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Antioxidant enzyme activities and potential biochemical indicators improve salt tolerance in four varieties of wheat (*Triticum aestivum L.*)

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ABSTRACT

Proline, glycine-betaine (GB) total phenolics, Na^+ and K^+ contents their ratio and some oxidative stress indices were studied in leaves of bread wheat cultivars namely Sehar-06, Lu-26, (salt-tolerant) and Miraj-08 and Wafaq-01 (salt-sensitive), grown under salinity treatments carried out in five levels (1< dS/m as control, 2, 4, 8, 16 dS/ m) via sodium chloride in order to find out the resistant cultivars of wheat against salt stress. Under high salt potency significant increase for activities of antioxidant enzymes such as ascorbate peroxidase (APX) and guiacol peroxidase (GPX), in salt tolerant varieties. On the other hand, in salt tolerant varieties, activity of (SOD) and (CAT) were not infected. Meanwhile, under salinity condition the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and (GPX) in sensitive cultivar was lower than control and no significant difference were recorded regarding (APX) activity. Salt tolerant varieties had more amounts of K^+ content, K^+/Na^+ ratio and RWC under salt conditions, and sensitive ones showed higher (CHL) and Na^+ content at tillering stage.

Results showed that the salinity tolerance in tolerant cultivars as manifested by lower decrease in grain yield is associated with the lower sodium accumulation and higher K^+/Na^+ compared to the sensitive cultivars. The mechanism of salt stress might be achieved due to low lipid peroxidation, assumingly lower changes in membrane stability index and evasion of Na^+ combination and amplified activity of antioxidant enzymes. These results indicate that these cultivars of wheat alleviate the deleterious effect of salt stress by the increased production of proline and betaine.

Key words: Antioxidant enzymes, Chlorophyll content, Glycine-betaine, K^+/Na^+ ratio, Proline, Salinity, Wheat

INTRODUCTION

All the environmental factors that effects life processes of the plants are referred to as stresses to plants. There are lots of biotic and abiotic stresses that inhibit productivity and destroy the biomass of the plants. Among these stresses drought, salinity, temperature and more elements are the prominent one that are the barriers in plant production. Immense soil salinity is one of the imperative environmental factors that bound distribution and efficiency of major crops (Ashraf *et al*. 2005; Chandan *et al*. 2006). Agricultural output in arid and semiarid regions of the world is very squat due to accumulation of salts in soils (Ashraf *et al.* 2002; Munns, 2002). Soil salinity causes many bad effects on plant growth, which is due to low osmotic potential of soil solution (osmotic stress), nutritional imbalance, specific ion effects (salt stress), or a combination of these factors (Marschner, 1995; Ashraf, 2004). All these factors cause adverse effects on plant growth and improvement at physiological and biochemical behavior (Ashraf and Sarwar, 2002; Munns and James, 2003).

Wheat is the chief cereal crop of Pakistan, which is cultivated all over the country. It is grown to meet up the food requirement of over growing population of Pakistan. However, per hectare

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yield of wheat is far beneath its yield potential, which may be due to different reasons i.e., lack of proper nutrient and water managements, unavailability of fertile soils, drought, salinity, and water logging . In Pakistan, salinity is a serious threat for wheat production. The most of underground water utilized for wheat cropping is brackish; however, some areas are irrigated with canal water but they are short of drainage system both the irrigation systems are increasing the soil salinity problem in the country due to which heavy losses in crop yields are reported (Khan *et al.,* 2006). This is essential to fulfill the wheat grain yield demands of ever growing population of Pakistan. Keeping in view the significance of wheat and salinity, the present study was aimed to estimate the changes that occurred in the biochemical constituents (glycine-betaine and proline) of wheat leaves and to screen out the potential wheat cultivars for better performance under salt stress as the increased levels of proline and glycine-betaine indicates resistant cultivars of wheat. Antioxidant capacity is contributed by a diverse array of bioactive compounds. These phytochemicals include phenolic acids, phytic acids, carotenoids, tocopherols, tocotrienols, phytosterols and flavonoids. Antioxidant properties vary with respect to wheat varieties (Zeliski and Kozlowska 2000) and percentage of bioactive compounds such as carotenoids (Adel-Aal *et al*., 2007), phenolic acids (Addel-Aal *et al.,* 2001) anthocyanins and tocopherols (Zhou *et al.,* 2004). Due to salinity growth reduction is also credited to ion toxicity and nutrient imbalance, which causes not only high sodium (Na^+) and chloride (Cl^-) accumulation in plants, but also destructively affects the uptake of essential nutrient elements such as potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) in rivalry with Na^+ and also nitrate (NO^{3-}) in contrast with Cl^- (Zörb *et al.,* 2004). Cations such as K^+ and Na^+ are recognized to be the main inorganic elements, which make available needed osmotic potential for water uptake by plant cells (Tejera *et al*., 2006). Regulation of K^+ uptake alongside avoidance of Na^+

