Antioxidant activity of enzymatic system of two different wheat (*Triticum aestivum* L.) cultivars growing under salt stress

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ABSTRACT

Soil salinity and semi-arid and arid climate of Pakistan is a major constraint in agriculture and predominantly in foodstuff production. It limits crop yield and use of land previously uncultivated. Wheat is moderately salt tolerant. A great variation was observed between and within the cultivars (genotypes: S-24 salt tolerant and DN-27 salt sensitive) in relationship to the choice of salinity level (control and treatments: in increment of 25 mol/m^3 NaCl/day to a final level of 80 and 160 mol/m^3 NaCl into the nutrient solution) that will be used for screening purpose. Relative water content (RWC), membrane stability index and the activities of some antioxidant enzymes were determined after 20 and 40 days of salt stress exposure. As a result of activity enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase increased in S-24 with the increase of salt stress, while in DN-27 all the enzymes showed constant activity at all the stress levels. Meanwhile, relative water content and membrane stability index decrease the value as well as they increases the stress levels. It can be concluded that all three antioxidant enzymes were limiting factors for these genotypes and these reasons also led to the salt sensitivity in DN-27. Different selection methods should be applied to improve different traits in different conditions in wheat.

Keywords: *Triticum aestivum* L.; osmotic stress; oxidative damage; defense response of plant; reactive oxygen species (ROS)

The spread of soil salinity in arid and semi-arid areas is posing a serious threat to crop production. The high rate of evapotranspirational loss of water under these environmental conditions results in the deposition of excess salts on the soils fraction in which the roots flourish. Salination affects all lands that receive little rain and in areas which are affected by deforestation. It can become even more detrimental in irrigated agriculture because long-term irrigation inevitably leads to salt accumulation in the soil. Also, irrigation is a necessity because, with only about 15% of all lands under irrigation, the area produces more than 30% of all food; yet, approximately half of this irrigated land is endangered by salination (Penning de Vries 2001). There is much archaeological evidence for the deterioration of soils by irrigation. For example, with the start of irrigation in the Euphrates valley (7000 years ago) a replacement of the salt-sensitive wheat by more salt-tolerant barley occurred in less than one hundred years. The understanding of the dilemma and the ways of land management were improved, the problem re-emerged nonetheless because we have used nearly all land that can be used for agriculture. Worse, in many poor countries farmers use poor, saline or degenerated soils that yield poor harvests to begin with and are destroyed by agriculture within a few years or a few decades. There are very few choices for civilized and fair societies. One of these is that we enable crops to grow in an increasingly saline environment, which always has a water deficit component associated, while technologies are developed by which soil deterioration might be reversed (Demiral and Turkan 2005). If one could, for example, increase yield in Sub-Saharan Africa from one ton/ha (which is lower approximately 10-fold than on prime farmland) to three tons this would be a significant improvement. This salinity stress like other abiotic soil stresses constitutes a major limiting factor, which hampers plant productivity throughout the world. Therefore,

strategies have to be developed to make use of these resources for food production. Because of their frequent potential for multiple cropping, the arid and semi-arid regions of the earth offer a considerable promise for development as major food producing regions. Many of the inhospitable deserts of such regions require only a source of water for conversion to prime agricultural land. A frequent problem with developing such lands is the accumulation of soluble salts, which imposes stress on growing crops that can lead to decreased yield and, in severe cases, complete crop failure (Kalaji and Piethiewicz 1993, Bhutta and Hanif 2008). Such salts originate from the relatively unweathered minerals in many soils of such regions, from the small amounts of salinity in rainfall that remains unleached and as residual (fossil) salts from former marine or lacustrine environments. These accumulated salts are redistributed on the landscape by irrigation and drainage waters. So, salinity is one of the major constraints responsible for low crop productivity in Pakistan. Salt tolerance is a complex quantitative, genetic character controlled by many genes. A few of these genes were identified and provided information that can be useful in screening and selection programs (Shannon 1997). Information is lacking on how most genes function in concert with other genes that may have influenced the mechanisms of salt tolerance, although, there was a great deal of work on plant responses to salinity (Maas and Hoffman 1977). Several mechanical, chemical and biological approaches are being pursued to cope with soil salinity. One of these is to modify the soil conditions to suit the crop plants but this is highly cost intensive and very little progress has been made in Pakistan with high financial costs. Despite the engineering solution to combat salinization of soil, efforts have also been made to tailor plants so that they can thrive grow well and when under such unfavorable conditions. Plant salt tolerance is generally thought of in terms of the inherent ability of the plant to withstand the effects of high salts in the root zone or on the plants' surface (Schachtman and Munns 1992). Salt resistance is another term that is often used for this phenomenon, and although some have tried to differentiate the two terms, they are used interchangeably. This study aimed at making comparison of the salt sensitive wheat mutants by comparing their physiological performance with salt tolerant wheat mutants under salinity stress and to appraise the contribution of the stimulating responses of the antioxidant enzymes in the lines tolerant to salt stress.

