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Research Paper

ACC deaminase and IAA producing growth promoting bacteria from the rhizosphere soil of tropical rice plants

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Beneficial plant-associated bacteria play a key role in supporting and/or promoting plant growth and health. Plant growth promoting bacteria present in the rhizosphere of crop plants can directly affect plant metabolism or modulate phytohormone production or degradation. We isolated 355 bacteria from the rhizosphere of rice plants grown in the farmers' fields in the coastal rice field soil from five different locations of the Ganjam district of Odisha, India. Six bacteria producing both ACC deaminase (ranging from 603.94 to 1350.02 nmol α-ketobutyrate mg^{-1} h^{-1}) and indole acetic acid (IAA; ranging from 10.54 to 37.65 μM ml^{-1}) in pure cultures were further identified using polyphasic taxonomy including BIOLOG(R), FAME analysis and the 16S rRNA gene sequencing. Phylogenetic analyses of the isolates resulted into five major clusters to include members of the genera *Bacillus*, *Microbacterium*, *Methylophaga*, *Agromyces*, and *Paenibacillus*. Seed inoculation of rice (cv. Naveen) by the six individual PGPR isolates had a considerable impact on different growth parameters including root elongation that was positively correlated with ACC deaminase activity and IAA production. The cultures also had other plant growth attributes including ammonia production and at least two isolates produced siderophores. Study indicates that presence of diverse rhizobacteria with effective growth-promoting traits, in the rice rhizosphere, may be exploited for a sustainable crop management under field conditions.

Keywords: Growth promoting rhizobacteria / Tropical rice / ACC deaminase / Indole acetic acid / Polyphasic taxonomy

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of free-living bacteria that live in the plant rhizosphere, aggressively colonize the root system, and have been studied as plant growth promoters for increasing agricultural production [1–3]. However, it is neither a single genus or species of bacteria nor a single trait rather it is a consortium of bacteria that possess various PGP properties that augments plant growth [4]. A diverse array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Xanthomonas*, and *Serratia* have been shown to promote plant growth [5].

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Plant–microbe interactions have been utilized to improve plant growth for the production of wood, fiber, bio-fuels, and key molecules [4]. In the era of sustainable agricultural production, plant–bacteria interactions in the rhizosphere play a pivotal role in transformation, mobilization, solubilization, etc. from the limited nutrient pool of the soil and subsequent uptake of essential plant nutrients by the crop plants to realize their full genetic potential [6]. PGPR stimulate the growth of plants by one or more of different direct and/or indirect mechanisms [7]. Indirect mechanisms are those related to the production of metabolites, such as siderophores which can sequester iron necessary for the growth of pathogens [8] and antifungal metabolites [9] that increase plant growth by controlling the activity of phytopathogens. Alternately, reported direct mechanisms include production of plant growth regulators (PGRs) such as auxins, cytokinins, gibberellins, and polyamines that can enhance various stages of plant growth [10],

enhancement of plant nutrient uptake especially phosphorus [11], nitrogen fixation [3], and stimulation of ion uptake or transport systems in plants [12].

Promotion of root growth is one of the markers of the beneficial effects of PGPR. The mechanism most often invoked to explain the various effects of PGPR on plants is the production of phytohormones, most notably auxins. There is ample evidence that several soil microorganisms are actively involved in the synthesis of IAA in pure culture and in soil [13]. Generally, microorganisms isolated from the rhizosphere and rhizoplane of various crops have revealed higher potential of IAA production than those from the root-free soil [14]. The levels of IAA produced by a bacterium in the rhizosphere determines its effect on the host plant, with high levels inducing developmental abnormalities and stimulating formation of lateral and adventitious roots, while low levels promoting root elongation.

In addition to these mechanisms of plant growth promotion, many PGPR has also been shown to stimulate plant growth through the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase that promotes plant growth by reducing plant ethylene levels. The enzyme hydrolyzes 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate biosynthetic precursor of the hormone ethylene in plant tissues to ammonia and α-ketobutyrate [7], thereby reducing the extent of the inhibitory effect of ethylene on root elongation, and thus promoting plant growth [15]. Plants treated with ACC-deaminase producing PGPR can be dramatically more resistant to the deleterious effects of ethylene synthesized as a consequence of stressful conditions, thereby inducing tolerance to several biotic and abiotic stresses [10, 16].

The beneficial effects of PGPR have been reported in many crops including cereals such as rice, wheat and sorghum [17–19], horticultural crops such as tomato [20], and oilseed crops such as canola [21]. The screening of the rhizobacteria for their PGP traits and growth-promoting activity could provide a reliable basis for the selection of effective PGPR strains and thereby buttress sustainable agriculture. For future development of commercial inoculants, it is therefore important to consider all potential metabolic activity of the phytohormone-producing bacteria. The objectives of the present study were (1) to isolate bacterial strains from the rhizosphere soil of rice plants grown in coastal Orissa (east coast of India), (2) assign taxonomic identity to the isolated strains by polyphasic method, (3) quantify *in vitro* production of IAA and ACC-deaminase activity, and (4) demonstrate plant growth promoting activity under controlled conditions to select efficient PGPR strains.

Materials and methods

Sampling and characterization of soils

The root adhering soil (RAS) samples were collected from coastal rice fields from five different locations of Ganjam district of Orissa (19°11′04.7″ to 20°06′48.7″N and 84° 48′06.3″ to 85°12′49.5″E), India, during September 2009 at the tillering stage of the monsoon season (*kharif*) rice. The rice plants were carefully uprooted along with the soil, placed in sterile polyethylene bags and brought to the laboratory after placing them in portable cool chambers (~4 °C). In the laboratory, the non-rhizosphere soil was removed by vigorously shaking the uprooted rice hills leaving behind the rhizosphere soil strongly adhering to the roots [22]. Physicochemical parameters of the soils including pH, EC, organic-C content and total C and N contents were determined according to Spark *et al.* [23] and reported in Table 1.

