



Triticum aestivum: antioxidant gene profiling and morpho-physiological studies under salt stress

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Abstract

Background Soil salinity drastically reduced wheat growth and production in Pakistan. It is a need of an hour to identify the best suitable salt tolerance or resistant wheat varieties which shows good growth under salinity affected areas. In presented study, two wheat varieties Johar (salt tolerant) and Sarsabaz (salt sensitive) were examined under NaCl stress conditions.

Methods Antioxidant enzyme activities were investigated in 10-days old wheat seedlings under 200 mM NaCl stress in hydroponic conditions. To investigate the various growth parameters, antioxidant enzyme activities such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6) and ascorbate peroxidase (APX: EC 1.11.1.11) were monitored and studied. Besides this various growth parameters such as length of the roots, shoots, as well as Physiological parameters likes lipid peroxidation by malondialdehyde (MDA), hydrogen peroxide (H₂O₂), and proline contents and antioxidant enzyme activities were estimated. The effect of salinity was also observed on gene transcription level and eventually expression level.

Results Shoot and root length were decreased in Sarsabaz variety while it showed opposite trend in johar at 200 mM salt concentration. The concentration of proline showed a noticeable rise in salt dependency. Higher concentrations of Proline in Johar were observed as compared to Sarsabaz. SOD showed the increase in activity for antioxidant enzymes. Significant increase of SOD levels were observed in shoot tissues as compared to root tissues. The results indicated that the shoots were more susceptible to salt stress. Activity of APX showed similar affects in both varieties. The production of CAT enzyme in the shoot and root tissues of both varieties showed substantial growth under increased salt stress. Furthermore, NaCl stress has increased the expression of certain genes coding for antioxidant enzymes such as catalase, superoxide dismutase, and peroxidase. Maximum expression of all the antioxidant enzyme coding genes were observed in Johar (tolerant) at 48 h exposure to salt. In contrast the expression of the all mentioned genes in Sarsabaz variety were found maximum at early hours (24 h) and gradually decreased at 48 h.

Conclusion The study showed that the selected salt tolerant wheat variety Johar is significantly resistant to 200 mM NaCl salt level as compared to Sarsabaz.

Keywords Proline · Antioxidants · RNA expression level · Catalase · Peroxidase

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Introduction

Wheat is the third most leading cereal crop in the world. Around 85 percent of the world's population depend on wheat [1]. The rapidly increasing population and decreasing agricultural lands in Pakistan demand higher productivity of wheat [2].

Salt stress caused oxidative damage by creating ionic imbalance and producing reactive oxygen species (ROS) like radical superoxide, hydrogen peroxide (H_2O_2) and radical hydroxyl [3, 4]. These are highly cytotoxic and can interfere with important biosynthetic pathways, resulting in peroxidation, protein denaturation, and DNA mutation [5–8]. The ROS in plant cells is also responsible for aggregation of membrane lipids, proteins and nucleic acids [9, 10].

Plants formed an efficient scavenging mechanism consisting of enzyme antioxidants such as peroxidase, catalase, ascorbate Xia [11]. These enzymes are also very effective metabolic stress indicators and their elevated expression which indicate a tendency for remediation. In an antioxidant system, antioxidant compounds (ascorbate, glutathione, tocopherols, salicylate) and antioxidant enzymes control the production of reactive oxygen species (ROS) [12, 13]. Several studies showed that, in an attempt to defend itself against oxidative damage, plants under different abiotic stresses alter the activity of antioxidant enzymes [4, 13–17].

SOD constitutes the first line of defense which resist oxidative damage in plants and catalyzes the disputation of two superoxide radicals to hydrogen peroxide and oxygen [11, 18]. There are three groups of SOD categorized by the metal cofactor, namely Cu/Zn-SOD, Fe-SOD and Mn-SOD [19]. CAT is a tetrameric heme protein and mostly located in peroxisomes [20]. CAT has the highest conversion efficiency of all antioxidant enzymes and one molecule of CAT can remove about six million molecules of H_2O_2 per minute [9, 21]. In stressed plants, the SOD genes are regulated by producing, tissue-specific and environmental signals [14, 22, 23]. The CATs genes respond differentially to various stresses conditions [24, 25]. The *APX* genes show differential modulation by several abiotic stresses in plants [26, 27]. The expression of wheat *GPX* genes were altered when wheat plants were submitted to salt, H_2O_2 and abscisic acid treatment. Moreover, other findings suggest that *GPX* genes not only act as scavenger of H_2O_2 to control abiotic stress responses but also play important roles in salt and ABA-signaling cascades [28].

The objectives of this study was to investigate the changes in antioxidant enzyme activities, osmotic adjustment and transcript levels of antioxidant enzyme-related genes against salt stress in two wheat varieties Johar and Sarsabaz at seedling stage.

Material and methods

The experiments were held at the Department of Botany, Islamia University of Bahawalpur. Seeds of 12 wheat varieties were obtained from Nuclear Institute of Agriculture (NIA) Tandojam Sindh, Pakistan.

Seed sterilization & germination

The seeds were surface sterilized for 2 min with 10% ethanol, then washed thrice with sterile H_2O and submerged into sterile H_2O for 24 h. Then seeds were moved to plastic pots lined with cheese cloth. Each pot contains eight seeds and 400 mL of half strength Hoagland's solution [29]. Pots were kept at 16 h light and 8 h dark cycle.

