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Tea seedlings growth promotion by widely distributed and stress-tolerant PGPR from the acidic soils of the Kangra valley

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Abstract

This is the first report of widespread and stress-tolerant PGPR from the tea rhizosphere of the Kangra valley. A total of 493 rhizobacteria were isolated from the major tea-growing regions of the Kangra valley. Molecular fingerprinting of 160 distinct morphotypes using ARDRA and ERIC techniques revealed intergenic and intragenic variability, resulting in the identification of 52 rRNA and 56 ERIC types belonging to 21 distantly related genera, identified by 16S rRNA gene sequencing. *Bacillus* constituted more than half of the genotypes, followed by *Pseudomonas*, *Burkholderia*, *Lysinibacillus*, *Citrobacter*, *Enterobacter*, and *Paenibacillus*. *Bacillus altitudinis*, *B. cereus*, *B. megaterium*, *B. subtilis* subsp. *inaquosorum*, *B. methylotropicus*, *Pseudomonas frederiksbergensis*, *P. mohnii*, and *P. moreiii* were found to be the most common in the tea rhizosphere across various locations. Quantitative assaying of 42 selected strains revealed significant variations in PGP activities ranging from 55–624 µg/ml for tri-calcium phosphate (TCP) solubilization, 4–3145 nM α-ketobutyrate h/mg/protein ACC-deaminase activity, 2–85 µg/ml IAA-like auxins production, and 2–83% siderophore production. Nine out of 42 PGPR also solubilized aluminium phosphate (Al-P) and iron phosphate (Fe–P). These efficient PGPR are suitable for application in tea soils, which are generally low in available phosphorus, a growth-limiting factor for tea cultivation. Five highly efficient PGPR also showed robust growth under different abiotic stresses under controlled conditions. Inoculum application of 5 efficient and abiotic stress tolerant PGPR showed a significant increment of 1.8–9.4%, 12–16.2%,18.1–30.3% and 21.4–39.2% in plant height, leaf number, fresh and dry weight of tea seedlings under the nursery conditions with 50% reduced NPK concentrations after one year of inoculations, respectively. These selected PGPR genotypes with multifarious PGP activities and natural ability to occur widely can be useful in developing plant microbial inoculants for improving tea productivity.

Keywords Microbial diversity, Phosphate solubilization, Tea rhizosphere, Multiple PGP attributes

Introduction

Tea is one of the most economically important crops and is commercially grown in countries like China, India, Kenya, Sri Lanka, Turkey, Indonesia, Vietnam, Japan, Iran and Argentina [21]. Tea cultivation is heavily dependent on the use of chemical fertilizers. Chemical fertilizers represent a critical factor to optimize tea growth and yield [53]. One of the primary reasons for the excessive use of chemical fertilizers is low availability of nutrients due to the acidic nature of tea soils [45]. In acidic soils, the majority of the phosphorous (P) is present in

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insoluble forms due to the presence of aluminium (Al) and iron (Fe)that leads to the formation of insoluble free oxides and hydroxides of P [11, 45]. In the long run, the persistent use of chemical fertilizers can lead to soil degradation and a reduction in rhizosphere biodiversity. Moreover, chemical fertilizers worsen the issue of soil acidification [55]. Continuous usage of chemical fertilizers has resulted in environmental pollution such as water contamination and soil toxicity [53]. To overcome these issues, sustainable practices that prioritize biodiversity, environmental health and soil health should be incorporated into tea cultivation.

Exploring the potential of soil microbes, particularly plant growth promoting rhizobacteria (PGPR), could offer sustainable solutions to improve tea cultivation. In recent years, PGPR have gain attention of many researchers as it can help the plant for soil nutrient acquisition as well as stress tolerance. PGPR have proven potential to improving the plant growth under controlled and field environmental conditions [10, 45, 57]. PGPR strains with multiple plant growth promoting (PGP) activities of phosphate solubilization, siderophore production, indole-3-acetic acid (IAA)-like auxins production and 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity are more effective than single PGP trait. Several studies showed the potential of phosphate solubilizing bacteria (PSB), such as *Acinetobacter* [17], *Arthrobacter* [26], *Azotobacter* [27], *Bacillus* [46], *Enterobacter* [9], *Halotalea* [18], *Priestia* [45], *Pseudomonas* [52], *Rhizobium* [43], *Viridibacillus* [47], and *Xanthomonas* [39] to solubilize P across several rhizospheres. However, in most studies, only single insoluble P substrate such as tri-calcium phosphate (TCP) has been used for solubilization. Relying solely on TCP as a method for selecting efficient PSB has proven to be weak and unreliable for enhancing plant growth [4]. For more effective selection, at least two or three different P sources should be considered.

In Himachal Pradesh, tea is cultivated over 2,348 hectares in the Kangra and Mandi districts (Indian Tea Association) but the information regarding rhizobacteria with PGP traits is scarce and the application of PGPR inoculants in tea cultivation remains largely unexplored in this region. Also, present data on the genetic diversity of rhizobacteria associated with tea possibly in the Kangra valley are limited to morpho-physiological characteristics and biochemical tests. The acidic nature and poor soil nutrients in tea soils make them an excellent source of isolating stress-tolerant rhizobacteria which can survive under harsh conditions. Nutrient scarcity of tea rhizosphere acts as a catalyst for rhizobacteria to adapt and evolve mechanisms to survive and function under harsh conditions [37].

This research addresses this critical gap of lackof data on the microbial diversity, widespread and stress-tolerant PGPR genotypes with significant PGP potential in the Kangra tea plantations. This work has reported the molecular characterization and comprehensive screening for multiple PGP activities and abiotic stress tolerance of tea rhizobacteria from the Kangra valley. The identified stress-tolerant strains exhibited great potential of growth promotion in tea seedlings and suitable for adapting to varying environmental conditions.

Material and Methods

Soil sampling and isolation of rhizobacteria

Soil samples were collected from 7 major locations, including Baijnath, Banuri, Bir, Dharamshala, Gopalpur, Joginder Nagar, and Palampur, covering the major tea-growing regions in the Kangra valley (Fig. 1). The samples were air dried and analyzed for different physicochemical properties. Electrical conductivity (EC) and soil pH were measured with a soil:water suspension (1:2.5) using the CyberScan 510 and CC 601 pH Meter, respectively [24]. Available N was assessed by alkaline permanganate method [38], available P by sodium bicarbonate method [32], available K by flame photometric method [24], organic matter (OM) by the potassium dichromate method [48] and micronutrients were assessed by atomic absorption spectrophotometer [41]. Strains were isolated by serial dilution plating on TSA tryptone soya agar (TSA), Reasoner's 2 agar, and Rhizosphere isolation medium (Supplementary Table S3), followed by purification on TSA medium.

Diversity analysis and PGP attributes screening

Amplified ribosomal DNA restriction analysis (ARDRA)

The 16S rRNA gene amplification was performed using the primers 27F (5'- AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (3′-ACG GCT ACC TTG TTA CGA CGT −5′) primers [15]. A 25 µl PCR reaction mixture was prepared containing primers (50 µM each), dNTPs (200 µM), PCR buffer (1x), Taq DNA polymerase (3U), and 100 ng of genomic DNA template [45]. Following PCR amplification, the product was digested with restriction endonucleases, including *Alu*I, *Hinf*I, *Hpa*II, *Rsa*I, and *Taq*I. Reaction mixture included 10x restriction enzyme buffer (1.5 µl), 1 unit restriction enzyme, 10 µl PCR product, and water to make a total volume of 25 µl. The reaction was carried out at 37 °C for 2 h with *Alu*I, *Hinf*I, *Hpa*II, and *Rsa*I, and at 65 °C for 2 h with *Taq*I. The digested products were visualized on a 2% (w/v) agarose gel and a100-bp DNA ladder (MBI Fermentas, Vilinius) was used to estimate the band size.

