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EVALUATION OF ROLE OF *Bacillus* AND *Halomonas* STRAINS IN IMPROVING THE GROWTH OF BARLEY CROP UNDER SALT STRESS

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Abstract

The main objective of this study was to assess the enhancement in the growth of barley crop through plant growth promoting rhizobacteria at different levels of salt stress. *Halomonas anticariensis* J6S4 exhibited the highest levels of auxin biosynthesis i.e., 25, 30, and 31 µg ml^{-1} when media was augmented with 0, 500 and 1000 µg ml^{-1} concentrations of L-tryptophan, respectively. *In-vitro* pot trials manifested statistically significant results for plant growth parameters when inoculated with halotolerant bacterial strains at different salt stress levels. The Highest fresh weight (120%) was recorded for *B. gradentensis* Rb9S4 and *H. smyremis* J2S7 at 300 mM salt concentration. For dry weight, shoot and root length significant results were recorded for *B. gradentensis* Rb9S4, in comparison with the respective control. Under natural conditions, *B. subtilis* Ra1S6 manifested significant results of up to 118% for dry weight at 300 mM concentration of salt. The significant increase in shoot length (48.95%) was recorded for *H. smyremis* J2S7. Highest spike length of 4.25% was documented for *B. subtilis* Ra1S6, whereas *B. zhangzhouensis* R7S6 presented significant increase in number of tillers. Finally, conclusion can be drawn that halotolerant bacteria have great potential to biosynthesize auxin and manifest plant growth-promoting traits under salt stress.

Keywords : Salt stress, halotolerant, barley, PGPR, auxin, chlorophyll content, proline, gibberellic acid

Introduction

Salinity stress is a topical global issue prevailing in semi-arid and arid areas around the globe affecting adversely agricultural productivity. A soil having electrical conductivity (ECe) > 4 dSm^{-1} is considered saline soil (Andrade Foronda & Colinet, 2023). Around 6% of the total cultivable world area i.e., 930 million hectares and 4.5 Mha of land in Pakistan is affected by salt stress. Due to natural and anthropogenic activities area size is increasing day by day (Hasanuzzaman et al., 2018; Ul Haq *et al*., 2023). Clearing of land, replacement of perennial vegetation with the annual crop, poor-quality irrigation water and poor drainage contribute to soil salinization (Naorem *et al*., 2023)

Salinity adversely affects cellular metabolism, biochemical and physiological mechanisms in plants. It causes osmotic stress, ion toxicity, metabolic imbalance, ROS generation and membrane instability. Ultimately, causing cellular oxidative damage leading to cell death which in turn decreases the yield of crops (Hoque *et al*., 2023). Salt stress affects the seed's viability and inhibits germination by hampering osmotic homeostasis and preventing water uptake. Owing to Na^+ and Cl^- ions accumulation, it also creates toxic effects on metabolic processes such as protein synthesis and DNA repair; consequently, it unfavorable affects seed germination (Krichen *et al*., 2023).

Salinity causes an imbalance in the ion flux inside plants. It creates higher Na^+ levels and lower K^+ concentrations. It also makes plant cells incapable of absorbing water from the environment, consequently leading to loss of cell osmotic pressure and a decrease in cytosolic volume. Due to a change in turgor pressure, stomatal closure occurs which leads to a decrease in CO_2 assimilation rate which restricts chlorophyll synthesis and its activity. Accumulation of Na^+ and Cl^- ions leads to a decline in the uptake of K^+ and Ca^{2+} and Mg^{2+} ions resulting in a decrease in root dry mass and length area during the tillering stage. It also causes a burned or bleached appearance of mature leaf tips and margins (Liu *et al*., 2023). Salinity stress also brings about decreased vegetative growth parameters, reduction in leaf thickness, leaf area and succulence, root necrosis, leaf abscission and reduction in internode lengths (Ervé *et al*., 2022).

Plants undergo several metabolic and physiological changes in response to salinity. Signal transduction mechanisms in plants allow them to modify their metabolism in response to environmental changes. Usually, they undergo a salt stress signaling pathway; for instance, the salt overly sensitive (SOS) that is well characterized in plants, especially cereals. Additionally, the mitogen-activated protein kinase (MAPK) cascade, converts stress signals to a variety of transcription factors. It further activates salt-responsive

genes that play a pivotal role in salt stress signaling (Nykiel *et al*., 2022).

To alleviate salinity stress in agriculture, several agricultural practices and the use of salinity-tolerant crops procured either by conventional breeding or genetic engineering are common. For an eco-friendly strategy use of PGPRs (Plant growth-promoting rhizobacteria) is considered a remarkable innovation to mitigate abiotic stress. PGPRs have several effects on promotion of plant growth by regulating plant hormones such as auxin, cytokinin, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, exopolysaccharide, siderophore, hydrogen cyanide; acquisition of nutrients from the environment, such as phosphate, nitrogen and iron; and by preventing harm caused by fungus and other microbes They also help in modulating antioxidants defense machinery and maintaining osmotic homeostasis; ion balance; and induce salt and drought-responsive genes, transcriptional reprogramming in ion transporter genes, etc. (Gupta *et al*., 2022). PGPRs have already been characterized as extraordinary candidates for lowering salinity stress effects. They are also known for the accumulation of compatible solutes like polyols, amino acids and their formatives e.g., betaine, glycine, and proline, and sugars and their formatives such as trehalose and sucrose (Rangseekaew *et al*., 2022).

Barley (*Hordeum vulgare* L.) is the fourth chief cereal crop that is known for its tolerance towards abiotic stress (salinity and drought). It may mitigate the salinity stress by accumulating Na^+ in the tissues. The use of PGPRs as adjuvants is considered to amplify the tolerance of barley under salinity stress by maximizing macronutrient absorption such as phosphate (Masrahi *et al*., 2023).

The primary objective of this research was to assess how salt-tolerant bacteria affected barley (*Hordeum vulgare* L.) growth under salt stress. Salinity tolerance, gibberellic acid and auxin production were tested in halotolerant bacterial strains. Finally, saline conditions were used to evaluate the ability of chosen strains to promote plant development.

Materials and Methods

Bacterial Strains: Around 7 Bacterial strains (*Bacillus zhangzhouensis* R7S6, *Halomonas smyremis* J2S7, *B. subtilis* Ra1S6, *H. anticariensis* J6S4, *B. hayneslli* R3S5, *B. gradentensis* Rb9S4, *B. licheniformis* Rb3S5) were collected from the "Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan". All strains were treated with Gram staining for morphological differentiation in accordance with Cappuccino & Sherman (2002) protocols.

Colorimetric Analysis of Indole-3-Acetic Acid: For auxin quantification, bacterial isolates were assessed for auxin synthesis at 0 µg ml^{-1}, 500 µg ml^{-1}, 1000 µg ml^{-1} concentrations of L-tryptophan. About 20 ml of L-broth media augmented with 0 M and 0.5 M NaCl with different concentrations of L-tryptophan were used to culture the isolates. Culture flasks were then incubated at 37°C for 3 days at 130 rpm. Afterward, the cultures were centrifuged for 10 minutes at 2300 g and the supernatant was utilized to estimate auxin production. For the color change from pink to red, 1 ml of supernatant was combined with 2 ml of Salkowski reagent and left in the dark for 30 minutes. At 535 nm, the optical density (OD) of each suspension was then measured. To develop a standard curve for determining the concentration of auxin biosynthesized by bacterial strains, various quantities of standard IAA were utilized.

Colorimetric Analysis of Gibberellic Acid: Gibberellic acid production from bacterial isolates was estimated according to Desai (2017). Bacterial strains were inoculated in 50 ml of L-broth and incubated at 30 ℃ for 10 days. Afterward, the culture was centrifuged (10,000 rpm) for 15 minutes at 4℃. With the help of 2N HCl, the pH of the supernatant was adjusted at 2.5. Later, 10 ml ethyl acetate was added and mixed by shaking for 10 min. These steps were carried out three times. The subsequent step was evaporating the ethyl-acetate layer that had been separated from the supernatant layer. A total 4ml of methanol and 1ml of 2,4-Dinitrophenylhydrazine (DNPH) were added to the remaining 0.2 ml of substance, which was then heated at 100 °C for 5 minutes before being cooled in a water bath. About 5ml of 10% KOH was then added. After 5 min development of a wine-red color was observed. Later, 15 ml of water was also added, and OD was taken at 430nm.

HCN Production: HCN producing ability of bacterial strains was assessed according to the protocol of Dhiman *et al*., (2023). Nutrient agar supplemented with glycine as well as 0 M and 0.5 M NaCl concentrations were used to analyze the HCN-producing activity of 7 bacterial strains tested. Picric acid (0.5%) was mixed with sodium carbonate (2%) solution. On top of plates, with bacterial isolates streaked over them, filter paper was positioned after being soaked in that solution. Para-films were used to seal the plates, which were then incubated for 3–4 days at 28°C. For positive test results, the development of orange to red color was observed.

