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INDUCTION OF SALT TOLERANCE IN WHEAT (*TRITICUM AESTIVUM* L.) SEEDLINGS THROUGH EXOGENOUS APPLICATION OF PROLINE

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Abstract

To appraise the potential role of foliar applied proline as an alternative shotgun approach to ameliorate the adverse effect of salinity on wheat, a pot experiment was conducted under controlled environmental conditions, two wheat genotypes; a salt tolerant strain ESW-9525 and a moderately tolerant cultivar kherman were used in this study. Factorial combination of treatments with three replications was arranged under completely randomized design. Seven days old wheat seedlings were exposed to various levels of salinity (0, 60 and 120 Mm NaCl) for one week and applied with foliar proline (0, 50 and 100 mM) one week later. Salinity stress caused a significant reduction in plant growth, leaf photosynthetic pigments, as well as alterations in ionic balance. Foliar applied proline significantly improved root and shoot length, seedling fresh and dry weight, photosynthetic pigments, K^+ contents and K^+: Na^+ ratio. Both genotypes varied considerably in endogenous level of proline (Pro), glycine betaine (GB), total soluble sugars (TSS) and total phenolic contents (TPC) in response to salinity and foliar proline as well. Foliar applied proline 50 mM and 100 mM were found as a stimulus for plant growth triggering the physiological and biochemical attributes, However, 100 mM proline was the most effective to ameliorate the toxic effects of salinity by improving root and shoot length, seedling fresh and dry weight, chlorophyll *a*, *b* contents, TSS, Pro, GB, TPC and K+ contents and K/Na ratio in both genotypes. These findings confirmed the ability of foliar applied proline to stimulate the salt tolerance in wheat plants.

Key words: Salinity; Salinity; Osmoprotactant; Growth; Total phenolics; Glycine betaine; Wheat.

Introduction

Soil salinity is a major aspect that restricts the yield of agricultural crops, jeopardizing the capacity of agriculture to sustain the burgeoning human population increase (Munns & Tester, 2008). Almost 7 percent of world's total land area is affected by salinity (Musyimi *et al.,* 2007). The problem of salinity in Pakistan is typical for irrigated agriculture where drainage is inadequate. In Pakistan, nearly 10 million ha area is badly affected by salinity, comprising 12.9 percent of country land (Anon., 2008).

Salinity impacts plants in two most important ways: elevated concentrations of salts in the soil perturb the capacity of roots to extract water, and high levels of salts within the plant itself can be toxic, resulting in a suppression of many physiological and biochemical processes such as nutrient uptake and assimilation (Hasegawa *et al.,* 2000; Munns & Tester, 2008). Like other crops, salinity adversely affects the growth and yield of wheat crop (Saboora & Kiarostami, 2006). It causes imbalance in nutrients uptake like K^+, Na^+, Ca^{2+} and Cl^- which alters the plant metabolism by affecting osmotic potential, enzymatic activities, membrane permeability and electrochemical potential (Hu & Schmidhalter, 2005; Khan *et al.,* 2010). Increasing Na+ and Cl^- contents in photosynthetic tissues can increase oxidative stress, which facilitate inhibition of photosynthesis by the loss of chlorophyll (Khosravinejad & Farboondia, 2008).

Plants have defense mechanisms for acclimatization to saline environment. The most common one is accumulation of various kinds of compatible organic solutes (Serraj & Sinclair, 2002). These osmolytes contribute to cellular osmotic adjustment, detoxification of ROS, stabilization of enzymes/proteins and determine adaptive ability of the photosynthetic apparatus of crops growing under stressed environment (Yancey *et al.,* 1982; Shahbaz *et al.,* 2011). Proline and quaternary ammonium compounds help plants to maintain the cell turgor (Huang *et al.,* 2000). Proline is one of the well-known osmoprotectants accumulated to high levels under saline conditions (Khatkar & Kuhad, 2000). When wheat plants undergo osmotic adjustment, endogenous proline level may increase in response to salt stress generally believed to role as a shield against salt damage (Wang *et al.,* 2007).

