Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Diversity and Functional Traits of Free-Living Rhizobacteria from the Agricultural Rhizosphere in Bangladesh: Implications for Biostimulation, Biocontrol, and Stress Resilience

Tasnia Nuzhat
University of Dhaka
Anowara Begum
University of Dhaka
Humaira Akhter
humaira@du.ac.bd

University of Dhaka

Research Article

Keywords: Agricultural rhizosphere, Free-living rhizobacteria, PGP and biocontrol, Phylogenetic diversity, Stress-tolerant diazotrophs

Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11482524/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Title: Diversity and Functional Traits of Free-Living Rhizobacteria from the Agricultural Rhizosphere in Bangladesh: Implications for Biostimulation, Biocontrol, and Stress Resilience

Running title: Free-living PGPR in Agricultural Rhizosphere

Names and Email Addresses of Authors:

Tasnia Nuzhat[1,2]	:	tasnianuzhat01@gmail.com
Anowara Begum, PhD[1]	:	anowara@du.ac.bd
Humaira Akhter[1*]	:	humaira@du.ac.bd

Authors' Affiliations:

[1] Department of Microbiology, University of Dhaka, Dhaka, Bangladesh

[2] Bangladesh Reference Institute for Chemical Measurements (BRiCM), Dhaka, Bangladesh

***Corresponding Author:**

Name: Humaira Akhter

Designation: Professor, Department of Microbiology, University of Dhaka, Ramna, Dhaka - 1000

Contact Number: 01552314010

Email Address: humaira@du.ac.bd

Abstract

Background: Plant Growth-Promoting Rhizobacteria (PGPR) are root-colonizing soil microbes that enhance soil fertility and disease resistance. The current study aimed to obtain and characterize free-living, nitrogen-fixing PGPR from agricultural rhizospheres to investigate their potential to enhance sustainable plant growth through nutrient acquisition, metabolite synthesis, phytopathogen suppression, and stress tolerance. The major goal is to promote sustainable plant growth and soil health using PGPR as eco-friendly biofertilizers. The primary traits screened in PGP strains were siderophore production, Indole-3-acetic acid (IAA) synthesis, and phosphate solubilization. Additional attributes assessed included zinc solubilization, production of ACC deaminase, ammonia, hydrogen cyanide (HCN), hydrolytic enzymes (cellulase and pectinase), and biofilm formation in capable isolates. The effects of abiotic stressors, including temperature variations, pH fluctuations, salinity stress, and pesticide exposure, were also examined.

Results: The study obtained 66 nitrogen-fixing strains from diverse agricultural soils, of which 48 were phylogenetically identified as belonging to the phyla Firmicutes (n=18 isolates), Actinobacteria (n=15 isolates), and Proteobacteria (n=15 isolates). The three most prevalent genera identified were *Bacillus* (n=9), *Priestia* (n=8), and *Arthrobacter* (n=4). Functional assessment of siderophore synthesis (54.54%), phosphate solubilization (37.88%), and IAA production (40.91%) identified 31 competent strains with at least two of these biostimulating traits, which were selected for further study. The efficient diazotrophic isolates also exhibited high ammonia production (83.87%) and zinc solubilization (51.61%), alongside moderate synthesis of HCN (29.03%), cellulase (51.61%), and limited pectinase (6.45%), with siderophore formation further enhancing nutrient acquisition and pathogen suppression. Stress tolerance was evident through salinity and alkalinity resistance, ACC deaminase activity in 25.8% of strains, and biofilm formation in 15.15%

of isolates, supporting their resilience under adverse conditions. Considering these combined traits, *Acinetobacter soli*, *Pantoea coffeiphila*, and *Burkholderia* sp. were identified as the most efficient bioinoculant candidates, offering strong potential for sustainable biofertilizer development across diverse agricultural regions.

Conclusion: Free-living PGPR harbor diverse functional attributes that make them promising biostimulant and biocontrol agents, serving as a promising alternative to synthetic fertilizers or pesticides. Bioinoculation of these diazotrophs into nutrient-deficient agroecosystems having diverse environmental stressors can improve soil fertility, suppress disease, and enhance stress tolerance while reducing environmental risk.

Keywords: Agricultural rhizosphere, Free-living rhizobacteria, PGP and biocontrol, Phylogenetic diversity, Stress-tolerant diazotrophs

1. Introduction

Crop yields in Bangladesh are drastically declining due to population explosion, rising food demand, and changing climate. Environmental risks include excessive use of agricultural land, dumping of unprocessed industrial waste, uncontrolled administration of synthetic fertilizers and pesticides, soil nutrient deficits, and changing abiotic conditions. This concerning phenomenon demands an eco-friendly approach to sustainable crop cultivation that uses Plant Growth-Promoting Rhizobacteria (PGPR) to enhance yield, suppress diseases, and improve stress tolerance across diverse crop varieties, such as rice, wheat, maize, onion, and tomato [1–4].

The rhizosphere is described as layers of soil located in the vicinity of plant roots and under the direct influence of plant-exuded substances [5], which act as a niche for a diverse microbial community. Joseph W. Kloepper introduced the term "PGPR" in the late 1970s, referring to microorganisms that inhabit the close proximity of plant roots and uphold plant development and disease resistance [6]. The author observed accelerated growth in potato plants by *Pseudomonas* sp. isolated from potato and celery roots [7], evidenced by enhanced phytohormone synthesis, nutrient acquisition, pathogen suppression, and stress resistance [8]. PGPR occur in nature in two varieties: extracellular PGPR (ePGPR), which occupy the rhizosphere, rhizoplane, and spaces external to plant cells, and intracellular PGPR (iPGPR), which persist inside specialized nodular forms within plant cells [8]. A wide range of bacterial and fungal communities, including *Acinetobacter*, *Arthrobacter, Enterobacter, Pseudomonas, Azotobacter, Micrococcus, Caulobacter, Aeromonas*, *Agrobacterium, Bacillus*, *Paenibacillus*, *Azorhizobium*, *Rhizobium, Azospirillum*, *Bradyrhizobium*, *Streptomyces, Mesorhizobium, Burkholderia*, *Chromobacterium*, *Serratia, Flavobacterium*, *Erwinia*, *Klebsiella, Gluconacetobacter*, and *Pantoea*, reside as free-living non-

symbiotic PGPR [8–10], whose growth-enhancing characteristics have been extensively studied over the years.

Free-living diazotrophs predominantly possess a molybdenum-associated nitrogenase enzyme system for biological nitrogen fixation (BNF) encoded by the *nif* gene cluster, which converts atmospheric nitrogen into plant-usable compounds, such as ammonia, nitrate, and nitrite [11]. A terrestrial ecosystem is a large reservoir of minerals, including phosphorus, potassium, zinc, iron, and magnesium, yet plants cannot access them in their insoluble form. PGPR, especially *Bacillus* spp. and *Pseudomonas* spp. synthesize low-molecular-weight organic acids, which aid in the mobilization and acquisition of minerals in usable forms by plants [12–16]. In addition, diverse soil microbes produce phytohormones, such as gibberellins, auxins, cytokinins, abscisic acid, and ethylene, which promote plant cell growth and root elongation [17, 18]. Therefore, inoculating these microbes as biostimulants in agricultural fields, particularly in nutrient-deficient soil under stress conditions, can support sustainable crop practices. “Plant biostimulants” were originally described as “materials that, in minute quantities, promote plant growth” [19], which has been further attributed to mineral sequestration and the elevation of plant hormonal mechanisms [20] by numerous researchers.

Various indigenous microbes exploit several inhibitory mechanisms to suppress soil-borne pathogens, such as the synthesis of antimicrobial substances (phenazines, amphysines, hydrogen cyanide, polymyxin, oomycins, cyclic lipopeptides, subtilin, and bacillisin), outcompeting pathogens by siderophore-mediated iron acquisition, production of hydrolytic enzymes (cellulase, protease, pectinase, lipase, chitinase), and inducing systemic resistance (ISR) [3, 12, 21–24]. Hence, many researchers have devoted considerable effort to studying the use of PGPR as

promising biocontrol agents for disease suppression, serving as substitutes for synthetic insecticides, pesticides, and herbicides while mitigating environmental burdens.

In addition to soil nutrient composition, agricultural productivity can be substantially affected by numerous abiotic factors. Extreme temperatures can interfere with enzyme activity, and an imbalance in pH can restrict nutrient accessibility to plants [25–27]. While salinity causes osmotic imbalance, drought reduces water availability [10, 28, 29]. As a consequence, numerous studies have examined the potential of diazotrophic organisms, such as *Bacillus* and *Pseudomonas* [30, 31], to help minimize stress factors through multiple metabolic pathways, particularly by synthesizing the ACC deaminase enzyme to reduce stress-induced ethylene content of soil [28, 29, 32]. Furthermore, various soil flora, including *Rhizobium, Bradyrhizobium, Zanthomonas, Bacillus, Enterobacter, Pseudomonas,* and *Agrobacterium*, form biofilms, a well-organized sessile bacterial encasement within an EPS matrix containing polysaccharides, proteins, lipids, and extracellular DNA [2]. Many reports on diazotrophs highlight the ability of biofilm-forming microbes to withstand environmental or antibacterial stressors, owing to their mechanical stability and intracellular communication [2, 4, 30, 33].

The present investigation was conducted to isolate and characterize free-living nitrogen-fixing PGPR from diverse agricultural soils and to examine their biostimulation and biocontrol potentials, as well as their tolerance to stress factors. The core aim of the work was to (i) isolate free-living diazotrophs from vegetables and crops rhizosphere, (ii) perform phenotypic and molecular characterization of the isolates through 16S rRNA sequencing, (iii) evaluate their potential plant growth-promoting and biocontrol attributes and compare them among different phylogenetic groups, and (iv) assess the influence of abiotic stress conditions (pH, temperature, salinity) and different pesticides (diazinon and dimethoate) on the growth of the strains.

2. Materials and Methods

2.1 Sampling of Agricultural Soil

A total of 22 rhizosphere soil samples were collected from various agricultural fields in Dhaka, Bangladesh, during November and December 2022. Samples were obtained from the rhizospheres of okra (*Abelmoschus esculentus*, n=2), Malabar spinach (*Basella alba*, n=4), pumpkin (*Cucurbita pepo*, n=1), bottle gourd (*Lagenaria siceraria*, n=4), bitter gourd (*Momordica charantia*, n=1), tomato (*Solanum lycopersicum*, n=2), eggplant (*Solanum melongena*, n=2), and maize (*Zea mays*, n=2), as well as from orchid garden soil (n=2) and aquatic soil (n=2). Soil adhering to plant roots at a depth of 20-25 cm [34] was collected in sterile zip-lock bags, transported to the Environmental Microbiology Laboratory, Department of Microbiology, University of Dhaka, and stored at 4°C until further analysis.

2.2 Isolation and Phenotypic Characterization of the Nitrogen-Fixing Bacterial Strains

One gram of the soil sample was mixed with 9 mL of sterile 0.85% NaCl solution, and the suspension was serially diluted tenfold up to a 10^{-5} dilution. From each dilution, 100µl aliquots were spread on Norris glucose nitrogen-free agar plates [Glucose, 10.0 g; Dipotassium hydrogen phosphate, 1.0 g; Magnesium sulfate, 0.2 g; Calcium carbonate, 1.0 g; Sodium chloride, 0.2 g; Sodium molybdate, 0.005 g; Ferrous sulfate, 0.1 g; Agar, 15.0 g; Distilled water, 1000 mL] [9] and were incubated at 30°C for 72 hours. Individual colonies capable of fixing atmospheric nitrogen were morphologically characterized by size, shape, color, elevation, margin, opacity, and texture. The colonies were purified on nutrient agar plates, and 2 sets of distinct isolates were preserved in sterile 50% glycerol at -40°C. Cellular morphology of the isolates was observed under the microscope using the Gram stain method [35]. The isolates were also biochemically analyzed for

citrate utilization, starch hydrolysis, Kligler's Iron Agar (KIA) test, MIU (motility, indole, urease) test, MR-VP (Methyl Red/Voges-Proskauer) test, oxidase test, catalase test, nitrate reduction, and gelatin hydrolysis [36].

2.3 Molecular Characterization of the Strains

Genomic DNA of rhizobacterial isolates was extracted using the boiling method [37], and the 16S rRNA gene (1467 bp) was amplified by PCR in a thermocycler (Applied BiosystemsTM, USA) with specific primers (Table 1a) under defined conditions (Table 1b). The amplified products were confirmed by agarose gel electrophoresis, purified (FavorPrep GEL/PCR Purification Mini Kit), and sequenced by Macrogen Inc. Consensus sequences were generated from forward and reverse reads using SeqMan software and analyzed through NCBI BLAST (Basic Local Alignment Search Tool) to determine sequence homology. Phylogenetic relationships among the strains were inferred using the Maximum Likelihood method in MEGA 7. Additionally, PCR targeting the *epsA* gene (451 bp) was performed on biofilm-forming isolates under the same conditions, with amplification confirmed by agarose gel electrophoresis.

2.4 Screening of the Isolates by Primary Plant Growth Promoting (PGP) Traits

2.4.1 Phosphate Solubilization

The ability of the rhizobacterial isolates to utilize insoluble tricalcium phosphate (TCP) was tested using Pikovskaya agar medium and the National Botanical Research Institute's phosphate (NBRIP) medium [38]. The plates were spot-inoculated and incubated at 30°C for up to 7 days. Colonies with a clear zone were identified as positive, and the Phosphate Solubilization Index (PSI) was calculated using the following formula:

Phosphate Solubilization Index (PSI) = (Colony diameter + Clearing zone) / (Colony diameter)

2.4.2 Indole-3-acetic acid (IAA) Production

Indole-3-acetic acid (IAA) production was quantified by inoculating strains in 5 mL of YEM broth supplemented with 0.5% L-tryptophan and incubating at 30 °C for 72 hours, with uninoculated broth serving as a negative control. After incubation, cultures were centrifuged at 4000rpm for 20 minutes at 4°C, and 1 mL of cell-free supernatant was mixed with Salkowski reagent [0.5 M Ferric chloride ($FeCl_3$) and 35% Perchloric acid ($HClO_4$)] in a 1:2 ratio [17]. The development of a pink color after 20 minutes at room temperature indicated IAA synthesis, which was measured spectrophotometrically at 535 nm, using the negative control for calibration.

