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Apoplastic hydrogen peroxide and superoxide anion exhibited different regulatory functions in salt-induced oxidative stress in wheat leaves

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Abstract

The present work aimed to investigate the mechanisms of nitric oxide (NO) and reactive oxygen species (ROS) generations and to explore their roles in the regulation of antioxidative responses in the wheat leaves under salinity. Except for an insignificant change of NO content and nitrate reductase (NR) activity due to 50 mM NaCl, NO, hydrogen peroxide, superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), chlorophyll and malondialdehyde content, as well as activities of nitric oxide synthase, NR, peroxidases (POD), catalase (CAT), and ascorbate peroxidase rose in response to different NaCl concentrations. Meanwhile, leaf superoxide dismutase activity lowered only at 50 mM NaCl. NaCl-stimulatory effects on NO content as well as POD and CAT activities could be partly alleviated by the application of 2-phenyl-4,4,5,5-tetrame-thylimidazoline-3-oxide-1-oxyl (PTIO, NO scavenger), exogenous CAT, or diphenylene iodonium (DPI, NADPH oxidase inhibitor). Native polyacrylamide gel electrophoresis also showed that the amount of POD (especially POD4, POD5, and POD7) and CAT (especially CAT1, CAT2, and CAT3) isozymes increased with increasing salinity but decreased by application of PTIO, CAT, or DPI. Furthermore, histochemical staining showed a similar change of $O_2^{\bullet-}$ generation. In addition, the inhibition of diamineoxidase (DAO), polyamine oxidase (PAO), and cell wall-bound POD (cw-POD) activities in NaCl-stressed seedlings seemed to be insensitive to the application of PTIO or DPI. Taken together, salinity-induced NO, H_2O_2, and $O_2^{\bullet-}$ generation influenced each other and played different roles in the regulation of antioxidant enzyme activities in the leaves of wheat seedlings under NaCl treatment.

Additional key words: antioxidants, nitric oxide, reactive oxygen species, salinity, *Triticum aestivum*.

Introduction

Salinity is among the major environmental factors that limit plant growth and cause significant losses in crop yield (Schroeder *et al.* 2013). Owing to shortages of irrigation water, shallow underground water tables, and groundwater salinization, soil salinity has become more serious and complicated worldwide (Xie *et al.* 2017). Globally, over one-third of the arable land is affected by salt stress, especially in arid and semiarid zones (Singh 2015). When the salt concentrations in the soil are higher than inside the plant root cells, the soil draw water from the roots, and plants wilt and sometimes die (Norbert and Tchiadje 2007). Generally, salinity reveals two major effects on plants, a rapid osmotic effect leading to cellular dehydration and plant wilting, and a cumulative ionic effect leading to penetration of toxic ions into cell cytoplasm (Munns and Tester 2008). In addition, high salinity also leads to nutritional disorders, oxidative stress, and damage to the photosynthetic apparatus.

Nitric oxide is recognized as an important signalling molecule regulating diverse physiological processes

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Abbreviations: APX - ascorbate peroxidase; ASA - ascorbic acid; CAT - catalase; Chl - chlorophyll; cPTIO - carboxy-2-phenyl-4,4,5,5-tetramethylimi-dazoline-3-oxide-1-oxyl; cw-POD - cell wall-bound POD; DAB - diaminobenzidine; DAO - diamineoxidase; DMTU - dimethylthiourea; DPI - diphenylene iodonium; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase; HbO_2 - oxyhaemoglobin; metHb - methaemoglobin; MDA - malondialdehyde; NBT - nitrobluetetrazolium; NO - nitric oxide; NOS-like protein - nitric oxide synthase-like protein; NR - nitrate reductase; $O_2^{\bullet-}$ - superoxide anion; $^{\bullet}OH$ - hydroxyl radical; PAO - polyamine oxidase; PBS - sodium phosphate buffer; POD - peroxidase; PTIO - 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA -trichloroacetic acid.

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including plant growth and development as well as responses to abiotic and biotic stresses (Saxena and Shekhawat 2013). The NO can be accumulated spontaneously in dependence on nitrite/nitrate ratio, but some is also generated enzymatically. Two main potential pathways depend on the involvement of nitric oxide synthase (NOS) activity and nitrate reductase (NR) activity, respectively (Rockel *et al.* 2002, Wilson *et al.* 2008). Thus, environmental stresses may regulate NO content in plants, which in turn affects the activities of these two enzymes. On the other hand, plants during growth and development experiencing salt stress can reveal the accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) (Chawla *et al.* 2011). The rapidly increasing ROS generation in response to salt stress can affect cellular functions by causing chlorophyll (Chl) degradation, lipid peroxidation, as well as the oxidation of nucleic acids and proteins (Foyer and Noctor 2005, Verma and Mishra 2005). Plants developed protective mechanisms against oxidative stress: mainly antioxidant enzymes superoxide dismutase (SOD), peroxidases (PODs), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). The SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2, which is then scavenged by CAT, POD, APX, and GR. Thus, the changes of these enzyme activities affect ROS accumulation in plants when exposed to different environment stresses. Besides, ROS accumulation is associated with other enzymatic reactions. For example, cell wall-bound POD (cw-POD) is important for H_2O_2 production *via* the oxidation of NADH (Achary *et al.* 2012). Diamine oxidase (DAO) and polyamine oxidase (PAO) preferentially catalyze the oxidation of polyamines, thus producing H_2O_2 in plant tissues (Cvikrová *et al.* 2012, Moschou *et al*. 2012).

More recently, Saxena and Shenkhawat (2013) reported that NO interacts with ROS in different ways and serves as an antioxidant during various stresses in plants. Yin *et al*. (2012) suggested that NO protected sweet potato roots against wound-induced oxidative damage by reacting with H_2O_2 directly or by modulating the activities of ROS-scavenging enzymes. Moreover, the interaction between NO and H_2O_2 generation relies on a mechanism that involves spatio-temporal organization and tissue-specific control at transcription, *e.g*., in citrus plants (Tanou *et al.* 2009).

Wheat is one of the main cereal crops and a major component of the human diet (Anita *et al.* 2010). The productivity of wheat is often adversely affected by salt stress which is associated with decreased germination percentage, reduced growth, altered reproductive behaviour, altered enzymatic activity, disrupted photosynthesis, and cell ultrastructure (Hasanuzzaman *et al.* 2017). Wheat cv. Xihan 3 is generally cultivated in northwest China and has high biomass production. Previous studies have been done on the effects of climate and planting density on its yield (Li *et al.* 2011). To better understand the function of NO and ROS in the salt-induced damage and/or plant tolerance to salt stress, wheat cv. Xihan 3 seedlings were used to explore interrelations among NO, H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$ generation and their roles associated with the regulation of antioxidant responses after NaCl treatment.