For better growth of crops under salt stressed conditions, various research tools are being tried to counteract the effects of salinity. Exogenous application of osmoprotectant such as proline is well-known to induce abiotic stress tolerance in plants (Claussen, 2005; Ashraf & Foolad, 2007). For instance, foliar spray of proline or glycinebetaine counteracted the growth restrictions induced by NaCl in rice (Rahman *et al.,* 2002), wheat (Talat *et al.,* 2013) and maize (Ali *et al.,* 2007). The exogenous proline applications efficiently adjust osmotic potential and play a critical role in sustaining plant growth under osmotic stress (Serraj & Sinclair, 2002, Ashraf & Foolad, 2007). However, the information regarding the role of exogenous proline on early seedling growth in wheat is scarce. Hence, present study conducted to evaluate the potential effects of the foliar applied proline on growth, physiological and biochemical parameters at seedling stage of two wheat genotypes under saline conditions.

Material and Methods

A pot experiment was conducted to examine the role of foliar applied proline to ameliorate adverse effects of salinity on seedling growth of two wheat genotypes in growth chamber (Vindon, England) at Plant Physiology Lab, Nuclear Institute of Agriculture, Tandojam, Pakistan. Day and night lengths were kept at 14/10 h, with 25°C and 20°C temperatures, respectively. Relative humidity was maintained at 60%. Seeds of wheat genotypes, Khirman and ESW-9525 were surface sterilized in 3%

sodium hypochlorite solution for 10 minutes, rinsed with distilled water and air-dried. Seeds were sown in plastic bowls (15 in each) containing acid/water washed sand. Hoagland's solution (50 ml) was applied to each bowl. The experiment was conducted in completely randomized design (CRD) in factorial arrangement using three replications. After completion of emergence, 10 plants were maintained in each replicate for imposition of salinity and collection of data. Salinity based on NaCl salt was imposed after uniform stand establishment to one week old seedlings. Three salinity levels were maintained at 0, 60 and 120 mM NaCl designated as control, moderate and high salinity stress respectively.

Stock solution of 1M proline was formulated by dissolving 11.513 g of extra pure proline (Mw = 115.13; Scharlau, Spain) in 100 ml distilled water mixed 0.1% (v/v) Tween-20. The working solutions of proline (0mM, 50mM and 100mM) were prepared through dilution method.

One week after salinity imposition and maintenance, proline (50mM and 100mM) against water spray as control were applied at seedling stage using 50 ml solution per replicate. After seven days of proline application, samples were collected to record the data.

Measurements

Growth characters: Three weeks old seedlings were evaluated for growth response after being carefully removed from the sand. Root and shoot length of five randomly selected seedlings was recorded per replicate and averaged. Seedling fresh weight was determined immediately after harvest; dry weight was taken after drying at 70°C for 72 h.

Biochemical analysis: The chlorophyll *a* and *b* content (mg g^{-1} F. wt.) were determined with the method as described by Arnon (1949) by using the following formulae:

Chlorophyll *a* (mg/100 mL) = 0.999 A663 - 0.0989A645
Chlorophyll *b* (mg/100 mL) = -0.328 A663+ 1.77 A645

Total soluble sugar, free proline and glycine betaine were measured according to the methods described by Riazi *et al.* (1985), Bates *et al.* (1973) and Grieve & Gartan (1983) respectively.

However, total phenolic contents were measured by using the method of Waterhouse (2001).

Determinations of ionic contents: Sodium and potassium contents were measured according to Ansari and Flowers, (1986). In order to estimate the inorganic salts (Na^+ and K^+), 0.1 g dried leaf sample was digested with 0.2 mM acetic acid (CH3COOH) in water bath for 1 h pre-heated at 95°C, the extracted solution was filtered and suitable dilution was made. Na^+ and K^+ concentrations were determined by flame photometer (Jenway, Model PFP7).

Statistical analysis: Graphical representation of seedling growth data was made and standard error was computed using Microsoft Excel program (Microsoft Corporation, Los Angeles, CA, USA) for comparison of treatments. The collected data of biochemical parameters was analyzed by implying Fisher analysis of variance technique and significant treatments means were analyzed using least significance difference (LSD) test at 0.05 probability levels (Steel *et al.,* 1997).

Results

An increase in NaCl concentrations to 60 or 120 mM significantly reduced the length of root and shoot, seedling fresh and dry weight compared to control. However, foliar applied proline significantly affected early seedling growth (Figs. 1&2).

