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RESEARCH ARTICLE

Molecular identification and effect of salt-tolerant plant growth-promoting rhizobacteria on the biochemical aspect and growth of rice

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ABSTRACT

Soil salinization, a rising issue globally, is a negative effect of the ever-changing climate, which has drawn attention to, and exacerbated problems related to soil degradation and the decline in wetland rice (*Oryza sativa* L.) production, leading to an unstable national economy. The use of rhizosphere inhabiting microorganisms (plant growth-promoting rhizobacteria, PGPR) is a viable method for boosting agricultural production on saline soils and reduce salt stress in rice crops. The objective of this study was to support the development of rice under salt stress by using a consortium of bacterial strains. 'Pokkali' rice plants inoculated with single *Bacillus tequilensis* and *B. aryabhattai* isolates were compared with consortium and non-inoculated plants while salinity was increased and by irrigation with tap water (control), 30 mM (5 dS m^{-1}) and 60 mM (10 dS m^{-1}) NaCl. The present study exhibited that inoculation of a mixed inoculum at 5 dS m^{-1} resulted in significantly higher dry weight of the shoots and roots of seedlings (9.29 and 1.24 g, respectively) which was due to the increased SPAD value, proline content (7.55 µmol g^{-1} FW), and antioxidant enzyme activity in the inoculated plants. The higher accumulation of osmoprotectants such as proline supported Na^+ ion reduction and antioxidant enzymes such as ascorbate peroxidase and reduced polyphenol oxidase content protect against higher cellular damage, eventually leading to increase plant growth performance in saline soil. This study demonstrates some positive effects of the locally isolated salt tolerant consortium PGPR strains on the growth of rice plants under salt stress conditions.

Key words: Antioxidants, *Bacillus tequilensis*, *Bacillus aryabhattai*, *Oryza sativa*, osmoprotectants, PGPR, plant growth, rice, salinity.

INTRODUCTION

Salinity is a significant issue limiting rice production globally. It is increasing dramatically in irrigated areas due to poor infrastructure maintenance and poor water quality. In the current global agricultural system, plant growth-promoting rhizobacteria (PGPR) are emerging as novel practices and successful biological solutions to restore degraded saline soils, boosting plant growth, development, and production, also reduce the negative impacts of high salinity. When plants are in their early growth stages, photosynthesis can be enhanced by PGPR through a variety of mechanisms, including biological N fixation, phosphate dissolution, enhancement of photosynthetic activities, chelation of Fe through the formation of siderophores, and enhancement of the synthesis of plant growth regulators such as indole-3-acetic acid (IAA) (Sapre et al., 2018).

It is interesting to note that findings have demonstrated that the beneficial microbiota of plants increases crop yields while supporting the ability of plants to withstand both biotic and abiotic stresses. Numerous reports show that crops can benefit from beneficial PGPR under various stress conditions (Rafique et al., 2022).

Halotolerant (HT)-PGPR are a type of microbe that can help plants cope with abiotic stresses such as salt stress and other environmental stressors by employing a variety of metabolic and genetic strategies. Both symbiotic plant and bacterial species (HT-PGPR) can produce a wide range of minor metabolites that support growth and protect plants in salinized environments (Sharma et al., 2022). Most of these metabolites are found exclusively in abiotic stress environments and confer the plant the ability to withstand harsh weather conditions by influencing essential survival processes such as ion transport networks and osmoprotectants uptake (Egamberdieva et al., 2019). Therefore, changes in soil microbial composition in terrestrial agricultural ecosystems are important markers of soil bioactivity and crop yields (Stefan et al., 2021). However, the results achieved in axenic experimental setups could not be replicated in the ground under certain stresses. The use of a single strain of bacteria may lead to inadequate enactment of agricultural microbiota in natural settings and in the rhizosphere of host plants. To obtain various benefits, it would therefore be possible to inoculate seeds or seedlings with multiple microbial consortia rather than a single bacterial strain possessing a single feature or traits. By preparing a multi-strain bacterial consortium with compatibility in mind, the individual strains can work together to reduce inhibitory products, progress plant growth in a variety of environments, and provide more balanced nutrition (Wani et al., 2007). Due to the low percentage of root colonization and low survival, inoculation with one strain occasionally not succeed to compete with native soil microflora.

A consortium consisting of multiple strains improves plant growth compared to a single strain by using a variety of processes, each adopted by a particular microbial strain (Khan and Zaidi, 2007). In greenhouse and field experiments, Jha and Saraf (2012) found that simultaneous application of three strains resulted in maximum improvement in jatropha (*Jatropha curca*) plant development. Consortia containing three strains showed superior performance in the growth characteristics of tomato. They exhibited that the benefit of mixed biofertilizers holding bacterial consortia is a highly effective inoculant for plant growth performance. Since there is not yet much research on salt stress, the inoculation of a bacterial consortium consisting of multiple strains instead of a single inoculation could be a convenient tactic to diminish the negative effects of salt stress on plant growth, which is one of the main factors limiting plant growth in arid and semiarid regions. Along with consortium inoculation, robust bacterial populations are required for fruitful plant-microbe interactions. The bacteria must also be able to overcome additional soil elements associated with the plant rhizosphere.

Numerous researchers have found inconsistent results when injecting bacteria into different crops in research laboratory, glasshouse, and field studies. Numerous factors, including soil conditions, population of local microbes, timing, and rate of administration of the bacterial population, and other external factors, may have contributed. Since there is not much research on salt stress, applying a bacterial consortium consisting of multiple strains instead of a single inoculation could be a useful strategy to reduce the negative effects of salt stress on plant growth, which is one of the main factors limiting plant growth in many regions. Therefore, the following objectives were considered in the design of the current study, a) to identify the selected salt tolerant *Bacillus* spp. strains obtained from previous isolation work of bacteria through molecular and phylogenetic identification, and b) to evaluate the efficacy of a multi-strain bacterial consortium to improve seedling growth and biochemical aspects of rice under salt stress conditions.

