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Effects of the interaction between ozone and carbon dioxide on gas exchange, ascorbic acid content, and visible leaf symptoms in rice leaves

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Abstract

Tropospheric ozone (O_3) decreases photosynthesis, growth, and yield of crop plants, while elevated carbon dioxide (CO_2) has the opposite effect. The net photosynthetic rate (P_N), dark respiration rate (R_D), and ascorbic acid content of rice leaves were examined under combinations of O_3 (0, 0.1, or 0.3 cm^3 m^{-3}, expressed as O^0, $O^{0.1}$, $O^{0.3}$, respectively) and CO_2 (400 or 800 cm^3 m^{-3}, expressed as C^{400} or C^{800}, respectively). The P_N declined immediately after O_3 fumigation, and was larger under $O^{0.3}$ than under $O^{0.1}$. When C^{800} was combined with the O_3, P_N was unaffected by $O^{0.1}$ and there was an approximately 20 % decrease when the rice leaves were exposed to $O^{0.3}$ for 3 h. The depression of stomatal conductance (g_s) observed under $O^{0.1}$ was accelerated by C^{800}, and that under $O^{0.3}$ did not change because the decline under $O^{0.3}$ was too large. Excluding the stomatal effect, the mesophyll P_N was suppressed only by $O^{0.3}$, but was substantially ameliorated when C^{800} was combined. Ozone fumigation boosted the R_D value, whereas C^{800} suppressed it. An appreciable reduction of ascorbic acid occurred when the leaves were fumigated with $O^{0.3}$, but the reduction was partially ameliorated by C^{800}. The degree of visible leaf symptoms coincided with the effect of the interaction between O_3 and CO_2 on P_N. The amelioration of O_3 injury by elevated CO_2 was largely attributed to the restriction of O_3 intake by the leaves with stomatal closure, and partly to the maintenance of the scavenge system for reactive oxygen species that entered the leaf mesophyll, as well as the promotion of the P_N.

Additional key words: dark respiration; net photosynthesis; *Oryza sativa*; photochemical oxidant; stomatal conductance.

Introduction

Stratospheric ozone (O_3) protects us from ultraviolet radiation. In contrast, tropospheric O_3 is a health hazard for us. It attacks all plant species and decreases their photosynthesis and growth rates, concomitantly with white-dot symptoms on the leaves of herbaceous species and reddish-to-black-dot symptoms on the leaves of arboreous, leguminous, and gramineous species (Hill and Littlefield 1969, Nouchi 2001, Izuta 2003, Xu *et al*. 2007) and, in the cell, it also damages organelles and biopolymers, including DNA (Roshchina and Roshchina 2003).

In Japan, ever since visible injury of plants by photochemical oxidants was first confirmed in 1970, the generation of such injury by high concentrations of photochemical oxidants became frequent in parallel with industrial development, especially around urban areas. The Japanese environmental quality standard for photochemical oxidant concentration is lower than 0.06 cm^3 m^{-3} h^{-1}, and when it exceeds 0.12 and 0.24 cm^3 m^{-3} h^{-1}, local public bodies must, under the regulations, dispatch a warning and an alarm, respectively (Izuta 2003). Recently, hourly peak values have often been close to 0.2 cm^3 m^{-3}. Because more than 90 % of photochemical oxidants consist of O_3, their appearance on sunny summer days during the rice growing season is harmful for the photosynthesis and yield of this staple crop. A considerable number of research studies on O_3-induced injury of rice crops have been conducted, and substantial anatomical, physiological, and biochemical evidence has been accumulated (Nakamura *et al*. 1975, Jeong *et al*. 1980, Toyama *et al*. 1989, Nouchi 1993), but without

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Abbreviations: AA – L-ascorbic acid; C_i – intercellular CO_2 concentration; DHA – dehydro-L-ascorbic acid; g_s – stomatal conductance; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; R_D – dark respiration rate; RDS – redox state of ascorbic acid.
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satisfactory implication and countermeasures (Nouchi 2001, 2003). On the other hand, the atmospheric CO_2 concentration is increasing due to the huge consumption of fossil fuels, deforestation, and other human activities. Because CO_2 is a substrate for the photosynthesis reaction, elevated CO_2 concentrations promote photosynthesis and yield of many C_3-crop species, including rice (Kimball 1983, Imai 1993, Horie *et al*. 2000). At the same time, elevated CO_2 concentrations induce stomatal closure due to increased intercellular CO_2 concentration (C_i), and this contributes to the restriction of the invasion of O_3 into the leaf cavity and mesophyll cells (Morison and Gifford 1983, Jaspers *et al*. 2005).