Catalase Test: The Catalase test for all bacterial strains was performed in accordance with the manual of Cappuccino and Sherman (2002). A drop of 3% hydrogen peroxide was applied to the glass slide containing the bacterial colony. Within a few seconds, bubble formation was observed.

Hydrolysis of Starch: Starch hydrolyzing capability of all bacterial isolates was determined according to the protocol of Cappuccino and Sherman (2002). Starch agar plates having 0 M and 0.5 M NaCl were streaked with bacterial strains. Iodine was utilized as an indicator after a 48 h incubation period. The formation of clear zones was recorded for positive test results.

Halophility Assay: To evaluate the salt tolerance of bacterial isolates Halophility assay was performed. L-broth supplemented with 0 M, 0.5 M, 1 M, 1.5 M and 2 M concentrations of NaCl was used for inoculation of each bacterial strain. Cultures were incubated at 37℃ and 130 rpm for 24 h. After that, optical density was recorded at 600 nm by keeping the blank as a control.

Proline Analysis Numerous salt stress conditions, including 0 M, 0.5 M, 1 M, and 1.5 M, were used to cultivate different bacterial strains over 24 hours. The culture was then extracted at 8000 g for 5 min. Total 5 ml of 3% sulfosalicylic acid was applied to cell pellets to extract them for 10 minutes at 100°C while being continuously shaken. 2 ml of the extract, 2 ml of glacial acetic acid, and 2 ml of the acid Ninhydrin reagent were mixed, and they were heated for 30 minutes at 100 °C. Following cooling, 4 ml of toluene was added. The absorbance was then measured at 520 nm.

***In-vitro* Pot Trials with Salt Stress:** *The In-vitro* pot experiment was conducted under axenic conditions in the laboratory. Firstly, with the help of 0.1% $HgCl_2$, seeds were surface sterilized and treated with suspensions of 7 bacterial strains (R7S6, J2S7, Ra1S6, J6S4, R3S5, Rb9S4 and Rb3S5) separately for 20-25 minutes. Water-treated seeds were used as a control. Autoclaved sand and vermiculite in 10:1 were mixed and added to pots. Five seeds were sown per pot for each treatment in duplicate. Various levels of salt stress were applied at the time of sowing i.e., “0 mM,100 mM, 200 mM and 300 mM NaCl conc”. After 2 weeks of germination vegetative growth parameters i.e., shoot length, root length, and fresh and dry weight were recorded.

Pot Trials under Natural Conditions with Salt Stress: The pot experiment was executed in the wirehouse of the “Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore.” Barley seeds procured from “Punjab Seed Corporation, Lahore, Pakistan” were sterilized and treated with suspensions of 5 bacterial isolates (R7S6, J2S7, Ra1S6, J6S4 and R3S5) as mentioned above. Water-treated seeds were utilized as a control. In each pot, approximately 10 seeds were sown for each treatment, in duplicate. Following two weeks of germination, salt stress conditions (0 mM, 100 mM, 200 mM, and 300 mM NaCl conc.) were administered. Following eight weeks of growth, the plants' roots, shoots and spike length, fresh and dried weights, and number of tillers per plant were all measured.

Proline analysis of salt-stressed plants: About 0.5 g of the fresh leaf was taken from a plant cultivated under various salt-stress conditions treated with different strains. About 5ml of 3% sulfosalicylic acid was added. The extract was filtered. Following that, 2 ml of aqueous extract, 2 ml of the acid Ninhydrin reagent and 2 ml of glacial acetic acid were mixed and heated for 30 minutes at 100 °C. After the reaction mixture had cooled, 4 ml of toluene was added. After that absorbance at 520 nm was measured through a spectrophotometer.

Chlorophyll Content Analysis: The chlorophyll content of plants was estimated according to Al-aghabary *et al*. (2005). The finest leaf blade that was fully formed after 10 and 27 days of salt treatment was chosen. After being thoroughly crushed, the leaf discs were extracted with 10 ml of 80% acetone. At 4 °C and 4000 g, the homogenate was centrifuged for 4 minutes. After 10 minutes, the supernatant was collected and used for chlorophyll analysis. At 663.6 and 646.6 nm, the concentrations of chlorophyll "a" and "b" were measured.

Catalase Test: Catalase produced in plants under salt stress was estimated according to Loreto & Velikova (2001). A total of 5 ml of 1% Trichloroacetic acid and an ice bath were used to homogenize about 70 mg of leaf tissue. It was then centrifuged for 15 minutes at 12,000 rpm. In 0.5 ml of 10 mM potassium phosphate buffer (pH 7), 500 µl of supernatant was added. Afterward, 1ml 1M potassium iodide was added and absorbance was recorded at 390 nm.

Statistical analysis: Using SPSS software, “analysis of variance” (ANOVA) was applied on the data. Using Duncan's multiple range test (DMRT), mean values were separated; $P \leq 0.05$.

Results

Bacterial strains Total 7 different *Bacillus* and *Halomonas* strains (*Bacillus zhangzhouensis* R7S6,

Halomonas smyremis J2S7, *B. subtilis* Ra1S6, *H. anticariensis* J6S4, *B. haynesllli* R3S5, *B. gradentensis* Rb9S4 and *B. licheniformis* Rb3S5) isolated from the rhizosphere of *Suaeda fruiticosa* at the saline area of "Khewra, Pakistan", were refreshed and maintained on L-Agar plates supplemented with NaCl. The morphology and color of strains were carefully observed. All strains were gram-positive rods except *H. smyremis* J2S7and *H. anticariensis* J6S4 which were gram-negative rods.

Colorimetric Analysis of Indole-3-Acetic Acid: Colorimetric analysis of bacterial auxin biosynthesis indicated significant levels of auxin irrespective of the concentration of L-tryptophan. The highest levels of auxin were recorded for *H. anticariensis* J6S4 (31 µg ml^{-1}) at 1000 µg ml^{-1} L-tryptophan concentration. *H. anticariensis* J6S4 also recorded 25 µg ml^{-1}, 30 µg ml^{-1} at 0 and 500 µg ml^{-1} of L–tryptophan conc. For all other strains, Statistically non-significant results were recorded (Fig 1)

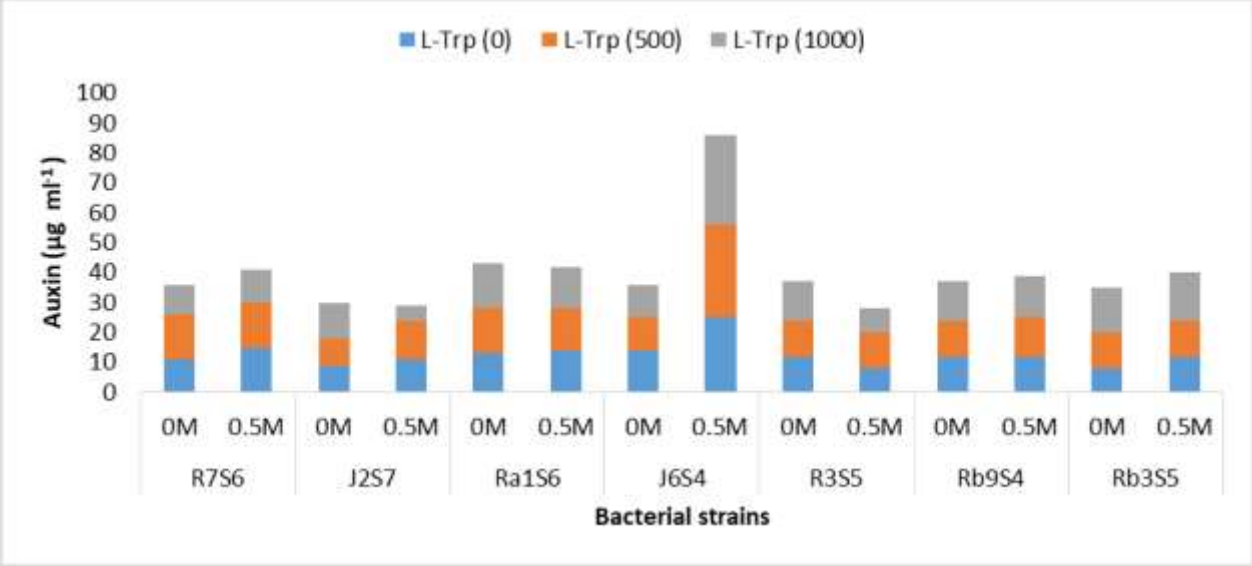


Fig. 1. Effect of different concentrations of NaCl and L-tryptophan on bacterial auxin production. Colored bars represent the mean of 4 replicates.

