

Effect of plant growth promoting rhizobacterial consortia on growth promotion of Mustard CS61 (*Brassica juncea*)

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Abstract

Plant growth-promoting rhizobacteria (PGPR) helps plants grow and develop by protecting them from abiotic and biotic stresses, increasing the synthesis of biochemicals that promote growth, and enabling the uptake of nutrients. Salinity is one of the biggest problems throughout the world. The identification of novel, salt-tolerant PGPR offers a promising strategy to mitigate the adverse effects of soil salinity. This study aimed to isolate and characterize PGPR strains from mangrove rhizosphere soils collected from **Koparkhairane (19.1045° N, 73.0033° E) to Belapur (19.0168° N, 73.0455° E), Navi Mumbai, Maharashtra, India**. A total of 1,263 bacterial isolates obtained from the rhizospheric zone of mangroves, among which 168 isolates were selected for further screening, 10% isolates showed upto 8% NaCl tolerance. Further upon the basis of purity, morphological characteristics and PGPR traits such as indole acetic acid (IAA), phosphate solubilization, ammonia production, carboxymethyl cellulase, and protease activity three isolates were selected for further study. Molecular identification by 16S rRNA sequencing revealed the PGPR potential isolates as *Micrococcus luteus* (Accession No. CP001628) and *Microbacterium barkeri* (Accession No. X77446). Plant growth promotion studies with potential PGPR consortia on mustard CS61 (*Brassica juncea* L.) under 1% saline conditions showed 100% germination, improved seedling vigor, increased growth, and biomass compared to controls.

This study represents the use of PGPR consortia in growth and augmentation of Mustard CS61 (*Brassica juncea*) under saline condition. Further studies using metagenomic approaches are needed to explore the wider uncultured microbial diversity, with the aim of discovering novel genes associated with stress tolerance and plant growth–promoting traits.

Introduction

The mangrove ecosystem is considered one of the most productive and ecologically relevant coastal habitats, as it contributes greatly to coastal biodiversity, shoreline stabilization, climate regulation through carbon sequestration, and nutrient cycling. Mangrove intertidal ecosystems continuously experience extreme environmental conditions due to high salinity, periodic tidal inundation, anoxic sediments, and fluctuating physicochemical parameters. Nonetheless, mangrove rhizospheres harbour diverse and metabolically active microbial communities that have evolved specialized physiological and biochemical adaptations, which play a primordial role in their ecology, given that they represent important reservoirs of stress-tolerant and functionally versatile plant-associated microorganisms (Donato et al., 2011; Alongi, 2014; Holguin et al., 2001).

Soil salinity and alkalinity are among the major abiotic stresses to agricultural productivity all over the world, especially in coastal and irrigated areas (Munns & Tester, 2008; Flowers & Colmer, 2015). The high accumulation of salts causes osmotic stress and ionic toxicity in plants, which ultimately reduces water uptake, disturbs nutrient balance, induces oxidative damage, and disrupts cellular metabolism (Parida & Das, 2005; Hasegawa et al., 2000). These effects are particularly pronounced during the early growth stages, such as seed germination and seedling establishment (Khan et al., 2016). Mustard (*Brassica*

juncea L.) cultivar CS-61, an important oilseed crop widely cultivated in India, is moderately sensitive to salinity stress, and exposure to elevated salt concentrations (approximately 1% NaCl, corresponding to ~ 145 mM) during early developmental stages significantly reduces germination percentage, seedling vigor, and overall growth performance (Richards, 1954; Munns & Tester, 2008). Therefore, enhancing salt stress tolerance at the initial growth stages is critical for sustaining mustard productivity under saline soil conditions.

Traditional methods of mitigating salinity stress, involving chemical modifications of soils and breeding salt-tolerant varieties of crops, generally yield poor, unreliable, and often not environment friendly results. In the recent past, utilization of beneficial soil microorganisms, especially plant growth-promoting rhizobacteria, has emerged as an ecofriendly and sustainable approach to enhance the growth of plants against abiotic stresses. PGPR enhance plant growth by both direct and indirect mechanisms, that include biological nitrogen fixation, phosphate solubilization, production of phytohormones like indole-3-acetic acid, ammonia production, and improvement of nutrient use efficiency (Vessey, 2003; Bhattacharyya and Jha, 2012).

Halotolerant-Plant Growth Promoting Rhizobacteria (HT-PGPR) assume greater significance in saline and alkaline environments due to their ability to survive and remain metabolically active under high salt concentrations. The bacteria mitigate salinity stress by maintaining the hormonal balance of the plant, reducing the levels of stress-induced ethylene by ACC deaminase activity, improving osmotic adjustment by accumulating compatible solutes, and enhancing antioxidant defense systems. Besides these, HT-PGPR produce hydrolytic enzymes-cellulase, pectinase, and amylase, which contribute towards nutrient cycling and improved rhizosphere health under stress conditions (Glick 2014; Etesami and Beattie 2018).

HT-PGPR associated with mangroves have great potential, in particular, owing to the fact that they are naturally adapted to extreme salinity and variable environmental conditions. Microorganisms isolated from mangrove rhizospheres often show superior halotolerance and multifunctional plant growth-promoting traits compared to their non-saline agricultural soil counterparts. Several reports confirm that PGPR from mangrove rhizosphere enhance seed germination, vigour, biomass accumulation, and physiological performance of crop plants under saline stress conditions. These features make mangrove PGPR important candidates for accomplishing sustainable agriculture in salt-affected soils (Dastager et al., 2010; Ramesh et al., 2014; Thatoi et al., 2013).

Single-strain PGPR inoculants, however, mostly behave inconsistently under field conditions because of environmental variability. On the other hand, PGPR consortia comprising compatible strains exhibiting complementary functional traits exhibit enhanced stability and wider stress mitigation potential. Such a consortium-based bioformulation enhances plant growth in saline–alkaline environments by mitigating nutrient limitation, hormonal imbalance, and stress tolerance simultaneously. Development of salt-tolerant PGPR consortia is one of the promising strategies for sustainable crop production in stress-prone agroecosystems (Malusá et al., 2012; Backer et al., 2018). The evaluation of PGPR, therefore, calls for a multi-disciplinary approach that encompasses culture-based screening, biochemical and enzymatic

characterization, molecular identification through 16S rRNA gene sequencing, and functional validation through plant-based assays. Germination tests and pot experiments under controlled saline conditions are very important for establishing the agronomic relevance of PGPR and their practical potential. More recently, metagenomics and functional genomics have opened even wider horizons to explore the uncultured microbial diversity and novel genes contributing to abiotic stress tolerance and plant growth promotion (Weisburg et al., 1991; Bulgarelli et al., 2013; Mendes et al., 2014). The present study covers isolation, characterization, and functional evaluation of halotolerant PGPR from the soil of mangrove rhizosphere from Navi Mumbai, Maharashtra, India. This study focuses on direct and indirect plant growth-promoting traits, biochemical and molecular characteristics, and the effect of selected PGPR and their consortia on the growth performance of salt-tolerant mustard under saline stress. This study thus constitutes one of the first comprehensive studies on the PGPR associated with mangrove from this region and provides a scientific basis for the formulation of efficient bioformulations to improve crop productivity in saline and alkaline soils.

Materials and Methods

Study area and Sample collection

This study was conducted in the mangrove ecosystems of Navi Mumbai, Maharashtra, India, covering sites from Koparkhairane (19.1045° N, 73.0033° E) to Belapur (19.0168° N, 73.0455° E), **Navi Mumbai, Maharashtra, India**. Rhizosphere soil samples were collected from eleven mangrove sites at a depth of approximately 5–10 cm below the pneumatophores of mangrove plants. The samples were collected aseptically using sterile tools, and the collected soil samples were transferred into sterile polyethylene bags. The samples were transported to the laboratory under refrigerated conditions and processed within 24 h of collection for microbial isolation.

Isolation of Rhizobacteria

One gram of rhizosphere soil was suspended in 10 mL of sterile saline (0.85% NaCl) and serially diluted up to 10^{-6} following standard microbiological procedures (Cappuccino and Sherman, 2014). Appropriate dilutions were spread-plated in 100 μ L aliquots on nutrient agar (NA) medium (Somasegaran and Hoben, 1994). Plates were incubated at $37 \pm 2^\circ\text{C}$ for 24–48 h to allow bacterial growth (Holt et al., 1994). A total of 1,263 bacterial isolates were obtained and preserved on NA slants at 4°C for further studies (Bergey's Manual Trust, 2010). Subsequently, the obtained isolates were screened for purity; pure cultures were selected by repeated streaking (Cappuccino and Sherman, 2014).

