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Comparison of antioxidant enzyme activity and gene expression in two new spring wheat cultivars treated with salinity

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Abstract

This study aimed to analyze element content, antioxidative response, and related gene expression in two new wheat (*Tritium aestivum* L.) cultivars Longchun 30 and Longchun 27 when exposed to different NaCl concentrations. Low NaCl concentration (25 mM) promoted root growth and decreased malondialdehyde (MDA) content and relative conductivity (REC) in Longchun 30. Differently, higher salinity stress (100 and 200 mM NaCl) inhibited root growth and increased MDA content and REC in both cultivars. Under salt stress, the increment of Na content in the roots and leaves and the reduction of Ca content in the roots were more remarkable in Longchun 27 than in Longchun 30. In contrast, the potassium content decreased in the roots but did not significantly change in the leaves in both cultivars under salinity. When the seedlings were exposed to salinity, the increases of superoxide dismutase (SOD) and catalase (CAT) activities in Longchun 27 roots were associated with high isoenzymes abundance and high *TaCu/ZnSOD*, *TaMnSOD* and *TaCAT* expression. Meanwhile, total peroxidase (POD) activity induced by NaCl treatment coincided with the changes of *TaPOD* expression and isoenzyme abundance in both cultivars. Besides, the inhibition of activities of apoplastic antioxidant enzymes, cell wall-bound POD, diamine oxidase, and polyamine oxidase was observed in salinity-stressed roots of both cultivars. Taken together, cv. Longchun 30 might be more suitable for growing in salinity environment in comparison with Longchun 27.

Keywords: catalase, diamineoxidase, malondialdehyde, NaCl, peroxidase, polyamine oxidase, superoxide dismutase, *Triticum aestivum*.

Introduction

Soil salinization is one of the most critical environmental problems and poses a threat to agricultural development (Setia *et al*. 2010). As one of the main factors limiting plant growth, salinity affects such different physiological and biochemical processes as ion imbalance, photosynthesis, and respiration, even causes oxidative stress in plants (Almeida *et al*. 2017, Todea *et al*. 2020). Several studies showed that salinity caused ion imbalance such as sodium (Na) accumulation, and potassium (K) and calcium (Ca) depletion in various plants (Hannachi and Van Labeke 2018, Zhang *et al*. 2018), and that the imbalance of Na, K, and Ca led to the reduction of plant height, the decrease of

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Abbreviations: CAT - catalase; cw-POD - cell wall-bound POD; DAO - diamine oxidase; EDTA - ethylenediaminetetraacetic acid; f.m. - fresh mass; MDA - malondialdehyde; NBT - nitroblue tetrazolium; $^{\bullet}OH$ - hydroxyl radical; $O_2^{\bullet-}$ - superoxide radical; PAGE - polyacrylamide gel electrophoresis; PAO - polyamine oxidase; PBS - potassium phosphate buffer; POD - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; *TaGAPDH* - *Triticum aestivum glyceraldehyde-3-phosphate dehydrogenase* gene; TCA - trichloroacetic acid.
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root profuseness and the loss of agricultural yield (Assaha *et al*. 2017). In addition, salt-induced accumulation of reactive oxygen species (ROS) can oxidize biological macromolecules and cause lipid peroxidation, membrane damage and enzyme inactivation (Meng *et al*. 2020). However, ROS are important signaling molecules at low levels (Cakmak *et al*. 2012). Therefore, the balance between the production and degradation of ROS is a fundamental requirement for the maintenance of cell function and survival. To protect themselves against oxidative stress, plants exposed to environmental stress develop antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase, and glutathione reductase. A series of studies have confirmed that antioxidant enzyme activities depend on plants species, stress factors, and even environmental conditions (Zhang *et al*. 2009, Hong *et al*. 2020, Mushtaq *et al*. 2020). Moreover, a great deal of evidence has demonstrated that antioxidant enzymes can effectively protect plants against salt stress damage by quenching the excessive ROS caused by salt stress (Castelli *et al*. 2010, Zhang *et al*. 2013, Mushtaq *et al*. 2020, Zhong *et al*. 2020).

Wheat is one of the most important grains planted around the world. The germination, emergence, and growth of wheat are sensitive to salinity, and soil salinization is one of the reasons for the decrease of agriculture production. For example, salinity caused a significant reduction in the biomass and yield of four wheat cultivars cultivated by Ayub Agricultural Research Institute, Faisalabad, Pakistan (Ali *et al*. 2017). Wheat cvs. Longchun 30 and Longchun 27 are the new cultivars of spring wheat cultivated by Gansu Academy of Agricultural Sciences, China. Longchun 30 is widely planted in the Hexi Corridor and along the Yellow River in Gansu Province (Hu *et al*. 2014). The irrigation fields in these areas are featured saline soil (Mao *et al*. 2016). Longchun 27, which is more tolerant to drought, is suitable for planting in Dingxi city, Yuzhong county and Huining county in Gansu Province (Jiang *et al*. 2013). Previous studies showed that extreme salinity and drought stresses always led to similar defense responses in plants (Khayatnezhad *et al*. 2010, Golldack *et al*. 2014). Even though the morphological characteristics of Longchun 27 during its growth process have been reported in a previous study (Huizhi *et al*. 2014), the reason for the survival of these two wheat species in abiotic stresses including salt environment has not been revealed. To better understand the antioxidant responses of crops to salinity stress and to reveal the mechanism of salt damage or salt tolerance, wheat Longchun 30 and Longchun 27 were used to investigate element content, antioxidant response and gene expression of related enzymes under salinity stress.

Materials and methods

Plants and growth conditions: Wheat (*Triticum aestivum* L.) cvs. Longchun 30 and Longchun 27 seeds, purchased from Gansu Academy of Agriculture Sciences, Lanzhou, China, were surface-sterilized with 0.1 % (m/v) $HgCl_2$ for 10 min, rinsed in running water for 10 h, and then germinated in the dark at 25 ± 1.5 °C for 24 h. The seedlings were treated with 1/4 Hoagland nutrient solution, which was composed of [g dm^{-3}] 0.945 $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.506 KNO_3, 0.08 NH_4NO_3, 0.439 $MgSO_4 \cdot 7H_2O$, 0.136 KH_2PO_4, 5.57 $FeSO_4 \cdot 7\ H_2O$ and microelements (Hoagland and Arnon 1950). Meanwhile, the solution was supplied with or without 25, 100, or 200 mM NaCl. The seedlings were cultivated at a temperature of 25 ± 1.5 °C, an irradiance of 300 µmol m^{-2} s^{-1} , and a 12-h photoperiod in an incubator *LRH-250-A* (*Medical Instruments Factory*, Guangdong, China), and the treatment solution was replaced every two days. All assays were replicated at least three times to minimize experimental errors.

Analysis of membrane lipid peroxidation: Membrane lipid peroxidation was measured by the method described by Sarker (2018b). Plant material (0.5 g) was homogenized in 5 cm^3 0.25 % (m/v) thiobarbituric acid (TBA) containing 10 % (m/v) trichloroacetic acid (TCA) and incubated in boiling water for 30 min. After cooling, it was centrifuged at 10 000 *g* for 10 min. The absorbance was read at 450, 532, and 600 nm, and then malondialdehyde (MDA) content was determined with the formula: $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}]/f.m.$

Measurement of relative conductivity: Fresh roots (0.5 g) were excised and placed in 10 cm^3 of deionized water for 30 min at room temperature. After it, the conductivity (value A) of the solution was measured with an electrical conductivity meter. The solution was then boiled for 30 min at 100 °C, and the conductivity (value B) was measured again. The relative conductivity (REC) was calculated as: value A/value B × 100 %.

Determination of element content: The amount of Na, K, and Ca in plant material was determined according to Achary *et al*. (2012). The roots and leaves were cleaned with distilled water, dried naturally, and 0.2 g samples were put into the microwave digestion tank (*CEM Mars6*, *Agilent*, Palo Alto, USA) to acidify with 8 cm^3 concentrated nitric acid (HNO_3) at 150 °C for 1.5 h. After being cooled, the residue was dissolved with 1 M HNO_3 to a constant volume of 50 cm^3, and was used for the determination of the element content by atomic absorption spectrophotometer (*5100 ICP-OES*, *Agilent*, Palo Alto, USA).

