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Isolation and characterization of salt-stress-tolerant rhizosphere soil bacteria and their effects on plant growth-promoting properties

Naveena Radhakrishnan & Chitra Krishnasamy[✉]

PGPR has a higher potential impact on agricultural crops. It enhances plant growth and development in a variety of adverse environmental conditions, including biotic and abiotic stresses. The PGPR is commercially vital since it is more efficient, safe for the environment, and beneficial to the economy. Nowadays, salt stress has an impact on the agricultural ecosystem. Salt-tolerant PGPR can directly stimulate plant growth and development by producing a variety of metabolites and phytohormones. The current study looked at the isolation of salt-tolerant bacterial species and their ability to stimulate plant development. Four bacterial species were chosen for their better salt stress tolerance (0–5%). They were identified by 16S rRNA sequencing: *Solibacillus silvestris* BR1, *Peribacillus frigoritolerans* BR2, *Paenibacillus taichungensis* CR1, and *Solibacillus isronensis* CR2. These strains were positive production of indole acetic acid with varying incubation periods (19.66 ± 1.528 to 646.111 ± 8.058 µg/mL), salt stress (ranging from 29.556 ± 1.171 to 147.8111 ± 2.086 µg/mL), phosphate solubilization (0.145 ± 0.011 to 0.921 ± 0.007 µg/mL), ammonium production (0.299 ± 0.047 to 1.202 ± 0.142 µg/mL), HCN production (0.308 ± 0.051 to 4.269 ± 0.069 µg/mL), and siderophore production (0.190 ± 0.064 to 1.543 ± 0.108 µg/mL) for control strains were used without salt stress. The production level was expressed using a standard curve containing various standards.

Keywords Nilgiri district, Rhizosphere soil, Rhizobacteria, Salt stress tolerance bacteria, Plant growth hormone, Plant growth activities

One of the most serious problems in agricultural soils is environmental stress, which includes both biotic and abiotic variables. Salinity stresses have negative consequences for the growth and production of many agricultural crops¹. Soil salinization is a critical issue that significantly affects the structure, processes, and functions of global ecosystems². In general, soil salinization is classified into two categories based on natural and human-induced actions: primary salinization and secondary salinization. Soil primary salinization is primarily influenced by the local climate, parent material, soil parameters, and groundwater. Insufficient rainfall, causing higher evapotranspiration and rising groundwater levels, can also lead to increased soil salinity³. Secondary or human-induced salinization occurs when humans use soil inappropriately. Saline irrigation, paired with poor drainage, may readily cause salt accumulation on the soil surface. In future climate change scenarios, seawater intrusion in coastal areas are expected to increases due to rising temperatures and fast development⁴. Soil salinization is one of the most adverse factors affecting plant development and causing land degradation and, as a result, diminishing potential net primary productivity and increasing desertification⁵. Increased soil salt content has a negative impact on both physical and chemical qualities, as well as microbiological processes. Electrical conductivity (EC) measures saline soil, which accumulates Na⁺, Mg²⁺, Ca²⁺, Cl⁻, HCO₃²⁻, SO₄²⁻, and vitamin B ions and negatively impacts plant growth⁶. The excessive accumulation of these ions induces osmotic stress and toxicity by increasing Na⁺ assimilation and reducing the Na⁺/K⁺ ratio⁷. These issues can be attributed to lower osmotic potential within plant roots, which affects almost all aspects of plant growth, including germination, vegetative growth, and reproduction. Salinity alters various morphological, biochemical, and molecular characteristics. Toxic ions frequently cause reactive oxygen species (ROS) accumulation in plant tissues. The produced ROS caused protein denaturation, DNA damage, lipid peroxidation, and carbohydrate oxidation, which impaired enzyme performance and pigment degradation in plants⁸.

The highest concentrations of salt (Na⁺ and Cl⁻ ions) cause a variety of agricultural problems, including porosity, aeration, and water conductivity⁹. In plants, they restrict plant metabolism and, to some extent,

Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu 641 046, India. [✉]email: Chitra@buc.edu.in

disrupt photosynthesis by creating reactive oxygen species (ROS), which can cause DNA damage, protein breakdown, and membrane injury. Salinity stress will also harm the physicochemical aspects of soil respiration as well as enzymatic and microbiological activity. Salt stress has been identified as the most harmful condition for agricultural soil¹⁰. Salinity has the greatest impact on the environment in terms of ecological change and soil variance in the future. This presents a challenge to agricultural crop productivity. Eco-friendly and non-hazardous alternative technology is required to overcome negative impacts of salt stress on crops. Furthermore, plant growth-promoting microbes can be used to implement novel tactics. Soil contains a broad range of microbiomes. Rhizospheric soil microorganisms are made up of various types of clustered bacteria. Their symbiotic interaction with plants helps to minimize salinity stress¹¹.

Applications of PGPR can be an effective elicitor to stimulate the inclusion of abiotic and biotic systematic tolerance in plants¹². Rhizobacteria that promote plant growth are usually found in the plant's root zone. This establishes a special symbiotic connection with plants. It will employ a direct mechanism to induce plant growth and development. The mechanisms involved are nutrient acquisition, HCN production, ammonium production, siderophore sequestration, and nitrogen fixation, synthesis of exo-polysaccharides and phytohormones, potassium and phosphate solubilization under salinity stress¹³, and upregulation of plant antioxidant defense enzymes to protect the plant from oxidative damage¹⁴. PGPR improves crop productivity and the production of plant growth regulators like auxin, gibberellins, cytokinin, ethylene, HCN, and antibiotics¹⁵. Many methods for PGPR-mediated salt stress resistance in plants have been documented in the literature. They specifically decrease the negative effects of high ethylene levels on plants, such as chlorosis, abscission, and senescence¹⁶. Furthermore, under salinity stress, NaCl causes rapid K⁺ loss from the plant's cellular cytosol, whereas PGPR retains more K⁺ in order to maintain a higher K⁺-Na⁺ ratio and avoid osmotic imbalance¹⁷. Furthermore, PGPR helps to reduce reactive oxygen species (ROS) produced by NaCl in plant cells by raising the amounts of ROS-scavenging enzymes¹⁸.

In high-salinity conditions, various PGPR can regulate and stimulate plant growth. According to various investigations, such bacteria belong to the following families: *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia*¹⁹. Tryptophan is a key precursor that contributes significantly to bacteria's biosynthetic pathway for the creation of IAA. During tryptophan metabolism, carbohydrates and sugar molecules are moved around. It will control physiological processes including cell proliferation, tissue differentiation, and tactic responses²⁰. IAA is produced by both bacteria and plants through Trp-dependent and Trp-independent mechanisms²¹. However, the majority of earlier investigations on IAA production focused on Trp-dependent pathways, with few studies examining Trp-independent processes. The intermediary stages and genes involved in Trp-independent pathways remain unknown. Bacteria's key IAA biosynthesis routes have been identified as IPA, IAM, and indole-3-acetonitrile (IAN)²². A higher percentage of phosphate-solubilizing bacteria have been isolated from several environmental sources, including rhizosphere soil, metal-contaminated soil, phosphate mines, composts, and macro fauna²³. Phosphate-solubilizing bacteria have been used as a novel biotechnology method. It has the ability to solubilize rock phosphate and serve as a fertilizer²⁴. The bacteria produce organic acid, which chelates the cations. Phosphate is bound through hydroxyl and carboxyl groups before being released as soluble phosphate²⁵. Bacterial solubilization lowers the pH of the media through either H⁺ extrusion or the secretion of organic acids such as gluconic, citric, oxalic, succinic, lactic, formic, and acetic acids. These microorganisms have increased the solubility of rock phosphate²⁶. Our research findings include rhizosphere soil bacteria isolation from the agricultural soil of beetroot and carrot vegetable cultivation areas in Nilgiri district, Tamil Nadu, India. The ability of the isolated bacterial species to tolerate salt stress was studied. In the synthesis of IAA, the phosphate solubilizing capability, ammonium, HCN, and siderophore must be examined.