So far, the effects of O_3 and CO_2 on the photosynthesis, growth, and yield of important economic plants such as wheat (Rao *et al*. 1995, Rudorff *et al*. 1996, Mulholland *et al*. 1997, Donnelly *et al*. 2000, Cardoso-Vilhena *et al*. 2004, Feng *et al*. 2007), potato (Lawson *et al*. 2001, Vandermeiren *et al*. 2002), soybean (Bernacchi *et al*. 2006), and peanut (Booker *et al*. 2007) have been studied. However, the underlying mechanisms of the interaction between O_3 and CO_2 still remain uncertain (Unthworth and Hogsett 1996, Lee 2000, Morita and Tanaka 2002, Vandermeiren *et al.* 2002, Cardoso-Vilhena *et al*. 2004, Weigel 2004). Therefore, we wanted to know how detrimental O_3 and promotive CO_2 interact and thereby affect the photosynthesis and production processes of rice, because observations of the combined effect of O_3 and elevated CO_2 on this Asian staple food crop are few and limited only to its growth response (Olszyk and Wise 1997, Ookoshi and Imai 1998). We hypothesized that the detrimental effects of O_3 would be ameliorated by elevated CO_2 through its main effect on the stomata. At the standard CO_2 concentration (*ca*. 400 cm^3 m^{-3}), a high O_3 concentration (higher than 0.1 cm^3 m^{-3}) would decrease rice photosynthesis by a direct and/or indirect oxidizing effect, O_3 being a reactive oxygen species. At elevated CO_2 concentrations (*ca*. 800 cm^3 m^{-3}), however, the stomatal closure caused by this concentration would protect the diffusion of O_3 into the stomatal cavity, while the interior leaf space would be kept at a relatively high CO_2 concentration, and this would maintain the photosynthetic activity at a normal level. As there is accumulating evidence that naturally occurring antioxidants, especially apoplastic ones, are involved in the mechanism of protection from O_3 injury (Barnes *et al*. 2002, Davey *et al*. 2002, Morita and Tanaka 2002, Baier *et al*. 2005, Jaspers *et al*. 2005), we measured the content of ascorbic acid as one of these antioxidants and compared it with the degree of visible symptoms on the leaves of rice plants grown under combinations of O_3 and CO_2.

Materials and methods

Experiment 1: Rice (*Oryza sativa* L. cv. Koshihikari) seeds were sown in 1/5 000-are plastic pots containing 2.5 kg of dry soil and 6 g of chemical fertilizer (N, P_2O_5, K_2O = 15, 15, 15, %) and watered sufficiently. The plants were grown in an artificially-lit growth cabinet (*HLN-35DA*, *Koito Industries*, Yokohama, Japan) under the following conditions: 12-h day/12-h night cycle, 28/23 ºC, and 60 % relative humidity (RH). Radiation (700 µmol m^{-2} s^{-1} PPFD at the plant height) was applied from metal-halide (*D400*, *Toshiba Lighting & Technology*, Tokyo, Japan + *MLBOC400C-U*, *Mitsubishi Electric Osram*, Yokohama, Japan) and fluorescent (*FLW40SW*, *Matsushita Electric Industrial Co.*, Tokyo, Japan) lamps. On the 11th day after sowing, the plants were thinned to one per pot and grown under submerged conditions (depth: 3 cm). Just after the 6th leaves expanded fully, the plants were transferred into natural-light gas-exposure chambers (*S-2003A*, *Koito Industries*, Yokohama, Japan) and acclimated for 24 h under the following conditions: 28/23 ºC, 60 % RH, 0 cm^3 m^{-3} O_3 (O^0) and 400 cm^3 m^{-3} CO_2 (C^{400}). Three-hour gas exposure treatments were conducted under combinations of O_3 and CO_2 at the following concentrations: O^0+C^{400} (control plot), O^0+C^{800}, $O^{0.1}$ or $O^{0.3}$+C^{400}, and $O^{0.1}$ or $O^{0.3}$+C^{800}. O_3 was supplied by a high-voltage ozone generator (*MO-5A*, *Ozone System*, Tokyo, Japan) and CO_2 was supplied from cylinders with liquid CO_2. The gases were injected into the air filtered through activated charcoal layers. The concentrations of O_3 and CO_2 were measured and computer-controlled by an ultraviolet absorption-type O_3 analyzer (*EG-2001F*, *Ebara Jitsugyo*, Tokyo, Japan) and an infrared CO_2 analyzer (*ZRC-1DF51*, *Fuji Electric Systems*, Tokyo, Japan), respectively. The actual concentration of O_3 in the O^0 plot was lower than 0.003 cm^3 m^{-3}.