Determination of ROS content: H_2O_2 content was determined according to Sarker (2018a). Fresh roots (0.5 g) were ground with 0.1 % TCA and then centrifuged at 12 000 *g* for 20 min. After that the supernatant was mixed with 10 mM potassium phosphate buffer (PBS, pH 7.0) and 1 M potassium iodide, and the absorbance was measured at 390 nm. H_2O_2 content, was calculated by the use of a standard curve prepared with known H_2O_2 concentrations.

The method of Achary *et al*. (2012) was used to analyze $O_2^{\bullet-}$ content. To develop colour resulting from the reduction of nitroblue tetrazolium (NBT), plant material was added to 50 mM Tris-HCl buffer (pH 6.4) containing 0.2 mM NBT, 0.2 mM nicotinamide adenine dinucleotide,

and 250 mM saccharose. It was vacuum-filtered for 20 min and then irradiated at 200 mmol $m^{-2}s^{-1}$ for 24 h. The absorbance was determined at 530 nm.

The content of ˙OH was measured according to Halliwell *et al.* (1987). Wheat roots (0.5 g) were homogenated with 10 mM PBS buffer (pH 7.4) supplied with 2 mM 2-deoxy-D-ribose, and the homogenate was centrifuged at 12 000 *g* for 15 min. The supernatant was collected and incubated at 75 °C for 2 h, then mixed with 3 cm^3 of 0.5 % thiobarbituric acid and 1 cm^3 of glacial acetic acid. The reaction mixture was boiled for 30 min and the absorbance was recorded at 532 nm.

The method of Córdoba-Pedregosa *et al*. (2005) was used to isolate apoplastic fluids with some modifications. Wheat roots (1 g) were placed in tubes containing 10 mM PBS buffer (pH 6.0), 1 % (m/v) polyvinylpyrrolidone (PVP), and 1 mM ethylenediaminetetraacetic acid (EDTA). After it was vacuumed for 1 h, plant tissues were dried with filter paper and placed in Eppendorf tubes with a small hole at the bottom. After it was centrifuged at 12 000 *g* for 10 min, the apoplastic fluids were collected at the bottom of the tubes and were used to measure apoplastic ROS content and antioxidant enzyme activities. H_2O_2, $O_2^{\cdot-}$ and ˙OH content in the apoplast was determined as described above.

Determination of antioxidant enzyme activities: About 0.5 g of plant material was ground with 50 mM PBS (pH 7.8) containing 0.1 % PVP and 0.1 mM EDTA. After the homogenate was centrifuged at 12 000 *g* for 30 min, the supernatant was collected for the assay of protein content and the measurement of antioxidant enzyme activities.

SOD activity was determined based on the method of Dhindsa and Matowe (1981). The reaction mixture consisted of 50 mM PBS (pH 7.6), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and an appropriate amount of enzyme extract, and the reaction was started by the addition of 2 µM lactochrome. After it was irradiated for 30 min at 25 °C, the absorbance was recorded at 560 nm. Failing to develop color, a non-irradiated reaction medium severed as dark control. One unit of enzyme activity was defined as the amount of enzyme bringing about 50 % inhibition in the reduction rate of NBT detected at 560 nm, and enzyme activity was expressed as U mg^{-1}(protein).

CAT activity was estimated by monitoring the decrease in absorbance at 240 nm due to decomposition of H_2O_2 (Aebi 1984). After being added to the enzyme extract, 3 cm^3 of 50 mM PBS buffer (pH 7.0) was incubated at 25 °C for 5 min and then 6 µM H_2O_2 was used to start the reaction. The changes of the absorbance were recorded at 240 nm at the interval of 20 s for 2 min. The activity of CAT was calculated from the decrease in absorbance per min when the coefficient of absorbance of H_2O_2 was 39.4 M^{-1} cm^{-1}.

POD activity was measured with the method of Rao *et al*. (1996). The enzyme extract was mixed with 3 cm^3 of PBS (50 mM, pH 7.0) containing 20 mM guaiacol. The mixture was incubated at 25 °C for 5 min and 6 µM H_2O_2 was added to initiate the reaction. The changes of the absorbance were recorded at 470 nm at the interval of 20 s for 2 min, and POD activity was calculated using the coefficient of absorbance of 26.6 mM^{-1} cm^{-1}.

The same methods were used for measurement of activities of antioxidant enzymes in apoplastic fluid.

Protein content was determined according to Bradford (1976), using bovine serum albumin as a standard.

Analyses of SOD, CAT, and POD isoenzymes were done on nondenaturing polyacrylamide gels as described (Laemmli 1970). The gels were run at 4 °C at 80 V for 20 min and subsequently at 120 V for 90 min. SOD isozymes were electrophoresed with 10 % separating and 4 % stacking gels, and CAT and POD isozymes were detected with 7.5 % separating and 4 % stacking gels.

The staining of SOD isoenzymes was performed as Beauchamp and Fridovich (1971) described with some modification. After being soaked with 50 mM PBS (pH 8.0) containing 0.24 mM NBT and 28 µM riboflavin for 20 min in the dark, the gel was incubated and color was developed in the solution of 50 mM PBS (pH 7.8) containing 28 mM N,N,N`,N′-tetramethylethylenediamine.

CAT isoenzyme staining was performed according to Chandlee and Scandalios (1984). After being incubated in H_2O_2 (0.01 %) solution for 20 min, the gel colour was developed with 1 % (m/v) ferric chloride and 1 % (m/v) potassium ferricyanide.

POD isoenzymes were stained based on the method of Huh *et al*. (1998). To visualize the band patterns, the gel was incubated in the dye solutions A: 2 % (v/v) benzidine; B: 4 % (m/v) NH_3Cl; C: 5 % EDTA; D: 0.3 % H_2O_2; A, B, C, D, and distilled water in a volume ratio of 1:1:1:1:8 at 37 °C for 5 - 10 min.

For determining the relative activity of different isoenzymes, *Gel Doc 2000* Gel imaging system and *Bio-Rad* (*Shanghai Shisen Vision Technology Co., Ltd*, Shanghai, China) *Quantity One* software were used to analyze the images.

Real-time quantitative PCR of gene expression: To investigate expressions of *TaCu/ZnSOD, TaMnSOD*, *TaCAT*, and *TaPOD*, wheat roots were collected after 6-d treatment with different NaCl concentrations. Total RNA extraction and first-strand cDNA synthesis were accomplished using the *TRIZOL* reagent and a *PrimeScript*™ RT reagent kit (*Takara*, Shiga, Japan). The PCR solution (20 mm^3) consisted of 10 mm^3 of 2 × *TransStart Tip Green* qPCR super mix (*TransGen*, Beijing, China), 0.4 mm^3 of 10 µM each primer, 1.5 mm^3 of cDNA and distilled water. Real-time quantitative PCR was completed on the detection system *Bio-Rad iQ5* (California, USA). The PCR conditions were as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. Gene-specific primers used for real-time quantitative PCR were designed using the primer design software *Primer Premier 5* from the wheat *Cu/ZnSOD* gene fragment (forward: 5′-TGTCGATAGCCAGATTCCTTTGA-3′; reverse: 5′-AGTCTTCCACCAGCATTTCCAG-3′), *MnSOD* gene (forward: 5′-CTGGAAGAACCTCAAGCCTATC-3′; reverse: 5′-GATCCTTGTAAAGCACCCTCTG-3′), *CAT* gene (forward: 5′-CAACCACTACGACGGGCTCA-3′;

reverse: 5′-GGGCTGCTTGAAGTTGTTCTCC-3′), *POD* gene (forward: 5′-CTTCGACAACGCCTACTACAC-3′; reverse: 5′-GGTTGGACGCAAAGTTCC-3′). *Triticum aestivum glyceraldehyde-3-phosphate dehydrogenase* (*TaGAPDH*) gene was used as a reference gene (forward: 5′-TTAGACTTGCGAAGCCAGCA-3′; reverse: 5′-AAATGCCCTTGAGGTTTCCC-3′). The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. The real-time quantitative PCR experiment was carried out thrice with three biological replicates.