Proline application under different levels of NaCl salinity significantly ($p<0.05$) improved the root and shoot length of both genotypes over control (non-sprayed). Plants sprayed with 100 mM proline exhibited maximum root and shoot length even under high salinity (120 mM NaCl) in both genotypes. Response to exogenous applied 50 mM proline was not too much effective for shoot length at 60 mM NaCl in ESW-9525 (Fig. 1a) and for root length at high level of salinity in both genotypes (Fig. 1b) and behaved similar to control. Seedling fresh and dry weight improved for both genotypes under various salinity levels and significantly improved over control ((Fig. 2a,b). Nevertheless foliar applied proline (100 mM) exhibited maximum seedling fresh and dry weight in both genotypes under control and high salinity (120 mM NaCl) as well. The genotype Khirman improved seedlings fresh weight for 50 mM applied proline at 60 mM NaCl stress (Fig. 2a) and for seedling dry weight behaved like control under 120 mM NaCl (Fig. 2b). Although both genotypes respond well towards the foliar application of proline, nevertheless ESW-9525 performed better than Khirman under various levels of salinity.

Proline treatments showed positive impact on chlorophyll *a* and *b* contents of both wheat genotypes under salinity (Table 1). Foliar applied proline (50 mM) improved chlorophyll *a* however; proline application (100 mM) had significant ($p<0.05$) effect on chlorophyll *a* and *b* contents in Khirman. In case of ESW-9525, 100 and 50 mM proline treatments significantly ($p<0.05$) improved contents of chlorophyll *a* and *b* respectively over non-sprayed plants under control and salt stress as well. Moreover minimum chlorophyll contents were found in untreated plants of ESW-9525 at 120 mM NaCl salinity.

Similarly proline treatments significantly affected the TSS, TPC, Pro and GB contents of both wheat genotypes under salinity (Table 1). Foliar applied proline significantly ($p<0.05$) improved TSS in both genotypes under salinity and revealed that plants of Khirman subjected to 50 mM proline showed maximum TSS followed by 100 mM proline at 120 mM NaCl stress. Similarly, in case of ESW-9525 highest TSS was observed in seedlings exposed to 100 mM proline at 120 mM NaCl. Whereas minimum TSS was observed in Khirman for control (under non-saline conditions) and behaved similar to 50 mM proline.

Table 1. Influence of foliar applied proline on Chlorophyll a, b contents, total soluble sugars (TSS), total phenolics contents (TPC) [mg/g (FW)], proline (Pro), glycine betaine (GB) [umol/g (FW)], Na^+, K^+ (%) and K^+/Na^+ of two wheat genotypes grown under salinity.

Genotypes	Salinity NaCl mM	Foliar proline	Chl *a*	Chl *b*	TSS	TPC	Pro	GB	Na^+	K^+	K^+/Na^+ ratio
Khirman	0	0 mM	1.86e	1.02cd	5.84j	3.25k	5.16k	6.35k	0.38f	1.893 e	4.893 e
Khirman	0	50 mM	1.97c	0.93de	5.87j	3.51k	5.39jk	7.63j	0.29hi	2.243 b	7.566 b
Khirman	0	100 mM	2.23a	1.37a	6.97i	5.20j	6.28i	9.06hi	0.34g	2.126 c	6.133d
Khirman	60	0 mM	1.43k	0.70g	6.32i	6.83de	6.65h	10.19g	0.48d	1.433ij	2.970jk
Khirman	60	50 mM	1.49j	0.69gh	7.88f	6.15fg	7.03g	8.73hi	0.40ef	1.5367h	3.77 gh
Khirman	60	100 mM	1.67g	0.96de	7.37gh	6.67def	8.17e	12.70d	0.51cd	1.760f	3.450hi
Khirman	120	0 mM	1.10m	0.54ij	7.74fg	9.22b	8.54d	9.56gh	0.68a	0.9933m	1.460 m
Khirman	120	50 mM	1.05n	0.44j	10.4b	8.17c	8.84d	12.99d	0.65a	1.0267m	1.886 l
Khirman	120	100 mM	1.57i	0.71g	9.15d	10.57a	10.33a	15.24b	0.54c	1.370jk	2.083 l
ESW 9525	0	0 mM	1.79f	0.96de	7.14h	4.94j	4.49l	7.81j	0.34g	2.010 d	5.800 d
ESW 9525	0	50 mM	1.92d	1.23b	7.21h	5.36ij	4.82l	8.19ij	0.28i	2.393 a	8.353 a
ESW 9525	0	100 mM	2.16b	1.11c	8.12ef	5.46hij	5.71j	11.23f	0.32 gh	2.193bc	6.713 c
ESW 9525	60	0 mM	1.42k	0.77fg	8.36e	6.6def	6.23i	9.40gh	0.51cd	1.6667g	3.270ij
ESW 9525	60	50 mM	1.62h	1.04cd	8.93d	7.16d	7.88ef	11.34ef	0.43e	1.7933f	4.1033g
ESW 9525	60	100 mM	1.63h	0.73fg	10.08b	5.97gh	6.54hi	14.13c	0.42e	1.9167e	4.493 f
ESW 9525	120	0 mM	0.94o	0.59hi	8.97d	5.9ghi	7.80f	12.19de	0.58b	1.2667 l	2.1600l
ESW 9525	120	50 mM	0.97o	0.84ef	9.52c	6.41defg	9.34c	17.05a	0.60b	1.460hi	2.820 k
ESW 9525	120	100 mM	1.24l	0.70g	12.09a	7.84c	9.77b	14.82bc	0.52c	1.3100kl	2.160 l
LSD value			0.0411	0.1171	0.0403	0.1171	0.4161	0.888	0.0363	0.0814	0.3607
S.E.			0.0198	0.0576	0.0198	0.0576	0.2048	0.4373	0.0179	0.0401	0.1775

