



# Characterization of plant growth-promoting, antifungal, and enzymatic properties of beneficial bacterial strains associated with pulses rhizosphere from Bundelkhand region of India

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## Abstract

The present study was conducted to characterize the native plant growth-promoting rhizobacteria (PGPRs) from the pulse rhizosphere of the Bundelkhand region of India. Twenty-four bacterial isolates belonging to nineteen species (*B. amyloliquefaciens*, *B. subtilis*, *B. tequilensis*, *B. safensis*, *B. haynesii*, *E. soli*, *E. cloacae*, *A. calcoaceticus*, *B. valezensis*, *S. macrescens*, *P. aeruginosa*, *P. fluorescens*, *P. guariconensis*, *B. megaterium*, *C. lapagei*, *P. putida*, *K. aerogenes*, *B. cereus*, and *B. altitudinis*) were categorized and evaluated for their plant growth-promoting potential, antifungal properties, and enzymatic activities to identify the most potential strain for commercialization and wider application in pulse crops. Phylogenetic identification was done on the basis of 16 s rRNA analysis. Among the 24 isolates, 12 bacterial strains were gram positive, and 12 were gram negative. Among the tested 24 isolates, IIPRAJCP-6 (*Bacillus amyloliquefaciens*), IIPRDSCP-1 (*Bacillus subtilis*), IIPRDSCP-10 (*Bacillus tequilensis*), IIPRRLUCP-5 (*Bacillus safensis*), IIPRCDP-2 (*Bacillus subtilis*), IIPRAMCP-1 (*Bacillus safensis*), IIPRMKCP-10 (*Bacillus haynesii*), IIPRANPP-3 (*Bacillus amyloliquefaciens*), IIPRKAPP-5 (*Enterobacter soli*), IIPRAJCP-2 (*Enterobacter cloacae*), IIPRDSCP-11 (*Acinetobacter calcoaceticus*), IIPRDSCP-9 (*Bacillus valezensis*), IIPRMKCP-3 (*Serratia macrescens*), IIPRMKCP-1 (*Pseudomonas aeruginosa*), IIPRCKPP-3 (*Pseudomonas fluorescens*), IIPRMKCP-9 (*Pseudomonas guariconensis*), IIPRMKCP-8 (*Bacillus megaterium*), IIPRMWCP-9 (*Cedecea lapagei*), IIPRKUCP-10 (*Pseudomonas putida*), IIPRAMCP-4 (*Klebsiella aerogenes*), IIPRCKPP-7 (*Enterobacter cloacae*), IIPRAMCP-5 (*Bacillus cereus*), IIPRSHEP-6 (*Bacillus subtilis*), IIPRRSba89 (*Bacillus altitudinis*) bacterial isolates, IIPRMKCP-9, IIPRAJCP-6, IIPRMKCP-10, IIPRAMCP-5, IIPRSHEP-6, and IIPRMKCP-3 showed the maximum antagonistic activity against *Fusarium oxysporum* f. sp. *ciceris* (FOC), *Fusarium oxysporum* f. sp. *lentis* (FOL), and *Fusarium udum* (FU) causing wilt disease of chickpea, lentil, and pigeonpea, respectively, and maximum plant growth-promoting enzyme (phosphatase), plant growth hormone (IAA), and siderophore production show promising results under greenhouse conditions. This study is the first report of bacterial diversity in the pulse-growing region of India.

**Keywords** Rhizobacteria · Pulses · Plant Growth promotion · *Fusarium* spp. · Biocontrol

## Introduction

Bundelkhand region of India, which comprises seven districts of Uttar Pradesh (Jhansi, Jalaun, Lalitpur, Mahoba, Hamirpur, Banda, and Chitrakoot) and six districts of

Madhya Pradesh (Datia, Tikamgarh, Chhatarpur, Panna, Damoh, and Sagar), is a semi-arid area with temperatures ranging between 3 and 47.8 °C [1]. Bundelkhand is the major pulse-growing region of India, commonly known as the “pulse bowl of the country” [2–4]. The Bundelkhand region contributes 8.4% of the total pulse production in the country. Chickpea is the most important pulse crop in the Bundelkhand region, followed by lentil, fieldpea, pigeonpea, and mungbean. Among the pulses, chickpea contributes about 30% of total pulse production in the world [5]. Pigeonpea (*Cajanus cajan* L.) is another major crop, and India is the largest producer and consumer in the world.

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Lentil (*Lens culinaris* Medik.) is the most nutritious and oldest pulse crop traced back to 13,000 and 7000 BC in the world and Asia [6]. These pulses are important because of the important role they play in atmospheric nitrogen fixation. They are also rich in dietary protein, minerals, and vitamins, which play an important role in human nutrition, especially for the vegetarian population worldwide. In terms of area and production, India is in first place, but in terms of productivity, it is far below Canada, Australia, Ethiopia, Mexico, and Myanmar.

The productivity of these crops is affected by a large number of pests and diseases at various crop stages. Among these diseases, wilt-causing *Fusarium* spp. is one of the most devastating soil-borne pathogens, causing up to 100% crop loss. Butler first reported that wilt of chickpea, pigeonpea, and lentil are caused by *Fusarium oxysporum* f. sp. *ciceris*, *F. udum*, and *F. oxysporum* f. sp. *lentis*, respectively [7]. The management of soil-borne pathogens is difficult because of their nature and wide host range. Biological control offers an environmentally friendly alternative to chemical fungicides [8, 9]. Among the biological approaches, plant growth-promoting rhizobacteria are considered an important and effective approach for crop protection [10]. The use of biocontrol products is increasing day by day, but their products on the market are available in very limited quantities. Several bacteria have been studied as plant growth-promoting rhizobacteria; in particular, the application of *Pseudomonas* and *Bacillus* has received particular attention because of their high root colonizing activity, metabolite (harzianic acid, viridin, etc.) production, and enzyme production [11, 12].

