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Responses of gas exchange, cellular membrane integrity, and antioxidant enzymes activities of salinity-stressed winter wheat to ozone pollution

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Abstract

A sand-culture experiment was conducted in open-top chambers which were constructed in a greenhouse to investigate the responses of salt-stressed wheat (*Triticum aestivum* L.) to O_3. Plant seeding of JN17 (a popular winter wheat cultivar) was grown in saltless (–S) and saline (+S, 100 mM NaCl) conditions combined with charcoal-filtered air (CF, < 5 ppb O_3) and elevated O_3 (+O_3, 80 ± 5 ppb, 8 h day^{-1}) for 30 d. O_3 significantly reduced net photosynthetic rate (P_N), stomatal conductance, chlorophyll contents and plant biomass in –S treatment, but no considerable differences were noted in those parameters between +O_3+S and CF+S treatments. O_3-induced loss in cellular membrane integrity was significant in –S plants, but not in +S plants evidenced by significant elevations being measured in electrolyte leakage (EL) and malondialdehyde (MDA) content in –S plants, but not in +S plants. Both O_3 and salinity increased proline content and stimulated antioxidant enzymes activities. Soluble protein increased by salinity but decreased by O_3. Abscisic acid (ABA) was significantly elevated by O_3 in –S plants but not in +S plants. The results of this study suggested that the specificity of different agricultural environments should be considered in order to develop reliable prediction models on O_3 damage to wheat plants.

Additional key words: abscisic acid; antioxidants; gas exchange; ozone; salinity; *Triticum aestivum* L.

Introduction

Ozone (O_3) is recognized as an important phytotoxic air pollutant worldwide due to rapid industrial development (Emberson *et al.* 2001, Fiscus *et al.* 2005, Zhang *et al.* 2011). It enters through stomata and impairs plant metabolism with deleterious effects on growth and yield of crops (Biswas *et al.* 2008, McKee and Long 2001, Shi *et al.* 2009). Salinity is another common stress which severely limits plant growth and productivity of winter wheat (Maggio *et al.* 2004, Chen *et al.* 2005). Saline injuries are mainly shown to lead to specific ion toxicity and peroxidative damage, decreasing stomatal conduction and photosynthetic capacity (Zheng *et al.* 2008a). High O_3 concentrations are associated with global warming which may cause elevation of sea level, as well as expanding saline farmlands (Titus 1990). Numerous studies have been conducted on lightening injuries induced by salinity or O_3 alone on wheat growth and productivity (Biswas *et al.* 2008, McKee and Long 2001, Zheng *et al.* 2009). However, little is known about the responses of salinity-stressed winter wheat to O_3 pollution.

Some authors reported that O_3 affects plant growth primarily by producing reactive oxygen species (ROS) (Ederli *et al.* 1997, Loreto and Velicova 2001, Ashmore

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Abbreviations: ABA – abscisic acid; CAT – catalase (EC 1.11.1.6); CF – charcoal-filtered; Chl – chlorophyll; EC – enzyme code; EL – electrolyte leakage; g_s – stomatal conductance; OTCs – open-top chambers; POD – peroxidase (EC 1.11.1.7); P_N – net photosynthetic rate; ROS – reactive oxygen species; RWC – relative water content; SOD – superoxide dismutase (EC 1.15.1.1).
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2005). ROS cause peroxidation of cellular membrane lipids or oxidation of cellular proteins (Calatayud *et al.* 2003). Under saline condition, plants synthesize anti-oxidants to scavenge ROS before cellular damages occur in chloroplast (Zheng *et al.* 2008b). Our hypothesis is that salinity-induced stomatal closure and enhancement of ROS-scavenging capacity may reduce O_3 flux into the cellular compartments and peroxidative damage, which protects wheat from O_3 injuries. Therefore, O_3-induced loss in plant growth may decrease under saline condition (Fiscus *et al.* 2005, Keutgen *et al.* 2005). In addition, salinity-induced overproduction of ABA may transfer a stress signal and increase the plant protective ability to O_3 (Zhu and Scandalios 1994).

