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INTERACTIVE EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) AND PLANT GROWTH PROMOTING BACTERIA (PGPB) ON THE ALLEVIATION OF SALT STRESS IN WHEAT

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Abstract

Present investigation was conducted to evaluate the effect of plant growth promoting rhizobacteria and Arbuscular mycorrhiza fungi, *Glomus* mixture alone and in combination to alleviate the salinity stress in wheat variety (AaS-2011). Sterilized seeds were soaked in culture of *Staphylococcus sciuri* and *Bacillus cereus* for 2 to 4 hours prior to sowing. Fungal inocula (*Glomus* mixture) was also mixed in pots with soil. Plants were grown in an incubator under controlled conditions. Both the PGPR have improved plant biomass, relative water content, leaf area, enhanced proline production and resulted in an increase of antioxidant enzymes SOD, POD and catalase activities. The inoculation with a mixture of *Glomus* species as well as its co-inoculation with bacterial strains was found to be the most effective in terms of growth, biochemical and physiological parameters of plants under salinity stress. It is inferred that *Glomus* species act in synergism with *Bacillus cereus* > *Staphylococcus sciuri* in the amelioration of adverse effects of salt stress.

Key words: Plant growth promoting rhizobacteria, Arbuscular mycorrhiza fungi, Salt stress, *Bacilluscereus, Staphylococcus.*

Introduction

Wheat plays an important role in food security by giving multiple benefits i.e., industrial use, human food as well as animal feed etc. (Ghorchiani *et al*., 2018). Wheat yield is reported to be reduced by 60% in presence of salinity (EL Sabagh *et al*., 2021). Imbalance of nutrient uptake, resulting in growth inhibition and alteration in plant physiology (Riaz *et al*., 2019). PGPR colonizes plant roots promoting plant growth also plays important role as biocontrol agent and assist in nutrient mobilization (Rincón-Molina *et al*., 2020). PGPR regulates the antioxidant machinery in plants and also induce systemic resistance in plants (Ghosh *et al*., 2018). The capability of PGPR in enhancing growth of plants by colonizing plant roots is of prime importance. Their effect is mediated by nutrient uptake, phytohormone production and induction of systemic resistance (Khoso *et al*., 2023).

PGPR strains *Pseudomonas putida, Lysinobacillus sphaericus* and *Bacillus pumilus* was demonstrated in inducing salt tolerance in maize (Asadullah *et al*., 2021). Three rhizobial strains *Xanthobacter autotrophicus BM13, Enterobacter aerogenes BM10* and *Bacillus brevis FK2* combat salinity stress. A rhizobial strain *Planococcus rifietoensis* improves the growth and yield of wheat (*Triticum Aestivum* L.) in saline soil (Egamberdieva *et al*., 2019).

Low P containing soil or P deficient soil are preferred by AM fungi which alleviate salt stress and enhance plant growth under salt stress (Frosi *et al*., 2018). The physiological and biochemical properties including photo assimilation and maintenance of osmotic potential were demonstrated following inoculation with AMF subsequently, inducing tolerance of plants against salt stress. This is mediated via increased uptake of water and nutrients (Zong *et al*., 2023). Different AMF were observed in saline environment like *Glomus versiform, G. intraradices, G. etunicatum*. At severely saline conditions AMF spores are present abundantly because these conditions inhibit the germination of spores which are inhabitant at 0-40 cm from the surface of rhizosphere. Usually, spores are found at a depth of 0-40 cm. Colonization of plant roots occurs through a network of fine thread-like structures arbuscules and vesicles effective in the uptake and transport of water and nutrients (Chandra *et al*., 2020). Single inoculation of microbes is lesser effective than that of combined inoculation e.g. *Rhizobium* + Phosphate solubilizing bacteria (PSB) and co inoculation with Arbuscular mycorrhizae fungi (AMF) (Zai *et al*., 2021). Combined treatment of PGPR and arbuscular mycorrhizal (AM) fungi induced salt tolerance and increased the efficiency of antioxidant enzymes in lettuce as compared to the single treatment of AMF fungi (Yasmeen *et al*., 2019). The P uptake from soil was increased when PSB was coinoculated with arbuscular mycorrhiza (Wahid *et al*., 2020).

The aim of the present study is to investigate the effects of PGPR isolates; *Bacillus cereus*, *Staphylococcus sciuri* and Arbuscular mycorrhizal fungi (Solarize mix.), alone and in combination on wheat variety AaS-2011 under normal and saline conditions.

Materials and Method

Isolation of bacteria: Soil samples were collected from 6 inches depth of rhizosphere soil of *Withania coagulens* commonly known as Indian rennet. The soil is from the mountainous region of D.G Khan. Samples were stored at 4ºC in a cold room till further analysis. PGPR (Plant Growth Promoting Rhizobacteria) isolation from soil rhizosphere (roots of *Withania coagulens*) has been carried out by serial dilution method and identified as B3 = *Bacillus cereus* (accession # LN714048) and B4= *Staphylococcus sciuri* (accession # HBXX06).