Materials and methods

Isolation of rhizosphere soil bacteria

The soil samples were collected from the beetroot and carrot rhizosphere soil in the Nilgiri district of Kilkothagiri, Tamil Nadu, India, at latitude 11.384° N and longitude 76.74305° E. The pH of the sampled soil was 7. The bacteria were isolated using the following procedure: 1 g of soil sample was obtained and mixed with approximately 10 mL of sterile distilled water (1:10 ratio). This diluted sample was taken up to 1 mL and serially diluted (10⁻¹ to 10⁻⁷). The pour plate method was used to pour approximately 1 mL of each diluted sample onto the nutrient agar medium (Himedia, India). Plates were incubated for 24 h at 37 °C. A single colony developed in dilution and was selected for a streak on sterilized nutrient agar medium. Four bacterial colonies were successfully isolated and preserved. They were tested for salt stress tolerance and plant growth-promoting properties.

Morphological characteristics of Bacterial isolates

After the cultures were screened out on the basis of colony morphology, distinct microbial colonies were counted and subsequently pure-cultured. The colony color, shape, and margin were noted. After that, the pure colonies were maintained solidified agar plates at 4 °C and used for further characterization studies. Next, the biochemical characterization was analyzed.

Biochemical characteristics of Bacterial isolates

Indole test

Take a sterile test tube and add 4 mL of tryptophan broth. Inoculate the tube aseptically by removing the growth from an 18- to 24-h culture. Incubate the tube at 37 °C for 24–28 h. Add 0.5 mL of Kovac's reagent to the broth culture. Check for the presence or absence of a ring²⁷.

Sugar utilization test

The medium includes proteose peptone (10.00 g). HM Peptone B (beef extract): 1 g; sodium chloride: 5.00 g. Mix 0.018 g of Phenol Red and 10 g of glucose in 1 L of clean water. Use a sterile inoculating loop to collect a well-isolated colony from a fresh culture of sample bacteria (18 to 24 h old) and add it to the broth. Incubate tubes at 35 ± 2 °C for 18–24 h. Check for color changes in the soup and trapped air bubbles in Durham's tube. If no color change or air bubbles appear, incubate the tubes for an additional 24 h before checking for color change and air bubbles²⁸.

Methyl red test

Allow the medium to reach room temperature before inoculating. Use organisms from an 18–24-hour pure culture to slightly inoculate the medium. Incubate aerobically at 37 °C for 24 h. After 24 h of incubation, transfer 1 ml of broth to a clean test tube. Add 2–3 drops of methyl red indicator to the aliquot. Check for red quickly²⁹.

Voges-Proskauer test

Allow the medium to reach room temperature before inoculating. Use organisms from an 18–24-hour pure culture to mildly inoculate the medium. Incubate aerobically at 37 °C for 24 h. After 24 h of incubation, transfer 2 mL of broth to a clean test tube. Re-incubate the remaining broth for another 24 h. Add 6 drops of 5% alpha-naphthol and mix thoroughly to aerate. Add 2 drops of 40% potassium hydroxide and mix thoroughly to aerate. Within 30 min, observe for a pinkish-red tint on the surface. Shake the tube aggressively for the entire 30-min time³⁰.

Citrate utilization test

Streak the slant back and forth with a mild inoculum obtained from the center of a well-isolated colony. The colony was inoculated onto simmons citrate agar. Incubate aerobically at 35 to 37 °C for 4–7 days. Observe the color change from green to blue³¹.

Catalase test

Using a loop or sterilized wooden stick, transfer a little bit of colony development to the surface of a clean, dry glass slide. Add a drop of 3% H₂O₂ to the glass slide. Observe the evolution of oxygen bubbles³².

Ammonia production test

Freshly developed cultures were injected into 10 mL of peptone water in each tube and incubated for 48–72 h at 28 ± 2 °C. Nessler's reagent (0.5 mL) was added to each tube. The development of a brown-to-yellow tint was a positive indicator of ammonia production³³.

Potassium hydroxide solubilization test

Apply one drop of 3% KOH to a microscope slide. Use the loop to transfer a generous number of bacteria (cultivated for 24–48 h) to a drop of KOH. Stir carefully. Gram-negative bacteria solutions are viscous and form a mucoid string within 30 s, whereas gram-positive bacteria solutions are not viscous.

Characterization of bacterial isolates

Sequencing and phylogenetic analysis

Bacterial genomic DNA was recovered via overnight culture using the CTAB (Cetyl trimethyl ammonium bromide) technique³⁴. The 16S rRNA gene was amplified using a forward primer (5' GAGTTGATCGTGG CTCAG 3') and a reverse primer (5' AGGGCTACCTGTTAGACTT 3'). PCR analysis using a reaction mixer volume of 20 µL. Emerald Amp GT PCR master mix contains MQ water, 10X Taq buffer, 15 mM MgCl₂, Taq polymerase, 4 mM DNTPs, genomic DNA, reverse and forward primers, and sterile water. PCR amplification was carried out in the following manner: initial denaturation at 96 °C for 5 min, followed by 35 cycles. They performed three steps: denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplified PCR products were sequenced on a 3500 genetic analyzer (Thermo Fisher, India). The ExoSAP-ITTM PCR product cleanup reagent was used to remove unincorporated PCR primers and dNTPs from the amplified PCR product. The thermocyclic reaction is then followed by ethanol precipitation of the PCR products. The DNA sample was then tested using a genetic analyzer. The 16S rRNA gene sequences were aligned using DNA laser gene software and compared to sequences available from GenBank using the BLASTN program (www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree constructed based on 16S rRNA sequences from the bacterial isolates using evolutionary history was inferred using the neighbor-joining method³⁵. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches³⁶ (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method³⁷ and are in units of the number of base substitutions per site. This analysis involved 35 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1573 positions in the final dataset. Evolutionary analyses were conducted in MEGA11³⁸.

National Center for Biotechnology Information (NCBI) database

The 16S rRNA sequences of the four bacterial species were examined at NCBI (National Center for Biotechnology Information) and deposited under the accession numbers BR1 ON819727.1, BR2 ON819728.1, CR1 OR478038.1, and CR2 OR478039.1.

Effects of incubation period on production of IAA

The incubation period for bacterial strains was tested for indole-acetic acid synthesis. When IAA production levels increased over time, the impacts of the incubation period were determined. The bacterial species were cultured in Erlenmeyer flasks with 100 mL of nutrient broth. Inoculated with 0.1 mL of culture and supplemented with 0.1% L-tryptophan. The culture was incubated for periods ranging from 1 to 10 days at 37 °C. Following incubation, the culture supernatant was centrifuged and tested for IAA synthesis using the colorimetric technique. The supernatant was combined with a 1:2 salkowski reagent (0.5 M FeCl₃ and 70% perchloric acid). Have incubated in the dark for 30 min and measured the absorbance of OD at 530 nm³⁹.

Determination of salt stress tolerance Bacteria for Indole-3-acetic acid production

The four bacterial strains have been examined for salt tolerance efficiency at various concentration levels. Each isolate was inoculated with 10 mL of nutritional broth containing 0, 1%, 2%, 3%, 4%, and 5% NaCl and 0.1 L tryptophan. The tubes were cultured for one week on an orbital shaker at 250 rpm and 37 °C. After that, the culture was centrifuged at 6000 rpm for 15 min. The turbidity of the assigned bacterial cells was evaluated using a spectrophotometer (Labtronics, India) at 600 nm. IAA production was measured using a calorimetric technique with a 1:2 salkowski reagent (0.5 M FeCl₃ and 70% perchloric acid). The concentration was calculated using a standard curve of IAA absorbance at 530 nm³⁹. They were successful in determining the bacteria with the bacteria with the highest salt tolerance and their IAA acid production.