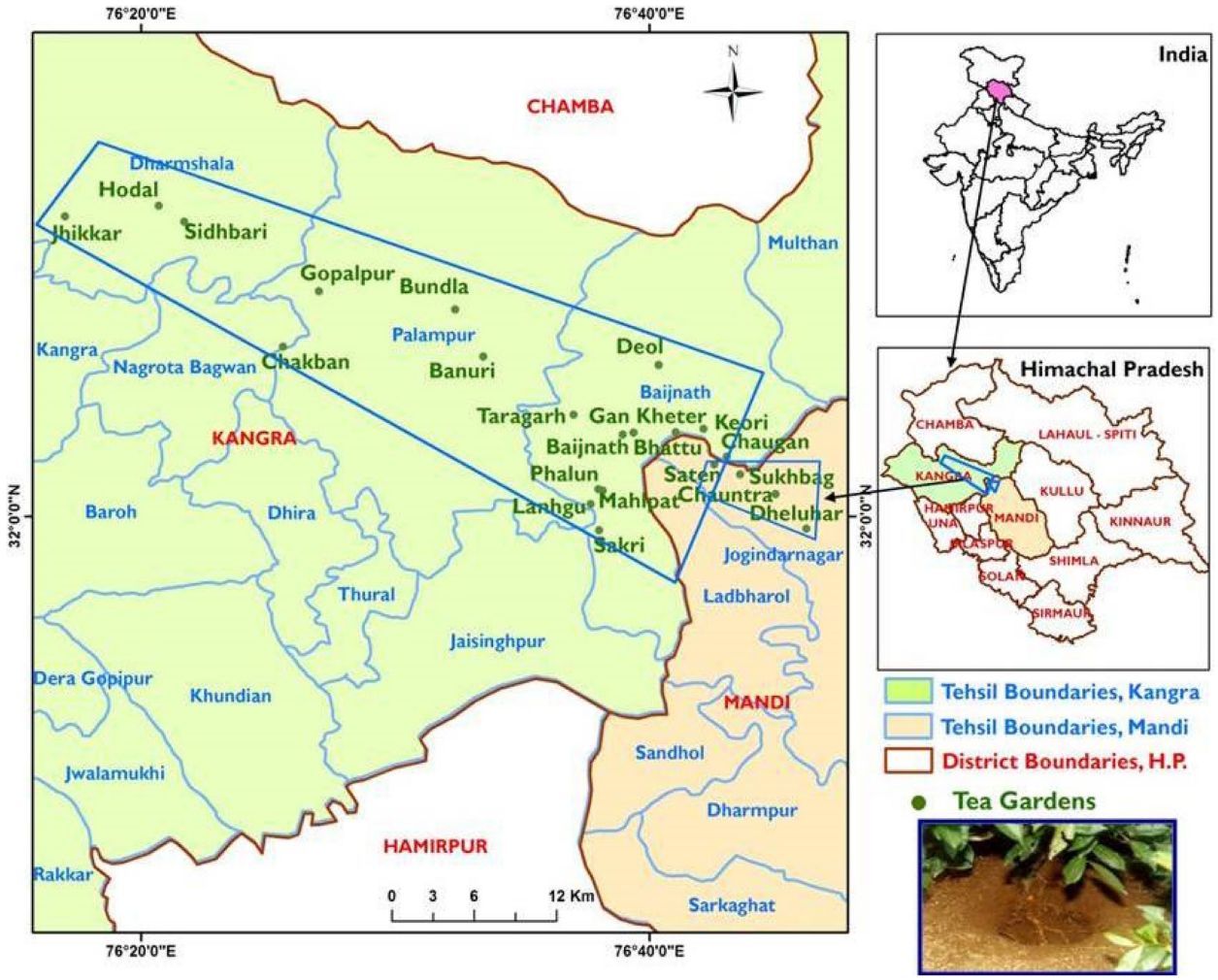

Fig. 1 Tea rhizosphere sampling locations in the Kangra valley

Enterobacterial repetitive intergenic consensus (ERIC-PCR)

A reaction mixture of 25 µl was prepared, comprising genomic DNA (50 ng), PCR buffer (1x), $MgCl_2$ (2.5 mM), dNTPs (2.5 mM), Taq DNA polymerase (1U) and primers (50 pmol each) ERICI (3'-CAC TTA GGG GTC CTC GAA TGT A-5') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') [51]. The thermocycling conditions started with an initial denaturation cycle at 95 °C for 7 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min. A final extension cycle at 65 °C for 16 min ensured complete amplification of the PCR products. The amplified ERIC-PCR products were visualized on a 2% agarose gel. Additionally, the evolutionary relationships among the bacterial strains and related taxa were assessed using MEGA 6.1 software. Carbon source utilization pattern was analyzed using the BIOLOG Microstation™ system. PAST software was utilized to determine the diversity indices. 16S rRNA gene sequencing was performed for the identification of the isolates [15].

Screening of rhizobacteria for multiple PGP attributes

The qualitative estimation of P-liberation, siderophore production, and ACC-deaminase activity PGP activities was performed on Pikovskaya's (PVK) agar, chrome azurol S (CAS), and DF salt minimal medium, respectively. For P-liberation estimation, cultures were grown in PVK broth supplemented with 0.5% TCP, Al-P and Fe–P at 28 ± 0.2 °C for 5 days. The P-liberation was estimated using Barton's reagent [52]. For siderophore production, CAS shuttle assay was performed as described by [1]. For IAA-like auxins estimation, cultures were grown in nutrient broth (NB) and Salkowski's reagent was used to estimate the IAA production [13]. ACC-deaminase activity was estimated in NB supplemented with ACC using 2,4-dinitrophenylhydrazine reagent [25].

Screening for tolerance against various abiotic stress parameters

Cultures were cultivated in 50 ml of tryptone soy broth (TSB) for 24 h at 28 °C and pH 7. The cultures were subjected to a various sets of abiotic stresses including pH (4 to 12), temperature (5 to 40 °C), NaCl (2.5, 5.0, 7.5, and 10.0%), polyethylene glycol (PEG) 6000 (10, 20, 30 and 40%) and $AlCl_3$ and $FeCl_3$ (2.5, 5, 7.5 and 10 mM). For each stress condition, the cultures were grown for 24 h, except for cultures exposed to 5 °C, 10% NaCl and 40% PEG 6000. Colonies were calculated as colony-forming units per milliliter (cfu/ml).

Growth promotion in maize and tea seedlings under controlled and nursery conditions

Inoculum were prepared by mixing 150 ml bacterial suspension with 300 g sterilized charcoal as a carrier to attain approximately 10^8 cfu/g. Pregerminated seeds of *Zea mays* var. Girija were surface sterilized using 2% sodium hypochlorite. These seeds were treated with a slurry prepared by mixing 300 g of a charcoal-based microbial inoculum (strains: IHB B 1045, IHB B 1059, IHB B 1702, IHB B 7048, and IHB B 7168) with 10% sterilized jaggery. Treated seeds was individually planted in plastic pots (15 cm diameter) filled with sterilized sand. Pots were followed randomized block design arrangement with 9 replicates under controlled conditions. The pots were irrigated periodically with sterilized water. Shoot length, root length, fresh weight and dry weights were recorded on the 30th day after sowing.

The experiment on tea seedlings was conducted at the State Agriculture Department of Himachal Pradesh Tea Plantation Nursery at Palampur. The seeds of Chinese variety Asha (HPKV1) were germinated in sand beds. Seeds treated with sterilized activated charcoal without inoculum served as a control. Seeds were inoculated at a depth of 4 cm in black poly-sleeves covered with 3 kg of soil, a mixture of coarse sand and tea garden soil (1:1 ratio, per sleeve). The tests were performed with 50 and 100% NPK, which is made up of 40 kg of single superphosphate (SSP), 40 kg of ammonium sulphate ($(NH_4)_2SO_4$), and 90 kg of muriate of potash (MOP). After one year of inoculation, we recorded data on growth parameters such as shoot height, leaf number, and plant biomass.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's test were performed to evaluate the significance differences among treatmenst. Principal component analysis (PCA) of growth promotion assay and carbon source utilization data were performed. All analysis were performed using SPSS data analysis software system version 28.0.1.