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entry and efflux of Na^+ from the cell, and further more confiscation of Na+ in vacuole for osmotic adjustment are the ordinary strategies for continuation of desirable K^+/Na^+ ratios in the cytosol. A soaring K^+/Na^+ ratio in the cytosol is necessary for usual cellular functions of plants (Chinnusamy *et al.,* 2005). Plants have a number of antioxidant enzymes system such as SOD, CAT, peroxidase (POX) that defend plant cells them from these prospective cytotoxic effects (Edreva, 2005). SOD is a major scavenger of O^{2-} and its enzymatic action consequences in the formation of H_2O_2 (Sen Gupta *et al*., 1993). CAT and POD catalyze the stop working of H_2O^2. Consequently, these enzymatic systems get rid of the destructive effects of toxic oxygen species (Katsuhara *et al*., 2005). Trust in view the importance of wheat and salinity, current study has been intended to inspect the role of glycine betaine, proline, phenolics, K^+ and Na^+ ratio, chlorophyll content and some oxidative stress indices in salt tolerance of wheat (*Triticum aestivum* L.).

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MATERIALS AND METHODS

The plant material (varieties) was selected on the base of their frequent cultivation in the areas. The selected wheat varieties i.e. Sehar-06, Lu-26, Miraj-08 and Wafaq-01 were collected from different research stations of Pakistan, authenticated and grown in the experimental field of Faculty of Agriculture, Rawalakot Azad Kashmir. The experiment was conducted in plastic pots, filled with equal amount of soil, sand and farm yard manure. The growing media was salinized by commercial NaCl salt to attain salinity level of (control i-e without any dose), 2.0 dS/m (desi Siemen's per meter) 4.0 dS/m, 8.0 dS/m and 16.0 dS/m. The doses of salt were applied at jointing stage and the electrical conductivity (EC) was calculated according to the prescribed method of USDA (1954). Growth observations were recorded at the time of maturity.

Proline estimation

The Proline content of the leaves was assessed according to the method of Bates *et al.* (1973). Proline content was calculated from a standard curve, using purified proline as a standard. Results were expressed in µmol/g of fresh weight (FW).

Glycine betaine estimation

The glycine-betaine content was estimated followed by the method of Grieve and Mass (1984). Glycine-betaine content was calculated from a standard curve, using purified glycinebetaine as a standard. Results were expressed in µmol/g of fresh weight (FW).

Determination of total phenolics

The total phenol content was determined by adding 0.5 ml of the aqueous extract to 2.5 ml, 10% Folin-Ciocalteau's reagent (v/v) and 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45 ^{0}C for 40 minutes and the absorbance was measured at 765 nm in the spectrophotometer. Gallic acid was used as a standard phenol (Singleton et al. 1999). The mean of three readings was used and the total phenol content was expressed as milligrams of gallic acid equivalents/g extract.

Extraction of enzymes

For CAT, GPX and SOD extraction, leaf samples (0.5 g) were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Centrifuge tubes was used for each homogenate was and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15000×g. For enzyme activity assay the supernatant was used (Esfandiari *et al*., 2007b).

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For the extraction of APX, leaf samples (0.5 g) were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA, 2mM ascorbate (AsA) and 5% polyvinylpyrrolidin (PVP 6000) with pre-chilled pestle and mortar. The other stages were similar to the extraction of other enzymes (Esfandiari *et al.,* 2007).

Enzyme activity assays

By recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme SOD activity was estimated (Sairam *et al.,* 2002). In test tubes about 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.01 ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml distilled water and 0.05 ml of enzyme extraction, were taken from each enzyme sample. Without enzyme extract two tubes were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 μM) and for 15 min placing the tubes below a light source of two 15 W florescent lamps. By switching off the light Reaction was stopped and covering the tubes with black cloth. Maximal color was developed in tubes without enzyme. A non-irradiated inclusive reaction mixture which did not develop color regarded as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which condensed the absorbance reading of samples to 50% in contrast with tubes lacking enzymes.

Activity of CAT was measured according to (Aebi, 1984). Reaction mixture limited 100 mM potassium phosphate buffer (pH 7), 75 mM H_2O_2, enzyme extract and distilled water. The reaction was started by adding H_2O_2, and the decrease in absorbance was recorded at 240 nm (ε= 36 mM-1cm-1) for 1 min. By calculating the amount of H_2O_2 decomposed enzyme activity was computed.

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The activity of APX was measured according to (Yoshimura *et al.,* 2002) by monitoring the rate of ascorbate oxidation at 290 nm (ε=2.8 mM-1cm^{-1}). The reaction mixture contained 25 mM phosphate buffer (pH 7), 0.1 mM EDTA, 1 mM H_2O_2, 0.25 mM reduced ascorbate (AsA) and the enzyme sample. In the absence of AsA in the test medium no change in absorption was found.

The activity of GPX was measured according to (Panda *et al.,* 2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and enzyme sample. By using H_2O_2 and guaiacol as substrates the enzyme created a colorful product. The absorbance of the product was monitored at 470 nm (ε= 26.6 mM-1cm-1), and peroxidase activity was expressed as units/mg protein. min.