Quantification of Bacterial phosphate solubilization

The phosphate solubility of bacteria was quantified using Pikovskaya's broth and tricalcium phosphate as a substrate⁴⁰. The PVK broth (10 mL) containing 0, 1%, 2%, 3%, 4%, and 5% NaCl was added to the culture tube and then screened for bacteria. Each sample was inoculated with 100 µL in triplicate. The culture was cultured for 7 days at 37 °C on an orbital shaker at 250 rpm. After incubation, the culture was centrifuged at 6000 rpm for 15 min, and the supernatant was collected. 0.5 mL of culture supernatant was combined with 10% trichloroacetic acid in an equal proportion. Add 3 mL of the reagent combination to the culture supernatant (1:1:1:2 ratios of 3 M H₂SO₄, 2.5% (w/v) ammonium molybdate, 10% (w/v) ascorbic acid, and distilled water). The culture was then allowed to incubate at room temperature for 15 min. The color-changing blue was used to assess the amount of soluble phosphorus in the sample. The absorbance at 820 nm was measured against the KH₂PO₄ standard curve (Himedia, India).

Ammonia (NH₃) production test

Ammonium production was analyzed. The bacterial isolates were inoculated in peptone water containing 10.0 g peptone, 5.0 g NaCl, 1,000 ml distilled water, and 7.0 pH, with NaCl stress concentrations of 0, 1%, 2%, 3%, 4%, and 5%, respectively. Incubate 100 µL of culture in 10 mL of peptone water at 28 ± 2 °C for 48–72 h on an orbital shaker at 350 rpm. Following incubation, the culture was centrifuged at 10,000 rpm for 15 min. To extract the supernatant, add 0.5 mL of Nessler's reagent. The change from brown to yellow was measured using OD at 450 nm. Ammonium sulfate was employed as a standard⁴¹.

Hydrogen cyanide (HCN) production

HCN production was estimated. The bacterial isolates were inoculated in nutritional broth at concentrations of 0, 1%, 2%, 3%, 4%, and 5% NaCl stress and supplemented with glycine (4.4 g/L). The culture was then inoculated, and a uniform-sized piece of filter paper was soaked in 2% sodium carbonate in a 0.5% picric acid solution before being suspended within the conical flasks. The reddish chemical originated in filter paper. The color was eluted by placing the filter paper in a test tube containing 10 mL of distilled water, and the absorbance was measured at 625 nm. Sodium cyanide was employed as the standard⁴².

Estimation of siderophore production

Siderophore production was quantified. The bacterial isolates were inoculated into nutritional broth with different concentrations of NaCl stress (0, 1%, 2%, 3%, 4%, and 5%). Fill 250-mL Erlenmeyer flasks with 100 mL of broth and sterilize thoroughly. After adding 1 mL of inoculum to the culture broth, it was incubated at 37 °C for 7 days in an orbital shaker at 300 rpm. Afterward, the bacterial inoculum was centrifuged at 10,000 rpm for 20 min. The culture supernatant was collected, and the pH was adjusted to 2.0 using diluted HCl. The supernatant was removed twice, and an equal volume of ethyl acetate (V/V) was added to the extract. To estimate the siderophore, one milliliter of 0.1 M ferric chloride and 1 mL of 0.1 N HCl were mixed with 100 mL of distilled water, followed by 1 mL of 0.1 M potassium ferricyanide. Add 5 mL of extract assay solution to 5 mL of Hathaway reagent and measure OD at 560 nm. The standard for salicylate-type siderophores was sodium salicylate⁴³.

Statistical analysis

The statistical analysis was carried out using Microsoft Office Excel (2007). Mean values for each treatment were determined, and the treatment means were compared using analysis of variance. The data in this study were statistically evaluated using one-way ANOVA and SPSS version 16. Mean values were expressed as mean ± SD. Values were compared using Duncan's multiple range test with a significance level of *p* < 0.05.

Results and discussion

Isolation and molecular characterization of Bacterial Isolates

The PGPR has positive properties that allow it to reduce the harmful effects of excessive salt concentrations. PGPR can increase plant development in a salt environment in two ways: (i) activating or regulating plant response systems during salt exposure, and (ii) manufacturing anti-stress molecules⁴⁴. To improve the growth

and resistance of plants exposed to salinity, the processes include (i) improving nutrient uptake (e.g., N₂ fixation, release of bound P and K⁺ from the soil, chelating iron) and maintaining the water balance; (ii) an influence on ion homeostasis; (iii) inducing the selective absorption of K⁺ and exclusion of Na⁺ to maintain a high K⁺/Na⁺ ratio; (iv) the formation of bio-film to reduce Na⁺ toxicity; (v) changes in root architecture; (vi) modulating the antioxidant system; (vii) modulating osmotic substances; (viii) modulating plant hormonal levels; and (ix) modulating the expression of salt-responsive genes⁴⁵. Recently, it has been demonstrated that PGPR inoculation causes DNA methylation modification, which regulates the expression of genes useful for plant growth promotion, and that these DNA methylation changes in the DNA of root tissues can persist even after the PGPR is removed from the root microbiome⁴⁶.

In this investigation, salt resistance and plant growth-promoting ability were studied. The bacterial species were screened using nutritional broth and various amounts of (0–5% NaCl stress) (Fig. 1). Furthermore, we observed only four bacterial species with great salt stress tolerance and higher potential plant development capabilities. In this investigation, these bacterial species only further classified morphologically and biochemically (Tables 1 and 2). Following this, the bacterial strains were genetically identified. The bacterial genomic DNA was extracted, and its purity was determined using Agarose gel electrophoresis and a comparison to the 1500 kbp ladder (Fig. 2). Following that, the DNA was amplified using a polymerase chain reaction. Then they were genetically identified using 16S rRNA sequencing. According to their sequence identification, BR1 is the *Solibacillus silvestris*; BR2 is *Peribacillus frigoritolerans*; *Paenibacillus taichungensis* CR1; and *Solibacillus isronensis* CR2. The bacterial isolates' sequences were placed in the gene bank of the nucleotide sequence database at the National Center for Biotechnology Information (NCBI) under strains BR1 ON819727.1, BR2 ON819728.1, CR1 OR478038.1, and CR2 OR478039.1.

Bacillus silvestris has been identified as a new *Bacillus* species⁴⁷. After that, it was classified as *Solibacillus silvestris*⁴⁸. Only a few studies on this bacterial characterization were available. The whole genome and characterization of AHL-degrading *S. silvestris* were isolated from potato leaf surfaces. They are related to our sequence studies⁴⁹. The study reclassified *Brevibacterium frigoritolerans* based on phylogenetic and molecular synapomorphies. The examination of DNA-DNA hybridization, nucleotide identity, and the difference in G+C content all support the phylogenetic inference that *Brevibacterium frigoritolerans* and the environmental isolate EB93 are the same. Based on data from numerous lines of study, it was reclassified as *Peribacillus frigoritolerans*⁵⁰. According to our findings, *Peribacillus frigoritolerans* BR2 was isolated from beetroot rhizosphere soil. The type strain is V10537T new species in the *Paenibacillus* genus, named *Paenibacillus taichungensis* sp. nov. due to its distinct characteristics from previous species.

Paenibacillus taichungensis, discovered in Taiwanese soil, is a distinct gram-variable, rod-shaped, motile, endospore-forming bacterial strain. Strain V10537T is proposed as a new species of the genus *Paenibacillus* nov., *Paenibacillus taichungensis* sp. based on phylogenetic, phenotypic, genotypic, and chemotaxonomic evidence. Phenotypic characteristics that are closely connected to novel *Paenibacillus* species⁵¹. Similarly, the *Paenibacillus taichungensis* CR1 strain was identified from carrot rhizosphere soil in the Nilgiri area of Tamil Nadu, India. *Solibacillus isronensis* and *Solibacillus silvestris* share a phylogenetic lineage that overlaps the phenotypic features of the *Solibacillus* genus⁴⁹ (Fig. 3).

