Inclination Angle Affects Ozone Injury in the Flag Leaf of Rice

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Abstract: To evaluate the effect of inclination angle of the leaf on the leaf injury of rice by O₃, we examined the effect of a 5-hr exposure to 0, 0.1, 0.3 cm⁻³ m⁻³ O₃, abbreviated as O⁰, O₀.₁, and O₀.₃, respectively, of the flag leaf using natural-light gas-exposure chambers. The middle portion of the flag leaf was set horizontally using lead weights (H plant) and it was compared with a non-treated, erect leaf (E plant). Gas exchange and chlorophyll fluorescence were measured immediately before, immediately after, and 1 and 3 d after the start of the exposure. The net photosynthetic rate, stomatal conductance and operating quantum efficiency of photosystem II (Fq'/Fm') were decreased by the exposure to O₃. Furthermore, the inhibitory effects of O₀.₁ and O₀.₃ on the H plants were severer than those on the E plants. The maximum quantum efficiency of photosystem II (Fv/Fm) in the leaf of H plants decreased immediately after exposure to O₃. These results indicate that the weaker inhibitory effect of O₃ on the erect leaf depends on the lower light intensity at the leaf surface, rather than the horizontal leaf position.

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

During windless, warm and sunny daytime periods, photochemical oxidants are generated by the reaction of ultraviolet rays to nitrogen oxides and hydrocarbons emitted from automobiles and factories. There is also a growing concern that the long distance transport of air pollution increases the background O₃ concentration (Ohara, 2011). Of the photochemical oxidant components, 90% or more is O₃ (Cabrera et al., 1988; Nouchi, 2001). The Japanese environmental quality standard for the concentration of photochemical oxidants is 0.06 cm⁻³ m⁻³ hr⁻¹. When the concentration exceeds 0.12 and 0.24 cm⁻³ m⁻³ hr⁻¹, local public bodies are obligated to issue a warning and an alarm, respectively (Nakanishi et al., 2009). Stratospheric O₃ protects the earth surface from ultraviolet rays. In contrast, tropospheric O₃ is a health hazard for humans. It attacks all plant species and decreases their photosynthesis and growth rates concurrently with white-dot symptoms on the leaves of herbaceous species and reddish-to-black dot symptoms on the leaves of arboresous, leguminous, and gramineous species. Within cells, it damages organelles and biopolymers, including DNA (Nouchi, 2001; Roshchina and Roshchina, 2003; Vandermeiren et al., 2009). In the Kanto region of Japan, where rice is cultivated as a staple summer crop, 10 – 20 warnings are issued every growing season. Hourly peak values of photochemical oxidants are sometimes close to 0.2 cm⁻³ m⁻³ (Environmental Improvement Division, Bureau of Environment, Tokyo Metropolitan Government, 2005). Kobayashi (1999) estimated that O₃ decreased rice yields by up to 10% in the Kanto region of Japan in 1981 – 1985. Therefore, the effects of exposure to O₃ on physiological processes of rice need to be studied as a basis of dry matter production and yield formation. When exposed to O₃, rice plants suffer damage such as the inhibition of net photosynthetic rate (Pₙ), stomatal conductance to CO₂ transfer (gₛ), photosystem II conductance to CO₂ transfer (gₛ), photosystem II (PSII) (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a), decreased ribulose 1,5-bisphosphate carboxylase / oxygenase (Ishioh and Imai, 2005), chlorophyll and carotenoid (Inada et al., 2008; Rai and Agrawal, 2008) contents and nitrite reductase activity (Kobayakawa and Imai, 2011b), in addition to breakdown of the cellular ultrastructure (Toyama et al., 1989) and visible leaf-related symptoms (Imai and Kobori, 2008). Furthermore, O₃ suppresses the growth rate (Imai and Ookoshi, 2011), alters photoassimilate partitioning (Nouchi et al., 1995) and ultimately decreases the grain yield (Reid and Fiscus, 2008; Yamaguchi et al., 2008; Pang et al., 2009; Rai et al., 2010; Imai and Ookoshi, 2011).

