

Full Length Research Paper

Alleviation of salt stress by *Halomonas* sp. and osmolytes in *Zea mays*

Aisha Waheed Qurashi* and Anjum Nasim Sabri

Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-i-azam Campus, Lahore, Pakistan-54590.

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Level of osmolytes accumulation to a variable extent in bacteria and plants is associated with the salt tolerance. We hypothesized that two previously isolated salt tolerant strains, *Halomonas meridiana* (PAa6) and *Halomonas aquamarina* (RT2), found to stimulate the growth of wheat also have the ability to accumulate osmolytes. These strains can favour plant growth due to their endogenous osmolyte accumulation. To ascertain this, strains were checked for osmolyte accumulation under salt stress. Growth and osmolyte accumulation of inoculated and non-inoculated seeds of the economically significant plant, *Zea mays* Var. EV.90, was recorded. Seeds were sown in soil supplemented with and without exogenous proline and glycine betaine and salt stress for 15 days. The results show that strains can accumulate osmolytes (preferably glycine betaine) at higher salt stress. Inoculation and exogenous osmolytes (10 mM) improved the plant growth at higher salt stress. However, concurrent application had an amplified effect. Endogenous level of osmolyte (preferably glycine betaine) on the fresh weight basis was significantly higher and improved bacterial and plant growth under stress. We conclude that accumulation of osmolytes in bacteria and plants significantly stimulated growth and protect them from the adverse effect of salt stress. This can be an alternate economic approach to increase crop productivity.

Key words: Osmolyte, glycine betaine, proline, salt stress.

INTRODUCTION

Dependence on multipurpose cereal crop maize is growing to a large extent throughout the world, while putting together all its uses. Sensitivity of maize to salt stress is of major concern for farmers all over the world (Maas et al., 1983; Jadhav et al., 2010). It is noteworthy for Pakistan (Nasim et al., 2008) and other countries where increasing population has markedly established the food security problems. Supporting a superior portion of the world's economy, it is vital to amplify maize yields even under the hostile conditions of salt stress. Salt stress, either due to badly chosen irrigation exercise, no drainage, poor soil management and higher evapotranspiration causes build-up of excess salts in the soil which is adverse to the crops yield (Afrasyab et al., 2010). Accumulation of osmolytes in bacteria and plants

is a stress-related phenomenon (Canovas et al., 1998). Although, much salt tolerant plants accumulate osmolytes in response to salt stress due to their genetic and biochemical advantage, they are still plants that can do this according to their metabolic capacity. Thus, higher accumulation of these osmolytes in response to salt stress is of prime importance in stress adaptation. The safer use of indigenous microorganisms for plant yield is among one of the cost effective technologies (Afrasyab and Hasnain, 2000a; Ahmed and Hasnain, 2010).

In this study, we worked on a number of bacteria isolated from the close vicinity of plants of saline soils in Pakistan; PAa6 from phylloplane of *Achyranthus aspera* (Afrasyab and Hasnain, 2000b) and *Halomonas aquamarina* (RT2) from rhizoplane of *Mazus* specie (Mirza et al., 1998). These have the innate ability to thrive under salt stress to a variable extent where existence of non-halophilic bacteria is difficult. These strains have also been reported to improve *Triticum aestivum* plant growth

*Corresponding author. E-mail: aieshawaheed@yahoo.com.

by increasing their auxin, alkaline phosphatases, acid phosphatase and proteins content (Mirza et al., 1998; Afrasayab and Hasnain., 2000b; Mehr et al., 2002; Afrasayab et al., 2010; Ahmed and Hasnain, 2010). Increased endogenous accumulation of osmolytes (proline, glycine betaine, ectoine, hydroxyectoine, choline and other amino acids) provide a salt tolerance to organisms (Roberts, 2005). Role of compatible solutes to bacteria as well as to plants resulted in improving their stress resistance (Canovas et al., 1998; Roberts, 2005; Vyrides and Stuckey, 2009). Other reports also highlight the beneficial impact of microorganisms isolated from stressed soil on plant growth (Sadowsky, 2005; Hirsch, 2010; Karlidag et al., 2011). From previous studies, considerable successful attempts have been made to ameliorate soil salinity with exogenous osmolytes like proline and glycine betaine.

With the aim of taking any measure toward economic and sustainable agriculture, we used salt tolerant bacteria and checked the potential for plant growth promotion for present research work. In our work, we studied growth and endogenous osmolyte accumulation from plants and bacterial point of view. Moreover, the effect of bacterial inoculation on salt sensitive maize plants were also compared with plants treated with exogenously applied osmolytes and varying salt concentrations.

MATERIALS AND METHODS

Growth conditions and osmolytes accumulation of bacterial strains

Previously isolated and characterized bacterial strains *H. meridiana* (PAa6) (accession no. HQ256515) (Afrasayab and Hasnain, 2000b) and *H. aquamarina* (RT2) (accession no. HQ107978) (Mirza et al., 1998), were used for the current study. Strains were maintained routinely in LB agar (Gerhardt et al., 1994) supplemented with 0.5 M NaCl.