2.4.2.1 Standard curve preparation for IAA quantification

To quantify IAA production of the strains, predetermined IAA concentrations (0, 2.5, 5.0, 10.0, 15.0, and 20.0 µg/ml) and their corresponding optical densities were used to construct a standard linear regression curve. The regression equation obtained from these data was: $Y = C + 44.8005X$, where X is the optical density of the sample, and Y is the concentration of IAA (µg/ml). The amount of IAA (µg/ml) secreted by the rhizobacterial isolates was determined from the equation.

2.4.3 Siderophore Formation

The siderophore-forming ability of the isolates was examined using the Universal Chrome Azurol Sulfonate (CAS) assay [39]. For iron decontamination, all glassware was cleaned with 6M HCl and then rinsed several times with ddH_2O. A fresh culture of the isolates was spot-inoculated onto the plate and incubated at 30°C for 72 hours. The appearance of an orange halo zone around the colonies indicated siderophore formation, which was measured as the Siderophore Index (SI) using the following formula:

Siderophore Index (SI) = (Colony diameter + halo zone diameter) / (Colony diameter)

2.5 Assessment of Indirect PGP Traits of the Competent Isolates

2.5.1 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

ACC deaminase activity was assessed by growing rhizobacterial isolates on modified DF [40] minimal salts medium supplemented with 3mM ACC as the sole nitrogen source [32]. A sterile stock solution of heat-labile ACC (0.5 M) was filter-sterilized and added to autoclaved medium, with cultures incubated at 30 °C for 72 hours. Growth on ACC-supplemented plates was compared to negative controls lacking ACC, and isolates capable of utilizing ACC as the nitrogen source were identified as ACC deaminase producers.

2.5.2 Ammonia Production

Rhizobacterial isolates were inoculated in 10 mL of peptone broth and incubated at 30°C for 72 hours under shaking conditions to study ammonia production [41]. Following incubation, 0.5 mL of Nessler's reagent was added to the culture broth, which developed a yellow-brown color, confirming ammonia production.

2.5.3 Hydrogen Cyanide (HCN) Production

Hydrogen cyanide (HCN) production by rhizobacterial strains was tested by streaking them on glycine-supplemented (4.4 g/L) nutrient agar slants in tightly capped test tubes containing Whatman Filter paper no. 1 strips saturated with Picric acid solution (0.5% Picric acid in 2% Sodium carbonate) [42]. After incubation at 30 °C for 48 hours, a color change in the filter paper from yellow to light brown, brown, or reddish-brown indicated weak (+), moderate (++), or strong (+++) HCN production, respectively, while no color change was recorded as negative (−).

2.5.4 Zinc Solubilization

Zinc solubilization ability of rhizobacterial isolates was tested using basal agar medium amended with 0.2% ZnO as an insoluble Zn source [2]. Fresh cultures were spot-inoculated and incubated at 30 °C for up to 7 days. The formation of clear zones around colonies indicated zinc solubilization, which was quantified as the Zinc Solubilization Index (ZnSI), following the same approach used for phosphate solubilization.

2.6 Hydrolytic Enzyme Production

Cellulolytic and pectinolytic activities of rhizobacterial strains were evaluated by spot-inoculating fresh cultures on carboxymethylcellulose (CMC) (0.5%) agar plates and pectin-fortified (0.5%) nutrient agar plates, respectively, followed by incubation at 30 °C for 48 hours [43]. After incubation, the plates were flooded with Gram's iodine for 3-5 mins, which revealed sharp, clear zones around the colonies, confirming enzyme hydrolysis and indicating cellulase and pectinase production [44]. The Cellulolytic Index (CI) and Pectinolytic Index (PI) of the positive strains were determined using the same approach as for phosphate solubilization.

2.7 Abiotic Stress Factors Tolerance of the Isolates

2.7.1 Temperature, Salinity, and pH Tolerance

The stress tolerance of efficient rhizobacterial strains was evaluated under varying temperature, salinity, and pH conditions [45]. Growth was tested on inoculated YMA plates incubated at different temperatures (4–52 °C) and with increasing NaCl concentrations (0.05–10%), while pH tolerance was assessed in YEM broth adjusted to pH 3–11 [31, 45]. After overnight incubation at 30 °C, isolates showing growth across these diverse conditions were identified as tolerant to multiple abiotic stresses.

2.7.2 Pesticide Tolerance

The ability of the strains to utilize pesticides as the sole carbon source was investigated using Bushnell-Hass agar media. The media was supplemented with Sabion 60 EC (Diazinon) and Tagfor 40 EC (Dimethoate) at different concentrations (0.05%, 0.075%, 0.1%, 0.3%, and 0.5%) [46]. Following overnight incubation at 30ºC, growth of the pesticide-tolerant isolates was observed on the plates.

2.8 Biofilm Formation Assay

The biofilm-forming ability of the strains was evaluated using the Microtiter Dish Biofilm Formation Assay [47]. Isolates cultured overnight in LB broth were diluted 100-fold and added to three consecutive wells of a microtiter plate. After overnight incubation, cells were washed out, and the biofilm formed in each well was stained with 125 µL of 0.1% crystal violet (CV) solution, then solubilized with 125 µL of 30% acetic acid in water. To determine the absorbance, solubilized CV was transferred to a new flat-bottomed microtiter dish and quantified in a plate reader at 550 nm using 30% acetic acid in water as the blank.

The following criteria [48] were used to categorize the biofilm-forming isolates:

OD_{cut} = OD_{avg} of negative control + 3 × standard deviation (SD) of ODs of negative control

1. $OD \leq OD_{cut}$ = non-biofilm-former (NBF)
2. $OD_{cut} < OD \leq 2 \times OD_{cut}$ = Weak biofilm-former (WBF)
3. $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ = Moderate biofilm-former (MBF)
4. $OD > 4 \times OD_{cut}$ = Strong biofilm-former (SBF)

2.9 Statistical analysis

All analyses were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Differences among PGP traits were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) as a post hoc analysis. IBM SPSS Statistics 20 was employed for statistical analysis, and a p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Isolation and Identification of Free-Living Diazotrophs and Their Distribution across Different Taxa

A total of 66 morphologically distinct bacterial isolates were obtained on Norris glucose medium, indicating potential nitrogen-fixing ability due to their growth on nitrogen-free medium (Figure 1A). Of these, 48 (72.72%) were Gram-positive and 18 (27.27%) were Gram-negative, exhibiting rod, coccoid, and filamentous morphologies (Figure 1B). The phenotypic characteristics of the isolates are presented in Supplementary Table 1 (Table S1). Based on 16S rRNA gene sequencing, 48 isolates were identified, showing 92.31-100% sequence similarity to their closest GenBank relatives (Table 2). The 16S rRNA nucleotide sequences from these isolates have been deposited in the NCBI GenBank database under accession numbers QB266539 to QB266586. These isolates belonged to three phyla: Firmicutes (18 strains), Actinobacteria (15 strains), and Proteobacteria (15 strains) (Figures 2 and 3). Firmicutes were dominated by *Bacillus* and *Priestia*, Actinobacteria by *Arthrobacter* and *Pseudarthrobacter*, and Proteobacteria by genera including *Rhizobium*, *Klebsiella*, *Ensifer*, *Metapseudomonas*, and others. The remaining 18 isolates could not be identified. The taxonomic distribution of all 66 isolates is presented in Figure 4.

3.2 Assessment of Primary PGP Properties

3.2.1 Phosphate Solubilization

After overnight incubation, 25 of 66 isolates (37.88%) showed phosphate-solubilizing ability by forming clear halos on Pikovskaya agar and/or NBRIP medium (Figure 5A). Of these, 14 isolates (21.21%) were positive only on Pikovskaya agar, 5 isolates (7.58%) only on NBRIP medium, and 6 isolates (9.09%) on both media. Statistical analysis showed significant differences in phosphate solubilization among the six common isolates ($p < 0.001$), while the overall effect of the two media was not significant ($p = 0.098$). However, the significant interaction between isolate and medium ($p = 0.013$) indicated that individual isolates responded differently depending on the medium used. The phosphate solubilization index (PSI) ranged from 1.1 to 4.667, with *Pantoea coffeiphila* (CP4) showing the highest phosphate-solubilizing activity on both media (Table 3). Pikovskaya agar contains yeast extract as a nitrogen source and supports the growth of a broad range of bacteria, whereas NBRIP is a chemically defined medium commonly used for evaluating phosphate-solubilizing bacteria, particularly in broth-based assays [38, 49, 50].

3.2.2 Indole-3-acetic Acid (IAA) Production

IAA production was confirmed by the development of a pink color upon addition of Salkowski reagent to tryptophan-supplemented cultures, with quantification performed spectrophotometrically at 535 nm using the regression equation $Y = 44.8005X$. Among 66 isolates, 27 (40.91%) produced at least 10 µg/mL IAA, while 13 exceeded 50 µg/mL, as shown in Figure 5B. The highest producers belonged to diverse genera, including *Pantoea* (CP4), *Rhizobium* (LS8, SL3, ZM2), *Ensifer* (LS12, SM8), *Pseudarthrobacter* (BA12), *Janibacter* (MC3), *and Bacillus* (GS1), representing members of Proteobacteria, Actinobacteria, and Firmicutes (Table 3).

3.2.3 Siderophore Formation

After 72 hours of incubation, 36 of 66 isolates (54.54%) produced siderophores, as indicated by orange halos on CAS agar (Figure 5C). This color change occurs when siderophores remove iron from the CAS–HDTMA dye complex [51]. The siderophore index (SI) ranged from 1.2 to 7.67, with *Acinetobacter soli* (BA16) and *Burkholderia* sp. (ZM6) showing the highest siderophore production, with SI values of 7.5 and 7.67, respectively (Table 3).

3.3 Selection of the Competent Isolates

In addition to nitrogen fixation, 31 isolates possessing at least two additional plant growth-promoting (PGP) traits were selected for further evaluation. Among them, seven isolates (10.6%) (BA16, CP4, LS12, LS18, ZM1, ZM3, and ZM6) exhibited all three key PGP traits: phosphate solubilization, IAA production, and siderophore synthesis. Another seven isolates (10.6%) (CP1, SL4, SM1, SM8, GS1, AS2, and AS4), showed both phosphate solubilization and IAA production, while eight isolates (12.12%), including AE9, BA2, BA17, SM4, SM5, ZM4, ZM7, and AS1, demonstrated phosphate solubilization and siderophore production. Additionally, nine isolates (13.64%) (BA19, CP3, LS8, LS14, SL1, SL2, SL3, ZM2, and GS8), produced both IAA and siderophores. These results highlight the multifunctional nature of several isolates, making them promising candidates for further study as potential biofertilizers.

3.4 Evaluation of Indirect PGP Traits

3.4.1 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

After 72 hours of incubation, eight of the 31 selected isolates (25.8%) grew on ACC-supplemented DF minimal salts medium, confirming their ability to produce ACC deaminase (Figure 6A). These strains belonged to diverse genera, including *Ensifer* (LS12, SM8), *Acinetobacter* (BA16), *Ralstonia* (ZM4), *Burkholderia* (ZM6), *Bacillus* (ZM3), *Priestia* (SL2), and *Arthrobacter* (BA8),

spanning the phyla Proteobacteria, Firmicutes, and Actinobacteria, respectively (Table 4). Since ACC is the direct precursor of the plant stress hormone ethylene, the bacterial enzyme ACC deaminase enhances plant tolerance to drought, salinity, and other biotic and abiotic stresses by hydrolyzing ACC into ammonia and α-ketobutyrate [32, 52].

3.4.2 Ammonia Production

After 72 hours of incubation, ammonia production was confirmed in 26 isolates (83.87%) by the appearance of a yellow to brown color in the culture broth (Figure 6B). The leading producers included Firmicutes such as *Bacillus* (ZM3, GS1) and *Priestia* (BA2, SL4, SM1), along with Proteobacteria including *Acinetobacter* (BA16), *Pantoea* (CP4), *Rhizobium* (LS8), and *Ensifer* (LS12) (Table 4). These results demonstrate the conversion of peptone, a nitrogenous compound, into ammonia during the nitrogen cycle, providing a source of usable nutrients that can enhance plant growth.

3.4.3 Hydrogen Cyanide (HCN) Production

After 48 h of incubation, 9 of 31 isolates (29.03%) produced volatile HCN, indicated by a color change in picric acid-soaked filter paper. Among these, 3 isolates showed moderate HCN production, while 6 showed weak production, and no strong producers were detected (Figure 6C). The main HCN-producing genera were *Rhizobium* (LS8), *Ensifer* (SM8), and *Priestia* (AS4) (Table 4), suggesting a possible role in suppressing soil-borne pathogens. Additionally, *Bacillus* (SM4, AS1) and *Priestia* (BA2) produced an unusual greenish pigment on the filter paper, the cause of which remains unknown.

3.4.4 Zinc Solubilization

Sixteen isolates (51.61%) showed a clear halo zone around the colonies on ZnO-fortified basal agar medium after 7 days of incubation (Figure 6D). The zinc solubilization index (ZnSI) of the

isolates ranged from 1 to 4.5, with isolate CP4 (*Pantoea coffeiphila*) being the highest zinc solubilizer (Table 4). The strain was also found to be a potent phosphate solubilizer, as mentioned earlier, indicating its ability to improve plant health by enhancing the bioavailability of minerals in the soil system and nutrient acquisition.

3.5 Hydrolytic Enzyme Production

In this study, 16 isolates (51.61%) demonstrated cellulase activity, producing clear zones on CMC agar after Gram's iodine treatment (Figure 7A), with cellulolytic indices ranging from 1.09 to 9.33. The strongest cellulase producers were isolates SL1 (CI = 9.33) and ZM7 (CI = 7.33), though they remained unidentified. In contrast, only two isolates (6.45%)—*Glutamicibacter* (CP3) and *Burkholderia* (ZM6)—exhibited pectinase activity, with pectinolytic indices of 5.33 and 1.067, respectively (Figure 7B, Table 4). These findings highlight the role of extracellular enzymes in pathogen suppression through cell wall degradation [53, 54].

