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DETECTION OF SALINE TOLERANT WHEAT CULTIVARS (*TRITICUM AESTIVUM* L.) USING LIPID PEROXIDATION, ANTIOXIDANT DEFENSE SYSTEM, GLYCINE-BETAINE AND PROLINE CONTENTS

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ABSTRACT

Salt produces free radicals in the plants and these free radicals are scavenged by the antioxidative capacity of the plant. Change in antioxidant activity, phenolic contents, lipid peroxidation, glycine betaine and proline contents were investigated in fifteen genotypes of wheat differing in salt tolerance. The results revealed that the aqueous extracts of wheat (300 µg/ml) in control and under salt stress (i.e. electrical conductivity, 2 EC, 4 EC, 8 EC and 16 EC) demonstrated antioxidant activity in DPPH radical assay and their increased scavenging percentage showed that AUQAB-2000, PIRSABAK-05, BAKHAR-2002 can be considered as salt tolerant varieties. The change of phenolic content suggests that wheat uses antioxidant properties of phenolics as a mechanism of salt stress. Whereas, the lipid peroxidation, proline and glycine betaine data has indicated that AUQAB-2000, PUNJAB-85, PIRSABAK-05, BAKHAR-2002, FARKHARE-SARHAD and KAGHAN-94 can be considered as salt tolerant varieties. Salt tolerant varieties also showed higher amount of yield at different salinity levels. These results indicate that these cultivars of wheat alleviate the deleterious effect of salt stress by their antioxidant activity and increased production of proline and betaine.

Key words: Wheat, salt stress, antioxidant activity, phenolic content, osmolytes, oxidative stress, salinity levels.

INTRODUCTION

Soil salinity is one of the environmental factors that bound distribution and efficiency of major crops (Ashraf and Fooland, 2005; Chandan *et al.*, 2006). The excess salt taken up by the plants is stored in older leaves: continued transport of salt into transpiration stream over extended period results in very high concentration of Na^+ Cl^- which results in the death of leaves. This injury is certainly caused by overloading the vacuolar capacity to catalog toxic salt species. Otherwise, they might accumulate in cell wall and cause dehydration (Munns, 2005).

Wheat is one of the foremost crops that have the chattels of anti-oxidation against the oxidation of important bio-molecules such as membrane lipids, protein and DNA. It stops the human LDL cholesterol per oxidation (Yu *et al.*, 2005), per oxide anion (O^{2-}). Free stable radicals like DPPH and $ABTS^+$ are also inhibited by wheat bran extracts (Zhou *et al.*, 2004) as well as phospholipid liposomes hydrogen peroxide (Martínez-Tomé *et al.*, 2004). The production of reactive oxygen species (ROS) is restricted or scavenged by an antioxidant system like antioxidant compounds (ascorbate, glutathione, tochopherols, salicylate, etc.) and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP_X) and catalase (CAT) (Foyer and Noctor, 2003).

Under stress, the generation of MDA (malondialdehyde) takes place in plants due to lipid peroxidation in membrane. It is a mean of assessing oxidative stress induced membrane damage (Sairam *et al.*, 2005) and cell membrane stability has been used to differentiate among crop cultivars with respect to grade of salt tolerance (Meloni *et al.*, 2003). Currently, a lot of research is being done to explain the role of various antioxidant metabolites in plants against stress tolerance. Antioxidant properties and behavior of phenolics have been explained to a great extent (Wang and Lin, 2000).

There is a strong evidence that glycine betaine and proline shield the sub cellular structures and mediate osmotic adjustment in stress. A positive correlation between these two osmolytes and stress tolerance in plants has been found in many studies (Garg *et al.*, 2002; Yang *et al.*, 2003).

Hence, the aim of this study was to screen (i) the potential wheat cultivars for better performance under salt stress (ii). pattern of accumulation and growth of glycine betaine, proline, MDA and total phenolic contents in the leaves of fifteen wheat cultivars under

different levels of salinity, and the roles of these phenolics in plant stress tolerance in terms of membrane lipid peroxidation, and the antioxidant capacity against the total production of free radicals.