Halotolerance Screening

Halotolerance of the selected pure isolates was evaluated using both solid and liquid media following standard protocols (Somasegaran and Hoben, 1994; Cappuccino and Sherman, 2014). For solid medium

screening, pure cultures were streaked on to nutrient agar plates supplemented with 5% to 10% (w/v) NaCl and incubated at $37 \pm 2^\circ\text{C}$ for 24–48 h. Bacterial growth was recorded based on visible colony formation on the agar plates (Ventosa et al., 1998).

For liquid medium screening, isolates were inoculated into nutrient broth containing increasing NaCl concentrations ranging from 2% to 16% (w/v) and incubated at $37 \pm 2^\circ\text{C}$ for 24–48 h. Bacterial growth in broth cultures was assessed based on turbidity as an indicator of cell proliferation (Kushner, 1978). Isolates exhibiting consistent growth at higher salinity levels were considered halotolerant and selected for further plant growth–promoting and functional characterization studies (Egamberdieva et al., 2019). All experiments were performed in triplicate.

The purity of the 97 halotolerant bacterial isolates was checked by repeated spreading (Cappuccino and Sherman, 2014). The spreading plate method was used to streak each isolate on nutrient agar and incubated at room temperature for 24–48 h (Holt et al., 1994). Spreading was done two to three times to obtain morphologically uniform colonies (Cappuccino and Sherman, 2014).

The salt-tolerant bacterial isolates were screened for pathogenicity using a hemolysis assay on blood agar medium composed of nutrient agar supplemented with 5% (v/v) defibrinated human blood (Atlas, 2010). The isolates were streaked on blood agar plates and incubated at $37 \pm 2^\circ\text{C}$ for 24–48 h (Brown AE, Benson HJ, Brown AE et al., 2007). Those isolates showing either a clear zone (β -hemolysis) or partial zones (α -hemolysis) around the colonies were considered potentially pathogenic and were discarded (Cappuccino and Sherman, 2014). Non-hemolytic (γ -hemolysis) isolates were considered safe and were selected for further studies (Ventosa et al., 1998).

Characterisation of the isolates for PGP traits

Nitrogen fixation

Nitrogen-fixing ability of the bacterial isolates was checked by inoculating them on nitrogen-free Waksman No. 77 medium containing (g L^{-1}): glucose, 10.0; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl, 0.2; CaCO_3 , 5.0; agar, 15.0. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48–72 h. Appearance of visible growth on the medium was checked and considered as indicative of nitrogen fixation ability (Cappuccino and Sherman, 2014).

Phosphate Solubilization

Phosphate solubilization was checked on Pikovskaya's agar medium containing (g L^{-1}): glucose, 10.0; tricalcium phosphate, 5.0; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; KCl, 0.2; yeast extract, 0.5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; agar, 15.0 (Pikovskaya, 1948). Plates were incubated at $28 \pm 2^\circ\text{C}$ for 5–7 days. Clear halo zones around colonies indicated phosphate solubilization.

Indole-3-Acetic Acid (IAA) Production

IAA production was evaluated by cultivating bacterial isolates in LB broth enriched with 1% w/v L-tryptophan at $28 \pm 2^\circ\text{C}$ for 3–5 days under shaking conditions (Gordon and Weber, 1951). The cultures were centrifuged at 10,000 rpm for 10 min and 1 mL of the resultant supernatant was mixed with 2 mL of Salkowski's reagent (1 mL of 0.5 M FeCl_3 in 50 mL of 35% perchloric acid). The resultant pink coloration was measured at 530 nm after incubation in the dark for 30 minutes (Patten and Glick, 2002).

Gibberellic Acid (GA) Production

Gibberellin production was measured by growing bacterial isolates in nutrient broth at $28 \pm 2^\circ\text{C}$ for 72 h under shaking conditions (Borrow et al., 1955). Cultures were centrifuged at 10,000 rpm for 10 min, and the supernatant was treated with a solution of ethyl acetate. The absorbance was read at 254 nm, and the concentration of gibberellic acid was calculated based on a standard curve of GA_3 (Holbrook et al., 1961).

Ammonia Production

Production of ammonia was observed by inoculating bacterial isolates into peptone water that contained (g L^{-1}): peptone, 10.0; sodium chloride, 5.0. Cultures were incubated at $28 \pm 2^\circ\text{C}$ for 48 h followed by the addition of Nessler's reagent (alkaline potassium tetraiodomercurate) (Cappuccino and Sherman, 2014). Development of yellow to brown coloration indicated ammonia production.

Biosurfactant Production (Emulsification Index, E24)

The emulsification index was used to analyze biosurfactant production (Cooper and Goldenberg, 1987). The bacterial isolates were grown in LB broth at $30 \pm 2^\circ\text{C}$ for 48 h under shaking conditions. Cultures were centrifuged at 10,000 rpm for 10 min to obtain cell-free supernatants. Equal volumes of culture supernatant and kerosene (each 2 mL) were mixed vigorously for 2 min with subsequent standing for 24 h at room temperature. The emulsification index was calculated as:

Total height of liquid

Height of emulsified layer

The isolates that show stable emulsions after 24 h were considered positive for biosurfactant production.

Cellulose Degradation

Cellulolytic activity was tested by growing the cultures on CMC agar medium composed of (g L^{-1}): carboxymethyl cellulose, 10.0; peptone, 5.0; yeast extract, 1.0; NaCl, 5.0; agar, 15.0 (Kasana et al., 2008).

Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48–72 h and then flooded with 0.1% Congo red solution, followed by rinsing with 1 M NaCl. Clear halo zones around the colonies demonstrated the cellulase activity.

Pectin Degradation

Pectinolytic activity was evaluated on Vincent's agar medium containing (g L^{-1}): pectin, 10.0; yeast extract, 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; K_2HPO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; agar, 15.0 (Vincent, 1970). Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48–72 h and flooded with iodine solution. The formation of clear halos around colonies indicated pectinase production.

Starch Hydrolysis

Amylolytic activity was determined by inoculation of bacterial isolates on nutrient agar added with 1% (w/v) soluble starch (Cappuccino and Sherman, 2014). The nutrient agar medium contained the following (g L^{-1}): peptone, 5.0; beef extract, 3.0; sodium chloride, 5.0; agar, 15.0, starch being added separately. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h and then flooded with iodine solution (0.3% iodine or 0.6% potassium iodide). Clear zones around the colonies showed the hydrolysis of starch.

Exopolysaccharide Production

Bacterial cultures were inoculated into nutrient broth supplemented with 1% (w/v) glucose and incubated at room temperature for 48 h. Cultures were centrifuged at 8,000 rpm for 10 min at 4°C to remove cells. The supernatant was mixed with three volumes of chilled ethanol (4 mL culture + 12 mL ethanol) and incubated to allow precipitation (Sutherland, 1972). The formation of a visible precipitate was considered indicative of EPS production.

Protease Activity Assay (Skim Milk Agar)

Protease activity of bacterial isolates was screened on skim milk agar medium. The medium was prepared with the following composition (g L^{-1}): peptone, 5.0; yeast extract, 3.0; sodium chloride, 5.0; skim milk powder, 10.0; agar, 15.0. Skim milk and basal agar were separately sterilized and then mixed aseptically after cooling (Smibert and Krieg, 1994). Bacterial isolates were spot-inoculated on skim milk agar plates and incubated at $28 \pm 2^\circ\text{C}$ for 48–72 h. Protease activity was indicated by the formation of clear zones around the colonies due to casein hydrolysis.

Antifungal Activity assay by Agar Well Diffusion Method

The antifungal activity of the isolates was evaluated using the agar well diffusion method (Perez et al., 1990). Fungal inocula of *Aspergillus niger* were prepared by suspending spores or mycelial fragments in sterile distilled water ($\sim 10^6$ spores/mL) and evenly spread on Potato Dextrose Agar (PDA) plates. Wells (6–8 mm) were punched into the agar using a sterile cork borer, and 50–100 μL of the test compounds, dissolved in sterile distilled water or DMSO, were added. Fluconazole served as positive controls, while the solvent alone acted as a negative control. Plates were incubated at $25\text{--}28^\circ\text{C}$ for 48–72 hours depending on the fungal species. Zones of inhibition around the wells were measured in millimeters using a ruler or caliper to assess antifungal activity.

Antagonistic activity assay

Antagonistic assay was performed between the selected strains. A horizontal streak of one strain was made in the central part of a nutrient agar Petri dish, and a vertical streak of the other strain was drawn, forming a cross. The plates were incubated at 28 °C for 24 h (Landa et al., 1997). The intersection zone was then examined to detect whether bacterial growth inhibition was present.