Our previous study (Kobayakawa and Imai, 2011a) revealed that the damage of Pₙ, gₛ and PSII by O₃ in the eighth (vegetative growth stage) and flag (16th, heading
stage) leaves of rice differed substantially. In the eighth leaves, these parameters were inhibited severely by O₃ (0.1 and 0.3 cm³ m⁻³). Their inhibition at higher O₃ concentrations did not recover for 3 d after exposure to O₃, but the inhibition in the flag leaves was less severe and their recovery was faster. This difference is ascribable to their respective leaf thicknesses and leaf inclination angles. Regarding leaf thickness, we have anticipated differences of O₃ inhibition because of the difference in antioxidant contents: the ascorbic acid and glutathione contents per unit of fresh weight in the seventh and eighth leaves (thinner than 15th and flag leaves) at the vegetative growth stage were lower than those in the 15th and flag leaves at the heading stage (Kobayakawa and Imai, 2011a). However, the effects of leaf inclination angle on inhibition of photosynthesis-related processes by O₃ were not clarified. Therefore, in this study, we examined the effect of the leaf inclination angle on the inhibition of P₇₀₀ and PSII in the flag leaf by O₃ at the heading stage. To test our hypothesis that the difference in leaf inclination angle is related to the difference in injury by O₃, we compared the inclination angle and relative light intensity on the adaxial surface, and the ascorbic acid contents of leaves at different positions on a stem.

Materials and Methods

1. Plant materials and gas exposure treatment

In mid-April 2010, seeds of a japonica rice (Oryza sativa L. cv. Koshihikari) were sown in Wagner pots (1/5000 a) 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 a glasshouse until the gas exposure experiment began. Just after the full expansion of the flag (16th) leaf, the plants were transferred to a natural-light gas-exposure chamber (width × depth × height = 2 m × 2 m × 1.9 m: S-2003A; Koito Industries, Ltd., Yokohama, Japan) and kept at 28/23ºC (12-hr day / 12-hr night), 60% RH and 400 cm³ m⁻³ CO₂. At the heading stage, the middle portion of the flag leaf was arranged horizontally by attaching small, globular lead weights at the tip as shown in Fig. 1 (H plant). The relative light intensity at the middle portion of adaxial leaf surface relative to the light intensity at the top of main stem was 91.4% in H plants and 24.7% in the elect-leaf plants (control E plant). Both H and E plants were exposed to 0 (<0.002), 0.1, and 0.3 cm³ m⁻³ O₃, expressed respectively as HO₀⁰ and EO₀, HO₀⁰¹ and EO₀¹, and HO₀⁰₅ and EO₀⁰₅ plants for 5 hr in the day-time (0800 – 1300; local time) using three chambers under isolated conditions with a minimum mutual shading. Ozone was supplied using a high-voltage ozone generator using dry air (ED-OG-R6, Ecodesign Inc., Ogawa, Saitama, Japan). CO₂ was supplied from cylinders containing liquid CO₂. These gases were injected into air that had been charcoal-filtered. Concentrations of O₃ and CO₂ were, respectively, measured and computer-controlled by an ultraviolet absorption-type O₃ analyzer (EG-2001F, Ebara Jitsugyo Co. Ltd., Tokyo, Japan) and an infrared CO₂ analyzer (ZRH, Fuji Electric Systems Co. Ltd., Tokyo, Japan). After the gas exposure treatment, all plants were kept in the same chamber for 3 d at 28 / 23ºC, 60% RH, and 400 cm³ m⁻³ CO₂ without O₃.

2. Gas exchange measurements

Using a portable photosynthesis and transpiration measurement system (LI-6400XT, LI-COR Biosciences, Lincoln, NE, USA), gas-exchange in the middle portion of flag leaves was measured in situ 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 O₃ during 1300 – 1430 (local time) for five replicate plants in each treatment. Environmental conditions within the LI-COR cuvette during measurements were set at 28ºC leaf temperature, 1.5 kPa VPD, and 1500 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD, mixed light from red and blue LEDs).
3. Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany), the chlorophyll fluorescence of PSII in the flag leaf was measured simultaneously with the gas exchange measurements for five replicate plants in each treatment. The respective chlorophyll fluorescence parameters were obtained by applying 0.2, 7000, and 1400 μmol m⁻² s⁻¹ of measuring light, saturating pulse (0.8 s flash), and actinic light. Before fluorescence measurements, the leaf was kept in the dark for 10 min to prevent the recovery from O₃ injury, since Sonoike (2009) indicated that the effect of acute stress recovered with time elapsed (cf., 30 min or more of dark acclimation time is necessary to examine potential value without stress). Then the minimum (F₀) and maximum (Fₘ) fluorescence were determined, respectively, by irradiating the measuring light and saturating pulse. Thereafter, the steady fluorescence (F') and maximum fluorescence in the steady state (Fₘ') were determined under actinic light irradiation. The maximum (Fᵥ/Fₘ) and operating (Fₚ'/Fₘ') quantum efficiencies of PSII were obtained using the following equations (Baker, 2008; Sonoike, 2009).

