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Antioxidant defense mechanism under salt stress in wheat seedlings

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Abstract

The present study was carried out to study the effect of salt stress on cell membrane damage, ion content and antioxidant enzymes in wheat (*Triticum aestivum*) seedlings of two cultivars salt-tolerant KRL-19 and salt-sensitive WH-542. Seedlings (4-d-old) were irrigated with 0, 50 and 100 mM NaCl. Observations were recorded on the 3rd and 6th day after salt treatment and 2nd day after salt removal. The relative water content declined with induction of salt stress, more in WH-542 than in cv. KRL-19. K^+/Na^+ ratio in KRL-19 was higher than in WH-542. WH-542 suffered greater damage to cellular membranes due to lipid peroxidation as indicated by higher accumulation of H_2O_2, MDA and greater leakage of electrolytes than KRL-19. The activities of catalase, peroxidase and ascorbate peroxidase and glutathione reductase increased with increase in salt stress in both the cultivars, however, superoxide dismutase activity declined. Upon desalanization, partial recovery in the activities of these enzymes was observed in KRL-19 and very slow recovery in WH-542.

Additional key words: ascorbate peroxidase, calatase, glutathione reductase, hydrogen peroxide, malondialdehyde, peroxidase, superoxide dismutase, *Triticum aestivum.*

Introduction

Salt stress is one of the most important environmental stresses, influencing the productivity of agricultural systems around the world. High salt concentration causes an imbalance of cellular ions resulting in ion toxicity, osmotic stress and production of active oxygen species (Cheeseman 1988, Alscher *et al.* 1997, Noctor and Foyer 1998). For mitigating their deleterious effects, plants have developed antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), peroxidase (POD) and non-enzymatic scavengers like glutathione, ascorbic acid and carotenoids (Vranova *et al.* 2002, Dalmia and Sawhney 2004).

A correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in several plant species (Gossett *et al.* 1994, Dionisio-Sese and Tobita 1998, Benavides *et al.* 2000). However, the definite mechanism of NaCl-tolerance still remains to be elucidated. Little information is available about capacity of antioxidants during NaCl stress and recovery in tolerant and sensitive cultivars of the same plant species. So the present investigation was undertaken to study the effect of NaCl on plant water status, cell membrane damage and antioxidant enzymes under salinity and during recovery in leaves of wheat seedlings.

Materials and methods

Seeds of *Triticum aestivum* L. cultivars WH-542 (salt-sensitive) and KRL-19 (salt-tolerant) were selected. Seedlings were raised in plastic pots containing dune sand at temperature 22 °C with 15-h photoperiod and irradiance of 250 μmol m^{-2} s^{-1}. Four days old seedlings were irrigated with 0, 50 and 100 mM NaCl in half strength Hoagland's nutrient solution. Their electrical conductivity (EC) at 25 °C was 0.3, 5.4 and 9.8 dS m^{-1}, respectively. For recovery studies, the salt stressed seedlings were flushed with tap water on eleventh day of

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DAR - days after removal of salt; EC - electrical conductivity; DAT - days after salt treatment; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase; GSSG - oxidised glutathione; MDA - malondialdehyde; NADPH - nicotinamide adenine dinucleotide phosphate (reduced); NBT - nitroblue tetrazolium; POD - peroxidase; RWC - relative water content; SOD - superoxide dismutase; TCA - trichloroacetic acid.
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growth to leach out the salts (EC of sand down to < 1.0 dS m^{-1}). The observations were recorded 3 and 6 d after salt treatment (3 and 6 DAT) and 2 d after removing the salts (2 DAR).

Relative water content (RWC) and osmotic potential were measured on fully expanded leaves of control, salinized and desalinized seedlings. The lipid peroxidation was measured in terms of malondialdehyde (MDA) contents, a product of lipid peroxidation, according to the method of Heath and Packer (1968). Na^+ and K^+ contents of the leaves were measured by flame photometer (*Systronics* 128, Ahemdabad, India) according to the method of Jackson (1973). The membrane stability was assessed by measuring leakage of electrolytes as described by Dionisio-Sese and Tobita (1998). Tissue was extracted in 5 % trichloroacetic acid (TCA) and H_2O_2 content was estimated by method of Sinha (1972).

For extraction and assay of enzymes, 500 mg of leaves of control and stressed seedlings were homogenized with 3 cm^3 of extraction buffer (0.1 M K-phosphate buffer, pH 7.5) containing 1 % polyvinylpyrrolidone and 0.1 mM ethylendiaminetetraacetic acid (EDTA) at 4 °C. However, for ascorbate peroxidase, 1 mM ascorbate was included but EDTA was excluded from extraction buffer. The homogenate was centrifuged at 10 000 *g* for 20 min. The supernatant was used for assaying the activities of enzymes.