Results

Isolation of rhizobacteria

A total of 493 rhizobacteria were isolated from the 7 distinct locations of the Kangra valley. Colonies exhibiting distinct morphological characteristics on TSA were subcultured, purified, and preserved in 25% glycerol. The soil samples collected from these locations exhibited varying physicochemical properties: pH ranged from 4.9 to 5.8, EC varied between 0.1 and 0.3 mmhos/cm, OM content ranged from 2.7 to 7.2%, available N levels were between 301 to 450 kg/ha, available P concentrations ranged from 44.8 to 111.5 kg/ha, available K levels varied from 145.1 to 432.5 kg/ha (Supplementary Table S1).

Diversity analysis and PGP attributes screening

ARDRA generated 52 distinct rRNA types, grouped into 19 clusters with 2 to 22 isolates, and 33 independent branches at a 30% similarity level (Fig. 2). ERIC-PCR fingerprinting further revealed 56 ERIC types, organized into 31 groups with 2 to 15 isolates each, and 17 independent branches also at the 30% similarity level (Fig. 3). The diversity of rhizobacteria was assessed using the Shannon diversity index. Gopalpur exhibited the highest diversity ($H' = 1.511$), followed by Joginder Nagar ($H' = 1.368$), Palampur ($H' = 1.311$), Baijnath ($H' = 1.386$), Banuri ($H' = 0.876$), Dharamshala ($H' = 0.867$), and Bir ($H' = 0.752$). Additionally, the Margalef index for richness (R) and the Pielou index for evenness (E) of community structure were calculated for each location (Baijnath: R = 1.6, E = 0.6; Bir: R = 0.9, E = 0.5; Banuri: R = 0.7, E = 0.7; Joginder Nagar: R = 1.7, E = 0.7; Palampur: R = 1.3, E = 0.9; Gopalpur: R = 1.6, E = 0.8; Dharamshala: R = 0.6, E = 0.7). Phylogenetic analysis based on 16S rRNA gene sequencing clearly differentiated between gram-positive and gram-negative bacteria (Fig. 4). NCBI GenBank accession numbers were obtained for all 104 rhizobacterial strains (Supplementary Table S2).

16S rRNA gene sequencing of representative strains showed that *Bacillus*, with 15 species, was the most common and widespread genus. *Bacillus* was represented by species including *B. altitudinis*, *B. amyloliquefaciens*, *B. anthracis*, *B. aryabhattai*, *B. cereus*, *B. drentensis*, *B. megaterium*, *B. methylotropicus*, *B. mycoides*, *B. niacin*, *B. pseudomycoides*, *B. simplex*, *B. subtilis*, *B. thuringiensis*, and *B. velezensis*. *Bacillus* was followed by *Pseudomonas* with 8 species, *Burkholderia*, with 6 species, *Lysinibacillus* with 4 species, *Citrobacter* with 3 species, *Paenibacillus* with 3 species, and *Enterobacter* with 2 species. *Pseudomonas* species such as *P. frederiksbergensis*, *P. koreensis*, *P. laurylsulfativorans*, *P. mohnii*, *P. moorei*, *P. palleroniana*, *P. plecoglossicida* and *P. protegens*, and *Burkholderia* species such as *B. ambifaria*, *B. arboris*, *B.*

