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Mitigation mechanism of ozone-induced reduction in net photosynthesis of Bangladeshi wheat under soil salinity stress

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Abstract

To clarify the combined effects of O_3 and soil salinity on net photosynthetic rate, stomatal conductance, and radical scavenging system of Bangladeshi spring wheat cv. BAW1059, plants were grown in two soil salinity levels (irrigated with 0 and 150 mM NaCl solutions) and exposed to three O_3 concentrations [charcoal-filtered air (CF), 1.0-fold the ambient O_3 concentration (1.0×O_3), and 1.5-fold the ambient O_3 concentration (1.5×O_3)]. The soil salinity mitigated adverse effects of O_3 on net photosynthesis in the 7th and flag leaves. The soil salinity did not induce stomatal closure, indicating no limitation of stomatal O_3 uptake. The activities of ascorbate peroxidase and catalase were stimulated by the soil salinity in the 7th and flag leaves, especially under 1.5×O_3 concentration. In the flag leaf, the soil salinity induced a significant increase in dehydroascorbate reductase activity and ascorbate concentration. These results suggest that the soil salinity activated detoxification capacity related to mitigation of O_3 damage on net photosynthesis in both 7th and flag leaves, while the activated enzymes and antioxidants were different between the leaves.

Additional key words: antioxidant; detoxification; leaf position; radical scavenging system; stomatal conductance.

Introduction

Elevated concentrations of ground-level ozone (O_3) result from photochemical reactions of the O_3 precursors, *i.e.*, nitrogen oxides (NO_x) and volatile organic compounds (VOC) (WHO 2006), and may pose a great threat to global food security and economy (Avnery *et al*. 2013). The current global surface O_3 concentration has more than doubled since the industrial revolution (WHO 2006). There is a probability that over the next 20–30 years, atmospheric concentrations of O_3 will increase especially in South Asia (Wild *et al*. 2012). Therefore, we need to evaluate the effects of O_3 on crops cultivated in South Asian countries such as Bangladesh, and implement countermeasure against the detrimental effects of O_3 on main crops such as rice and wheat.

Ozone absorbed into the leaves of crops through stomata produces reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\bullet}OH$) (Pell *et al*. 1997, Ainsworth *et al*. 2012). Although a part of ROS generated by O_3 can be detoxified in the leaves by radical-scavenging system, the remaining ROS cause oxidative stress in the leaf cells (Gill and Tuteja 2010). As a result, O_3 leads to reductions in net photosynthetic rate and other biochemical processes in the leaves, and finally in the growth and yield of crops (Akhtar *et al*. 2010, Gerosa *et al*. 2014). There are controversial empirical data on O_3-induced alteration of detoxification

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Abbreviations: 1.0×O_3 – 1.0-fold the ambient O_3 concentration; 1.5×O_3 – 1.5-fold the ambient O_3 concentration; AOT40 – accumulated O_3 exposure over a threshold of 40 ppb; APX – ascorbate peroxidase; AsA – reduced ascorbate; CAT – catalase; CF – charcoal-filtered air; DHA – oxidized ascorbate; DHAR – dehydroascorbate reductase; FM – fresh mass; GR – glutathione reductase; g_s – stomatal conductance to H_2O; GSH – reduced glutathione; GSSG – oxidized glutathione; MDAR – monodehydroascorbate reductase; NO_x – nitrogen oxides; P_N – net photosynthetic rate; PVPP – polyvinyl polypyrrolidone; ROS – reactive oxygen species; SOD – superoxide dismutase; VOC – volatile organic compounds.
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capacity in plants. The O_3-induced ROS scavenging mechanisms are mostly a matter of the concentrations or dose, frequency of the ozone exposure in relation to the genetic base of plants (Bellini and Tullio 2019). Gillespie *et al*. (2012) suggested that total antioxidant capacity of soybean plant was increased with chronic exposure to elevated O_3. Bortolin *et al*. (2014) found that elevated O_3 significantly decreased antioxidant defense capacity in red pepper plant. Moreover, in a FACE study, elevated O_3 consistently showed a declining trend of antioxidant pools and key enzymes of two different O_3-sensitive wheat cultivars (Wang *et al*. 2014). Although there is a lot of information on the effects of O_3 on physiological functions and detoxification capacity against ROS of crops cultivated in the USA and Europe, very limited information is available for crops cultivated in South Asian countries, such as Nepal, Pakistan, Bangladesh, Afghanistan, and Sri Lanka to date (Akhtar *et al*. 2010, Saitanis *et al*. 2014).

In Bangladesh, in addition to atmospheric O_3, soil salinity is recognized as a serious problem for production of crops such as wheat, which is one of the major grain crops in this country. Over 30% of cropland in Bangladesh is under various levels of soil salinity (Haque 2006). In general, soil salinity stress induces stomatal closure (Sharma *et al*. 2012), which can mitigate detrimental effects of O_3 on crops due to the limitation of stomatal O_3 uptake. On the other hand, the adverse effects of O_3 on crops might be enhanced by soil salinity-induced reduction in leaf antioxidant capacity (Zheng *et al*. 2012). Several studies reveal that increased salinity level enhances the activities of both enzymatic and nonenzymatic antioxidants in plants (Ghosh *et al*. 2011, Hasanuzzaman *et al*. 2011). Moreover, Zheng *et al*. (2011) found that the combined effects of O_3 and salinity increased antioxidant enzyme activity significantly only in the salt-sensitive wheat cultivar and projected a complementary defense for both stresses. Still, there is uncertainty about the antioxidant defense mechanisms under O_3 and salinity stress. Furthermore, information on the combined effects of O_3 and soil salinity on Bangladesh wheat cultivar and its defense mechanism are extremely limited to date.

In our previous study, we clarified the exposure to O_3 singly decreased net photosynthetic rate of flag leaves of BAW1059, which is the cultivar with relatively high yield under salinity stress (Kamal *et al*. 2015). On the other hand, there was no significant interactive effect of O_3 and soil salinity on net photosynthetic rate of the leaves at anthesis stage (Kamal *et al*. 2015). Several investigations also reported inconsistent effects of O_3 and/or salinity on different biochemical attributes and defense mechanisms at different growth stages (Ashraf *et al*. 2012, Rai and Agrawal 2014). Interactive effects of O_3 and soil salinity may be observed in the other growth stages, *e.g*., at the vegetative stage. Therefore, further research is needed to clarify the combined effects of O_3 and soil salinity on net photosynthesis of Bangladeshi wheat at different growth stages and across leaf positions.