Isolation of bacteria

After manually shaking to dislodge the large soils aggregates, the roots were carefully washed with sterile distilled water to remove the medium soil aggregates. The soil still adhering to the roots after washing (rhizosphere soil) was suspended in sterile distilled water to prepare the rhizosphere soil suspension [22]. Dry

Table 1. Characteristics of the soils used in the study.

	Soil samples				
Properties	**Arunpur**	**Chilika**	**Haripur**	**Humma**	**Indrakhi**
GPS location	19°28′01.4″N 85°08′52.3″E	19°44′48.7″N 85°12′49.5″E	20°06′48.7″N 84°54′57.4″E	19°24′51.5″N 85°04′03.7″E	19°11′04.7″N 84°48′06.3″E
pH	6.59	5.57	6.08	6.7	6.8
EC (dS m^{-1})	0.047	0.82	1.00	1.75	0.98
Organic C (%)	0.690	0.600	0.800	1.290	0.570
Total C (%)	0.092	0.094	0.097	0.142	0.079
Total N (%)	0.793	0.776	0.968	1.515	0.693

weight of the soil present in the suspension was determined. Ten-fold dilutions were made in sterile distilled water and plated on seven media viz. nutrient agar (NA), Luria–Bertani (LB) agar, tryptose soy yeast extract (TSY) agar, 1/1000 dilution of TSY (TSY/1000) agar, casein–peptone–starch–glycerol (CPSG) agar, soil extract (SE) agar and Jensen's N-free agar, and incubated for 72 h at 30 °C [24]. Bacterial isolates with IAA production potential (see IAA Production Assay Section) were further screened for ACC deaminase activity by plating on DF salt minimal medium [25] containing ACC as the sole nitrogen source [26]. Bacterial colonies that developed were chosen based on their morphology and further purified by streaking agar plates of the same medium. Bacterial cultures were maintained onto the respective medium slants at 4 °C and/or in 65% glycerol at −80 °C till further use.

Characterization of the bacterial isolates

Morphological and biochemical characterization: Morpho-physiological and biochemical characteristics of the bacterial isolates were examined according to the Bergey's Manual of Determinative Bacteriology [27]. Individual cultures grown on nutrient agar at 30 °C were examined for the colony morphological features viz. color, form, elevation, margin, diameter, surface, opacity and texture. Motility and morphology were evaluated by performing phase contrast microscopy (Olympus BX-51, Olympus America Inc., USA). Gram staining was performed as per standard procedure with exponentially growing cultures. Plate assays were performed to determine different sugar source utilization by the isolates using 21 different sugar discs (Hi Media, India). Resistance profile of the bacterial isolates against different antibiotics was carried out on nutrient agar plates using eleven different antibiotic discs (Hi Media, India) placed on inoculated plates and incubated at 30 ± 2 °C. The results were recorded after 5 days of incubation.

Physiological characterization: The ability of the bacterial isolates to grow in diverse temperature range was studied by growing individual isolates in nutrient broth and incubated separately at 25, 30, 35, and 40 °C. Growth was recorded at 600 nm after 5 days of incubation by turbidimetry (Specord 200, Analytic Jena, Germany). Intrinsic tolerance of the rhizobacterial isolates against salinity was evaluated by observing their growth in nutrient broth amended with various concentrations of NaCl (0.34, 0.68, 1.02, 1.36, 1.71, 2.05, and 2.4 M) and incubated for 48 h at 30 ± 2 °C. Growth in NaCl amended broth was compared with those on unamended medium [28]. Ability of the isolates to grow in acid or alkaline media was studied by plating on nutrient agar with pH adjusted from 4 to 8.5. The plates were incubated for 72 h at 30 ± 2 °C and growth recorded.

BIOLOG$^{(R)}$ analysis: Potential carbon source utilization of the isolates was assessed via community level physiological profiling (CLPP) using the BIOLOG$^{(R)}$ GEN III MicroPlates (Biolog Inc., Hayward, CA). The isolates were grown at 33 °C on TSA (Tryptose Soy Agar) medium and then suspended in a special "gelling" inoculation fluid (IF) to get the transmittance of 90–98%. The cell suspension was then inoculated into the GEN III MicroPlate$^{(R)}$, 100 µl per well, and the MicroPlate was incubated at 33 °C for 22 h to allow the phenotypic fingerprint to form. After incubation the readings were obtained using automated BIOLOG$^{(R)}$ MicroStation Reader.

FAME analysis: The cellular fatty acid profiles of the isolates were analyzed by fatty acid methyl ester (FAME) analysis using MIDI$^{(R)}$ Sherlock Microbial Identification System. Isolates were grown on TSA medium at 28 °C for 24 h and then 20 mg of bacterial cell was harvested for extraction. The fatty acids were extracted by a procedure which consists of saponification in dilute sodium hydroxide/methanol solution followed by derivatization with dilute HCl/methanol to give the respective methyl esters which are more volatile. The FAMEs were then extracted from the aqueous phase by the use of the organic solvent hexane:methyl-*tert* butyl ether (1:1) and the resulting extract was analyzed by gas chromatography (GC; Agilent Technologies, USA) with flame ionization detector (FID) and Ultra 2 capillary column (25 m × 0.2 mm cross linked, 5% phenylmethyl silicon filled silica capillary column). FAMEs were identified according to their retention time, as compared to a commercial standard mixture (MIS standard calibration, Part no. 1200-A) [29].