$$\frac{F'_v}{F_m} = \frac{(F_m - F_0)}{F_m}$$
$$\frac{F'_p}{F'_m} = \frac{(F'_m - F')}{F'_m}$$

4. Leaf inclination angle and relative light intensity measurements

The leaf inclination angle at the bottom and middle portion of leaf blade between the main stem (vertical axis) were measured using a protractor. The relative light intensity, which was the intensity relative to that of the top of main stem at the middle portion of leaf blade, was measured using a photon sensor attached to the fluorometer. Both measurements were conducted at the heading stage (for 16th to 10th leaves) each with five replicate plants.

5. Ascorbic acid measurements

At the heading time, each of four flag (16th), 15th, 14th, and 13th leaves were sampled and used for measurements of ascorbic acid (reduced form, AA; oxidized form, DHA). Immediately after measurements of the fresh weight and leaf area, leaves were frozen in liquid N₂ and ground with a mortar and pestle by adding metaphosphoric acid to obtain leaf extracts. Then AA and DHA were determined by the hydrazine method (Fujita and Yamada, 2006) using a spectrophotometer (Ubest-30, JASCO Corp., Tokyo, Japan), with L(+)ascorbic acid (Kanto Chemical Co. Inc., Tokyo, Japan) 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 is obtainable. The AA content was calculated by subtraction of DHA from total ascorbic acid. The redox state (RDS) of ascorbic acid was calculated as AA / (AA + DHA). Using five separate set of plants, the leaf area was measured with an automatic area meter (LI-3100C, LI-COR Biosciences, Lincoln, NB, USA) and the leaf dry weight, after oven-drying at 80°C for 48 hr.

6. Statistical analysis

All photosynthesis-related data were subjected to a two-way analysis of variance (ANOVA) using a software package (Excel Statistics 2010 for Windows, Social Survey Research Information Co., Ltd., Tokyo, Japan). Because of the lack of chamber replications, the O₃ effect was not able to separate from the chamber effect and therefore, the former could be biased. However, the chamber effect did not seem large from our experience on environment regulation. The significance among treatments in each time interval was
determined using the least significant difference test (LSD, \( P \leq 0.05 \)).

Results

1. Effects of O₃ and leaf inclination angle on gas exchange parameters

Comparison of the HO⁰ plants with the EO⁰ plants in Fig. 2 revealed that the \( P_N \) and \( g_s \) were unaffected by attaching lead weights during the measurement periods. At AE-0, the \( P_N \) of the EO⁰.1, HO⁰.1, EO⁰.3, and HO⁰.3 plants decreased to 75, 71, 63, and 55%, respectively, of that at BE. Thereafter, the O₃-inhibition of \( P_N \) began to recover at AE-1: the EO⁰.1, HO⁰.1, EO⁰.3, and HO⁰.3 plants recovered to 92, 89, 89, and 80%, respectively, of that at BE. At AE-3, the O₃-inhibition recovered fully in the EO⁰.1 and HO⁰.1 plants. However, the inhibition remained slightly (-4% relative to BE) in the EO⁰.3 plants and substantially (-14% relative to BE) in the HO⁰.3 plants, respectively. The two-way ANOVA (Table 1) for \( P_N \) indicated clear negative effects of O₃ from AE-0 to AE-3. In contrast, the vertical leaf inclination gave positive effects on \( P_N \) and mitigated the O₃-inhibition of \( P_N \) at AE-1 and AE-3.

The \( g_s \) of the EO⁰.1, HO⁰.1, EO⁰.3, and HO⁰.3 plants at AE-0 decreased to 54, 54, 39, and 34%, respectively, of that at BE. Thereafter, the O₃-inhibition of \( g_s \) began to recover at AE-1 as in the case of \( P_N \). The EO⁰.1, EO⁰.3, and HO⁰.3 plants attained 89, 87, and 83%, respectively, of that at BE. At AE-3, the O₃-inhibition recovered fully in the EO⁰.1 and HO⁰.1 plants. However, the inhibition remained in the EO⁰.3 and HO⁰.3 plants (-17 and -18% of BE) (Fig. 2b). The two-way ANOVA (Table 1) for \( g_s \) indicated a clear negative effect of O₃ from AE-0 to AE-3.