Determination of activities of cell wall-bound POD, DAO, and PAO: In order to isolate cell walls, wheat roots (0.5 g) were homogenized with 50 mM PBS (pH 5.8) as described by Lee and Lin (1995). The homogenate was centrifuged at 4 000 *g* for 10 min and washed at least twice with 50 mM PBS buffer (pH 5.8), and then the pellets were dissolved and incubated in 1 M NaCl for 2 h. After it was centrifuged at 1 000 *g* for 10 min, the supernatant containing cell wall-bound POD (cw-POD) was collected. Enzyme activity was determined with the method of Dos Santos *et al*. (2008). The reaction mixture consisted of 25 mM PBS (pH 6.8), 10 mM H_2O_2, 2.58 mM guaiacol, and the enzyme extract. The changes of the absorbance at 470 nm within 2 min were recorded to calculate enzyme activity. One unit of cw-POD activity was defined as an absorbance change of 0.01 per min.

DAO activity in the fractions of cell walls was analyzed according to Naik *et al*. (1981). The reaction mixture consisted of 50 mM PBS buffer (pH 7.8), 0.1 mM pyridoxal phosphate, 10 mM putrescine, and enzyme extract. After it was incubated at 30 °C for 1 h, the reaction was stopped with 1 cm^3 20 % TCA. 30 min later, the incubation mixture was centrifuged at 5 000 *g* for 15 min. A ninhydrin (1 mm^3) was added to the supernatant and the colour was developed at 100 °C for 30 min. After 1 cm^3 of acetic acid was added, the absorbance was determined at 510 nm. In contrast to the tratment group, TCA was added to the enzyme solution in advance.

PAO activity was measured by using spermidine as a substrate (Asthir *et al*. 2002). Roots (1 g) were homogenized with 2 cm^3 100 mM PBS (PH 7.0) containing 5 mM dithiothreitol, and then it was centrifuged at 16 000 *g* for 20 min. The pellet was sequentially extracted with 100 mM PBS (pH 7.0) containing 1 mM NaCl. The reaction mixture was made from 450 mm^3 enzyme extract, 250 mm^3 2-amino benzaldehyde (0.1 %, m/v) and 50 U CAT, and then the reaction was started with 250 mm^3 10 mM spermidine. After the reaction mixture was incubated at 30 °C for 3 h, the reaction was terminated with 1 cm^3 of 10 % (v/v) perchloric acid. After it was centrifuged at 6 500 *g* for 10 min, the formation of Δ^1-pyrroline product was measured by assaying the absorbance at 430 nm. Control reactions were carried out without the enzyme extract.

Statistical analysis: All experiments were repeated at least three times. Data were analyzed by univariate analysis of variance (one-way *ANOVA*) and Duncan's multiple comparisons with *SPSS 13.0*, and they were expressed as the means ± standard errors (SEs), and the differences were considered significant at $P \leq 0.05$.

Results

As shown in Fig. 1*A-B*, 25 mM NaCl promoted the growth of roots and shoots in Longchun 30 seedlings, while 100 and 200 mM salt inhibited the growth with the strongest inhibitory effect due to 200 mM NaCl. In comparison with the control, Longchun 27 seedlings showed insignificant changes of root and shoot lengths at low NaCl concentration (25 mM), but significant reduction at 100 and 200 mM NaCl (Fig. 1*A-B*).

Compared with the control, MDA content in Longchun 30 roots was reduced about 16 and 20 % under 25 and 100 mM NaCl treatment, respectively. When salt concentration was enhanced to 200 mM, this parameter

Table 1. Changes of Na, K, and Ca content [μg g^{-1}(d.m.)] in roots and leaves of two wheat seedlings under NaCl treatment. Means ± SEs of at least five experiments with replicated measurements; values within each line marked with different small letters show significant difference at 0.05 level.

Element amount			NaCl concentration [mM]			
			0	25	100	200
Longchun 30	Na	root	78.87 ± 1.73a	95.83 ± 3.26b	106.32 ± 3.86c	109.69 ± 2.8c
		leaf	37.54 ± 1.97a	39.7 ± 0.78a	52.79 ± 2.28b	81.52 ± 2.44c
	K	root	519.39 ± 5.11c	501.64 ± 5.80b	497.56 ± 4.34b	440.39 ± 5.02a
		leaf	646.27 ± 4.36b	645.48 ± 4.07b	630.00 ± 8.05ab	625.97 ± 6.40a
	Ca	root	309.67 ± 5.50c	276.95 ± 9.57b	275.58 ± 2.86b	205.82 ± 10.59a
		leaf	934.45 ± 38.12d	792.77 ± 38.31c	627.55 ± 32.51b	348.56 ± 22.11a
Longchun 27	Na	root	79.75 ± 1.88a	109.98 ± 5.52b	116.55 ± 5.27bc	128.93 ± 2.57c
		leaf	46.48 ± 2.6a	53.56 ± 2.65a	64.13 ± 2.55b	68.91 ± 1.67b
	K	root	947.18 ± 14.68b	910.18 ± 13.91b	837.57 ± 7.43a	815.29 ± 13.23a
		leaf	875.23 ± 19.8b	835.54 ± 22.89b	743.65 ± 23.33a	843.01 ± 23.47b
	Ca	root	422.06 ± 20.54c	334.42 ± 10.69b	145.99 ± 3.27a	120.13 ± 6.04a
		leaf	1000.36 ± 31.41d	824.28 ± 25.78c	700.24 ± 34.42b	400.27 ± 12.36a

increased significantly. The difference of the change of MDA content was found between Longchun 27 and Longchun 30 roots. About 16 % decrease in MDA content was observed in response to 25 mM NaCl stress, and an insignificant reduction to 100 mM NaCl, while Longchun 27 roots accumulated a high MDA due to 200 mM NaCl

Table 2. Changes of total and apoplastic SOD [U mg^{-1}(protein)], CAT [mmol g^{-1}(f.m.) min^{-1}], and POD [µmol g^{-1}(f.m.) min^{-1}] in roots of two wheat seedlings under salinity treatment. Means ± SEs of at least five experiments with replicated measurements; values within each line marked with different small letters show significant difference at 0.05 level.

Cultivar	NaCl concentration [mM]		0	25	100	200
Longchun 30	total antioxidative enzymes	SOD	43.69 ± 1.60b	43.35 ± 2.82b	31.57 ± 1.95a	55.93 ± 1.34c
		CAT	4.57 ± 0.74a	6.04 ± 0.93b	10.63 ± 1.85c	18.45 ± 1.84d
		POD	9.88 ± 4.39a	12.54 ± 1.79b	22.24 ± 6.61c	34.07 ± 1.10d
	apoplastic antioxidative enzymes	SOD	2.18 ± 0.06b	1.95 ± 0.04ab	2.00 ± 0.05ab	1.75 ± 0.04a
		CAT	0.30 ± 0.10b	0.33 ± 0.08b	0.10 ± 0.04a	0.08 ± 0.01a
		POD	0.44 ± 0.01d	0.40 ± 0.01c	0.39 ± 0.03b	0.35 ± 0.01a
Longchun 27	total antioxidative enzymes	SOD	38.86 ± 3.20a	47.68 ± 2.24b	40.19 ± 1.79a	52.99 ± 1.67c
		CAT	9.16 ± 1.04a	15.36 ± 3.70b	22.03 ± 2.52c	29.47 ± 4.84d
		POD	15.86 ± 7.81c	17.67 ± 3.18d	15.81 ± 1.26b	15.17 ± 2.35a
	apoplastic antioxidative enzymes	SOD	2.57 ± 0.01d	1.88 ± 0.04b	1.99 ± 0.04c	1.70 ± 0.03a
		CAT	0.71 ± 0.06c	0.46 ± 0.06b	0.33 ± 0.05a	0.34 ± 0.05a
		POD	0.44 ± 0.05b	0.51 ± 0.03c	0.43 ± 0.04b	0.34 ± 0.10a