In the present study, we hypothesized that soil salinity can affect the sensitivity of wheat to O_3 through its effects on stomatal O_3 uptake and/or leaf antioxidant capacity depending on the growth stages and leaf positions. In order to clarify this, we investigated net photosynthetic rate, stomatal conductance, and radical scavenging system of Bangladeshi wheat cultivar, BAW1059, under elevated O_3 and soil salinity stresses.

Materials and methods

Plant materials: In the present study, we used Bangladeshi spring wheat (*Triticum aestivum* L.) cultivar, BAW1059, which is one of the widely grown spring wheat cultivars in Bangladesh. Seeds of this wheat cultivar were obtained from Bangladesh Agricultural Research Institute (BARI). The cultivar has potential yields of 3.6–5.0 t ha^{-1} in Bangladesh (Barma *et al*. 2011). We adopted standard cultivation methods, such as preparation of the soil and fertilization, performed commonly in wheat fields in Bangladesh. On 31 March 2014, the seeds were sown in Wagner pots (volume: 12 L, diameter: 240 mm, and depth: 258.5 mm) filled with horticultural soil at three hills and three seeds per hill. The seedlings were thinned to leave one seedling per hill on 15 May. Accordingly, the final number of plants per pot was three. The textural class of the studied soil was sandy loam, having an initial soil pH 5.73. The potted soil contained similar amounts of N, P, and K (384 mg L^{-1}). Before sowing the seeds, 5 g of $CaCO_3$ mixed thoroughly in each pot to a depth of 15 cm, was added for correction of soil acidity. Before the gas exposure and soil salinity treatment, the seedlings were raised in open-air condition for 26 d from 31 March to 25 April 2014. During this period, average 12-h O_3 concentration (06:00–18:00 h) was 47.1 nL L^{-1} (ppb), and air temperature and relative air humidity fluctuated between 0–22.8°C and 13.8–97.6%, respectively.

Gas-exposure chambers: The experiment was conducted at the Field Museum Tamakyuryo of Tokyo University of Agriculture and Technology (35°38'N, 139°22'E) situated in Hachioji, Tokyo, Japan, from March to July 2014. Nine commercial-type vinyl greenhouses were used as gas-exposure chambers in the present study. The fumigation chambers (length: 3.6 m, width: 2.2 m, height: 2.0 m) were built with steel pipe construction covered with soft transparent polyvinyl chloride film (average light transmittance of 73%). PPFD under the ambient conditions (outside the chambers) was recorded at 1-min intervals using a quantum sensor (*LI-190SA*, *Li-Cor Inc*., Lincoln, NE, USA). In three of the nine chambers, temperature and humidity sensors (*TR-72U*, *T&D Corporation*, Nagano, Japan) were set to monitor the air temperature and relative air humidity inside the chambers at 10-min interval.

The air passed through an activated charcoal filter in the fan box (*EF-40DTB1*, *Mitsubishi Electric Co*., Tokyo, Japan) in order to remove O_3 from the ambient air, and introduced in each chamber at a flow rate of 1.03 m^3 s^{-1}. Ozone was produced by a silent electrical discharge O_3 generator (*OZC-05A*, *Dylec Inc*.) from oxygen-enriched dry air generated from the ambient air by a pressure-swing adsorption-type oxygen enricher (*SO-008S*, *Sanyo Electronic Inc*., Okayama, Japan). The O_3 was delivered

to the chamber after quick mixing with charcoal-filtered air through a high-speed fan. The O_3 concentrations in each chamber and in the ambient air were recorded at 30- and 10-min intervals, respectively, using UV absorption O_3 analyzer (*Model-1210*, *Dylec Inc*., Ibaraki, Japan) throughout the experimental period. For the control of atmospheric O_3 concentration in the O_3-exposure chambers, the ambient O_3 concentration was used as the standard level. Based on the O_3 concentration in the chambers, mean concentration and accumulated O_3 exposure over a threshold of 40 ppb (AOT40) were calculated.

Experimental design and treatment exposure: The experiment was designed as a split-split plot with three gas-exposure chamber replications. The whole-plot treatment comprised of three different O_3 concentrations using nine gas-exposure chambers: charcoal-filtered air (CF) (mean O_3 removal efficiency: 54%), 1.0-fold the ambient O_3 concentration ($1.0\times O_3$), and 1.5-fold the ambient O_3 concentration ($1.5\times O_3$). Two levels of saline irrigation (0 or 150 mM NaCl solution) embraced as sub-plot treatment. Wheat seedlings established in an open space were transferred to the nine gas-exposure chambers on 26 April 2014 for introducing gas-exposure and soil salinity treatment until 12 June 2014. In each gas-exposure chamber, four pots (*i.e*., 12 seedlings) were assigned to each soil salinity treatment. One liter of 0 or 150 mM NaCl solution was applied to each pot at 4-d intervals during the exposure period. There is strong evidence that irrigation water with an EC ≥ 13 dS m^{-1} or 130 mM NaCl solution significantly suppressed the growth and yield of Bangladeshi wheat under field conditions (Mojid *et al.* 2013, Bilkis *et al.* 2016). During the growing period, a total of 15 saline irrigations (4-d interval) were done using 150 mM NaCl solution. The 4-d interval was selected to prevent the washout of salt from the soil. To avoid soil drying and prevent salt accumulation, deionized water was applied as necessary. The soil EC was 0.5 and 6.1 dS m^{-1} in 0 mM and 150 mM NaCl treatments, respectively.

