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Characterization of Plant-Growth-Promoting Traits of *Acinetobacter* Species Isolated from Rhizosphere of *Pennisetum glaucum*

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A total of 31 *Acinetobacter* isolates were obtained from the rhizosphere of *Pennisetum glaucum* and evaluated for their plant-growth-promoting traits. Two isolates, namely *Acinetobacter* sp. PUCM1007 and *A. baumannii* PUCM1029, produced indole acetic acid (10–13 μg/ml). A total of 26 and 27 isolates solubilized phosphates and zinc oxide, respectively. Among the mineral-solubilizing strains, *A. calcoaceticus* PUCM1006 solubilized phosphate most efficiently (84 mg/ml), whereas zinc oxide was solubilized by *A. calcoaceticus* PUCM1025 at the highest solubilization efficiency of 918%. All the *Acinetobacter* isolates, except PUCM1010, produced siderophores. The highest siderophore production (85.0 siderophore units) was exhibited by *A. calcoaceticus* PUCM1016. Strains PUCM1001 and PUCM1019 (both *A. calcoaceticus*) and PUCM1022 (*Acinetobacter* sp.) produced both hydroxamate- and catechol-type siderophores, whereas all the other strains only produced catechol-type siderophores. *In vitro* inhibition of *Fusarium oxysporum* under iron-limited conditions was demonstrated by the siderophore-producing *Acinetobacter* strains, where PUCM1018 was the most potent inhibitor of the fungal phytopathogen. *Acinetobacter* sp. PUCM1022 significantly enhanced the shoot height, root length, and root dry weights of pearl millet seedlings in pot experiments when compared with controls, underscoring the plant-growth-promoting potential of these isolates.

Keywords: *Acinetobacter*, plant-growth-promoting rhizobacteria, *Pennisetum glaucum*, siderophore, mineral solubilization, pot experiment

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The rhizosphere contains large bacterial populations capable of exerting beneficial or detrimental influences on plant growth. Rhizobacteria that exert positive effects on plant development are called plant-growth-promoting rhizobacteria (PGPR) [5, 8] and are commonly used to improve the growth and yield of agricultural crops. Thus, the screening of effective strains from the rhizosphere of crop plants is critical [7]. In this context, there is some evidence that *Acinetobacter* strains play an important role in plant-growth promotion [9, 21, 23, 43], as certain strains of this genus are known to be involved in phytostimulation based on the production of plant-growth-promoting hormones [9, 21], solubilization of phosphate [17, 36], and production of siderophores [23, 43, 46]. Meanwhile, other *Acinetobacter* strains exhibit indirect PGPR activity *via* the growth suppression of phytopathogenic fungi, such as *Cryphonectria parasitica*, *Phytophthora capsici*, and *Rhizoctonia solani* [21, 32], and potential biocontrol properties against pathogenic bacteria like the *Ralstonia solanacearum* related to the wilt of tomatoes [56].

Pearl millet (*Penisetum glaucum*) was used for the present study owing to its increasing worldwide importance as a food and forage crop [19, 38, 39, 48]. Moreover, whereas various recent studies on the use of PGPR have focused on different economically important agricultural crops, such as wheat [44, 46], rice [3], and maize [12, 48], there is little available information on the rhizobacteria that could promote the growth of pearl millet [7, 19, 48]. Hence, indigenous *Acinetobacter* strains were isolated from the rhizosphere of pearl millet to investigate their plant-growth-promoting properties, such as indole acetic acid (IAA) production, phosphate and zinc oxide solubilization, and siderophore production.

Materials and Methods

Isolation of *Acinetobacter* sp. from Rhizosphere of Pearl Millet

Different fields of pearl millet were selected in the Pune district, India, including Kamshet, Rajgurunagar, and Paud. Healthy pearl millet plants were uprooted during different growth stages (seedling, flowering, and reproductive). The roots of the plants with their rhizosphere soil were kept in sterile plastic bags in an icebox and carried to the laboratory. The rhizosphere soil samples were then suspended in sterile saline (5 g of soil in 45 ml) and Bauman's enrichment medium (5 g in 75 ml) [6], and incubated on a shaker (200 rpm) at 30°C for 1 h and 48 h, respectively. Thereafter, the soil was allowed to settle by gravity and the supernatant serially diluted and plated on an *Acinetobacter* minimal medium (AMM) [25], Cysteine Lactose Electrolyte Deficient agar (CLED; HiMedia, Mumbai, India), Holton's medium without cefsulodin [20, 44], and violet red bile agar (VRBA; HiMedia, Mumbai, India) [21]. Preliminary characterization of the isolates included the colony morphology, Gram staining, capsule staining, motility, and oxidase and catalase reactions. These tests were performed as described by Gerhardt *et al.* [16].

Identification of Bacterial Isolates

The putative *Acinetobacter* isolates obtained from the rhizosphere of pearl millet were identified using a polyphasic identification approach, which included a chromosomal DNA transformation assay, biochemical profiling by API ID32GN, 16S rRNA gene sequencing, a FAME analysis, and determination of the G+C content.

Chromosomal DNA transformation assay (CTA). A naturally competent tryptophan auxotrophic mutant of *Acinetobacter baylyi* (BD4 *trp*E27) was transformed with the total DNA of a putative *Acinetobacter* isolate, as described previously [4, 25, 58], and the transformation mixture plated on a brain heart infusion agar. The growth was then harvested after incubation for 24 h at 30°C, plated on an *Acinetobacter* minimal agar, and incubated at 30°C for 108 h. Growth on the minimal agar medium indicated a positive transformation assay and confirmed the isolate as a member of the genus *Acinetobacter*. *E. coli* HB101 and *A. calcoaceticus* MTCC1921^T were used as the negative and positive controls, respectively.

Biotyping by API 32GN system. API ID32GN (bioMerieux, France), a standard system with 32 miniaturized assimilation tests and a database of Gram-negative bacteria, was used for automatic identification of putative *Acinetobacter* sp. [51].