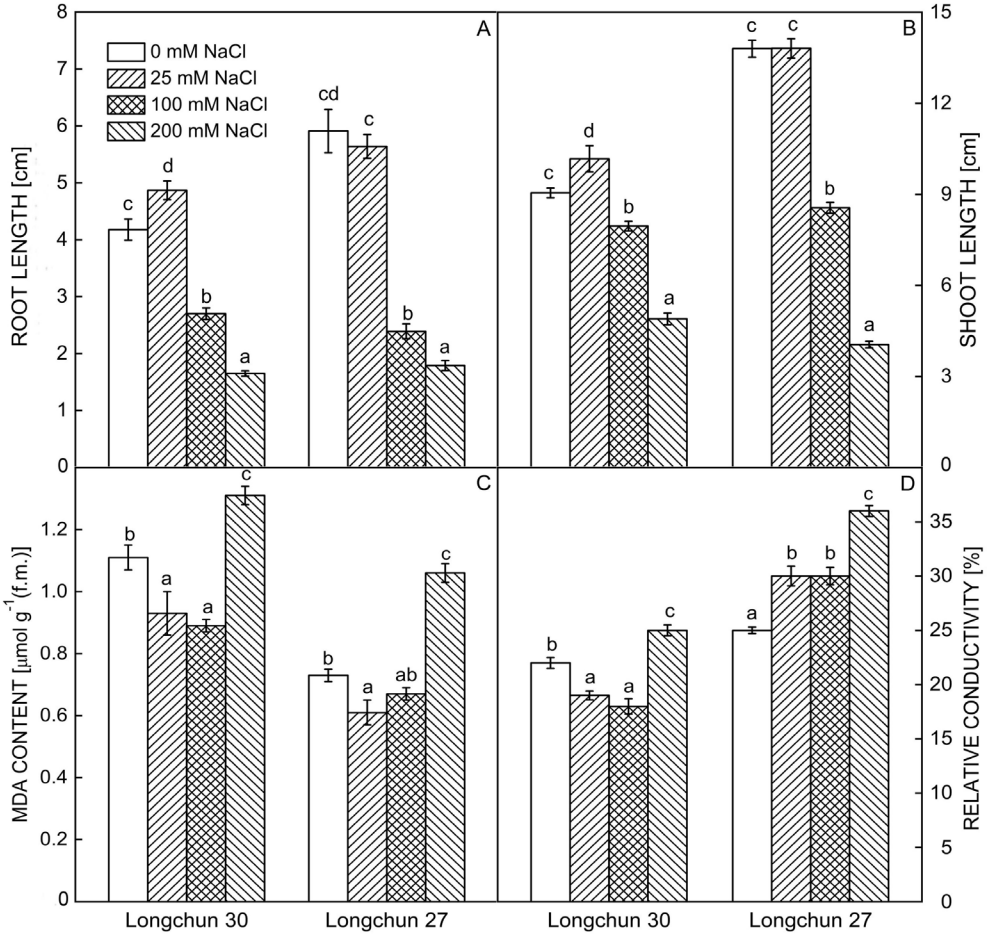

Fig. 1. Changes of root (*A*) and shoot (*B*) growth in wheat seedlings, as well as MDA content (*C*) and relative conductivity (*D*) in the roots under different NaCl concentrations. Means ± SEs based on at least three experiments, and different lowercase letters indicate significant difference ($P < 0.05$) according to Duncan's test.

Table 3. Changes of cell wall-bound peroxidase (cw-POD), diamine oxidase (DAO), and polyamine oxidase (PAO) activities [U mg^{-1}(protein)] in roots of two wheat seedlings in response to different NaCl concentrations. Means ± SEs of at least five experiments with replicated measurements; values within each line marked with different small letters show significant difference at 0.05 level.

NaCl concentration [mM]		0	25	100	200
Longchun 30	cw-POD	10.61 ± 0.11d	7.49 ± 0.12c	5.31 ± 0.08b	3.66 ± 0.17a
	DAO	8.03 ± 0.37c	5.96 ± 0.25b	5.00 ± 0.12a	4.45 ± 0.05a
	PAO	4.87 ± 0.03c	3.85 ± 0.20b	3.18 ± 0.17a	3.10 ± 0.09a
Longchun 27	cw-POD	12.56 ± 0.05b	12.12 ± 0.20b	8.05 ± 0.09a	8.48 ± 0.10a
	DAO	7.30 ± 0.28b	7.27 ± 0.28b	4.65 ± 0.20a	4.76 ± 0.21a
	PAO	5.24 ± 0.09c	5.23 ± 0.06c	2.67 ± 0.10a	3.06 ± 0.17b

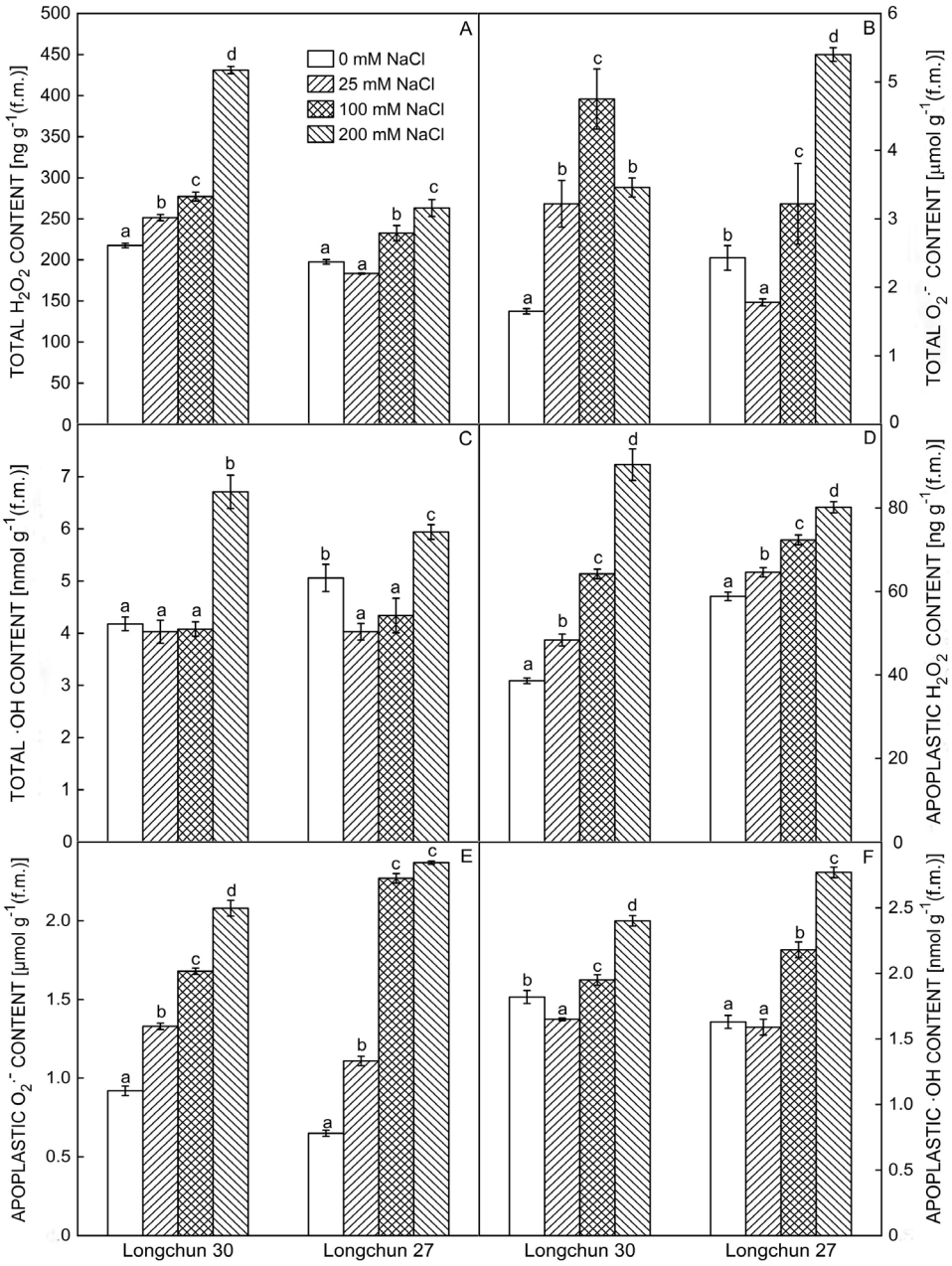

Fig. 2. Changes of both total H_2O_2 (*A*), $O_2^{\cdot-}$ (*B*) and $^{\cdot}OH$ (*C*) and apoplastic H_2O_2 (*D*), $O_2^{\cdot-}$ (*E*) and $^{\cdot}OH$ (*F*) content in wheat roots under salinity treatments. Means ± SEs of at least three experiments with replicated measurements, and different lowercase letters indicate statistically significant difference ($P < 0.05$).

treatment (Fig. 1*C*).

