

## ISOLATION OF PLANT GROWTH-PROMOTING *PSEUDOMONAS* SP. PPR8 FROM THE RHIZOSPHERE OF *PHASEOLUS VULGARIS* L.

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**Abstract:** *In vitro* screening of plant growth-promoting (PGP) traits was carried out using eight *Pseudomonas* spp., PPR1 to PPR8, isolated from the rhizosphere of *Phaseolus vulgaris* growing on the Uttarakhand Himalayan range in India. All the isolates were fast growers, positive for catalase, oxidase and urease activities, and utilized lactose and some amino acids. All the isolates were indole acetic acid (IAA) positive, however PPR8 solubilized potassium and zinc along with various other types of inorganic (tricalcium, dicalcium and zinc phosphate) and organic (calcium phytate) phosphates, as well as producing siderophore and ACC deaminase. PPR8 also produced cyanogens, extracellular chitinase,  $\beta$ -1,3-glucanase,  $\beta$ -1,4-glucanase and oxalate oxidase. Based on the PGP traits of all isolates, PPR8 was found to be the most potent plant growth-promoting rhizobacteria (PGPR). Further, PPR8 was identified as *Pseudomonas* sp. PPR8, based on 16 S rRNA gene sequencing analysis. Moreover, the PGP activities of PPR8 confirmed it to be a potent biocontrol agent, inhibiting the growth of various plant pathogenic fungi. This study reveals the potential of *Pseudomonas* sp. PPR8 to be used as a good bioinoculant for growth promotion of common bean and for the protection of important legume crops from various deleterious phytopathogens.

**Key words:** *Pseudomonas* sp. PPR8; *Phaseolus vulgaris*; plant growth promotion; biocontrol

## INTRODUCTION

The rhizosphere represents a thin layer of soil surrounding plant roots, which supports the large active groups of bacteria known as plant growth-promoting rhizobacteria (PGPR) [1]. PGPR are known to rapidly colonize the rhizosphere and suppress soil-borne pathogens at the root surface. These organisms can also be beneficial to the plants by stimulating their growth [2,3]. Among PGPR, pseudomonads are considered to be the most promising group of PGPR involved in growth promotion and biocontrol of plant pathogens [4]. In previous studies, *Pseudomonas* spp. have shown increased production of indole acetic acid (IAA), which has a significant role in the root

development system [5]. IAA stimulates the elongation of plant cells and helps in cell division, thus helping in root growth directly or by the influence on the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity of bacteria indirectly [6]. Levels of potassium in the soil have dropped due to rapid developmental changes in world agriculture, including leaching, runoff, crop removal and erosion. Potassium can increase the plant height, fresh plant weight and also increase herbage and oil yield in patchouli [7]. As reported previously, potassium solubilizing bacteria (KSB) and phosphate solubilizing bacteria (PSB) have been found to increase the uptake of primary macronutrients such as nitrogen, phosphate and potassium

in eggplant, pepper and cucumber, leading to a higher crop yield [8]. Indian soils are generally low in zinc and as much as half of the country's soils are categorized to be zinc-deficient. Total and available zinc content in Indian soils ranged between 7-2960 mg/kg and 0.1-24.6 mg/kg, respectively. Zinc is one of the eight essential trace elements or micronutrients required for the normal healthy growth and reproduction of crop plants. Iqbal et al. [9] reported growth enhancement of mung bean by zinc solubilizing PGPR, which could be utilized to improve the growth of economically important cash crops. In nature, several microorganisms have the ability to solubilize phosphorus (organic and inorganic) and make it available for the growth and development of plants [10,11]. Pseudomonads have conferred several primary mechanisms as biocontrol strategies, including the production of antibiotics and secondary metabolites, cell wall-degrading enzymes, as well as production of hydrogen cyanide (HCN) and siderophores [12,13].