Measurement of Potassium and sodium contents was taken by flame photometry method. Leaf samples were dried out and ground. Powdered leaf materials (1 g) were kept at 560°C for 4 h for ash preparation. To these samples, 20 ml 1N HCl was added and the mixtures were heated at 90°C to drive off the hydrochloric acid. The digested ash was dissolved in 100 ml distilled water and then filtered. The filtrate was stored in a refrigerator until analysis. Concentrations of potassium and sodium ions were estimated by referring to 0, 5, 10, 20, and 30 ppm standard working solution.

Estimation of protein and chlorophyll contents

Samples of Protein content was determined by method of (Bradford, 1976). Bovine serum albumin was used as a standard and the enzyme activity was expressed in mg of protein. Following extraction of liquid-nitrogen frozen leaf with 80% acetone, the concentration of chlorophyll was determined according to the spectrophotometer method of Porra *et al.* (1989).

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Statistical Analysis

The results were expressed as means ± standard deviation. The data was analyzed by two way ANOVA and different group means were compared by Duncan's multiple range test where necessary. $P < 0.05$ was considered significant in all cases. The software Package Statistica was used for analysis of data.

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RESULTS

The estimation of glycine-betaine serves as physiological marker of salt stress. Under saline conditions an overall increasing trend in glycine-betaine contents was noted in all genotypes (Fig. 1). Treatment with different doses of salt (2 EC, 4 EC, 8 EC and 16 EC) caused a significant ($P < 0.05$) increase in glycine-betaine. The glycine-betaine contents increased in the order 2 EC < 4 EC < 8 EC < 16 EC. Maximum increased production of glycine-betaine was observed in Sehar-2006 (34.7 µmol/g), Lu-26 CTR (33.2 µmol/g), showing obvious tolerance under salt stress. In control condition these genotypes were having glycine-betaine contents 6.7 µmol/g, 6.3 µmol/g respectively (Fig.1). There was also a significant difference ($P < 0.05$) among different cultivars of wheat for glycine-betaine. It is obvious in this study that Sehar-06 and Lu-26 showed excellent results of salt tolerance over all other genotypes and thus they are resistant genotype and sustains well in the soils that are saline in nature up to the maximum salinity level of 16 dS/m. It increases its glycine-betaine production with the increase in salinity level gradually with a linear behavior. Minimum increase was observed in Wafaq-01 that is 24.6 µmol/g, and in Meraj-08 that is 26.1 µmol/g. It means there is less production of glycine-betaine in these two genotypes as compared to resistant genotypes and so these two genotypes are salt sensitive. Thus, the parameter of glycine-betaine production demonstrated these two genotypes are not fit for better production in such soil condition which is considered as saline.

Secondly, Proline plays an important role in protecting the sub cellular structures and mediating osmotic adjustment in stressed condition. The calculated amount of proline in Sehar-06 is 26.98 µmol/g, in Lu-26 26.14 µmol/g. This is an adequate production of proline, as we increased the salinity in all the genotypes and the genotypes such as Wafaq-01 released 23.99 µmol/g, and Meraj-08 24.17 µmol/g of proline showed less production under salt stress conditions (Fig. 2). A

positive correlation between the accumulation of these two osmolytes and stress tolerance in plants has been found in many studies.

Total phenolic content in Sehar-06 and Lu-26 measured by Folin-Ciocalteu,s reagent method ranged from (59.6-50.56 mg/g) of gallic acid equivalent of aqueous wheat leaf extract (Fig. 3). While, in Wafaq-01 and Meraj-2008 ranged from (49-39 mg/g) from control to highest level of salinity. Treatment with different salt doses caused a concentration dependent decrease in phenolic content. However, this decrease was not significant ($P > 0.05$). The cultivar Sehar-06 relatively contained high quantity of phenolic content compared to other cultivars (Fig. 3).

The results of enzymatic assays showed that the activity of antioxidant enzymes such as SOD, CAT and GPX in varieties Wafaq-01 and Meraj-2008 (salt-sensitive) were lower than control under salinity condition, (Fig. 4). APX value got higher activity in adverse condition of salinity. In the meantime, there was no significant difference between salinity situation and control ones regarding APX activity (Fig. 4d). Mean while, in Sehar-06, Lu-26 (salt-tolerant) the activity of SOD and CAT was about unchanged (Fig. 4a and 4b). Interestingly, APX and GPX showed significantly amplified activity under salinity compared to control treatment and it is almost three times than the control condition (Fig. 4c and 4d).