MATERIALS AND METHODS

Bacterial growth and pure colony collection

The selected two locally isolated salt tolerant bacteria strains (UPMRB9 and UPMRE6) were resuscitated from glycerol storage at the Soil Microbiology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia. The two isolates were sub-cultured throughout tryptic soy agar (TSA) media to produce a single pure colony. The pure single colonies of the isolates were examined using the spreading technique. A loopful of the locally isolated salt-tolerant bacteria was streaked onto a fresh plate of TSA medium from the original strains. Large-scale bacterial subcultures were duplicated and incubated for 24 h at 32 °C in an incubator (SD-310 RL, Dasol Scientific, Hwaseong-si, Korea).

Bacterial identification by 16S rRNA gene sequence technique and phylogenetic analysis

Partial sequencing of the 16S rRNA gene was performed tracking universal protocol to identify the two selected isolates (Singh et al., 2015). Bio Sune Biotechnology Co. Ltd. (Shanghai, China) provided universal forward (5′-GAGTTGATCCTGCTCAG-3′) and reverse (5′-GTTACCTTGTTACGACTT-3′) primers for PCR amplification of a 16S rRNA gene fragment from whole genomic DNA. The PCR product was sent to Apical Scientific Sdn. Bhd. in Selangor, Malaysia, for sequencing after being purified using the Gel/PCR DNA Mini Kit (Real Biotech, New Taipei City, Taiwan). The basic local alignment search tool (BLAST) (National Centre for Biotechnology Information, (NCBI), Maryland, USA) was used to align and process the sequence data to identify the bacteria and their nearest neighbors.

Experiment setup

The experiment was operated in glasshouse, Ladang 15, Faculty of Agriculture, Universiti Putra Malaysia. The rice (*Oryza sativa* L.) cultivar used in this study was Pokkali. Seeds were collected from Malaysian Agricultural Research and Development Institute (MARDI) and stored in freezer at-20 °C to maintain freshness and viability. The trial was set up in the completely randomized design (CRD).

Soil preparation and fertilizer application

The soils were obtained from the farmer's plot in Sungai Besar, Kuala Selangor, Malaysia, and the soil series is Bernam; pH 4.23. After air drying for 2 wk, the soil was sieved using a 2 mm sieve. Then, 2 kg soil were weighed, cleaned, and placed in undrained plastic pots 15 cm high and 20 cm wide. Fertilization of the soil was done according to the amount recommended by the Department of Agriculture, Kuala Selangor, Selangor, Malaysia. Phosphate and K fertilizers were applied in the form of potassium chloride (52% K) and monobasic sodium phosphate (44% P) at a rate of 80 kg P ha^{-1} and 150 kg K ha^{-1}, respectively, as base fertilizers. During seed placement, inorganic N fertilizer in the form of urea (46% N) was applied at a rate of 170 kg N ha^{-1} for the full N treatment and 56.1 kg N ha^{-1} for the one-third N treatment.

Seed germination and surface sterilization

Surface sterilization of seeds was performed using a modified technique developed by Miche and Balandreau (2001) by immersing them in 95% ethanol for 10 s. After discarding the ethanol, seeds were shaken in 1% sodium hypochlorite (Chlorox) for 2 min and then rinsed six times with sterile distilled water. For germination of 50 surface sterilized seeds, three layers of moistened filter paper were placed on glass Petri plates. The filter paper (Whatman N°1) was frequently moistened with sterile dH_2O, and for a maximum of 4 d, the germination percentage of the seed was noted each day. The seedlings were transplanted after 5 d.

Bacterial inoculum preparation and rice seedling application

Bacterial isolates UPMRB9, UPMRE6, and mixed strains (a combination of UPMRB9, UPMRE6) were chosen based on their ability to resist salt and promote plant growth (Shultana et al., 2021). Tryptic soy broth (TSB) medium was injected into a single dish containing 24 h old bacterial cultures, and cultures were shaken for a full day (using an orbital shaker, model 722-2T, Hotech Instruments, New Taipei City, Taiwan). After soaking the germinated rice seedlings for 1 h in either distilled water (as a control) or in almost 10^8-10^9 CFU mL^{-1} of the overnight grown bacterial solution, they were allowed to rest. The single seedling was transferred to a plastic pot containing 2 kg disinfected soil. At 14 d after planting (DAP), plants in the inoculated treatments received a subsequent inoculation of 5.0 mL microbial cells (harvested by centrifugation and then washed) in their roots; in the uninoculated treatment, cells were substituted with sterile distilled water.

Salinity applications

Soil was salinized 14 DAP with sodium chloride (NaCl) at 3 and 5 g L^{-1} to achieve electrical conductivity (EC) of 30 mM (5 dS m^{-1}) and 60 mM (10 dS m^{-1}). The EC was maintained throughout the growth period by adding additional NaCl. The effect of inoculum UPMRB9, UPMRE6, and mixed strains on rice grown in saline soils was studied with four replicates and 12 combinations (12 × 4 = 48). The individual treatments were as follows: Uninoculated + control (T1); uninoculated + 30 mM salinity (T2); uninoculated + 60 mM (T3); UPMRB9 + control (T4); UPMRB9 + 30 mM (T5); UPMRB9 + 60 mM (T6); UPMRE6 + control (T7); UPMRE6 + 30 mM (T8); UPMRE6 + 60 mM (T9); mixed strains + control (T10); mixed strains + 30 mM (T11); and mixed strains + 60 mM (T12).

Measurement of rice growth parameters

The growth parameters of rice, namely chlorophyll content, plant height (cm), number of functional leaves, root length (cm), dry weight of shoot and root (g) were recorded 7, 14, 21, 28, 35 and 42 DAP. Chlorophyll content of plants was measured with a portable SPAD meter (SPAD-502 Plus, Konica Minolta, Tokyo, Japan). Plant height (cm) was measured with a normal tape measure. After 42 d, roots of the harvested plants were repeatedly cleaned under running tap water. After separation, height of root and shoot of 12 plants of each replicate were determined separately with a ruler and dried in an oven at 72 °C for up to 48 h. The dry weight of root and shoot was assessed using an electric balance (1620 C, Precisa, Dietikon, Switzerland).

Leaf sample collection and preparation for biochemical analysis

Forty-two-day-old rice leaf samples were used for biochemical analysis. Three developed leaves from every plant were randomly selected, stored in sealed plastic bags, and transported from the greenhouse to the laboratory. We used liquid nitrogen to rapidly crush the leaves. For further biochemical studies, ground leaf samples were preserved in sealed synthetic bags and kept in the freezer at-80 °C.

