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RESEARCH ARTICLE

Endophytic Microbes from Diverse Wheat Genotypes and Their Potential Biotechnological Applications in Plant Growth Promotion and Nutrient Uptake

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Abstract Endophytic microbes residing inside the tissues of plants play a significant role to enhance the growth and health of plants by different plant growth-promoting mechanisms. In the present investigation, N_2-fixing endophytic bacteria were isolated and characterized by plant growth. A total of one hundred fifty-nine endophytic bacteria were isolated from surface-sterilized roots and stem of different genotypes of wheat growing in the Divine Valley of Baru Sahib, Himachal Pradesh. The isolated bacterial endophytes were screened in vitro for plant growth-promoting attributes. Out of one hundred fifty-nine, thirteen endophytic bacteria were selected based on multifarious plant growth-promoting attributes. Among plant growth-promoting activities, hydrogen cyanide producers (19%) were higher when compared to siderophores producers (16%) and P-solubilizers (16%), ammonia producers (14%), K-solubilizers (14%), IAA producers (12%), Zn-solubilizers (5%), N_2-fixers (2%) and biocontrol (2%). One of the isolates EU-B2RT.R1 demonstrated that a significant level of nitrogenase activity, P-solubilization and IAA production was identified as *Acinetobacter guillouiae* EU-B2RT.R1 based on 16S rRNA gene sequencing and BLAST analysis. *Acinetobacter guillouiae* EU-B2RT.R1, exhibiting multifarious beneficial traits, is further evaluated for plant growth promotion of wheat cultivar PBW 343+Lr24+GPC in pot experiment under greenhouse conditions. The *Acinetobacter guillouiae* EU-B2RT.R1 with multifarious plant growth-promoting activity has emerged as one of the efficient biofertilizers that need to be explored for sustainable agriculture.

Keywords *Acinetobacter* · Endophytic bacteria · PGP attributes · Plant growth promotion · Wheat

Kusam Lata Rana and Divjot Kour contributed equally to the present work.

Significance Statement The significance of present work is the isolation of nitrogen-fixing endophytic bacterium isolated from wheat, having huge potential role in plant growth promotion under in vitro condition and in future can be developed as nitrogen biofertilizers.

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Introduction

Globally, wheat (*Triticum aestivum* L.) is one of the most important cereal crops grown worldwide. The United States Department of Agriculture (USDA) estimated the production of wheat (2019) in India is 100.0 million metric tons with an area harvested which is estimated at 29.8 million hectares. In India, the second most important cultivated food crop is wheat which feeds hundred of million populations every day. During the time of independence, India was facing the problem of food shortages; then, the initiative of Green Revolution or Third Agricultural Revolution occurred between 1950 and 1960s in India resulted with a higher yield of cereals, superior quality of food, disease-resistant varieties of wheat, as well as implementation of better methods of farming practices. The extensive usage of chemical fertilizers provides nutrients to plants, though their overuse has also negative impact on the environment, animals and humans health, decreases the fertility of soil, polluted air and water, emitted greenhouse gases, causes soil acidification, depletes essential nutrients from soil, degrades the soil and loss of equilibrium and accumulates toxic substances in the soil [1].

With the increase in population, one of the most important priorities is enhanced agricultural productivity. Recently, the researchers are focusing more on microbial bioinoculants as biofertilizers and biopesticides for sustainable agriculture and environments. Plant growth-promoting (PGP) microbe will be as efficient and environmentally friendly solutions for replacing chemical fertilizers [2]. The microbes residing within the plant tissues known for > 120 years without causing any substantive harm to their host are endophytes [3]. Endophytic microbes could have a major impact on the health of the plant as they enhance crop productivity and improve the adaptation capability of the host plant and provide tolerance against various biotic and abiotic stresses. Endophytic microbes promote the growth of the plant by utilizing direct or indirect mechanism such as fixation of atmospheric nitrogen; solubilization of phosphorus, potassium and zinc; production of Fe-chelating molecule (siderophores); and secretion of various phytohormones (phytostimulation), for example auxins, cytokinins, gibberellins and ethylene [4]. In the endophytic relationship, microbes provide a unique opportunity indirectly for control of the deleterious impact of phytopathogens on health of plant and soil, via synthesis of innumerable compounds, for example antibiotics, immunosuppressants, biocontrol agents, hydrogen cyanide (HCN), and ammonia, induced systemic tolerance, production of hydrolytic enzymes [5].

The interactions of the endophytic microbe with plant range from mutualism to latent pathogenicity; in mutualistic interactions, the plant provides protective niches and endophytic microbes produce various useful metabolites that enhance the uptake of nutrients which further effects on the growth and development of plants [6]. Endophytic microbes are capable of maintaining sustainable agriculture, *i.e*., enhanced the health, yield, productivity of plants via numerous independent or linked mechanisms. There are many reports on isolation of endophytic bacteria from roots, stems, leaves, seeds, needles, twigs and barks of different plant species and their agricultural applications [7]. The wheat plant inoculated with endophytic *Acinetobacter, Azospirillum brasilense, Bacillus subtilis, Herbaspirillum hiltneri, Klebsiella, Streptomyces coelicolor, Pantoea alhagi*, and *Paenibacillus* reported to exhibit higher seed yield, improved accumulation of soluble sugars, decreased degradation of chlorophyll [8–10]. A huge diversity of endophytic bacteria belonging to different genera have been identified including *Achromobacter, Acinetobacter, Arthrobacter, Bacillus, Chitinophaga, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Leifsonia, Microbispora, Micrococcus, Micromonospora, Mycobacterium, Paenibacillus, Pantoea, Pseudomonas, Roseomonas, Staphylococcus, Streptomyces* and *Xanthomonas* isolated from wheat.

In the present investigation, bacterial endophytes using the culture-dependent technique have been isolated from different genotypes of wheat growing in Baru Sahib "Valley of Divine Peace" Himachal Pradesh, India. Using 16S rRNA gene sequencing, representative strains were undertaken for identification. The present investigation aimed to determine the plant growth-promoting activities of endophytic bacteria with wheat in vitro growing in Baru Sahib, Himachal Pradesh, India. The selected strains with multiple PGP attributes were evaluated for the growth of wheat under the controlled conditions.