Compared with the control, REC significantly lowered in Longchun 30 roots treated with 25 or 100 mM NaCl, while this parameter increased approximately 14 % in 200 mM NaCl-stressed roots. Differently, REC strengthened obviously in response to all NaCl concentrations in Longchun 27 roots as compared with the control (Fig. 1*D*).

The effects of different NaCl concentrations (0, 25, 100, 200 mM) on the amount of Na, K, and Ca in Longchun 30 and Longchun 27 seedlings were shown in Table 1. In Longchun 30 seedlings, Na content in the roots increased about 22, 35, and 39 % in response to 25, 100 and 200 mM NaCl, respectively; this parameter in the leaves did not change due to 25 mM NaCl treatment, but significantly enhanced to 100 and 200 mM NaCl. Similar changes of Na content were observed in the roots and leaves of Longchun 27 under salinity stress (Table 1). However, the increment of Na content in Longchun 27 roots was significantly higher than that in Longchun 30 under the same NaCl concentration.

In Longchun 30, the reduction of K content was found in the roots in response to all NaCl concentrations but only to 200 mM salinity stress in the leaves. In contrast, 25 mM NaCl did not affect K content in the roots and leaves of Longchun 27, wheres 100 and 200 mM NaCl treatments significantly reduced this parameter in Longchun 27 roots (Table 1).

In comparison with the control, when the seedlings were exposed to 25, 100 and 200 mM NaCl treatments, root Ca content decreased approximately 11, 11, and 34 % in Longchun 30 as well as 21, 65, and 72 % in Longchun 27, respectively (Table 1). Salt stress resulted in significant decrease of Ca content in the leaves of two wheat seedlings in a concentration-dependent manner. In addition, the reduction of Ca content induced by salt stress at the same concentration was notably lower in Longchun 30 roots than in Longchun 27 roots (Table 1).

Compared with the control, all NaCl concentrations caused significant enhancements of total H_2O_2 generation in Longchun 30 roots, with the highest content due to 200 mM NaCl. Differently, 25 mM NaCl treatment did not obviously affect total H_2O_2 content in the roots of Longchun 27, while about 18 and 33 % increase of this parameter was observed in response to 100 and 200 mM NaCl, respectively (Fig. 2*A*).

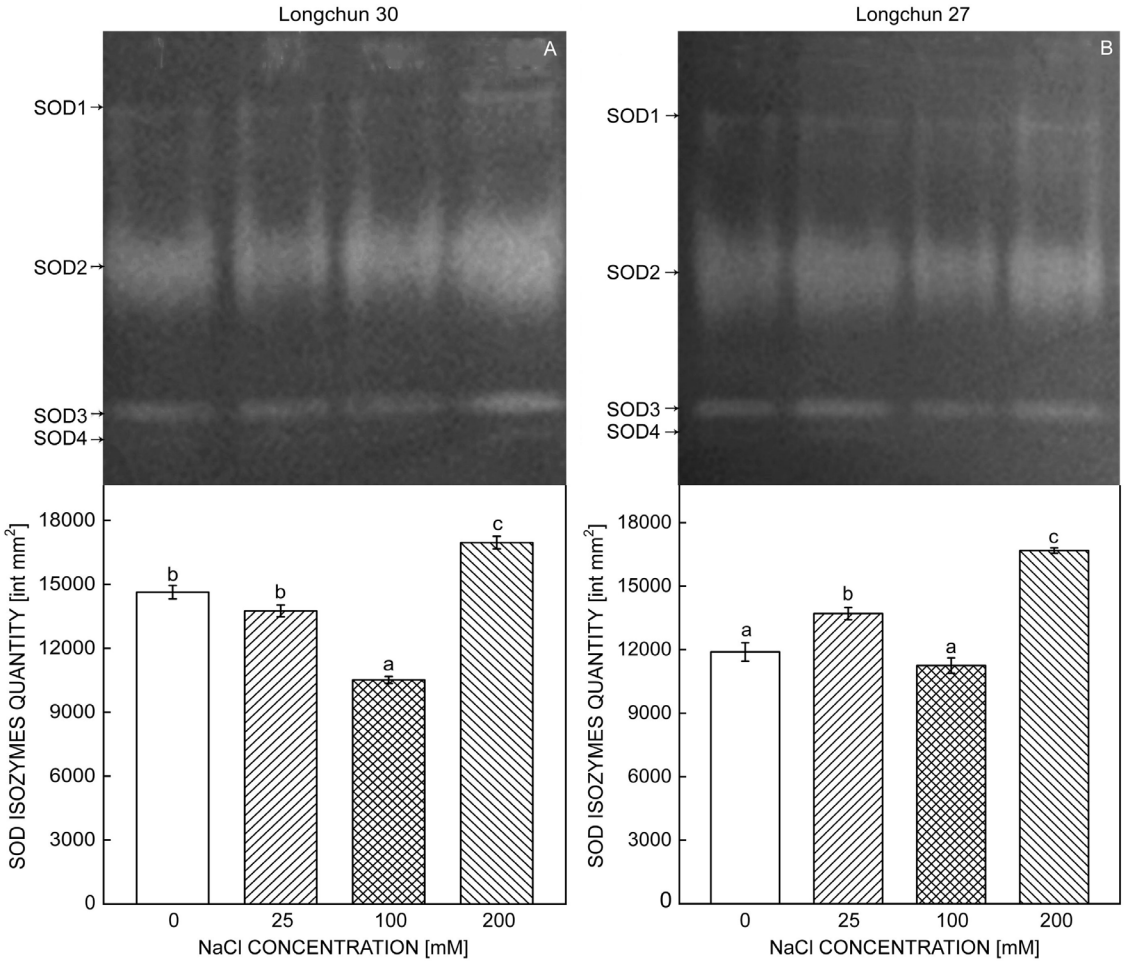

Fig. 3. Native gel analysis and quantity of SOD isoenzymes in response to different NaCl concentrations in roots of wheat cultivars. Int mm^2 - intensity × mm^2. Means ± SEs based on three repeated analysis, and different lowercase letters indicate statistically significant difference ($P < 0.05$).

