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BACTERIAL-MEDIATED MITIGATION OF SALINITY TOLERANCE IN LETTUCE THROUGH COORDINATED PHYSIOLOGICAL, ANTIOXIDANT, AND STRUCTURAL ADAPTATIONS

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Abstract

Salinity stress has a drastic effect on the productivity of vegetables; the current study aimed to explore the beneficial role of soil-applied *Bacillus* strains (*Bacillus megaterium* and *Bacillus subtilis*) to alleviate NaCl-induced stress in lettuce. Different salinity levels (0, 50, 100, and 150 mM NaCl) were applied to lettuce plants. Salinity stress reduced plant height (24-17 cm), leaf number (18-13), total plant fresh weight (42-30 g), leaf area (360-250 cm²), and root length (19-13 cm), alongside marked declines in chlorophyll contents (1.30-1.40 mg g^{-1}). Salinity stress also intensified oxidative stress, as indicated by elevated malondialdehyde (MDA) and hydrogen peroxide levels (H_2O_2). Soil application of *Bacillus* strains significantly mitigated the oxidative stress in plants. The combined application of *B. megaterium* and *B. subtilis* improved plant height, fresh biomass, restored photosynthetic pigments, reduced MDA and H_2O_2, and enhanced antioxidant enzyme activities and vascular development under salinity. Hence, Microbial Synergy is an effective approach to mitigate salt toxicity in lettuce.

Key words: Salinity stress; Lettuce; Oxidative stress, *Bacillus* strains; Physiology

Introduction

Crop productivity is affected by salinity stress, particularly in semi-arid and arid regions (Ahmad *et al*., 2023; Huq *et al*., 2025). Salinity induces both osmotic and ionic stress, with oxidative stress occurring as a downstream. response; These physiological interruptions impair plant growth (Hasanuzzaman *et al*., 2020). Salinity stress can severely affect germination, leaf growth and development, photosynthetic activity, and total biomass accumulation in leafy vegetables, particularly in lettuce (Ahmed *et al*., 2019).

Lettuce is a widely consumed leafy vegetable, valued for its low-calorie content, high levels of vitamins, minerals, and bioactive compounds such as sesquiterpene lactones and flavonoids (Fatima *et al*., 2025; Ishaq *et al*., 2025). Although lettuce is economically important, but it is salt-sensitive vegetable (Fernández *et al*., 2016). As salinity is an ever-present threat to agricultural lands, there is a need for a sustainable, cost-effective approach for mitigation and improving the productivity of lettuce on salt-affected lands (Yelni *et al*., 2026).

Application of plant growth-promoting rhizobacteria (PGPR) has emerged as an innovative approach to mitigate salinity stress in vegetables, as these are capable of modifying various mechanisms, like modulation of phytohormones, stabilization of ion homeostasis, stimulation of antioxidant systems, and ACC-deaminase activity (Ghani *et al*., 2025; Khawula *et al*., 2025; Ali *et al*., 2026). *Bacillus* species are considered most suitable for agricultural applications because they can form biofilms and show broad stress tolerance in plants grown under salinity stress (Pudova *et al*., 2022; Jhanzab *et al*., 2026).

Salinity tolerance can be enhanced in lettuce through *Bacillus* strains, as the application of *Bacillus thuringiensis* along with silicon enhanced yield, water status tolerance, and antioxidant defense in lettuce grown on salt-affected soil (AlKahtani *et al*., 2021). The modifications in physiological and biochemical attributes using *Bacillus* strains can be effective as reported in lettuce grown in hydroponic conditions (Moncada *et al*., 2020). However, most researchers have focused on application via root or nutrient solution inoculation. Limited comparative information is available on the responses of lettuce to *B. megaterium* and *B. subtilis* under different levels of salinity.

The present study aimed to investigate the individual and interactive effects of salinity and Bacillus megaterium and Bacillus subtilis treatments on lettuce growth, physiological and biochemical characteristics, and nutrient accumulation, and to assess the potential of these bacterial inoculants for improving lettuce performance under saline conditions.

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Materials and Methods

Experimental site and plant material: The current experiment was conducted in the Department of Botany, the Government Sadiq College Women University, Bahawalpur. For this experiment, *Bacillus* strains were obtained from the Microbiology Laboratory, Department of Soil Science, Faculty of Agriculture and Environment, the Islamia University of Bahawalpur, Pakistan. Seeds of lettuce (Grand Rapids) were purchased from a local vegetable seed market in Bahawalpur and sown in plug trays; the trays were kept in a greenhouse. Uniform seedlings of lettuce were transplanted into plastic pots (8 × 10 inches) filled with homogenized growing media comprised pond soil and compost (1:1). Pots were kept under optimal light, temperature, and relative humidity conditions throughout the experimental period. The reported climatological averages were approximately 30.4°C mean temperature, 32.8% relative humidity, and 11.0 h day^{-1} sunshine duration. Plants were irrigated regularly to maintain adequate soil moisture until salinity treatments were imposed.

Experimental design and arrangement of treatments: The experiment followed a completely randomized design (CRD) under factorial arrangements, i.e., salinity levels and *Bacillus* treatments. Salinity was applied at four sodium chloride (NaCl) concentrations (0, 50, 100, and 150 mM), while soil treatments consisted of four levels: untreated control (distilled water), BS1, BS2, and a combined BS1 + BS2 treatment. Each treatment combination was replicated three times. Each treatment was comprised of fifteen plants. The normal irrigation was only applied to control plants, while others were irrigated with saline water as described by Munns & Tester (2008).

The bacterial strains *Bacillus megaterium* and *Bacillus subtilis* were cultured separately in an appropriate growth medium until the desired cell density was achieved. The bacterial suspensions were adjusted to approximately 10^8 CFU mL^{-1} using sterile distilled water. The prepared inoculum was applied to lettuce plants as a soil/root-zone drench, with 50 mL of bacterial suspension applied to each plant. Plants receiving an equivalent volume of sterile distilled water were maintained as the control. The same inoculation procedure was followed for both bacterial strains.

