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Full Length Research Paper

Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress

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Establishment of biofilm, production of exopolysacharides (EPS) and accumulation of endogenous osmolytes under varying stress conditions are significant strategies adopted by bacterial strains for their successful survival in plant rhizosphere. Our studies focus on determining the osmoadaptation strategies used by two native salt-tolerant strains *Oceanobacillus profundus* (Pmt2) and *Staphylococcus saprophyticus* (ST1) and their plant growth promoting abilities. The ability of these strains to be used as inoculants for *Lens esculenta* Var. masoor 93 under salt stress was tested in the laboratory. We found that unlike the bacterial growth, biofilm formation, exopolysaccharide production and endogenous osmolyte (proline and glycine betaine) accumulation increased at higher salt stress. Biofilm formation and endogenous osmolytes increased with increasing salt concentrations. The maximum increase in EPS accumulation was observed at maximum NaCl stress for ST1. Bacterial inoculation improved growth parameters and endogenous osmolytes accumulation of plants under salt stress compared to noninoculated control plants. The ST1 strain in this work efficiently produced biofilm and exopolysacharide and accumulated osmolytes in response to NaCl stress. It could be speculated that these strategies reverse the detrimental effects of high osmolarity in soil and helpful for improving crop under salt stress.

Key words: Biofilm, exopolysaccharide, endogenous osmolytes, *Lens esculenta,* salinity.

INTRODUCTION

Farmers around the globe have to deal with challenges of various biotic and abiotic stress factors that reduce plant growth and productivity (Nemati et al., 2011). Salt stress is one of the major dilemmas that also cause a decline in fertile land productivity. Problems associated with salinity not only effects agriculture but also the biodiversity of that environment. This situation is more alarming in arid and semi arid environments (Fernandez-Aunión et al., 2010). The significance of lentils for its higher proteins, vitamins and minerals contents can not be denied. Moreover, its consumption is increasing through out the world due to high protein components. Besides nutritional value, it is also significant for its sustainable crop production as it adds nitrogen to the soil. Unfortunately, like other leguminous crop lentil is also most salt-sensitive and severely affected by soil salinity throughout the world. To improve its yield it is mandatory to enhance crop yield using safe biological measures (Sarker and Erskine, 2006). To improve the productivity of legumes, efforts should object in improving the soil management and symbiotic relationships (Fernandez-Aunión et al., 2010).

Beneficial plant growth promoting bacteria are found in the rhizosphere of soil and involved in plant growth promotion (Fernandez-Aunión et al., 2010). Bacteria in the rhizosphere are not only involved in plant growth promotion but also the adherence of the bacteria with the environmental surfaces and removing toxicants and contaminants from water, soil and the atmosphere (Afrasayab et al., 2010; Nawaz and Ashraf, 2010). Previous studies show that roots with attached microbial biofilms significantly contribute to soil fertility

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(Ashraf and Foolad, 2007). This association of bacteria with the roots of the plants through these exopolysacharides (EPS) results in formation of sheath or biofilm around the roots and the soil. We are working on number of salt-tolerant bacteria isolated from plant vicinity (Mirza et al., 1998; Mehr et al., 2002). Bacteria in the rhizosphere have to deal with varying stress factors in the soil. For the successful survival of bacteria under salt stress, it is mandatory to have potential of salt tolerance in bacteria. Halophiles have different mechanisms to respond in salt stressed environments at molecular level. They have the ability to establish biofilms containing extracellular polymeric substances with high water content (Xiang et al., 2008). Besides inducing competitive advantage to bacteria under salt stress, osmotolerance also helps the effective colonization of roots as well. Previous studies prove that increased salinity did not effect the root colonisation and plant growth promoting activity of PGPR (Paul and Nair, 2008).

Osmotolerance is induced either by the production of exopolysaccharides, accumulating intracellular osmolytes or biofilm formation. Compatible solutes are used for osmotic adjustment of bacterial cells and protect the cells against desiccation, high temperature and oxygen radicals (Fernandez-Aunión et al., 2010). Excretion of EPS as a boundary between cells and surrounding environment also acts as a protective mechanism against desiccation, salt stress and UV radiations and helps their survival under desiccation conditions (Chen et al., 2008). Biofilm protect the bacterial cells within the EPS layer and protect them from hostile conditions like antibiotics, salinity and radiations (Wijman et al., 2007).

