



Plant Growth-Promoting Rhizobacteria of *Curcuma longa* L. and Their Impact on its Growth

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Abstract Plant growth-promoting rhizobacteria are known to have stimulating effects on plant growth and have become an emerging field of research in sustainable agriculture. The present study aims to select potent strains with a diverse set of in vitro plant growth-promoting (PGP) traits from the rhizosphere of *Curcuma longa* L cv. Lakadong and evaluates their growth-promoting ability in greenhouse experiment. A total of 33 isolates were obtained, purified, and subjected to preliminary in vitro screening for the production of IAA, ammonia, siderophore and phosphate solubilization. The isolates were further evaluated quantitatively for the production of IAA, solubilized phosphate, ammonia, and siderophore. Molecular identification of four selected isolates (BS1, BS4, IJ2, and IJ10) which showed promising in vitro PGP activity was done based on 16S rRNA gene analysis and their plant growth promotion was evaluated in greenhouse conditions. The isolates produced 2.67–52.88 $\mu\text{g ml}^{-1}$ IAA, 31.56–320.50 $\mu\text{g ml}^{-1}$ solubilized phosphate, 0.52–2.07 $\mu\text{mol ml}^{-1}$ ammonia, and 16.99–43.30% of siderophore. The partial 16S rRNA sequence analysis identified the selected isolates as *Arthrobacter* sp. BS1, *Pseudarthrobacter chlorophenolicus* BS4, *Pseudomonas* sp. IJ2, and *Bacillus subtilis* IJ10. All the plants inoculated

with *Pseudomonas* sp. IJ2 and *B. subtilis* IJ10 showed a significant difference at $p \leq 0.05$ in all the growth parameters. These isolates, therefore, may provide a viable alternative to chemical inputs for improved growth and yield of *C. longa*.

Keywords *Bacillus subtilis* IJ10 · *Curcuma longa* L. · In vitro · Plant growth-promoting rhizobacteria · *Pseudomonas* IJ2 · 16S rRNA

Introduction

Turmeric (*Curcuma longa* L.), a rhizomatous perennial herb belonging to the family Zingiberaceae is widely grown in India. Because of its high curcumin content (6.8–7.3%) [1], Lakadong may be considered as one of India's finest varieties of turmeric. In addition to flavoring food, it is also used in medicines, cosmetics and as a dyeing agent [2]. Aggarwal and Sung [3] reported that curcumin has antioxidant, antimicrobial, anti-inflammatory, and anti-mutagenic properties.

The use of plant growth-promoting rhizobacteria (PGPR) covers a small yet growing niche in organic farming and is expected to increase in the future [4]. PGPR may induce plant growth and yield by either direct or indirect modes of action [5]. Direct mechanisms include the production of phytohormones, fixation of nitrogen, solubilization of inorganic phosphate, production of siderophores, regulating ethylene concentration, etc. [6], and indirect modes include the production of antibiotics, hydrogen cyanide, lytic enzymes such as chitinase, anti-fungal metabolites, competition, and systemic resistance induction [7].

Significance Statement The study found that *Pseudomonas* sp. IJ2 and *B. subtilis* IJ10 were able to significantly enhance the growth of *C. longa*. These isolates, therefore, may provide a viable alternative to chemical inputs for improved growth and yield of *C. longa*.

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Chemical fertilizers have been extensively used for many years and are detrimental to soil and human health, impacting soil fertility and altering the microbial population [8]. As an alternative solution to these chemicals, different agricultural practices have employed new biotechnological methods, which not only increase crop production but also preserve soil quality [9]. The use of PGPR as bioinoculants would be an ideal alternative for minimizing the indiscriminate use of chemical fertilizers [10].

The present study aims to select potent strains with a diverse set of *in vitro* plant growth-promoting (PGP) traits from the rhizosphere of *C. longa* and tests them under greenhouse conditions for their growth-promoting ability toward the crop.

Material and Methods

Sample Collection and Isolation of Rhizobacteria

Rhizospheric soil sample of *C. longa* cv. Lakadong was collected from Shangpung village, West Jaintia Hills District of Meghalaya (25°18' N latitudes and 92°22' E longitudes). Briefly, the soil attaching the root system was removed by shaking and subsequent brushing of the remaining soil particles in autoclaved plastic bags. The soil sample was then brought to the laboratory, labeled, kept at 4 °C in the refrigerator, and processed within 48 h after collection.

