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ORIGINAL ARTICLE

Stress-Tolerant *Viridibacillus arenosi* Strain IHB B 7171 from Tea Rhizosphere as a Potential Broad-Spectrum Microbial Inoculant

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Abstract *Viridibacillus arenosi* strain IHB B 7171 identified based on 16S rRNA gene sequence produced colony forming units (cfu/ml) ranging from 3.3×10^4 to 1.2×10^{10} under pH 5–11, 2.2×10^2 to 1.4×10^{10} for temperature 5–40 °C, 2.4×10^2 to 1.1×10^{10} for PEG 6000 10–30%, 2.2×10^2 to 1.4×10^{10} for 2.5–10% NaCl, 3.1×10^3 to 1.7×10^9 for 2.5–7.5 mM $CaCl_2$, 2.2×10^2 to 1.4×10^7 for 2.5–7.5 mM $AlCl_3$, and 3.2×10^2 to 1.2×10^7 for 2.5–7.5 mM $FeCl_3$. The activities of plant growth-promoting attributes with the increasing acidity, desiccation and salinity ranged from 408 to 101, 20 to 8, 14 to 5 µg/ml P-liberated from tri-calcium phosphate, aluminium phosphate and iron phosphate, 20–9% siderophore units, 14–4 µg/ml IAA and 190–16 α-ketobutyrate h/mg protein ACC-deaminase activity. Plant height, leaf number, and leaf weight on treatment with bacterial inoculum showed an increment of 9.5, 17.6, 54.5 and 31.0% in tea seedlings, respectively. The bacterium also enhanced plant height and yield by 10 and 13% in pea and 2.8 and 13.9% in wheat. The results exhibited stress-tolerance and plant growth-promoting activities by the strain under stressed growth-conditions with potential as a broad-spectrum plant growth-promoting rhizobacterium.

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Keywords *Viridibacillus arenosi* · Abiotic stress-tolerance · PGPR activities · Plant growth promotion · Broad-spectrum PGPR

Introduction

Exploring plant growth-promoting rhizobacteria (PGPR) as a useful biological tool has gained an immense interest for improving growth and productivity of plants [1, 2]. Their metabolic versatility manipulates the rhizosphere for plant growth promotion mainly by liberation of bound P into utilizable forms by plants, secretion of auxins involved directly in plant growth and development, production of siderophores which facilitate iron-uptake by plants, and synthesis of ACC (1-aminocyclopropane-1-carboxylase) deaminase responsible for lowering the levels of plant stress-hormone ethylene [3, 4]. However, the outcome of PGPR application is impacted by environmental conditions and soil properties. The stress-tolerance against acidity/alkalinity, temperature, desiccation, salinity, and metal ions has been tested only for a few PGPR to ensure their field performance as the microbial inoculants [5, 6]. This paper reports the efficacy of *Viridibacillus arenosi* strain IHB B 7171 selected for abiotic stress-tolerace and multiple plant growth-promoting attributes as a broad-spectrum microbial inoculant.

Materials and Methods

Isolation and Characterization

The isolate IHB B 7171 purified on tryptone soya agar (TSA) from the several hundred bacterial colonies obtained by spread plating the serial soil dilutions of tea rhizosphere soils collected from a depth of 15–30 cm from Zen Tea Estate Gopalpur (latitude 32°11′30″N and longitude 76°20′54″E) in the Kangra valley located in the Western Himalayas. The isolate was identified by amplifying 16S rRNA gene using the standard methods.

Stress Tolerance and PGPR Activities

The effect of stress parameters on culture growth was studied in TSB: temperature—5, 10, 15, 30, 35 and 40 °C at pH 7; acidity/alkalinity—pH 4, 5, 6, 7, 8, 9, 10, 11 and 12 at 28 °C; desiccation—10, 20 and 30% PEG 6000 at pH 7 under 28 °C; salinity—2.5, 5.0, 7.5 and 10% NaCl at pH 7 under 28 °C; and salt tolerance—2.5, 5.0, 7.5, and 10.0 mM $CaCl_2$, $FeCl_3$ or $AlCl_3$ at pH 7 under 28 °C. The growth was measured by determining cfu/ml by plating the serial dilutions after 24 h incubation under various conditions, excepting the cultures subjected to 5 °C were incubated up to 72 h. All cfu values represent 1 ml of the culture.