Knowledge of the native microbial population is required for understanding the distribution and diversity of rhizosphere microbes in specific crops. With the increasing popularity of chemical fertilizer in agriculture, there is a need to search for region specific microbes that can be utilized for better soil and crop health management in a sustainable manner. Recently, the bacterial diversity in the forest soil of Kashmir has been reported [13], but there is no data regarding the bacterial population of Uttar Pradesh. Keeping this in mind, the native strains from the rhizosphere of the pulse-growing regions of Bundelkhand were isolated. These bacterial strains were characterized and screened in vitro for plant growth-promoting (PGP) and antagonistic potential, and representative potential isolates were identified by 16SrRNA gene analysis. Further, the plant growth potential was also evaluated on major pulses (chickpea, lentil, and pigeonpea) under in vivo conditions. The main objectives of the present investigation were to identify and characterize indigenous PGPR from the crop niches and to prove their efficacy for plant growth promotion potential and disease suppression ability against *Fusarium udum*, *F. oxysporum* f. sp. *ciceris*, and *F. oxysporum* f. sp. *lentis* which cause wilt disease of pigeonpea, chickpea, and lentil worldwide.

## Material and methods

### Survey and sampling

Field survey of 13 districts (Jhansi, Jalaun, Panna, Banda, Lalitpur, Chhatarpur, Hamirpur, Damoh, Tikamgarh, Mahoba, Chitrakoot, Datia, and Sagar) was conducted to collect healthy plant samples from pigeonpea, chickpea, and brinjal crops of Bundelkhand region (Table 1). Ten rhizospheric samples were collected with a shovel to a depth of 30 cm to cut any of the lateral roots holding the plant in the soil from each district [14]. Collected plant samples were kept in plastic bags and stored in the refrigerator until used for the isolation.

### Isolation of rhizospheric bacteria

Bacterial species were isolated from the rhizosphere of healthy plants collected from Bundelkhand region of India (Fig. 1). Roots were excised and placed into 10 ml sterilized distilled water and vortexed to detach soil. Serial dilutions of the prepared mixture are plated on the nutrient agar and incubated for 28 °C for 24 h [15, 16].

### Isolation and characterization of *Fusarium* spp. associated with chickpea, lentil, and pigeonpea

Fungal phytopathogens, *Fusarium oxysporum* f. sp. *ciceris* (FOC), *Fusarium oxysporum* f. sp. *lentis* (FOL), and *Fusarium udum* (FU), were isolated from the wilt infected chickpea, lentil, and pigeonpea plants by surface sterilization methods [17]. The plates were incubated at 28 °C, and primary identification was done by microscope. Isolated fungal cultures were stored in PDA slants, and pathogenicity tests were performed with hydroponics techniques. The desired *Fusarium* conidial suspensions ( $3.3 \times 10^6$  conidia/ml) were added to each hydroponic test tube, and 15-day-old seedlings of chickpea, pigeonpea, and lentil were inoculated. After 7 days of inoculation, wilt disease symptoms were examined, and the plant became wilted and dry. The infected plants were brought in the laboratory for *Fusarium* isolation. Isolated cultures were further identified by molecular techniques using ITS universal primers ITS-1 (TCCGTAGGT GAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATAT GC) [18] were used as molecular markers.

### Characterization of rhizospheric bacteria

Bacterial characterization was done on the colony morphology, gram staining, oxidase test, and glucose fermentation test. Genomic DNA of bacteria was isolated by using

**Table 1** Morphological characters of different bacteria isolated from rhizosphere of different crop plants of Bundelkhand region of India

Strains	Isolated from	Geographical locations	Plant rhizosphere	Cell motility	Bacterial cell shape
IIPRAJCP-6	Jhansi	25.4484° N, 78.5685° E	Chickpea	Highly motile	Small rod
IIPRDSCP-1	Jalaun	26.1459° N, 79.3297° E	Chickpea	Less motile	Medium rod
IIPRDSCP-10	Panna	24.7180° N, 80.1819° E	Chickpea	Less motile	Medium rod
IIPRRLUCP-5	Banda	25.4763° N, 80.3395° E	Chickpea	Highly motile	Small rod
IIPRCDCP-2	Lalitpur,	24.6912° N, 78.4138° E	Chickpea	Less motile	Medium rod
IIPRAMCP-1	Chhatarpur	24.9168° N, 79.5910° E	Chickpea	Medium motile	Medium rod
IIPRMKCP-10	Jalaun	26.1459° N, 79.3297° E	Chickpea	Slow motile	Medium rod
IIPRANPP-3	Hamirpur	31.6862° N, 76.5213° E	Pigeonpea	High motile	Medium rod
IIPRKAPP-5	Jhansi	25.4484° N, 78.5685° E	Pigeonpea	High motile	Small oval
IIPRAJCP-2	Damoh	24.7456° N, 78.8321° E	Chickpea	Slow motile	Large oval
IIPRDSCP-11	Tikamgarh	24.7456° N, 78.8321° E	Chickpea	Medium motile	Large oval
IIPRDSCP-9	Mahoba	25.2921° N, 79.8724° E	Chickpea	Medium motile	Medium rod
IIPRMKCP-3	Tikamgarh	24.7456° N, 78.8321° E	Chickpea	Slow motile	Small rod
IIPRMKCP-1	Chitrakoot	25.1094° N, 81.0755° E	Chickpea	High motile	Small oval
IIPRCKPP-3	Mahoba	25.2921° N, 79.8724° E	Pigeonpea	Slow motile	Medium oval
IIPRMKCP-9	Datia	25.6653° N, 78.4609° E	Chickpea	Less motile	Medium oval
IIPRMKCP-8	Mahoba	25.2921° N, 79.8724° E	Chickpea	Medium motile	Medium rod
IIPRMWCP-9	Chitrakoot	25.1094° N, 81.0755° E	Chickpea	Medium motile	Medium rod
IIPRKUCP-10	Jalaun	26.1459° N, 79.3297° E	Chickpea	High motile	Small oval
IIPRAMCP-4	Panna	24.7180° N, 80.1819° E	Chickpea	High motile	Small oval
IIPRCKPP-7	Damoh	24.7456° N, 78.8321° E	Pigeonpea	Medium motile	Small oval
IIPRAMCP-5	Sagar	23.8388° N, 78.7378° E	Chickpea	High motile	Small rods
IIPRSHEP-6	Mahoba	25.2921° N, 79.8724° E	Brinjal	High motile	Small rods
IIPRRSBa89	Damoh	24.7456° N, 78.8321° E	Pigeonpea	Motile	Small rods