Pakistan is an agricultural country. Poor irrigation practices and expanded canal system results in development of soil salinity. The role of salt-tolerant bacteria in plant growth promotion makes them ideal candidates for improving crop yield (Nasim et al., 2008). We describe the effect of salt stress on the bacterial biofilm formation and EPS accumulation at varying salt concentrations. Microorganisms growing in the free planktonic state are sticked together by exopolysacharide linkages. EPS produced by surface-colonizing microorganisms are generally rich in monosaccharide like glucose and mannose and serve as signal for the settlement and colonization of surfaces (Ashraf and Foolad, 2007). Since salinity is a major limitation to crop yield so it would be meaningful to study whether bacterial inoculation is useful in supporting seed survival in the presence of salts in the soil.

The present study aims to determine the osmotolerance of bacterial strains by determining biofilm formation, EPS accumulation and osmolyte accumulation under salt stress. Secondly we focused on the effect of these strains on plant growth promotion under varying levels of salinity and compared the growth and biochemical parameters of plants under salinity.

MATERIALS AND METHODS

Two previously isolated bacterial strains Pmt2 (accession no. HQ256514) from phylloplane of plant *Malvestrum tricupsidatum* (Mehr et al., 2002) and ST1 (accession no. GU057989) from rhizosphere of maize species in Pakistan (Mirza et al., 1998) were used for the present study. These strains are maintained routinely in LB agar (Gerhardt et al., 1994) supplemented with 0.5 M NaCl.

Biofilm formation assay

Bacterial growth and biofilm formation were measured under different molar NaCl (0, 0.5, 1, 1.5, 2 and 2.5) concentrations following microtitre plate assay (Fujishige et al., 2006a). Briefly, strains were incubated at 37°C for 24 h to exponential phase in LB (Gerhardt et al*.,* 1994) and M9 media (Kahn et al., 1979), with different concentrations of NaCl added, respectively. Cells were harvested, washed and resuspended in same respective medium to get optical densities OD_{600} 0.3 (10^8 ml^{-1} CFUs) using a spectrophotometer (Model S-300 DL, R&M marketing, and England). To each well of flat bottom 96 well microtitre plate (5530100 made of crystal Polystyrene orange scientific), 200 µl of bacterial culture or medium was added to individual wells. Plates were covered with lid and incubated at 37°C for 48 h. After incubation, bacterial cells were homogenized by repeated pipetting. Bacterial growth from each well was determined by measuring the absorbance at 590_{nm}. To measure bacterial adherence, the medium from each well was gently removed by pipetting. Wells were washed twice with 200 µl of sterile distill water to remove unbound bacteria. Microtitre plates were emptied, air dried and stained with 200 µl of 0.01% crystal violet dye per well. Stained wells were rinsed twice with sterile distill water and biofilm formation was quantified by measuring the absorbance at 570 nm and read in a Microtitre plate reader (Bio-Rad Model No. 680 XR serial no 10298, Japan Experiments were repeated twice and average and standard errors of means for each strain was calculated under the tested conditions.

Determination of endogenous proline and glycine betaine accumulation in bacterial strains

Endogenous osmolyte accumulation in bacterial strains was also checked. Bacterial strains were grown in LB and M9 medium added with varying (0, 0.5, 1, 1.5, 2 and 2.5) molar concentrations of NaCl. Both free living and sessile bacteria, from overnight cultures were obtained by centrifugation (2000 rpm for 5 min). Cells were weighed and suspended in 1 ml sterile distill water. The suspensions were boiled for 20 min in a water bath (100°C) and endogenous proline from cells was determined following (Tonon et al., 2004). Endogenous glycine betaine accumulation was determined following Grieve and Grattan (1983). Calibration of proline and glycinebetaine was made using reference L-proline and glycine betaine, respectively. The experiments were carried out with three replicates.

Exopolysaccharide production assay

For optimized exopolysaccharide production previously reported medium Verhoef et al. (2003) was used. Flasks (250 ml) containing 100 ml of medium added with and without varying NaCl concentrations (0.5, 1, 1.5, 2, 2.5 M) were inoculated with freshly prepared bacterial culture (OD_{600} 0.3; 10^8 ml^{-1} CFUs) and incubated at 160 rpm (Sanyo Orbital Shaker) on shaker. Cultures were incubated for 48 h at 37°C. Bacterial growth was monitored by estimating