The ability of PGPR to produce siderophores that bind to most of the Fe III present around the plant root, has proven to be a significant factor in reducing the devastation caused by plant pathogenic fungi. A deficiency of iron in their vicinity inhibits the growth and proliferation of pathogens, and as a result, PGPR out-compete the pathogens for available iron, thus causing their death. In addition, when siderophores are produced by *Pseudomonas* spp. in limited iron conditions, they have been found to show antagonistic phenomenon against several plant pathogens, including *Pythium* spp., and are also associated with root rot disease of many crop plants [14]. In addition, hydrogen cyanide, a secondary metabolite produced by various PGPR such as *P. fluorescens*, has been found to suppress root rot disease of *Coleus forskohlii*, caused by *Macrophomina phaseolina* [15,16].

*Phaseolus vulgaris* L. (common bean) is an important legume for human nutrition and a major source of protein and calories in the world [17]. It belongs to the subfamily Papilionaceae of the family Fabaceae. Common bean is widely cultivated in the tropics and subtropics as well as in temperate regions of the world [18]. It is an important pulse crop of India, cultivated

in Sub-Himalayan and Higher-Himalayan ranges at 1200-1800 m, mainly in Maharashtra, Himachal Pradesh, Jammu & Kashmir and Uttarakhand. In India, the common bean is cultivated under an area of 9700 million hectares. Due to its significant nutritive value, it has been known as one of the most important pulse crops worldwide [19]. An increase in the production of common bean may be one of the best alternatives to meet the demand for pulse crops, which can be achieved either by the use of chemical fertilizers or of rhizosphere-competent microorganisms [20]. However, the continuous use of chemical fertilizers has detrimental effects on host plants and soil [21]. A potential way to decrease the negative impacts caused by chemical fertilizers is the introduction of PGPR inoculants; therefore, the current research was designed to isolate and characterize PGPR (*Pseudomonas* spp.) from common bean and to evaluate their antagonistic properties against phytopathogens associated with common bean.

## MATERIALS AND METHODS

### Isolation of bacteria

The isolation of bacteria from the rhizosphere of *P. vulgaris* was carried out using standard microbiological techniques [22]. A total of eight isolates (PPR1-PPR8) was selected based on preliminary screening, and was maintained on nutrient agar (NA) at 4°C for further use. Identification of the culture strains was based on the morphological, physiological and biochemical characterization as followed by Bergey's Manual of Determinative Bacteriology [20], and compared against standard strains, *Pseudomonas fluorescens* MTCC-103, *Pseudomonas* sp. MTCC-129 and *P. aeruginosa* MTCC-1934, procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

### Isolation of fungal pathogens

The diseased root and seed samples were collected from the rhizosphere of *P. vulgaris* growing area. Two plant pathogenic fungi, *Macrophomina phaseolina* and

*Fusarium oxysporum*, were isolated using the water agar and blotter techniques, respectively. In addition, *F. solani*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Colletotrichum* sp., available in our laboratory culture collection, were also used in this study.

### HiMedia Enterobacteriaceae identification kit

The HiMedia Enterobacteriaceae identification kit obtained from the HiMedia Co. Ltd. (Mumbai, India) was used according to the manufacturer's instructions. The following tests were performed using kits: ONPG (ortho-Nitrophenyl- $\beta$ -galactoside), lysine decarboxylase, ornithine decarboxylase, deamination, nitrate reduction, H<sub>2</sub>S production, Voges-Proskauer, methyl red, indole, malonate and esculin hydrolysis [20].

### 16 S rRNA gene sequencing

The genomic DNA from PPR8 was isolated following the method of Sambrook and Russel [23]. Amplification of the 16 S rRNA gene was carried out by PCR (PTC 100, M.J. Research, USA) using universal eubacterial primers FD1 5' CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TC AG 3' and RD1 5' CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC 3' [20,24]. The 16 S rRNA gene sequence was aligned for similarity using the BLAST program for the GenBank database (NCBI).

### Production of indole-3-acetic acid (IAA)

Production of IAA from bacterial isolates was determined by the method as described previously [25]. The growth and culture of bacteria were maintained on Luria-Bertani (LB) broth and incubated at 28°C for 24 h at 120 rpm [20]. To obtain the cell-free supernatant, culture from the exponential phase was centrifuged at 10000 g for 15 min at 4°C. For confirmation of IAA production by bacterial isolates, 2 drops of o-phosphoric acid were added to the supernatant of each bacterial isolate to generate a pink color as the confirmatory measure of IAA production.