To study the effect of different NaCl concentrations (0.5, 1, 1.5, 2 and 2.5 M) on the growth and osmolyte accumulation of bacterial strains, 10 ml LB (Gerhardt et al., 1994) and M9 broth (Kahn et al., 1979) added with respective molar concentrations of NaCl were poured in test tubes and autoclaved. 24-h old bacterial suspension (absorbance adjusted 0.3 A at 600 nm; 10^8 ml⁻¹ CFUs) was inoculated in each test tube containing L broth and M9 media. One test tube in each case was used as a control. All the test tubes were incubated at 37°C for 24 h at 150 rpm on the shaker (orbital incubator Model I-4000 serial number 104 A IRMECO GmbH, Goesthacht/Germany) at pH 8.0. After 24 h incubation, bacterial growth was recorded by measuring absorbance (OD₆₀₀) using the spectro-photometer (Model S-300 DL, R & M marketing, and England).

Also, bacterial cells were harvested from cultures after 24 h by centrifugation (2000 rpm for 5 min), weighed and suspended in 1 ml sterile distilled water. Cells were used for determination of endogenous proline following the method of Tonon et al. (2004). The level of endogenous glycine betaine and choline was determined following the method of Grieve and Grattan (1983). Calibration of proline and glycine betaine was made using reference L-proline and glycine betaine, respectively. The experiments were carried out with three replicates and mean and standard errors of means were calculated. In all the Figures, the values are represented with standard errors of the means.

Inoculation of plants, harvesting and biochemical analysis

For inoculum preparation, bacterial strains were incubated overnight (0.5 M NaCl stress, pH and temperature 37°C) at 150 rpm shaker in LB medium. Overnight cultures were centrifuged and bacterial pellet was suspended in sterile water. Optical density of this suspension was measured on spectrophotometer at 600 nm. To ensure the equal cell population of each bacterial strain, optical density of the suspension was adjusted to 10^8 cells ml⁻¹. Healthy and certified seeds of *Zea mays* Var. EV-90 were obtained from Punjab Seed Corporation, Lahore, Pakistan and used in experiments. Seeds were disinfected with 0.1% HgCl₂ solution for 10 minutes and followed by five to six times rinsing with sterile water to remove all traces of HgCl₂ completely. After surface sterilization, seeds were inoculated with 10 ml of bacterial suspension for 30 minutes. Uninoculated seeds were sown as control treatments. For control, seeds were soaked in sterile water of same volume and for the same period of time in disposable sterile plastic pots filled with 120 g sieved and sterilized garden soil with 95% water- holding capacity.

After seed were sown, equal volume of salt solution was added to each pot so that the final concentrations of the NaCl in the pots were 50, 100 and 200 mM per gram weight of soil. Pots without any salt solution (0 mM NaCl) were treated as control. Similarly, filter sterilized solutions of proline and glycine betaine were added to each pot making the final concentrations 10 mM per gram weight of soil. To prevent fluctuations in salt concentrations of soil, pots were watered with care so that salt or soil was not drained out of the pot. Seeds were allowed to germinate in the dark in a growth chamber at 28 ± 30°C for three days, and 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. The seedlings were harvested after 15 days and different growth parameters including germination, shoot length, root length, fresh weight, dry weight and Chlorophyll (a, b and carotenoid content) were recorded. Endogenous proline from harvested plants was determined following Tonon et al. (2004); endogenous glycine betaine and choline were determined following Grieve and Grattan (1983), while chlorophyll a, b and carotenoid contents of the plants were determined following Lichtenthaler and Wellburn (1983). The experiment was carried out in three replicates. Mean and standard errors of means were calculated, and the difference between the means was tested using the least significant difference test ($p < 0.05$).

RESULTS

Determination of osmolyte accumulation in bacterial strains

The effect of varying concentrations of NaCl on the growth of the bacterial strains was recorded. With increasing salt concentrations, decrease in bacterial growth was observed. Growth was comparatively better in LB as compared to M9 medium (Figure 1). Optimum growth of strain *H. meridiana* (PAa6) and *H. aquamarina* (RT2) was observed at 0.5 M NaCl stress after which growth decreased for both strains in LB and M9 media, respectively. Although, both strains of *H. meridiana* (PAa6) and *H. aquamarina* (RT2) showed a comparable growth profile in LB and M9 media, however, growth was comparatively less in M9 media compared to LB (Figure 1). Unlike growth pattern, cellular osmolyte accumulation

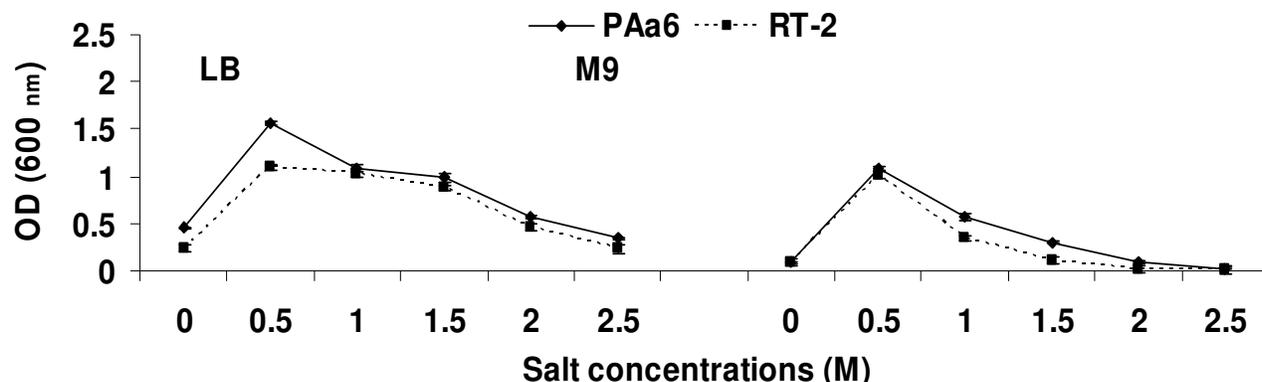


Figure 1. Effect of varying NaCl concentrations (M) on bacterial growth in LB and M9 media.

