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Inducing salinity tolerance in red pepper (*Capsicum annuum* L.) through exogenous application of proline and L-tryptophan

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Abstract

Deterioration of agricultural lands due to salinity is one of the serious threats in irrigated areas of the world. Confronting the influence of salinization in agriculture is a key for achieving food security. Red pepper (Capsicum annuum L.) is not only economically but also nutritionally, important for human diet. A study was conducted to ameliorate the effect of salinity on the production and quality of red pepper by exogenous application of osmolytes. Two potential osmolytes i.e. proline and L- tryptophan were exogenously applied solely and in combination (@ 50 mM and 25 ppm, respectively). There were three salinity levels i.e. EC_e; 0.6, 4.04 and 6.11 dS m^{-1}. Results showed that plant height, root length, plant biomass and yield were significantly decreased, while significant increase in Na^+ and Na^+/K^+ ratio was observed with increasing level of salinity. A significant improvement in growth, yield and quality was observed when both osmolytes were exogenously applied under salinity stress. The combined use of proline and L-tryptophan was more effective for reducing the inhibitory effect of salinity as compared to sole application of these osmolytes. It is concluded that the combined application of proline and L-tryptophan (@ 50 mM and 25 ppm, respectively) was more effective for improving growth of red pepper under salinity stress (0.6 dS m^{-1}).

Keywords: Salinity, osmolytes, ionic balance, physiology, pepper

Introduction

Land deterioration due to salinity is a major problem in arid regions of the world, causing about 30-50% reduction in crops yield (Munns, 2005; Keshtehgar *et al.*, 2013). About 19.5% of world's agricultural land is affected by salinity fuelled by aridity (Ashraf and Foolad, 2007). Many of physico-chemical processes of plant development, like photosynthesis, respiration, nutrient uptake, hormonal balance, transpiration, vegetative and reproductive growth, are adversely affected by salinity stress (Munns, 2005; Munns and Tester, 2008; Ahmad *et al*., 2014). Salinity could result in the onset of other stresses and different physiological imbalances in plant (Jampeetong and Brix, 2009). These include the induction of osmotic stress due to ex-osmosis, disturbance in hormonal balance due to excessive uptake of toxic ions and salt-induced oxidative stress due to the disturbance in equilibrium of antioxidant and reactive oxygen species (ROS) (Talei *et al*., 2012; Khan *et al*., 2013).

Red pepper is an important cash crop of arid and semi-arid zones. It is not only important from economic point of view, but also important from nutritional and medicinal purpose (Howard *et al*., 1994). Nutritionally, red pepper is a rich source of vitamins, antioxidants and phenolic compounds. Salinity is one of the most important limiting factors of crop production in pepper growing regions of the world. The threshold level of EC for red pepper is 1.5 dS m^{-1} thus it is considered as moderately sensitive crop to salinity stress (Bernstein, 1954). In recent years, the production of red pepper is adversely affected by salinity (Tabur and Demir, 2010).

Under stressed environments, plants protect themselves by adopting different alterations within their morphological and physiological traits. An important physiological mechanism that plants adopt widely under salinity stress is the biosynthesis of different metabolites or osmolytes at cellular level (Tester and Davenport, 2003). These osmolytes help plant to cope with salinity stress by protecting its cellular machinery (Hasegawa *et al*., 2000). These osmolytes regulate the uptake of toxic ions in cells, regulate osmotic status, maintain equilibrium between antioxidants and ROS and adjust hormonal balance to help plant in continuing its development under adverse conditions (Parida *et al*., 2003).

Not all plants are capable of producing the optimum level of osmolytes under stress conditions. While the

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exogenous application of these metabolites can enhance their cellular concentration and plant can acquire stress tolerance (Ashraf and Foolad, 2007). Osmolytes i.e., proline and L-tryptophan may helpful in inducing the salinity stress in red pepper (*Capsicum annuum* L.). Keeping in view the above discussion, present study was conducted to evaluate the effectiveness of two potential osmolytes, i.e. proline and L-tryptophan, to induce salinity tolerance in red pepper through their exogenous application.