Rhizobacteria were isolated by serial dilution technique [11]. For isolation, 1 g of soil was transferred to a culture tube containing 10 ml of sterile distilled water. The tube was vortexed for 5 min. Dilutions of up to 10⁻⁶ grades were made. Hundred microliters each of 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilution was spread on nutrient agar plates and incubated at 30 °C for 24–48 h. Bacterial isolates with different morphological appearances were purified, maintained and stored for further studies.

In vitro Evaluation of PGP Traits

Indole Acetic Acid (IAA) Production

The production of IAA by bacterial isolates was determined using Salkowski's reagent based spectrophotometric method [12]. Bacterial isolates were grown in Luria Bertani broth supplemented with Tryptophan (100 µg ml⁻¹) at 30 ± 2 °C for 72 h. Culture supernatants from each of these isolates were mixed with Salkowski's reagent (49 ml of 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution) in the ratio of 1:2. The pink color development indicates positive production of IAA and its optical density was

measured at 530 nm using Lambda 35 UV–VIS spectrophotometer (PerkinElmer, USA). The concentration of IAA produced was estimated against the standard curve of IAA (Himedia, India).

Phosphate Solubilization

For determining phosphate solubilizing activity, rhizobacterial isolates were spot inoculated on Pikovskaya's agar medium containing 0.04% Bromo Cresol Green as a pH indicator (Himedia, India). Isolates that developed a clear zone around it were considered positive after 3–5 days of incubation at 30 ± 2 °C. Quantitative estimation of solubilized P was determined by the method described by Subba Rao [13] in Pikovskaya broth containing 1000 µg ml⁻¹ tricalcium phosphate. Using the Lambda 35 UV–VIS spectrophotometer (PerkinElmer, USA), absorbance for each isolate was measured at 430 nm and the concentration of solubilized P was extrapolated from the standard curve of potassium dihydrogen phosphate.

Ammonia Production

Ammonia production was determined both qualitatively and quantitatively using the method of Cappuccino et al. [14]. Bacterial isolates were inoculated in test tubes containing 5 ml of peptone water and incubated for 48 h at 30 ± 2 °C. The culture was centrifuged at 10,000 rpm for 5 min after incubation and 1 ml of Nessler's reagent was added to the supernatant in each tube. The formation of a yellow to brownish color is positive for the production of ammonia. The production of ammonia was measured spectrophotometrically at 450 nm using the Lambda 35 UV–VIS spectrophotometer, and the amount of ammonia produced was extrapolated from the standard curve of ammonium sulfate.

Siderophore Production

Siderophore production was analyzed using Chrome Azurol S (CAS) agar plate assay [15]. Each rhizobacterial isolate was spot inoculated on CAS agar plates and incubated at 30 ± 2 °C. After 48–72 h of incubation, an orange halo around the colonies indicates the production of siderophores. Quantitatively, siderophore content was estimated by CAS-Shuttle assay [16], in which 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS reagent and the absorbance was recorded at 630 nm against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. The quantitative production of siderophores was calculated using the formula:

$$\text{Siderophore (\%)} = \frac{A_{\text{reference}} - A_{\text{sample}}}{A_{\text{reference}}} \times 100$$

where $A_{\text{reference}}$ = absorbance of the reference, and A_{sample} = absorbance of the sample.

Screening of Hydrolytic Enzymes

Cellulase activity: The production of cellulase was assessed using the method described by Cattelan et al. [17]. Pure culture isolates were inoculated in nutrient agar amended with 1% carboxymethylcellulose (CMC) and incubated at 30 ± 2 °C for 48–72 h. Extracellular cellulase was detected by flooding the plates with 0.1% congo red solution for 15 min and the plates were destained with 1 M NaCl solution for 15 min. The plates were then observed for positive cellulase production.

Protease activity: Protease activity was detected according to Smibert et al. [18]. Skim milk agar was used as the medium containing 0.04% of Bromo Cresol Green as a pH indicator (Himedia, India). Isolates forming a halo zone around the colonies specify positive protease activity after 48–72 h of growth at 30 ± 2 °C.