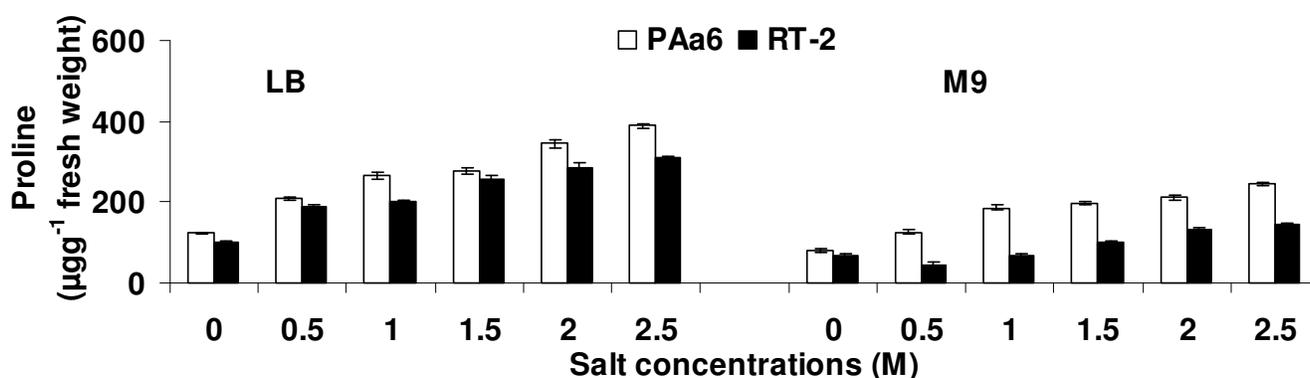


Figure 2. Effect of varying NaCl concentrations (M) on endogenous proline ($\mu\text{g g}^{-1}$ fresh weight of bacterial strains) accumulation in LB and M9 media.

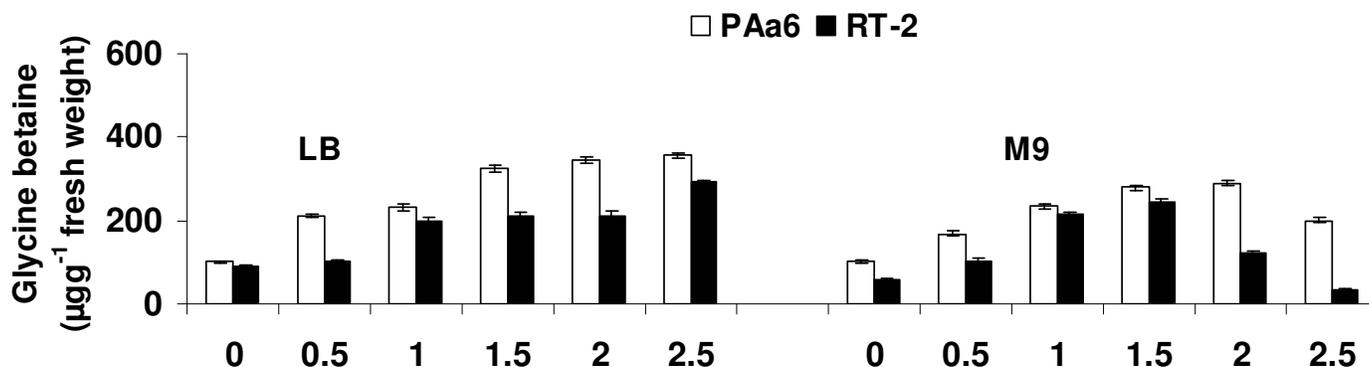


Figure 3. Effect of varying NaCl concentrations (M) on endogenous glycine betaine ($\mu\text{g g}^{-1}$ fresh weight of bacterial strains) accumulation in LB and M9 media.

on the fresh weight basis tends to increase at increasing salt stress. However, in general, accumulation of choline (Figure 4) was less as compared to proline (Figure 2) and glycine betaine (Figure 3) osmolytes. At 0 mM salt stress, accumulation of osmolytes was lower (Figures 2 to 4). *H. meridiana* (PAA6) was found to be more efficient in

accumulating both osmolytes than *H. aquamarina* (RT2) strain. Accumulations of glycine betaine (Figure 3) were higher in *H. aquamarina* (RT2) at 0 and 0.5 M NaCl supplemented media. Maximum choline accumulation was observed at 1.5 M to 2.5 M NaCl stress in LB medium.