Material and Methods

The different genotypes of wheat samples were collected from the different locations of Baru sahib in sterile polythene bags, properly labeled and transported to the laboratory immediately. The roots of freshly collected plants were washed under running tap water to eliminate the adhering soil. Freshly collected roots and stem were weighed 1 g and cut into 1–2 cm, and the freshly collected roots and stems were sterilized using 70% of ethanol for 30 s, followed by 0.1% mercuric chloride for 2 min, and again sterilized with 70% of ethanol for 30 s and rinsed three times in sterilized distilled water. The surface-

sterilized roots and stem segments were ground in a mortar with a pestle, and 100 µL of aliquots was plated on different growth media [11]. Plant extract agar medium [2 g glucose; 1 g yeast extract; 0.5 g K2HPO4; 100 ml plant extract (250 g plant sample +1 L H_2O, to autoclave and filter); 20 g agar; pH 7 ± 0.2] was formulated for the first time in the present investigation. The plates were incubated at 30–37 °C for 7–14 days. By repeated streaking on their respective medium, bacterial colonies which were morphologically different were picked and purified. The pure cultures were maintained on nutrient agar slants at 4 °C and glycerol stock (25%) at − 80 °C for further experiments. All the isolates were screened for tolerance to temperature (10–50 °C), salinity (5–15% NaCl) and pH (4–8) according to methods described earlier [12].

Functional Characterization of Endophytic Bacteria

The functional characterization of endophytic bacteria provides vital information about the ability of endophytic bacteria to exhibit plant growth-promoting attributes was studied by qualitative and quantitative screening. The endophytic bacteria were screened for solubilization of phosphorus, potassium, and zinc; nitrogen fixation; production of siderophore; indole-3-acetic acid; HCN; and ammonia. The bacterial isolates were screened both qualitatively and quantitatively for phosphorus-solubilizing activity. The qualitative estimation of all the phosphate-solubilizing bacteria was carried according to methods described by Pikovskaya [13] using Pikovskaya agar (glucose 1%, tricalcium phosphate 0.5%, $(NH_4)_2SO_4$ 0.05%, KCl 0.02%, $MgSO_4{\cdot}7H_2O$ 0.01%, $MnSO_4$ trace, $FeSO_4$ trace, yeast extract 0.05%, pH-7.2). Quantitative estimation of P-solubilization was performed at an incubation temperature of 30 °C in the NBRIP broth medium [14] supplemented with tricalcium phosphate as sole source of phosphorus [15]. The qualitative analysis of the solubilization of potassium by bacterial isolates was studied on modified Aleksandrov agar medium (pH 7.2 0.2). The plates containing Aleksandrov medium [16] inoculated with bacterial culture were incubated at 30 °C for 48–96 h [16]. The zinc-solubilizing activity was carried according to methods described by Fasim et al. [17] on nutrient agar medium supplemented with 1% of insoluble zinc compounds (ZnO, ZnS, $Zn_3(PO_4)_2$). The bacterial culture was spot inoculated on agar medium plates and incubated for 48–96 h at 30 °C. The clear zone around the culture indicates the capability of bacteria to solubilize zinc. The bacterial cultures were screened for production of IAA using the Salkowski reagent [18] and quantitatively screened as the method described by Patten and Glick [19], production of siderophores [20], HCN [21] and ammonia [22]. All assays were done in triplicate.

The in vitro antagonistic activity of bacterial isolates was evaluated against fungal pathogens *Fusarium graminearum* ITCC 1856 according to the method described by Sijam, Dikin [23]. The fungal pathogens responsible for root–rot complex in crops were obtained from the culture stock preserved at the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, 110012, India. The plates poured with potato dextrose agar [11] were inoculated with bacterial strains and with the fungal colony and incubated at 30 °C for 3–5 days. Control plates with only the mycelia plug were set up, and when the pathogen had grown across these control plates, the diameter of growth in the challenge plates was measured. Triple culture assays were repeated three times per isolate [24].

Acetylene Reduction Assay

The nitrogen-fixing activity of the bacterial isolates was determined by acetylene reduction assay (ARA). A loopful of bacteria was grown for 24–36 h at 30 °C in a nitrogen-free medium. The cotton plugs of the tubes were replaced with Suba-Seal in laminar air flow. The air inside the tubes was replaced with acetylene (10% v/v) and incubated at 30 °C for 6 h. After incubation, 1 ml of gas sample was withdrawn and injected into the gas chromatograph. The amount of ethylene produced by acetylene reduction was measured by a PerkinElmer F-11 gas chromatograph (Model Hewlett Packard Series II-5890) [25]. At the end of the experimental period, the cell protein contents of the cultures were determined following the method described by Bradford [26].

Evaluation of Plant Growth-Promoting Ability of Endophytic Bacteria

The experiments with wheat cultivar PBW 343+Lr24+GPC were conducted in the greenhouse. Greenhouse experiments were run in pots. One of the potential bacterial endophyte cultures isolated from different wheat genotypes was selected for their positive influence on wheat cultivar PBW 343+Lr24+GPC, and the experiment was planned in triplicate. Wheat cultivar PBW 343+Lr24+GPC seeds were sterilized properly and then soaked in the bacterial suspension. The treatment used in the present study consisted of control (wheat cultivar seeds treated in nutrient broth without bacterial culture), *Azotobacter chroococcum* (positive control), *Acinetobacter guillouiae* (EU-B2RT.R1), 50% N (half dose of urea) and 100% N (full dose of urea). For the reduction in cross-contamination of the controls, the pots were placed with the proper distance from each other. Seeds treated with individual bacterial suspension, uninoculated seeds and

chemically treated were sown in the pots, and each treatment in triplicate was arranged in a completely randomized design (CRD). In each pot after germination, three plants were maintained till their harvesting. The plants were uprooted for analyzing various parameters such as root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, root and shoot length, with their number of tillers being recorded. Physiologically available Fe and Zn in wheat seeds were determined according to Jorhem and Engman [27].

PCR Amplification of 16S rRNA and Phylogenetic Profiling

Genomic DNA isolation of the bacterial strains obtained was conducted as per the method described by Pospiech and Neumann [28] with minor modifications. The bacterial isolates were grown overnight in nutrient broth [29], and cells are harvested by centrifugation for the separation of supernatant from pellet for 10 min. One milliliter of TE buffer (10 mM Tris–HCl and 1 mM EDTA pH 8.0) was added 3 times for the washing process of the bacterial pellet. The lysis of bacterial cell was achieved by using 0.5 ml SET buffer (75 mM NaCl, 25 mM EDTA and 20 mM Tris) with 10 µl of lysozyme (10 mg mL^{-1}) for 1 h at 37 °C and 10% SDS with 20 mg ml^{-1} proteinase K, and mixture was mixed well and incubated for 2 h at 55 °C. The lysate was mixed thoroughly using phenol/chloroform/isoamyl alcohol for the extraction of DNA, mixed well by inverting and incubated at room temperature for 5 min and centrifuged at 8000 rpm for 10 min at 4 °C. The aqueous phase which is a highly viscous jellylike supernatant is transferred to a fresh tube, and an equal volume of isopropanol was added then mixed gently by inversion till white strands of DNA precipitates out and centrifuged again at 8000 rpm for 10 min. At the final step, the pellet was washed in 70% ethanol (v/v) incubated at 37 °C for 25–30 min for the complete elimination of ethanol and after air drying at room temperature finally resuspended in 100–200 µL of TE buffer. On a 0.8% agarose gel (w/v), the quality of genomic DNA was checked.