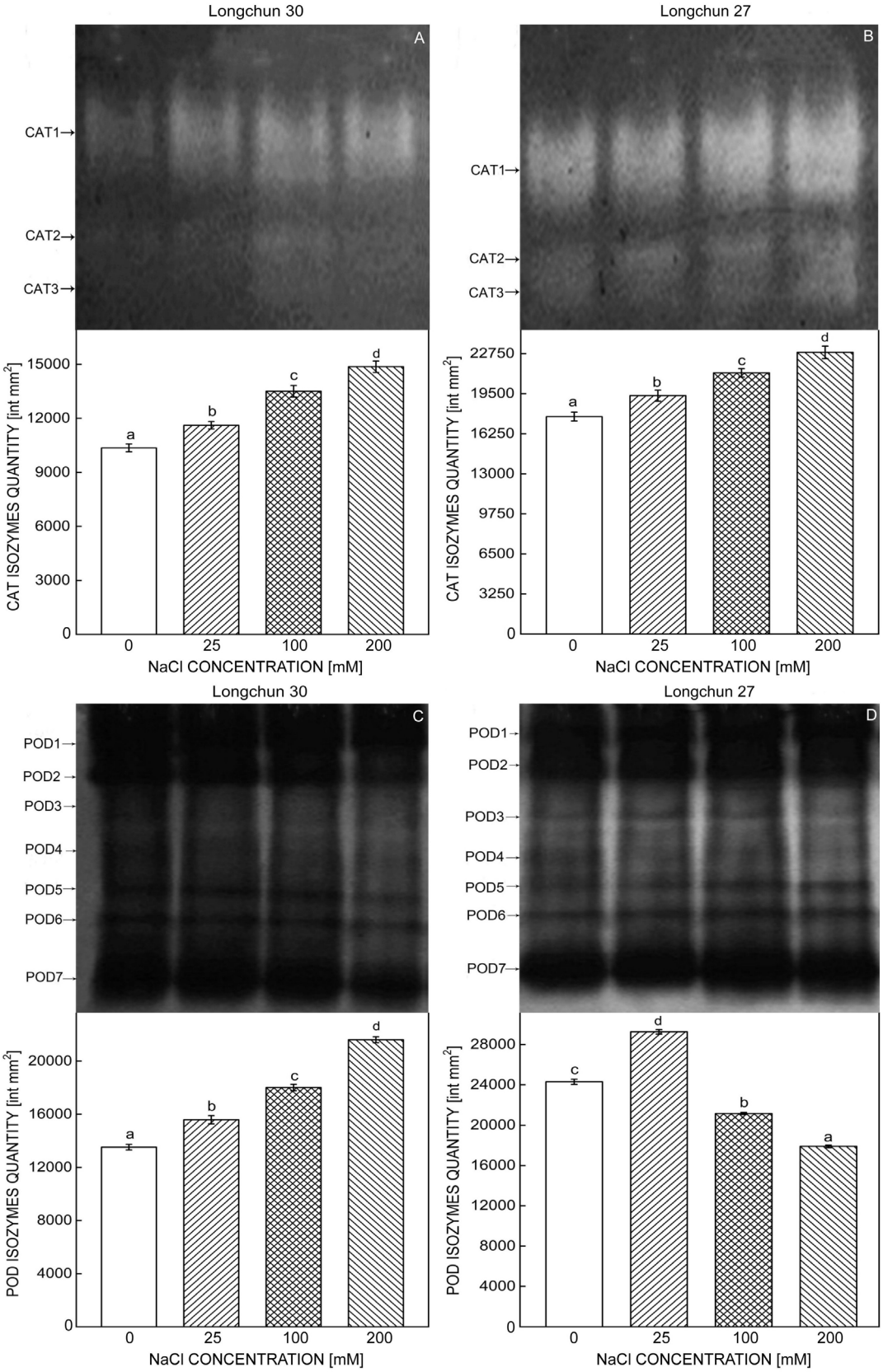

Fig. 4. Native gel analysis and quantity of CAT isoenzymes (*A* and *B*) and POD isoenzymes (*C* and *D*) in response to different NaCl concentrations in roots of wheat cultivars. Int mm^2 - intensity × mm^2. Means ± SEs based on three repeated analysis, and different lowercase letters indicate statistically significant difference ($P < 0.05$).

As shown in Fig. 2*B*, total $O_2^{\bullet-}$ production obviously rose in Longchun 30 roots exposed to salt treatment. In addition, this parameter notably lowered in Longchun 27 roots treated with 25 mM NaCl stress, but increased about 33 and 122 % under 100 and 200 mM NaCl, respectively, in comparison with the control (Fig. 2*B*).

An insignificant change of total ·OH content was found in 25 and 100 mM NaCl-treated roots of Longchun 30, but 200 mM NaCl led to obvious elevation of this parameter, compared with the control. In Longchun 27 roots, 25 and 100 mM NaCl induced about 20 and 14 % reduction of ·OH content, respectively, while approximately 17 % increase in this parameter was observed due to 200 mM NaCl stress (Fig. 2*C*).

Salinity enhanced apoplastic H_2O_2 content in Longchun 30 roots, with about 25, 66, and 134 increment due to 25, 100 and 200 mM NaCl, respectively. Similarly, 25, 100, and 200 mM NaCl-treated roots exhibited significant elevations of apoplastic H_2O_2 content in Longchun 27 seedlings, in comparison with the untreated ones (Fig. 2*D*).

As shown in Fig. 2*E*, 25, 100 and 200 mM NaCl resulted in approximately 45, 83, and 126 % increase of apoplastic $O_2^{\bullet-}$ content in Longchun 30 roots, respectively. Additionally, about 71, 249, and 265 % enhancement of this parameter was detected in Longchun 27 roots exposed to 25, 100 and 200 mM NaCl, respectively (Fig. 2*E*).

In comparison with the control, 25 mM NaCl induced a significant reduction of apoplastic ·OH content in Longchun 30 roots, but 100 and 200 mM NaCl-stressed roots exhibited approximately 1.07- and 1.32-fold increase of this parameter. Apoplastic ·OH in Longchun 27 roots did not react to 25 mM NaCl treatment, but rose significantly in response to 100 and 200 mM NaCl (Fig. 2*F*).

In comparison with the control, total SOD activity in Longchun 30 changed insignificantly in response to 25 mM NaCl treatment. However, it reduced markedly due to 100 mM NaCl, but increased approximately 28 % due to 200 mM NaCl. This parameter in Longchun 27 roots increased up to 123 and 136 % of control value under 25 and 200 mM NaCl treatment, respectively, while 100 mM NaCl stress did not significantly affect total SOD activity (Table 2). Further study was to explore NaCl effect on the isoenzyme profiles. Native PAGE coupled with activity localization showed four SOD isoenzymes in wheat roots, with the predominant abundance due to SOD2 and SOD3

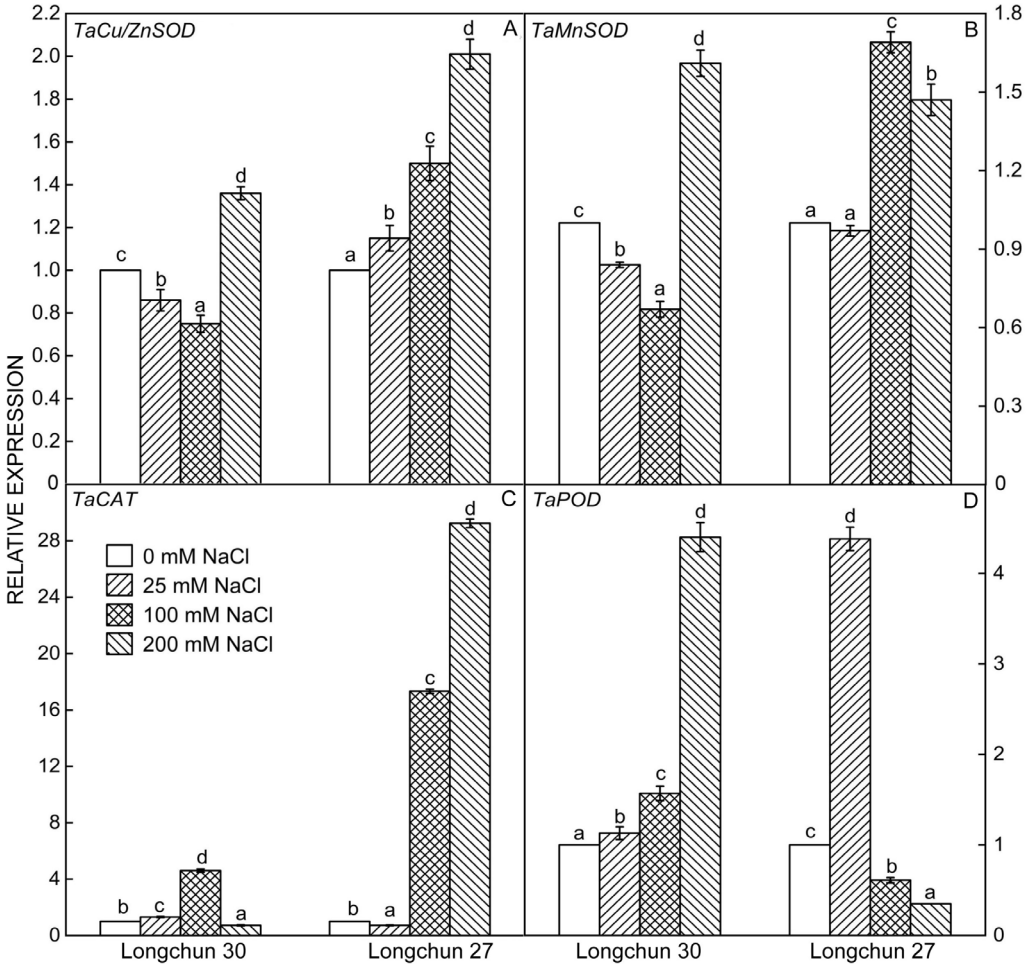

Fig. 5. Relative expression levels of *TaCu/ZnSOD* (*A*), *TaMnSOD* (*B*), *TaCAT* (*C*) and *TaPOD* (*D*) in roots of wheat cultivars under salinity treatments. Values represent Means ± SEs of at least three experiments with replicated measurements, and different lowercase letters on bars indicate significant difference ($P < 0.05$) according to Duncan's test.

