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Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes

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Abstract

Effects of long-term sodium chloride salinity (100 and 200 mM NaCl; ECe = 6.85 and 12.3 dS m^{-1}) were studied in tolerant (Kharchia 65, KRL 19) and susceptible (HD 2009, HD 2687) wheat genotypes. NaCl decreased relative water content (RWC), chlorophyll content (Chl), membrane stability index (MSI) and ascorbic acid (AA) content, and increased the contents of hydrogen peroxide, thiobarbituric acid reactive substances (TBARS), and activities of superoxide dismutase (SOD), ascorbate peroxidase (APOX) and glutathione reductase (GR). Kharchia 65 showed lowest decline in RWC, Chl, MSI and AA content, lowest increase in H_2O_2 and TBARS contents and higher increase in SOD and its isozymes, APOX and GR, while HD2687 showed the highest decrease in AA content, highest increase in H_2O_2 and TBARS contents and smallest increase in activities of antioxidant enzymes. KRL 19 and HD 2009 showed intermediate response both in terms of oxidative stress and antioxidant activity.

Additional key words: ascorbate peroxidase, ascorbic acid, chlorophyll, glutathione reductase, lipid peroxidation, superoxide dismutase, *Triticum aestivum.*

Introduction

Salinity stress exerts many symptoms similar to those observed under water deficit. Similar to water stress, reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are also produced during salinity stress, and are responsible for the damage to membranes and other essential macromolecules such as photosynthetic pigments, proteins, DNA and lipids (Hernandez *et al.* 1993, 1995, Fadzilla *et al.* 1997, Gueta-Dahan *et al.* 1997, Fahmy *et al.* 1998). In plant cells chloroplasts, mitochondria and peroxisomes are important intracellular generators of ROS (Rich and Bonner 1978, Del Rio *et al.* 1991, Elstner 1991, Salin 1991). ROS produced as a result of various abiotic stresses need to be scavenged for maintenance of normal growth. The primary scavenger is superoxide dismutase (SOD) (EC 1.15.1.1), which converts $O_2^{\bullet -}$ to H_2O_2 which is eliminated by ascorbate peroxidase (APOX) (EC 1.11.1.11) in association with dehydroascorbate reductase (EC 1.8.5.1) and glutathione reductase (GR) EC 1.6.4.2), and regenerate the ascorbic acid (Asada and Takahashi 1987, Salin 1991, Asada 1994). H_2O_2 is also scavenged by catalase (EC 1.11.1.6) (Dhindsa *et al.* 1981, Anderson *et al.* 1995, Comba *et al.* 1998), though the enzyme is less efficient than ascorbate peroxidase-glutathione reductase system. Changes in activities of various antioxidant enzymes under salinity stress have been reported (*e.g.* Gueta-Dahan *et al.* 1997, Fahmy *et al.* 1998, Hernandez *et al*. 2000). However, little information is available on the effects of salt stress on the activated oxygen species metabolism and antioxidant enzymes activity in tolerant/susceptible genotypes of wheat. This knowledge can supply information on the possible involvement of antioxidants as a defense against reactive oxygen species in the mechanism of salt sensitivity, thus allowing an insight in to the molecular mechanism of plant tolerance to salt induced oxidative stress. Hence the objective of the present investigation was to study the effect of two levels of salt stress on various antioxidant enzymes activity and oxidative stress (H_2O_2 and TBARS contents) and their relevance in terms of salinity stress tolerance in differentially tolerant and susceptible wheat genotypes.

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Abbreviations: AA - ascorbic acid; APOX - ascorbate peroxidase; Chl - chlorophyll; GR - glutathione reductase; MSI - membrane stability index; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances.

