Pak. J. Bot., 50(5): 1735-1744, 2018.

BENEFICIAL ROLE OF FOLIAR-APPLIED PROLINE ON CARROT (*DAUCUS CAROTA* L.) UNDER SALINE CONDITIONS

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Abstract

A pot experiment was conducted to appraise the beneficial role of foliar application of proline on two cultivars of carrot (*Daucus carota* L.) cv. Arwa red long and cv. Red corl grown in pots under salt-stress conditions. There were two levels of salinity i.e. non-stress (0 mM) and NaCl stress (150 mM) and three levels of foliar applied proline (0, 5, 10 mM). Growth, gas exchange and chlorophyll fluorescence parameters decreased, while enzymes activities and proline contents increased in carrot plants under saline conditions. Proline treatment as foliar spray significantly enhanced growth, gas exchange, chlorophyll fluorescence, activity of peroxidase, root K^+ and Ca^{2+} and shoot K^+ contents, while decreased Na^+ contents of root in both carrot cultivars. Arva Red Long showed better performance as compared to cultivar Red Corl due to improved growth, gas exchange characteristics, antioxidant enzymes activities (SOD, POD), high free proline and K^+ and Ca^{2+} contents.

Key words: Chlorophyll florescence, Free proline, Gas exchange, Peroxidase, Superoxide dismutase.

Introduction

Abiotic factors such as soil salinity adversely affects yield of horticultural crops throughout the world (Chen *et al.*, 2014). Seed germination, seedling growth and various physiochemical attributes inhibited under salinity stress in different vegetable crops, like cabbage (*Brassica oleracea* capitata L.), mustard (*Brassica juncea*) (Sarker *et al.*, 2014), tomato (*Lycopersicum esculentum* L.) and cauliflower (*Brassica oleracea* L.) (Wahid *et al*., 2014) etc. Moreover, salinity stress reduces fresh and dry weight, photosynthetic pigments and photosynthesis rate of different crop species (Kanwal *et al.*, 2013; Xiaochuang *et al*., 2017).

Proline exerts its effect in lowering the oxidative stress and enhancing tolerance to salinity stress (Gurmani *et al*., 2014). For example, under stress proline can scavenge free radicals, protect antioxidant enzymes and reduce oxidation of cellular membrane system (Wutipraditkul *et al*., 2015). There are many techniques to increase endogenous proline level e.g., by over expression of proline biosynthesis gene (s) (Zhu *et al*., 1998), or knock-out of degradation gene (s) (Nanjo *et al*., 1999) and via foliar application of proline on under unfavorable conditions (Ashraf & Foolad, 2007). Proline application (0.5 g/L) lead to increased biomass (34.6%) and accumulation of osmolytes such as alanine, glutamate, N-acetyl-tryptophan, mannitol, and citrulline in *Tetragenococcus halophilus* under salt stress (He *et al*., 2017). Proline alleviated Na^+ toxicity in sainfoin seedling by maintaining nutrients level and increased synthesis of free proline (Wu *et al*., 2017). Seed priming with proline has been reported to increase salt stress tolerance in maize cultivar (cv. Safaid Afgoi) (Perveen *et al*., 2018). Similarly, proline treatment at the rate of 0.8 mM could increase growth, photosynthetic rate, activities of enzymes under salt stress in chilli (Bhutt *et al*., 2016).

Carrot (*Daucus carota* L.) is ranked at 10^{th} position among commercially important vegetables worldwide (Simon *et al*., 2008). It has marvelous medicinal importance as it is rich in nutrients, carotene, vitamin A and C, mineral nutrients (sodium, potassium), fiber, carbohydrates and proteins (Ahmad *et al*., 2005).

Various abiotic stresses including salinity are the major threats to carrot production. In order to assess the beneficial effect of proline under abiotic stresses this study was appraised to identify that whether or not foliar application of proline can ameliorate adverse effects of salt stress on carrot plants. To achieve this, we observed the beneficial roles of the foliar application of proline on growth, photosynthesis, antioxidant defense system and mineral nutrients of carrot plants.

Materials and Methods

To assess the beneficial roles of proline on carrot plants when applied as foliar spray a pot experiment was executed under saline conditions under natural climatic conditions. Temperature of day and night was 27 ± 4°C and 12 ± 3 °C, respectively; day and night lengths were 8.8 h and 16 h respectively and relative humidity of day was 60%. A completely randomized design was used for this experiment. There were two carrot cultivars (Arwa Red Long and Red Corl), two levels of NaCl i.e., control (0 mM) and salt stress (150 mM) and three proline levels i.e., 0, 5 and 10 mM. Ten seed of each cultivar were sown in pots. Salt stress was applied after 4-weeks of germination. Salt treatment was applied by daily increasing salt in aliquots of 50 mM so that the final level of 150 mM was achieved on the 3^{rd} day. Plants were irrigated with 200 ml water daily to keep moisture in pots. Three proline levels were foliarly applied on the shoots of 38 days old plants. Data collection was performed of 52 days old plants of single time foliar application of proline.