MATERIALS AND METHODS

Plant material. A genotype S-24 reported to be salt tolerant and the other DN-27 declared salt sensitive by other researcher. The seeds of two genotypes were grown in iron trays filled with acid washed gravel. These trays were placed in glasshouse with natural temperature of 10–15°C and 10 h photoperiod. The young seedlings at the two-leaf stage were transferred to aerated half strength Hoagland solution (Hoagland and Arnon 1950) in 3 iron containers internally lined with polythene sheet.

Salt stress applications. After 3 days of transplanting, salt was added to the nutrient solution starting on the 15 days after germination, in increment of 25 mol/m^3 NaCl/day to a final level of 80 and 160 mol/m^3 NaCl. $CaCl_2$ was also added to maintain Na: Ca ratio of 20:1. The pH was adjusted at 6.0 to 6.5 daily by adding HCl or NaOH. Solutions were changed after 8 days during the entire experimental period.

Growth parameters. After 0, 20 and 40 days of NaCl treatments, 8 pants from each group were divided into separate root and shoot fractions. Fresh weights of shoots and roots were weighed and shoot lengths were measured. The samples were then dried in oven at 80°C for 72 h and dry weights were determined.

Superoxide dismutase (SOD). SOD activity was estimated by recording the decline in the absorbance of superoxide nitro blue tetrazolium complex by means of the enzyme. As regards 3 ml of reaction combination, containing 0.1 ml of 3 mmol EDTA, 0.1 ml of 200 mmol methionine, 1.5 ml of 100 mmol potassium phosphate buffer, 0.01 ml of 2.25 mmol nitro-blue tetrazolium (NBT), 0.05 ml of enzyme extraction and 1 ml distilled water were use within the test tubes in double in number for each enzyme model. Two tubes were used as control, not including enzyme. The effect progressed with the addition of 0.1 ml riboflavin (60 µmol) and insertion of these tubes underneath the light supply of two 18 W lamps for 20 min. Reaction was stopped up by switching off the light. These tubes were covered with black cloth. Tubes used as control developed maximal colour. A non-irradiated complete reaction mixture which did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme movement was taken as the amount of enzyme. The tubes lacking enzymes compact the absorbance reading of samples up to 50%.

Catalase (CAT) and peroxidase (POD). Catalase action was precise according to Aebi

(1998). As concerning for CAT 3 ml reaction mixture containing 1.5 ml of 100 mmol potassium phosphate buffer (pH = 7.2), 0.5 ml of 75 mmol H_2O_2, 0.05 ml enzyme extraction. The distilled water was used to construct the volume up to 3 ml. Reaction in progress by the addition of H_2O_2. The absorbance recorded in decrease at 240 nm for 60 s. The enzyme action was accounted by calculating the quantity of decomposed H_2O_2.

Peroxidase (POD) activity was assayed by recording 3 ml reaction mixture. The reaction mixture contained 0.1 mmol EDTA, 1 ml of 0.2 mol/m^3 potassium phosphate buffer with pH = 7.6, 0.1 ml of 2 mmol (NADPH), 0.5 ml of 3 mmol DTNB, 0.1 ml enzyme extract. The distilled water was used to create a closing volume of 2.9 ml. Reaction initiated by adding of one unit of POD activity. The raise within absorbance at 412 nm was recorded at 25°C in excess of a period of 5 min on a spectrophotometer. Protein extract was quantified using the technique of Bradford (1976).