Molecular Identification of Selected PGPR

For molecular identification, DNA was extracted using the lysozyme method. Briefly, 2 ml overnight culture was taken in a 2 ml centrifuge tube and centrifuged at 13,000 rpm for 5 min. The pellet was washed twice with 0.5 ml sterile distilled water. To the pellet, 3.3 μl of 3 mg ml^{-1} lysozyme and 96.7 μl sterile water were added and mixed by vortexing. The tube was incubated at 37 °C for 1 h and 95 °C for 5 min. Centrifugation was done at 13,000 rpm for 5 min and the supernatant was transferred to a fresh tube. The 16S rRNA gene PCR amplification was performed using universal primers 27F 5' AGAGTTT-GATCMTGGCTCAG 3' and 1492R 5' TACGGY-TACCTTGTTACGACTT 3' [19]. The PCR mixture of 25 μl final volume consists of 1X PCR buffer with 1.5 mM MgCl_2 , 200 μM dNTPs, 0.2 μM of each primer, 3 U Taq DNA polymerase and 50 ng template DNA was performed in a thermal cycler (Bio-Rad C1000 touch, USA). The amplicons were separated on 0.8% agarose gel containing ethidium bromide (10 mg ml^{-1}) in 1X TBE buffer. The amplified products were sequenced at Macrogens Inc., Korea. The sequences obtained were identified by using NCBI nucleotide BLAST analysis.

Preparation of PGPR Inoculum and Treatment

Four potent isolates were used as PGPR inoculum. Briefly, each bacterial isolate was cultured in a 500ml Erlenmeyer flask containing 250 ml of nutrient broth (Himedia, India) and incubated in an orbital rotary incubator at 150 rpm for

48–72 h at 30°C. For the treatment, bacterial inoculum load was adjusted to 10^8 CFU ml^{-1} using sterile distilled water. The sprouted rhizomes were surface sterilized using 2% sodium hypochlorite solution for 1 min and soaked in the bacterial solution for 12 h. The treated rhizomes were air-dried under a sterile airflow. The rhizomes were then planted in polythene bags containing 5 kg of autoclaved soil. 250 ml of each PGPR inoculum (10^8 CFU ml^{-1}) was added 30 and 60 days after planting (DAP) as a booster dosage. The growth parameters were analyzed after 180 DAP.

Statistical Analysis

The statistical analysis was performed using SPSS, v.25 (Chicago, IL, USA) and MS-Excel v.2007 (Microsoft, Washington, DC, USA). The data obtained were subjected to analysis of variance (ANOVA) and Tukey's HSD test at $p \leq 0.05$ using replicates to evaluate the effects of PGPR on the growth of *C. longa*.

Results and Discussion

Isolation of Rhizobacteria

A total of 33 isolates were obtained from the rhizosphere of *C. longa*. The number of rhizobacteria isolated is summarized in Table 1.

In vitro Evaluation of PGP Traits

Preliminary Screening of PGP Traits

All the isolates were screened for different PGP traits and it was found that 22 isolates were positive for IAA production, 14 for phosphate solubilization, 29 for ammonia production and 21 for siderophore production. Moreover, eight isolates were positive to all the PGP traits tested while one isolate did not exhibit any of the traits. All the PGP traits tested are summarized in Table 1.

Quantitative Estimation of PGP Traits

The 32 isolates which were positive to at least one of the PGP traits were further quantified for the production of IAA, solubilized phosphate, ammonia, and siderophore. The isolates produced 2.67–52.88 $\mu\text{g ml}^{-1}$ IAA, 31.56–320.50 $\mu\text{g ml}^{-1}$ solubilized phosphate, 0.52–2.07 $\mu\text{mol ml}^{-1}$ ammonia, and 16.99–43.30% of siderophore (Table 1, Fig. 1).

The production of IAA by PGPR is responsible for an increased number of adventitious roots, length and root

Table 1 Screening of rhizobacterial isolates for their plant growth promoting traits