In order to validate the above hypothesis, an experiment was conducted in open top chambers (OTCs) which were located in a greenhouse. Physiological and biochemical responses of salinity-treated winter wheat to O_3 were investigated to offer a technical support for developing reliable prediction models under interaction of O_3 and salinity.

Materials and methods

Plant culture and salinity treatments: The experiment was carried out in four open-top chambers (OTC, 1.2 m in diameter, 1.6 m in height) which were placed in a temperature-controlled double-glazed greenhouse. On 25 October 2007, 30 seeds of JN17, a popular winter wheat cultivar, were sown in each of 12 plastic boxes (length × width × height = 26 × 16 × 10 cm^3) in each OTC. The boxes were filled with 3 kg of sterilized and distilled-water-washed sand. Six boxes in each OTC were irrigated with full-strength Hoagland nutrient solution (–S, no NaCl), and the other boxes were irrigated with modified Hoagland solution with NaCl (+S, 100 mM NaCl). We used 100 mM NaCl as salt treatment which was found to have significant effects on wheat in our previous study (Zheng *et al.* 2008a). The water lost by evapotranspiration was replenished every day during the experiment. Plants were thinned to 20 per box on 15th d after sowing.

The chambers were illuminated by natural day light supplemented with fluorescence light, providing a maximum photosynthetic photon flux density (PPFD) of approximately 1,600 μmol m^{-2} s^{-1} at canopy height during the 14-h photoperiod. The temperature in the OTCs fluctuated from 17°C (night) to 36°C (day) and the relative humidity (RH) was 75–86% during the experiment.

O_3 fumigation and treatments: The gas-dispensing system of the OTCs was similar to the method described by Biswas *et al.* (2008). The OTCs were ventilated continuously (24 h day^{-1}) with charcoal-filtered air (CF, < 5 ppb O_3). The average air-flowing velocity corresponded to approximately one complete air exchange per minute. O_3 was generated by electrical discharge using ambient air with an O_3 generator (*JQ-6A, Telihuo Co*., Beijing, China). The air stream was bubbled through distilled water before its entry to the chambers to remove harmful compounds other than O_3 (Vollsnes *et al.* 2009). Elevated O_3 (+O_3, 80 ± 5 ppb) was dispensed into the CF air stream entering two of the four chambers for 8 h day^{-1} (09:00–17:00 h) for 30 days from sowing to harvest. O_3 concentrations in the OTCs were continuously monitored by an O_3 analyzer (*APOA-360, Horiba*, Japan). To minimize chamber effect and the impact of environmental heterogeneity, plants were switched among chambers with the same O_3 treatment every other day, with location of plants within the chambers being also randomized.

Gas exchange was measured on the most recently fully expanded leaves, using a portable *Gas Exchange Fluorescence System* (*GFS-3000, Heinz* Walz, Germany) on 30th d after O_3 fumigation. There were six replications. Relative humidity was maintained at 70% and leaf temperature was set at 25°C in the leaf chamber. The flow rate was set at 600 μmol s^{-1} and CO_2 concentration in the leaf chamber was maintained at 400 μmol mol^{-1}. The leaf was illuminated with a PPFD of 1,500 μmol m^{-2} s^{-1} of internal light source of the leaf chamber. After the humidity, flow rate, light, and temperature in leaf chamber stabilized, P_N and stomatal conductance (g_s) were simultaneously recorded.

Cellular membrane permeability was estimated by measuring EL of leaves following the method described by Dionisio-Sese and Tobita (1998). Ten 4-cm pieces taken from the middle of the leaves were placed in test tubes containing 10 cm^3 of distilled deionized water. The tubes were incubated in a water bath at 25°C for 2 h in order to measure the initial electrical conductivity of the medium (EC_1). The samples were autoclaved at 100°C for 20 min to release all electrolytes, cooled to 25°C and then the final electrical conductivity (EC_2) was measured. The EL was calculated using the formula:

$$EL\ [\%] = EC_1/EC_2 \times 100 \quad (1)$$

Plant harvest, leaf sampling, and growth parameters: Thirty days after treatments, plants were harvested and separated into two parts, one part for fresh leaf sampling for biochemical analysis and another part for biomass measurement. Leaf area was measured with a *CI-203* portable laser leaf area analyzer (*CID Company*, USA), which was calibrated with conventional manual method.