Biochemical and molecular identification of selected bacterial isolates

Potential Halotolerant PGPR bacterial isolates were selected for biochemical and molecular characterisation. Total genomic DNA was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. 27F and 1492R primers were used to amplify almost entire 16S rRNA gene in a standard PCR reaction carried out on Eppendorf Gradient Master cycler system with a cycle of 94°C for 3 min; 32 cycles of 94°C for 45 sec, 51°C for 1 min and 72°C for 1.30 min and final extension at 72° C for 10 mins, and the mixture was held at 4°C. The PCR product was cleaned using Magnetic bead-based method. BigDye™ Terminator v3.1 Cycle Sequencing Kit (applied biosystems) was used for the sequencing of the PCR product using internal primers. The cycle sequencing products were cleaned using BigDye XTerminator™ Purification Kit (applied biosystems). Samples were run on Seqstudio 232002103 (Applied Biosystems). The sequencing output was analyzed using the accompanying DNA Analyzer computer software version 1.1.4 (applied biosystems). Raw data was manually curated and assembled using ChromasPro software v2.1. Assembled sequence was subjected to database search for identification on EZBioCloud server. The assembled 16S rRNA gene sequences of the selected bacterial isolates were compared with closely related reference sequences retrieved from public databases. Multiple sequence alignment was performed using the MAFFT online server with default parameters, followed by manual trimming to remove ambiguously aligned regions. Phylogenetic relationships were inferred using MEGA software by constructing a Neighbor-Joining tree based on the Tamura–Nei (TN93) nucleotide substitution model. The reliability of the tree topology was evaluated by bootstrap analysis with 1000 replicates, and bootstrap values greater than 50% are shown at the respective nodes. *Rothia dentocariosa* was used as an outgroup to root the phylogenetic tree. Further for potential isolated biochemical tests were performed according to the standard protocols, including catalase, citrate, oxidase, urease, starch hydrolysis and sugar fermentation test.

Seed Germination Assay

A seed germination assay was conducted to evaluate the effect of bacterial inoculation on salinity tolerance using mustard CS61 (*Brassica juncea*) seeds obtained from the Kharland Research Center, Panvel, India (ISTA, 2015). Uniform and healthy seeds were surface sterilized and rinsed thoroughly with sterile distilled water (Cappuccino and Sherman, 2014). The sterilized seeds were treated with one-day-

old bacterial cultures adjusted to approximately 10^8 CFU mL⁻¹ for 30–60 min (Glick et al., 2007). Control seeds were treated with sterile distilled water.

Treated and control seeds were put on sterile filter paper in Petri dishes and moistened with 0.85% (w/v) NaCl solution to impose salinity stress (Egamberdieva et al., 2019). The Petri dishes were incubated at 25–28°C for 5–7 days under controlled laboratory conditions (ISTA, 2015). Each treatment was performed in triplicate. Germination percentage was noted and the growth parameters of seedlings like root length, shoot length, and seedling vigour index were measured under salinity stress due to bacterial inoculation (Abdul-Baki and Anderson, 1973).

Plant growth promotion study

A pot experiment study was carried out to assess the role of bacterial inoculation in enhancing salinity tolerance in mustard (*Brassica juncea*) (Egamberdieva et al., 2019). Seeds and soils were obtained from the Kharland Research Center, Panvel, India. Pots were surface sterilized using sodium hypochlorite solution and filled with autoclaved soil (50–100 g per pot) (Cappuccino and Sherman, 2014). Seeds treated with bacterial inoculum (10^8 CFU mL⁻¹) were sown at a density of 30 seeds per pot, while untreated seeds served as controls (Glick, 2012).

Salinity stress was imposed by irrigating the pots with saline soil having an electrical conductivity of 4.69 dS m⁻¹, supplemented with additional NaCl to achieve a final salinity concentration of 1% (Richards, 1954; Munns and Tester, 2008; Flowers and Colmer, 2015). The pots were maintained under controlled conditions at 25–30°C with regular watering to sustain salinity stress. All the treatments were arranged in triplicate. Plant emergence percentage, plant height, root length, shoot length, and biomass were recorded after 7–14 days of growth (Abdul-Baki and Anderson, 1973). Comparisons with untreated controls were made to assess the effect of bacterial inoculation on plant growth as well as salinity tolerance (Egamberdieva et al., 2019).

Statistical analysis

The data generated during the pot experiments were subjected to analysis of variance (ANOVA) by Dunnett's test multiple range test at $p < 0.05$ in order to compare the treatments with the control (uninoculated plants). Experimental data obtained from this study were statistically analysed using Graphpad Prism software.

Results

Isolation of Rhizobacteria

A total of 1,263 isolates were obtained from rhizosphere soil samples collected from eleven mangrove sites of the Navi Mumbai region shown in Fig. 1. Thus, the density of the bacterial population in each location indicated that the sites possessed unique physicochemical and ecological characteristics. In addition, all the isolates showed variation in their colony morphology regarding size, pigmentation,

margin, elevation, and texture on nutrient agar. Pure cultures of these isolates were obtained after repeated subculturing and were preserved for further screening and characterization.

Halotolerance Screening

The rhizobacterial isolates were screened for halotolerance on both nutrient agar and nutrient broth supplemented with an increasing concentration of NaCl. Among 1,263 bacterial isolates recovered from mangrove rhizosphere soils, 168 isolates showed consistent growth at an NaCl concentration of 5% w/v, showing moderate salt tolerance.

On being exposed to higher saline conditions, 97 isolates showed growth on solid medium containing 10% (w/v) NaCl with continued turbidity in liquid culture. The growth response showed a progressive decline with further increase in the concentration of NaCl beyond 8%, and only few isolates survived at 10% salinity.

These 97 highly halotolerant isolates, which showed stable and reproducible growth under both solid and liquid culture at 10% NaCl, were selected for further biosafety evaluation and detailed assessment of plant growth–promoting attributes. The recovery of a large number of salt-tolerant bacteria indicates the high selective pressure exerted by the saline mangrove rhizosphere and points to this ecosystem as a promising reservoir of halotolerant plant growth-promoting rhizobacteria.

97 bacterial isolates showed consistent growth in 10% NaCl and were further checked for purity. Among these, 36 isolates were confirmed as pure cultures, while 61 isolates were identified as mixed cultures and were excluded from study. Only the 36 pure halotolerant isolates were retained for subsequent biosafety assessment and plant growth–promoting trait evaluation.

Hemolytic activity on blood agar was carried out for a preliminary biosafety assessment. Among the 36 salt tolerant isolates, several revealed either α - or β - hemolysis and were, therefore excluded. A total of 17 isolates exhibited γ -hemolysis (non-hemolytic behavior), and were considered as non-pathogenic. Such isolates were finally selected for further studies.

Screening of bacterial isolates for the in vitro plant growth promotion activities

A total of 17 bacterial isolates were chosen for plant growth promotion characteristic analysis (*Table 1*). Nitrogen fixation activity was detected in 82.4% isolates, while DJ-3, S-14(14), and BF-3 isolates did not indicate nitrogen fixation activity. Phosphate solubilization was observed in 82.4% isolates, as indicated by the formation of clear halo zones on the assay medium. Indole-3-acetic acid production was recorded in all the studied isolates (100%), with variation in production intensity; five isolates (DJ-4, J1, J4, J5, and BF-3) exhibited strong IAA production (+++), while all the other isolates produced IAA at a moderate (++) or low (+) level. Gibberellin production detected in 64.7% of the isolates. Ammonia production was observed in all isolates, with 47.1% showing strong activity (+++), including NRI-2(20), DJ-3, DJ-12, J1, J2, J4, VT-6, and VT-7. Further screening revealed that 100% of the isolates were able to produce

biosurfactants. Cellulase activity was present in 58.8% of isolates, pectinase activity in 35.3%, and amylase activity in 23.5% of isolates. Exopolysaccharide production was detected only in 11.8% isolates, which were BJ-2 and S-14(14). Protease activity was observed in 94.1% of the isolates, while one isolate (DJ-17) did not exhibit detectable protease activity.

Table 1. *In vitro* screening of bacterial isolates for plant growth-promoting (PGP) traits.