Foliar applied proline has significant influence on total phenolics in wheat genotypes under salinity. Maximum phenolics contents were observed in Khirman plants exposed to 100 mM proline followed by untreated plants at high level of salinity (120 mM NaCl). Similarly, ESW-9525 subjected to 100 mM proline showed maximum TPC under high salinity (120 mM NaCl) followed by 50 mM proline treatment at moderate saline stress (60 mM NaCl). However minimum TPC was observed in Khirman plants without proline spray under control and behaved like 50 mM proline.

Nevertheless, exogenously applied proline treatments significantly ($P < 0.05$) enhanced endogenous proline and glycine betaine contents in both genotypes under salinity as compared with control (Table 1). Foliar applied proline (100 mM) enhanced the endogenous level of proline in Khirman followed by ESW-9525 treated with 100 and 50 mM proline at 120 mM salinity. Conversely least proline contents were observed in untreated ESW-9525, followed by 50 mM proline under control. Likewise, proline application (50 and 100 mM) resulted in significant enhancement in GB contents under salinity (Table 1). Nonetheless maximum GB contents were observed in ESW-9525 subjected to 50 mM proline at 120 mM NaCl salinity followed by 100 mM proline in Khirman at same level of stress. Minimum GB was observed in Khirman without proline application followed by 50 mM proline and statistically at par with same level of proline in ESW-9525 under control.

Exogenously applied proline also affected the Na^+ and K^+ concentration in leaves of both wheat genotypes significantly ($p<0.05$) and also showed the considerable interaction with salinity for both Na^+ and K^+ concentration (Table 1). Minimum Na^+ and maximum K^+ concentration and K^+/Na^+ ratio was observed in leaves of ESW-9525 followed by Khirman, applied with 50 mM proline under control (without salinity). However, under various levels of salinity 100 mM foliar proline was more effective to improve K^+ contents and K^+/Na^+ ratio, accompanied by reduced Na^+ uptake in both genotypes. All the proline treatments resulted in better K^+/Na^+ ratio as compare to non-sprayed (control) in both genotypes apart from ESW- 9525 plants subjected to 100 mM proline at 120 mM level of salinity.

Discussion

The present study showed that NaCl stress reduced growth. These inhibitory effects of NaCl stress on plant growth and biomass production are well known (Ma *et al.,* 2013). The reason for low shoot and root length, their fresh and dry masses may be due to increase in osmotic potential by increasing salts, which leads to dehydration, ionic imbalance in transpiring leaves that caused reduction in meristem activity and cell elongation, consequently inhibit the growth of wheat plant (Zhu, 2001; Munns, 2005; Huang *et al.,* 2006). However, the results explored that exogenous proline applications had significant impact on root and shoot growth in wheat. This increase in growth by foliar proline may be due to its role as osmoprotectant (Yancey, 1994), membrane stabilizing (Bandurska, 2001), ROS scavenger (Matysik *et al.,* 2002), maintained turgidity in leaves (Huang *et al.,* 2000) and improved salt tolerance of wheat (Talat *et al.,* 2013), barley (Agami, 2013) and sunflower (Khan *et al.,* 2014).