Proline content measurement

Proline content in leaves was determined considering the protocol stated by Bates et al. (1973) with a slight adjustment of chemical concentrations. The source for all chemicals was Merck, Germany. After homogenization of 0.1 g fresh leaves with 2 mL 5% (w/v) sulfosalicylic acid, the mixture was centrifuged at 10 000 rpm for 10 min (centrifuge 3K30, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). A test tube containing 1 mL acid ninhydrin (including the standard) and 1 mL glacial acetic acid was filled with 1 mL supernatant. The test tubes were cooled in an ice bath before being placed in a water bath (95 °C) for 1 h. Toluene (2 mL) was mixed into each tube, including the reference tube. Using the 800 TS microplate reader (BioTek, Winooski, Vermont, USA) absorbance measurement at 520 nm was used to calculate proline concentration. By using L-proline as a standard (Sigma-Aldrich, St. Louis, Missouri, USA) and plotting the value on a standard curve, the proline content was determined.

$$\text{Proline } (\mu\text{mol g}^{-1}\text{ FW}) = \frac{(\text{Proline } (\mu\text{g mL}^{-1}) \times \text{toluene (mL)}/115.5\ \mu\text{g } \mu\text{mol}^{-1})}{\text{Sample (g)}/5}$$

Determination of ascorbate peroxidase (APX)

The activity of the antioxidant enzyme ascorbate peroxidase (APX) was assessed using the previously mentioned method (Tang et al., 2007). In a pre-cooled mortar and pestle, leaf tissue was ground without centerline in a 50 mM potassium phosphate solution. The material was centrifuged for 15 min at 4 °C and 11180 rpm. The resulting supernatant was used as the enzyme source. Then, 50 mM potassium phosphate buffer (PPB), 0.5 mM ascorbic acid, 1.0 mM H_2O_2, and 0.1 mM EDTA were combined to form a 0.9 mL reaction mixture. 0.1 mL crude enzyme extract was added to start the reaction. After an exposure time of 30 s, the absorbance at 290 nm was measured every 15 to 60 s using a UV spectrophotometer (Multiskan SkyHigh Microplate Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, USA). The reference reaction mixture was one that did not contain H_2O_2 and crude enzyme. There were two approaches to determine the enzyme activity. The amount of enzyme required to oxidize 1.0 µmol ascorbate min^{-1} mg^{-1} protein is a unit of enzyme activity.

Initially, a standard curve analysis was used to calculate ascorbic acid concentrations at the initial and final points. Instead of using crude enzyme, the standard curve was constructed using the known amounts of ascorbic acid in a reaction mixture. The following formula was then used to determine the oxidized ascorbic acid min^{-1} mg^{-1} protein concentration:

Ascorbic acid concentration = Quantity of ascorbic acid oxidized ¼ Initial absorbance- Final absorbance.

The following formula can be used to determine ascorbate peroxidase (APX) activity:

$$\text{APX activity (Units L}^{-1}) = (\Delta\text{Abs} \times \text{Total assay volume})/(\Delta t \times \varepsilon \times l \times \text{Enzyme sample volume})$$

where, ΔAbs is the change in absorbance, Δt is the time of incubation (min), ε is the extinction coefficient of substrates in units of 2.8 mM^{-1} cm^{-1}, and l is the cuvette diameter (1 cm). The quantity of enzyme that oxidized one micromole of substrate per minute was defined as enzyme activity (Unit).

Polyphenol oxidase measurement

Polyphenol oxidase (PPO) was calculated according to the method described by Peixoto et al. (1999) with a slight adjustment of chemical concentrations. The fresh leaf samples were ground with liquid nitrogen. Then, 1 g pulverized leaf material was placed in a 2 mL Eppendorf tube and dissolved in 2 mL cold 50 mM phosphate buffer at pH 6.5. Centrifugation (Sigma 3K30) at 16000 g for 15 min at 4 °C removed the filtrate. The enzyme was recovered from the resulting filtrate. To prepare the reaction mixtures, 2.6 mL phosphate buffer solution pH 6.5, 0.1 mL 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA), 0.1 mL 2.1 mM ascorbic acid, and 1 mL 0.065 mM EDTA were added and mixed thoroughly. The mixture was mixed with 0.1 mL crude enzyme and allowed to stand at room temperature for 10 min. The combination without enzyme extract served as a control. The reaction mixtures used as blanks were stored at room temperature and did not contain enzyme extract. A spectrophotometer (Multiskan SkyHigh Microplate Spectrophotometer) was used to record the change in absorbance after 3 min to determine the activity of PPO at 265 nm.

$$\text{PPO activity U mL}^{-1} = \text{Abs at 3 min- Abs at 0 min} \times \text{total reaction vol/Time interval} \times 0.1$$

Total reaction volume = 3.9 mL, time interval = 3 min, crude enzyme = 0.1 mL in reaction mixture.

Statistical analysis

The R programming software (R version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/), a bioinformatics tool, was used to perform ANOVA for all rice plant development and biochemical property assessment data. The least significant difference (LSD) test was used to compare means with a probability threshold of 0.05%. The tests were performed in triplicate.

RESULTS

Partial sequencing of 16S rRNA gene sequence for molecular identification of certain PGPR isolates

The PGPR isolates UPMRB9 and UPMRE6 were chosen for molecular identification due to their salt tolerance and PGPR characteristics (Shultana et al., 2021). The 16S rRNA fragments were successfully amplified using polymerase chain reaction (PCR). Approximately 1490 bp for UPMRB9 and 1493 bp for UPMRE6 were sequenced (Figure 1).

Isolates UPMRB9 and UPMRE6 had 99% similarity to *B. tequilensis* (NCBI accession number: NR 104919.1) and *B. aryabhattai* B8W22 (NCBI accession number: NR 115953.1), respectively, as indicated by a BLAST-comparison search of the NCBI nucleotide database as seen in (Figures 2a, 2b), neighbor-joining bootstrap analysis (1000 replications) was also used to do the phylogenetic analysis of the PGPR isolates.

Figure 1. UPMRB9 and UPMRE6 DNA fragment viewed UV-transilluminator.