(Fig. 3*A-B*). The staining intensities of SOD2 and SOD3 decreased in 100 mM NaCl-treated roots of Longchun 30 seedlings significantly, but increased notably in the roots of two wheat seedlings exposed to 200 mM NaCl concentration.

In comparison with the control, about 33, 133, and 305 % enhancement of root CAT activity was found due to 25, 100, and 200 mM NaCl stress in Longchun 30, respectively. Similarly, all NaCl concentrations caused the remarkable increase in total CAT activity in Longchun 27 roots, with approximately 1.68-, 2.41-, and 3.22-folds of the control value, respectively (Table 2). Three CAT isoenzymes were observed in wheat roots, with the maximum of CAT1 (Fig. 4 *A-B*). The intensities of CAT2 and CAT3 in Longchun 27 roots were much higher than in Longchun 30 roots, which was also why total CAT activity in Longchun 27 was higher than that in Longchun 30.

Compared with the control, 25, 100 and 200 mM NaCl-stressed roots exhibited about 1.27-, 2.25-, and 3.45-fold enhancement in total POD activity in Longchun 30, respectively. In addition, total POD activity increased approximately 11 % in Longchun 27 roots treated with 25 mM NaCl, but significantly decreased in 100 and 200 mM NaCl-stressed roots, in comparison with the control (Table 2). As indicated in Fig. 4*C-D*, seven POD isoenzymes were detected in both cultivars. The band abundance of POD isoenzymes increased significantly in Longchun 30 roots under different NaCl concentrations. Differently, the intensity of isoenzymes in Longchun 27 rose due to 25 mM NaCl, but decreased to 100 or 200 mM salinity stress (Fig. 4*C-D*).

As shown in Table 2, 25 and 100 mM NaCl did not affect apoplastic SOD activity, while about 20 % reduction of this enzyme activity due to 200 mM NaCl was observed in Longchun 30 roots. Compared with the control, about 27, 23, and 34 % decrease of this parameter was caused by 25, 100, and 200 mM NaCl treatment in Longchun 27 roots, respectively (Table 2).

There was an insignificant increase of apoplastic CAT activity in Longchun 30 roots under 25 mM NaCl treatment, but compared with the control, 100 and 200 mM NaCl stress resulted in about 67 and 73 % reduction of this parameter, respectively. Additionally, all NaCl concentrations induced the significant decrease of apoplastic CAT activity in Longchun 27 roots (Table 2).

NaCl treatment inhibited apoplastic POD activity in Longchun 30 roots significantly, with the maximal reduction due to 200 mM NaCl. Differently, in comparison with the untreated roots, approximately 16 % elevation and 23 % decrease in apoplastic POD activity were detected in Longchun 27 roots treated with 25 and 200 mM NaCl, respectively, while 100 mM NaCl did not affect this enzyme activity (Table 2).

In Longchun 30 roots, 25 and 100 mM NaCl inhibited the expressions of *TaCu/ZnSOD* and *TaMnSOD*, while the expressions of these two parameters were up-regulated in response to 200 mM NaCl (Fig. 5*A,B*). In Longchun 27, 25 mM NaCl stimulated *TaCu/ZnSOD* expression, but did not affect the expression of *TaMnSOD*. Differently, the expressions of these two genes were up-regulated in response to high NaCl concentrations (100 and 200 mM NaCl) in Longchun 27 roots.

Compared with the control, the relative expression of *TaCAT* in Longchun 30 roots significantly elevated about 1.31- and 4.61-folds due to 25 and 100 mM NaCl, but declined at 200 mM NaCl (Fig. 5*C*). However, in comparison with the control, the *TaCAT* expression in Longchun 27 roots significantly lowered due to 25 mM NaCl treatment but increased about 17- and 29-folds at 100 and 200 mM NaCl stress, respectively (Fig. 5*C*). Moreover, high NaCl concentrations (100 and 200 mM NaCl) led to higher expression of *TaCAT* in Longchun 27 roots than that in Longchun 30 ones.

TaPOD expression in Longchun 30 roots was notably stimulated by salinity, with about 13, 57, and 340 % increase in response to 25, 100, and 200 mM NaCl compared with the control, respectively. In addition, *TaPOD* expression increased in 25 mM NaCl-stressed roots of Longchun 27, but decreased significantly due to higher NaCl concentrations (Fig. 5*D*).

About 29, 50, and 66 % decrease of cw-POD activity was observed in Longchun 30 roots treated with 25, 100, and 200 mM NaCl, respectively. Differently, 25 mM NaCl did not notably affect cw-POD activity in Longchun 27 roots, but compared with the control, this parameter lowered approximately 36 and 32 % in response to 100 and 200 mM NaCl, respectively (Table 3).

DAO activity in Longchun 30 decreased by 74, 62, and 55 % of the control value under 25, 100, and 200 mM NaCl treatment, respectively. In comparison with untreated Longchun 27 seedlings, DAO activity was insignificantly lower in 25 mM NaCl-treated roots, but decreased approximately by 36 and 35 % under 100 or 200 mM NaCl, respectively.

Similarly, PAO activity decreased in two wheat roots treated with different NaCl concentrations (Table 3). Compared with the control, about 21, 35, and 36 % reduction of PAO activity was observed in Longchun 30 exposed to 25, 100, and 200 mM NaCl, respectively. Any change of this enzyme activity in Longchun 27 roots did not take place under 25 mM NaCl treatment, but it lowered about 49 % and 42 % in response to 100 and 200 mM NaCl stress, respectively, in comparison with the control.

Discussion

As primary organs which come into contact with salt environment, roots are considered to play a critical role in plant salt tolerance (Chen and Heuer 2013). In the present study, 25 mM NaCl promoted root and shoot growth in Longchun 30 seedlings, but high salt concentrations inhibited significantly the seedling growth in both wheat species. Moreover, inhibitory effect on the growth of Longchun 30 seedlings was weaker compared with that on Longchun 27. MDA is generally considered a good indicator of oxidative stress when plants are exposed to a stressful environment. Additionally, plasma membrane damage or deterioration in plants may lead to a substantial increase in electrolyte leakage (Yabuta *et al.* 2002). The

enhancement of REC and MDA content was found in cotton or mung bean seedlings under salt stress (Nazar *et al*. 2011, Wang *et al*. 2017). According to the present study, the reduction of MDA content and REC in response to 25 and/or 100 mM NaCl treatment suggested that wheat cultivars Longchun 30 and Longchun 27 were tolerant to low NaCl concentrations. Besides, high NaCl concentration did greater damage to Longchun 27 than to Longchun 30, which could be supported by the higher REC in Longchun 27 roots as compared with that in Longchun 30 ones. These results seemed to indicate that Longchun 30 seedlings were more suitable for growing in salt environment, which also provided an explanation for the fact that Longchun 30 is widely planted in the irrigation area in the Hexi Corridor and along the Yellow River, the main distribution area of saline land in Gansu Province.

An excessive amount of NaCl in the environment may compete with nutrient elements and further interfere with the balanced absorption of various ions. Various studies suggested that NaCl toxicity was associated with the increase of Na uptake and the inhibition of K and Ca absorption and content in different plant tissues (Arif *et al*. 2020, Attia *et al*. 2020, Gogna *et al*. 2020). In this study, increased Na content and reduced Ca content were caused in the roots and leaves of two wheat seedlings under NaCl treatment. Differently, the response to salt stress was accompanied by the increase of Ca content in the roots of wheat and rice (Zhang *et al*. 2018, Terletskaya *et al*. 2019). Meanwhile, the significant reduction of K content was also observed in salt-stressed roots of both cultivars. Potassium is an essential macronutrient related to enzyme activation and osmotic adjustment in plants (Barragan *et al*. 2012). The toxicity of Na accumulation associated with salinity stress is one of the primary mechanisms of cell damage in most plants. Na accumulation in shoot or leaf was used as physiological marker to assess salt tolerance in many plants (Feng *et al*. 2018). For example, *Hordeum marinum* showed higher salt tolerance than *Hordeum vulgare*, which might be associated with low Na content in shoot under high salt conditions (Huang *et al*. 2018). NaCl-induced Ca decline leads to loosening of cell wall and disruption of Ca ion homeostasis in plants (Rengel 1992), thus affecting plant growth and development. Furthemore, the Na increment in the roots and leaves in Longchun 27, together with the Ca depletion in the roots, was more notably under the same NaCl concentration than that in Longchun 30. These Na and Ca content changes were more unfavourable to the growth of Longchun 27 seedlings compared with that of Longchun 30. Contrarily, the accumulation of Na and K might not account for the greater tolerance of rice Dongdao-4 than Jigeng-88 to salt stress (Li *et al*. 2017).