### Determination of phosphate solubilization

A method of spotting of isolates on Pikovskaya agar plates was applied to confirm the ability of bacterial isolates for phosphate solubilization as reported previously [26]. Two media, namely phosphate solubilization-bromophenol blue medium (PSBPBM) and rhizospheric phosphate solubilizing medium (PSM) were used separately, with dicalcium phosphate (DCP), tricalcium phosphate (TCP) and zinc phosphate (ZP) as the major sources of insoluble inorganic phosphate [27]. Bromophenol blue was used as a pH indicator. Finally, spot-inoculated plates were incubated at 28±1°C for 3 days and observed for the appearance of the clearing zone around the colonies [20]. Quantitative estimation of water extractable free inorganic P (P<sub>i</sub>) using National Botanical Research Institute Phosphate (NBRIP) broth [20] was done as described by Nautiyal [27].

### Determination of phytase production

To confirm the ability of bacterial isolates to produce phytase, the log phase cultures of each bacterial isolate were spot-inoculated on phytase screening medium containing calcium and sodium phytate as the sole sources of organic phosphate [20]. Thereafter, inoculated plates were examined for phytase activity by observing a halo zone formation around the colonies following an incubation of 3 days at 28±1°C.

### Determination of organic acid production

To confirm the production of organic acid, cultures of bacterial isolates were grown in MM9 agar medium and observed for the decline in pH using methyl red as an indicator. The color changes from yellow to pink below pH 5.0. Isolates with the ability to produce organic acid formed a pink zone around the bacterial colony.

### Determination of potassium solubilization

A method described by Hu et al. [28] was adopted to determine the ability of each bacterial isolate for potassium solubilization, and the log phase cultures of each bacterial isolate were spot-inoculated on Aleksandrov

agar plates containing potassium aluminum silicate as a sole source of insoluble inorganic potassium [20]. Bromophenol blue was used as a pH indicator. The spot-inoculated plates were then examined for the appearance of clear halo zones around the developed colonies following a 3-5-day incubation at  $28\pm 1^\circ\text{C}$ .

### **Determination of zinc solubilization**

The zinc solubilization ability of bacterial strains was determined by spotting the log phase cultures of each bacterial isolate on Tris-minimal medium [29] containing zinc phosphate and zinc carbonate as the sole sources of insoluble inorganic zinc. Bromophenol blue was used as a pH indicator. The spot-inoculated plates were then examined for the appearance of clear halo zones around the developed colonies following a 3-5-day incubation at  $28\pm 1^\circ\text{C}$ .

### **1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity**

ACC deaminase enzymatic activity of bacterial isolates was determined by the previously reported method [30]. The log phase cultures of each bacterial isolate were used to determine the activity of ACC deaminase. The cultures were harvested to obtain the pellets after centrifugation at 10000 rpm (Remi C-30). Pellets were washed two times using sterilized saline water and resuspended, followed by spot-inoculation on agar plates in minimal medium containing ACC as the sole source of nitrogen. Ammonium sulphate served as a positive control, and the medium without a nitrogen source served as a negative control, and the plates were incubated at  $28\pm 1^\circ\text{C}$  for 72 h [20].

The bacterial isolates were further inoculated in the minimal medium containing ACC as a sole source of nitrogen [20]. Bacterial growth in minimal medium was recorded by measuring the absorbance spectrophotometrically at 600 nm at different time intervals. A serial dilution spread plating method was also used to determine the viable counts, and the multiplication in bacterial cell counts of the test organisms in the minimal medium showed their ability to produce

ACC deaminase while utilizing ACC as a sole source of nitrogen.

A previously reported method was adopted to measure ACC deaminase enzyme activity [31]. Briefly, a 0.2-mL enzymatic extract and ACC (50 mM) were mixed in 0.2 mL of Tris buffer (0.1 M; pH 7.5), and the reaction was stopped by the addition of 1.8 mL of HCl (0.56 N). Further, 0.3 mL of 2, 4-dinitrophenylhydrazine (1% DNPH) solution was added to the reaction mixture, followed by a 30-min incubation at  $30^\circ\text{C}$ , followed by the addition of 2 mL of 2 N NaOH to the reaction mixture, and spectrophotometric measurement of absorbance of the reaction mixture was done at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that liberates 1  $\mu\text{g}$  of  $\alpha$ -ketobutyrate per min. Each experiment was performed 4 times [20].

