

RHIZOBIA AND ARBUSCULAR MYCORRHIZAL FUNGI ENHANCE SOYBEAN (*GLYCINE MAX* (L.) Merr.) TOLERANCE TO SALINITY STRESS

Urooj Muhammad Sharif, Afshan Rahman and Fouzia Naseer

Agricultural Biotechnology & Phytopathology Laboratory, Department of Botany, University of Karachi, Karachi 75270, Pakistan

Corresponding author's email: a.rahman@uok.edu.pk

ABSTRACT

Soybeans are cultivated worldwide due to their economic importance as a major source of oil and protein. The study aims to mitigate the salinity by inoculating VAM spores with six rhizobia isolates. Exposure to salinity causes significant growth retardation of soybean as compare to normal conditions. In this study, soybean plants were inoculated with VAM spores and six rhizobium isolates under salt stress conditions to evaluate their effects on primary and secondary growth parameters and biochemical responses. The coupled inoculation of rhizobia and VAM led to a considerable increase in soybean mass and length under salt stress. The inoculation significantly improved the concentration of potassium (K) and phosphorus (P). The inoculation notably increased soybean polyphenol and protein levels, which contributed to improving salt tolerance. It also led to a significant increase in the number of VAM spores and nodules after harvest. The coupled inoculation with rhizobia and VAM effectively enhanced soybean growth and stress tolerance. This strategy holds promise for sustainable soybean cultivation in salt-affected soils.

Key-words: Coupled-inoculation, rhizobia, salinity, soybean, VAM.

INTRODUCTION

Soil salinity is the most threatening environmental factor imposing hyper-osmotic and ionic stresses in plants. Approximately 50% of the agricultural land area is badly affected and it reduces the production of crop plants (Ullah *et al.*, 2021). Salinity alters many metabolic and physiological processes, inhibits photosynthesis, reduces growth and other biosynthesis mechanisms, and changes seed germination behavior (Hasanuzzaman and Fujita, 2022). Soybean (*Glycine max* L.) is one of the main oilseeds and an economically significant leguminous crop because it provides 20% oil content and 40% protein for human and animal consumption (Asad *et al.*, 2020). The crop is badly influenced by salt stress through poor seed germination, reduced plant growth, Na⁺ and Cl⁻ ions imbalance, osmotic stress, and inaccessibility of essential nutrients elements particularly phosphorus (P) because of its precipitation that leads to a decrease of 40% crop yield hence agricultural production is declined (Hasanuzzaman *et al.*, 2022).

Microorganisms play a vital role in promoting plant growth, particularly through their ability to enhance stress tolerance and fulfill nutritional requirements, making them ideal for use in innovative commercial biofertilizer products (Lin *et al.*, 2025). Among these beneficial microbes, rhizobia and vesicular-arbuscular mycorrhizal (VAM) fungi are particularly notable for their well-established symbiotic relationships with plants (Dabré *et al.*, 2022). VAM fungi, as natural root symbionts, contribute significantly to plant development and productivity by improving the uptake of key macronutrients, especially phosphorus (Begum *et al.*, 2019). Likewise, biological nitrogen fixation—considered the second most crucial process after photosynthesis—is facilitated by rhizobia, which form mutually beneficial associations with the roots of legumes such as beans (Han *et al.*, 2020). These bacteria have a tremendous ability to convert unavailable atmospheric nitrogen into available form for leguminous plants (Sharif *et al.*, 2024). The symbiotic associations have gained global popularity due to their low cost, eco-friendly nature, and ease of application (Spagnoletti *et al.*, 2020).

This study elucidated the coupled effects of rhizobia and vesicular-arbuscular mycorrhizae (VAM) fungi on soybean growth under salt stress. The research focused on discovering the mutualistic benefits of these microorganisms in enhancing plant growth, improving nutrient uptake and mitigating the stress.

MATERIAL AND METHODS

Rhizobia cultures and Isolation of VAM spores:

The rhizobial isolates utilized in this investigation were previously documented by Farhat *et al.* (2017) and Sharif *et al.* (2024). The Bokhari *et al.* (2023) wet sieving and decanting process was used to isolate VAM spores.

PLANT GROWTH-PROMOTING TRAITS OF BACTERIA:

Bacterial growth on salt concentration:

Bacterial growth under salinity stress was assessed using the method of Chakraborty *et al.* (2011), with media containing 0 mM, 50 mM, 100 mM, 150 mM, and 200 mM NaCl. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48–72 hours.

Determination of indole acetic acid production by bacteria:

Bacteria were cultured in YEMA broth for 48 hours. Salkowski's reagent was added to the bacterial supernatant and left at room temperature for 30 minutes. Absorbance was measured at 530 nm using a spectrophotometer, with 2 mL of uninoculated broth as the reagent blank (Patten and Glick, 2002; Noreen *et al.*, 2018).

Cyanide production:

Hydrogen cyanide production was evaluated using the method of Noreen *et al.* (2018). Bacteria were streaked on glycine-amended YEMA agar, and a sodium picrate-soaked Whatman No. 1 filter paper was placed inside the lid. Plates were sealed with parafilm and incubated at 28°C for 4 days.

Phosphate solubilization:

Rhizobial cultures were stabbed onto Pikovskaya's agar plates for phosphate solubilization, following the method of Noreen *et al.* (2018). A clear halo zone around the bacterial growth indicated phosphate solubilization.

Ammonia (NH₃) production:

Ammonia production was tested by inoculating freshly grown cultures in peptone water and incubating for 72 hours. Nessler's reagent was then added to detect ammonia production (Cappuccino & Sherman, 1992).

PLANT GROWTH AND EXPERIMENTAL DESIGN:

Screen house experiments were conducted at the Department of Botany, University of Karachi, using a randomized complete block design with three replicates. Six surface-sterilized soybean seeds were sown in each pot. 25 mL of Rhizobium YEMA broth was added to the soil at sowing. Another set of pots was inoculated with 100 VAM spores and rhizobium suspension. The study consisted of two parts: one with unstressed plants and another irrigated with 100 mM NaCl. After four weeks, plants were uprooted, and growth parameters—such as shoot and root length, fresh and dry weights, number of nodules (Rehman *et al.*, 2010), leaf area, relative water content, and post-harvest VAM spore count—were recorded.

SECONDARY GROWTH PARAMETERS:

Plant mass fraction:

Seedling weight was used to calculate plant mass fractions (Sánchez-Gómez *et al.*, 2010). The following formula was used to calculate FSMF, DSMF, FRMF, DRMF, SLF, and RLF.

Fresh shoot mass fraction (FSMF) (g/g) = Fresh shoot mass/fresh seedling mass

Dry shoot mass fraction (DSMF) (g/g) = Fresh shoot mass/dry seedling mass

Fresh root mass fraction (FRMF) (g/g) = Fresh root mass/fresh seedling mass

Dry root mass fraction (DRMF) (g/g) = Dry root mass/dry seedling mass

Shoot length fraction (SLF) (cm/cm) = Shoot length/seedling length

Root length Fraction (RLF) (cm/cm) = Root length/seedling length

Total mass and length of seedling:

Total fresh weight (TFW) was calculated by adding the fresh weights of the shoot (including leaves), roots, and nodules. Total dry weight (TDW) was determined by summing the dry weights of the roots, nodules, and shoot (including leaves) (Zhang *et al.*, 2020). Seedling length was the sum of root and shoot lengths in centimeters (Paudel & Gupta, 2008). TSDFW, TSDDW, and TSDL were calculated using the following formulas:

Total seedlings fresh weight (TSDFW) (g) = Shoot fresh weight + root fresh weight + legume fresh weight

Total seedlings dry weight (TSDDW) (g) = shoot dry weight + root dry weight + legume dry weight

Total seedling length (TSDL) (cm) = shoot length + root length

Plant growth ratio:

Root length, shoot length (above-ground part), and total leaf area relative to seedling mass were measured and expressed as ratios (Goergen *et al.*, 2019). LAR, SLR, and RLR were calculated using the following formula:

Leaf area ratio (LAR) (cm^2/g) = leaf area/seedling mass

Shoot length ratio (SLR) (cm/g) = Shoot length/seedling mass

Root length ratio (RLR) (cm/g) = Root length/seedling mass

PLANT BIOCHEMICAL ANALYSIS:**Chlorophyll:**

Chlorophyll a and b were determined using the method of Arnon (1949). Fresh leaf samples were extracted in acetone, and the mixture was centrifuged. The supernatant was used to measure absorbance at 645 nm and 663 nm. Photosynthetic pigments were calculated using the following formulas:

Chlorophyll a (mg/g fresh wt.) = $[(12.7 \times A663) - (2.69 \times A645)] \times V / W \times 1000$

Chlorophyll b (mg/g fresh wt.) = $22.9(A645) - 4.68(A663) \times V / W \times 1000$

Total chlorophyll (mg/g fresh wt.) = $20.2 (A675) + 8.02 (A663) \times V / W \times 1000$

Protein determination:

Protein was estimated using the Bradford method (1976) with Coomassie Brilliant Blue dye. Bovine serum albumin (BSA) was used to prepare the calibration curve in the range of 0 to 100 $\mu\text{g}/\text{mL}$.