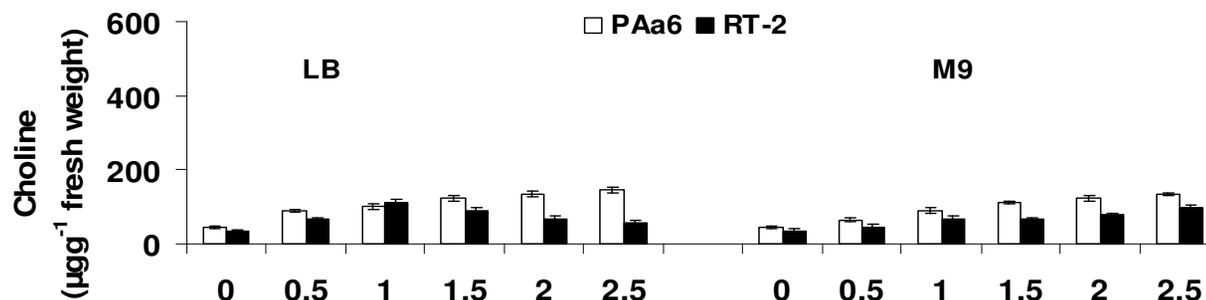


Figure 4. Effect of varying NaCl concentrations (M) on endogenous choline (µg⁻¹fresh weight of bacterial strains) accumulation in LB and M9 media.

Table 1. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on germination (%) of *Zea mays* Var. EV.90.

Treatment	Germination (%)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	100 ± 1.23	34 ± 13	28 ± 12	17 ± 9	75 ± 0.89	85 ± 1.33	65 ± 1.32	65 ± 0.99	65 ± 0.98	55 ± 1.11	55 ± 2.22	45 ± 1.29
PAA6	100 ± 1.23	78 ± 15	56.09 ± 23	53 ± 14	75 ± 1.99	85 ± 1.45	90 ± 2.33	75 ± 1.24	85 ± 0.98	75 ± 2.22	75 ± 2.53	60 ± 1.34
RT-2	100 ± 2.23	56 ± 20	48.56 ± 23	47 ± 12	65 ± 1.23	65 ± 2.30	40 ± 2.43	55 ± 1.45	75 ± 1.23	65 ± 1.23	45 ± 2.33	59 ± 1.92

LSD at p = 0.05
 Between strains 35.58 12.13 16.32
 Between treatments 30.81 8.57 11.54

Effect of bacterial inoculation on growth parameters of *Zea. mays* seedlings

Severe reduction in germination of seedlings (Table 1) at NaCl stress was observed especially at 200 mM NaCl (83%) as compared to non inoculated control plants grown without NaCl stress. However, noteworthy increase (211 %) was observed with *H. meridiana* (PAA6) and *H. aquamarina* (RT2) (176%) at 200 mM NaCl stress. Exogenous application of 10 mM proline and 10 mM glycine betaine also improved seed germination. However, bacterial inoculation along with exogenous osmolytes significantly increased

the germination. However, addition of proline with bacterial inoculation *H. aquamarina* (RT2) reduced the germination with and without salt stress compared to non-inoculated controls. Salt stress resulted in the maximum reduction in shoot length (33%) and root length (38%) at 200 mM NaCl stress, respectively over noninoculated plants at 0 mM NaCl stress. Bacterial inoculation caused an increment (58.3 with PAA6 and 17% with RT2) in shoot length (Table 2) at no salt stress. Both strains *H. meridiana* (PAA6) and *H. aquamarina* (RT2) showed maximum 40% increment at 50 mM NaCl stress. Similar response was seen in case of root length (Table 3); strain

H. meridiana (PAA6) showed 46% increment, while *H. aquamarina* (RT2) showed 39% increment at 0 mM NaCl stress. Exogenous application of glycine betaine improved root length compared to exogenous proline. Root length also increased (87%) with exogenous proline and (137%) with glycine betaine at 200 mM salt stress compared to non-osmolyte supplemented control plants. With the combination of exogenous osmolytes and bacterial strains, the effect of glycine betaine was slightly higher than proline. Addition of proline resulted in 30% and 19% increase in shoot length with *H. meridiana* (PAA6) and *H. aquamarina* (RT2) strain,

Table 2. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on Shoot length (cm) of *Z. mays* Var. EV.90.

Treatment	Shoot length (cm)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	12 ± 0.45	10 ± 0.78	8 ± 0.89	8 ± 0.89	10 ± 0.89	14.4 ± 0.45	13 ± 0.79	10 ± 0.56	18 ± 0.67	16 ± 0.68	15 ± 1.89	15 ± 3.89
PAa6	19 ± 0.89	14 ± 0.98	12 ± 0.89	10 ± 0.89	20 ± 0.63	18 ± 0.56	14 ± 0.34	13 ± 0.89	25 ± 0.56	23.5 ± 0.89	20 ± 2.0	18.5 ± 2.09
RT-2	14 ± 0.88	14 ± 0.34	11 ± 1.09	10.9 ± 0.98	22 ± 0.34	17 ± 0.23	15 ± 0.34	11.9 ± 0.45	21 ± 0.49	19.12 ± 0.78	16.5 ± 1.79	13.5 ± 2.23
LSD at p = 0.05												
Between strains 2.046 3.86 1.738												
Between treatments 1.772 3.35 1.505												

Table 3. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on root length (cm) of *Z. mays* Var. EV90.