### **Determination of HCN production**

For the confirmation of the production of hydrogen cyanide (HCN), a previously reported method was adopted with some modifications [32]. The cultures of bacterial isolates growing in the log phase were streaked on agar plates supplemented with or without 4.4 g glycine/L separately with simultaneous addition of filter paper soaked in 0.5% picric acid in 1%  $\text{Na}_2\text{CO}_3$  in the upper lid of the plate, and the plates were incubated at  $28\pm 1^\circ\text{C}$  and sealed with parafilm [20]. The uninoculated plates served as a control. The plates were examined putatively for the color change from yellow to light brown, moderate brown or strong brown for the confirmation of HCN production.

### **Determination of siderophore production**

For the determination of siderophore production, a method followed by Schwyn and Neilands [33] was adopted utilizing Chromeazurol S (CAS) medium. The log phase-grown cultures of bacterial isolates were spot-inoculated on CAS medium followed by incubation at  $28\pm 1^\circ\text{C}$  for 48-72 h. Plates were examined for the formation of orange to yellow halo zones around the developed colonies as confirmation of siderophore production.

### Determination of cell wall-degrading enzymes

The method of Dunne et al. [34] was followed for the quantitative determination of chitinase production. The log phase-grown cultures of bacterial isolates were spot-inoculated on minimal medium agar plates containing chitin as a sole source of carbon, followed by incubation for 7 days at  $30\pm 2^\circ\text{C}$ . The plates were observed for the development of clear halo zones around the developed colonies, confirming chitinase production. Enzymatic activities of  $\beta$ -1, 3-glucanase and  $\beta$ -1, 4-glucanase were also measured using chitin minimal medium (CMM) containing laminarin azure and cellulose powder, respectively. Bacterial isolates were grown on CMM agar plates utilizing laminarin azure, and cellulose powder as the sole sources of carbon [20].

### Determination of oxalate oxidase (OO) enzyme production

The method of Dickman and Mitra [35] was followed to assess the production of oxalate oxidase enzyme. The log phase cultures (12 h) of bacterial isolates were spot-inoculated on agar plates of oxalic acid degradation selective medium containing ammonium oxalate as a sole source of carbon, followed by incubation for 7 days in the dark at  $30\pm 1^\circ\text{C}$ . The plates were examined for the development of clear zones around the developed colonies on agar plates as confirmation of the production of OO. Oxalic acid is a known pathogenicity factor of certain phytopathogens [36].

### Antagonism against phytopathogens

All *Pseudomonas* isolates were tested for their antagonistic activities against various plant pathogenic fungi, including *F. oxysporum*, *F. solani*, *M. phaseolina*, *S. sclerotiorum*, *R. solani* and *Colletotrichum* sp., in the agar medium. *Pseudomonas* isolates were spot-inoculated on the agar medium 2 cm away from the center of fungal disk showing mycelial growth. Antagonistic activity was recorded on the 7<sup>th</sup> day after incubation at  $28^\circ\text{C}\pm 1^\circ\text{C}$ . The zones of inhibition were measured and the plates without bacterial inoculum were used as a

control. The percentage of inhibition of fungal growth was calculated by the following formula:  $100\times C-T/C$  (T, treatment; C, control).

### Antagonism using cell-free culture filtrate (CFCF)

To obtain CFCFs, the cultures of all the bacterial isolates growing in the log phase were centrifuged at 8000 rpm, for 10 min, at  $4^\circ\text{C}$ , and filtered with a 0.45-Millipore filter. Further, a 5-day-old mycelia disk cut from actively growing margins of the plant pathogenic fungi such as *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *R. solani* and *Colletotrichum* sp., were separately transferred to the center of the YEMA-PDA (1:1) media agar plates. A 0.5-mm well was made in each agar plate, 2 cm away from the fungal disk, and the CFCF of each bacterial isolate was aliquoted into each well, followed by further incubation for 5-6 days at  $28\pm 1^\circ\text{C}$ . Clearing of a fungal zone, if any, was recorded.