Growth and biomass measurements: Plants were carefully harvested, brought to the laboratory, and washed to remove soil particles. The growth attributes, including plant height, number of leaves, plant fresh weight, leaf area, root fresh weight, root length, and root dry weight, were measured at the time of crop harvesting. Plant height and root length were measured using a measuring scale, while fresh weights were determined using a digital analytical balance. Root samples were oven-dried at 70°C until constant weight to determine the dry biomass of roots.

Determination of photosynthetic pigments: For measurement of chlorophyll a, chlorophyll b, and total chlorophyll contents, around 0.5 g of fresh leaf tissue was used, followed by washing to remove dust particles. For the extraction of pigments, weighed leaves were ground in 80% acetone using a mortar and pestle, followed by centrifugation at 10,000 × g for 10 minutes to collect the supernatant. This supernatant was used for spectrophotometer analysis. Absorbance was recorded at 480, 645, and 663 nm, and concentrations were calculated using standard equations (Lichtenthaler, 1987). Results were expressed as mg g^{-1} fresh weight (FW).

$$\text{Chlorophyll a} = [(12.7 \times \text{OD } 663) - (2.69 \times \text{OD } 645)] \times V/1000 \times W \quad (1)$$

$$\text{Chlorophyll b} = [(22.9 \times \text{OD645}) - (4.68 \times \text{OD663})] \times V/1000 \times W \quad (2)$$

$$\text{Total chlorophyll} = [(20.2 \times \text{OD645}) + (8.02 \times \text{OD663})] \times V/1000\ W \quad (3)$$

Leaf gas exchange parameters: Leaf gaseous exchange traits, including photosynthetic rate, transpiration rate, and stomatal conductance, were measured on fully expanded leaves using a portable photosynthesis system under ambient environmental conditions. An infrared gas analyzer (IRGA), such as LI-6400XT/LI-6800 (LI-COR Biosciences, Lincoln, NE, USA) was used to measure the leaf gas exchange by., following the method described by Long (2003).

Antioxidant enzyme assays: To calculate enzymatic activity, leaf samples (1 g) were washed and ground in sodium phosphate buffer (50 mM: 1 mL: 7 pH); 0.5 mmol of EDTA was also added, followed by centrifugation of the mixture at 12000 × g to separate debris at 4°C for 10 minutes. The supernatant was removed, and SOD, POD, and CAT activities were determined spectrophotometrically, following the protocol as described by Sardar *et al*., (2022), and were expressed in U mg^{-1} protein.

Determination of oxidative stress markers: For the assessment of oxidative stress in leaf tissues of lettuce, quantification of lipid peroxidation and hydrogen peroxide accumulation was performed. Lipid peroxidation was calculated by determining malondialdehyde (MDA) content using the thiobarbituric acid (TBA) reaction method, while hydrogen peroxide (H_2O_2) content was measured by using a spectrophotometer following the protocol of Velikova *et al*., (2000), which was based on the formation of a stable titanium peroxide complex. Malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) reaction. Fresh leaf tissue (0.5 g) was homogenized in 5 mL of 0.1% TCA and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was reacted with 20% TCA containing 0.5% TBA, heated at 95°C for 30 min, cooled, and centrifuged. Absorbance was recorded at 532 and 600 nm, and MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW. Hydrogen peroxide (H_2O_2) was determined from TCA extracts using the potassium iodide-based colorimetric method. Absorbance was measured at 390 nm, and H_2O_2 concentration was calculated against a standard curve and expressed as µmol g^{-1} FW.

Estimation of leaf and root anatomical changes: Fresh plant samples of lettuce were harvested from each treatment randomly, followed by washing and thin slicing with a sharp surgical blade approximately 1cm. These thin slices were washed with alcoholic solutions (30%-50%) for dehydration, followed by staining with 1–2 drops of safranin for 1–2min. These stained sections were dried using blotting paper after removal of excessive stain with the help of 70% and 90% alcohol. After the drying process, these stained sections were placed on slides, covered with glycerin, and sealed with a coverslip. Observations were made at low magnification (4X) for leaf and root length, width, and surface area, followed by higher magnifications (10X and 40X) for detailed examination of leaf and root anatomy, with images captured via a camera-equipped light microscope, as performed by Maryam *et al*., (2026).

Statistical analysis

Data were recorded and arranged to apply statistical analysis to assess the significance among the treatments. Statistix 8.1 (https://statistix.informer.com/8.1/) was used to compute the analysis of variance (ANOVA) and least significant difference (LSD) test at $p \leq 0.05$ was applied to separate treatment means. Results are presented as mean values with their corresponding standard errors.

Results

Effect of *Bacillus* strains on growth traits of lettuce: Plant height (cm) declined gradually with increasing salinity (0–150 mM NaCl) in all treatments (Fig. 1A). However, inoculation with BS1 (*Bacillus megaterium*) and BS2 (*Bacillus subtilis*) alleviated the adverse effects of salt stress, with combined inoculation with *B. megaterium B. subtilis* (BS1+BS2) progressively producing the tallest plants across all salinity levels. The beneficial effect of bacterial inoculation was more pronounced under 100 and 150 mM NaCl. A similar trend was also observed in case of number of leaves/ plant (Fig. 1B), significant decrease was recorded as NaCl concentration increased. Compared with the uninoculated control, plants treated with single-strain inoculation BS1 or BS2 maintained a higher leaf number under saline conditions, while with combine application BS1+BS2 resulted in the greatest improvement, indicating a synergistic effect of the combined bacterial inoculation. Plant fresh weight/plant (g) was notably reduced with increasing salinity level (Fig. 1C). Nevertheless, inoculation with BS1 and BS2 improved fresh biomass compared with the uninoculated (control) at all salinity levels, with BS1+BS2 producing the highest fresh weight, particularly under 100 and 150 mM NaCl, suggesting its superior performance to mitigate adverse effects of salinity on growth traits of lettuce.