16S rRNA gene sequencing. The *Acinetobacter* isolates were identified up to the species level on the basis of 16S rRNA gene sequencing as follows: Total genomic DNA was extracted from the putative *Acinetobacter* isolates using a Gen-Elute DNA isolation kit (Sigma-Aldrich, USA) according to the manufacturer's instructions (http://www.sigmaaldrich.com). The 16S rRNA gene was then amplified by a polymerase chain reaction (PCR) using the universal primers FDD2 (5'-CCGGATCCGTCCACAGAGTTTGATCITGGCTCAG-3') and RPP2 (5'-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3') on a GeneAmp 9700 PCR System (Applied Biosystems Inc., USA) [41]. All the PCR reaction mixtures (20 µl) contained 2 pmol of the forward and reverse primers, 0.2 mM dNTPs, 1 U of *Taq* polymerase (Sigma-Aldrich Ltd, USA), and *ca.* 50 ng of genomic DNA. The following time–temperature profile was used: initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing of the primers for 1 min at 62°C, and an extension for 1 min at 72°C, with a final extension for 20 min at 72°C for the last cycle. The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels in a 1× TAE buffer (40 mM Tris acetate and 1 mM sodium EDTA, pH 7.9, adjusted by glacial acetic acid), visualized under ultraviolet transillumination, and documented using an Alphaimager Gel Documentation System (Alpha Innotech, USA). The unincorporated dNTPs and primers were removed from the PCR products using the polyethylene glycol (PEG) precipitation method. The cycle sequencing was performed using a Big Dye terminator kit (Applied Biosystems Inc., USA). The reaction mixture (20 µl) contained 3 µl of Ready mix (ABI, USA), 2.5 µl of the sequencing buffer (ABI, USA), the sequencing primer (0.2 mM), and 2 µl of the PCR product. The following time–temperature profile was used: initial denaturation for 1 min at 96°C, followed by 25 cycles of denaturation for 10 s at 96°C, annealing of the primers for 5 s at 50°C, and an extension for 4 min at 60°C. A Big Dye Terminator Cycle Sequencing Clean up was performed using the sodium acetate–ethanol precipitation method. In brief, 2 µl of 125 mM EDTA and 2 µl of 3 M sodium acetate (pH 4.6) were added to 20 µl of the cycle sequencing product and the contents mixed by tapping. After adding 50 µl of 95% (v/v) ethanol, the tubes were incubated at room temperature for 15 min and centrifuged at 12,600 ×*g* for 20 min at room temperature. The supernatant was then discarded and the pellet washed twice with 70% (v/v) cold ethanol. Finally, the pellet was air dried, dissolved in 20 µl of formamide, and denatured at 95°C for 4 min. The automated fluorescent DNA sequencing was performed using a 3100 Avant DNA sequencer (Applied Biosystems Inc., USA), whereas the sequence alignment with reference sequences available in the GenBank database was conducted using the advanced Blastn search program (http://www.ncbi.nlm.nih.gov/BLAST) [10, 43].

FAME analysis. The methyl esters of fatty acid extracted from each isolate were analyzed on a Hewlett-Packard 5890A gas chromatograph (Avondale, PA, USA) equipped with a flame ionization detector, automatic sampler, integrator, and computer. Separation of the FAMEs was achieved using a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5% (v/v) phenylmethyl silicone. The specific operating parameters of the instrument were controlled and set automatically by the computer software. The operating parameters used in this study were as follows: injector temperature, 250°C; detector temperature, 300°C; and oven (column) temperature, programmed from 170 to 300°C at 5°C/min and held at 300°C for 5 min prior to recycling. Chromatograms with the peak retention times and areas were produced by a recording integrator and electronically transferred to a computer for analysis, storage, and report generation. The peak naming and column performance were achieved through the use of calibration samples (Microbial ID) containing straight-chain saturated and hydroxyl fatty acids. A fresh calibration mixture was used daily prior to the analysis of the samples, and was automatically rerun and evaluated after every 10 samples [28].

Determination of DNA base composition. The DNA (50 ng in 10 µl of sterile distilled water) was mixed with 80 µl of a 0.1× SSC solution (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). A SYBR Green I solution (1 µl of 1:4,000 dilution) was then added to 9 µl of the DNA solution. Next, the entire mixture (10 µl) was placed in a capillary tube and heated using a LightCycler 2.0 thermal cycler (Roche, USA). The temperature was maintained at 70°C for 15 s and then increased to 98°C at a ramping rate of 0.1°C per second. The fluorescence intensity was monitored sequentially. The fluorescence data for the melting curves were acquired on a

plot of the derivative of the fluorescence with respect to temperature (dT/dF), and the data also converted into melting peaks using the Light Cycler software 4.05. The center of the melting peak was the T_m. The G+C contents were then calculated using the T_m values measured by the Light Cycler as follows:

$$[\text{mol\% G+Cx}=\text{mol\% G+Cr}+1.4652(T_m x - T_m r)+0.0063(T_m x^2 - T_m r^2)]$$

where x is the unknown organism and r is the reference organism [55].

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of 31 *Acinetobacter* strains were submitted to GenBank. The accession numbers are described in Table 1.

Phylogenetic Analysis of Strains

The sequences from this study and reference sequences were aligned using the program DNAMAN (version 5.1; Lynnon Biosoft) [54]. A phylogenetic tree was then constructed from a matrix of pairwise genetic distances using the maximum-parsimony algorithm and the neighbor-joining method using the DNAMAN program, plus 1,000 trials of bootstrap analyses were used to provide confidence estimates for phylogenetic tree topologies [26, 43, 54].

Detection and Quantification of Indole Acetic Acid (IAA)

The *Acinetobacter* strains were grown in an M9 minimal medium with 0.3 mM tryptophan at 30°C for 96 h. After adjusting the bacterial suspension to an optical density of 0.5 (10^6–10^7 CFU/ml of cells) at 530 nm [21], twice the volume of the Salkowski reagent [0.01 M $FeCl_2$ in 35% (w/v) $HClO_4$] was added to each cell-free supernatant. IAA was visually detected by the development of a pink to red color. For quantification, one colony of the bacterial isolate was inoculated in 5 ml of an LB medium and incubated at 30°C for 24 h at 200 rpm. Next, 1% (v/v) of the bacterial suspension was transferred to 5 ml of an LB medium containing 1 mg/ml tryptophan and incubated at 200 rpm, 30°C, for 96 h. After adding 4 ml of the Salkowaski reagent to 1 ml of the supernatant, positive results were measured using a spectrophotometer at 540 nm. The level of IAA produced was estimated using a standard IAA (HiMedia Laboratories Pvt. Ltd, Mumbai, India) graph [21].