Materials and Methods

A pot experiment was conducted in the wire house, to evaluate the potential of proline and L-tryptophan in reducing the adverse effects of salinity stress on red pepper (*Capsicum annuum* L). The experiment was conducted with different sets of treatments and three levels of salinity viz. 0.6, 4 and 6 dS m^{-1}. Two osmolytes proline and L-tryptophan were used as T_1: No application (Control), T_2: Proline @ 50 mM, T_3: L-Tryptophan @ 25 ppm and T_4: Proline and L- tryptophan @ 50 mM and @ 25 ppm, respectively

Pot experiment

The soil used for the experiment was collected from the experimental field of the Department of Soil Science, University College of Agriculture and Environmental Sciences, IUB. Before filling the pots, the soil sample was collected and analyzed for physico-chemical properties by following the protocols as described by Ryan et al. (2001). Before development of salinity levels, the soil had pHs 7.23, EC 0.6 dS m^{-1}, organic matter 0.53%, total nitrogen 0.03%, available P 14.3 mg kg^{-1}, and extractable K 103 mg kg^{-1}. Different salinity levels 0.6 (control / original), 4.04 and 6.11 dS m^{-1} were developed by adding calculated amounts of mixed salts (NaCl, $MgSO_4$, $CaCl_2$ and Na_2SO_4) using quadratic equation as described by Haider and Ghafoor (1992). The pots were lined with polythene sheets and filled with soil having pre-developed salinity levels. The pots were arranged in wire house of the Department at ambient light and temperature according to completely randomized design (CRD) in factorial fashion. Seed of red pepper (*Capsicum annuum* L.) variety Green Star (Sher Ali and Brothers Peshawar) was obtained from local vegetable market and nursery was raised. When plants reached 4 leaves stage, they were transferred in pots with 12 kg of soil with different salinity levels as described above. A population of 4 plants per pot was maintained after establishment of seedlings. Another set of three pots having same EC levels was maintained as reference pots to check the effect of irrigation water on salinity levels.

Recommended dose of N, P, K fertilizers (100: 40: 40 kg ha^{-1}) was applied in each pot as urea, diammonium phosphate and sulphate of potash, respectively. Full dose of P and K along with 1/4th of N was applied as basal dose at the time of sowing. The remaining N was applied in three splits at 15 days interval. Pots were irrigated with good quality irrigation water meeting the irrigation quality criteria for crop. Tap water was used for irrigation 500 ml water was applied after every 5 days interval. Osmolytes were applied every 15 days interval up to fruiting by foliar application. Fully matured leaves were analyzed. Roots were collected by flooding the pots and soil was washed on sieve. After establishment of seedlings, thinning was done to maintain the uniform plant population. The fresh plant leaves were sampled after 50 days of transplanting for determination of physio-biochemical attributes and yield parameters were recorded at harvest of the crop.

Plant analyses

After 50 days of transplanting, fresh leaf samples were collected from each pot and Roots were collected by flooding the pots and soil was washed on sieve than analyzed for different physiological and morphological parameters. Leaf samples were digested according to the method of Wolf (1982) and potassium and sodium were determined by using standard protocols as described by Ryan *et al*. (2001). Relative water content (RWC) of leaves was determined by using the formula as described by Mayak *et al.* (2004).

Chlorophyll and carotenoid contents were determined by extracting leaf material (0.05 g) in 10 cm^3 dimethyl sulfoxide (Hiscox and Israelstam, 1979). Briefly, the samples were heated at 65°C for 4 h and then the absorbance of extract was recorded at 660 and 645 nm. Chlorophyll contents were calculated as per standard method (Arnon, 1949). Carotenoid content was estimated according to the method of Wellburn (1994).

Statistical analysis

Analysis of variance techniques (ANOVA) were applied to evaluate the data using CRD-factorial design and means were compared by Least Significance Difference Test (Steel *et al*., 1997).