3.6 Abiotic Stress Factors Tolerance

3.6.1 Temperature, Salinity, and pH Tolerance

PGPR isolates (n=31) demonstrated resilience to multiple abiotic stresses, with optimal growth observed at 20–37 °C, 0.5–2% NaCl, and pH 7.0 (Figure 8, Supplementary Figure S1). Survival under extreme conditions varied: 12 strains (38.71%) tolerated 4 °C, while 14 strains (45.16%) thrived at elevated temperatures (45–52 °C) (Figure 8A). Salinity tolerance was moderate, with 24 isolates (77.42%) growing at 4% NaCl, but only 9 (29.03%) surviving at 10% NaCl, indicating limited adaptation to highly saline soils (Figure 8B). In terms of pH, 24 isolates (77.42%) grew well at pH 5.0; none survived at pH 3.0, while strong alkaline adaptability was evident as 29 isolates (93.55%) grew at pH 9.0 and 17 (54.84%) at pH 11.0 (Figure 8C). These findings highlight

the ability of PGPR strains to withstand diverse stress conditions, particularly alkaline environments, making them promising bioinoculants for arid and challenging soils.

3.6.2 Pesticide Tolerance

The rhizobacterial isolates demonstrated their ability to tolerate and utilize hazardous organophosphate insecticides. When grown on media supplemented with Diazinon (Sabion 60 EC) and Dimethoate (Tafgor 40 EC), the strains showed similar tolerance up to 0.1% (Supplementary Figure S2). However, only 11 isolates were able to grow at concentrations as high as 0.5% Diazinon, while none survived beyond 0.3% Dimethoate (Figure 8D). These findings suggest that certain isolates can metabolize toxic pesticides as carbon and energy sources, enabling them to proliferate in chemically contaminated soils while maintaining plant growth-promoting activities.

3.7 Biofilm Formation Assay

Biofilm formation was evaluated to determine the root-colonizing ability of rhizobacteria. The study revealed that out of 58 isolates, 7 (12.07%) were strong biofilm formers, 3 (5.17%) moderate, and 20 (34.48%) weak, while the rest showed no biofilm activity (Figure 9). The presence of the *epsA* gene in strong and moderate formers such as *Acinetobacter* (BA16*)*, *Metapseudomonas* (BA18*)*, and *Pantoea* (CP4) confirmed their genetic capacity for exopolysaccharide and amyloid-like fiber biosynthesis (Figure 9C, Table 5). This trait enhances root adhesion, nutrient uptake, and protection against pathogens and stress, underscoring their potential as effective PGPR bioinoculants.

4. Discussion

The present investigation recovered 66 nitrogen-fixing bacterial isolates with diverse morphotypes from crop rhizospheres, highlighting *Bacillus* (9), *Priestia* (8), and *Arthrobacter* (4) as the most

391 prevalent genera. These findings align with earlier reports from maize rhizospheres in Cameroon
392 and other agricultural systems, where *Bacillus, Arthrobacter,* and *Pseudomonas* were dominant
393 PGPR groups [55–57]. In addition, the present work identified other diazotrophs such as
394 *Pseudarthrobacter, Metapseudomonas, Klebsiella, Rhizobium, Microbacterium, Pantoea,* and
395 *Burkholderia*, further supporting previous observations of their widespread occurrence in soil
396 ecosystems and reinforcing their significance as indigenous PGPR communities.

397 The study identified 31 promising rhizobacterial strains (46.97%) that expressed at least two major
398 plant growth–promoting (PGP) traits—phosphate solubilization, IAA production, and siderophore
399 formation—and were selected for further analysis. Most strains, particularly *Bacillus* and *Priestia,*
400 showed strong nutrient-solubilizing abilities, including phosphorus and zinc. High IAA production
401 (>50 µg/mL) was observed in isolates from *Bacillus, Pantoea, Rhizobium, Ensifer,*
402 *Pseudarthrobacter,* and *Janibacter*, while ammonia production was moderate across most strains,
403 supporting nitrogen assimilation. These findings were consistent with reports of diverse PGPR
404 genera (e.g., *Paenibacillus, Lysinibacillus, Pseudomonas, Klebsiella, Enterobacter, Burkholderia,*
405 *Serratia, Stenotrophomonas)* [2, 4, 14, 18, 22, 34, 49, 50, 58] contributing to nutrient
406 solubilization, phytohormone synthesis, and ammonia production in agricultural soils. Studies on
407 wheat and maize rhizosphere revealed that *Pseudomonas, Pantoea, Burkholderia, Enterobacter*,
408 and *Rhizobium* can solubilize Zinc oxide [3, 15, 22]. Free-living bacteria, including *Bacillus,*
409 *Klebsiella*, *Burkholderia, Pseudomonas, Rhizobium*, and *Stenotrophomonas* isolates from diverse
410 crop-cultivating soils, have been mentioned as ammonia producers in several reports [2, 22, 34,
411 59]. Collectively, these traits enhance soil fertility and plant health, positioning the isolates as
412 effective bioinoculants that could reduce reliance on synthetic chemical fertilizers.

Indigenous rhizobacteria employ diverse biocontrol strategies to suppress pathogens, including competition for nutrients, secretion of volatile antimicrobials, and production of hydrolytic enzymes. In this study, *Burkholderia* and *Acinetobacter* were identified as dominant siderophore producers, while some *Metapseudomonas* and *Bacillus* strains showed minor siderophore activity. Siderophore-forming bacteria, including *Pseudomonas, Bacillus, Rhizobium, Pantoea, Enterobacter, Burkholderia, Arthrobacter,* and *Beijerinckia*, chelate iron from soils and deprive other pathogens of nutrition [22, 31, 34, 51, 60–62]. The study identified *Priestia, Ensifer*, and *Rhizobium* as HCN-secreting organisms, highlighting their potential to disrupt pathogen respiration, a trait observed in several other strains including *Pseudomonas, Bacillus, Burkholderia, Providencia,* and *Serratia* [21, 22, 41, 63]. Additionally, extracellular enzyme activity was noted, with *Klebsiella* and *Glutamicibacter* exhibiting cellulase and pectinase production. These findings, consistent with reports of diverse soil bacteria secreting lytic enzymes, such as *Paenibacillus, Arthrobacter, Pseudomonas, Enterobacter, Staphylococcus, Azorhizobium,* and *Brevibacillus* [2, 34, 43, 44, 53, 54, 64, 65], underscore the isolates' ability to suppress deleterious microbes and position them as promising candidates for biocontrol applications in agriculture.

Among the 31 potential isolates, *Bacillus* (5) and *Priestia* (6) were the dominant genera, while none of the *Arthrobacter* strains qualified due to lacking sufficient PGP traits. Figure 10 illustrates the comparison of these leading PGP properties, along with other indirect PGP traits, including synthesis of ACC deaminase, ammonia, HCN, and zinc mobilization across the *Bacillus* and *Priestia* genera. Phosphate solubilization was consistently observed in all *Bacillus* (100%) and most *Priestia* (83.33%) isolates. Siderophore production was detected in three *Bacillus* (60%) and two *Priestia* (33.33%) strains, with additional activity noted in four other *Bacillus* and *Priestia*

strains beyond the selected ones and in one non-selected *Arthrobacter* isolate. IAA production was more frequent in *Priestia* (83.33%) compared to *Bacillus* (40%). Indirect PGP traits were less common, with hydrogen cyanide synthesis found in two *Bacillus* and two *Priestia* strains, and ACC deaminase activity in one *Bacillus* and two *Priestia* isolates. Zinc solubilization was predominant in *Bacillus* (80%), while ammonia production was universal in *Priestia* (100%) but lower in *Bacillus* (60%). These findings highlight the strong nutrient-solubilizing and ammonia-producing potential of *Priestia,* alongside *Bacillus*'s dominance in phosphate and zinc mobilization.

The selected rhizobacterial isolates demonstrated notable tolerance to diverse abiotic stresses, including salinity, pH, temperature, and pesticide exposure. About 29.3% of strains survived up to 10% NaCl, while most showed strong growth under alkaline conditions, supporting their suitability for arid and semi-arid soils. Research on diverse soils, including the wheat rhizosphere, demonstrated that *Bacillus* sp. expressed PGP traits more strongly under high salinity up to 8% [10, 31, 55]. Biofilm-forming strains exhibited enhanced resilience and growth promotion compared to planktonic ones, consistent with reports of *Bacillus* and *Pseudomonas* thriving under high salinity and elevated temperatures [2]. The identified isolates also tolerated organophosphate pesticides at concentrations of at least 0.1%, indicating their ability to persist in chemically treated soils. Furthermore, several strains in this study synthesized ACC deaminase, including *Priestia, Bacillus, Acinetobacter, Ensifer, Ralstonia,* and *Burkholderia*, which reduces stress-induced ethylene levels and enhances plant tolerance to drought, salinity, and other abiotic stresses. Several reports mentioned ACC deaminase production by soil-dwelling *Bacillus, Lysinibacillus, Pseudomonas, Staphylococcus, Micrococcus, Arthrobacter, Burkholderia, Citrobacter, Enterobacter, Pantoea, Klebsiella,* and *Ralstonia* [28, 29, 34, 52]. Collectively, these traits

highlight the adaptability of the isolates and their potential as robust bioinoculants for challenging agricultural environments.

In this current study, three isolates, BA16 (*Acinetobacter soli*), CP4 (*Pantoea coffeiphila*), and ZM6 (*Burkholderia* sp.), emerged as the most promising bioinoculant candidates, as they expressed multiple plant growth–promoting and biocontrol traits, including phosphate and zinc solubilization, phytohormone (IAA) production, siderophore formation, and ammonia synthesis. Notably, CP4 uniquely secreted HCN, while ZM6 exhibited pectinolytic activity, enhancing pathogen suppression. ACC deaminase activity in BA16 and ZM6 further supports their resilience under stress conditions such as salinity, temperature extremes, alkaline pH, and pesticide exposure. These multifunctional traits highlight their strong potential for *in vitro* and *in vivo* applications in crop growth enhancement. So far, many researchers have made significant, successful attempts to investigate the role of these free-living rhizobacteria in supporting the growth of rice and tomato under laboratory conditions, such as *Acinetobacter* sp. for tomato [66, 67], onion [68]; *Pantoea* sp. for capsicum [13], maize, rice, wheat [69]; and *Burkholderia* sp. for maize [16, 70, 71], rice [72], sugarcane [73], reinforcing their suitability as sustainable biofertilizers.

5. Conclusion

Bangladesh's agriculture faces mounting challenges from population pressure, rising food demand, land overuse, pesticide misuse, industrial waste, and climate change. To address these issues, this study explored rhizobacterial isolates with diverse plant growth–promoting (PGP) traits. Out of 66 diazotrophic strains screened, 31 efficient candidates were identified, showing capabilities such as nutrient mobilization, metabolite production for growth and pathogen control, biofilm formation, and resilience under stress. Among them, *Acinetobacter soli* (BA16), *Pantoea*

coffeiphila (CP4), and *Burkholderia* sp. (ZM6) stood out as the most promising bioinoculant strains. Their introduction into nutrient-deficient soils could enhance crop yields while reducing environmental burdens. These findings expand our knowledge of free-living diazotroph diversity and provide scientific evidence of their functional value as bioinoculants. However, before broad agricultural application, further research is needed on quantitative analysis of functional traits under diverse stress conditions, bacterial population stability, formulation design, biosafety, and season-specific field trials to ensure safe and effective integration into sustainable cultivation practices.

List of abbreviations

ACC, 1-aminocyclopropane-1-carboxylate

ANOVA, Analysis of variance

BLAST, Basic Local Alignment Search Tool

BNF, Biological nitrogen fixation

CAS, Chrome Azurol Sulfonate

CI, Cellulolytic index

CMC, Carboxymethylcellulose agar

CV, Crystal violet

DMRT, Duncan's Multiple Range Test

EPS, Extracellular polymeric substance

501 HCN, Hydrogen cyanide

502 IAA, Indole-3-acetic acid

503 MBF, Moderate biofilm-former

504 MR-VP, Methyl Red/Voges-Proskauer

505 NBF, Non-biofilm-former

506 NBRIP, National Botanical Research Institute's phosphate medium

507 PGPR, Plant growth promoting rhizobacteria

508 PI, Pectinolytic index

509 PSI, Phosphate solubilization index

510 PVK, Pikovskaya agar

511 SBF, Strong biofilm-former

512 SI, Siderophore index

513 TCP, Tricalcium phosphate

514 WBF, Weak biofilm-former

515 YEM, Yeast extract mannitol broth

516 YMA, Yeast extract mannitol agar

517 ZnSI, Zinc solubilization index

518

Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All relevant data supporting the findings of this study are included in the article and its supplementary materials, where applicable.

The 16S rRNA nucleotide sequences have been deposited in the NCBI GenBank database under accession numbers QB266539 to QB266586.

Competing interests

The authors declare that they have no competing interests

Funding

The research is funded by the Centre for Advanced Studies and Research in Biological Sciences, University of Dhaka, Dhaka-1000, Bangladesh (2023-24).

Authors' contributions

TN conducted experiments, collected and analyzed the data, and was a major contributor to writing the manuscript. AB provided scientific guidance and critically reviewed and edited the manuscript.

HA was responsible for the study design and supervision of the research and contributed to the final revision. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to the Centre for Advanced Studies and Research in Biological Sciences University of Dhaka, Dhaka-1000, Bangladesh, for funding the present project.

References

1. Aasfar A, Bargaz A, Yaakoubi K, Hilali A, Bennis I, Zeroual Y, et al. Nitrogen fixing Azotobacter species as potential soil biological enhancers for crop nutrition and yield stability. Front Microbiol. 2021;12:628379.

2. Haque MM, Mosharaf MK, Khatun M, Haque MA, Biswas MS, Islam MS, et al. Biofilm producing rhizobacteria with multiple plant growth-promoting traits promote growth of tomato under water-deficit stress. Front Microbiol. 2020;11:542053.

3. Batykova Z, Kistaubayeva A, Abdulzhanova M, Doktyrbay G, Saidullayeva L, Baimirzayeva Z, et al. Multifunctional Maize Rhizobacteria: Isolation, Characterization and Prospects for Sustainable Agriculture. International Journal of Plant Biology. 2026;17:2. https://doi.org/10.3390/ijpb17010002.

4. Zhao X, Ding Z, Tao Z, Lyu D, Qin S. Isolation and Identification of plant growth promoting rhizobacteria from Malus hupehensis Rehd and Their Promoting Effects on the Horticultural Plants. Sci Hortic (Amsterdam). 2025;346:114165.

https://doi.org/https://doi.org/10.1016/j.scienta.2025.114165.

5. Mahaffee WF, Kloepper JW. Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (Cucumis sativus L.). Microb Ecol. 1997;34:210–23.