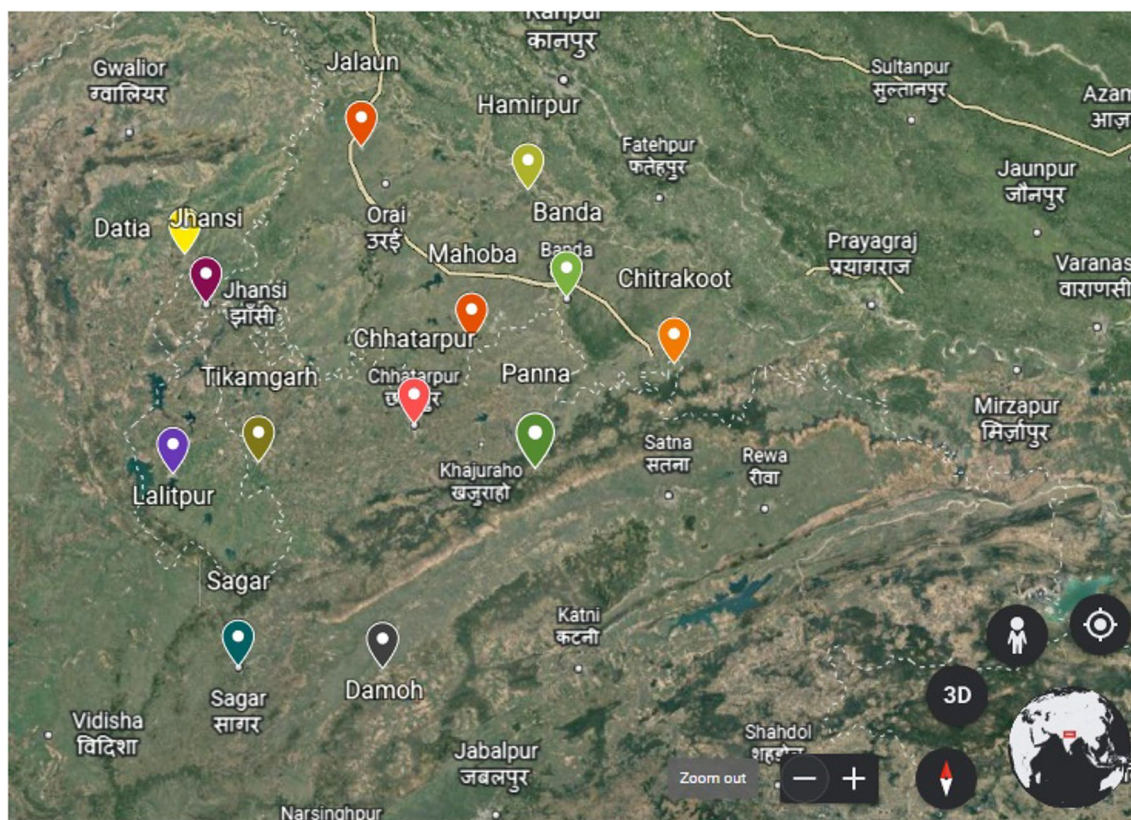
alkaline lysis method [19], and 16S rRNA gene was amplified using the universal primer pair (27F: 5'-AGA GTT TGA TC[A/C] TGG CTC AG-3', 1492R: 5'-G[C/T]T ACC TTG TTA CGA CTT-3') [17]. PCR conditions used for amplification were 1 min DNA denaturation at 94 °C, 1 min annealing at 57 °C, and 1 min extension at 72 °C for 35 cycles. The amplified PCR products were sent for sequencing to Chromus Biotech, and obtained sequences were run on BLAST program for the identification. After identification, the obtained sequences were trimmed and submitted to NCBI database for accession number allotment (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic tree was constructed with the obtained 16SrRNA gene sequences and reference sequences obtained from NCBI. The phylogenetic relationship among bacterial isolates and reference sequences was constructed using the maximum likelihood method and Tamura-Nei model [20].

### In vitro estimation of plant growth-promoting traits and extra-cellular enzymatic abilities of PGPRs

For proteolytic enzyme production, 0.1% CMC was amended in the YEMA medium, and 24-hour-old bacterial culture was inoculated. After 3 days, petri plates

were flooded with 0.1% Congo red solution; after 10 min, plates were washed with 1M NaCl, and plates were observed for the hydrolysis zone formation [22]. Measurement of proteolytic activity was done by the method of Uman, 1968 [21]; 1 ml of culture supernatant was added into a MCT containing 1 ml solution of 1% casein and incubated at 37 °C for 30 min. After incubation, 3 ml of 5% TCA solution was added and shaken and kept at RT for 30 min. After incubation, contents of the tube were centrifuged for 15 min. Colloidal chitin amended media (glucose, 0.5%; peptone, 0.2%; colloidal chitin, 0.2%; K<sub>2</sub>HPO<sub>4</sub>, 0.1%; MgSO<sub>4</sub>, 0.05%; and NaCl, 0.05%) was used for chitinase activity determination. For chitinase activity, determination method of Reissig, Strominger, and Leloir (1955) [22] was used. One unit of chitinase was determined as 1 nmol of GlcNAc released per minute per mg of protein as described by Miller method [23]. The method described by García et al. [24] was used for the siderophore production. CAS media plates were prepared, and spot inoculation of tested bacterial strains was done. After 5 days of incubation at 28 °C, the plates were observed for the appearance of orange halo zone. For IAA screening, Salkowaski reagent was used [25], and 2 ml of Luria Bertani broth was inoculated by bacterial





**Fig. 1** Geographical map location of bacterial isolates

strains and incubated for 48 h. After 48 h, 1 ml of Salko-waski reagent was added and observed for color change. Appearance of pink color indicates the IAA production. For quantification, the optical density (OD) was recorded at 530 nm, and IAA standard curve was used for the calculation of IAA content. Phosphate solubilization activity of PGPR bacteria was studied on Pikovskaya's agar media plates. Bacterial cultures were streaked on the Pikovskaya plates and incubated for 5 days for the appearance of clear zone formation around the colony [26]. For quantification, the bacterial strains were cultured on LB broth and incubated on shaker at 30 °C for 3 days. After incubation, cultures were centrifuged for 5 min at 12,000 rpm at 4 °C, and supernatant was collected to measure the amount of solubilized phosphate by method of Kovacs, 1956 [27]. Ammonia production was estimated by growing rhizobacterial strains in peptone broth (10 ml) and incubated at 30 °C for 48 to 72 h. After incubation, 0.5 ml of Nessler's reagent was added to bacterial suspension. The development of brown to yellow color indicated ammonia production [25]. For oxidase test, a loopful of bacterial inoculums was picked up and smeared on the filter paper containing oxidize reagent. Appearance of purple color indicates that the isolates are oxidase positive [28].