Effect of various incubation periods on IAA producing Bacterial Isolates

The incubation period was the most beneficial characteristic for producing Indole acetic acid. The bacterial isolates were cultured in a nutritional medium containing L-tryptophan. The bacterial strains were incubated for various incubation periods, such as 1, 3, 7, and 10 days, to produce indole acetic acid. Our findings demonstrated that *Solibacillus silvestris* BR1, *Peribacillus frigoritolerans* BR2, *Paenibacillus taichungensis* CR1, and *Solibacillus isronensis* CR2 have the potential to produce the higher amount of IAA after 7 and 10 days of incubation (Fig. 4a). *Solibacillus isronensis* CR2 produced the highest amount of Indole acetic acid (646.111 ± 8.058^a µg/mL) after 10 days of incubation, while the BR1 strain produced (268.33 ± 0.33^b µg/mL) after 7 days. At one day of incubation, the BR2 strain produced less IAA (19.66 ± 1.528^n µg/mL). After one day of incubation, the BR1 produced (35.66 ± 1.764^m µg/mL) (Table 3). The bacteria *Bacillus subtilis*, like our findings, produced a significant amount of production during the 96-h incubation period⁵². IAA synthesis in *S. silvestris* decreased with longer incubation periods (46.556 ± 4.718^l µg/mL). *P. frigoritolerans* (115.66 ± 0.577^j µg/mL) levels were reduced after 10 days of incubation. During the 3-day incubation period, IAA production increased progressively in CR2 (188.333 ± 2.186^e µg/mL), followed by CR1 (181.222 ± 1.644^f µg/mL), BR1 (68.22 ± 1.530^k µg/mL), and BR2 (46.11 ± 3.15^l µg/mL) (Fig. 4a). IAA synthesis levels differ among microbes⁵⁴. Bacterial species, strains, culture conditions, substrate availability, and growth stage may all have an impact on indole acetic acid synthesis.

Screening of salt tolerant Bacteria for the production of IAA

The rhizo-microbiome produces phytohormones in low concentrations, which act as chemical messengers to regulate the metabolic pathways of growth simulators in plants and alter root shape when stressed. Several published study findings support the concept that phytohormones synthesis is one of the primary plant stress relief methods used by microorganisms. Auxin and IAA stimulate cellular division, growth, and differentiation in plant roots, as well as the formation of lateral roots, both of which help plants to absorb nutrients and retain water^{54,55}. Salt stress has influenced plants' physiological and biochemical systems and pathways, causing sufficient food absorption, altering growth-inducing regulators, and inhibiting protein synthesis⁵⁶. Bacterial isolates were grown in a nutritional broth medium containing L-tryptophan at varying concentrations (0, 1%, 2%, 3%, 4%, and 5% NaCl). The culture was then incubated for 7 days at ambient temperature. The ability to produce IAA was then determined using the IAA standard curve. *S. isronensis* CR2 produced the largest quantity of IAA (147.8111 ± 2.086^d µg/mL) in 1% and 2% of NaCl stress (120.333 ± 0.882^e µg/mL), followed by *Paenibacillus taichungensis* CR1, which showed (106.889 ± 3.339^f µg/mL) at 1% and (86 ± 3.333^g µg/mL) at 2% NaCl stress.

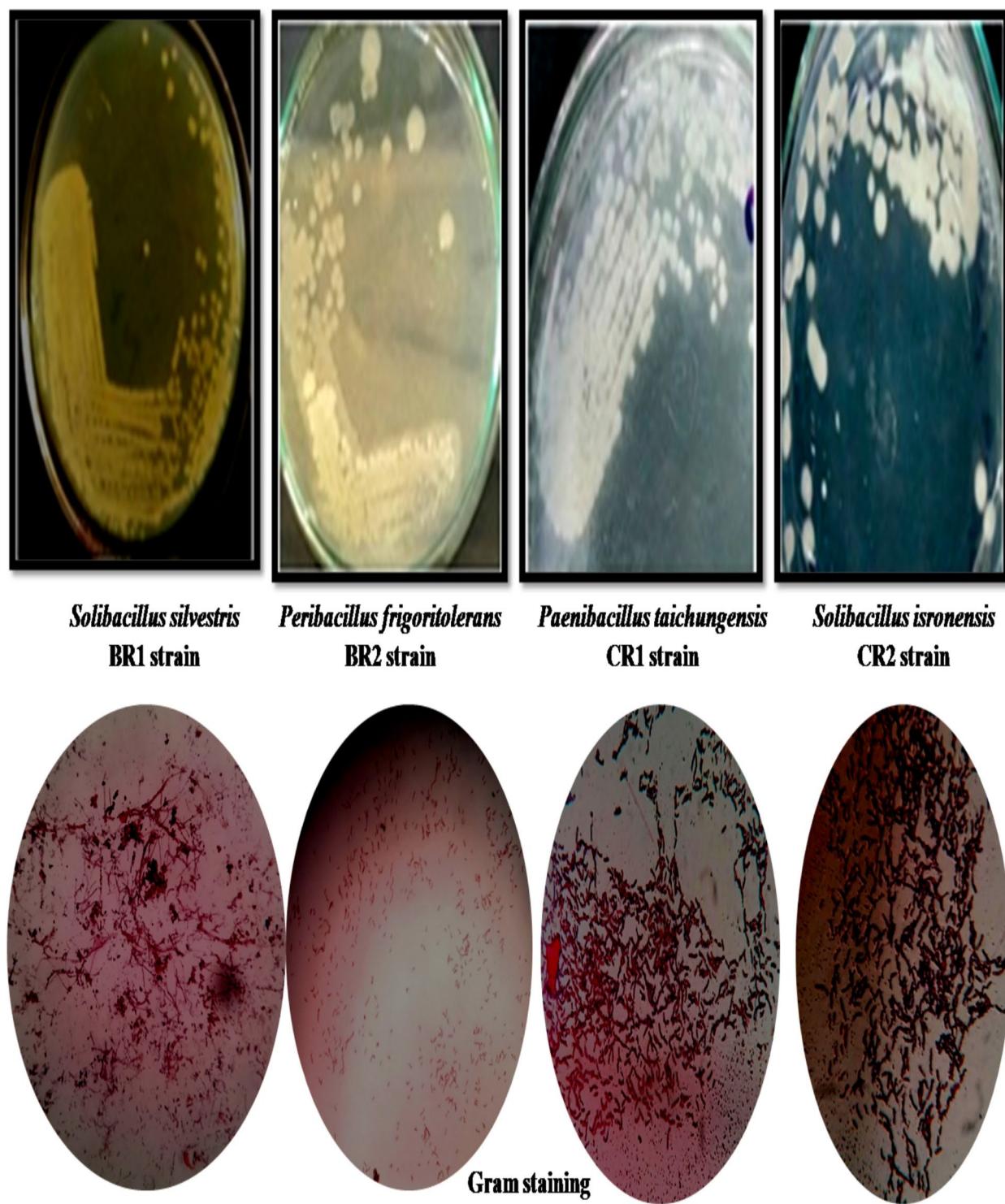


Fig. 1. Screening of salt-stress-resistant plant growth-promoting rhizobacterial strains.

mL) in 2% of NaCl stress (Table 4). At 1% NaCl stress, *Solibacillus silvestris* BR1 produced an average of $(76.889 \pm 0.192^{\text{h}}$ µg/mL), and *Peribacillus frigoritolerans* BR2 produced $(65 \pm 0.57^{\text{h}}$ µg/mL) of IAA. At 5% NaCl stress, *Peribacillus frigoritolerans* BR2 produced $(29.556 \pm 1.171^{\text{n}}$ µg/mL), while *Solibacillus silvestris* BR1 produced $(38.111 \pm 2.411^{\text{m}}$ µg/mL), the least amount of IAA shown in Table 4. *Paenibacillus taichungensis* CR1 $(48.111 \pm 1.575^{\text{l}}$ µg/mL) and *Solibacillus isronensis* CR2 $(52.889 \pm 1.171^{\text{j,k}}$ µg/mL) produced the least amount of indole acetic under 5% NaCl stress. BR2 *P. frigoritolerans* produced significantly less IAA than other strains (Fig. 4b). Similar to our studies, *Hallobacillus* strains produced the highest quantity of IAA compared to SK-Pbt 01 (25.07 µg/mL), SK-Pbt 03 (24.47 µg/mL), and SK-Pbt 01 (19.58 µg/mL) in salinity conditions⁵⁷. Salinity stress

S. No	Morphological characteristics	<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Gram stain	Gram positive	Gram positive	Gram variable	Gram positive
2	Cell shape	Rod shape	Rod shape	Rod shape	Rod
3	Motility	Motile	Non-motile	Motile	Motile
4	Colony	Whitish and shiny	Beige	Shiny and round	Beige
5	Growth	Aerobic	Aerobic	Facultative anaerobe	Mesophilic

Table 1. Morphological characterization of Bacterial Isolates.