Growth parameters: Fresh weight and length of roots and shoots of two plants were obtained. Then same samples dried in oven (at 65ºC) and dry matter was examined.

Gas exchange and chlorophyll fluorescence: Gas exchange parameters were measured during 9.30 a.m. to 1.00 p.m. under PPFD of 100 µmol (photon) m^{-2} s^{-1}, relative humidity of 54 ± 5%, and 20/6ºC day/night temperature cycle with an infrared gas analyzer (LCA-4ADC; UK). A Multi-Mode Chlorophyll Fluorometer (Winn Avenue Hudson, USA, Model, OS5P Opti-Sciences, Inc.) was used for measuring the efficiency of photosystem II (PSII) according to the protocol of Strasser *et al*., (1995).

Total soluble proteins: Proteins were measured using the method of Bradford (1976).

Extraction of enzymes: Fresh leaves (0.5 g) were placed in 10 ml 50 mM phosphate buffer (pH 7.8), centrifuged (15000 × *g*) at 4ºC for 20 min. and supernatant was used for enzymes activities measurement (Fridovich, 1974).

Superoxide dismutase (SOD) activity: Activity of SOD was determined by Giannopolitis & Ries (1977) method.

Catalase (CAT) and Peroxidase (POD) activity: Chance & Maehly (1955) protocol was used for measuring activities of these enzymes with UV-visible spectrophotometer (IRMECO U2020).

Leaf free proline determination: The method of Bates *et al*., (1973) was applied for the determination of proline contents. Fresh leaf tissue (500 mg) was extracted in 10 ml of 3% sulfosalicylic acid. To 2 ml of extract added 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. Then mixture was incubated at 95ºC for 60 min. After cooling added 4 ml of toluene, vortexed and read the absorbance of chromophore layer at 520 nm with spectrophotometer (IRMECO U2020).

Determination of mineral elements: The determination of mineral ions in shoots and roots of carrot plants was according to Allen *et al*., (1985) method.

Statistical analysis: Three-factor factorial analysis of variance (ANOVA) of data was calculated using the Co-STAT computer software according to Snedecor and Cochran (1980) method.

Results

Growth parameters: Fresh and dry matter significantly reduced in both carrot cultivars (Arwa Red Long and Red Corl) under 150 mM NaCl stress (Fig. 1A, B). The two carrot cultivars exhibited significant difference as Arwa Red Long was higher in shoots and roots weights than those of Red Cort under salt stress or non-stress conditions. Foliar-applied proline significantly increased biomass and the two carrot cultivars also differed significantly in shoot dry weight under varying levels of foliar treatment with proline as cv. Arwa Red Long showed more positive response particularly at 10 mM concentration. The fresh and dry mass of the roots of cultivar Arwa Red Long were higher than those of Red Corl cv. Foliar-applied proline slightly enhanced the root weights that were decreased under salt stress of carrot plants (Fig. 1C, D).

Salt stress decreased length of shoot while effect was not prominent on root length of both carrot cultivars (Fig. 1E, F).Both carrot cultivars showed a variable response with respect to root or shoot length as cultivar Arwa Red Long was high and Red Corl low in these root growth attributes. Although, statistically non-significant, however**,** 10 mM proline concentration seemed to performed better in increasing shoot and root lengths.

Effect on photosynthetic characteristics: Under salt stress, photosynthetic and transpiration rates (*A* and *E*) were significantly decreased in both carrot cultivars (Fig. 2A, B). Cultivar Arwa Red Long showed higher '*A'* than Red Corl particularly under normal conditions. Proline increased '*A'* and '*E'* values of both carrot cultivars.

Water use efficiency (WUE) (Fig. 2C) remained unchanged, while stomatal conductance (g_s) (Fig. 2D) was decreased under salt stress. C_i (Fig. 2E) and C_i/C_a ratio (Fig. 2F) did not change, however, the two cultivars varied with regard to A/E and g_s, where Arwa Red Long was higher in '*A/E*' and 'g_s' than cv. Red Corl, while reverse was true for cv. Red Corl in terms of *C*i, and C_i/C_a ratio. Moreover, Arwa Red Long excelled in g_s value than cv. Red Corl. Foliar spray of proline did not incorporate any prominent significant effect on WUE, however, g_s, C_i and C_i/C_a ratio enhanced in carrot plants.