200 mM NaCl is used for Salt treatment. Among 12 varieties, two varieties of wheat Johar and Sarsabz were selected on the bases of preliminary salt tolerance tests. These selected varieties were then sprouted in petri dishes [30]. Three-day old seedlings (5 replicates) were moved to Hoagland solution. One week old seedlings (3 replicates) were moved to the same medium with 200 mM of NaCl and without NaCl as control for next 3 days. Ten days old seedlings were taken to perform the enzyme assays and mRNA extraction. These plantlets were kept in liquid nitrogen immediately for further studies.

Estimation of growth

Growth analysis of wheat seedlings were carried out after 10 days. The uprooted plants were washed with distilled water. Growth attributes such as root length, shoot length, were measured and various plant parts were separated for further experimentation.

Estimation of chlorophyll content

For spectrophotometric estimation of Chlorophyll a, Chlorophyll b, and the carotenoids, a 50 mg sample in mortar was crushed and homogenized with 4 ml of 80% (v/v) acetone. The solution is filtered through a paper filter. The filtrate volume was made up to 5 mL by adding cold acetone. The absorption of solution was detected at 663.3, 646.8, and 470 nm, and the pigment concentrations were calculated as defined by Lichtenthaler [31].

Estimation of electrolyte leakage (EL) in leaves

The completely formed 1–2 leaves were cut in 0.5 cm pieces and dipped into 7 ml sterilize H_2O in glass tubes and incubated for 24 h in rotatory shaker at 250 rpm. After the initial reading of the primary leaf conductivity (E_c-i) was noted

then samples were heat at 120 °C for thirty minutes and cooled to room temperature for final conductivity (Ec–f) recording by EC meter. The leakage of electrolytes was estimated by the formula given [32],

$$EL = (EC - i)/EC - f) 100$$

Estimation of proline

Proline content was determined taking 0.5 g of fresh leaves and 5 ml of 3% sulfosalicylic acid were added into a test tube and then kept into boiling water for 10 min. The extract was filtered into a clean test tube. 2 ml of extract was put into a test tube with a stopper, and then 2 ml of glacial acetic acid and 2 ml of acidic ninhydrin were added. The mixture was incubated at boiling water for 30 min. After cooling the mixture 4 ml of toluene was added into the test tube. The supernatant was collected into a 10 ml centrifuge tube after centrifuging for 5 min at 3000 rpm. The absorbance of the supernatant was recorded at 520 nm using toluene as a blank [33].

Estimation of H₂O₂ and MDA content

Patterson [34] method was employed for the detection of hydrogen per oxide content. The content of MDA was calculated using the Hodges et al. [35] test.

Estimation of potassium and sodium ions

The concentrations of Na⁺ and K⁺ in leaf and root were measured according to the methods of Weimberg [36]. Briefly, the samples were washed with distilled water to remove any traces of the Na⁺ from the leaf and root tissues, then dried at 50 °C for 4 days. Thereafter, the dried leaf and root tissues were ground into a fine powder in liquid nitrogen. The powder was then placed in 5 mL nitric acid overnight. Thereafter, the digested solution was diluted to 25 mL with double-distilled water. The concentration of Na⁺ and K⁺ in the acid-digested tissue was measured using a flame photometer.

Determination of osmotic adjustment substances

Protein contents were measured using the [37]. Bovine serum was used as standard. The level of total free amino acids in wheat leaf was estimated by the method described by Misra et al. [38] and Moore & Stein [39].

Evaluation of antioxidant enzymes

SOD activity was determined according to Beyer and Fridovich [40]. Twenty microlitres (20 ul) of enzyme solution was mixed with 3 ml SOD reaction solution (pH 7.8 phosphate buffer 1.5 ml, 750 mol⁻¹ NBT 0.3 ml, 130 mmol⁻¹ Met 0.3 ml, 20 mol⁻¹ FD 0.3 ml, 100 mol⁻¹ EDTA-Na 2 0.3 ml, distilled water 0.3 ml). The control and enzyme solution were placed for 30 min in 4000 l × light. The blank was placed in the dark and read at 560 nm.

Peroxidase (POD) activity was determined using Hernández et al. [41] method. Twenty microliters (20ul) of enzyme solution was mixed with 3 ml POD reaction solution (1.4 μl guaiacol, 0.85 μl 30% H₂O₂ and 0.1 mol⁻¹ pH 6.0 phosphate buffer). The absorbance values was recorded every 30 s at 470 nm.

Catalase (CAT) activity was assayed as a decrease in absorbance at 240 nm for 1 min following the decomposition of H₂O₂ according to Aebi [42]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H₂O₂.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada [43]. The assay mixture consisted of 0.5 mM ASA, 0.1 mM H₂O₂, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0), and 0.15 ml enzyme extract.