Relative water content (RWC). Relative water content (RWC) was estimated by taking leaf sample (0.5 g) in 100 ml distilled water. Leaf samples were placed in kraft's bag and oven dried at 70°C for 36 h. On concordant dry weight achievement, the weight of sample was noted.

RWC = (fresh weight – dry weight)/(turgid weight – dry weight)

Membrane stability index (MSI). Determination of MSI was done by employing electrical conductivity (EC) of leaf leachates. For this, double distilled water is utilized as medium at temperatures 40°C and 100°C. Two same sized discs were taken in standard test tubes having 10 ml double distilled water. Two sets of discs were maintained for comparison being kept for 30 min. at 40°C and for 15 min at 100°C in water bath respectively. Electric conductivity's EC_1 and EC_2 were measured by conductivity meter (Sairam 2002).

$$MSI = [1 - EC_1/EC_2]/100$$

Analysis of variance. All the analyses were done in a completely randomized design. All the data were subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by *LSD* test.

RESULTS AND DISCUSSION

The growth of shoots was followed by measuring length, fresh weight and dry weight. The effect of 80 mmol NaCl shows lower results on the shoot length through out the experimental period in both the wheat genotypes. After the exposure of wheat genotype S-24 plants to 160 mmol NaCl for 20 and 40 days, shoot length decreased by 16% and 18%; in DN-27, the decrease in shoot length was 25% and 30% (Table 1), respectively.

The fresh weight of shoots was affected under both salinity levels on day 20 at 80 mmol NaCl and 160 mmol NaCl applications for 40 days; it caused 40% and 69% decrease in shoot fresh weight in S-24 and this ratio is increased in DN-27 at both salinity levels (80 mmol NaCl and 160 mmol NaCl) after 20 and as well as 40 days caused, the decrease was 51% and 72% decreased (Table 2), respectively. The shoot dry weight decreased by 70% after 40 days treatments of 160 mmol NaCl. In wheat, genotypic differences in biomass production under salt stress were observed only after 3 weeks of stress (James et al. 2002).The decrease in shoot fresh weight might be due to low uptake of water by plants as well as due to toxicity of Na^+ and $C1^-$ because of their high concentration in the nutrient solution (Silveira et al. 2003, Bhutta and Hanif 2010), combination of slower growth and development as a result of osmotic stress (Shani and Ben-Gal 2005), inhibition of photosynthesis as a result of direct effects of salinity on the photosynthetic apparatus or indirect effects as a result of a reduction in sink capacity. Leaf relative water content of wheat seedling decreased 30% under the both salinity levels after 20 days. Whereas leaf relative water content was decreased by 22% under the both 80 mmol NaCl and 160 mmol NaCl treatments after 40 days in S-24 as well as it decreased in DN-27 (Figure 1).

Under the non saline environment membrane stability index increases in the leaves of wheat seedling. Whereas under the low saline levels 80 mmol NaCl 18% and 23% decreases after the 20 and 40 days treatments the wheat genotypes S-24 and 22% and 29% in DN-27, respectively. The level of membrane stability index decreases under the high saline levels 160 mmol NaCl 27% for 40 days treatments. Membrane stability index

Table 1. Effect of salt stress on shoot length (cm) of wheat seedlings

	Cultivars			
	S-24		DN-27	
	20 days	40 days	20 days	40 days
Control	33.47	39.81	22.34	26.29
80 mmol NaCl	29.31	31.12	18.74	20.48
160 mmol NaCl	22.89	24.52	13.29	15.33

Table 2. Effect of NaCl treatments on fresh (FW) and dry (DW) weight (g) of shoot in wheat seedlings

	FW				DW			
	S-24		DN-27		S-24		DN-27	
	20 days	40 days	20 days	40 days	20 days	40 days	20 days	40 days
Control	4.23	6.14	3.27	4.13	2.14	2.82	1.66	2.08
80 mmol NaCl	3.84	4.44	2.61	3.13	1.82	2.11	1.14	1.52
160 mmol NaCl	3.31	3.86	2.27	2.85	1.53	1.77	0.95	1.22

in leaves of wheat seedling exposed to 160 mmol NaCl for 40 days reached the lowest levels in both the wheat genotypes S-24 and DN-27.