Salt stress decreased *F*v/*F*m (Fig. 3A), q_P (Fig. 3B) and q_N (Fig. 3C) in both carrot cultivars (Table 1). For example, the values of Fv/Fm and qN of cultivar Red Corl were higher than those of the Arwa Red Long, while the reverse was true for Arwa Red Long which had higher value of qp. *F*v/*F*m, q_P and q_N values significantly increased by proline in carrot plants. However, an exception was observed in the case of cv. Red Corl grown under normal conditions and sprayed with 10 mM proline, where *F*v/*F*m was lower than the values measured in plants sprayed with 5 mM proline and of the non-sprayed plants. Moreover, proline enhanced q_P values more in cv. Arwa Red Long than in Red Corl.

Effect on leaf free proline and total soluble protein contents: Salt stress significantly increased leaf free proline contents in both carrot cultivars (Fig. 3D). The contents of proline were higher in Arwa Red Long than Red Corl. Proline application (10 mM) significantly increased proline in carrot plants under NaCl stress or non-stress conditions. Under salt stress, total soluble protein contents (Fig. 3E) significantly decreased, while increased by proline in both carrot cultivars (Table 1).

Effect on activities of antioxidants: Activities of catalase (CAT) (Fig. 3F) and peroxidase (POD) did not change (Fig. 3G). However, the application of proline as foliar spray enhanced activity of POD in both carrot cultivars and decreased CAT activity in cv. Arwa Red Long (Fig. 3F, G; Table 1). Activity of superoxide dismutase (SOD) (Fig. 3H) was markedly increased in carrot cv. Red Corl. Superoxide dismutase activity increased in cv. Red Corl under salt stress conditions. Proline treatment decreased SOD activity in Arwa Red Long plants and caused the same enzyme to increase in Red Corl cultivar under saline conditions.