6. Kloepper JW. Plant growth-promoting rhizobacteria on radishes. In: Proc. of the 4th Internet. Conf. on Plant Pathogenic Bacter, Station de Pathologie Vegetale et Phytobacteriologie, INRA, Angers, France, 1978. 1978. p. 879–82.

7. Kloepper JW, Schroth MN, Miller TD. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology®. 1980;115:1078–82.

8. Gray EJ, Smith DL. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol Biochem. 2005;37:395–412.

9. Stella M, Suhaimi M. Selection of suitable growth medium for free-living diazotrophs isolated from compost. J Trop Agric Food Sci. 2010;38:211–9.

10. Upendra S, Sk M, Singh DP. Salinity tolerance in free living plant growth promoting Rhizobacteria. Indian J Sci Res. 2012;03:73–8.

11. Kim J, Rees DC. Nitrogenase and biological nitrogen fixation. Biochemistry. 1994;33:389–97. https://doi.org/10.1021/bi00168a001.

12. dos Santos RM, Diaz PAE, Lobo LLB, Rigobelo EC. Use of plant growth-promoting rhizobacteria in maize and sugarcane: characteristics and applications. Front Sustain Food Syst. 2020;4:136.

580 13. Omar AF, Abdelmageed AHA, Al-Turki A, Aggag AM, Rehan M, Abdelhameid NM.
581 Assessment of the Plant Growth-Promoting Potential of Three Pseudomonas and Pantoea
582 Isolates to Promote Pepper Growth. Agronomy. 2025;15:2419.
583 https://doi.org/10.3390/agronomy15102419.

584 14. Leiva-Mora M, Mera Guzmán PE, Mera-Andrade RI, Zabala Haro AM, Saa LR, Loján P, et
585 al. Isolation and Characterization of Phosphate-Solubilizing Rhizobacteria from Solanum
586 tuberosum with Plant Growth-Promoting Activity. Applied Microbiology. 2026;6:8.
587 https://doi.org/10.3390/applmicrobiol6010008.

588 15. Kamran S, Shahid I, Baig DN, Rizwan M, Malik KA, Mehnaz S. Contribution of Zinc
589 Solubilizing Bacteria in Growth Promotion and Zinc Content of Wheat. Front Microbiol.
590 2017;Volume 8-.

591 16. Baghel V, Thakur JK, Yadav SS, Manna MC, Mandal A, Shirale AO, et al. Phosphorus and
592 Potassium Solubilization From Rock Minerals by Endophytic Burkholderia sp. Strain FDN2-1 in
593 Soil and Shift in Diversity of Bacterial Endophytes of Corn Root Tissue with Crop Growth
594 Stage. Geomicrobiol J. 2020;37:550–63. https://doi.org/10.1080/01490451.2020.1734691.

595 17. Shoukry AA, El-Sebaay HH, El-Ghomary AE. Assessment of indole acetic acid production
596 from rhizobium leguminosarum strains. Curr Sci Int. 2018;7:60–9.

597 18. Alemneh AA, Cawthray GR, Zhou Y, Ryder MH, Denton MD. Ability to produce indole
598 acetic acid is associated with improved phosphate solubilising activity of rhizobacteria. Arch
599 Microbiol. 2021;203:3825–37. https://doi.org/10.1007/s00203-021-02364-w.

600 19. Zhang X, Schmidt RE. Hormone-Containing Products' Impact on Antioxidant Status of Tall
601 Fescue and Creeping Bentgrass Subjected to Drought. Crop Sci. 2000;40:1344–9.

https://doi.org/https://doi.org/10.2135/cropsci2000.4051344x.

20. Yakhin OI, Lubyanov AA, Yakhin IA, Brown PH. Biostimulants in Plant Science: A Global Perspective. Front Plant Sci. 2016;7:2049. https://doi.org/10.3389/fpls.2016.02049.

21. Abdelall MF, El-Gamal S, Abdelghany WR. Exploiting hydrogen cyanide producing Rhizobacteria to combat Fusarium equiseti and improve sugar beet performance. Physiol Mol Plant Pathol. 2026;142:103069. https://doi.org/https://doi.org/10.1016/j.pmpp.2025.103069.

22. Heo AY, Koo YM, Choi HW. Biological Control Activity of Plant Growth Promoting Rhizobacteria Burkholderia contaminans AY001 against Tomato Fusarium Wilt and Bacterial Speck Diseases. Biology. 2022;11:619. https://doi.org/10.3390/biology11040619.

23. Saberi Riseh R, Vatankhah M, Hassanisaadi M, Barka EA. Unveiling the Role of Hydrolytic Enzymes from Soil Biocontrol Bacteria in Sustainable Phytopathogen Management. Front Biosci (Landmark Ed. 2024;29:105. https://doi.org/10.31083/j.fbl2903105.

24. Kloepper JW, Ryu C-M, Zhang S. Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology. 2004;94:1259–66.

25. Zhang X, Yang Z, Wang L, Yue Y, Wang L, Xiulian Yang. The effects of plant growth-promoting rhizobacteria on plants under temperature stress:A meta-analysis. Rhizosphere. 2023;28:100788. https://doi.org/https://doi.org/10.1016/j.rhisph.2023.100788.

26. Sarkar J, Chakraborty B, Chakraborty U. Plant Growth Promoting Rhizobacteria Protect Wheat Plants Against Temperature Stress Through Antioxidant Signalling and Reducing Chloroplast and Membrane Injury. J Plant Growth Regul. 2018;37:1396–412. https://doi.org/10.1007/s00344-018-9789-8.

623 27. Wang M, Han T, Huang F, Li X, Shan J, Zhang D, et al. Saline–Alkaline Stress-Driven
624 Rhizobacterial Community Restructuring and Alleviation of Stress by Indigenous PGPR in
625 Alfalfa. Plants. 2025;14:3844. https://doi.org/10.3390/plants14243844.

626 28. Orozco-Mosqueda M del C, Glick BR, Santoyo G. ACC deaminase in plant growth-
627 promoting bacteria (PGPB): An efficient mechanism to counter salt stress in crops. Microbiol
628 Res. 2020;235:126439. https://doi.org/https://doi.org/10.1016/j.micres.2020.126439.

629 29. Niu X, Song L, Xiao Y, Ge W. Drought-Tolerant Plant Growth-Promoting Rhizobacteria
630 Associated with Foxtail Millet in a Semi-arid Agroecosystem and Their Potential in Alleviating
631 Drought Stress. Front Microbiol. 2018;Volume 8-.

632 30. Ashry NM, Alaidaroos BA, Mohamed SA, Badr OAM, El-Saadony MT, Esmael A.
633 Utilization of drought-tolerant bacterial strains isolated from harsh soils as a plant growth-
634 promoting rhizobacteria (PGPR). Saudi J Biol Sci. 2022;29:1760–9.
635 https://doi.org/https://doi.org/10.1016/j.sjbs.2021.10.054.

636 31. Upadhyay SK, Singh DP, Saikia R. Genetic Diversity of Plant Growth Promoting
637 Rhizobacteria Isolated from Rhizospheric Soil of Wheat Under Saline Condition. Curr
638 Microbiol. 2009;59:489–96. https://doi.org/10.1007/s00284-009-9464-1.

639 32. Ali SZ, Sandhya V, Venkateswar Rao L. Isolation and characterization of drought-tolerant
640 ACC deaminase and exopolysaccharide-producing fluorescent Pseudomonas sp. Ann Microbiol.
641 2014;64:493–502.

642 33. Rafique M, K. H, Mukhtar T, . A, Abdullah Khan A, Afridi M, et al. Bacterial biofilm
643 formation and its role against agricultural pathogens. 2015.

644 34. Bhattacharyya C, Banerjee S, Acharya U, Mitra A, Mallick I, Haldar A, et al. Evaluation of
645 plant growth promotion properties and induction of antioxidative defense mechanism by tea
646 rhizobacteria of Darjeeling, India. Sci Rep. 2020;10:15536.

647 35. Beveridge TJ. Use of the Gram stain in microbiology. Biotech Histochem. 2001;76:111–8.

648 36. Bergey DH. Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins;
649 1994.

650 37. Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Heat treatment of bacteria: a simple
651 method of DNA extraction for molecular techniques. Kuwait Med J. 2009;41:117–22.

652 38. Nautiyal CS. An efficient microbiological growth medium for screening phosphate
653 solubilizing microorganisms. FEMS Microbiol Lett. 1999;170:265–70.

654 39. Louden BC, Haarmann D, Lynne AM. Use of blue agar CAS assay for siderophore detection.
655 J Microbiol Biol Educ. 2011;12:51–3.

656 40. Dworkin M, Foster J. Experiments with some microorganisms which utilize ethane and
657 hydrogen. J Bacteriol. 1958;75:592–603.

658 41. Agbodjato NA, Noumavo PA, Baba-Moussa F, Salami HA, Sina H, Sèzan A, et al.
659 Characterization of potential plant growth promoting rhizobacteria isolated from Maize (Zea
660 mays L.) in central and Northern Benin (West Africa). Appl Environ Soil Sci.
661 2015;2015:901656.

662 42. Patil N, Inchanalkar M, Desai D, Landge V, Bhole BD. Screening of 1-aminocyclopropane-
663 1-carboxylic acid (ACC) deaminase producing multifunctional plant growth promoting
664 rhizobacteria from onion (Allium cepa) rhizosphere. Int J Curr Microbiol Appl Sci. 2016;5:118–

665 27.

666 43. Reetha S, Selvakumar G, Bhuvaneswari G, Thamizhiniyan P, Ravimycin T. Screening of
667 cellulase and pectinase by using Pseudomonas fluorescens and Bacillus subtilis. Int Lett Nat Sci.
668 2014;8.

669 44. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection
670 of microbial cellulases on agar plates using Gram's iodine. Curr Microbiol. 2008;57:503–7.

671 45. Singh RK. Study on abiotic tolerance of plant growth promoting rhizobacteria (PGPR)
672 isolated from wheat rhizospheric soils at different agroclimatic zones. J Biotechnol Crop Sci.
673 2020;9:4–12.

674 46. Hassanshahian M. Isolation and characterization of diazinon degrading bacteria from
675 contaminated agriculture soils. Iran J Toxicol Vol. 2016;10.

676 47. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp JoVE. 2011;:2437.

677 48. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different
678 detection methods of biofilm formation in the clinical isolates. Brazilian J Infect Dis.
679 2011;15:305–11.

680 49. Amri M, Rjeibi MR, Gatrouni M, Mateus DMR, Asses N, Pinho HJO, et al. Isolation,
681 identification, and characterization of phosphate-solubilizing bacteria from Tunisian soils.
682 Microorganisms. 2023;11:783.

683 50. Rfaki A, Zennouhi O, Aliyat FZ, Nassiri L, Ibijbijen J. Isolation, selection and
684 characterization of root-associated rock phosphate solubilizing bacteria in moroccan wheat
685 (Triticum aestivum L.). Geomicrobiol J. 2020;37:230–41.

686 51. Kumar V, Menon S, Agarwal H, Gopalakrishnan D. Characterization and optimization of
687 bacterium isolated from soil samples for the production of siderophores. Resour Technol.
688 2017;3:434–9.

689 52. Han L, Zhang H, Xu Y, Li Y, Zhou J. Biological characteristics and salt-tolerant plant
690 growth-promoting effects of an ACC deaminase-producing Burkholderia pyrrocinia strain
691 isolated from the tea rhizosphere. Arch Microbiol. 2021;203:2279–90.

692 53. Riseh RS, Vatankhah M, Hassanisaadi M, Ait Barka E. Unveiling the role of hydrolytic
693 enzymes from soil biocontrol bacteria in sustainable phytopathogen management. Front Biosci.
694 2024;29:105.

695 54. Jabiri S, Legrifi I, Benhammou M, Laasli S-E, Mokrini F, Amraoui MB, et al. Screening of
696 rhizobacterial isolates from apple rhizosphere for their biocontrol and plant growth promotion
697 activity. Appl Microbiol. 2023;3:948–67.

698 55. Tchakounté GVT, Berger B, Patz S, Fankem H, Ruppel S. Community structure and plant
699 growth-promoting potential of cultivable bacteria isolated from Cameroon soil. Microbiol Res.
700 2018;214:47–59.

701 56. Almaghrabi OA, Abdelmoneim TS, Albishri HM, Moussa TA. Enhancement of maize
702 growth using some plant growth promoting rhizobacteria (PGPR) under laboratory conditions.
703 Life Sci J. 2014;11:764–72.

704 57. Alaylar B, Güllüce M, Karadayı M, Koç TY, Karadayı G. Rhizospheric PGPR strains of
705 wheat, barley and trefoil grown in Ağrı province. Türk Doğa ve Fen Derg. 2021;10:182–90.

706 58. Melini F, Luziatelli F, Bonini P, Ficca AG, Melini V, Ruzzi M. Optimization of the growth

conditions through response surface methodology and metabolomics for maximizing the auxin production by Pantoea agglomerans C1. Front Microbiol. 2023;Volume 14.

59. Singh RK, Singh P, Li H-B, Guo D-J, Song Q-Q, Yang L-T, et al. Plant-PGPR interaction study of plant growth-promoting diazotrophs Kosakonia radicincitans BA1 and Stenotrophomonas maltophilia COA2 to enhance growth and stress-related gene expression in Saccharum spp. J Plant Interact. 2020;15:427–45. https://doi.org/10.1080/17429145.2020.1857857.

60. Ghosh SK, Pal S, Chakraborty N. The qualitative and quantitative assay of siderophore production by some microorganisms and effect of different media on its production. Int J Chem Sci. 2015;13:1621–9.

61. Arora NK, Verma M. Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech. 2017;7:381. https://doi.org/10.1007/s13205-017-1008-y.

62. Marques MPC, Walshe K, Doyle S, Fernandes P, de Carvalho CCCR. Anchoring high-throughput screening methods to scale-up bioproduction of siderophores. Process Biochem. 2012;47:416–21. https://doi.org/https://doi.org/10.1016/j.procbio.2011.11.020.

63. Olagunju OO, Ogunnusi TA, Akpor OB. Evaluation of Indole acetic acid and hydrogen cyanide production by plant growth promoting rhizobacteria. Vegetos. 2026;39:582–9. https://doi.org/10.1007/s42535-025-01347-2.

64. Bhadrecha P, Bala M, Khasa YP, Arshi A, Singh J, Kumar M. Hippophae rhamnoides L. rhizobacteria exhibit diversified cellulase and pectinase activities. Physiol Mol Biol Plants. 2020;26:1075–85. https://doi.org/10.1007/s12298-020-00778-2.