Leaf area/ plant (cm²) declined gradually with an increase in salinity level (0, 50, 100, and 150 mM NaCl). However, soil inoculation with BS1 (*Bacillus megaterium*), BS2 (*Bacillus subtilis*), and combined inoculation with BS1+BS2 mitigated the adverse effects of salt stress compared with the uninoculated control (Fig. 2A). A significant reduction in root length (cm) was observed with increasing NaCl concentration (Fig. 2B). Inoculation with BS1, BS2, and BS1+BS2 alleviated the inhibitory effects of salinity, resulting in longer roots than the control (uninoculated) at all salinity levels suggesting *Bacillus* strains have positive effect on growth traits. Among the treatments, the performance of BS1 was the most effective in upholding root growth, particularly under 100 and 150 mM NaCl, indicating its superior role in improving salinity tolerance.

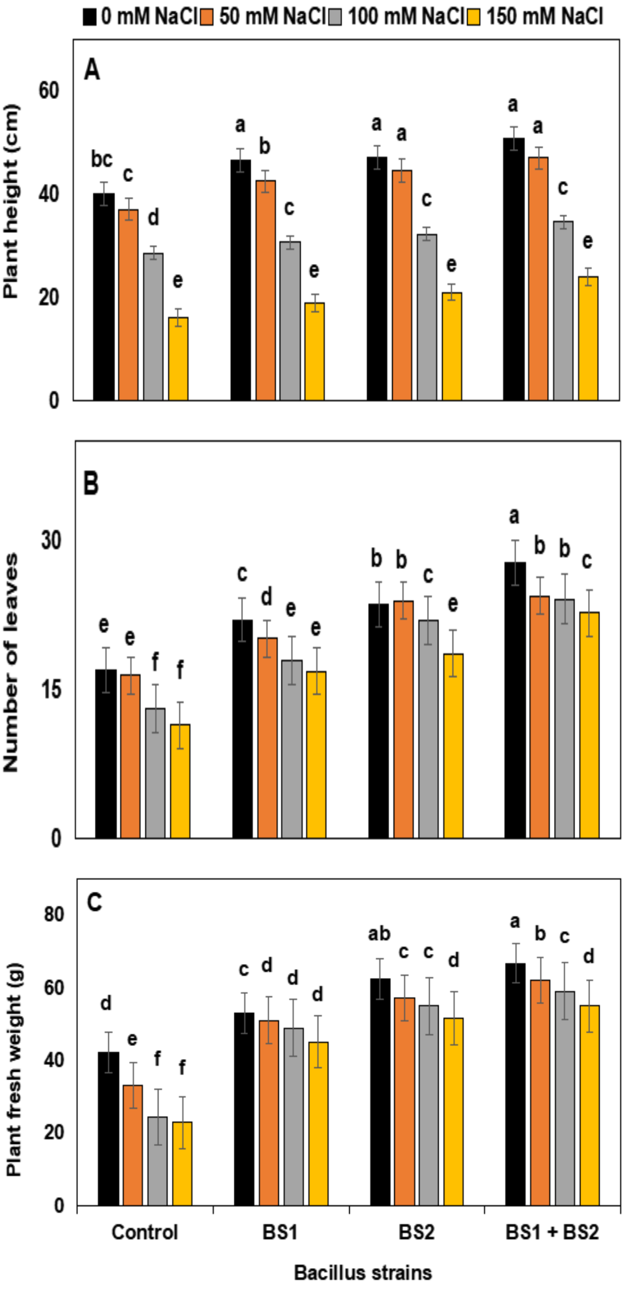

Fig. 1. Impact of Soil application of *Bacillus Strains* on lettuce plant height (A), number of leaves (B), and plant fresh weight (C). where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*.

Effect of *Bacillus* strains on photosynthetic pigments: A progressive decrease in Chlorophyll *a* (mg g^{-1} FW) was recorded with increasing salinity (Fig. 3A). However, inoculation with BS1 (*Bacillus megaterium*), BS2 (*Bacillus subtilis*), and combined inoculation (BS1+BS2) mitigated salinity-induced reduction, with BS1 consistently maintaining the highest chlorophyll *a* content, particularly under 100 and 150 mM NaCl, suggesting superior ability to mitigate adverse effects of salinity. Similarly, chlorophyll *b* was also declined with increasing salinity level (Fig. 3B). Inoculation with *Bacillus* strains improved chlorophyll *b* content as compared to the uninoculated (control). Total chlorophyll, a continuous decline was measured with an increase in salinity level. However, inoculation with BS1 mitigated salinity effects at all salinity levels as compared to the control (Fig. 3C).