Results

The results (Table 1) showed that the exogenous application of osmolytes significantly improved plant height at all salinity levels. Maximum improvement in plant height was observed at EC level 0.6 dS m^{-1} where proline and L-tryptophan were applied in combination, as compared to the untreated stressed plants. Similarly shoot dry weight was significantly reduced by salinity stress and the exogenous application of proline and L-tryptophan were resulted in significant increase in shoot dry weight at all levels of

Table 1: Effect of exogenous application of proline and L-tryptophan on plant height and shoot dry weight under salinity stress (n = 3)

Treatment	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}
	Plant height (cm)			Shoot dry weight (g plant^{-1})		
Control	21.73 c	11.16 e	5.73 f	8.69 de	5.36 fg	3.93 g
Proline	28.50 b	16.96 d	9.80 e	11.22 bc	7.88 e	5.87 f
L-tryptophan	30.73 b	17.30 d	10.33 e	11.63 b	7.88 e	5.35 fg
Proline + L-tryptophan	35.03 a	22.06 c	15.16 d	14.68 a	9.83 cd	7.71 e
LSD value	**3.7325**			**1.6451**		

Table 2: Effect of exogenous application of proline and L-tryptophan on root length and root dry weight under salinity stress (n = 3)

Treatment	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}
	Root length (cm)			Root dry weight (g plant^{-1})		
Control	6.40 c	5.50 d	3.53 e	4.33 cd	2.66 ef	1.13 g
Proline	9.63 b	6.83 c	5.43 d	6.66 b	3.66 de	1.95 fg
L-tryptophan	9.30 b	6.76 c	5.13 d	6.80 b	3.70 d	1.65 fg
Proline + L-tryptophan	11.23 a	9.13b	6.53 c	8.66 a	4.80 c	2.46 f
LSD value	**0.8270**			**1.0106**		

Table 3: Effect of exogenous application of proline and L-tryptophan on Na^+\\K^+ ratio in leaves and roots under salinity stress (n = 3)

Treatment	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}
	Na^+\\K^+ ratio in leaves			Na^+\\K^+ ratio in roots		
Control	0.69 d	1.24 b	2.67 a	0.66 d	1.27 b	2.20 a
Proline	0.34 e	0.62 cd	1.19 b	0.38 e	0.66 d	1.17 b
L-tryptophan	0.37 c	0.70cd	1.31 b	0.39 e	0.76 cd	1.22 b
Proline + L-tryptophan	0.24 f	0.47 de	0.80 c	0.26 f	0.48 e	0.84 c
LSD value	**0.2445**			**0.1136**		

Table 4: Effect of exogenous application of proline and L-tryptophan on relative water contents and fruit weight under salinity stress (n = 3)

Treatment	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}	0.6 dS m^{-1}	4 dS m^{-1}	6 dS m^{-1}
	Relative water contents (%)			Fruit weight (g fruit^{-1})		
Control	60.53 d	41.67 g	21.66 i	8.44 c	5.17f	-
Proline	73.19 b	55.68 e	39.01 g	10.45 b	6.83 de	4.17 g
L-tryptophan	72.61 b	53.6 e	33.6 h	10.18 b	7.03 d	4.37 g
Proline + L-tryptophan	89.52 a	63.43 c	48.7 f	12.63 a	10.00 b	6.40 e
LSD value	**4.7118**			**0.6031**		

salinity. Maximum shoot dry weight was observed with the combined use of L-tryptophan and proline under EC level of 0.6 dS m^{-1}.

Data showed that root length and root dry weight (Table 2) was significantly decreased with increasing levels of salinity. The exogenous application of proline and L-tryptophan significantly improved root growth under saline as well as normal conditions. Maximum increase in root length (84.9%) and root dry weight (117%) was observed at 0.6 dS m^{-1} where both osmolytes were applied together.