Colorimetric Estimation of Gibberellic Acid: Significant results for gibberellic acid production were recorded for *H. anticariensis* J6S4 i.e., 0.41 µg ml^{-1} at 0 M salt stress. While for other strains at 0.5 M salt stress significant levels of gibberellic acid were noted for "*H. smyremis* J2S7, *B. subtilis* Ra1S6, *B. haynesllli* R3S5 and *B. gradentensis* Rb9S4" (Fig 2).

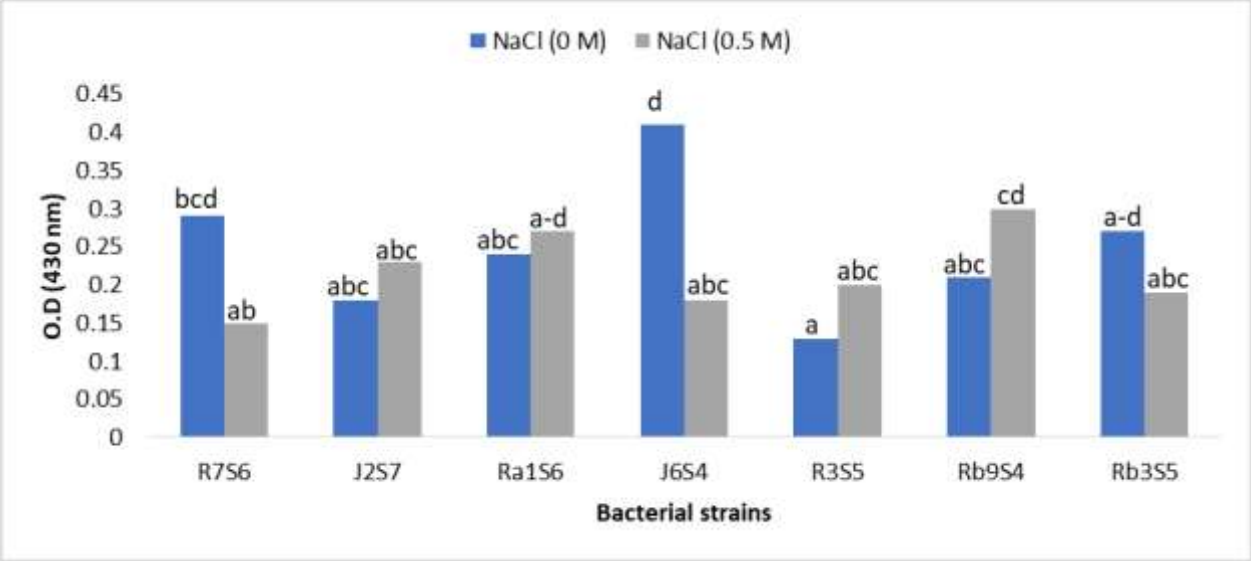


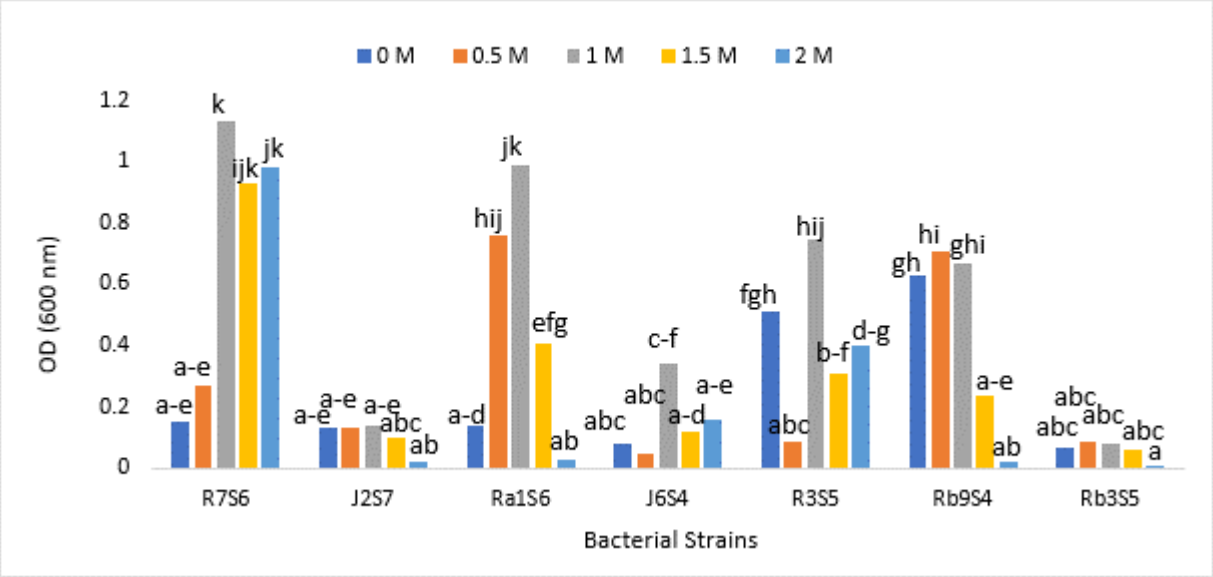
Fig. 2. Gibberellic acid production at 0 and 0.5 M NaCl. Each bar represents the mean of 4 replicates. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

HCN Production: As HCN producing activity was assayed against 0 M and 0.5 M NaCl conc., variability of response was observed in each case. *B. licheniformis* Rb3S5 showed no color change and was negative at both 0 and 0.5 M salt concentrations. While *H. anticariensis* J6S4 showed development of orange color after incubation and was positive at both concentrations. While *B. zhangzhouensis* R7S6 exhibited orange color development only at 0 M NaCl, thus positive at 0 M but negative at 0.5 M NaCl. *H. anticariensis* J6S4, *B. gradentensis* Rb9S4, *B. hayneslli* R3S5 and *B. subtilis* Ra1S6 showed negative results at 0 M and positive results at 0.5 M salt concentrations.

Catalase Test: Bubble formation was observed for *B. gradentensis* Rb9S4, *H. smyremis* J2S7, *B. zhangzhouensis* R7S6, *B. licheniformis* Rb3S5, *B. subtilis* Ra1S6 and *B. hayneslli* R3S5 were positive for catalase test. While *H. anticariensis* J6S4 produced no bubbles and waswas considered negative for catalase test.

Hydrolysis of Strach: After the addition of iodine different results were observed at different salt concentrations. *B. zhangzhouensis* R7S6 showed no zone formation and was negtive for starch hydrolyzation at both 0 and 0.5 M salt stress. *B. licheniformis* Rb3S5, *B. gradentensis* Rb9S4, *B. hayneslli* R3S5, *H. smyremis* J2S7 showed zone formation only at 0 M NaCl, thus positive at 0 M but negative at 0.5 M salt concentration. *B. subtilis* Ra1S6 was recorded negtive at 0 M NaCl while positive at 0.5M. However, *H. anticariensis* J6S4 showed starch hydrolysis and clear zone formation at both 0 and 0.5M salt stress conditions.

Halophility: Assay From 0-1 M NaCl increasing salt concentration positively affected the bacterial growth. The highest tolerance response was recorded for *B. zhangzhouensis* R7S6 at 1 M NaCl (Fig 3). Albeit, up to 2 M concentration of salt inhibits bacterial growth. The lowest levels of growth were recorded for *B. licheniformis* Rb3S5 at 2 M salt stress.



Proline Analysis: At different salt concentrations all bacterial strains showed different levels of proline production. Significant levels of proline were recorded by *B. hayneslli* R3S5 at 1.5 M, as compared to the control. For all other strains, inhibitory effects were recorded against different salinity levels. The lowest levels of proline were observed with *B. hayneslli* R3S5 at 0.5 M (Fig 4).