729 65. Tirry N, Kouchou A, El Omari B, Ferioun M, El Ghachtouli N. Improved chromium
730 tolerance of Medicago sativa by plant growth-promoting rhizobacteria (PGPR). J Genet Eng
731 Biotechnol. 2021;19:149. https://doi.org/10.1186/s43141-021-00254-8.

732 66. Hoang DM, Ngo DT, Huynh ON, Nguyen TKM, Pham TT, Le TTT. Impact of Acinetobacter
733 soli on Tomato Plants. Chem Eng Trans. 2024;113:73-78 SE-Research Articles.
734 https://doi.org/10.3303/CET24113013.

735 67. Senga R, Alexandros M, Saira A, Aileen C, Rodrigo AT, Catherine A, et al. Acinetobacter
736 enrichment shapes composition and function of the bacterial microbiota of field-grown tomato
737 plants. mSphere. 2026;11:e00842-25. https://doi.org/10.1128/msphere.00842-25.

738 68. Kour D, Kaur T, Devi R, Chaubey KK, Yadav AN. Co-inoculation of nitrogen fixing and
739 potassium solubilizing Acinetobacter sp. for growth promotion of onion (Allium cepa). Biologia
740 (Bratisl). 2023;78:2635–41. https://doi.org/10.1007/s11756-023-01412-8.

741 69. Suman A, Shukla L, Marag PS, Verma P, Gond S, Prasad JS. Potential use of plant
742 colonizing Pantoea as generic plant growth promoting bacteria for cereal crops. J Environ Biol.
743 2020;41:987–94.

744 70. Young L-S, Hameed A, Peng S-Y, Shan Y-H, Wu S-P. Endophytic establishment of the soil
745 isolate Burkholderia sp. CC-Al74 enhances growth and P-utilization rate in maize (Zea mays L.).
746 Appl Soil Ecol. 2013;66:40–7. https://doi.org/https://doi.org/10.1016/j.apsoil.2013.02.001.

747 71. dos Santos IB, Pereira AP de A, de Souza AJ, Cardoso EJBN, da Silva FG, Oliveira JTC, et
748 al. Selection and Characterization of Burkholderia spp. for Their Plant-Growth Promoting
749 Effects and Influence on Maize Seed Germination. Front Soil Sci. 2022;Volume 1-.

750 72. Singh MK, Singh DP, Mesapogu S, Babu BK, Bontemps C. Concomitant colonization of
751 nifH positive endophytic Burkholderia sp. in rice (Oryza sativa L.) promotes plant growth.
752 World J Microbiol Biotechnol. 2011;27:2023–31. https://doi.org/10.1007/s11274-011-0664-z.

753 73. Luvizotto DM, Marcon J, Andreote FD, Dini-Andreote F, Neves AAC, Araújo WL, et al.
754 Genetic diversity and plant-growth related features of Burkholderia spp. from sugarcane roots.
755 World J Microbiol Biotechnol. 2010;26:1829–36. https://doi.org/10.1007/s11274-010-0364-0.

756 74. Dos Santos HRM, Argolo CS, Argôlo-Filho RC, Loguercio LL. A 16S rDNA PCR-based
757 theoretical to actual delta approach on culturable mock communities revealed severe losses of
758 diversity information. BMC Microbiol. 2019;19:74.

759 75. Zeighami H, Valadkhani F, Shapouri R, Samadi E, Haghi F. Virulence characteristics of
760 multidrug resistant biofilm forming Acinetobacter baumannii isolated from intensive care unit
761 patients. BMC Infect Dis. 2019;19:629.

762

763

764

765

766

767

768

769

Tables

Table 1. Primers and their corresponding reaction conditions for 16S rRNA and *eps*A gene amplification

(a) Primers utilized for amplification of 16S rRNA and *eps*A genes				
Target Gene	**Primer Name**	**Primer Sequence**	**Amplicon Size**	**Reference**
16S rRNA	16SF	5'-AGAGTTTGATCCTGGCTCAG-3'	1467bp	[74]
	16SR	5'-TACCTTGTTTACGACTT-3'		
*eps*A	epsA-F	5'-AGCAAGTGGTTATCCAATCG-3'	451bp	[75]
	epsA-R	5'-ACCAGACTCACCCATTACAT-3'		

(b) Reaction conditions for 16S rRNA and epsA gene PCR amplification					
Target Gene	**Initial Denaturation**	**30 cycles**			**Final Extension**
		Denaturation	**Annealing**	**Extension**	
16S rRNA	96 °C, 4 min	94°C, 30 s	52°C, 30 s	72°C, 1 min	72°C, 10 min
*eps*A	94°C, 5 min	94°C, 1 min	60°C,1 min	72°C, 1 min	72°C, 10 min

779 **Table 2.** List of the obtained rhizobacterial isolates and their closest related bacterial strains

Isolate ID	Identified Strains	NCBI GenBank Accession Number	Closest Reference Accession Number	Query Cover	Percent Identity
AE1	*Arthrobacter* sp.	QB266539	KY072895.1	96%	97.12%
AE7	*Pseudarthrobacter enclensis*	QB266540	MH178357.1	99%	99.70%
AE9	*Streptomyces* sp.	QB266541	MH645731.1	97%	99.42%
BA1	*Bacillus* sp.	QB266542	KJ944108.1	99%	99.37%
BA2	*Priestia aryabhattai.*	QB266543	KF208483.1	97%	100%
BA4	*Bacillus* sp.	QB266544	MK426628.1	99%	93.91%
BA6	*Metabacillus indicus*	QB266545	KP408217.1	97%	98.70%
BA8	*Arthrobacter ramosus*	QB266546	KJ124592.1	96%	99.64%
BA9	*Pseudarthrobacter enclensis*	QB266547	MH178357.1	98%	99.13%
BA12	*Pseudarthrobacter enclensis*	QB266548	MH178357.1	99%	99.78%
BA16	*Acinetobacter soli*	QB266549	OP216732.1	99%	99.71%
BA17	*Klebsiella pneumoniae*	QB266550	MK571203.1	99%	92.72%
BA18	*Metapseudomonas resinovorans*	QB266551	KT380602.1	99%	99.71%
CP3	*Glutamicibacter mishrai*	QB266552	CP101461.1	99%	99.06%
CP4	*Pantoea coffeiphila*	QB266553	OP986729.1	99%	99.28%
LS4	*Terrabacter terrigena*	QB266554	MF681978.1	94%	99.55%
LS5	*Priestia megaterium*	QB266555	CP035094.1	99%	99.93%
LS6	*Priestia aryabhattai*	QB266556	KR085881.1	99%	99.86%
LS8	*Rhizobium* sp.	QB266557	MT577595.1	99%	97.78%
LS10	*Escherichia fergusonii*	QB266558	CP042946.1	98%	95.37%
LS12	*Ensifer adhaerens*	QB266559	KX507144.1	98%	99.85%
LS13	*Bacillus* sp.	QB266560	MT673835.1	99%	99.93%
LS15	*Acidovorax* sp.	QB266561	HQ694760.1	94%	99.85%
MC1	*Metapseudomonas resinovorans*	QB266562	MT757965.1	98%	99.64%
MC2	*Microbacterium pollutisoli*	QB266563	MW346024.1	95%	99.63%
MC3	*Janibacter terrae*	QB266564	KJ768435.1	93%	98.19%
MC4	*Arthrobacter* sp.	QB266565	KU597219.1	95%	96.75%
SL2	*Priestia aryabhattai*	QB266566	MH748616.1	99%	99.79%
SL3	*Rhizobium* sp.	QB266567	MK318781.1	99%	99.63%
SL4	*Priestia megaterium*	QB266568	MG430231.1	99%	100%
SL5	*Klebsiella pneumoniae*	QB266569	MK571203.1	100%	92.31%
SM1	*Priestia megaterium*	QB266570	MG430222.1	98%	99.86%
SM2	*Bacillus* sp.	QB266571	MG309330.1	98%	99.86%
SM4	*Bacillus* sp.	QB266572	MG388170.1	99%	100%
SM5	*Bacillus* sp.	QB266573	MF102042.1	98%	99.22%
SM8	*Ensifer adhaerens*	QB266574	KX507144.1	98%	99.78%
SM9	*Micrococcus endophyticus*	QB266575	MT225652.1	99%	99.71%
SM10	*Arthrobacter silvisoli*	QB266576	MH063845.1	99%	97.58%
ZM2	*Rhizobium* sp.	QB266577	MN211545.1	98%	99.93%
ZM3	*Bacillus* sp.	QB266578	KF933670.1	98%	99.72%
ZM4	*Ralstonia* sp.	QB266579	KY203969.1	99%	100%
ZM6	*Burkholderia* sp.	QB266580	MK691483.1	98%	99.71%
GS1	*Bacillus zanthoxyli*	QB266581	OQ786977.1	99%	99.86%
GS2	*Glutamicibacter nicotianae*	QB266582	KR152305.1	96%	99.71%
GS7	*Micrococcus endophyticus*	QB266583	MT214326.1	99%	99.93%
AS1	*Bacillus aerophilus*	QB266584	KC171990.1	99%	99.93%
AS2	*Priestia megaterium*	QB266585	OR077483.1	98%	99.93%
AS4	*Priestia aryabhattai*	QB266586	MN543785.1	97%	96.77%

780 **Table 3.** Phosphate solubilization, Indole-3-acetic acid (IAA) production, and siderophore
781 production attributes of the rhizobacterial strains

Phosphate Solubilization			IAA Production		Siderophore Production	
Isolate ID	**PSI (PVK)**	**PSI (NBRIP)**	**Isolate ID**	**Concentration (µg/mL)**	**Isolate ID**	**SI**
AE8	1.25 ± 0	-	**BA12**	59.002 ± 4.21	**AE9**	2 ± 0.42
AE9	1.25 ± 0.25	1.25 ± 0	**BA16**	11.872 ± 0.31	**BA2**	2 ± 0.5
BA2	-	1.14 ± 0.14	**BA19**	20.967 ± 0.538	**BA4**	2 ± 0
BA7	-	1.25 ± 0.25	**CP1**	66.977 ± 2.01	**BA6**	3 ± 1
BA14	-	1.2 ± 0	**CP3**	20.608 ± 1.34	**BA16**	7.5 ± 0.43
BA16	2.75 ± 0.25	3.03 ± 0.29	**CP4**	70.202 ± 1.79	**BA17**	3 ± 1
BA17	-	1.09 ± 0.08	**LS8**	68.5 ± 1.88	**BA18**	1.67 ± 0.33
CP1	1.2 ± 0.03	-	**LS12**	66.574 ± 2.28	**BA19**	1.5 ± 0.1
CP4	4.67 ± 0.58	3.67 ± 0.58	**LS14**	16.442 ± 1.75	**CP3**	1.25 ± 0.25
LS12	1.2 ± 0	-	**LS18**	57.972 ± 1.16	**CP4**	3.2 ± 0.2
LS18	1.23 ± 0.06	-	**MC3**	56.628 ± 2.42	**LS5**	1.25 ± 0
SL4	1.1 ± 0.01	1.33 ± 0.17	**SL1**	23.655 ± 4.93	**LS6**	1.33 ± 0
SM1	1.1 ± 0.01	-	**SL2**	19.085 ± 3.27	**LS8**	1.53 ± 0.06
SM4	2 ± 0.33	-	**SL3**	64.154 ± 5.11	**LS12**	1.14 ± 0
SM5	1.75 ± 0.25	1.52 ± 0.13	**SL4**	37.453 ± 4.26	**LS14**	1.67 ± 0.33
SM8	1.12 ± 0.01	-	**SM1**	28.762 ± 2.46	**LS15**	3 ± 0
ZM1	1.33 ± 0.17	-	**SM8**	73.652 ± 3.36	**LS18**	3.8 ± 0.2
ZM3	-	1.14 ± 0	**SM9**	11.469 ± 1.57	**MC1**	1.71 ± 0.26
ZM4	2.55 ± 0.18	2.23 ± 0.25	**ZM1**	72.667 ± 4.93	**MC4**	2 ± 0
ZM6	1.33 ± 0.01	-	**ZM2**	71.546 ± 5.38	**MC5**	1.5 ± 0
ZM7	1.52 ± 0.17	-	**ZM3**	43.456 ± 1.43	**SL1**	2 ± 0
GS1	1.1 ± 0.11	-	**ZM6**	21.101 ± 3.23	**SL2**	1.5 ± 0.5
AS1	1.4 ± 0.17	-	**GS1**	57.837 ± 4.12	**SL3**	2.5 ± 0.5
AS2	1.1 ± 0.1	-	**GS6**	29.389 ± 7.39	**SM2**	2 ± 0
AS4	1.13 ± 0	-	**GS8**	66.887 ± 4.84	**SM4**	1.5 ± 0
			AS2	47.847 ± 1.52	**SM5**	1.42 ± 0.3
			AS4	44.621 ± 5.69	**SM6**	2 ± 0.5
					ZM1	1.33 ± 0.3
					ZM2	1.68 ± 0.03
					ZM3	1.33 ± 0.29
					ZM4	4.02 ± 0.29
					ZM6	7.67 ± 0.58
					ZM7	1.57 ± 0.4
					GS2	2.08 ± 0.38
					GS8	1.34 ± 0.29
					AS1	1.21 ± 0.04

782 IAA: Indole-3-acetic acid, PSI: Phosphate Solubilization Index, NBRIP: National Botanical Research Institute's phosphate
783 medium, SI: Siderophore Index. Data are mean ± SD.