The reduced growth in plants subjected to NaCl stress is often associated with a decrease in photosynthetic pigments and this reduction in chlorophyll contents due to NaCl stress revealed in wheat, maize and canola (Ali *et al.,* 2007; Raza *et al.,* 2006). Foliar applied proline significantly improved the chlorophyll contents of salt-stressed wheat plants (Table 1), either through stimulating its biosynthesis and/or inhibiting its degradation and consequently augment the rate of CO_2 diffusion and permitted higher photosynthetic rate (Ali *et al.,* 2007; Sharkey *et al.,* 2007). Similar results were also reported by Khan *et al.* (2010) in *Brassica campestris,* Abdelhamid *et al.* (2010) in bean, Abd El-Samad *et al.* (2011) in maize and Agami, (2013) in barely.

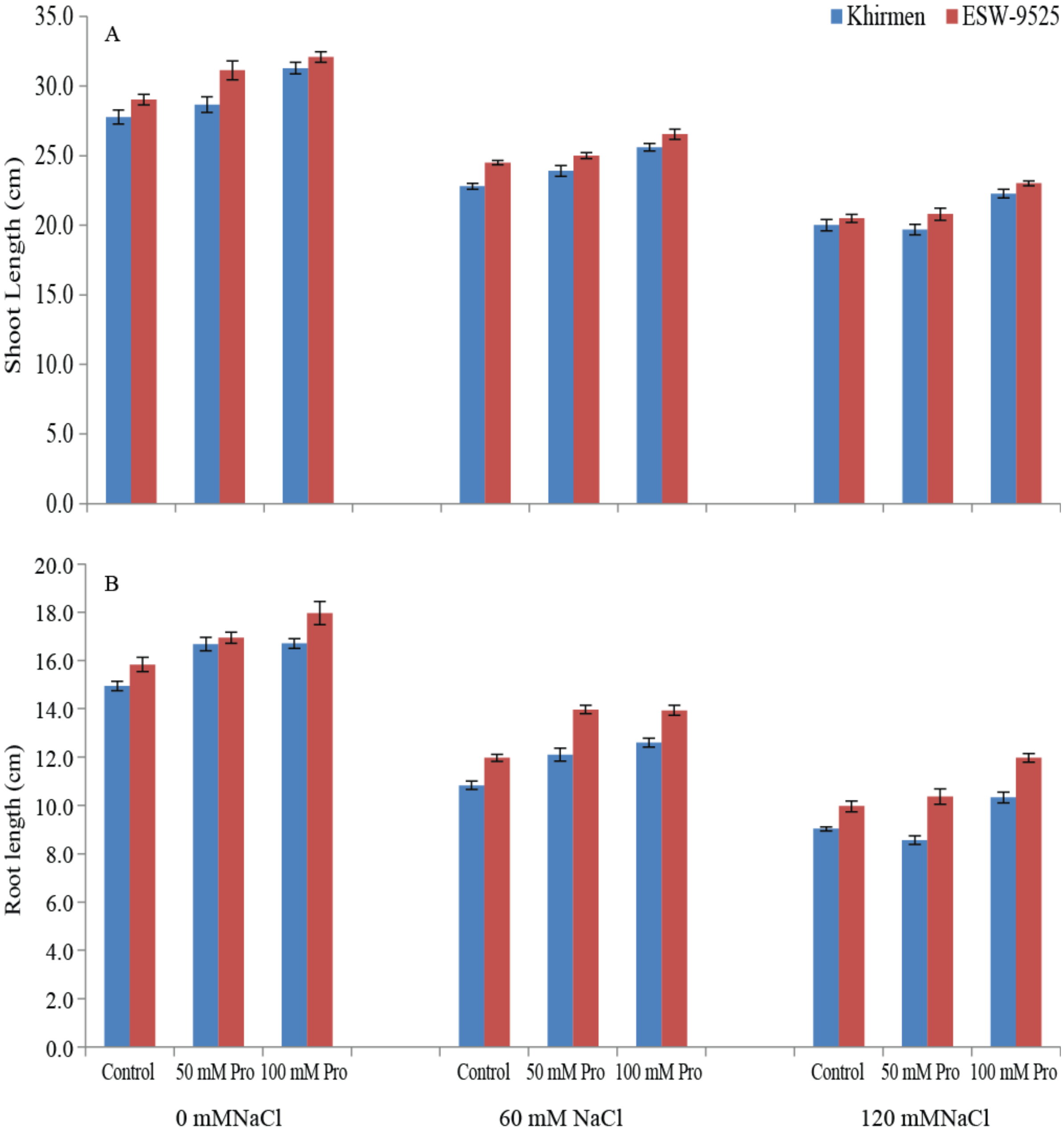

Fig. 1. Influence of foliar applied proline on (a) shoot length and (b) root length ± S.E. in two wheat genotypes under salinity.