S. No	Biochemical tests	<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Indole test	–	–	–	–
2	Sugar utilization test	+	–	+	–
3	Methyl red test	–	+	+	+
4	Voges prokauer test	–	–	+	+
5	Citrate utilization test	–	–	+	–
6	Catalase test	+	+	–	+
7	Ammonium production test	+	+	+	+
8	Potassium hydroxide solubility	+	+	+	+

Table 2. Biochemical characterization of Bacterial Isolates. (+) Indicates the positive and (–) Indicates the negative.

is a big issue for plants since it inhibits their growth and development. High levels of salt raise ethylene levels, cause senescence, chlorosis, and abscission processes, and result in plant death⁵⁸. Halo-tolerant microorganisms have a high potential for agricultural crop productivity in arid and semi-arid settings⁵⁹.

Determination of Phosphate solubilization of Rhizobacteria

Phosphorus stress causes the plant to be unable to absorb nutrients. Phosphate-solubilizing bacteria may help plants cope with stress⁶⁰. In our study, we examined the phosphate solubilization capability of IAA salt-stress-tolerant bacteria on Pikovskayas agar medium using a tricalcium phosphate source. The primary mechanism of phosphate solubilization is the formation of organic acids. Organic acids generated by microorganisms include gluconic, formic, 2-ketogluconic, citric, oxalic, lactic, isovaleric, succinic, glucolic, and acetic acids⁵⁸. *Bacillus* species is the most abundant bacteria in the rhizosphere, have evolved various methods to promote plant growth and boost nutrient availability. Phosphate solubilization was quantified in the liquid broth of a Pikovskay's medium culture with various doses of sodium chloride (0, 1%, 2%, 3%, 4%, and 5%) after 7 days. At 5% NaCl stress, the *Solibacillus isronensis* CR2 strain (0.921 ± 0.007^a µg/mL) and *Paenibacillus taichungensis* CR1 strain ($0.851 \pm 0.012^{a,b}$ µg/mL) exhibited the highest phosphate solubilization levels. Phosphate solubilization was strong at 5% NaCl stress (Table 5). Because the bacterial isolates are exposed to hyper-osmotic-adaptation pathways, they synthesize suitable solutes or accumulate potassium in order to overcome Na⁺ ion toxicity. Many non-halophilic bacteria have also been shown to accumulate potassium in response to sodium stress⁶¹. High NaCl concentrations often disrupt membrane transport systems and denature proteins; however, some microbes have atypical plasma membranes and many peculiar enzymes that allow them to live for prolonged periods of time under such harsh stress circumstances. It has been discovered that osmo-tolerance responses, de novo synthesis of osmolytes, and excess production of salt stress proteins effectively eliminate the negative effects of excessive osmolarity⁶². At 1% NaCl stress, *Solibacillus silvestris* BR1 and *Peribacillus frigoritolerans* BR2 had higher average phosphate solubilization ($0.789 \pm 0.039^{b,c}$ µg/mL and 0.588 ± 0.035^f µg/mL, respectively) (Fig. 4c). Similarly to our findings, the three different *Bacillus* bacteria have rock phosphate solubilization. *Bacillus cereus* S0B4 has been shown to have a high solubilization capacity of 338.5 mg/L. Following other species, *Bacillus amyloliquefaciens* (287.6 mg/L) and *Solibacillus isronensis* S0B8 (271.7 mg/L) have been reported to have the highest amount⁴². The CR2 strain had the highest phosphate solubilization, while the BR2 strain had a smaller quantity (0.145 ± 0.011^e µg/mL) at 5% NaCl stress (Table 5). Phosphorus plays a crucial role in plant photosynthesis, respiration, energy storage and transfer, cell division and growth. Soil microorganisms have the ability to transform inorganic phosphate into an insoluble form due to their solubility capacity. This bacterium is highly beneficial for plant nutrient uptake from phosphorus^{63,64}. It can serve as a future option for organic bio fertilizers.

Ammonium production determination of Rhizobacteria

Ammonium is a significant nutrient for plants and aids in atmospheric nitrogen fixation. Salt stress is a serious issue for ammonium formation in soil. It affects nitrification and ammonification in soil because chloride (Cl⁻) competes with nitrate (NO₃⁻), causing ion toxicities and ionic inequalities that can specifically limit N uptake,

