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OSMOLYTE ACCUMULATION IN MODERATELY HALOPHILIC BACTERIA IMPROVES SALT TOLERANCE OF CHICKPEA

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Abstract

Salinity results in poor crop yield round the globe. We hypothesized those strategies of endogenous osmolytes proline, glycine betaine and choline accumulation in moderately halophilic bacterial strains *Staphylococcus haemolyticus* (ST-9) and *Bacillus subtilis* RH-4 isolated from saline rhizosphere have a role in improving bacterial and plant growth by alleviating salt stress. We checked the effect of varying salt stress (0, 0.5, 1, 1.5, 2, 2.5 M NaCl) on bacterial growth and osmolyte accumulation. The data showed that bacterial growth was affected by increasing salt stress however, cells cultured in the presence of 1.5 M NaCl stress showed higher accumulation of osmolytes. Seeds of *Cicer arietinum* Var. CM 98 were inoculated and grown up to full maturity at different salinity levels (0, 50, 100, 200 mM NaCl) in wire house under full sunlight. Inoculated plants showed significantly improved plant growth (germination, length, chlorophyll contents, total soluble sugars and protein content) and accumulation of endogenous osmolytes at 200 mM NaCl stress as compared to uninoculated control plants. We concluded that increased osmolyte accumulation can overturn the negative effects of high osmolarity in bacteria and plants. Both strains can be effectively used as bio inoculants for improving *Cicer arietinum* Var. CM98 growth under salt stress.

Introduction

Soil salinity around the globe reduces the agricultural crop productivity. This problem is most serious for agricultural based countries like Pakistan. Osmotic stress bring forth by high salinity manipulates the growth of all living cells either disrupting the normal physiological activities or intracellular macromolecular structures (Vogel *et al*., 2010). Salinity imposed osmotic stress is worth mentioning for symbiotic relationships of plants as well (Sohrabi *et al*., 2008; Kouas *et al*., 2010). Accumulation of small organic molecules also known as compatible solutes in response to salinity is reported in all living groups to a variable extent. Accumulation of these osmolytes in bacteria and plants is an indicator of salt tolerance in response to salt stress (Bremer & Kraemer, 2000; Gul & Khan, 2008). These strategies may confer salt tolerance to bacteria as well as plants in saline rhizosphere and results in better growth and survival. Effective existence of bacteria in saline environment due to excessive accumulation of secondary metabolites may result in better root colonisation and plant growth (Hirsch, 2010; Karlidag *et al*., 2011).

Chick-pea (*Cicer arietinum* L.) is a leguminous crop significantly important for its high protein and carbohydrate contents (Soussi *et al*., 1998). However, this crop is severely affected by soil salinity. We hypothesised that moderately halophilic bacterial strains can improve the overall plant tolerance toward varying levels of salinity by elevating endogenous osmolyte accumulation. Keeping in view the significance of chickpea as potent legume for its nutritional benefits, the present research work is an endeavor to explore the potential of two previously isolated moderately halophilic bacterial strains in accumulating endogenous osmolytes i.e., proline, glycine betaine and choline in improving chickpea growth in saline soil.

Material and Methods

Two previously isolated bacterial strains (Afrasayab, 2004), *Staphylococcus haemolyticus* (ST-9) accession no. JN542715 (from rhizosphere of *Astragallus* Plant sp.) and *Bacillus subtilis* (RH-4) accession no. JN559390 (isolated from rhizosphere of *Heleochloa schocnoides* Plant) were used for present study. These bacterial strains were routinely maintained on LB agar plates (Gerhardt *et al*., 1994) containing 0.5 M NaCl. The effect of different NaCl concentrations (0.5, 1, 1.5, 2, 2.5 M) on the growth and endogenous osmolyte accumulation of bacterial strains was studied. Cultures were incubated for 24 hours in a shaker at 150 rpm (orbital incubator Model I-4000 serial number 104 A IRMECO GmbH, Goesthacht/Germany) at 37˚C temperature. Bacterial growth was recorded in term of optical density at 600 nm using spectrophotometer (Model S-300 DL, R & M marketing, and England). After taking OD 600 nm bacterial cells were harvested from the overnight cultures (optimized conditions) by centrifugation (2000 rpm for 5 minutes), weighed and suspended in 1 mL sterile distilled water to measure endogenous level of osmolyte accumulation. For determination of endogenous proline accumulation method of Tonon *et al*., (2004) was followed. Suspension of freshly harvested cells was boiled for 20 minutes. After boiling, (150 µL) supernatant from centrifugation (2000 rpm for 5 minutes) was mixed with 100µL water and 1mL of ninhydrin reagent (0.35% in ethanol). This mixture was heated for 20 minutes and absorbance was noted at 520 nm. Calibration of proline was made using standard curve prepared with varying concentrations (µg mL^{-1}) of reference L-proline. Endogenous accumulation of glycine betaine was determined following method of Grieve & Grattan, (1983). Supernatant from freshly harvested cells was diluted 1:1 ratio with 2 N H_2SO_4 for glycine betaine determination while for choline estimation the supernatant was diluted 1:1 with KPi buffer (0.2 M, pH 6.8) and further proceeded same. The mixture (0.50 mL) was cooled in ice water for 60 minutes and added with 200 µL cold KI-I_2 reagent and gently vortexed. The mixture was placed at 4˚C for 16 hours and finally centrifuged at