Poly-Phenol determination:

Samples were homogenized in 80% ethanol, and 100 μL aliquots were mixed with 7.5% Na_2CO_3 and incubated at room temperature for two minutes. After incubation, freshly prepared Folin-Ciocalteu reagent (0.2 N) was added, and the optical density was measured at 720 nm (Chandini *et al.*, 2008).

Estimation of carbohydrates:

Dry leaf samples were homogenized in distilled water and centrifuged. The supernatant was mixed with 0.2% Anthrone and boiled for 30 minutes in a water bath. The reaction was stopped by placing the mixture in an ice bath. Carbohydrate content was determined using the method of Hedge and Hofreiter (1962), and optical density was measured at 620 nm.

Estimation of phosphorus:

Phosphorus content was determined using the method of Noreen *et al.* (2019). Oven-dried leaves were digested in concentrated H_2SO_4 , heated on a hot plate, and re-digested with dropwise addition of H_2O_2 . An aliquot was transferred to a test tube, and ammonium vanadomolybdate was added, followed by a 30-minute incubation. Absorbance was measured at 420 nm, and phosphorus percentage was calculated using the following formula:

Phosphorus (%) = $\frac{\mu\text{mol/mol P (from the standard curve)} \times R \times 100}{\text{Wt.} \times 10000}$

Where: R= Ratio between the total volume of the digestion

Wt.= oven-dried weight of leaf sample

Estimation of nitrogen:

Nitrogen content was estimated using Nessler's method (Noreen *et al.*, 2019). A 0.1 mL aliquot of the digested sample was mixed with Nessler's reagent and incubated for 20–30 minutes at room temperature. Absorbance was measured at 410 nm, and nitrogen percentage was calculated using the following formula:

Nitrogen (%) = $\frac{\mu\text{mol/mol N (from the standard curve)} \times R \times 100}{\text{Wt.} \times 10000}$

Where: R= Ratio between the total volume of the digestion

Wt.= oven-dried weight of leaf sample

DATA ANALYSIS:

All the data were statistically analyzed by SPSS Software and all graphs were plotted by OriginLab 2019 software.

RESULTS

In this study all the selected rhizobial strains tested, were showed positive results for cyanide, phosphate solubilization, and ammonia production. IAA was produced with varying levels of concentration. Strain NFB-301 produced the highest concentration of IAA, while strains NFB-305 and NFB-306 produced the lowest concentrations (Table 1). A significant difference was observed in the growth of bacteria under salt-amended media. NFB-301, NFB-302, and NFB-303 strains exhibited the highest tolerance to increasing salinity, maintaining strong growth even at 100 mM NaCl and moderate growth at 200 mM NaCl. NFB-304 showed strong tolerance up to 100 mM NaCl but growth reduced at higher concentrations, with minimal growth at 200 mM NaCl. NFB-305 and NFB-306 strains showed good tolerance up to 100 mM NaCl, but their growth significantly diminished as the NaCl concentration increased, showing only minimal growth at 200 mM NaCl. The results suggested that NFB-301, NFB-302, and NFB-303 were the most salt-tolerant strains, while NFB-305 and NFB-306 were the least tolerant under high salinity conditions.

Coupled inoculated treatments under control conditions showed the highest shoot length. Treatments of rhizobia strains and VAM, especially NFB-302 + VAM and NFB-303 + VAM, exhibited significant shoot length under both conditions, suggesting that these treatments helped in mitigating the negative effects of salinity on shoot growth. The reduction in shoot length due to NaCl was less pronounced in these treatments compared to others, indicating their effectiveness in combating salt stress. Like shoot length, root length was generally reduced under 100 mM NaCl compared to the control across all treatments. Maximum root length achieved in coupled inoculated treatment (rhizobia 302 and 303 with VAM), which exhibited the longest root lengths under both stressed and unstressed conditions, highlighting their potential to enhance root development even in saline environments (Fig 1). The combination of rhizobia strains with VAM generally showed better plant growth including shoot and root weight under normal and saline environments. Both microorganisms appeared to enhance the plant's ability to cope with salinity, reducing the negative impacts of salt stress on growth. These coupled inoculated treatments were considered as the most effective, consistently showing higher values across all parameters, even under saline conditions. This suggested that these treatments could be particularly useful in enhancing plant growth in salt-affected soils (Fig. 1).

Coupled inoculated treatments showed higher total seedling fresh and dry weight under non-saline conditions. Under saline conditions, weight dropped, but still outperformed many other treatments regarding total seedling fresh and dry weight under salt stress. This indicated that rhizobia and VAM might help to alleviate some effects of salinity stress on fresh and dry weight fractions. There was a less significant difference observed in shoot length fraction between saline and non-saline conditions while root length fraction was significantly ($p < 0.05$) increased under combined treatment (rhizobia and VAM) and improvement was found maximum in NFB-303+VAM (Fig. 2).

When plants were subjected to 100 mM NaCl stress photosynthesis efficiency was mitigated. The reduction in a, b, and total chlorophyll directly affected growth and biomass accumulation. The coupled inoculation treatments showed significant enhancement in photosynthetic pigments even under saline conditions, as shown in Fig. 4. This suggested that these inoculants helped in alleviating the negative effects of salinity on photosynthesis. All three combinations showed positive effects on photosynthetic activity under salt stress. These treatments improved the efficiency of the photosynthetic process by maintaining chlorophyll levels, which allowed the plants to continue growth under salt stress.

The Carbohydrate content is a crucial indicator of plant energy storage and reflects overall plant health. The highest carbohydrate content was observed in treatments NFB-301 + VAM and NFB-302 + VAM. Other treatments, like all NFB strains, also showed higher carbohydrate content compared to the control, but these were still not as high as the combined treatments of rhizobia with VAM. Salinity significantly reduced carbohydrate content, but the combined treatments with VAM were able to mitigate the damaging effect of salinity, showing the best results in carbohydrate content under both conditions. Similarly, the highest protein and phenol content were observed in the combined treatments of NFB-301 + VAM, NFB-302 + VAM, and NFB-303 + VAM. Other NFB strain treatments such as NFB-304, NFB-305, and NFB-306 also showed relatively high protein and phenol content compared to respective controls, but effectiveness was not as in the combination treatments with VAM as shown in Fig 6.

The number of post-harvested VAM spores without stress was significantly highest in the treatments involving VAM (both with and without NFB), particularly in coupled inoculated treatments of NFB-301 + VAM and NFB-302 + VAM, whereas other NFB treatments without mycorrhizal showed very low numbers of VAM spores. A

slight reduction was observed under saline stress, but the combined treatments involving mycorrhizal fungi showed the highest number of VAM spores. Similarly, the post-harvested number of nodules was significantly increased in all three coupled inoculated combinations. These rhizobia treatments promoted mycorrhizal colonization, aiding nutrient uptake and stress tolerance (Fig. 5).