RNA extraction from plant tissues

200 mg lypholysed plant was weighed and finely ground with liquid nitrogen. Homogenized tissues were allowed to thaw at room temperature. 1 ml of Trizol was added in homogenized tissue in 1.5 ml microfuge tubes. Tubes were invert mix for 4–5 times. Incubate it at room temperature for 5 min. 400 μL of chloroform was added and incubated at RT for 3 min. Mixture was allowed to centrifuge at 12,000 rpm for 10 min at four degree centigrade for phase dispersion. (During this centrifugation step, new microfuge tubes were labeled and placed in ice box to cool down). Supernatant was transferred in new 1.5 ml tube placed on ice and iso-propanol was added in equal ratio. Tubes were incubated on ice (– 20 °C) for 10 min in horizontal form to precipitate the RNA. Samples were centrifuged at 4 °C centigrade with 12,000 rpm for 10 min. Supernatant was discarded. Pellet was washed 2 times with 1 ml of 70% ethanol at 7500 rpm for 5 min at 4 °C. Pellet was allowed to dry fully. 40 μL of RNase free DEPC treated water is used to dissolve the RNA pellet Store at – 80 °C.

RNA evaluation and quantification

RNA standard and quantity were evaluated by using Nano drop. Absorbance at 260, 280 and 320 nm were calculated.

The 260/280 ratio was from 1.9 to 2.2 denoting good quality of RNA whereas RNA quantity was about 800–1200 ng/ μ L.

cDNA synthesis

cDNA was synthesized from isolated RNA using cDNA kit (Vivantis cDSK01-050). For primer preparation mix the reagents i.e., RNA template (7 μ L), primer oligos (T)₈ (1 μ L) dNTPS (1 μ L), Nuclease free water (1 μ L) and centrifuge the tubes. Incubate the mixture at 65 °C for 5 min and chill on ice for 2 min. Briefly spin down the mixture.

For cDNA Synthesis prepare the Mix according to instructions, Reverse transcriptase (0.2 μ L), Buffer 2X (M-MULV, 2 μ L, nuclease free H₂O (7.8 μ L) and make 10 μ L final volume. Add 10 μ L of the cDNA Synthesis Mix into each RNA-primer mixture. Mix gently and centrifuge briefly. Incubate at 42 °C for 60 min. Terminate the reaction by incubate the tubes at 85 °C for 5 min. Chill the tubes on ice and centrifuge the tube briefly. The synthesized cDNA can be directly used for downstream application or stored at – 20 °C. The list of primers is given in Table 1.

Real time—PCR

Real time polymerase chain reactions (40 cycles) were performed on Mic PCR (Bio Molecular System). To illuminate

Table 1 List of primers used in presented study

Actin F: R:	GTGCCCATTTACGAAGGATA GAAGACTCCATGCCGATCAT
CAT_F: R:	CGACTTCGACCCGCTGGACGTGAC GCGTCGATCCATCTGTTGATGAATC
SOD_F: R:	GAAGCACCACGCCACCTACGTCGC TCACGCAAGCACTTTTCATACTCT
APX_F: R:	ATGGCGGCTCCGGTGGTGGACGCC TACAATATCTTTGTCTGTAAACCCCA

each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. The relative level of gene expression was calculated using the Klein method [44]. The gene transcription levels in control plants were normalized as 1. Following primers were used in Table 1.

Statistical analysis

All of the data was analyzed using IBM SPSS Statistics 2.3 software and a One-way analysis of variance and honest significant difference (HSD) test was done. Principal component analysis was also measured and constructed by Origin 2b software.

Results

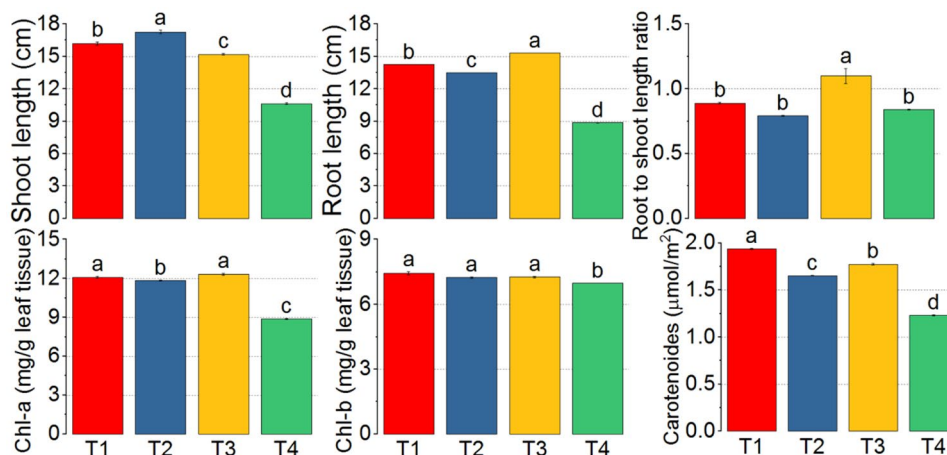
Effect of NaCl on growth

It was observed that increased salt concentration decreased the shoot development duration of both varieties. The 200 mM NaCl salt concentration increased the shoot length of Johar by 15.2 cm while the shoot length of susceptible variety Sarsabaz decrease (10.6 cm). Root length of tolerant Johar increased (15.32 cm) under 200 mM NaCl stress whereas root length of susceptible Sarsabaz showed decrease. The physiological changes due to the salinity stress were assessed through calculating the ratio of root and shoot length (Fig. 1).