Fungal inoculum: Fungal inoculum consists of a granular mixture of various *Glomus* species (Solarize mix.). This inoculum was purchased from company Agrauxine (ZA Troyalach-2 Rue Louis Lumiere 29170 SAINT EVARZEC, France). The granular mixture was mixed in the soil in a ratio of 1:9.

Preparation of inocula and method of inoculation using PGPR strains and fungal inoculum: Seeds of wheat (*Triticum aestivum*) variety-AaS-2011 were collected from NARC, Islamabad. AaS-2011 originated from a cross [PRL/PASTOR//2236(V.6550/Sutlej-86)] attempted at Regional Agricultural Research Institute (RARI), Bahawalpur during 1997-1998. Bacterial fresh cultures were inoculated in LB media and allowed to grow in shaker. After 48h it was centrifuged at 10,000 rpm for 10 min. Bacterial pellet was suspended in autoclaved distilled water and the optical density was adjusted to 1 by spectrophotometer at O.D 600 nm. Seeds were surface sterilized using 95% ethanol for 3 min followed by soaking in 10% chlorox for 3 min. with constant shaking and successively washed with autoclaved water. Prior to sowing the sterilized seeds were soaked in fresh culture of PGPR inocula for 3 to 4 hours. The control seeds were soaked in autoclaved distilled water for the same period of time. Pots were filled with autoclaved soil and sand in 3:1 mixture and the control and treated seeds were sown in pots. Fungal inocula was also mixed in pots with soil in 1:9 ratio. Seven different treatments (consisting of two PSB's and fungal inoculum solely and in combination with each other) were applied with five replicates per treatment. Pots were placed for 21 days in growth chamber maintained at 27°C-29°C with RH 65%. Seven days after seed germination, the seedlings were subjected to salinity stress. The rhizosphere soil was treated with 100 mM and at 200 mM of NaCl separately. The seedlings were watered with autoclaved distilled water. Seedlings were harvested after two weeks of salt stress.

Determination of P solubilization index (SI): The ability of the isolated bacteria to solubilize phosphate was determined as p solubilization. The Solubilization Index was measured using the following formula (Pikovskaya, 1948; Edi-Premono *et al*., 1996).

$$SI = \frac{\text{(Colony diameter + Halozone diameter)}}{\text{Colony diameter}}$$

Soil moisture content: 20g of fresh weight of soil was taken which was measured by digital balance. These measured soil samples were dried to constant weight in oven at 70°C. Then dry weight of soil sample was taken. The % moisture content of soil was determined following the formula (Li *et al*., 2022).

$$SMC\ (\%) = \frac{FW\text{-}DW}{DW} \times 100$$

Relative water content of leaves (RWC): After induction of salt stress (21d) the relative water content of flag leaf was measured following the method of Weatherley (1950).

Root/Shoot length: Measuring tape was used to measure the length (cm) of freshly harvested roots and shoots.

Root and shoot biomass: Fresh weight of roots and shoots were measured. These samples were then placed in an oven for 72 h at 70°C. Dry weight of roots and shoots were taken using digital balance.

Leaf area: Leaf area was measured by taking the length and width of the leaf sample. Following formula was used to determine leaf area introduced by (McKee an, 1964).

$$LA = \text{Length x breadth x } 0.75$$

where 0.75 is constantly used for cereals.

Chlorophyll content of leaves: Chlorophyll content was determined following the method of Kirk (1968)

Total chlorophyll = (20.2 x A645) + (8.02 x A663)
A645: Absorbance at 645nm
A663: Absorbance at 663nm

Leaf protein contents: Leaves protein content was determined according to Lowry *et al*., (1951). BSA (Bovine Serum Albumen) was used to prepare standard curves. The absorbance of leaves sample and the standard BSA was measured spectrophotometrically at 650 nm.

Determination of leaf proline: For proline content of leaves estimation Bates method was used (Bates *et al*., 1973).

Antioxidant enzymes extraction: Leaves were extracted in phosphate buffer and the supernatant of the centrifuged extract was used to determine peroxidase (POD), Superoxide Dismutase (SOD) and Catalase activity (CAT) by the method of Gorin & Heidema (1976). Beauchamp & Frodovich (1971) and Chandlee *et al*., (1984) respectively.

Statistical Analyses

Analysis of variance (ANOVA) was applied followed by factorial design test to determine significance of the variation in experiments according to Steel & Torrie (1980). Statistix version 8.1 was used.

Results

Morphology of bacterial colony: Bacterial colonies were obtained and are subjected to morphological tests. The colony of B3 was *Bacillus cereus*, margin was undulate under light microscope it was Gram negative. Colony diameter was about 3mm, shows elevation and was shiny opaque with off white colour. However, B4 *Staphylococcus sciuri* strain was yellow in color, cocci, gram positive, with a colony diameter of 2mm, shiny appearance with entire margins (Table 1).