Treatment	Root length (cm)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	13 ± 0.89	12 ± 0.34	8 ± 0.78	8 ± 1.09	22 ± 0.34	19 ± 0.78	19 ± 0.65	15 ± 0.99	26 ± 0.76	23 ± 0.67	21 ± 0.29	19 ± 0.57
PAa6	19 ± 0.82	14 ± 0.24	13 ± 0.79	11 ± 0.59	25 ± 0.32	21 ± 0.34	18 ± 0.56	16 ± 0.34	29 ± 0.68	25 ± 0.54	28 ± 0.38	25 ± 0.78
RT-2d	18 ± 0.56	13 ± 0.89	10 ± 1.67	9 ± 0.78	21 ± 0.89	21 ± 0.89	20 ± 0.89	18 ± 0.79	26 ± 0.98	23.5 ± 0.59	29 ± 0.56	20 ± 2.39
LSD at p = 0.05												
Between strains 2.059 6.35 2.22												
Between treatments 1.786 5.506 1.928												

respectively at 200 mM NaCl stress, compared to non-inoculated control plants grown at same treatment. Combination of strain *H. meridiana* (PAa6) and exogenously applied glycine betaine also increased the root length by 33% at 100 mM and 31.5% at 200 mM NaCl compared to non-inoculated control plants.

In addition, maximum reduction (96 %) in fresh weight (Table 4) was observed at 200 mM NaCl stress compared to control plant. However, both strains improved fresh and dry weight (Table 5) over noninoculated control plants at same stress. Strain *H. meridiana* (PAa6) showed 144% increase and strain *H. aquamarina* (RT2) showed

77.7% increase in fresh weight, over respective non-inoculated control plants at 0 mM salt stress. Both strains *H. meridiana* (PAa6) and *H. aquamarina* (RT2) showed upper limit increment in fresh weight (1100 and 1111%) and dry weight (261% and 206%) at 200 mM NaCl stress, respectively. Bacterial inoculation and exogenously applied osmolytes showed maximum increase in fresh weight and dry weight of plant at 100 mM NaCl stress. Exogenously applied glycine betaine showed significant increase in the dry weight values at 200 mM NaCl stress.

Furthermore, salt stress improved the plant growth by improving chlorophyll contents of plants

(Table 6). Salt stress resulted in 40% increase at 50 mM NaCl, and 33 and 33% reduction in Chlorophyll a contents at 100 and 200 mM NaCl stress, respectively over noninoculated plants at 0 mM NaCl stress. Similarly, 18%, 49% and 69% reduction at 50, 100 and 200 mM NaCl stress in chlorophyll b contents (Table 7) was recorded over noninoculated plants at 0 mM NaCl stress. Carotenoid contents (Table 8) increased 0.47 at 50 mM and 43.5% at 100 mM, respectively over noninoculated plants at 0 mM NaCl stress. No change was however observed for chlorophyll b contents of inoculated plants at 200 mM NaCl stress. Inoculation of *Halomonas meridiana*

Table 4. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on fresh weight (g) of *Z. mays* Var. EV90.

Treatment	Fresh weight (g)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	2.7 ± 0.12	1.8 ± 0.09	1.8 ± 0.12	0.09 ± 0.01	3.8 ± 0.56	2.8 ± 0.19	1.9 ± 0.89	1.5 ± 0.89	4.6 ± 0.67	3.6 ± 0.89	2.5 ± 0.89	2.0 ± 1.89
PAa6	6.6 ± 0.15	5.0 ± 0.12	3.8 ± 0.12	1.08 ± 0.02	6.9 ± 0.65	4.5 ± 0.52	4.5 ± 0.56	2.9 ± 0.56	10.9 ± 2.09	7.1 ± 0.22	6.3 ± 1.89	4.5 ± 0.89
RT-2	4.8 ± 0.89	2.8 ± 0.23	2.0 ± 0.09	1.09 ± 0.09	4.5 ± 0.19	3.5 ± 0.83	2.0 ± 0.56	2.5 ± 0.34	10.9 ± 1.98	5.4 ± 0.67	5.4 ± 3.78	3.9 ± 0.67
LSD at p=0.05												
Between strains 3.10 0.995 1.34												
Between treatments 2.68 0.703 0.94												

Table 5. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on dry weight (g) of *Z. mays* Var. EV90.

Treatment	Dry weight (g)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	0.14 ± 0.09	0.2 ± 0.01	0.27 ± 0.01	0.34 ± 0.03	0.32 ± 0.1	0.29 ± 0.1	0.18 ± 0.1	0.13 ± 0.02	0.29 ± 0.07	0.19 ± 0.09	0.12 ± 0.09	0.09 ± 0.09
PAa6	0.8 ± 0.09	0.9 ± 0.04	1.06 ± 0.1	1.23 ± 0.09	1.2 ± 0.15	0.65 ± 0.1	0.49 ± 0.5	0.2 ± 0.06	0.98 ± 2.0	0.29 ± 0.02	0.25 ± 0.09	0.10 ± 0.01
RT-2	0.7 ± 0.01	0.8 ± 0.09	1.09 ± 0.2	1.04 ± 0.23	0.9 ± 0.19	0.45 ± 0.2	0.29 ± 0.5	0.19 ± 0.04	0.65 ± 1.9	0.23 ± 0.07	0.19 ± 0.08	0.19 ± 0.01
LSD at p = 0.05												
Between strains 3.10 0.518 0.274												
Between treatments 2.68 0.4488 0.237												

(PAa6) caused 97.5%, 48 %, 122 % and 75 % increase in chlorophyll a, 143 %, 200 %, 196 % and 300 % increase in chlorophyll b, while 143 %, 90.4%, 33% and 50% increase in carotenoid contents at 0, 50, 100 and 200 mM NaCl stress, respectively.