Salinity treatment led to the increased Na^+ content in both sets of varieties. This increase in the case of salt-sensitive varieties was almost four-time higher than salt-tolerant varieties as it is 9.5-22 mg/g and 10.3-23.6 mg/g in Meraj-08 and Wafaq-01 respectively (Fig. 5a). What is more, salinity harmfully exaggerated K^+ content in both cultivars its amount decreased about one fourth in case of salt tolerant and about half in case of salt sensitive varieties from control to the maximum stress level (Fig. 5b). In case of Sehar-06 the most salt tolerant, its value is 88 at

control level and devalued to 67.8 mg/g at salinity level of 16 ds/m and in case of Wafaq-01 at control its value is 78.4 and lowered to 41.1 at maximum salinity level. K^+/Na^+ ratio of both types of cultivars reduced while travelling from control towards maximum salinity, in case of tolerant varieties it devalued with ratio: from 12.4 to 3.2 in case of Sehar-06 and from 7.1 to 2.3 in case of WAFAQ-01 (Fig. 5c). However, there was no statistical difference between varieties in view of this ratio. Grain yield of different wheat varieties was significantly influenced by the salinity (Table. 1). The genotype Sehar-06 and Lu-26s showed minimum reduction, when compared with control, whereas maximum reduction over control was recorded in MERAJ-2008 and WAFAQ-01. The genotypes Sehar-06 and Lu-26 were successful in maintaining grain yield per plant more than 60% under salinity stress (16 dS/m).

DISCUSSION AND CONCLUSION

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Different free radicals in whole plant disturb its most of the biochemical and metabolic reactions and ultimately result in low grain yield or the death of whole plant in severe conditions of salt stress (Gara *et al.,* 2003). It is the antioxidant enzyme system of the plant that protects the plant against the free radicals. Since, various physiological and biochemical processes are regulated by the plant hormones , the investigation of the role of new plant growth regulators in crop abiotic stress tolerance is being much alert these days (Peleg and Blumwald, 2011). As we increased the salinity level there is continuous increase in proline production. Glycine-betaine and proline are two important osmolytes that significantly increase under the salt stress. It is concluded that on the basis of osmolytes production, two genotypes viz., Sehar-2006, LU-26, were found to be salt tolerant whereas genotypes, Wafaq-01, and Meraj-2008 could be designated as sensitive ones. Several workers have proved that tolerance of plants in their rooting medium is under genetic control (Munns *et al.,* 2000). Genetic variabilities are basis for improvement in plants (Akber *et*

al., 2009). A strong positive correlation between total phenolic contents and antioxidant enzyme activity (r = 0.97) indicates that antioxidant enzyme activity is highly contributed by phenolics. Such strong correlation between total phenolics and antioxidant enzyme activity of wheat has already been reported (Trust *et al.,* 2005). The phytochemicals responsible for the antioxidant activity of wheat are mainly due to phenolic acids and flavonoid compounds (Cao *et al.,* 1997). It has been recurrently reported that one of the major causes of oxidative damage to plant tissues is salt stress (Jalali-e-Emam *et al.,* 2011). However, plants protect themselves by developing a strong defense system including antioxidant enzymes like CAT, POD and SOD (Joseph and Jini, 2011).

In present work, salinity stress increased the activities of CAT, GPX, APX and decreased the activity of SOD, while the POD activity remained unchanged. The increase in SOD activity has been reported in two wheat cultivars (Banysoif 1 and Sakha 68). Such a disparate expression of SOD was ascribed to different expression of SOD isozymes under control or saline conditions (Abdel, 2010). The accumulation of $O2^{\cdot -}$ in leaf cells is result of reduced SOD activity and consequently results the decreased activity of CAT and peroxidases (Fridivich, 1989). Under salinity conditions any increase in above mentioned cycle's activity goes to stability in cell mechanisms and reduced occurrence of oxidative stress (Edreva, 2005). In 'Wafaq-01, and Meraj-2008, in spite of oxidative stress, SOD activity was not increased. Mehler cycle operation under stress conditions help the plant to turn on the xanthophyl cycle by acidification of lumen space (Ort, 2001). In addition, in Wafaq-01, and Meraj-08, CAT and GPX activities were significantly decreased. These enzymes have the prospective to neutralize H_2O_2 by means of its conversion to H_2O molecules (Edreva, 2005, Ahmed *et al*., 2009). In leaf cells any reduction in H_2O_2 scavenging enzymes activity causes the accumulation of these oxidants. Ionic stress

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caused by Salinity conditions i.e. intensified absorption of Na^+ and Cl^- antagonistically affect K^+, Ca^{2+} and Mg^{2+} absorbtion and metabolism (El-Hendawy *et al*., 2005; Mansour *et al*., 2005).

In conclusions, the examined wheat varieties exhibited a significant difference in their glycine-betaine, proline, total phenolics and antioxidant enzymes contents showing the biochemical variation among different genotypes. Besides, the results revealed that Sehar-06 and Lu-26 plants were able to escape the ionic toxicity under saline sodic conditions via scavenging of ROS molecules and concurrently controlled Na^+ absorption and translocation. Those qualities potentiate the plants endurance and efficiency under stressful conditions.