Phosphate solubilization: Solubilization of phosphorus is a key characteristic of plant growth promoting rhizobacteria. These rhizobacteria form halo-zones around the colonies (Table 1).

Table 1. Phosphorus solubilized by PGPR isolates from the rhizospheric soil and their test for catalase and oxidase activity.

Bacterial isolates	Colony diameter(cm)	Halozone diameter(cm)	Oxidase activity	Catalase activity	Solubilization index (SI)
Bacillus cereus	1	3	-	-	4
Staphylococcus sciuri	0.8	0.7	+	+	1.8

- Stands for absence; + Stands for presence

Relative water content: The results in (Fig. 1) revealed significant decrease in RWC of leaves of plants treated with NaCl. However, Inoculation with B3 alleviated the inhibitory effects of 100mM NaCl similar was with *Glomus* sp., the combined treatment i.e., PGPR B3 and B4 along with *Glomus* has shown highest RWC value under unstressed conditions. Exposure to 200mM NaCl stress significantly reduced RWC of leaves and B3 and B4 alone and in combination with *Glomus sp.* was not effective against increased salt stress. The combined treatment of both the PGPR B3 and B4 with Glomus sp. resulted in decrease in RWC at 100mM and 200mM NaCl.

Root fresh and dry weight: The results in (Fig. 2) showed that salinity stress has non-significant effects on fresh weight of roots. However, plants inoculated with PGPR (B3>B4) condition showed significant increases (150% and 65% respectively) in root fresh weight as compared to unstressed control (C 0). No significant difference can be seen in treatments under salinity stress in control and other Rhizobacterial and *Glomus* treated plants. But B4 in combination augmented the stimulatory effects of Glomus sp. mixture under unstressed as well as at 100mM NaCl stress.

The results demonstrated in (Fig. 3) revealed that plants inoculated with PGPR (B3 0) and (B4 0) has shown significant increases of 54% and 44% in root dry weight respectively as compared to unstressed control (C0). Plants treated with B3 and B4 inhibited the inhibitory effects of 100mM NaCl stress and B4 was effective at 200mM NaCl also. has shown amelioration of salinity stress as compared to 100mM NaCl treatment but B3 and B4 inhibited the stimulatory effects of *Glomus* sp., under unstressed condition and more so under NaCl stress.

Chlorophyll content: The results in (Fig. 4) showed that NaCl decreased the chlorophyll content equally at the concentration of NaCl. B3 = *Glomus* sp., significantly enhanced the chlorophyll content and sustained at 100mM NaCl. *Glomus* species alone has also ameliorated the effect of salinity stress at 100mM as compared to control.

Protein content of leaves: The results in (Fig. 5) revealed that the concentration dependent decrease on protein content of leaves was observed with the increase in NaCl concentration. Under unstressed condition, B3= B4 has shown maximum increase in protein content as compared to control (C0). The *Glomus* mixture under unstressed condition was lesser effective compared to that of B3 and B4. B4>B3 assisted *Glomus* mixture to enhance protein content both under unstressed and NaCl stressed conditions.

Proline content of leaves: Proline content was increased significantly linearly with respect to the salt concentration applied (Fig. 6). Maximum proline content was observed at 200mM NaCl treatment as compared to control. B3 at 0mM NaCl had significantly lower proline content compared to control but on exposure to NaCl showed highly significant increase in contrast, B4 had higher proline at 0 mM NaCl and showed further increase at 200mM NaCl>100mM NaCl treatment. *Glomus* mixture under unstressed condition exhibited proline content similar to control and showed increase in response to NaCl. B3 augmented the effect of *Glomus* mixture in enhancing proline content but B4 suppressed the effect of *Glomus* mixture. The combined effect of B3+B4+glomus mixture had least proline content but on NaCl stress the proline content was enhanced.

Antioxidant enzymes in leaves

Superoxide dismutase (SOD): Superoxide dismutase was increased at 200mM NaCl>100mM NaCl. Both the rhizobacteria enhanced SOD activity significantly over control (Fig. 7). *Glomus* mixture under unstressed condition had SOD activity similar to control. Under salt stressed conditions, maximum level of SOD was observed in leaves of plants inoculated with Bacterial isolate B4 (200) i.e., 65% followed by co inoculation of *Glomus* mixture and bacterial isolates (G+B3+B4) at 200mM NaCl.