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Materials and methods

Wheat (*Triticum aestivum* L.) genotypes HD 2009, HD 2687 (salinity stress sensitive), KRL 19 (moderately tolerant) and Kharchia 65 (salinity tolerant) were grown in earthen pots (30 × 30 cm) lined with polythene bags and filled with a 10 kg mixture of air dried clay-loam soil and FYM in 6:1 ratio, supplemented with 60, 60 and 60 kg ha^{-1} of N, P, K at the time of sowing. Remaining 60 kg N ha^{-1} was given after one month of sowing. Before sowing pots were supplied with 2.5 dm^{-3} of 100 (S_1) and 200 (S_2) mM sodium chloride (NaCl). The electrical conductivity of extract (ECe), which is mean of 4 estimations made at three stages, viz., one month after sowing, anthesis and after harvest was 1.7, 6.85 and 12.3 dS m^{-1} for control and saline (S_1 and S_2) treatments. Four plants were maintained in each pot. All observations are means of 2 estimations made in each of 4 replicates at two stages clubbed together (30 and 40 d after sowing) ($n = 16$). Samples for various assays were collected between 10:00 - 10:30 h from 1st fully expanded leaf (2nd leaf from top), and brought to the laboratory in ice buckets. Assays for relative water content (RWC), chlorophyll (Chl) content, membrane stability index (MSI), hydrogen peroxide, lipid peroxidation (thiobarbituric acid reactive substances) and ascorbic acid were done in fresh samples.

Leaf relative water content (RWC) was estimated according to Wheatherley (1950) and calculated as follows: RWC = [(fresh mass - dry mass)/(saturated mass - dry mass)] × 100. Chlorophyll contents were estimated by extracting 0.05 g of the leaf material in 10-cm^3 dimethylsulfoxide (DMSO) (Hiscox and Israelstam 1979). The samples were heated at 65° C for 4 h and than the absorbance of extract recorded at 665 and 645 nm. Chlorophyll contents were calculated as per standard method (Arnon 1949). Membrane stability index (MSI) was estimated as per Sairam *et al*. (1997). Plant material (0.1 g) was taken in 10 cm^3 of double distilled water in two sets. One set was subjected to 40 °C for 30 min and its conductivity was recorded using a conductivity meter (C_1). Second set was kept in a boiling water bath (100 °C) for 10 min and its conductivity was also recorded (C_2). Membrane stability index (MSI) = [1 - (C_1/C_2)] × 100.

Hydrogen peroxide contents were estimated by measuring the titanium-hydroperoxide complex at 415 nm (Mukherjee and Choudhuri 1983). For titanium reagent preparation 1 g titanium dioxide and 10 g potassium sulphate were digested in 150 cm^3 conc. sulphuric acid over a hot plate for 4 h. The digested mixture was diluted to 500 - 600 cm^3 and stirred with a magnetic stirrer cum heater at 70 - 80 °C till a clear transparent solution was obtained. This solution was diluted to 1.5 dm^3 and stored in dark brown bottle. Fresh leaf sample (0.5 g) was macerated with 10 cm^3 cooled acetone in a cold room (10 °C). Mixture was filtered with *Whatman No. 1* filter paper followed by the addition of 4 cm^3 titanium reagent and 5 cm^3 ammonium solution to precipitate the titanium-hydro peroxide complex. Reaction mixture was centrifuged at 10 000 *g* for 10 min in the centrifuge (*J2-21*, *Beckman*, Geneva, Switzerland). Precipitate was dissolved in 10 cm^3 2 M H_2SO_4 and than recentrifuged. Supernatant was read at 415 nm against reagent blank in UV-visible spectrophotometer (model *M 36*, *Beckman*, CA, USA). The concentration of hydrogen peroxide was calculated from a standard curve plotted with range of 100 to 1000 nmol H_2O_2.

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBARS) (Heath and Packer 1968). Fresh leaf samples (0.5 g) were homogenized in 10 cm^3 of 0.1 % trichloracetic acid (TCA), and the homogenate was centrifuged at 15 000 g for 15 min. To 1.0 cm^3 aliquot of the supernatant 4.0 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 °C for 30 min in the laboratory electric oven (*Scientific*, New Delhi, India) and than cooled in an ice bath. After centrifugation at 10 000 *g* for 10 min the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to its coefficient of absorbance 155 mM^{-1} cm^{-1}.