Leaf gas-exchange rates: Net photosynthetic rate (P_N) and stomatal conductance to H_2O (g_s) were measured *ex situ* in the chamber using a portable photosynthetic measurement system (*LI-6400*, *Li-Cor Inc*., Lincoln, NE, USA). The measurements of gas-exchange rates were made for the fully expanded 7th leaf from the plant bottom at the vegetative stage on 10–13 May 2014 and flag leaves at the anthesis stage on 7–11 June 2014 of three randomly selected plants per treatment × chamber combination (nine measurements per treatment and one plant per pot). The P_N and g_s were measured at stable environmental conditions: atmospheric CO_2 concentration of 390 µmol mol^{-1}, air temperature of 25 ± 1°C, relative air humidity of 70 ± 5%, indicating VPD ≈ 1.0 kPa, and PPFD of 1,500 µmol m^{-2} s^{-1}.

Radical-scavenging enzyme assays: After a few days from the measurement period of gas-exchange rates, the 7th and flag leaves from three randomly selected plants per treatment × chamber combination were collected on 15 May and 12 June 2014, respectively (nine plants per treatment). Immediately after the measurements of leaf area and fresh mass (FM), the leaves were frozen in liquid nitrogen and then stored at –80°C until the measurements of radical-scavenging enzymes.

For the determination of activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, 1.8.1.7), the stored leaf samples (FM of 100 mg) were homogenized to fine powder with a mortar and pestle in liquid nitrogen with a small amount of quartz sand and polyvinyl polypyrrolidone (PVPP). Subsequently, 1 ml of extraction buffer containing 50 mM potassium phosphate (pH 7.8) and 1 mM Na-EDTA was added to the leaf samples. The crude homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant of the sample was used for the assay of the activities of MDAR, DHAR, and GR. The activity of MDAR was determined by the method described by Hossain and Asada (1984a). The supernatant (80 µl) was added to 500 µl of 100 mM Tris-HCl buffer (pH 7.5), 100 µl of 25 mM L-ascorbic acid solution, 25 µl of 4 mM NADPH, and 294 µl of deionized water in a disposable cuvette for blank measurement. The reaction of NADPH consumption by MDAR was activated by adding 1 µl of ascorbate oxidase and monitored by absorbance at 340 nm using spectrophotometer (*UVmini-1240*, *Shimadzu*, Japan). The MDAR activity was calculated from consumption rate of NADPH using molar extinction coefficient of 6.30 mM^{-1} cm^{-1}, and expressed as µkat of NADPH m^{-2}(leaf area). For the measurement of DHAR activity, 20 µl of supernatant was added to 500 µl of 100 mM potassium phosphate buffer (pH 6.5), 330 µl of deionized water, and 100 µl of 25 mM reduced glutathione (GSH). To activate the reaction of reduction of dehydroascorbic acid (DHA) by DHAR, 50 µl of 10 mM DHA was added, and the absorbance at 265 nm was measured for 2 min using the above spectrophotometer. The activity of DHAR was calculated from reduction rate of DHA using molar extinction coefficient of 14.3 mM^{-1} cm^{-1} (Hossain and Asada 1984b), and expressed as µkat of DHA m^{-2}(leaf area).

The activity of GR was determined by the method of Foyer and Halliwell (1976). For the measurement of GR activity, 200 µl of the supernatant was added to 700 µl of assay buffer containing 100 mM KH_2PO_4 (pH 7.8) and 0.2 mM Na-EDTA (pH 7.8), and 50 µl of 2 mM NADPH in a disposable cuvette for blank measurement. Subsequently, to activate the reaction of reduction of oxidized glutathione (GSSG) by GR, 10 mM GSSG was added, and the absorbance at 340 nm was recorded for 2 min with the above spectrophotometer. The activity of GR was calculated from the consumption rate of NADPH using molar extinction coefficient of 6.10 mM^{-1} cm^{-1}, and expressed as µkat of NADPH m^{-2}(leaf area).

For the measurement of activities of SOD and CAT, 500 µl of the supernatant was desalted by applying it to *PD-10* desalting column filled with *Sephadex G-25*. The activity of SOD was assessed according to McCord and Fridovich (1969). The reaction mixture contained

650 µl of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM Na-EDTA, 100 µl of 1 mM xanthene solution, and 10 µl of 1 mM cytochrome *c*, and 220 µl of desalted supernatant or extraction buffer. After blank quantify, the reaction was activated by adding 20 µl of xanthene oxidase solution and absorbance was recorded at 550 nm for 2 min with the above spectrophotometer. Single unit of SOD activity (*i.e.*, µmol min^{-1}) was defined as the amount of the enzyme required for inhibiting cytochrome *c* reduction by 50%. The activity of CAT was determined by H_2O_2 elimination rate monitored by absorbance at 240 nm with a molar extinction coefficient of H_2O_2 at 240 nm being 0.04 mM^{-1} cm^{-1} (Aebi 1983). For CAT assay, an aliquot of 20 µl of desalted supernatant was added to 970 µl of assay buffer containing 50 mM KH_2PO_4 (pH 7.8) and 1 mM EDTA in a quartz cuvette for blank measurement. The reaction was started by adding 10 µl of 880 mM H_2O_2. The CAT activity was expressed as µkat of H_2O_2 m^{-2}(leaf area).

To extract ascorbate peroxidase (APX), the stored leaf samples (FM of 100 mg) were homogenized to fine powder with a mortar and pestle in liquid nitrogen with a small amount of quartz sand and PVPP. Subsequently, 1 ml of extraction buffer containing 50 mM potassium phosphate (pH 7.8), 1 mM Na-EDTA, and 5 mM reduced ascorbic acid was added to the leaf samples. The crude homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant of the sample was used for the assay of the APX activity. The activity of APX was assessed by measurement of reduced ascorbic acid oxidation *via* monitoring the decrease in absorbance at 290 nm and calculated using extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981). The reaction mixture contained 10 µl of crude supernatant, 920 µl of 50 mM potassium phosphate buffer (pH 7.0), and 50 µl of 5 mM L-ascorbic acid solution. Activation of reaction was started by adding 20 µl of 50 mM H_2O_2 and subsequently recorded the decrease in absorbance at 290 nm for 2 min with the spectrophotometer. The activity of APX was expressed as µkat of ascorbate m^{-2}(leaf area).

Concentration and redox state of antioxidants: At the same time of leaf sampling for the measurement of radical scavenging enzyme activity, the 7th and flag leaves (FM of 30 mg) were collected from three randomly selected plants per treatment × chamber combination. Immediately after the measurements of leaf area and FM, the leaves were frozen in liquid nitrogen and then stored at –80°C until the measurements of radical scavenging compounds.