CAT activity was estimated using slightly modified procedure of Sinha (1972). The reaction mixture contained 0.4 cm^3 of 0.1 M phosphate buffer (pH 7.0), 0.4 cm^3 of 0.1 M H_2O_2 and 0.2 cm^3 of enzyme extract. The absorbance was measured after cooling at 570 nm. One enzyme unit is defined as 1.0 µmol (H_2O_2) min^{-1}.

POD activity was determined by the method of Shannon *et al.* (1966). The reaction mixture contained 2.9 cm^3 of 0.1 M phosphate buffer (pH 7.0), 0.04 cm^3 of 0.1 M H_2O_2, 0.04 cm^3 of 0.2 % *O*-dianisidine and 0.02 cm^3 of enzyme extract. The change in absorbance was read at 470 nm for 4 min. One enzyme unit is defined as change in 1 unit of absorbance min^{-1}.

APX was estimated by the procedure of Nakano and Asada (1981). The reaction mixture contained 1.2 cm^3 of 0.1 M phosphate buffer (pH 7.0), 0.04 cm^3 of 0.1 M H_2O_2, 0.02 cm^3 of 0.5 mM ascorbate and 0.04 cm^3 of cell free extract. The decrease in absorbance was recorded at 290 nm. Ascorbate peroxidase activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

GR activity was assayed by the method of Halliwel and Foyer (1978). The assay mixture consisted of 1.6 cm^3 of 0.1 M phosphate buffer (pH 7.5), 0.1 cm^3 of 5 mM oxidized glutathione (GSSG), 0.1 cm^3 of 3.5 mM of NADPH, 0.1 cm^3 of 1% (m/v) albumin and 0.1 cm^3 of enzyme extract. The decrease in absorbance was measured at 340 nm (coefficient of absorbance of 16.2 $mM^{-1}cm^{-1}$). One unit of GR was defined as 0.1 µmol(NADPH oxidized) min^{-1} at 30 °C.

Superoxide dismutase was estimated by measuring inhibition of the reduction of nitroblue tetrazolium (NBT) by the method of Giannopolitis and Ries (1977). The reaction mixture contained 1.6 cm^3 of 0.1 M Tris-HCl, buffer (pH 7.8), 0.2 cm^3 of 120 µM NBT and 0.3 cm^3 of 13 mM methionine, 0.5 cm^3 of 0.1 mM EDTA, 0.2 cm^3 of 10 µM riboflavin and 0.2 cm^3 of enzyme extract. The reaction mixture was irradiated (250 µmol m^{-2} s^{-1}) for 5 min and the absorbance was read at 570 nm. One enzyme unit is defined as the amount of enzyme which could cause 50 % inhibition of NBT reduction.

The data were analyzed using analysis of variance (*ANOVA*) for complete randomized design where each observation was replicated thrice. To compare the treatments, critical difference ($P = 0.05$) was calculated.

Results and discussion

The relative water content declined significantly with induction of salt stress and duration of salt stress. However, decline in RWC was lower in salt-tolerant cultivar KRL-19 as compared to salt-sensitive WH-542. At 50 mM NaCl stress, there was 0.75 and 1.6 % decline in relative water content at 3 DAT and 6 DAT, respectively in the former cultivar as compared to corresponding 1.1 and 6.7 % in the later. Similarly the reduction in RWC values at 100 mM NaCl in KRL-19 was 2.2 and 3.5 % at 3 DAT and 6 DAT as compared to corresponding 5.6 and 6.7 % in WH 542 with respect to their controls. Leaf osmotic potential also became more negative with increasing NaCl concentration and duration of salt stress. The osmotic potential in leaves of WH-542 was -0.63 MPa at 3 DAT and -0.73 MPa at 6 DAT at 50 mM NaCl concentration. At 100 mM NaCl stress it was lowered to -0.79 and -0.91 MPa at 3 and 6 DAT, respectively. The osmotic potential in KRL-19 was -0.60 and -0.66 MPa at 3 and 6 DAT, respectively, at 50 mM NaCl and -0.70 and -0.77 MPa after 3 and 6 DAT, respectively, at 100 mM NaCl. WH-542 suffered greater damage to cellular membranes due to lipid peroxidation as indicated by higher accumulation of H_2O_2 (Fig. 1*A*) and malondialdelyde content (Fig. 1*B*) and greater leakage of electrolytes than KRL-19 (Fig. 1*C*). Lower content of H_2O_2 and MDA and less electrolyte leakage in both the cultivars was observed during recovery, however, decrease was higher in KRL-19.