Ascorbic acid content was estimated as described by Mukherjee and Choudhuri (1983). Fresh leaf sample (0.5 g) was extracted with 10 cm^3 of 6 % trichloroacetic acid. Four cm^3 of this extract was mixed with 2 cm^3 of 2 % dinitrophenylhydrazine (in acidic medium) followed by the addition of 1 drop of 10 % thiourea (in 70 % ethanol). The mixture was boiled for 15 min in a water bath and after cooling to room temperature, 5 cm^3 of 80 % (v/v) H_2SO_4 was added to the mixture at 0 °C (in an ice bath). The absorbance was recorded at 530 nm. Ascorbic acid concentration was calculated from a standard curve plotted with range of 100 to 1500 nmol ascorbic acid.

Enzyme extract for superoxide dismutase, ascorbate peroxidase and glutathione reductase was prepared by first freezing the weighed amount of leaf samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 cm^3 of cold extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). Brie was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15 000 *g* and the supernatant was used as enzyme. Superoxide dismutase activity was estimated by recording the decrease in absorbance of formazan produced by superoxide and nitro-blue tetrazolium by the enzyme (Dhindsa *et al.* 1981). Three cm^3 of the reaction mixture contained 13 mM methionine, 25 mM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 cm^3 enzymes. Reaction was started by adding 2 μM

riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. To distinguish SOD isoforms, viz., Cu/Zn-SOD, Fe-SOD and Mn-SOD, the sensitivity of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to H_2O_2 (5 mM) were used, whereas Mn-SOD was unaffected (Yu and Rengel 1999). Separate controls (lacking enzymes) were used for total SOD and inhibitor studies. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme.

Ascorbate peroxidase was assayed by recording the decrease in absorbance at 290 nm due to a decrease in ascorbic acid content (Nakano and Asada 1981). Reaction mixture (3 cm^3) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, and 1.5 mM H_2O_2 and 0.1 cm^3 enzyme extract. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm.

Glutathione reductase was assayed as per the method of Smith *et al*. (1988). The reaction mixture contained 66.67 mM potassium phosphate buffer (pH 7.5), 0.333 mM EDTA, 0.5 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.0667 mM NADPH, 0.1 cm^3 enzyme extract and distilled water to make up a final volume of 2.9 cm^3. Reaction was initiated by adding 0.667 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min.

Results were analyzed by analysis of variance and LSD values were calculated for cultivars, treatments and their interactions. Standard error of mean was also calculated for presentation in figures.