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Figures

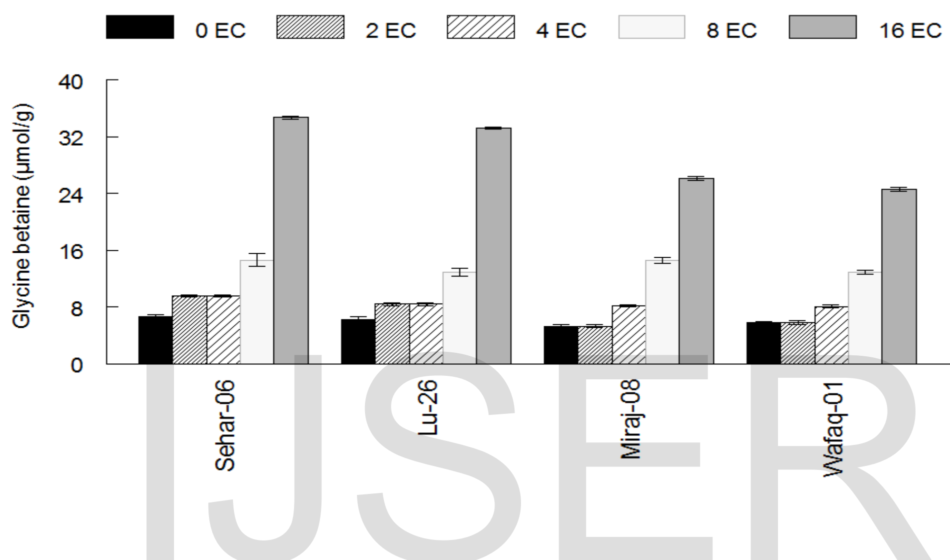

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Fig. 1. The effect of salt stress on glycine-betaine content in different cultivars of wheat. Values are mean ± SD (n=3). The salt stress causes a significant increase ($p < 0.05$) in glycine betaine at different doses (Control, 2 EC, 4 EC, 8 EC and 16 EC) of salt.

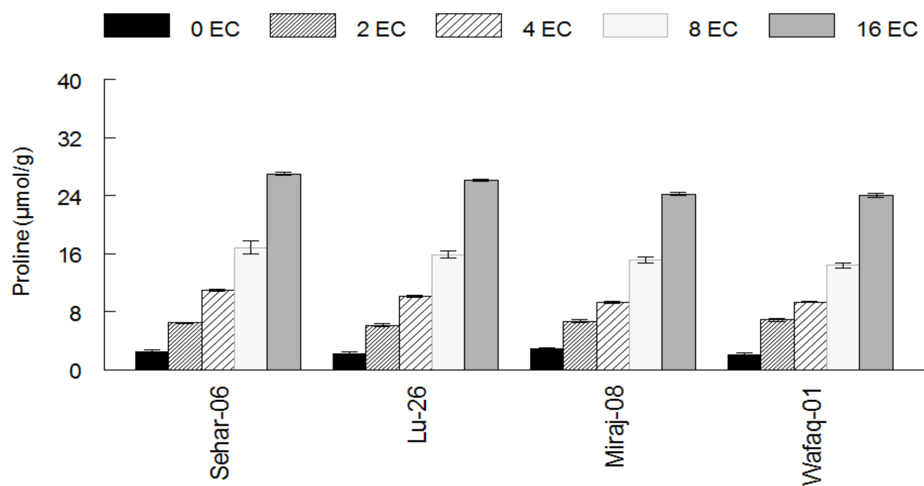

Fig. 2. The effect of salt stress on proline content in different cultivars of wheat. Values are mean ± SD (n=3). The salt stress causes a significant increase ($P < 0.05$) in proline at different doses (Control, 2 EC, 4 EC, 8 EC and 16 EC) of salt.

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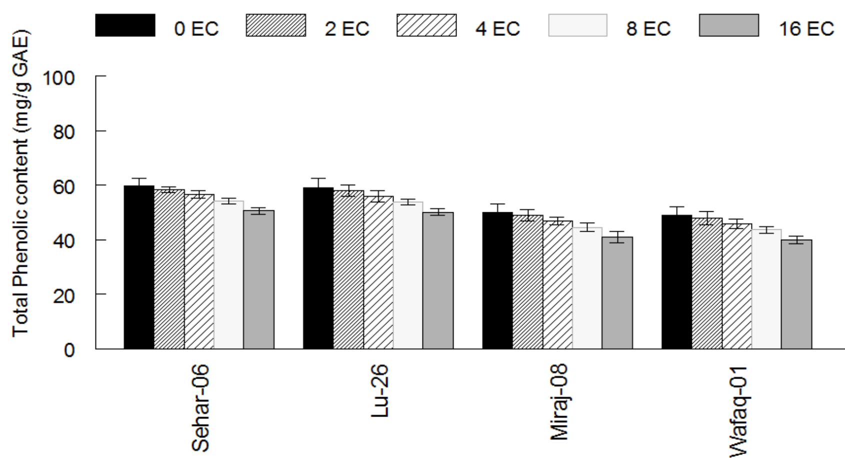

Fig. 3. Effects of salinity on Total phenolic contents in leaf of different cultivars of wheat. The result is means ±. SD (n=3).