## RESULTS

### Isolation of bacterial strains

All *Pseudomonas* isolates (PPR1-PPR8) were Gram-negative, non-spore forming, non-capsulated, motile and rod-shaped in structure. The colonies were smooth, translucent, large, low convex, 2-4 mm in diameter with regular spreading edges on the nutrient agar plate (Table 1).

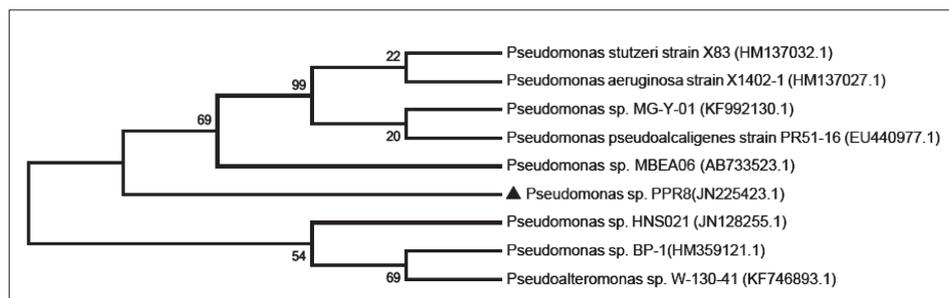
### 16 S rRNA gene sequencing

The 16 S rRNA gene sequencing of PPR8 was carried out comprising a 904-bp-long sequence (NCBI gene bank accession number JN225423). A neighbor-joining dendrogram was constructed using 16 S rRNA-gene sequencing of PPR8 and representative sequences from NCBI databases, which showed 100% sequence similarity to *Pseudomonas* sp. BP-1 (HM359121) and 99% to *Pseudomonas* sp. MBEA06 (AB733523) and *Pseudomonas* sp. HNS021 (JN128255) (Fig. 1).

**Table 1.** Morphological, physiological and biochemical characters of *Pseudomonas* spp. from rhizosphere of *Phaseolus vulgaris*.

| Characteristics                          | PPR1 | PPR 2 | PPR 3 | PPR 4 | PPR 5 | PPR 6 | PPR 7 | PPR 8 | MTCC 103 | MTCC 129 | MTCC 1993 |
|--|------|-------|-------|-------|-------|-------|-------|-------|----------|----------|-----------|
| Gram reaction                            | -    | -     | -     | -     | -     | -     | -     | -     | -        | -        | -         |
| Shape                                    | Rod  | Rod   | Rod   | Rod   | Rod   | Rod   | Rod   | Rod   | Rod      | Rod      | Rod       |
| Motility                                 | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Capsule                                  | -    | -     | +     | -     | -     | -     | -     | -     | -        | -        | -         |
| Endospore                                | -    | -     | -     | -     | -     | -     | -     | -     | -        | -        | -         |
| Fluorescence                             | -    | +     | -     | -     | -     | -     | -     | -     | +        | -        | -         |
| Pigment                                  | -    | -     | +     | +     | -     | -     | +     | +     | +        | +        | +         |
| PHB accumulation                         | +    | -     | +     | -     | -     | +     | -     | +     | -        | +        | +         |
| Generation time (h)                      | 1.3  | 1.3   | 1.4   | 1.3   | 1.4   | 1.4   | 1.3   | 1.3   | 1.2      | 1.3      | 1.4       |
| 8% KNO <sub>3</sub> tolerance            | -    | -     | -     | -     | +     | -     | -     | -     | +        | -        | +         |
| Catalase                                 | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Ca-glyc                                  | +    | +     | -     | +     | +     | +     | +     | +     | +        | +        | +         |
| Gelatin hydrolysis                       | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Starch hydrolysis                        | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| ONPG <sup>K</sup>                        | -    | +     | +     | -     | -     | -     | +     | +     | -        | +        | +         |
| Lysine decarboxylase <sup>K</sup>        | +    | +     | +     | -     | +     | +     | +     | +     | +        | +        | -         |
| Ornithine decarboxylase <sup>K</sup>     | +    | +     | +     | -     | +     | +     | +     | +     | -        | +        | +         |
| Urease                                   | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Phenylalanine deamination <sup>K</sup>   | -    | -     | -     | -     | -     | -     | -     | -     | -        | -        | -         |
| Nitrate reduction <sup>K</sup>           | -    | +     | -     | -     | +     | +     | +     | -     | +        | -        | +         |
| H <sub>2</sub> S production <sup>K</sup> | -    | -     | -     | -     | -     | -     | -     | -     | -        | -        | -         |
| Citrate utilization <sup>K</sup>         | -    | -     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Voges-Proskaur <sup>K</sup>              | -    | +     | +     | -     | +     | +     | -     | -     | +        | -        | +         |
| Methyl red <sup>K</sup>                  | -    | -     | -     | -     | -     | -     | -     | -     | -        | -        | -         |
| Indole <sup>K</sup>                      | -    | +     | -     | -     | +     | -     | -     | +     | +        | +        | +         |
| Malonate <sup>K</sup>                    | -    | +     | -     | +     | +     | +     | -     | -     | +        | -        | +         |
| Esculin hydrolysis <sup>K</sup>          | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |
| Oxidase <sup>K</sup>                     | +    | +     | +     | +     | +     | +     | +     | +     | +        | +        | +         |