Soluble sugars are main category of organic compatible solutes increased by salinity and play critical role in mitigating the effects of salinity either by osmotic adjustment or by conferring some desiccation resistance in plant cells (Hassanein *et al.*, 2009). Exogenous proline application might counteract the harmful effects of raised salinity on carbohydrate metabolism, resulting in improved entire plant growth (Nessim *et al.*, 2008; Abd El-Samad 2011). Similar results were also reported by Agami, (2013) in barely seedlings which concurred with our findings. These improvements in growth could be due to the role of proline in minimizing the harmful effects of salinity, by reducing Na^+ and accumulation of high K^+ in wheat leaves (Table 1).

The leaf phenolics content increased in response to NaCl stress and after the proline application (Table 1). The proline application caused higher accumulation of phenolics (Hayat *et al.*, 2013) which are known for their antioxidant potential (Ashraf, 2010), possible mechanism of proline-induced salt stress tolerance of present study. Lattanzio *et al.*, (2009) reported that an application of 0.5 mM proline to in vitro grown oregano seedlings elicit the accumulation of phenolics compound in that plant. Similarly, total phenolics concentrations significantly increased in faba bean leaves under salinity stress (Dawood *et al.*, 2014).

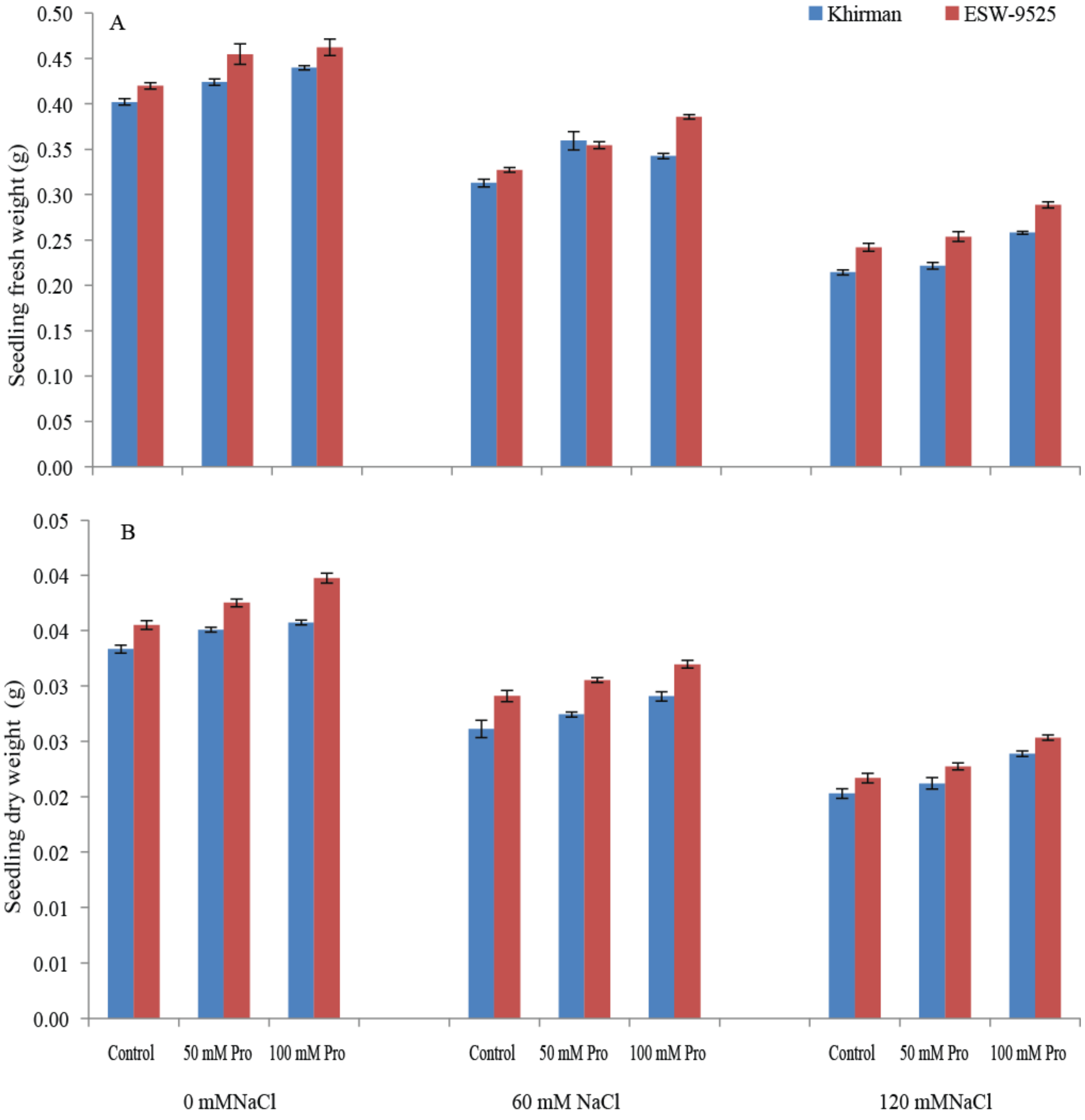

Fig. 2. Influence of foliar applied proline on (a) seedling fresh weight and (b) seedling dry weight ± S.E. in two wheat genotypes under salinity.

It has been found that a salt stress up-regulated the enzymes involved in proline biosynthesis (Munns, 2005). The results of this investigation (Table 1) imply that saline stress enhanced the accumulation of proline and further improvement was noted by exogenous application of proline in the leaves of both wheat genotypes. The better proline accumulation may result from augmented proteolysis or by reduced protein synthesis. Similarly increased levels of Pro under salt stress were also reported in two wheat cultivars (Khatkar & Kuhad, 2000). Shahid *et al.* (2014) support present observation that level of free proline contents elevated in response to NaCl stress which is further augmented by foliar spray of proline in pea plants. Likewise, exogenous application of proline to *Pancratium maritimum*, caused significant increase in endogenous level of proline in shoots of both stressed and non-stressed plants (Khedr *et al.,* 2003) which is also coherent with current findings.