Inoculation of *H. aquamarina* (RT2) caused 97.5%, 48.2%, 122.5% and 50% increase in chlorophyll a contents while 22.4, 62.5, 100 and 229% increase in chlorophyll b contents at 0, 50, 100 and 200 mM NaCl stress, respectively over noninoculated plants. While 33.9 and 47.6% increase in carotenoid contents at 0 and 50 mM NaCl stress, and 6% and 40% reduction in

carotenoid content was observed in non-inoculated plants at 100 and 200 mM NaCl stress, respectively. Application of exogenous glycine betaine (10 mM) also increased 125% chlorophyll a at 100 mM NaCl stress while 433% increase in chlorophyll b was recorded as compared to control at 200 mM NaCl stress. Combination of seed inoculation with exogenously applied osmolytes increased both chlorophyll a and b contents.

Level of endogenous osmolytes accumulation was increased by (178% and 123%) with a combination of bacterial strain *Halomonas meridiana* (PAa6) and *Halomonas aquamarina*

(RT2), respectively and exogenous osmolytes at 200 mM NaCl stress.

Endogenous accumulation of proline (Table 9), glycine betaine (Table 10) and choline (Table 11) in seedlings were also found to be affected by bacterial inoculation. Increment in proline and glycine betaine accumulation was observed at increasing salt stress in non-inoculated control plants. However, this accumulation was further stimulated in inoculated plants. In non-inoculated plants (134%) maximum increase in proline and boost (286%) in glycine betaine at 200 mM NaCl was observed. Inoculation of strain *H. meridiana* (PAa6) caused maximum (156%) increase at 200

Table 6. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on Chlorophyll "a" ($\mu\text{g/g}$ fresh weight) of *Z. mays* Var. EV90.

Treatment	Chlorophyll "a" ($\mu\text{g/g}$ fresh weight)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	12 \pm 0.89	16.78 \pm 0.89	8 \pm 0.12	8 \pm 0.89	18 \pm 2.59	18 \pm 1.78	16 \pm 1.12	14 \pm 0.69	22 \pm 0.69	22 \pm 2.89	18 \pm 0.82	16 \pm 0.79
PAa6	23.7 \pm 0.34	24.88 \pm 0.98	17.8 \pm 0.9	14 \pm 1.09	32 \pm 1.94	29 \pm 1.23	20 \pm 1.9	20 \pm 1.89	39 \pm 1.84	30 \pm 1.98	25 \pm 0.99	23 \pm 1.39
RT-2	22.4 \pm 0.12	23 \pm 0.99	12 \pm 0.89	12 \pm 1.34	26 \pm 1.92	22 \pm 1.78	19 \pm 2.89	19 \pm 0.94	25 \pm 1.02	25 \pm 1.99	20 \pm 0.99	18 \pm 1.24
LSD at p = 0.05												
Between strains 3.54 5.226 4.872												
Between treatments 3.06 3.69 3.445												

Table 7. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on Chlorophyll "b" ($\mu\text{g/g}$ fresh weight) of *Z. mays* Var. EV90.

Treatment	Chlorophyll "b" ($\mu\text{g/g}$ fresh weight)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	9.8 \pm 0.23	8 \pm 0.89	5 \pm 0.89	3 \pm 0.89	10 \pm 0.45	14 \pm 1.89	12 \pm 2.89	12 \pm 2.89	23 \pm 0.78	25 \pm 1.89	18 \pm 0.69	16 \pm 1.89
PAa6	23.8 \pm 0.8	24 \pm 1.00	14.8 \pm 0.8	12 \pm 0.12	32 \pm 0.67	29 \pm 2.00	29 \pm 1.8	20 \pm 1.12	35.8 \pm 0.98	32 \pm 2.00	32 \pm 0.78	30 \pm 2.12
RT-2	12 \pm 0.12	13 \pm 1.09	10 \pm 1.09	9.89 \pm 0.89	29 \pm 1.09	25 \pm 2.09	20 \pm 2.09	12 \pm 1.89	22 \pm 1.89	30 \pm 2.09	19 \pm 1.29	22 \pm 2.89
LSD at p = 0.05												
Between strains 4.42 7.47 5.14												
Between treatments 3.83 5.28 3.63												

mM NaCl, while *H. aquamarina* (RT2) caused (183%) increase at 50 mM NaCl in proline content. Inoculation of strain *H. meridiana* (PAa6) caused 224% increase at 200 mM NaCl, while *H. aquamarina* (RT2) caused increase 187% at 200 mM increase in glycine betaine content of the plants. Exogenously applied osmolytes further stimulated the accumulation of osmolytes. It was observed that addition of glycine betaine reduced the endogenous level of proline when applied in combination with bacterial inoculation and also reduced the choline level.