Sequencing of 16S rDNA for identification of rhizobacterial isolates: Six bacterial strains efficient in plant growth promotion and preserving multiple beneficial traits were identified by partial sequencing of the 16S rDNA. Genomic DNA was isolated from the culture by using Genomic DNA isolation kit (Sigma, India). Amplification of 16S rRNA gene was carried out using forward (5′-AGAGTRTGATC MTYGCTWAC-3′) and reverse (5′-CGY-TAMCTTWTTACGRCT-3′) primers. The reaction was carried out in a 100 µl mixture containing 1 µl DNA template, 400 ng of each primer, 4 ml dNTP (2.5 mM each), 10 µl 10× Taq DNA polymerase assay buffer, 1 µl Taq DNA polymerase (3 U ml^{-1}). PCR reactions were carried out in a thermal cycler (Model ABI 2720, Applied Biosystems International, Foster City CA, USA). The PCR cycle used to amplify the 16S rRNA gene was as follows:

5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 2 min at 72 °C and a final extension of 5 min at 72 °C. The amplified 16S rDNA was purified with a PCR purification kit (Qiaquick PCR purification kit, Qiagen, India) and outsourced for sequencing (Chromus Biotech Pvt. Ltd., Bangalore, India). The sequence data was aligned with System Software aligner and analyzed to identify the bacterium and its closest neighbours by using BLAST (NCBI, USA). The partial 16S rDNA sequences were deposited in GenBank database.

Phylogenetic and molecular evolutionary analyses with the 16S rDNA sequences of six most effective bacterial isolates were conducted by using software MEGA4 [30]. All the six sequences were aligned using CLUSTAL-X. The pair-wise evolutionary distance matrix was generated and the evolutionary tree was inferred using the Neighbor-Joining method [31, 32]. The bootstrap test has been done to cluster together the associated taxa [32]. The evolutionary distances were compared using the Maximum Composite Likelihood method [33].

Screening for plant growth promoting activities

Indole acetic acid (IAA) production assay: IAA production by the isolated PGPB, both in the presence and absence of L-tryptophan (L-Trp), was detected by the method of Salkowski [34]. Uninoculated control was kept for comparison. The quantity of IAA produced was determined by UV–VIS spectrophotometry (Specord 200, Analytic Jena, Germany) against a standard curve of IAA ranging from 0.01 to 0.1 mM [35]. All measurements were made in five replicate samples and averaged.

ACC deaminase activity assay: To determine the amount of ACC deaminase activity, the amount of α-ketobutyrate generated from the cleavage of ACC was monitored [26]. The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per mg of protein per hour. All measurements were made in five replicate samples and averaged.

NH_3 production: Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 28 ± 2°C. Development of brown to yellow color following nesslerization was a positive test for ammonia production [36].

HCN production: All the isolates were screened for the production of hydrogen cyanide by adopting the method of Lorck [37]. Briefly, nutrient broth was amended with 4.4 g glycine L^{-1} and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2 °C for 4 days. Development of orange to red color indicated HCN production.

Siderophore production: Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by [38]. Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (10 μl of 10^6 CFU ml^{-1}) and incubated at 28 ± 2 °C for 48–72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production.

Phosphate solubilisation: For studying phosphate solubilization, 5 μl of overnight grown culture was spotted onto Pikovskaya's agar plates containing 2% tricalcium phosphate. The plates were incubated at 28 °C for 24–48 h and observed for the appearance of the solubilization zone around the bacterial colonies [39].

Plant growth-promotion activity by bacterial isolates

Seed treatment and root elongation assay with rice (cv. Naveen) were performed according to Penrose and Glick [26]. Primary plant growth parameters were measured on the fifth day of growth. The bacterial cell pellet of the six selected strains were washed and resuspended in 0.5 ml sterile 0.03 M $MgSO_4$ and placed on ice. The absorbance of the sample was adjusted evenly at 600 nm.

For the greenhouse experiment, soil sample was collected from the experimental fields at CRRI, Cuttack, air-dried, sieved (2-mm/10-mesh) and analyzed for physico-chemical characteristics The soil was alluvial having pH 6.16, EC 0.5 dS m^{-1}, CEC 15.0 meq g^{-1} soil, organic carbon 0.86%, total nitrogen 0.09% and contained 25.9% clay, 21.6% slit, and 52.5% sand. The air-dried soil was sterilized by autoclaving at 121.1 °C for 1 h for three consecutive days and 200 g portions of sterilized soil were filled in thermocol cups (4.5 cm × 4.5 cm × 5.5 cm).

Rice seeds (cv. Naveen) were surface-sterilized by dipping in 95% ethanol in glass petri dishes and then immersed in 0.2% $HgCl_2$ solution for 3 min, which were subsequently washed thoroughly with sterile distilled water for at least five times to make them free of $HgCl_2$ [10]. Each dish was incubated at room temperature for 1 h with the appropriate treatment – sterile 0.03 M $MgSO_4$ (used as a negative control) or bacterial suspensions in sterile 0.03 M $MgSO_4$.

Following inoculation, six seeds were placed in each cup with sterile forceps and 10 cups were used for each treatment. The cups were incubated in a growth chamber with maximum and minimum temperatures maintained at 28 and 20 °C, respectively with a cycle beginning with 12 h of dark followed by 12 h of light. The primary root lengths were measured on the fifth day of growth and the

data were analyzed. Seeds that failed to germinate by 2 days after they were sown were marked and the roots that subsequently develop from these seeds were not measured. The plants were harvested after 15 days and growth parameters (plant height including root and shoot length and plant biomass including fresh and dry weight) were recorded [40]. For measuring the chlorophyll content, 100 mg of finely chopped fresh leaves were placed in a capped measuring tube containing 25 ml of 80% acetone, and placed inside a refrigerator (4–8 °C) for 28 h. The chlorophyll content was measured at 646.6 and 663.6 nm in a spectrophotometer and calculated using the equation of Porra [41].