absorbance at 600 nm (R&M Marketing England S 300 D Spectrophotometer). All experiments were performed in triplicate. In order to extract exopolysaccharide, method of De Vuyst et al. (1998) was followed. Following centrifugation (10,000 rpm for 15 min at 4°C), exopolysaccharide fraction from bacterial supernatant was precipitated using three volumes of pre chilled acetone. After 48 h, precipitated EPS was separated by centrifugation at 15000 rpm for 20 min at 4°C. Dry weight of exopolysaccharide was calculated after drying it (58°C) in oven for 24 h in the same glass centrifuge tubes to minimize the loss of exopolysaccharide. Exopolysaccharide was quantified (mg 100 ml^{-1} culture) in terms of total carbohydrate by the phenol-sulphuric acid method (Dubois et al., 1956). Experiments were performed in triplicates.

Inoculation of plants, harvesting and biochemical analysis

Healthy and certified seeds of *Lens esculenta* (Var. Masoor-93) from Punjab Seed Corporation, Lahore, Pakistan were disinfected with 0.1% $HgCl_2$ solution for 10 min followed by five to six times rinsing with sterile water to remove all traces of $HgCl_2$ completely. After surface sterilization, seeds were inoculated with bacterial suspension prepared in autoclaved distilled water (OD_{600} 0.3; 10^8 cells ml^{-1}) for 30 min. For control, seeds were soaked in sterile water for the same period of time. Seeds were then sown in pots containing 120 g sieved garden soil. After seed sowing, equal quantity of salt solution containing respective NaCl concentrations (50, 100 and 200 mM) per gram weight of soil were added to each pot. Seeds were allowed to germinate in the dark in a growth chamber at 28±30°C for three days. Germination was recorded daily. After seed germination, pots were transferred to light intensity of 10 Klux, photoperiod of 16 h light/dark and temperature 37°C ±1. After 15 days, the seedlings were harvested and different growth parameters (germination, shoot length, root length, fresh weight, dry weight and chlorophyll (a, b and carotenoid content) were recorded. Determination of endogenous osmolytes i.e., proline (Tonon et al., 2004), glycine betaine (Grieve and Grattan, 1983) from harvested plants were determined. Chlorophyll a, b and carotenoid contents of the plants were determined (Lichtenthaler and Wellburn, 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.

Statistical analysis

In all above experiments, results obtained from these experiments were analyzed statistically. Mean, standard deviations and standard errors of the means were calculated. In all the figures, the spread of the values is shown as error bars representing standard errors of the means.

RESULTS AND DISCUSSION

Biofilm formation assay in microtiter plates

The challenge of improving growth on persistent basis and alleviation of salt stress by microorganisms depends on quick and efficient response to the salt stress. Halophilic bacteria have ability to survive in salt stress environment. High salt concentrations can cause major imbalance in osmotic and water relations of organisms (Zhao et al., 2009). Halophilic bacteria in the present studies that is, *O. profundus* (Pmt2) and *S. saprophyticus* (ST1) have been tested for determining their strategies for successful survival under salt stress.

We have focused on *in vitro* biofilm formation on abiotic surface in two different media the rich and minimal (LB & M9) added with varying salt concentrations. Bacterial growth was determined by measuring OD_{595} nm. Bacterial growth declined at higher salt concentration. Strain *S. saprophyticus* (ST1) showed optimum growth at 0.5 M NaCl in LB and M9 media. Strain Pmt2 showed optimum growth at 1 M to 1.5 M NaCl in LB and M9 media. For monitoring biofilm, culture was removed from wells and biofilm was stained using 0.01 % crystal violet. Results indicate that both strains were variable in biofilm formation. Strain ST1 showed peaked biofilm at 1.5 M to 2.5 M NaCl in LB and M9 media. However, in case of *O. profundus* (Pmt2) biofilm formation was maximum at no salt stress and consistently decreased at higher salt stress in LB. In M9 medium, biofilm formation increased gradually from 0 M to 1 M NaCl stress, decreased at 1.5 M and 2 M NaCl and again become higher at 2.5 M NaCl stress (Figure 1).