For biochemical analysis, the leaves exhibiting no visible symptoms of O_3 damage were sampled and immediately frozen in liquid nitrogen and transferred to an ultrafreezer at –80°C until the time of assay. For

biomass measurement, plant samples were well washed with distilled water, then whole plants were dried with tissues and separated into shoots and roots. Ten plants were sampled for each treatment. Samples were oven-dried at 75°C to constant mass before the dry masses were recorded.

For relative water content (RWC) determination, 30 d after O_3 and salinity treatments, 5 most recently fully expanded leaf samples were washed in distilled water and wiped with paper towels before taking fresh mass (FM), then placed in distilled water again under dark conditions at 22°C for 6 h. After 6-h hydration, the turgid leaf samples were blotted dry, and weighed to gain the turgid mass (TM). All the leaf samples were then dried at 75°C to constant mass, with their dry masses (DM) being determined. Leaf RWC was calculated by the following formula (Muranaka *et al.* 2002):

$$RWC\ [\%] = [(FM - DM)/(TM - DM)] \times 100 \quad (2)$$

Chlorophyll (Chl) and proline contents: Chl content was determined following the method described by Maggio *et al.* (2004). Frozen leaf samples (0.2 g) were ground into fine homogenate and extracted in 95% ethanol. The absorbance of the extract was recorded at 663 and 645 nm. Chl content was calculated by the following formula:

$$Chl\ content\ [\%] = 8.02 \times OD_{663} + 20.20 \times OD_{645} \quad (3)$$

where OD_{663} and OD_{645} is the absorbance at 663 and 645 nm, respectively.

Free proline was determined according to the method of Wang *et al.* (2004). Each sample (0.5 g) was suspended in 5 cm^3 of water and 5 cm^3 of 8% (w/v) sulfosalicylic acid, sonicated and centrifuged at 10,000 × *g* for 20 min using a centrifuge (*Sigma 3k15*, Heidelberg, Germany). Proline concentrations were determined in the supernatant with an amino acid analyzer (*Hitachi 835*, *Hitachi Co. Ltd*., Japan).

Soluble protein content, lipid peroxidation, and antioxidant enzymes activities: Frozen leaf samples (about 0.5 g) were homogenized in a prechilled mortar and pestle placed in ice with 5 cm^3 0.05 M potassium phosphate buffer (pH 7.8) containing 8.5% (v/v) 0.2 M KH_2PO_4 and 91.5% 0.2 M K_2HPO_4. The homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatant was further used for measurement of soluble protein, MDA contents, and antioxidant enzymes activities. All the processes were carried out at 0–4 °C. Soluble protein content was determined following the method of Bradford (1976). Each 0.02 cm^3 protein extract was homogenized with 3 cm^3 reaction solution containing 100 mg L^{-1} Coomassie brilliant blue G-250, 90% ethanol, and 85% phosphoric acid. The absorbance was recorded at 595 nm after 2-min reaction. Soluble protein content was calculated by comparing the standard curve utilizing bovine serum albumin as a standard.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to Mittova *et al.* (2004). A mixture of 1 cm^3 of extracts and 2 cm^3 of 0.6% thiobarbituric acid (TBA) was produced, boiled for 15 min, cooled and centrifuged for 10 min (10,000 × *g*). The content of MDA was determined from the absorbance at 600, 532, and 450 nm, and then calculated using the following formula:

$$MDA\ [\mu mol\ g^{-1}(FM)] = [6.45 \times (D_{532} - D_{600}) - 0.56\ D_{450}] \times V/FM) \quad (4)$$

where D_{532}, D_{600}, and D_{450} is the absorbance at 600, 532, and 450 nm, respectively, and V is the volume of extraction; FM is the fresh mass of sample.