Detection and Quantification of Phosphate Solubilization

The solubilization of insoluble phosphate was examined in the cultures of *Acinetobacter* using a modified Pikovskaya (PVK) medium that consisted of (g/l) $Ca_3(PO_4)_2$ (5.0), glucose (10.0), $(NH_4)_2SO_4$ (0.5), KCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.1), trace of $MnSO_4$ and $FeSO_4$, yeast extract (0.5), and agar (15.0), in distilled water. An aliquot (10 µl) of each suspension with approximately 1×10^7 colony forming units/ml (CFU/ml) from a 2-day culture was spot inoculated. A clear zone around each spot during 1 week at 28°C was considered positive evidence of phosphate solubilization [1]. The phosphate solubilization efficiency (SE) was calculated according to the following formula [23]: SE=[Halo zone (z)/Diameter of colonies (n)]×100. The experiment was duplicated for each isolate.

The phosphate solubilization was quantified as follows: an actively growing bacterial suspension [1% (v/v)] was transferred to 5 ml of a Pikovskaya (PKV) solution and incubated at 200 rpm, 30°C. After 7 days, 5 ml of a chloromolybdic acid solution was added to 5 ml of the supernatant, followed by the addition of 10 ml of distilled water and 1 ml of a chlorostannous acid solution. The total volume was adjusted to 25 ml using distilled water. After 5 min, the development of a blue color was measured using a spectrophotometer at 660 nm. The quantity of solubilized phosphate was determined using a standard graph and achieved using known quantities of calcium phosphate solutions and reading the absorbance at 660 nm. Uninoculated PKV was used as the blank [37, 40].

Detection of Zinc Oxide Solubilization

The solubilization of zinc oxide was examined in the cultures of *Acinetobacter* using a medium that consisted of (g/l) ZnO (12.0); glucose (10.0); $(NH_4)_2SO_4$ (0.5); KCl (0.2); $MgSO_4 \cdot 7H_2O$ (0.1); trace of $MnSO_4$ and $FeSO_4$, yeast extract (0.5); and agar (15.0), in distilled water. An aliquot (10 µl) of each suspension with approximately 1×10^7 CFU/ml from a 2-day culture was spot inoculated. A clear zone around each spot after incubation for 24 h at 28°C was considered positive evidence of ZnO solubilization [23]. The test was duplicated for each isolate. The zinc oxide solubilization efficiency (SE) was calculated according to the following formula [23]: SE=[Halo zone (z)/Diameter of colonies (n)]×100.

Detection of Siderophore Production Using CAS Blue Agar Method

The chrome azurol S (CAS) Blue agar was prepared as follows: a stock solution of CAS (60.5 mg in 50 ml of milli Q water) was mixed with 10 ml of an Fe^{3+} solution (1 mmol/l $FeCl_3 \cdot 6H_2O$, 10 mmol HCl), and then mixed with an HDTMA solution (72.9 mg of hexadecyltrimethyl ammonium bromide in 40 ml of milli Q water) with continuous stirring. The resulting dark-blue solution (100 ml) was autoclaved, cooled to 50°C, and mixed with 900 ml of a sterile MM9 medium [29] containing 15 g/l agar (also maintained at 50°C). This medium was allowed to gel on Petri dishes. The bacterial cultures (*ca.* 10^7 cells) were spot inoculated on the CAS Blue agar plates and incubated in the dark at 28°C for 5 days. Each assay was performed in triplicate. The colonies surrounded by an orange halo were scored as siderophore producers [29, 35, 47].

Quantification of Siderophores Using CAS Solution Assay

In the CAS solution assay, one colony of the bacterial isolate was inoculated in 5 ml of an LB medium and incubated at 30°C for 24 h with agitation (200 rpm). The bacterial suspension [1% (v/v)] was then transferred to 5 ml of a succinate broth and incubated for 7 days at 30°C with agitation (200 rpm). The growth was harvested by centrifugation at 8,014 ×*g* for 10 min, whereas the supernatant was transferred to fresh tubes, an equal volume of the CAS reagent added to each tube, and the mixture incubated in the dark for 30 min at room temperature. The absorbance was then measured at 630 nm [29, 35] and the siderophore units calculated using the following formula [33, 44]:

$$\text{Siderophore unit}=(A_C - A_S/A_C)\times100$$

where A_C represents the absorbance of the CAS solution plus the medium, and A_S is the absorbance of the CAS solution plus the culture supernatant of the respective sample.

Siderophore unit percentages of less than 10 were considered negative [33, 35, 47].

Determination of Siderophore Type

The siderophore type was detected using two chemical assays:

Table 1. Biotyping of *Acinetobacter* strains by FAME analysis and molecular identification by 16S rRNA gene sequencing.