Advantages associated with biofilm formation at higher salt concentration might seem to protect bacterial cells from stress and nutrient deficiency (Sandasi et al., 2011). Across a range of NaCl (0, 0.5, 1, 1.5, 2, 2.5 M) concentrations tested, the reduced biofilm development suggests the role of inhibitory osmotic stress that inhibits or reduced the bacterial growth and these lead bacteria from planktonic to sessile stage. Unlikely to other reports (Fujishige et al., 2006a, b) our results show that bacterial growth was better in nutrient rich LB and likewise the better biofilm formation ability under salt stress. This might be speculated that development of biofilm was better in LB as it was added with varying concentrations of NaCl. To cope with salt stress bacteria utilize nutrients from the medium and revert to sessile stage as compared to M9 media with same concentration of NaCl. Environmental conditions greatly effect the fate of cells that is, planktonic or sessile. It can be suggested that reduction in the biofilm formation is due to depletion of nutrients in M9 medium (Fujishige et al., 2006b). These observations suggest the possible role of nutrient in the medium and their possibility in regulating the establishment of biofilm. Strain *S. saprophyticus* (ST1) was more efficient in biofilm formation. Previous studies also report the role of strain *S. Saprophyticus* in colonising plant roots and stimulating plant growth against biotic stress factors (Egamberdieva et al., 2008; Egamberdieva, 2010). It may be speculated that these strains have ability to form biofilm and this gives the benefit of better root colonisation. However, further genetic studies must be needed before using these strains in plant growth promotion.

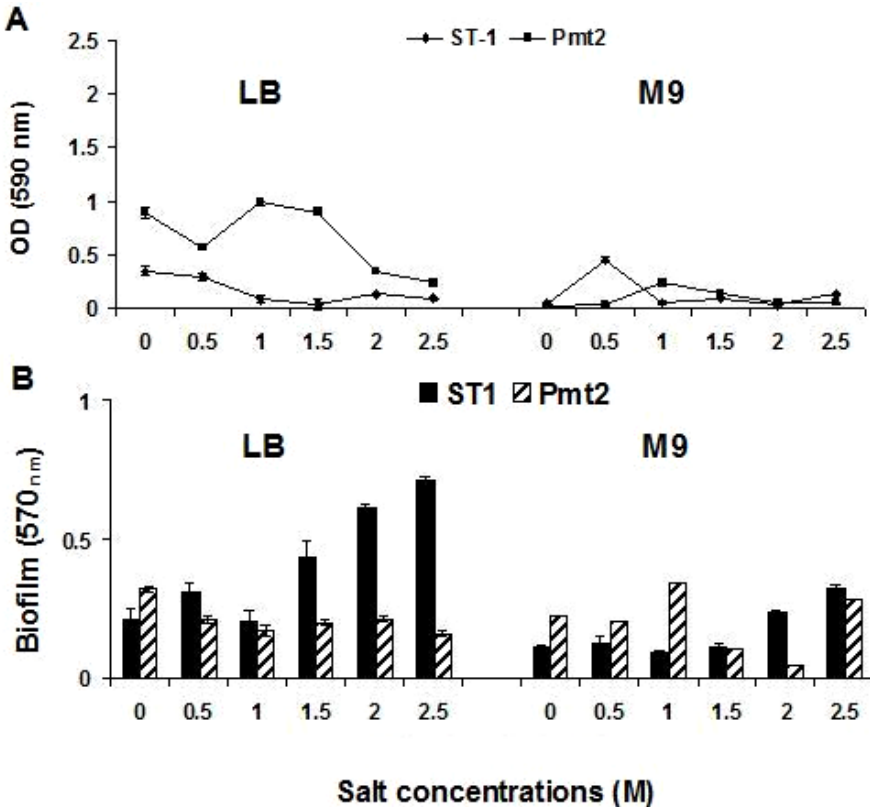

Figure 1. Effects of varying salt concentrations on the (A) Bacterial growth (B) biofilm formation of bacterial strains in LB and M9 media. Error bars show standard errors of the mean values.

Determination of osmolyte accumulation in bacterial strains

To determine the effect of increased osmolarity on bacterial cells, we also determined the intracellular accumulation of endogenous osmolytes in bacterial strains. Cellular osmolyte accumulation on fresh weight basis tends to increase at increasing salt stress at a comparable rate in both strains. Accumulation of proline in ST1 was maximum 325.49 µg/g fresh weight at 1.5 M NaCl (190% increase) in LB compared to media with no salt stress. Maximum 378.89 µg/g fresh weight accumulation (246%) of proline for Pmt2 was observed at 0.5 M NaCl stress that slightly decreased or become constant at higher salt stress in LB (Figure 2).

Maximum accumulation 205.13 µg/g fresh weight (109%) of Glycinebetaine in ST1 was observed in LB at 2.5 M NaCl stress while in M9 medium maximum 223.78 µg/g fresh weight (156%) accumulation at 0.5 M NaCl stress was noticed. Accumulation of cellular glycinebetaine on fresh weight basis for strain Pmt2 was maximum (194%) at 1.5 M NaCl stress in both LB and M9 medium.