The isolated genomic DNA was used as a template for PCR amplification of 16S rDNA using the primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) [30]. PCR was performed at a final volume of 100 µL reaction containing 50–100 ng of genomic DNA, 10 pmol of each primer (pA and pH), 5 U of Taq DNA polymerase, 0.2 mM of each dNTPs mixture, 1.5 mM $MgCl_2$ and 1X Taq buffer. The PCR was performed using the following conditions as initial denaturation step of 5 min at 94 °C further followed by 40 cycles consisting of 40 s at 95 °C as the denaturation step, annealing for 40 s at 52 °C and primer extension for 2 min at 72 °C with final extension for 8 min at 72 °C. The amplification of the PCR product was confirmed by agarose gel electrophoresis (1.2%) and visualized on a gel documentation system (Alpha–Imager), and gel images were digitalized.

16S rRNA Gene Sequencing and Phylogenetic Analysis

The amplified PCR products 1540 bp of partial 16S rRNA gene were purified and sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer at Xcelris Labs Ltd., Ahmedabad (India). Using the codon code aligner v.4.0.4, 16S rRNA gene sequences were analyzed. For the identification of closely related bacterial species, the partial 16S rRNA gene sequences of the isolated strains were BLAST searched on the NCBI GenBank website. The bacterial isolates were identified based on the percentage of sequence similarity with that of a prototype strain sequence in the GenBank. The phylogenetic tree was constructed on the aligned datasets using the neighbor-joining method implemented in the program MEGA 4.0.2 software [31].

Results and Discussion

Isolation and Enumeration of Endophytic Bacteria

A total of one hundred fifty-nine endophytic bacteria were isolated from roots and stem of different wheat genotypes grown in the experimental farm of Baru Sahib, Sirmaur-173001, Himachal Pradesh, India (Table 1). The abundance of endophytic bacteria in the root and stem of different wheat genotypes varied from 1×10^6 to 4.4×10^7 CFU g^{-1} sample. The highest value found in root samples of wheat crosses (79-1-2-14-1-6-1-11) is 4.16×10^7 CFU g^{-1} root, and the lowest value is 1×10^6 CFU g^{-1} in root samples of wild wheat (*Aegilops longissima* 3506). The population of endophytic bacteria in wheat stem also ranges from lowest to highest as 2.5×10^6 CFU g^{-1} stem in wild wheat (*Aegilops kotschyi* 396) to 4.4×10^7 CFU g^{-1} stem in wheat crosses (1-1-7-13-15). Among the media used, nutrient agar supported the highest growth of endophytic bacteria, whereas plant extract agar supported the least population of endophytic bacteria (Table 2).

Table 1 Isolation of bacterial endophytes from different growing at Baru Sahib "Valley of Divine Peace" Himachal Pradesh

S. no.	Plant ID	Pedigree	Plant type
1	*Aegilops kotschyi* 396	*Aegilops* species	Wild
2	*Aegilops Peregrina* 3774	*Aegilops* species	Wild
3	*Aegilops longissima* 3506	*Aegilops* species	Wild
4	Wheat Chinese Spring	*Triticum aestivum* Chinese Spring	Cultivar
5	Wheat PBW 343 (Lr24+GPC B1)	*Triticum aestivum* cv. PBW 343	Cultivar
6	Wheat (HD 2967)	*Triticum aestivum* cv. HD 2967	Cultivar
7	Wheat (46-1-15-15-3)	CS (Ph^I)/*Aegilops Kotschyi* 396//PBW 343-3///WL 711-1-15-15-3	Wheat–*Aegilops* substitute line
8	Wheat (1-1-7-18-15)	CS (Ph^I)/*Aegilops Peregrine*//55-5-1//PBW 373-////WL 711-70-18-5	Wheat–*Aegilops* substitute line
9	Wheat (79-1-2-14-1-6-1-11)	HD 2687/*Aegilops Longissima* 3506//WL 711-2-1-14-1-6-1-1	Wheat–*Aegilops* substitute line

16S rRNA Gene Amplification and Phylogenetic Analysis

Based on the molecular chronometer, the ribosomal operon, especially 16S rRNA the phylogeny of endophytic bacteria was characterized. PCR amplification of the 16S rRNA gene was carried out to look for the species. The taxonomic position of the endophytic bacterium was determined by comparing the 16S rRNA gene sequence with different strains obtained from the NCBI database using the BLAST search. The strain EU-B2RT.R1 was sequenced and BLAST analyzed within the GenBank database which further characterize and validate the taxonomic position. The strain EU-B2RT.R1 showed maximum identity with *Acinetobacter guillouiae*. The 16S rRNA gene sequence revealed that strain EU-B2RT.R1 belongs to the Proteobacteria phylum and Moraxellaceae family. The sequence of strain EU-B2RT.R1 was deposited in the NCBI GenBank database under the following accession numbers MN294536 (Fig. 1).

Phenotypic Characterization of Endophytic Bacteria

A total of 13 bacterial isolates were screened for different abiotic stress factors such as temperature, salinity and pH. About 5, 5 and 8 bacterial isolates could grow at a

Table 2 Total viable counts of endophytic microbes isolated from different wheat genotypes