			FAME analysis		Molecular identification			G+C	
Site of sampling	Plant stage	*Acinetobacter* strain	MIDI identification	SI[a]	Closest match	Similarity identity (%)	GenBank accession number[b]	T_m	Content (mol%)
Kamshet	Flowering	PUCM1001	*A. calcoaceticus*	0.816	*A. calcoaceticus*	99	FJ816048	85.00	37.97
Paud road	Flowering	PUCM1002	Genomospecies 3	0.292	*A. calcoaceticus*	99	FJ816049	84.96	37.86
Rajgurunagar	Flowering	PUCM1003	No match	-	*A. calcoaceticus*	99	FJ816050	85.00	37.97
		PUCM1004	Genomospecies 3	0.851	*A. calcoaceticus*	99	FJ816051	85.21	38.50
		PUCM1005	No match	-	*Acinetobacter* sp.	96	FJ816052	85.31	38.75
		PUCM1006	Genomospecies 3	0.843	*A. calcoaceticus*	99	FJ816053	85.71	39.76
Field no.2	Control soil	PUCM1007	*A. lwoffii*	0.346	*Acinetobacter* sp.	98	FJ816054	86.95	42.92
		PUCM1008	*A. calcoaceticus*	0.481	*A. calcoaceticus*	99	FJ816055	85.37	38.90
		PUCM1009	*A. calcoaceticus*	0.170	*A. calcoaceticus*	99	FJ816056	84.84	37.56
		PUCM1010	Genomospecies 3	0.688	*A. calcoaceticus*	98	FJ816057	85.06	38.12
		PUCM1011	Genomospecies 3	0.830	*A. calcoaceticus*	99	FJ816058	85.84	40.09
Field no.1	Seedling	PUCM1012	*A. calcoaceticus*	0.781	*A. calcoaceticus*	99	FJ816059	85.80	39.99
		PUCM1013	*A. calcoaceticus*	0.642	*A. calcoaceticus*	99	FJ816060	85.62	40.06
Field no.2	Seedling	PUCM1014	Genomospecies 3	0.834	*A. calcoaceticus*	99	FJ816061	85.06	38.98
		PUCM1015	Genomospecies 3	0.688	*A. calcoaceticus*	99	FJ816062	85.59	39.46
		PUCM1016	*A. hemolyticus*	0.100	*A. calcoaceticus*	99	FJ816063	84.9	38.58
		PUCM1017	*A. calcoaceticus*	0.181	*A. calcoaceticus*	98	FJ816064	84.78	38.27
	Flowering	PUCM1018	*A. haemolyticus*	0.237	*A. calcoaceticus*	99	FJ816065	84.78	38.27
		PUCM1019	*A. calcoaceticus*	0.711	*A. calcoaceticus*	100	FJ816066	84.68	38.02
		PUCM1020	Genomospecies 3	0.799	*A. calcoaceticus*	99	FJ816067	84.59	37.79
		PUCM1021	Genomospecies 3	0.469	*Acinetobacter* sp.	99	FJ816068	84.87	38.50
		PUCM1022	*A. calcoaceticus*	0.670	*Acinetobacter* sp.	99	FJ816069	84.37	37.23
		PUCM1023	*A. calcoaceticus*	0.156	*Acinetobacter* sp.	97	FJ816070	85.04	38.59
Field no.1	Reproductive	PUCM1024	Genomospecies 3	0.911	*A. calcoaceticus*	99	FJ816071	84.37	36.38
		PUCM1025	*A. calcoaceticus*	0.477	*A. calcoaceticus*	99	FJ816072	84.94	38.34
		PUCM1026	*A. calcoaceticus*	0.673	*A. calcoaceticus*	99	FJ816073	85.16	38.89
		PUCM1027	*A. calcoaceticus*	0550	*A. calcoaceticus*	98	FJ816074	84.51	37.25
		PUCM1028	Genomospecies 3	0.543	*Acinetobacter* sp.	97	FJ816075	83.97	35.89
		PUCM1029	No match	-	*A. baumannii*	99	FJ816076	84.75	37.86
Field no.2	Reproductive	PUCM1030	Genomospecies 3	0.897	*A. calcoaceticus*	99	FJ816077	84.83	38.06
		PUCM1031	*A. calcoaceticus*	0.630	*Acinetobacter* sp.	94	GQ469890	86.16	41.43

[a]Similarity Index (SI) is a numerical value that expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strain used to create the library entry listed as its match. Similarity value between 0.300 and 0.500 could be an acceptable match, yet would indicate an atypical strain. Values lower than 0.300 suggest that the species is not included in the database.

[b]16S rRNA gene partial sequences determined in this study are deposited in the GenBank database, NCBI. Strains with % similarity ≥ 97 are kept as *Acinetobacter* species.

I. Csaky assay (hydroxamate-type detection). A cell-free supernatant of each actively-growing culture was hydrolyzed with an equal volume of 6 N H_2SO_4 in a boiling water bath for 6 h and subsequently at 130°C for 30 min. The solution was then buffered by adding 3 ml of a 3.5% (w/v) sodium acetate solution, followed by the addition of 1 ml of a sulfanilic acid solution (1.3 g of sulfanilic acid in 100 ml of acetic acid) and 0.5 ml of an iodine solution, and incubation for 5 min. The excess iodine was removed with 1 ml of a sodium arsenite solution (2 g of Na_2ASO_4 in 100 ml of distilled water) and 1 ml of an α-naphtyl amine solution (3 g of α-naphtyl amine dissolved in 100 ml of acetic acid). Milli Q water was then added to make a total volume of 10 ml and the solution allowed to stand for 30 min at room temperature. The development of a purple color indicated the presence of a hydroxamate type of siderophore [11, 35].

II. Arnow assay: (catechol-type detection). The supernatant of each actively growing bacterial culture (1 ml) was hydrolyzed with 1 ml of 1 N HCl. Thereafter, 1 ml of a nitrite molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of milli Q water) was added to the hydrolyzed culture supernatant. The presence of a catechol type of siderophore was indicated by the development of a yellow color and confirmed when the yellow color changed to a red color within 5 min of the addition of 1 ml of 0.5 N NaOH [2, 29, 35]. The uninoculated medium was used as the negative control.

Bioassay of Siderophores Using *Fusarium oxysporum* as Target Fungus

Fusarium oxysporum MTCC284 was used as the test plant pathogen. The ability of the *Acinetobacter* isolates to inhibit the growth of the test plant pathogen was evaluated on King's B medium (20 g Peptone, 10 ml glycerol, 2.25 g aspargine, 1.5 g K_2HPO_4, 4.4 g glycine, 1,000 ml distilled water, pH 7.2). An agar plug (1 cm^2) with an actively growing *F. oxysporum* culture was placed on the King's B medium ensuring contact between the fungal growth and the agar medium. The *Acinetobacter* strains were streaked in the form of a "V" and 3 cm away from the agar plug at the side towards the edge of Petri plates. The agar medium inoculated with only the plant pathogen was used as the control. The plates were then incubated at 30°C until fungal mycelia completely covered the agar surface in the control plate. The inhibition of fungal growth in the vicinity of the *Acinetobacter* isolates on the King's B medium under iron-limited conditions was considered as inhibition of the plant pathogen mediated by the siderophores produced by the *Acinetobacter* isolates [27, 44].