Our data reveals that bacterial strains show higher production of biofilm and maximum accumulation of endogenous osmolytes proline and glycine betaine under salt stress, also in line with previous reports suggesting that *S.saprophyticus* need amino acids for growth and successful survival and biofilm formation (Zhu et al., 2007).

Exopolysacharides production

Comparing EPS production under varying salt concentrations, strain ST1 was found to be efficient in accumulating EPS as compared to Pmt2 strain. Maximum increase in EPS accumulation was observed at 2 M to 2.5 M NaCl for ST1. Maximum EPS accumulation at 2.5 mM NaCl stress was 243% for ST1 and 203 % for Pmt2 compared to media at 0 M NaCl stress. Strain ST1 seemed more efficient in accumulating EPS as compared to Pmt2 strain. There was a general trend of gradual increase in EPS with increasing salt concentrations (Figure 3). Better EPS production leads to making of better biofilm development. Reducing sugars (mg100 ml^{-1} culture) are major components of EPS that are increased

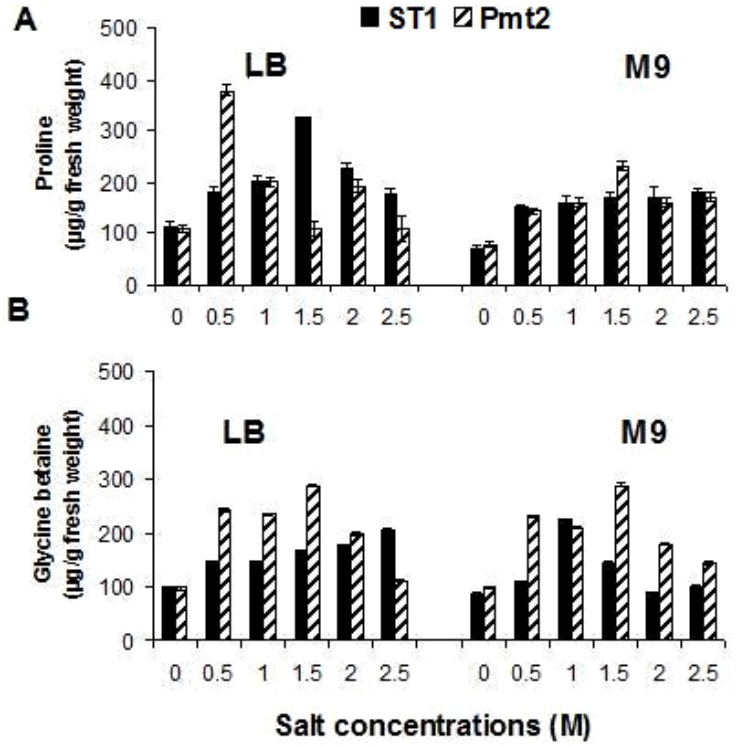

Figure 2. Effects of varying salt concentrations on the (A) Bacterial growth (B) Endogenous osmolytes (µg 100 ml^{-1} culture) of bacterial strains in LB and M9 media. Error bars show standard errors of the mean values.