In situ gas exchange measurements were conducted just before gas exposure and 1, 2, and 3 h after the start of gas exposure with a portable photosynthesis and transpiration measurement system (*LI-6400*, *LI-COR*, Lincoln, NE, USA) for the 6th leaves on the main stem. The conditions other than those of the gas exposure were as follows: 28 °C leaf temperature, 1.5 kPa vapour saturation deficit, and 1 500 µmol m^{-2} s^{-1} PPFD (mixed light from red and blue LEDs). The above mentioned treatments and measurements were performed during sunny daytimes, and 4 replicated data sets were obtained for each of the following parameters: net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and C_i.

Experiment 2: Rice plants were solution cultured using 1/5 000-are plastic pots containing 3 500 cm^3 of Kimura's B solution at pH 5.5 (Imai 1981). The combinations of environmental factors were the same as in Exp. 1. In Exp. 2, the plants were exposed to O_3 and/or CO_2 for 3 h, and afterward they were kept under charcoal-filtered, clean air (O^0+C^{400}) for 21 h. The measurement of P_N in

attached, fully-expanded 6th leaves was conducted just before gas exposure, and 0, 3, and 21 h after the termination of gas exposure. The dark respiration rate (R_D) was examined 6 h after the termination of gas exposure. In this experiment, the contents of ascorbic acid as a naturally occurring antioxidant (reduced form: AA; oxidized form: DHA) were determined by the hydrazine method (Roe and Oesterling 1944, Kubo *et al.* 1994). The 4th and 6th rice leaves from the bottom of the main stem were harvested just before the gas exposure, and 0, 3, and 21 h after the termination of gas exposure. After measuring the areas and fresh masses of the leaves, the ascorbic acid was extracted and determined spectrophotometrically (*Ubest-30*, *JASCO Co.*, Tokyo, Japan).

Experiment 3: Soil-cultured rice plants at the 9th fully-expanded-leaf stage were treated under the same conditions as those in Exp. 2. Photographs of adaxial and abaxial leaf surfaces were taken at 0, 21, and 72 h after the termination of gas exposure.

Statistical analysis of data, including the means, standard errors, and least significant difference (LSD) test, was made using a statistical package (*Excel Statistics 2004 for Windows*, *Social Survey Research Information Co.*, Tokyo, Japan).

Results

Exp. 1.

P_N* and *g_s: As expected, the P_N of the control plot remained fundamentally unchanged during the 3-h gas exposure (Fig. 1*A*). The P_N of the O^0+C^{400} plot decreased to 88 %, and afterward recovered to 94 % of the initial value. The P_N of the O^0+C^{800} plot increased by 16 % of the initial value during the first hour and thereafter maintained this elevated level. The P_N of the $O^{0.1}$+C^{800} plot increased by 14 % of the initial value during the first hour and became similar to that of the O^0+C^{800} plot. At 1 and 2 h of gas exposure, the P_N values of all plots were significantly different from that of the control plot, but at 3 h the difference between the $O^{0.1}$+C^{400} plot and control plot was reduced. Throughout the treatment time, no difference in P_N between the O^0+C^{800} plot and the $O^{0.1}$+C^{800} plot was observed. The g_s of the control plot gradually increased, and after 3 h of treatment, the increment was 8 % of the initial value (Fig. 1*C*). Under $O^{0.1}$+C^{400}, the g_s declined to 68 % of the initial value after 1 h of gas exposure, but gradually recovered and reached 86 % during the next 2 h. The g_s of the O^0+C^{800} plot decreased to 80 % of the initial value after 1 h of gas exposure, and further decreased to 71 % during the subsequent 2 h. The g_s of the $O^{0.1}$+C^{800} plot decreased to 44 and 54 % of the initial value after 1 and 3 h of treatment, respectively. Statistical differences in g_s were observed between all plots until 3 h from the start of gas exposure, except between the $O^{0.1}$+C^{400} plot and the O^0+C^{800} plots at 1 and 2 h.

The $O^{0.3}$ concentration had severe effects on the P_N and g_s of the rice leaves compared to $O^{0.1}$. The P_N values of the $O^{0.3}$+C^{400} and $O^{0.3}$+C^{800} plots decreased to 44 and 87 %, respectively, of the initial values, and afterward recovered slightly (Fig. 1*B*). At 1 and 2 h, the P_N values of the control plot and the $O^{0.3}$+C^{800} plot were significantly different, but at 3 h the significance disappeared. The g_s values of both the $O^{0.3}$+C^{400} plot and the $O^{0.3}$+C^{800} plot immediately decreased to 20 % of the initial values (at 1 h) and gradually recovered to 26 % during the next 2 h (Fig. 1*D*). Throughout the gas exposure treatments, significant differences in g_s were observed between the plots, except between the $O^{0.3}$+C^{400} plot and the $O^{0.3}$+C^{800} plot.