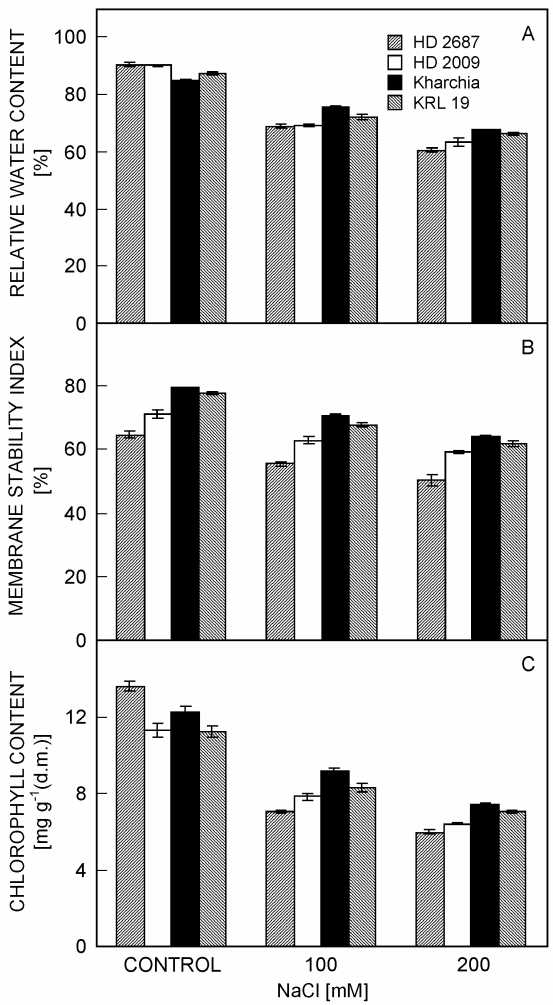

Fig. 1. Effect of salinity stress on relative water content (*A*), membrane stability index (*B*) and chlorophyll content (*C*) in tolerant and susceptible wheat genotypes. *Vertical bars* indicate SE of mean. LSD for cultivars and treatments were significant at $P = 0.05$.

Results

RWC decreased under salinity in all the genotypes. Though HD 2687 had higher RWC under normal condition, salinity tolerant genotype Kharchia 65 and KRL 19 maintained relatively higher RWC under salt stress as well as showed less decline as compared to HD 2009 and HD 2687 (Fig. 1*A*). Membrane stability index, estimated as electrolyte leakage, decreased under salt stress, lowest being at 200 mM NaCl. Kharchia 65 followed by KRL 19 showed fewer declines as compared to HD 2687 and HD 2009 (Fig. 1*B*). Total chlorophyll contents under control condition were higher in HD 2687 than in other genotypes. However, the decrease under salt stress was also higher in HD 2687 and HD 2009, while Kharchia 65 and KRL 19 maintained comparatively higher Chl content than HD 2687 and HD 2009 (Fig. 1*C*).

Ascorbic acid content (AA) decreased under salt stress in all the genotypes, and the magnitude of decline increased with salinity levels (Fig. 2*A*). However, Kharchia 65 followed by KRL 19 maintained higher AA content under all the treatments than HD 2687 and HD 2009. H_2O_2 content increased with increasing NaCl concentrations in all the genotypes (Fig. 2B). HD 2687 followed by HD 2009 showed higher H_2O_2 content in control and stressed plants than other genotypes. Lipid peroxidation estimated as thiobarbituric acid reactive substances (TBARS) also increased with salinity in all

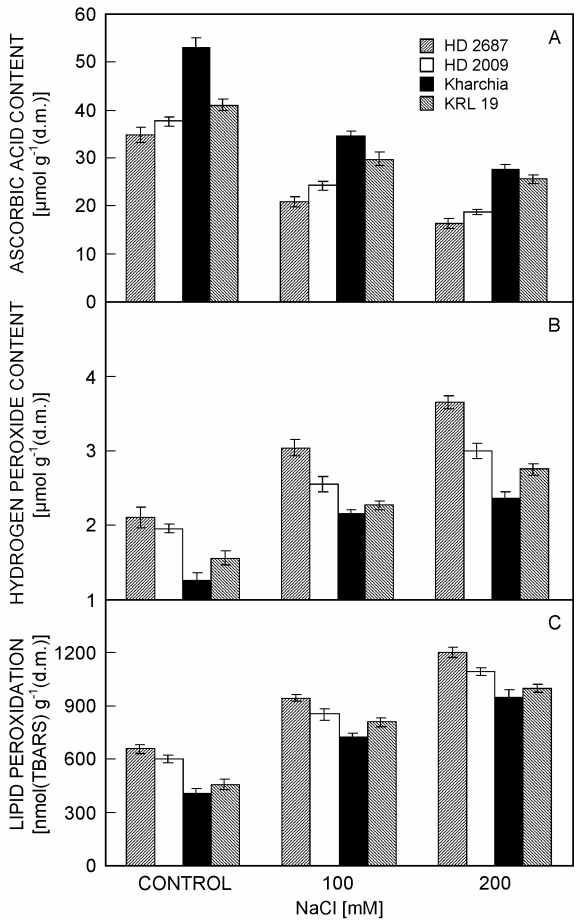

Fig. 2. Effect of salinity stress on ascorbic acid contents (*A*), hydrogen peroxide (*B*) and thiobarbituric acid reactive substances (*C*) in tolerant and susceptible wheat genotypes. *Vertical bars* indicate SE of mean. LSD for cultivars and treatments were significant at $P = 0.05$.

