants were, respectively, 47% and 39% of those at BE. The $O_3$-inhibition of $g_s$ in the $O^{0.3} + C^{800}$ plants was slightly intensified by ethephon but was ameliorated by STS. The $g_s$ in the $O^{0.3} + C^{800} + S$ plants at AE-0 was 60% of that at BE. The $g_s$ of $O^{0.5} + C^{800} + E$ plants at AE-3 was 70% of that at BE (Fig. 3b). Three-way ANOVA for $P_N$ and $g_s$ revealed clear interactions between $O_3$ and $CO_2$ during AE-0 to AE-3 ($P \leq 0.001$ or 0.05, Table 1).

The $F_v/F_m$ values in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants at AE-0 were, respectively, 95% and 84% of those at BE. At AE-1, the $O^{0.1} + C^{400}$ plants fully recovered from $O_3$-
inhibition, but the O\(^{0.3} + C_4\) plants at AE-1 and AE-3 respectively remained 83% and 95% of BE. Ethephon worsened the O\(^3\)-inhibition of \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) plants at AE-0. However, the O\(^3\)-inhibition of \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) plants at AE-3 was ameliorated by STS. At AE-3, \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) plants at AE-0 decreased to 81% of that at BE. At AE-1, they recovered fully from O\(^3\)-inhibition. The O\(^3\)-inhibition of \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) plants were unaffected by ethephon or STS (Fig. 3c). The \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) plants at AE-0 decreased to 81% of that at BE. At AE-1, they recovered fully from O\(^3\)-inhibition. The O\(^3\)-inhibition of \(F_\text{in}^'/F_\text{in}\) in the O\(^{0.3} + C_4\) and O\(^{0.5} + C_4\) plants were unaffected by ethephon or STS (Fig. 3d). Three-way ANOVA for \(F_\text{in}^'/F_\text{in}\) and \(F_\text{in}^'/F_\text{in}\) indicated clear interactions between O\(^3\) and CO\(_2\) at AE-0 to AE-3 (\(P \leq 0.001\) or 0.05, Table 1).

### Table 1. Results of statistical analyses of the effects of O\(^3\), CO\(_2\), and ethylene-related plant growth regulators on photosynthesis-related parameters and ascorbic acid contents per unit of fresh weight in rice leaves shown in Figs. 1, 2, and 3 (Exp. 1). AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, and 1 d and 3 d after gas exposure. *\(P \leq 0.05\), **\(P \leq 0.01\), ***\(P \leq 0.001\), n.s. — not significant, by three-way ANOVA.

| Time  | Factor          | \(P_\alpha\) | \(g\) | \(F_\text{in}^'/F_\text{in}\) | \(F_\text{in}^'/F_\text{in}\) | AA+DHA | RDS |
|-------|-----------------|--------------|-------|-----------------|-----------------|--------|-----|
| AE-0  | O\(^3\)         | ***          | ***   | ***             | ***             | ***    | *** |
|       | CO\(_2\)        | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | Ethylene        | ***          | n.s.  | n.s.            | ***             | n.s.   | n.s.|
|       | O\(^3\) × CO\(_2\) | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × Ethylene | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | CO\(_2\) × Ethylene  | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × CO\(_2\) × Ethylene | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| AE-1  | O\(^3\)         | ***          | ***   | ***             | ***             | ***    | *** |
|       | CO\(_2\)        | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | Ethylene        | *            | n.s.  | *               | n.s.            | *      | *** |
|       | O\(^3\) × CO\(_2\) | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × Ethylene | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | CO\(_2\) × Ethylene  | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × CO\(_2\) × Ethylene | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| AE-3  | O\(^3\)         | ***          | ***   | ***             | ***             | ***    | *** |
|       | CO\(_2\)        | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | Ethylene        | *            | n.s.  | *               | n.s.            | *      | *** |
|       | O\(^3\) × CO\(_2\) | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × Ethylene | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | CO\(_2\) × Ethylene  | ***          | ***   | ***             | ***             | ***    | n.s.|
|       | O\(^3\) × CO\(_2\) × Ethylene | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

4. Effects of O\(^3\) and ethylene on ascorbic acid contents under different CO\(_2\) concentrations (Exp. 1)

The effects of O\(^3\) and ethylene on total ascorbic acid (AA + DHA), AA, and DHA contents expressed per unit of fresh weight (FW) were similar to those expressed per unit of leaf area. Figure 4 shows values obtained on a FW basis.