10,000 rpm for 15 minutes. The supernatant was carefully separated and resulting pellet was dissolved in 9.0 ml of 1, 2-dichloroethane and placed for 2-2.5 hours. The absorbance was recorded at 365 nm. Standard curve was prepared using varying concentrations (µg mL^{-1}) of reference glycinebetaine and choline as standards.

Healthy and certified seeds of *Cicer arietinum* Var. CM 98 were obtained from Punjab Seed Corporation, Lahore, Pakistan and disinfected with 0.1% $HgCl_2$ solution for 10 minutes. After three to four times rinsing with sterile water to remove all traces of $HgCl_2$, seeds were inoculated with pure culture suspension in autoclaved distilled water (OD_{600} = 0.5) for 30 minutes. For control plants, seeds were soaked in sterile water for the same period of time. Large pots (12 cm diameter) were filled with approximately 3 Kg of thoroughly sieved and dried garden soil in each pot and placed in wire house, Microbiology and Molecular Genetics Department, University of the Punjab, Lahore under full sunlight during November 2010 to March 2011. Seeds were sown in soil and NaCl solution was added to each pot making final concentrations 50, 100 and 200 mM for each pot (per gram weight of soil). At maturity, the plants were harvested and different parameters marker of growth i.e., shoot length (cm), root length (cm), fresh weight (mg $plant^{-1}$) dry weight (mg $plant^{-1}$) and germination (%) were recorded. Photosynthetic activity of plants in term of chlorophyll a, b and carotenoid was determined following Lichtenthaler & Wellburn, (1983). Total soluble sugars (mg $gram^{-1}$ dry weight of plant) and soluble protein (µg $gram^{-1}$ fresh weight of plant) were also determined following Afrasayab *et al*., (2010). Endogenous proline accumulation (µg $gram^{-1}$ fresh weight of plant) was determined following (Tonon *et al*., 2004). Endogenous glycine betaine and choline was determined following Grieve & Grattan, (1983). The experiment was carried out in three replicates. The difference between the means were tested using the least significant difference test ($p<0.05$) by subjecting data to Two-way ANOVA. In all figures, the reported values are the mean of three replicates.

Results

Soil salinity poses serious threat to plant productivity. Plant growth promoting bacteria must be able to deal with such osmotic challenges and have ability to improve crop growth. The influence of varying salt concentrations was checked on the accumulation of salt in bacterial cells as well as on the level of intracellular osmolytes proline, glycine betaine and choline accumulation. Data showed that growth of bacterial strains *Staphylococcus haemolyticus* (ST-9) and *Bacillus subtilis* **(**RH-4) was peaked at 0.5 to 1 M NaCl stress (Fig. 1A). However, maximum accumulation of proline (298 µg/g fresh weights) was observed at 1.5 M NaCl stress for strain *Staphylococcus haemolyticus* (ST-9) and further accumulation decreased towards increasing salt concentrations. However, for strain RH-4, there was a general trend of increase in glycine betaine accumulation towards increasing salt concentrations (Fig. 1B). Maximum accumulation of glycine betaine was observed at 1.5 M NaCl stress while maximum accumulation of choline was observed at 1 M NaCl stress. Choline accumulation becomes consistent at higher salt stress. Generally there was maximum accumulation of proline as compared to glycinebetaine and choline in bacterial strains except few cases, i.e., 0.5 M for *Staphylococcus haemolyticus* (ST-9) and *Bacillus subtilis* (RH-4) and 1 M for *Bacillus subtilis* (RH-4) where glycine betaine accumulation was maximum.