the genotypes, and similar to H_2O_2 accumulation TBARS content was highest in HD 2687 in both control and salt stressed plants and minimum in Kharchia 65, while HD 2009 and KRL 19 showed intermediate response (Fig. 2*C*).

Total SOD activity mostly increased in salt stressed plants, but it was higher at 100 mM NaCl than at 200 mm NaCl. Exception was HD 2687, where activity decreased below its control levels at 200 mM NaCl. Kharchia 65 showed highest SOD activity and HD 2687 the lowest. Among the SOD isoforms, Cu/Zn-SOD was most abundant, followed by Mn-SOD, and the two isoforms followed the trends similar to total SOD both in terms of genotypes and salinity stress. Fe-SOD activity was very low in all genotypes and did not show any significant salinity induced changes (Fig. 3*B-D*).

Ascorbate peroxidase (APOX) activity slightly increased under salt stress up to 100 mM NaCl in all the genotypes and up to 200 mM NaCl only in Kharchia 65, while in KRL 19, HD 2687 and HD 2009 the activity decreased at 200 mM NaCl. APOX activity was highest in Kharchia 65 in control and salt stressed plants, followed by KRL 19 and HD 2009, which had similar level of activities, and minimum in HD 2687 (Fig. 4*A*). Glutathione reductase (GR) activity was also highest in Kharchia 65 and increased up to 200 mM NaCl. KRL 19, HD 2687 and HD 2009 showed increase in GR activity only at 100 mM NaCl and decline at 200 mM NaCl. In HD 2687 the GR activity at 200 mM NaCl decreased even below that in controls (Fig. 4*B*).

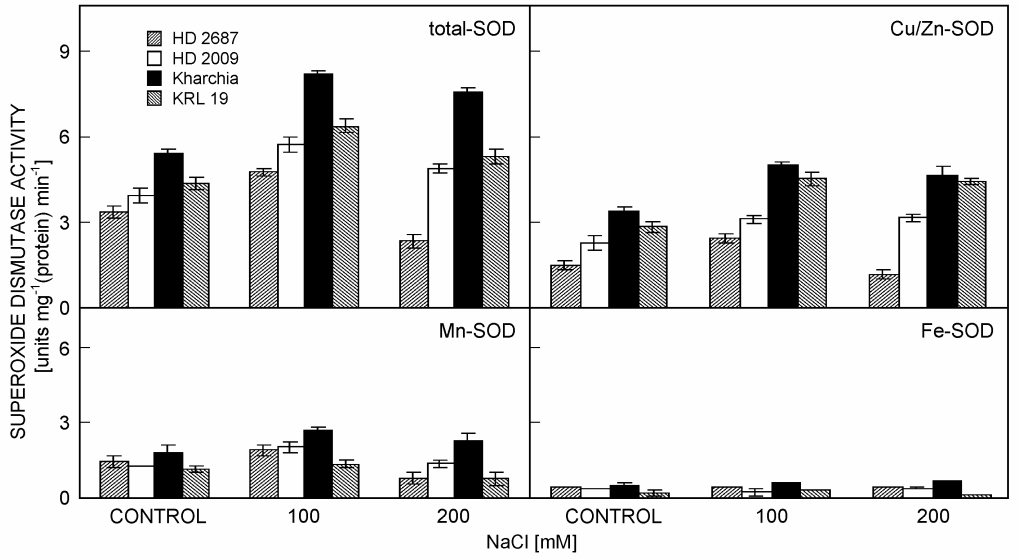

Fig. 3. Effect of salinity stress on the activities of total superoxide dismutase and its isozymes in tolerant and susceptible wheat genotypes. *Vertical bars* indicate of mean. LSD for cultivars and treatments were significant at $P = 0.05$.