Material and methods

Plants and experimental design: Wheat (*Triticum aestivum* L. cv. Xihan 3) seeds were purchased from Gansu Agricultural University. Seeds were surface-sterilized with 0.1 % (m/v) $HgCl_2$ for 10 min, then soaked in water for 10 h and germinated in the dark at 25 ± 1.5 °C. The seedlings were cultured in Petri dishes and treated with 1/4 Hoagland solution containing 0, 50, and 150 mM NaCl with or without 250 μM 2-phenyl-4,4,5,5-tetramethyl-imidazoline-3-oxide-1-oxyl (PTIO), 500 unit dm^{-3} catalase (CAT), or 10 μM diphenylene iodonium (DPI). After culturing at a temperature of at 25 ± 2.5 °C, an irradiance of 300 μmol m^{-2} s^{-1}, and 12-h photoperiod for 6 d, leaves were harvested for further analyses.

Chlorophyll content was determined according to Arnon (1949). Plant material (0.5 g) was ground and extracted with 4 cm^3 of 95 % (v/v) ethanol, and then centrifuged at 10 000 *g* for 10 min. Absorbance of the supernatant was measured at 663 and 646 nm using spectrophotometer (*UV-Vis, Agilent 8453*, Palo Alto, USA).

Quantification of nitric oxide: The amount of NO was estimated according to Murphy and Noack (1994). Leaves (0.2 g) were homogenized with 1 cm^3 of cooled buffer [pH 6.0, containing 0.1 M sodium acetate, 1 M NaCl and 1 % (m/v) ascorbic acid (ASA)]. After centrifugation (10 000 *g*, 4 °C, 20 min), the supernatant was collected and pre-treated with CAT (100 U) and SOD (100 U) for 5 min. Oxyhaemoglobin (HbO_2) was added to a final concentration of 10 μM and then the mixture was incubated for 5 min. NO was quantified spectrophotometrically at 401 and 421 nm by following the conversion of HbO_2 to methaemoglobin (coefficient of absorbance, ε, of 77 mM^{-1} cm^{-1}).

Measurement of nitric oxide synthase (NOS) and nitrate reductase (NR) activities: Determination of NOS activity was based on the method of Guo *et al.* (2003). Fresh leaves (1 g) were ground with 50 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 7 mM reduced glutathione and 0.2 mM phenylmethylsulfonyl fluoride, and then centrifuged at 10 000 *g* for 30 min. Immediately,

the extract was mixed with 100 U of CAT and 100 U of SOD and incubated for 5 min according to HbO_2 capture method (Murphy and Noack 1994) in order to remove the endogenous ROS that affected the measurement. In addition, HbO_2 was added to a final concentration of 10 μM. After incubation at 37 °C for 5 min, absorbance of the mixture was measured at 401 and 421 nm. One unit (U) of NOS activity was defined as the amount of NO which react with 1 μmol HbO_2 per hour.

Assay for NR activity was according to Botrel and Kaiser (1997). Leaves (0.5 g) were ground in 5 cm^3 of 25 mM sodium phosphate buffer (PBS; pH 7.8) containing 5 mM EDTA and 5 mM cysteine at 4 °C. The homogenate was centrifuged at 12 000 *g* for 15 min. NR activity was measured in a reaction mixture containing 50 mM K-phosphate buffer (pH 7.5), 100 mM KNO_3, and 0.25 mM NADH. The reaction started with the addition of 0.4 cm^3 of the supernatant. After incubation at 25 °C for 30 min, 0.5 cm^3 of 1 % (m/v) sulphanilamide in 1.5 M HCl and 0.5 cm^3 of 0.01 % (m/v) α-naphthylamine were added to the reaction mixture. After the mixture was centrifuged at 18 000 *g* for 5 min, the resulting supernatant was used to detect absorbance at 540 nm and one unit of NR was defined as the amount of enzyme required for catalyzing the conversion of 1 μmol NO_2 within 1 h.

The content of H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$: H_2O_2 content was measured according to Sergiev *et al.* (1997). H_2O_2 was extracted by homogenizing 0.5 g leaves with 1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000 *g* for 20 min. The solution was mixed with 0.7 cm^3 of 10 mM PBS (pH 7.0) and 1.4 cm^3 of 1 M KI, absorbance of the supernatant was measured at 390 nm. H_2O_2 content was calculated using a standard curve. The $O_2^{\bullet-}$ content was determined according to Achary *et al.* (2012). Fresh leaves (0.15 g) were dipped into solution containing 50 mM Tris-HCl buffer (pH 6.4), 0.2 mM nitrobluetetrazolium (NBT), 0.2 mM NADH, and 250 mM sucrose, then vacuum-infiltrated for 10 - 15 min, and kept in the solution under an irradiance of 200 μmol m^{-2} s^{-1} for 24 h. Absorbance of the reaction mixture was measured at 530 nm and the amount of $O_2^{\bullet-}$ was calculated using ε = 12.8 mM^{-1} cm^{-1}. The $^{\bullet}OH$ content was measured according to Halliwell *et al.* (1987) as follows: 0.5 g leaves were homogenized with 10 mM PBS (pH 7.4) containing 15 mM 2-deoxy-D-ribose and centrifuged at 12 000 *g* for 15 min. The mixture containing 0.5 % (m/v) thiobarbituric acid (TBA) and 0.5 % (m/v) glacial acetic acid was heated in boiling water for 30 min, after which the supernatant was added. Absorbance was read at 532 nm.

The histochemical determination of $O_2^{\bullet-}$ was done by NBT staining (Romero-Puertas *et al.* 2004). Leaves isolated from the seedlings were vacuum-infiltrated with a water-circulating vacuum pump (*SHZ-DIII*, *Zihua Instruments*, Gongyi, China) for 10 min, and then incubated with 0.1 % (m/v) NBT solution (pH 7.4) in darkness until the appearance of dark blue insoluble formazan. Subsequently, leaves were bleached in 96 % boiling ethanol, and images were captured with digital acquisition device from *Olympus* (Tokyo, Japan) microscope.

Analysis of lipid peroxidation: The lipid peroxidation was determined according to malondialdehyde (MDA) content using thiobarbituric acid (TBA) reaction (Zhou 2001). Fresh leaves were homogenized and extracted with 5 cm^3 solution of 0.25 % (m/v) TBA and 10 % trichloroacetic acid (TCA) and then incubated in boiling water for 30 min, then cooled to room temperature and centrifuged at 10 000 *g* for 10 min. MDA content was calculated from absorbances at 440, 532, and 600 nm.

Measurement of antioxidant enzyme activities: Extracts for determination of SOD, peroxidase (POD), and CAT activities were prepared from leaves (0.5 g) homogenized under ice-cold conditions in extraction buffer (containing 50 mM PBS, pH 7.8, 0.1 mM EDTA, and 1 % PVP). The homogenates were centrifuged at 15 000 *g* for 30 min and the supernatants were used for the assays. All steps were performed at 4 °C.

The activity of SOD was determined by using the method of Dhindsa and Matowe (1981), measuring its ability to inhibit the photochemical reduction of NBT. The assay medium contained 50 mM PBS (pH 7.6), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA-Na_2, 2 μM riboflavin, and the enzyme extract. The reaction was initiated when the light was switched on and allowed to run for 10 min at 25 °C. The non-enzyme solution was irradiated as reference. One unit of enzyme activity was defined as the amount of enzyme required to inhibit the NBT reduction by 50 % measured at 560 nm.