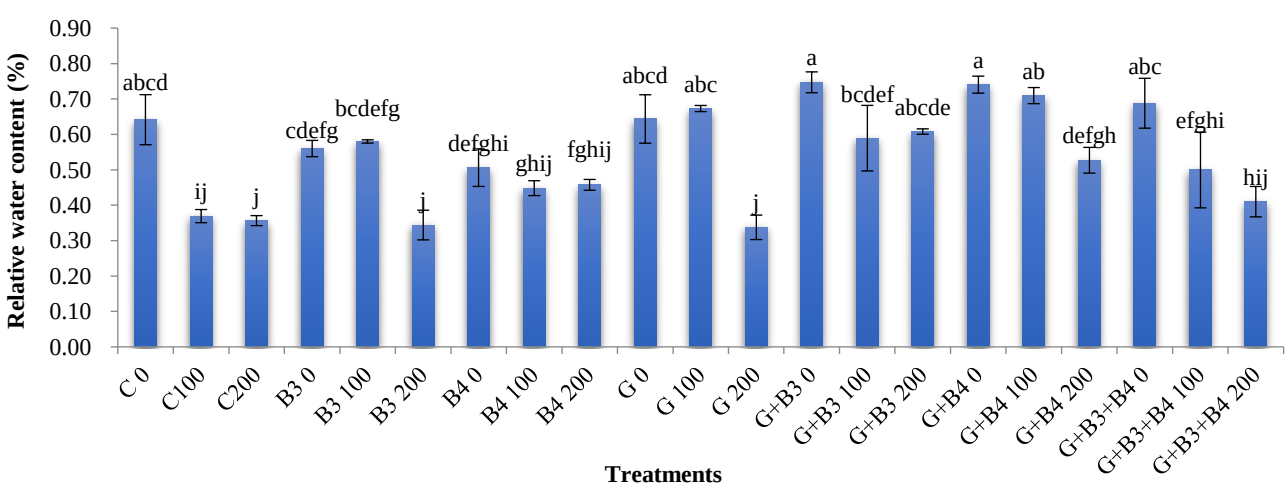

Fig. 1 Effect of PGPR alone and in combination with *Glomus* species on relative water content of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

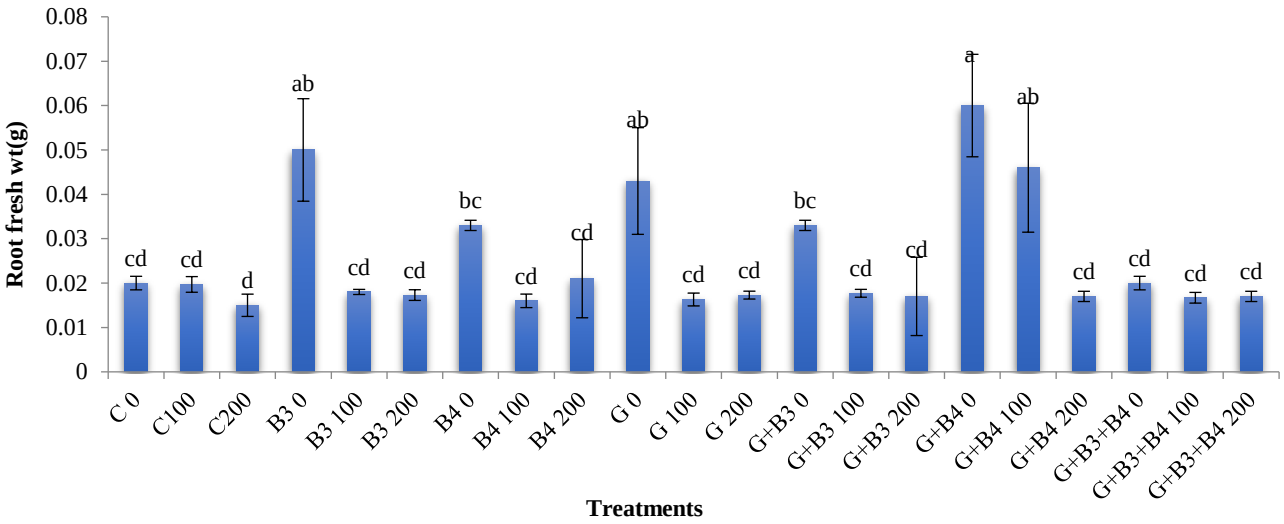

Fig. 2. Effect of PGPR alone and in combination with *Glomus* species on root fresh weight of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