Guaiacol peroxidase (POD) was determined by measuring of the oxidation of guaiacol. The assay mixture contained 50 cm^3 of 0.1 M sodium phosphate (pH 6.0), 28 mm^3 guaiacol and 19 mm^3 of 30% H_2O_2. The absorbance was continuously recorded five times at 470 nm at 30-s intervals. Variation of absorbance per minute per gram FM [ΔA_{470}, g^{-1} min^{-1}(FM)] stands for POD activity. Superoxide dismutase (SOD) and catalase (CAT) activities were determined following the method of Giannopotitis and Ries (1997). The supernatant was desalted by Sephadex G-25 gel filtration to remove interfering materials and served for the crude enzyme extract. One unit of SOD activity (U) was defined as the amount of crude enzyme extract that required for inhibiting the reduction rate of nitroblue tetrazolium (NBT) by 50%. Unit of CAT activity [ΔA_{240}, g^{-1} min^{-1}(FM)] expressed variation of absorbance per minute per gram FM. All spectrophotometric analyses were performed at 0–4°C with an UV/visible light spectrophotometer (*UV-365, Shimadzu*, Japan).

ABA content: Frozen leaf samples (about 0.5 g) were pulverized in liquid nitrogen and extracted in 80% methanol containing 1 mmol L^{-1} of butylated hydroxytoluene (BHT). After 4-h extraction, the homogenate was centrifuged at 10,000 × *g* and 4°C for 15 min. The supernatant was used for ABA measurement by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRCMAC 252) according to the method of Asch (2000). Six replicates were carried out for each treatment.

Statistical analysis: Data were analyzed using one-way *ANOVA* of *SPSS* package (*Ver. 11, SPSS*, Chicago, IL, USA). The effect of O_3 was tested against the error variance for the main plot. The effects of salinity were tested against the error variance for the subplots. *Tukey-Kramer* method was applied to assess pair-wise comparisons among treatments. Differences between treatments were considered significant if $p \leq 0.05$.

Results

Plant growth and biomass yield: Seedling emergence rate was not significantly affected by O_3, evidenced by only 0.3% reduction being found in $+O_3$–S than CF-S treatment and 0.2% decrease in $+O_3$+S than CF+S. Significant reductions induced by salinity were measured, with 23% lower in CF+S than in CF–S and 16% lower in $+O_3$+S than in $+O_3$–S treatment (Table 1). Plant height significantly decreased in $+O_3$–S than in CF–S treatment, while nonsignificant differences were measured between $+O_3$+S and CF+S treatment. Both O_3 and salinity significantly reduced root length, number of leaves, leaf areas, shoot and root masses (Table 2). The reductions caused by O_3 were smaller in +S plants than in –S condition. Significantly higher O_3-caused reductions were noted in roots than in shoots. Significant interactions were noted between O_3 and salinity in leaf area, RWC, and shoot mass.

Table 1. Effects of ozone and salinity stress on plant growth parameters and leaf relative water content (RWC). CF, charcoal filtered air and $+O_3$, CF+O_3 (80 ± 5 ppb); –S, no salinity and +S, salinity-treated. Data were mean ± SE (*n = 6*). *Different letters* behind data within a row indicate significant differences ($p \leq 0.05$, *t*-test).

Treatments	CF–S	$+O_3$–S	CF+S	$+O_3$+S
Seedling emergence rate [%]	95.5 ± 4.1^a	92.7 ± 5.7^a	62.3 ± 4.8^b	61.4 ± 4.7^b
Plant height [cm]	7.4 ± 0.8^a	6.2 ± 0.7^b	4.7 ± 0.5^c	4.6 ± 0.6^c
Root length [cm]	28.3 ± 1.8^a	22.7 ± 1.3^b	19.1 ± 1.1^c	18.4 ± 0.9^c
Leaves [plant^{-1}]	5.5 ± 0.7^a	5.1 ± 0.3^a	4.6 ± 0.6^b	4.1 ± 0.4^b
Roots [plant^{-1}]	46.1 ± 3.5^a	44.5 ± 3.7^a	39.2 ± 2.8^b	38.4 ± 3.1^b
Leaf area [cm^2 plant^{-1}]	20.5 ± 1.5^a	16.6 ± 2.3^b	9.0 ± 0.8^c	8.5 ± 1.7^c
Leaf RWC [%]	86.5 ± 4.1^a	72.7 ± 5.8^b	63.2 ± 4.8^c	60.9 ± 4.7^c

Table 2. Effects of ozone and salinity stress on plant shoot, root and total dry biomasses. CF, charcoal filtered air and $+O_3$, CF+O_3 (80 ± 5 ppb); –S, no salinity and +S, salinity-treated. Data were mean ± SE (*n = 6*). *Different letters* behind data within a row indicate significant differences ($p \leq 0.05$, *t*-test). DM – dry mass.