Isolates	IAA production ($\mu\text{g ml}^{-1}$)	Ammonia production ($\mu\text{mol ml}^{-1}$)	Phosphate solubilization ($\mu\text{g ml}^{-1}$)	Siderophore production (%)	Protease activity	Cellulase activity
IJ1	3.7 \pm 0.14	0.98 \pm 0.03	–	18.76 \pm 1.45	+	–
IJ2	35.98 \pm 1.98	1.55 \pm 0.08	320.50 \pm 7.39	27.75 \pm 1.53	+	+
IJ3	3.42 \pm 0.25	1.32 \pm 0.03	–	23.68 \pm 1.54	+	–
IJ4	6.63 \pm 0.21	0.52 \pm 0.04	–	–	–	–
IJ5	–	0.77 \pm 0.03	151.50 \pm 3.73	–	–	+
IJ6	9.67 \pm 1.15	0.71 \pm 0.02	–	34.77 \pm 1.67	+	–
IJ7	2.67 \pm 0.29	1.09 \pm 0.03	–	21.60 \pm 0.72	–	+
IJ8	9.34 \pm 0.97	–	175.64 \pm 5.38	19.40 \pm 0.90	–	–
IJ9	–	1.18 \pm 0.03	44.62 \pm 2.72	–	–	–
IJ10	33.05 \pm 1.61	1.31 \pm 0.03	130.43 \pm 4.13	43.30 \pm 1.92	+	+
IJ11	–	0.94 \pm 0.06	–	18.31 \pm 1.12	+	+
IJ12	21.27 \pm 1.04	–	130.49 \pm 3.62	–	+	–
IJ13	–	1.57 \pm 0.04	–	22.49 \pm 1.66	+	+
IJ14	27.80 \pm 0.89	1.99 \pm 0.02	98.45 \pm 2.85	16.99 \pm 1.24	+	–
IJ15	30.55 \pm 0.69	1.29 \pm 0.02	89.26 \pm 2.09	23.14 \pm 1.19	+	+
IJ16	19.57 \pm 0.85	1.38 \pm 0.03	–	–	–	–
IJ17	13.35 \pm 1.12	1.40 \pm 0.04	103.53 \pm 2.05	25.36 \pm 1.08	–	+
BS1	17.79 \pm 0.52	2.07 \pm 0.03	101.02 \pm 3.47	34.01 \pm 0.82	+	–
BS2	–	0.62 \pm 0.04	–	19.16 \pm 0.63	–	+
BS3	–	0.99 \pm 0.03	–	–	–	–
BS4	52.88 \pm 2.10	1.49 \pm 0.04	104.15 \pm 2.82	18.32 \pm 0.71	–	+
BS5	–	0.74 \pm 0.03	–	19.21 \pm 0.59	–	–
BS6	7.03 \pm 0.23	1.53 \pm 0.03	174.54 \pm 4.17	35.73 \pm 1.39	–	–
BS7	–	–	–	–	+	+
BS8	–	1.24 \pm 0.06	–	–	+	–
BS9	16.56 \pm 1.26	–	–	–	–	+
BS10	31.72 \pm 1.3	1.64 \pm 0.06	–	17.56 \pm 0.69	+	+
BS11	22.57 \pm 0.63	1.22 \pm 0.04	57.38 \pm 1.90	–	–	–
BS12	–	0.83 \pm 0.04	–	–	+	+
BS13	25.44 \pm 1.90	1.02 \pm 0.030	–	28.26 \pm 0.93	–	–
BS14	29.05 \pm 1.29	1.47 \pm 0.03	31.56 \pm 1.47	–	+	+
BS15	–	1.66 \pm 0.03	–	21.84 \pm 1.22	–	–
BS16	33.18 \pm 1.20	0.69 \pm 0.02	–	24.16 \pm 0.95	–	–

Values are means of three replicates \pm SD. (+) positive, (–) negative

volume through which roots can provide the plant with a large number of nutrients [20]. In our study, *Pseudarthrobacter chlorophenolicus* BS4 exhibits the highest IAA production (52.88 $\mu\text{g ml}^{-1}$). Forni et al. [21] documented that, using tryptophan as a precursor, all *Arthrobacter* species were able to produce IAA. Another important PGP trait is inorganic phosphate solubilization in which bacteria produced several organic acids that lower the pH and released the phosphates that are attached to inorganic calcium complexes. *Pseudomonas* sp. IJ2 was found to solubilize the highest inorganic phosphate (320.50 $\mu\text{g ml}^{-1}$). Different free-living rhizospheric

bacteria like *Bacillus*, *Pseudomonas* and *Azotobacter* have been documented as excellent phosphate solubilizers and siderophore producers [7]. Siderophores are low molecular weight molecules which bind to Fe^{3+} and chelate Fe^{3+} making it less accessible to other microorganisms. *Bacillus subtilis* IJ10 produced the highest (43.30%) siderophore among all the isolates. O'Sullivan and O'Gara [22] showed that by depriving the pathogen of iron nutrition, efficient siderophore producers can regulate several plant diseases, resulting in increased crop yield. In the study, *Arthrobacter* sp. BS1 produced the highest ammonia (2.07 $\mu\text{mol ml}^{-1}$) content. The production of ammonia by PGPR also