Discussion

Salinity sensitive wheat genotype HD 2687 showed significantly higher decline in RWC, Chl and MSI than tolerant genotypes Kharchia 65 and KRL 19 under salt stress, that reflects their tolerant nature. Salinity induced decrease in RWC (Gadallah 1999), Chl (Salama *et al.* 1994) and MSI (Bhattacharjee and Mukherjee 1996) have been reported earlier. Srivastava *et al.* (1988) reported chlorophyll content as one of the parameters of salt tolerance in crop plants. Hernandez *et al.* (1995) observed higher chlorophyll degradation in sodium chloride sensitive pea cultivar as compared to tolerant one.

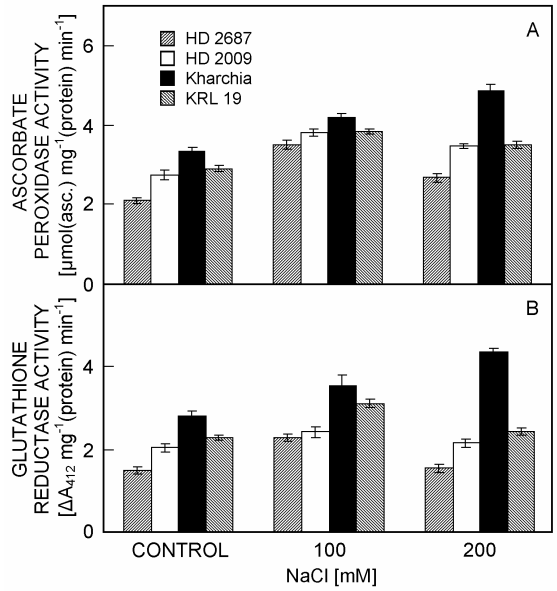

Fig. 4. Effect of salinity stress on the activities of ascorbate peroxidase (*A*), and glutathione reductase (*B*) in tolerant and susceptible wheat genotypes. *Vertical bars* indicate SE of mean. LSD for cultivars and treatments were significant at $P = 0.05$.

We observed an increase in H_2O_2 and TBARS contents (a measure of lipid peroxidation) under salt stress. Tolerant genotype Kharchia 65 showed lower H_2O_2 and TBARS contents than HD 2687 under salt stress, while HD 2009 and KRL 19 were intermediate. Hydrogen peroxide is a toxic reactive oxygen species and has deleterious effects in plant tissue (Asada and Takahashi 1987, Salin 1988, Sairam *et al.* 1998). Higher H_2O_2 accumulation and lipid peroxidation in sensitive cultivars of pea (Hernandez *et al.* 1993) and rice (Dionisio-Sese and Tobita 1998) have been reported earlier. Salinity induced increase in lipid peroxidation has also been reported by Ying *et al.* (1995).

In spite of a decrease in ascorbic acid (AA) content under salinity stress, Kharchia 65 and to some extent KRL 19 maintained higher AA content compared to HD 2687 and HD 2009. Ascorbic acid is an antioxidant, which can scavenge $O_2^{\bullet-}$ and H_2O_2 non-enzymatically, and also takes part in ascorbate peroxidase mediated scavenging of H_2O_2 (Asada 1992). Ascorbic acid is also involved in the regeneration of another important non-enzymatic antioxidant, α-tocopherol (Kunert and Ederer 1985, Hess 1993).