PGP traits	Nomenclature of Isolates																
	NRI-2 (2)	NRI-2 (12)	NRI-2 (20)	BJ-2	DJ-3	DJ-4	DJ-12	DJ-17	J1	J2	J4	J5	S-14 (14)	VT-2	VT-6	VT-7	BF-3
Nitrogen fixation	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	–
Phosphate solubilization	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–
IAA production	+	+	+	++	++	+++	+	++	+++	+	+++	+++	++	++	+	+	+++
Starch hydrolysis	–	–	–	–	–	+	+	–	–	–	–	+	–	–	+	–	–
Cellulose degradation	+	+	–	+	–	+	+	+	–	–	+	–	–	+	+	–	+
Pectin degradation	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Ammonia production	++	++	+++	++	+++	++	+++	++	+++	+++	+++	++	++	++	+++	+++	++
Biosurfactant production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Exopolysaccharide production	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–
Gibberellin production	+	+	+	+	+	+	–	+	+	–	+	–	+	–	–	+	–
Protease activity	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+

Antifungal Activity

The antifungal activities of the selected mangrove-associated bacterial isolates were assayed against *Aspergillus niger* using the agar well diffusion method. Among 17 bacterial isolates screened for antifungal activity, 16 (94.12%) isolates exhibited detectable antifungal activity, while one isolate, BF-3 (5.88%), failed to produce any zone of inhibition and was thus considered antifungal negative (-). The high frequency of antifungal-positive isolates indicated the good biocontrol potential of mangrove-derived PGPR.

The bacterial isolates that had shown consistent and strong inhibition were selected according to the antifungal activity assay against *Aspergillus niger*. BJ-2, DJ-12, and J-4 were chosen as promising biocontrol candidates because of their reproducible antifungal activity, as shown by the distinct zones of inhibition in the agar well diffusion assay. These isolates showed higher antagonistic potential than the rest of the strains and thus were selected as suitable for further functional validation and plant growth-

promoting studies. The selection of these isolates was further justified based on their halotolerance, non-pathogenicity, and multiple plant growth-promoting traits, indicating their potential applicability as a bioinoculant under saline stress.

Antagonistic activity assay

In the cross-streak assay, the selected antifungal isolates BJ-2, DJ-12, and J-4 did not show any kind of antagonistic interaction, reflecting complete mutual compatibility and suitability for the consortium formulation.

Biochemical and molecular characterization of bacterial isolates

The identification and characterization of selected rhizospheric isolates were carried out based on morphological, physiological, biochemical characteristics, and PCR amplification of the 16S rRNA gene. The bacterial strains were selected due to their pronounced salt tolerance and positive plant growth-promoting (PGP) traits. Sequence similarity analysis of the 16S rRNA gene using the EzBioCloud/NCBI database revealed that the successfully sequenced isolates showed high similarity (97–100%) with reference sequences belonging to the genera *Micrococcus* and *Microbacterium*. Isolate BJ2 exhibited 99.49% sequence similarity with *Micrococcus luteus* (Accession No. CP001628), while isolates DJ12 and J4 showed 99.88% and 99.90% similarity, respectively, with *Microbacterium barkeri* (Accession No. X77446). The corresponding 16S rRNA gene sequences of these isolates were submitted to the NCBI GenBank database. The phylogenetic analysis confirmed the molecular identification and evolutionary relationships of the studied bacterial isolates based on 16S rRNA gene sequence analysis, supporting their taxonomic assignment at the genus and species levels.

Table 2. Colony and Cell Morphological Characteristics of Selected Bacterial Isolates

Isolate	Colony Color	Colony Shape	Margin	Elevation	Opacity	Gram Reaction	Cell Shape	Spore Formation
BJ 2	White	Circular	Entire	Flat	Opaque	Gram-positive	Rod (bacilli)	Absent
DJ 12	Yellow	Circular	Entire	Raised	Opaque	Gram-negative	Rod (coccobacilli)	Absent
J 4	Yellow	Circular	Entire	Flat	Opaque	Gram-negative	Rod (bacilli)	Absent

Table 3.*Biochemical characteristics of bacterial isolates*

Isolates	Biochemical Test								
	Catalase	Citrate utilization	Oxidase	Urease	Starch hydrolysis	Glucose fermentation	Sucrose fermentation	Lactose fermentation	Mannitol fermentation
BJ-2	+	–	+	–	+	–	–	–	–
DJ-12	+	–	–	+	–	+	+	–	+
J-4	+	–	–	+	–	+	+	–	+

Seed Germination Assay

The effect of bacterial inoculation on mustard seed germination and early seedling growth under salt stress is presented in Table 4. All treatments showed 100% germination within 2 days, indicating that the applied salinity level did not inhibit seed germination.

One-way ANOVA showed a highly significant effect of bacterial treatments on root length ($F(4,10) = 28.78$, $p < 0.0001$), shoot length ($F(4,10) = 57.89$, $p < 0.0001$), seedling length, and seed vigour index under salinity stress ($p < 0.001$). Dunnett's test showed that all bacterial treatments significantly increased seedling growth parameters compared to the salt control, with BJ2 (*Micrococcus luteus*) being the most effective and the consortium treatment ($p < 0.05$ – 0.001).

Root length was significantly promoted by all bacterial treatments over the salt control. Among the individual isolates, the maximum root length was observed in BJ2 (*Micrococcus luteus*) and DJ12 (*Microbacterium barkeri*), which showed highly significant increases ($p < 0.001$). The J4 isolate also showed a significant increase over the control ($p < 0.05$). The consortium treatment showed a significant increase in root length over the salt control, but its efficacy was lower than BJ2 (*Micrococcus luteus*).

Shoot length was also significantly impacted by bacterial inoculation. The maximum shoot length was found in BJ2 (*Micrococcus luteus*) and the consortium treatment, which were significantly longer than the salt control ($p < 0.001$). J4 and DJ12 were moderately but significantly longer ($p < 0.05$).

Consistent with the trends observed in root and shoot growth, seedling length and seed vigour index were significantly higher in all bacterial treatments than the salt control. The highest seedling length and seed vigour index were observed in BJ2 (*Micrococcus luteus*) and the consortium treatment.

Overall, bacterial inoculation significantly enhanced early seedling growth of mustard under salinity stress with BJ2 (*Micrococcus luteus*) emerging as the most effective isolate and the consortium, indicating their potential use as plant growth-promoting rhizobacteria for saline soil management.

Table 4 *Effect of bacterial inoculation on seed germination and seedling growth of mustard under salinity stress.*

Treatment Description	No. of Seeds Germinated (Out of 5)	Days taken for Germination	Germination %	Avg Root Length (mm)	Avg Shoot Length (mm)	Seedling Length	Seed Vigour index
Salt Control	5	2	100	7.23 ± 2.25	35.8 ± 2.36	43.03	4303
BJ2	5	2	100	14.37 ± 2.15	53.6 ± 1.76	67.97	6797
DJ12	5	2	100	14.20 ± 2.95	44.2 ± 1.76	58.40	5840
J4	5	2	100	7.30 ± 2.45	53.5 ± 2.25	60.80	6080
Consortium (BJ2+DJ12+J4)	5	2	100	24.63 ± 2.02	57.4 ± 1.78	82.03	8203

Plant growth promotion study

The three potential PGPR isolates and their consortium, previously identified as effective for salt tolerance and in vitro plant growth–promoting (PGP) traits, were selected for further evaluation of their impact on mustard seedling growth under controlled conditions. Growth parameters including root length, shoot length, seedling length, seed vigour index, and chlorophyll content were assessed and compared with the control and autoclaved treatments (Table 5).

All treatments showed 100% germination within 2 days, indicating uniform seed viability across all treatments. One-way ANOVA revealed a highly significant effect of bacterial inoculation on root length, shoot length, seedling length, and seed vigour index of mustard seedlings ($p < 0.001$). Dunnett's post hoc test showed that all PGPR treatments significantly enhanced growth parameters compared to the control, whereas the autoclaved treatment did not show any significant improvement.

Root growth was significantly enhanced in PGPR-inoculated plants compared to the control (35.43 ± 3.16 mm). The maximum root elongation was observed in DJ12 (58.37 ± 2.02 mm) and BJ2 (57.43 ± 2.25 mm), followed by J4 (56.57 ± 2.35 mm) and the consortium treatment (55.60 ± 2.11 mm), all of which showed highly significant increases ($p < 0.001$). In contrast, the autoclaved treatment recorded a lower root length (31.63 ± 3.03 mm), suggesting the absence of biologically active growth-promoting factors.

Shoot length was also significantly influenced by bacterial inoculation. The highest shoot length was recorded in J4 (107.9 ± 2.30 mm), followed by the consortium (105.93 ± 3.46 mm) and BJ2 (104.3 ± 2.07 mm), all of which were significantly higher than the control (96.8 ± 1.80 mm; $p < 0.001$). The autoclaved treatment showed comparatively reduced shoot growth (89.4 ± 1.80 mm).