The POD activity was measured using the method of Rao *et al.* (1996). Leaf extract was added to a reaction solution containing 50 mM PBS (pH 7.0) and 20 mM guaiacol. H_2O_2 was added to initiate the reaction after pre-incubation at 25 °C for 5 min. The enzyme activity was measured at 460 nm for 1 min. One unit was defined as an absorbance change of 0.01 min^{-1}.

Determination of the CAT activity was performed by the method of Aebi (1974) with some modifications. The mixture containing 50 mM PBS buffer (pH 7.0) and the enzyme extract was incubated at 25 °C for 5 min and then 6 μM H_2O_2 was added. CAT activity was measured as the decline in absorbance at 240 nm. An absorbance change of 0.1 min^{-1} was defined as one unit of CAT activity.

The APX activity assay was performed according to Nakano and Asada (1981). Plant material was ground with 1 cm^3 of 50 mM PBS buffer (pH 7.0) containing 1 mM EDTA and 1 mM ascorbate (ASA), then it was centrifuged at 15 000 *g* for 30 min. Reaction was initiated by addition the tissue extract to the reaction buffer containing 50 mM PBS (pH 7.0), 0.5 mM ASA, and

3 μM H_2O_2. Ascorbate oxidation was monitored by measuring the decrease in absorbance at 290 nm. One unit of APX activity was defined as an absorbance change of 0.1 min^{-1}.

The glutathione reductase (GR) activity assays were performed following the method of Schaedle and Bassham (1977). Leaves (0.5 g) were ground with 2 cm^3 of 50 mM Tris-HCl (pH 7.5) buffer supplied with 0.1 mM EDTA and 0.1 % (m/v) PVP, and then centrifuged at 13 000 *g* for 30 min. Tissue extracts were placed in a buffer containing 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, and 0.15 mM NADPH. The decrease in absorbance at 340 nm was continuously monitored and one unit of GR activity was defined as a change in absorbance of 0.1 min^{-1}.

Polyacrylamide gel electrophoresis for POD and CAT isozymes: Native polyacrylamide gel electrophoresis (PAGE) was prepared according to Laemmli (1970). The samples were loaded into gel lanes composed of 4 % stacking gels and 7.5 % resolving gel for POD and CAT. Electrophoresis was performed at 120 V and 4 °C.

For determination of POD activity by the method of Huh *et al.* (1998), the gel was after washing in distilled water transferred to the dye solution [A: 0.4 g benzidine was added to 3 cm^3 of glacial acetic acid, and then mixed with 17 cm^3 of warm (80 °C) distilled water; B: 4 % (m/v) NH_4Cl; C: 5 % EDTA; D: 3 % H_2O_2; A, B, C, D and distilled water in a volume ratio of 1:1:1:1:9], and kept at 37 °C for 5 - 10 min until the enzyme bands cleared. Then the gel was rinsed with distilled water for 30 min.

CAT activity staining was performed according to Chandlee and Scandalios (1984). After incubation in a solution of H_2O_2 (0.01 %, v/v) for 20 min, the gel was soaked in the solution supplying 1 % ferric chloride and 1 % potassium ferricyanide, and CAT bands was developed. Vertical electrophoresis system (*Bio-Rad Mini-Protean Tetra Cell 164-8001*, Hercules, CA, USA) was used to run gel electrophoresis, *Gel Doc 2000* (*Bio-Rad*) gel imaging system and *Quantity One* software were used to capture and analyse the images.

Determination of DAO, PAO and cell wall-bound POD activities: Extraction of cell wall was performed following the method of Lee and Lin (1995). Fresh leaves were homogenized in 50 mM PBS (pH 5.8), and the homogenate was centrifuged at 1 000 *g* for 10 min and washed at least twice with 50 mM PBS (pH 5.8). The pellets were dissolved and incubated in 1 M NaCl for 2 h. After centrifugation at 1 000 *g* for 10 min, the supernatant was used for the estimation of diaminooxidase (DAO) and cell wall-bound (cw) POD activities.

The DAO activity in cell wall fraction was carried out spectrophotometrically at 510 nm as described by Naik *et al.* (1981). The enzyme extract was mixed with 50 mM PBS buffer (pH 7.8), 10 mM putrescine, 0.1 mM pyridoxal phosphate, and the reaction mixture was incubated at 30 °C for 1 h. After the addition of 1 cm^3 of 20 % (m/v) TCA, the reaction mixture was centrifuged at 5 000 *g* for 15 min. Then, the supernatant was mixed with 1 cm^3 of ninhydrin and the colour was developed at 100 °C for 30 min. Absorbance was measured at 510 nm after the addition of 1 cm^3 of acetic acid. Additionally, TCA was added prior to the enzyme solution in the control.

Roots were ground with 2 cm^3 of 100 mM PBS (pH 7.0) containing 5 mM dithiothreitol. After centrifugation at 16 000 *g* for 20 min, the residue pellet was sequentially extracted with 100 mM PBS (pH 7.0) containing 1 mM NaCl. The supernatant was used for the determination of polyamineoxidase (PAO) activity following the procedure described by Asthir *et al.* (2002). The enzyme extract was mixed with 50 U of CAT and 250 mm^3 of 2-aminobenzaldehyde (0.1 %, m/v), then 250 mm^3 of 10 mM spermidine was added to initiate the reaction. The reaction mixture was incubated at 30 °C for 3 h followed by the addition of 1 cm^3 of 10 % (v/v) perchloric acid. After centrifugation at 6500 *g* for 10 min, absorbance (formation of Δ^1-pyrroline product) was recorded at 430 nm.

The activity of cw-POD was assayed by the method of Dos Santos *et al.* (2008). The enzyme extract was mixed with 25 mM PBS buffer (pH 6.8) containing 2.58 mM guaiacol, and 10 mM H_2O_2. Absorbance was measured at 470 nm for 2 min. One unit of enzyme activity was defined as an absorbance change of 0.01 min^{-1}.

Protein content in the supernatant was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

Data analysis: The data were expressed as the mean ± standard error (SE) of three independent replicates of every treatment. Statistical analysis was carried out with *SPSS 17.0* software. After analysis of variance (one-way *ANOVA*), Duncan's multiple comparisons were performed and the significant differences were indicated by different letters ($P < 0.05$). Moreover, Pearson's correlation analysis was used to identify the relationship among different parameters.