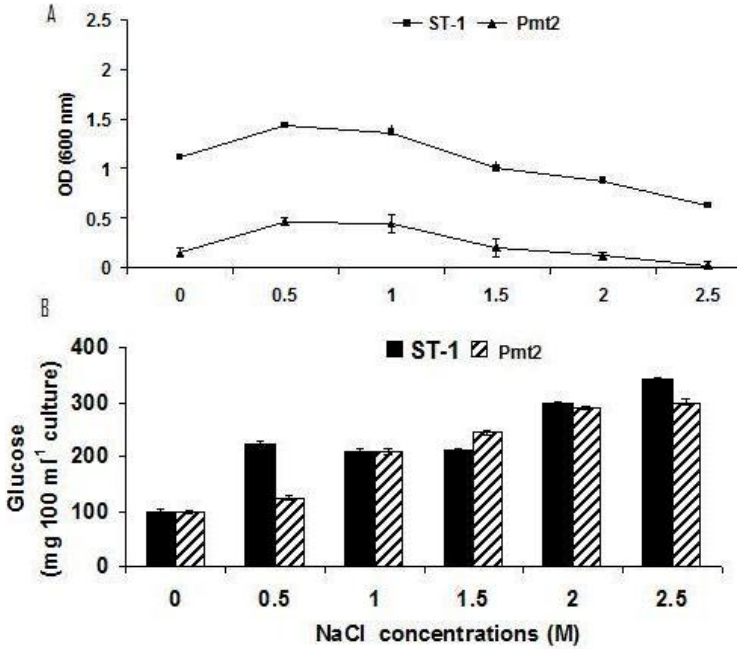

Figure 3. Effects of varying salt concentrations on the A. bacterial growth and B. carbohydrate content (mg 100 ml^{-1} culture) of bacterial strains in EPS media. Error bars show standard errors of the mean values.

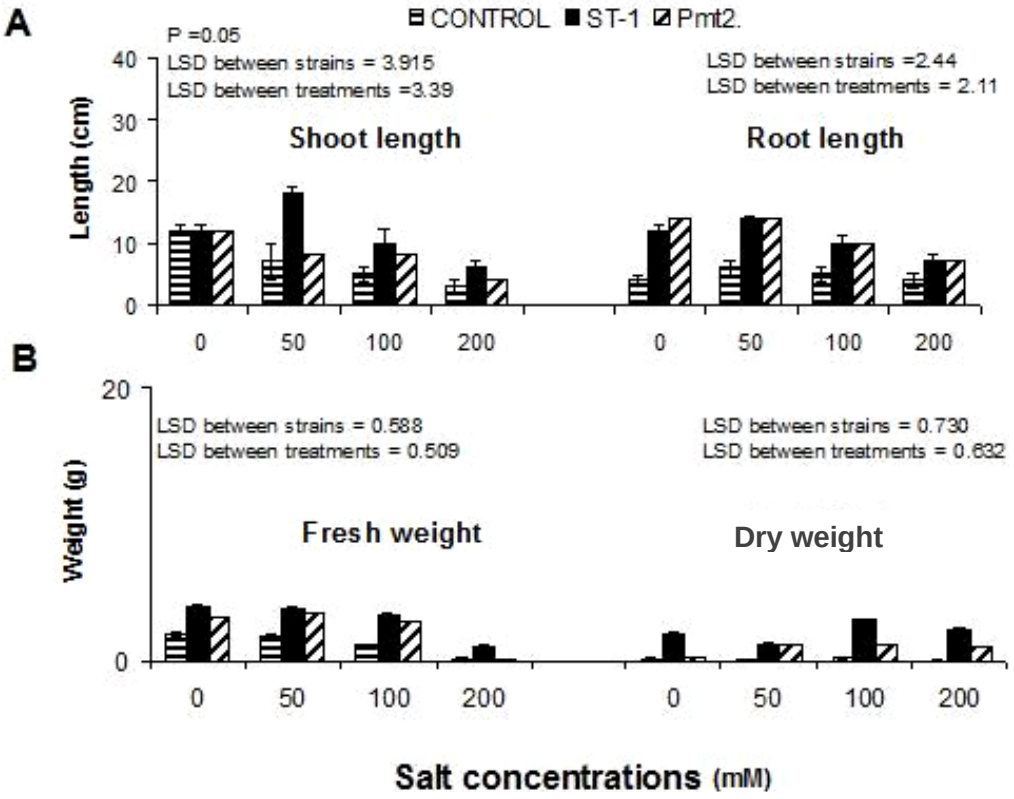

Figure 4**.** Effects of varying salt concentrations on the (A) Shoot Length and root length (cm) and (B) fresh weight and dry weight (g) parameters of *L. esculenta* Var. masoor 93. Error bars show standard errors of the mean values.

in the presence of higher salt stress and increases the biofilm stability of bacterial cells. Both the coupled analysis of biofilm formation and EPS production at higher salt stress gives a better clue for bacterial survival strategies in rhizosphere. Efficient colonization of *Azospirillum brasilense* with wheat roots involves the sugars helping its adherence (Bacilio-Jiménez et al., 2001). Bacterial EPS under salt stress can bind sodium ions and reduces its toxicity in the soil. These may be in line with recent studies showing the role of EPS to bind the sodium Na^+ and alleviate its toxic effect (Arora et al., 2010). Salt free soil thus stimulates the plant growth by providing the nutrients in the soil (Afrasayab, 2010; Ahmed and Hasnain, 2010). Exopolysaccharides (EPS) production under salt stress has been reported to play a significant role in providing protection to the cell under salt stress (Arora et al., 2010). With these results we may speculate the effect of these strains on better symbiotic relationship of plants.