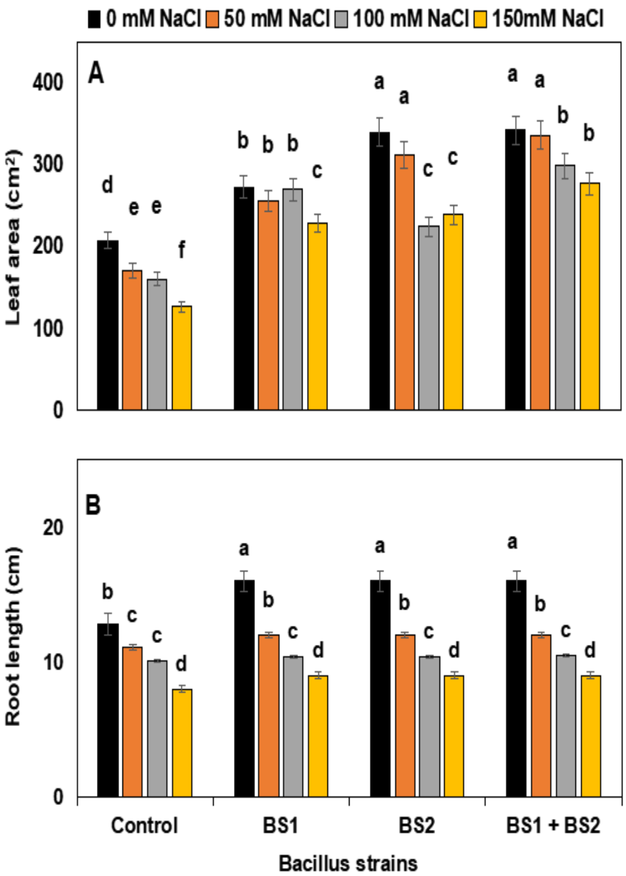

Fig. 2. Impact of soil application of *Bacillus Strains* on leaf area (A) and root length (B) of lettuce. Where BS1 =*Bacillus megaterium* and BS2= *Bacillus subtilis*.

Effect of *Bacillus* strains on antioxidative enzymes: SOD activity increased progressively with increasing salinity (Fig. 4A). Soil inoculation with individual *Bacillus* strains BS1 (*Bacillus megaterium*), BS2 (*Bacillus subtilis*), and combined (BS1+BS2) further improved SOD activity, results revealed that combined inoculation (BS1+BS2) showed the maximum SOD activity, particularly under 100 and 150 mM NaCl levels. Similarly, POD activity was also increased under salinity stress (Fig. 4B), but inoculation with *Bacillus* strains significantly improved POD activity compared with the uninoculated (control), with combined inoculation (BS1+BS2) showing maximum POD activity. Likewise, CAT activity also improved with the increase in NaCl level (Fig. 4C). Soil inoculation with *Bacillus* strains individually (BS1, BS2) and combined (BS1+BS2) consistently improved CAT activity under saline conditions, but combined soil inoculation (BS1+BS2) showed maximum CAT activity as compared to uninoculated (control).

Effect of *Bacillus* strains on oxidative stress indicators: Rapid increase in MDA content was observed as salinity level increased (Fig. 5A), demonstrating enhanced membrane lipid peroxidation under salinity stress. Soil inoculation with Bacillus strains reduced MDA accumulation as compared to the uninoculated (control), however, the combined inoculation (BS1+BS2) performed superior as minimum MDA content was observed, particularly at higher salinity levels. Thess results suggest that inoculation with the combined BS1+BS2 was more effective in protecting membrane integrity against salinity stress as compared to individual strains. Likewise, H_2O_2 content also progressively increased with the increase in salinity level (Fig. 5B); however, inoculation with Bacillus strains mitigated H_2O_2 accumulation under saline conditions. The performance of combined BS1+BS2 inoculation was superior as minimum H_2O_2 was produced even under moderate and severe salinity stress, which indicates that combined inoculation has a stronger capacity to alleviate oxidative stress by limiting the accumulation of reactive oxygen species compared with the individual bacterial treatments.

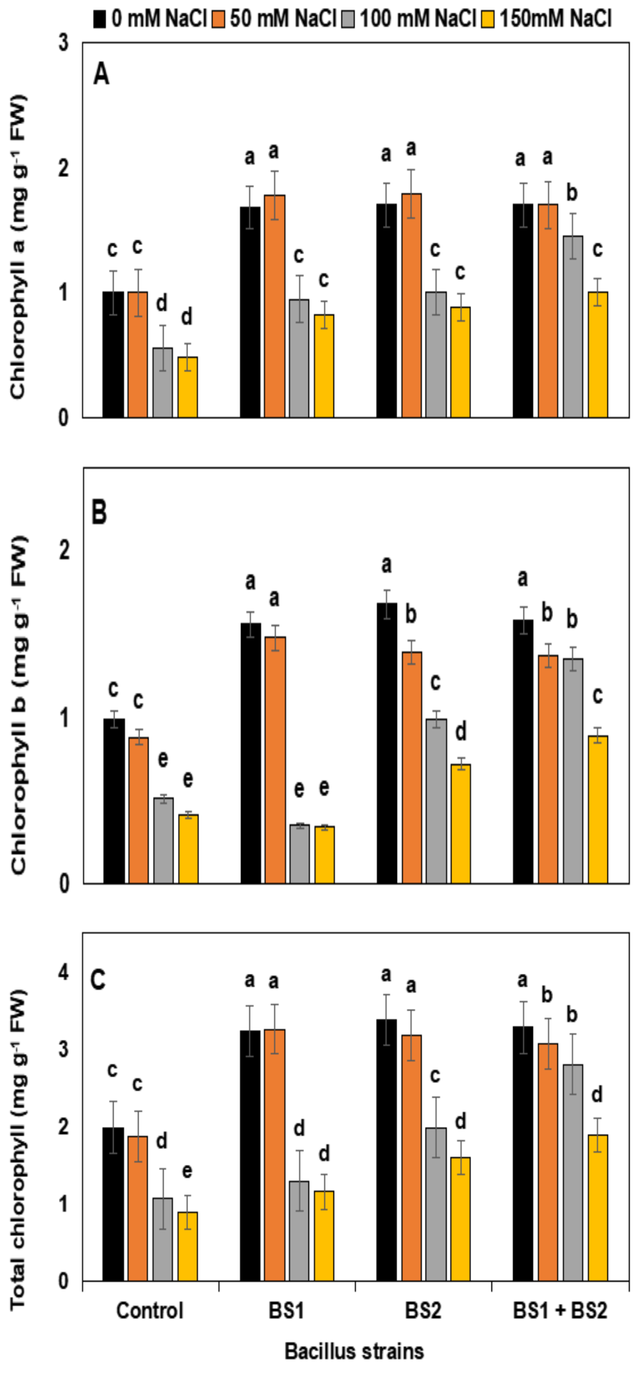

Fig. 3. Impact of soil application of *Bacillus strains* on chlorophyll a (A), chlorophyll b (B), and chlorophyll c (C) of lettuce. where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*.