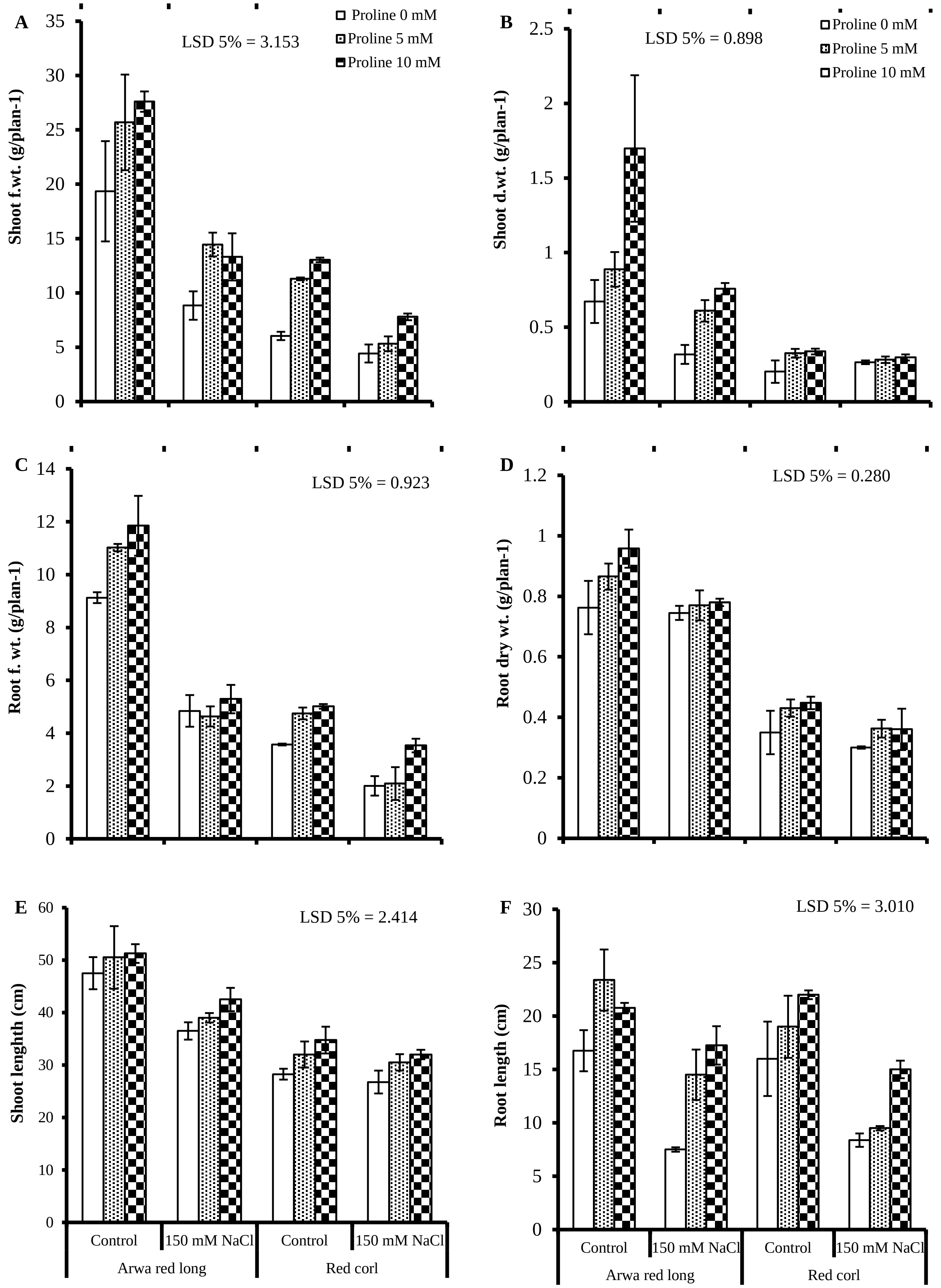

Fig. 1. Shoot and root fresh and dry weights, and shoot and root length of carrot (*Daucus carota* L.) subjected to foliar applied proline under salt stress. LSD = least significance difference.

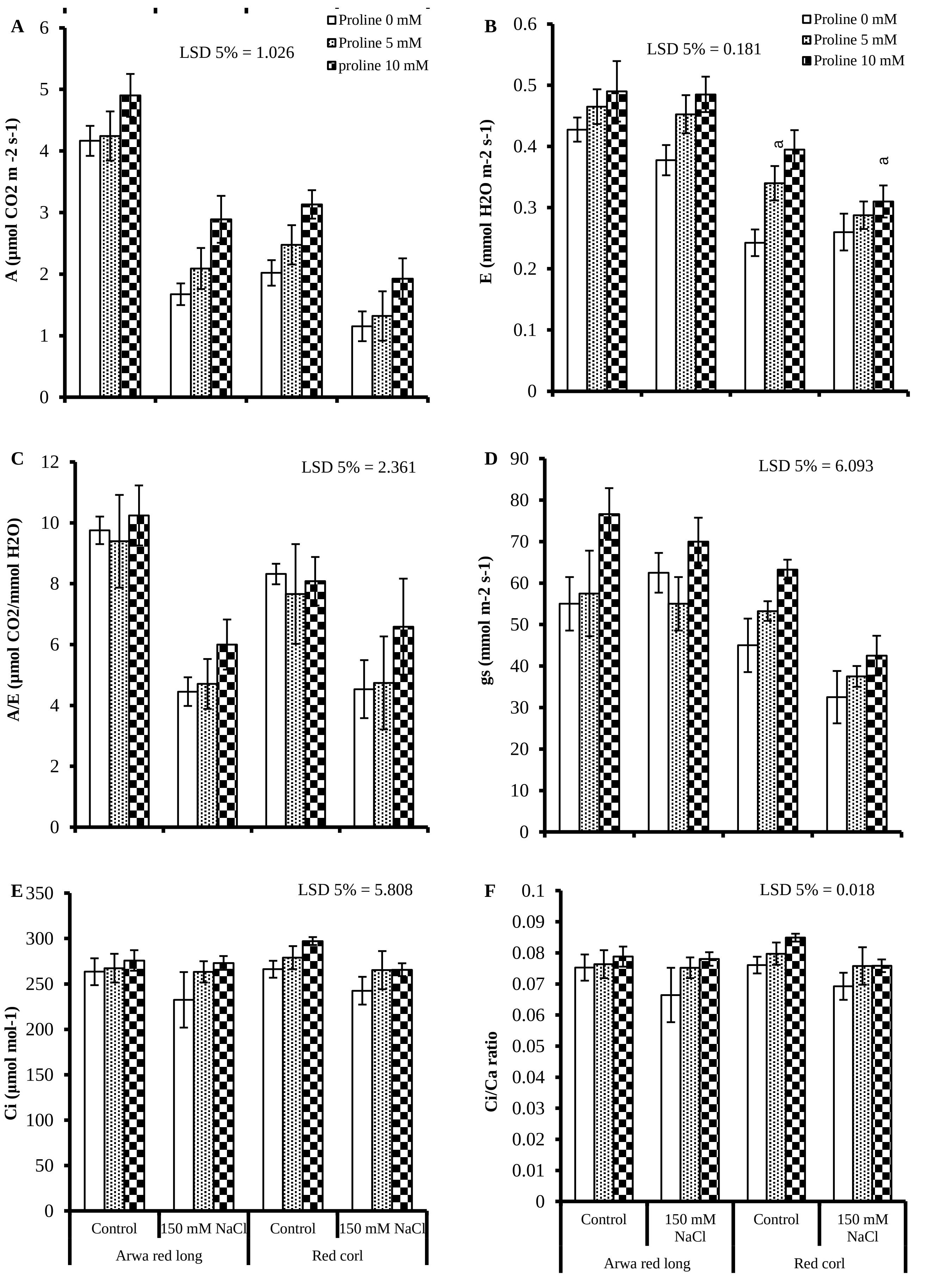

Fig. 2. Photosynthetic rate (*A*), water use efficiency (*A/E*), stomatal conductance (g_s), sub-stomatal CO_2 concentration (C_i) and C_i/Ca ratio of carrot (*Daucus carota* L.) subjected to foliar applied proline under salt stress. LSD = least significance difference.