Table 4. Indirect PGP properties (ACC deaminase, ammonia, and hydrogen cyanide production, zinc solubilization, cellulase and pectinase enzyme production) of the competent rhizobacterial strains

Isolate ID	Identified Genus	ACC Deaminase	NH_3	HCN	ZnSI	CI	PI
AE9	*Streptomyces*	-	+	-	1.21 ± 0.03	3.52 ± 0.09	-
BA2	*Priestia*	+	++	-	1.41 ± 0.08	2.5 ± 0.19	-
BA16	*Acinetobacter*	+	++	-	2.67 ± 0.58	-	-
BA17	*Klebsiella*	-	+	-	1.5 ± 0.17	2.6 ± 0.53	-
BA19	(Unidentified)	-	+	-	-	-	-
CP1	(Unidentified)	-	++	+	1.16 ± 0.01	1.09 ± 0.1	-
CP3	*Glutaicibacter*	-	+	-	-	2.42 ± 0.25	5.33 ± 0.58
CP4	*Pantoea*	-	++	+	4.5 ± 0.5	-	-
LS8	*Rhizobium*	-	++	++	-	-	-
LS12	*Ensifer*	+	++	-	-	-	-
LS14	(Unidentified)	-	+	-	-	-	-
LS18	(Unidentified)	-	+	+	1.86 ± 0.13	-	-
SL1	(Unidentified)	-	-	-	-	9.33 ± 1.15	-
SL2	*Priestia*	+	+	+	-	2.21 ± 0.12	-
SL3	*Rhizobium*	-	+	-	-	-	-
SL4	*Priestia*	-	++	-	-	2.37 ± 0.03	-
SM1	*Priestia*	-	++	-	1.42 ± 0.15	2.25 ± 0.17	-
SM4	*Bacillus*	-	-	-	1.75 ± 0.21	-	-
SM5	*Bacillus*	-	-	+	-	2.82 ± 0.16	-
SM8	*Ensifer*	+	-	++	-	-	-
ZM1	(Unidentified)	-	+	-	-	-	-
ZM2	*Rhizobium*	-	+	-	1.3 ± 0.12	-	-
ZM3	*Bacillus*	+	++	-	2.06 ± 0.42	5.33 ± 0.33	-
ZM4	*Ralstonia*	+	+	-	1.86 ± 0.13	-	-
ZM6	*Burkholderia*	+	+	-	2.14 ± 0.14	-	1.07 ± 0.03
ZM7	(Unidentified)	-	-	-	1.33 ± 0.33	7.33 ± 0.33	-
GS1	*Bacillus*	-	++	+	1.75 ± 0.25	2.56 ± 0.21	-
GS8	(Unidentified)	-	+	-	-	2.33 ± 0.15	-
AS1	*Bacillus*	-	+	-	1.55 ± 0.18	-	-
AS2	*Priestia*	-	+	-	-	1.69 ± 0.14	-
AS4	*Priestia*	-	+	++	-	2.87 ± 0.49	-

ACC deaminase production: + positive, - negative; NH_3 (ammonia) and HCN (hydrogen cyanide) production: - negative reaction, + weak reaction, ++ moderate reaction; ZnSI: Zinc Solubilization Index; CI: Cellulolytic Index, PI: Pectinolytic Index. Data are mean ± SD.

Table 5. Biofilm-forming capability of the isolates along with the presence of the *epsA* gene

Categories	Isolate ID	Optical Density at 550 nm	Presence of *epsA* gene
Strong Biofilm-Former	BA16	0.898	Present
	CP3	0.769	-
	CP4	1.16	Present
	SM5	1.768	-
	ZM5	0.338	-
	GS7	1.157	-
	AS1	0.548	-
Moderate Biofilm-Former	BA18	0.139	Present
	LS18	0.162	-
	ZM6	0.256	-
Weak Biofilm-Former	AE1, AE7, AE9, BA1, BA4, BA8, BA9, BA12, CP1, LS4, LS5, LS6, LS8, LS10, LS14, MC3, SL4, SM6, GS8, AS4		

Figures

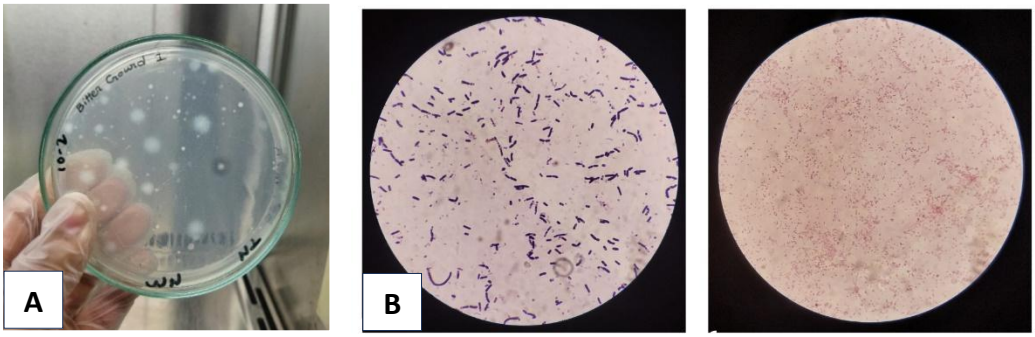


Figure 1. Isolation of nitrogen-fixing bacteria from rhizosphere samples. (A) Appearance of nitrogen-fixing isolates of different morphotypes on Norris glucose nitrogen-free agar plates. (B) Gram reaction of the isolates (Left: Gram-positive, bacilli; Right: Gram-negative, cocci)

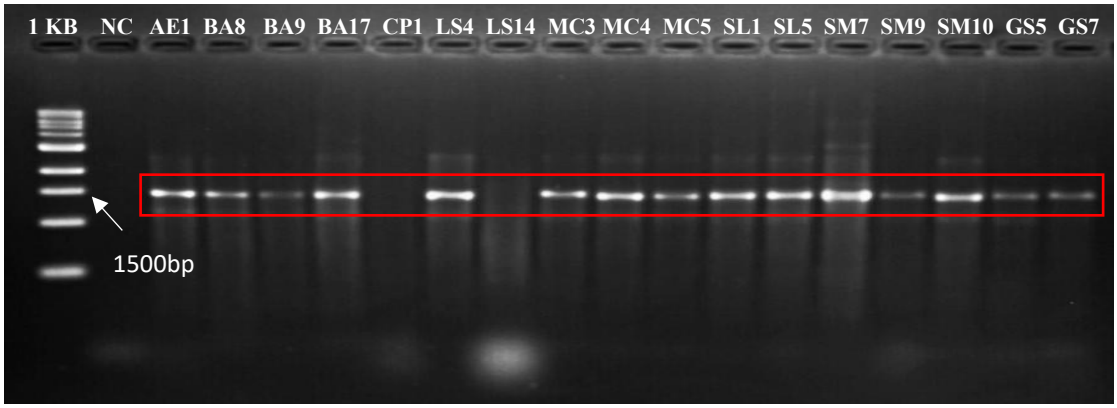
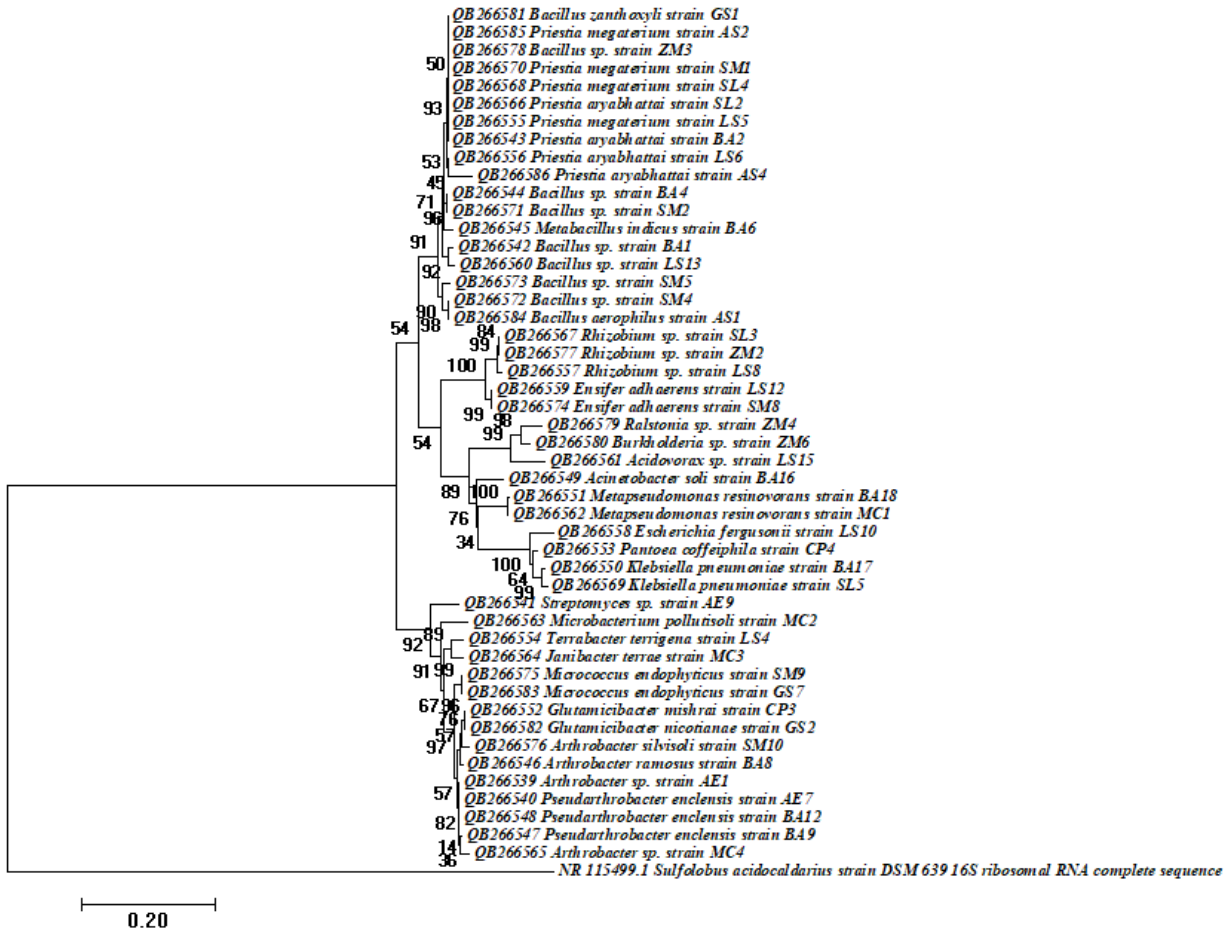


Figure 2. Molecular detection of the rhizobacterial strains. Detection of 16S rRNA amplicons (1467 bp) by PCR using universal primers 16S-F and 16S-R; a 1 kb ladder is used as a DNA marker (New England Biolabs); Lane NC indicates the negative control



811

812 **Figure 3. Phylogenetic relatedness of the rhizobacterial strains.** The phylogenetic relationship among
813 forty-eight rhizobacterial strains is illustrated based on 16S rRNA gene sequences, with *Sulfolobus*
814 *acidocaldarious* assigned as an outgroup species. Tamura 3-parameter model and the neighbor joining
815 method were utilized to construct the Maximum Likelihood tree, with bootstrap analyses of 1000 replicates.

816

817

818

819

820

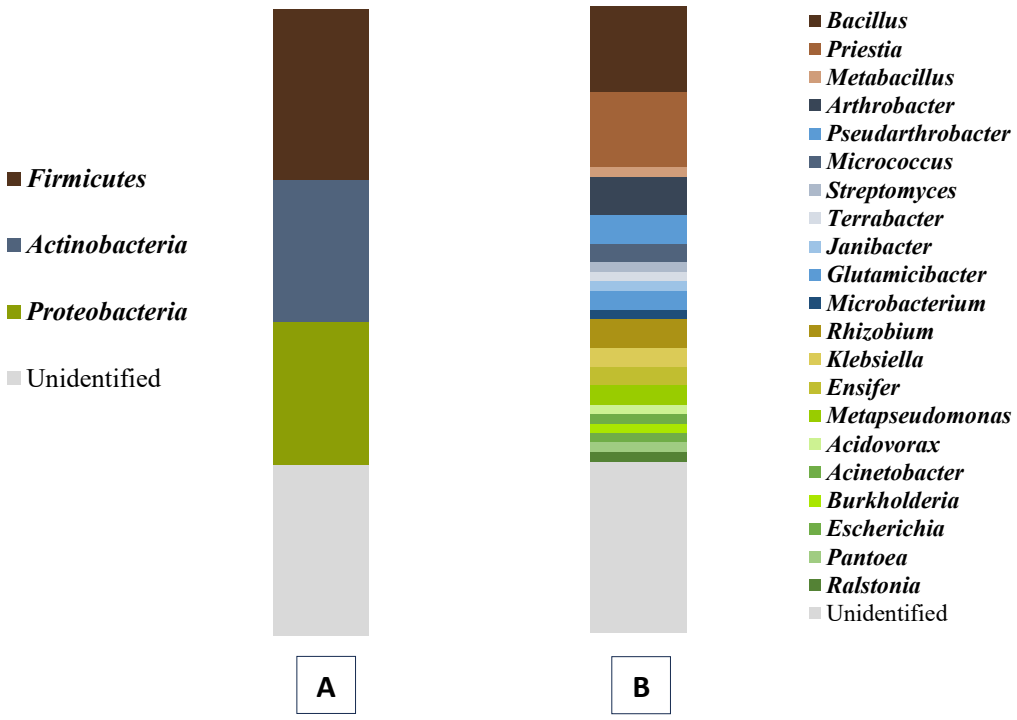
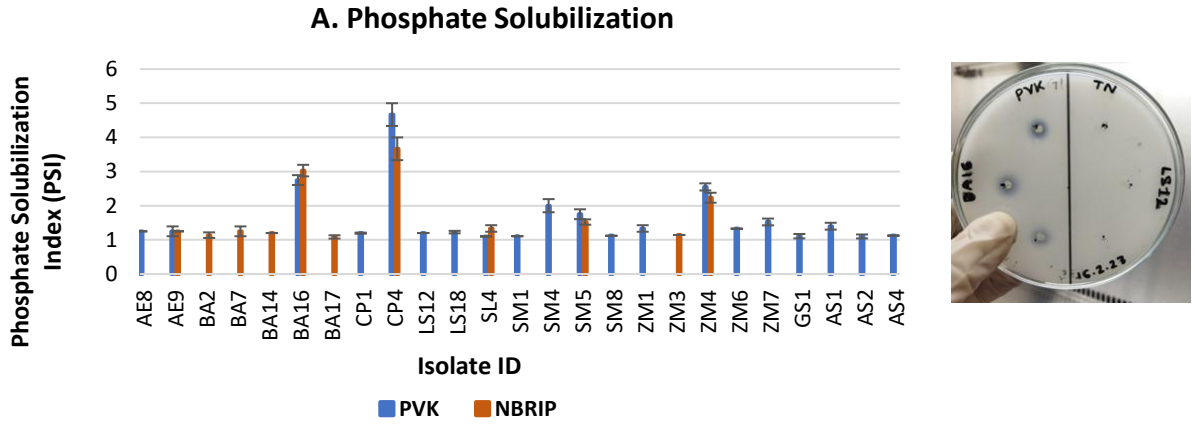
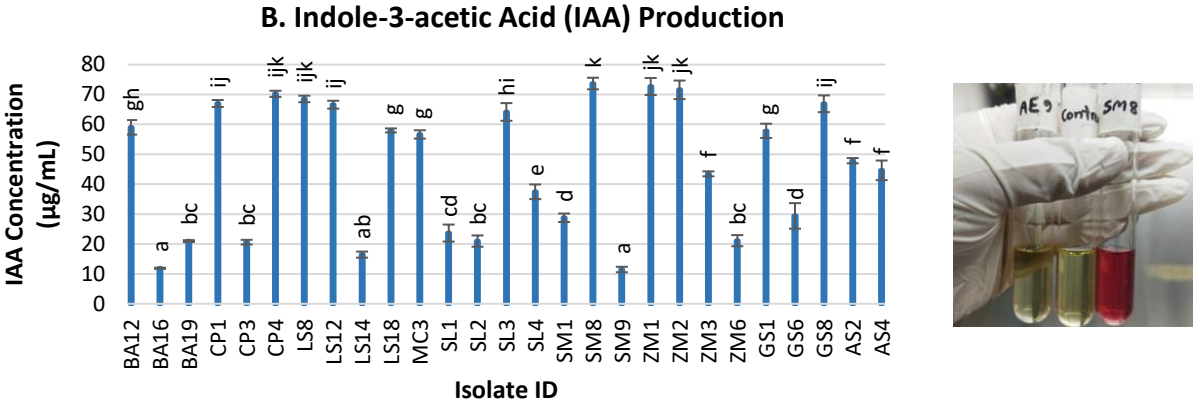


Figure 4. Relative abundance of nitrogen-fixing rhizobacteria in soil samples. The relative abundance of all sixty-six nitrogen-fixing rhizobacteria inhabiting vegetable and crop rhizosphere is shown at (A) phylum; and (B) genus levels. The occurrence of the 48 identified isolates across the phyla Firmicutes, Actinobacteria, and Proteobacteria, as well as across different genera, is represented by blocks of varying shades. The remaining 18 unidentified isolates were presented in a grey block.