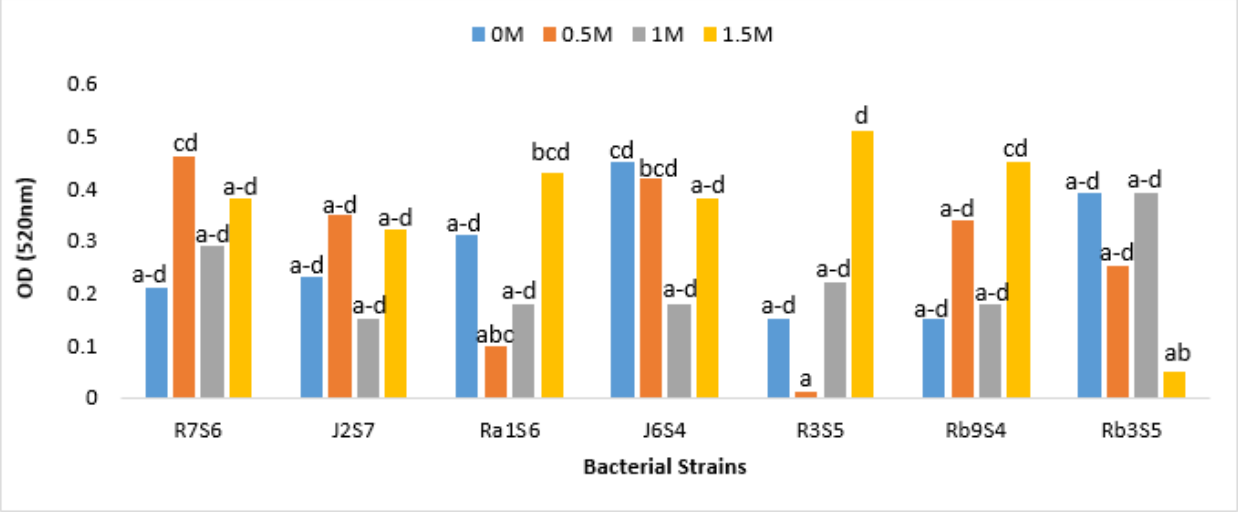


Fig. 4. Proline production at 0 M, 0.5 M, 1 M and 1.5 M NaCl. Each bar represents the mean of 4 replicates. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

***In Vitro*, Pots Trials with Salt Stress:** Inoculation of bacterial strains affected positively the growth of plants with increasing salt stress levels. For fresh weight, a significant level of increase was observed for *B. gradentensis* Rb9S4 (184% at 0 mM and 120% at 300 mM NaCl) and *H. smyremis* J2S7 (120% at 300 mM NaCl), as compared to respective control (Fig 5). For dry weight, 950% and 575%
the increase was recorded for *B. gradentensis* Rb9S4 at 300 mM and 200 mM NaCl, as compared to the control (Fig 6). While *Halomonas smyremis* J2S7 showed an increase of 350% at 100 mM NaCl. All bacterial strains showed significant enhancement in shoot length at 300

mM NaCl (Fig 7). The highest increase (42.44%) was recorded for *B. haynesllli* R3S5 with respect to control. *B. gradentensis* Rb9S4 manifested an increase of 107.55% in root length at 0 mM NaCl, while *H. anticariensis* showed an enhancement of 62.33% at 300 mM NaCl, as compared to the control (Fig 8).

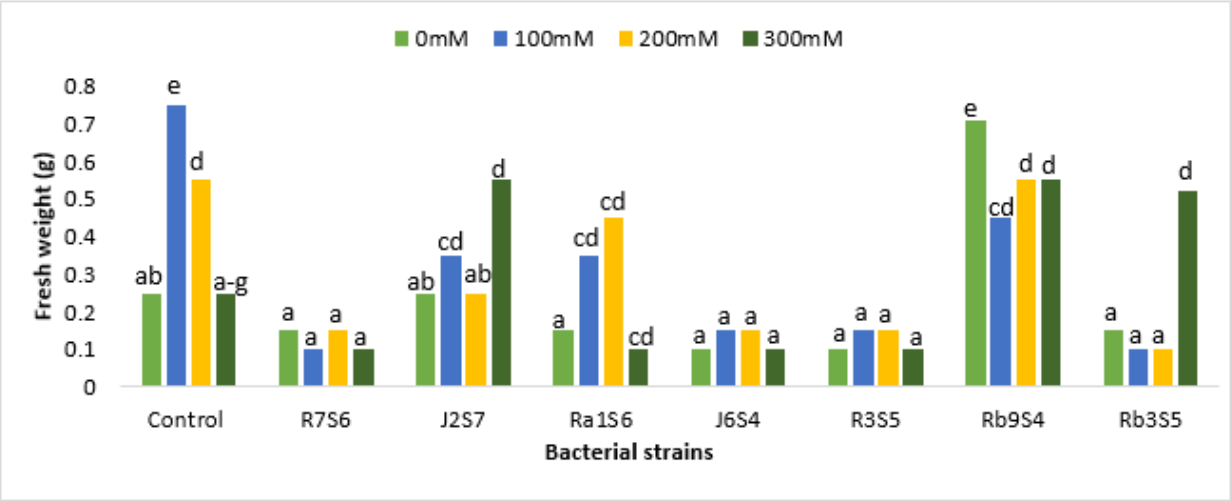


Fig. 5. Effects of *Bacillus* and *Halomonas* strains on the fresh weight (0-300mM) of barley grown under laboratory conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

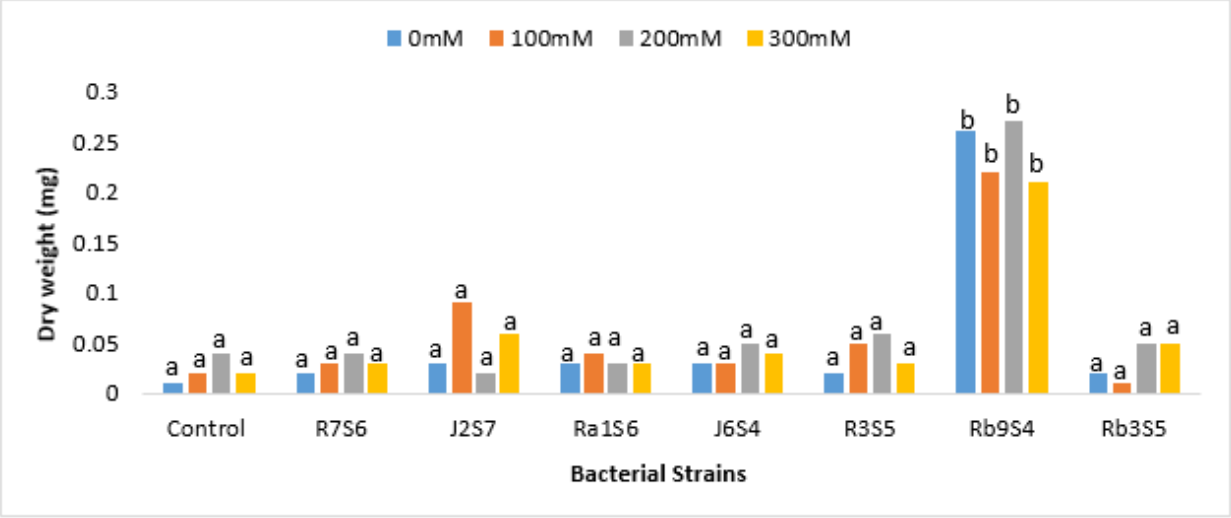


Fig. 6. Effects of *Bacillus* and *Halomonas* strains on the dry weight (0-300 mM) of barley grown under laboratory conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

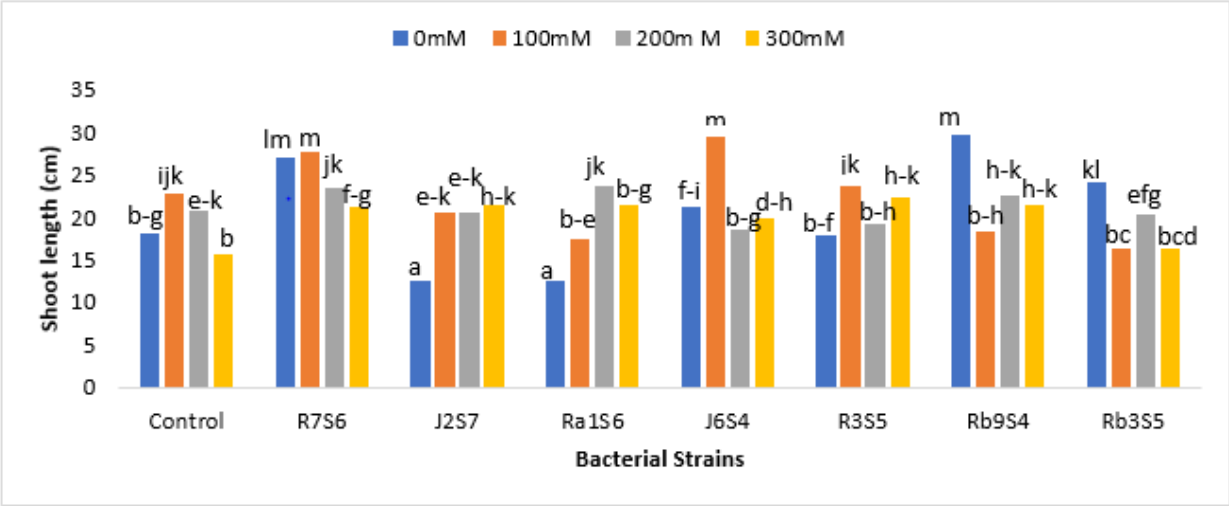


Fig. 7. Effects of *Bacillus* and *Halomonas* strains on the shoot length (0-300 mM) of barley grow under laboratory conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