Total nitrogen and phosphorus percentages were significantly enhanced across all plants treated with combined inoculations. Although salinity stress caused a noticeable reduction in nitrogen and phosphorus content in all treatments, the plants subjected to coupled inoculations managed to maintain comparatively higher nitrogen levels. This demonstrated the effectiveness of these combined treatments in increasing phosphorus nitrogen uptake, even under stressful saline conditions. The ability of the coupled inoculated plants to sustain elevated nutrient levels suggests their capacity to mitigate the adverse effects of salinity, thereby supporting more robust growth and nutrient assimilation. These findings highlighted the potential benefits of using microbial inoculations to improve nutrient availability, particularly in challenging environments like saline soils. The increased nutrient content highlighted the resilience of plants treated with both nitrogen-fixing bacteria and mycorrhizal fungi, emphasizing their role in enhancing plant health under salt stress Fig. 6.

The coupled treatments, especially NFB-101 + VAM and NFB-102 + VAM, consistently showed higher values across shoot length ratio, root length ratio, and seedling total length in both control and stressed conditions (Fig. 3). Stress conditions generally reduced the shoot length ratio and total seedling length across treatments. Leaf area ratio showed more variable responses to stress, with some treatments showing increased values under stress (Fig. 5). The individual treatment of NFB-101 + VAM and NFB-102 + VAM performed better than the control, especially under stressed conditions. These observations suggested that the combined treatments provided stress tolerance or growth promotion, particularly in stressful conditions.

Table 1. Characteristics of six rhizobial strains (NFB-301 to NFB-306) to their ability to produce indole acetic acid (IAA), cyanide, solubilize phosphate, and produce ammonia.

S.no	Rhizobial strains	IAA	Cyanide production	Phosphate solubilization	Ammonia production	Indole acetic acid Concentration $\mu\text{g/ml}$
1.	NFB – 301	+VE	+VE	+VE	+VE	7.33 \pm 0.66
2.	NFB – 302	+VE	+ VE	+VE	+VE	4.33 \pm 0.66
3.	NFB – 303	+VE	+ VE	+VE	+VE	5.33 \pm 0.66
4.	NFB – 304	+VE	+VE	+VE	+VE	3.67 \pm 0.33
5.	NFB – 305	+VE	+ VE	+VE	+VE	3.33 \pm 0.88
6.	NFB – 306	+VE	+ VE	+VE	+VE	3.33 \pm 0.33

Table 2. Growth response of six rhizobial strains (NFB-301 to NFB-306) under different concentrations of sodium chloride (NaCl).

S.No	Rhizobial strains	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl
1.	NFB – 301	++++	++++	++++	+++	++
2.	NFB – 302	++++	++++	++++	+++	++
3.	NFB – 303	++++	++++	++++	+++	++
4.	NFB – 304	++++	++++	+++	+++	+
5.	NFB – 305	++++	++++	+++	++	+
6.	NFB – 306	++++	++++	+++	++	+

The different levels of NaCl concentration are 0 mM, 50 mM, 100 mM, 150 mM, and 200 mM. The growth response is indicated by a series of plus signs, with "++++" representing the strongest growth and "+" representing the weakest.

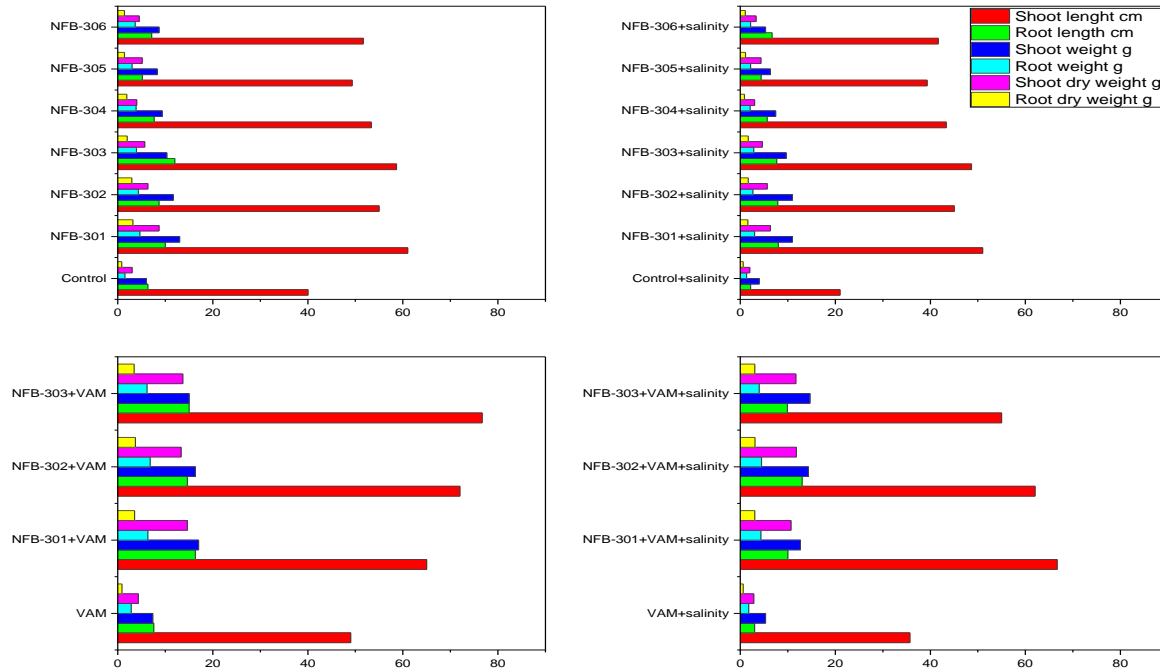


Fig. 1. Comparative analysis of plant growth parameters under NaCl stressed (100 mM) and (0 mM) unstressed. The bar charts displayed measurements for shoot length (cm), root length (cm), shoot weight (g), root weight (g), shoot dry weight (g), and root dry weight (g). Each parameter is color-coded to illustrate the impact of each treatment on plant growth.

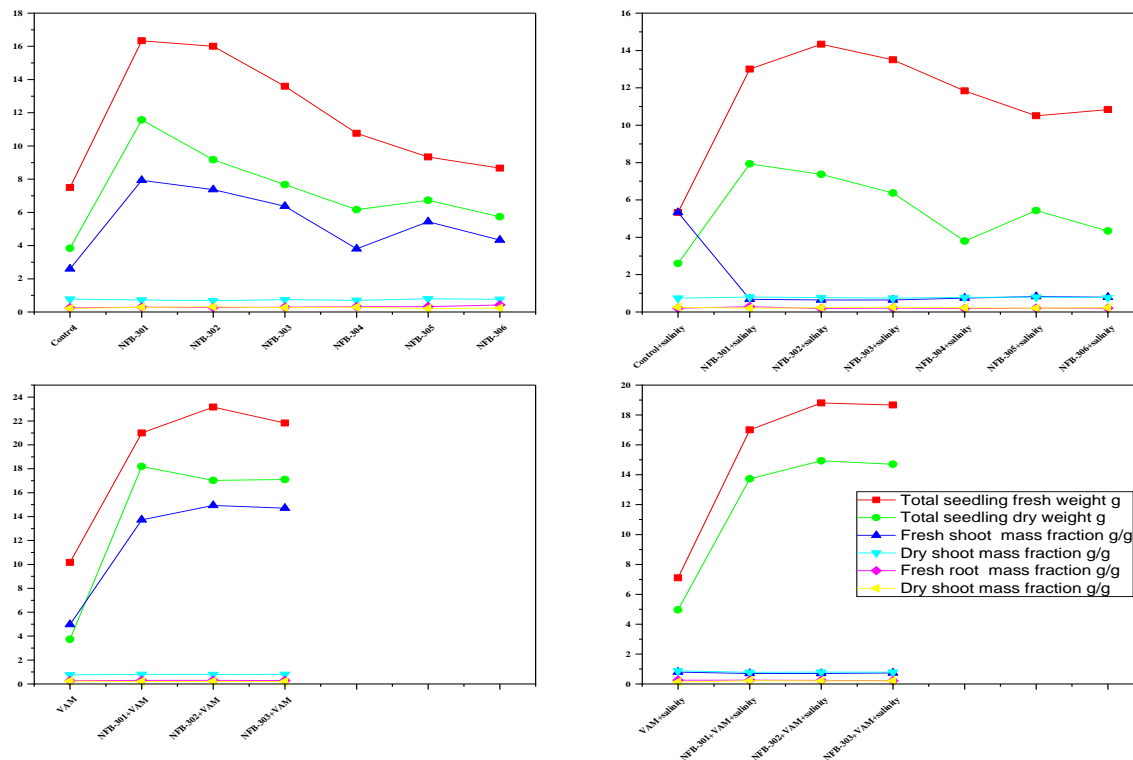


Fig. 2. Effect of rhizobia and VAM on seedling biomass and mass fraction. The line charts showed changes in total seedling fresh weight (g), total seedling dry weight (g), fresh shoot mass fraction (g/g), dry shoot mass fraction (g/g), fresh root mass fraction (g/g), and dry root mass fraction (g/g) across different treatments under unstressed and 100 mM NaCl. Each growth parameter was represented by a unique color and symbol.