The stored leaf samples were homogenized to fine powder in liquid nitrogen and subsequently 1.5 ml of 5% (w/v) metaphosphoric acid was added. The crude homogenate was centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant of the sample was used in the assay of the concentrations of ascorbate and glutathione.

The ascorbate concentration was determined by the modified method of Luwe and Heber (1995). For quantification of reduced ascorbate (AsA), 50 µl of the supernatant was added to 949 µl of 100 mM potassium phosphate buffer (pH 7.0). The reaction was activated by adding ascorbate oxidase (1 unit $µl^{-1}$, *Sigma-Aldrich*), and the absorbance at 265 nm was monitored for 2 min. The oxidized ascorbate (DHA) assay was done in a quartz cuvette containing 100 µl of the supernatant, 899 µl of 100 mM potassium phosphate buffer (pH 7.7), and 1 µl of 200 mM dithiothreitol (DTT). The absorbance was monitored at 265 nm for 10 min. The ascorbate concentration was calculated using the molar extinction coefficient of 14.3 mM^{-1} cm^{-1}. The redox state of ascorbate was expressed as the concentration ratio of AsA to total ascorbate (sum of AsA and DHA).

The concentration of glutathione was determined by the method of Griffith (1980). For the measurement of total glutathione (sum of reduced and oxidized glutathione), 195 µl of neutralized supernatant was added to 660 µl of assay buffer containing 125 mM KH_2PO_4 and 6.3 mM Na-EDTA (pH 7.7), 100 µl of 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and 40 µl of 5 mM NADPH in a cuvette. After blank reading, 5 µl of GR (1.81 units $µl^{-1}$, *Sigma-Aldrich*) was added to activate the reaction, and increase rate of the absorbance at 412 nm was monitored with the spectrophotometer. To determine oxidized glutathione (GSSG) concentration, the supernatant was pretreated with 2-vinylpyridine and incubated at 25°C for 1 h to eliminate reduced glutathione (GSH). The incubated sample was centrifuged at 16,000 × *g* for 5 min at 25°C and used for measurement of GSSG by following total glutathione protocol. A standard curve between increase rate of 412 nm and concentrations of GSH and GSSG was constructed using GSH and GSSG standard series, and used for the calculation of the amounts of total glutathione and GSSG concentration, respectively. The concentration of GSH was calculated from subtracting GSSG from total glutathione concentration. The redox state of glutathione was expressed as the ratio of GSH concentration to total glutathione concentration.

Data interpretation: We clarified soil salinity-induced change in O_3 damage on P_N: mitigation, no effect, or enhancement. We defined mitigation effects as follow: there was significant O_3-induced reduction in P_N at the 0 mM NaCl treatment but no significant effect of O_3 on P_N in the 150 mM NaCl treatment. Then, to consider the mechanisms underlying the soil salinity-induced mitigation of O_3 effect on P_N (*i.e.*, protective mechanism), we focused on the salinity effects on g_s, antioxidative enzymes, and antioxidants, especially under elevated O_3 treatments.

Statistical analyses: In the present study, we have three factors, *i.e.*, gas exposure, soil salinity, and leaf position, to perform analysis of variance (*ANOVA*). In the present case, however, it was impossible to distinguish between the effects of leaf positions and the effects of plant development stages. Thus, the data were subjected to split-plot *ANOVA* to examine the effects of O_3 and/or soil salinity on data for the net photosynthetic rate, stomatal conductance to H_2O, and radical-scavenging enzymes and nonenzymatic substances in each leaf position. The results presented are the means ± standard deviation of three replicates and each grand mean is the average of

nine plants. When a significant interaction between O_3 and salinity was detected ($p<0.05$), *Tukey*'s HSD test was performed to identify significant differences between the gas and salinity treatments. Significant differences at $p<0.05$ by *Tukey*'s HSD are shown by the differences in letters in the figures and tables. All statistical analyses were performed with the *SPSS* statistical package (*SPSS 16.0*, *SPSS Inc*., USA).

Results

Meteorological parameters: The daily mean, daily maximum, daily minimum, and 12-h mean air temperature were 18.5, 25.6, 13.1, and 21.5°C, respectively (Table 1). The daily mean, daily maximum, daily minimum, and 12-h mean relative air humidity were 78.5, 97.2, 52.1, and 67.1%, respectively.

Ozone exposure: The daily mean O_3 concentration in the CF, 1.0×O_3, and 1.5×O_3 treatments were 11.7, 36.4, and 52.7 nl L^{-1}, respectively (Table 2). The 12-h mean O_3 concentrations in the CF, 1.0×O_3, and 1.5×O_3 treatments were 13.3, 41.2, and 61.3 nl L^{-1}, respectively. The AOT40 in the CF, 1.0×O_3 and 1.5×O_3 treatments was 0.0, 4.8, and 13.3 µl L^{-1} h, respectively.

Leaf gas-exchange rates: There were significant interactions between O_3 and soil salinity for P_N in both leaves (Table 3). In both leaves, the exposure to O_3 at 1.5×O_3 significantly reduced P_N in the 0 mM NaCl treatment while P_N was not significantly reduced in the 150 mM NaCl treatment (Fig. 1*A*,*B*). The exposure to O_3 significantly reduced g_s in 7th and flag leaves. Salinity had no significant impact on the g_s in the 7th and flag leaves (Fig. 1*C*,*D*). Furthermore, in both leaves, no significant interactive effect of O_3 and salinity was noticed on the g_s of BAW1059.

Table 1. Daily mean, daily max., daily min. daylight hour (12-h) air temperature and daily mean, daily max., daily min. daylight hour (12-h) relative air humidity inside the chambers during the treatment period in 2014. Each value is the mean of three chambers ± SD. Daily max. – mean of daily 1-h maximum value; Daily min. – mean of daily 1-h minimum value; 12-h mean – 6:00–18:00 h.