Relationship between *P_N* and *C_i*: To eliminate the effect of the stomata and to evaluate the inhibitory effect of O_3 on mesophyll activity, we obtained the C_i-response curves of P_N for the control plot by changing the CO_2 concentration from 30 to 1 200 cm^3 m^{-3} immediately after the 3-h gas exposure and compared the P_N values of the $O^{0.1}$ plot and the $O^{0.3}$ plot.

Under $O^{0.1}$, there was no actual decline in P_N in any of the treated plots (Fig. 2*A*). Under $O^{0.3}$, on the other hand, there were substantial declines in P_N in both the C^{400} and the C^{800} plots (Fig. 2*B*). However, the decline in the $O^{0.3}$+C^{800} plot was about half of that in the $O^{0.3}$+C^{400} plot.

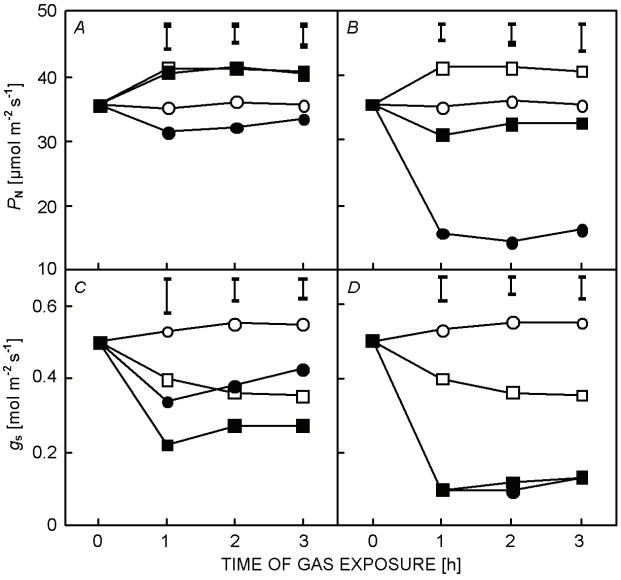

Fig. 1. Effect of various O_3 and CO_2 concentrations [cm^3 m^{-3}] on the net photosynthetic rate (P_N) and stomatal conductance (g_s) in rice leaves (Exp. 1). *Vertical bars* indicate LSD 0.05. *A, C*: ○, O^0+C^{400}; ●, $O^{0.1}$+C^{400}; □, O^0+C^{800}; ■, $O^{0.1}$+C^{800}. *B, D*: ○, O^0+C^{400}; ●, $O^{0.3}$+C^{400}; □, O^0+C^{800}; ■, $O^{0.3}$+C^{800}. For the combinations of O_3 and CO_2, see Materials and methods.

Exp. 2.

Inhibition and recovery of P_N and g_s: Table 1 shows the inhibition rates of P_N and g_s after 3 h of O_3 and CO_2 treatments and their recovery rates under charcoal-filtered, clean air (O^0+C^{400}) at 3 and 21 h after the termination of treatment.

The P_N underwent a 12.0 % inhibition under $O^{0.1}$ and a 40.5 % inhibition under $O^{0.3}$, both with C^{400}. However, under C^{800}, the P_N underwent a 14.9 % boost under $O^{0.1}$ and an 8.8 % boost under $O^{0.3}$. With time and under normal air (O^0+C^{400}), the inhibition of P_N in the $O^{0.1}$+C^{400} plot recovered rapidly, but the $O^{0.3}$+C^{400} plot did not regain the value before the treatment. These facts indicate that the photosynthetic ability was hardly affected by $O^{0.1}$ and was substantially suppressed by $O^{0.3}$ but ameliorated by C^{800} (Fig. 2*A,B*).

The g_s declined under both the $O^{0.1}$ and $O^{0.3}$ treatments. Under $O^{0.1}$, the g_s declined further in combination with C^{800}, but when the plants were exposed to charcoal-filtered air (O^0+C^{400}), it almost recovered to the value before the treatment. Under $O^{0.3}$, the initial decline of g_s was not different between C^{400} and C^{800} but the recovery process was different. The $O^{0.3}$+C^{400} plot did not reach the value before the treatment, but the $O^{0.3}$+C^{800} plot recovered almost completely 21 h after the termination of treatment (Table 1).

***R_D* and *g_s* in the dark**: With increasing O_3 concentration during the photoperiod, the R_D was affected substantially (Fig. 3). When the plants were kept in clean air (O^0+C^{400}) for 6 h after the termination of gas exposure, elevated O_3 concentration increased the R_D whereas C^{800} suppressed it. The g_s was similarly affected by O_3 and CO_2, and the after-effect of $O^{0.3}$ was larger than that for R_D.