S. no.	Samples details		CFU per g of sample (root and shoot) on different media ($\times 10^7$)									
	Wheat genotypes	Code	BM	TSA	JA	KB	YMA	NA	AIA	PEA	T_3A	LBA
1	*Aegilops kotschyi* 396	A_1R	0.84	3.08	0.56	2.40	0.48	0.56	0.31	0.35	0.46	0.52
2	*Aegilops kotschyi* 396	A_1S	0.44	0.46	3.60	3.20	1.04	0.80	0.25	-	0.30	0.50
3	*Aegilops Peregrina* 3774	A_2R	0.72	0.80	0.68	1.20	2.00	0.33	0.30	0.72	0.31	0.30
4	*Aegilops Peregrina* 3774	A_2S	2.72	2.20	3.20	3.80	1.20	1.00	0.40	-	0.35	0.43
5	*Aegilops longissima* 3506	A_3R	0.84	2.40	0.60	0.80	3.80	0.37	0.43	0.10	3.12	1.24
6	*Aegilops longissima* 3506	A_3S	3.00	3.20	2.40	2.84	0.50	0.55	0.50	-	0.33	0.49
7	Wheat Chinese Spring	B_1R	0.76	3.40	0.13	2.39	1.20	0.33	3.82	-	0.36	0.42
8	Wheat Chinese Spring	B_1S	0.88	0.40	0.66	4.00	3.40	0.76	0.57	-	0.32	0.36
9	Wheat [PBW 343 (Lr24+GPC B1)]	B_2R	0.56	0.80	0.46	0.33	0.72	0.62	0.65	0.33	0.43	0.38
10	Wheat PBW 343 (Lr24+GPC B1)	B_2S	2.68	3.80	1.80	3.36	0.43	1.00	0.50	0.32	3.15	1.60
11	Wheat HD 2967	B_3R	0.56	0.10	0.32	0.40	0.43	0.38	0.35	-	3.20	2.00
12	Wheat HD 2967	B_3S	3.48	0.40	0.31	0.48	0.40	0.60	0.47	-	2.28	2.60
13	Wheat 46-1-15-15-3	C_1R	3.92	0.30	0.40	0.32	0.34	0.30	0.40	-	0.39	0.39
14	Wheat 46-1-15-15-3	C_1S	0.52	0.30	0.32	0.43	0.35	0.32	0.68	-	0.37	0.35
15	Wheat 1-1-7-18-15	C_2R	1.24	0.40	0.31	0.53	0.34	0.36	0.70	0.55	0.45	0.63
16	Wheat 1-1-7-18-15	C_2S	2.68	2.80	1.32	1.36	0.30	4.4 0	4.00	-	3.37	2.00
17	Wheat 79-1-2-14-1-6-1-11	C_3R	1.40	3.60	4.16	1.05	0.90	0.51	0.60	0.42	0.52	0.45
18	Wheat 79-1-2-14-1-6-1-11	C_3S	0.40	1.20	2.40	3.20	3.34	2.00	9.70	-	1.20	3.00

NA nutrient agar, *PEA* plant extract agar, *AIA Azotobacter* isolation agar, *LBA* Luria–Bertani Agar, *YMA* Yeast mannitol agar, *KB* King's B agar, *JA* Jensen agar, *TSA* tryptic soy agar, *BM* Burks media

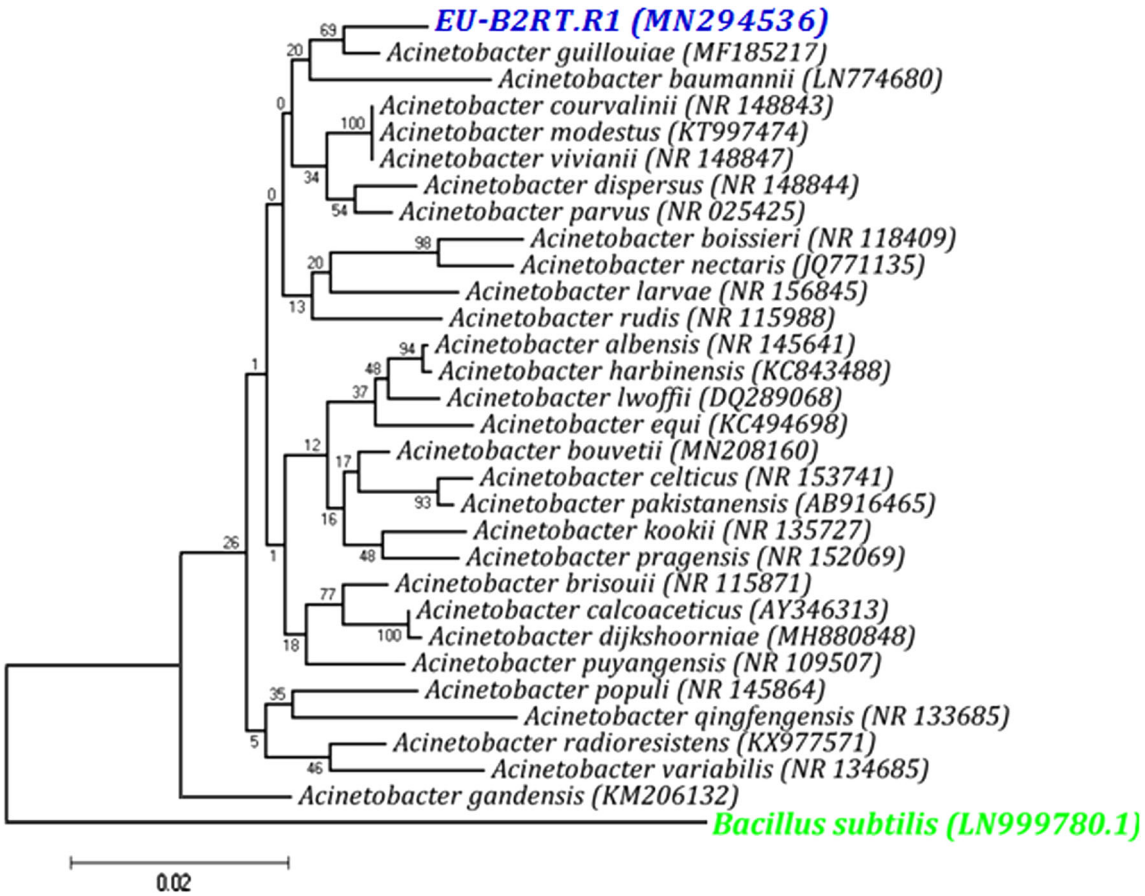

Fig. 1 Phylogenetic tree showing the relationship of **EU-B2RT.R1** with reference sequences obtained from NCBI through BLAST search. The sequence alignment was performed using the CLUSTAL W program, and tree was constructed using neighbor joining (NJ) with algorithm using MEGA4 software

temperature of 5 °C, 10 °C, and 20 °C respectively indicating that they were psychrophiles, whereas 8 and 4 bacterial isolates could grow at a temperature of 40 °C and 50 °C respectively indicating that they were mesophiles and thermophiles. To a varying concentration of NaCl, bacterial isolates also exhibited tolerance. Three bacterial isolates showed tolerance against 5% of NaCl and against 10% of NaCl, and none of the bacterial isolates showed tolerance against 15% of NaCl. Bacterial isolates were also screened for varying ranges of pH from 4–9. Out of 13 bacterial isolates, none of the bacterial isolates showed growth at 4 and 5 pH, whereas all the 13 bacterial isolates demonstrated that their growth at pH 6 and 8 and only 3 bacterial cultures could grow at pH of 9 (Table 3).