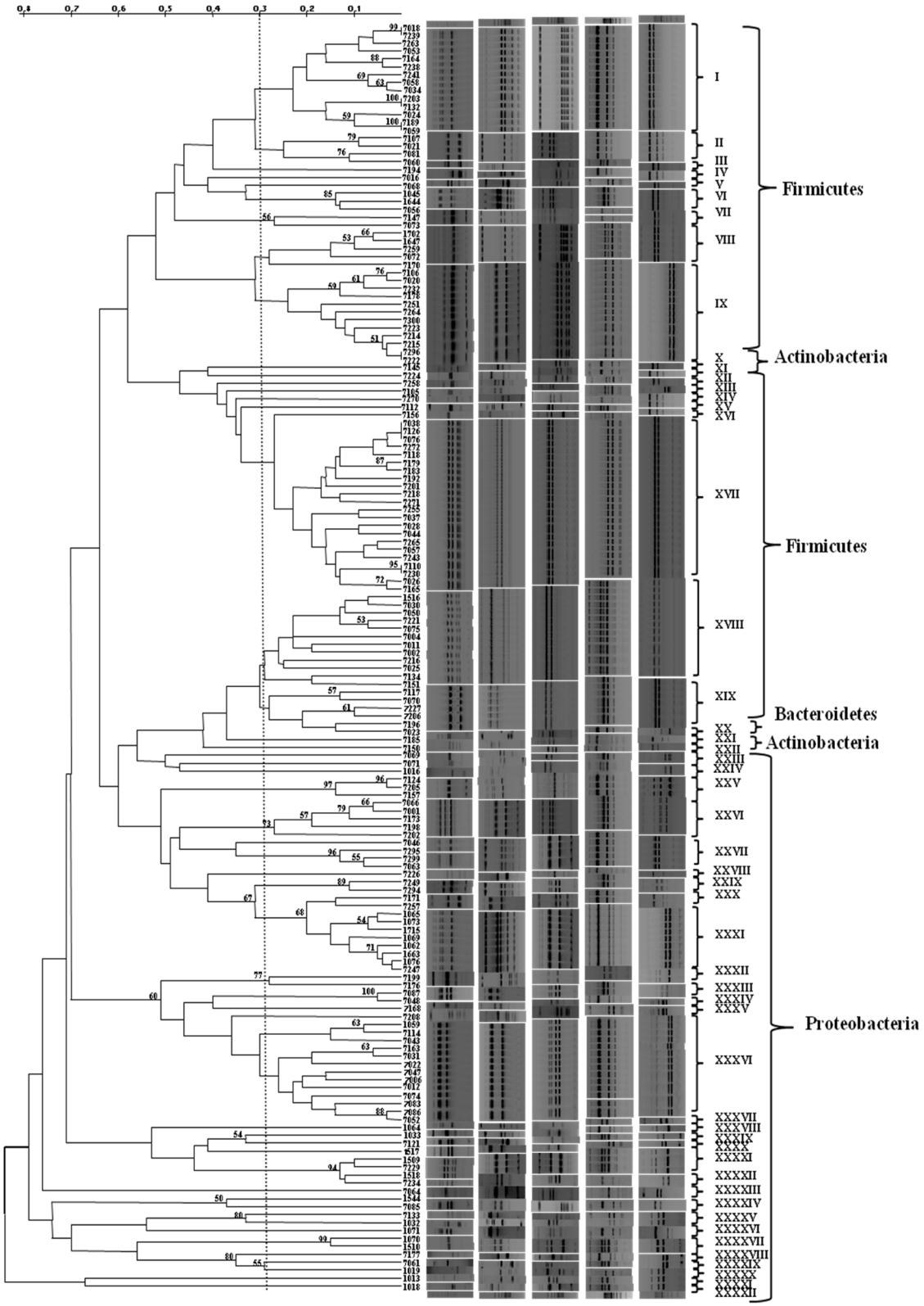

Fig. 2 UPGMA dendrogram based on ARDRA fingerprinting of tea rhizobacteria

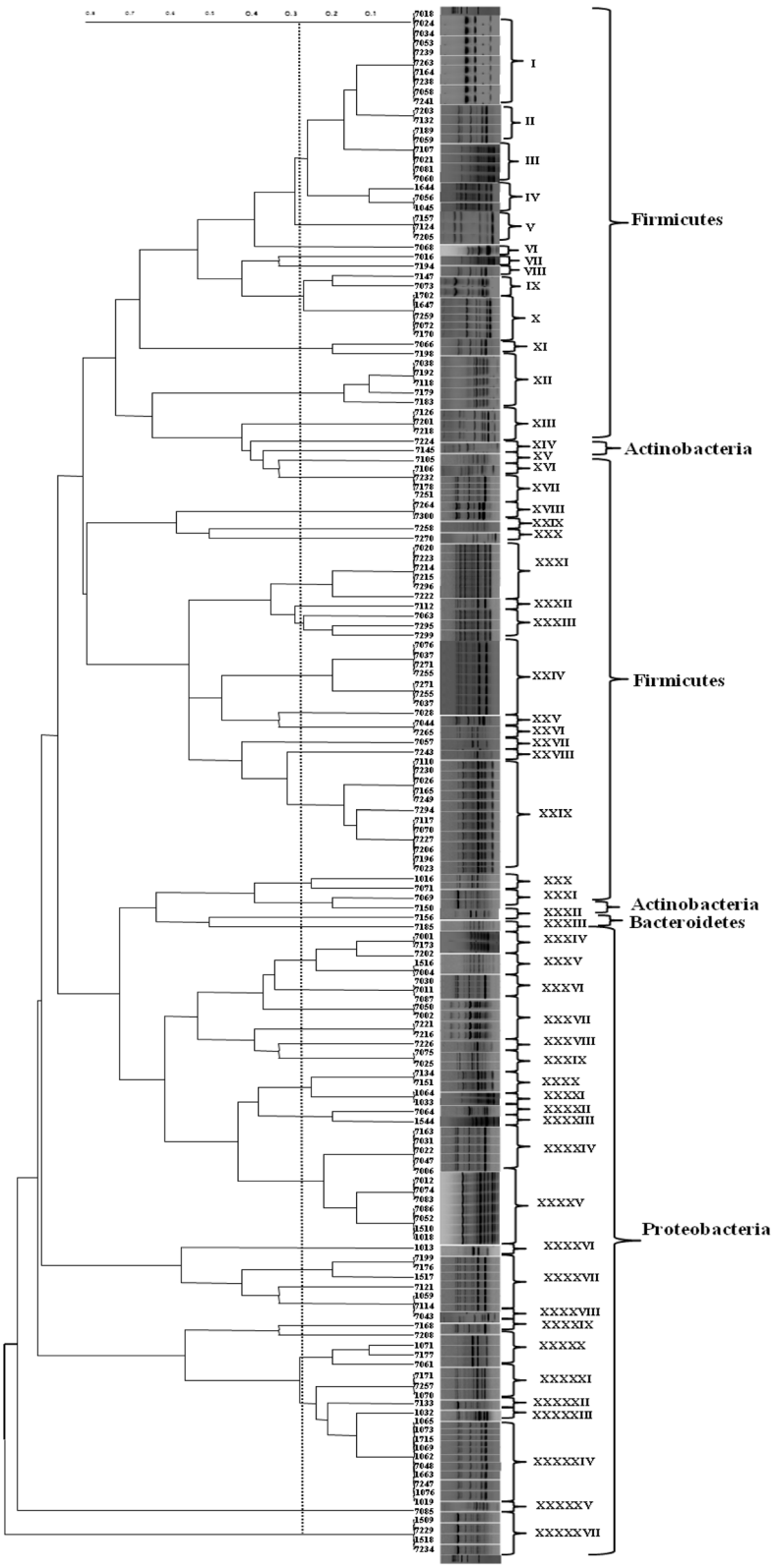

Fig. 3 UPGMA dendrogram based on ERIC-fingerprinting of tea rhizobacteria

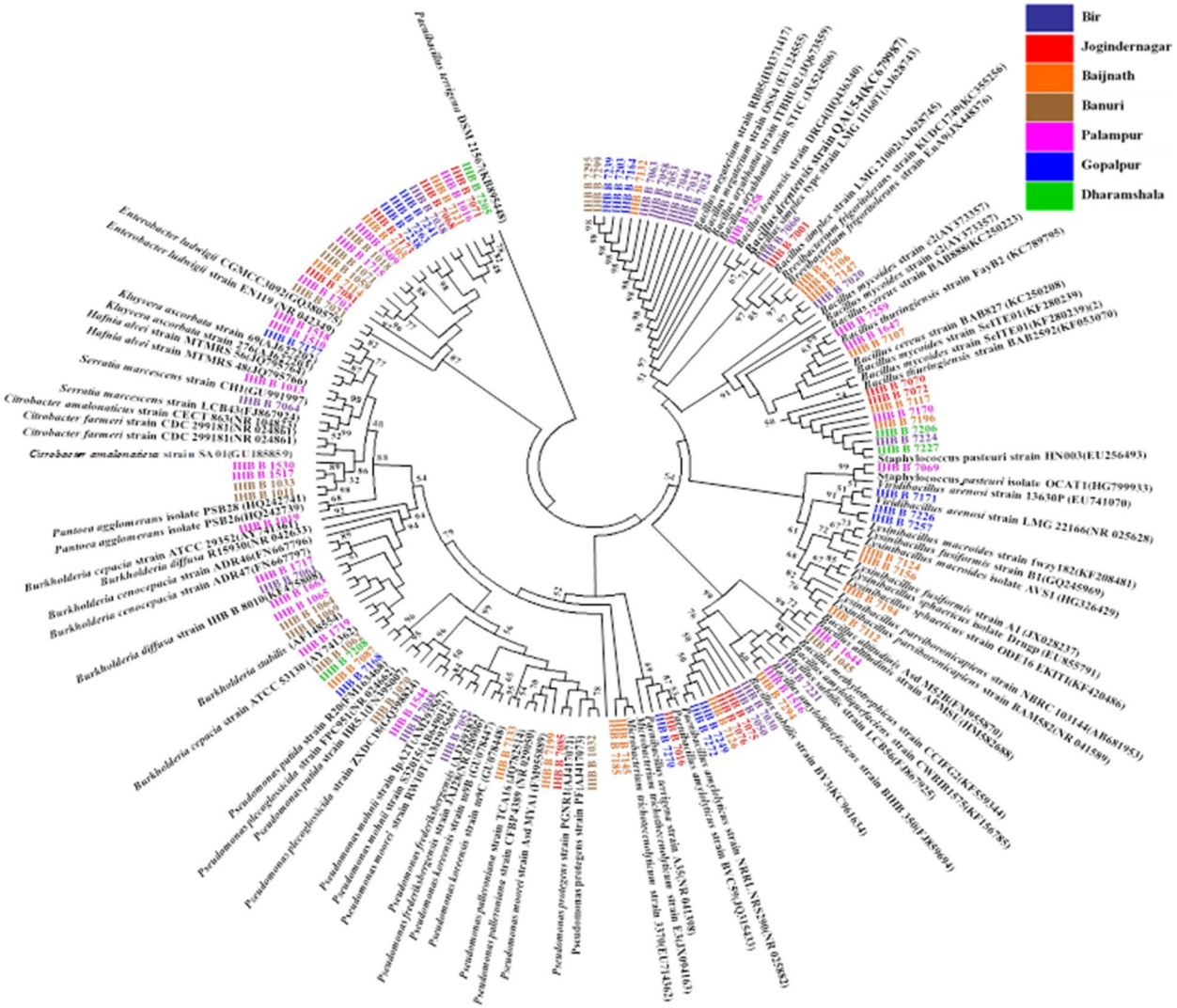

Fig. 4 Phylogenetic analysis based on 16S rRNA gene sequences of tea rhizobacterial isolates representing different rRNA types constructed by using the Neighbor-Joining method. The evolutionary distances computed using the Kimura 2-parameter method

cepacia, *B. diffusa*, *B. paludis*, and *B. stabilis* were also found in large number among tea rhizospheres.

PCA analysis of carbon source utilization patterns revealed significant variation, with PC1 explaining 11.31% and PC2 explaining 6.6% of the total variance (Fig. 5). This analysis also highlighted distinctions between gram-positive and gram-negative bacterial groups based on their metabolic profiles.

Screening for multiple PGP attributes

Among 493 isolates, 42 demonstrated multiple PGP activities (Tables 1 and 2). Quantification of 42 isolates revealed P-liberation ranging from 55–624 µg/ml. Among them, 9 strains showed the solubilization of Al-P and Fe–P, demonstrating P liberation levels ranging from 13 to 116 µg/ml and 15 to 96 µg/ml, respectively (Fig. 6). Siderophore production ranged from 2 to 83% siderophore units in succinate broth. Additionally, 42 rhizobacterial isolates showed the production of IAA-like auxins, ranging from 2 to 85 µg/ml in tryptophan-supplemented medium. Strains also showed enzyme activity levels ranging from 4 to 3145 nM α-ketobutyrate/h/mg/protein.