MATERIALS AND METHODS

Plant growth conditions: The plant material (varieties and lines) was chosen on the base of their repeated cultivation in the area. The selected wheat varieties i.e. SULEMAN-96, SHAHEEN-94, PIRSABAK-05, PASHKOO-03, PUNJAB-85, MANTHAR-03, BAKHAR-2002, AUQAB-2000, FAKHAR-E-SARHAD, SALEEM-2002, ZARDANA, SAUGHAT-90, PASTOR, ROHTAS-90 and KAGHAN-94 were collected from different research centers of Pakistan and grown in the experimental field of Faculty of Agriculture, Rawalakot Azad Jammu and Kashmir. The leaves of all varieties grown in pots at comparable conditions were taken for aqueous extraction. The experiment was carried out in two factors factorial RCBD design with three replications. Equal amount of soil, farm yard manure and sand (FYM) was mixed and mixture was used to fill these pots. Four doses of the salt (NaCl) were functional to the wheat varieties. The doses integrated, 2 ds/m (desi Siemen's per meter), 4 ds/m, 8 ds/m and 16 ds/m against control (No salt applied) in the soil. The doses of salt were applied at jointing stage and the electrical conductivity (EC) was calculated according to the approved method of USDA (1954).

Preparation of wheat extracts: For the measurement of antioxidant activity and total phenolic contents the preparation of wheat extract was carried out. The leaves at full maturity (1 g) were finely ground and extracted in boiling water (250 ml) for 15 minutes, cooled and filtered using whatman filter paper No. 1. The obtained residue was extracted twice and finally the whole extract was concentrated. The serial dilution of the extracts was done to achieve the desired concentration of wheat extract for experiment.

Antioxidant activity by scavenging of DPPH radical: The antioxidant activity of the wheat extracts were determined using the stable DPPH radical according to the method of Hatano *et al.*, (2002). Briefly 0.25 mM solution of DPPH (0.5 ml) was added to the sample solution in ethanol (1 ml) at a concentration (300 µg/ml). The mixture was shaken and kept for 30 minutes in the dark, and the absorbance was read at 517 nm. The scavenging capacity of the DPPH radical was calculated by the following equation: (%) = $[(Ao - A_1)/Ao)] \times 100$, Where, Ao is the absorbance of the control reaction and A_1 is the absorbance of the sample. The experiments were carried out in triplicate.

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

Estimation of Lipid peroxidation: Lipid peroxidation was determined in leaves as malondialdehyde (MDA) by Carmak and Horst (1991) method. The content of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} by using the formula:
MDA level (nmol) = Δ (A 532nm-A 600nm)/1.56×10^5

Determination of Glycine betaine and Proline: Glycine betaine and proline contents were estimated spectrophotometrically according to Grieve and Mass (1984) and Bates (1973) methods respectively. Standard curves of Glycine-betaine and proline and were used to estimate the amount of proline and glycine betaine.

Statistical analysis: The results were shown as means ± standard deviation. The data was analyzed by two way ANOVA; $P < 0.05$ was significant in all cases. The software Package Statistica was used.

RESULTS AND DISCUSSION

DPPH radical scavenging activity of wheat: The antioxidant potential of wheat cultivars in terms of percentage scavenging of DPPH radical is given in Figure 1. The results showed that the control of each variety displayed higher scavenging percentage that was gradually and significantly ($p<0.05$) decreased by increasing the salt concentration. Among different varieties the AUQAB-2000 showed the highest scavenging percentage of 75.67% which was reduced significantly to 62.72 ± 2.1 % at salt stress of 8 EC and 47.4 ± 3.1% at 16 EC. PIRSABAK-05 and BAKHAR-2002 showed the highest antioxidant activity by discoloring the DPPH radical at the highest concentration (300 µg/ml). These wheat cultivars were found to be salt tolerant as they showed higher percentage of scavenging even at maximum dose of salt (16 EC). On the other end, the wheat varieties (SAUGHAT-90, PASTOR, ROHTAS-90 and KAGHAN-94) showed less scavenging capacity of 46.02, 45.69, 44.87 and 43.67 ± 3.1%, (Figure 1). High level of DPPH activity has been correlated with tolerance to different stress conditions (Kang and Salveit, 2000) but they might point a source of easy accessible food supplement or its pharmaceutical use in industries. Our results are in accordance to Sunil *et al.* (2006) where wheat grass has indicated the antioxidant activity on DPPH, ferric reducing antioxidant power (FRAP) and

ABTS assays. Several authors have reported that the antioxidant properties of wheat or wheat products are appreciably predisposed by the genotype and or/ the environment in which wheat is grown (Sairam *et al.*, 2005; Jeffrey *et al.*, 2006).