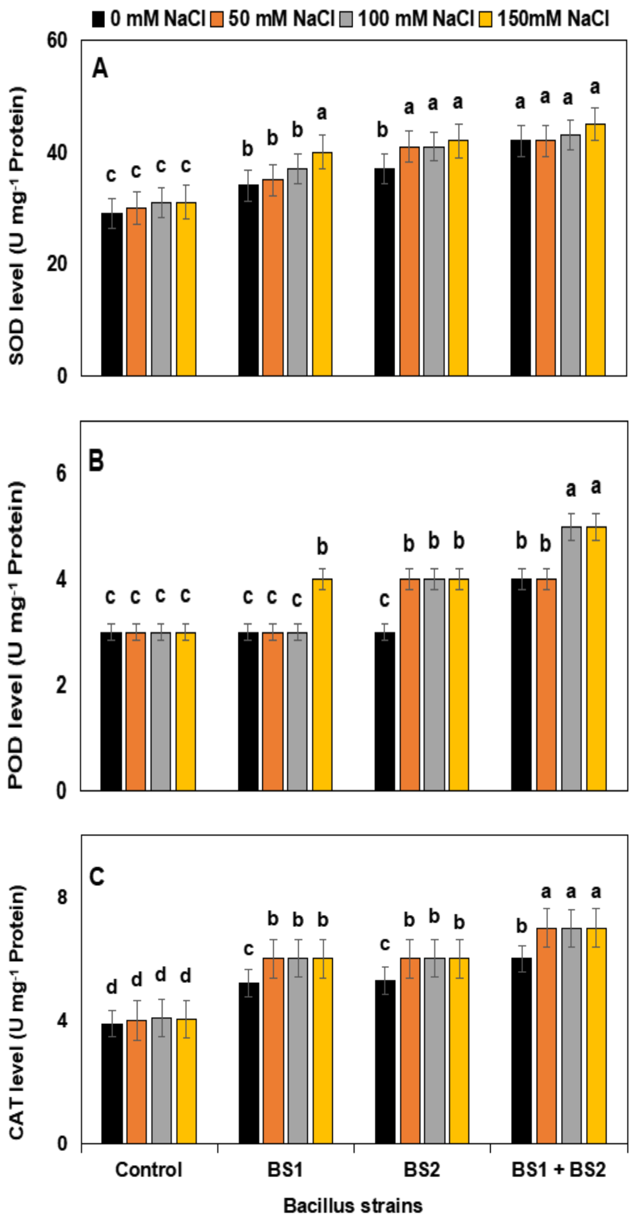

Fig. 4. Impact of soil application of *Bacillus Strains* on SOD (A), POD (B), and CAT (C) activities of lettuce. Where BS1 =*Bacillus megaterium* and BS2= *Bacillus subtilis*

Effect of soil application of *Bacillus* Strains on leaf anatomical traits: Leaf midrib area declined gradually as salinity level increased (Fig. 6A); this may be due to osmotic stress restricting cell expansion. However, Soil inoculated with *Bacillus* strains mitigated this reduction; among treatments, inoculation with BS1 showed the greatest improvement, indicating better safeguarding of leaf vascular structure under salt stress. A similar trend was also recorded in the case of leaf epidermal thickness as exhibited in leaf midrib area (Fig. 6B). However, inoculation with BS2 individually and combined application (BS1+BS2) improved structural adaptation in lettuce under saline conditions Leaf vascular bundle area affected under induced-salinity level (Fig 6C). However, inoculation with *Bacillus* strains proved better for structural adaptation under salinity stress. It was observed that inoculation with BS1 produced the highest leaf vascular bundle area among all treatments. Leaf phloem thickness also increased with an increase in salinity level (Fig. 6D). Inoculation with *Bacillus* strains slightly improved thickness, although marginal differences among bacterial treatments were exhibited.

Effect of soil application of *Bacillus* Strains on root anatomical traits: Application of *Bacillus* strains significantly improved root anatomical traits of lettuce grown under salinity stress. Root protoxylem diameter (Fig. 7A) increased from 22–24 µm in control plants to 30–33 µm in plants treated with *Bacillus megaterium* and 27–30 µm in plants treated with *Bacillus subtilis* at the highest salinity level, respectively, which indicates that *Bacillus* strains have a mitigating effect at the cellular level in lettuce plants. A similar trend was observed in the case of root metaxylem diameter (Fig. 7B), as the diameter of root metaxylem was recorded as 28–30 µm in untreated plants, which increased up to 38–41 µm with application of *B. megaterium* and 34–36 µm with *B. subtilis*, respectively. The minimum root epidermal thickness was observed to be 18-20 µm in plants grown under saline conditions without receiving Bacillus strains. However, it was improved with the application of (Fig. 7C) *B. megaterium* and *B. subtilis* to 26–28 µm and 24–26 µm under *B. subtilis,* respectively. Likewise, root phloem thickness (Fig. 7D) was 15–17 µm in plants grown under salinity stress, but without Bacillus strains, root phloem thickness was improved with application of *B. megaterium* and *B. subtilis* up to 23–25 µm and 21–23 µm, respectively at higher salinity levels, suggesting a mitigating effect of Bacillus strains at the cellular level.