Growth under various abiotic stress parameters

Out of 11 isolates, 5 isolates showed a robust growth under various stress parameters. Optimal growth was observed between 20–30 °C, with a maximum of 10.72 $\log_{10}$ cfu/ml recorded within this range (Supplementary Fig S1). Growth varied across a wide pH range from 4 to 12. The highest growth (10.78 $\log_{10}$ cfu/ml) was recorded

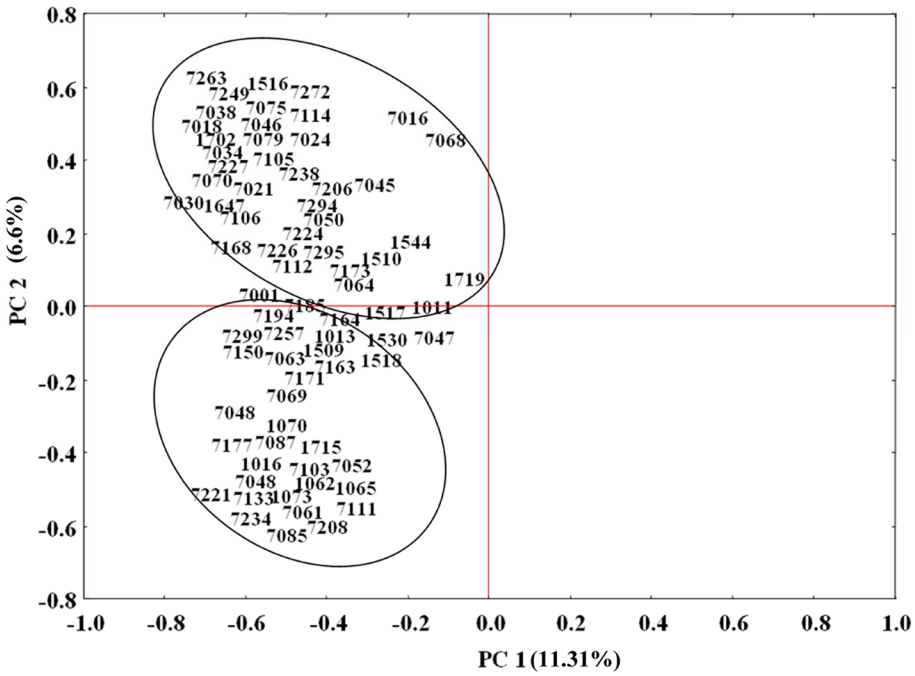

Fig. 5 Principal component analysis based on carbon source utilization of 104 tea rhizobacterial isolates with one or more PGP attributes

Table 1 Descriptive statistics of plant growth-promoting activities of 42 efficient tea rhizobacteria

Descriptive statistic	P-solubilization (µg/ml)			Siderophore (%)	IAA-like auxin production (µg/ml)	ACC-deaminase activity (nMα-ketobutyrate h/mg/protein)
	TCP	Al-P	Fe-P			
Min	55	13.20	15	2	2	4
Max	624	116	96	83	85	3145
Mean	304.3	75.8	49.6	16.57	32.40	1758.23
Std.error	23	10.3	7.3	2	4.0	143.07
Std.deviation	154.5	34.4	24.5	13.2	26.5	938.2
Median	261	80	48.2	12	21	2152.3
Variance	23889.4	1187.8	600.5	176.06	707.3	880266

at pH 7, 8 and 9. Strains exhibited varying degrees of growth under desiccation stress. Growth ranged from 6.24 to 10.57 $\log_{10}$ cfu/ml were recorded with 10% and 6.34 to 9.11 $\log_{10}$ cfu/ml with 20% PEG 6000. Increase in salinity levels (2.5 to 7.5% NaCl) affected culture growth, with growth ranging from 10.71 to 2.33 $\log_{10}$ cfu/ml. Cultures showed differential growth responses to metal ion stress.

Growth promotion in maize and tea seedlings under controlled nursery conditions

Five efficient PGPR strains were showed varying degrees of plant growth promotion (Table 3). Among these strains, *P. moreii* IHB B 7168 exhibited the highest level of plant growth promotion, statistically differing from *B. altitudinis* IHB B 1045, *B. cereus* IHB B 1702, *P. frederiksbergensis* IHB B 1059, and *P. mohnii* IHB B 7048 (Table 4). The combined application of 50% NPK + *B. altitudinis* IHB B 1045, 50% NPK + *B. cereus* IHB B 1702, 50% NPK + *P. frederiksbergensis* IHB B 1059, 50% NPK + *P. mohnii* IHB B 7048 and 50% NPK + *P. moreiii* IHB B 7168 resulted in significantly higher plant height, leaf number, fresh weight, and dry weight compared to individual treatment with 50% NPK alone.

In maize, PCA described 94.34% of the total variation, with PC1 hold for 81.98% and principal component

Table 2 PGPR activities of 42 efficient isolates and characterization based on 16S rRNA gene sequencing