	Daily mean	Daily max.	Daily min.	12-h mean
Air temperature [°C]	18.5 ± 0.2	25.6 ± 0.6	13.1 ± 0.2	21.5 ± 0.5
Relative air humidity [%]	78.5 ± 0.8	97.2 ± 0.9	52.1 ± 0.5	67.1 ± 0.7

Table 2. Daily mean and 12-h mean O_3 concentration and accumulated exposure over the threshold of 40 nl L^{-1} of O_3 (AOT40) during daylight hours (solar radiation > 50 W m^{-2}) during the treatment period in 2014. Each value is the mean of three chambers ± SD. CF – charcoal-filtered air; 1.0×O_3 – 1.0-fold the ambient O_3 concentration; 1.5×O_3 – 1.5-fold the ambient O_3 concentration; 12-h – 6:00–18:00 h.

Treatment	O_3 concentration [nl L^{-1}]		AOT40 [µl L^{-1} h]
	Daily mean	12-h mean	
CF	11.7 ± 3.2	13.3 ± 3.7	0.0 ± 0.0
1.0×O_3	36.4 ± 0.2	41.2 ± 0.2	4.8 ± 0.0
1.5×O_3	52.7 ± 0.4	61.3 ± 0.2	13.3 ± 0.2

Table 3. The results of split-plot *ANOVA* of the effects of O_3 and salinity on the leaf gas-exchange rates and activity of radical scavenging enzymes at vegetative and anthesis growth stage of Bangladeshi wheat cultivars BAW1059. Result of split-plot *ANOVA* indicates *p*-values. Asterisks indicate levels of significance at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***), whereas ns = not significant. APX – ascorbate peroxidase; DHAR – dehydroascorbate reductase; GR – glutathione reductase; g_s – stomatal conductance to H_2O; MDAR – monodehydroascorbate reductase; P_N – net photosynthetic rate; SOD – superoxide dismutase.

Parameter	7th leaf			Flag leaf		
	O_3	Salinity	O_3×Salinity	O_3	Salinity	O_3×Salinity
P_N	0.026*	0.904^{ns}	0.018*	0.006**	0.098^{ns}	0.006**
g_s	0.044*	0.213^{ns}	0.438^{ns}	0.043*	0.920^{ns}	0.139^{ns}
SOD	0.075^{ns}	0.017*	<0.001***	0.029*	0.977^{ns}	0.779^{ns}
CAT	0.025*	0.002**	0.752^{ns}	0.004**	0.843^{ns}	0.002**
APX	<0.001***	<0.001***	0.048*	0.014*	0.002**	0.005**
MDAR	0.660^{ns}	0.581^{ns}	0.242^{ns}	0.046*	0.910^{ns}	0.048*
DHAR	0.025*	0.498^{ns}	0.044*	0.571^{ns}	0.008**	0.924^{ns}
GR	0.566^{ns}	0.197^{ns}	0.384^{ns}	0.428^{ns}	0.760^{ns}	0.493^{ns}

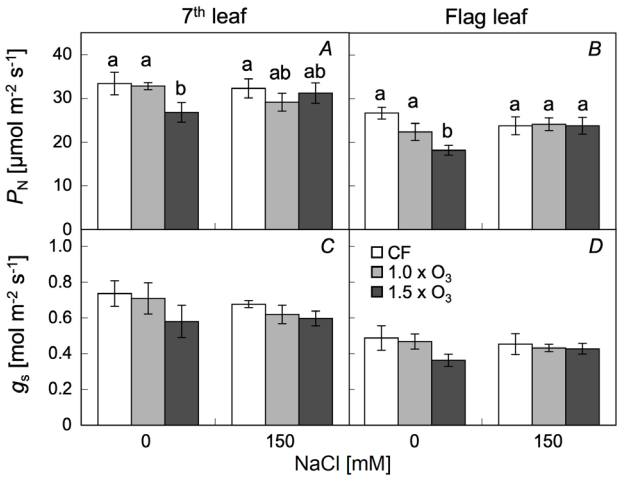

Fig. 1. Effects of O_3 and soil salinity on the net photosynthetic rate (P_N) (*A*,*B*) and stomatal conductance to H2O (g_s) (*C*,*D*) in the 7th (*A*,*C*) and flag (*B*,*D*) leaves of Bangladeshi wheat cultivar BAW1059. Values are means ± SD, *n* = 3. *Different letters* above the bars indicate significant difference between the six treatments (*Tukey*'s HSD test, $p<0.05$).

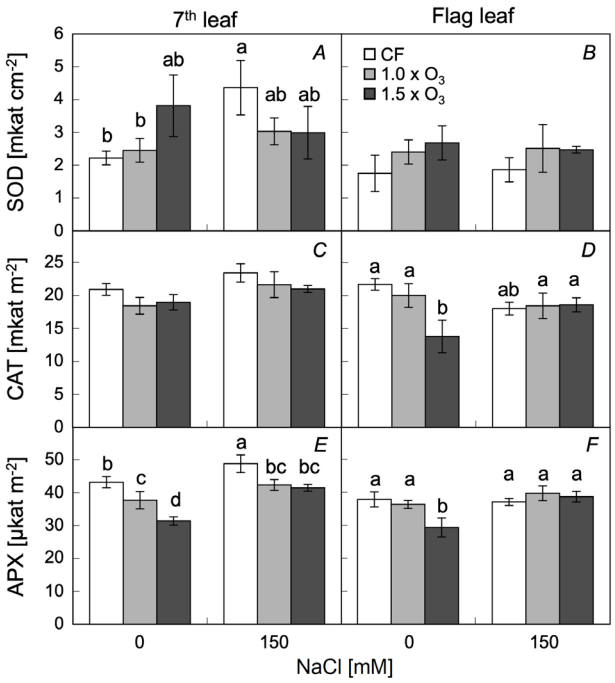

Fig. 2. Effects of O_3 and soil salinity on the activities of superoxide dismutase (SOD) (*A*,*B*), catalase (CAT) (*C*,*D*) and ascorbate peroxidase (APX) (*E*,*F*) in the 7th (*A*,*C*,*E*) and flag (*B*,*D*,*F*) leaves of Bangladeshi wheat cultivar BAW1059. Values are means ± SD, *n* = 3. *Different letters* above the bars indicate significant difference between the six treatments (*Tukey*'s HSD test, $p<0.05$).