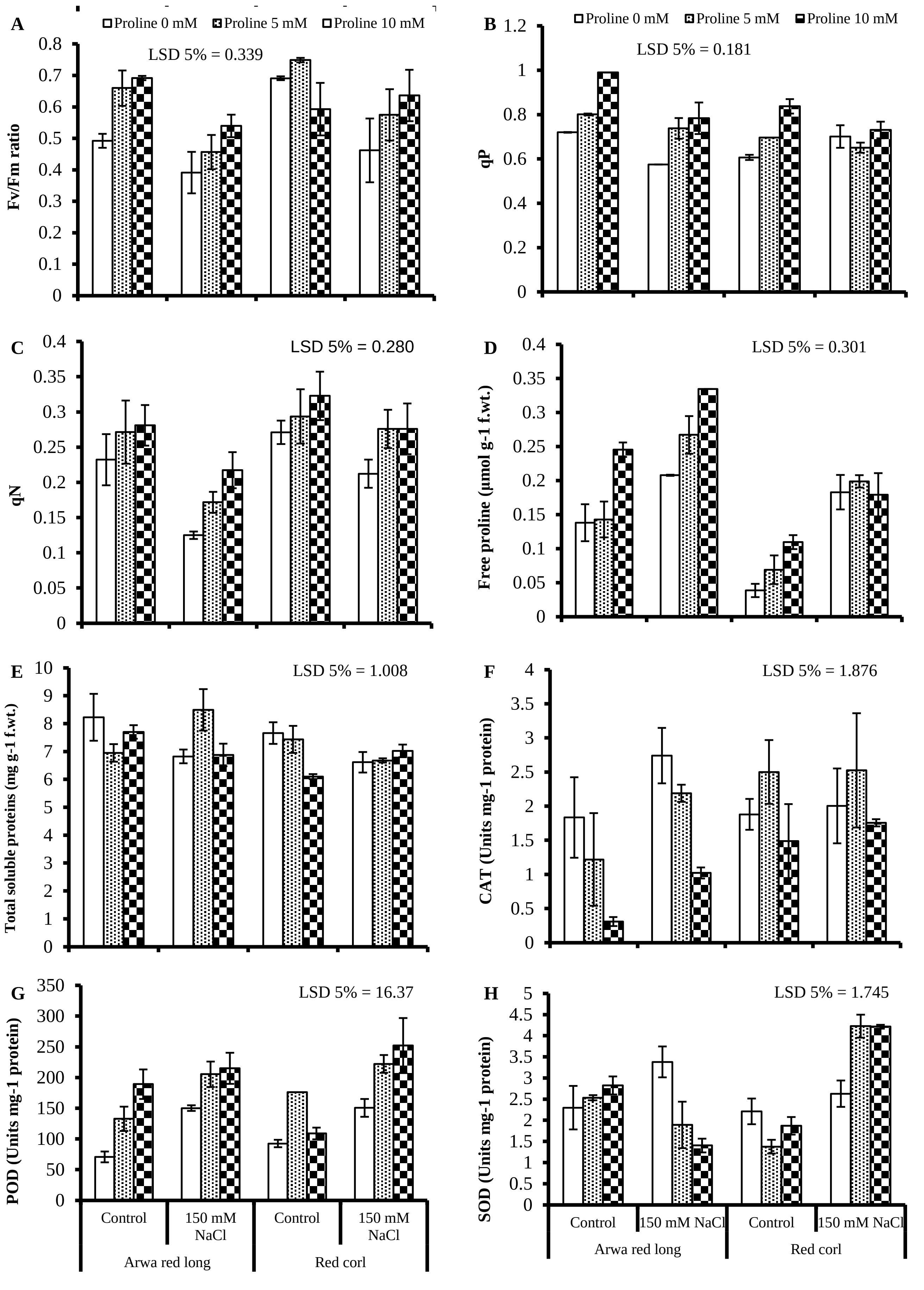

Fig. 3. Chlorophyll fluorescence, free proline, total soluble proteins, and activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) of carrot (*Daucus carota* L.) subjected to foliar applied proline under salt stress. LSD = least significance difference.