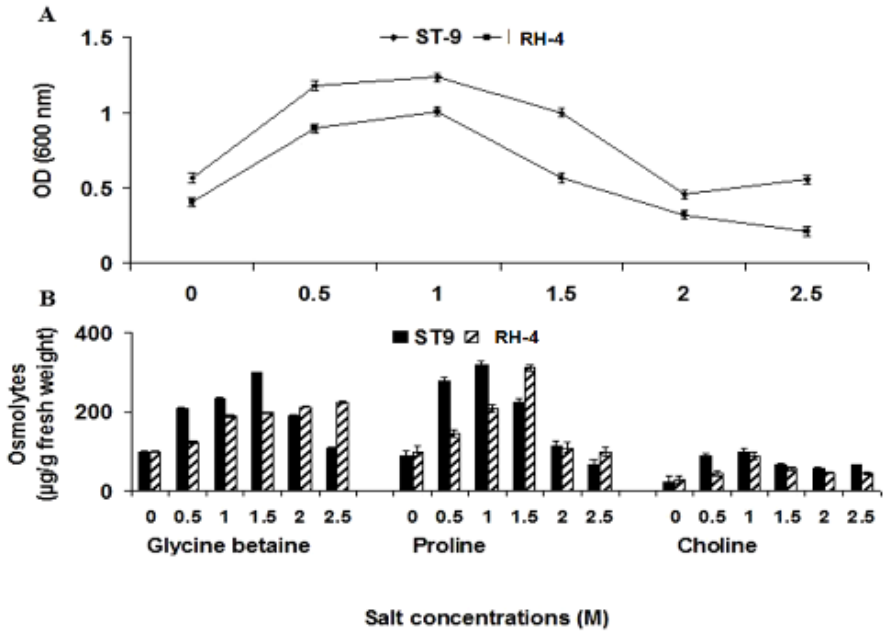

Fig. 1. Effect of varying salt concentrations on bacterial growth and endogenous osmolytes (µg/ g fresh weight of bacterial cells) i.e., proline, glycine betaine and choline accumulation in LB medium adjusted at optimized condition of pH 7.0 and temperature 37˚C.

Salt treatment influenced the germination stage of plant at a significant rate however, more pronounced effect was observed at 200mM NaCl stress where 72% reduction in seed germination was observed compared to noninoculated control plants (Fig. 2). Inoculation of both strains improved the germination of seeds at same rate i.e., 125% as compared to control noninoculated plants at 100 mM salt stress. Generally salt stress reduced the shoot length and root length (cm) towards increasing salt concentrations i.e., 50, 100 and 200 mM NaCl stress, respectively (Fig. 3A). Maximum reduction in shoot length (68%) and root length (65%) was observed at 200mM NaCl treated plants. However, *Staphylococcus haemolyticus* (ST-9) inoculation significantly ($p<0.05$) stimulated the plant shoot length (56%) and root length (21%) compared to noninoculated control plants. Inoculation of *Bacillus subtilis* RH-4 improved (21%) shoot length but reduced (14 %) root length in control treatment. However, Maximum increase in shoot length with *Staphylococcus haemolyticus* (ST-9) inoculation (194%) and *Bacillus subtilis* (RH-4) (125%) was observed at 200 mM NaCl stress. Both strains improved the root length by 70% and 54% for *Staphylococcus haemolyticus* (ST-9) and *Bacillus subtilis* (RH-4), respectively.