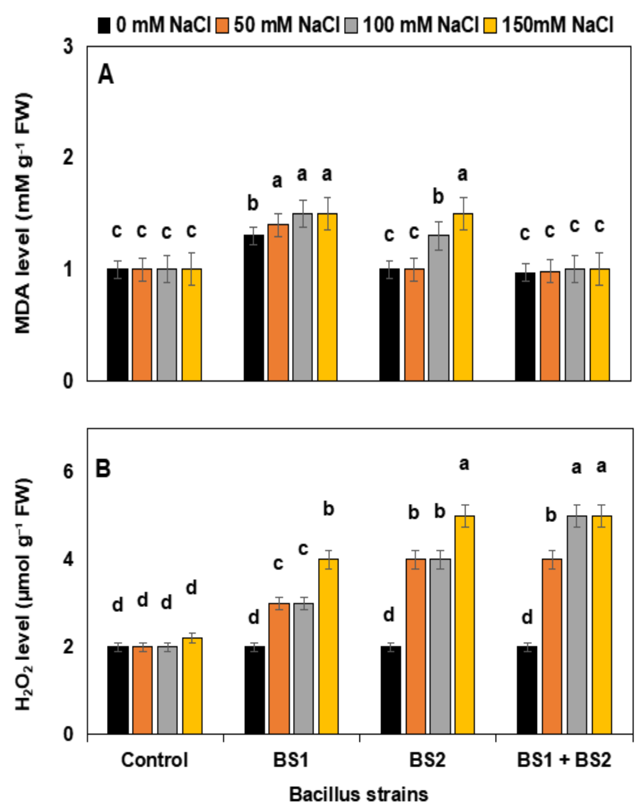

Fig. 5. Impact of soil application of *Bacillus Strains* on MDA (A) and H_2O_2 (B) of lettuce. where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*.

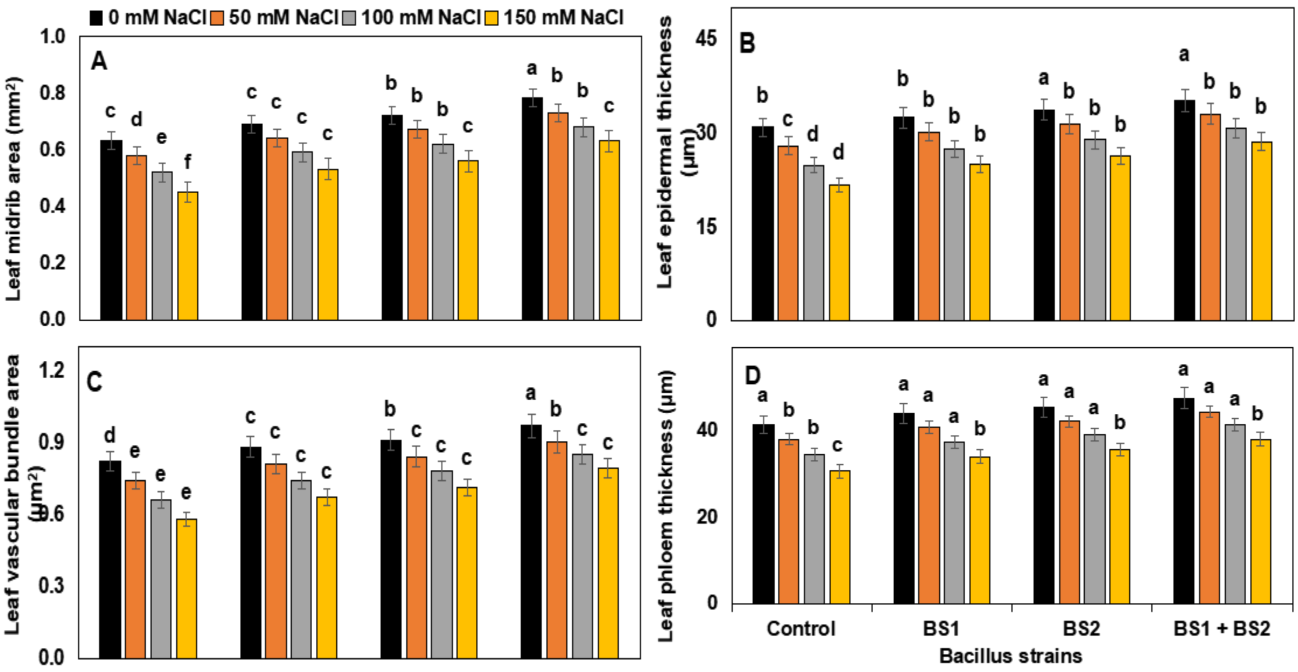

Fig. 6. Impact of soil application of *Bacillus Strains* on leaf midrib area (A), leaf epidermal thickness (B), leaf vascular bundle area (C), and leaf phloem thickness (D) of lettuce, where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*

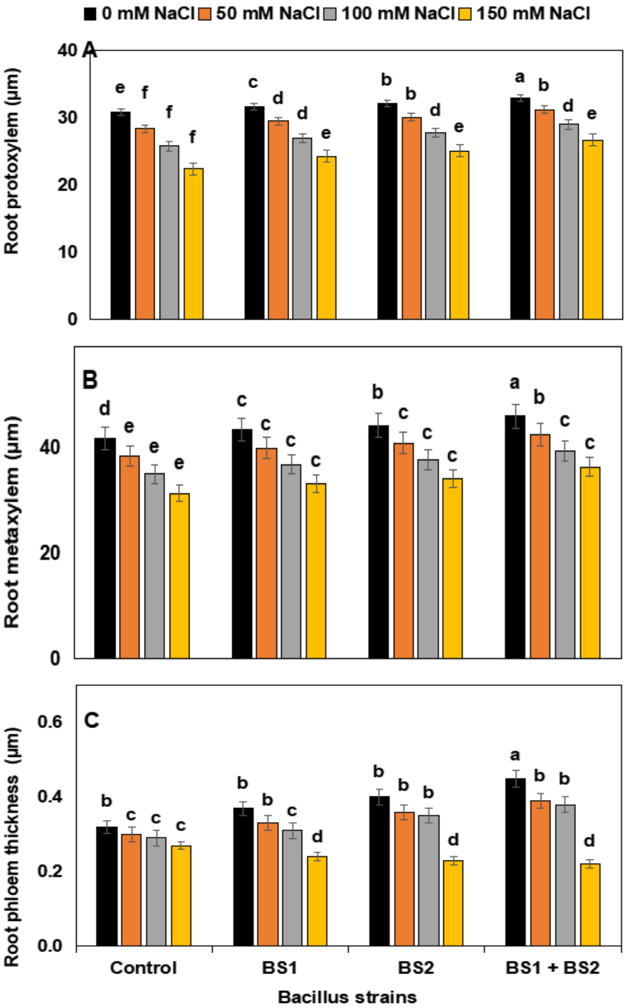

Fig. 7. Impact of soil application of *Bacillus Strains* on root protoxylem (A), root metaxylem (B), and root epidermal thickness (C) of lettuce, where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*