Results

Compared with the control, the amount of total chlorophyll (Chl), Chl *a* and Chl *b* per fresh mass unit rose and the Chl *a*/Chl *b* ratio decreased in the leaves of wheat seedlings under 50 mM and 150 mM NaCl (Table 1). Salinity stress together with PTIO or CAT did not affect these three parameters in comparison with

Table 1. Content of total chlorophyll (Chl), Chl *a*, Chl *b* [mg g^{-1}(f.m.)] contents and Chl *a*/Chl *b* ratio in wheat leaves under different treatments. Means ± SEs, $n = 3$, values within the same column followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

	Total Chl	Chl *a*	Chl *b*	Chl *a*/Chl *b*
Control	1.30 ± 0.002a	1.10 ± 0.001a	0.20 ± 0.001a	5.45 ± 0.018f
50 mM NaCl	1.65 ± 0.007b	1.34 ± 0.001b	0.31 ± 0.006b	4.31 ± 0.076e
150 mM NaCl	1.85 ± 0.002c	1.46 ± 0.003c	0.39 ± 0.001c	3.68 ± 0.018d
150 mM NaCl + PTIO	1.84 ± 0.005c	1.44 ± 0.004c	0.41 ± 0.004c	3.52 ± 0.039c
150 mM NaCl + CAT	1.88 ± 0.001c	1.43 ± 0.004c	0.45 ± 0.003c	3.16 ± 0.028b
150 mM NaCl + DPI	2.07 ± 0.002d	1.47 ± 0.004c	0.60 ± 0.003d	2.47 ± 0.020a

150 mM NaCl stress alone. However, the addition of DPI led to significant increases in total Chl and Chl *b* content in the leaves of salinity-stressed seedlings. Additionally, the Chl *a*/Chl *b* ratio decreased in response to NaCl + PTIO, NaCl + CAT, or NaCl + DPI treatments as compared with 150 mM NaCl alone. Leaf NO content did not change due to 50 mM NaCl, but it significantly increased at 150 mM NaCl exposure, with about 20 % enhancement in comparison with the control. However, compared with 150 mM NaCl alone, the application of PTIO, CAT, or DPI suppressed the increase of NO generation (Fig. 1).

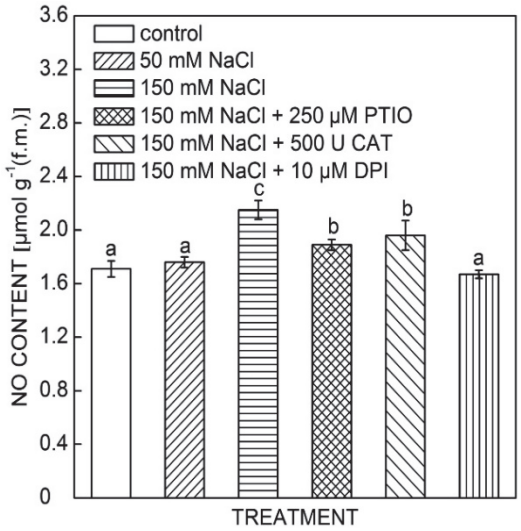

Fig. 1. Changes of NO content in wheat leaves under different treatments. Means ± SEs, $n = 3$, different letters indicate significant difference according to Duncan´s multiple range test ($P < 0.05$).

Wheat leaves exhibited significant elevations of NOS activity in response to both NaCl concentrations (Fig 2*A*). In comparison with 150 mM NaCl alone, the NOS activity increased less after addition of PTIO, CAT, or DPI. Compared to the control, 50 mM NaCl treatment did not affect NR activity, whereas a notable increase of this enzyme activity was observed in response to 150 mM NaCl (Fig. 2*B*). In addition, leaf NR activity decreased by about 35 % in response to NaCl + PTIO treatment, but insignificantly changed to NaCl + CAT or NaCl + DPI treatments in comparison with 150 mM NaCl stress alone.

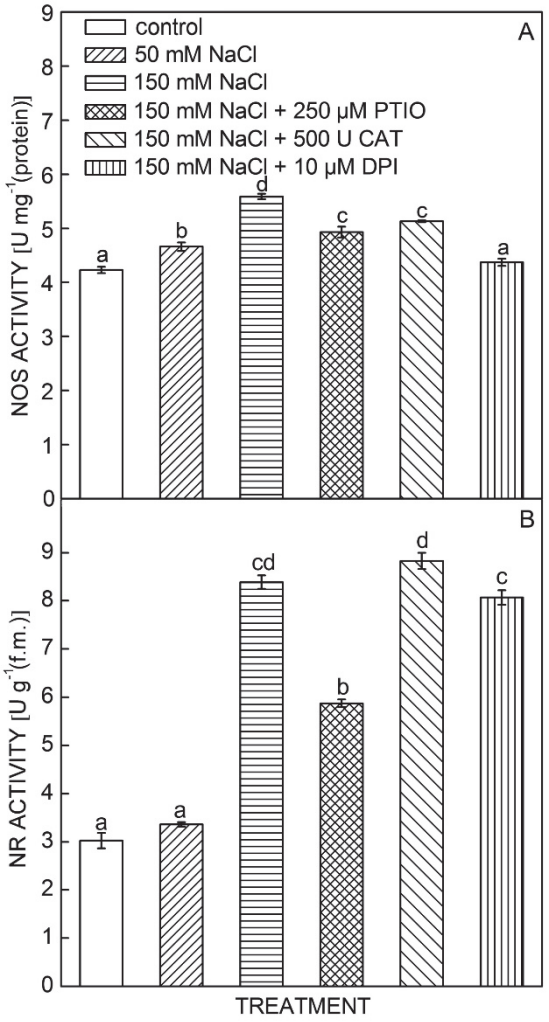

Fig. 2. Effects of different treatments on the activities of NOS (*A*) and NR (*B*) in wheat leaves. Means ± SEs, $n = 3$, different letters indicate significant difference according to Duncan´s multiple range test ($P < 0.05$).

Table 2. Effects of different treatments on the content of H_2O_2 [ng g^{-1}(f.m.)], $O_2^{\bullet-}$ [μmol g^{-1}(f.m.)] and $^{\bullet}OH$ [(nmol g^{-1}(f.m.)] in wheat leaves. Means ± SEs, n = 3, values within the same column followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

Treatments	H_2O_2	$O_2^{\bullet-}$	$^{\bullet}OH$
Control	467.58 ± 10.08a	1.15 ± 0.02a	39.46 ± 1.35b
50 mM NaCl	527.62 ± 4.84b	1.69 ± 0.07b	45.67 ± 1.00c
150 mM NaCl	619.41 ± 9.36d	2.04 ± 0.02bc	46.14 ± 0.79c
150 mM NaCl + PTIO	612.73 ± 8.28d	2.40 ± 0.13cd	51.20 ± 0.95d
150 mM NaCl + CAT	668.08 ± 7.10e	2.75 ± 0.18d	40.84 ± 0.83b
150 mM NaCl + DPI	556.74 ± 4.77c	0.98 ± 0.05a	27.34 ± 0.37a