Effect of NaCl on chlorophyll content

Triticum aestivum varieties grown under high salt stress; Sarsabaz variety showed lower values of chlorophyll a, chlorophyll b compared to the Johar variety (Fig. 1) The Johar genotype was proved most tolerant variety by enhanced

Fig. 1 Effect of NaCl stress (200 mM) on plant growth parameters (upper row), Chlorophyll a, Chlorophyll b and Carotenoid (lower row), of tolerant (Johar) and sensitive (Sarsabaz) varieties of *Triticum aestivum*. Period of stress application (48 h): T1 = control Johar plant, T2 = control Sarsabaz plant, T3 = NaCl treated Johar plant and T4 = NaCl treated Sarsabaz plant. Data are means \pm SE at $P \leq 0.01$



chlorophyll “a” and chlorophyll “b” under 200 mM salt stress. S arsabaz variety showed lower carotenoid levels as compared to the Johar variety (Fig. 1). The Johar proved to be the most tolerant variety by enhanced carotenoids percentage under 200 mM salt stress.

Effect of NaCl on electrolyte leakage

The impact of salt stress on plants was estimated by the measurement of the electrolyte leakage test. Significant increase of EL percent was observed in Sarsabaz sensitive plant under 200 mM NaCl. However, resistant variety (Johar) cultivar produced decreased electrolyte leakage (Fig. 2).

Effect of NaCl on H₂O₂ and MDA content

Determining the MDA content, that is the result of lipid peroxidation, has established the impact of salt stress on plant tissues. MDA content in the roots of both of varieties showed significant changes under increased salt concentrations. Concentrations of MDA in shoot and roots increased under 200 mM NaCl stress in the Sarsabaz variety (Fig. 2).

Measuring the content of H₂O₂ has established the impact of salt stress on plant tissues. H₂O₂ concentrations decreased in Sarsabaz variety under NaCl stress of 200 mM (Fig. 2). Results revealed that regulation of increasing variety

indicates limited oxidative stress that has been decreased slowly and dramatically ($p > 0.01$).

Osmotic adjustment substance contents in response to NaCl stress

Effect of NaCl on proline content

Proline is the remarkable organic osmolyte which accumulates in plants as a result of response to environmental stresses i.e., severe temperature, H₂O deficiency conditions, salt stress and heavy metals. The proline estimation serves as a physiological marker for salt stress. Proline content varies from 2.3 to 3.5 in the shoot and 2.6–4 in the root under control conditions. Maximum increased proline output was observed in Johar (3.2 $\mu\text{g g}^{-1}$ and 4.2 $\mu\text{g g}^{-1}$) in the shoot and root respectively. Minimal proline production was observed in Sarsabaz variety (Fig. 3).

Total soluble sugars

Figure 3 shows that wheat variety, salinity and their association had major effects on overall soluble sugar ($p > 0.01$) The two salinity-stressed wheat varieties showed a significantly lower accumulation of sugars in roots relative to shoot tissues. The reducing sugars of shoot decreased in Sarsabaz.

Total soluble amino acids

Varieties, salinity and association of varieties had major effects on total soluble amino acids ($p > 0.01$) among the two salinity stressed wheat varieties. Root tissues displayed significantly more amino acid accumulation than shoot. The amino acids of shoot decreased as salinity increased in only Sarsabaz (Fig. 3).

Determination of potassium and sodium ions

After 48 h of stress measurement of sodium aggregation revealed two significant variations in Na transport between Johar and Sarsabaz. Sarsabaz sequestered more root sodium than shoot under 200 mM NaCl stress. In comparison, more potassium was stored in the leaf of Johar than Sarsabaz. In both tested varieties, the roots exhibited higher sodium compared to shoots. The highest sodium content (6.82 mg g⁻¹ DW) was observed in roots of Sarsabaz plants under 200 mM NaCl (Fig. 4).

Determination of Na/k Ratio

Higher ratio of Na/K in the salt sensitive cultivar Sarsabaz was observed. On the contrary, Johar had less Na/K

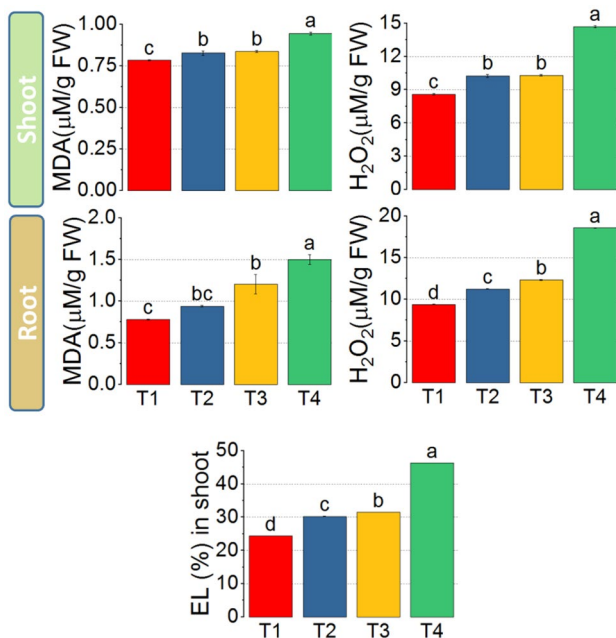


Fig. 2 Effect of NaCl stress (200 mM) on oxidative stress parameters in shoot and root of tolerant (Johar) and sensitive (Sarsabaz) varieties of *Triticum aestivum*. Period of stress application (48 h): T1 = control Johar plant, T2 = control Sarsabaz plant, T3 = NaCl treated Johar plant and T4 = NaCl treated Sarsabaz plant. Data are means \pm SE at $P \leq 0.01$