**Abbreviations:** + – positive; - – negative; PHB – polyhydroxybutyrate; Ca-glyc – precipitation in calcium glycerophosphate; ONPG – *o*-nitrophenyl- $\beta$ -D-galactoside; K – Performed using Hi25™ Hi-Media Enterobacteriaceae Identification Kit along with standard phenotypic test following Bergey's Manual of Determinative Bacteriology, *Pseudomonas fluorescens* MTCC-103; *Pseudomonas* sp. MTCC-129; *Pseudomonas aeruginosa* MTCC-1934.



**Fig. 1.** Phylogenetic analysis of *Pseudomonas* sp. PPR8 (JN225423) (904bp) isolated from the rhizosphere of *P. vulgaris* based on 16 S rRNA gene sequences available online from the National Center for Biotechnology Information (NCBI) database. The tree was constructed after multiple alignments of sequence data by ClustalW. Distance and clustering with the neighbor-joining method was performed using Mega 4. Bootstrap values based on 1000 replications, listed as percentages at the branching points.

## IAA production

All the isolates of *Pseudomonas* spp. (PPR1-PPR8) produced IAA. The results confirmed that all the bacterial isolates were able to develop a pink color in their respective culture broth with or without the addition of tryptophan along with their cell-free culture supernatants. Development of pink color was common in all the strains; however, *Pseudomonas* sp. PPR8 produced the maximum IAA of 39 µg/mL (Table 2).

## Phosphate solubilization

All the isolates of *Pseudomonas* spp. were able to form clear halo zones around their spot-inoculations by solubilizing tricalcium phosphate (TCP) on Pikovskaya agar. A decline in the pH of Pikovskaya medium containing bromothymol blue (BTB) resulted in the color change of the medium. Similar experiments were performed using Pikovskaya agar medium by replacing TCP by DCP and ZP. However, no bacterial

isolates solubilized DCP, while PPR3, PPR4 and PPR8 solubilized ZP (Table 2).

The Pikovskaya agar plate assay is used for screening phosphate solubilization bacteria, although it provides variable results. The bromophenol blue phosphate solubilization medium (BPBM) and rhizospheric phosphate solubilizing medium (PSM) were used separately to confirm the results of P solubilization. In NBRIP, four isolates of *Pseudomonas* spp. (PPR3, PPR5, PPR7 and PPR8) solubilized P of TCP. The advantage of using this method is that it assists in the provision of comparatively quick and better results on P solubilization than the Pikovskaya agar plate method, as the pH shift and zones could be visible overnight and initialize after 12-14 h; in Pikovskaya's assay, it takes from 48 h to several days to obtain results. In BPBM (DCP used as a sole source of insoluble inorganic P), only three isolates of *Pseudomonas* spp. (PPR1, PPR4 and PPR8) solubilized phosphate. In BPBM (ZP used as a sole source of insoluble inorganic P), only two isolates of *Pseudomonas* spp. (PPR5 and