### Abiotic stress tolerance of bacterial isolates

All the isolated bacteria were checked for their salt tolerance on nutrient agar media plates supplemented with different NaCl concentrations (1, 2, 4, 8, and 10%) [28]. For temperature tolerance test, all the isolated bacteria were incubated at different temperature 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, and 42 °C [29]. All the experiments were performed in three replicates.

### Antagonism assay

The pathogenic fungus culture was streaked on the PDA plate after 48 h on the opposite direction, bacterial culture was streaked, and plates were incubated for 7 days at  $28 \pm 2$  °C in incubator. After 7 days, mycelial growth inhibition percentage was calculated. For control plates, bacteria were not inoculated with pathogen [30]. Inhibition percentage is calculated by using the following formula:

$$\% \text{inhibition} = [(R - r/R)] \times 100$$

where  $R$  is the radius of fungal growth from the center of the plate towards the control treatment and  $r$  is the radius of fungal growth towards the bacterial treatment.

## Greenhouse assay

On the basis of antagonistic potential and PGPR tests, species selection of bacterial strains was done for greenhouse assay. Experiments were conducted in pots containing dried and sieved soil. For seed treatment, 5 ml of log phase bacterial cell suspension ( $1 \times 10^8$  CFU/ml) was added on seeds (10 seeds per pot) placed on a filter paper in petri dish and kept for 4 h. Each treatment was replicated three times. Plants were kept in greenhouse for 16 weeks with a 12-h photoperiod, 70% relative humidity, and 35 °C for light and 20 °C for dark. At the end of the experiment, bacterial isolation was done to check that introduced strains in the plants were the same. Bacterial identification was done by microscopic, cultural, and molecular techniques.

## Results

### Survey, sampling, isolation, and identification

A total of 24 bacterial isolates were obtained from the rhizosphere of different crop plants. Population density of the isolates in the rhizosphere was estimated, and it was found  $3.1 \pm 2.20 \times 10^6$  to  $5.6 \pm 1.21 \times 10^{10}$ , respectively. Based on the gram staining and KOH test, it was found that 52.1% were gram positive, while the remaining isolates were gram negative. Details of all the isolated bacterial strains are given in Table 1.

### Molecular characterization

The results indicated 94.5 to 100% similarity homology with various *Bacillus*, *Acinetobacter*, *Enterobacter*, *Cedecea*, *Serratia*, and *Pseudomonas* species available in NCBI database (Table 2). Two clades are present in Fig. 2. Clade second presents a single bacterial isolate IIPRMKCP-8 (MT436399). Clade first is divided into two subclades. Subclade first represents IIPRMWCP-9 (MT436400), IIPRCKPP-7 (MT436403), IIPRAJCP-2 (MT436392), IIPRKUCP-10 (MT436401), IIPRMKCP-1 (MT436396), IIPRMKCP-3 (MT436395) IIPRAMCP-4 (MT436402), IIPRLUCP-5 (MK863577), IIPRCDP-2 (MK863578), and *Bacillus subtilis* (ON872223). Subclade second is further divided into two parts representing IIPRAMCP-5 (MT436404), IIPRDSCP-1 (MK863575), IIPRANPP-3 (MK863581), IIPRMKCP-9 (MT436398), IIPRCKPP-3 (MT436397), IIPRDSCP-11 (MT436393), IIPRKAPP-5 (MT436391), IIPRMKCP-10 (MK863580), IIPRDSCP-10

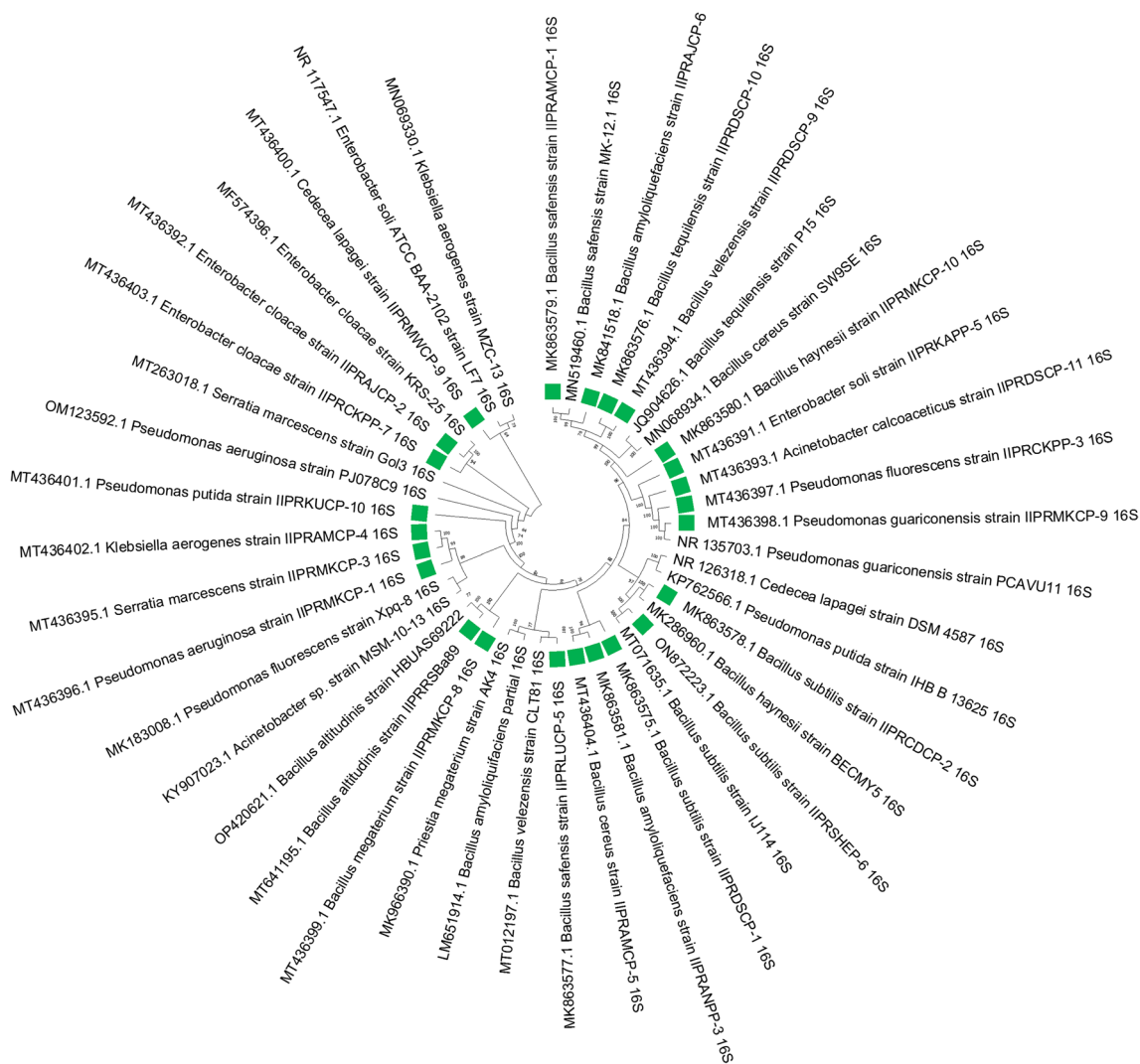
**Table 2** Molecular analysis of isolated bacterial strains