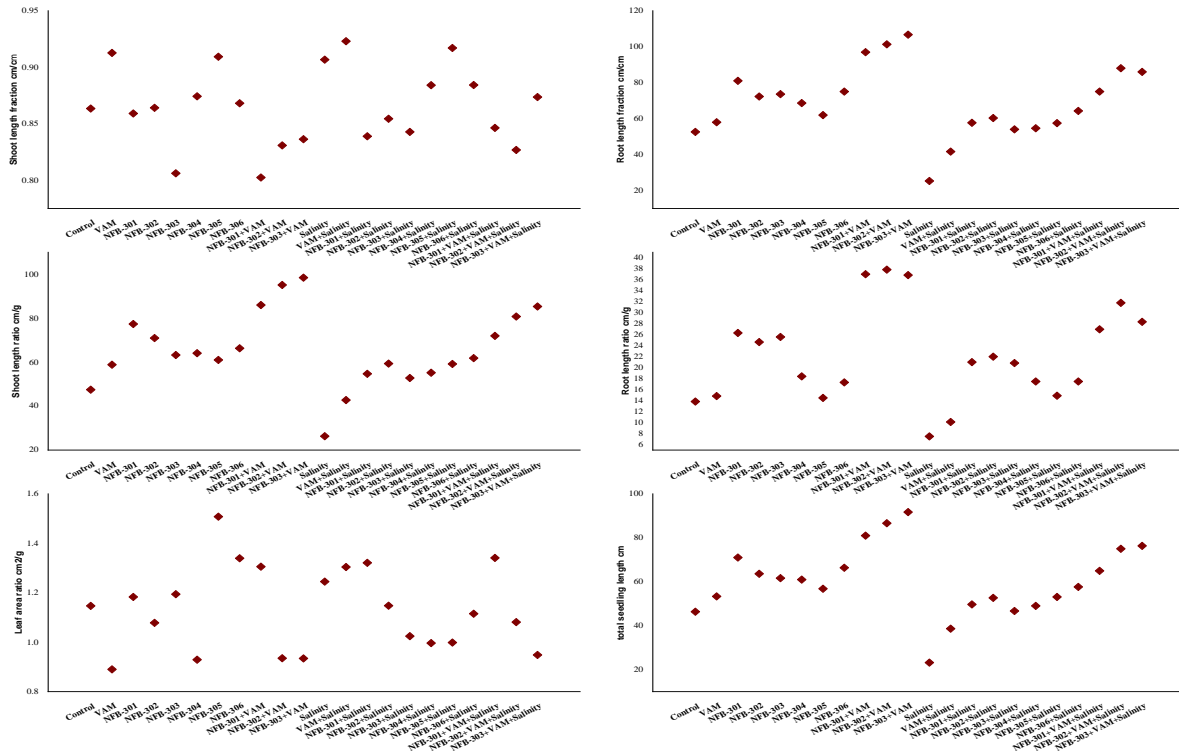


Fig. 3. Effect of rhizobia and VAM on plant total seedling length, seedling fraction and seedling ratio under unstressed and 100 mM NaCl.

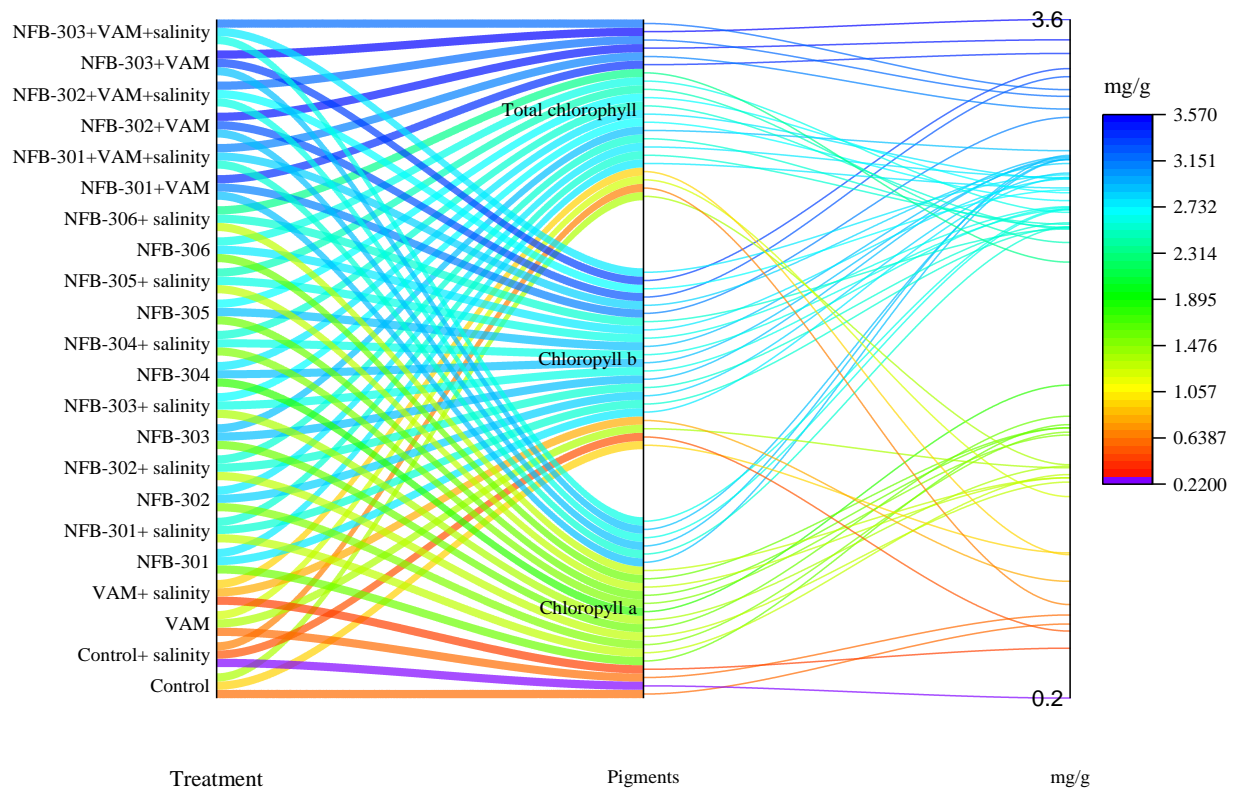


Fig. 4. Effect of rhizobia and VAM, represented by a parallel plot, illustrates the relative proportions of total chlorophyll, chlorophyll (b), and chlorophyll (a) under-stressed and unstressed.