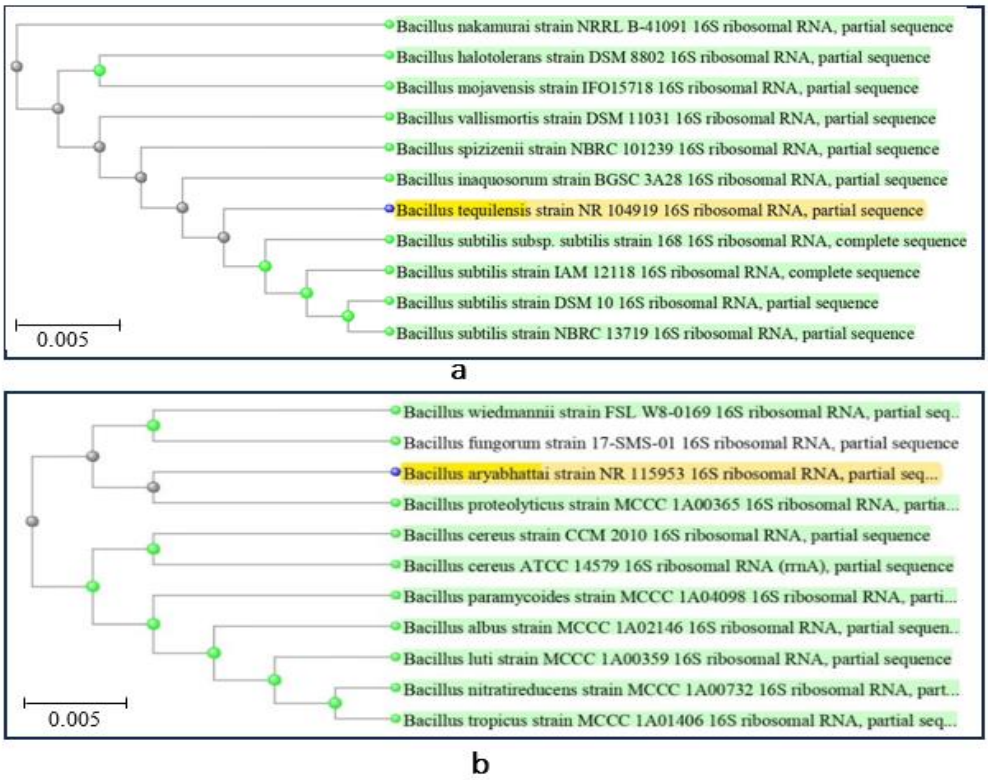

Figure 2. Bacterial isolates UPMRB9 (a) and UPMRE6 (b) are phylogenetically related to isolates as demonstrated by a neighbor-joining tree based on 16S rRNA gene sequences; 1000 replicates of bootstrap analysis were performed; nucleotide divergence value is indicated by the bar.

Effect of treatment interactions on rice plant growth characteristics

Significant interactions between PGPR strains and salt concentrations ($p < 0.001$) were observed for various plant growth parameters such as SPAD value, root length, shoot length, root weight, and shoot weight.

Effect of salinity on functional SPAD value

The effects of bacterial inoculation on SPAD value were more pronounced at the early stage (28 DAP) under both normal and stressed conditions (Figure 3).

Bacterial inoculation under non-stressed conditions resulted in the highest SPAD values (7, 14, 21, 28, 35, and 42 DAP) compared to the salt-stressed conditions. Inoculation with mixed bacterial strains or a consortium resulted in significantly higher SPAD values at 14, 28, and 42 DAP with values of 37.43, 46.03, and 50.43, respectively. Under salt-stressed conditions (5 dS m^{-1}), the bacterial consortium also produced higher SPAD values at 42 DAP (except for single inoculation). Results demonstrated that inoculations with individual strains UPMRB9 and UPMRE6 significantly increased chlorophyll (SPAD) which was greater than the control under both non-stressed and salt-stressed circumstances.

Effects of salinity on plant height and root length

Without salt application, significant differences were observed in the later growth stage, which was 42 DAP. All bacterial inoculations significantly increased plant height and root length at the later vegetative growth stage under salt stress conditions. Significant changes in shoot and root length were observed above a salinity level of 5 dS m^{-1} in response to normal and salt stress conditions.

In general, all bacterial inoculations increased rice shoot and root length production with or without salinity at 42 DAP, although some of the increases were nonsignificant (Figures 4a, 4b). Bacterial inoculation with mixed strains at 5 dS m^{-1} salinity resulted in significantly greater shoot and root length (148.7 and 31.76 cm,

respectively), whereas single inocula UPMRE6 and UPMRB9 produced comparatively shorter shoot and root lengths (146.8 and 144.5 cm; 31.13 and 28.96 cm, respectively). Rhizobia inoculated plants supplied with different salinity showed significant difference in shoot and root growth compared to the non-inoculated plants.

There were nonsignificant differences between treatments in shoot length at 42 DAP, as the rice plants had the same maximum shoot stage.

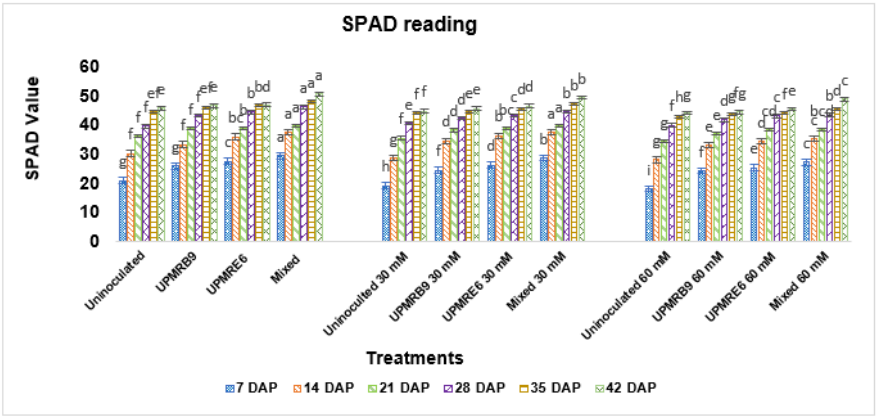

Figure 3. Effect of bacterial inoculation on total chlorophyll content (SPAD) of 'Pokkali' rice plants under non-stress and salt-stress condition measured 7, 14, 21, 28, 35 and 42 d after planting (DAP). Means with the same letter in each variety do not differ significantly using Tukey's test at $P > 0.05.0$.