Pot Experiments

Pot experiments were conducted to determine the influence of the rhizospheric *Acinetobacter* isolates on the growth of pearl millet. Four *Acinetobacter* strains (PUCM1001, PUCM1007, PUCM1022, and PUCM1029) with the desired *in vitro* plant-growth-promoting traits were selected for this study. NATH Hybrid seeds "NBH-05" [Nath Biogene (I) Ltd. Aurangabad, India] of pearl millet were used. The seeds were surface-sterilized with 0.02% (w/v) sodium hypochlorite for 2 min and rinsed thoroughly with sterile milli Q water. The seeds were then soaked in a cell suspension of the *Acinetobacter* isolates (10^8 CFU/ml) for 10 min and dried under aseptic conditions. Thereafter, the bacterized and non-bacterized seeds (control) were sown at a depth of 5 cm in pots (15 cm diameter) containing 2 kg of sterile soil [18]. The experiments were performed in duplicate. The pots were kept in the sunlight and watered daily. The shoot height and root length were measured after 21 days. The roots were shade dried to evaporate the moisture and measure the dry weight, where the shade drying was continued until the root weight remained constant on three consecutive days. The data were statistically analyzed using ANOVA. A Least Significant Difference (LSD) test at a probability level of 0.05 and 0.01 was used to separate the means when the ANOVA F-test indicated a significant effect from the treatments [12, 18].

RESULTS

Acinetobacter spp. Associated with Rhizosphere of Pearl Millet

Gram-negative, nonmotile, short rods or coccobacilli that were oxidase negative and catalase positive were considered as putative *Acinetobacter* isolates. A total of 31 putative *Acinetobacter* isolates were obtained from the rhizosphere of pearl millet. Preliminary identification of these isolates as members of the genus *Acinetobacter* was on the basis of morphological, cultural, and biochemical characterizations (API 32GN identification system), as well as a chromosomal transformation assay. The isolates were identified up to the species level by a FAME analysis, which revealed that the 31 isolates included 13 strains of *A. calcoaceticus*, 12 strains of *Acinetobacter* genospecies 3, one strain of *A. lwoffii*, and two strains of *A. haemolyticus*. Three *Acinetobacter* isolates did not show any acceptable match (Table 1). However, these isolates were conclusively identified up to the species level on the basis of 16S rRNA gene sequencing. Molecular identification by 16S rRNA gene sequencing revealed that 24 isolates belonged to *A. calcoaceticus*, one isolate to *A. baumannii*, and six isolates to *Acinetobacter* sp. (Table 1). The G+C content of the *Acinetobacter* strains was within a range of 36.38–41.43 mol% (Table 1). The phylogenetic analysis of the *Acinetobacter* strains isolated from the rhizosphere of pearl millet and reference *Acinetobacter* strains is illustrated in Fig. 1.

Plant-Growth-Promoting Traits of *Acinetobacter* Strains

Only two isolates, *Acinetobacter* sp. PUCM1007 and *A. baumannii* PUCM1029, were able to produce IAA (Table 2). Only 26 of the 31 *Acinetobacter* isolates displayed phosphate solubilization in the plate assay (Table 2). The most efficient phosphate solubilization was exhibited by *A. calcoaceticus* PUCM1006 (84 mg/ml) and *A. calcoaceticus* PUCM1005 (70 mg/ml). A total of 27 *Acinetobacter* strains were able to solubilize zinc oxide *in vitro* (Table 2). The highest solubilization of zinc oxide was demonstrated by strain PUCM1005 (SE: 918), followed by PUCM1005 (SE: 344) and PUCM1009 (SE: 333).

The ability of the *Acinetobacter* isolates to produce siderophores was evaluated qualitatively and quantitatively by a CAS Blue agar assay and broth assay, respectively. The CAS blue agar plate assay revealed that 29 of the *Acinetobacter* isolates were able to produce siderophores.

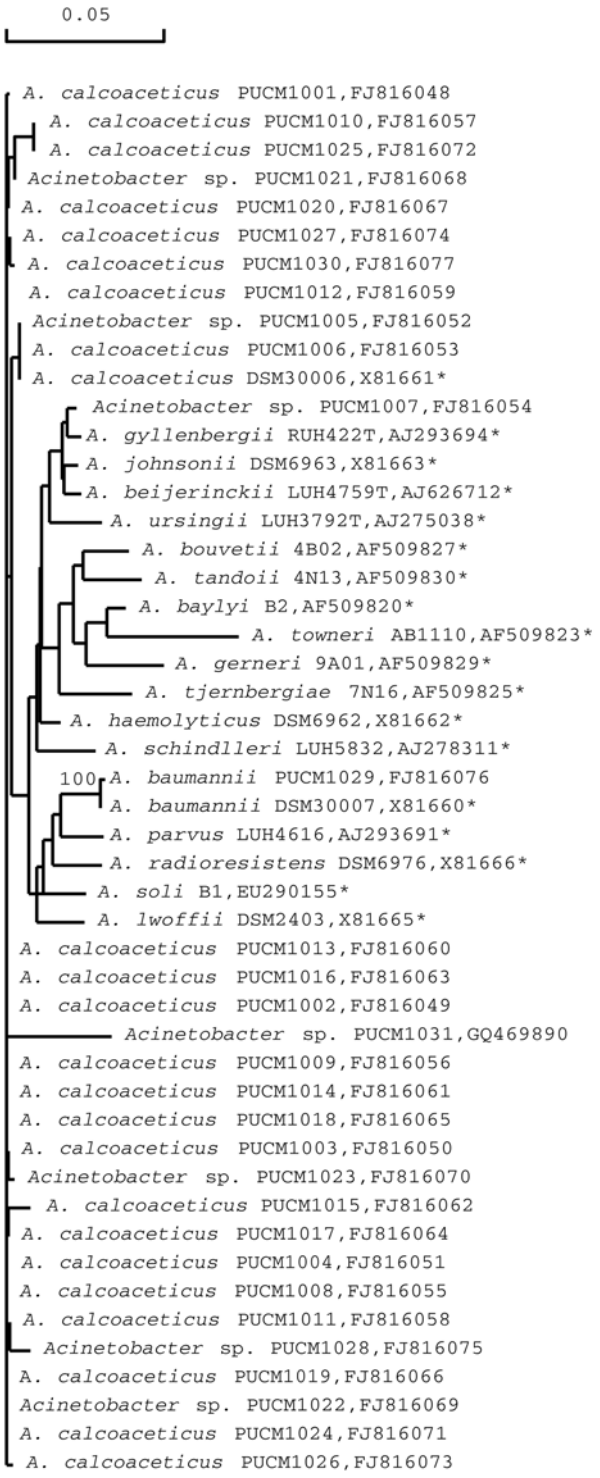

Fig. 1. Phylogeny of 16S rRNA gene sequences of *Acinetobacter* strains used in this study and reference sequences taken from GenBank.