Statistical analysis

All results presented are the means of five independent replicates. Data were subjected to statistical analysis [42] by a statistical package (IRRISTAT version 3.1; International Rice Research Institute, Los Baños, Philippines). The mean difference comparison between the treatments was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p < 0.05$.

Results

Isolation and characterization of bacteria

A total of 355 bacteria were isolated from the coastal rice field soils from five different locations of Ganjam district of Orissa, India. The number of isolates obtained on seven different media viz., NA, LB agar, TSY agar, TSY/1000 agar, CPSG agar, SE agar, and Jensen's N-free agar medium were 76, 56, 53, 29, 47, 61, and 33, respectively. Out of the total 355 isolates, 66 isolates were selected for further study. Among the 66 bacteria, 11 were IAA-producing and of the IAA producing isolates 7 were ACC deaminase producing bacteria. Biochemical characterization, physiological stress tolerance and PGPR activities were examined for six isolates (AR-ACC1, AR-ACC2, AR-ACC3, ANR-ACC1, ANR-ACC2, and ANR-ACC3) having both the highest IAA and ACC deaminase production abilities.

Table 2. Morphological features, biochemical characters and plant growth promotion traits of the isolates.

	Bacterial isolates					
Characters	**AR-ACC1**	**AR-ACC2**	**AR-ACC3**	**ANR-ACC1**	**ANR-ACC2**	**ANR-ACC3**
Morphological						
Gram reaction	+	+	−		+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Cell length (μ)	3.6 ± 0.2	2.1 ± 0.1	2.0 ± 0.2	4.5 ± 0.2	2.0 ± 0.1	3.1 ± 0.2
Colony color	White	Yellow	White	White	Yellow	Cream
Motility	+	+	−	+	+	+
Biochemical						
MR	+	−	+	+	+	−
MRVP	+	−	−	+	+	−
Citrate utilization	−NG	+	−NG	+	−NG	−NG
Nitrate reduction	+	+	−	+	−	+
Oxidase	+	−	−	+	−	+
Catalase	−	−	−	−	−	−
Starch hydrolysis	−	−	−	−	−	+
Tributyrin hydrolysis	+	−	+	+	+	+
Tween 80 hydrolysis	−	−	+	−	−	+
Gelatin hydrolysis	+	+	−	+	+	+
Casein hydrolysis	+	+	−	+	+	+
Lysine decarboxylase	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+
Urease	−	−	−	+	−	−
Phenylaline deamination	−	−	−	−	−	+
Plant growth promoting traits						
IAA production	+++	++	+	++	+++	+
P-solubilization	−	−	−	−	−	−
HCN production	−	−	−	−	−	−
Ammonia production	+	+	+	+	+	+
Siderophore production	−	+	−	−	−	+
ACC deaminase production	+	+	+	+	+	+

NG, non growth. Values are the mean ± SE of three replicates, +++, high; ++, moderate; +, low. Cell lengths of exponential phase cultures grown in nutrient broth, were recorded. Colony color were recorded after growing the isolates on nutrient agar (NA).

Physiological and biochemical characterization

Morphological and physiological characteristics of six bacterial strains are given in Table 2. Following morphological characterization, motility and gram staining, the isolates were compared with those of the standard species using Bergey's Manual of Determinative Bacteriology [27].

All the isolates grew at the temperature ranging from 25 to 35 °C. Only two isolates AR-ACC1 and ANR-ACC1 could tolerate up to 45 °C. Most of the isolates tolerated salt concentrations ranging from 0.51–1.37 M NaCl excepting ANR-ACC2 that could tolerate up to 1.54 M NaCl. All the isolates were able to grow at the pH levels tested.

Carbon substrate utilization profile of the isolates was obtained using BIOLOG(R) system, which employs a redox reaction by tetrazolium dye to test the ability of isolates to utilize different carbon sources. All the six isolates were tested for their carbon source utilization profile using BIOLOG(R) GEN III MicroPlate which provide phenotypic fingerprint. Each assay was scored as growth or no growth (Table 3). Out of the 71 carbon sources assayed, at least one isolate (AR-ACC2) utilized 43 carbon sources followed by isolates ANR-ACC2, ANR-ACC1, and ANR-ACC3 which utilized 41, 40, and 34 carbon sources respectively. Isolate ARA-CC3 utilized only two carbon sources, L-glutamic and acetoacetic acid. Out of the 71, all the tested isolates grew on only 2 of the carbon sources (L-glutamate and acetoacetic acid).

The predominant FAMEs for each pure culture (Table 4) were selected on the basis of those FAMEs that comprised greater than 5% of the total area of FAMEs in each respective database file of FAME profiles (Sherlock Software, Microbial ID, Newark, NJ, USA).

Identification and phylogenetic analysis of bacterial isolates

Full length (about 1270–1460 bp) 16S rDNA of the six isolates were sequenced and the closest affiliation according to sequencing is shown in Table 5. The phylogenetic analyses of the isolates based on neighbor joining method with 100 bootstrap sampling resulted into five major clusters. The isolates AR-ACC1 and ANR-ACC1 clustered with the members of the genus *Bacillus*. The isolates AR-ACC2, AR-ACC3, ANR-ACC2, and ANR-ACC3 formed clusters with the members of the genera *Microbacterium*, *Methylophaga*, *Agromyces*, and *Paenibacillus*, respectively (Fig. 1).