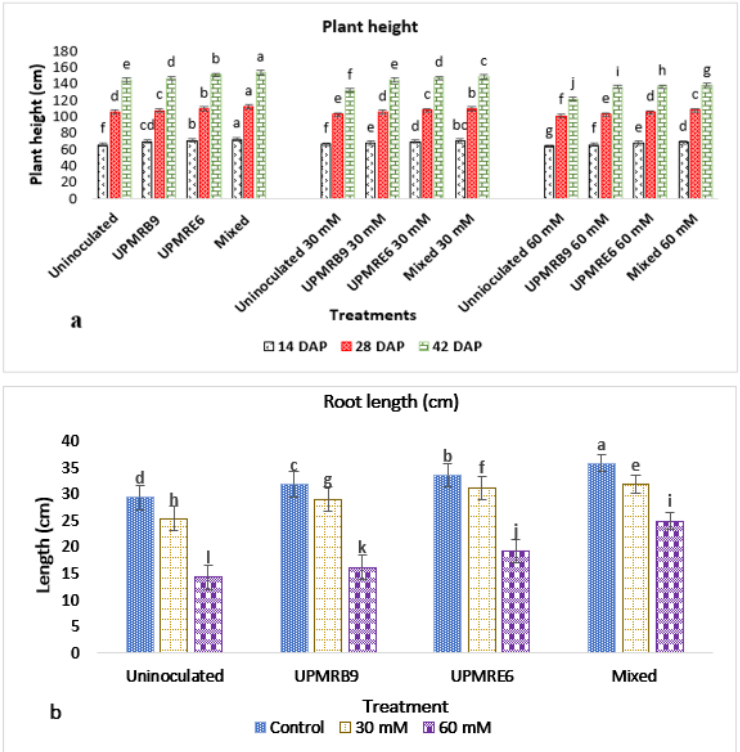

Figure 4. Effect of bacterial inoculation on plant height (a) and root length (b) (42 DAP) of 'Pokkali' rice plants under non-stress and salt-stress condition. Means with the same letter in each variety do not differ significantly using Tukey's test at $P > 0.05$.

Effects of salinity on shoot and root dry weight

The effect of the consortium strains resulted in significantly higher dry weight of the top plant and roots compared to the sole and non-inoculated treatments under normal and salt stress conditions. Single inoculation with rhizobial strain UPMRE6 and consortial inoculations with (UPMRB9+UPMRE6) stimulated shoot and root dry weight production compared to the control treatment at 42 DAP with an increment of (27.40% and 35.84%) and (23.38% and 41.12%), respectively (Figures 5a, 5b).

The reduction in dry weight of shoots when inoculated with mixed strains increased slightly at 5 dS m^{-1}, followed by the smallest reduction at 10 dS m^{-1} (72.25%) compared to single inoculations UPMRE6 and UPMRB9 (74.04% and 76.85%) compared to control treatments. A comparable tendency was monitored for root dry weight, which was 66.844% higher for inoculation with mixed inocula compared to single inocula UPMRB6 (69.64%) compared to control. Inoculations with other single bacterial strains also resulted in significantly higher shoot and root dry weights than the non-inoculated control.

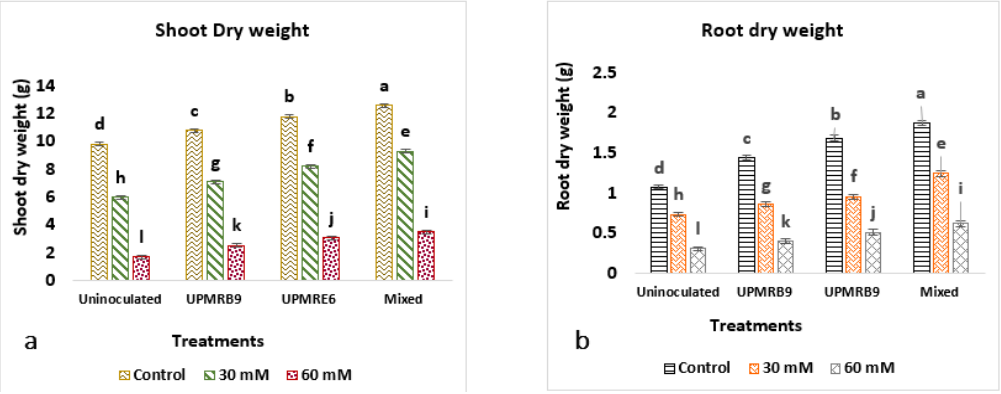

Figure 5. Effect of bacterial inoculation on plant height (a) and root length (b) (42 DAP) of 'Pokkali' rice plants under non-stress and salt-stress condition. Means with the same letter in each variety do not differ significantly using Tukey's test at $P > 0.05$.

Effects of salt-tolerant PGPR inoculation on proline content under salt stress condition

Bacterial inoculations had a significant effect on increasing osmoprotectants in plants, namely proline content, independent of bacterial isolates compared to non-inoculated plants under normal and salt stress conditions. Inoculation with consortium strains resulted in significantly higher production of proline content (7.55 µmol g^{-1} FW) compared to single strains UPMRE6 and UPMRB9 (7.05 and 5.94 µmol g^{-1} FW), respectively and uninoculated plants at 5 dS m^{-1} salinity level (Figure 6).

However, the effect of proline accumulation persisted nonsignificant for the uninoculated control and plants at 60 mM salt stress.

Effects of salt-tolerant PGPR inoculation on antioxidant enzyme production under salt stress condition

Information on the effects of PGPR application on various biochemical properties is shown in Figure 7. The PGPR application had a significant effect on the accumulation of ascorbate peroxidase (APX) and polyphenol oxidase (PPO) in the salt tolerant 'Pokkali'.

Inoculation of a mixed bacterial strain resulted in higher APX activity (1.42 units g^{-1} FW) compared to single inoculation UPMRE6 (1.25 units g^{-1} FW) and UPMRB9 (1.07 units g^{-1} FW) at 60 mM salinity level. Similar trends were observed for bacterial inoculation and non-inoculation under normal and stressed conditions (Figure 7a).