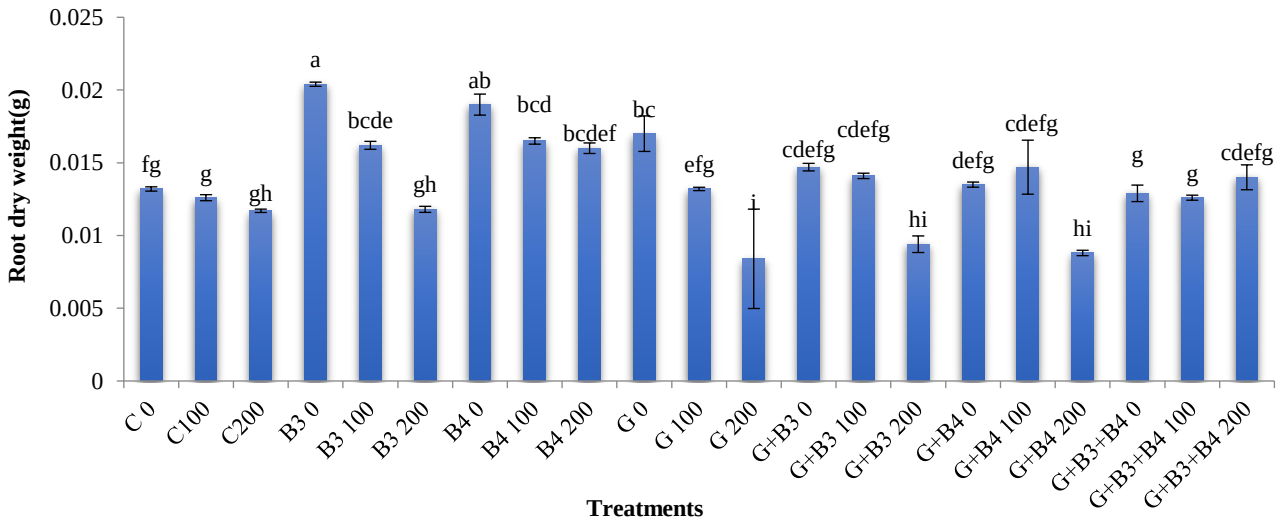

Fig. 3. Effect of PGPR alone and in combination with *Glomus* mixture on root dry weight (g) of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

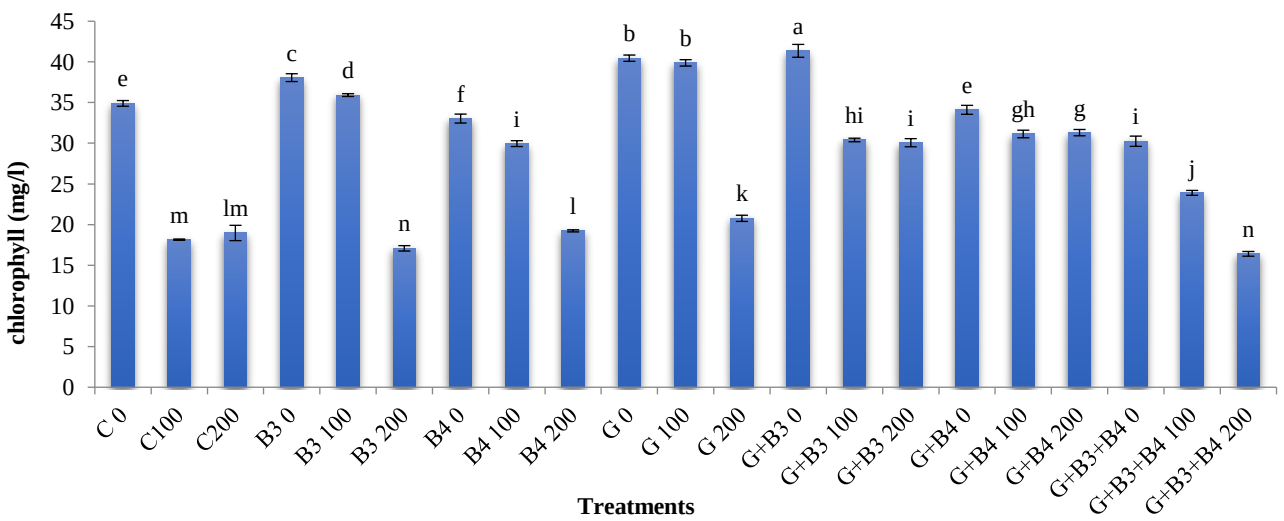

Fig. 4. Effect of PGPR alone and in combination with *Glomus* mixture on chlorophyll content in leaves of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