Functional Characterization of Endophytic Bacteria

The endophytic bacteria were screened for different plant growth-promoting attributes. Out of 159 bacterial isolates, 13 bacterial isolates were screened for direct and indirect PGP traits. About 7, 6 and 2 of bacterial isolates exhibited the ability to solubilize phosphorus, potassium, and zinc respectively. Among 13 isolates, a total of 7, 6 and 8 of the isolates exhibited the siderophore, ammonia, and HCN production, respectively. About 5 isolates produced indole-3-acetic acid, and only 1 isolate showed antagonistic activity against *Fusarium graminearum*. Among plant growth-promoting activities, the HCN-producing strains were higher, followed by siderophore-producing and phosphorus-solubilizing strain. The strain *Acinetobacter guillouiae* EU-B2RT.R1 demonstrated with nitrogenase activity of 25.84 n moles C_2H_4 mg^{-1} protein h^{-1} and also showed phosphorus-solubilizing activity 59.8 ± 0.01 mg L^{-1}. The strain EU-C3SJ1 also exhibited the phosphorus-solubilizing attribute 33.3 ± 0.02 mg L^{-1}. The strain *Acinetobacter guillouiae* EU-B2RT.R1 demonstrated the production of IAA 11.40 ± 0.00 mg L^{-1} in the presence of tryptophan and 13.6 ± 0.01 mg L^{-1} in the absence of tryptophan (Table 4).

Table 3 Tolerance of bacterial endophytes for different abiotic stresses

Code	Temperature (°C)					Salinity (% NaCl)			pH				
	5	10	20	40	50	5	10	15	4	5	6	8	9
EU-A2RK2	+	+	++	+	−	−	−	−	−	−	+	+	++
EU-A2SN1	−	−	−	−	−	−	−	−	−	−	+	+	−
EU-A1SA1	−	−	++	+	+	+	−	−	−	−	+	+	−
EU-A1SA2	−	−	−	−	−	−	−	−	−	−	+	+	−
EU-A1SJ2	−	−	−	−	−	−	−	−	−	−	+	+	−
EU-A2SJ1	−	−	++	+	−	−	−	−	−	−	+	+	−
EU-A2SB1	−	−	−	−	−	−	−	−	−	−	+	+	−
EU-B2RT.R1	+	+	+	+	−	−	−	−	−	−	+	+	−
EU-B2RK1	+	+	+	+	+	−	−	−	−	−	+	+	−
EU-B3RY1	+	+	+	+	+	+	+	−	−	−	+	+	−
EU-B2RP1	+	+	+	+	+	+	+	−	−	−	+	+	++
EU-C1RJ1	−	−	+	+	−	−	−	−	−	−	+	+	+
EU-C3SJ1	−	−	−	−	−	−	−	−	−	−	+	+	−

Table 4 Plant growth-promoting attributes of bacterial endophytes

Bacterial Strain	N_2 Fix.	Solubilization			Production					BC
	ARA	P	K	Zn	Sid	NH_3	HCN	IAA		
								(C+T+Lb)	(C−T+Lb)	
EU-A_2RK_2		+	+		+					
EU-A_2SN_1		+	+							
EU-A_1SA_1							+			
EU-A_1SA_2		+			+		+			
EU-A_1SJ_2					+		+			
EU-A2SJ1		+		+	+		+	+	+	
EU-A2SB1		+	+	+						
EU-B2RT.R1	25.84	59.8 ± 0.01	+		+	+	+	11.40 ± 0.00	13.6 ± 0.01	
EU-B_2RK_1			+		+	+	+	+	+	
EU-B_3RY_1						+	+			+
EU-B_2RP_1					+	+	+			
EU-C_1RJ_1						+		+	+	
EU-C3SJ1		33.3 ± 0.02	+			+		+	+	

ARA acetylene reduction assay (nmoles C_2H_4 mg^{-1} protein h^{-1}), *P* phosphorus (mg L^{-1}), *K* potassium, *Zn* zinc, *Sid.* siderophore, *NH_3* ammonia, *HCN* hydrogen cyanide, *IAA* indole acetic acid (mg L^{-1}), *C+T+Lb* media supplemented with tryptophan, *C−T+Lb* media without tryptophan, *BC* biocontrol

(+) positive for the attributes

Evaluation of Plant Growth-Promoting Ability of Endophytic Bacteria

In the present investigation, the bacterized wheat seedlings with endophytic bacteria under the greenhouse conditions showed a significant result in enhancing the growth of wheat plants after 90 days. After 90 days, the *Acinetobacter guillouiae* EU-B2RT.R1 demonstrated a significant enhancement in length of shoot (66.4 cm) and root (32.5 cm) over uninoculated control (59.2 cm and 30 cm, respectively). There was also enhanced biomass of shoot (34 g) and root (17 g) over uninoculated control (20 g and 10.6 g, respectively). Treatment with a full dose of 100% NP also enhanced the shoot and root (64.4 and 33 cm) length along with fresh biomass of shoot and roots. It was interesting to note that fresh biomass was obtained maximum after the application of full dose of urea. The numbers of tillers were 2.0-fold higher in *Acinetobacter guillouiae*