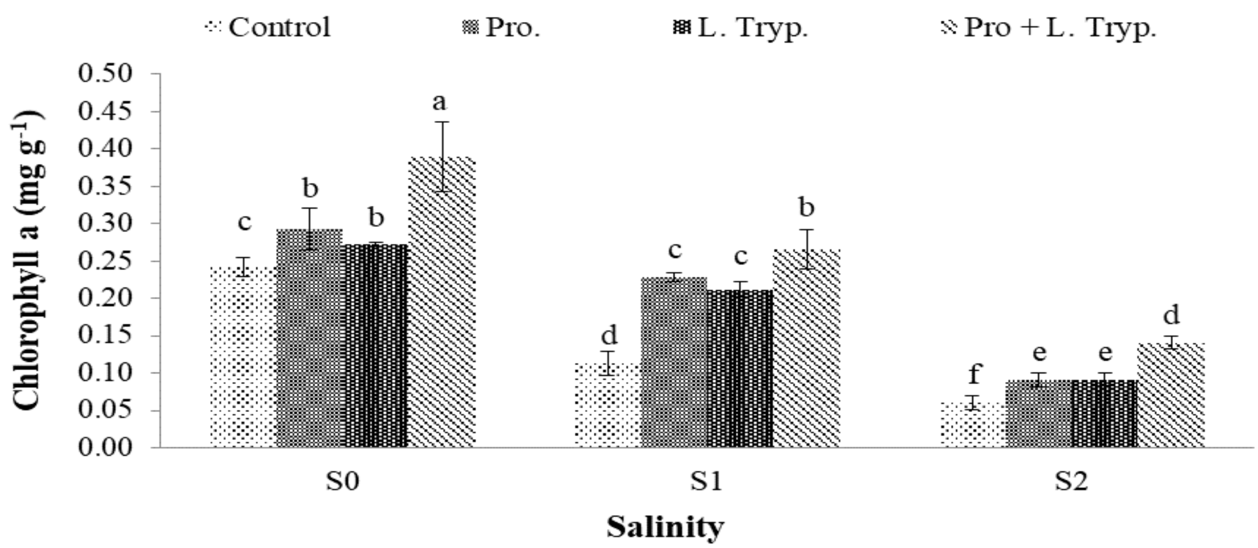

Figure 1: Effect of exogenous application of proline and L-tryptophan on chlorophyll a content of red pepper (*Capsicum annuum* L) under salinity stress [S0 - control 0.6 dS m^{-1}; S1- 4 dS m^{-1}; S2 - 6 dS m^{-1}; n = 3]