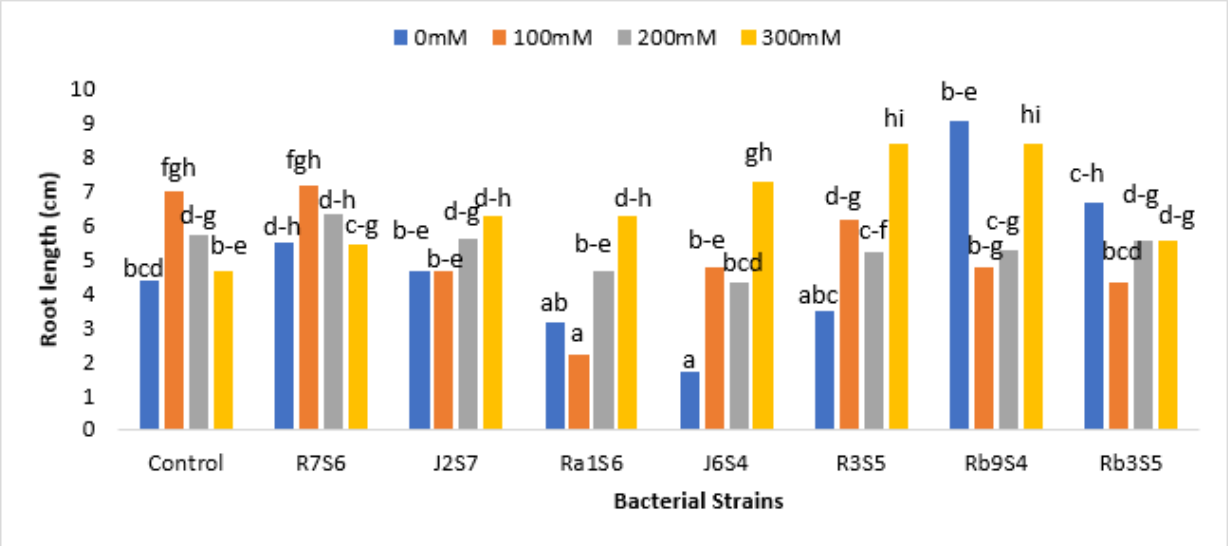


Fig. 8. Effects of *Bacillus* and *Halomonas* strains on the root length (0-300 mM) of barley grown under laboratory conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

Pot Trials under Natural Conditions with Salt Stress: *B. subtilis* Ra1S6 exhibited 6% and 8% enhancement in fresh weight at 100 mM and 300 mM NaCl concentrations as compared to respective controls (Fig 9). For dry weight, the highest increase was observed for *B. subtilis* Ra1S6 (118.62% at 100 mM and 34.7% at 300 mM) and *H. anticariensis* J6S4 (29.07% at 300 mM) as compared to control (Fig 10). A significant increase in shoot length was recorded for *H. smyremis* J2S7 i.e., 48.95% at 200 mM, while *B. zhangzhouensis* R7S6 showed an increase of 47.95% at 100 mM NaCl; as compared to control (Fig 11). The highest increase in root length (103.90%) was recorded for *H. anticariensis* J6S4 at 300mM NaCl). However, *B. zhangzhouensis* R7S6, *H. smyremis* R7S6 and *B. haynesllii* R3S5 also showed a significant increase of 68.96%, 50.57% and 72.41% at 300 mM NaCl concentration (Fig 12).

B. subtilis Ra1S6 exhibited the highest increase (4.25%) in spike length at 100 mM salt conc. (Fig 13). Albeit, it has been observed that increasing salinity negatively influenced a number of tillers. For instance,

the highest no of tillers was recorded for *B. zhangzhouensis* (172.72%) at 300 mM NaCl, as compared to control (Fig 14).

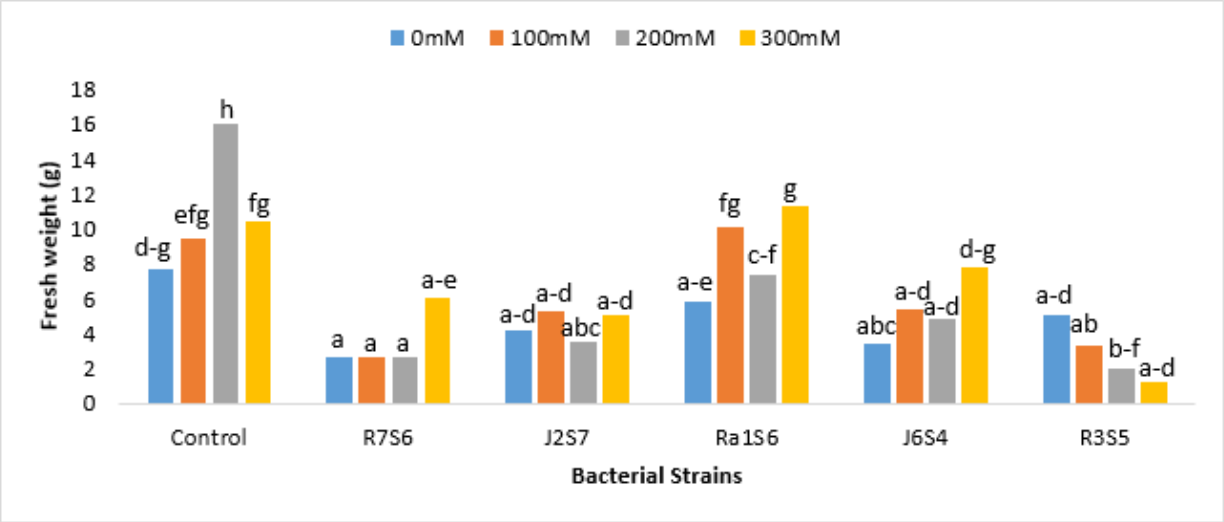


Fig. 9. Effects of *Bacillus* and *Halomonas* strains on the fresh weight (0-300mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

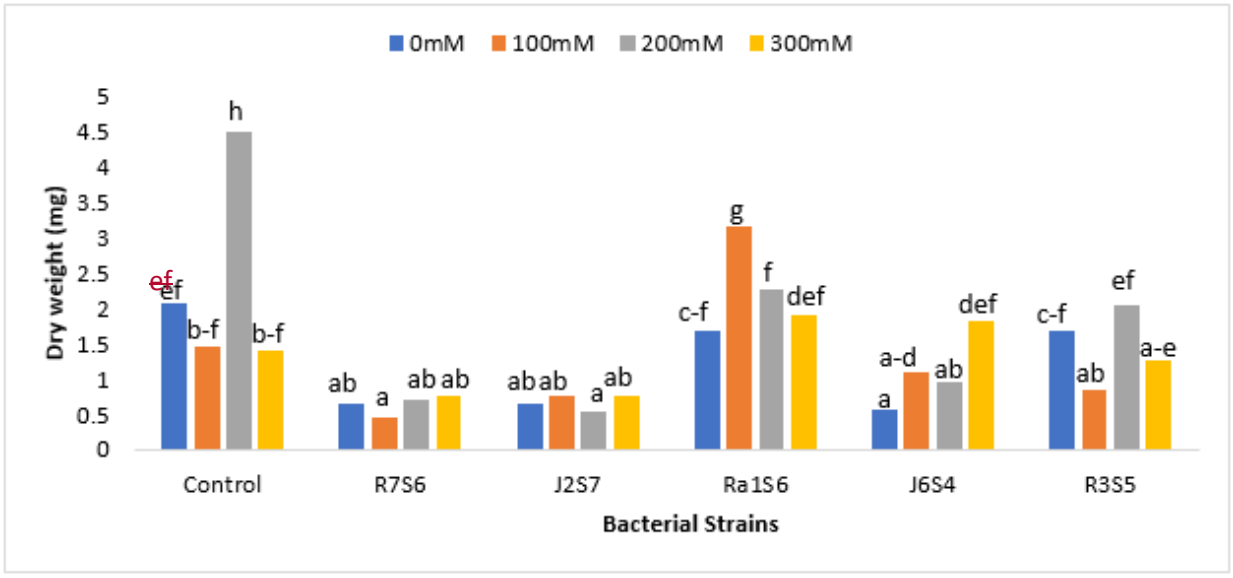


Fig. 10. Effects of *Bacillus* and *Halomonas* strains on the dry weight (0-300mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