Accumulation of Na^+ was observed in leaves of both the cultivars (Fig. 1*D*) and its amount was related to concentration and duration of NaCl application. Higher concentration of Na^+ (163 % at 100 mM NaCl) as compared to control was observed in WH-542. The KRL-19 has the ability to avoid high uptake/ accumulation of Na^+ in its tissue. Upon removing of salt, there was a decrease in the content of sodium ions. The decrease was only 2 % in WH-542 but 16 % in KRL-19. So better recovery was observed in KRL-19. On the other

hand, K^+ (Fig. 1*E*) accumulated more in this cultivar so that KRL-19 had higher K^+/Na^+ ratio than WH-542 (Fig. 1*F*).

In WH-542, at 50 mM NaCl stress K^+/Na^+ ratio decreased by 2.3 times as compared to their respective control at 3 DAT and it was further lowered by 2.7 times at 6 DAT. The decrease of K^+/Na^+ ratio was still higher at 100 mM of salt concentration, *i.e.*, 3 times decreased than the respective control at 3 DAT and 3.8 times decreased at 6 DAT. In KRL-19, this ratio decreased by 2.1 and 2.3 times (at 100 mM NaCl) as compared to their respective controls at 3 and 6 DAT, respectively (Fig.1F). The removal of salt from growth media resulted in increased K^+/Na^+ ratio in both cultivars. However, KRL-19 had better recovery on desalinization from salt stress.

The activities of CAT (Fig. 2*A*), GR (Fig. 2*B*), APX (Fig. 2*D*) and POD (Fig. 2*E*) increased in both the cultivars due to salt stress. The increase was proportional to NaCl concentration and treatment duration. In contrast, leaves of WH-542 exhibited a gradual and substantial decline in the SOD activity (Fig. 2*C*). Enzyme activity decreased 1.3 and 1.8 times at 50 mM NaCl and 1.9 and 2.1 times at 100 mM NaCl as compared to respective control at 3 DAT and 6 DAT, while in KRL-19 it increased 2.6 and 3.7 times at 3 DAT and 6 DAT, respectively, at 50 mM NaCl but at 100 mM NaCl the stimulation of activity was much higher, *i.e.*, 3.2 and 4.7 times at 3 DAT and 6 DAT.

The increase in the content of H_2O_2 due to salt stress caused peroxidation of the lipid membrane, thus disrupting its permeability. Our results are in agreement with those of Pandey and Ganapathy (1984), Lutts *et al.* (1999) and Panda and Upadhyay (2003), who reported higher increase in amount of electrolyte leakage with the increase in salt stress in the salt-sensitive cultivar as compared to tolerant cultivar of rice and in roots of *Lemna minor*. There is lesser uptake of Na^+ and higher uptake of K^+ in KRL-19 than in WH-542. High Na^+ accumulation in salt-sensitive foxtail millet cultivar and in tomato roots have been reported to result in an enhanced electrolyte leakage and oxidative damage, whereas significantly lower Na^+ accumulation in salt-tolerant cultivar showed maintenance of cellular intactness (Sreenivasulu *et al.* 2000, Dureja 2003, Racagni *et al.* 2003/4). There is much evidence from various plants showing that the amounts and activities of enzymes involved in scavenging active oxygen species are altered by environmental stresses such as drought and salinity (Sairam *et al.* 2002, 2005, Dalmia and Sawhney 2004). The NaCl induced enhancement of peroxidase activity in salinized cells of KRL-19 indicates that these cells have a higher capacity for the decomposition of H_2O_2 generated by SOD.