Fig. 3 Effect of NaCl stress (200 mM) on osmoprotectants in shoot and root of tolerant (Johar) and sensitive (Sarsabz) varieties of *Triticum aestivum*. Period of stress application (48 h): T1 = control Johar plant, T2 = control Sarsabz plant, T3 = NaCl treated Johar plant and T4 = NaCl treated Sarsabz plant. Data are means ± SE at $P \leq 0.01$

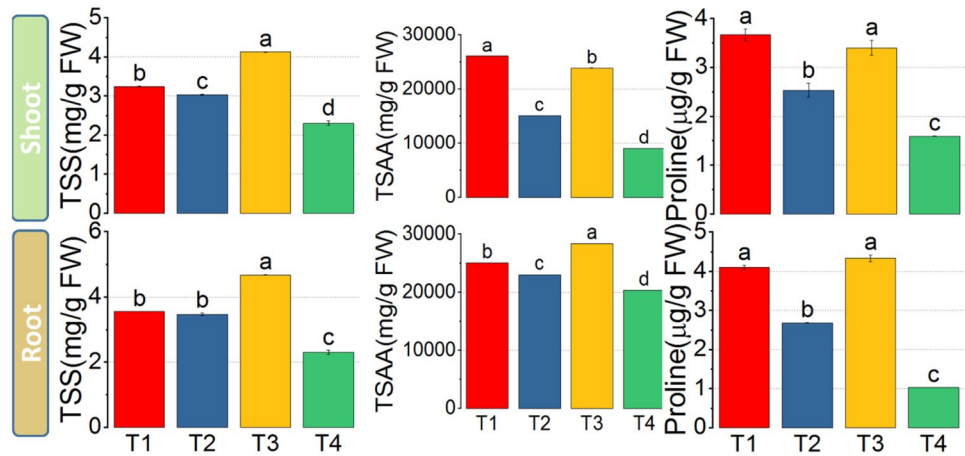
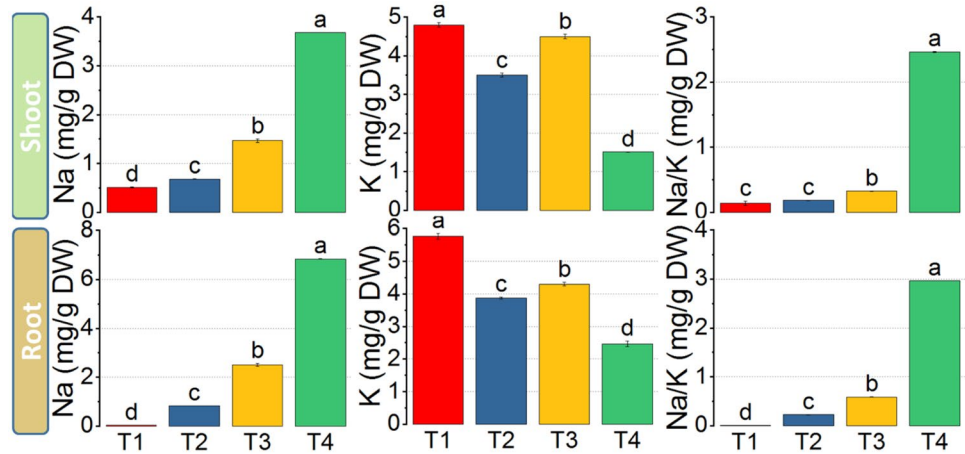


Fig. 4 Effect of NaCl stress (200 mM) on ion contents in root and shoot of tolerant (Johar) and sensitive (Sarsabz) varieties of *Triticum aestivum*. Period of stress application (48 h): T1 = control Johar plant, T2 = control Sarsabz plant, T3 = NaCl treated Johar plant and T4 = NaCl treated Sarsabz plant. Data are means ± SE at $P \leq 0.01$



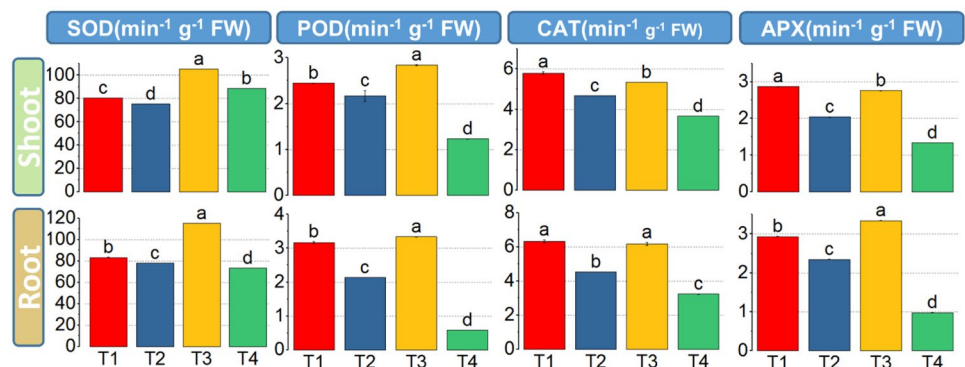
ratio than Sarsabaz (Fig. 4). The highest ratio of Na/K was observed in Sarsabaz roots under 200 mM NaCl.