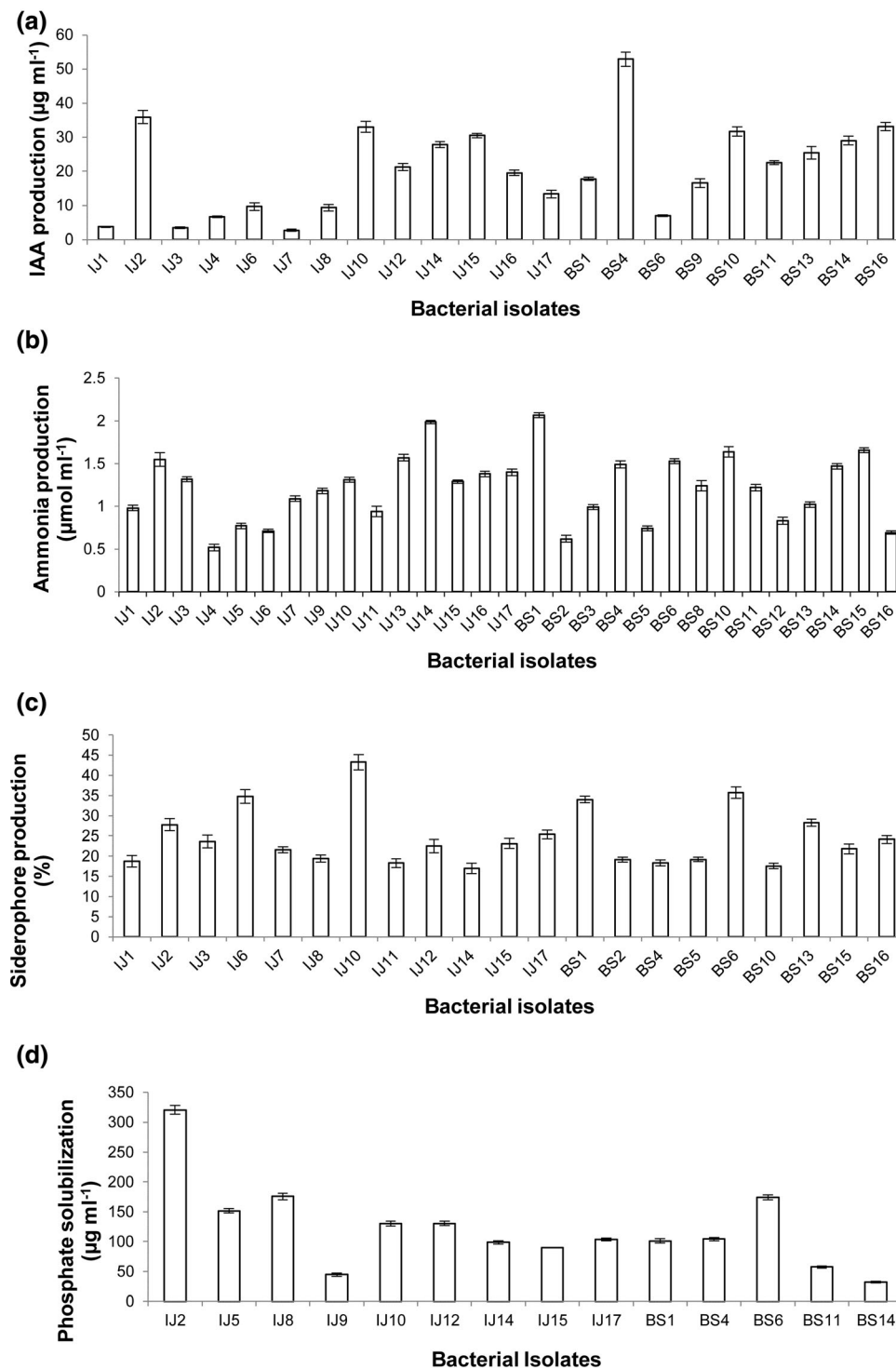


Fig. 1 Quantitative estimation of **1a** IAA production, **1b** Ammonia production, **1c** Siderophore production, **1d** Phosphate solubilization

contributes to an increase in the root, shoot growth and biomass production through nitrogen accumulation [23].