Ascorbic acid content (Table 2): The total content [reduced (AA) + oxidized (DHA)] in 6th leaves tended to decrease with increasing O_3 concentrations at 0, 3, and 21 h. It decreased especially under $O^{0.3}$+C^{400}. However, this decline was partly ameliorated by C^{800}. The AA showed a trend similar to that of the total content. Under C^{400}, the content of the DHA increased slightly with $O^{0.1}$, but the tendency was not obvious with $O^{0.3}$. Under C^{800}, the DHA content tended to increase with O^0 but decreased with $O^{0.1}$ and $O^{0.3}$. The content of DHA was hardly ameliorated by C^{800}. The redox state of ascorbic acid (RDS = AA/total) was generally steady, but decreased slightly in all the treatment plots 21 h after the gas exposure as compared to the control plot (O^0+C^{400}). We found in the 4th leaves exposed to O_3 and CO_2 rather low ascorbic acid contents and responses similar to those obtained in the 6th leaves (data not shown).

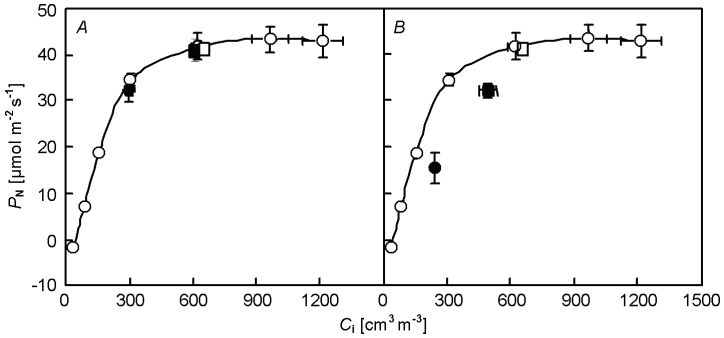

Fig. 2. Effect of various O_3 and CO_2 concentrations on the relationship between the intercellular CO_2 concentration (C_i) and the P_N in rice leaves just after 3 h of gas exposure (Exp. 1). *Vertical and horizontal bars* indicate standard errors of the mean. *A*: ○, O^0+C^{400}; ●, $O^{0.1}$+C^{400}; □, O^0+C^{800}; ■, $O^{0.1}$+C^{800}. *B*: ○, O^0+C^{400}; ●, $O^{0.3}$+C^{400}; □, O^0+C^{800}; ■, $O^{0.3}$+C^{800}.

Table 1. Inhibitions of net photosynthetic rate (P_N) and stomatal conductance (g_s) under various combinations of O_3 and CO_2 concentrations, and their recovery under clean air (Exp. 2). [1] [(A – B)/A]×100, [2] (C/A)×100, [3] (D/A)×100. P_N and g_s of rice leaves were kept and measured under (A) O^0+C^{400} just before gas exposure; (B) combinations of O_3 and CO_2 after 3 h of gas exposure; (C), (D) O^0+C^{400} 3 and 21 h after the termination of gas exposure, respectively. For explanation of symbols see the text.

O_3	CO_2	Inhibition [%][1]		Recovery [%][2]		Recovery [%][3]	
		P_N	g_s	P_N	g_s	P_N	g_s
$O^{0.1}$	C^{400}	12.0±7.0	25.1±14.6	90.5±9.0	103.6±20.3	101.7±5.0	110.5±9.6
$O^{0.1}$	C^{800}	–14.9±2.4	34.3±10.7	96.9±3.1	101.9±15.7	98.1±4.0	95.9±7.5
$O^{0.3}$	C^{400}	40.5±4.1	66.1±10.3	75.9±7.5	66.2±15.4	85.6±8.8	86.0±20.2
$O^{0.3}$	C^{800}	–8.8±10.6	63.3±8.9	87.6±5.7	87.4±19.7	103.7±4.9	96.2±13.1

Exp. 3.

Visible leaf symptoms: No visible symptoms on the 9th leaves were observed just after O_3 exposure (0 h) in any of the treatments. Under C^{400}, slight visible symptoms with $O^{0.3}$ appeared at 21 h and developed further at 72 h (Fig. 4). However, these symptoms were not observed under C^{800}. The observed symptoms were red-brown dots located in the area from the middle portion to the tip of the leaf, and this pigmentation looked more severe on the adaxial leaf surface than on the abaxial surface.