Plant microbe interaction experiments

Our results show that salinity greatly affected the growth and biochemical parameters of plants. This is in accord with several recent and previous reports where depressive effect of salt on leguminous crops has been reported (Soussi et al., 1998; Chookhampaeng et al., 2011). However, bacterial inoculation improved the growth parameters of *L. esculenta* plants under salt stress. Like other leguminous crops *L. esculenta* seems very tempting for soil microorganisms by releasing phytohormones that stimulate bacterial root colonization and EPS formation (Ahmed et al., 2010). Previous studies also highlight the significance of biofilm formation on plant roots and regulation of plant growth under salt stress (Fujishige et al., 2006; Hirsch, 2010). Indeed strains used in the present study have plant association and they have been reported to increase the growth of *Triticum aestivum* under NaCl stress (Afrasayab and Hasnain, 2000a, b).

Shoot length (Figure 4) of seedlings was significantly ($p<0.05$) reduced. Salinity reduced maximum shoot length reduction at 200 mM NaCl stress by 75%. Maximum reduction in root length was 50% at 200 mM NaCl stress. However, bacterial inoculation caused noteworthy improvements in shoot and root length of *Lens esculenta* with and without salt stress. Noteworthy

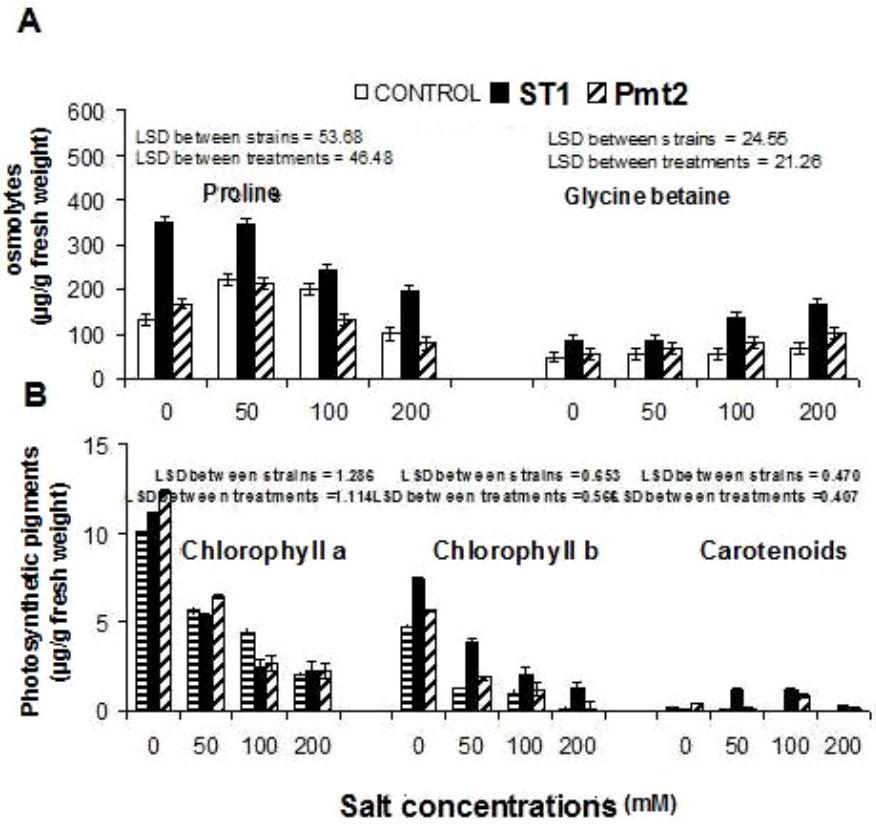

Figure 5. Effects of varying salt concentrations on the (A) Endogenous osmolyte (µg/g fresh weight) accumulation and (B). photosynthetic pigments (Chlorophyll a, Chlorophyll b and carotenoids (µg/g fresh weight) of *L. esculenta* Var. masoor 93.

improvement 157% in shoot length by ST1 under 50 mM NaCl stress and 100% by Pmt2 fewer than 200 mM NaCl stress respectively, compared to control plants (Figure 4). Salinity causes cells to lose water and shrinkage of cells gradually resulting in death at cellular level (Munns, 2002). Salinity also induces morphological and developmental changes e.g. lifecycle inhibition and reduction in the leaves and root growth (Hasegawa et al., 2000). Many workers reported stretching of root tissue causing reduction in length parameters (Stephenson et al., 2000).