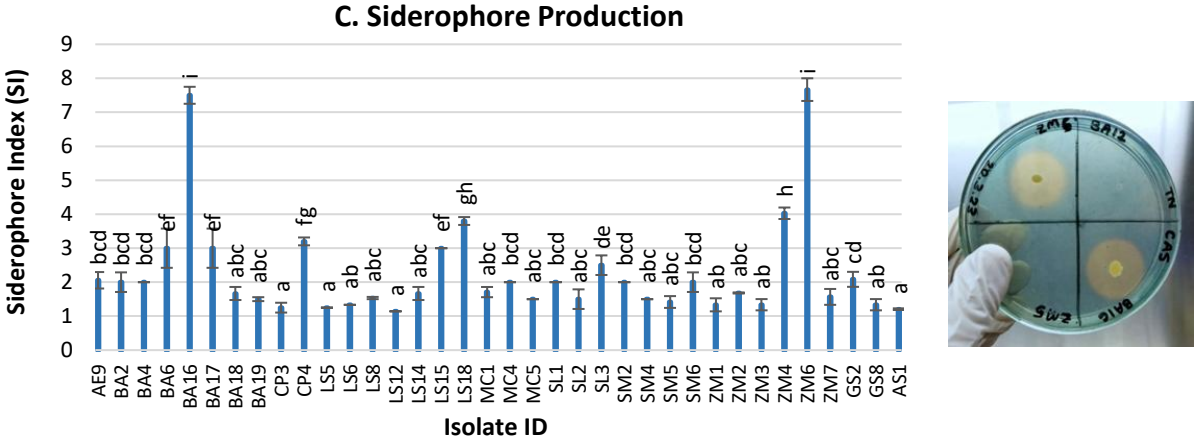


834



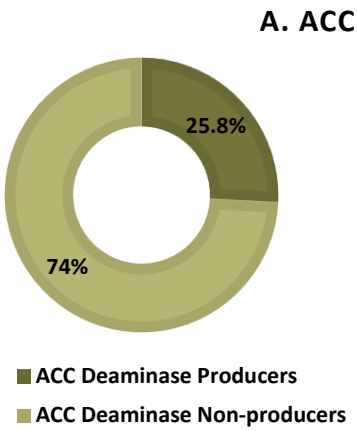
835

836

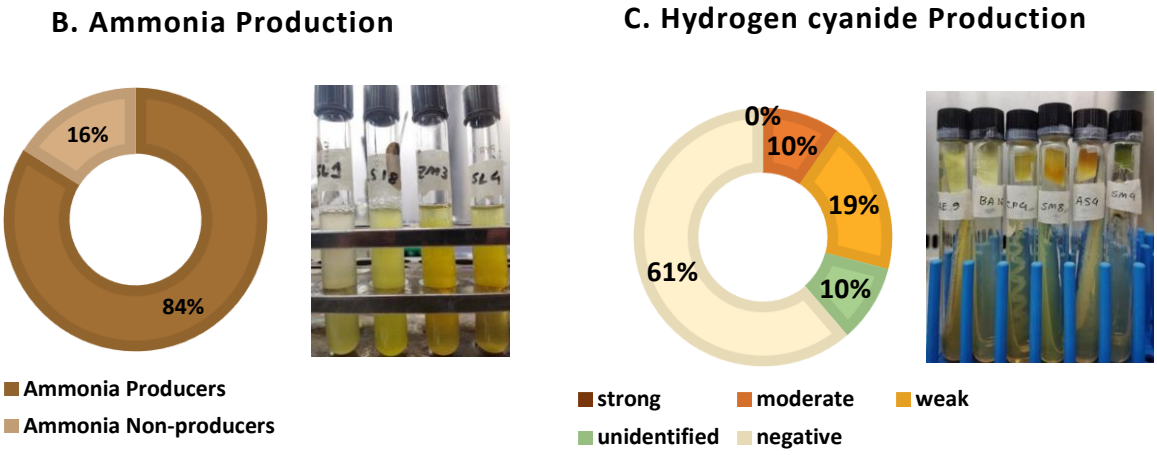


837

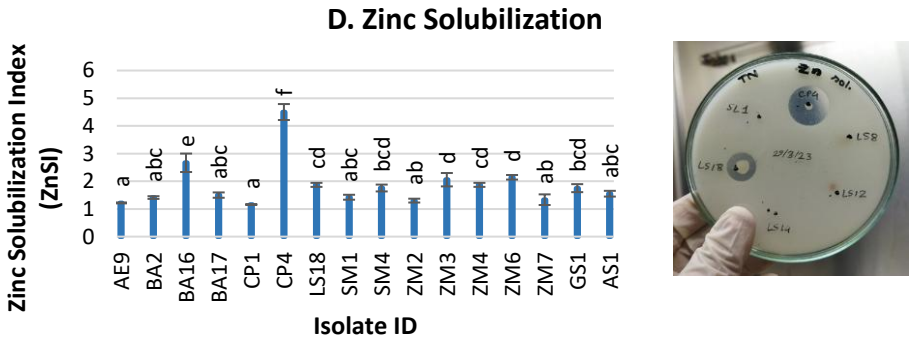
838 **Figure 5. Primary plant growth-promoting properties of the rhizobacterial strains.** (A) Phosphate
839 solubilization; (B) Indole-3-acetic acid (IAA) production; and (C) Siderophore production. Data are mean
840 ± SE. Different letters indicate significant differences in zinc solubilization among isolates using Duncan's
841 test.



842



843



844

845 **Figure 6. Indirect PGP traits of the selected rhizobacterial isolates.** Production of (A) ACC deaminase;
846 (B) ammonia; (C) hydrogen cyanide (HCN); and (D) Zinc solubilization. Data are mean ± SE. Different
847 letters indicate significant differences in zinc solubilization among isolates using Duncan's test.

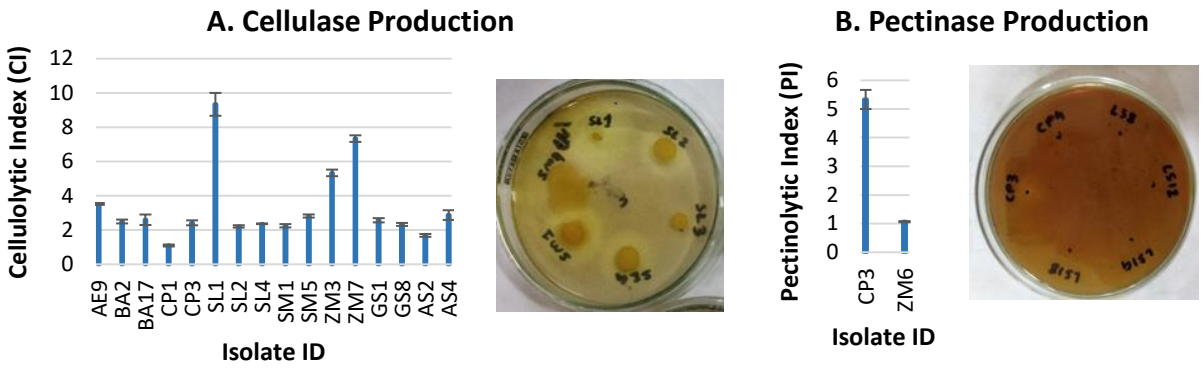


Figure 7. Hydrolytic enzyme production by the rhizobacterial strains. (A) Cellulolytic activity; and (B) pectinolytic activity of the isolates. Data represent mean ± SE.

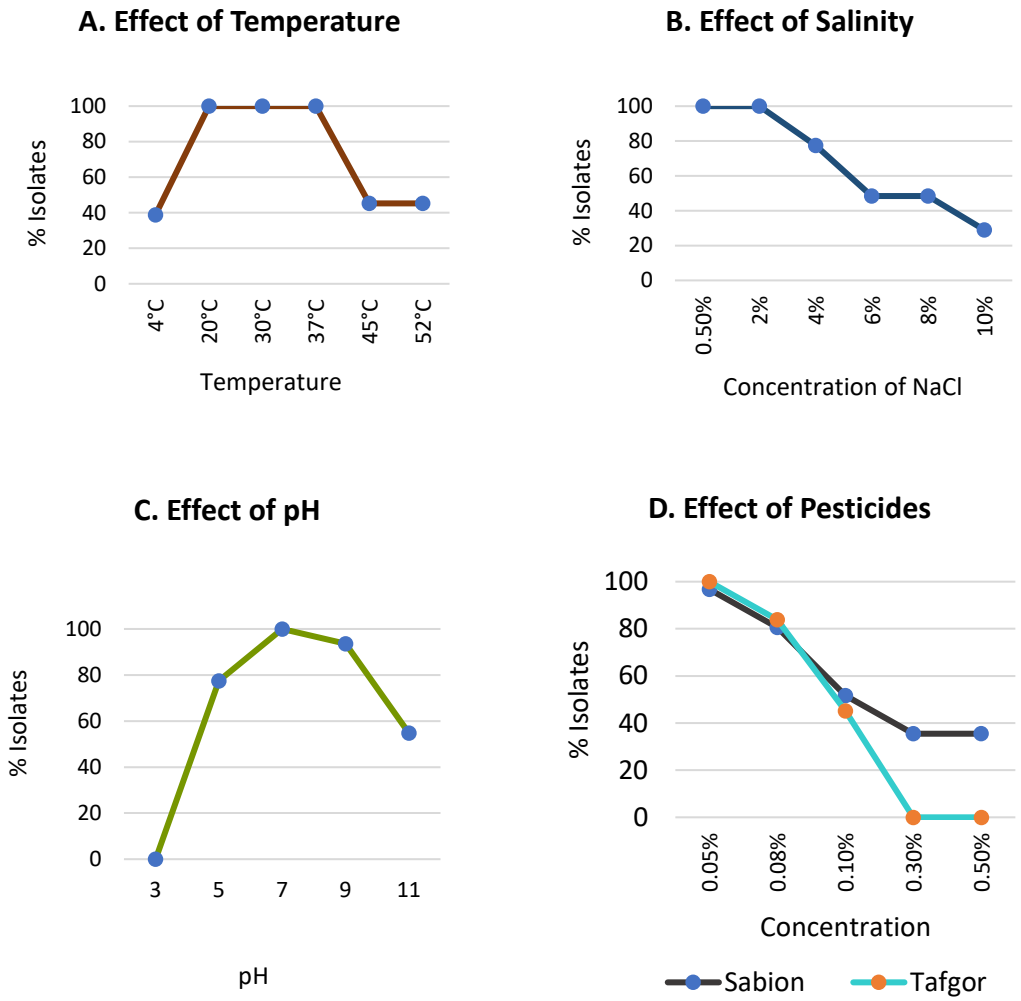
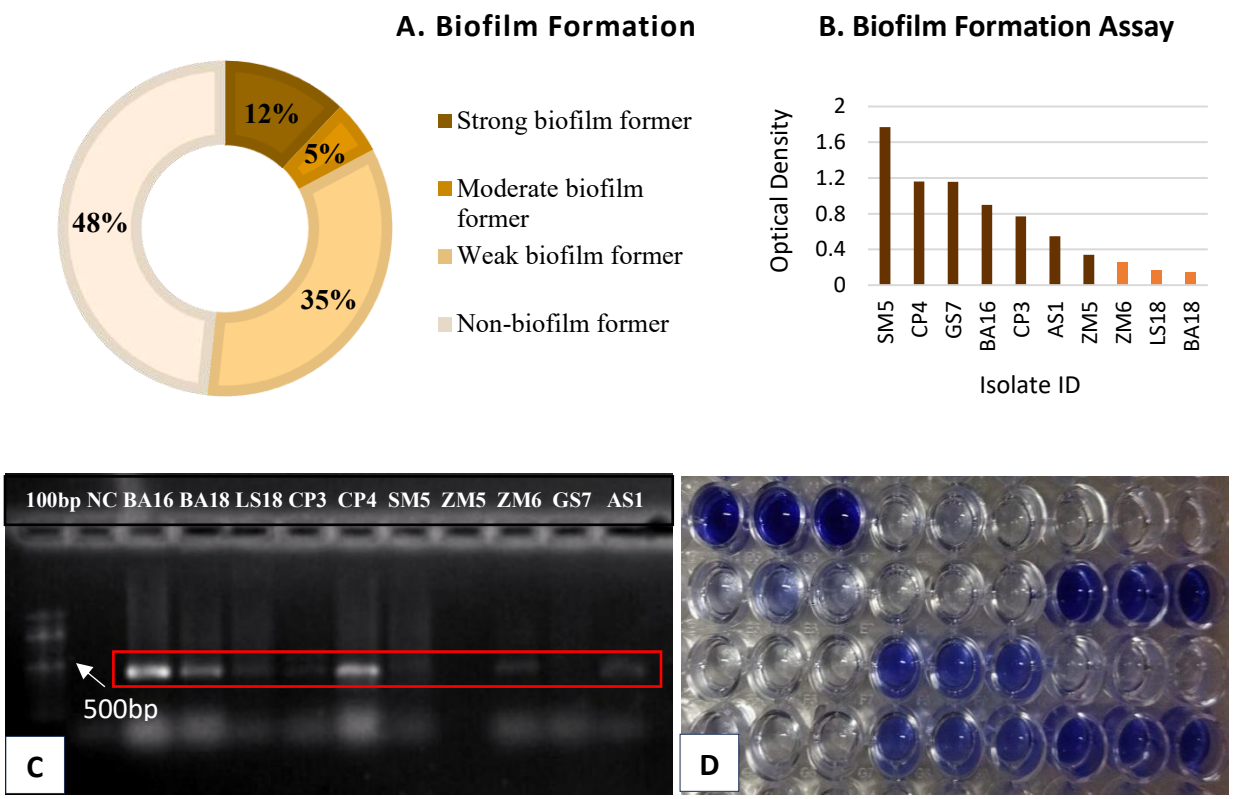


Figure 8. Effect of abiotic stress factors and pesticides on growth of the isolates. Percentages of isolates able to grow at various (A) temperatures; (B) NaCl concentrations; (C) pH conditions; and (D) pesticides, including Sabion (Diazinon) and Tafgor (Dimethoate).