Sl No	Isolate name and their scientific name	NCBI Acc. No
1.	IIPRAJCP-6 ( <i>Bacillus amyloliquefaciens</i> )	MK841518
2.	IIPRDSCP-1 ( <i>Bacillus subtilis</i> )	MK863575
3.	IIPRDSCP-10 ( <i>Bacillus tequilensis</i> )	MK863576
4.	IIPRRLUCP-5 ( <i>Bacillus safensis</i> )	MK863577
5.	IIPRCDP-2 ( <i>Bacillus subtilis</i> )	MK863578
6.	IIPRAMCP-1 ( <i>Bacillus safensis</i> )	MK863579
7.	IIPRMKCP-10 ( <i>Bacillus haynesii</i> )	MK863580
8.	IIPRANPP-3 ( <i>Bacillus amyloliquefaciens</i> )	MK863581
9.	IIPRKAPP-5 ( <i>Enterobacter soli</i> )	MT436391
10.	IIPRAJCP-2 ( <i>Enterobacter cloacae</i> )	MT436392
11.	IIPRDSCP-11 ( <i>Acinetobacter calcoaceticus</i> )	MT436393
12.	IIPRDSCP-9 ( <i>Bacillus vazezensis</i> )	MT436394
13.	IIPRMKCP-3 ( <i>Serratia macrescens</i> )	MT436395
14.	IIPRMKCP-1 ( <i>Pseudomonas aeruginosa</i> )	MT436396
15.	IIPRCKPP-3 ( <i>Pseudomonas fluorescens</i> )	MT436397
16.	IIPRMKCP-9 ( <i>Pseudomonas guariconensis</i> )	MT436398
17.	IIPRMKCP-8 ( <i>Bacillus megaterium</i> )	MT436399
18.	IIPRMWCP-9 ( <i>Cedecea lapagei</i> )	MT436400
19.	IIPRKUCP-10 ( <i>Pseudomonas putida</i> )	MT436401
20.	IIPRAMCP-4 ( <i>Klebsiella aerogenes</i> )	MT436402
21.	IIPRCKPP-7 ( <i>Enterobacter cloacae</i> )	MT436403
22.	IIPRAMCP-5 ( <i>Bacillus cereus</i> )	MT436404
23.	IIPRSHEP-6 ( <i>Bacillus subtilis</i> )	ON872223
24.	IIPRRSBA89 ( <i>Bacillus altitudinis</i> )	MT641195

(MK863576), IIPRDSCP-9 (MT436394), IIPRAJCP-6 (MK841518), and IIPRAMCP-1 (MK863579). For *Fusarium* isolates, reference sequences were retrieved from the NCBI, and neighbor-joining based phylogenetic tree was constructed (Fig. 3). Two clades are present in the tree; clade first is divided into two subclades. Subclade first presents *F. oxy. lentis* strains, and subclade second presents *F. oxy. ciceris* strain. Clade second presents *F. udum* strains.

### In vitro estimation of plant growth-promoting traits and extra-cellular enzymatic abilities of PGPRs

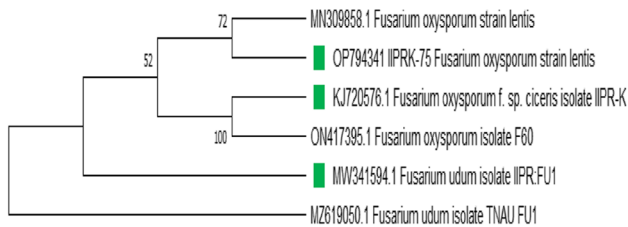
In vitro plant growth-promoting traits of the isolated rhizobacteria are described in the Table 3. All the tested 24 isolates produced IAA. The maximum IAA production was shown by IIPRSHEP-6 ( $78.90 \text{ ug ml}^{-1}$ ) followed by IIPRMKCP-9 ( $69.09 \text{ ug ml}^{-1}$ ) and IIPRANPP-3 ( $65.11 \text{ ug ml}^{-1}$ ). All the tested bacteria except IIPRLUCP-1 showed the formation of clear zone on Pikovaskaya media. Quantification of phosphate enzyme was done spectrophotometrically, and IIPRSHEP-6 showed maximum phosphate enzyme production ( $28 \text{ ug ml}^{-1}$ ). The remaining other isolates showed the P solubilization from 2 to 22 ug/ml (Table 3). Siderophore test revealed that 9 (IIPRMKCP-9, IIPRMKCP-10, IIPRAJCP-6,



**Fig. 2** Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16S rRNA gene sequence of PGPR bacterial isolates and related sequences obtained from NCBI

IIPRSHEP-6, IIPRCDP-2, IIPRMKCP-3, IIPRDSCP-10, and IIPRAMCP-5) bacterial isolates were able to produce orange colored zone on the CAS media. Ammonia production test was also done for all the isolates. All the isolates showed ammonia production. All the isolates were tested for

the hydrolytic enzyme (chitinase and proteolytic enzyme) production. Quantification of chitinase enzyme production showed that IIPRSHEP-6, IIPRMKCP-9, IIPRMKCP-10, and IIPRAMCP-3 produced maximum chitinase enzyme, while for proteolytic enzyme, production range was 2.33 to 24.33 mg/ml (Table 3).