Total phenolic content of wheat: To assess the whole wheat grains in relation to their health benefits and in the credentials of potential constituents for the development of grain based functional diet the total antioxidant scavenging capacity and total phenols content (TPC) have extensively been used. Total phenolic content ranged from 29.33-50.34 mg/g of gallic acid equivalent in leaves (Figure 2). Treatment with different salt doses caused a concentration dependent decrease in phenolic content. However, this decrease was not significant (P> 0.05). The cultivars PIRSABAK-05, BAKHAR-2002, PUNJAB-85 and PASHKOO-03 relatively contained high quantity of phenolic content compared to other cultivars (Figure 2) which might be responsible for their high antioxidant activity. At control level, the phenolic content ranges from 29.33-50.34 mg/g and at maximum salt application, it was found between 21.33-41.43 mg/g. The results of our studies have shown that phenolic contents were decreased with different doses of salts. Such decrease in phenolic content has already been reported in wheat leaves (Muhammad *et al.*, 2010, Rao *et al.*, 2013).

Lipid peroxidation: Lipid peroxidation expressed as MDA was found to be different among different wheat cultivars (Figure 3). Among different cultivars (control) MDA level varied from 7.7-12.7 nmol/g of leaves. The minimum MDA was detected in PUNJAB-85 (8.4 nmol/g) followed by BHAKAR-2002 (8.9 nmol/g), PIRSBAK-05 (9.5 nmol/g) whereas the maximum MDA was found in SULEMAN-96 (13.3 nmol/g) at control level (Figure 3). Whereas, at maximum level of salt stress it ranges from 16.2–21.1 nmol/g in BHAKAR 2002 and SULEMAN-96 respectively. Lipid peroxidation showed increasing trend with increasing doses of salt. The lower lipid peroxidation shows the tolerance of genotypes to salt stress. Under salt stress conditions an increase in lipid peroxidation was found to be higher in cultivars, ZARDANA (55.9%), ROHTAS-90 (42.26%), SAUGHAT-90 (50.88%) and SHAHEEN-94 (52.47%). Thus these genotypes were found to be salt sensitive. Whereas PUNJAB-85 (33.37%), BHAKAR 2002 (34.68%), PIRSBAK-05 (30.72%) and AUQAB (27.9%) showed decreased levels of lipid peroxidation and were found to be salt tolerant (Figure 3). Oxidative stress is induced in plant tissues by salt stress (Hernandez *et al.*, 1994). Lipid peroxidation requires uptake and involves the production of superoxide radical (O^{2-}). The other highly reactive species are singlet oxygen ($^1O_2$), hydroxyl frees radical (OH) and H_2O_2, which stimulates lipid peroxidation (Fridovic, 1986). Higher membrane stability (lower ion leaching) and lower lipid peroxidation and have also been reported in tolerant genotypes of wheat (Kraus *et al*., 1995) and rice (Tijen and Ismail, 2005). On the basis of lipid peroxidation PUNJAB-85, BHAKAR 2002, PIRSBAK-05 and AUQAB showed decreased levels of lipid peroxidation and were found to be salt tolerant.

Glycine betaine and proline contents: Glycine betaine (GB) and proline are the most important organic osmolytes that accumulate in different plant species in response to environmental stresses like extreme temperatures, drought, salinity, UV radiation and heavy metals. A lot of research and articles showed a positive relationship between accretion of proline and glycine betaine and stress tolerance (Hamdia and Shaddad, 2010; Ashraf and Fooland, 2006). The estimation glycine betaine serves as physiological marker for salt stress. Under saline conditions an overall increasing trend in glycine-betaine contents was found in all genotypes (Figure 4). At control conditions the production of glycine betaine ranges from 4.6 µmol/g (SAUGHAT-90) to 6.0 µmol/g (AUQAB-2000) and at the maximum level of salt application it ranges from 24.1 µmol/g (ROHTAS-90) to 34.3 µmol/g (AUQAB-2000) and in middle levels of salt application there is more diversity shown in the Figure 4. Maximum increased production of glycine betaine was observed in AUQAB-2000 (34.3 µmol/g), PIRSBAK-05 (29.8 µmol/g), BHAKAR-02 (29.1 µmol/g) and PUNJAB-85 (27.3 µmol/g) showing the obvious tolerance under salt stress.