S. no.	Isolate	P-solubilization (µg/ml)	Siderophore (%)	IAA-like-auxins (µg/ml)	ACC-deaminase activity nM α-ketobutyrate h^{-1} mg $protein^{-1}$	Closely related species in NCBI GenBank
1	IHB B 1644	420^{q}±0.3	10^{h}±0.08	46^{t}±0.3	2251^{f}±0.3	*Bacillus altitudinis* 41KF2b
2	IHB B 1045	624^{w}±0.3	18^{n}±0.03	30^{p}±0.3	1360^{c}±0.03	*Bacillus altitudinis* FM955870
3	IHB B 7239	420^{q}±0.3	23^{r}±0.03	5^{c}±0.1	1453^{c}±0.06	*Bacillus aryabhattai* B8W22
4	IHB B 7079	117^{b}±0.8	83^{aa}±0.06	22^{l}±0.1	2426^{g}±0.1	*Bacillus aryabhattai* strain MDSR14
5	IHB B 1647	252^{k}±0.4	20^{p}±0.08	2^{a}±0.05	2051^{e}±0.1	*Bacillus cereus* AY138271
6	IHB B 1516	442^{r}±0.2	3^{b}±0.2	5^{c}±0.03	23^{a}±0.06	*Bacillus subtilis* EF428238
7	IHB B 7075	113^{b}±0.3	6^{e}±0.05	15^{g}±0.1	2303^{g}±0.2	*Bacillus subtilis* subsp. *inaquosorum* S6I18
8	IHB B 7070	393^{p}±0.3	21^{q}±0.05	85^{ae}±0.05	2958^{j}±0.08	*Bacillus thuringiensis* strain IAM 12077
9	IHB B 1702	416^{q}±0.5	32^{x}±0.03	26^{n}±0.1	189^{a}±0.05	*Bacillus cereus* strain BCT-7112
10	IHB B 7126	335^{m}±0.3	11^{i}±0.1	21^{k}±0.08	302^{a}±0.03	*Bacillus velezensis* strain FZB42
11	IHB B 7103	130^{c}±0.6	9^{g}±0.03	58^{x}±0.06	2406^{g}±0.08	*Brevibacterium frigoritolerans* DSM 8801
12	IHB B 7111	140^{d}±0.3	10^{h}±0.03	83^{ad}±0.08	3145^{l}±0.1	*Burkholderia ambifaria* MC40-6
13	IHB B 1069	194^{h}±0.6	27^{u}±0.1	14^{f}±0.06	2928^{i}±5.3	*Burkholderia ambifaria* strain AMMD
14	IHB B 1073	251^{k}±0.9	33^{y}±0.05	14^{f}±0.03	2563^{h}±0.08	*Burkholderia ambifaria* strain AMMD
15	IHB B 1064	575^{v}±0.9	28^{v}±0.1	11^{e}±0.06	982^{b}±0.03	*Burkholderia arboris* R-24201
16	IHB B 1715	148^{e}±0.6	21^{q}±0.1	5^{c}±0.03	1737^{d}±0.1	*Burkholderia cepacia* AF097533
17	IHB B 7061	183^{h}±0.6	13^{k}±0.08	43^{s}±0.03	2205^{f}±0.3	*Burkholderia diffusa* R-15930$^{(T)}$
18	IHB B 1062	120^{b}±0.6	35^{z}±0.06	5^{c}±0.03	2915^{i}±0.08	*Burkholderia stabilis* AF148554
19	IHB B 1719	261^{k}±0.3	17^{m}±0.03	15^{g}±0.06	1564^{c}±0.0	*Burkholderia stabilis* strain LMG 14294
20	IHB B 1517	205^{i}±0.3	2^{a}±0.03	21^{k}±0.08	11^{a}±0.33	*Citrobacter amalonaticus* strain SA01
21	IHB B 1033	570^{v}±0.3	23^{r}±0.06	4^{b}±0.05	2348^{g}±0.08	*Citrobacter farmeri* CDC 2991-81
22	IHB B 1011	442^{r}±0.3	11^{i}±0.1	51^{v}±0.06	2204^{f}±0.0	*Citrobacter telavivensis* strain 6105
23	IHB B 1019	55^{a}±0.1	17^{m}±0.1	14^{f}±0.06	1242^{c}±0.3	*Cupriavidus basilensis* strain T103
24	IHB B 1510	342^{n}±0.2	12^{j}±0.06	85^{ae}±0.05	2990^{k}±0.06	*Enterobacter asburiae* HQ455820
25	IHB B 1070	166^{g}±0.1	26^{t}±0.05	28^{o}±0.08	$1323^{cv0.33}$	*Enterobacter asburiae* JCM6051
26	IHB B 1518	292^{l}±0.2	11^{i}±0.0	21^{k}±0.08	1149^{b}±0.1	*Enterobacter ludwigii strain* K9
27	IHB B 7106	323^{m}±0.3	10^{h}±0.08	78^{ab}±0.07	571^{a}±0.06	*Enterobacter ludwigii* strain K9 EF175735
28	IHB B 1013	233^{j}±0.3	16^{l}±0.03	75^{aa}±0.03	1210^{b}±0.3	*Hafnia alvei* M59155
29	IHB B 7177	454^{t}±0.3	25^{s}±0.08	16^{h}±0.06	320^{a}±0.1	*Kluyvera ascorbata* CDC 0648-74$^{(T)}$
30	IHB B 1016	166^{g}±0.3	6^{e}±0.03	11^{e}±0.1	2410^{g}±0.06	*Lysinibacillus sphaericus* CP000817
31	IHB B 1018	441^{r}±0.3	6^{e}±0.08	49^{u}±0.1	1426^{c}±0.1	*Pantoea aqqlomerans* strain DSM 3493
32	IHB B 1059	539^{u}±0.3	21^{q}±0.05	25^{m}±0.06	2251^{f}±0.1	*Pseudomonas frederiksbergensis* PSB37
33	IHB B 1065	168^{g}±0.3	31^{w}±0.08	7^{d}±0.03	2152^{l}±0.03	*Pseudomonas frederiksbergensis* PSB37
34	IHB B 7114	114^{b}±0.2	8^{f}±0.05	81^{ac}±0.03	2622^{h}±0.04	*Pseudomonas frederiksbergensis* strain M60
35	IHB B 7048	443^{r}±0.1	11^{i}±0.1	55^{w}±0.03	4^{a}±0.01	*Pseudomonas mohnii* Ipa-2$^{(T)}$
36	IHB B 7168	344^{n}±0.2	18^{n}±0.03	42^{r}±0.03	2387^{g}±0.5	*Pseudomonas moorei* RW10$^{(T)}$
37	IHB B 7133	467^{s}±0.3	11^{i}±0.05	66^{y}±0.03	1251^{c}±0.6	*Pseudomonas palleroniana* CFBP 4389$^{(T)}$
38	IHB B 1544	452^{r}±0.1	12^{j}±0.0	74^{z}±0.1	2711^{h}±0.8	*Pseudomonas plecoglossicida* FPC951
39	IHB B 1032	368^{o}±0.2	19^{o}±0.07	18^{l}±0.03	2234^{f}±1.2	*Pseudomonas protegens* AJ417073
40	IHB B 1071	154^{f}±0.2	5^{d}±0.06	5^{c}±0.03	1020^{b}±0.2	*Pseudomonas laurylsulfativorans* AP3_22
41	IHB B 7234	456^{t}±0.5	6^{e}±0.06	36^{q}±0.0	2828^{i}±0.8	*Pseudomonas tolaasii* strain LMG 12215
42	IHB B 7064	157^{f}±0.4	4^{c}±0.07	20^{j}±0.05	2210^{f}±0.4	*Serratia marcescens* strain MH6

Values are the mean of three replicates ± standard error of mean. Values with same letters were not significantly different from each other in each column-based on duncan's post hoc test at $p \leq 0.01$.

2 for 12.36% of the variance (Fig. 7). These results indicated that PGPR treatment were positively associated with growth parameters while control treatment was negatively associated with all growth parameters. For tea

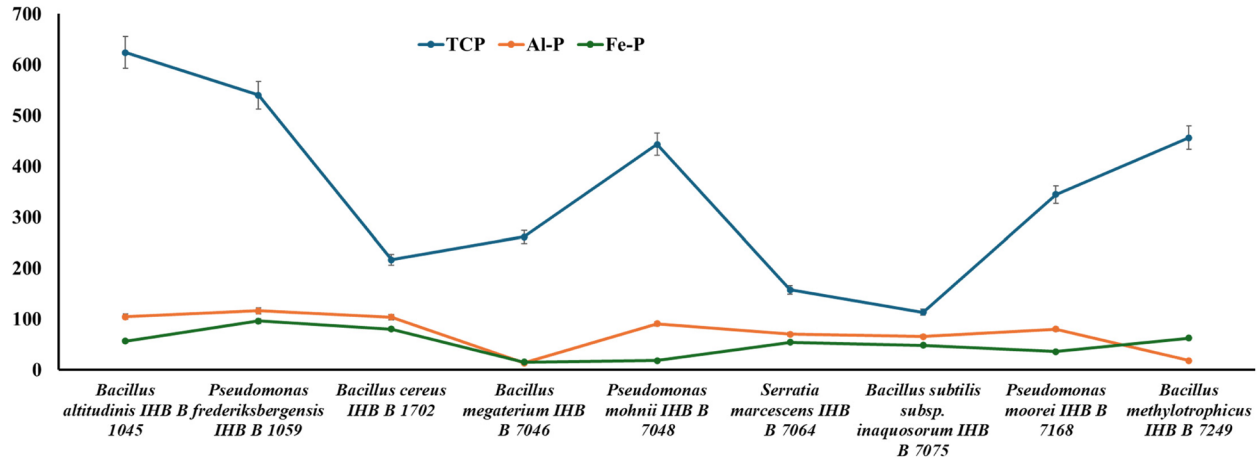

Fig. 6 Tricalcium phosphate (TCP), aluminium phosphate (AL-P) and iron phosphate (Fe-P) solubilization by 9 efficient PGPR

Table 3 Growth promotion by 5 efficient PGPR in maize under controlled environment

Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Control	55.3^{a}±0.08	12.3^{a}±0.05	2.3^{a}±0.1	0.4^{a}±0.03
IHB B 1045	60.7^{c}±0.06	14.7^{b}±0.1	3.3^{c}±0.05	0.7^{c}±0.00
IHB B 1059	60.7^{c}±0.03	17.7^{d}±0.2	3.0^{b}±0.05	0.6^{b}±0.03
IHB B 1702	60.0^{b}±0.03	15.1^{c}±0.1	3.7^{d}±0.05	0.7^{c}±0.0
IHB B 7048	61.3^{d}±0.05	15.3^{c}±0.1	3.1^{b}±0.05	0.6^{b}±0.0
IHB B 7168	60.7^{c}±0.01	17.7^{d}±0.2	3.7^{d}±0.02	0.7^{c}±0.03

Values are the mean of nine replicates±standard error of mean. Values with same letters were not significantly different from each other in each column-based on duncan's post hoc test at $p \leq 0.01$. IHB B 1045= *Bacillus altitudinis*; IHB B 1702= *B.cereus*, IHB B 1059= *Pseudomonas frederiksbergensis*, IHB B 7048= *P. mohnii*, and IHB B 7168= *P. moreii*

seedlings, PC 1 account for 87.45% and PC 2 for 9.30% of the variance (Fig. 8).