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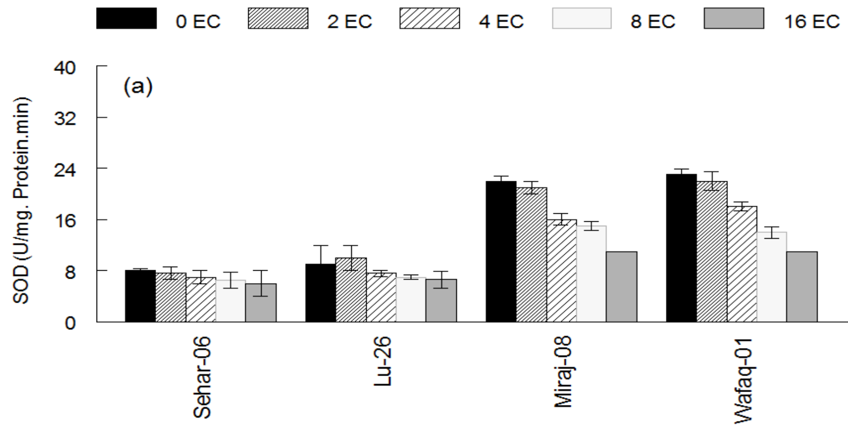

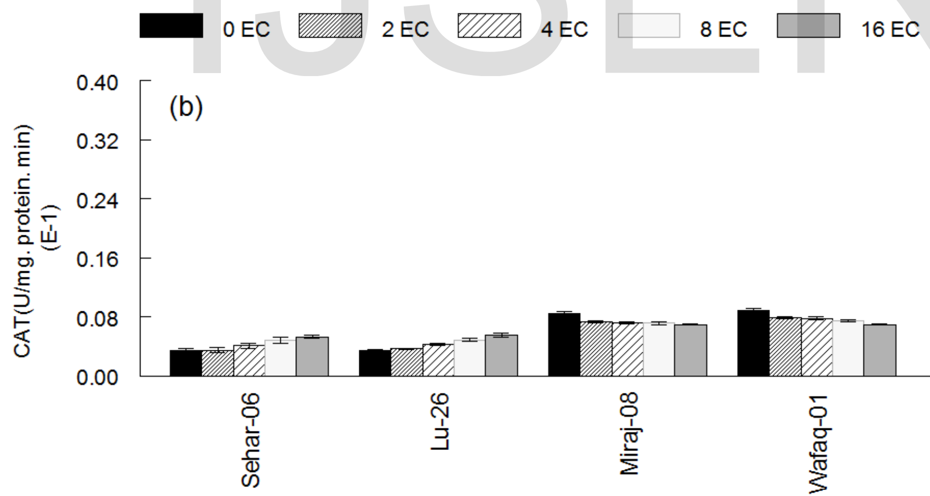

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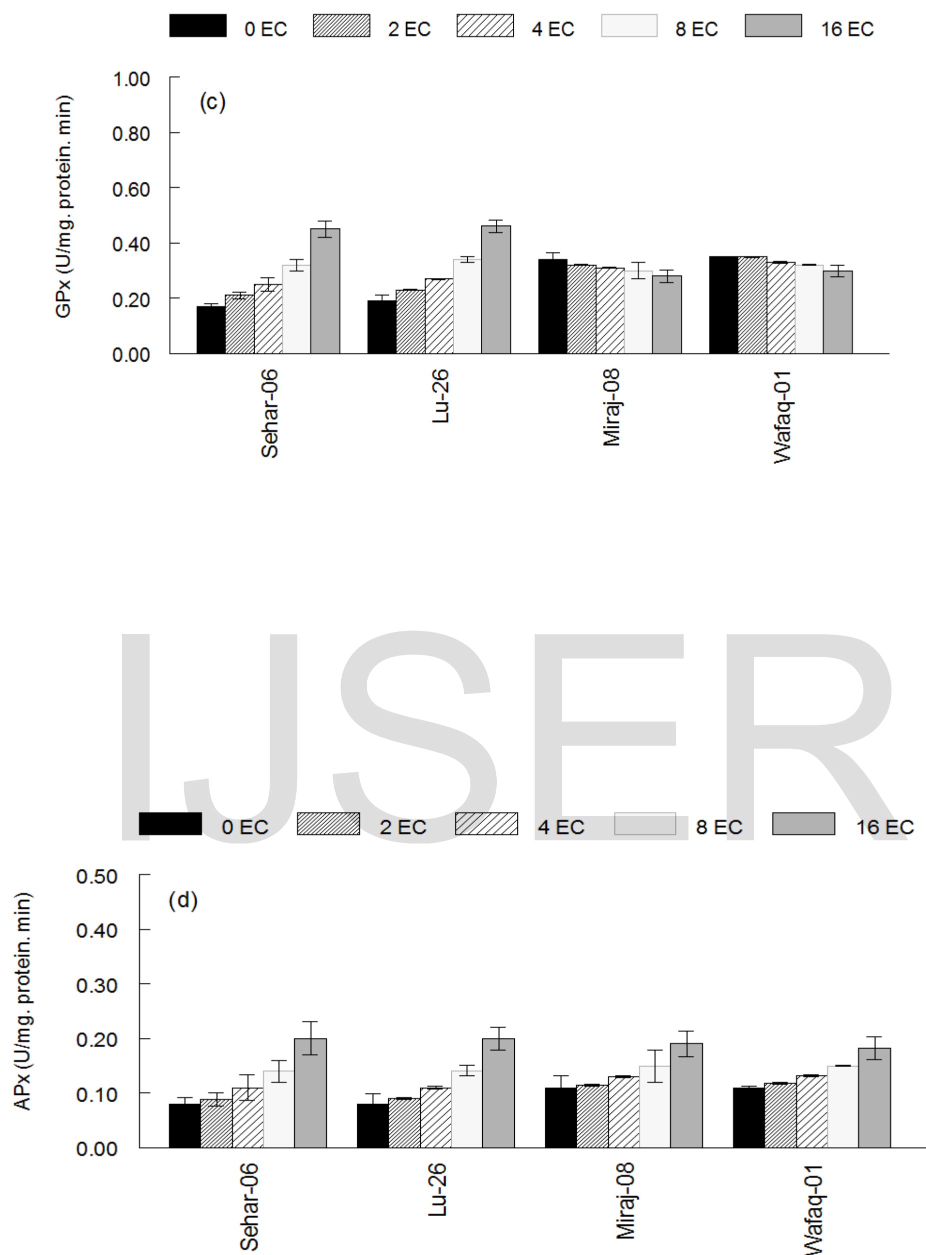