The wheat plants from both genotypes, exhibited a significant rise in endogenous level of glycine betaine in leaves, upon exposure to foliar proline and NaCl stress as well (Table 1). Chen & Murata, (2008) reported that plants known to accumulate GB naturally grow well under saline environment also observed in our study. However, some reports showed that exogenous proline in *Atriplex halimus* had no effect on endogenous glycine betaine content which might be the result of feedback inhibition (Hassine *et al.,* 2008). The present observation is in accordance with Shahid *et al.* (2014) who reported that foliar applications of proline or LP leaf extract showed a significant elevation in endogenous organic osmolytes, including glycine betaine in plants grown under salinity and/or $NiCl_2$ stress and consequently caused an improvement in osmotic adjustment capacity. This improved osmotic adjustment potential in terms of enhanced level of organic osmolytes might be resulted in better photosynthetic activity.

The salinity levels caused significant and gradual increase in Na^+ content, reduction in K^+ content and K^+/Na^+ ratio, compared with control plants (Table 1). Similar outcomes have also been reported in different plant species (Abdelhamid *et al.,* 2010; Doğan, 2011). While foliar application of proline significantly restricted Na^+ uptake and improved the uptake of K^+ and caused K^+/Na^+ ratio high in both wheat genotypes under control and NaCl stress. These observations are coherent with Nessim ***et al.*** (2008) in corn, Abd El-Samad ***et al.*** (2011) in broad bean, Talat ***et al.*** (2013) in wheat and Dawood ***et al.*** (2014) in faba bean plants.

Conclusions

It may be concluded that salt-induced adverse effects on photosynthetic capacity in wheat can be alleviated by the exogenous application of proline. Foliar applied 100 mM proline not only improved root and shoot length, seedling fresh and dry weight but also significantly enhanced the level of chlorophyll contents, osmoprotectants, antioxidant phenolics and improved osmotic adjustment. Moreover, protecting photosynthetic machinery of wheat against salt induced oxidative stress by proline might have contributed to better growth of both wheat genotypes under salinity. However, genotype ESW-9525 was comparatively better in osmotic adjustment and accumulation of osmoprotectants than Khirman, which could explain its ability to grow well under salinity than Khirman. The present study suggests that exogenously applied proline can improve seedling growth under salt stress which explores its potential to mitigate adverse effects of salinity.

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