Antioxidant enzymes activities

Effect of NaCl on catalase activity

Data in the Fig. 5 showed that salinity had a significant impact on the antioxidant enzyme activities. The salt stress produced a significant increase in catalase activity in the

Fig. 5 Effect of NaCl stress (200 mM) on antioxidant enzymes in shoot and root of tolerant (Johar) and sensitive (Sarsabz) varieties of *Triticum aestivum*. Period of stress application (48 h): T1 = control Johar plant, T2 = control Sarsabz plant, T3 = NaCl treated Johar plant and T4 = NaCl treated Sarsabz plant. Data are means ± SE at $P \leq 0.01$



Johar plant at 200 mM NaCl concentration. Catalase activity was slightly higher in root ($6.0 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$) and ($5.33 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$) in shoot of Johar variety relative to Sarsabaz variety showing limited catalase activity in both tissues of plant.

Effect of NaCl on superoxide dismutase activity

It indicates that SOD is inducible under higher salt concentration. SOD behavior increased dramatically under 200 mM NaCl concentration for Johar. Higher percent increase was observed in the shoot ($105.2 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$). In the Johar, the root SOD activity was $115.5 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ (Fig. 5).

Effect of NaCl on ascorbate peroxidase activity

APX activity indicates a rise under 200 mM NaCl concentration in the Johar variety. A higher percentage increase was observed in the shoots tissues of the Johar ($2.77 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$) (Fig. 5).

Effect of NaCl on peroxide dismutase activity

POD tends to induce under higher salt concentration. POD behavior dramatically increased under 200 mM NaCl concentration for Johar variety. Higher percent rise was observed in Johar shoot tissue ($2.83 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$). Whereas in root tissues POD activity was $3.33 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ (Fig. 5).

Gene expression of antioxidant enzymes in response to salt stress

Expression of CAT gene

According to the analysis of variance, it was observed that the level of transcripts of the genes of CAT, SOD, and APX enzymes in wheat seedlings subjected to 200 mM NaCl salt stress significantly change in period of stress (Fig. 6) In this study, maximum expression of CAT gene was observed in Johar at 48 h compared to sensitive variety (Sarsabaz) which shows highest gene expression only at early hours (24 h) and gradually decrease at 48 h (Fig. 6).

Expression of MnSOD gene

The expression profile of the MnSOD gene under salt stress (200 mM NaCl) was observed. In this study minimum expression of MnSOD gene was observed at early hours (24 h) in both tolerant (Johar) and sensitive variety (Sarsabaz) and it gradually increased after 24 h which shows highest expression at 48 h (Fig. 6).

Expression of APX gene

In this study, we determine a differential response of APX activity in two wheat varieties under 200 mM NaCl stress. APX activity was observed highest at early hours (24 h) in both salt tolerant variety (Johar) and sensitive variety (sarsabaz) and it gradually decrease to show minimum expression at 48 h (Fig. 6).

Correlation between antioxidant enzyme activity and relevant gene expressions

According to CAT enzyme activity and gene expression profile of CAT gene (Fig. 6), we observed that both CAT enzyme activity and expression was greater in Johar variety compared to Sarsabaz. SOD enzyme efficiency of the salt-tolerant varieties (Johar) increases in both root and shoot tissues. In contrast, Super dismutase activity in variety Sarsabaz slightly increased relative to the control plants. According to gene expression we observed that MnSOD gene expression highest at 48 h in Johar followed by Sarsabaz.