Consistent with improvements in root and shoot growth, seedling length and seed vigour index (SVI) were significantly higher in all PGPR-treated seedlings compared to the control and autoclaved treatments. The maximum seedling length was observed in J4 (164.47 mm), followed by BJ2 (161.73 mm), the consortium (161.53 mm), and DJ12 (156.70 mm). Similarly, the highest SVI was recorded in

BJ2 (16,173), followed by J4 (16,447), the consortium (16,153), and DJ12 (15,670), indicating enhanced seedling vigour and early establishment.

Chlorophyll content analysis revealed that there was an increased amount of chlorophyll in PGPR-treated mustard plants compared to control plants. The highest total chlorophyll content was observed in J4 ($0.365 \pm 0.0070 \text{ mg g}^{-1}$), followed by BJ2 ($0.284 \pm 0.0060 \text{ mg g}^{-1}$), consortium treatment ($0.249 \pm 0.0065 \text{ mg g}^{-1}$), and DJ12 ($0.203 \pm 0.0055 \text{ mg g}^{-1}$) whereas the control ($0.131 \pm 0.0035 \text{ mg g}^{-1}$) and autoclaved treatment ($0.109 \pm 0.0030 \text{ mg g}^{-1}$) showed comparatively lower chlorophyll levels. Increased contents of chlorophyll a and chlorophyll b in PGPR-treated plants indicated enhanced photosynthetic efficiency.

Overall, PGPR inoculation significantly improved mustard seedling growth compared to uninoculated and autoclaved controls. BJ2 and DJ12 were most effective in enhancing root elongation, while J4 promoted maximum shoot growth and chlorophyll accumulation, demonstrating isolate-specific growth-promoting effects. The consortium treatment also showed consistent improvement across all parameters, indicating a synergistic influence of combined bacterial inoculation on early mustard seedling growth.

Table 5 Effect of bacterial inoculation on growth of mustard (CS61) in pot assay under salinity stress.

Treatment Description	No. of Seeds Germinated (Out of 30)	Days taken for Germination	Germination %	Avg Root Length (mm)	Avg Shoot Length (mm)	Seedling Length	Seed Vigour Index
Control	30	2	100	35.43 ± 3.16	96.8 ± 1.80	132.23	13,223
Autoclave	30	2	100	31.63 ± 3.03	89.4 ± 1.80	121.03	12,103
BJ2	30	2	100	57.43 ± 2.25	104.3 ± 2.07	161.73	16,173
DJ12	30	2	100	58.37 ± 2.02	98.33 ± 1.80	156.70	15,670
J4	30	2	100	56.57 ± 2.35	107.9 ± 2.30	164.47	16,447
Consortium (BJ2+DJ12+J4)	30	2	100	55.60 ± 2.11	105.93 ± 3.46	161.53	16,153

Discussion

Salinity is a major abiotic constraint that adversely affects seed germination, early seedling establishment, and overall plant productivity, particularly in salt-sensitive crops like mustard (*Brassica juncea*). High salt concentrations induce osmotic stress and ion toxicity, leading to reduced water uptake, nutrient imbalance, and impaired metabolic activity during early growth stages (Munns and Tester, 2008; Shrivastava and Kumar, 2015; Ismail and Horie, 2017). In the present study, halotolerant plant growth-promoting rhizobacteria (PGPR) isolated from mangrove rhizosphere soils significantly

improved mustard growth and early seedling establishment under saline conditions, demonstrating their strong potential to alleviate salt-induced growth inhibition.

The study of halotolerant bacterial isolates capable of surviving upto 8% NaCl concentrations reflects the intense selective pressure of the mangrove ecosystem. Mangrove rhizospheres are known to harbor metabolically diverse and stress-adapted microorganisms due to fluctuating salinity, periodic tidal inundation, and oxygen-limited conditions (Holguin et al., 2001; Alongi, 2014; Thatoi et al., 2013). Previous studies have similarly reported that PGPR isolated from mangrove and saline environments exhibit superior stress tolerance and plant growth-promoting abilities compared to isolates from non-saline agricultural soils (Dastager et al., 2010; Ramesh et al., 2014).

The selected bacterial isolates in this study exhibited multiple direct and indirect plant growth-promoting traits, including indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation, ammonia production, biosurfactant synthesis, and hydrolytic enzyme activity. These traits are well documented to enhance nutrient availability, root system development, and plant stress tolerance under saline conditions (Vessey, 2003; Bhattacharyya and Jha, 2012; Glick, 2014). The consistent IAA production by all selected isolates is particularly important, as auxins stimulate root elongation and lateral root formation, thereby improving water and nutrient uptake under salt stress (Patten and Glick, 2002; Glick et al., 2007).

Exopolysaccharide (EPS) production by isolate BJ2 (*Micrococcus luteus*) plays a crucial role in salinity stress mitigation by improving soil aggregation, limiting Na⁺ ion availability in the rhizosphere, and enhancing bacterial root colonization. EPS-producing PGPR can form protective biofilms on root surfaces, reducing ionic toxicity and osmotic stress experienced by plants (Glick, 2014; Etesami and Beattie, 2018). Furthermore, the ability of isolates to exhibit antifungal activity highlights their dual role in nutrient acquisition and biocontrol, which is essential for sustainable crop production under stress conditions (Schwyn and Neilands, 1987; Backer et al., 2018).

Although all treatments showed 100% germination at 1% salinity, bacterial inoculation significantly enhanced post-germination seedling growth. One-way ANOVA revealed a highly significant influence of PGPR treatments on root length, shoot length, seedling length, and seed vigour index ($p < 0.001$), while Dunnett's test confirmed that all PGPR-treated seedlings performed significantly better than the saline control. Early seedling vigor is a critical determinant of plant establishment under salt stress (Abdul-Baki and Anderson, 1973; Egamberdieva et al., 2019).

Among the tested isolates, BJ2 (*Micrococcus luteus*) and the bacterial consortium showed the highest growth-promoting effects. Enhanced root elongation in PGPR-inoculated seedlings is an adaptive advantage that allows plants to explore deeper soil layers for water and nutrients under saline stress (Rojas-Tapias et al., 2012; Egamberdieva et al., 2019). Pot experiments further confirmed the effectiveness of the selected PGPR under controlled saline conditions. The absence of growth promotion in autoclaved treatments clearly indicates that the observed improvements were due to active bacterial metabolism rather than residual nutrients (Glick, 2012).

Isolate-specific responses were evident, with BJ2 (*Micrococcus luteus*) and DJ12 (*Microbacterium barkeri*) promoting greater root growth, while J4 significantly enhanced shoot growth. These variations likely result from differences in phytohormone production, nutrient solubilization capacity, and stress-alleviating mechanisms among the isolates (Bhattacharyya and Jha, 2012; Backer et al., 2018). The consortium treatment resulted in balanced improvement across all growth parameters, suggesting synergistic interactions among compatible strains, which may improve consistency and effectiveness under variable environmental conditions (Malusá et al., 2012).

Increased chlorophyll content observed in PGPR-treated plants indicates improved photosynthetic efficiency and reduced stress-induced chlorophyll degradation. Enhanced chlorophyll levels under salinity stress have been linked to improved nutrient uptake and reduced oxidative damage (Ismail and Horie, 2017; Egamberdieva et al., 2019), ultimately contributing to higher biomass accumulation and improved plant performance.

Overall, the statistically significant improvements observed in this study ($p < 0.001$) shows the strong potential of mangrove-derived halotolerant PGPR as bioinoculants for improving mustard growth under saline conditions. These findings highlight mangrove ecosystems as valuable reservoirs of stress-tolerant beneficial microbes and provide a strong scientific foundation for developing eco-friendly PGPR-based strategies for saline agriculture (Thatoi et al., 2013; Backer et al., 2018).

Conclusion

The present study demonstrated that mangrove rhizosphere soils of Navi Mumbai serve as a rich reservoir of halotolerant plant growth-promoting rhizobacteria (PGPR) with significant potential to enhance the growth of mustard CS61 (*Brassica juncea*) under saline conditions. From 1,263 bacterial isolates recovered, 97 exhibited strong halotolerance up to 10% NaCl, and 17 non-hemolytic isolates displayed multiple plant growth-promoting traits.

These selected isolates demonstrated key functional attributes, including nitrogen fixation, phosphate solubilization, IAA production, ammonia production, biosurfactant synthesis, siderophore production, hydrolytic enzyme activity, and antifungal potential, collectively contributing to improved plant nutrition and stress tolerance. Seed germination assays and pot experiments conducted under 1% salinity stress revealed that PGPR inoculation significantly enhanced root length, shoot length, seedling length, seed vigour index, and chlorophyll content compared to uninoculated controls.