**Fig. 3** Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the ITS gene sequence of *Fusarium* isolates (highlighted in green color) and related sequences obtained from NCBI

**Abiotic stress tolerance**

Twenty-four bacterial isolates of Bundelkhand region were screened for the salt tolerance. The bacterial isolates were analyzed ranging from 2 to 10%; only one isolate MKCP-9 was able to survive up to 6% of salt concentration. Thermophilic bacteria have the ability to survive in high temperature conditions. In the present study, a survey was conducted in Bundelkhand to collect and isolate plant growth



**Table 3** Biochemical characterization of isolated plant growth promoting rhizobacteria

Isolate name	IAA ( $\mu\text{g ml}^{-1}$ )	Siderophore	KOH	Ammonia	Phosphate ( $\mu\text{g ml}^{-1}$ )	Chitinase (mg/ml)	Proteolytic (mg/ml)	Gram reaction
IIPRAJCP-6	35.9	+	+	+	12.4	0.31	18.78	+
IIPRDSCP-1	56.2	-	-	+	21.8	0.30	10.23	+
IIPRDSCP-10	17.3	+	-	+	19.7	0.27	9.22	+
IIPRLUCP-5	22.9	-	-	+	-	0.30	2.33	+
IIPRCDCP-2	56.2	+	+	+	16.5	0.37	14.76	+
IIPRAMCP-1	29.7	-	+	+	20.8	0.35	17.98	+
IIPRMKCP-10	20.3	+	+	+	12.5	0.41	20.11	+
IIPRANPP-3	65.1	-	+	+	14.3	0.39	16.45	+
IIPRKAPP-5	27.3	-	+	+	11.2	0.38	11.43	-
IIPRAJCP-2	32.1	-	+	+	19.6	0.38	9.34	-
IIPRDSCP-11	12.3	-	+	+	18.7	0.23	5.45	-
IIPRDSCP-9	17.4	-	+	+	20.9	0.29	16.87	+
IIPRMKCP-3	34.6	-	+	+	11.3	0.19	12.98	+
IIPRMKCP-1	40.2	-	+	+	12.6	0.40	17.54	-
IIPRCKPP-3	43.1	-	+	+	20.5	0.31	6.43	-
IIPRMKCP-9	69.0	+	+	+	23.5	0.38	23.12	-
IIPRMKCP-8	56.4	-	+	+	16.3	0.19	5.54	+
IIPRMWCP-9	44.3	-	+	+	15.6	0.11	19.80	+
IIPRKUCP-10	65.3	-	-	+	14.3	0.14	20.33	-
IIPRAMCP-4	50.8	-	-	+	23.5	0.18	17.65	-
IIPRCKPP-7	48.5	-	+	+	20.4	0.24	15.55	-
IIPRAMCP-5	50.3	+	+	+	13.2	0.27	14.74	+
IIPRSHEP-6	78.9	+	+	+	28.2	0.45	24.33	+
IIPRRSBa89	55.1	+	+	+	17.8	0.34	11.32	+

and disease-suppressing rhizobacteria for the wilt disease of major pulses. Twenty-four bacteria were isolated, and out of the 23 bacteria, only one bacterium IIPRMKCP-9 was able to survive at 42 °C of temperature.

### Biocontrol ability of bacterial isolates against *Fusarium* isolates of pulses

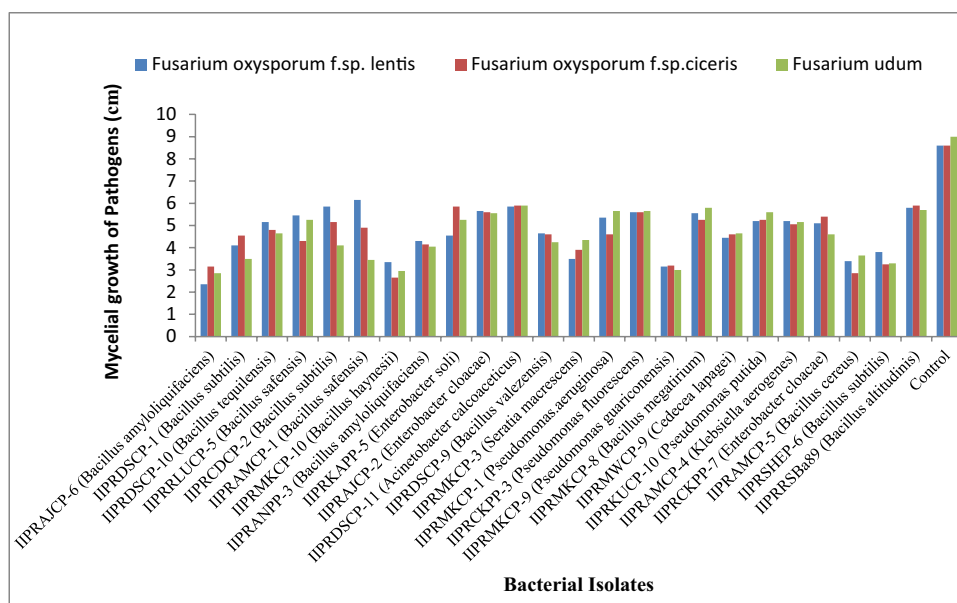
Screening of all the bacterial isolates revealed significant reduction in the mycelial growth of all the tested wilt pathogens (*F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *lentis*, and *F. udum*) (Figs. 4 and 5). Among all the tested bacterial isolates, five isolates showed IIPRMKCP-9 (*Pseudomonas guariconensis*), IIPRAJCP-6 (*Bacillus amyloliquefaciens*), IIPRMKCP-10 (*Bacillus haynesii*), IIPRAMCP-5 (*Bacillus cereus*), IIPRSHEP-6 (*Bacillus subtilis*), and IIPRMKCP-3 (*Serratia macrescens*) and showed the maximum inhibition of all the tested *Fusarium* isolates.