Discussion

Hill and Littlefield (1969) exposed 13 crop species to O_3 for 30–120 min and observed the suppression of P_N, E, and stomatal aperture; the responses were reversible if the O_3 exposure was terminated, with the recovery process faster at lower O_3 than at higher O_3. They proposed three possible O_3 effects: (*a*) the suppression of the P_N and E during O_3 fumigation induces stomatal closure, (*b*) O_3 suppresses P_N and the stomatal aperture independently, or (*c*) O_3 counteracts CO_2 utilization and increased CO_2 concentration in the guard cell decreases stomatal aperture. Our experiments using rice were roughly similar to their research results in relation to the effects of O_3. We found that both the P_N and g_s decreased immediately after O_3 exposure (Fig. 1). This decline reflected the concentration of O_3 and was more severe under $O^{0.3}$ than under $O^{0.1}$. Therefore, we assume that the guard cells contacted with O_3 lost their turgor pressure, a process mediated by the inhibition of the K^+ channel (Torsethaugen *et al*. 1999), and the stomatal closure limited the intake of atmospheric CO_2, thereby suppressing the P_N, although, in wheat, the stomatal regulation of the O_3 influx is not sufficient under elevated CO_2 (Mulholland *et al*. 1997). In addition, cuticles are completely impermeable to O_3 as long as environmentally relevant concentrations go (Kerstiens 1996). Jeong *et al*. (1980) protected rice leaves from O_3-induced visible injury by applying abscisic acid (ABA) which induced stomatal closure, although Jaspers *et al*. (2005) have suggested that ABA plays roles other than stomatal regulation in the tolerance to O_3. As shown in Table 1, the P_N just after the 3 h of $O^{0.1}$+C^{400} exposure was 88.0 % of that before O_3 exposure, recovered to 90.5 % at 3 h, and reached 101.7 % 21 h after the termination of O_3 exposure. The 3-h exposure to $O^{0.3}$+C^{400} had a severe after-effect on P_N, whose values were 59.5, 75.9, and 85.6 % at 0, 3, and 21 h, respectively. The behaviour of g_s was similar to that of P_N (Table 1). Under $O^{0.1}$+C^{400}, the decline in P_N was caused by the temporal closure of the stomata, and by the slight damage, if any, to mesophyll function. However, under $O^{0.3}$+C^{400}, the photosynthetic ability was temporarily but substantially decreased. This was further clarified when we compared the C_i-response curves of the P_N, in which the stomatal effect was eliminated (Fig. 2). High concentrations of O_3, such as $O^{0.3}$, caused damage to the photochemical mechanisms and/or carbon reduction systems of chloroplasts as well as damage to stomatal mechanics (Rao *et al*. 1995, Unthworth and Hogsett 1996, Torsethaugen *et al*. 1999, Oksanen *et al*. 2001, Wittig *et al*. 2007).

The increased R_D after O_3 exposure, especially in the $O^{0.3}$+C^{400} plot, seemed to reflect the restoration of cell components from injury (Fig. 3*A*). However, increased g_s was observed simultaneously (Fig. 3*B*). Therefore, these changes may mark the paralysis of guard-cell function such as the disorder of osmotic adjustment by O_3 as

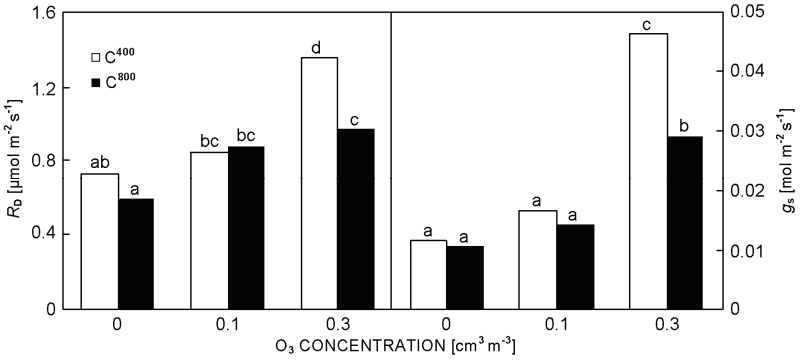

Fig. 3. Effect of the 3-h exposure to O_3 and CO_2 on the respiration rate (R_D) and g_s in rice leaves, which were kept for another 6 h in clean air with 400 cm^3 m^{-3} CO_2 (C^{400}) and measured in the dark (Exp. 2). The means followed by the same letter are not significantly different at LSD 0.05.

Table 2. Effects of O_3 and CO_2 concentrations on the ascorbic acid content [mmol kg^{-1}(FM)] and its redox state in rice leaves (Exp. 2). Before the gas exposure, the total ascorbic acid (Total), L-ascorbic acid (AA), and dehydro-L-ascorbic acid (DHA) contents and the redox state of ascorbic acid (RDS = AA/Total) were 12.45, 11.19, 1.25, and 0.90, respectively. In each column, the means followed by the same letter are not significantly different at the 0.05 LSD probability level.