The plant growth-promoting activities were measured using Barton's reagent for P-liberated in Pikovskaya's broth (PVK) supplemented with 0.5% TCP, Al–P or Fe–P [7], CAS reagent for siderophore production in succinate broth [8], Salkowski reagent for IAA (indole-3-acetic acid)-like auxins in nutrient broth (NB) supplemented with 0.1% tryptophan [9], and 2,4-dinitrophenylhydrazine reagent for ACC-deaminase activity in NB supplemented with ACC [10]. Organic acids produced during phosphate solubilization were quantified using Lichrosphere RP-18 column (Merck, Germany) on Waters 996 High Performance Liquid Chromatography system (HPLC) equipped with PDA detector and Waters 717 Plus Autosampler. The mobile phase consisted of 0.1% ortho-phosphoric acid (Merck, Germany) with gradient flow of 0.4 ml min^{-1} for 0–8 min, 0.5 ml min^{-1} for 8–4 min and 1.2 ml min^{-1} for 14–25 min [7].

Plant Growth Promotion Under Controlled Environment and Field Conditions

The carrier-based inoculum on activated charcoal at approximately 10^8 cfu/g was applied to the surface-sterilized uniformly pre-germinated seeds of maize (*Zea mays* var. Girija) sown in the pots kept in a Randomized Block Design (RBD) under temperature 25 ± 2 °C, R.H. 65–70% and photoperiod 16 h in Controlled Environment Chamber. Data on growth parameters were recorded after 30 days of treatment. Likewise, the seeds collected from bushes of chinary tea (*Camellia sinensis* var. HPKV1) were germinated, selected for uniform radicle growth, and treated with the inoculum. The inoculum treated seeds along with the seeds treated with the carrier-base without inoculum to serve as the control were grown in polysleeves in RBD in the Tea Plantation Nursery, State Agriculture Department of Himachal Pradesh at Diffarpatt. Data on growth parameters were recorded after 1 year of inoculum application.

The field evaluations were done in the Experimental Farms of Krishi Vigyan Kendra, CSKHPKV at Bajaura in RBD in the plots measuring 4.08 m^2 with 90 plants each for pea (*Pisum sativum* var. Punjab 89) and 9.9 m^2 with 900 plants each for wheat (*Triticum aestivum* var. HPW-155). The inter-row and intra-plant distances were 45 and 10 cm for pea and 22 and 5 cm for wheat, respectively. Data were recorded for growth parameters for 10 plants randomly taken from each plot and for yield for all the plants plot-wise.

Statistical Analysis

The STATISTICA data analysis software system version 7 (StatSoft Inc., Tulsa, OK, USA 2004) was used for the analysis of variance (ANOVA).

Results and Discussion

Isolation and Characterization

The isolate was identified as *V. arenosi* strain IHB B 7171 based on 99.5% identity of 16S rRNA gene sequence with *V. arenosi* LMG 22166 reported from a soil sample from the Netherlands [11]. The phylogenetic analysis revealed close relatedness with *V. arenosi* LMG 22166 and *V. arvi* LLP-44 (ESM_1).

Stress Tolerance and PGPR Activities

Viridibacillus arenosi IHB B 7171 exhibited tolerance to various abiotic stresses as evidenced by the culture growth under stressful regimes of acidity/alkalinity, temperature, desiccation, salinity, and Ca, Al and Fe salts (Fig. 1). Growth over the wide pH range of 5–11 suggested its suitability for application under both acidic and alkaline soils [12–14]. High cfu values of 1.8×10^4 to 1.4×10^{10} of the cultures under 10–40 °C further indicated its tolerance to diurnal and seasonal temperature regimes through the different cropping seasons [15]. Endurance to desiccation revealed by the high cfu of 2.4×10^4 to 1.1×10^{10} under 10–30% PEG 6000 was comparable to the high desiccation tolerance reported for *Bacillus pumilus, B. firmus* and *Pseudomonas poae* strains [13, 16]. The culture