At AE-0, total ascorbic acid content in the O\(^{0.3} + C_4\) plants (▱) decreased to 85% of that at BE. At AE-1, the content decreased further. Total ascorbic acid contents in the O\(^{0.1} + C_{400}\) (▲), O\(^{0.1} + C_{300}\) (▲), O\(^{0.3} + C_{400}\) (▱), and O\(^{0.3} + C_{300}\) plants were, respectively, 58%, 93%, 55%, and 69% of those at BE. At AE-3, the content in the O\(^{0.1} + C_{400}\) plants had recovered fully but in the O\(^{0.1} + C_{400}\), O\(^{0.3} + C_{400}\), and O\(^{0.3} + C_{300}\) plants, the recoveries remained at low levels (16%, 1%, and 4% of those at BE). The O\(^3\)-inhibition of total ascorbic acid content was unaffected by ethephon but was ameliorated by STS. Ascorbic acid contents in the O\(^3\) + C\(_{400}\) + S plants (□) increased to 133–161% by STS from AE-0 to AE-3 (Figs. 4a and 4b).

At AE-0, the redox state of ascorbic acid (RDS) in the O\(^{0.3} + C_{400}\), O\(^{0.3} + C_{300}\), and O\(^{0.3} + C_{300}\) plants decreased, respectively, to 92%, 88%, and 91% of those at BE. At AE-1, RDS decreased further: RDS in the O\(^{0.1} + C_{400}\), O\(^{0.3} + C_{400}\), and O\(^{0.3} + C_{300}\) plants were, respectively, 75%, 62%, and 85% of those at BE. Thereafter, the plants recovered from O\(^3\)-inhibition at AE-3. The O\(^3\)-inhibition of RDS was ameliorated by STS at AE-1 under ambient CO\(_2\) (C\(_{400}\)). At AE-1, the O\(^{0.1} + C_{400}\) + S plants (△) recovered fully, but the O\(^{0.3} + C_{400}\) + S plants (◇) remained at 79% of BE (Figs. 4c and 4d). Three-way ANOVA for total ascorbic acid contents and RDS showed clear negative effects of O\(^3\) at AE-0 to AE-3 (\(P \leq 0.001\), Table 1). In addition, because STS ameliorated the negative effect of O\(^3\) on ascorbic acid...
Fig. 4. Effects of O₃ and ethylene-related plant growth regulators on total ascorbic acid and redox state of ascorbic acid (RDS) at ambient (a, c) and elevated (b, d) CO₂ concentrations in rice leaves (Exp. 1). 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 d and 3 d after gas exposure. O₀, O₀.1, O₀.3: 0, 0.1, 0.3 cm⁻³ m⁻³ O₃; C₄₀₀, C₈₀₀: 400, 800 cm⁻³ m⁻³ CO₂; E, S: ethephon and STS application. ●: O₀ + C₄₀₀ or C₈₀₀; ○: O₀.1 + C₄₀₀ or C₈₀₀; ▲: O₀.1 + C₄₀₀ or C₈₀₀ + S; △: O₀.1 + C₄₀₀ or C₈₀₀; ▲: O₀.3 + C₄₀₀ or C₈₀₀; △: O₀.3 + C₄₀₀ or C₈₀₀ + S.

Fig. 5. Effects of O₃ and CO₂ on maximum ($F_v/F_m$) and operating ($F'_v/F'_m$) quantum efficiencies of photosystem II (a, b) and ethylene emission rate in rice leaves (Exp. 2). Vertical bars represent Tukey’s HSD at 5% on each day. BE, AE-0, AE-1, AE-3: before, immediately after, 1 d and 3 d after gas exposure. O₀, O₀.1, O₀.3: 0, 0.1, 0.3 cm⁻³ m⁻³ O₃; C₄₀₀: 800 cm⁻³ m⁻³ CO₂; ●: O₀ + C₄₀₀; ○: O₀ + C₄₀₀; ▲: O₀.1 + C₄₀₀; △: O₀.1 + C₄₀₀; ▲: O₀.3 + C₄₀₀; △: O₀.3 + C₄₀₀; ▲: O₀.3 + C₄₀₀ or C₈₀₀; △: O₀.3 + C₄₀₀ or C₈₀₀.
contents and RDS, three-way ANOVA for those showed clear effects of ethylene during AE-0 to AE-3, except for RDS at AE-3 (Table 1).