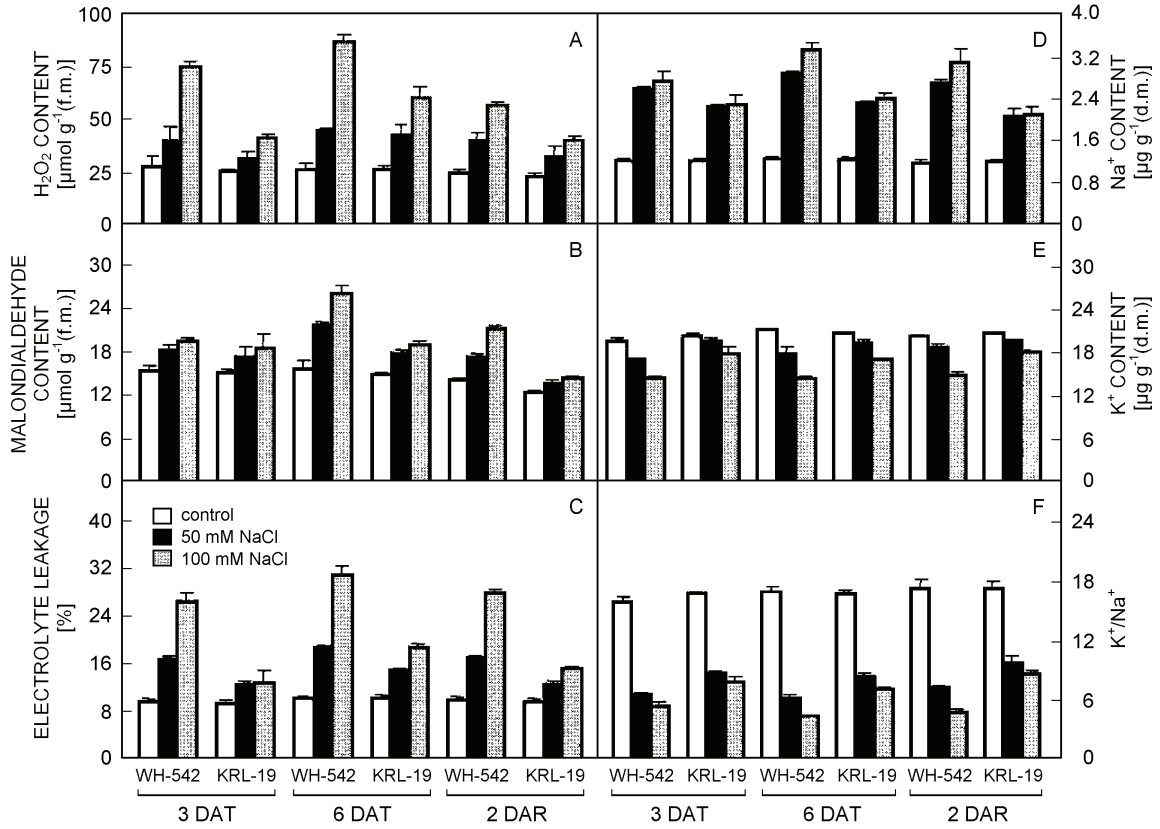

Fig. 1. Effect of salt stress and revival on hydrogen peroxide content (*A*), malondialdelyde content (*B*) and electrolytes leakage(*C*), changes in intracellular levels of Na^+ (*D*), K^+ (*E*) and K^+/Na^+ ratio (*F*) in leaves of KRL-19 (salt-tolerant) and WH-542 (salt-sensitive) wheat cultivars under salt stress and desalinization. DAT - days after salt treatment, DAR - days after removal of salt.