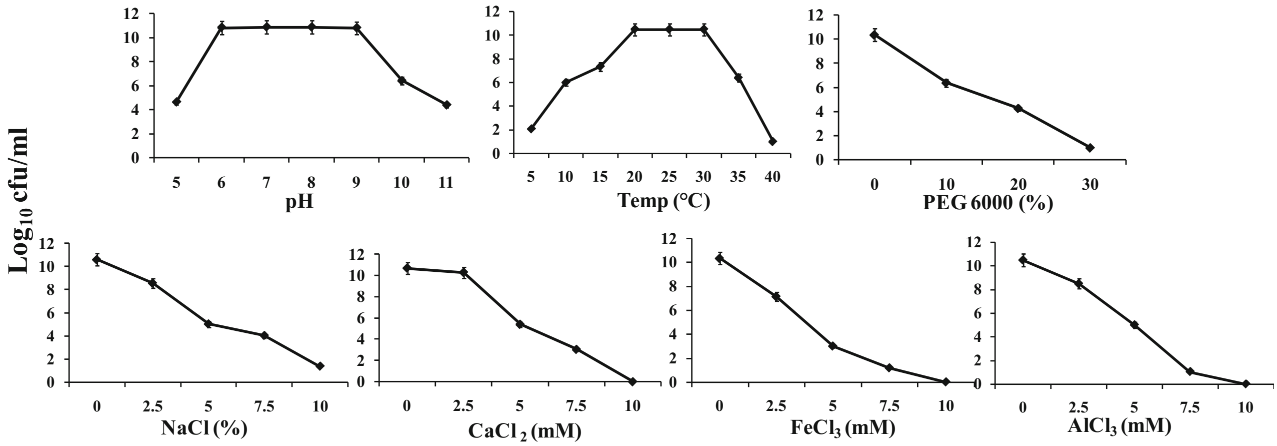

Fig. 1 Growth of *Viridibacillus arenosi* strain IHB B 7171 cultures under different levels of pH, temperature, PEG 6000, NaCl (%), $CaCl_2$, $FeCl_3$ and $AlCl_3$ (mM). Values are the mean of three replicates, *error bars* indicate standard deviation

growth producing cfu of 1.4×10^{10} under 2.5% NaCl and 2.3×10^5 under 5% NaCl suggested its tolerance to the saline growth conditions prevailing in the alkaline soils. Likewise, the culture growth of 3.1×10^3 to 1.7×10^9, 2.2×10^2 to 1.4×10^7 and 3.2×10^2 to 1.2×10^7 cfu/ml under the increasing concentrations of 2.5–7.5 mM $CaCl_2$, $AlCl_3$ and $FeCl_3$, respectively, revealed tolerance of the strain to high concentrations reported for Ca in the alkaline soils and Al and Fe in the acidic soils [17, 18].

Viridibacillus arenosi IHB B 7171 also exhibited the multiple plant growth-promoting functions of phosphate solubilization, siderophore production, production of IAA-like auxins, and ACC-deaminase activity similar to the efficient PGPR strains of *Acinetobacter rhizosphaerae*, *Arthrobacter* sp., *Bacillus* sp., *Burkholderia phytofirmans*, *P. putida*, *Pseudomonas* sp. and *Serratia liquefaciens* (Fig. 2) [19–21]. The solubilization of different inorganic phosphates indicated its suitability for application in the acidic soils rich in Al and Fe-bound P and the alkaline soils rich in Ca-bound P [20, 21]. In particular, the solubilization of Al–P and Fe–P shown has been reported among the rhizobacteria only for *Arthrobacter* sp., *Bacillus* sp., *Micrococcus* sp. and *P. fluorescens* [22–24]. Under the culture growth at pH 5 which prevails in the acidic soils of tea plantations, the PGPR activities recorded P-liberation of 101.2, 21.2 and 14.2 µg/ml from TCP, Al–P and Fe–P solubilization, respectively, 9% siderophore units, 4.2 µg/ml IAA-like auxins and 16 nM α-ketobutyrate h/mg protein ACC-deaminase activity. Likewise, the culture growth at pH 8 which occurs widely in the cultivable alkaline soils showed PGPR activities of 105, 20 and 14 µg/ml P-liberation from TCP, Al–P and Fe–P solubilization, respectively, 16% siderophore units, 11 µg/ml IAA-like auxins and 595 nM α-ketobutyrate h/mg protein ACC-deaminase activity (14). The test strain also exhibited the PGPR activities to varying extent under the stressed growth conditions (Fig. 2), recording 109 µg/ml TCP solubilization, 20% siderophore units, 5 µg/ml IAA-like auxins and 190 nM α-ketobutyrate h/mg protein ACC-deaminase activity under the salinity of 2.5% NaCl, and 408 µg/ml P-liberation from TCP and 20% siderophore units under the desiccation regime of 10% PEG 6000. Only TCP solubilization has been reported under the stressed culture conditions of acidity and alkalinity for *Arthrobacter* sp. and *Bacillus* sp., acidity, alkalinity and temperature for *Acenitobacter* sp., and acidity and salinity for *Pseudomonas* spp. [21, 22].