Proline plays an important role in protecting the sub cellular structures and mediating osmotic adjustment in stressed condition. The accumulation of proline in different genotypes showed very close result to glycine betaine under salt stress conditions (Figure 5). The amount of proline found in AUQAB-2000 was (27.98 µmol/g), PIRSBAK-05 (24.67 µmol/g) and BHAKAR-02 (26.66 µmol/g) and thus AUQAB-2000 ranked the best among all genotypes. This enhanced production of proline increased linearly with the salinity. Similarly, FAKHAR-E-SARHAD (25.76 µmol/g) and KAGHAN-94 (24.98 µmol/g) also showed increased production of proline at maximum dose of salt. ZARDANA and SAUGHAT-90 also showed intermediate levels of proline. These results also favor the results of glycine betaine production with very small differences. It is concluded on the basis of osmolytes production, four genotypes viz., BHAKAR-02, PIRSBAK-05, FAKHAR-E-SARHAD and AUQAB-2000 were found to be salt tolerant whereas genotypes ZARDANA, SAUGHAT-90 and ROHTAS-90 could be considered as sensitive ones.

Grain yield of different wheat varieties was significantly subjective by the salinity (Table 1). The genotype AUQAB-2000, PIRSABAK-05, SHAHEEN, and KAGHAN-94 showed minimum reduction, when

compared with control, whereas maximum reduction over control was recorded in SHAHEEN-94 and ZARDANA-89. The genotypes AUQAB and PIRSBAK were successful in maintaining grain yield more than 60% under salinity stress (16 dS/m).

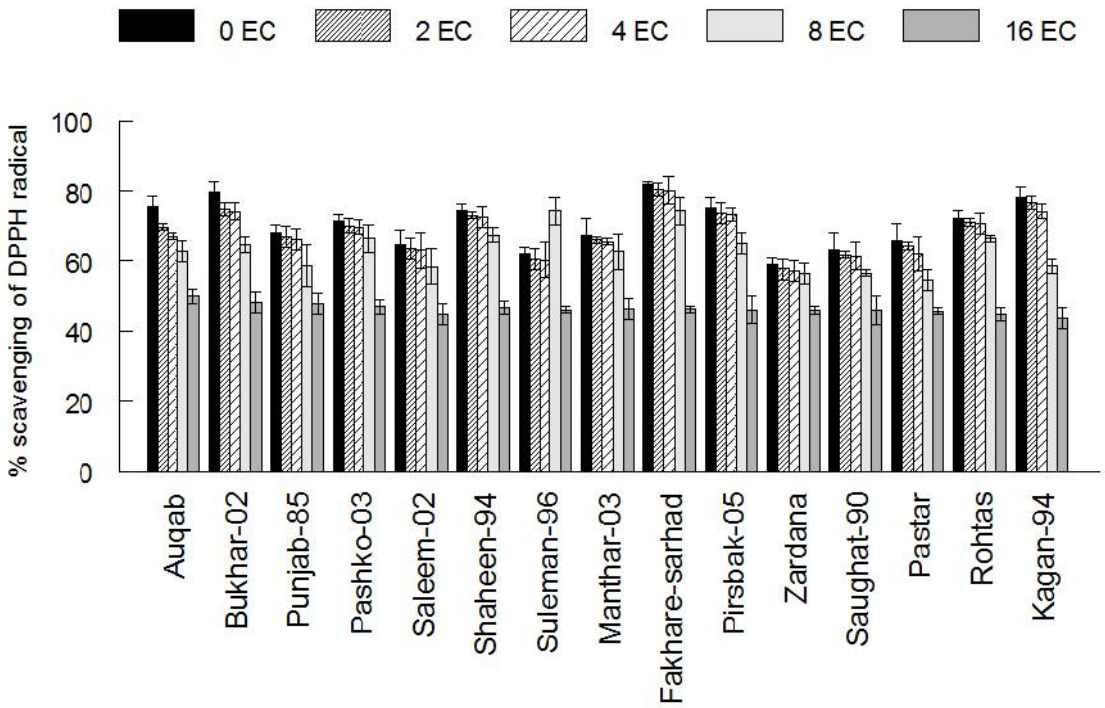

Figure 1. Antioxidant activity of extract of wheat leaves at 300 µg/ml. Values are mean ± SD (n=3). The stressed varieties are significantly different (p<0.05) from control