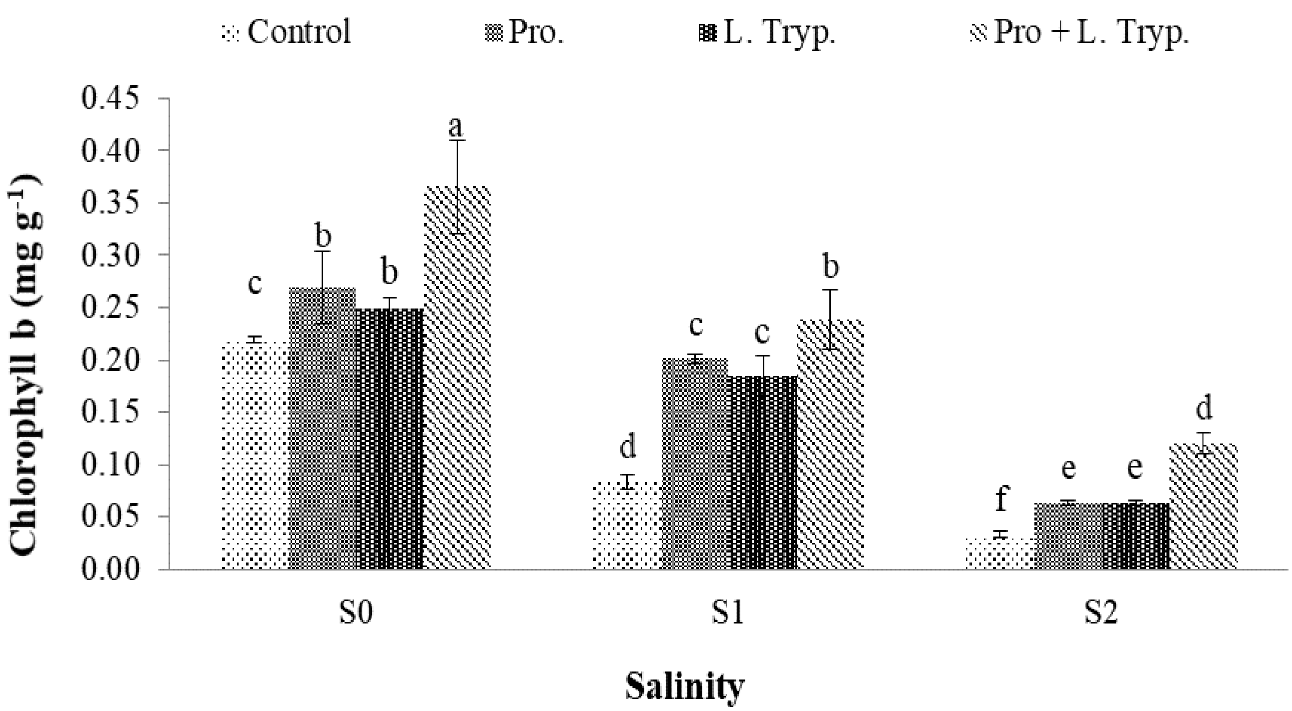

Figure 2: Effect of exogenous application of proline and L-tryptophan on chlorophyll b contents of red pepper (*Capsicum annuum* L) under salinity stress [S0 - control 0.6 dS m^{-1}; S1- 4 dS m^{-1}; S2 - 6 dS m^{-1}; n = 3]

The analysis of leaves and roots showed that salinity stress significantly increased the Na^+/K^+ in leaves and roots of red pepper (*Capsicum annuum* L) plant. The results (Table 3) showed that exogenous application of proline and L-tryptophan, solely as well in combination, significantly decreased the Na^+/K^+ ratio in leaves and roots as comparison to control. In both cases, combined application of proline and L-tryptophan (Na^+/K^+ ratio in leaves 0.24 & in roots 0.24 @ EC level 0.6 dS m^{-1}) gave better results as compared to sole application of proline and L-tryptophan.

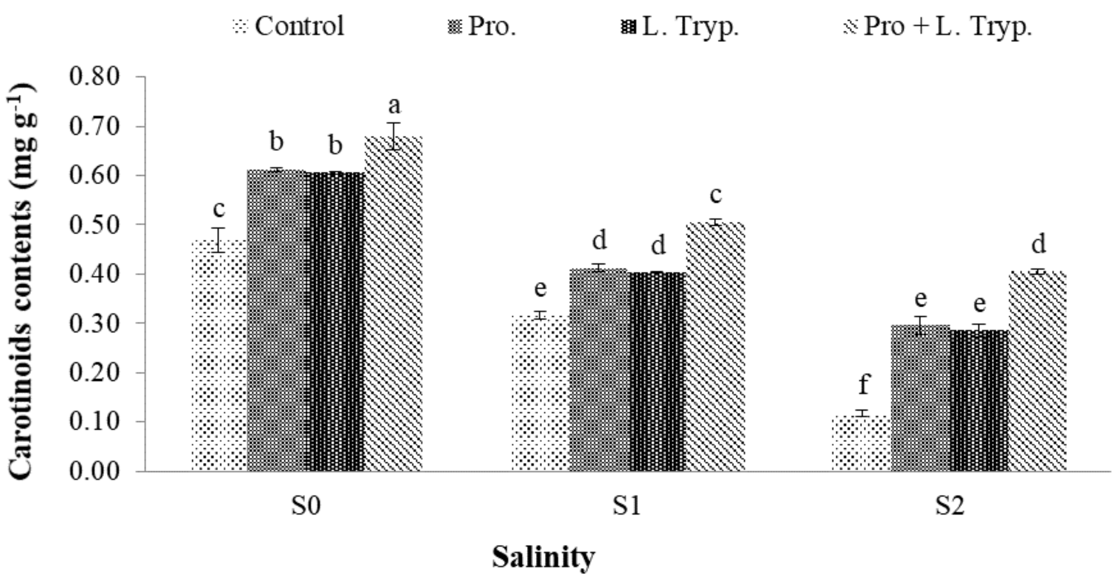

Figure 3: Effect of exogenous application of proline and L-tryptophan on carotenoid contents in leaves of red pepper (*Capsicum annuum* L) under salinity stress [S0 - control 0.6 dS m^{-1}; S1- 4 dS m^{-1}; S2 - 6 dS m^{-1}; n = 3]