Activities of radical-scavenging enzymes: In both soil salinity treatments, the exposure to O_3 significantly increased SOD activity in the flag leaf, but not in the 7th leaf (Table 3, Fig. 2*A*,*B*). In the 7th leaf, soil salinity significantly increased SOD activity in the CF treatment, but not in the 1.0×O_3 and 1.5×O_3 treatments. In the flag leaf, there was no significant effect of soil salinity on SOD activity.

The exposure to O_3 significantly reduced CAT activity in the 7th flag leaf in both soil treatments and in the flag leaf at the 0 mM NaCl treatment (Table 3, Fig. 2*C*,*D*). The soil salinity significantly increased CAT activity in the 7th leaf in all the gas treatments, and in the flag leaf in the 1.5×O_3 treatment.

The exposure to O_3 significantly reduced APX activity in the 7th leaf in the both soil salinity treatments, and in the flag leaf in the 0 mM NaCl treatment (Table 3, Fig. 2*E*,*F*). The soil salinity significantly increased APX activity in the 7th leaf in the CF and 1.5×O_3 treatments, and in the flag leaf in the 1.5×O_3 treatment.

In the 7th leaf, there were no significant effects of O_3 and soil salinity on the MDAR activity (Table 3, Fig. 3*A*,*B*). In the flag leaf, the MDAR activity was significantly reduced by the exposure to O_3 in the 0 mM NaCl treatment, but not in the 150 mM NaCl treatment.

The exposure to O_3 significantly increased DHAR activity in the 7th leaf in the 0 mM treatment, but not in the 150 mM NaCl treatment (Table 3, Fig. 3*C*,*D*). There was no significant effect of O_3 on DHAR activity in the flag leaf. The DHAR activity was significantly increased by soil salinity treatment in the flag leaf, but not in the 7th leaf in any gas treatment.

There were no significant effects of O_3 and soil salinity and their interaction on GR activity in the 7th and flag leaves (Table 3, Fig. 3*E*,*F*).

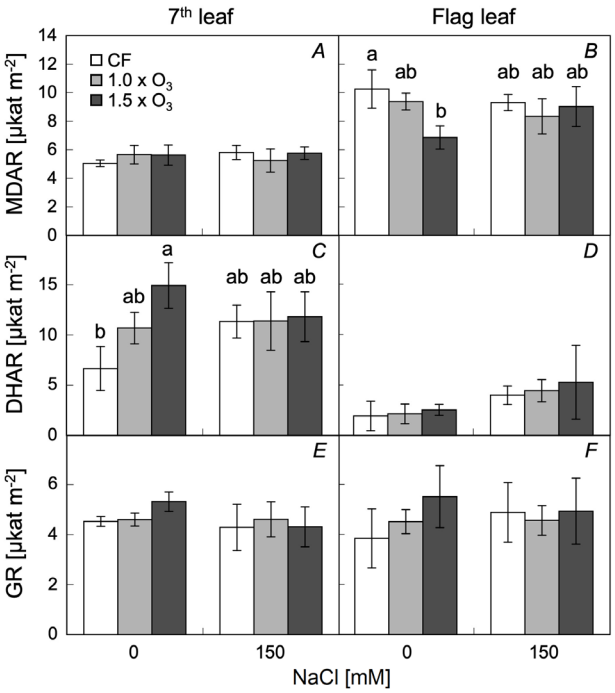

Fig. 3. Effects of O_3 and soil salinity on the activities of monodehydroascorbate reductase (MDAR) (*A*,*B*), dehydroascorbate reductase (DHAR) (*C*,*D*), and glutathione reductase (GR) (*E*,*F*) in the 7th (*A*,*C*,*E*) and flag (*B*,*D*,*F*) leaves of Bangladeshi wheat cultivar BAW1059. Values are means ± SD, *n* = 3. *Different letters* above the bars indicate significant difference between the six treatments (*Tukey*'s HSD test, $p<0.05$).

Concentration and redox state of antioxidants: Exposure of O_3 significantly increased the reduced ascorbate (AsA) and total ascorbate concentration in the 7th leaf (Table 4). In the flag leaf, in contrast, concentrations of AsA and total ascorbate were significantly reduced by the exposure to O_3. The soil salinity significantly increased concentrations of AsA and total ascorbate in the flag leaf, but not in the 7th leaf. The exposure to O_3 significantly reduced dehydroascorbate (DHA) concentration in the flag leaf, but not in the 7th leaf. The DHA concentration was significantly increased by soil salinity in the flag leaf in the 1.5×O_3 treatment, but not in the 7th leaf by any gas treatment. There were no significant effects of O_3 and soil salinity and their interaction on redox state of ascorbate in the 7th and flag leaves.

The exposure to O_3 significantly increased concentration of reduced glutathione (GSH) in the 7th leaf (Table 5). In the flag leaf, in contrast, the GSH concentration was significantly reduced by the exposure to O_3. The total glutathione concentration was significantly increased by the exposure to O_3 in the 7th leaf in the 0 mM NaCl treatment, but not in the 150 mM NaCl treatment. In the flag leaf, on the contrary, the exposure to O_3 significantly reduced total glutathione concentration. There was no significant effect of soil salinity on the total glutathione concentration in both leaves in any gas treatment. There were no significant effects of O_3 and soil salinity and their interaction on oxidized glutathione concentration and redox state of glutathione in the 7th and flag leaves.