Salt stress caused an increase in total SOD activity at lower salinity (100 mM NaCl), and tolerant genotype Kharchia 65 recorded highest enzyme activity in control and salt stressed plants, while HD 2009 and KRL 19 were intermediate and HD 2687 was lowest. SOD is responsible for the scavenging of toxic $O_2^{\bullet-}$ in different cell organelles (Fridovich 1986, Salin 1988). Salinity induced increase in SOD activity has been reported by various workers (Sehmer 1995, Hernandez *et al.* 2000, Sreenivasulu *et al.* 2000). Cu/Zn-SOD, which has been reported in cytosol (Hernandez *et al.* 1994, 1999, 2000) and chloroplast (Hernandez *et al.* 1994, 1999, Gomez *et al.* 1999) accounted for most of the total SOD, and seems to be responsible for the observed tolerance of Kharchia 65 and KRL 19. Mn-SOD activity, which is reported to be mostly localized in mitochondrial fraction (Hernandez *et al.* 1999) was comparatively less, but nevertheless was higher in tolerant cultivar Kharchia 65, and therefore, contributing to scavenging of ROS produced in mitochondria. Fe-SOD activity, which was very rudimentary, did not change due to salinity stress in various genotypes, though Kharchia 65 has slightly higher activity than other genotypes. It is apparent that Fe-SOD did not contribute to the scavenging of salinity induced ROS in wheat genotypes in this study.

Ascorbate peroxidase (APOX) and glutathione reductase (GR) were highest in Kharchia 65. While in KRL 19, HD 2009 and HD 2687 APOX and GR activities decreased at higher salinity (200 mM NaCl), Kharchia 65 showed enhanced induction of both the enzymes still at higher salinity level. The increase in H_2O_2 scavenging capacity of Kharchia 65 could be one of the reasons of its tolerance to higher salinity (200 mM NaCl). Salinity induced increase in APOX and GR activities in salinity tolerant cultivars have been reported by Hernandez *et al.* (1995), Fahmy *et al.* (1997), Gueta-Dahan *et al.* (1997), Comba *et al.* (1998), and Hernandez *et al.* (2000). Secondly the susceptibility of HD 2009 and HD 2687 to salinity could be due to inhibition of APOX activity at higher salinity level. Glutathione reductase, responsible for the reduction of oxidized glutathione, which in turn reduces dehydroascorbic acid to ascorbic acid, and thus is a key enzyme of Asada-Halliwell pathway responsible for the detoxification of H_2O_2, is limiting in susceptible cvs. HD2687 and HD 2009, and this could be one of the factors of their observed susceptibility to salt stress.

A perusal of the foregoing discussion reveals that SOD isoforms Mn-SOD and Cu/Zn-SOD contributed mainly to the total-SOD activity and scavenging of $O_2^{\bullet-}$ in wheat genotypes. Secondly even the enzyme (SOD)

present in tolerant cv. Kharchia 65 is sensitive to higher salinity levels, though the degree of sensitivity may differ. However, Kharchia 65 has much better hydrogen peroxide scavenging mechanism as manifested by continuous increase in APOX and GR activities up to highest salinity level, resulting in lower H_2O_2 content, lipid peroxidation, and higher chlorophyll content and membrane stability. Thus salt stress tolerance of Kharchia 65 as indicated by lower H_2O_2 content and lipid peroxidation (TBARS content), and higher membrane stability and chlorophyll content under salt stress was mainly due to constitutively higher activity as well as salinity induced increase in SOD, APOX and GR than HD 2687 and HD 2009. The results show that in HD 2687, scavenging systems are very limiting, and the genotype is thus ill equipped to face salt stress as it fails to respond in a manner similar to Kharchia 65, resulting in higher H_2O_2 content, lipid peroxidation (TBARS content), and lower membrane stability and chlorophyll content under salt stress. From the above results it can be concluded that both constitutive as well as salt induced increase in antioxidant enzymes activities are important for providing protection against ROS. While constitutive levels provide protection from oxidative stress arising from normal oxidative metabolism, the salinity or the abiotic stress induced increase in antioxidant activity in response to increase in oxidative stress actually decide the level of tolerance of a plant.

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