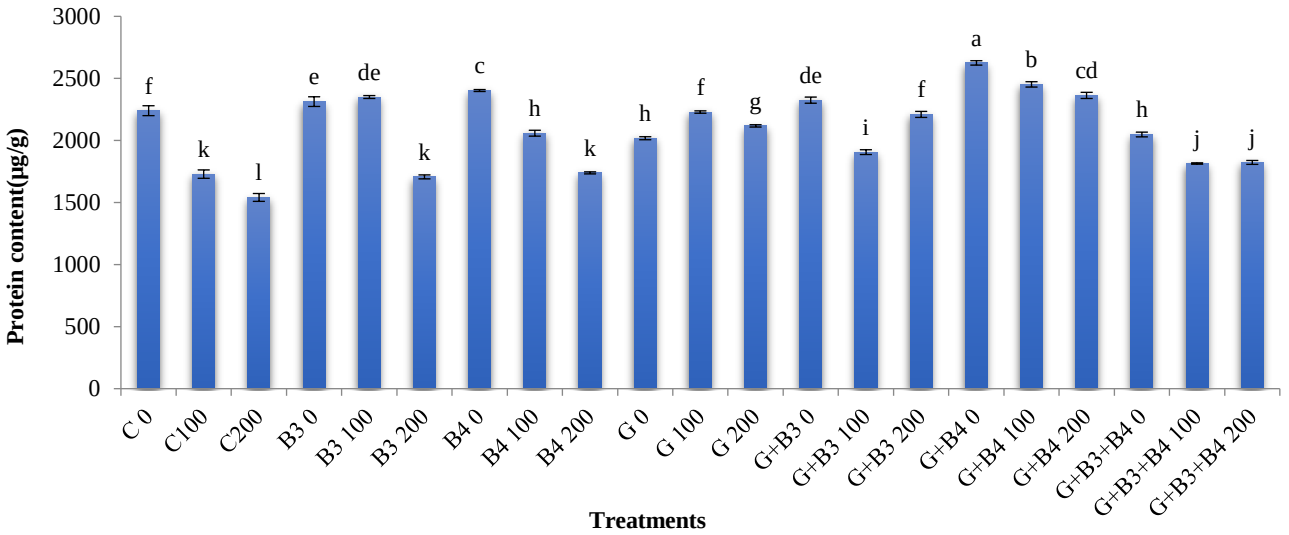

Fig. 5. Effect of PGPR alone and in combination with *Glomus* mixture on protein content in leaves of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

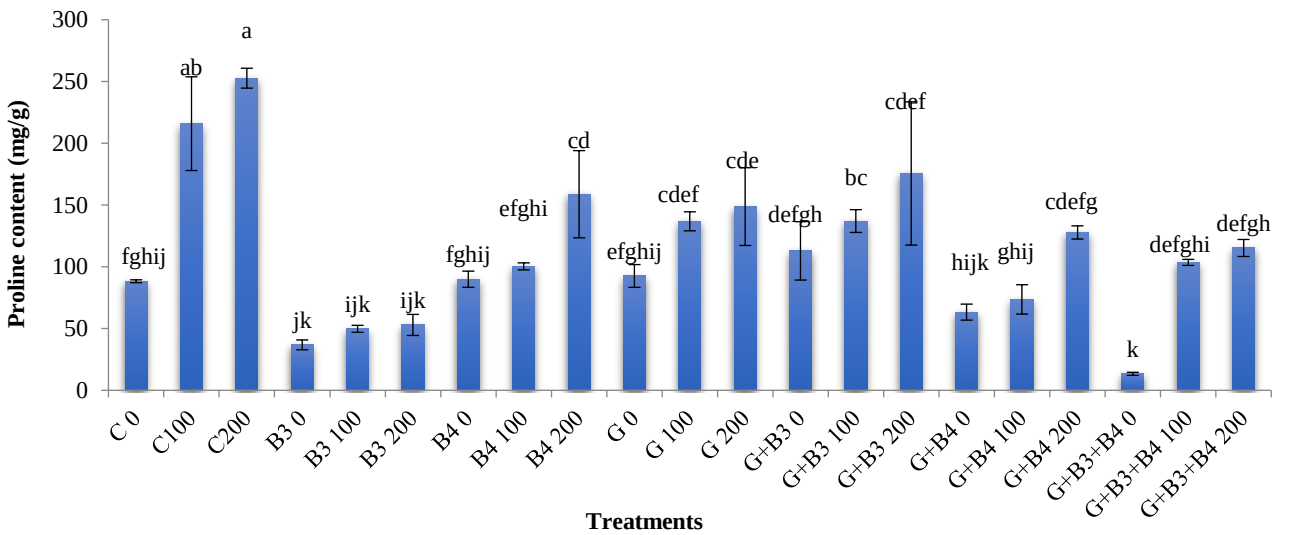

Fig. 6. Effect of PGPR alone and in combination with *Glomus* mixture on proline content in leaves of wheat plants under saline and non-saline conditions. Treatment details as in Fig. 1.