Discussion

In the present study, the adverse effects of O_3 on net photosynthetic rate in the 7th and flag leaves of Bangladeshi wheat cultivar (BAW1059) were mitigated by soil salinity stress (Fig. 1*A*,*B*). In general, the degree of O_3 damage is determined by the balance between stomatal O_3 uptake and detoxification capacity in the leaves (Musselman *et al*. 2006). The stomatal O_3 uptake is determined by atmospheric O_3 concentration and stomatal conductance (Laisk *et al*. 1989). The stomatal conductance in the 7th and flag leaves was not reduced by soil salinity stress (Fig. 1*C*,*D*). The upholding of stomatal conductance under salinity stress might be due to the fact that the cultivar with high tolerance to salinity maintains a better osmotic balance under such stress (Acosta-Motos *et al.* 2017). Moreover, salinity-tolerant cultivars can retain high stomatal conductance by suppressing sodium uptake (Muranaka *et al.* 2002). The cultivar BAW1059 was considered as salt-tolerant cultivar, due to its comparatively better protection capacity of stomatal conductance, osmotic and ionic balance, lower visual leaf injury, and delayed leaf

Table 4. Reduced ascorbate (AsA), dehydroascorbate (DHA), and total ascorbate concentrations and redox states of ascorbate in the 7th and flag leaves of Bangladeshi wheat cultivar BAW1059, exposed to different O_3 concentrations (CF – charcoal-filtered air; 1.0×O_3 – 1.0-fold the ambient O_3 concentration; 1.5×O_3 – 1.5-fold the ambient O_3 concentration) and soil salinity (0 mM, 150 mM) treatments up to flag leaves stage. Each value is the mean of three chambers ± SD. Each grand mean is the average of nine plants. Result of split-plot *ANOVA* indicates *p*-value and asterisks indicate levels of significance at $p<0.05$ (*), $p<0.01$ (**), whereas ns = not significant. Values with *different letters* are significantly different at $p<0.05$ between the six treatments (*Tukey*'s HSD test).

Gas	NaCl	AsA [μmol m^{-2}]	DHA [μmol m^{-2}]	Total [μmol m^{-2}]	Redox state [%]
7th leaf					
CF	0 mM	974.3 ± 87.2	216.9 ± 3.0	1190.2 ± 88.5	82.9 ± 1.0
1.0×O_3		1112.5 ± 76.4	209.3 ± 34.6	1321.8 ± 78.5	84.2 ± 2.8
1.5×O_3		1237.4 ± 41.0	232.4 ± 25.8	1470.8 ± 31.2	84.1 ± 1.8
CF	150 mM	1094.8 ± 98.1	243.2 ± 24.6	1337.9 ± 116.5	81.6 ± 1.3
1.0×O_3		1074.6 ± 97.3	222.5 ± 41.1	1295.1 ± 123.0	82.7 ± 2.8
1.5×O_3		1158.0 ± 95.0	236.4 ± 37.0	1394.3 ± 132.0	83.2 ± 1.1
ANOVA	O_3	0.020^{*}	0.408^{ns}	0.023^{*}	0.439^{ns}
	Salinity	0.986^{ns}	0.235^{ns}	0.727^{ns}	0.195^{ns}
	O_3×Salinity	0.141^{ns}	0.711^{ns}	0.129^{ns}	0.970^{ns}
Flag leaf					
CF	0 mM	631.7 ± 57.0	89.9 ± 12.1^{a}	720.6 ± 54.9	86.2 ± 1.5
1.0×O_3		496.2 ± 12.1	71.4 ± 3.6^{abc}	568.6 ± 8.5	86.4 ± 0.2
1.5×O_3		480.7 ± 50.5	56.1 ± 8.5^{c}	536.8 ± 59.0	88.9 ± 0.7
CF	150 mM	772.4 ± 86.4	80.4 ± 7.2^{a}	853.7 ± 86.0	89.9 ± 2.5
1.0×O_3		580.3 ± 86.4	60.8 ± 7.4^{bc}	640.1 ± 92.9	89.7 ± 0.9
1.5×O_3		628.7 ± 204.6	76.3 ± 5.0^{ab}	704.0 ± 202.6	87.8 ± 4.2
ANOVA	O_3	0.042^{*}	0.002^{**}	0.023^{*}	0.971^{ns}
	Salinity	0.031^{*}	0.999^{ns}	0.030^{*}	0.109^{ns}
	O_3×Salinity	0.847^{ns}	0.007^{**}	0.731^{ns}	0.196^{ns}

Table 5. Reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione concentrations and redox states of glutathione in the 7th and flag leaves of Bangladeshi wheat cultivar BAW1059, exposed to different O_3 concentrations (CF – charcoal-filtered air; 1.0×O_3 – 1.0-fold the ambient O_3 concentration; 1.5×O_3 – 1.5-fold the ambient O_3 concentration) and soil salinity (0 mM, 150 mM) treatments up to flag leaves stage. Each value is the mean of three chambers ± SD. Each grand mean is the average of nine plants. Result of split-plot *ANOVA* indicates *p*-value and asterisks indicate levels of significance at $p<0.05$ (*), $p<0.01$ (**), whereas ns = not significant. Values with *different letters* are significantly different at $p<0.05$ between the six treatments (*Tukey*'s HSD test).

Gas	NaCl	GSH [μmol m^{-2}]	GSSG [μmol m^{-2}]	Total [μmol m^{-2}]	Redox state [%]
7th leaf					
CF	0 mM	354.5 ± 13.8	104.9 ± 0.9	458.4 ± 14.7^{b}	77.3 ± 0.5
1.0×O_3		389.8 ± 22.5	134.3 ± 13.9	523.0 ± 9.6^{ab}	73.6 ± 3.8
1.5×O_3		514.7 ± 116.5	125.0 ± 21.8	638.3 ± 96.1^{a}	79.8 ± 5.5
CF	150 mM	426.9 ± 3.1	139.3 ± 4.5	565.2 ± 2.7^{ab}	73.6 ± 3.6
1.0×O_3		447.7 ± 63.3	150.0 ± 28.7	597.7 ± 53.5^{a}	74.5 ± 5.6
1.5×O_3		455.8 ± 47.3	120.8 ± 11.6	575.6 ± 57.4^{ab}	78.6 ± 1.2
ANOVA	O_3	0.026*	0.084^{ns}	0.019*	0.072^{ns}
	Salinity	0.351^{ns}	0.069^{ns}	0.111^{ns}	0.457^{ns}
	O_3×Salinity	0.098^{ns}	0.138^{ns}	0.025*	0.546^{ns}
Flag leaf					
CF	0 mM	581.9 ± 104.8	60.6 ± 17.5	640.6 ± 120.0	91.0 ± 1.3
1.0×O_3		433.3 ± 52.8	63.7 ± 20.0	496.0 ± 32.9	86.6 ± 5.7
1.5×O_3		441.0 ± 4.9	37.7 ± 11.0	478.7 ± 15.7	92.1 ± 2.2
CF	150 mM	539.8 ± 58.8	46.8 ± 10.9	585.6 ± 49.2	92.1 ± 2.5
1.0×O_3		497.3 ± 100.3	36.8 ± 13.6	533.1 ± 86.8	93.1 ± 3.7
1.5×O_3		504.3 ± 19.8	43.0 ± 1.9	547.3 ± 19.0	92.6 ± 0.8
ANOVA	O_3	0.047*	0.312^{ns}	0.032*	0.371^{ns}
	Salinity	0.361^{ns}	0.118^{ns}	0.574^{ns}	0.084^{ns}
	O_3×Salinity	0.287^{ns}	0.176^{ns}	0.252^{ns}	0.213^{ns}