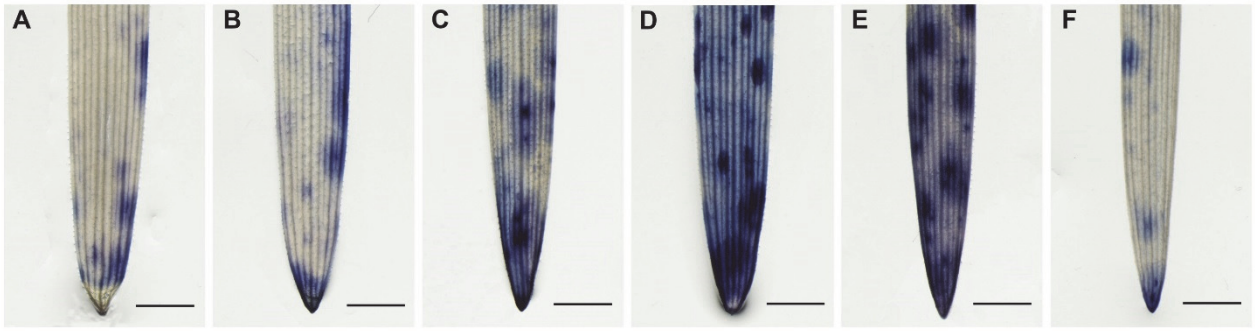

Fig. 3. Histochemical localization of $O_2^{\bullet-}$ by NBT staining. Wheat leaves were exposed to different treatments. *A* - control; *B* - 50 mM NaCl; *C* - 150 mM NaCl; *D* - 150 mM NaCl + 250 μM PTIO; *E* - 150 mM NaCl + 500 U dm^{-3} CAT; *F* - 150 mM NaCl + 10 μM DPI. *Scale bars* = 2 mm.

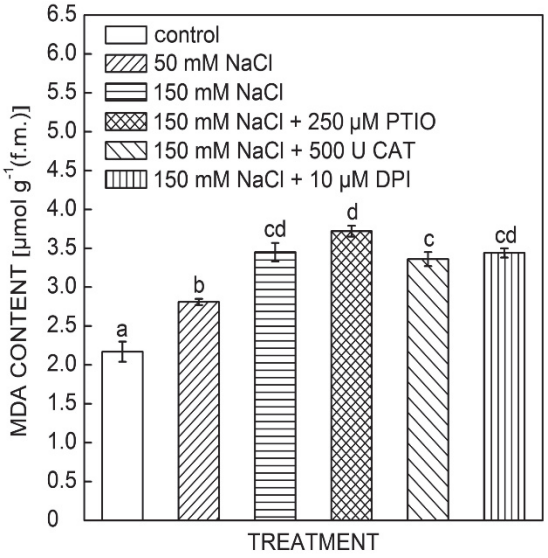

Fig. 4. Changes of MDA content in wheat leaves under different treatments. Means ± SEs, n = 3, different letters indicate significant difference according to Duncan´s multiple range test ($P < 0.05$).

Leaf H_2O_2 content was enhanced in response to both NaCl concentrations (Table 2). The presence of PTIO did not affect this parameter, whereas H_2O_2 content reduced about 10 % in the leaves of NaCl + DPI stressed seedlings, in comparison with 150 mM NaCl alone. In contrast, a further enhancement in leaf H_2O_2 content was found when the seedlings were exposed to NaCl together with CAT.

Compared with the control, leaf $O_2^{\bullet-}$ generation rose by about 47 and 77 %, respectively, after treatment with 50 and 150 mM NaCl. The leaves of seedlings treated with NaCl + PTIO or NaCl + CAT showed a significant increase in $O_2^{\bullet-}$ content compared with 150 mM NaCl alone, and the maximal increase was observed at NaCl + CAT. However, this parameter decreased by about 52 % in NaCl + DPI treated seedlings (Table 2). Additionally, the production of $O_2^{\bullet-}$ was monitored by NBT histochemical staining, and results were consistent with the results of spectrophotometry. As shown in Fig. 3*A*-*C*, the enhanced distribution of blue $O_2^{\bullet-}$-NBT precipitates were observed in the leaves exposed to 50 and 150 mM NaCl in comparison with the control. Compared with 150 mM NaCl treatment alone, the addition of PTIO or CAT resulted in a higher density of precipitates, but the density was significantly reduced in the leaves treated with NaCl + DPI (Fig. 3*C*-*F*).

Similarly, $^{\bullet}OH$ content rose about 16 and 17 % in the leaves under 50 and 150 mM NaCl stress, respectively, compared to the control. A further enhancement in leaf $^{\bullet}OH$ content was observed due to 150 mM NaCl and PTIO treatment, whereas this parameter declined due to NaCl + CAT or NaCl + DPI treatments, with the lowest content after NaCl + DPI treatment (Table 2).

Compared with control, the amount of MDA significantly rose in the leaves of wheat seedlings under 50 and 150 mM NaCl stresses. 150 mM NaCl treatment combined with PTIO slightly increased MDA content but CAT slightly decreased it in comparison with NaCl alone (Fig. 4). The application of DPI did not affect NaCl-induced change of MDA content.

Leaf SOD activity decreased in 50 mM NaCl treated seedlings, but insignificantly altered in 150 mM NaCl-stressed ones (Table 3). NaCl treatment combined with CAT did not affect leaf SOD activity, but in comparison with 150 mM NaCl treatment alone, the addition of PTIO or DPI led to about 15 and 20 % decreases in this enzyme activity in NaCl-stressed seedlings, respectively.

POD activity in wheat leaves did not alter significantly in response to 50 mM NaCl but it was stimulated by about 64 % under 150 mM NaCl (Table 3). NaCl treatment combined with PTIO, CAT, or DPI resulted in about 23, 56, and 20 % decreases in this enzyme activity, respectively, compared with 150 mM NaCl alone. Besides, we detected at least seven bands of POD isozymes in wheat leaf. Among them, mainly POD4, POD5, and POD7 isozymes contributed to total POD activity (Fig. 5*A-F*). Compared with control, 50 mM NaCl treatment did not change the six bands, but the treatment with 150 mM NaCl generally led to increases in all band size. We also noticed that the bands intensity declined due to 150 mM NaCl + PTIO, NaCl + CAT, or NaCl + DPI treatments, in comparison with the 150 mM NaCl alone. These results are consistent with the changes of total POD activity.

As shown in Table 3, 50 mM NaCl treatment did not affect leaf CAT activity, but exposure to 150 mM NaCl led to about 41 % increase in this enzyme activity. Compared with 150 mM NaCl stress alone, CAT activity was insignificantly changed in response to NaCl + PTIO, while the presence of CAT and DPI resulted in about 19 and 11 % decreases, respectively. Fig. 6*A-F* reveals three CAT isozymes and compared with control these three bands did not change obviously in response to 50 mM NaCl. However, 150 mM NaCl brought about significant increases in band size, especially the band of CAT3 isozyme. In comparison with 150 mM NaCl alone, the band intensity of CAT1, CAT2, and CAT3 decreased in response to NaCl + PTIO, NaCl + CAT, or NaCl + DPI, with minimum band intensity under NaCl + PTIO.

Compared with the control, an insignificant decrease in leaf APX activity was observed under 50 mM NaCl, but the activity increased by about 25 % in response to 150 mM NaCl, NaCl + CAT, or NaCl + DPI. Addition of PTIO slightly decreased leaf APX activity (Table 3). As shown in Table 3, all treatments did not affect leaf GR activity in wheat seedlings.