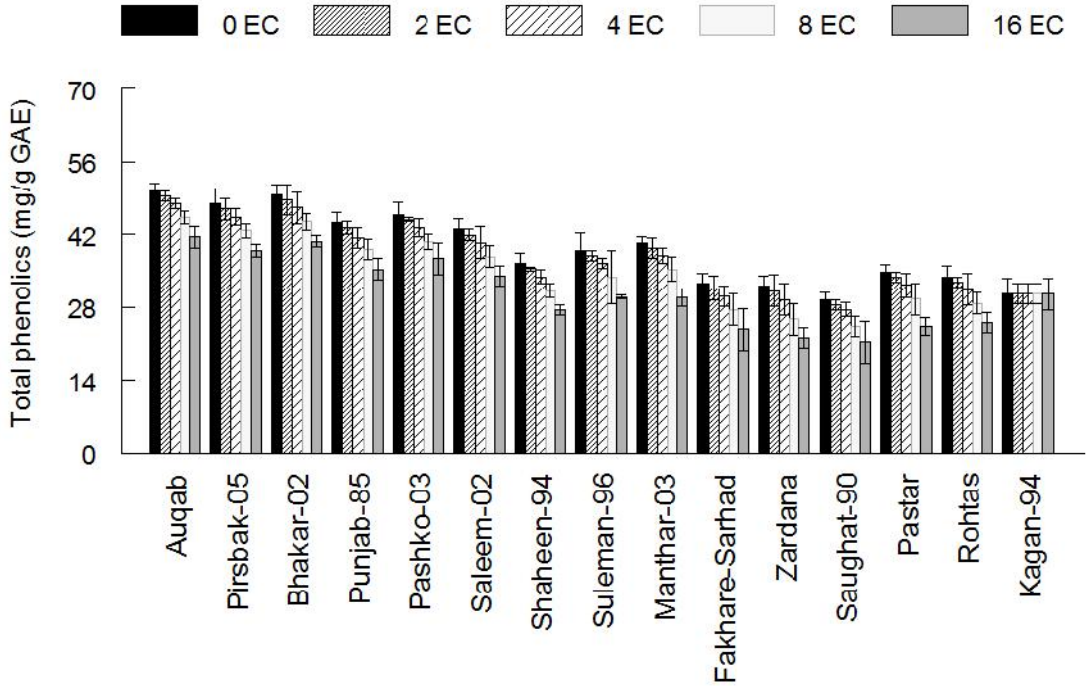

Figure 2. Total phenolics among non stress and stressed varieties of wheat. Results are mean ± SD (n=3). The stressed varieties are non significantly different (p>0.05) from control