In contrast, a decrease in PPO was observed with the increase in NaCl salt concentration (0, 30 mM, and 60 mM). Under salt stress conditions, inoculation with combined inocula significantly increased the PPO activity of the salt-tolerant 'Pokkali' (Figure 7b). The highest increase was observed at a salinity of 30 mM with 3.92 mol g^{-1} compared to single strains UPMRB9 (3.90 mol g^{-1}) and UPMRE6 (3.86 mol g^{-1}) and the non-inoculated control. Nonsignificant differences were observed between treatments for PPO content at 60 mM salt stress.

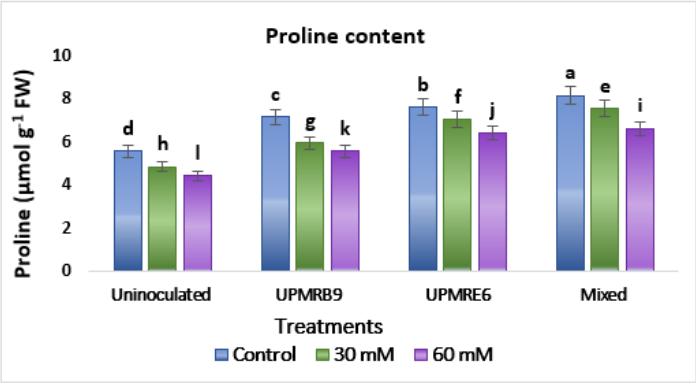

Figure 6. Effect of bacterial inoculation on accumulation of osmotically active metabolites of single and combined inocula proline content in 'Pokkali' rice plants under non-stress and salt-stress condition. Means with the same letter in each variety do not differ significantly using Tukey test at P > 0.05.

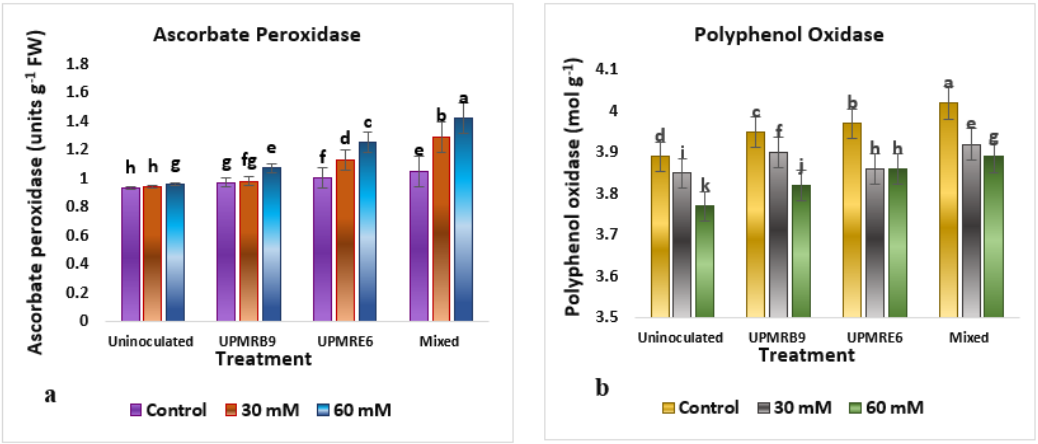

Figure 7. Effect of bacterial inoculation on enzymatic antioxidant content ascorbate peroxidase (a) and polyphenol oxidase (b) of single and combined inocula in 'Pokkali' rice plants under non-stress and salt-stress condition. Means with the same letter in each variety do not differ significantly using Tukey's test at P > 0.05.

DISCUSSION

A major environmental factor limiting agricultural production and food security is soil salinity. The sensitivity of rice plants to soil salts varies among cultivars. Because of the increased concentration of salts, especially Na^+ and Cl^-, salt stress leads to a reduction in soil water potential. The rate at which Na^+ and Cl^- ions are transported from plant cells to leaves is used to determine the physiological differences between salt-tolerant and salt-sensitive cultivars. Both ions cause a decrease in the osmotic potential of the roots, which is exacerbated by their accumulation (Ehsan et al., 2010).

In addition, salt stress leads to physiological and biochemical changes in plant cells through the regulation of various antioxidants such as PPO, APX, and glycine betaine, as well as non-toxic low molecular weight solutes such as proline. Under salt stress conditions, plants produce more proline and antioxidants, compatible solutes that help coordinate transport and metabolic processes.

Therefore, to increase the output of such challenging soils, it is essential to use innovative sustainable methods in addition to the application of organic or inorganic soil amendments together with salt-resistant plant species (Egamberdieva et al., 2019). The use of salt-tolerant PGPR has recently been shown to be a successful tactic to address the above circumstances. De novo production or uptake of osmoprotectants and specialized

ion transport systems such as NaCl/HCl antiporters are the chief procedures accountable for their existence in stressful environments (Egamberdieva et al., 2019).

The salinity problem can be addressed in several ways. Although PGPR inoculation has been the subject of numerous studies to increase plant growth, the multi-strain consortium was formed to reduce the uncertainty associated with this strategy, as individual bacterial strains provided different results. The objective of this work was to discover the potential effects of a salt-tolerant multi-strain PGPR consortium on the growth of rice seedlings by altering their morphological and biochemical processes.

The findings of the salt tolerance experiment flourished that the different strains exhibited different levels of effectiveness in maintaining their growth under saline conditions. Certain strains also proliferate in the presence of elevated salt concentrations. One or more processes of a strain may be responsible for allowing it to continue to expand in the existence of high salt content. These include the synthesis of polysaccharides such as exopolysaccharides and osmolytes. For example, to develop in a saline environment, the bacteria create and maintain an internal pressure that is higher than that of the environment.

In this study, the two isolates selected (UPMRB9 and UPMRE6) were identified as members of genera reported to be beneficial to various crops. UPMRB9 was identified as *B. tequilensis*, which is used as a salt-tolerant agent against various abiotic conditions in different crops. The 16S rRNA gene sequence of these two isolates revealed their identity as *B. tequilensis* and *B. aryabhattai*, respectively. A similar bacterial strain was also isolated and identified from saline soil by Zhao et al. (2016), who identified the isolates as *B. tequilensis* by sequencing the 16S rRNA gene. The salt-tolerant strain *B. aryabhattai* RS341 was identified by Hong et al. (2017), who reported improved vigor index and higher fresh weight of red bell pepper plants by its inoculation under salt stress. The bacterial strain *B. tequilensis* was also identified by Shultana et al. (2021) through sequencing the 16s rRNA gene isolated from salt-damaged rice plants in sodium-rich soils, which have various PGPR characteristics and can ingest and survive at different NaCl concentrations that produce phytohormones (IAAs) and exhibit salt-tolerant traits at high salt concentrations.