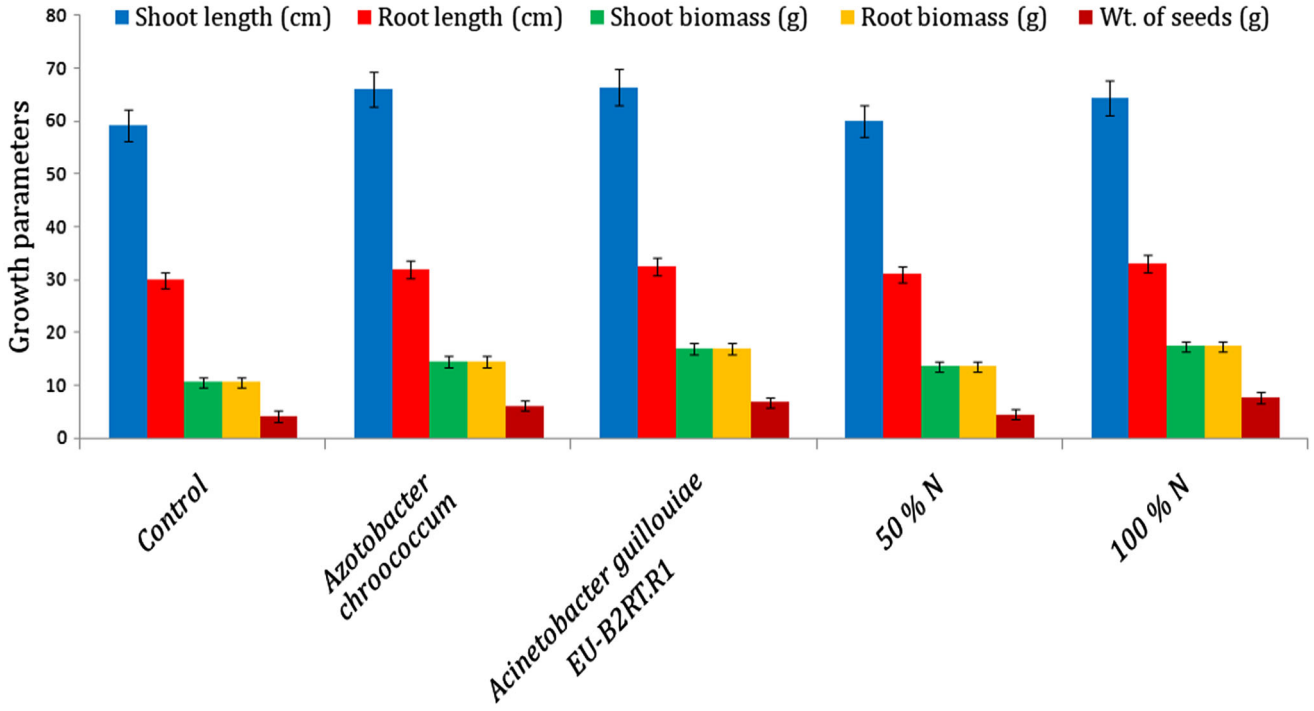

Fig. 2 Effect of inoculation of endophytic bacteria (*Acinetobacter guillouiae* EU-B2RT.R1) on growth parameters of wheat

EU-B2RT.R1 as compared to uninoculated control. Increment of seed weight was also observed in *Acinetobacter guillouiae* EU-B2RT.R1 inoculated wheat (Fig. 2).

The visible growth promotion of wheat reflected the positive impact of endophytic bacterial cultures. There is an enhanced increase in the concentration of Fe and Zn as the seeds of wheat inoculated with bacterial endophytes as compared to control which remain uninoculated. Maximum physiologically available Fe uptake about 123.18 mg kg^{-1} was observed in *Acinetobacter guillouiae* EU-B2RT.R1 about 3.7-fold higher over uninoculated control plants 33.20 mg kg^{-1}. Further, the full (100% N) dose of urea confirmed with a 2.7-fold increase in physiologically available Fe uptake. *Acinetobacter guillouiae* EU-B2RT.R1 also resulted in enhancement of Zn 59.91 mg kg^{-1} content as compared to uninoculated wheat seeds with a concentration of Zn 29.33 mg kg^{-1} (Fig. 3).

Endophytic bacterial strains by their active colonization mostly colonize the microniches within the tissues of plants and have and ecological significance. Endophytic bacteria have a stronger interaction with tissues of plants than rhizospheric microbes. A total of one hundred fifty-nine endophytic bacteria allied with different genotypes of wheat were obtained from the experimental field of Baru Sahib, Sirmour-173101, Himachal Pradesh (Table 1). The different factors affect the number of endophytic bacteria in specific niches within the tissues of plants [32]. In the present work, nutrient agar media supported the higher endophytic bacterial CFU count. The present findings of CFU count were found to be slightly higher than the data provided in an earlier report by Verma et al. [33] where the endophytic bacterial CFU count from tissues of wheat root and stems were lower. Further, bacterial endophytes isolated from plant roots of non-sterile soil reported containing a higher number of bacterial isolates as compared to other tissues of plants [34]. The increased colonization of bacterial endophytes in the root apical zone is due to different mechanisms of colonization and interaction with the host plant [35]. The sequencing of the 16S rRNA gene of the bacterial strain was taken up for phylogenetic analysis. Partial sequencing of the smaller subunit of 16S rRNA gene assigned and representative *Acinetobacter* belongs to family Moraxellaceae.

To best of the existing knowledge, *Acinetobacter guillouiae* EU-B2RT.R1 isolated from plant extract agar medium from wheat and also exhibited multifarious PGP attributes. *Acinetobacter radioresistens* isolated from Cotton and Soil [36]. *Acinetobacter lwoffi* have been isolated previously from shoot tip of banana [37]. In another study, Verma et al. [38] reported *Acinetobacter lwoffi* from the endosphere of wheat growing under the low-temperature conditions. The endophytic bacteria *Acinetobacter lwoffi* solubilizes the phosphorus at a concentration of 21.6 ± 1.0 mg L^{-1}, produces IAA at a 15 ± 0.4 µg mg^{-1} protein day^{-1} and produces siderophores, gibberellic acid, HCN and ammonia. *Acinetobacter lwoffi* was the fixation

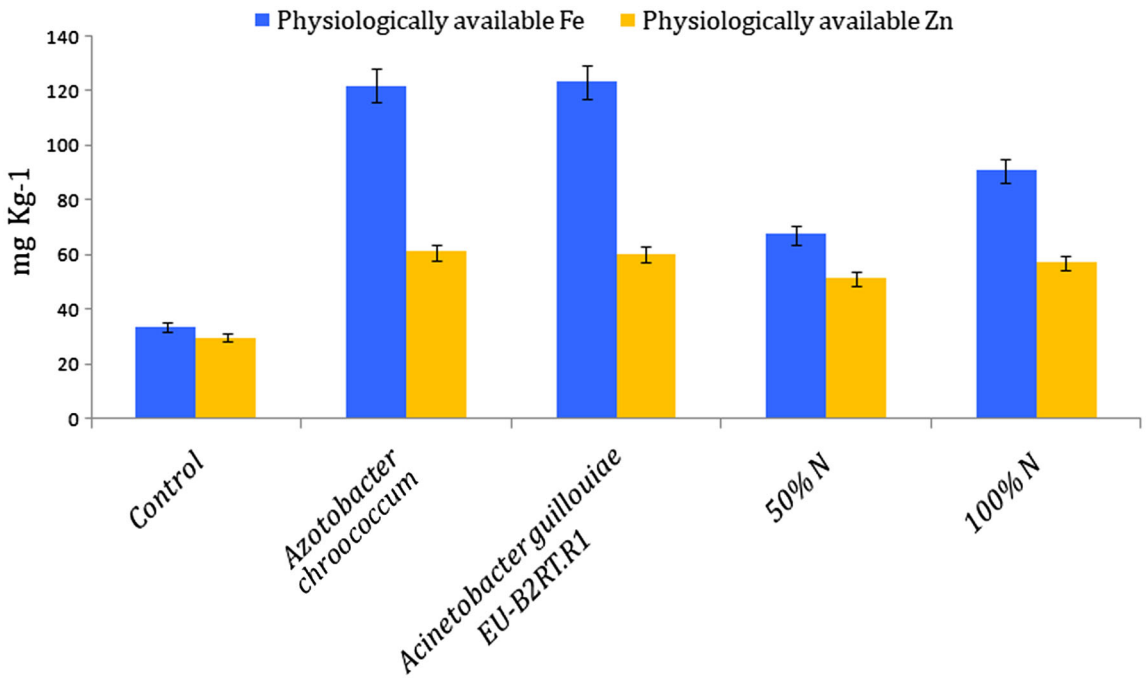

Fig. 3 Effect of inoculation of endophytic bacteria (*Acinetobacter guillouiae* EU-B2RT.R1) on physiologically available iron and zinc content in seeds of wheat