### Plant growth parameters

In vitro plant growth-promoting traits of six most effective rhizobacteria are described in Fig. 6. The application

of PGPRS has a profound effect on the lentil, chickpea, and pigeonpea plants. The relative increase in shoot and root length due to bacterial inoculation ranged from 25 to 50% over uninoculated plants. Similarly, bacterial isolates increase the root and shoot biomass up to 65% and 70%. *Pseudomonas guariconensis* (IIPRMKCP-9) and *Bacillus subtilis* (IIPRSHEP-6) found significantly better than the other tested rhizobacteria. Rhizobacteria strains with plant growth potential and high antifungal potential were evaluated for the disease suppression and pulse plant growth under greenhouse conditions. All the tested bacterial strains showed enhanced seed germination (87–91%) compared to the control (76%). Seed mortality caused by *Fusarium* isolate was reduced by PGPR bacteria isolates. The maximum seed germination was shown by *Bacillus subtilis* (IIPRSHEP-6) followed by *Pseudomonas guariconensis* (IIPRMKCP-9) and *Bacillus amyloliquefaciens* (IIPRAJCP-6). Plant growth characteristics, viz., shoot length, root length, fresh weight, and dry weight, were significantly increased by the bacterial seed inoculation compared to the untreated control. All the tested bacterial strains enhanced the root and shoot length compared to the control. All the tested strains showed strong ability to produce IAA, siderophore, and phosphatase

**Fig. 4** In vitro antifungal activity of rhizobacteria against pulse wilt pathogen (*Fusarium oxysporum lentis*, *Fusarium oxysporum ciceris*, and *Fusarium udum*)



enzyme. Dry plant weight was also significantly higher in the treated plants over control plants (Fig. 6).

## Discussion

*Fusarium* wilt is a most destructive plant disease which poses a serious threat to plants by infecting the host plant at any growth stage and caused seedling death. It causes loss in many crops including fruits, cereal, pulses, and vegetable crops. *Fusarium* wilt pathogen is soil borne in nature and survives in soil for longer periods. In the present study, *Fusarium* spp. was isolated from the infected plants of pigeonpea, chickpea, and lentil. Rhizobacteria living in the rhizosphere interact with plants in many ways by increasing the plant growth and controlling the plant diseases. Rhizobacteria with plant growth and disease-suppressing potential are used as an alternative to chemical pesticides in the agriculture. In our study, 24 rhizobacterial isolates were recovered from the rhizosphere belonging to the *Bacillus*, *Acinetobacter*, *Enterobacter*, *Cedecea*, *Serratia*, and *Pseudomonas* genera. All identified bacterial isolates are frequently used for the management of wilt disease in chickpea, lentil, and pigeonpea. Results revealed that *Pseudomonas guariconensis* IIPRMKCP-9, *Bacillus amyloliquefaciens* IIPRAJCP-6, *Bacillus haysnesii* IIPRMKCP-10, *Bacillus cereus* IIPRAMKCP-5, *Bacillus subtilis* IIPRSHEP-6, and *Serratia macrescens* IIPRMKCP-3 showed the mycelial growth inhibition greater than 80% (Fig. 7). Mycelial growth inhibition could be attributed due to the process of antibiosis [31, 32]. It has been reported that production of various antimicrobial compounds (antimicrobial peptides, secondary metabolites,  $\beta$ -lactams, aminoglycoside, macrolides, quinolones and

flouroquinolones, streptogramin antibiotics, sulphonamides, tetracyclines, nitroimidazoles) are responsible for the phytopathogenic fungal growth inhibition. Most of the tested bacteria exhibit multiple PGP traits which aid in the growth promotion and disease reduction potential of PGPR. It is important to explore the potential of native rhizobacteria for their plant growth-promoting traits. In the present investigation, 7 bacterial strains showed strong positive response to IAA, siderophore and phosphate, and chitinase enzyme production. IAA production in bacteria generally depends on their ability to utilize tryptophan via indole pyruvic acid pathway. IAA helps in the root development, elongation, and proliferation and helps the plant to take up water and nutrients. All the bacterial strains produced IAA in varying concentration because of their different physiological characters (culture condition, growth stage, and substrate availability). However, it has been investigated that indole pyruvic decarboxylase is the enzyme which determines IAA biosynthesis. Siderophore production is the major biocontrol mechanisms exhibited by various plant growth-promoting rhizobacteria under iron-limiting conditions. PGPR produces a wide variety of siderophores, most of the bacterial siderophores are catecholates (i.e., enterobactin), and some are carboxylates (i.e., rhizobactin) and hydroxamates (i.e., ferrioxamine B). However, there are also certain types of bacterial siderophores that contain a mix of the main functional groups (i.e., pyoverdine). Microbial siderophores provide plants with Fe nutrition to enhance their growth when the bioavailability of Fe is low. Siderophores also play an important role in disease suppression by limiting iron which is necessary for virulence, which make iron unavailable for pathogens, thus inhibiting their growth. 16 s rRNA sequencing technique has been widely used for the classification and identification of



**Fig. 5** In vitro antifungal activity of Rhizobacteria against wilt pathogen pulses (*Fusarium oxysporum lentis*, *Fusarium oxysporum ciceris*, and *Fusarium udum*)