senescence under salinity stress (Barma *et al.* 2011, Hasan *et al.* 2015, Kamal *et al*. 2015). That is why the stomatal conductance was not affected by soil salinity in the present study. In any case, this result indirectly suggests that the mitigation of O_3 damage under soil salinity stress was not caused by reduction in stomatal O_3 uptake, but by changes in nonstomatal factors, such as increase in detoxification capacity by soil salinity.

In the leaves, reactive oxygen species (ROS) are metabolized and detoxified through the radical-scavenging system to cope with oxidative stress such as O_3 (Ashmore 2005, Gill and Tuteja 2010, Liu *et al.* 2015). SOD acts as the first line of defense against ROS by dissimulating superoxide (O_2^{-}) to H_2O_2. CAT and APX convert H_2O_2 into O_2 and H_2O, while APX uses reduced ascorbate (AsA) as an electron donor (Asada 2006). In the 7th leaf, the soil salinity significantly increased SOD activity in the CF treatment, APX activity in the CF and 1.5×O_3 treatments and CAT activity in all the gas treatments (Fig. 2*A*,*C*,*E*). In the flag leaf, on the other hand, the activities of APX and CAT were increased by the soil salinity in the 1.5×O_3 treatments (Fig. 2*D*,*F*). These results suggest that enzymes activated by soil salinity mitigated adverse O_3 effect on net photosynthesis under relatively high salt concentration in both leaves.

The activity of enzymes catalyzing regeneration of antioxidants is one of the important factors to eliminate ROS (Asada 2006, Gill and Tuteja 2010, Mishra *et al.* 2013). The AsA is oxidized to MDHA, which is reduced back to AsA by the activity of MDAR or disproportionate to DHA and AsA. The DHA is reduced by the reaction with reduced glutathione (GSH) catalyzed by DHAR forming oxidized glutathione (GSSG). The GSH in turn is regenerated from GSSG by GR (Asada 2006). In the 7th leaf, there were no significant effects of soil salinity on the activities of MDAR, DHAR, and GR (Fig. 3*A*,*C*,*E*), suggesting that the soil salinity stress did not mitigate O_3 damage *via* acceleration of reduction rate of antioxidants. In the flag leaf, on the other hand, DHAR activity was stimulated by soil salinity stress in all the gas treatments. Thus, in the flag leaf, soil salinity-induced higher regeneration rate of DHA might result in the mitigation of adverse O_3 effect on net photosynthesis by the soil salinity stress.

In addition to the activity of enzymes catalyzing elimination of ROS and regeneration of antioxidants, the concentration of antioxidants is important for antioxidant system (Asada 2006). In the 7th leaf, the soil salinity did not significantly affect the concentrations of ascorbate and glutathione in any gas treatments (Tables 4, 5). In the flag leaf, on the other hand, the soil salinity significantly increased the concentrations of reduced and total ascorbate, but did not significantly affect glutathione concentration

(Tables 4, 5). These results suggest that the soil salinity-induced increases in the concentration of ascorbate and its reduction rate were related to the mitigation of O_3 damage under relatively high soil salinity level in the flag leaf. We found soil salinity induced a higher tolerance to absorbed O_3 *via* stomata in both the 7th and flag leaves, while the mechanisms were different. Previous studies reported the response of O_2^- and H_2O_2-elimination capacity to soil salinity was changed due to the duration of salinity stress (Parida and Das 2005, Miller *et al.* 2010). Similarly, in the present study, the leaf-dependent difference in the mechanism of salinity-induced higher tolerance to O_3 of net photosynthesis of the wheat cultivar may be due to the difference in leaf age and/or the duration of treatments.

According to Kamal *et al*. (2015), the yield of BAW1059 is relatively sensitive to O_3 as compared to the American and European wheat cultivars. Furthermore, in a meta-analysis by Feng *et al*. (2008), elevated O_3 (the daily mean concentration of 73 ppb) significantly decreased net photosynthetic rate by *ca*. 20% as compared with control (*i.e*., CF) treatment in wheat. In the present study, O_3-induced reductions in P_N in the 7th and flag leaves were approximately 20 and 32%, respectively, by the exposure to 1.5×O_3 (*i.e*., 61 ppb of O_3) in the 0 mM NaCl treatment (Fig. 1*A*,*B*). These results suggest that BAW1059 would be sensitive or moderately sensitive to O_3. On the other hand, Sikder *et al*. (2010) reported that the monthly daytime averaged O_3 concentration in Bangladesh sometimes exceeded 60 ppb (*ca*. 120 ppb at a maximum). Such O_3 concentration could adversely affect the physiology of wheat, which was clearly demonstrated in our present study at low soil salinity level (Table 2, Fig. 1*A*,*B*). To prevent the adverse effects of O_3 on Bangladesh wheat, exogenous application of agrochemicals, such as ethylenediurea, might be useful (*e.g.*, Agathokleous 2017, Ghosh *et al.* 2018).

In conclusion, the soil salinity mitigated adverse effects of O_3 on net photosynthesis in the 7th and flag leaves of Bangladeshi wheat cultivar (BAW1059), although soil salinity did not induce stomatal closure. The mitigation of the effects of O_3 was due to the change in nonstomatal factors, such as an increase in the detoxification capacity of O_3 in the leaves by soil salinity. Therefore, Bangladeshi wheat cultivar with high salt tolerance, BAW1059, is expected to maintain net photosynthesis high to some extent under high levels of soil salinity and atmospheric O_3.

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