It has been well documented that exposure of plants to such unfavourable conditions as salt stress results in ROS generation. In particular, ROS accumulation can take place in the cell walls or the outer side of the plasma membrane in plants under abiotic stresses (Gupta *et al*. 2016, Sharma *et al*. 2019). In the present study, the amount of total H_2O_2 and $O_2^{\bullet-}$ as well as these two parameters in the apoplast increased in salt-stressed roots of two wheat cultivars significantly. Meanwhile, high salinity stress resulted in the remarkable increase of total $^{\bullet}OH$ and apoplastic $^{\bullet}OH$ content in both cultivars. SOD, the first enzyme in the detoxifying process, catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Wang *et al*. 2012), and CAT and POD are the primary H_2O_2-scavenging enzymes in plant cells. High activity of antioxidant enzymes is considered as one of the salt tolerance mechanisms in most plants (Ashraf 2009). However, the findings of some studies showed that salt-tolerant cultivars had lower antioxidant enzyme activities than salt-sensitive ones under salinity stress (Senandheera *et al*. 2012, Ali *et al*. 2017, Siddiqui *et al*. 2017). This could be supported by a series of studies, which demonstrated that the responses of plant antioxidant enzymes to salinity environment is dependent on the plant species, the tissues, and even subcellular localizations (Raza *et al*. 2007, Castelli *et al*. 2010, Jan *et al*. 2017). In this study, total SOD activity lowered under 100 mM NaCl concentration and enhanced under 200 mM NaCl in Longchun 30, but significantly elevated under salt stress in Longchun 27. Additionally, total CAT activity was higher in untreated and salinity-treated Longchun 27 roots than in Longchun 30 ones. These results suggested that SOD and CAT played more important roles in alleviating excessive ROS accumulation induced by salinity stress in Longchun 27 roots than in Longchun 30 ones. Comparative studies of salt-tolerant and salt-sensitive wheat cultivars (Ali *et al*. 2017, Siddiqui *et al*. 2017) and tomato cultivars (Amjad *et al*. 2020) under salinity stress suggested that salt tolerance is correlated with increased POD activity. In this study, total POD activity was significantly enhanced in Longchun 30 roots but decreased in Longchun 27 ones with the increase of NaCl concentration. Therefore, Longchun 30 seedlings were capable of tolerating salt stress by increasing the activity of POD to minimize oxidative damage evoked by salt stress. However, the inhibition of apoplastic CAT and POD in response to salinity stress was not beneficial to apoplastic H_2O_2 elimination in the roots of both cultivars. Besides, H_2O_2 can be synthesized by cw-POD, DAO, and PAO. Salt stress increased DAO and PAO activities in the roots of salt-resistant rice cultivars and in the shoots of salt-sensitive ones (Quinet *et al*. 2010). However, the activities of cw-POD and PAO decreased in hyperhydricity of garlic plantlets under oxidative stress treatment (Tian *et al*. 2017). Moreover, cw-POD-dependent and PAO-dependent H_2O_2 generation was the major source of H_2O_2 accumulation in the roots of cucumber under salt stress (Wu *et al*. 2018). The significant decrease of cw-POD, DAO, and PAO activities in the present study suggested that these three enzymes might not be responsible for salinity-induced H_2O_2 accumulation in both wheat cultivars.

It was reported that the elevated expressions of antioxidant enzyme genes were involved in the adaptive pathways to salt stress in plants (Kaur *et al*. 2016, Saini *et al*. 2018). To understand the role of antioxidant enzyme genes in conferring salt tolerance in both wheat cultivars, their expressions were assayed. The present data showed that total SOD activity was correlated with *TaCu/ZnSOD* and *TaMnSOD* expressions in response to 200 mM NaCl, and that the changes of total POD activity was consistent with *TaPOD* expression in salinity-stressed roots of

both cultivars. Similarly to our results, higher SOD and POD activities were attributed to the higher expression of *MnSOD* and *POD* genes (Ben Saad *et al*. 2019) and the higher expressions of *Fe-SOD* and *Cu/ZnSOD* genes in several rice cultivars (Saini *et al*. 2018). Therefore, the changes of total SOD and POD activities induced by salinity stress were mainly dependent on their gene expressions in both wheat cultivars. However, it was found that the change of antioxidant enzyme activities was not always consistent with their gene expression (Chen *et al*. 2020, Saini *et al*. 2018). For example, in Kentucky bluegrass cultivar Kenblue, salt stress increased SOD, CAT, and POD activities, but inhibited their gene expressions (Puyang *et al*. 2015). High NaCl concentrations led to higher CAT activity and higher *TaCAT* expression in Longchun 27 roots than those in Longchun 30 roots, while 200 mM NaCl-stressed Longchun 30 roots and 25 mM NaCl-treated Longchun 27 ones exhibited the decrease of *TaCAT* expression and the increase of CAT activity. These results indicated that the post-transcriptional regulation might be associated with CAT activity induced by salinity treatment. As an important indicator of oxidative stress, the change of isoenzymes activities in plant cells can be generally used to study plant tolerance to environmental stress (Kumar *et al*. 2012). Four SOD isoenzymes in leaves and five ones in stems were found in *Broussonetia papyrifera* (Zhang *et al*. 2013) and two CAT isoenzymes were detected in barley (Pérez-López *et al*. 2009). Puyang *et al*. (2015) reported that five SOD isoenzymes enhanced their intensity in two cultivars of Kentucky blue grass under 200 mM NaCl stress. In addition, the band intensities of POD1 and POD5 were higher in salt-tolerant cultivar while POD4 was higher in salt-sensitive one under 250 mM NaCl stress (Hu *et al*. 2012). Differently, POD isoenzymes did not change in *P. euphratica* (salt-resistant) but decreased in *P. popularis* (salt-sensitive) under salt exposure (Wang *et al*. 2007). These findings suggested that the activities of SOD, CAT, and POD and the expression patterns of their isoenzymes depended on the difference of plant species, plant organs, and even stress conditions. When the roots of both cultivars were exposed to salinity environment, the intensities of four SOD isoenzymes were correlated with the change of total SOD activity (Fig. 3*A-B*), and the increase of total CAT activity could be attributed to the increased abundance of CAT1 and CAT2 (Fig. 4*A-B*). Additionally, among seven distinct POD isoenzymes, the intensities of POD2, POD5, and POD7 increased in salt-stressed roots of Longchun 30, and the changes of the abundance of POD isoenzymes coincided with the decrease of total POD activity in Longchun 27 roots exposed to high NaCl concentrations (Fig. 4*C-D*).

In conclusion, the increase of REC, the increment of Na content, and the reduction of Ca content was more sensitive to NaCl treatment in Longchun 27 roots than in Longchun 30 ones, which might be the reason why Longchun 30 seedlings grew better than Longchun 27 ones in salt environment. ROS accumulation was associated with the inhibition of apoplastic SOD, CAT, and POD but not with the reduction of cw-POD, DAO, and PAO activities in both cultivars under salinity stress. Moreover, the stimulation of SOD and CAT in Longchun 27 roots was consistent with high activities of SOD and CAT isoenzymes accompanying with high expression of *TaCu/ZnSOD*, *TaMnSOD*, and *TaCAT*. Additionally, total POD activity coincided with *TaPOD* expression and POD isoenzymes abundance in the roots of both wheat seedlings under salinity stress. As an initial attempt, this study will build up a basis for further investigation of the mechanism of salinity tolerance in wheat. Our further studies will focus on signal regulation pathways in plant adaptation to salinity environment.

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