Among the tested isolates, BJ2 (*Micrococcus luteus*) and the bacterial consortium consistently exhibited superior performance. Molecular identification based on 16S rRNA gene sequencing confirmed that the effective isolates belonged to beneficial bacterial taxa such as *Micrococcus luteus* and *Microbacterium barkeri*, which are recognized for their stress tolerance and plant growth-promoting potential.

Overall, this study provides compelling evidence that mangrove-derived halotolerant PGPR can function as sustainable and eco-friendly bioinoculants for improving crop productivity in salt-affected soils, supporting their potential application in saline agriculture as alternatives to chemical fertilizers.

Future Study

Large-scale, multi-location field trials in naturally saline and coastal agricultural soils are warranted to evaluate the long-term effectiveness, stability, and commercial feasibility of the selected halotolerant PGPR consortium on mustard and other economically important crops. These trials should also include detailed, multi-season assessments of soil health, microbial community dynamics, and crop yield. Metagenomic approaches can be further employed to investigate uncultured microbial diversity and identify novel genes associated with stress tolerance and plant growth promotion.

Declarations

Data availability statement:

Data sets of the this manuscript, 16S rRNA gene sequences generated during this study have been submitted to the NCBI genbank database and the accession number will be made available to the reviewer as it will be received.

All the required data sets are included in this manuscript. Any other data required will be made available as it is required during review process.

GenBank accession number(s) for submitted nucleotide sequence(s):

SUB15995336 ML_BJ2 – Accession Number -PX974661

SUB15995336 MB_DJ12 – Accession Number -PX974662

SUB15995336 MB_J4– Accession Number -PX974663

As we received this today – as per NCBI – This will be available on NCBI website by this weekend.

Ethical approval:

The plant material used in this study consisted of cultivated mustard (*Brassica juncea* L.) cultivar CS61 seeds obtained from the Kharland Research Center, Panvel, Maharashtra, India with required permissions.

The use of this plant material complied with all relevant institutional, local, and national guidelines for research involving cultivated plants. No endangered or protected plant species were involved in this study, and no special permits or licenses were required for the use of the cultivated plant material.

Rhizosphere soil samples were collected from wild mangrove ecosystems of Navi Mumbai, Maharashtra, India, following standard scientific sampling procedures and in accordance with local environmental and

institutional guidelines. Soil sampling was conducted without causing damage to protected plant species or habitats, and no specific permissions or licenses were required for soil sample collection at the sampling sites.

This study did not involve human participants or animals, and therefore formal ethical approval was not applicable.

Clinical Trials:

Not applicable. This study does not involve human participants or medical interventions; it focuses on plant-microbe interactions in an agricultural context.

Consent to Participate and Publish:

Consent to participate: Not applicable.

Consent to publish: Not applicable.

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Authors' contributions:

Mayur Auti and Prajval Poojary prepared the manuscript and Manish Bhat, helped in the revisions of the manuscript. He supervised the experiments carried out by Mayur Auti and Prajval Poojary. All authors read and approved the final manuscript.

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Figures

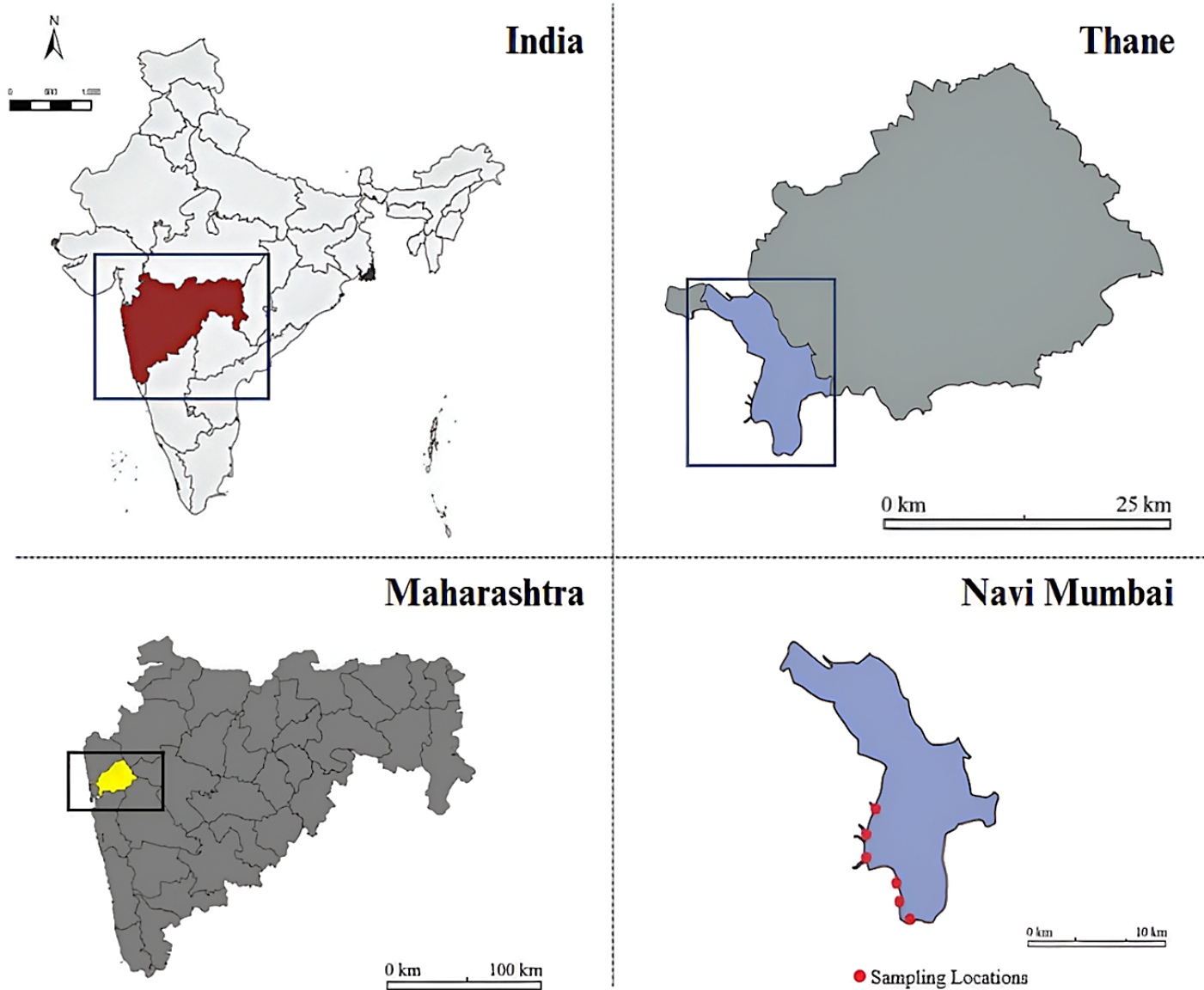


Figure 1

Location map of India and Maharashtra showing the sampling site in the Navi Mumbai region, Thane district, India.