Screening for plant growth promoting activities

Screening results of plant growth promoting activities are depicted in Table 2. All the six isolates were able to produce IAA, ammonia and having ACC deaminase activity. Production of siderophore was exhibited by only two isolates, AR-ACC2 and ANR-ACC3. But none of the isolate was capable of solubilizing tri-calcium phosphate or produce HCN.

Indole acetic acid (IAA) production assay

IAA production was recorded in all the isolates but their production efficiency varied, both in the presence or absence of L-Trp (Table 6). Among the six isolates AR-ACC1 produced highest amount of IAA followed by ANR-ACC2 > ANR-ACC1 > ANR-ACC3 > AR-ACC3 > AR-ACC2. In the presence of L-Trp, bacterial efficiency for IAA synthesis was enhanced several folds (ranging from 94.16 to 187.93 μM ml^{-1}). All the isolates produced high quantity of IAA during their stationary phase of growth.

ACC deaminase activity assay

The bacterial isolates differed in their potential for ACC-deaminase activity to degrade ACC into α-ketobutyrate (Table 6). Highest total ACC-deaminase activity per hour was exhibited by the isolate AR-ACC1 (1350.02 nmol α-ketobutyrate mg^{-1} h^{-1}) and was followed by AR-ACC2 > ANR-ACC3 > AR-ACC3 > ANR-ACC2 > ANR-ACC1 in that order.

Plant growth-promotion activity by bacterial isolates

Statistical analysis of data recorded after fifteen days of seed germination is summarized in Table 7. Results revealed that seed inoculation by the six PGPR isolates had a considerable impact on different growth parameters of rice compared to the uninoculated control (0.03 M $MgSO_4$). Data regarding root length indicate that inoculation with all the isolates except ANR-ACC1 enhanced root length significantly ($p = 0.05$) in comparison to the control after 15 days of growth. Maximum root length (16.20 cm) was obtained upon inoculation with the isolate AR-ACC1. Root elongation was positively correlated with ACC deaminase activity ($r = 0.91$) and IAA production ($r = 0.41$).

All the isolates promoted root fresh weight significantly ($p = 0.05$) in comparison with the control. The maximum root fresh weight was observed by isolate ANR-ACC3 that was 57% more than the uninoculated control. Likewise, all the isolates upon inoculation increased root dry weight significantly ($p = 0.05$) which followed the order of ANR-ACC3 > AR-ACC3 > ANR-ACC1 > ANR-ACC2 > AR-ACC1 and AR-ACC2.

Comparison of mean shoot length for the six isolates showed that inoculation with all the isolates significantly ($p = 0.05$) promoted the shoot length in comparison with the uninoculated control. The highest shoot length was

Table 3. Carbon source utilization profile of the isolates using the BIOLOG GEN III MicroPlates.

	Gelatin	Glycyl-L-proline	L-Alanine	L-Arginine	L-Aspartate	L-Glutamate	L-Histidine	L-Pyroglutamate	L-Serine	Pectin	D-Galacturonate	D-Gluconate	D-Glucoronate	Glucuronamide	p-Hydroxy-phenylacetate	Methyl pyruvate	L-Lactic acid	Citric acid	α-Keto-glutaric Acid	D-Malic acid	L-Malic acid	Bromo-succinic acid	Tween 40	γ-Amino-butyric acid	α-Hydroxy butyric acid	β-Hydroxy-D,L-butyrate	α-Keto-bButyric acid	Acetoacetic acid	Propionic acid	Acetic acid	Formic acid
AR-ACC1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AR-ACC2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AR-ACC3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ANR-ACC1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ANR-ACC2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ANR-ACC3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-D-glucoside	D-Salicin	N-Acetyl-D-glucosamine	N-Acetyl-D-galactosamine	N-Acetyl- β-D-mannosamine	N-Acetyl neuraminate	α-D-glucose	D-Mannose	D-Fructose	D-Galactose	L-Fucose	L-Rhamnose	Inosine	D-Sorbitol	D-Mannitol	D-Arabitol	myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-PO4	D-Serine
AR-ACC1	+	+	+	+	+	+	+	+	+	+			+	+	+		+	+	+	+		+	+	+	+	+		+	+	+	+
AR-ACC2	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+		+	+	+	+			+	+	+	
AR-ACC3	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+				+	+			+		+	
ANR-ACC1	+	+	+	+	+	+	+	+	+		+	+	+	+	+		+	+	+	+		+	+	+	+	+	+	+	+	+	+
ANR-ACC2	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+		+	+	+	+			+	+	+	
ANR-ACC3	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+				+	+			+		+	

Table 4. Percentage of total fatty acids presents in isolates.