### 5. Effects of O₃ and CO₂ on ethylene production and photosystem II (Exp. 2)

The trends of $F_v/F_m$ and $F_{m}'/F_{m}$* resemble those in Exp. 1. At AE-0, $F_v/F_m$ in the $O^{8/1} + C^{800}$, $O^{8/1} + C^{400}$, and $O^{8/3} + C^{400}$ plants decreased, respectively, to 85%, 63%, and 67% of those at BE. The $F_{m}'/F_{m}$* in the $O^{8/1} + C^{800}$, $O^{8/1} + C^{800}$, $O^{8/3} + C^{400}$, and $O^{8/3} + C^{400}$ plants decreased, respectively, to 66%, 75%, 55%, and 56% of those at BE. Thereafter, the $F_v/F_m$ and $F_{m}'/F_{m}$* recovered, more or less, from O₃-inhibition: At AE-3, $O^{8/1} + C^{800}$ and $O^{8/3} + C^{800}$ plants recovered fully, but the $O^{8/3} + C^{400}$ plants remained inhibited (86% and 79% of those at BE, respectively) (Figs. 5a and 5b).

At AE-0, the ethylene production was increased by O₃. The production in the $O^{8/3} + C^{400}$ and $O^{8/3} + C^{800}$ plants at AE-0 increased drastically (302% and 389%, respectively) compared to those at BE. In the $O^{8/1} + C^{800}$ and $O^{8/1} + C^{800}$ plants, it increased slightly ($P \leq 0.10$). Thereafter, the increase disappeared from AE-1 to AE-3 (Fig. 5c). Two-way ANOVA for ethylene production indicated a clear effect of O₃ at AE-0 ($P \leq 0.001$, Table 2).

#### Discussion

Coincident with our previous studies in rice (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a), the photosynthesis-related parameters and ascorbic acid content and its RDS were degraded by O₃ exposure. They were ameliorated by elevated CO₂ ($C^{800}$) (Figs. 2 – 5). Generally, the amelioration of O₃-induced damage of plants by elevated CO₂ is largely attributed to the restriction of CO₂ intake through stomatal closure (Booker and Fiscus, 2005; Imai and Kobori, 2008), although undetermined additional factors might be concerned. In this study, STS application ameliorated the O₃-induced visible injury (Fig. 1) and O₃-inhibitions of $P_{N}$ and PSII ($F_v/F_m$ and $F_{m}'/F_{m}$*), as in the cases of MeJA and SA applications (Kobayakawa and Imai, 2012b, 2013b). In contrast, ethephon application slightly intensified the O₃-inhibition of photosynthesis-related parameters. These results demonstrated that ethylene functions as a positive regulator of O₃ injury, in contrast to JA in rice leaves (Kobayakawa and Imai, 2012b), as seen in Arabidopsis (Rao and Davis, 2001). The amelioration of O₃-induced damage of plants by MeJA application was ascribed to the induction of stomatal closure and increased antioxidant capacity (Kobayakawa and Imai, 2012b). In contrast to MeJA, SA and ethylene-related plant growth regulator (STS and ethephon) application did not affect $g$ (Figs. 2 and 3; Kobayakawa and Imai, 2013b). Nevertheless, the amelioration of O₃-inhibition of photosynthesis-related parameters by STS was more evident than that by SA, and was even equal to that by MeJA. With respect to endogenous ethylene, its production in rice leaves increased immediately after exposure to O₃ (AE-0) and decreased rapidly at AE-1 (Fig. 5). These are coincident with the case of Arabidopsis (Rao et al., 2002). Therefore, ethylene is expected to be an important factor in the enlargement of O₃ injury in rice leaves.

The inhibition of ethylene action did not affect stomatal function, but slightly increased the total ascorbic acid contents and ameliorated O₃-induced decrease of total ascorbic acid and RDS (Fig. 4). Therefore, the amelioration of O₃-inhibition by STS is attributed to the activation of antioxidant capacity, although it does not fully explain it. Although antioxidant ability is an important factor for the amelioration of O₃ injury (Didyk and Blum, 2011), Kobayakawa and Imai (2013b) demonstrated that the increase of ascorbic acid by exogenous AA and SA could not completely prevent O₃ injury in rice leaves. However, STS ameliorated O₃-induced injury more effectively than ascorbic acid and SA applications. These results suggest that ethylene affects other factors for preventing O₃ injury. Ethylene induces programmed cell death (PCD) and senescence (Trobacher, 2009). In addition, levels of chronic and acute O₃ exposure respectively induce premature senescence and hypersensitive reaction-like PCD (Rao and Davis, 2001). Therefore, it is predicted that O₃-induced rapid ethylene production and thereby cell death and/or senescence later appeared on the leaf surface as visible leaf symptoms. Nakamura and Saka (1978) demonstrated that kinetin and benzimidazole applications ameliorated O₃-induced chlorophyll degradation in rice leaves. Because these cytokinins suppress leaf senescence in contrast to ethylene content.