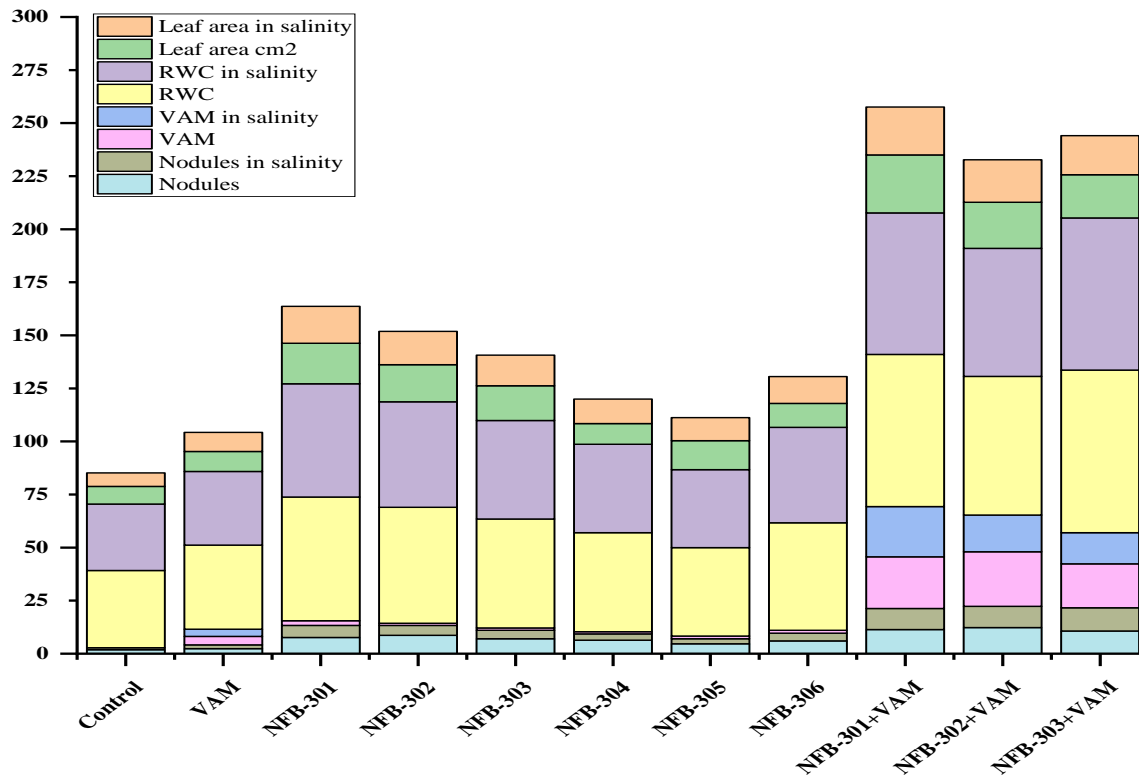


Fig. 5. Effect of rhizobia and VAM on the leaf area, relative water content, post-harvested VAM spores and nodules number in salinity stress and unstressed. The stacked bar showed differences in performance among the rhizobia strains which are statistically analyzed at alpha level 0.05.

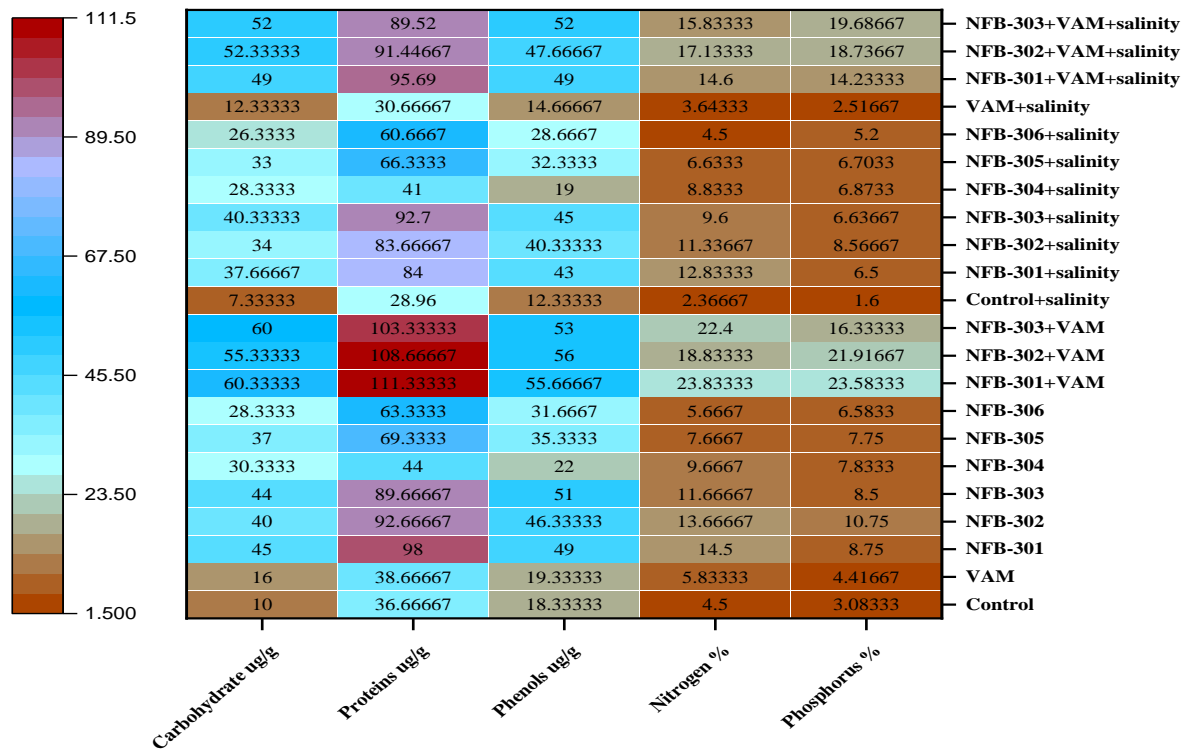


Fig. 6. Effect of rhizobia and VAM on leaf protein content, phenols content, nitrogen%, and phosphorus % under 100 Mm NaCl stress.

DISCUSSION

In vitro investigations of rhizobial strains (NFB-301, NFB-302, NFB-303) demonstrated a high level of salt tolerance, as evidenced by their growth on salt-supplemented media at concentrations of 0 mM, 50 mM, 100 mM, 150 mM, and 200 mM NaCl. In contrast, other strains (NFB-304, NFB-305, NFB-306) showed moderate tolerance, sustaining growth at up to 200 mM NaCl. These strains are categorized as plant growth-promoting rhizobacteria (PGPR), possessing essential traits such as nitrogen fixation and phosphate solubilization. Both traits are crucial for enhancing plant growth by increasing the availability and uptake of nitrogen and phosphorus, respectively (Wang *et al.*, 2020).

Rhizobia play a vital role in mobilizing insoluble soil phosphorus, thereby improving its availability to plants either directly or indirectly (Ngosong *et al.*, 2022). Phosphate solubilization is closely associated with the production of indole-3-acetic acid (IAA), a phytohormone that enhances root development and nutrient uptake (Waday *et al.*, 2022). Additionally, rhizosphere-associated and nodule-inhabiting bacteria often produce hydrogen cyanide (HCN), a secondary metabolite known to promote plant growth by suppressing root pathogens (Saeed *et al.*, 2021). Bacterial strains adapted to saline soils have also been reported to produce HCN under saline conditions (Shahid *et al.*, 2022). All rhizobial strains evaluated in this study were found to produce both ammonia and HCN under in vitro conditions, though the concentrations varied among strains (Devi *et al.*, 2023). These functional traits underscore the potential of these rhizobia as bio-inoculants for improving plant resilience and productivity in saline environments.

In this study, plants subjected to salinity stress exhibited a significant reduction in both root and shoot growth. Previous research has shown that salt stress disrupts metabolic activities and hinders plant development due to osmotic imbalances and ion toxicity (Ait-El-Mokhtar *et al.*, 2020). We evaluated six rhizobial isolates, among which three—NFB-301, NFB-302, and NFB-303—when combined with vesicular-arbuscular mycorrhizal (VAM) fungi, demonstrated the most promising results under saline conditions.

Our findings revealed that inoculation with rhizobia significantly enhanced plant growth parameters, even in salinity. These results are consistent with previous studies, such as that of Ashwin *et al.* (2022), who reported increases in shoot and root length, as well as in fresh and dry biomass, in plants inoculated with rhizobia. Similarly, VAM fungi have been shown to promote plant growth by enhancing the uptake of essential nutrients, particularly phosphorus, which supports improved shoot and root branching (Jager *et al.*, 2022).