Negative effects of salt stress was also observed for fresh weight and dry weight (Fig. 3B) of plant at 200mM NaCl stress where 57% reduction in fresh weight and 61% reduction in dry weight was observed at 200 mM NaCl stress. In all non stressed plants, inoculation significantly improved the fresh weight and dry weight. Maximum increase in fresh weight (116% and 39% for ST-9 and RH-4, respectively) and dry weight (121% and 108% for ST-9 and RH-4, respectively) was observed at 50 mM NaCl stress. Data recorded for plant photosynthetic pigments (Fig. 4) it was observed that salt stress negatively effected the plant growth by reducing chlorophyll content. Significant reduction in Chlorophyll a, b and carotenoid content (33, 75 and 98%, respectively) at 200 mM NaCl stress was observed. Maximum improvement in Chlorophyll a with inoculation of both strains was noteworthy at 100 mM NaCl stress while significant improvement in Chlorophyll b and carotenoid content was observed at 200 mM NaCl stress (Fig. 4). Total soluble sugars (mg/g fresh weight) (Fig. 5) increased in plant at 0 mM NaCl stress and 50 mM NaCl stress by 43% and 24%, respectively. However, plant inoculation with *Staphylococcus haemolyticus* (ST-9) at 200 mM NaCl stress significantly increased (323%) total soluble sugar content. Inoculation of *Bacillus subtilis* (RH-4) increased (343 %) total soluble sugar content at 100 mM NaCl stress. The total soluble protein content (µg/g fresh weight) (Fig. 5) also significantly increased (177% and 63% by (ST-9) and *Bacillus subtilis* RH-4respectively) at 50 mM NaCl stress, however, 83% increase was observed at 0 mM NaCl stress noninoculated plants (Fig. 5). Data obtained from level of endogenous osmolyte accumulation (µg/g fresh weight) (Fig. 6) in *Cicer arietinum* plants, marked increase in endogenous proline accumulation was observed at 50 mM NaCl stress. However, maximum accumulation of glycine betaine (Fig. 6B) and choline accumulation (Fig. 6C) was observed at 100 mM NaCl stress. Inoculation of *Staphylococcus haemolyticus* (ST-9) and *Bacillus subtilis* RH-4 significantly augmented the level of glycine betaine (138% and 54% respectively for each strain) and choline accumulation (135% and 112% respectively for each strain) at 100 mM NaCl stress.

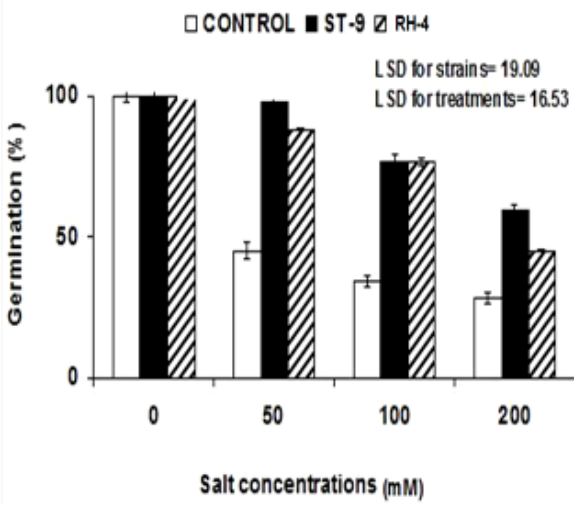

Fig. 2. Effect of varying salt concentrations on seed germination of *Cicer arietinum* Var. CM 98.

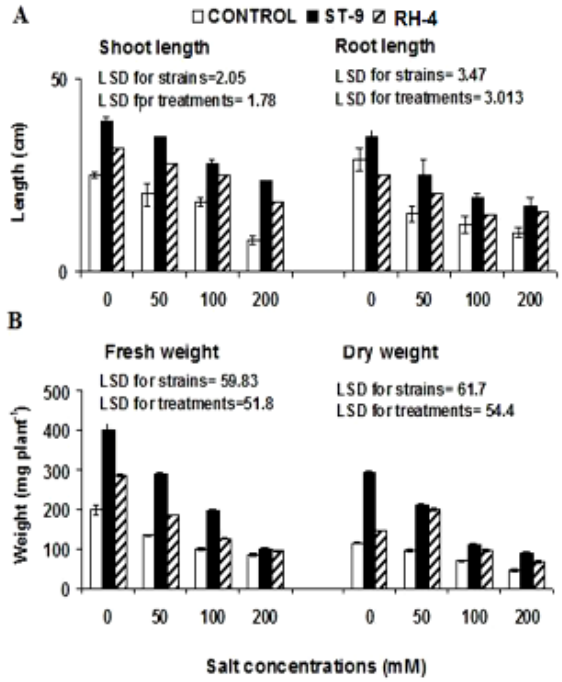

Fig. 3. Effect of varying salt concentrations on plant shoot length (cm), root length (cm), fresh weight (mg plant^{-1}) and dry weight (mg plant^{-1}) of *Cicer arietinum* Var. CM 98.