	Percentage of total fatty acids							Percentage of total fatty acids					
Fatty Acids	AR-ACC1	AR-ACC2	AR-ACC3	ANR-ACC1	ANR-ACC2	ANR-ACC3	Fatty Acids	AR-ACC1	AR-ACC2	AR-ACC3	ANR-ACC1	ANR-ACC2	ANR-ACC3
12:00	ND	ND	0.63	0.39	ND	0.37	17:0 iso	12.11	2.69	ND	7.38	ND	4.31
11:0 2OH	ND	0.04	ND	ND	ND	0.72	17:0 anteiso	2.49	24.11	0.41	1.62	ND	7.22
13:0 anteiso	ND	0.15	0.42	1.37	ND	1.38	17:1 ω5c	ND	0.05	ND	ND	0.5	ND
12:0 3OH	ND	ND	ND	ND	0.59	ND	17:00	0.22	0.16	3.35	ND	ND	ND
14:0 iso	ND	0.64	ND	2.9	ND	1.79	18:0 iso	0.45	0.13	ND	ND	ND	ND
14:1 ω5c	0.68	ND	0.21	ND	ND	ND	18:1 ω9c	0.6	0.05	7.31	0.43	ND	0.71
14:00	3.44	0.06	4.71	3.18	ND	1.72	18:1 ω5c	ND	ND	ND	0.85	ND	ND
15:0 iso	33.69	9	ND	32.82	ND	11.54	18:00	0.66	0.08	1.5	0.83	ND	0.98
15:0 anteiso	6.93	40.68	0.62	4.42	ND	43.4	17:0 iso 3OH	0.41	0.06	ND	0.37	ND	ND
15:1 ω6c	ND	ND	ND	ND	0.98	ND	18:0 10-methyl, TBSA	ND	0.07	21.91	ND	ND	ND
15:1 ω5c	0.5	ND	0.18	0.29	ND	ND	19:0 iso	0.88	ND	ND	ND	ND	ND
14:0 iso 3OH	0.49	ND	ND	0.33	ND	ND	19:0 anteiso	ND	0.09	ND	ND	ND	ND
16:1 ω7c alcohol	0.47	ND	ND	0.86	0.51	0.89	20:1 ω9c	ND	ND	1.61	ND	ND	ND
16:0 N alcohol	ND	ND	ND	ND	ND	0.45	20:00	ND	ND	0.72	ND	ND	ND
16:0 iso	5.8	20.45	0.34	3.82	ND	6.13	Sum. feature 1[a]	ND	ND	ND	0.22	68.45	ND
16:1 ω11c	0.25	ND	ND	0.54	ND	4.84	Sum. Feature 2[b]	3.42	ND	ND	2.92	ND	ND
16:1 ω9c	ND	ND	ND	ND	0.24	ND	Sum. feature 3[c]	8.95	ND	11.02	9.66	0.34	0.7
16:00	3.56	1.29	32.45	2.68	ND	9.43	Sum. Feature 4[d]	ND	ND	ND	ND	ND	1.16
15:0 2OH	0.69	ND	ND	0.66	ND	ND	Sum. feature 6[e]	ND	ND	1.08	ND	ND	ND
17:1 iso ω10c	2.91	ND	ND	2.55	ND	1.19	Sum. feature 7[f]	ND	ND	1.32	ND	ND	ND
17:1 iso ω5c	7.87	0.09	ND	5.69	ND	ND	Sum. feature 8[g]	0.26	ND	ND	0.3	ND	0.5
17:1 anteiso A	1.53	ND	ND	0.98	28.39	ND	Sum. feature 9[h]	ND	ND	6.29	ND	ND	ND

ND, fatty acid was not detected.
[a]13:0 3OH/15:1 iso H.
[b]14:0 3OH/16:1 isoI.
[c]16:1 ω6c/16:1 ω7c.
[d]17:1 anteiso/isoI.
[e]19:1 ω11c/19:1 ω9c.
[f]19:1 ω7c/19:1 ω6c, 19:0 cyclo ω10c/19ω6.
[g]18:1 ω7c.
[h]17:1 iso ω9c.

Table 5. Identity of isolates AR-ACC1, AR-ACC2, AR-ACC-3, ANR-ACC1, ANR-ACC2, and ANR-ACC3 as established by BIOLOG® GenIII, FAME, and molecular identification.

			Molecular identification			
Isolates	Biolog GEN III identification	FAME identification	NCBI accession number	16S rDNA fragment length (bp)	Closest relatives and NCBI accession number	Similarity (%)
AR-ACC1	*Bacillus* sp.	*Bacillus* sp.	HM063033	1456	*Bacillus* sp. cp-h16; EU584534	99
AR-ACC2	*Microbacterium* sp.	*Microbacterium* sp.	HM063034	1428	*Microbacterium paraoxydans* strain M2; GQ200829	99
AR-ACC3	Unassigned	Unassigned	HQ222610	1272	*Methylophaga sp.* DMS048; DQ660930	95
ANR-ACC1	*Bacillus* sp.	*Bacillus* sp.	HM063030	1446	*Bacillus* sp. eg4; DQ278903	99
ANR-ACC2	*Corynebacterium* sp.	*Corynebacterium* sp.	HM063031	1431	*Agromyces* sp. MJ21; GQ241325	96
ANR-ACC3	*Paenibacillus* sp.	*Paenibacillus* sp.	HM063032	1439	*Paenibacillus elgii* strain SD17; AY090110	99