Gluconic acid and oxalic acids commonly detected during the solubilization of these phosphate substrates by *V. arenosi* strain IHB B 7171 strain have been reported among the major organic acids produced during the microbial solubilization of phosphate substrates (Table 1). Detection of lactic and succinic acids only during TCP solubilization corroborated the influence of phosphate substrates on the nature of organic acids produced by microorganisms [25, 26]. However, only quantitative decrease was marked in the production of various organic acids by the test strain during solubilization of TCP, Al–P and Fe–P under the stressed growth conditions. The production of organic acids accompanied by the pH drop of cultures corroborated the involvement of organic acids in phosphate solubilization [7, 25, 26].

Plant Growth Promotion Under Controlled Environment and Field Conditions

A significant improvement in plant growth with an increment of 9, 49.3, 30.7 and 93.3% of plant height, shoot dry

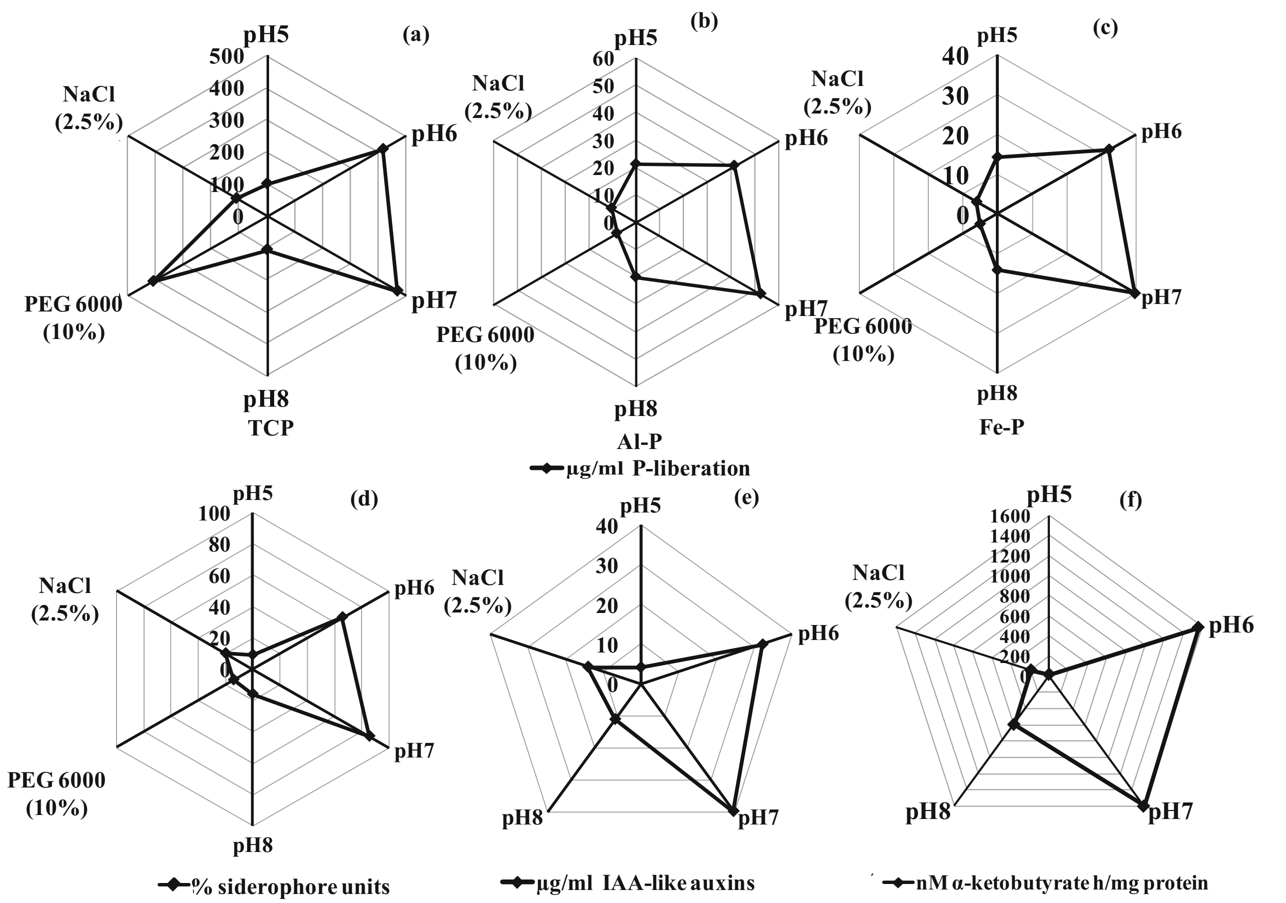

Fig. 2 Radar pattern illustrating the PGPR activities of *Viridibacillus arenosi* strain IHB B 7171 under pH 5–8, PEG 6000 10% and 2.5% NaCl. TCP, Al–P and Fe–P solubilization (**a**–**c**), siderophore production (**d**), IAA-like auxins production (**e**), and ACC-deaminase activity (**f**). Values are the mean of three replicates. *Scale-bars* represent activity levels