SOD activity in the leaves of wheat seedling under different salinity levels was shown in Figure 1a. Under normal growth environments, SOD activity in control groups decreased throughout the experimental period. The 80 mmol NaCl treatments for 20 days cause a remarkable change in SOD activity whereas 160 mmol NaCl caused a 30% increase in SOD activity after 40 days in S-24. The increase in SOD activity was noticed in both salinity levels in DN-27. Salt stress increased the superoxide level in cells. If this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler 2000). Moreover, it inactivates antioxidant enzymes which are very important for H_2O_2 scavenging such as catalases and peroxidases, while the minimum SOD activity was in DN-27. In these genotypes superoxide radical (O_2^-) production increases with the increase of salt stress. But SOD activity was constant at all salt stress levels. For this reason scavenging of this dangerous radical was not done perfectly. Consequently, this radical attacks vital biomolecules that mentioned before and damage to membranes happens in this cultivar. Esfandiari et al. (2007) and Zhao et al. (2007) had similar findings and expressed that the increase in SOD activity and decrease in oxidative damage were closely related.

The activity of CAT started to increase with the rise of NaCl content in comparison with the control in S-24 and as well as in DN-27; at 80 mmol NaCl and 160 mmol NaCl it showed a significant difference compared to control. The CAT activity increases with increased salinity levels in both the wheat genotypes. CAT activity had the highest activity at 160 mmol NaCl stress level in S-24 in 40 days. Furthermore, there is a significant difference between 80 mmol NaCl and