Data from our screen house experiments confirmed that single and combined inoculations of rhizobia and VAM fungi improved overall plant growth and development. Compared to non-inoculated controls, soybean plants treated with these microbial associations showed increased root and shoot lengths, along with greater fresh and dry biomass. These microorganisms act as effective biofertilizers: the plant supplies carbohydrates to VAM fungi, while the fungi and bacteria enhance nutrient availability, especially phosphorus, nitrogen, and water absorption for the plant (Khaliq *et al.*, 2022).

Moreover, the co-inoculation of rhizobia and VAM fungi was particularly effective in alleviating the negative effects of salinity. This improvement is likely due to the ability of these beneficial microbes to reduce internal ethylene levels in plants under stress (Fracetto *et al.*, 2023). Plants treated with the combined inoculation showed greater plant height and fresh weight compared to those exposed to salinity stress alone. Supporting this, Begum *et al.* (2022) reported that co-inoculation helps protect plant cells from oxidative damage caused by salinity stress.

In addition to increased plant height and biomass, the number of root nodules and VAM spores also significantly increased under co-inoculation compared to the control. These findings are consistent with the observations of Revanna *et al.* (2021), who reported similar enhancements in microbial colonization and plant-microbe symbiosis. Furthermore, several studies (Sun *et al.*, 2020; Allito *et al.*, 2020; Gebremariam and Tesfay, 2021) have demonstrated that the application of rhizobia enhances nitrogen uptake in the soil, which in turn promotes greater root nodulation in plants compared to uninoculated treatments.

Alrajhei (2022) reported that co-inoculation with rhizobia and VAM fungi reduced the negative effects of salinity by enhancing root nodulation and VAM sporulation. This increase in symbiotic activity led to higher counts of both nodules and VAM spores, ultimately boosting the internal levels of nitrogen and phosphate in the plant and indirectly contributing to increased protein content.

Salinity stress negatively impacts plants by causing stomatal closure, reducing leaf relative water content (RWC), and limiting photosynthetic activity (Das and Biswas, 2022). However, Dobo (2022) demonstrated that inoculation with VAM fungi and rhizobia improved both leaf surface area and RWC. Consistent with these findings, our study showed that both single and combined applications of rhizobial isolates and VAM fungi significantly enhanced RWC and leaf area in both stressed and non-stressed plants.

These benefits can be attributed to the mechanisms employed by the microbes. Mycorrhizal fungi form extensive hyphal networks that expand the effective surface area for water absorption, enhancing moisture availability to roots (Seyahjani *et al.*, 2020). Meanwhile, rhizobia contribute to plant resilience by producing secondary metabolites that help mitigate salt-induced stress (Sunita *et al.*, 2020) and by modifying the NaCl concentration in the rhizosphere to create more favorable conditions for plant growth (Dastogeer *et al.*, 2020).

Compared to the non-inoculated control, plants receiving co-inoculation with rhizobia and VAM fungi exhibited significantly higher total carbohydrate and protein contents under both saline and non-saline conditions. Notably, under salinity stress, the co-inoculated plants outperformed those receiving single inoculations, a finding consistent with the work of Khan *et al.* (2022), who reported that co-inoculation enhances both protein and sugar accumulation in plants.

According to Jabborova *et al.* (2022) and Khan *et al.* (2022), this synergistic effect results from the complementary functions of VAM fungi and rhizobia: VAM fungi improve sugar and oil content by enhancing phosphorus uptake, while rhizobia increase protein content through nitrogen fixation, especially under salinity stress. Additionally, Jabborova *et al.* (2021) noted that co-inoculation under saline conditions improves soil nutrient availability and enzymatic activity, which supports osmotic balance and enhances photosynthetic efficiency in plants.

In the present study, total phenolic content also increased significantly under salinity, with higher values observed in both single and co-inoculated treatments compared to the control. Phenolic compounds play critical roles as signaling and defense molecules; they are secreted by roots to facilitate and maintain rhizobial associations in the rhizosphere (Misra *et al.*, 2023). Karimi *et al.* (2020) also reported elevated phenolic content under salinity stress, particularly in plants receiving microbial inoculation.

The present study demonstrated that the coupled association of rhizobia and VAM fungi is highly beneficial for soybean growth, particularly by enhancing mineral nutrition. Sagar *et al.* (2021) emphasized that the combined application of beneficial bacteria and VAM fungi is an effective, affordable, and sustainable strategy for increasing plant resilience to salinity stress. His findings confirmed that co-inoculation improved nutrient uptake and crop productivity under both normal and stressed conditions. Similarly, Kavadia *et al.* (2021) reported that the synergistic interaction between rhizobia and VAM fungi significantly promotes plant growth and development through the mutual sharing of essential nutrients—phosphorus by VAM and nitrogen by rhizobia—establishing them as effective biofertilizers.

Further supporting this, studies by Bourles *et al.* (2020) and Turrini *et al.* (2018) concluded that the consortium of VAM and rhizobia enhances mineral uptake, plant yield, and nodulation. Dobo (2022) also documented improvements in nodulation due to this symbiotic interaction. Kamau *et al.* (2020) noted that soybean, in particular, relies heavily on VAM for phosphorus acquisition and rhizobia for nitrogen fixation. In agreement with Alinia *et al.* (2022), our study found that rhizobial inoculation under salinity stress led to increased carbohydrate and protein content, as well as higher uptake of nitrogen, phosphorus, and phenolic compounds, compared to non-inoculated plants.

Additionally, the study observed a notable reduction in photosynthetic pigments, particularly chlorophyll, at 100 mM NaCl. This decline aligns with earlier research by Qados (2011) on *Vicia faba*, which showed that salt stress inhibits chlorophyll biosynthesis and reduces the uptake of magnesium, a key component of chlorophyll (Mushtaq *et al.*, 2021). However, *Rhizobium* inoculation significantly improved chlorophyll content in both salt-stressed and control plants, enhancing photosynthetic efficiency. These findings are consistent with the results of Abd-Alla *et al.* (2019), who reported improved pigment production due to rhizobial treatment under salinity.

Moreover, mycorrhizal colonization under saline conditions has been shown to facilitate improved micronutrient absorption, contributing to increased chlorophyll synthesis (Giri and Mukerji, 2003). Zhu *et al.* (2010) observed that *Glomus etunicatum* enhanced photosynthetic activity, transpiration, and chlorophyll a and b content in maize under salinity. Correspondingly, the current study demonstrated a significant increase in chlorophyll a, chlorophyll b, and total chlorophyll content in soybean plants treated with the combined inoculation of rhizobia and VAM fungi under salinity stress. These results are consistent with those of Nisha *et al.* (2014).

CONCLUSION

The research effectively created a nutrient-rich, cost-effective, and eco-friendly treatment for plants that greatly improved their growth and development in salty environments. Beneficial microorganisms are used as organic fertilizers in place of dangerous chemicals in this treatment. The encouraging findings of this study show that these microbial-based treatments can be a useful substitute for expensive synthetic inorganic fertilizers. Farmers in areas affected by salinity can increase crop output while lowering input costs and limiting environmental damage by implementing this sustainable strategy.

ACKNOWLEDGEMENT:

I am greatly indebted to Prof. Dr. Ehtesham-ul-Haque (late), Ex-Chairman, Department of Botany, University of Karachi, for his constructive guidance, motivation, and consolation throughout the research.

CONTRIBUTION:

Urooj Muhammd Sharif: Investigation, validation, data curation, writing—original draft preparation.

Afshan Rahman: Conceptualization, methodology, software, writing, review, and editing, resources.

Fouzia: Formal analysis, visualization.