The sequences were aligned using the DNAMAN program. The phylogenetic tree was constructed from a matrix of pairwise genetic distances using the maximum parsimony algorithm and neighbor-joining method using the DNAMAN program. The scale bar represents 0.05 substitutions per nucleotide. The sequences marked with "*" represent the reference gene sequences taken from the GenBank database.

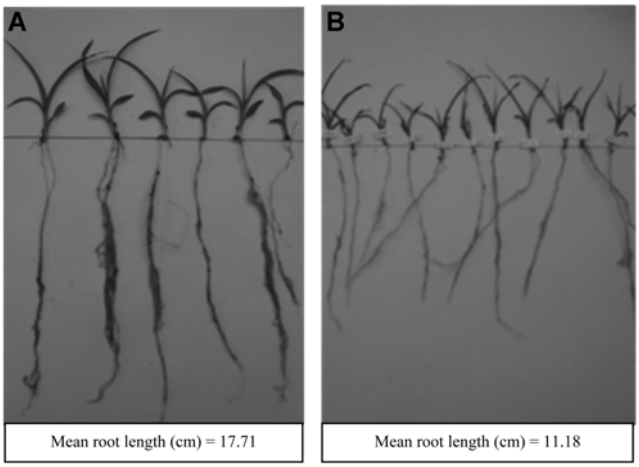

Fig. 2. Effect of *Acinetobacter* sp. PUCM1022 on shoot height and root length of *P. glaucum.*

A. Treated plants by *Acinetobacter* sp. PUCM1022; **B**. Control plants without bacterial treatments.

Seven of the *Acinetobacter* strains, namely PUCM1001, PUCM1002, PUCM1003, PUCM1008, PUCM1015, PUCM1016, and PUCM1029, produced significantly higher quantities of siderophores when compared with the other strains investigated in the present study, as evident from the quantitative assay. *A. calcoaceticus* PUCM1016 (85.0% siderophore units) showed the maximum siderophore production (Table 2). Two strains, *Acinetobacter* sp. PUCM1007 and *A. calcoaceticus* PUCM1010, did not grow on the CAS medium. Strains PUCM1001 and PUCM1019 (both *A. calcoaceticus*) and PUCM1022 (*Acinetobacter* sp.) produced both hydroxamate- and catechol-type siderophores, whereas all the other strains only produced catechol-type siderophores (Table 2). An evaluation of the antifungal activity of the *Acinetobacter* isolates revealed that *A. calcoaceticus* PUCM1018 was the most potent inhibitor of plant pathogen growth among all the isolates tested. Interestingly, the growth of *F. oxysporum* was only arrested by the isolates under iron-limited conditions (Table 3).

Promotion of Pearl Millet Growth by Rhizospheric *Acinetobacter* Strains

Pot experiments were conducted to determine the influence of the rhizospheric presence of the *Acinetobacter* isolates on the early growth of pearl millet. The results of the pot experiments showed a significant increase in the shoot height, root length, and root dry weight of seedling plants bacterized with *A. calcoaceticus* PUCM1022 when compared with control plants (Tables 4, 5, and 6).

Discussion

The present study was initiated to investigate the prevalence of cultivable *Acinetobacter* species in the rhizosphere of

Table 2. Plant-growth-promoting traits of *Acinetobacter* strains from rhizosphere of pearl millet.

Acinetobacter strain	IAA production	Phosphate solubilization		Zinc oxide solubilization	CAS liquid assay	Siderophore type	
	μg/ml	mg/ml	SE (%)	SE (%)	Siderophore unit (%)	Arnow assay	Casky assay
PUCM1001	-	27.0	129	316.6	79.2	+	+
PUCM1002	-	-	-	295.0	70.9	+	-
PUCM1003	-	51.0	139	306.0	72.7	+	-
PUCM1004	-	53.5	130	328.3	46.0	+	-
PUCM1005	-	70.0	143	344.3	59.4	+	-
PUCM1006	-	84.0	150	306.0	55.4	+	-
PUCM1007	13	-	-	-	69.0	+	-
PUCM1008	-	10.0	155	316.6	78.8	+	-
PUCM1009	-	-	-	333.0	69.1	+	-
PUCM1010	-	-	-	332.3	9.95	-	-
PUCM1011	-	37.0	145	315.6	54.6	+	-
PUCM1012	-	62.0	145	-	55.1	+	-
PUCM1013	-	10.0	134	-	79.6	+	-
PUCM1014	-	16.0	139	285.0	30.2	+	-
PUCM1015	-	28.0	145	270.3	83.1	+	-
PUCM1016	-	35.0	124	265.6	85.0	+	-
PUCM1017	-	-	-	-	35.8	+	-
PUCM1018	-	34.0	120	286.0	62.9	+	-
PUCM1019	-	50.0	154	268.6	68.4	+	+
PUCM1020	-	14.0	124	275.6	69.6	+	-
PUCM1021	-	17.5	133	290.0	66.6	+	-
PUCM1022	-	46.0	144	286.6	64.1	+	+
PUCM1023	-	28.0	162	247.0	64.8	+	-
PUCM1024	-	34.0	145	271.0	68.7	+	-
PUCM1025	-	26.0	125	918.0	65.4	+	-
PUCM1026	-	32.5	132	275.6	66.1	+	-
PUCM1027	-	24.0	139	285.0	69.4	+	-
PUCM1028	-	28.0	145	257.0	60.2	+	-
PUCM1029	10	64.0	170	261.6	74.2	+	-
PUCM1030	-	46.0	153	271.0	62.3	+	-
PUCM1031	-	32.0	137	300.0	66.3	+	-