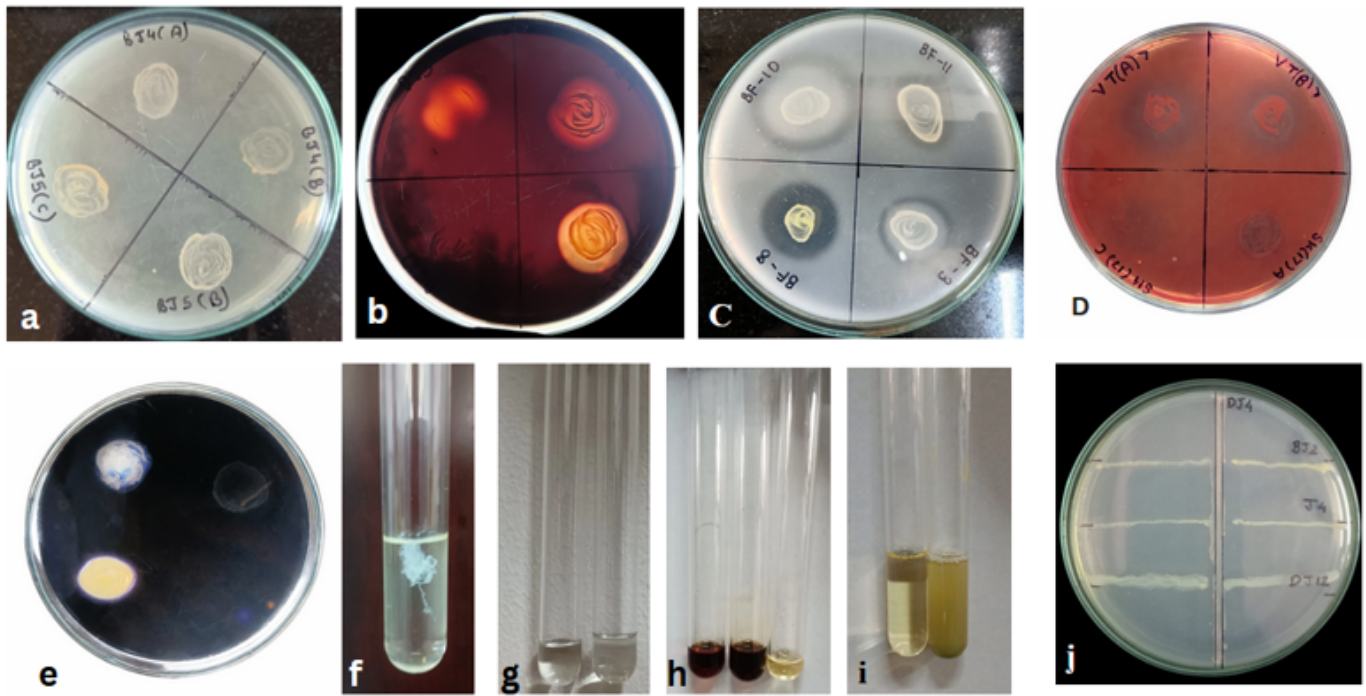


Fig 2. a) Nitrogen Fixation; b) Pectin degradation; c) Protease activity; d) Cellulose degradation; e) Starch hydrolysis; f) Exopolysaccharide production; g) Gibberellin production; h) Indole acetic acid production; i) Ammonia Production; j) Antagonistic activity

Figure 2

See image above for figure legend.

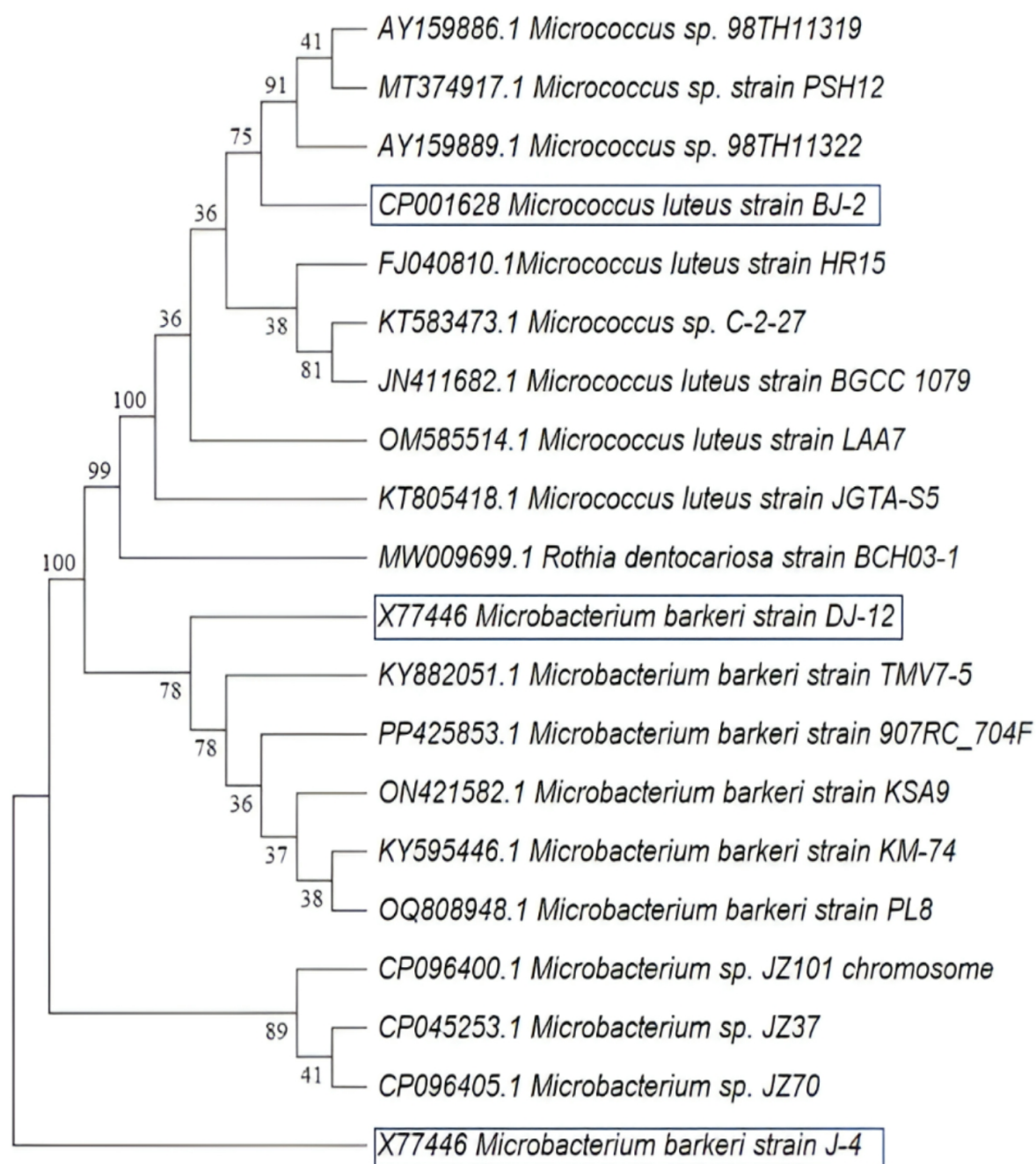
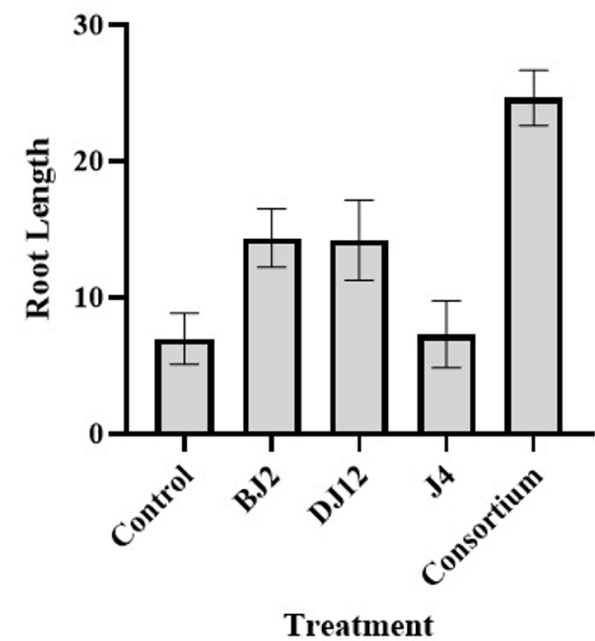


Figure 3

Phylogenetic relationships of six bacterial isolates based on 16S rRNA gene sequences. Sequences were aligned using the MAFFT online server, and the phylogenetic tree was constructed using the Neighbor-Joining (NJ) method using the Tamura–Nei (TN93) substitution model with 1000 bootstrap replicates. Bootstrap support values (expressed as percentages) are indicated at the branch nodes. The tree is drawn to scale, and branch lengths represent 0.05 substitutions per nucleotide position. Rothia dentocariosa was used as an outgroup to root the tree.

Effect of Treatments on Seed Germination



Effect of Treatments on Seed Germination

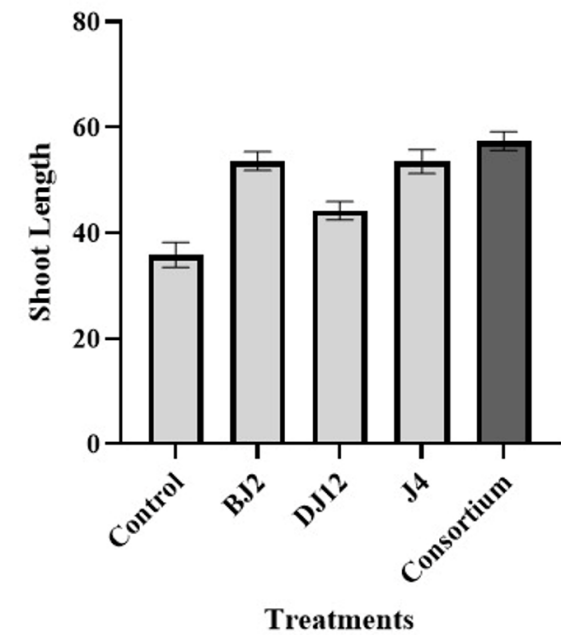


Figure 4

Effect of bacterial inoculation on average root and shoot length of mustard (CS61) under salinity stress.