		Time from the termination of 3-h gas exposure [h]											
		0				3				21			
		Total	AA	DHA	RDS	Total	AA	DHA	RDS	Total	AA	DHA	RDS
O^0	C^{400}	13.34a	12.29a	1.05abc	0.92ab	13.04a	12.27a	0.77ab	0.94a	11.49a	10.62a	0.88bc	0.92a
	C^{800}	12.71a	11.50ab	1.21ab	0.90ab	12.48ab	11.65ab	0.83ab	0.93a	11.07a	9.80ab	1.27a	0.88b
$O^{0.1}$	C^{400}	12.57ab	11.20b	1.36a	0.89b	12.56ab	11.57ab	0.99a	0.92a	10.07ab	8.87bc	1.20ab	0.88b
	C^{800}	12.36ab	11.51ab	0.82bc	0.93a	11.70b	10.71b	0.99a	0.92a	10.14ab	8.84bc	1.30a	0.87b
$O^{0.3}$	C^{400}	10.86c	9.94c	0.91bc	0.92ab	9.35c	8.53c	0.82ab	0.91a	6.40c	5.63d	0.77c	0.88b
	C^{800}	11.43bc	10.70bc	0.73c	0.94a	9.64c	9.06c	0.58b	0.94a	8.56b	7.75c	0.81c	090ab

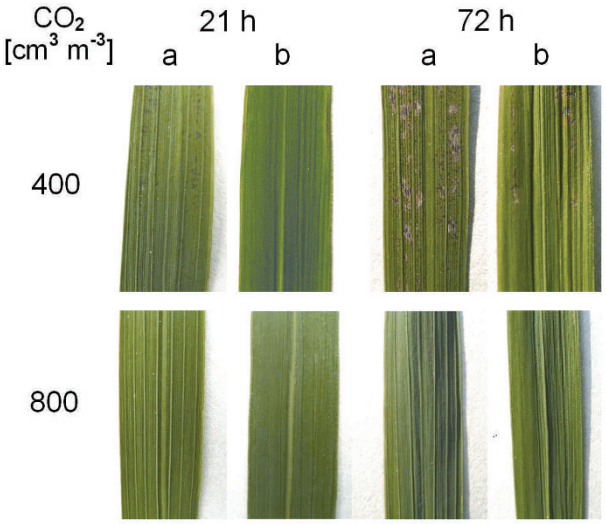

Fig. 4. Visible symptoms on the adaxial (a) and abaxial (b) surfaces in rice leaves exposed to 0.3 cm^3 m^{-3} O_3 ($O^{0.3}$) with 400 (C^{400}) or 800 cm^3 m^{-3} CO_2 (C^{800}) for 3 h and afterward kept in clean air with C^{400} for 21 and 72 h (Exp. 3).

observed by scanning electron microscopy (Nakamura *et al*. 1975), where the stomatal opening in the dark increases CO_2 flux from the inside to the outside of the leaf.

Under C^{800}, the abovementioned situation changed depending on the O_3 concentration. As shown in Fig. 1*A*, C^{800} boosted the P_N and $O^{0.1}$ did not interfere with this boost. This was due primarily to the effect of C^{800} as a substrate. When the P_N values of the $O^{0.1}$+C^{400} plot and the $O^{0.1}$+C^{800} plot were plotted to the P_N-C_i curve obtained for normal air, no actual depression of mesophyll photosynthesis followed (Fig. 2*A*). However, this positive effect of C^{800} was nullified by $O^{0.3}$, although the absolute P_N was not very different from that of the control plot (Fig. 1*B*). When the P_N values of the $O^{0.3}$+C^{400} and $O^{0.3}$+C^{800} plots were plotted to the P_N-C_i curve obtained from the normal air (Fig. 2*B*), substantial depressions of mesophyll photosynthesis were found. The depression of P_N under C^{400} was about twice that under C^{800}. However, the recovery of P_N at 21 h after exposure to $O^{0.3}$+C^{400} was substantial, and that after exposure to $O^{0.3}$+C^{800} was perfect (Table 1).

Elevated CO_2 induces stomatal closure through increased C_i levels (Morison and Gifford 1983), thereby limiting the O_3 invasion inside the leaf mesophyll and reducing O_3 injury (Unthworth and Hogsett 1996, Olszyk and Wise 1997, Vandermeiren *et al*. 2002, Cardoso-Vilhena *et al*. 2004). In our experiment, both elevated O_3 and elevated CO_2 reduced the g_s, which decreased even further when these two gases were combined (Fig. 1*C,D*). However, under $O^{0.3}$, there were no actual differences in g_s when the gas was applied singly or in combination with C^{800}. If the decline in g_s caused by $O^{0.3}$ and C^{800} is additive and very large, it is possible that the effect of O_3 masks the effect of CO_2, and *vice versa*. The increase in R_D caused by $O^{0.3}$ was substantially suppressed by C^{800}, and this was coupled with suppressed g_s, *i.e*. with stomatal closure (Fig. 3*A,B*). The recovery of the P_N and g_s from the inhibition caused by $O^{0.3}$ was completed when concomitant C^{800} was present (Table 1). Therefore, elevated CO_2 protected the plants from guard-cell dysfunction, as well as promoting the mesophyll P_N. However, elevated CO_2 may have other roles to play during O_3 fumigation, such as the protection of the ribulose-1,5-bisphosphate carboxylase/oxygenase protein (Weigel 2004).