873

874

875 **Figure 9. Biofilm formation capacity of the rhizobacterial isolates.** (A) Percentage of strong, moderate,
876 weak and non-biofilm forming isolates; (B) Optical density of the strong and moderate biofilm-formers
877 (shown with different colored bars); (C) Detection of *epsA* gene (451 bp) using universal primers epsA-F
878 and epsA-R; 100bp ladder is used as DNA marker (New England Biolabs); Lane NC indicates negative
879 control; (D) Representative figure of biofilm formation of the isolates on microtiter plate.

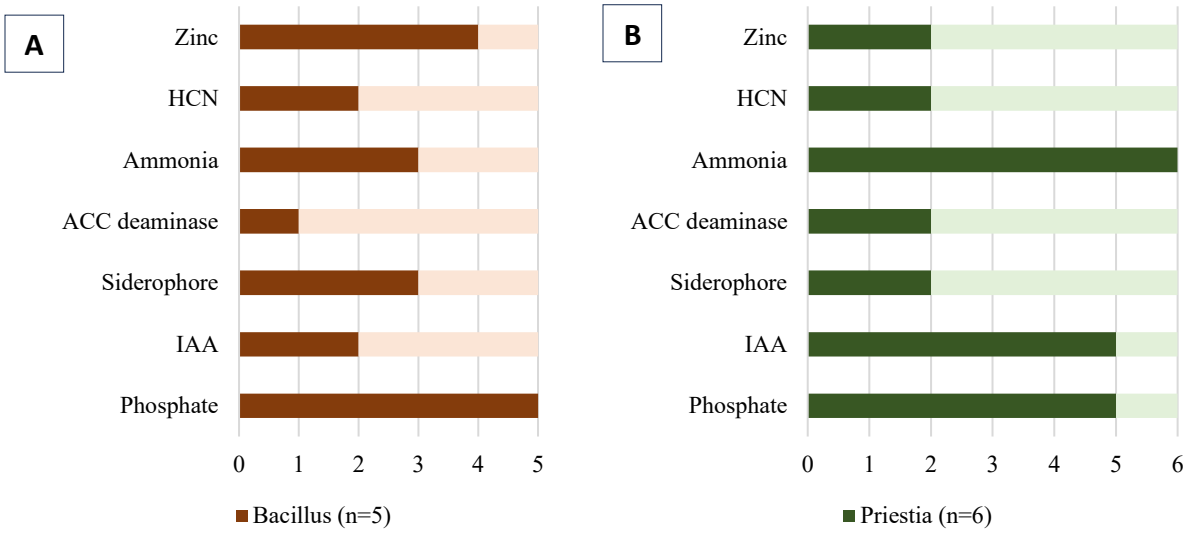
880

881

882

883

884



885

886 **Figure 10.** Occurrence of PGP attributes, including phosphate solubilization, production of indole-3-acetic
887 acid (IAA), siderophore, ACC deaminase, ammonia, hydrogen cyanide, as well as Zinc solubilization
888 among the isolates from (A) *Bacillus* (n=5) and (B) *Priestia* (n=6) genera.

889

890

891

892

893

894

895

896

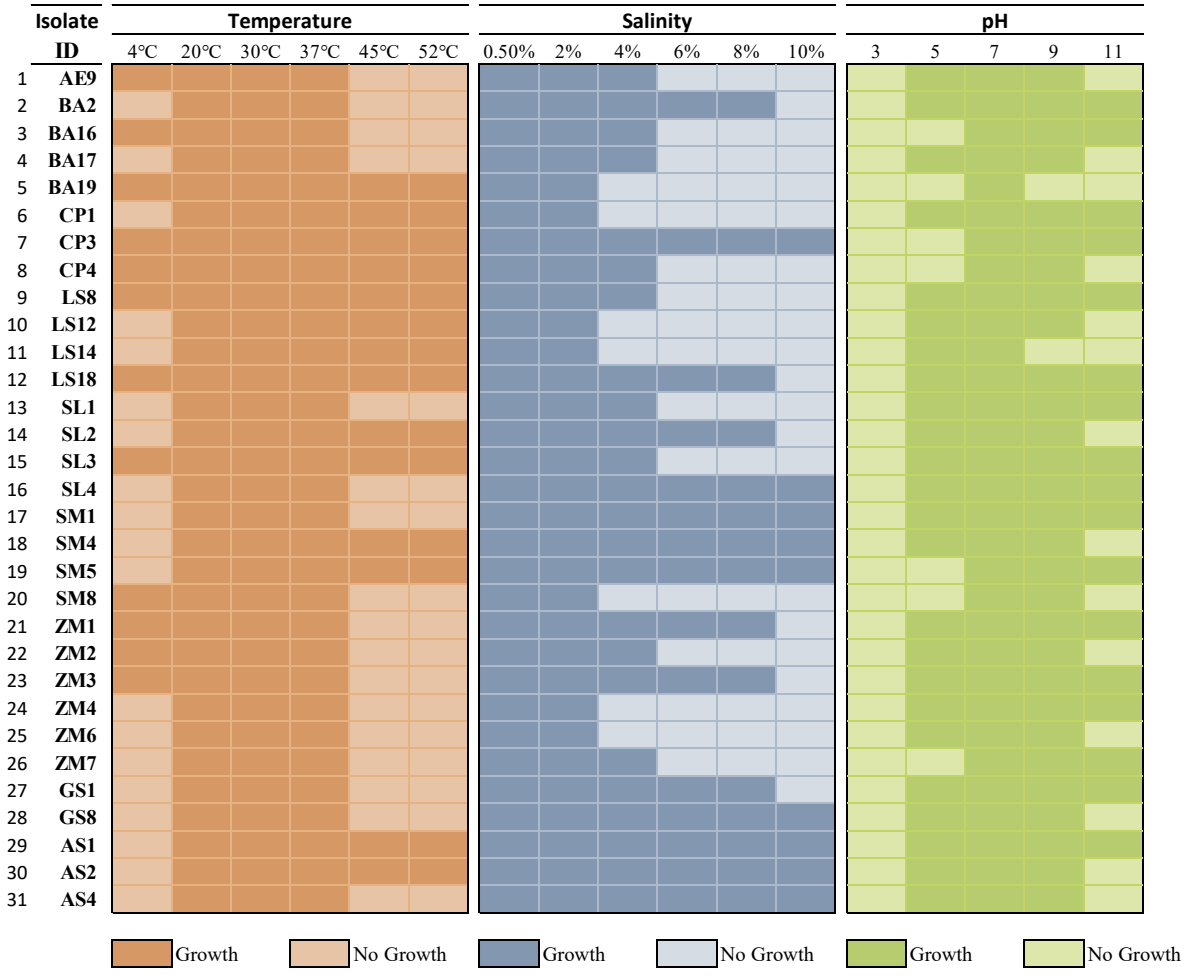
897

898 **Supplementary Materials**

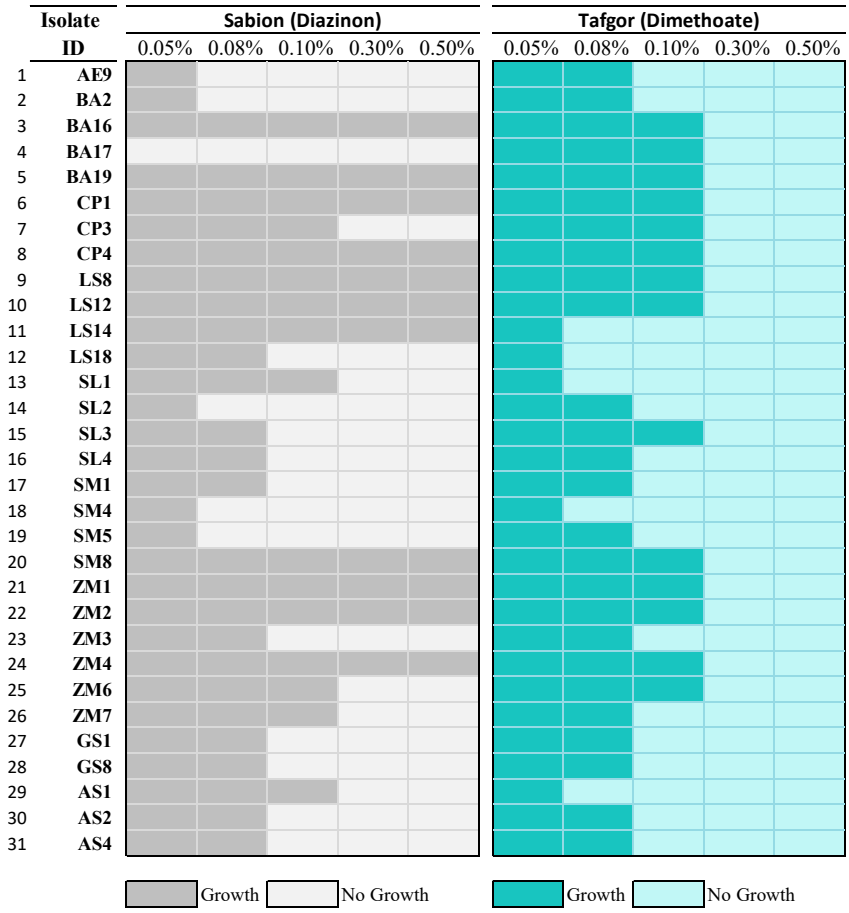
899 **Supplementary Table 1 (Table S1)**. Phenotypic characterization of the rhizobacterial isolates

Isolate ID	Citrate	Starch	Glucose	Lactose	H2S	Gas	Motility	Indole	Urease	MR	VP	Oxidase	Catalase	Nitrate	Gelatinase
AE1	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
AE7	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
AE8	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
AE9	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
BA1	-	+	+	-	-	-	+	-	-	+	-	-	+	+	+
BA2	+	+	+	-	-	-	+	-	-	-	-	-	+	-	+
BA4	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
BA6	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
BA7	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-
BA8	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
BA9	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-
BA12	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
BA14	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-
BA16	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-
BA17	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
BA18	+	-	-	-	-	-	-	-	+	-	-	+	+	+	-
BA19	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-
CP1	-	-	-	-	-	-	+	+	+	-	-	+	-	+	-
CP3	+	+	-	-	-	-	+	-	+	-	-	+	+	-	+
CP4	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-
LS4	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
LS5	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+
LS6	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+
LS8	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-
LS9	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
LS10	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
LS12	-	-	-	-	-	-	-	+	+	-	-	+	-	+	-
LS13	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
LS14	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-
LS15	+	-	-	-	-	-	-	+	+	-	-	+	+	+	-

LS18	+	-	+	+	-	+	+	-	-	-	+	+	+	+	-
MC1	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-
MC2	-	+	-	-	-	-	+	+	-	-	-	+	+	-	-
MC3	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
MC4	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-
MC5	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-
SL1	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
SL2	-	+	+	-	-	-	+	-	-	+	-	-	+	-	+
SL3	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-
SL4	+	+	-	-	-	-	+	+	-	+	-	-	+	-	+
SL5	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
SM1	+	+	-	-	-	-	+	+	-	-	-	-	+	-	+
SM2	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-
SM4	+	-	-	-	-	-	-	+	-	+	+	-	+	-	-
SM5	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-
SM6	-	+	+	-	-	-	+	-	-	+	-	-	+	+	-
SM7	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-
SM8	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
SM9	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
SM10	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
ZM1	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-
ZM2	-	-	-	-	-	-	+	+	+	-	-	+	+	-	-
ZM3	-	+	+	-	-	-	+	+	-	-	-	-	+	-	+
ZM4	+	-	-	-	-	-	+	+	+	-	-	+	-	+	-
ZM5	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-
ZM6	+	+	-	-	-	-	+	+	+	-	-	+	-	-	-
ZM7	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-
GS1	-	+	-	-	-	-	+	+	-	-	-	-	+	-	+
GS2	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-
GS5	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-
GS6	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-
GS7	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-
GS8	+	+	-	-	-	-	+	+	-	-	-	-	+	-	+
AS1	+	-	-	-	-	-	+	+	-	-	-	-	+	-	-
AS2	+	+	-	-	-	-	+	+	-	-	-	-	+	-	+
AS4	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+



Supplementary Figure 1 (Figure S1). Growth of isolates at various temperatures (4°C, 20°C, 30°C, 37°C, 45°C, and 52°C), concentrations of NaCl (0.5%, 2%, 4%, 6%, 8%, and 10%), and pH conditions (pH 3, 5, 7, 9, and 11).



909

910

911 **Supplementary Figure 2 (Figure S2).** Growth of isolates on organophosphate pesticides, including Sabion

912 (Diazinon) and Tafgor (Dimethoate), at up to 0.5% concentration.

913