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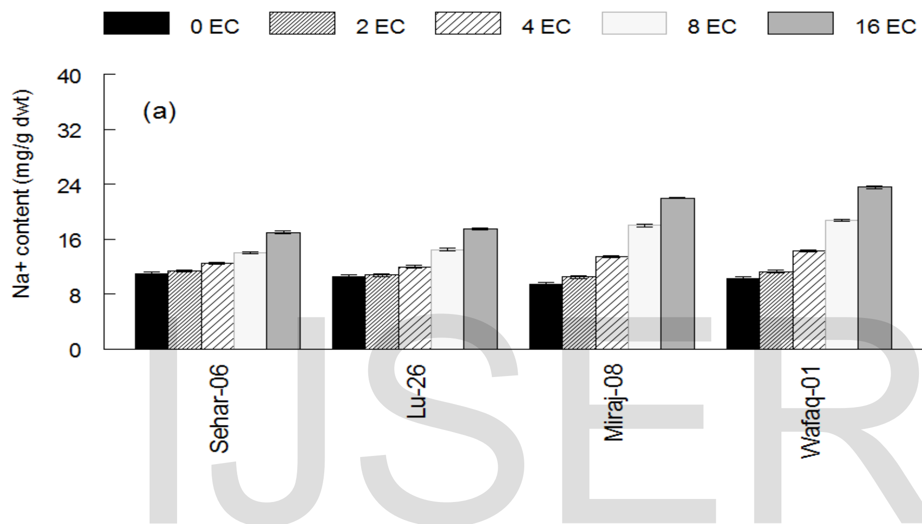
Fig. 4. The activities of antioxidant enzymes in different cultivars of wheat in control and under salt stress (a). superoxide dismutase activity among different cultivars of wheat (b).

catalase activity among different cultivars of wheat (c). guaiacol peroxidase activity among different cultivars of wheat (d). ascorbic acid peroxidase activity among different cultivars of wheat. The results are means ±SD (n=3).



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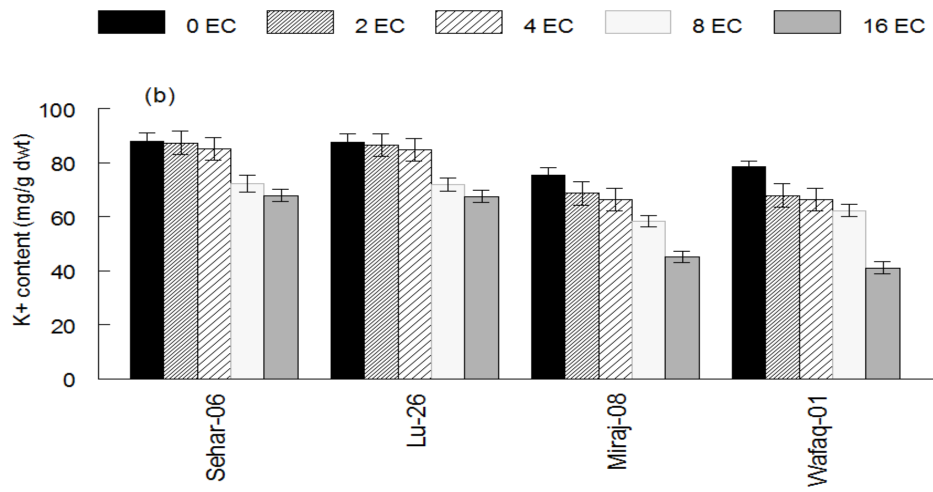
0 EC
2 EC
4 EC
8 EC
16 EC
(b)
K+ content (mg/g dwt)
100
80
60
40
20
0
Sehar-06
Lu-26
Miraj-08
Wafaq-01