A significant decrease of leaf DAO activity was also observed in wheat seedlings treated with NaCl (Table 4). Leaf DAO activity declined to 88 and 78 % of control

Table 3. Changes of antioxidant enzyme activities [U mg^{-1}(protein)] in wheat leaves under different treatments. Means ± SEs, $n = 3$, values within the same column followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

Treatments	SOD	POD	CAT	APX	GR
Control	20.31 ± 0.25d	217.16 ± 3.37b	29.07 ± 0.20a	121.70 ± 2.23a	4.34 ± 0.18a
50 mM NaCl	18.66 ± 0.12c	230.52 ± 3.40b	31.77 ± 0.14a	111.89 ± 3.12a	4.36 ± 0.06a
150 mM NaCl	19.96 ± 0.05d	356.73 ± 1.89d	41.04 ± 0.33d	162.60 ± 8.42c	4.50 ± 0.32a
150 mM NaCl + PTIO	17.03 ± 0.18b	274.91 ± 5.29c	37.84 ± 0.37cd	142.64 ± 5.69b	4.17 ± 0.14a
150 mM NaCl + CAT	19.88 ± 0.15d	158.39 ± 1.24a	33.27 ± 0.25ab	166.56 ± 5.49c	4.68 ± 0.07a
150 mM NaCl + DPI	15.91 ± 0.08a	286.98 ± 4.70c	36.40 ± 0.64bc	169.85 ± 4.10c	4.82 ± 0.11a

Table 4. Effects of different treatments on the activities of DAO, PAO, and cw-POD activities [U mg^{-1} (protein)] in wheat leaves. Means ± SEs, $n = 3$, values within the same column followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

Treatments	DAO	PAO	cw-POD
Control	4.98 ± 0.03d	4.60 ± 0.15d	25.79 ± 1.26b
50 mM NaCl	4.37 ± 0.10c	4.25 ± 0.06c	17.69 ± 0.28a
150 mM NaCl	3.87 ± 0.01b	3.83 ± 0.05b	17.22 ± 0.59a
150 mM NaCl + PTIO	3.84 ± 0.06b	3.80 ± 0.08b	18.44 ± 0.68a
150 mM NaCl + CAT	3.51 ± 0.08a	3.57 ± 0.06a	19.02 ± 0.66a
150 mM NaCl + DPI	3.70 ± 0.09b	3.87 ± 0.05b	17.43 ± 0.71a

value in 50 and 150 mM NaCl-treated seedlings, respectively. Addition of PTIO or DPI did not affect this enzyme activity, but NaCl + CAT treatment further inhibited leaf DAO activity compared with 150 mM NaCl stress alone (Table 4).

Similarly, PAO activity lowered in response to 50 and 150 mM NaCl stress in the leaves of wheat seedlings (Table 4). The addition of PTIO or DPI did not affect NaCl-inhibitory effect on leaf PAO activity, whereas CAT application further decreased this enzyme activity in 150 mM NaCl-stressed seedlings.

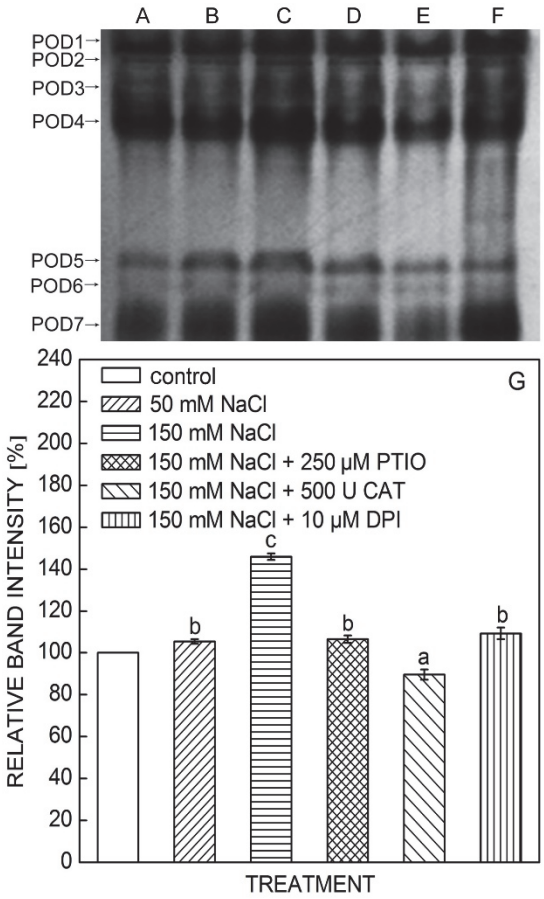

Fig. 5. Native gel analysis of peroxidase (POD) isozymes (*A*-*F*) and relative band intensity of POD isozymes (*G*) in wheat leaves in response to different treatments. *A* - control; *B* - 50 mM NaCl; *C* -150 mM NaCl; *D* - 150 mM NaCl + 250 µM PTIO; *E* - 150 mM NaCl +500 U dm^{-3} CAT; *F* - 150 mM NaCl + 10 µM DPI. Means ± SEs, $n = 3$, different letters indicate significant difference at $P < 0.05$.

Compared with the control, cw-POD activity in the leaves maintained a stable and significant decrease when the seedlings were exposed to different NaCl concentrations (Table 4). The application of PTIO, CAT, or DPI did not significantly affect 150 mM NaCl-inhibitory effect on cw-POD activity in the leaves of wheat seedlings (Table 4).

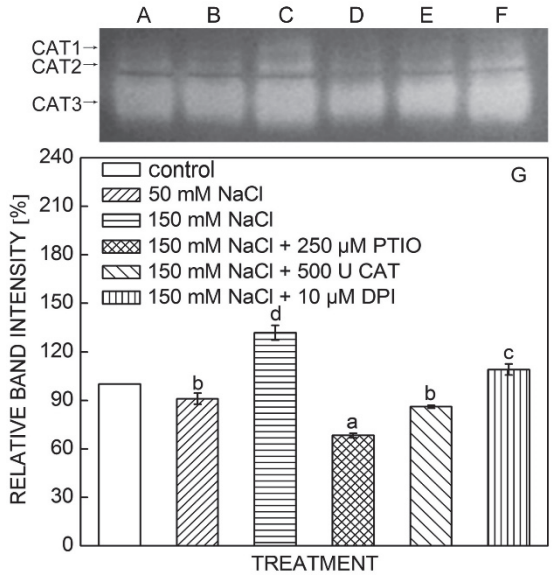

Fig. 6. Native gel analysis of catalase (CAT) isozymes (*A*-*F*) and relative band intensity of CAT isozymes (*G*) in wheat leaves in response to different treatments. *A* - control; *B* - 50 mM NaCl; *C* - 150 mM NaCl; *D* - 150 mM NaCl + 250 µM PTIO; *E* - 150 mM NaCl + 500 U dm^{-3} CAT; *F* - 150 mM NaCl + 10 µM DPI. Means ± SEs, $n = 3$, different letters indicate significant differences at $P < 0.05$.