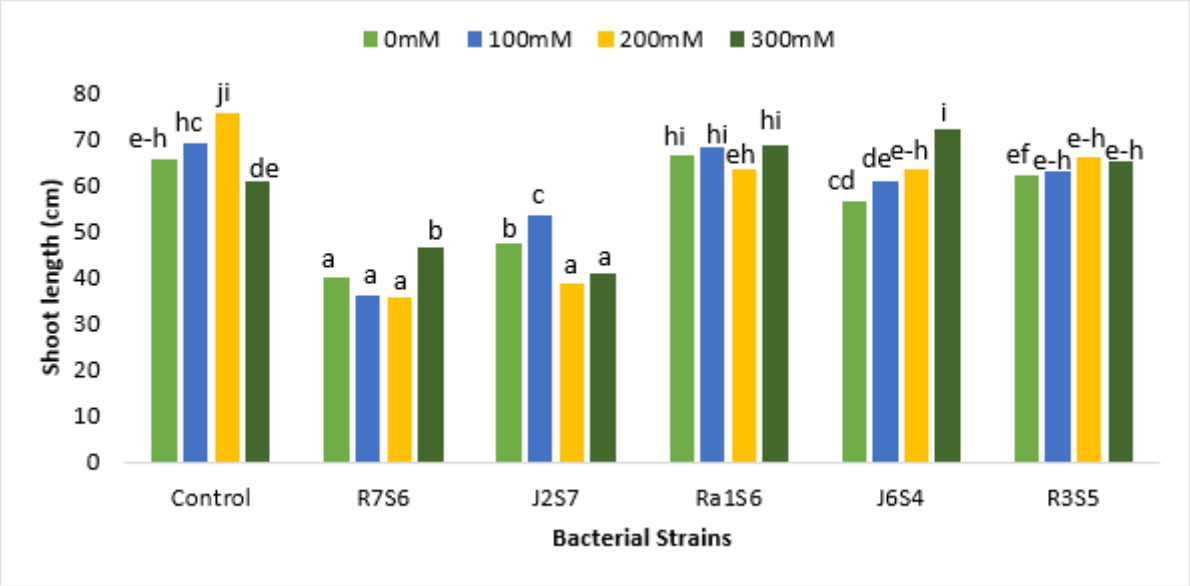


Fig. 11. Effects of *Bacillus* and *Halomonas* strains on the shoot length (0-300 mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

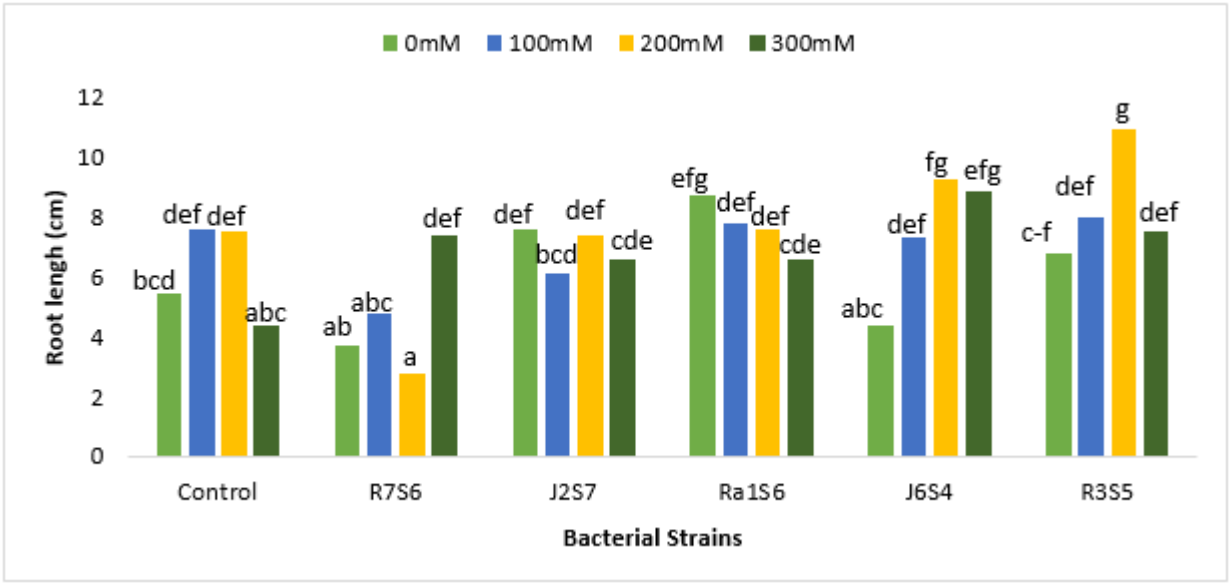


Fig. 12. Effects of *Bacillus* and *Halomonas* strains on the root length (0-300mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

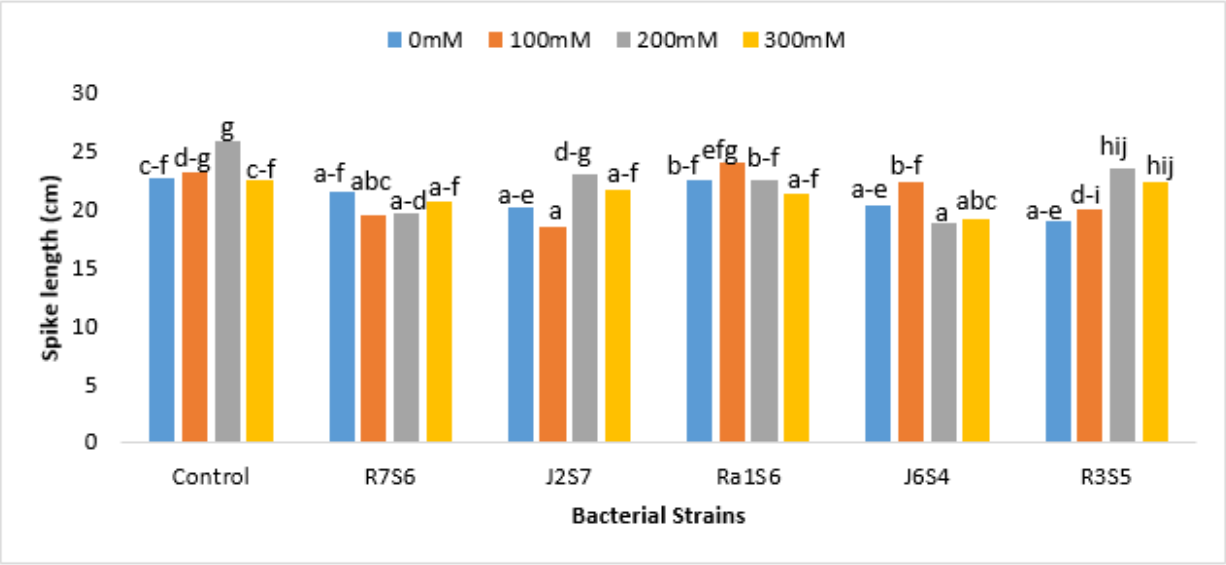


Fig. 13. Effects of *Bacillus* and *Halomonas* strains on spike length (0-300mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

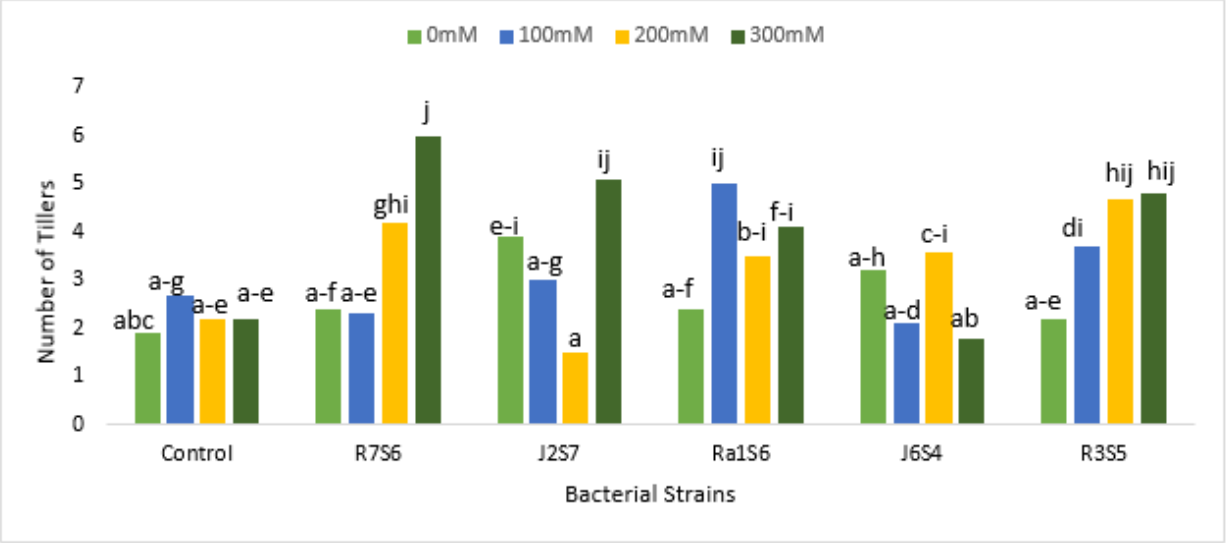


Fig. 14. Effects of *Bacillus* and *Halomonas* strains on the number of tillers (0-300 mM) of barley grown under natural conditions amended with different levels of salinities. Color bars show the different levels of salt stress. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

Chlorophyll Content: Bacterial inoculation positively affected the chlorophyll content of plants with increasing levels of salinity. To illustrate, the highest level of chlorophyll (a) was recorded for *B. subtilis* Ra1S6 i.e., 42.25% at 300 mM NaCl, as compared to control. Similarly, a significant level of chlorophyll (b) production was recorded for *H. anticariensis* J6S4 i.e., 44.73% at 300 mM NaCl (Fig 15).