2. Effects of O₃ and leaf inclination angle on photosystem II

Under the O₃-free condition, the \( F_v/F_m \) and \( F_q'/F_m' \) were unaffected by attaching lead weights during the measurement periods (Figs. 3a and 3b; HO⁰ vs. EO⁰). At AE-0, the \( F_v/F_m \) of the HO⁰.3 plants decreased to 92% of that at BE, but no significant effect was found in other treatments (Fig. 3a). The two-way ANOVA (Table 1) for \( F_v/F_m \) indicated that the O₃ had a negative effect at AE-0 and then recovered to the standard level by AE-1 in clean air. The vertical leaf inclination interacted positively with O₃ at AE-0.

The \( F_q'/F_m' \) of the EO⁰.3 and HO⁰.3 plants decreased to 78 and 74%, respectively, of that at BE at AE-0. These remained, but were not significantly different from those at BE at AE-1 and AE-3 (Fig. 3b). The two-way ANOVA (Table 1) for

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### Table 1. Results of statistical analyses of the effects of O₃ and leaf inclination angle 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 PSII in the flag leaf of rice shown in Figs. 2 and 3. AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, and 1 and 3 d after gas exposure.

<table>
<thead>
<tr>
<th>Time Factor</th>
<th>( P_N )</th>
<th>( g_s )</th>
<th>( F_v/F_m )</th>
<th>( F_q'/F_m' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE-0 O₃</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Leaf inclination</td>
<td>n.s.</td>
<td>n.s.</td>
<td>**</td>
<td>n.s.</td>
</tr>
<tr>
<td>O₃ × Leaf inclination</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
</tr>
<tr>
<td>AE-1 O₃</td>
<td>**</td>
<td>**</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leaf inclination</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>O₃ × Leaf inclination</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>AE-3 O₃</td>
<td>**</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leaf inclination</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>O₃ × Leaf inclination</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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Fig. 3. Effects of O₃ and inclination angle on the maximum quantum efficiency (\( F_v/F_m \)) (a) and operating efficiency (\( F_q'/F_m' \)) (b) of PSII in rice leaves (\( n = 5 \)). Vertical bars represent LSD (\( P \leq 0.05 \)). BE, AE-0, AE-1, and AE-3 respectively indicate before, immediately after, and 1 and 3 d after gas exposure. ●, EO⁰; ○, HO⁰; ▲, EO⁰.1; △, HO⁰.1; ■, EO⁰.3; □, HO⁰.3.
Fq'/Fm' indicated a negative effect of O₃ at AE-0 and AE-1.

**Positioned leaves (not significant at**

** Contents per leaf area were not significantly different**

** The DHA and RDS of ascorbic acid expressed per leaf area,**

** Generally, the photoinhibition of photosynthesis is**

** Induction of reactive oxygen species (e.g., superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical) and cause oxidative stress to plants (Taiz and Zeiger, 2010), the stress-induced damage is more severe if such factors occur concomitantly (Mittler, 2006).**

** Table 2. Content (mmol m⁻²) of total, reduced form (AA) and oxidized form (DHA) of ascorbic acid and its redox state (RDS) in the 16th (flag leaf) – 13th leaves. In each column, means (n=4) followed by the same letter are not significantly different at P ≤ 0.05 level as inferred from results of one-way ANOVA.**

<table>
<thead>
<tr>
<th>Leaf position</th>
<th>Total AA</th>
<th>DHA</th>
<th>RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>per leaf area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16th (flag leaf)</td>
<td>1.38 a</td>
<td>1.03 a</td>
<td>0.36 a</td>
</tr>
<tr>
<td>15th</td>
<td>1.17 b</td>
<td>0.77 b</td>
<td>0.39 a</td>
</tr>
<tr>
<td>14th</td>
<td>1.15 b</td>
<td>0.63 b</td>
<td>0.52 a</td>
</tr>
<tr>
<td>13th</td>
<td>1.14 b</td>
<td>0.65 b</td>
<td>0.48 a</td>
</tr>
</tbody>
</table>

**Discussion**

Imai and Kobori (2008) reported that the inhibition of P₅ in rice leaves by short exposure to O₃ was explained considerably by decreased gₛ, and at a high O₃ concentration, irreversible dysfunction of guard and mesophyll cells occurred. Furthermore, Kobayakawa and Imai (2011a) showed that the P₅, PS II and gₛ of flag (16th) leaf at the heading stage were inhibited by O₃ treatment but their damage was smaller and their recovery was faster than those in the topmost, fully expanded eighth leaf at the vegetative stage. Therefore, they interpreted that the difference in leaf inclination angle and / or antioxidant content were associated with the difference in inhibition by O₃. In the present experiment, the total ascorbic acid content per leaf area of the flag leaf was slightly higher than that of the lower (15th – 13th) leaves (Table 2). However, such small difference was unable to explain the mitigation of inhibition by O₃, although it seemed not meaningless.