Discussion

The present study provides insights into the widespread and dominant genotypes of tea rhizobacteria possessing multiple PGP attributes and growth enhancement under controlled and nursery conditions. To best of our knowledge, this is a first comprehensive report on the molecular characterization and diversity analysis of tea rhizobacteria from the Kangra valley. Tea rhizosphere has a unique combination of environmental challenges such as acidification and poor nutrient content [30] which can serve as a natural selector for the selection of stress tolerant rhizobacteria. Such

Table 4 Growth promotion by 5 efficient PGPR in tea seedlings under nursery conditions

Treatment	Plant height (cm)	Leaf number	Fresh weight (g)	Dry weight (g)
Control	22.2^{a}±0.01	16^{a}±0.2	10.2^{a}±0.01	0.28^{a}
IHB B 1045	23.3^{b}±0.01	18^{b}±0.01	12.1^{b}±0.02	0.34^{b}
IHB B 1059	23.3^{b}±0.01	18^{b}±0.02	13.1^{c}±0.03	0.36^{b}
IHB B 1702	23.2^{b}±0.02	18^{b}±0.1	13.2^{c}±0.02	0.35^{b}
IHB B 7048	23.6^{b}±0.1	18^{b}±0.05	13.2^{c}±0.01	0.35^{b}
IHB B 7168	23.1^{b}±0.2	18^{b}±0.06	13.3^{d}±0.02	0.37^{c}
50% NPK	24.4^{b}±0.3	18^{c}±0.01	14.2^{e}±0.03	0.39^{d}
50% NPK+IHB B 1045	24.9^{d}±0.02	19^{d}±0.02	16.2^{f}±0.01	0.39^{d}
50% NPK+IHB B 1059	24.7^{c}±0.02	19^{d}±0.02	16.4^{f}±0.02	0.39^{d}
50% NPK+IHB B 1702	24.2^{c}±0.01	20^{e}±0.01	17.1^{h}±0.01	0.41^{e}
50% NPK+IHB B 7048	23.3^{b}±0.02	21^{e}±0.05	16.2^{g}±0.02	0.42^{f}
50% NPK+IHB B 7168	24.3^{b}±0.02	20^{f}±0.02	17.2^{h}±0.02	0.41^{e}
100% NPK	25.5^{d}±0.02	21^{g}±0.02	17.4^{h}±0.01	0.44^{f}

Values are the mean of nine replicates±standard error of mean. Values with same letters were not significantly different from each other in each column-based on duncan's post hoc test at $p \leq 0.01$. IHB B 1045= *Bacillus altitudinis*; IHB B 1702= *B.cereus*, IHB B 1059= *Pseudomonas frederiksbergensis*, IHB B 7048= *P. mohnii*, and IHB B 7168= *P. moreii*.

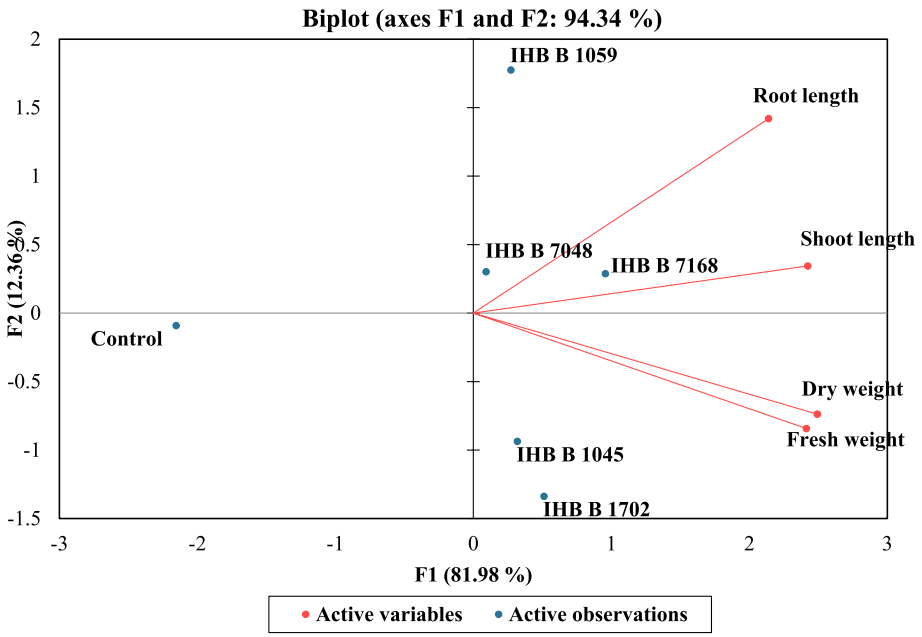

Fig. 7 Principal component analysis of growth promotion of 5 efficient strains including *Bacillus altitudinis* IHB B 1045, *B. cereus* IHB B 1702, *Pseudomonas frederiksbergensis* IHB B 1059, *P. mohnii* IHB B 7048, and *P. moreii* IHB B 7168 in maize under controlled conditions

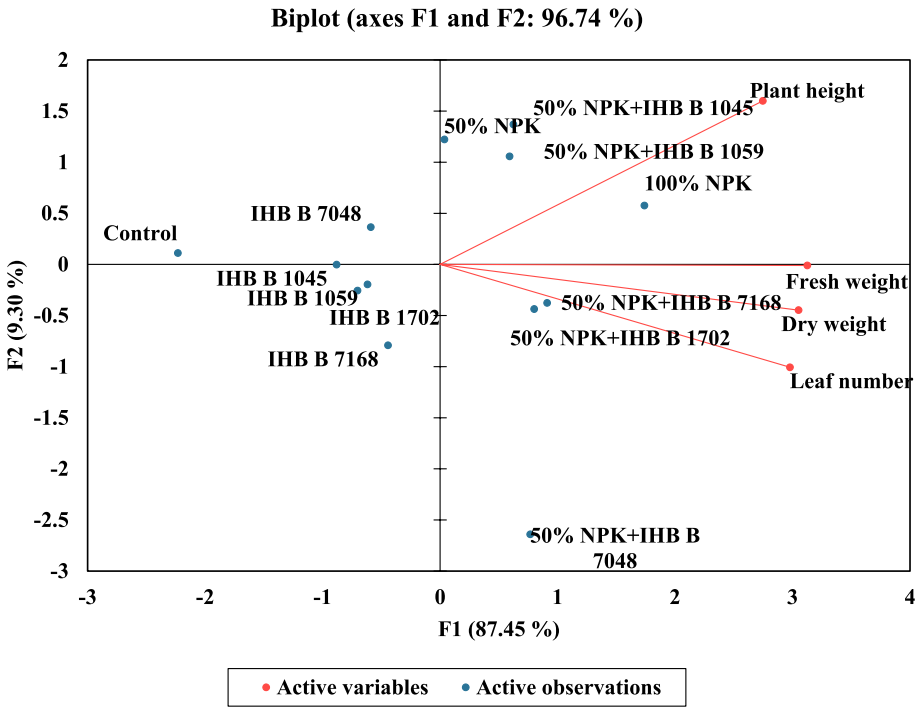

Fig. 8 Principal component analysis of growth promotion of 5 efficient strains including *Bacillus altitudinis* IHB B 1045, *B. cereus* IHB B 1702, *Pseudomonas frederiksbergensis* IHB B 1059, *P. mohnii* IHB B 7048, and *P. moreii* IHB B 7168 in tea seedlings under nursery condition

stressful conditions can act as a catalyst for the occurrence of resilient microbes with advanced mechanisms to thrive in such harsh conditions. Soil acidification significantly decreases the diversity and functionality of rhizobacteria [14]. In this study, soil pH ranged from 4.9 to 5.8 and was significantly different between the locations. Highest diversity index was observed in Gopalpur followed by Joginder Nagar, Palampur, Baijnath, Banuri,

Dharamshala and Bir. Several factors such as soil type, OM content, availability of essential nutrients like N, P, and K, and other applied amendments and fertilizers could influence the distribution and abundance of rhizobacteria in soil [28, 42, 54].