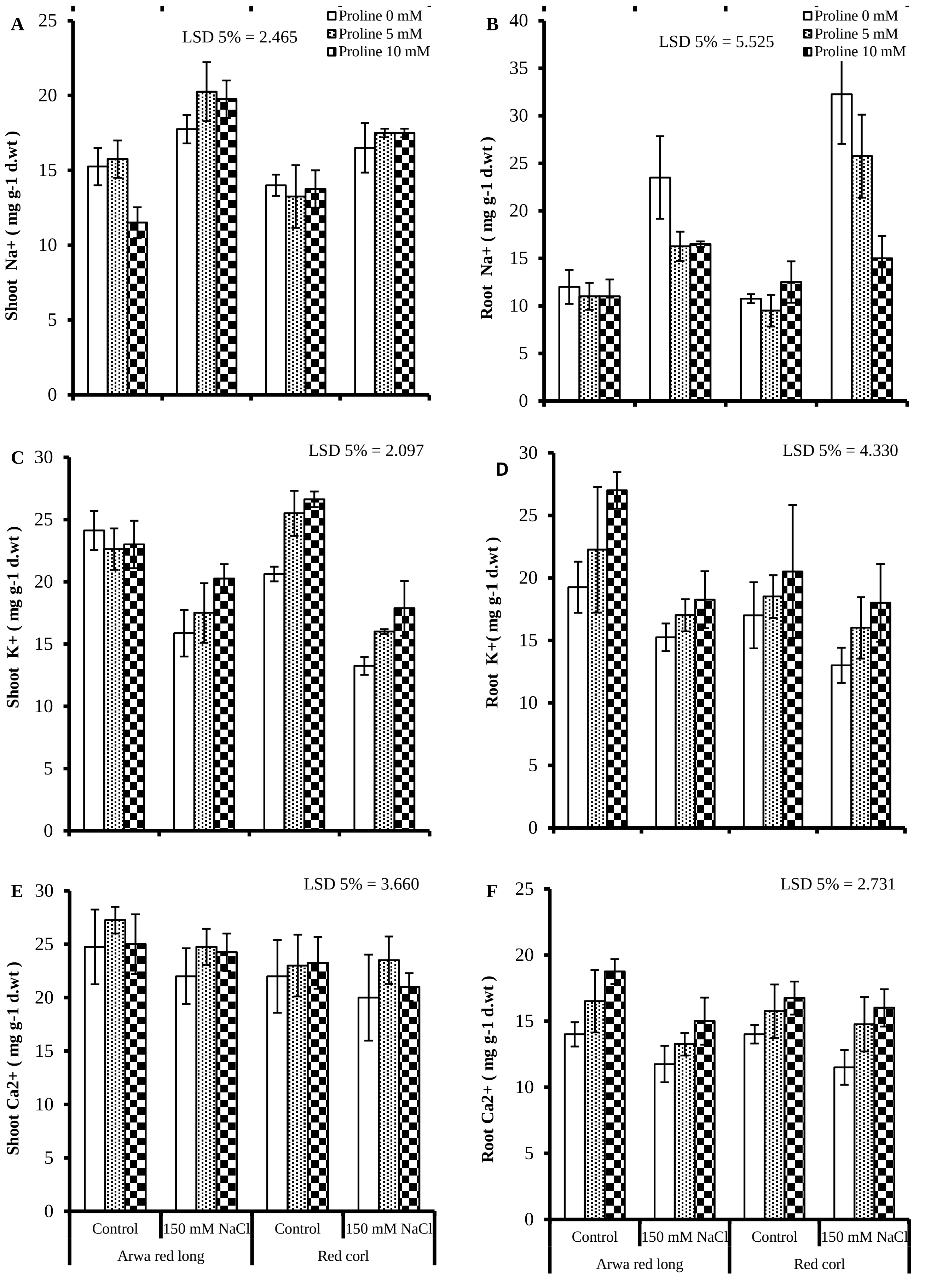

Fig. 4. Shoot and root mineral contents of carrot (*Daucus carota* L.) subjected to foliar applied proline under salt stress.
LSD = least significance difference.

Table 1. Mean squares from analysis of variance of the data for various growth, gas exchange characteristics, chlorophyll fluorescence, free proline, soluble proteins and activities of antioxidant enzymes of carrot (*Daucus carota* L.) plants foliary-applied with proline under non-saline saline and saline conditions.