IAA: Indole acetic acid; SE: Solubilization efficiency; -: absence of activity; +: presence of activity.

pearl millet and evaluate the potential of such *Acinetobacter* strains to promote plant growth. Any isolate that was Gram-negative, nonmotile, oxidase negative, catalase positive, coccobacillus or short rods, and transformed auxotrophic *A. baylyi* BD4 *trp*E27 to prototrophy in a chromosomal transformation assay was considered a putative *Acinetobacter* isolate. All the putative *Acinetobacter* isolates were identified up to the species level on the basis of 16S rRNA gene sequencing. The genus *Acinetobacter* has already been delineated into a total of 31 species on the basis of 16S rRNA gene sequences as well as DNA hybridization studies [52]. However, not all these species can be identified by an API 32GN identification system or FAME analysis. Hence, in the present investigation, the isolates were identified up to the species level mainly on the basis of their 16S rRNA gene sequences. The identification was further supported by data obtained from a FAME analysis, as well as biochemical profiling using an API 32GN identification system. The API test provided important biochemical characterization of the *Acinetobacter* species [30], whereas the FAME analysis was performed to determine the differences between strains [28]. It was observed that the species level identification of the isolates by FAME and 16S rRNA gene sequencing was not always consistent. However, in such cases, the identification based on 16S rRNA gene sequencing was considered as conclusive. Accordingly, most isolates were confirmed as strains of *A. calcoaceticus.* Thus, the prevalence of *Acinetobacter* sp. in the rhizosphere of pearl millet was clearly evident in the present study and also indicated the ability of these isolates to thrive on the exudates released by the roots of the same plant.

The *Acinetobacter* strains obtained in this study were further investigated for their *in vitro* PGP traits. One of the most important mechanisms involved in plant-growth

Table 3. Percent mycelia inhibition of *Fusarium oxysporum* by *Acinetobacter* strains under iron-limited conditions (siderophore-mediated antifungal activity).

Genospecies	Strains	Diameter of fungal colony (mm)	Growth inhibition of mycelia[a] (%)
A. calcoaceticus	PUCM1001	39.6	26.66
A. calcoaceticus	PUCM1002	37.3	30.92
A. calcoaceticus	PUCM1003	42.3	21.66
A. calcoaceticus	PUCM1004	42.7	20.92
Acinetobacter sp.	PUCM1005	38.6	28.51
A. calcoaceticus	PUCM1006	38.3	29.07
Acinetobacter sp.	PUCM1007	38.6	28.51
A. calcoaceticus	PUCM1008	35.6	34.07
A. calcoaceticus	PUCM1009	35.0	35.18
A. calcoaceticus	PUCM1010	44.7	17.22
A. calcoaceticus	PUCM1011	39.6	26.66
A. calcoaceticus	PUCM1012	40.0	25.92
A. calcoaceticus	PUCM1013	35.6	34.07
A. calcoaceticus	PUCM1014	37.6	30.37
A. calcoaceticus	PUCM1015	36.0	33.33
A. calcoaceticus	PUCM1016	37.0	31.48
A. calcoaceticus	PUCM1017	34.6	35.92
A. calcoaceticus	PUCM1018	7.3	86.48
A. calcoaceticus	PUCM1019	35.0	35.18
A. calcoaceticus	PUCM1020	36.0	33.33
Acinetobacter sp.	PUCM1021	33.0	38.88
Acinetobacter sp.	PUCM1022	34.0	37.03
Acinetobacter sp.	PUCM1023	34.6	35.92
A. calcoaceticus	PUCM1024	35.0	35.18
A. calcoaceticus	PUCM1025	35.0	35.18
A. calcoaceticus	PUCM1026	34.0	37.03
A. calcoaceticus	PUCM1027	36.6	32.22
Acinetobacter sp.	PUCM1028	34.3	36.48
A. baumannii	PUCM1029	32.3	40.18
A. calcoaceticus	PUCM1030	32.6	39.62
A. calcoaceticus	PUCM1031	20.0	62.96
Control (dc)	-	54.0	0

[a]Percent mycelia growth inhibition (%)=(dc−dt/dc)×100
dc=Average diameter of fungal colony in control (54 mm)
dt=Average diameter of fungal colony in treatment group

promotion is the rhizobacterial excretion of phytohormones, such as IAA. Auxin is a well-known plant-growth regulator because of its important role in the initial processes of lateral and adventitious root formation [14] and root elongation [57]. The ability of bacteria producing IAA *in vitro* is apparently a quantitative rather than qualitative trait [31]. The amounts of IAA produced *in vitro* by the *Acinetobacter* strains varied greatly, ranging from nearly undetectable to significantly high quantities (Table 2). There are a few reports on the involvement of the genus *Acinetobacter* in the production of IAA and its effect on plant growth [17, 21]. Therefore, the multiplication of these bacterial populations in the rhizosphere with the release of IAA may enhance rhizogenesis. Alternatively, IAA production may be an important strategy for detoxifying excess tryptophan released in the rhizosphere [49].

Mineral solubilization and increasing mineral availability are regarded as the most important traits directly associated with PGPR [48, 50]. In general, the maximum agriculture yield can only be obtained when there is sufficient phosphate available to plants. Furthermore, extractable inorganic phosphates are considered a nonrenewable depleting resource, and natural ecosystems are increasingly being exposed to the detrimental effects of deposited phosphates and associated heavy metals. Hence, exploring alternative approaches to increase the availability of inorganic phosphate has become a necessity. Phosphate-solubilizing bacteria (PSB) play an important role in improving the availability of phosphorus to plants and increasing the crop yield [24, 50, 53]. The isolation of PSB from endogenous soils has always been preferred, as such bacteria are well adapted to the natural climates of their habitat [36]. Some of the *Acinetobacter* strains in this study showed higher phosphate-solubilization efficiencies (Table 2) than those previously reported for *Pseudomonas* and *Serratia* species [1, 44, 48]. Moreover, in the present investigation, increased phosphate solubilization was observed to be associated with a decreased pH for the growth medium, indicating a direct correlation between phosphate solubilization and acid production. This observation is consistent with previous reports [22, 43]. A drop in the pH of the growth media subsequent to bacterial growth was monitored in the present study using Pikovskaya's medium supplemented with the pH indicator bromocresol purple dye, as reported in previous literature [13, 15] (data not shown).