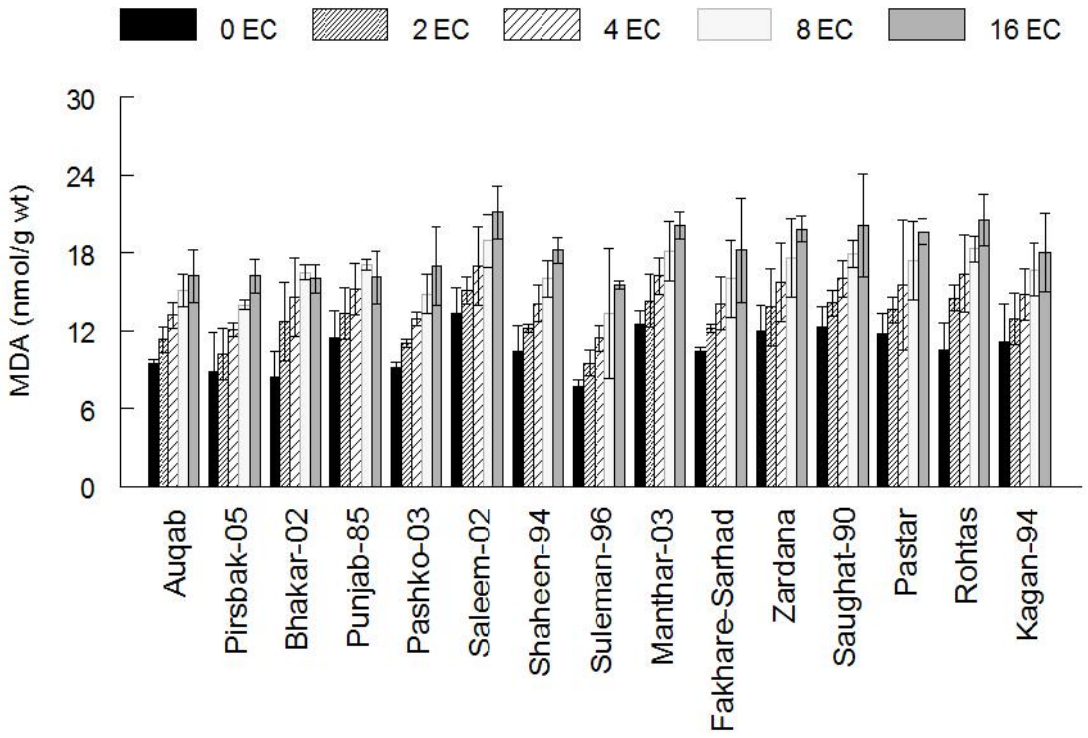

Figure 3. Lipid peroxidation among non stress and stressed varieties of wheat. Results are mean ± SD (n=3). The stressed varieties (8 EC and 16 EC) are significantly different ($p<0.05$) from control

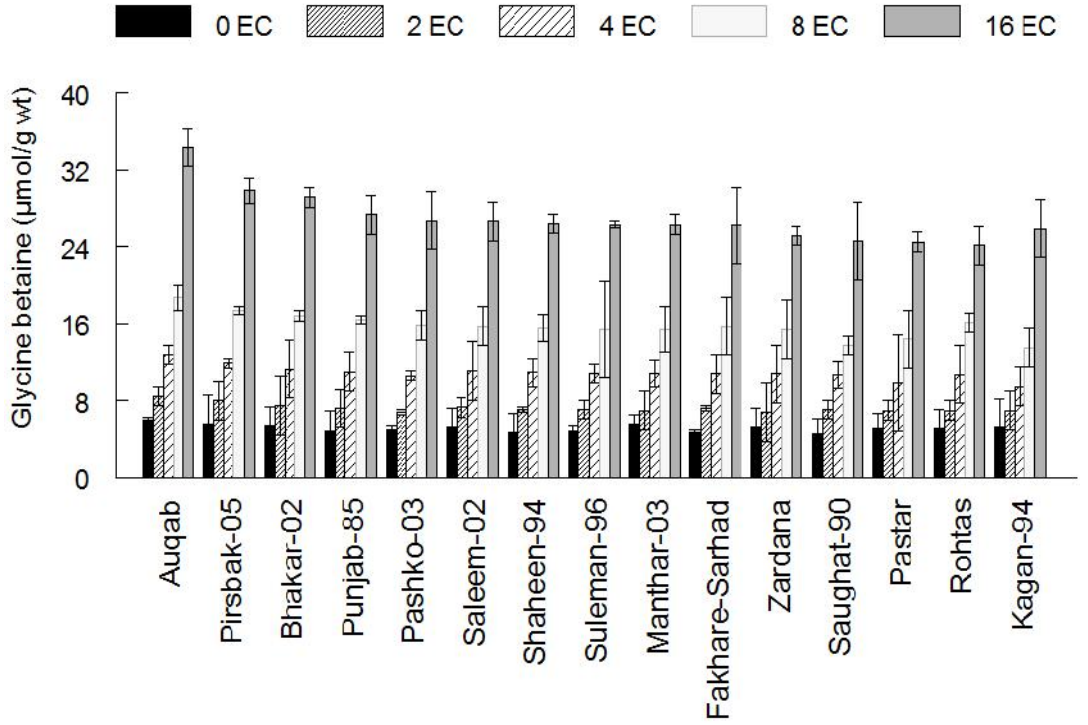

Figure 4. The effect of salt stress on glycine-betaine in different cultivars of wheat. Results are mean ± SD (n=3). The salt stress causes a significant increase ($p<0.05$) in glycine betaine (Control, 4 EC, 8 EC and 16 EC)