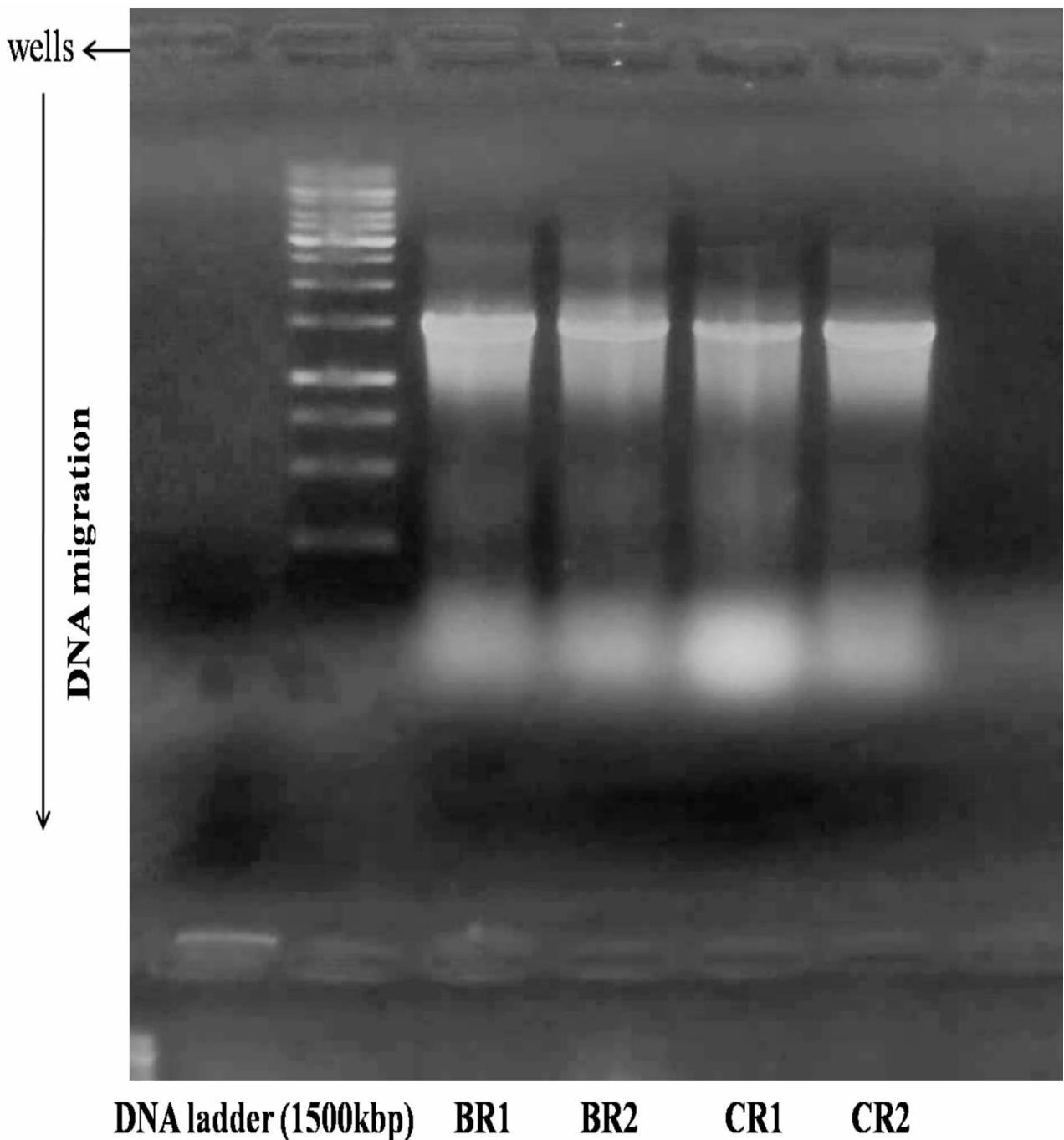


Fig. 2. Genomic DNA isolated from the isolated salt stress resistant plant growth promoting bacterial strains Lane 1 (DNA ladder), Lane 2 (BR1 strain), Lane 3 (BR2 strain), Lane 4 (CR1 strain), and Lane 5 (CR2 strain).

transport, and assimilation processes^{65,66}. The current study demonstrates ammonium generation at various NaCl stress levels using the approach of¹⁹. Ammonium sulfate was used as a standard curve. At 1% NaCl stress, the *Solibacillus isronensis* CR2 strain produced the more production of ammonium (1.202 ± 0.142^a µg/mL), while the *Paenibacillus taichungensis* CR1 strain produced (1.09 ± 0.071^b µg/mL) (Fig. 4d). *Solibacillus silvestris* BR1 ($0.849 \pm 0.003^{f,g}$ µg/mL) and *Peribacillus frigoritolerans* BR2 ($0.814 \pm 0.032^{f,g}$ µg/mL) were later detected. When compared with other strains, BR2 produced the least quantity of ammonium. The BR2 strain had the lowest amount (0.299 ± 0.047^h µg/mL) under 5% NaCl stress (Table 6). Ammonium nitrogen fixation occurred with the help of the nitrogenase enzyme, which reduced H⁺ to H₂. Because the liberated hydrogen gas is lost to the atmosphere, the ATP used to produce is wasted. If any of the strains include the enzyme hydrogenase, they can recover H₂ from the atmosphere and convert it back into H⁺ for the synthesis of ATP, which can then be used for more nitrogen fixation. These strains preserve energy while also fixing nitrogen⁶⁷.

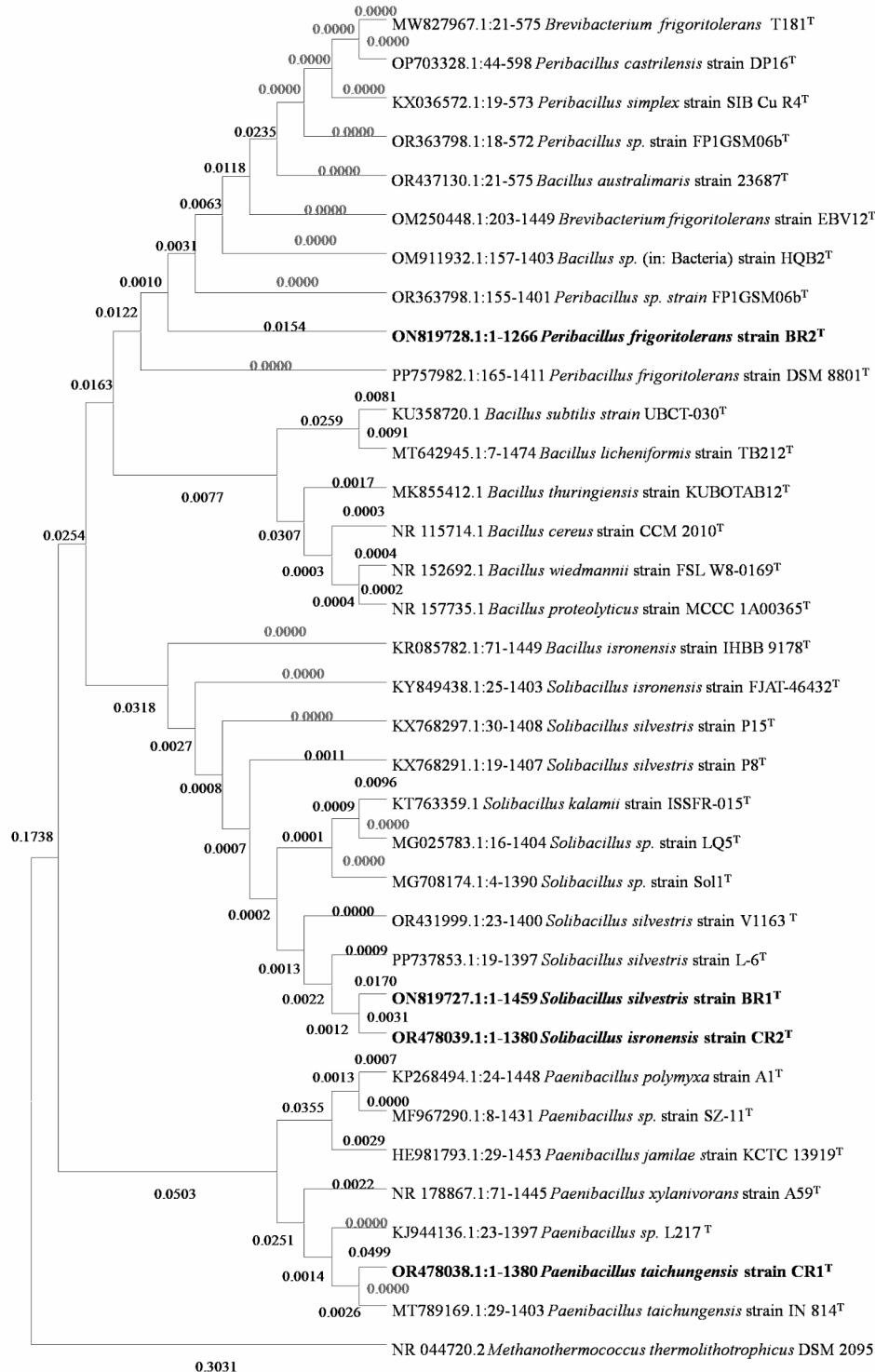


Fig. 3. Phylogenetic tree based on 16S rRNA of a BR1, BR2, CR1 and, CR2 (Bold) closely related species. *Methanothermococcus thermolithotrophicus* DSM 3610 was used as out-group. The percentages of replicate trees (> 50%) in which the associated taxa clustered together in the bootstrap test (1000 replicates). The gene bank accession numbers of 16S rRNA gene sequences and scale bars are shown.