Screening for the Production of Hydrolytic Enzymes

All the 33 isolates were screened for protease and cellulase enzyme activity, of which 16 isolates were protease positive and 15 were cellulase positive (Table 1,

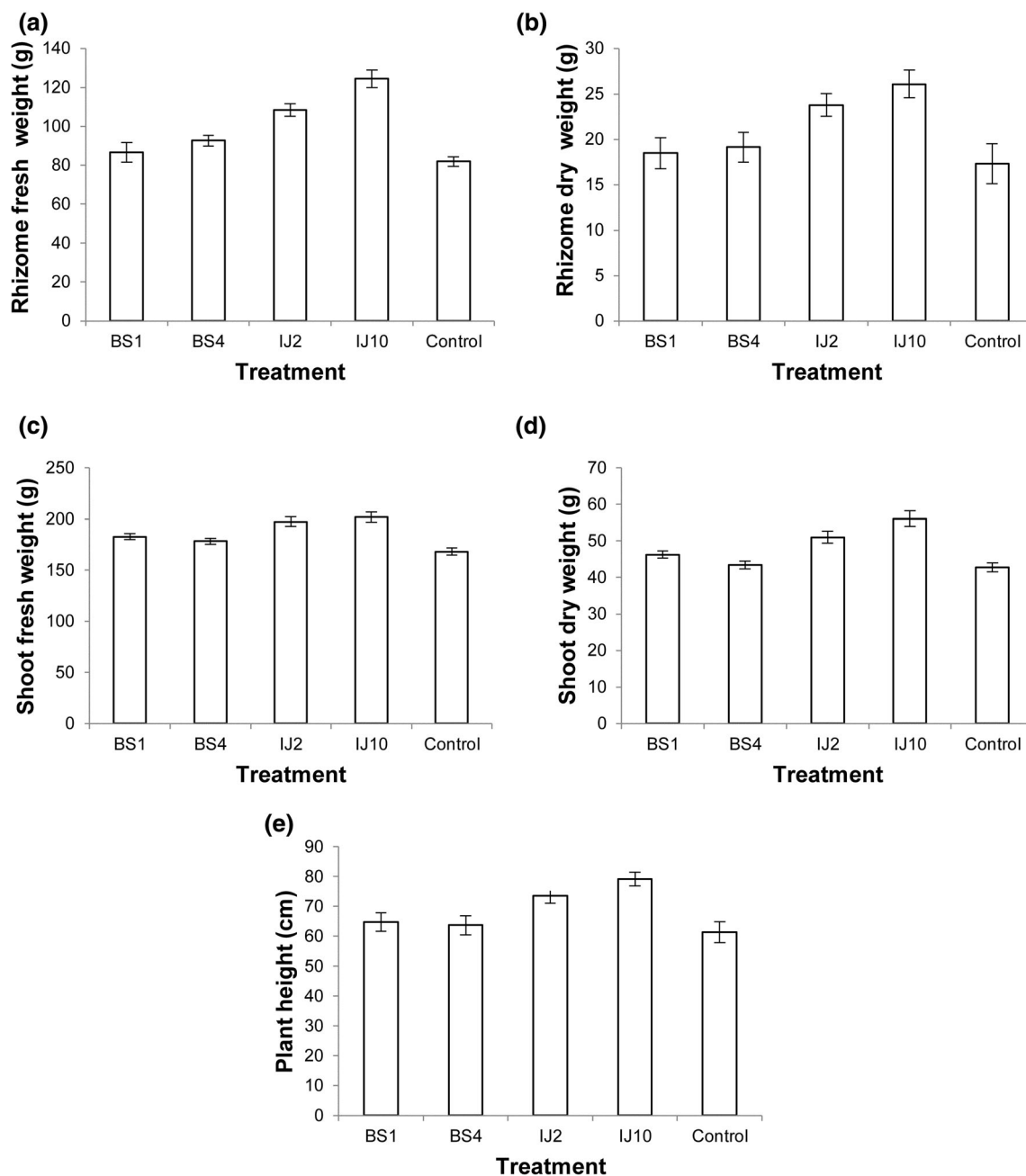


Fig. 2 Evaluation of PGPR treatment on various plant growth parameters under greenhouse environment **2a** Rhizome fresh weight, **2b** Rhizome dry weight, **2c** Shoot fresh weight, **2d** Shoot dry weight, **2e** Plant height

Supplementary Fig. 1). The production of hydrolytic enzymes such as cellulase and protease by PGPR may play an important role in the decomposition of organic matter and nutrient mineralization and may also help to promote the entry of microorganisms into host tissues [24].