Source of variation	df	Shoot f. wt.	Shoot dry wt.	Root f. wt.	Root d. wt.	Shoot length
Salinity (S)	1	1254***	0.845**	221.8***	2.305***	2296***
Cultivars (Cvs)	1	797.0***	3.483***	175.3***	0.082**	456.3***
Proline (Pro)	2	147.6***	0.675**	9.429***	0.039*	117.3*
S × Cvs	1	179.4**	0.083**	44.33**	0.002ns	216.8**
Pro x Cvs	2	8.476ns	0.447*	0.044ns	0.002ns	1.333ns
S × Pro	2	14.36ns	0.151ns	2.662ns	0.009ns	0.583ns
S × Cvs × Pro	2	4.599ns	0.118ns	1.400ns	0.004ns	4.75ns
Error	36	16.91ns	0.098	0.931ns	0.009	26.22
Source of variation	**df**	**Root length**	***A***	***E***	***A/E* (WUE)**	**g_s**
Salinity (S)	1	35.02ns	20.98***	0.248***	7.112ns	3512***
Cultivars (Cvs)	1	697.7***	32.57***	0.012ns	167.6***	855.1*
Proline (Pro)	2	180.8***	3.907***	0.036***	5.776ns	963.1**
S × Cvs	1	2.083ns	3.921**	9.188ns	12.12ns	747.3*
Pro × Cvs	2	26.94ns	0.009ns	2.688ns	0.032ns	116.8ns
S × Pro	2	17.48ns	0.005ns	8.313ns	2.817ns	126.8ns
S × Cvs × Pro	2	6.599ns	0.183ns	0.006ns	0.424ns	13.52ns
Error	36	14.32	0.384	0.004	4.744	135.4
Source of variation	**df**	***Ci***	**C_i/C_a**	***Fv/Fm***	**q_P**	**q_N**
Salinity (S)	1	536.9ns	4.383ns	0.075*	0.049**	0.041**
Cultivars (Cvs)	1	3815*	3.114*	0.222***	0.075***	0.052***
Proline (Pro)	2	2959*	2.416*	0.057*	0.138***	0.017*
S × Cvs	1	326.2ns	2.663ns	0.003ns	0.043**	0.007ns
Pro × Cvs	2	0.278ns	2.273ns	0.020ns	0.015*	2.189ns
S × Pro	2	341.3ns	2.786ns	0.021ns	0.019*	.170ns
S × Cvs × Pro	2	325.0ns	2.653ns	0.026ns	0.013ns	0.001ns
Error	36	906.3	7.399	0.014	0.004	0.003
Source of variation	**df**	**Free proline**	**Soluble proteins**	**SOD**	**POD**	**CAT**
Salinity (S)	1	0.104***	4.212*	1.605*	513.0 ns	2.688ns
Cultivars (Cvs)	1	0.131***	0.806ns	7.172***	6019***	3.008ns
Proline (Pro)	2	0.023***	1.021ns	0.060ns	2754***	4.998 **
S × Cvs	1	0.001ns	0.014ns	14.47***	1623ns	1.571ns
Pro × Cvs	2	0.008**	0.132ns	1.959**	2725ns	2.031ns
S × Pro	2	0.002ns	2.919*	0.413ns	640.8ns	7.013ns
S × Cvs × Pro	2	0.002ns	4.230**	6.152***	6671*	0.065ns
Error	36	0.001	0.742	0.370	1563	0.856

***, **, and *= significant at 0.001, 0.01, and 0.05 levels respectively; df= Degrees of freedom
ns= Non-significant *A* = Net CO_2 assimilation rate; *E* = Transpiration rate; g_s = Stomatal conductance
*C*i = Sub-stomatal CO_2 conc.; *A/E* (WUE) = water use efficiency; Fv/Fm = Efficiency of photosystem II
q_P = Photochemical quenching; q_N = Co-efficient of non-photochemical quenching
SOD = Superoxide dismutase; POD = Peroxidase; CAT = Catalase

Table 2. Mean squares from analysis of variance of the data for shoot and root mineral contents of carrot (*Daucus carota* L.) plants foliary-applied with proline under non-saline saline and saline conditions.

Source of variation	Df	Shoot Na^+	Root Na^+	Shoot K^+	Root K^+	Shoot Ca^{2+}	Root Ca^{2+}
Salinity (S)	1	20.02ns	80.08ns	4.083 ns	85.33ns	77.52ns	0.083ns
Cultivars (Cvs)	1	2210***	1302***	581.0***	243**	31.69ns	60.75*
Proline (Pro)	2	4.938ns	144.1*	48.35*	92.67ns	23.77ns	58.77**
S × Cvs	1	7.521ns	108ns	30.08ns	27ns	1.686ns	8.333ns
Pro × Cvs	2	6.895ns	20.08ns	18.44ns	1.521ns	0.146ns	0.771ns
S × Pro	2	12.27ns	156.6**	4.630ns	3.813ns	1.938ns	0.063ns
S × Cvs × Pro	2	6.396ns	61ns	12.91ns	9813ns	5.063ns	2.896ns
Error	36	6.647	29.69	9.628	31.92	27.69	9.069

***, **, and *= Significant at 0.001, 0.01, and 0.05 levels respectively; df= Degrees of freedom; ns= Non-significant

Effect on mineral nutrients: The contents of shoot and root Na^+ (Fig. 4A, B) were slightly increased (non-significantly) in carrot plants under 150 mM level. Foliar application of proline (10 mM) significantly decreased root Na^+ in both carrot cultivars (Table 2). Shoot and root K^+ (Fig. 4C, D) and Ca^{2+}contents (Fig. 4E, F) did not vary in carrot plants under salt stress. Examination of Fig. 4C indicated that at 150 mM NaCl, the shoot K^+ content of Arwa Red Long was much higher than that of Red Cort cultivar under the same conditions. However, various levels of foliar-applied proline increased shoot K^+ (Fig. 4C) and root Ca^{2+} (Fig. 4F) contents in both carrot cultivars. Potassium and calcium contents of Arwa Red Long were higher than those in the cv. Red Cort.