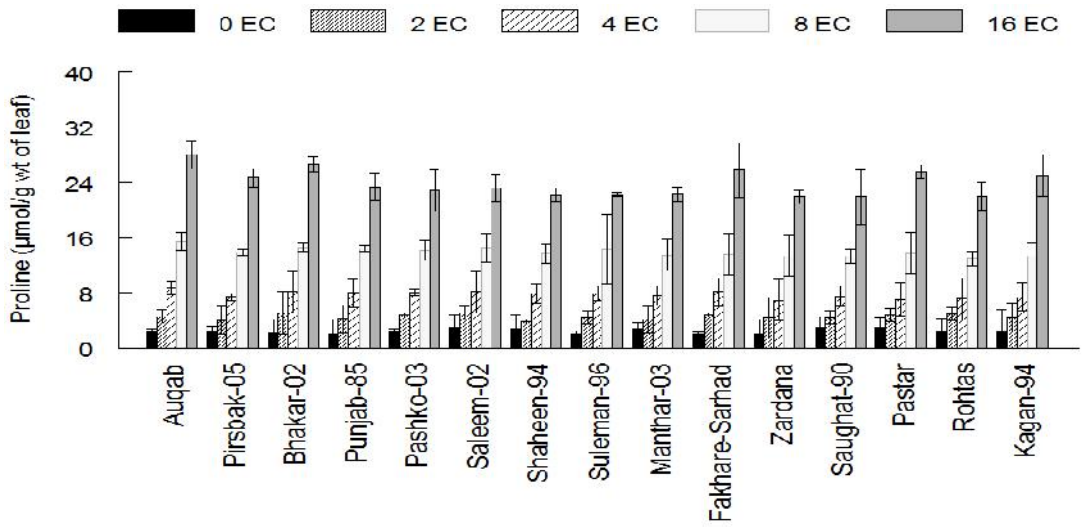

Figure 5. The effect of salt stress on proline in different cultivars of wheat. Results are mean ± SD (n=3). The salt stress causes a significant increase ($p<0.05$) in proline (2 EC, 4 EC, 8 EC and 16 EC)

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

Wheat Cultivars	Control	2 EC	4 EC	8 EC	16 EC
Auqab	5.1±0.4	5.0±0.41	4.81±0.31	3.67±0.21	3.16±0.18
Pirsbak-05	4.22±0.38	3.91±0.32	4.0±0.23	3.36±0.23	2.58±0.09
Bhakar-02	5.6±0.43	5.42±0.42	3.89±0.17	3.17±0.14	2.50±0.19
Punjab	4.36±0.31	4.02±0.32	3.50±0.21	2.76±0.24	1.87±0.1
Saleem-02	3.87±0.29	3.31±0.2	2.65±0.12	2.08±0.9	1.38±0.8
Shaheen-94	4.25±0.31	3.89±0.23	3.57±0.13	2.69±0.7	2.08±1.1
Suleman-96	5.27±0.42	4.99±0.31	4.72±0.3	3.30±0.2	2.27±0.3
Manthar-03	4.82±0.29	4.52±0.24	3.94±0.9	3.31±0.7	2.24±0.4
Fak-e-Sarhad	4.38±0.26	4.12±0.24	3.91±0.21	3.04±0.14	2.38±0.3
Zardana-89	4.44±0.2	4.23±0.25	3.49±0.31	2.38±0.12	3.16±0.18
Saughat-90	4.49±0.2	4.32±0.12	3.81± 0.13	3.32± 0.21	2.58±0.09
Pastar	3.51± 0.3	3.30± 0.13	3.0± 0.12	2.7 ± 0.23	1.9±0.2
Rohtas	3.71± 0.1	3.51± 0.2	3.2 ± 0.13	2.6 ± 0.13	2.09±0.3
Kagan-94	4.65±0.12	4.51±0.14	3.12±0.13	2.85±0.10	2.70±0.13
Pashko-03	3.61±0.14 *	3.25±0.13 *	2.75±0.12 *	2.25±0.21 *	1.89±0.22 *

*Marked differences are significantly different ($p< 0.05$) from each other

Conclusion: In conclusion, the examined wheat varieties exhibited a significant difference in their scavenging capacities against free radicals, proline, glycine betaine, MDA production and yield. Certain wheat varieties, AUQAB-2000, PIRSBAK-05, BHAKAR-2000, PUNJAB-85, PASHKOO-03, and SALEEM-02 were found to contain high concentration of phenolics and showed high antioxidant potential. On the basis of lipid peroxidation, glycine betaine and proline contents, AUQAB-2000, PIRSBAK-05, BHAKAR-2000, PUNJAB-85 were found to be salt tolerant genotypes. FAKHARE-SARHAD and KAGHAN-94 also showed salt tolerance as evidenced by increased DPPH activity, glycine betaine and proline contents. However, further biochemical and molecular studies are in progress to indicate the salt resistant genotypes among these cultivars of wheat.

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