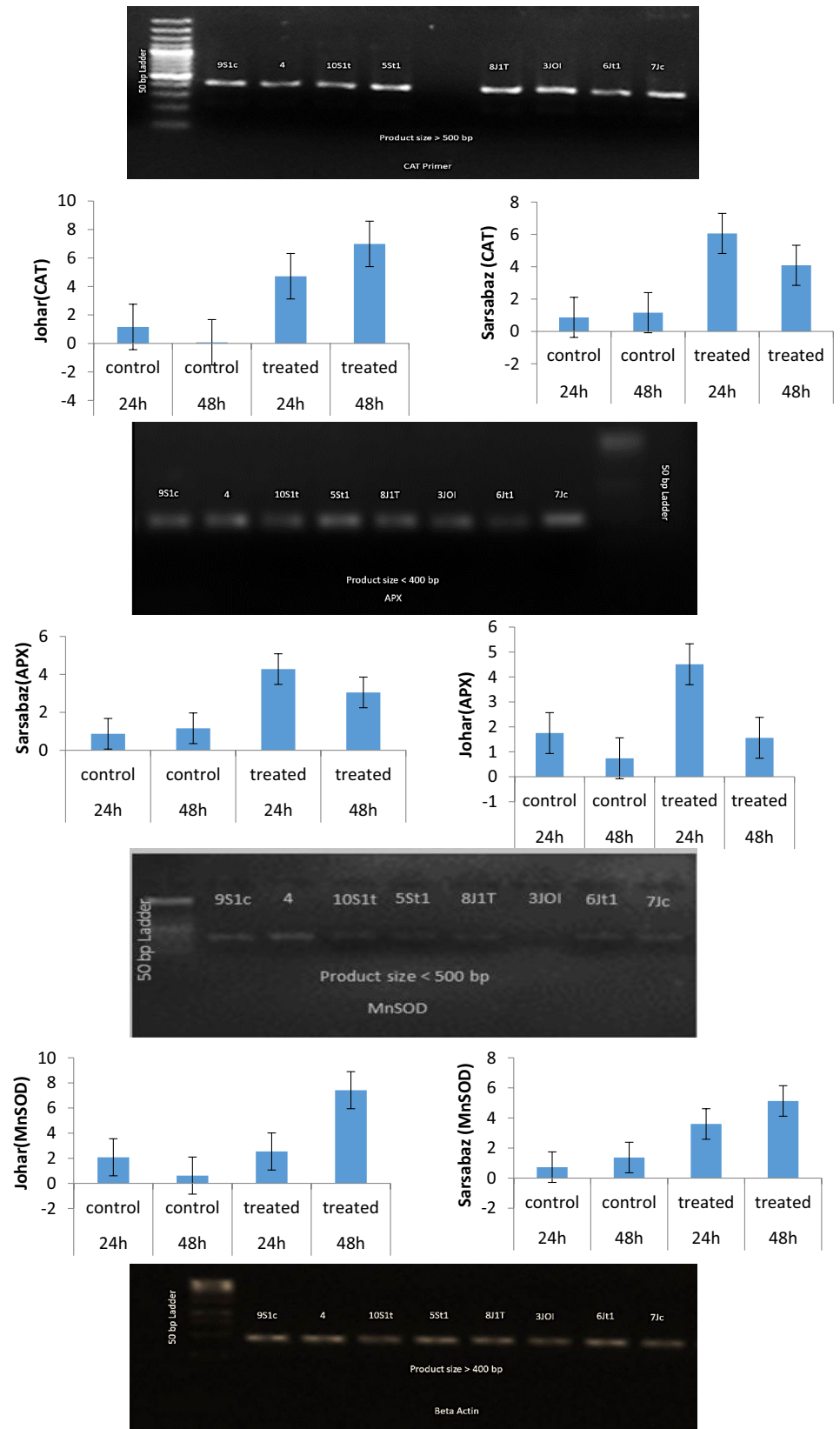
In the Johar cultivar APX activity increased under salt stress with respect to control plants. Moreover, this increase was more in Johar than in Sarsabaz. In the present study we observed maximum APX enzyme activity in shoot and roots of Johar compared to Sarsabaz variety. In the presence of high NaCl concentration (200 mM), APX gene expression of the salt -tolerant variety was higher relative to untreated plants and higher in Sarsabaz variety.

Discussion

In presented study, salt tolerance of two wheat varieties were examined. At germination stage, Johar cultivar was more tolerant to salinity than Sarsabaz. In Sarsabaz, the higher concentration of sodium chloride reduced the plant growth is higher in particular relative water content.

Plant growth particularly plant biomass and root and shoot length were reduced under saline conditions (Fig. 1). The reduction in growth at high salt stress resulted from a reduction in osmotic reliability might be due to saturated ion uptake systems, [45, 46]. It was observed that salt stress affects plant development and growth by causing nutrient deficiency [potassium (K^+), calcium (Ca) and Magnesium (Mg^+)]. Excessive growth decrease was seen in leaf tissues as compared to roots under 200 mM of salinity stress [47]. Development of the shoot in most crop species is highly susceptible to salinity as compared to root growth [48]. Hernandez et al. [41] found that salt stress contributed to a decrease in the area of the leaves as well as a decrease in dry and fresh

Fig. 6. Antioxidant enzyme gene expression in Johar and Sarsabaz varieties of wheat. **a** Catalase gene expression **b** Ascorbate peroxidase gene expression **c** Mn superoxide dismutase gene expression and **d** Beta actin gene as reference gene



weight in both the pea roots and leaves. Salt stress causes decreased wet and dry weight in Maize genotypes [49].

Two varieties of wheat that we observed seemed to have same sodium storage ability in roots and shoots, Johar showed different Na⁺ accumulation efficiency in both tissues (Fig. 4). Sana et al. [50] reported that the storage capacity of Na⁺ in roots was comparable in tolerant and sensitive genotypes of wheat but higher in leaf blades of sensitive varieties.

Proline is an important osmolyte [51, 52] that plays a special role in exposure to various stress conditions as an antioxidant protection compounds and a signaling molecule in the plant system [6]. Although Proline action mechanism in response to stress is not well understood, this may be involved in water stress membrane stabilization [5]. The excessive presence of proline in plants is known to retard development and adversely impacts many metabolisms of tissues [53]. Build up of well suited solutes such as Proline and GB under salinity is hypothesized to raise plant salt resistance [7, 48].