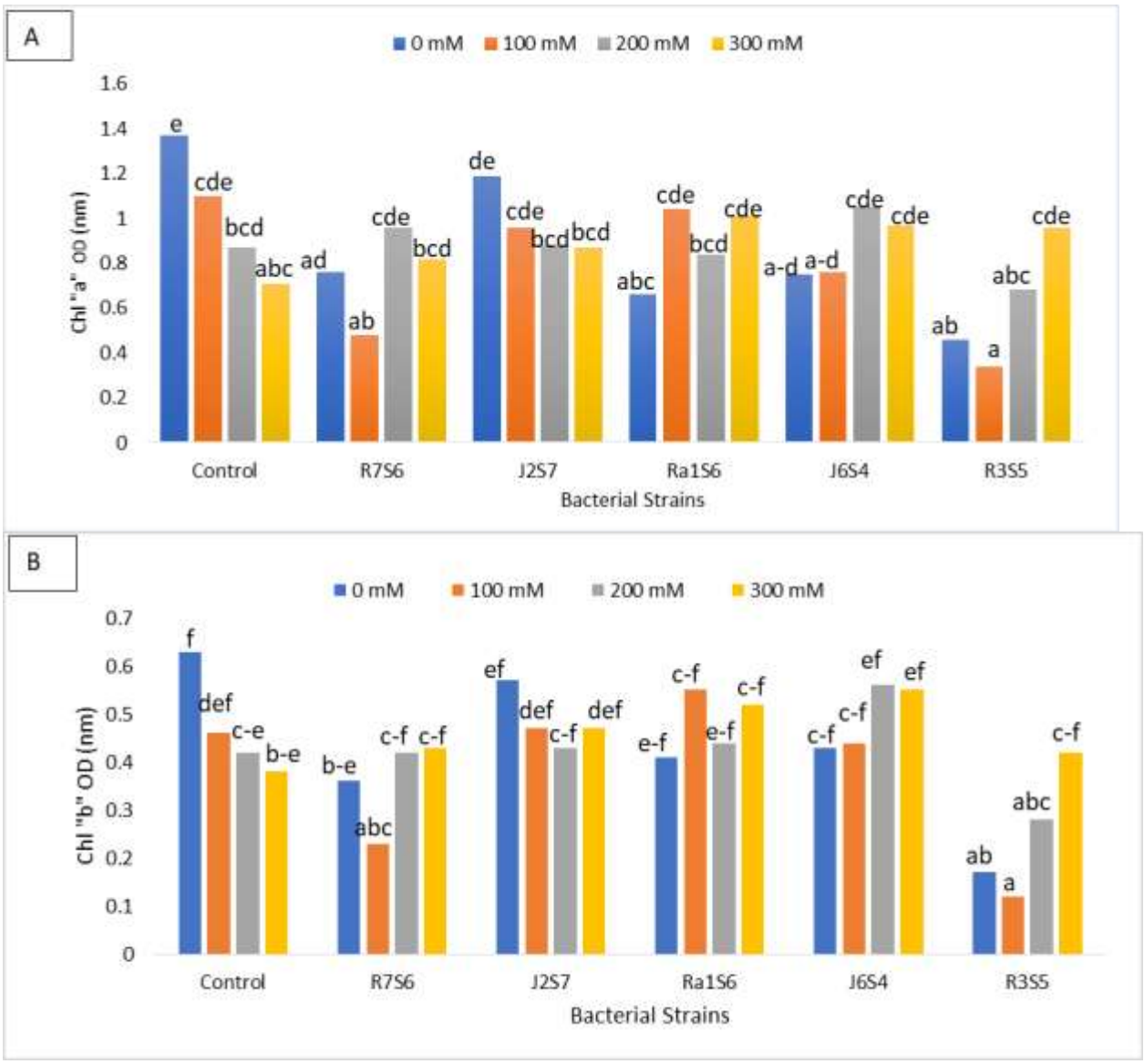


Fig. 15. Effect of different NaCl concentrations (0-300 mM) on chlorophyll content of plants (A) Chl "a" (B) Chl "b". Bars represent the mean of 8 replicates. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

Catalase Test: A significant level (41.17%) of catalase formation was observed for "*H. anticariensis* J6S4, *H. smyremis J2S7*, and *B. haynesllli* R3S5" at 200 mM NaCl, with respect to control (Fig 16). For all other strain treatments, non-significant levels of results were recorded

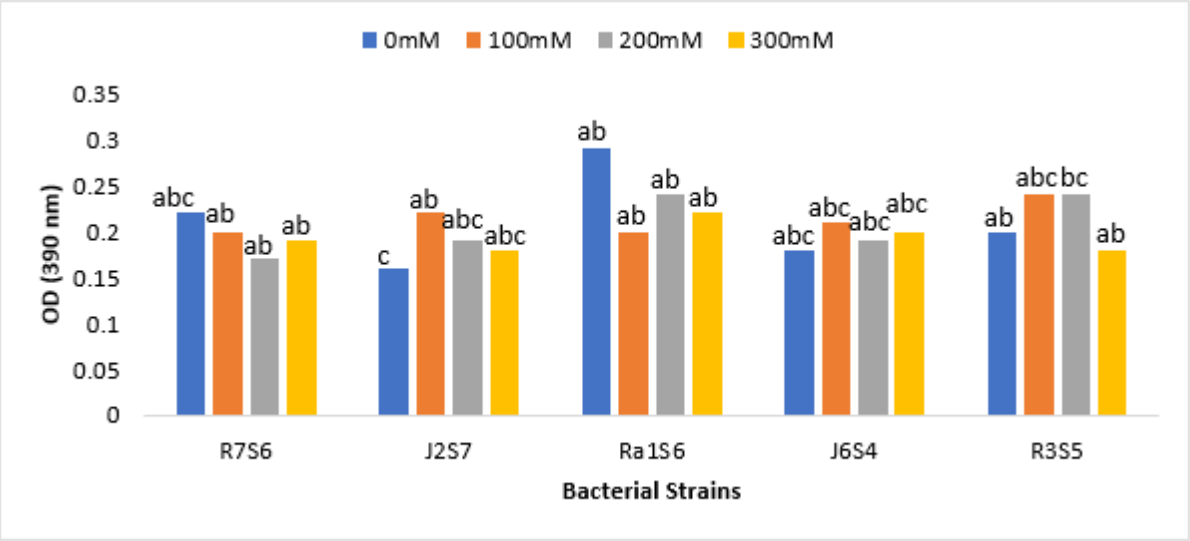


Fig. 16. Effects of 0 mM, 100 mM, 200 mM and 300 mM NaCl conc. on catalase content of barley. Color bars show the mean of 8 replicates. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

Proline Analysis: The highest level of proline content was recorded for *B. subtilis* Ra1S6 (180%) at 100 mM NaCl. While for all other strains treatments, statistically non-significant results were reported (Fig 17).