REFERENCES

- Abd-Alla, M. H., N. A. Nafady, S. R. Bashandy and A. A. Hassan (2019). Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. *Rhizosphere*, 10: 100148.
- Ait-El-Mokhtar, M., M. Baslam, R. Ben-Laouane, M. Anli, A. Boutasknit, T. Mitsui and A. Meddich (2020). Alleviation of detrimental effects of salt stress on date palm (*Phoenix dactylifera* L.) by the application of arbuscular mycorrhizal fungi and/or compost. *Frontiers in Sustainable Food Systems*, 4: 131.
- Alinia, M., S. A. Kazemeini, M. Sepehri and A. Dadkhodaie (2022). Simultaneous application of rhizobium strain and melatonin improves the photosynthetic capacity and induces antioxidant defense system in common bean (*Phaseolus vulgaris* L.) under salinity stress. *Journal of Plant Growth Regulation*, 41(3): 1367-1381.
- Allito, B. B., N. Ewusi-Mensah and V. Logah, (2020). Legume-rhizobium strain specificity enhances nutrition and nitrogen fixation in faba bean (*Vicia faba* L.). *Agronomy*, 10(6): 826.
- Alrajhei, K. N. (2022). Influence of arbuscular mycorrhizal fungi (AMF) isolated from arid zones on plant growth, protein concentrations and response to salinity conditions in alfalfa (*Medicago sativa*) (doctoral dissertation).
- Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant physiology*, 24(1): 1.
- Asad, S. A., M. A. Wahid, S. Farina, R. Ali and F. Muhammad (2020). Soybean production in Pakistan: experiences, challenges and prospects. *International Journal of Agriculture and Biology*, 24(4): 995-1005.
- Ashwin, R., D. J. Bagyaraj and B. M. Raju (2022). Dual inoculation with rhizobia and arbuscular mycorrhizal fungus improves water stress tolerance and productivity in soybean. *Plant Stress*, 4: 100084.
- Begum, N., L. Wang, H. Ahmad, K. Akhtar, R. Roy, M. I. Khan and T. Zhao (2022). Co-inoculation of arbuscular mycorrhizal fungi and the plant growth-promoting rhizobacteria improve growth and photosynthesis in tobacco under drought stress by up-regulating antioxidant and mineral nutrition metabolism. *Microbial ecology*, 1-18.
- Bokhari, S. S., H. Farhat, S. A. Ali, F. Urooj, A. Rahman, J. Ara and S. Ehteshamul Haque (2023). Role of mycorrhizospheric fluorescent *Pseudomonas* in suppressing the root rot disease, enhancement of vesicular arbuscular mycorrhizal (VAM) population and phosphorus uptake in sunflower. *Pak. J. Bot.*, 55(2): 779-790.
- Bourles, A., L. Guentas, C. Charvis, S. Gensous, C. Majorel, T. Crossay and H. Amir (2020). Co-inoculation with a bacterium and arbuscular mycorrhizal fungi improves root colonization, plant mineral nutrition, and plant growth of a Cyperaceae plant in an ultramafic soil. *Mycorrhiza*, 30(1): 121-131.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2): 248-254.
- Cappuccino, J. G., and N. Sherman (2005). *Microbiology: a Laboratory Manual* (p. 507). San Francisco: Pearson/Benjamin Cummings.
- Chakraborty, U., S. Roy, A. P. Chakraborty, P. Dey and B. Chakraborty (2011). Plant growth promotion and amelioration of salinity stress in crop plants by a salt-tolerant bacterium. *Recent Research in Science and Technology*, 3(11): 61-70.
- Chandini, S. K., P. Ganesan and N. Bhaskar (2008). In vitro antioxidant activities of three selected brown seaweeds of India. *Food chemistry*, 107(2): 707-713.
- Dabré, É. E., J. Brodeur, M. Hijri and C. Favret (2022). The effects of an arbuscular mycorrhizal fungus and rhizobium symbioses on soybean aphid mostly fail to propagate to the third trophic level. *Microorganisms*, 10(6): 1158.
- Das, R. and B. Saikat (2022). Influence of abiotic stresses on seed production and quality. In: *Seed Biology Updates*. (Jimenez-Lopez J. C., Eds.). IntechOpen. doi 10.5772/intechopen.106045.
- Dastogeer, K. M., M. I. Zahan, M. Tahjib-Ul-Arif, M. A. Akter and S. Okazaki (2020). Plant salinity tolerance conferred by arbuscular mycorrhizal fungi and associated mechanisms: a meta-analysis. *Frontiers in plant science*, 11: 588550.

- Devi, S., S. Sharma, A. Tiwari, A. K. Bhatt, N. K. Singh, M. Singh and A. Kumar (2023). Screening for multifarious plant growth promoting and biocontrol attributes in bacillus strains isolated from indo gangetic soil for enhancing growth of rice crops. *Microorganisms*, 11(4): 1085.
- Dobo, B. (2022). Effect of arbuscular mycorrhizal fungi (AMF) and rhizobium inoculation on growth and yield of Glycine max L. varieties. *International Journal of Agronomy*, 2022(1): 9520091.
- Farhat, H., F. Urooj, H. A. Shafique, V. Sultana and S. Ehteshamul-Haque (2017). Rhizobia suppress the root knot nematode and root rotting fungi on mungbean. *Int. J. Biol. Res.*, 5(2): 71-75.
- Fracetto, G. G. M., F. J. C. Fracetto, E. R. de Souza, V. L. V. P. de Araújo, M. Lidélias, J. P. M. Júnior and C. C. G. da Silva (2023). Microbial inoculants and their potential application in salinity management. In: *Microbial Inoculants* (pp. 53-74). Academic Press.
- Gebremariam, M. and T. Tesfay (2021). Effect of P application rate and rhizobium inoculation on nodulation, growth, and yield performance of chickpea (*Cicer arietinum* L.). *International Journal of Agronomy*, 2021(1): 8845489.
- Giri, B., R. Kapoor and K. G. Mukerji (2003). Influence of arbuscular mycorrhizal fungi and salinity on growth, biomass, and mineral nutrition of *Acacia auriculiformis*. *Biology and Fertility of Soils*, 38: 170-175.
- Goergen, P. C. H., I. Lago, A. Durigon, G. F. M. Roth, L. G. Scheffel and T. Slim (2019). Performance of chia on different sowing dates: characteristics of growth rate, leaf area index, shoot dry matter partitioning and grain yield. *Journal of Agricultural Science*, 11(9): 252-252.
- Han, Q., Q. Ma, Y. Chen, B. Tian, L. Xu, Y. Bai, and X. Li (2020). Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *The ISME journal*, 14(8), 1915-1928.
- Hasanuzzaman, M. and M. Fujita (2022). Plant responses and tolerance to salt stress: physiological and molecular interventions. *International Journal of Molecular Sciences*, 23(9): 4810.
- Hasanuzzaman, M., K. Parvin, T. I. Anee, A. A. C. Masud and F. Nowroz, (2022). Salt stress responses and tolerance in soybean. *Plant Stress Physiology-Perspectives in Agriculture. IntechOpen: London*, 47-82.
- Hedge, J. E., B. T. Hofreiter and R. L. Whistler, (1962). *Carbohydrate chemistry*. 17, Academic Press, New York, pp. 371-380.
- Jabborova, D., A. Kannepalli, K. Davranov, A. Narimanov, Y. Enakiev, A. Syed and A. Gafur (2021). Co-inoculation of rhizobacteria promotes growth, yield, and nutrient contents in soybean and improves soil enzymes and nutrients under drought conditions. *Scientific Reports*, 11(1): 22081.
- Jäger, T., A. Mokos, N. I. Prasianakis and S. Leyer (2022). First_page settings order article reprints open accessarticle-pore-level multiphase simulations of realistic distillation membranes for water desalination. *Membranes*, 12(11): 1112
- Kamau, N. N., J. B. Kungu and D. Mugendi (2020). Effects of mycorrhizal and rhizobium inoculation on soybean growth in acidic soils of Gatanga, Kenya. *Cell Biology and Development*, 4(1): 1-16
- Karimi, G., L. Pourakbar, S. S. Moghaddam and J. Popović-Djordjević (2020). Integrated effects of bacteria and fungi biofertilizers on morphological traits, antioxidants indices, and polyphenol compounds of quinoa (*Chenopodium quinoa* Willd.) under salinity condition. *Res. Sq.*, (2020): 1-32.
- Kavadia, A., M. Omirou, D. A. Fasoula, F. Louka, C. Ehaliotis and I. M. Ioannides (2021). Co-inoculations with rhizobia and arbuscular mycorrhizal fungi alters mycorrhizal composition and lead to synergistic growth effects in cowpea that are fungal combination-dependent. *Applied Soil Ecology*, 167: 104013.
- Khaliq, A., S. Perveen, K. H. Alamer, M. Zia Ul Haq, Z. Rafique, I. M. Alsudays and H. Attia, (2022). Arbuscular mycorrhizal fungi symbiosis to enhance plant–soil interaction. *Sustainability*, 14(13): 7840.
- Khan, Y., S. Shah and H. Tian (2022). The roles of arbuscular mycorrhizal fungi in influencing plant nutrients, photosynthesis, and metabolites of cereal crops—A review. *Agronomy*, 12(9): 2191.
- Lai, H., F. Gao, H. Su, P. Zheng, Y. Li and H. Yao (2022). Nitrogen distribution and soil microbial community characteristics in a legume–cereal intercropping system: A review. *Agronomy*, 12(8): 1900.
- Lin, T., F. U. Haider, T. Liu, S. Li, P. Zhang, C. Zhao and X. Li (2025). Salt Tolerance Induced by Plant Growth-Promoting Rhizobacteria Is Associated with Modulations of the Photosynthetic Characteristics, Antioxidant System, and Rhizosphere Microbial Diversity in Soybean (*Glycine max* (L.) Merr.). *Agronomy*, 15(2): 341.
- Misra, D., W. Dutta, G. Jha and P. Ray (2023). Interactions and regulatory functions of phenolics in soil-plant-climate nexus. *Agronomy*, 13(2): 280.
- Mushtaq, Z., S. Faizan, B. Gulzar and K. R. Hakeem (2021). Inoculation of Rhizobium alleviates salinity stress through modulation of growth characteristics, physiological and biochemical attributes, stomatal activities and antioxidant defence in *Cicer arietinum* L. *Journal of Plant Growth Regulation*, 40: 2148-2163.
- Ngosong, C., B. N. Tatah, M. N. E. Olougou, C. Suh, R. N. Nkongho, M. A. Ngone and S. Ruppel (2022). Inoculating plant growth-promoting bacteria and arbuscular mycorrhiza fungi modulates rhizosphere acid