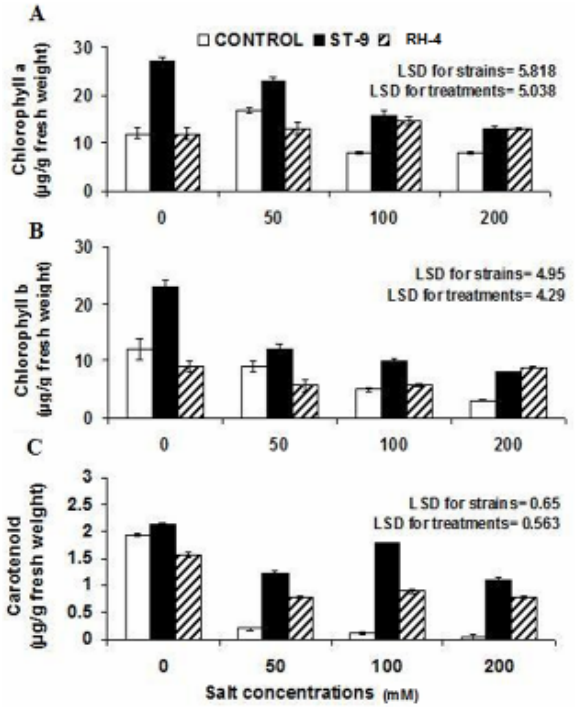

Fig. 4. Effect of varying salt concentrations on plant Chlorophyll a, b and carotenoid contents (µg gram^{-1} fresh weight) of *Cicer arietinum* Var. CM 98.

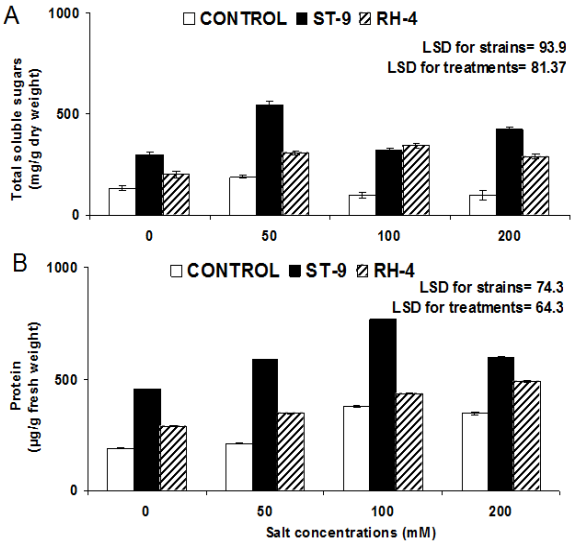

Fig. 5. Effect of varying salt concentrations on total soluble sugars (mg gram^{-1} dry weight) and soluble proteins (µg gram^{-1} fresh weight) of *Cicer arietinum* Var. CM 98.

Discussion

Soil salinity is a very important issue of the day. Jadhav *et al*., (2010) reported 40% of the world's land to be effected by soil salinity. Consequences of soil salinity on plants and microbial community are visible as stunted growth and the continued existence of the fittest microbial species (Ibekwe *et al*., 2010).

Under salinity Na^+ toxicity and alkaline pH microrganisms can not perform their normal physiological activities. The use of indigenous halophilic bacteria that are better adapted to saline environment can be a useful and eco friendly approach for sustainable agriculture (El-Ghany *et al*., 2010). In the present study, the depressive effect of NaCl on the growth of moderately halophilic bacterial strains and *Cicer arietinum* var. CM 98 plants was observed. Intracellular accumulation of endogenous osmolytes i.e., proline, glycine betaine and choline has been found to elevate toward increased salinity in both bacterial strains *Staphylococcus haemolyticus* ST-9 and RH-4. Many previous and recent studies are in line with our results where the significance of these osmolytes to cope with osmotic stress has been reported (Ventosa *et al*., 1998; Afrasayab, 2010). The level of endogenous choline was found to be lower than proline and glycine betaine in both bacterial strains as well as in plants under salt stress. Accumulation of osmolytes as an osmoprotectants in bacteria and plants has been variously reported. They do not upset the macromolecules cells even when accumulated at molar concentrations (Yancey, 1994; Ventosa *et al*., 1998). Smith *et al*., (1988) has reported the role of choline in osmoprotection as well as its use to be converted to glycinebetaine under higher level of salinity. Accumulation of osmolytes in bacterial strains at higher salinity might be involved for their adaptation to saline environments in soil in improving plant growth. Data obtained from our results showed that salinity had drastically influenced the plant growth from germination stage to overall growth parameters. This is in line with several recent and previous reports (Roychoudury *et al*., 2008; Summart *et al*., 2010; Moradi *et al*., 2011; Shereen *et al*., 2011; Fahad & Bano, 2012; Jamil *et al*., 2012) where increased production of reactive oxygen species results in poor metabolic activities of plants (Roychoudury *et al*., 2008). Bacterial strains *Staphylococcus haemolyticus* (ST-9) used in the present research work have been previously reported to improve wheat plant growth under soil salinity (Afrasayab *et al*., 2010). The unfavorable consequences of salinity results in accumulation of excessive Na^+ toxicity in soil that leads to stunted and poor plant growth (Summart *et al*., 2010). Role of Na^+ toxicity in leaves senescence and reduction in photosynthetic activities of plants is well documented (Munns, 2002; Chaum & Kirdmanee, 2009; Hussain *et al*., 2010). From our results, it can be speculated that reduction in photosynthetic activities at increasing soil salinity causes overall reduction of total soluble sugars and protein content of plant. Salt tolerant bacteria accumulate excess Na^+ ions in the cells to maintain their normal metabolic activities (Afrasayab *et al*., 2010) and helpful in reducing soil salinity.