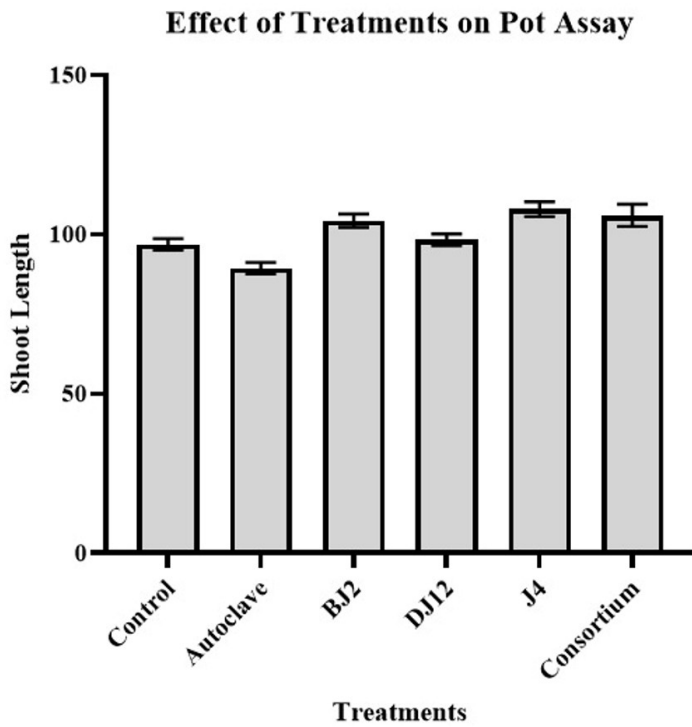
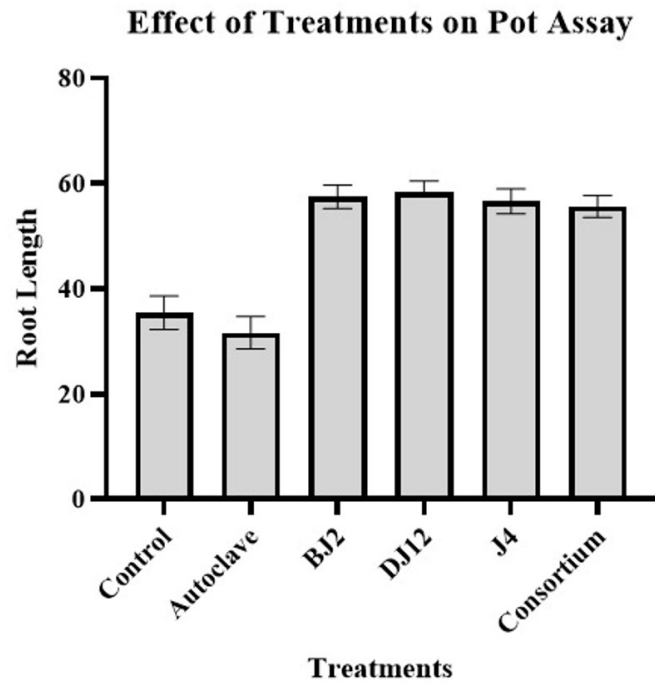


Figure 5

Effect of bacterial inoculation on root and shoot length of mustard (CS61) in pot assay under salinity stress.

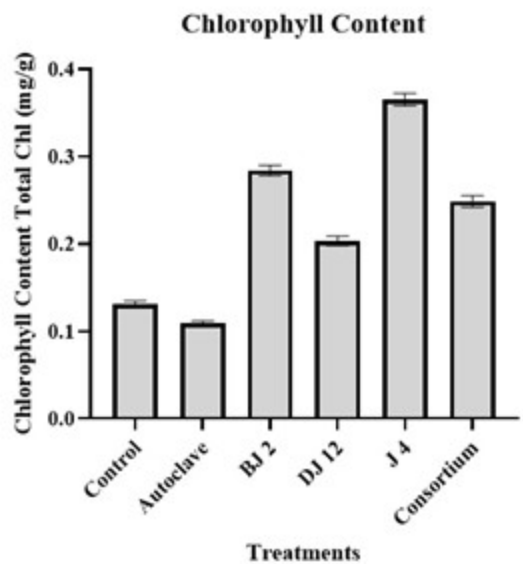


Figure 6

*Effect of different treatments on total chlorophyll content (mg g^{-1} FW) in mustard (*Brassica juncea*).*

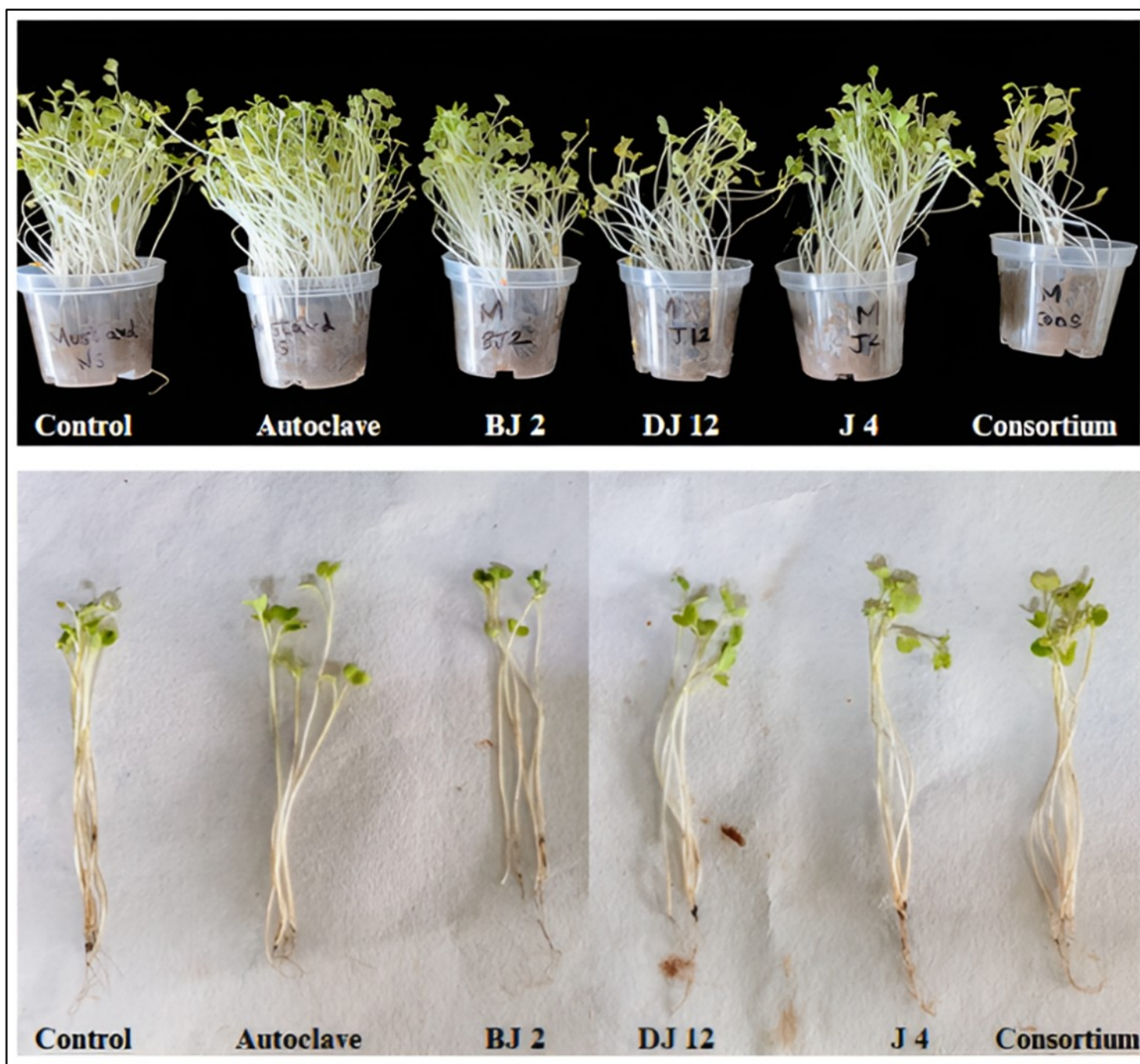


Figure 7

Effect of bacterial treatments on root and shoot development of mustard (CS61) seedlings in pot assay under salinity stress.