DISCUSSION

The results in the present study supports previous reports showing that bacterial growth and osmolyte accumulation are strongly affected by varying media and salt concentrations (Roberts, 2005; Kouas et al., 2010). M9 medium and higher salt stress resulted in reduced growth of both strains. Association of increased osmolytes accumulation at higher salt stress suggests the possibility that osmolytes have a protective role in stabilizing macromolecules to extreme conditions

and thus regulating metabolic activities of cells (Munns, 2002). Recently, accumulation of osmolytes and bacterial growth has been reported to be effected by culture conditions (Fallet et al., 2010). However, the influence of salt stress was not same for bacterial growth and osmolyte accumulation. Also, it is found that endogenous accumulation of glycine betaine is more than accumulated proline and choline at same culture conditions. The osmoprotective role of glycine betaine has been discussed previously (Zhao et al., 2009). There are many other evidences where

Table 8. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on carotenoids (µg/g fresh weight) of *Z. mays* Var. EV90.

Treatment	Carotenoids (µg/g fresh weight)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	2.09 ± 0.8	2.1 ± 1.23	3.0 ± 1.23	2.0 ± 1.23	2.09 ± 0.3	22 ± 1.09	12 ± 1.03	12 ± 1.7	4.05 ± 0.3	23 ± 3.09	32 ± 2.03	42 ± 1.88
PAa6	5.09 ± 0.3	4.0 ± 1.23	4.0 ± 1.23	3.0 ± 1.23	12.1 ± 0.3	89 ± 1.09	65 ± 1.03	42 ± 1.67	8.12 ± 0.9	112 ± 3.09	89 ± 1.93	65 ± 1.87
RT-2	2.8 ± 0.12	3.1 ± 2.23	2.8 ± 2.23	1.2 ± 2.23	3.85 ± 1.7	65 ± 3.23	45 ± 2.29	32 ± 2.93	4.52 ± 1.2	75 ± 3.93	65 ± 2.19	65 ± 2.03
LSD at p = 0.05												
Between strains 1.58 23.27 34.17												
Between treatments 1.374 16.45 24.16												

Table 9. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on proline (µg/g fresh weight) of *Z. mays* Var. EV90.

Treatment	Proline (µg/g fresh weight)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	100 ± 1.23	109 ± 9.89	209 ± 7.8	234 ± 9.8	56 ± 5.23	78 ± 12.89	100 ± 7.8	123 ± 12	123 ± 10.3	234 ± 9.89	256 ± 12.9	312 ± 23.8
PAa6	209 ± 0.98	423 ± 12.9	489 ± 12	600 ± 9.8	120 ± 11.9	189 ± 22.9	200 ± 5.2	200 ± 12.8	289 ± 12.8	312 ± 12.9	332 ± 19	345 ± 12.8
RT-2	189 ± 1.23	309 ± 12.2	509 ± 7.8	765 ± 3.8	100 ± 10.2	200 ± 32.2	189 ± 9.8	198 ± 5.8	200 ± 18.3	298 ± 12.2	300 ± 12	345 ± 23.8
LSD at p = 0.05												
Between strains 176.12 62.28 126												
Between treatments 152.53 44.04 89.35												

endogenous proline and glycine betaine accumulation at higher salt concentrations have osmoprotective role coping with increasing salt concentrations (Munns, 2002; Nagata et al., 2009). It is also relevant to mention that bacterial growth requirements are increased in the presence of salt stress so bacteria prefer LB medium for optimum growth and osmolytes accumulation unlike minimal media (Gouffi and Blanco, 2000).

Following this microbial work, we tested the comparative effect of bacterial inoculations and exogenously applied osmolytes on the salt

tolerance of maize seedlings at 50, 100 and 200 mM NaCl concentrations separately along with their simultaneous application to seedlings. Increasing salt stress inhibited all growth parameters of plants. Moreover, plant growth at higher level of salinity showed significant reduction in germination, length, weight and chlorophyll contents. However, accumulation of osmolytes was higher in the plants grown at increasing salt concentrations. In general, it is accepted that salt sensitive plants are unable to compartmentalize the salt in vacuoles (Ashraf and Harris, 2004). It reduces the amount of available water needed for

essential metabolic activities and ultimately causes yellowing of leaves, reducing photosynthetic activities and reduction in plant growth (Hussain et al., 2010). Decrease in fresh weight and increase in dry weight at high salinity is due to accumulation of inorganic ions (Na⁺/K⁺ ions). The increased level of endogenous compatible solutes has also been reported to be involved with osmotic adjustment mechanism of plants (Ashraf and Harris, 2004).

Bacterial inoculation improved the plant growth and elevated their endogenous osmolytes accumulation at higher salt stress. Bacterial

Table 10. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on glycine betaine ($\mu\text{g/g}$ fresh weight) of *Z. mays* Var. EV90.

Treatment	Glycine betaine ($\mu\text{g/g}$ fresh weight)											
	Salt concentrations (mM)				Salt concentrations (mM) + 10 mM proline				Salt concentrations (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	23 \pm 9.89	45 \pm 12	69 \pm 9.8	89 \pm 9	123 \pm 23.9	134 \pm 12	234 \pm 9.8	298 \pm 9	156 \pm 9.89	165 \pm 12	345 \pm 9.8	389 \pm 9
PAa6	100 \pm 8.78	145 \pm 12	156 \pm 8.8	289.98 \pm 9	300 \pm 12.78	378 \pm 8.8	435 \pm 12	534 \pm 15	245 \pm 8.78	565 \pm 8.8	600 \pm 12	678 \pm 15
RT-2	87 \pm 6.09	90 \pm 12	209 \pm 23	256 \pm 23	278 \pm 12.09	367 \pm 12	423 \pm 23	512 \pm 23	224 \pm 6.09	389 \pm 12	509 \pm 23	645 \pm 23
LSD at p = 0.05												
Between strains 85.9 30.02 74												
Between treatments 74.47 21.22 52.6												

Table 11. Effect of varying NaCl concentrations (mM) and exogenously applied osmolytes (proline 10 mM and glycine betaine 10 mM) on choline ($\mu\text{g/g}$ fresh weight) of *Z. mays* Var. EV90.