In plant tissues, there are scavenge systems for oxidants which induce injury to the plant body. Ascorbic acid is the principal antioxidant, and its role is not only to respond to free-radicals but also to affect enzyme activities related to oxidant scavenge systems (Foyer 1993, Smirnoff 2005). There is accumulating evidence that high contents of leaf ascorbic acid protect the plant from O_3 fumigation (Zheng *et al*. 2000, Barnes *et al*. 2002, Maddison *et al*. 2002, Morita and Tanaka 2002, Chen and Gallie 2005, Burkey *et al*. 2006). Nouchi (1993) exposed rice to 0.5 cm^3 m^{-3} O_3 and observed that, 2 h after the exposure, AA content had decreased and DHA content had increased, and this situation became more pronounced with time. In our experiment, the AA content decreased after 3 h of O_3 exposure and continued to decrease thereafter (Table 2). However, the DHA content was low, and its change did not affect the change in total ascorbic acid. Differences in growth environment, as well as plant and/or leaf age, may affect the ascorbic acid metabolism.

The contents of total ascorbic acid and AA under elevated O_3 and CO_2 were maintained relatively high, but they did not change appreciably after the termination of treatment (Table 2). Lee (2000) reviewed an experiment in which anti-oxidative enzyme activities and total ascorbic acid content were maintained under elevated O_3 and CO_2. As the plants grown under elevated CO_2 adjust their photosynthesis-related electron transfer system and produce more NADPH, the increased flow of NADPH to the antioxidative system may activate the ascorbate-glutathione cycle, and finally the RDS may be steadily high (Rao *et al*. 1995). In our experiment, the RDS was maintained close to the initial level when the rice leaves were treated with C^{800}, irrespective of O_3 concentration (Table 2). The protection of signal transduction toward hormonal and enzymatic regulation induced by O_3 may be one important role of elevated CO_2, in addition to the regulation of the ascorbate-glutathione cycle (Lee 2000, Morita and Tanaka 2002, Kangasjärvi *et al*. 2005).

Toyama *et al*. (1989) found in young rice plants exposed to $O^{0.1}$+C^{400} no symptom in the chloroplast structure of the 3rd leaves on the 1st day, but found a swelling of the thylakoid membrane on the 2nd day. Previously, we exposed rice plants to $O^{0.1}$ at the flowering stage and observed slightly visible symptoms on the leaves on the

2nd day of exposure (Ookoshi and Imai 1998). The symptoms induced by O_3 start to appear in the mesophyll tissues facing the stomatal cavity, after which they become visible (Nakamura *et al*. 1975). In the present experiment, such visible symptoms did not develop to any great extent under elevated O_3 and CO_2 (Fig. 4); similarly, Rao *et al*. (1995) reported a decrease in the injured leaf area and the suppression of chlorophyll destruction under elevated O_3 and CO_2. Thus, elevated CO_2 counteracts O_3 in terms of the development of visible symptoms on rice leaves. In a Mediterranean shrub, *Arbutus unedo*, visible symptoms caused by $O^{0.1}$ are not necessarily associated with mesophyll injury (Bussotti *et al*. 2003), but in rice visible symptoms did not accompany the decline in P_N, as observed 21 h after the termination of O_3 exposure (Table 2).

In conclusion, we confirmed that: (*a*) Elevated CO_2 counteracts with the inhibitory effect of O_3 by promoting the P_N under limited g_s, and its action is larger under low O_3 concentrations; (*b*) elevated O_3 concentrations such as $O^{0.3}$ induce visible leaf symptoms but if C^{800} is concomitant, the P_N and g_s recover almost completely after the termination of O_3 exposure; (*c*) after exposure to O_3, the R_D increases with increasing g_s, which indicates the partial disorder of guard cells, but if elevated CO_2 is concomitant, such changes are limited; (*d*) rapid AA increment does not occur during O_3 fumigation, and the RDS is also steady under elevated CO_2. Our current results include acute responses to fumigation with O_3, a photochemical oxidant, which often occurs in paddy fields around urban areas during the summer season. The suppression of P_N caused by elevated O_3 will reduce the production of photosynthates, and this will lead to poor rice biomass and yield, depending on the frequency of the appearance of this photochemical oxidant. However, such situations may be improved by a rapid increase in the atmospheric CO_2 concentration and the interaction of that CO_2 with O_3. Finally, this type of approach tends to yield clear results, and the analyses are easier to perform than those of cumulative, long-term exposure to O_3 and CO_2; this compensates for our lack of knowledge and makes future crop production more feasible.

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