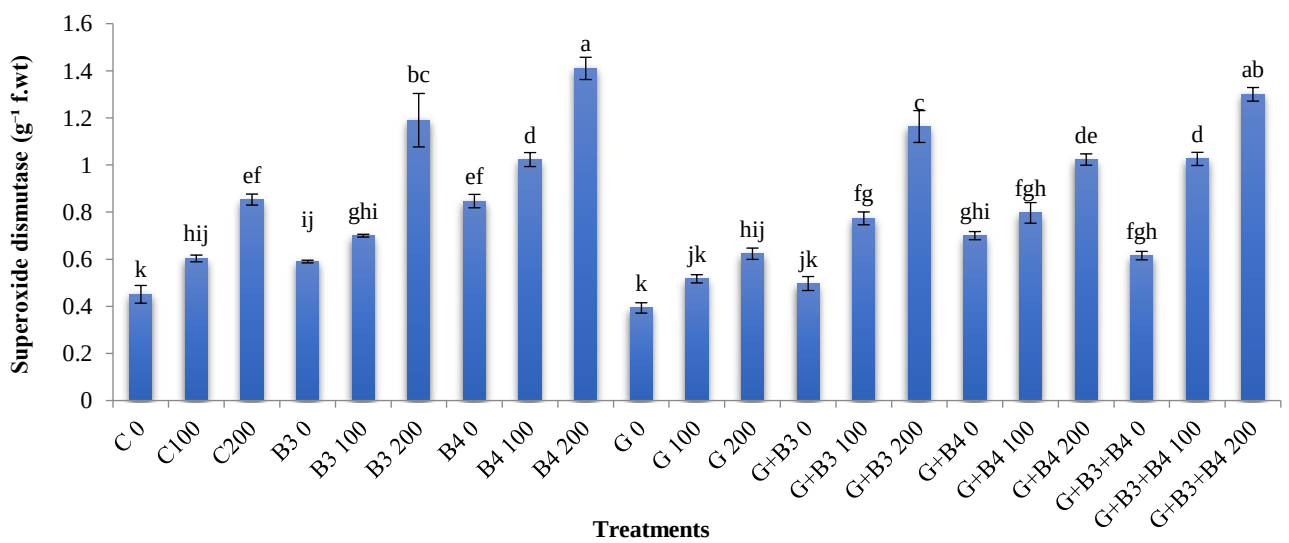

Fig. 7. Effect of PGPR alone and in combination with *Glomus* mixture on superoxide dismutase. Treatment details as in Fig. 1.

Discussion

One of the major non biotic factor that hampers annual productivity of staple crops by limiting plant growth and production of plants is exposure of plants to salt stress (EL Sabagh *et al*., 2021). According to the data provided by FAO (2005), 2% of dry lands and 20% of the world's irrigated land have been under the negative impact of salinity (Salwan *et al*., 2019). The microbiota which has proved to be beneficial to plants includes plant growth promoting rhizobacteria (PGPR), and arbuscular mycorrhizal fungi (AMF), which because of its natural occurrence in the soil and those introduced to combat salinity stress play a pivotal role in the survival of plants, to mitigate salinity stress and in promoting plant growth (Trivedi *et al*., 2020). A marked improvement has been recorded in the crop yields when inoculated with salt tolerant PGPRs (Tsegaye *et al*., 2022).

B3 *(Bacillus cereus*) and B4 (*Staphylococcus sciuri*) showed phosphorus solubilization on Pikovskaya media. Studies on cereal crops including rice, wheat and maize have confirmed that bacteria with potential to solubilize P exhibit positive significant effects on plant development (Wen *et al*., 2019).

Results revealed that *Glomus* mixture retain maximum moisture content of rhizosphere soil under unstressed but under salt stress B3 *(Bacillus cereus*) was found more effective, but their combined application was inhibitory. The PGPR suppresses the stimulatory effects of *Glomus* mixture on soil moisture contents. Contrary to the present results, Karimi *et al* (2022) found that the combined inoculation with *Glomus mosseae* and *Streptomyces rimosus* markedly improved moisture content of rhizospheric soil of grapewine plants under saline soil conditions.

An important indicator to measure water status in plants is relative water content which represents a balance between transpiration rate and water supply to the leaf tissue (Basak *et al*., 2020). The relative water content is an important determinant of water status of plants (Ichsan *et al*., 2022). *Bacillus cereus* (B3) and *Glomus* mixture (GB) maintained this trait even at high concentration of salt but in combined treatment B3 and B4 stimulated *Glomus* effect. Combination of B3 enhanced the efficiency of GB to maintain the RWC of leaves similar to control both at 100 mM and 200mM NaCl. Whereas the B4 was effective with GB only at 100 mM NaCl and induced plant growth. This may be attributed to the bio-inoculants induced increased in root absorptive area and the enhanced uptake of ions and nutrients, particularly phosphorus even under water deficit condition (Aine *et al*., 2019). The present findings corroborate the previous reports where maximum values of leaf relative water content were obtained with combined treatment of *Bacillus megaterium* and Arbuscular Mycorrhizal fungi even at higher concentrations of salt (Motaleb *et al*., 2020).

The salt induced inhibition in root and shoot length was alleviated by both the PGPR B3, B4 and *Glomus* mixture alone and in combination with B4 is noteworthy and can be attributed to growth promoting phytohormone production by these microbes. B3 sand B4 assisted *Glomus* mixture under unstressed condition and also at 100mM NaCl but the effect was lesser at 200mM NaCl. But the synergistic effect was more pronounced for shoot length in this context B4 (*Staphylococcus sciuri*) was at par to *Glomus* mixture. Pan *et al*., (2020) also reported the similar trend for root length and shoot length and noticed that *Elaeagnus angustifolia* plants were more responsive to specific inoculation by AMF than by a combination of AMF and PGPR or by solely PGPR in saline soils.