Treatment	Choline ($\mu\text{g/g}$ fresh weight)											
	Salt concentration (mM)				Salt concentration (mM) + 10 mM proline				Salt concentration (mM) + 10 mM glycine betaine			
	0	50	100	200	0	50	100	200	0	50	100	200
Control	13 \pm 1.8	23 \pm 16.23	33 \pm 12.3	32 \pm 1.73	56 \pm 6.8	100 \pm 9.23	100 \pm 9.23	67 \pm 9.23	65 \pm 1.8	76 \pm 9.23	100 \pm 19.23	78 \pm 9.23
PAa6	57 \pm 0.3	123 \pm 12.23	167 \pm 23	209 \pm 10.93	73 \pm 8.3	89 \pm 9.23	100 \pm 9.23	109 \pm 12.23	87 \pm 5.93	96 \pm 10.23	100 \pm 10.23	112 \pm 8.23
RT-2	39 \pm 0.12	132 \pm 22.23	123 \pm 3.23	145 \pm 12.3	56 \pm 9.12	67 \pm 9.03	100 \pm 8.23	98 \pm 8.23	65 \pm 8.12	89 \pm 20.23	100 \pm 8.23	100 \pm 8.23
LSD at p = 0.05												
Between strains 3.10 31.186 22												
Between treatments 2.68 22.052 15.95												

inoculations helped the plants to survive under salt stress due to better osmotic adjustment of plants (Nemati et al., 2011). The results obtained in the present study are also in agreement by many other previous and recent reports (Al-Sobhi, 2006; Jaleel et al, 2008; Chookhampaeng, 2011). Similar results were noted with fresh and dry weight where bacterial inoculations enhanced the biomass production that in turn increased the weight of plant (Rajkumar et al, 2009). Previous studies were also in agreement with the fact that

increasing salt stress causes accumulation of proline and glycine betaine in germinating seeds (Gul and Khan, 2008; Nawaz and Ashraf, 2010).

We also evaluated the effect of exogenous osmolytes on the growth of plants. However, simultaneous application of exogenous osmolytes and bacterial inoculation significantly improved the plant growth parameters. Application of exogenously applied glycine betaine (10 mM) was more effective than proline in alleviating salt stress for plant growth. There are also a number

of other studies reporting proline and glycine betaine to increase the tolerance level of plants at high levels of salinity acting as an osmoregulator in the cytoplasm or as an osmoprotectant of proteins (Gul and Khan, 2008). The exogenous application of choline chloride (Table 11) had non-significant effects as compared to proline and glycine betaine treated plants. It may also be speculated from our results that improved plant growth and photosynthetic activity is favoured by inoculation. Endogenous glycine betaine and

proline, either taken from the soil, or up regulated by bacteria is one of the additional factors that may have contributed to a higher germination and other growth parameter under saline conditions. It is noteworthy that endogenous level of proline was higher in control seedlings treated with glycine betaine in the soil compared with those control seedlings treated with proline (Table 9).

Although, accumulation of glycine betaine as an osmoprotectant has been variously described, but under salt stress plants also accumulate proline as an osmoprotectant to overcome soil salinity. Plants treated with exogenous proline showed reduced level of accumulation of proline.

Previous studies are also in line with this finding, where proline overproduction in bacteria and plants has been found to reduce the level of proline accumulation due to feedback inhibition (Chen et al., 2007).

It is evident from the aforementioned results that plants accumulate osmolytes to cope with NaCl stress to a variable extent. This variable accumulation is not enough to maintain their osmotic potential under salt stress. From the results, it is also clear that endogenous level of osmolytes or both proline and glycine betaine were increased in plants treated with exogenously applied glycine betaine and proline. Accumulation of endogenous glycine betaine was more than accumulated proline. The increased accumulation of glycine betaine at higher salt stress indicates salt tolerance status of plants (Nawaz and Ashraf, 2010). It may be speculated that bacterial inoculation to plants might be involved in causing upregulation of osmolyte biosynthesis that tends to keep osmolytes level higher in plant cells. It was also observed that the higher the endogenous osmolytes accumulation, the lesser the inhibitory effects of salt on the growth parameters of plants, and as a result, seeds germinate as if they were at the lower levels of the NaCl (Ashraf and Harris, 2004)..

In view of the discussion, it is evident that osmolytes accumulation in bacteria and plants helps to alleviate salt stress. Accumulation of osmolytes to alleviate salinity in agricultural crops can be increased by exogenous osmolytes like proline and glycine betaine. However, increased demand of these osmolytes in future will not be economical for sustainable agriculture. Inoculated plants also have high capacity for osmolyte accumulation. Both strains *H. meridiana* (PAa6) and *H. aquamarina* (RT2) strains can accumulate osmolytes and exert stimulatory effects on the plant growth. Thus, these strains can be effective and suitable candidate for plant growth improvement, hence becoming an alternate economical approach for increasing crop productivity.

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