Discussion

In this study, proline application at vegetative stage improved growth parameters under NaCl stress. Similar studies were carried out in different crop species (Huang *et al*., 2009; Deivanai *et al.,* 2011; Nounjan & Theerakulpisut, 2012; Hasanuzzaman *et al*., 2014; Khan *et al*., 2014), in which proline showed positive role in enhancing growth under saline conditions. Proline treatment might have contributed in the osmoregulation (accumulation of inorganic and organic osmolytes) that could have played role in H_2O and minerals uptake, increased CO_2 assimilation and consequently improved growth of carrot plants.

Salinity stress adversely affected gas exchange characteristics, however, foliar application of proline significantly increased all gas exchange characteristics of both carrot cultivars in the current study. Chlorophyll fluorescence indices such as F_v/F_m provide a rapid and valuable tool for monitoring physiological status of plants (Kautz *et al*., 2014; Hannachi *et al*., 2014). In the current study, low F_v/F_m despite it occurs under control conditions in cv. Red Corl in comparison to F_v/F_m at 150 mM NaCl level of the same cv., which is higher than the control treatments. The data of current study indicated that under 150 mM salinity the value of F_v/F_m was less reduced in Red Corl than in Arwa Red Long cv. This indicates higher stability of PSII of Red Corl. Ali *et al*., (2007) and Deivanai *et al*., (2011) have also observed similar findings in maize and rice by treatment with proline. Proline accumulation lowers the level of free radicals (singlet oxygen species) generated in chloroplast thylakoids membranes and protected plants from photoinhibition under environmental stresses (Rejeb *et al*., 2014). Proline accumulation under stress might be directly involved in the scavenging of free radicals or activate antioxidant defense system (Rejeb *et al*., 2014). Similar to previous studies in mustard (Iqbal *et al*., 2014) and wheat (Gurmani *et al*., 2014; Konotop *et al*., 2017) free proline contents were accumulated in the present study under both salt stress and proline application in both the carrot cultivars.

Total soluble protein was decreased in carrot plants under salt stress, while foliar applied proline did not change proteins significantly in the current study. However, total soluble proteins were decreased in Red Corl cultivar. Similarly, Deivanai *et al*., (2011) reported that proline decreased protein content in rice plants.

Proline has been considered to be responsible for changing the antioxidant-related genes expression under environmental stresses (de Carvalho *et al*., 2013). So, it might be take part in the detoxification of O_2^{-} radical by enhancing SOD activity (Xu *et al*., 2009) or increasing the level of chloroplast Cu/Zn isoforms of SOD (de Carvalho *et al.*, 2013). Foliar application of proline increased POD activity, decreased CAT activity in both cultivars, while SOD activity decreased in Arwa Red Long and increased in Red Corl under salt stress in the current study. Haung *et al*., (2009) reported an enhanced antioxidant enzymes activities under proline treatment in cucumber. However, Konotop *et al*., (2017) reported no significant effect of proline on SOD activity under cadmium stress.

Low uptake and restricted transport of Na^+ to shoots help the plant to combat negativities of salt stress (Perveen *et al*., 2012). Gilberti *et al*., (2014) were of the view that proline metabolism was responsible for restoring energy balance in cellular compartments. In current study, foliar application of proline might have protected carrot plants from deleterious effects of salt stress through maintaining ions homeostasis (via increased uptake of essential K^+, Ca^{2+} and especially exclusion of toxic root Na^+) and osmoregulation i.e., high accumulation of inorganic (K^+, Ca^{2+}) and organic (free proline, total soluble proteins) osmolytes. Accordingly, the salt tolerance of the carrot cultivars is enhanced, which improves photosynthetic activity via stabilization of thylakoid membranes and consequently increased growth and yield. It has been reported that proline protects plants through ROS scavenging, protection of enzymes structure and integrity of membranes and osmotic adjustment (Ozden *et al*., 2009).

Conclusions

Salinity stress adversely affected growth and photosynthetic characteristics, while increased enzymes activities (SOD and POD) and contents of proline in both carrot cultivars in the present investigation. Overall, proline treatment as foliar spray improved growth, photosynthesis, activities of antioxidant enzymes, K^+ of root and shoot and root Ca^{2+}, while decreased Na^+ contents of root of both carrot cultivars. Of the two carrot cultivars, Arwa Red Long performed better than Red Corl due to improved growth, photosynthetic attributes, antioxidant defense system and osmoregulation under saline or non-saline conditions.

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(Received for publication 29 November 2017)