Treatments	Shoot DM [g plant^{-1}]	Root DM [g plant^{-1}]	Total DM [g plant^{-1}]
CF–S	2.24 ± 0.21^a	1.21 ± 0.12^a	3.45 ± 0.21^a
$+O_3$–S	1.95 ± 0.19^b	0.87 ± 0.09^b	2.49 ± 0.19^b
CF+S	1.27 ± 0.17^c	0.64 ± 0.05^c	2.31 ± 0.17^b
$+O_3$+S	1.18 ± 0.16^c	0.49 ± 0.03^c	1.66 ± 0.16^c

Gas exchange and leaf Chl content: As shown in Fig. 1*A*,*B*, O_3 significantly decreased P_N and g_s in –S plants, but nonsignificant reductions were noted in +S plants. Intercellular CO_2 concentration (C_i) drastically increased by O_3 in –S plants, while no statistical difference was noted in +S plants (Fig. 1*C*). Leaf Chl contents significantly decreased in –S plants, but not in +S plants (Fig. 1*D*). However, both gas exchange and Chl content were significantly reduced by salinity in both CF and $+O_3$ treatments.

Membrane permeability, lipid peroxidation and osmo-regulatory ability: Both EL and MDA content of wheat leaves were significantly elevated by O_3 in –S plants, but not in +S plants (Fig. 2*A*,*B*). Salinity significantly enhanced EL and MDA contents in both CF and $+O_3$ conditions.

Significant enhancements in both soluble protein and proline contents of leaves were induced by salinity in CF and $+O_3$ conditions (Fig. 2*C*,*D*). Ozone stress elevated proline content in –S plants, but not in +S plants. Soluble protein content significantly decreased by O_3 in both –S and +S plants.

Antioxidant enzymes activities and ABA content: Antioxidant enzymes (SOD, POD, and CAT) activities were significantly elevated by individual O_3 or salinity (Fig. 3*A*,*B*,*C*). Salinity stimulated those enzymes activities in both CF (SOD 103%, POD 53%, CAT 69%) and $+O_3$ (SOD 91%, POD 36%, CAT 63%) conditions. The variational extents caused by salinity/O_3 were smaller in POD/CAT activity than SOD activity. No significant elevation induced by O_3 was noted in POD activity in +S plants. Salinity drastically increased ABA content in both $+O_3$ and CF plants (Fig. 3*D*). A considerable O_3-caused increase in ABA content was observed in –S plants, but not in +S plants.

Discussion

ABA accumulation and stomatal closure: The elevation of ABA content is an adaptive response of plants to stress conditions (Xiang *et al.* 2008). This stress signal transfer may lead to stomatal closure under saline condition. And stomatal closure may enhance the resistance of wheat to O_3 pollution by reducing O_3 uptake and diffusing in the intercellular spaces (Loreto and Fares 2007, Warren and Dreyer 2006). In this study, salinity-induced ABA accumulation in leaves considerably decreased g_s. Ozone induced significant reduction of g_s in –S plants, but not in +S plants. The result was consistent with the report on field-grown tomato (Maggio *et al.* 2007). Salinity-induced stomatal closure may limit plant O_3 uptake and minimized the O_3 damages. However, saline condition protects winter wheat from O_3 damage should be considered only in a relative term since salinity itself negatively affects plant growth. In addition, it is worth pointing out that O_3 may also mitigate the effects of salinity, evidenced by smaller reductions of g_s being found in $+O_3$+S plants than in $+O_3$–S plants (Fig. 1*B*).