### Table 2. Results of statistical analyses of the effects of O₃ and CO₂ on maximum ($F_v/F_m$) and operating ($F_{m}'/F_{m}$*) quantum efficiencies of photosystem II and ethylene production in rice leaves portrayed in Fig. 4 (Exp. 2).

| Time | Factor | $F_v/F_m$ | $F_{m}'/F_{m}$* | ethylene |
|------|--------|-----------|-----------------|----------|
| AE-0 | O₃     | ***       | ***             | ***      |
|      | CO₂    | n.s.      | *               | n.s.     |
|      | O₃×CO₂| n.s.      | n.s.            | n.s.     |
| AE-1 | O₃     | ***       | ***             | n.s.     |
|      | CO₂    | *         | n.s.            | n.s.     |
|      | O₃×CO₂| *         | n.s.            | n.s.     |
| AE-3 | O₃     | ***       | ***             | n.s.     |
|      | CO₂    | n.s.      | n.s.            | n.s.     |
|      | O₃×CO₂| n.s.      | n.s.            | n.s.     |
(Taiz and Zeiger, 2010), O$_3$-induced rapid ethylene production might induce premature leaf senescence.

This study showed that ethylene production was unaffected by elevated CO$_2$ (Fig. 5), but Abeles et al. (1992) reported that elevated CO$_2$ promoted, inhibited, or had no effect depending on the plant species and tissues. However, the effects of STS on photosynthesis-related parameters were significant under both CO$_2$ conditions, and the effects of ethephon were noted under elevated CO$_2$, as evidenced by significant CO$_2$ × ethylene or O$_3$ × CO$_2$ × ethylene interactions (Table 1, Figs. 2 and 3). These results suggest that elevated CO$_2$ may reduce the levels or function of ethylene and thereby ameliorate the O$_3$-inhibition of photosynthesis. Kobayakawa and Imai (2013c) found that JA contents in rice leaves were decreased slightly by elevated CO$_2$, as reported previously (DeLucia et al., 2012). Likewise, rice plants grown under elevated CO$_2$ are more susceptible to leaf blast than those grown under ambient CO$_2$ (Kobayashi et al., 2006). In addition, elevated CO$_2$-grown soybean plants exhibited higher sensitivity to acute exposure to O$_3$ because of the lowered antioxidant capacity under elevated CO$_2$ (Gillespie et al., 2011). Elevated CO$_2$ may alter the physiological response in plants including the defense response and sensitivity to various stresses through changes in plant hormone levels.

Results of present and previous (Kobayakawa and Imai, 2012b, 2013b, c) studies show that JA and SA function as negative regulators of O$_3$-inhibition, and that ethylene functions as a positive regulator of O$_3$-inhibition. In addition, jasmonates (JA and MeJA) and ethylene increased immediately (AE-0) and SA increased with a lag time (from AE-1 to AE-3) after exposure to O$_3$. Furthermore, the amounts of jasmonates and ethylene increased more than that of SA. Therefore, JA and ethylene may be more important for the determination of O$_3$ inhibition than SA in rice leaves. This phenomenon might be attributed to high SA contents in rice leaves (8–30 µg g$^{-1}$ FW), which is higher than other plants such as Arabidopsis (0.01–0.1 µg g$^{-1}$ FW) (Ogawa et al., 2006). According to Yang et al. (2004), rice had two orders of magnitude higher levels of SA than tobacco and Arabidopsis, and was insensitive to exogenous SA. In addition, Kanno et al. (2012) reported that JA and SA contents in rice leaves were increased by the feeding damage of white-back planthopper (Sogatella furcifera), but the increment of SA was less than that of JA because rice plants contain much higher levels of endogenous SA in healthy tissues. Therefore, we conclude that the role of SA in rice is less pronounced than in other plants, and that JA and ethylene are major components of signal transduction causing O$_3$ injury.

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