Pearson's correlation was applied to assess the association between different parameters, and the correlations were identified based on the Pearson's correlation coefficient value (Table 5). Chl content was closely linked to H_2O_2 generation as well as NR, SOD, CAT, and APX activities, while significant negative correlations were observed between the amount of Chl and the activities of DAO, PAO, and cw-POD. DAO, PAO and cw-POD activities also showed strong negative correlations with parameters NOS, NR, H_2O_2, $O_2^{\bullet-}$, CAT and APX, respectively. These results suggest that parameters NO, NOS, NR, H_2O_2 and CAT were significantly related to each other.

Discussion

Photosynthesis, as the basis of plant productivity, is a major determinant of crop yield and extremely sensitive to salt stress (Ogbonnaya *et al.* 2013, Silveira and Carvalho 2016). The variation in photosynthetic pigment content can provide valuable insight into the physiological performance of leaves and indicate their photosynthetic capacity (Boquera and Morales 2010). Many reports indicated that amount of photosynthetic pigments decrease in some plants when exposed to increasing salt stress (Taïbi *et al.* 2016, Bacha *et al.*

2017). However, in the present study, the significant increases in the Chl *a*, Chl *b* and total Chl amounts were observed in the leaves of wheat seedlings under salt stress, probably due to its expression per fresh mass unit and decrease in water content in fresh mass. This was in agreement with previous studies in plants *Ricinus communis* (Li *et al.* 2010) and *Amaranthus* (Wang and Nil 2000) under salt stress. The study of Lutts *et al.* (1996) indicated that Chl content can be an indicator of tissue tolerance to NaCl. Thus, the stability of Chl might play an important role in salt tolerance capability of wheat seedlings. Additionally, the increased Chl content in wheat leaves was accompanied by the reduction of Chl *a*/Chl *b* ratio, suggesting that Chl *b* might be more sensitive to salt stress than Chl *a*. Apostolova *et al*. (2006) reported that a reduction in Chl *a*/Chl *b* in salt-treated melon plants was a criterion for reduction in light absorption capacity of photosystem II.

It is commonly observed, that both NO and ROS are generated in plants under stress (Qiao *et al.* 2014). Moreover, excessive ROS formation in different organelles induces oxidative damage that is usually characterized by increased lipid peroxidation (Noctor and Foyer 2016). In wheat leaves, except for an insignificant change of NO content under 50 mM NaCl, salinity stress induced the increases of NO, H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$ generation (Fig. 1 and Table 2) and led to MDA accumulation (Fig. 4), a biomarker of lipid peroxidation (Koca *et al.* 2007). Differently, 250 mM NaCl reduces NO content but stimulate higher $O_2^{\bullet-}$ and H_2O_2 accumulation in tomato roots (Poór *et al*. 2015).

Accumulation of NO induced by 150 mM NaCl (Fig. 1) was the result of the enhancement of NOS and NR activities (Fig. 2*A*,*B*) in wheat leaves because NOS and NR are two key enzymes for NO synthesis (Wang *et al.* 2010). A similar change of NR activity and the increase of NO content were found in response to salt stress in watermelon seedlings (Yang *et al.* 2013). However, NOS activity in *Arabidopsis* decreases with increasing NaCl concentrations (Zhao *et al.* 2007).

The coordination of antioxidant enzymes plays an important protective role in ROS scavenging when plants are exposed to environment stresses. SOD can catalyze the dismutation of two molecules of $O_2^{\bullet-}$ into O_2 and H_2O_2 (Zhang *et al.* 2007), and the stimulation of this enzyme is correlated with increased protection from damage associated with oxidative stress (Anjum *et al.* 2011). Here, we observed that SOD activity decreased or did not change under NaCl stress, and in consequence $O_2^{\bullet-}$ content increased. However, the stimulation of POD, CAT, and APX by NaCl treatment (Table 3) played positive roles in detoxifying H_2O_2 and alleviating oxidative damage in wheat leaves. Our observations were in agreement with the findings of Singh *et al*. (2015), who showed decreased SOD activity and increased POD, APX, CAT, and GR activities in two wheat genotypes exposed to 200 mM NaCl. Antioxidant enzymes have multiple molecular forms (isozymes), and isozymes are located in different cellular compartment (Hu *et al*. 2005). Changes of isozyme activities in plant cells are important indicators of oxidative stress (Kumar *et al.* 2012). In fact, the discrepancy of isozyme bands and activities may be attributed to the difference of plant species, plant organs, and even stress conditions. For instance, at least six CAT isozymes were reported in *Arabidopsis thaliana* (Jaleel *et al.* 2008), and three CAT isozymes were found in shoots and two in roots of rice (Shah and Nahakpam 2012). Additionally, native polyacrylamide gel assays showed the abundance of low molecular mass POD isozymes in the leaves and roots of salt-sensitive rice seedlings under salt treatment (Lee *et al.* 2013). In wheat leaves, the electrophoretic pattern revealed seven POD isozymes and three CAT ones (Figs. 5 and 6). Furthermore, analyses of isozyme activities were consistent with the changes of total POD and CAT activities. Meanwhile, with increasing NaCl concentration, the enhancement of the total POD activity mainly depended on isozymes POD4, POD5, and POD7 (Fig. 5), and the stimulation of the total CAT activity was due to CAT1, CAT2, and CAT3 isozymes (Fig. 6).

The removal of NO due to presence of PTIO in salinity-treated wheat leaves (Fig. 1) was accompanied with the inhibition of NOS and NR activities (Fig. 2*A*,*B*). Hsu and Kao (2004) suggested that the protective effect of NO against abiotic stresses is closely associated with NO-mediated ROS reduction. Moreover, NO abolishment by cPTIO in cold-stress fruit results in significant increases of $O_2^{\bullet-}$ and H_2O_2 content (Xu *et al.* 2012). The present data support these studies, because NO scavenging in NaCl-treated seedlings by using PTIO further increased $O_2^{\bullet-}$ and $^{\bullet}OH$ content (Table 2). Thus, salinity-induced increase of NO content probably played an important role in alleviating ROS accumulation in wheat leaves. Nevertheless, PTIO application did not affect lipid peroxidation in salt-stressed wheat leaves. Similar results were observed in tall fescue (Xu *et al*. 2010) and rye grass (Liu *et al.* 2008). In *Chorispora bungeana* cell suspension, NO exerts a protective function against chilling-induced oxidative damage by enhancing APX, CAT, GR, POD, and SOD activities (Liu *et al.* 2010). Lin *et al.* (2012) also found that exogenous NO increases SOD, CAT, APX, and GR activities in cucumber under salt stress. Similar effects of endogenous NO generation were found in cadmium-stressed *Brassica juncea* seedlings (Verma *et al.* 2008) and ABA-treated transgenic tobacco plants (Lu *et al.* 2014). Consistent with these observations, blocking NO accumulation using PTIO negatively affected SOD, POD, CAT, and APX activities in wheat leaves, indicating that salinity-induced NO generation plays an important function in maintaining SOD activity and stimulating POD, CAT, and APX activities. Furthermore, the intensity of POD and CAT isozyme bands was reduced in response to NaCl + PTIO treatment. According to the

above results, when wheat seedlings were treated with salinity, leaf NO accumulation resulted in the increases of $O_2^{\bullet-}$ and $^{\bullet}OH$ content together with CAT, POD, and APX activities, which prevented the excessive H_2O_2 accumulation, thus alleviating strong oxidative damage caused by salt stress.