Membrane integrity and gas exchange: EL and MDA content are two parameters indicating the cellular membrane integrity and lipid peroxidation (Calatayud *et al.* 2003, Zheng *et al.* 2008b). Exposure to O_3 resulted in marked increase in EL and MDA content in wheat grown in –S condition, but not in +S plants (Fig. 2*A*,*B*). This may be explained that salinity enhanced the plant adaptation to O_3 and mitigated the O_3-induced loss of cellular integrity and lipid peroxidation. Lower O_3 flux in +S plants due to stomatal closure alleviated O_3-induced decrease in chlorophyll content.

O_3-induced loss in P_N may be not only due to stomatal limitation, but also due to impaired activity of mesophyll cells as evidenced by an increase in C_i (Fig. 1*C*) along with a reduction in soluble protein (Fig. 2*C*). O_3-induced significant increase in C_i of –S plant may be caused by the decrease of the photosynthetic capacity as well as the increase of the plant respiration (Grantz *et al.* 2003, Xu *et al*. 2007). In saline condition, no significant differences were measured in C_i between CF and $+O_3$ plants, indicating plant organs has adapted to the stress. Anderson and Carol (2004) reported that Rubisco makes up as much as 70% of the soluble protein in the chloroplast. Therefore, O_3-induced reduction in soluble protein may result from the loss of Rubisco content and activity, which further decline photosynthesis. O_3-induced loss in P_N may also be caused by chloroplast membrane damages resulting from lipid peroxidation (Biswas *et al.* 2008). However, no significant O_3-induced decrease in P_N was found in +S plants. It indicates that salinity may protect mesophyll cells and chloroplast membrane integrity from O_3 damages (Maggio *et al.* 2009).