Table 1 Production of organic acids by *Viridibacillus arenosi* strain IHB B 7171 during TCP, Al–P and Fe–P solubilization under different stress parameters

Parameter			Organic acids production (μg/ml)							
			TCP				Al–P		Fe–P	
		Final pH	GA	LA	OA	SA	GA	OA	GA	OA
pH	5	3.9	217.8	629.3	117.8	434.3	16.2	125.3	24.8	14.8
	6	4.1	1022.1	952.4	122.1	46.4	22.4	806.5	24.7	24.7
	7	3.9	1125.2	722.1	125.2	172.1	55.7	822.1	34.4	24.2
PEG 6000 (%)	10	4.2	16.2	345.3	342.4	ND	16.8	111.3	ND	ND
NaCl (%)	2.5	4.1	211.9	132.3	56.6	ND	ND	ND	ND	ND
$CaCl_2$ (mM)	2.5	4.0	121.9	154.3	111.3	ND	ND	ND	ND	ND
$AlCl_3$ (mM)	2.5	3.8	168.8	111.7	14.9	ND	ND	ND	ND	ND
$FeCl_3$ (mM)	2.5	3.9	145.9	15.4	29.4	ND	ND	ND	ND	ND

Values are the mean of three replicates and significantly different from the control at $p < 0.05$

ND not detected, *TCP* tricalcium phosphate, *Al–P* aluminium phosphate, *Fe–P* iron phosphate. *OA* oxalic acid, *GA* gluconic acid, *SA* succinic acid, *LA* lactic acid

weight, root length, and root dry weight in maize employed as the quick screen demonstrated plant growth-promotion potential of the strain (Table 2). Likewise, a significant improvement in various growth parameters in tea seedlings exhibited the usefulness of the strain for promoting growth in tea [27, 28]. This is the first stress-tolerant PGPR with

Table 2 Plant growth promotion by *Viridibacillus arenosi* strain IHB B 7171 in maize and tea under controlled conditions and pea and wheat in field conditions

Growth parameters	Control	Inoculated	Increase over control (%)
Maize seedlings (controlled environment)[a]			
Plant height (cm)	55	60	9.0
Shoot dry weight (g)	2.59	3.86	49.3
Root length (cm)	13	17	30.7
Root dry weight (g)	0.15	0.29	93.3
Tea (nursery conditions)[b]			
Plant height (cm)	21	23	9.5
Leaves (No.)	17	20	17.6
leaf fresh weight (g)	11	17	54.5
leaf dry weight (g)	0.29	0.38	31.0
Pea (field conditions)[c]			
Plant height (cm)	40	44.3	10.7
Yield (q/ha)	46	52	13.0
Fresh weight (q/ha)	12.4	14.5	16.9
Dry weight (q/ha)	8.2	9.9	20.7
Wheat (field conditions)[d]			
Plant height (cm)	91	93.6	2.8
Yield (q/ha)	25.8	29.4	13.9
Fresh weight (q/ha)	56.18	70.01	25
Dry weight (q/ha)	11.0	14.07	27

Values are the mean of three replicates and significantly different from the control at $p < 0.05$

[a] Temperature 25 ± 2 °C, R.H. 65–70% and photoperiod 16 h

[b] Average minimum and maximum temperatures 15–34 °C and R.H. 45–75%, and overall rainfall 290–1250 mm

[c] Average minimum and maximum temperatures 1–27 °C and R.H. 35–94%, and overall rainfall 9.5–153.8 mm

[d] Average minimum and maximum temperatures 1–32 °C and R.H. 35–95%, and overall rainfall 95–290 mm

multiple plant growth-promoting traits with the ability to solubilize Al and Fe bound P for utilization by tea plants for their growth limited by the binding of applied P with Al and Fe cations in the acidic soils required for tea cultivation [28]. A significant increment in plant height, fresh and dry weight and yield to the extent of 10, 16.9, 20.7 and 13% in pea and 2.8, 25, 27 and 13.9% in wheat demonstrated the broad-spectrum growth-promotion by the test strain considered important for undertaking bulk production at commercial-scale (Table 2).

The stress-tolerant *V. arenosi* strain IHB B 7171 with multiple plant growth-promoting activities and broad-spectrum plant growth promotion is a prospective microbial inoculant for improving productivity in tea plantations and agricultural crops under stressed farming systems.

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