Molecular Identification of Selected PGPR

Molecular identification of four selected isolates was performed by amplifying the 16S rRNA gene and the sequences were obtained from Macrogen Inc., Korea. The sequences obtained were identified using NCBI BLAST analysis. The analysis identified the isolates as *Arthrobacter* sp. BS1, *Pseudarthrobacter chlorophenolicus* BS4, *Pseudomonas* sp. IJ2, *Bacillus subtilis* IJ10, and the

Table 2 Effects of PGPR treatment on the growth parameters of *C. longa*

Treatment	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Rhizome fresh weight (g)	Rhizome dry weight (g)
Isolate BS1	64.7 ± 3.08 ^b	182.71 ± 2.94 ^b	46.25 ± 0.99 ^c	86.70 ± 5.15 ^{cd}	18.48 ± 1.72 ^b
Isolate BS4	63.7 ± 3.20 ^b	178.32 ± 2.99 ^{bc}	43.42 ± 1.12 ^c	92.70 ± 2.81 ^c	19.14 ± 1.63 ^b
Isolate IJ2	73.6 ± 2.60 ^a	197.45 ± 4.51 ^a	50.99 ± 1.62 ^b	108.48 ± 3.31 ^b	23.80 ± 1.26 ^a
Isolate IJ10	79.2 ± 2.30 ^a	201.73 ± 5.05 ^a	56.10 ± 2.18 ^a	124.48 ± 4.55 ^a	26.09 ± 1.50 ^a
Control	61.3 ± 3.53 ^b	168.25 ± 3.66 ^c	42.78 ± 1.15 ^c	81.93 ± 2.54 ^d	17.30 ± 2.19 ^b

Data are means of three replicates ± SD. Data with the same letter within each column are not significantly different at $p \leq 0.05$ as determined by Tukey's HSD tests

sequences were deposited in GenBank database (NCBI) under the respective accession numbers MT192804, MT192880, MT193010, and MT219296.

Effects of PGPR Treatment on Plant Growth

Four selected isolates (BS1, BS4, IJ2, and IJ10) which showed promising in vitro PGP activity were considered for the pot trial experiment. Plants inoculated with the selected isolates exhibit better growth performance than uninoculated (control) plants (Fig. 2 and Supplementary Fig. 2). All the plants inoculated with *Pseudomonas* sp. IJ2 and *B. subtilis* IJ10 showed a significant difference at $p \leq 0.05$ in all the growth parameters relative to control (Table 2, Fig. 2). This may be due to their different PGP traits. The primary factors in enhancing plant growth and yield have been attributed to these multiple PGP characteristics [25]. In a similar study, Kumar et al. [26] found that *P. fluorescens* CL12 inoculation resulted in better growth and yield of turmeric rhizome. Upon inoculation with *Pseudomonas* sp., chickpea and green gram resulted in an enhanced growth [27]. The species complex of *B. subtilis* is well known for its ability to promote plant growth, especially *B. subtilis* and *B. amyloliquefaciens* [28]. Such organisms encourage plant growth directly through the production of phytohormones, siderophores, organic acids involved in P-solubilization, and/or nitrogen fixation [29]. The root-colonizing *B. subtilis* subsp. *subtilis* PTS-394G is capable of persisting on the roots and of stimulating tomato plant growth [30].

Conclusion

The study established the value of isolating and screening rhizobacteria for various PGP traits and evaluating them for their ability to promote plant growth toward the crop in greenhouse environment. On the basis of greenhouse experiment, *Pseudomonas* sp. IJ2 and *B. subtilis* IJ10 were able to significantly enhance the growth of *C. longa*. These

isolates, therefore, may provide a viable alternative to chemical inputs for improved growth and yield of *C. longa*.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40011-021-01268-5>.

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Author contributions All the authors contributed to the study's conception and design. The first author (FK) performed sample collection, laboratory experiments, data analysis and writing of the manuscript. The second author (AK) assists in experiments related to molecular identification of the isolates. The third author (HK) supervised the research work and experimental design. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors have declare no conflicts of interest.

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