Presence of salt in soil caused 11, 43 and 96% reduction in fresh weight at 50, 100 and 200 mM NaCl stress, respectively over non-inoculated control plants (Figure 4). Bacterial inoculation of ST1 caused maximum increase (147%) at 200 mM NaCl stress and Pmt2 inoculation caused maximum stimulation (154%) at 100 mM NaCl stress. Dry weight values were 50% reduced at 200 mM NaCl stress compared to other concentration of salt. Bacterial inoculation significantly ($p<0.05$) improved the dry weight with and without salt stress. However, most spectacular increase was observed at 200 mM NaCl by ST1 and Pmt2 (Figure 4). Decrease in fresh weight with higher salinity is due to accumulation of inorganic ions (Na^+ / K^+ ions), which resulted in decreased water level or accumulation of increased compatible solutes. According to previous studies water and salt stress had detrimental effects on fresh weight accumulation in young leaves of different tomato cultivars (Sturz et al., 2000). Increased fresh weight with bacterial inoculations reflects that bacteria might be involved in taking up (Na^+) from the media making availability of water to plants or increasing the water status of plants (Afrasayab et al., 2010). Dry weight of plants increased over time scale Hasegawa et al. (2000). Increase dry weight parameters under salt stress have variously been reported with osmotic adjustment mechanism of plants by augmented level of organic and inorganic solutes in the tissues (Ashraf and Harris, 2004).

Salinity in the rhiozosphere results in leaves senescence. Both quantitative as well as qualitative changes in photosynthetic pigments composition was observed under salt stress. Salinity affected the photosynthetic activities of plants. Maximum reduction in Chlorophyll a, b and carotenoid was observed at 200 mM NaCl stress by 80, 98 and 95% at (Figure 5). Bacterial inoculation significantly improved the photosynthetic pigments. Strain ST1 caused most

significant increment in chlorophyll a, chlorophyll b and carotenoid contents at 200 mM NaCl stress. Salinity diminishes the photosynthetic activities of plants under salt stress. In general, bacterial inoculations improved the chlorophyll pigments over respective non inoculated treatments. Bacterial inoculation improves the water status of plants thus increasing plant vigor (Yang and Lu, 2005). Moreover, improved water status of the plant in inoculated plants causes stomatal openings and increased CO_2 assimilation in plants (Dubey, 2005).

Higher level of endogenous osmolytes accumulation in plants is a halophytic characteristic of plants. Salinity causes water deficit in plants at a very first level. In an effort to overcome this stress, plants increase their endogenous osmolytes accumulation (Nemati et al., 2011). Accumulation of endogenous proline in plants increased by 68 and 51% at 100 and 200 mM NaCl stress. Maximum accumulation of proline (98%) in plant with inoculation of ST1 and (33%) with inoculation of Pmt2 was observed at 100 mM NaCl stress. Most significant level of endogenous glycine betaine accumulation was observed in plants inoculated with both strains at 100 mM NaCl stress. In the present study, a relationship is observed between inoculated and noninoculated plants and their osmotic levels as salt tolerance. It is likely that inoculation oppressed NaCl to maintain normal metabolic activities required for growth, thereby sustain in defending salt effects from plants. Endogenous osmolytes include various compounds like proline, glycine betaine, choline, salicyclic acids, brassinosteroids, silicates and total soluble sugars etc (Munns, 2002). These compounds play a fundamental role in osmotic adjustment under saline condition and create the hindrance in the way of ion toxicity for plants. Thus elevated level of endogenous osmolytes in inoculated plants compared to noninoculated control plants under salt stress show salt tolerance status of plants.

Conclusion

From above results, it can be speculated that the strains establish biofilm and produce extracellular EPS to cope with salt stress. Endogenous accumulation of intracellular osmolytes in bacterial cells is also elevated at high salinity that protects the cells from adverse effects of salinity in soil. It can be speculated that same mechanism might be adopted by bacterial cells under salt stress in the rhizosphere that help their successful survival in the rhizosphere. Biofilm and exoploysacchride not only protects bacterial cells but also help them to reduce toxicity of sodium in soil that increases the plant growth under salinity. However, further study of osmo-adaptation at molecular level is still needed for understanding detailed mechanism. It will also be helpful for continuing purpose of plant-microbe interaction for the improvement of lentil crops in saline soil.

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