Significant improvement in the photosynthetic activities (Chlorophyll a, b, and carotenoid contents), total soluble sugar and higher level of endogenous osmolytes accumulation i.e., proline, glycine betaine and choline of inoculated plants is observed in our results compared to uninoculated control plants at 200 mM NaCl stress. Level of protein and carbohydrate changes in plant are closely related to the photosynthetic activities of plants (Chookhampaeng, 2011). Our results showed that higher content of total soluble sugars (mg gram^{-1} dry weight of plant) in inoculated plants are similar to the one in the recent study showing that more accumulation of total soluble sugars in salt tolerant lines are present as

compared to salt sensitive ones indicating the salinity tolerance status of plants (Nemati *et al*., 2011). Inoculated plants also showed higher accumulation of endogenous osmolytes at salt stress as compared to control plants without bacterial inoculation indicating improved ability to tolerate salt stress (Klahn & Hagemann., 2011). This might play a role to forestall the deficiency of nitrogen supply put forth by soil salinity in salt stressed plants (Munns, 2002; Tonon *et al*., 2004; Nagata *et al*., 2009; Zhao *et al*., 2009; Nemati *et al*., 2011).

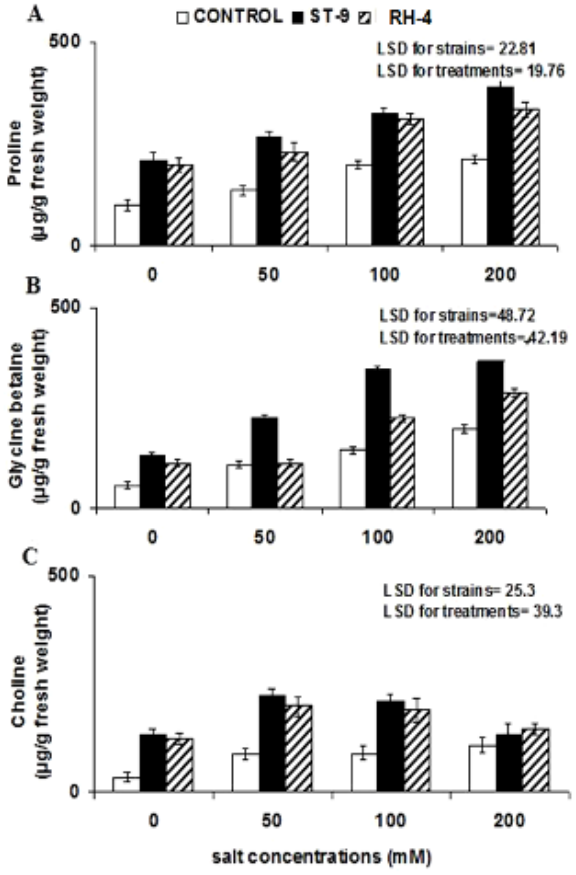

Fig. 6. Effect of varying salt concentrations on endogenous osmolytes accumulation (µg gram^{-1} fresh weight) of *Cicer arietinum* Var. CM 98.

Significant increase in plant biomass in term of weight and length parameters is an indication of improved and better functioning of inoculated plants under soil salinity. From the ongoing discussion it can be concluded that inoculation improved the overall growth of plant and endogenous osmolyte level of plant. This augmented level of osmolyte accumulation resulted in better plant growth.

Conclusion

Our results validate the hypothesis that application of halotolerant bacterial strains to seeds can be a promising approach for curtailing the toxic effect of salinity on plant. This can be a significant and beneficial endeavour for saline agriculture in Pakistan.

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