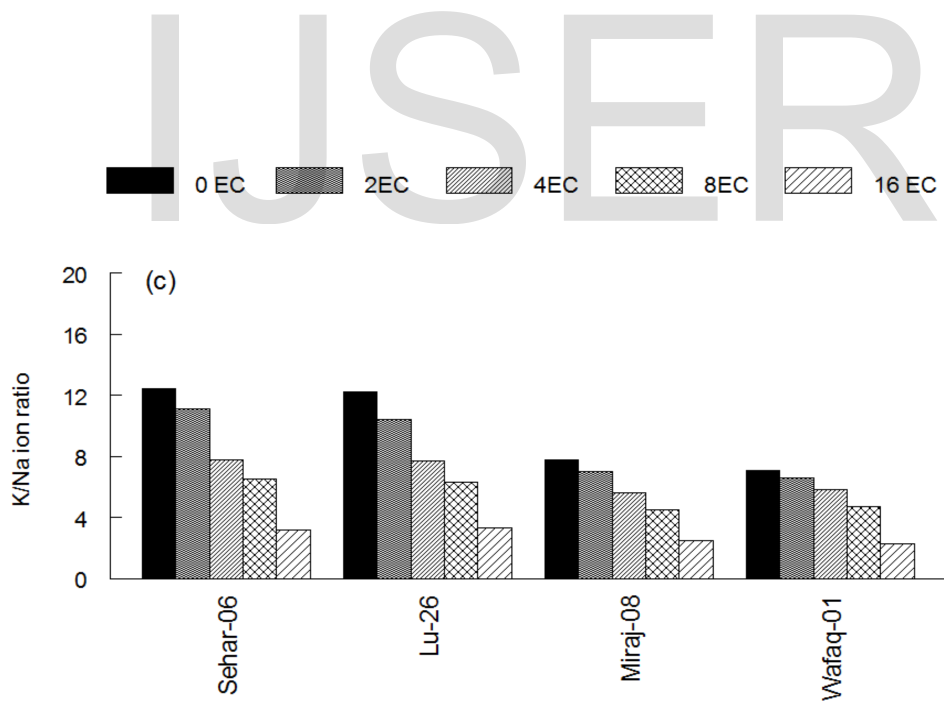
0 EC
2EC
4EC
8EC
16 EC
(c)
K/Na ion ratio
20
16
12
8
4
0
Sehar-06
Lu-26
Miraj-08
Wafaq-01

Fig. 5. Effect of salinity on the contents of sodium and potassium among different cultivars of wheat (a). Sodium content (mg/g) in wheat leaves (b). potassium content (mg/g) in wheat leaves (c). The ratio of K and Na in wheat leaves. The result are means ±SD (n=3).

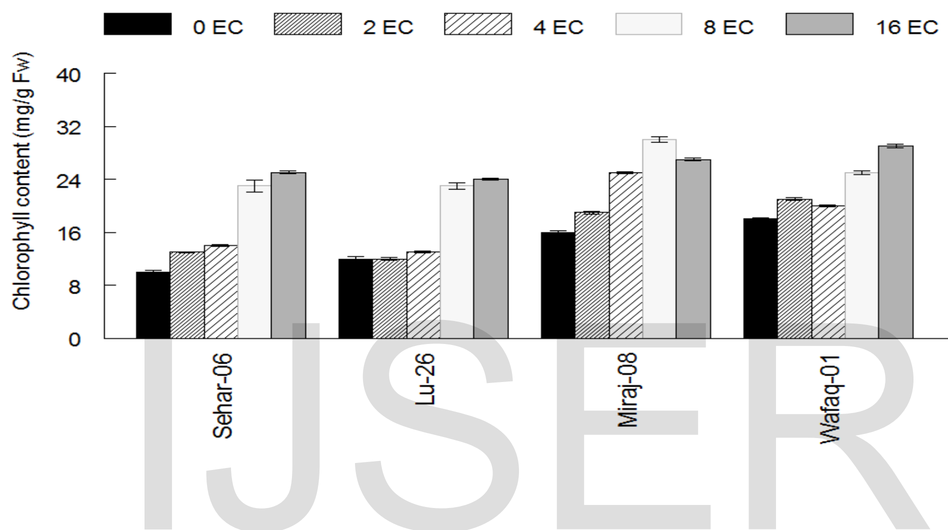

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Fig. 6. Effect of salinity on chlorophyll content in leaf of different cultivars of wheat. The result are means ±SD (n=3).

Table 1. Grain yield (g) among four cultivars of wheat in control and under salt stress

Wheat Cultivars	Control	EC2	EC4	EC8	EC16
Sehar-06	5.05±0.4	5.05±0.41	4.8±0.31	3.69±0.21	3.14±0.18
Lu-26	4.32±0.38	4±0.32	4.02±0.23	3.38±0.23	2.57±0.09
Miraj-08	5.27±0.42	4.98±0.31	4.72±0.3	3.23±0.2	2.16±0.3
Wafaq-01	5.55±0.29	5.51±0.24	4.92±0.9	3.41±0.7	2.25±0.4

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