The proline content under osmotic stress save plants by controlling osmotic equilibrium, Preventing EL and ROS concentrations to control oxidative bursts in plants [52]. Similar results were observed in this research, higher Proline values in roots and shoots of tolerant variety Johar (Fig. 3). Environmental stresses increase superoxide development efficiency depending on the species, stress duration and severity, and plant age. There is growing evidences that membrane wound under salinity is associated with high production of AOS [52–56], Hernandez et al. 1998. In accordance with these studies, our findings demonstrate that salt stress produced oxidative stress in leaves of two wheat varieties shown by a collection of oxygen ions, rise in MDA and hydrogen peroxide content. However, the amount of lipid peroxidation and the content of hydrogen peroxide in the Sarsabaz were higher than in the other varieties under NaCl stress. These data indicate that the Johar under NaCl stress was better protected against oxidative damage. According to our findings, low MDA is significant in respect of salt resistance as shown in various observations. Salt resistant tobacco 58 and wheat varieties [56, 57] also showed low MDA levels, that is a significant sign of increasing oxidative retardation limiting potential salt stress.

For the estimation of lipid peroxidation level the MDA content is used as an indicator. The determination of the MDA level and the membrane lipid peroxidation extent, which has been used as a method to evaluate magnitude of the oxidative stress and the extent of non-tolerance of the plant [5, 59]. Many studies have recorded an increase in H₂O₂ concentration after exposure to stress, it is related to stress intensity and duration. In addition, the amount of H₂O₂ varies between different cellular compartments and is correlated with stress form [60].

Tolerance to the stress of salinity in higher plants is associated with antioxidant systems and substrates [61]. The increases in concentration of antioxidant compounds are indicators of plant resistance/adaptation to salinity condition. Variation in the efficiency of the enzymes is therefore associated with plant oxidative stress resistance [13]. Changes in antioxidant concentration may act as a signal for modulating reactive oxygen species scavenging mechanisms and transduction of reactive oxygen species signals [17].

Comparison of the enzymatic efficiency for two wheat varieties; the salt tolerant Johar showed the higher activity as compared to Sarsabaz. This result indicated that the Johar is more able to scavenge ROS than the Sarsabaz. The decrease in Johar's H₂O₂ content is thus the product of superoxide dismutase reaction; that is followed by an increased enzymatic capability to decompose it. This was especially evident in Johar, where under low or high salinity a significant increase in SOD, CAT, POD and APX activities were observed (Fig. 5).

SOD level raised under 200 mM NaCl in the shoot tissues of Johar (Fig. 5). This link between salinity resistant and increased Superoxide dismutase activity was demonstrated in several studies. [14, 18, 62, 63]. High production of superoxide dismutase in Johar at higher salt level was able to cope with injurious effects of reactive oxygen. Ascorbate peroxidase uses AsA as an electron donor for reduction of hydrogen peroxide to H₂O with monodehydro ascorbate generation, [23] and plays a major role in controlling reactive oxygen species during stress [61].

It is observed in several salt resistant varieties that salinity increase ascorbate peroxidase activity [26, 27, 64]. The Sarsabaz's vulnerability to salinity may be due to ascorbate peroxidase efficiency inhibition at a high salt stress level.

In wheat, the superoxide dismutase transcript was high in response to differential heat sensitive experimental treatments. [51], suggested an increased resistance to environmental stresses. In plants, the superoxide dismutase genes are regulated by growth, tissue and environmental stresses [25, 65, 66].

In Johar, higher CAT activity was observed in the presence of a high level of salt stress (200 mM NaCl) (Fig. 5), resulting in good coping with ROS. Higher CAT activity, close to our findings, has been observed in many salt resistant varieties i.e., sesame, maize, and wheat [67, 68]. The maximum expression of the CAT gene was observed in Johar in at 48 h relative to the susceptible variety (Sarsabaz) showing the highest gene expression only at the early hours (24 h) and steadily decreasing with increase in time till 48 h Fig. 6. Similar results were observed when the wheat CAT gene expressed in transgenic rice increases resistance compared to non-transgenic plants to low-temperature stress [69]. The catalase genes react to many stress conditions differently [27, 70].

In this analysis, APX activity was observed to be highest in early hours (24 h) and steadily decreased to show minimum expression at 48 h. Ascorbate peroxidase proteins are dispersed in chloroplasts, mitochondria, cytosol and peroxisomes. The ascorbate peroxidase genes show differential modulation in plants through various environmental stresses [26, 64, 71, 72].

Conclusion

The findings of this study indicate that the ability of *Triticum aestivum* to cope with salt stress depends on antioxidant stress defense mechanisms. An increase in antioxidant enzyme activity and the resulting increase in ROS-scavenging capacity can improve the salt tolerance of plants. Our results show that there were increases in antioxidant enzyme activity and in the transcript levels of genes encoding these enzymes under salt stress. These findings suggest that the response of salt tolerant variety Johar to salt stress involves CAT, SOD, APX, POD, and GPX. These results increase our understanding of how *T. aestivum* responds to salt stress and provide evidence for the effectiveness of its detoxification mechanisms. Thus, we concluded that insight into any plant's antioxidant enzyme mechanism implies tolerance efficiency.

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Data availability All data are available when need.

Declarations

Conflict of interest The authors declare no conflict of interest.

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