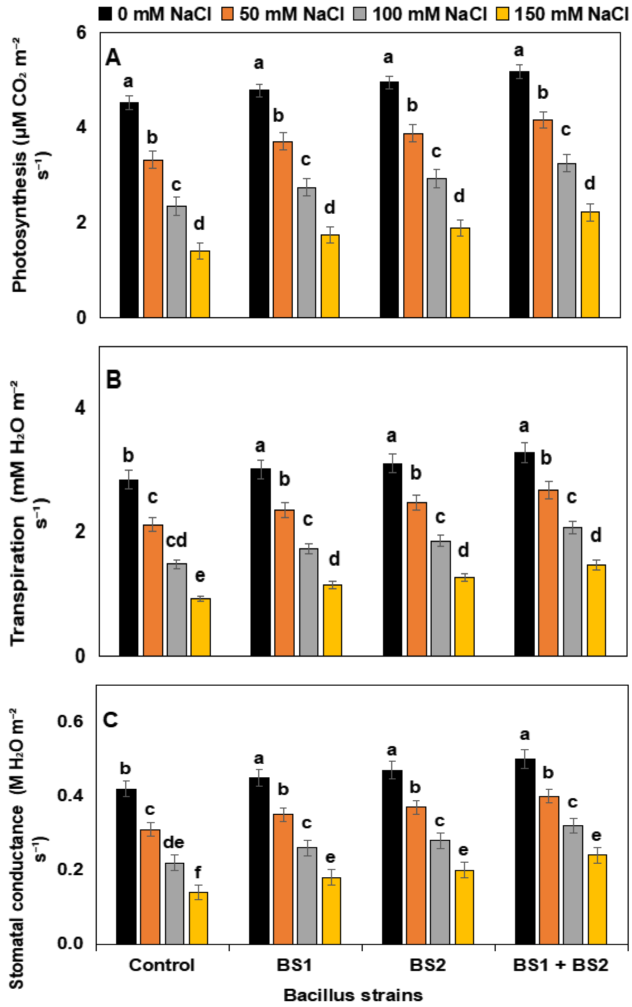

Fig. 8. Impact of soil application of *Bacillus Strains* on photosynthesis (A), transpiration rate (B), and stomatal conductance (C) of lettuce, where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*.

0 mM NaCl (S_1+S_2) 50 mM NaCl (S_1+S_2) 100 mM NaCl (S_1+S_2) 150 mM NaCl (S_1+S_2)

Fig. 9. Physiological view of the effect of different concentrations of NaCl and *Bacillus* strains on the lettuce plant, where BS1= *Bacillus megaterium* and BS2= *Bacillus subtilis*.

Effect of soil application of *Bacillus* Strains on leaf gaseous exchange traits: Bacillus inoculation enhanced gas-exchange traits of lettuce grown under salinity stress. The photosynthetic rate, as presented in Fig. 8A, in control plants increased from 0.28 µmol CO_2 m^{-2} s^{-1} at 0 mM NaCl to 0.38 µmol CO_2 m^{-2} s^{-1} at 150 mM NaCl, whereas plants that received *B. megaterium* and *B. subtilis* maintained relatively stable values ranging from 0.27 - 0.33 µmol CO_2 m^{-2} s^{-1} across all salinity levels. The combined application of *B. megaterium* + *B. subtilis* sustained photosynthetic rates between 0.29 and 0.31 up to 100 mM NaCl, followed by a slight reduction at 150 mM NaCl. Transpiration rate, as presented in Fig. 8B, in untreated plants increased from 0.18-0.30 µmol H_2O m^{-2} s^{-1} with increasing salinity, while bacterial treatments moderated transpiration, with values of 0.19–0.24 µmol H_2O m^{-2} s^{-1} in *B. megaterium* and 0.20–0.24 µmol H_2O m^{-2} s^{-1} in *B. subtilis* and the combined treatment. Results related to stomatal conductance presented in Fig. 8C indicated that there was a decline in control plants from 0.28-0.25 µmol m^{-2} s^{-1} as salinity increased, whereas plants receiving *B. megaterium* and *B. subtilis* maintained higher conductance at low salinity (0.30–0.32 µmol m^{-2} s^{-1}) before decreasing to 0.21–0.22 µmol m^{-2} s^{-1} at 150 mM NaCl. Remarkably, combined application of *B. megaterium* + *B. subtilis* showed stomatal conductance at 0 mM NaCl (0.40 µmol m^{-2} s^{-1}) and maintained elevated values (0.34–0.36 µmol m^{-2} s^{-1}) at 50–100 mM NaCl, indicating mitigation of salinity stress through improved regulation of gas exchange (Fig. 9).