The results also showed that salinity stress significantly affected the photosynthetic pigments in leaves of red pepper resulting in significant decrease in chlorophyll-a (Figure 1), chlorophyll-b (Figure 2) and carotenoid contents (Figure 3). Maximum reduction in photosynthetic pigments was observed at 6.11 dS m^{-1} as comparison to control, where plants showed severe decrease in chlorophyll-a, chlorophyll-b and carotenoid contents in leaves. The exogenous application of proline and L-tryptophan significantly improved chlorophyll-a, chlorophyll-b, and carotenoid contents. At all salinity levels, combined application of proline and L-tryptophan resulted in better results than the sole application of osmolytes.

Salinity stress also disturbed the water relations in red pepper which is indicated by the significant reduction in relative water contents in leaves. The data (Table 4) showed that salinity stress significantly decreased relative water contents, while the exogenous application of proline and L-tryptophan resulted in significant increase in relative water contents under normal as well as in axenic conditions. The overall results revealed that combined application of osmolytes gave better results in improving relative water contents in leaves of red pepper. The result of Table 4 showed the combined application of proline and L-tryptophan at EC level 0.6 dS m^{-1} improved the relative water content and fruit weight as sole application of proline and L-tryptophan. The combined application of proline and L-tryptophan at EC level 0.6 dS m^{-1} also improved the fruit weight and RWC as compared to control and all other salinity level.

Discussion

In present study, the effect of proline and L-tryptophan was evaluated under salinity stress on red pepper. Proline and L-tryptophan are the potential osmolytes that plants biosynthesize under salinity stress (Ashraf and Foolad, 2007, Khalid *et al*., 2013). Proline is considered as an osmo-protectant and regulates the scavenging of free radicals in plants under salt stress. The exogenous application of proline results in the induction of salt tolerance in plants which are sensitive to salinity (Mansour, 2000). L-tryptophan is also an important biomolecule which is involved in the synthesis of auxins in plant. Auxins are important plant hormone that is involved in stress regulation (Taiz and Zeiger, 2006).

In present study, the growth attributes i.e. plant height, root length, root, shoot dry weight, significantly decreased under salinity stress and the exogenous application of osmolytes (proline and L-tryptophan) resulted in significant increase in all growth parameters. Talat *et al*. (2013) reported that all growth attributes were significantly decreased when plants were subjected to high salt concentrations, but the exogenous application of osmolytes significantly improved growth attributes of red pepper. Similar results have also been reported by Khalid *et al*. (2013).

Salt stress reduces plant vegetative growth by effecting morphological and physiological characteristics (Shabani *et al*., 2012). Under salt stress, due to the excessive uptake of toxic ions like Na^+ and Cl^-, plant growth is restricted due to inhibition of cell division. These ions disturb the osmotic potential of vacuoles and cause cytoplasmic dehydration. In addition, high salt concentration in root zone decreases water potential of rhizosphere which results in low availability of water and onset of osmotic stress (Chartzoulakis *et al*., 2002). The exogenous application of osmolytes results in the regulation of osmotic potential that leads to the amelioration of toxic effects of ions and plant can grow normally (Le-Rudulier, 2005). The improvement in plant growth might be due to the ability of osmolytes to restrict the activity of Na^+ and Cl^- ions in cytoplasm. The exogenous application of osmolytes results in improvement in ion balance and the regulation of water relations within plant's cell (Ashraf and Foolad, 2007: Deivanai *et al*., 2011).