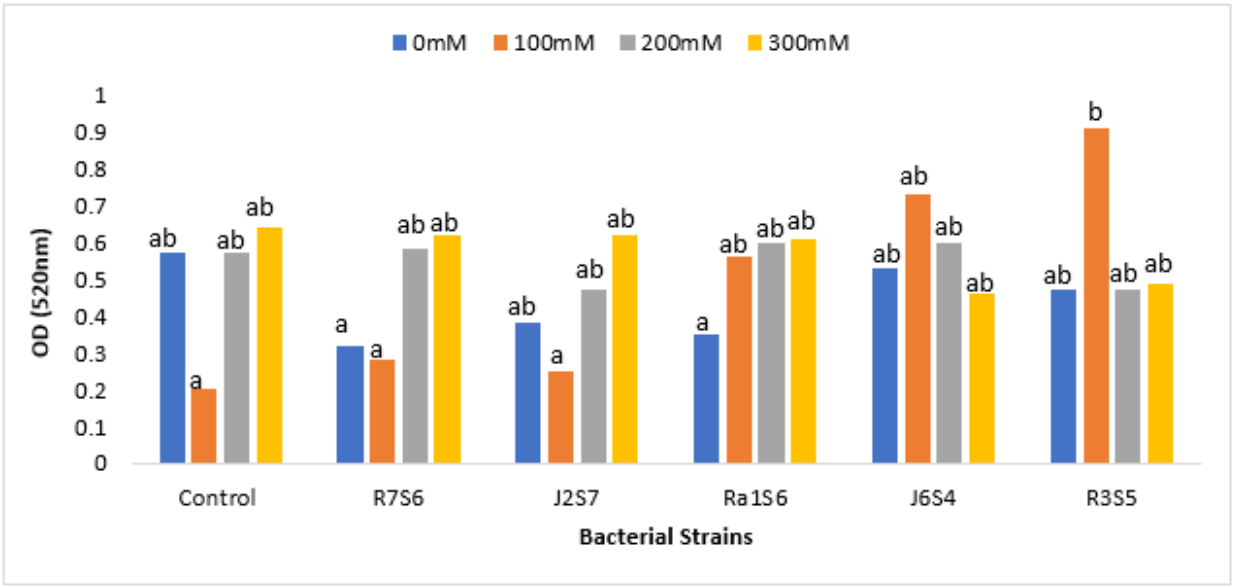


Fig. 17. Effects of 0 mM, 100 mM, 200 mM and 300 mM NaCl conc. on proline content of barley. Color bars show the mean of 8 replicates. Different alphabets represent a significant difference in mean values by using Duncan's multiple range test (P≤0.05).

Discussion

Halotolerant PGPRs role in mitigating salt stress and promoting the growth of plants is incredible. It has been delineated that bacteria isolated from saline soil are likely to promote the growth of plants under salt stress (Latif *et al*., 2023). In the current study, 5 strains of *Bacillus* (*B. zhangzhouensis. subtilis, B. haynesllli, B. gradentensis* and *B. licheniformis*) while two species of *Halomonas (H. smyremis, H. anticariensis*) were used. Asadullah & Bano (2023) have also reported *Halomonas* and *Bacillus* sp. as

extraordinary salt-tolerant strains with plant growth-promoting potential.

Bacterial isolates screening for auxin production revealed that bacteria biosynthesize auxin in the presence or absence of L- tryptophan. *H. anticriensis* produces 31µg ml^{-1} of auxin at 1000 µg ml^{-1} L-tryptophan while *H. anticariensis* biosynthesized 25 and 30 µg ml^{-1} of auxin at 0 and 500 µg ml^{-1} L-tryptophan at different salt concentrations Oliva *et al*., (2023) has also revealed synthesis of auxin by *Halomonas* and *Bacillus* at different concentrations of L-tryptophan with or without salt supplemented media. Gibberellins help in alleviating salt stress by enhancing the water availability to crops and promoting leaf and stem growth. In this study, *H. anticariensis* showed the highest gibberellic acid production at 0 M NaCl stress. It has been observed in several studies that after inoculation of gibberellic acid-producing *H. desiderata*, *B. licheniformis* and *Acinetobacter calcoaceticus* plant growth has been boosted under salt stress (Etesami & Glick, 2020).

In this research, *B. subtilis* and *H. smyremis* were found to be positive for hydrogen cyanide activity (HCN) on both 0M and 0.5 M NaCl stress. Mukhtar *et al*., (2020) have observed that most of the strains of *Halomonas* isolated from the rhizosphere of haplotypes were positive for HCN activity.

In the current study, *H. smyremis* and *B. subtilis* show starch hydrolysis in the medium amended with NaCl. It was demonstrated that halotolerant bacterial strains isolated from the rhizospheric soils of Bangalore have the ability of starch hydrolysis. This emphasized their potential for promoting the growth of roots and germination of seedlings (Vekataramappa *et al*., 2022). Bacterial strains studied to check their ability of NaCl tolerance showed maximum tolerance by *B. zhangzhouensis* at 1M NaCl stress. However, the lowest tolerance was observed to be at 2 M conc. Aslam & Ali (2018) have reported 1.5 M NaCl conc. as the maximum tolerance level for halotolerant strains isolated from the rhizospheric saline soil of Khewra, Pakistan.

Proline estimation for bacteria and plants was evaluated. A significant level of proline was reported for *B. haneslli* at 1.5 M NaCl. However, for plant proline analysis, the highest response was recorded from the inoculation of *B. subtilis* i.e., 180% at 100 mM NaCl stress as compared to the respective control. It has been observed that PGPRs inoculation upregulated the expression of proline under salt stress conditions (200 mM), thus helping in growth enhancement and mitigation of salt stress (Ayaz *et al*., 2022).

Our study showed the highest level of chlorophyll (a) content for plants inoculated with *B. subtilis* Ra1S6 i.e., 42.25% at 100 mM NaCl. While for chlorophyll (b) the highest level was recorded for *H. anticariensis* J6S4 i.e., 44.73% at 300 mM NaCl. Hajiabadi *et al*., (2021) have also proved significant levels of enhancement in chlorophyll (a) and chlorophyll (b) content of wheat after treatment with *B. safensis*, *B. pumilus*, and *Zhihengliuella halotolerans* at 80 and 160 mM NaCl conc. PGPRs role in scavenging reactive oxygen species (ROS) through antioxidant defense enzymes like catalase is well known. In this research, the highest level of catalase formation was observed for "*H. anticariensis* J6S4 and *H. smyremis* J2S7, *B. haneslli* R3S5" at 200 mM NaCl. Similarly, catalase production in plants was also studied under salt stress and an increase in catalase activity was recorded. Several studies report that halotolerant bacteria increase the growth of plants by upregulating catalase and ascorbate peroxidase (Etesami & Glick, 2020).

PGPRs play a pivotal role in the enhancement of vegetative growth parameters under abiotic stress. *In-vitro* pot trials indicated plant growth enhancement after salt-tolerant PGPRs treatment under salinity stress. *B. gradentensis* Rb9S4 and *H. smyremis* J2S7, *B. haneslli* R3S5 manifested an enhancement in root length, fresh and dry weight, and shoot length of *H. vulgare* at 300 mM NaCl conc., respectively. Pot trials under wirehouse conditions also indicated a positive correlation between some bacterial strains' treatments and plant growth under salt stress. *B. subtilis* Ra1S6, *H. smyremis* J2S7, *H. anticariensis* J6S4 exhibited an enhancement of fresh weight, shoot and root length under salt stress, respectively. Similarly, significant levels of increase in spike length and number of tillers were recorded after treatment of barley with *B. subtilis* Ra1S6 and *B. zhangzhouensis* at 100 and 300 mM NaCl stress levels. Under controlled conditions, an increase in root length, shoot length, fresh weight and dry weight of wheat after treatment with *Bacillus strains* in 2% NaCl soil was well demonstrated by Zahra *et al*., (2023). It was further reported that under field environment, certain *Bacillus* strains improved spike length and number of spikelets significantly. Albeit, a decline in growth was also evident that may be attributed to the detrimental lowering of osmotic potential reported. Previously Albdaiwi *et al*., (2019) also reported a reduction in the number of roots of wheat with increasing salinity level of up to 200 mM. However, seeds treated with combinations of 6 and 7 strains showed promising results of growth at ≥80

mM NaCl concentration, as compared to non-inoculated seeds. IAA and proline-producing *Bacillus* and *Pseudomonas* have also been reported to mitigate salt stress in barley by decreasing the concentration of Na^+ in roots and shoots and promoting vegetative growth parameters (Mahmoud *et al*., 2020)

Conclusion

Our study concludes that inoculation of salt-tolerant *Bacillus* and *Halomonas* strains to barley (*Hordeum vulgare* L.) showed promising results. These bacterial strains showed plant growth-promoting traits like auxin, gibberellic acid, proline and HCN production under different levels of salt stress. Significant growth response in pot trails was recorded for *B. gradentensis* at 0 mM and 300 mM NaCL conc. The highest fresh, dry weight and spike length was recorded for *B. subtilis* at 0-300 mM NaCl stress. A significant increase of 103.90% in shoot and root length was recorded for *H. smyremis* and *H. anticariensis* J6S4 at 300 mM, respectively. While the highest no of tillers was recorded for *B. zhangzhouensis* at 300 mM NaCl conc. Finally, *Bacillus* and *Halomonas* strains have the capability to increase the growth of barley under salt stress.

Contribution of authors:

Habiba Amin conducted the experimental work and collected the data. Sana Tanveer prepared the graphs and draft of the manuscript. Basharat Ali conceived this study, performed the statistical analysis and checked the final draft of this study.

Conflicts of interest:

Here are no conflicts of interest among the authors."

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