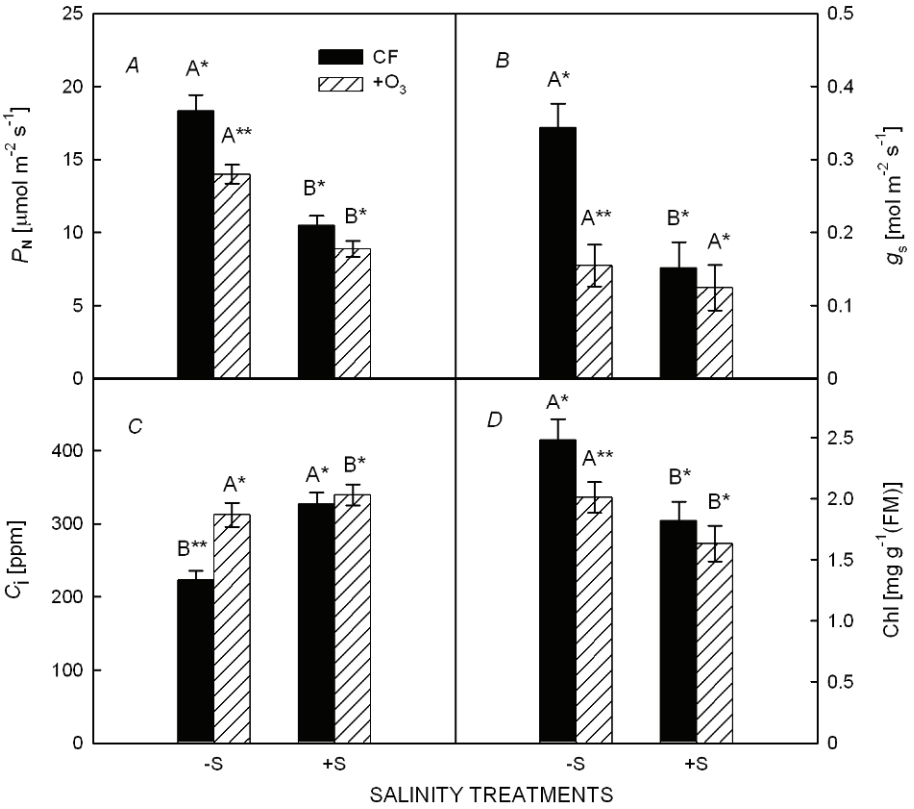

Fig. 1. Light-saturated net photosynthetic rate (P_N, *A*), stomatal conductance (g_s, *B*), intercellular CO_2 concentration (C_i, *C*) and chlorophyll content (Chl, *D*) of most recently fully expanded leaves of JN17 in responses to O_3 and salinity. CF – charcoal filtered air and $+O_3$ – CF+O_3 (80 ± 5 ppb) open-top chamber; –S – no salinity; +S – salinity-treated. *Vertical bars* indicate SE ($n = 6$). Within each treatment, significant differences between CF and $+O_3$ are marked by * while –S and +S treatments are marked by letters at $p \leq 0.05$.

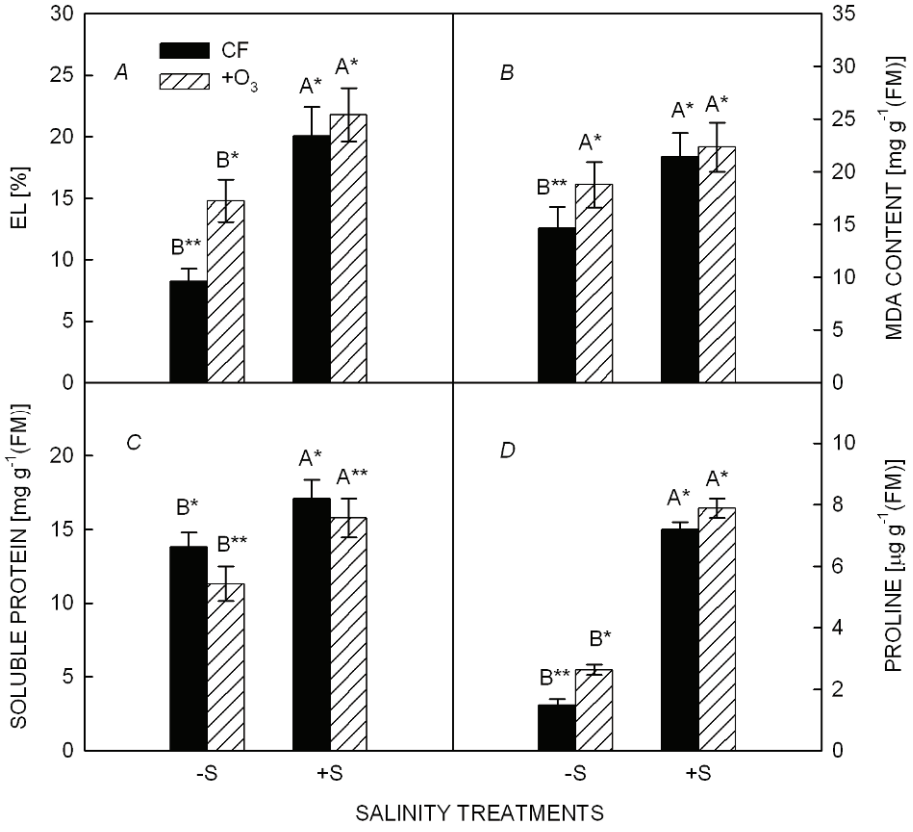

Fig. 2. Electrolyte leakage (EL, *A*), malondialdehyde (MDA) content (*B*), soluble protein (*C*) and proline contents (*D*) of JN17 in responses to O_3 and salinity. CF – charcoal filtered air and $+O_3$ – CF+O_3 (80 ± 5 ppb) open-top chamber; –S – no salinity; +S – salinity-treated. *Vertical bars* indicate SE (*n* = *6*). Within each treatment, significant differences between CF and $+O_3$ are marked by * while –S and +S treatments are marked by letters at $p \leq 0.05$.