Determination of Hydrogen cyanide production of Rhizobacteria

Hydrogen cyanide (HCN) is one of the secondary metabolites and a volatile molecule. Many rhizobacteria produce them, and they have a potent effect on a wide range of species. Many bacterial genera can produce HCN, including species of *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas*, and *Rhizobium*⁶⁸. In the present study, BR1,

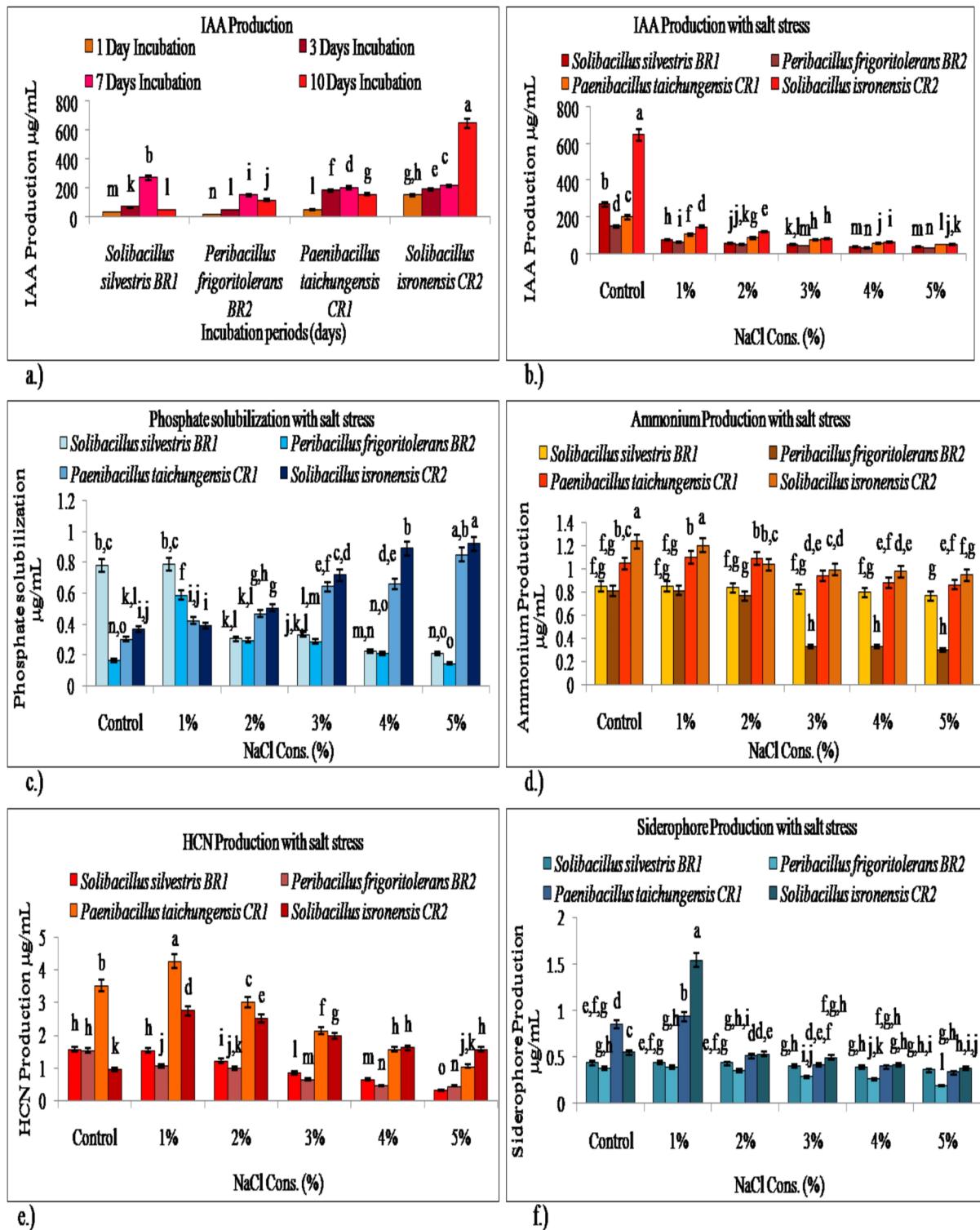


Fig. 4. Effect of salt stress on plant growth promoting properties of rhizobacterial strains BR1, BR2, CR1 and CR2.

BR2, CR1, and CR2 were examined for HCN generation using the sodium cyanide as a standard curve. The CR1 strain produced the most HCN (4.269 ± 0.069^c $\mu\text{g/mL}$) at 1% NaCl stress, followed by (3.013 ± 0.166^c $\mu\text{g/mL}$) at 2% NaCl stress (Fig. 4e). The CR2 strain produced the second-highest amount of HCN (2.756 ± 0.029^d $\mu\text{g/mL}$) at 1% NaCl stress. The BR2 strain had the lowest quantity (0.308 ± 0.051^n $\mu\text{g/mL}$) at 5% NaCl concentration (Table 7). As salt stress increased, HCN production eventually decreased. In plants, HCN indirectly improved

S. No	Incubation periods	IAA production Con. $\mu\text{g/mL}$			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
	1 day	35.66 \pm 1.764 ^m	19.66 \pm 1.528 ⁿ	51.444 \pm 1.262 ^l	150.222 \pm 3.006 ^{g,h}
2	3 days	68.22 \pm 1.503 ^k	46.11 \pm 3.151 ^l	181.222 \pm 1.644 ^f	188.333 \pm 2.186 ^e
3	7 days	268.33 \pm 0.33 ^b	148.55 \pm 1.644 ⁱ	199.778 \pm 3.151 ^d	212.556 \pm 0.509 ^c
4	10 days	46.556 \pm 4.718 ^j	115.66 \pm 0.577 ^l	155.555 \pm 3.006 ^g	646.111 \pm 8.058 ^a

Table 3. Rhizobacteria Indole acetic acid production effect on various Incubation periods. Values represent the mean SE. Means \pm standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

S. No	NaCl (Con. %)	Sodium chloride stress IAA production Con. $\mu\text{g/mL}$			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Control	268.33 \pm 0.33 ^b	148.55 \pm 1.644 ^d	199.77 \pm 3.151 ^c	646.111 \pm 8.058 ^a
2	1%	76.889 \pm 0.192 ^h	65 \pm 0.577 ⁱ	106.889 \pm 3.339 ^f	147.8111 \pm 2.086 ^d
3	2%	57.111 \pm 0.962 ^j	53 \pm 1.856 ^{j,k}	86 \pm 3.333 ^g	120.333 \pm 0.882 ^e
4	3%	49.77 \pm 1.540 ^{k,l}	42 \pm 1.732 ^m	78.667 \pm 1.764 ^h	80.889 \pm 1.678 ^h
5	4%	39.444 \pm 0.839 ^m	32.22 \pm 1.171 ⁿ	56.111 \pm 0.839 ^j	63.222 \pm 0.509 ⁱ
6	5%	38.111 \pm 2.411 ^m	29.556 \pm 1.171 ⁿ	48.111 \pm 1.575 ^l	52.889 \pm 1.171 ^{j,k}

Table 4. Sodium chloride stress on Indole acetic acid production of Rhizobacteria. Values represent the mean SE. Means \pm standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

S. No	NaCl (Con. %)	Phosphate solubilization Con. $\mu\text{g/mL}$			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Control	0.779 \pm 0.110 ^{b,c}	0.163 \pm 0.054 ^{h,o}	0.301 \pm 0.001 ^{k,l}	0.368 \pm 0.001 ^{j,k}
2	1%	0.789 \pm 0.039 ^{b,c}	0.588 \pm 0.035 ^f	0.423 \pm 0.012 ^{i,j}	0.390 \pm 0.010 ⁱ
3	2%	0.331 \pm 0.103 ^{k,l}	0.296 \pm 0.031 ^{k,l}	0.465 \pm 0.011 ^{g,h}	0.506 \pm 0.004 ^g
4	3%	0.305 \pm 0.044 ^{j,k,l}	0.285 \pm 0.096 ^{l,m}	0.642 \pm 0.011 ^{e,f}	0.717 \pm 0.002 ^{c,d}
5	4%	0.224 \pm 0.029 ^{m,n}	0.210 \pm 0.002 ^{n,o}	0.662 \pm 0.003 ^{d,e}	0.892 \pm 0.014 ^b
6	5%	0.208 \pm 0.015 ^{n,o}	0.145 \pm 0.011 ^o	0.851 \pm 0.012 ^{a,b}	0.921 \pm 0.007 ^a

Table 5. Effect of inorganic Phosphate solubilization of Rhizobacteria with salt stress. Values represent the mean SE. Means \pm standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