The results of present study showed that plant physiological attributes, i.e. relative water contents (RWC), carotenoids and chlorophyll-a, chlorophyll-b contents were negatively affected by salinity stress. The reduction in these parameters might be due to the degradation of chloroplasts and other photosynthetic pigments (Moradi and Ismail, 2007; Kafi, 2009). Salinity effects photosynthesis by reducing efficiency of different plant mechanisms including water use efficiency, efficiency of stomata, water regulation and translocation of carbohydrates and protein compounds (Sultana *et al*., 1999). Under salt stress, the concentration of chlorophyll-a, chlorophyll-b and total chlorophyll significantly decreases. Reduction in these, results in inhibition of photosynthetic activity (Xinwen *et al*., 2008; Amuthavalli and Sivasankaramoorthy, 2012). In present study, RWC, chlorophyll and carotenoid contents were improved as compared to control by the exogenous application of osmolytes in red pepper. The results are in line with those of Kahlaoui *et al.* (2013), where they reported that exogenous application of proline significantly increased leaf chlorophyll pigments and RWC. The findings are also supported by Kamran *et al.* (2009) and Talat *et al.* (2013). They reported that exogenously applied proline and L-tryptophan improved all physiological attributes of plant. It is also reported that exogenous application of L-tryptophan under salinity stress resulted in significant improvement in RWC.

Under salinity stress, plants showed higher concentration of toxic ions and deficiency essential nutrients. The results are parallel with the study by Khan *et al*. (2009) where they reported that under salinity stress, there was high concentration of Na^+ and Cl^- ions and decrease in the concentration of Ca^+, K^+ and Mg^{+2}. Zhu (2007) reported that Na^+ due to its similar ionic nature competes with K^+ is excessively up taken by plant in place of K^+, resulting in K^+ deficiency and toxicity of Na^+. Decrease in K^+ uptake and increase in Na^+ uptake result in increase in Na^+/K^+. This Na^+/K^+ ratio decreases the soluble sugars resulting in disruption of solute biochemistry in cells. Our results showed that when proline and L-tryptophan were applied exogenously, significant decrease in Na^+ concentration and Na^+/K^+ ratio, and increase in K^+ concentration was observed. The improvement in physiological attributes is considered due to the regulation and alteration in the uptake of toxic ions due to the enhanced levels of osmolytes into plant cells (Jesus *et al*., 2009: Deivanai *et al*., 2011).

The yield parameters of pepper were significantly affected by salinity. The results showed that under salinity stress, red pepper fruit weight was adversely affected. The results are parallel with the findings of Khan and Abdullah (2003). Huez-Lopez *et al*. (2011) also reported that fruit production of red pepper was severely inhibited by the salinity. They attributed the reduction in fruit development due to the disturbance in hormonal balance and decreased water uptake. Our findings showed that the exogenous application of proline and L-tryptophan significantly increased the yield attributes of pepper. These results were supported by the work of Kahlaoui *et al*. (2013) on tomato and Jemaa *et al*. (2011) on arabidopsis. They reported that the exogenous application of proline and L-tryptophan resulted in a significant increase in yield parameters. Sudadi (2011) also reported that exogenous application of L-tryptophan significantly enhanced the yield of soybean when subjected to salinity stress. The exogenous application of L-tryptophan results in the improvement in the phytohormonal balance under salinity stress, especially of abscisic acid and auxin, which results in better flowering, fruit formation and maturation (Guilfoyle *et al*., 1998), This might be due to the reason that exogenous application of proline and L-tryptophan improve cell division and elongation which leads to rapid and improved development of reproductive parts of plant (Yamada *et al*., 2005; Chaudhry and Khan, 2007). Exogenously applied osmolytes improves the photosynthetic activity by scavenging oxygen radicals in chloroplasts. The increase in photosynthetic activity also results in the increased production and translocation of carbohydrates and sugars which improves the final quality and quantity of fruits (Hala and Bassiouny, 2005; Yang and Lu, 2005).

Conclusion

It was concluded that salinity stress significantly decreased growth and yield of red pepper (*Capsicum annuum* L) plant. The exogenous application of osmolytes, i.e. proline and L-tryptophan, significantly improved plant growth, yield and physiology, resulting in the induction of salinity tolerance in red pepper. Thus, it may be concluded that combined application of proline and L-tryptophan (@ 50 mM and 25 ppm, respectively) was more effective in reducing the inhibitory effect of salinity on plant growth therefore; this combination may be evaluated in extensive field trials before its recommendation to the farmers.

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