- phosphatase and nodulation activities and enhance the productivity of soybean (*Glycine max*). *Frontiers in Plant Science*, 13: 934339.
- Nisha Kadian, N. K., K. Yadav and A. Ashok (2014). Application of AM Fungi with Bradyrhizobium japonicum in improving growth, nutrient uptake and yield of Vigna radiata L. under saline soil. *Journal of Stress Physiology & Biochemistry*, 10(3): 134-152.
- Noreen, R., S. A. Ali, K. A. Hasan, F. U. Habiba, A. Tariq, J. Ara and S. Ehteshamul-Haque (2019). Role of fluorescent Pseudomonas associated with root nodules of mungbean in the induction of nodulation by the rhizobia in mungbean. *Pak J Bot.*, 51(3): 1161-1168.
- Patten, C. L., and B. R. Glick (2002). Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Applied and environmental microbiology*, 68(8): 3795-3801.
- Paudel, V. R., M. A. Q. Amira (2011). Effect of salt stress on plant growth and metabolism of bean plant Vicia faba (L.). *Journal of the Saudi Society of Agricultural Sciences*, 10(1): 7-15.
- Qados, A. M. A. (2011). Effect of salt stress on plant growth and metabolism of bean plant Vicia faba (L.). *Journal of the Saudi Society of Agricultural Sciences*, 10(1): 7-15.
- Rehman, A., N. Hamid, and F. Jawaid (2010). Growth and nitrogen dynamics of Glycine max inoculated with Bradyrhizobium japonicum and exposed to elevated atmospheric carbon dioxide. *Pak. J. Bot.*, 42(3): 1977-1982.
- Rubina Noreen, H., F. Urooj, H. Farhat, H. A. Shafique, A. Rahman and S. Ehteshamul-Haque (2018). Impact of endo-nodule fluorescent Pseudomonas and Rhizobia on root rotting fungi and growth of soybean (*Glycine max* L. Merr.). *Int. J. Biol. Res.*, 6(1): 27-33.
- Saeed, Q., W. Xiukang, F. U. Haider, J. Kučerik, M. Z. Mumtaz, J. Holatko and A. Mustafa (2021). Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: a comprehensive review of effects and mechanisms. *International Journal of Molecular Sciences*, 22(19): 10529.
- Sagar, A., P. Rathore, P. W. Ramteke, W. Ramakrishna, M. S. Reddy and L. Pecoraro (2021). Plant growth promoting rhizobacteria, arbuscular mycorrhizal fungi and their synergistic interactions to counteract the negative effects of saline soil on agriculture: Key macromolecules and mechanisms. *Microorganisms*, 9(7): 1491.
- Sánchez-Gómez, D., J. Majada, R. Alía, I. Feito and I. Aranda (2010). Intraspecific variation in growth and allocation patterns in seedlings of Pinus pinaster Ait. submitted to contrasting watering regimes: can water availability explain regional variation?. *Annals of Forest Science*, 67(5): 505.
- Seyahjani, E. A., M. Yarnia, F. Farahvash, M. B. K. Benam and H. A. Rahmani (2020). Influence of rhizobium, pseudomonas and mycorrhiza on some physiological traits of red beans (Phaseolus vulgaris l.) under different irrigation conditions. *Legume Research-An International Journal*, 43(1): 81-86.
- Shahid, M., M. T. Zeyad, A. Syed, U. B. Singh, A. Mohamed, A. H. Bahkali and J. Pichtel (2022). Stress-tolerant endophytic isolate Priestia aryabhatai BPR-9 modulates physio-biochemical mechanisms in wheat (Triticum aestivum L.) for enhanced salt tolerance. *International Journal of Environmental Research and Public Health*, 19(17): 10883.
- Sharif, U. M., A. Rahman, R. Noreen, and S. Mansuri (2024). Root nodule bacteria and arbuscular mycorrhizae in soybean (*Glycine Max*) promote plant growth and suppress charcoal rot fungus (*Macrophomina Phaseolina*). *FUUAST J. Biol.*, 14(2): 25 – 42.
- Spagnoletti, F. N., M. Cornero, V. Chiochio, R. S. Lavado and I. N. Roberts (2020). Arbuscular mycorrhiza protects soybean plants against Macrophomina phaseolina even under nitrogen fertilization. *European Journal of Plant Pathology*, 156: 839-849.
- Sun, Q., Y. Liu, H. Liu and R. K. Dumroese (2020). Interaction of biochar type and rhizobia inoculation increases the growth and biological nitrogen fixation of Robinia pseudoacacia seedlings. *Forests*, 11(6): 711.
- Sunita, K., I. Mishra, J. Mishra, J. Prakash and N. K. Arora (2020). Secondary metabolites from halotolerant plant growth promoting rhizobacteria for ameliorating salinity stress in plants. *Frontiers in microbiology*, 11: 567768.
- Turrini, A., L. Avio, M. Giovannetti and M. Agnolucci (2018). Functional complementarity of arbuscular mycorrhizal fungi and associated microbiota: the challenge of translational research. *Frontiers in Plant Science*, 9: 1407.
- Ullah, A., A. Bano and N. Khan (2021). Climate change and salinity effects on crops and chemical communication between plants and plant growth-promoting microorganisms under stress. *Frontiers in Sustainable Food Systems*, 5: 618092.
- Waday, Y. A., E. G. Aklilu, M. S. Bultum and V. R. Ancha (2022). Optimization of soluble phosphate and IAA production using response surface methodology and ANN approach. *Heliyon*, 8(12): doi.org/10.1016/j.heliyon.2022.e12224.

- Wang, J., R. Li, H. Zhang, G. Wei and Z. Li (2020). Beneficial bacteria activate nutrients and promote wheat growth under conditions of reduced fertilizer application. *BMC microbiology*, 20: 1-12.
- Zhang, Y., J. Ding, H. Wang, L. Su and C. Zhao (2020). Biochar addition alleviate the negative effects of drought and salinity stress on soybean productivity and water use efficiency. *BMC Plant Biology*, 20: 1-11.
- Zhu, X. C., F. B. Song and H. W. Xu (2010). Arbuscular mycorrhizae improves low temperature stress in maize via alterations in host water status and photosynthesis. *Plant and Soil*, 331: 129-137.

(Accepted for publication April 2025)