S. No	NaCl (Con. %)	Ammonium production Con. $\mu\text{g/mL}$			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Control	0.850 \pm 0.002 ^{f,g}	0.811 \pm 0.031 ^{f,g}	1.047 \pm 0.052 ^{b,c}	1.239 \pm 0.031 ^a
2	1%	0.849 \pm 0.003 ^{f,g}	0.814 \pm 0.032 ^{f,g}	1.099 \pm 0.071 ^b	1.202 \pm 0.142 ^a
3	2%	0.838 \pm 0.007 ^{f,g}	0.768 \pm 0.062 ^g	1.087 \pm 0.095 ^b	1.037 \pm 0.006 ^{b,c}
4	3%	0.825 \pm 0.040 ^{f,g}	0.329 \pm 0.015 ^h	0.939 \pm 0.006 ^{d,e}	0.994 \pm 0.074 ^{c,d}
5	4%	0.797 \pm 0.031 ^{f,g}	0.327 \pm 0.001 ^h	0.878 \pm 0.028 ^{e,f}	0.979 \pm 0.030 ^{d,e}
6	5%	0.765 \pm 0.008 ^g	0.299 \pm 0.047 ^h	0.865 \pm 0.034 ^{e,f}	0.947 \pm 0.140 ^{f,g}

Table 6. Sodium chloride stress on Ammonium production of Rhizobacteria. Values represent the mean SE. Means \pm standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

S. No	NaCl (Con. %)	HCN production Con. µg/mL			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Control	1.526 ± 0.011 ^h	1.577 ± 0.051 ^h	3.519 ± 0.051 ^b	0.952 ± 0.023 ^k
2	1%	1.058 ± 0.038 ^j	1.538 ± 0.051 ^h	4.269 ± 0.069 ^a	2.756 ± 0.029 ^d
3	2%	0.981 ± 0.084 ⁱ	1.218 ± 0.029 ^{j,k}	3.013 ± 0.166 ^c	2.526 ± 0.062 ^e
4	3%	0.635 ± 0.038 ^l	0.846 ± 0.019 ^m	2.128 ± 0.113 ^f	1.987 ± 0.029 ^g
5	4%	0.449 ± 0.048 ^m	0.641 ± 0.048 ⁿ	1.571 ± 0.022 ^h	1.615 ± 0.022 ^h
6	5%	0.436 ± 0.062 ^o	0.308 ± 0.051 ⁿ	1.045 ± 0.011 ^{j,k}	1.571 ± 0.011 ^h

Table 7. Sodium chloride stress on Hydrogen cyanide production of Rhizobacteria. Values represent the mean SE. Means ± standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

S. No	NaCl (Con. %)	Siderophore production Con. µg/mL			
		<i>Solibacillus silvestris</i> BR1 Strain	<i>Peribacillus frigoritolerans</i> BR2 Strain Strain	<i>Paenibacillus taichungensis</i> CR1 Strain	<i>Solibacillus isronensis</i> CR2 Strain
1	Control	0.437 ± 0.007 ^{e,f,g}	0.381 ± 0.005 ^{g,h}	0.549 ± 0.096 ^d	0.852 ± 0.071 ^c
2	1%	0.443 ± 0.002 ^{e,f,g}	0.387 ± 0.013 ^{g,h}	0.936 ± 0.036 ^b	1.543 ± 0.108 ^a
3	2%	0.432 ± 0.004 ^{e,f,g}	0.352 ± 0.006 ^{g,h,i}	0.512 ± 0.019 ^{d,e}	0.531 ± 0.027 ^d
4	3%	0.400 ± 0.076 ^{g,h}	0.288 ± 0.012 ^j	0.418 ± 0.006 ^{f,g,h}	0.493 ± 0.067 ^{d,e,f}
5	4%	0.389 ± 0.003 ^{g,h}	0.262 ± 0.003 ^{j,k}	0.394 ± 0.091 ^{g,h}	0.414 ± 0.011 ^{f,g,h}
6	5%	0.358 ± 0.002 ^{g,h,i}	0.190 ± 0.064 ^l	0.332 ± 0.060 ^{h,i,j}	0.378 ± 0.011 ^{g,h}

Table 8. Sodium chloride stress on Siderophore production of Rhizobacteria. Values represent the mean SE. Means ± standard error. The *p* value is less than 0.05 statistically significant, the null hypothesis rejected.

phosphorus availability through metal chelation and sequestration, boosting nutrient availability for both rhizobacteria and host plants⁶⁹.

Determination of Siderophore production of Rhizobacteria

Rhizosphere soil microorganisms have the potential to create siderophores. They are bacterial molecules that are organic in nature and weigh 500 to 1500 Da. They are specialized metal-chelating Fe³⁺ compounds generated by microorganisms under constrained conditions to boost their iron absorption ability^{70,71}. In this work, we determined the catecholate type of siderophore manufacturing ability using sodium salicylate as the reference. The *Solibacillus isronensis* CR2 strain (1.543 ± 0.108^a µg/mL) and *Paenibacillus taichungensis* CR1 (0.936 ± 0.036^b µg/mL) produced the most siderophores at 1% NaCl concentration (Table 8). The *Peribacillus frigoritolerans* BR2 stain revealed the lowest amount (0.190 ± 0.064^l µg/mL) when NaCl stress was increased by 5%. Our research found that increasing the salt concentration level resulted in the lowest amount of siderophore formation. The BR2 strain produced the least quantity, followed by CR1, BR1, and CR2 at 5% NaCl stress (Fig. 4f). Siderophores are classified into three groups: hydroxamate, catecholate, and carboxylate⁷². However, some siderophores include ligands that contain a combination of lysine, ornithine, and histamine derivatives⁷³. Siderophore synthesis may be regulated by environmental factors such as pH, metal pollution, and microbe-Fe₃⁺ interactions⁷⁴. Plant growth and development involve colonizing the plant roots, eliminating iron from the surface area, and producing an environment conducive to root development⁷⁵. Our studies showed that rhizobacteria have the ability to create plant growth-promoting compounds. In the future, beneficial microorganisms from rhizosphere soil will be extracted and turned into bio fertilizers. It will promote plant growth and development as non-hazardous and environmentally friendly manner.

Conclusion

Salinity stress is a major issue with agricultural crops. These issues are primarily attributable to environmental causes and ecological changes. Eco-friendly and non-hazardous alternative technology is required to overcome salt stress. From these additional viewpoints, plant growth-promoting microorganisms can be used to implement novel tactics. Rhizosphere soil microorganisms serve a range of functions in plants. It transfers nutrients from the earth to the plant. This microorganism is useful for regulating soil pollution, fertility, erosion, heavy metal contamination, and organic and inorganic contaminants. So, it can be utilized to participate in the plant growth and development process in a non-hazardous way. Our research indicated that the isolated salt-stress-tolerant bacteria have the ability to produce plant growth compounds. The four salt-tolerant bacterial species were screened out. In this study, the bacterial strains *Solibacillus silvestris* BR1, *Peribacillus frigoritolerans* BR2, *Paenibacillus taichungensis* CR1, and *Solibacillus isronensis* CR2 were identified. In comparison, *S. isronensis* CR2 has a higher capacity for salt tolerance and produces a large number of plant growth-promoting chemicals such as IAA, phosphate solubilization, ammonium synthesis, HCN, and siderophores. Our research found that *Solibacillus isronensis* CR2 and *Paenibacillus taichungensis* CR1 are more effective and capable of producing high-

volume of plant growth-promoting activities. It can also boost agricultural crop yields. The future perspective is that microbes' genes can be conveyed to plants. It can also improve the agricultural production of specific crops in salt stressed condition.

Data availability

The DNA sequences generated in this study have been deposited on the public database NCBI (National Centre for Biotechnology Information), and they were also deposited under the accession numbers of the strains BR1 ON819727.1, BR2 ON819728.1, CR1 OR478038.1, and CR2 OR478039.1.

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Author contributions

NR and CK-these two authors have equally contributed to this work. Both authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

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The authors have given consent for publication.

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Correspondence and requests for materials should be addressed to C.K.

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