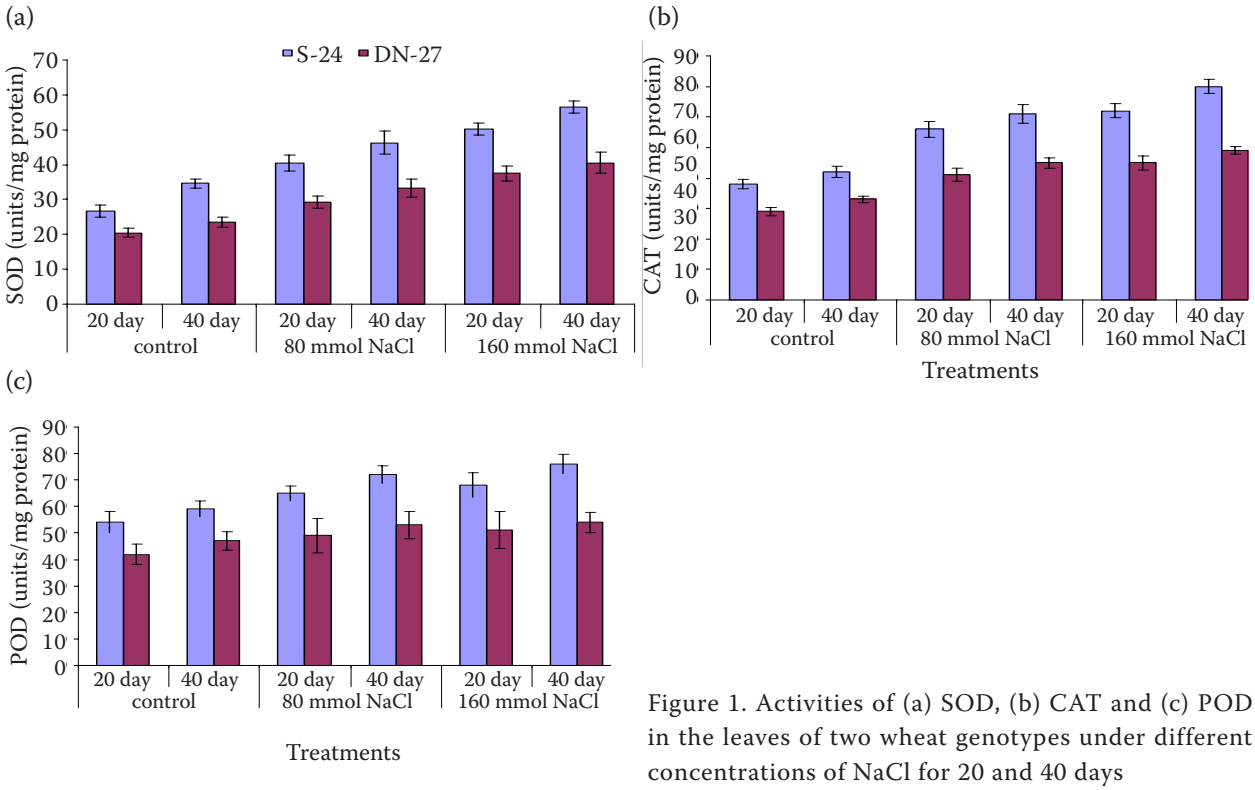

Figure 1. Activities of (a) SOD, (b) CAT and (c) POD in the leaves of two wheat genotypes under different concentrations of NaCl for 20 and 40 days

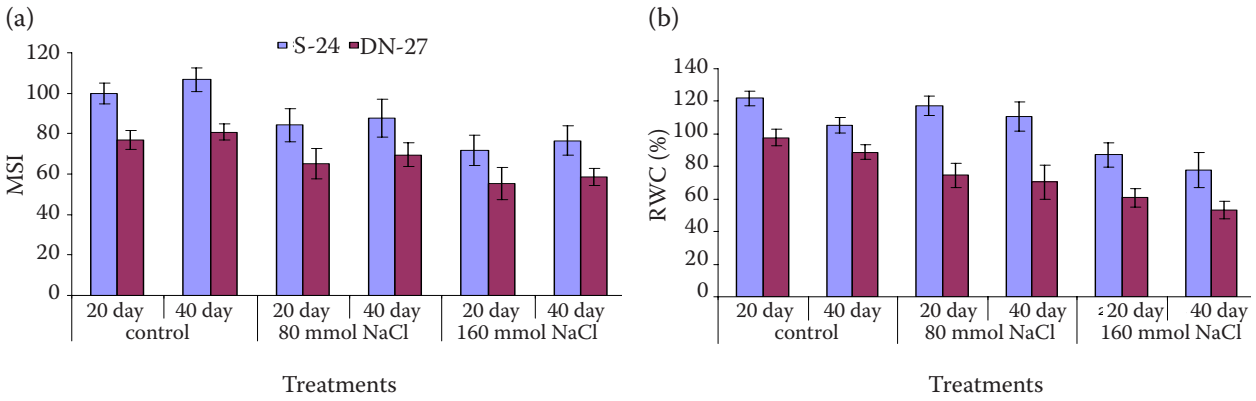

Figure 2. MSI (a) and RWC (b) of two wheat genotypes under different concentrations of NaCl for 20 and 40 days

160 mmol NaCl in days 20 and 40 in both the wheat genotypes (Figure 2). CAT is an important antioxidant enzyme that converts H_2O_2 to water in the peroxysomes. In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration. Higher activity of CAT and APX decreased H_2O_2 level in cell and increased the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki et al. 2003). There was a significant different between varieties at all treatments. CAT activity at both 80 mmol and 160 mmol NaCl was significantly higher in S-27 when compared to control. Although its activity increased with the increase of salt stress, it was not sufficient for the complete scavenging of H_2O_2. The results are in accordance with previous observations of Esfandiari et al. (2007) and Shao et al. (2005 a,b,c).