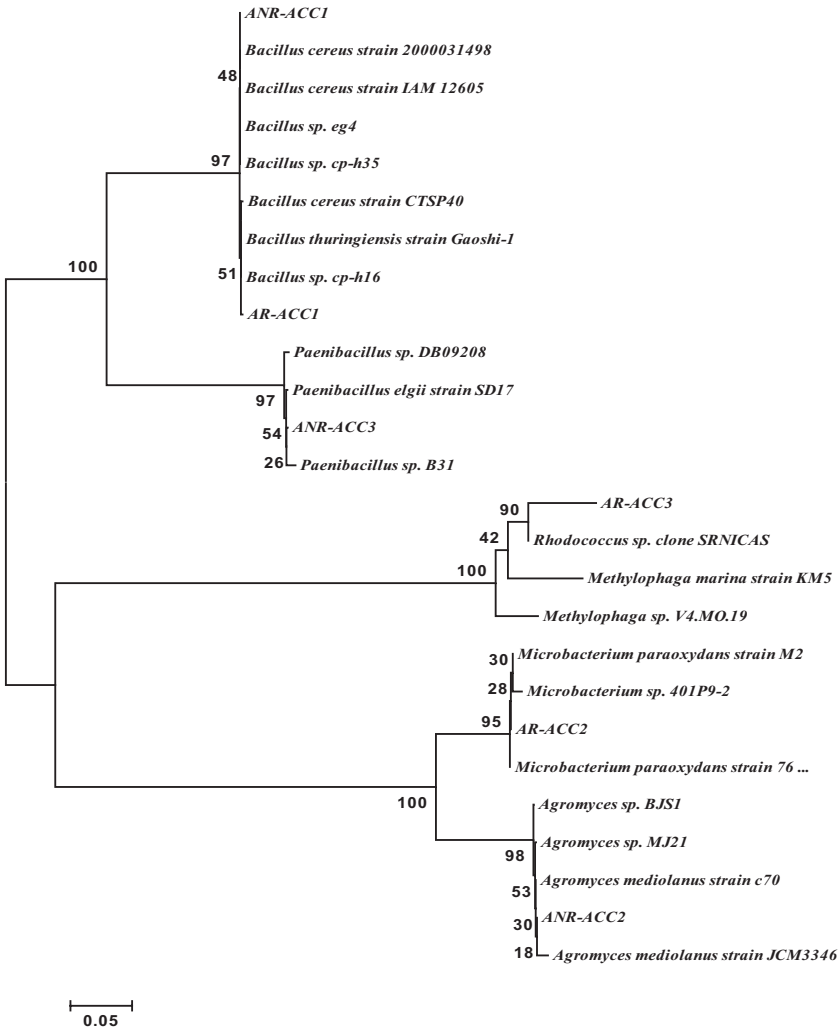

Figure 1. Neighbor joining tree showing phylogenetic relationship based on the 16S rDNA sequences of the selected PGPR isolates from rice rhizosphere soil and their representative species from NCBI database. The bar represents 0.05 substitutions per site. Bootstrap value ($n = 100$) are displayed at the node.

observed with the isolate ANR-ACC3. Significant increase in shoot fresh weight of rice as compared to the uninoculated control was also recorded with the most promising increase of 51% over control was observed on inoculation with ANR-ACC3. The results for shoot dry weight showed that all the isolates significantly ($p = 0.05$) increased the shoot dry weight in comparison to uninoculated control. ANR-ACC2 and AR-ACC3 were

Table 6. Indole acetic acid production and ACC deaminase activity of the isolated bacterial cultures.

Bacterial isolates	Indole acetic acid produced (μM ml^{-1})[a]		ACC deaminase activity (nmol α-ketobutyrate mg^{-1} h^{-1})
	Without L-Trp	With L-Trp	
AR-ACCI	37.65 ± 1.03	187.93 ± 5.35	1350.02 ± 42.5
AR-ACC2	10.54 ± 1.23	94.16 ± 2.41	961.84 ± 27.8
AR-ACC3	15.23 ± 1.05	105.87 ± 1.26	819.55 ± 30.2
ANR-ACC1	25.64 ± 2.76	132.26 ± 2.08	603.94 ± 29.3
ANR-ACC2	32.24 ± 1.32	173.80 ± 3.68	762.24 ± 37.9
ANR-ACC3	22.24 ± 4.23	125.83 ± 2.97	862.48 ± 41.1

[a]Mean of five replicate values ± SD.

Table 7. Effect of inoculation of six PGPR isolates on different growth parameters of rice (*cv.* Naveen) 15 days after seed germination.

Treatment	RL (cm)	RFW (g)	RDW (g)	SL (cm)	SFW (g)	SDW (g)	Chl a (mg g^{-1} fresh wt)	Chl b (mg g^{-1} fresh wt)	Chl a + b (mg g^{-1} fresh wt)
Control ($MgSO_4$)	14.52^{b*}	0.168^{d}	0.018^{d}	30.54^{c}	0.285^{d}	0.049^{d}	2.781 f	0.869^{d}	3.650^{e}
AR-ACC1	16.20^{a}	0.210^{c}	0.022^{c}	32.64^{b}	0.339^{c}	0.054^{c}	2.775 f	0.997^{c}	3.771^{e}
AR-ACC2	15.48^{a}	0.210^{c}	0.021^{c}	32.74^{b}	0.335^{c}	0.053^{c}	3.782^{e}	1.057^{c}	4.839^{d}
AR-ACC3	15.54^{a}	0.255^{ab}	0.025^{ab}	35.27^{a}	0.429^{a}	0.069^{a}	4.984^{c}	1.792^{a}	6.777^{b}
ANR-ACC1	14.74^{b}	0.239^{b}	0.023^{bc}	33.30^{b}	0.359^{b}	0.060^{b}	4.509^{d}	1.544^{b}	6.054^{c}
ANR-ACC2	15.44^{a}	0.254^{ab}	0.023^{bc}	34.80^{a}	0.418^{a}	0.069^{a}	5.252^{b}	1.888^{a}	7.140^{a}
ANR-ACC3	15.63^{a}	0.263^{a}	0.027^{a}	35.77^{a}	0.431^{a}	0.067^{a}	5.536^{a}	1.875^{a}	7.412^{a}
LSD (5%)	0.796	1.095	0.027	0.026	0.004	0.003	0.267	0.130	0.370

In a column, means followed by a common letter are not significantly different ($p < 0.05$) by Duncan's multiple range test.
*Mean of five replicate observations.

the most effective isolate which enhanced shoot dry weight by 41% over control.

Inoculation with all the isolates excepting AR-ACC1 increased the total chlorophyll content of rice plants significantly ($p = 0.05$) in comparison with the uninoculated control. The highest chlorophyll content was observed in the plants treated with the isolate ANR-ACC3 that was 103% higher than the uninoculated control. Isolate AR-ACC1 caused the least increase in chlorophyll content and was statistically at par with the control.