Leaf chlorophyll content and leaf number data underscored the importance of using salt-tolerant beneficial microbes as a growth-promoting factor for rice plants growing and interacting positively under salt stress conditions. These increased parameters indicated the beneficial interactions between these salt-tolerant inocula and plants that received higher salt concentration than the non-inoculated control plants. It is possible that these phenomena are since the inoculated bacteria can survive better under highly saline conditions due to ion exchange and ion homeostasis mechanisms. Another possible reason is the combined beneficial properties such as solubilization of phosphate and production of phytohormones (Martynenko et al., 2022), as well as various salt-tolerant properties such as exopolysaccharides, flocculation, and biofilm production that prevent Na^+ from attaching to roots, thereby inhibiting their uptake by plants. Leaf chlorophyll content in stressed plants is an index of leaf damage as it affects the photosynthetic process. The SPAD is the indirect measure of chlorophyll content in plant leaves, which is an index of salt tolerance screening. Since the value of SPAD is positively correlated with leaf area production, the highest value of SPAD was measured with a mixed strain of *Bacillus* spp. inoculated with the rice genotype 'Pokkali', the middle value was produced by UPMRE6 inocula and the lowest value by UPMRB9 bacterial strains at salinity levels of 5 and 10 dS m^{-1}. Nevertheless, the current study demonstrated that the application of a multi-strain bacterial consortium under salinity stress proved to be an efficient technique to increase growth parameters, which in turn improved plant growth rate. A similar observation was reported by Fitriatin et al. (2018), who found that a halotolerant PGPR consortium can live, survive, and engage near plant roots to create a rhizospheric microbiome to enhance rice plant growth.

Since the salt tolerance variability of different PGPR isolates was studied at salinity of 5 and 10 dS m^{-1}, the morpho-physiological and biochemical properties of all rice genotypes were discussed at these levels. At this salinity, 'Pokkali' inoculated with mixed strains exhibited the maximum plant height and root length, while plants inoculated with UPMRE6 exhibited the second greatest plant growth under both normal and stress conditions. The lowest plant height and root length were obtained when inoculated with a single UPMRB9 bacterium. This is due to the high salt tolerance of 'Pokkali' at high salinity. 'Pokkali' is a genetically long-growing plant with lower tillering ability. These results support the findings of Sen and Chandrasekhar (2014), who reported that *Pseudomonas* strains increased rice plant height and leaf area index and improved plant and soil productivity under high salinity. Similarly, Jha et al. (2011) mentioned that combined inoculation with salt-tolerant PGPR stimulated rice root and dry biomass growth through the production of IAA and phytohormones.

In *Zea mays* inoculated with different PGPR strains, plant growth parameters were found to be enhanced by the production of osmoprotectants. In a remarkable work, Ali et al. (2022) revealed that application of *Enterobacter cloacae* PM23 increased plant growth and biomass under salt stress and increased proline, glycine betaine, free amino acids, and soluble sugars. Compared to the non-inoculated control and to single inoculation (Figure 4b), promoting root growth advances the plant's capacity to captivate water and nutrients under saline conditions, resulting in stronger plant growth and more greenery.

It was suggested that the combined effects of bacterial inoculations on early plant expansion, greenery, and shoot and root development were responsible for the remarkable increase in plant dry weight. Greater shoot and root growth results in greater surface area for plant uptake of water and nutrients, which promotes overall plant growth and increases shoot and root dry weight (Figure 5). This is favorably associated with bacterial inoculation leading to an increase in rice grain yield. In this study, bacterial inoculation increased the production of DM in the plants, which is like the conclusions of Mahmood et al. (2016), who found that the highest increases in plant biomass were obtained from mung bean plants that were inoculation with *B. drentensis* and *Enterobacter cloacae*. Tomato plants injected with specific plant growth-promoting *A. chroococcum* strains (67B and 76A) exhibited higher growth and biomass accumulation under salt stress conditions, according to a similar study by Viscardi et al. (2016). Further studies discovered that injection of PGPR, which included *Kocuria erythromyxa*, *B. subtilis*, *B. atrophaeus*, *B. sphaericus*, and *Staphylococcus kloosii*, increased fresh and dry root weights and shoot weights of strawberry plants grown in high salinity environments (Karlidag et al., 2013). The PGPR multi-strain consortium significantly improved shoot length, root length, shoot fresh weight, and root fresh weight of wheat in both pot and glass experiments at maximum salinity, as reported by Khan et al. (2022).

The accumulation of proline content in plant tissue was significantly higher in the inoculated plants, as the increase in growth parameters compared to the non-inoculated control plants at a different salinity. The contributions of the individual bacterial strains and the combined inocula were associated with the increased availability of proline content. Plants generally store suitable solutes such as proline in response to stress as a type of adaptive response. However, it is still unclear whether this adaptive response has anything to do with how plants interact with microorganisms in their rhizosphere, particularly their ability to synthesize proline under both positive and stressful conditions (Nabti et al., 2015). After the studied combined bacterial strains were inoculated into the rice leaves, the proline content of the leaves increased. The proline buildup observed in the inoculated plants under salt stress was more pronounced than in the non-inoculated control plants. This suggests that inoculation of bacteria increased the proline content of plants, improving their resistance to salt stress. This is due to the synergistic interactions between rice plants and bacteria. The plants themselves have a defense mechanism triggered by microbial exopolysaccharide (EPS) production that reduces the upregulation of Na^+ in the plants, resulting in higher resistance to salinity. Similarly, Sziderics et al. (2007) found that treatment with PGPR strains EZB4 of *Arthrobacter* sp. and EZB8 of *Bacillus* sp. intensified proline concentration in *Capsicum annuum*, even in the privation of abiotic stress. These authors suggested that proline production in plants was probably stimulated by bacteria. By promoting proline synthesis, which functions as an osmo-regulator, a building block for the synthesis of stress response proteins (Metoui et al., 2020), and an important source of N and power for plant growth and tolerance to salt stress, PGPR bacteria promote plant growth.