Fertilizer application, leaf litter, root exudates and continual cropping of tea may also affect microbial composition [3, 16]. The OM content varied between 2.7 and 7.2%, maximum at Joginder Nagar and minimum at Banuri. However, high OM (7.2%) probably was one of the reasons for its higher diversity than those observed from other locations. Long-term application of fertilizers might change organic matter content and potentially lead to different microbial compositions in the soil [23]. We have not observed any correlation between soil available N, P, and K levels and the diversity index. Similar trends are observed in Northeastern Indian tea soils, which are also acidic, have a high OM content and moderate to high availability of N, P, and K, much like the conditions we observed in the Kangra valley [6].

A total of 108 genotypes were generated by ARDRA and ERIC cluster analyses. Firmicutes were the dominant phylum, followed by Proteobacteria, Actinobacteria and Bacteroides. Several studies on tea rhizobacterial diversity consistently showed the dominance of *Bacillus* species such as *B. cereus*, *B. megaterium*, *B. mycoides*, and *B. subtilis* [7, 35, 49]. A study conducted in Darjeeling also showed the dominance of *Bacillus* species followed by *Staphylococcus*, *Ochrobactrum*, *Pseudomonas*, *Lysinibacillus*, *Micrococcus*, *Leifsonia*, *Exiguobacterium*, and *Arthrobacter* [5]. Dominance of the genus *Bacillus* supports the notion of selective buildup of bacterial populations influenced by tea rhizosphere effect [35]. *Bacillus* is known for their ability to survive under extreme conditions, rapid growth, endospore formation and excellent competitive colonization potential that makes them a suitable candidate for microbial bioformulations [22]. Nevertheless, there are innumerable influences that might affect the occurrence and predominance of *Bacillus*. Time to collect the soil samples and method of isolation could influence the occurrence of the rhizobacteria. In general, *Bacillus* dominates the rhizosphere of many crops like wheat, rice, and maize. [2, 31, 50, 56].

Besides *Bacillus*, *Pseudomonas* and *Burkholderia* were found to be dominant. Similar prevalences of *Burkholderia* and *Pseudomonas* species have been recorded from the tea rhizosphere of other regions such as Turkey and Assam [7, 11]. Only one or two locations in the Kangra valley showed the presence of genera *Acetobacter*, *Chryseobacterium*, *Cupriavidus*, *Hafnia*, *Kluyvera*, *Microbacterium*, *Pantoea*, *Serratia*, and *Staphylococcus*. Reports from Turkish tea rhizosphere also showed that some genera such as *Achromobacter*, *Paenibacillus* and *Stenotrophomonas* are not widespread [7]. It is important to note that the findings of this study are restricted to culturable methadology. A comprehensive approach that includes both culturable and nonculturable is required to capture the complete microbial diversity of this region.

One of the key challenges in tea cultivation lies in the presence of insoluble forms of Al, Fe, and Ca phosphates) in the soil [36]. P availability is low in acid soils as a large amount of P is fixed by metal ions. PSB can secrete organic acids such as citric acid, oxalic acid, lactic acid gluconic and acetic that could solubilize inorganic P [29]. PSB also excretes several enzymes like phosphatase, phytases and C-P lyases to mineralisee P [34]. However, majority of the previous studies have not thoroughly explored PGPR ability to solubilize Al-P and Fe–P. A few strains such as *Priestia megaterium* and *Viridibacillus arenosi* are reported to solubilize Ca, Fe and Al phosphates under controlled conditions [45, 47]. In this study, most of the isolates that were able to solubilize TCP belonged to *Bacillus*, followed by *Pseudomonas*, *Burkholderia*, and *Enterobacter*. In addition to TCP solubilization, these strains also demonstrated Fe–P and Al-P solubilizing abilities. The ability to solubilize different inorganic P suggests their potential applicability in both acidic soils rich in Al and Fe-bound phosphates and alkaline soils rich in Ca-bound phosphates.

Strains exhibited IAA-like auxins production ranging from 2 to 85 µg/ml. Several studies on tea rhizobacterial isolates reported IAA production levels of 22 µg/ml by *B. megaterium*, 17.5 µg/ml by *Kurthia* sp., 92.5 µg/ml by *E. lignolyticus*, and 31.47 µg/ml by *B. altitudinis* [8, 11, 40]. Our isolates also exhibited siderophore production ranging from 2 to 83%. Rhizobacteria control plant pathogens in an iron limited conditions through secretion of siderophores [10]. Species of *Burkholderia*, *Enterobacter* and *Pseudomonas* isolated from Assam tea estates also exhibited the siderophore production which ranges between 32–39% units [11]. In our study strains were also found to be positive for ACC-deaminase activity which aids lowering of stress-induced plant hormone ethylene level and provide resistance against different abiotic stresses like drought, salinity and flooding [33]. Also, strains ability to utilize diverse carbon sources such as different acids and salts, suggesting their capability to survive under acidic conditions. These traits would also seem likely to be an important asset in communities of microbes that reside within acidic soils. Also, their abilities to grow in high temperature, desiccation and salinity levels make these strains suitable for all the growing seasons from different agricultural settings.

Under controlled and nursery conditions, *P. moreii* IHB B 7168 exhibited the highest level of PGP, statistically differing from *B. altitudinis* IHB B 1045, *B. cereus* IHB B

1702, *P. frederiksbergensis* IHB B 1059, and *P. mohnii* IHB B 7048 exhibited great potential for growth promotion. Researchers have previously conducted several studies on the impact of PGPR on promoting growth in tea and other crops under controlled environment. Thirty tea rhizobacterial isolates from Darjeeling, India, demonstrated growth promotion in rice and maize seedlings, indicating their potential use as inoculants for microbe-based bio-formulations [5]. Another study from Darjeeling found that applying PGPR consortia to tea nursery plants significantly improved growth under nursery conditions [12]. Four indigenous strains including *Enterobacter lignolyticus*, *Burkholderia* sp., *Bacillus pseudomycoides*, and *Pseudomonas aeruginosa* isolated from tea rhizosphere showed their potential of be used as microbial inoculation for growth promotion of tea [11]. A Chinese study showed the potential of salt tolerant and ACC deaminase-producing tea rhizobacterium *Burkholderia pyrrocinia* in peanut seedling growth under saline conditions [20].

Our selected strains showed a significant increase in the growth of tea seedlings with 50% NPK. These enhanced attributes in terms of plant height, leaf number and weight recorded under acidic soil conditions with lower NPK dosages indicate that the strains could serve as significant microbial inoculants with a useful implication for growth promotion in tea cultivation.

Conclusion

This is a first-ever report on the genetic and functional diversity of tea rhizobacteria in Kangra valley. This study can serve as a solid foundation for the subsequent research and field application of PGPR-based approaches in tea. The observed genetic and functional diversity, multiple PGP attributes, resilience against various abiotic factors, and growth promotion in maize and tea seedlings highlight the possibility of their use in acidic as well as alkaline soils and under different climatic conditions [19, 44]. Selected PGPR strains have great potential for improving tea cultivation not only in the Kangra valley, but also in other agroclimatic conditions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03811-0.

Supplementary Material 1.

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Authors' contributions
RT: Conducted lab and field experiments, wrote the original manuscript; PR: Supervision; Ashu Gulati: Resources, Supervision; Arvind Gulati: Conceptualization, Supervision, Resources, Manuscript editing.

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Data availability
The accession numbers for the 16S rRNA gene sequences of rhizobacteria are provided in Supplementary Table S2.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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