PGPR and *Glomus* alone and in combination enhanced the root fresh weight under controlled (unstressed) conditions but were unable to sustain at 100 or 200mM NaCl. This observed lack of ameliorative effect may be attributed to the proline production ability of the microbes which was lower than control and hence decrease in water uptake as measured by fresh weight may be an adaptive strategy to combat osmotic stress. There appears also a correlation between root fresh weight and proline content of these treatments. Noteworthy, association of PGPR B4 (*Staphylococcus sciuri*) with *Glomus* mixture was effective at 100mM NaCl to improve root fresh weight. Previously reported that co-inoculation of *Acacia gerradii* with AMF + *Bacillus subtilis* had higher fresh root weight and fresh shoot weight than plants inoculated with AMF or *B. subtilis* alone under salt stress (Hashem *et al*., 2016). Both the PGPR, B3 and B4 were stimulatory and improved the root dry weight significantly over control even at 100mM NaCl but B4 was found more effective even at 200mM NaCl that relates to the differences in proline content of B3 and B4. Noteworthy, the percentage increase in root dry weight ranked as B3>B4>G. Combined effect of B3 and B4 have not been significant with GB. Most studies have focused on the effects of exogenous inoculation of PGPR or AMF on host plants as little research has been done on the synergistic effects of salt-tolerant PGPR with other microorganisms in the root zone on saline soils and crops (Ji *et al*., 2022).

The salt induced decrease in growth yield was mainly attributed to the inhibition of cell division and elongation and have been reported earlier (Sadak *et al*., 2020). Former report demonstrated an ameliorative effect of bio inoculation on plant growth parameters of bean under salt stress (Bhat *et al*., 2020). Previously, the involvement of proline in tolerance mechanism against oxidative stress was reported which help the plants to cope with detrimental effects of salinity stress (Albdaiwi *et al*., 2019). Proline content significantly increased due to salt treatment. The observed lack of significant effects of microbes, B3>B4 > *Glomus* mixture on proline production may be that they maintain the osmotic balance adversely affected by salinity and thereby sustain the bioenergetics of cell for proline production. Proline production is an energy intensive process. Earlier it was demonstrated that AM symbiosis assist plants to overcome osmotic stress by ion homeostasis but there was a lack of significant increase in proline production (Augé *et al*., 2014). PGPR synthesize osmotically active metabolites like amino acids, sugars, polyols and betaines and detoxification of reactive oxygen species by antioxidants (Nawaz *et al*., 2020).

Co-inoculation with PGPR strains B3, B4 and *Glomus* mixture has increased protein contents of leaves but could not sustain at high concentration (200 Mm NaCl). Present finding of an increase in protein content of leaves following mycorrhizal inoculation was also supported by the previous experiment (Parihar *et al*., 2020). Chlorophyll content also follows the same pattern as that of protein

under salinity stress. *Glomus* mixture was effective even at 200 mM NaCl. *Glomus* mixture>B3 was found more stimulatory. The protection of photosynthetic machinery under abiotic stress can be attributed to bioinoculants (Khan & Bano, 2019).

Antioxidant enzymes play a vital role in the defense system of the plant against various oxidative stresses imposed by salinity. Alteration in the activity of antioxidant enzymes is a defense mechanism adopted by plants to deal with oxidative stress imposed by environmental stresses (Rajput *et al*., 2021). The induction of antioxidant enzyme such as peroxidase and catalase can be considered as a salt tolerance mechanism in plants (Mishra *et al*., 2023). The SOD shows its role as a first line of defense to deal with abiotic stress and is very efficient in scavenging the superoxide radicles (Ighodaro *et al*., 2018). It can be observed from the results that an increase in SOD activity occurred in salt stressed treatments. However, its level was significantly higher in PGPR inoculated salt affected plants than uninoculated salt affected plants. The bacterial association with plants results in the activation of antioxidant scavenging machinery. These antioxidants ameliorate the harmful effects of oxidative stress caused by the over production of reactive oxygen species (Desoky *et al*., 2020). The observed increase in the antioxidant enzyme SOD under salt stress was further augmented by PGPR B3 and B4 at 200mM NaCl but the *Glomus* miuxture was found less effective. Previous data for other investigations showed that the best results were found by plants inoculated with *Bacillus megaterium* followed by the treatment of Arbuscular Mycorrhizal fungi (Motaleb *et al*., 2020).

The CAT and POD behave as a second line of defense. Acclimation of these enzymes can be correlated with the production of SOD (Jincy *et al*., 2017). B4 co-inoculated with a mixture of *Glomus* species enhances peroxidase and catalase under salt stress particularly at 200mM NaCl. The bacterial association with plants results in activation of antioxidant scavenging machinery (El-Esawi *et al*., 2018).

Conclusion

Current findings revealed that *Bacillus cereus* (B3) was more effective to alleviate the salinity stress on plant physiology and growth as compared to *Bacillus cereus* (B3). *Glomus* mixture effectively retained soil moisture and RWC of leaves but under salt stress the effects of PGPR were more pronounced. PGPR particularly *Bacillus cereus* (B3) assisted *Glomus* mixture in promoting root and shoot growth, chlorophyll production and antioxidant enzymes production. The dual effects of PGPR particularly phosphate solubilizing bacteria and AMF may be more beneficial as an adaptive strategy to combat salinity.

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