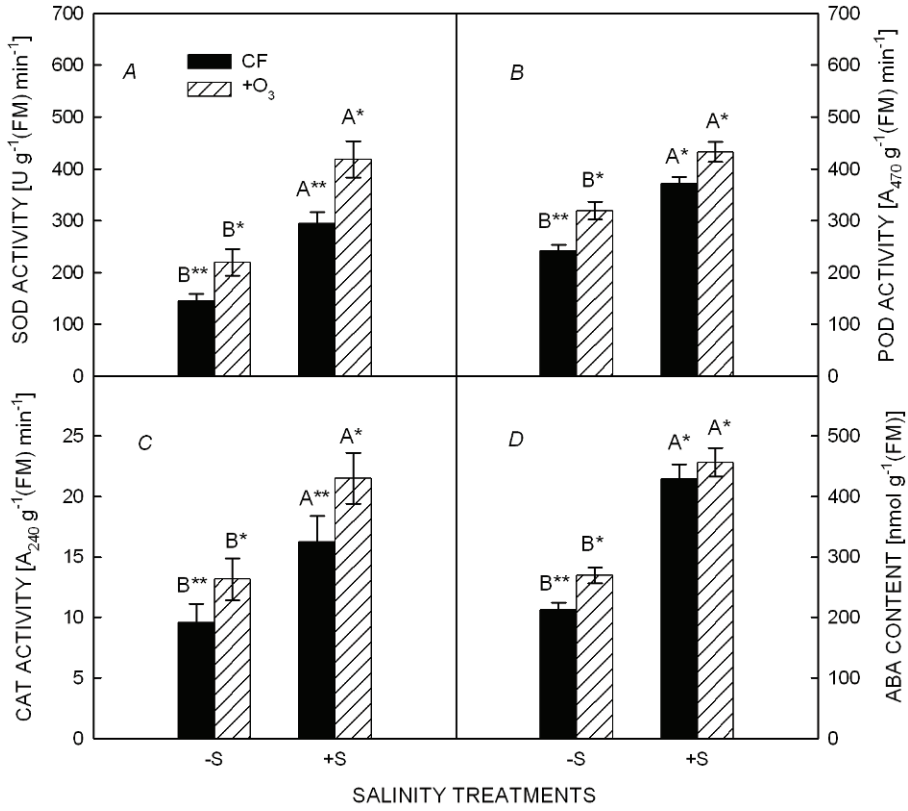

Fig. 3. Antioxidant enzymes (SOD, *A*; POD, *B*; CAT, *C*) activities and abscisic acid (ABA, *D*) content of JN17 in responses to O_3 and salinity. CF – charcoal filtered air and $+O_3$ – CF+O_3 (80 ± 5 ppb) open-top chamber; –S – no salinity; +S – salinity-treated. *Vertical bars* indicate SE (*n* = *6*). Within each treatment, significant differences between CF and $+O_3$ are marked by * while –S and +S treatments are marked by letters at $p \leq 0.05$. SOD – superoxide dismutase; POD – peroxidase; CAT – catalase.

Proline content and antioxidant enzymes activities: Plant adaptation to stress conditions has been correlated to increased levels of proline content and antioxidant enzymes activities (D'Haese *et al.* 2005). Salinity- and O_3-induced increases of proline content may improve the plant osmoregulatory ability (Mittova *et al.* 2004, Wang *et al.* 2004).

Salinity-induced stimulation of antioxidant enzymes (Zheng *et al.* 2008b, Zhu and Scandalios 2001) may enhance the ability of scavenging ROS caused by O_3. Significant interactions existed between salinity- and O_3-induced increases of antioxidant enzymes activities (Fig. 3*A*,*B*,*C*). Therefore it is believable that not only salinity-induced responses may protect wheat from O_3 damages, but also O_3-induced changes may increase the ability of plants against salinity. Nevertheless, those saline protections from O_3 injury are relative because both stresses themselves cause injuries to wheat plants (Massman 2004).

Future perspectives and conclusions: The results of this study pointed out that salinity-induced physiological response could alleviate O_3 damage by reducing O_3 uptake and increasing antioxidants capacities. Therefore, the effects of salinity counteracting O_3 damages in crops should be well considered in assessing potential O_3 damages in agricultural ecosystems.

In conclusion, saline condition significantly protected winter wheat from O_3 injuries. However, the negative effects of salinity on winter wheat were more serious than those of O_3. Consequently, the specificity of different agricultural environments should be involved in order to develop reliable prediction models on O_3 damage to wheat plants.

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