Discussion

The plant rhizosphere is a versatile and dynamic ecological environment of intense microbe–plant interactions for harnessing essential micro- and macronutrients from the limited nutrient pool [5]. Several factors viz. root morphology, the stage of plant growth, root exudates, and the physico-chemical properties of the soil are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere.

In the present investigation, six bacterial isolates were screened *in vitro* for their PGP abilities. The isolates, AR-ACC1, AR-ACC2, AR-ACC3, ANR-ACC1, ANR-ACC2, and ANR-ACC3 were identified as *Bacillus* sp., *Microbacterium* sp., *Methylophaga* sp., *Bacillus* sp., *Agromyces* sp., and *Paenibacillus* sp., respectively by 16S rDNA sequencing. The substrate utilization profile by BIOLOG and FAMEs profile of the isolates AR-ACC1, AR-ACC2, ANR-ACC1, and ANR-ACC3 matched with the result of 16S rDNA sequencing for identification. But AR-ACC3 was identified as *Arthrobacter* sp. by substrate utilization profile analysis whereas it was identified as *Rhodococcus* sp. by FAME analysis. ANR-ACC2 was identified as *Microbacterium* sp. by substrate utilization profile but could not be identified by using the existing database of FAME profile.

Rhizosphere bacteria referred to as PGPR include the well-known *Azotobacter* and *Azospirillum* sp., and also a number of other bacteria, including various species of *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Alcaligenes*, *Klebsiella*, *Enterobacter*, *Herbaspirillum*, *Xanthomonas*, and *Bacillus* [1]. *Microbacterium* species having PGPR activities have been reported earlier [43], so also *Paenibacillus* sp. [44], and *Bacillus* sp. [45] as effective PGP bacteria.

Indole-3-acetic acid (IAA) is a quantitatively important phytohormone produced by several PGPR and treatment with such auxin-producing rhizobacteria increases plant growth [13]. All the isolates listed in the present study are capable of producing IAA with the isolate AR-ACC1, a *Bacillus* sp. showing the highest IAA production. IAA production by all the isolates increased several fold when culture medium was supplemented with L-Trp (Table 6). Following addition of L-Trp, IAA production of PGPR strains range from 5–10 to 100–200 μg IAA ml^{-1} culture medium or higher depending on exogenous L-Trp concentration [46].

ACC is an immediate precursor of ethylene in higher plants, and the production of ethylene is highly and positively dependent on the endogenous levels of ACC [15]. The enzyme ACC-deaminase present in some microorganisms hydrolyzes ACC into ammonia and α-ketobutyrate, limiting the substrate availability for ethylene production and thereby indirectly stimulating plant growth [15]. *In vitro* ACC-deaminase activity of the rhizobacterial isolates used in the present study varied substantially but compared suitably with reported values [47]. Plant growth-promoting *Bacillus* sp. with ACC deaminase activity was first reported by Ghosh *et al.* [45].

Root elongation assay was used for the selection of the most effective PGPR. Data revealed that the selected

isolates significantly differed in their potential to promote root elongation. There was positive correlation ($r = 0.41$) between *in vitro* IAA production by the isolates and plant root elongation under controlled condition. Mechanism most commonly invoked to explain the various effects of PGPR on plant growth is the production of phytohormones, and IAA may play the most important role in plant growth promotion. Khalid *et al.* [17] suggested that majority of the rhizobacteria (~89%) active in IAA production showed stimulatory effect on root elongation (up to 233%) and weight (150%) of rice seedlings. The ACC-deaminase activity of the isolates used in the present study showed significant positive correlation ($r = 0.91$) with root elongation in the inoculated plants, than IAA production. Studies indicate that inoculation with bacteria carrying the ACC-deaminase enzyme promotes root growth of seedlings of various crops [2]. The differences in plant growth promotion among the isolates are also attributed to their individual rhizospheric competencies and hydrolyzing the ACC synthesized in roots.

Siderophore production by bacteria in a plant–bacteria association is beneficial to the plant in mobilizing different metal ions and has also some bio-control properties by helping a particular microorganism to compete effectively against others for available iron [8]. ACC deaminase and siderophore producing PGPB can help plants to overcome many of the effects of metal stresses [48]. Bacterial isolates AR-ACC2 and ANR-ACC3 were tested positive for siderophore production. Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia and all the isolates in the present study were able to produce ammonia. Further, at least two isolates were able to tolerate higher temperature while two other isolates were capable of tolerating high salinity mediated by NaCl. Under conditions of intensive agriculture involving irrigation and in coastal areas, salinity is a major problem affecting crop productivity. High salinity also adversely affects microbial inoculants from expressing their full potential. Similarly, high temperature also affects both crop production and microbial activity. While several PGPR are known to alleviate abiotic stresses [16] caused by salinity [20, 49] or high temperature, any microbial incoulant including PGPR to be used as biofertilizer [44] is likely to be more successful under field conditions with additional characteristics like higher salt or high temperature tolerance.

Plant growth-promoting bacteria have been shown to positively impact plant performance through different mechanisms [5, 6]. For future development of commercial inoculants it is important to consider all potential physiological and biochemical properties of the phytohormone-producing bacteria. In the present study, six potential PGPR with multiple PGP traits isolated from rice rhizosphere soil indicated that plant root length was positively correlated with both IAA production and ACC deaminase activity of the isolates. Different plant growth promoting techniques (e.g., growth promotion under gnotobiotic conditions, *in vitro* production of PGRs and screening for ACC deaminase activity) are used for the selection of beneficial PGPR. In addition to these approaches, novel molecular and biochemical technologies are needed to screen rhizobacteria as PGPR in promoting the yield of agronomic crops and sustainable agriculture. For successful application of plant-growth promotion using bacterial inoculants, various aspects of the plant environment need to be considered under exacting field situations.

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