Discussion

Soil is composed of various essential nutrients and many microbes; i.e., bacteria and fungi have growth-promoting capability to mitigate abiotic stress conditions (Abou Fayssal *et al*., 2024; Manzoor *et al*., 2025). From the current results, *Bacillus* strains improved plant height, number of leaves, plant fresh weight, leaf area, root fresh weight, root length, and root dry weight of lettuce grown under salinity stress. *Bacillus* sp. is well-known for its growth-promoting nature (Patani *et al*., 2024). Moreover, *Bacillus* strains improved the growth traits of lettuce grown under all salinity levels. =Lettuce attained the maximum height (28 cm) under the combined effect of *B. megaterium* and *B. subtilis*. Previous research also confirmed our results as reported by Valencia-Marin *et al*., (2024). Salinity stress hampers the physiological processes in plants, which leads to restricted plant growth because excessive sodium and chloride ions can disrupt the uptake and assimilation of nutrients (Fatima *et al*., 2025; Nadeem *et al*., 2025). *Bacillus* sp., is effective in alleviating salinity stress, as these bacteria improve nutrient uptake by accelerating enzymatic activity (Mahmoud *et al*., 2024; Muhammad *et al*., 2025). The performance of *Bacillus sp*. was also found to be practical, while minimizing salinity stress and maintaining root growth traits (Tatar *et al*., 2025). Significant root elongation was observed, which aligned with another study where root elongation was measured under abiotic stress (Sharifi *et al*., 2021).

Photosynthetic pigments are very important for plant bioactive processes; maintaining total chlorophyll content is a crucial factor while managing abiotic stress, as it is severely damaged due to oxidative stress (Chauhan *et al*., 2023; Muhammad *et al*., 2024). The present investigation revealed that application of *Bacillus* strains improved chlorophyll content, as values were stable while increasing salinity levels. Plants receiving combined application of *B. megaterium* and *B. subtilis* showed significant chlorophyll content. The results of the present investigation were consistent with previous findings as reported by Curá *et al*., (2017) and Altaf *et al*., (2026). The accumulation of photosynthetic pigments depends on the type of bacteria, kind of stress, and plant species (Dube *et al*., 2024).

Production of reactive oxygen species (ROS) massively increased with salinity stress, which leads to serious oxidative damage to cell membranes and vital biomolecules if not effectively controlled by the plant antioxidant system (Wang*et al*., 2024; Cortez Espinoza *et al*., 2026). Application of *Bacillus megaterium* and *Bacillus subtilis* improved SOD, POD, and CAT in lettuce under salinity stress, which signifies a strengthened enzymatic defense against oxidative stress. SOD is a key enzyme that helps in mitigating oxidative stress and strengthening plant resilience through scavenging excessive ROS (Jiang *et al*., 2019). There are certain *Bacillus* species that improve abiotic stress tolerance through accelerating the production of SOD, POD, and CAT, which neutralize free radicals (AlHosani *et al*., 2025; Song *et al*., 2026). The current findings confirm that reduced midrib and vascular bundle area is evident in plants treated with the application of *B. megaterium* and *B. subtilis,* which had significantly higher enzymatic activity under various levels of salinity (Kumar *et al*., 2021). Similar results were also reported by Sajid *et al*. (2025) in wheat and maize. Moreover, another investigation also confirmed that the application of *Sphingobacterium* BHU-AV3 in tomato enhanced antioxidant enzyme activity (Vaishnav *et al*., 2020).

Salinity stress induced higher MDA and H_2O_2 levels, as these are indicators of oxidative stress, which confirms membrane damage in lettuce. Such increases are well-reported penalties of salt-induced ionic and osmotic imbalance that disrupt cellular redox homeostasis (Zhang *et al*., 2022). On the contrary, application of *Bacillus* strains substantially mitigated oxidative damage, demonstrated by reduced MDA and H_2O_2 levels (Radhakrishnan *et al*., 2017). This protective effect can be credited to the ability of *Bacillus megaterium* and *Bacillus subtilis* to enhance ROS detoxification, stabilize membranes, and improve stress signaling through modulation of antioxidant machinery and phytohormone balance (Chieb *et al*., 2023). Findings of the current investigation were confirmed, as results presented by Mazzoli *et al*., (2019), who found that *Bacillus megaterium*helps manage oxidative stress.

Reduced midrib and vascular bundle area is evidence that salinity stress noticeably disrupted leaf and root anatomical organization of lettuce, with a thinner epidermis and compromised xylem–phloem development, reflecting impaired transport efficiency and structural integrity. Application of *Bacillus megaterium* and *B. subtilis* effectively alleviated these cellular-level damages by enhancing leaf midrib area, epidermal thickness, vascular bundle size, and root xylem–phloem dimensions. Results of the current investigation indicate that *Bacillus* strains accelerated phytohormone production and osmotic adjustments necessary for better cambial activity under saline conditions. Similarly, Abdullah *et al*., (2025) reported that improvement in the transport machinery of plants leads to an increase in crop yield.

Salinity stress also disturbed the regulation of gas exchange in *Lactuca sativa*, as reflected by unstable photosynthetic rates, increased transpiration, and reduced stomatal conductance. However, application of *B. megaterium* and *B. subtilis*, in combination, adjusted these lethal effects through stabilizing photosynthesis, restricting excessive transpiration, and maintaining higher stomatal conductance under various levels of salinity. The findings of Kumawat *et al*., (2023) and Faiq *et al*. (2025) were aligned with our findings, as disruption in stomatal conductance affects nutrient uptake and photosynthetic activity, which leads to yield losses.

Conclusion

Salinity stress markedly impaired growth, photosynthetic capacity, gas exchange, antioxidant balance, and anatomical integrity of lettuce. Application of *Bacillus megaterium* and *Bacillus subtilis* significantly mitigated these adverse effects of salinity stress. The combined bacterial treatment consistently outperformed individual applications by enhancing biomass accumulation, chlorophyll stability, antioxidant enzyme activities, and regulation of oxidative stress, while reducing malondialdehyde and hydrogen peroxide accumulation. Moreover, improvements in leaf and root anatomical traits indicated enhanced vascular development and structural adaptation under saline conditions. Collectively, these findings demonstrate that combined application of *B. megaterium* and *B. subtilis* is an effective and sustainable strategy to improve lettuce tolerance to salinity stress.

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