of nitrogen 10.23 ± 1.2 n moles C_2H_4 mg^{-1} protein h^{-1} by acetylene reduction assay. In a study, Kang et al. [39] reported that *Acinetobacter calcoaceticus* converts the insoluble soil phosphorus to soluble form with the decrease in pH and demonstrated that gluconic acid and 2-keto-gluconic acid were responsible for phosphorus solubilization. Among different species of *Acinetobacter* reported, the *Acinetobacter guillouiae* EU-B2RT.R1 solubilizes the higher concentration of phosphorus 59.8 ± 0.01 mg L^{-1} as compared to *Acinetobacter lwoffi* which was reported earlier by Verma et al. [38].

A wide range of plants nitrogen-fixing endophytic bacteria has been isolated, and they exhibit the ability to transfer fixed nitrogen to the host. Earlier bacteria fixing the atmospheric nitrogen were reported as symbiotic associations with higher plants [40]. Recently, more focus is laid on the isolation and identification of nitrogen-fixing endophytic bacteria in cereal crops [33, 38]. *Acinetobacter calcoaceticus* reported by Doty et al. [41] isolated from *Populus trichocarpa* and *Salix sitchensis* confirmed with the nitrogenase activity and enhances the capability of growing these tree species under nitrogen-limited conditions. Endophytic bacteria actively enhance the physiology of the host plant by fixing nitrogen. The presence of N_2-fixing endophytic *Acinetobacter oryzae* has been reported from wild rice, *Oryza alta.* Apart from phosphorus solubilization, nitrogen fixation, the production of IAA by endophytic bacteria adds a beneficial trait for enhancing the growth and productivity of the host plants [42]. The enhancement in IAA production provides protection against the cellular defense systems and against various stress conditions [43]. Various studies reported the bacteria also produce IAA in the absence of L-tryptophan [44]. *Acinetobacter guillouiae* EU-B2RT.R1 supplemented with L-tryptophan produces a higher concentration of IAA in the absence of media supplemented with L-tryptophan.

Microbes synthesizing a sufficient amount of siderophores can enhance the growth of the host plant by acquiring a sufficient amount of Fe [45]. The previous literature reported that *Acinetobacter haemolyticus* grow in an iron-depleting medium with the production of hydroxymate siderophore [46]. In a report, *Acinetobacter baumannii* also grows under the iron-limited condition and synthesizes baumannoferrin, a novel hydroxyamate [47].

Conclusion

In the present study, *Acinetobacter guillouiae* EU-B2RT.R1 showed enhancement of Fe content in the seeds of wheat. The present study demonstrated for the first time inoculation of endophytic *Acinetobacter guillouiae* EU-B2RT.R1 led to an increase in the biomass of wheat plant and enhancement in the content of Fe and Zn. The integrated use of *Acinetobacter guillouiae* EU-B2RT.R1 demonstrated one of the sustainable options to reduce the usage of chemical fertilizers for sustainable agriculture and environments

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Savci S (2012) An agricultural pollutant: chemical fertilizer. Int J Environ Sci Dev 3(1):73. https://doi.org/10.7763/IJESD.2012.V3.191
2. Smith P, House JI, Bustamante M, Sobocká J, Harper R, Pan G et al (2016) Global change pressures on soils from land use and management. Glob Change Biol 22(3):1008–1028. https://doi.org/10.1111/gcb.13068
3. Hallmann J, Quadt-Hallmann A, Mahaffee W, Kloepper J (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43(10):895–914. https://doi.org/10.1139/m97-131
4. Jha PN, Gupta G, Jha P, Mehrotra R (2013) Association of rhizospheric/endophytic bacteria with plants: a potential gateway to sustainable agriculture. Gr J Agric Sci 3(2):73–84. https://doi.org/10.15580/GJAS.2013.2.010313354
5. Yadav AN, Kumar R, Kumar S, Kumar V, Sugitha T, Singh B et al (2017) Beneficial microbiomes: biodiversity and potential biotechnological applications for sustainable agriculture and human health. J Appl Biol Biotechnol 5(6):45–57. https://doi.org/10.7324/JABB.2017.50607
6. Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16(10):463–471. https://doi.org/10.1016/j.tim.2008.07.008
7. Yadav AN, Singh J, Rastegari AA, Yadav N (2020) Plant microbiomes for sustainable agriculture. Springer, Cham
8. Rana KL, Kour D, Yadav AN (2019) Endophytic microbiomes: biodiversity, ecological significance and biotechnological applications. Res J Biotechnol 14:142–162
9. Chen C, Xin K, Liu H, Cheng J, Shen X, Wang Y et al (2017) *Pantoea alhagi*, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat. Sci Rep 7:41564. https://doi.org/10.1038/srep41564
10. Liu B, Qiao H, Huang L, Buchenauer H, Han Q, Kang Z et al (2009) Biological control of take-all in wheat by endophytic *Bacillus subtilis* E1R-j and potential mode of action. Biol Control 49(3):277–285. https://doi.org/10.1016/j.biocontrol.2009.02.007
11. Verma P, Yadav AN, Kumar V, Singh DP, Saxena AK (2017) Beneficial plant-microbes interactions: biodiversity of microbes from diverse extreme environments and its impact for crop improvement. In: Singh DP, Singh HB, Prabha R (eds) Plant-microbe interactions in agro-ecological perspectives: microbial interactions and agro-ecological impacts, vol 2. Springer, Singapore, pp 543–580. https://doi.org/10.1007/978-981-10-6593-4_22
12. Yadav AN, Verma P, Kumar M, Pal KK, Dey R, Gupta A et al (2015) Diversity and phylogenetic profiling of niche-specific Bacilli from extreme environments of India. Ann Microbiol 65(2):611–629. https://doi.org/10.1007/s13213-014-0897-9
13. Pikovskaya R (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya 17:362–370
14. Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170(1):265–270. https://doi.org/10.1111/j.1574-6968.1999.tb13383.x
15. Mehta S, Nautiyal CS (2001) An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr Microbiol 43(1):51–56. https://doi.org/10.1007/s002840010259
16. Hu X, Chen J, Guo J (2006) Two phosphate-and potassium-solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. World J Microbiol Biotechnol 22(9):983–990. https://doi.org/10.1007/s11274-006-9144-2
17. Fasim F, Ahmed N, Parsons R, Gadd GM (2002) Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol Lett 213(1):1–6. https://doi.org/10.1111/j.1574-6968.2002.tb11277.x
18. Salkowski E (1885) Ueber das Verhalten der Skatolcarbonsäure im Organismus. Z Physiol Chem 9(1):23–33. https://doi.org/10.1515/bchm1.1885.9.1.23
19. Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microbiol 68(8):3795–3801. https://doi.org/10.1128/AEM.68.8.3795-3801.2002
20. Schwyn B, Neilands J (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160(1):47–56. https://doi.org/10.1016/0003-2697(87)90612-9
21. Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant growth-stimulation. Soil Biol Biochem 19(4):451–457. https://doi.org/10.1016/0038-0717(87)90037-X
22. Cappucino J, Sherman N (1992) Nitrogen cycle. Microbiology: a laboratory manual, 4th edn. Benjamin/Cumming, New York, pp 311–312
23. Sijam K, Dikin A (2005) Biochemical and physiological characterization of *Burkholderia cepacia* as biological control agent. Int J Agric Biol 7(3):385–388
24. Kour D, Rana KL, Sheikh I, Kumar V, Yadav AN, Dhaliwal HS et al. (2019) Alleviation of drought stress and plant growth promotion by *Pseudomonas libanensis* EU-LWNA-33, a drought-adaptive phosphorus-solubilizing bacterium. In: Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. https://doi.org/10.1007/s40011-019-01151-4
25. Han SO, New P (1998) Variation in nitrogen fixing ability among natural isolates of *Azospirillum*. Microb Ecol 36(2):193–201
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254. https://doi.org/10.1016/0003-2697(76)90527-3
27. Jorhem L, Engman J (2000) Determination of lead, cadmium, zinc, copper, and iron in foods by atomic absorption spectrometry after microwave digestion: NMKL1 collaborative study. J AOAC Int 83(5):1189–1203
28. Pospiech A, Neumann B (1995) A versatile quick-prep of genomic DNA from gram-positive bacteria. Trends Genet 11(6):217–218. https://doi.org/10.1016/S0168-9525(00)89052-6
29. Yadav AN, Sachan SG, Verma P, Saxena AK (2015) Prospecting cold deserts of north western Himalayas for microbial diversity and plant growth promoting attributes. J Biosci Bioeng 119(6):683–693. https://doi.org/10.1016/j.jbiosc.2014.11.006
30. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17(19):7843–7853

31. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599. https://doi.org/10.1093/molbev/msm092
32. Kumar G, Kanaujia N, Bafana A (2012) Functional and phylogenetic diversity of root-associated bacteria of *Ajuga bracteosa* in Kangra valley. Microbiol Res 167(4):220–225. https://doi.org/10.1016/j.micres.2011.09.001
33. Verma P, Yadav AN, Khannam KS, Mishra S, Kumar S, Saxena AK et al (2019) Appraisal of diversity and functional attributes of thermotolerant wheat associated bacteria from the peninsular zone of India. Saudi J Biol Sci 26(7):1882–1895. https://doi.org/10.1016/j.sjbs.2016.01.042
34. Sun L, Qiu F, Zhang X, Dai X, Dong X, Song W (2008) Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. Microb Ecol 55(3):415–424. https://doi.org/10.1007/s00248-007-9287-1
35. Botta AL, Santacecilia A, Ercole C, Cacchio P, Del Gallo M (2013) *In vitro* and in vivo inoculation of four endophytic bacteria on *Lycopersicon esculentum*. New Biotechnol 30(6):666–674. https://doi.org/10.1016/j.nbt.2013.01.001
36. Nishimura Y, Ino T, Iizuka H (1988) *Acinetobacter radioresistens* sp. nov. isolated from cotton and soil. Int J Syst Evol Microbiol 38(2):209–211. https://doi.org/10.1099/00207713-38-2-209
37. Thomas P, Soly TA (2009) Endophytic bacteria associated with growing shoot tips of banana (*Musa* sp.) cv. Grand Naine and the affinity of endophytes to the host. Microb Ecol 58(4):952–964. https://doi.org/10.1007/s00248-009-9559-z
38. Verma P, Yadav AN, Khannam KS, Panjiar N, Kumar S, Saxena AK et al (2015) Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India. Ann Microbiol 65(4):1885–1899. https://doi.org/10.1007/s13213-014-1027-4
39. Kang S-M, Joo G-J, Hamayun M, Na C-I, Shin D-H, Kim HY et al (2009) Gibberellin production and phosphate solubilization by newly isolated strain of *Acinetobacter calcoaceticus* and its effect on plant growth. Biotechnol Lett 31(2):277–281. https://doi.org/10.1007/s10529-008-9867-2
40. Zahran HH (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63(4):968–989
41. Doty SL, Oakley B, Xin G, Kang JW, Singleton G, Khan Z et al (2009) Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47(1):23–33
42. Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol 2(5):a001446. https://doi.org/10.1101/cshperspect.a001446
43. Khan Z, Doty SL (2009) Characterization of bacterial endophytes of sweet potato plants. Plant Soil 322(1–2):197–207. https://doi.org/10.1007/s11104-009-9908-1
44. Lee S, Flores-Encarnacion M, Contreras-Zentella M, Garcia-Flores L, Escamilla J, Kennedy C (2004) Indole-3-acetic acid biosynthesis is deficient in *Gluconacetobacter diazotrophicus* strains with mutations in cytochrome c biogenesis genes. J Bacteriol 186(16):5384–5391. https://doi.org/10.1128/JB.186.16.5384-5391.2004
45. Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica. https://doi.org/10.6064/2012/963401
46. Okujo N, Sakakibara Y, Yoshida T, Yamamoto S (1994) Structure of acinetoferrin, a new citrate-based dihydroxamate siderophore from *Acinetobacter haemolyticus*. Biometals 7(2):170–176. https://doi.org/10.1007/BF00140488
47. Penwell WF, DeGrace N, Tentarelli S, Gauthier L, Gilbert CM, Arivett BA et al (2015) Discovery and characterization of new hydroxamate siderophores, baumannoferrin A and B, produced by *Acinetobacter baumannii*. ChemBioChem 16(13):1896–1904. https://doi.org/10.1002/cbic.201500147