We examined whether or not the leaf inclination angle was related to the difference in inhibition as the other possibility of the mitigation of O₃-inhibition in the present experiment. Because the leaf inclination of rice becomes steeper at late growth stages (Ito et al., 1973), it must affect the radiation interception of individual leaves, especially when plants were isolated situation such like the present experiment. To date, few studies show that the light intensity on the leaf surface is involved in the response to O₃ of plants. In spring wheat, Mulholland et al. (1997) reported that the O₃-induced inhibition of P₅ in the eighth (flag) leaf was lighter than that in the fifth to seventh leaves. They ascribed this difference to the higher contents of active ribulose 1, 5-bisphosphate carboxylase / oxygenase and the absence of shade acclimation of the flag leaf. In addition, in primary leaves of pinto bean (Phaseolus vulgaris), Guidi et al. (2000) observed that the inhibition of P₅ and PS II activities by O₃ was more pronounced under higher light intensities (30 – 1000 μmol m⁻² s⁻¹ PPFD). Because abiotic stresses such as high light intensity and O₃ induce the production of reactive oxygen species (e.g., superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical) and cause oxidative stress to plants (Taiz and Zeiger, 2010), the stress-induced damage is more severe if such factors occur concomitantly (Mittler, 2006). Generally, the photoinhibition of photosynthesis is induced under high intensity light. However, if the plant is
exposed to some other stress that induces the oxidative one, such inhibition may be induced even at low light intensity. Therefore, the light intensity might be an important factor to determine the sensitivity to O₃ of a plant (Fiscus et al., 2005). Along with more erect leaves, the relative light intensity of the leaf surface decreases. Therefore, the relative light intensity of the flag leaf is lower than that of other leaves during daytime. The damage of leaf photosynthesis in the horizontal leaf (H) plant must be severer than that in the erect leaf (E) plant if the difference in intercepted radiation is involved in the extent of O₃ inhibition.

The Pₙ of the O₆¹ and O₆² plants decreased, but damage of the HO₆¹ and HO₆³ plants was severer than that of the E plants (Fig. 2a). Coincident with our previous study (Kobayakawa and Imai, 2011a), the Fₚ'/Fₚ was unaffected by O₃ in the flag leaf of the EO₆¹ and EO₆² plants, but that in the HO₆¹ plants decreased at AE-0. Furthermore, the Fₚ'/Fₚ of the EO₆³ or H plants decreased at AE-0, and the value of HO₆² plants was lower than that of the EO₆³ plants, although it was not significantly different (Fig. 3). These results indicated that the damage of PSIⅡ incurred by the flag leaf in the H plants was severer than that in the E plants (erect flag leaf). Consequently, the damage of carbon fixation reaction in the H plants was also severer, and the recovery was slower than that in the E plants. However, under the same O₃ concentration, the gₑ of E and H plants was similarly inhibited at AE-0 (Fig. 2b). This indicated that the O₃ negatively affected the guard cell function, as reported by Torsethaugen et al. (1999) who clarified that O₃ inhibited the opening of stomata through the guard cell K⁺ channel.

As shown in Fig. 4, the leaf inclination angle and relative light intensity of the flag leaf were lower than that of the lower leaves (15th to 10th). The O₃-induced damage of photosynthesis in the flag leaf of HO₆⁻³ plant was severer than that of the EO₆⁻³ plant in this study (Figs. 2 and 3). Thus, we conclude that the leaf inclination angle is a major determinant of the O₃ inhibition in our case. In paddy field conditions, however, individual rice plants are affected by mutual shading during growth and receive considerably different incident light within the canopy (San-oh et al., 2008). Furthermore, the profile of canopy O₃ concentration may be complex. Therefore, to elucidate actual O₃-inhibition of photosynthesis-related processes in rice, especially that growing in urban fringe areas, further studies under canopy levels are required.

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* In Japanese.
** In Japanese with English abstract.
*** In Japanese with English summary.