In this study, the regulation of antioxidant enzymes in the leaf extracts of PGPR-treated rice plants was significantly higher than that in the untreated control plants. Ascorbate peroxidase (APX) is a key enzyme abundant in plants and algae. Overall, APX production improved under salt stress conditions regardless of rice cultivars. Among the two different bacterial isolates, the combined application showed a stronger response to APX production than inoculation alone under salt stress conditions. The APX is identified as an important antioxidant enzyme and mainly scavenges H_2O_2 in the cytosol and chloroplasts. Every part of the plant contains APX, which scavenges H_2O_2 through the ASC-GSH pathway (also known as the Asada-Halliwell-Foyer pathway) (Pandey et al., 2017). Since APX is more widely distributed and has a higher affinity for H_2O_2, it is a stronger H_2O_2 scavenger in stress situations. Thus, it protects intercellular molecules from the toxicity of H_2O_2.

The higher antioxidant production of rice proves that rice plants become more salt tolerant with the application of PGPR. A comparable statement was made by Ullah and Bano (2015), who found that salt-resistant PGPR from the rhizosphere of the halophyte *Haloxylon salicornicum* promoted the growth of maize plants grown under salinity (5.5 dS m^{-1}), with an extent of synthesis of osmolytes (e.g., sugars and proline) and also increased the activity of the antioxidant system (e.g., superoxide dismutase [SOD], peroxidase, catalase [CAT], and APX)

compared with the untreated plants. Moreover, this is also reliable with the discoveries of Ali et al. (2022) who stated that inoculation of *E. cloacae* PM23 contributes to the upregulation of stress-related genes (APX and SOD) in maize and helps to alleviate salt stress and improve plant growth. According to El-Esawi et al. (2018), another study using *B. firmus* SW5 increased the activity of antioxidant enzymes (APX, CAT, SOD and POD) in soybean by up to 48%. It has been reported by Chen et al. (2022) that the APX activities increased when salicylic acid and abscisic acid treatments were applied to rice plants.

The Cu-containing phenolase known as polyphenol oxidase (PPO) is present primarily in developing tissues and is found in plastids, including the leucoplasts of storage cells and the chloroplasts of photosynthetic cells. Plant secondary metabolites known as phenolic compounds can be released in various biotic and abiotic stresses. The process of photosynthesis is assisted by PPOs, which catalyze the conversion of monophenols and/or o-diphenols into favorably reactive *o*-quinones. These compounds then react with O_2 and proteins to create reactive oxygen species (ROS) and characteristic brown pigmented compounds (Boeckxy et al., 2015).

Reduced PPO content in cells increases membrane permeability and defense against higher cellular damage. The rice plant inoculated with the mixed strain could produce ROS under salt stress conditions. This suggests the production of photosynthetic pigments and protection from the harmful effects of NaCl, which can be protected by the application of rhizobacteria. In addition, salt tolerance helps the plant to selectively uptake ions. These results indicate the study conducted by many researchers that plants have an enzyme called PPO, which controls plant growth and is essential for security against biotic and abiotic stressors. According to a study by Sarkar et al. (2018), PPO activities were significantly lower in P23-inoculated seedlings than in the non-inoculated control seedlings, indicating that when rice seeds were inoculated with *Enterobacter* sp. strain P23, it increased the germination rate and seedling vigor index than under saline conditions. Tomato seedlings inoculated with *B. megaterium* A12 (BMA12) can increase PPO enzyme and other antioxidant enzymes (SOD, CAT, APX, PPO) by 10% at 2000 mM NaCl salinity evaluated to the non-inoculated and control plants (Akram et al., 2019). The present study agrees with the findings of Kang et al. (2014) that the inoculated *Cucumis sativus* plants (e.g., *Burkholdera cepacia* SE4, *Promicromonospora* sp. SE188, or *Acinetobacter calcoaceticus* SE370) exhibited lower PPS and PPO, CAT, and POD enzyme activities over the non-inoculated plants under salt stress (120 mM NaCl). The influence of consortium application (*Pseudomonas fluorescence*, *B. pumilus* and *Exiguobacterium aurantiacum*) observed by Nawaz et al. (2020) resulted in lower antioxidant activity in the treated salt-sensitive wheat genotype Galaxy-13 compared to the treated salt-tolerant genotype Aas-11.

CONCLUSIONS

Most people agree that plant growth-promoting rhizobacteria (PGPR) are safe, effective, and suitable bioinoculants for use in agricultural practices. The present study demonstrated that the multi strain salt-tolerant biofertilizer containing the locally isolated PGPR (UPMRB9 and UPMRE6) successfully promoted rice plant SPAD reading (5%-15%), root growth (20%-50%), plant dry weight (10%-19%) and substantially increased antioxidant enzymatic activity of the plant under high salinity stress. However, inoculation of mixed strain to tolerant 'Pokali' rice highly contributed to achieving its potential growth parameter components which is due to the high proline content, low osmoprotectants content, higher regulation of antioxidant ascorbate peroxidase and a reduced polyphenol oxidase content. This may be due to the synergistic effects produced by the cumulative beneficial properties of the salt-tolerant strains. The interactions between PGPR in consortia that have antagonistic or synergistic effects on defense mechanisms and plant growth are frequently observed in terrestrial soil ecosystems and need to be carefully analyzed. The combined inocula reduced salt stress and contributed to maximum utilization of biofertilizer efficiency for sustained rice plant growth.

Author contribution

Conceptualization: S.S.C., A.T.K.Z., A.M.A., T.G.H., A.H.A.G. Methodology: S.S.C., A.T.K.Z. Software: S.S.C., M.E.R., H.O.R. Validation: S.S.C., A.T.K.Z. Formal analysis: S.S.C., A.T.K.Z. Investigation: S.S.C., A.T.K.Z. Resources: S.S.C., B.M.S., A.A. Data curation: S.S.C., B.M.S., A.A. Writing-original draft: S.S.C., A.T.K.Z. Writing-review & editing: S.S.C., A.T.K.Z., H.O.R. Visualization: S.S.C., A.T.K.Z., H.O.R. Supervision: A.T.K.Z., A.M.A., T.G.H., A.H.A.G. Project administration: A.T.K.Z. Funding acquisition: A.T.K.Z., A.M.A., T.G.H. All authors have read out and consented to the available version of the manuscript.

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