Literature related to bacterial zinc solubilization is limited and there is paucity of reports describing zinc solubilization

Table 4. Statistical analysis of shoot height of pearl millet treated with *Acinetobacter* strains.

Sr. No.	Treatment	Mean shoot height (cm)	Standard error (SE)	Difference from control	P-value
-	Controls	5.81	0.300	-	-
1	PUCM1001	6.54	0.371	0.74ns	0.196
2	PUCM1007	6.30	0.341	0.49ns	0.362
3	PUCM1022	7.41	0.324	1.61**	0.003
4	PUCM1029	6.84	0.389	1.04ns	0.053

**: Significant at 1% level of probability; ns: not significant.

Table 5. Statistical analysis of root length of pearl millet treated with *Acinetobacter* strains.

Sr. No.	Treatment	Mean root length (cm)	Standard error (SE)	Difference from control	P-value
-	Controls	11.18	0.65419	-	-
1	PUCM1001	13.21	0.66410	2.02 ns	0.051
2	PUCM1007	12.79	0.63509	1.60 ns	0.105
3	PUCM1022	17.71	0.72086	6.52 **	0.000
4	PUCM1029	12.96	0.87941	1.77 ns	0.068

**: Significant at 1% level of probability; ns: not significant.

by *Acinetobacter* strains [23, 43]. Bacterial genera such as *Bacillus*, *Pseudomonas*, and the diazotrophic *Gluconacetobacter* have already been reported to exhibit zinc-solubilizing activity. In the present study, a majority of the *Acinetobacter* isolates solubilized both phosphates as well as zinc oxide. However, a direct correlation between the two properties could not be established. Additionally, at least five of the isolates demonstrated soubilization of either zinc oxide or phosphates, but not both (Table 2). This observation was not consistent with a previous report, where a direct correlation between phosphate and zinc oxide solubilization was observed [50]. The zinc solubilization efficiency (918% SE) displayed by strain *A. calcoaceticus* PUCM1025 was among the highest reported in the literature.

The siderophores of rhizobacteria can significantly influence the ability of plants to acquire iron from soil [46]. In the present study, the siderophore production by *A. lwoffii* PUCM1007 was only revealed by a CAS liquid assay (Table 2). PUCM1007 did not show growth on a CAS agar medium containing dextrose and mannitol, possibly due to nonsaccharolytic behavior and/ or its inability to grow in the presence of HDTMA. Hence, it would appear prudent to ascertain the ability of isolates to produce siderophores by employing more than one type of assay. *A. calcoaceticus* PUCM1016 demonstrated the highest production of siderophores (85% siderophore units). Among the 31 *Acinetobacter* isolates, only three (PUCM1001, PUCM1019, and PUCM1022) produced both types of siderophore (Table 2). It has been reported that the amount and types of siderophore produced by bacteria depend on the availability of organic and inorganic nutrients [34], and thus the synthesis of siderophores by bacteria may differ when grown in a defined culture medium instead of a nutrient-deficient rhizosphere ecosystem [46]. The siderophores produced by root-colonizing rhizobacteria have been reported to suppress the growth of certain plant pathogenic fungi. Some reports have indicated that siderophore-mediated competition for iron may be a major factor in the suppression of plant pathogenic *Fusarium oxysporum* [27, 44]. The siderophore-producing *Acinetobacter* strains from the rhizosphere of pearl millet may serve the same purpose. To the best of our knowledge, this study is the first to demonstrate the *in vitro* inhibition of *F. oxysporum* growth by siderophore-producing *Acinetobacter* strains under iron-limited conditions (Table 3).

The ability of four *Acinetobacter* isolates to promote the growth of pearl millet seedlings was evaluated in pot experiments. As a result, *Acinetobacter* sp. PUCM1022 significantly promoted the growth of the pearl millet seedlings, as evident from an increased shoot height, root length, and root dry weight (Tables 4, 5, and 6). Similarly, promotion of the growth parameters of several crop plants in response to inoculation with PGPR has also been reported by other researchers [12, 17, 21, 43, 49, 56]. Interestingly, strain PUCM1022, which did not exhibit *in vitro* IAA production, showed better results in the pot experiments when compared with strains PUCM1007 and PUCM10029 that tested positive for IAA. Earlier studies also reported plant-growth promotion of pearl millet when using a consortium of *Bacillus* species [38] or a consortium of three *Bacillus* species, namely *B. amyloliquefaciens*, *B. pumilus*, *B. subtilis*; and *Brevibacillus brevis* [39]. Both of these studies were performed in a greenhouse, and the results reported in the present study were similar.

The presence of *Acinetobacter* species in the rhizosphere of pearl millet has also been reported in a few other studies [19, 49]. However, the present investigation revealed that *Acinetobacter* strains have interesting plant-growth-promoting

Table 6. Statistical analysis of root dry weight of pearl millet roots treated with *Acinetobacter* strains.

Sr. No.	Treatment	Mean root dry weight (mg)	Standard error (SE)	Difference from control	P-value
-	Controls	38.55	4.42276	-	-
1	PUCM1001	36.42	3.99429	9.93ns	0.086
2	PUCM1007	29.80	2.89732	3.32ns	0.546
3	PUCM1022	42.91	3.97303	16.42**	0.002
4	PUCM1029	30.12	2.96340	3.63ns	0.502

**: Significant at 1% level of probability; ns: not significant.

traits, such as siderophore and IAA production, as well as phosphate and zinc oxide solubilization. Thus, the present data corroborates the hypotheses that *Acinetobacter* strains have the potential to act as plant-growth-promoting rhizobacteria and can enhance the growth of pearl millet, particularly under stressed environmental conditions where the iron, phosphate, or zinc sources may be limited.

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