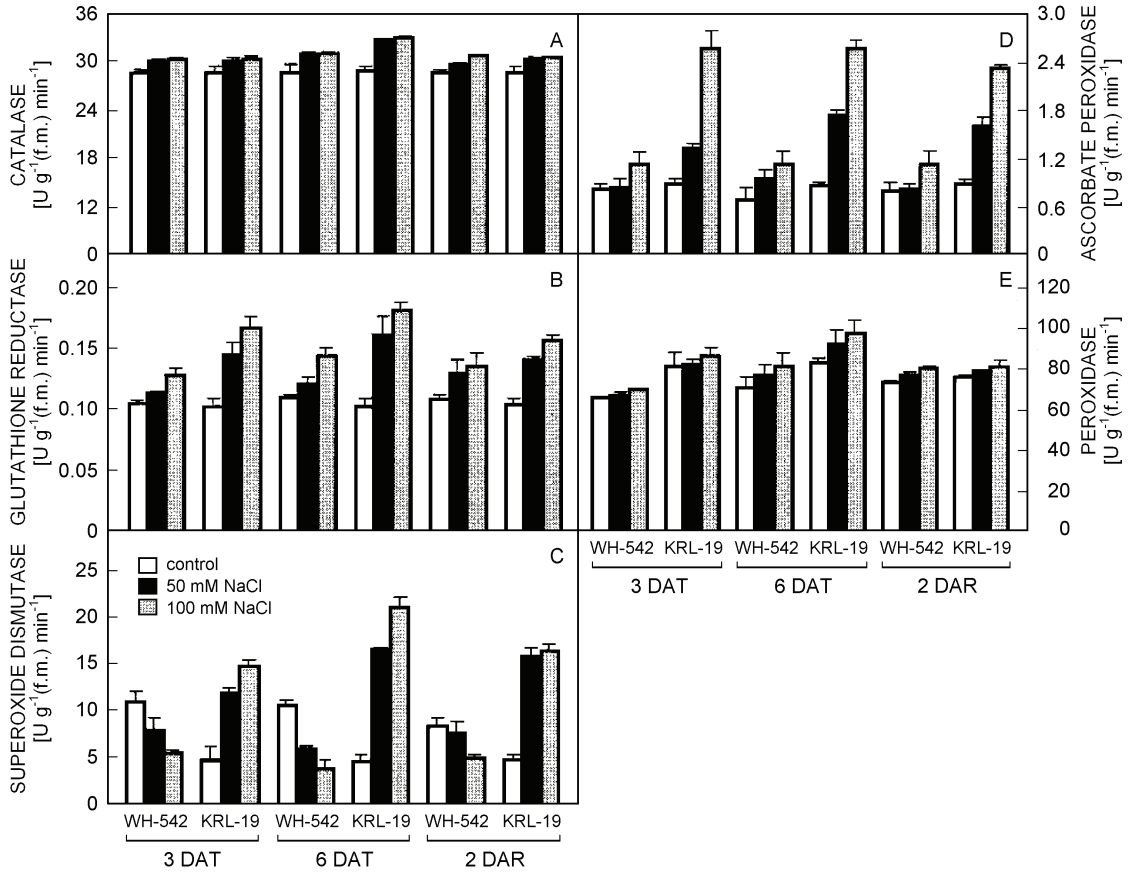

Fig. 2. Changes in activities of calatase (*A*), glutathione reductase (*B*), superoxide dismutase (*C*), ascorbate peroxidase (*D*) and peroxidase (*E*) in leaves of KRL-19 (salt-tolerant) and WH-542 (salt-sensitive) wheat cultivars under salt stress and desalinization. DAT - days after salt treatment, DAR - days after removal of salts.

CAT is another enzyme which is also involved in H_2O_2 detoxification converting H_2O_2 into water and oxygen. The results obtained in this study are in accordance with those of Sairam *et al.* (2002) who reported enhancement in CAT activity in both salt-sensitive and salt-tolerant cultivars of wheat. Similar observation was also reported by Gueta-Dahan *et al.* (1997).

The stimulation of APX activity by salt stress was much higher in salt-tolerant cultivar. The result of the present study are in conformity with those of Sreenivasulu *et al.* (2000) and Benavides *et al.* (2000) who also reported higher enzyme activity in leaves of salt-tolerant than salt-sensitive cultivar of foxtail millet.

The greater GR activity in salt stressed tolerant cultivar KRL-19 indicated that the tolerant plant exhibit a more active ascorbate-glutathione cycle than the non-tolerant cultivar. This cycle has been implicated in mitigating the effect of reactive oxygen species (Molina *et al.* 2002) and like other environmental stresses, salt stress affects the activities of these enzymes. The enhanced activities of these enzymes at mild stress may be related with increased pool of antioxidant metabolites which may act as scavengers of various reactive species and influence the gene expression as proposed by Foyer *et al.* (1997), Panda and Upadhyay (2003). SOD activity declined gradually and substantially in salt-sensitive cultivar and increased in salt-tolerant cultivar. The decline in SOD activity observed in the present data suggests a lesser O_2^- scavenging and dismutating capacity in salt-sensitive cultivar (WH-542) and signifies a possible involvement of this enzyme in salt-tolerance. Our results are in accordance with those of Rout and Shaw (2001) and Dureja (2003).

Upon desalting, partial recovery was observed in the activities of these enzymes in salt-tolerant cultivar. Smaller changes were observed in salt-sensitive cultivar, thus indicating slower recovery similar changes were observed in chickpea (Dureja 2003, Kukreja 2003).

The better NaCl tolerance in KRL-19 observed during present investigation may be due to restriction of Na^+ accumulation and ability to maintain high K^+/Na^+ ratio in tissue. It also restricted the damage to cellular membranes due to lower MDA and H_2O_2 under stress. In addition to all these, much higher activities of some of the enzymes involved in scavenging active oxygen species may explain the higher NaCl-tolerance of KRL-19 than WH-542. These results also indicate that though there was

some improvement in the plant water status, activities of the antioxidant enzymes, metabolites (carotenoides and ascorbate) and ions after two days of removal of salt from the growth media, yet, the seedlings were still suffering from the salt stress.

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