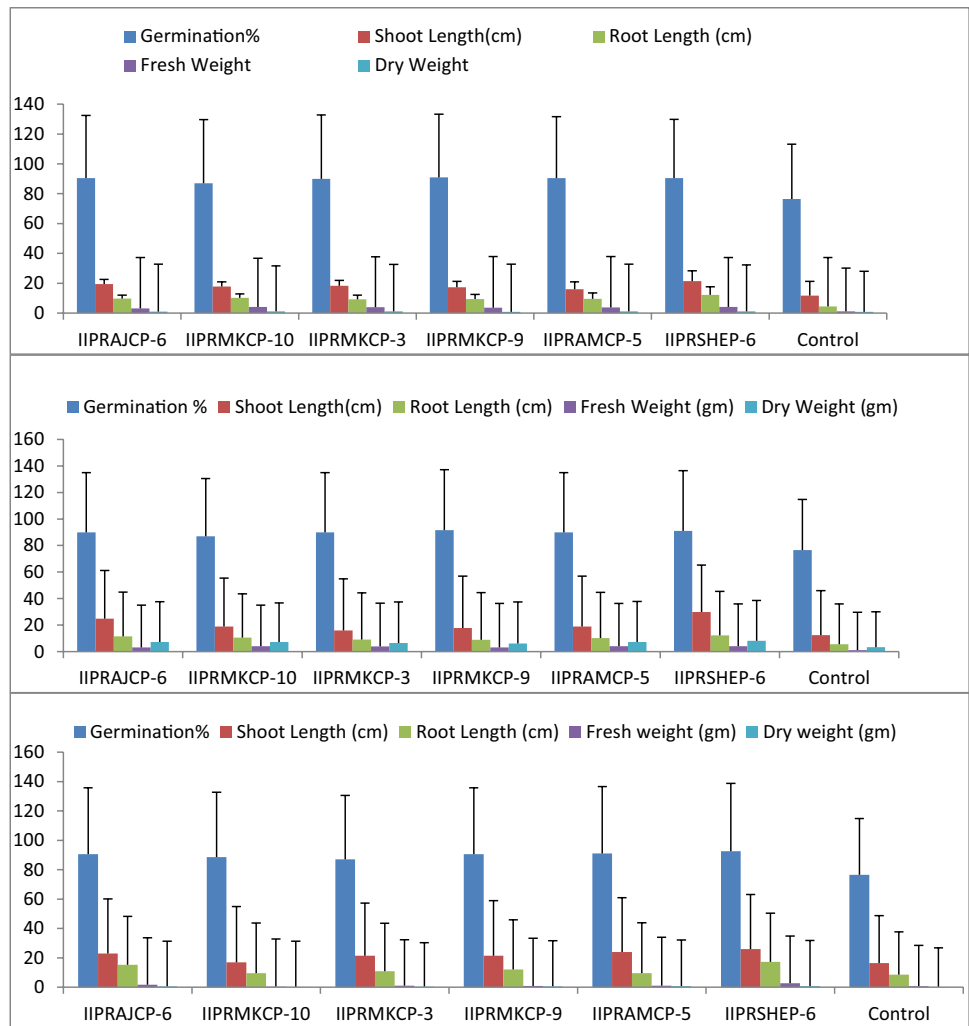
bacteria [32]. In the present study, 16 s rRNA-based maximum likelihood tree indicates that isolated bacteria belong to the genus *Bacillus*, *Acinetobacter*, *Enterobacter*, *Cedecea*, *Serratia*, and *Pseudomonas*.

In pulse crops, salinity affects the production of pulses [33]. It adversely affects the root nodulation, symbiotic relationship, and finally the nitrogen fixation. Many workers have demonstrated the negative effects of soil salinity on soybean, pigeonpea, and common bean, mungbean [34–40]. Salt-tolerant species of *Pseudomonas* have been found to increase the health of chickpea under saline conditions [41]. The application of *Agrobacterium*, *Pseudomonas*, and *Ochrobactrum* has been found to increase the salt tolerance of groundnut crop [42]. Co-inoculation of *Bacillus japonicum* and *Pseudomonas putida* has been found to increase the N and P content in soybean plant under salt stress [43].

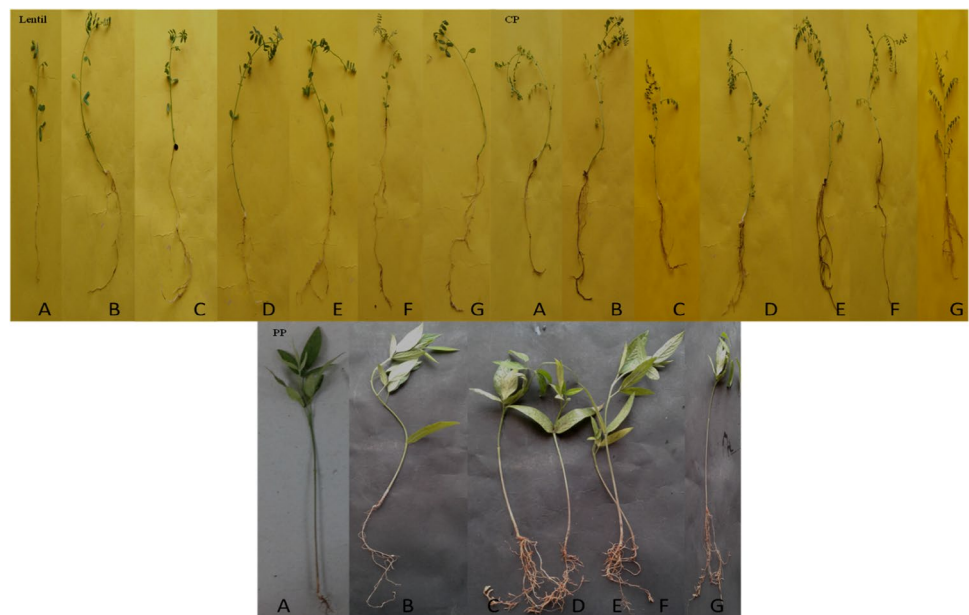
The present research clearly indicates that seed treatment with bacteria results in the decreased mortality rate of chickpea, pigeonpea, and lentil crops. Various studies have reported that rhizobacteria belonging to *Bacillus* and *Pseudomonas* genera are efficient in controlling the wilt disease in pulses [44]. The greenhouse study results revealed that all the tested PGPR strains significantly differ in their nature to control disease and PGP activity. All the tested bacterial strains performed better under greenhouse conditions compared to the control. Many studies have reports that PGP bacteria decrease the disease incidence in treated plants [45–50].

It has been observed that field application of bacteria-based products has been hampered because of their low performance due to various climatic and soil factors [51]. It was investigated that bacterial sporulation is greatly influenced by environmental factors. Development of multi-trait

**Fig. 6** Effect of selected plant growth-promoting bacteria (PGPB) on **A** lentil, **B** chickpea, and **C** pigeonpea growth characters (values are mean  $\pm$  SD of three replicates. Significant at  $p \leq 0.05$ )



**Fig. 7** Effect of different bacterial isolates on the growth characteristics of lentil, chickpea, and pigeonpea (**A** control, **B** IIPRAJCP-6 (*Bacillus amyloliquefaciens*), **C** IIPRMKCP-10 (*Bacillus haynesii*), **D** IIPRMKCP-3 (*Serratia macrescens*), **E** IIPRMKCP-9 (*Pseudomonas guariconensis*), **F** IIPRAMCP-5 (*Bacillus cereus*), **G** IIPRSHEP-6 (*Bacillus subtilis*))



bioformulation as well consortia is under process as a series of experiments with different soil types under greenhouse conditions, carrier material assessment etc. are going on.

## Conclusion

The findings of the present investigation suggest that identified most potential indigenous rhizobacteria strains could be utilized for making multi-trait biocontrol formulation as well as consortia as an eco-friendly alternative to the chemical fertilizers for soil and plant health management which enhances the production and productivity of pulse crops in a sustainable manner to increase the income of farming communities.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

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