In plant tissues, the apoplast is mainly composed of cell walls and intercellular spaces, and is often regarded as a key site of important events regulating physiological and developmental processes (Fernández *et al.* 2012). The present data showed that apoplastic H_2O_2 scavenging by exogenous CAT resulted in the increases of H_2O_2 and $O_2^{\bullet-}$ content as well as the decrease of NO and $^{\bullet}OH$ content in the leaves of wheat seedlings under 150 mM NaCl. Similarly, in cucumber seedlings, diamino-benzidine (DAB) staining indicates the increase in leaf H_2O_2 content imparted by dimethylthiourea (DMTU; H_2O_2 scavenger) treatment (Li *et al.* 2013), and ABA-induced H_2O_2 production mediates NO generation in bermudagrass (Lu *et al.* 2009). The apoplastic $O_2^{\bullet-}$ is mainly produced by plasma membrane localized NADPH oxidases because this enzyme is able to transfer electrons from cytoplasmic NADPH to an O_2 molecule to produce $O_2^{\bullet-}$ (Rejeb *et al.* 2015). DPI has been widely used to inhibit NADPH oxidase. Even though apoplastic $O_2^{\bullet-}$ from NADPH oxidase can be disproportionated to apoplastic H_2O_2 by SOD (Park and Doke 2005), in contrast with the use of CAT, the application of DPI negatively affected the generation of NO, H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$ in the leaves of wheat seedlings under salinity stress (Fig. 1 and Table 2). Thus, the accumulation of apoplastic H_2O_2 and the generation of $O_2^{\bullet-}$ depended on NADPH oxidase playing the opposite signal function in regulating H_2O_2 and $O_2^{\bullet-}$ generation in salt-treated wheat leaves. These different responses of total H_2O_2 and $O_2^{\bullet-}$ accumulation to exogenous CAT or DPI addition could be explained by different distribution of H_2O_2-generating enzymes in the apoplast of wheat leaves. Moreover, several previous studies (Pastori and Foyer 2002, Vranova *et al.* 2002) demonstrated that $O_2^{\bullet-}$ itself from NADPH oxidase could trigger an independent series of signalling events. However, application of exogenous CAT and DPI inhibited high irradiance induced H_2O_2 accumulation in vascular and perivascular area in variegated *Pelargonium zonale* leaves (Vidović *et al.* 2016). Wheat leaves treated with NaCl and CAT or DPI in combination exhibit an insignificant change of MDA content in comparison with NaCl alone. According to Bechtold *et al.* (2008), the production of H_2O_2 from extracellular sources is an important component in the regulation of *APX2* expression in heat-treated *Arabidopsis.* Moreover, NADPH oxidase-dependent H_2O_2 production is required for salt stimulation of CAT, APX, and GR in *Arabidopsis* (Rejeb *et al.* 2015). The study of Hu *et al.* (2005) showed that pre-treated maize leaves with DPI fully prevents the enhancement of SOD, APX, and GR activities induced by ABA treatment. Similarly, exogenous CAT application reduced POD and CAT activities (Table 3) and the presence of DPI significantly inhibited SOD, POD, and CAT activities in the leaves of salt-stressed seedlings (Table 3). However, the addition of these two reagents did not affect APX and GR activities (Table 3). In addition, the application of CAT or DPI decreased the intensity of POD isozyme bands, especially with the most notable reduction of isozymes POD4, POD5, and POD7 due to NaCl + CAT treatment (Fig. 5). Meanwhile, in comparison with NaCl alone, the activities of CAT1, CAT2, and CAT3 isozymes decreased when wheat seedlings were exposed to NaCl + CAT or NaCl + DPI (Fig. 6). These observations indicated that salt-induced H_2O_2 accumulation and $O_2^{\bullet-}$ generation from NADPH oxidase in the apoplast played an important role in the regulation of antioxidative enzyme activities.

Apart from antioxidative enzymes and NADPH oxidase, enzymes DAO, PAO, and cw-POD are involved in the regulation of H_2O_2 content. For example, mannitol treatment stimulates DAO activity and H_2O_2 enhancement in the roots of rice seedlings (Lin and Kao 2001). In addition, DAO and PAO might be responsible for NO production resulted from polyamines (Wimalasekera *et al.* 2011). Haghighi *et al.* (2014) showed that the activity of cw-POD rises in leaves of *Aeluropus littoralis* under salt treatment. Likewise, salt stress in maize clearly increases the activity of POD in cell wall extracts (Uddin *et al*. 2014). However, the inhibition of DAO, PAO, and cw-POD in response to NaCl treatment suggested that these three enzymes were not responsible for the increases of NO and H_2O_2 content in wheat leaves (Table 4). This could be supported by previous studies in NH_4Cl-treated roots of rice seedlings (Lin and Kao 2001) and in NaCl-stressed maize seedlings (Rodriguez *et al.* 2009). Fan *et al.* (2013) reported that exogenous NO can increase salt tolerance in cucumber seedlings by DAO activation but PAO inhibition in cucumber roots. The correlation between DAO and PAO activities and respective gene expressions were also observed after application of sodium nitroprusside dihydrate (an NO donor) in leaves of *Medicago truncatula* (Filippou *et al.* 2013). NO scavenging by cPTIO and $O_2^{\bullet-}$ decreasing by DPI did not affect DAO, PAO, and cw-POD activities in the leaves of 150 mM NaCl-treated seedlings (Table 4), demonstrating that endogenous NO and $O_2^{\bullet-}$ generation was not associated with activities of these three enzymes. However, extracellular H_2O_2 scavenging with CAT further decreased DAO and PAO activities in wheat leaves under 150 mM NaCl (Table 4), revealing that apoplastic H_2O_2 might serve as messenger to regulate DAO and PAO activities.

In conclusion, NO and ROS production influenced each other and interactively regulated antioxidant scavenging system in the leaves of wheat seedlings exposed to NaCl. Increased POD and CAT activities were

associated with NO accumulation and apoplastic H_2O_2 and $O_2^{\bullet-}$ content in wheat leaves under salinity. Moreover, NaCl-triggered stimulation of total POD activity mainly depended on isozymes POD4, POD5, and POD7, and total CAT activity on CAT1, CAT2, and CAT3 isozymes. Additionally, apoplastic H_2O_2 accumulation and $O_2^{\bullet-}$ generation from NADPH oxidase played the different regulatory functions for total H_2O_2 and $O_2^{\bullet-}$ generation as well as DAO and PAO activities in NaCl-stressed wheat seedlings.

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