POD activity in control groups of wheat seedling significantly increased throughout the experimental period (Figure 1c). POD activity increased three times and two times after 20 and 40 days of low salinity treatments 80 mmol NaCl in S-24, respectively; it increased one and two times in DN-27. The 160 mmol NaCl treatment caused a 25% increase in POD activity after 20 days. This trend is continuing in DN-27. When SOD activity was high, ROS, especially superoxide radical (O_2^-), scavenging was done properly and thus, damage to membranes and oxidative stress decreased, leading to the increase of tolerance to oxidative stress. POD catalyses the reduction of oxidized glutathione, an important endogenous antioxidant (Zhang et al. 2006, Gao et al. 2008). Although POD acquires the reduction of power from NADPH, H^+, it dissipates this power and, in turn, increases $NADP^+$/NADPH, H^+ ratio. Results indicated that in S-24, however, POD activity decreased with the rise of salt stress, but not so significantly as compared to other genotypes. In DN-27 POD activity decreased significantly upon salt stress treatments as well. In these genotypes, however, there was a significant difference ($P <$ 5%) between the control, 80 and 160 mmol NaCl. Furthermore, there was a considerable difference between POD activities of all the cultivars. On the other hand, the rate of POD activity decrease with increasing salt stress was quicker in S-24. These reasons lead to the limitation of glutation-ascorbat cycle and $NADP^+$/NADPH, H^+ ratio in S-24 and damage of membranes. Consequently, in S-24, POD activity was sensitive to salt stress and the decrease of its activity influenced ROS scavenging systems. The results in this study are in confirmation with the findings of the Telesiński et al. (2008).

K^+/Na^+ concentration under control conditions had no significant differences in both wheat seedlings in 20 and 40 days analysis. As we increase the salinity levels, the K^+/Na^+ ratio gradually decreases. The reduction in K^+/Na^+ ratio is 11% and 16% under the low saline levels 80 mmol NaCl in 20 and 40 days in S-24. The other wheat genotype DN-27 is salt sensitive. The decrease in the K^+/Na^+ ratio is more as compared to salt tolerant. The reduction in DN-27 is the 21% and 30% in the high saline levels of 160 mmol NaCl. The increase in Na^+ content was due to the increased amount of sodium in the plant medium. The decrease in K^+ uptake under salinity could be due to the antagonism of Na^+ and K^+ at sites of uptake in roots, an effect of Na^+ on the K^+ transport into the xylem (Flowers 2004, Willenborg et al. 2004). Tolerant genotypes (S-24) maintained substantially greater biomass, shoot length under salt stress, lower Na^+ concentration and higher K^+/Na^+ ratio in leaves than the sensitive genotypes (DN-27) during both stages. In the majority of crops, salt

tolerance is allied with the amassing of only a squat quantity of Na^+ and Cl^- in the shoots. Yet, K^+ and Ca^+ amassing in salt strained plants were significantly reduced. The tolerable quantity of equally K^+ and Ca^+ is essential for veracity and utility of cell membranes and additional cellular processes (Davenport et al. 2005). It is recognized that with the aim of salinity tolerance, it was classically characterized by means of attractive Na^+ elimination as well as rising inclusion of K^+ ion to sustain most advantageous K^+/Na^+ ratio, which may be an applicable assortment criteria on behalf of assessing salinity tolerance of dissimilar crop genus. The maintenance along with attainment of K^+ may be an imperative determinant of salt tolerance. During the present research, accretion of K^+/Na^+ in the shoots of six generations is appreciably improved appropriate to salt stress, while K^+ accretion was decreased; plants make use of low level in addition to elevated resemblance transporters in attendance in genetic membranes in favor of uptake of K^+ as of the growth standard. It has imperative role during maintaining cellular K^+/Na^+ ratio (Amtmann and Sanders 1999). It is currently established with the purpose of instant oxygen genus created in salt stress and may be capable of demolishing the typical metabolism from side to side oxidative spoil of lipids, protein, nucleic acids along by way of eventually destructive cell arrangement (Vranova et al. 2002). The imprudent oxygen species are on the way to be scavenged as a result of enzymatic or non-enzymatic antioxidant mechanisms in favor of preservation of regular growth. Salt tolerance is often correlated with a more efficient antioxidative system (Juan et al. 2004).

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