**Table 2.** Plant growth-promoting attributes of *Pseudomonas* spp. isolated from *P. vulgaris*.

| Isolates                 | IAA <sup>A</sup> | HCN <sup>B</sup> | Siderophore <sup>C</sup> | Phosphate solubilization <sup>D</sup> |     |    |                 |     |    |                         |     |    |                    |        |  |
|--------------------------|------------------|------------------|--------------------------|---------------------------------------|-----|----|-----------------|-----|----|-------------------------|-----|----|--------------------|--------|--|
|                          |                  |                  |                          | Inorganic phosphate                   |     |    |                 |     |    | Organic phosphate       |     |    |                    |        |  |
|                          |                  |                  |                          | Pikovskaya's medium                   |     |    | NBRI-BPB medium |     |    | Rhizospheric BCG medium |     |    | Phytase production |        |  |
|                          |                  |                  |                          | TCP                                   | DCP | ZP | TCP             | DCP | ZP | TCP                     | DCP | ZP | Ca-Phy             | Na-Phy |  |
| PPR1                     | +                | -                | +                        | +                                     | -   | -  | -               | +   | -  | +                       | -   | -  | -                  | -      |  |
| PPR2                     | +                | +                | +++                      | +                                     | -   | -  | -               | -   | -  | -                       | -   | -  | -                  | -      |  |
| PPR3                     | ++               | +                | +                        | +                                     | -   | +  | +               | -   | -  | +                       | -   | -  | -                  | -      |  |
| PPR4                     | -                | +                | +                        | +                                     | -   | +  | -               | +   | -  | +                       | +   | -  | -                  | -      |  |
| PPR5                     | ++               | -                | +                        | +                                     | -   | -  | +               | -   | +  | -                       | -   | +  | -                  | -      |  |
| PPR6                     | +                | -                | ++                       | ++                                    | -   | -  | -               | -   | -  | -                       | -   | -  | +                  | -      |  |
| PPR7                     | ++               | +                | ++                       | +                                     | -   | -  | +               | -   | -  | -                       | -   | -  | -                  | -      |  |
| PPR8                     | +++              | ++               | +++                      | +++                                   | -   | ++ | +++             | +   | ++ | ++                      | ++  | ++ | ++                 | +      |  |
| <b>Standard cultures</b> |                  |                  |                          |                                       |     |    |                 |     |    |                         |     |    |                    |        |  |
| MTCC 103                 | +                | -                | -                        | +                                     | -   | -  | +               | -   | -  | +                       | -   | -  | -                  | -      |  |
| MTCC 129                 | +                | +                | ++                       | +                                     | -   | -  | +               | -   | -  | +                       | -   | -  | -                  | -      |  |
| MTCC 1934                | -                | -                | +                        | -                                     | -   | -  | +               | -   | -  | +                       | -   | -  | -                  | -      |  |

**Abbreviations:** A -, IAA negative, +, IAA positive; B -, HCN negative, +, HCN positive; C -Siderophore production negative; +, Siderophore production positive; D -, Phosphate solubilization negative; +, phosphate solubilization positive, -, Absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0 cm wide surrounding colonies; NBRI-BPB medium (National Botanical Research Institute Phosphate Solubilization Media with bromophenol blue (0.025%) as pH indicator); Rhizospheric BCG medium (Rhizospheric Phosphate Solubilization Media with bromocresol green (0.01%) as pH indicator); TCT (tricalcium phosphate), DCT (dicalcium phosphate), ZP (zinc phosphate); *Pseudomonas fluorescens* MTCC 103; *Pseudomonas* sp. MTCC-129; *Pseudomonas aeruginosa* MTCC-1934; All experiments were done in triplicate with three independent trials.

PPR8) solubilized phosphate as shown by the halo zone formation due to the decrease in the pH. In rhizospheric PSM, only four isolates of *Pseudomonas* (PPR1, PPR3, PPR4 and PPR8) solubilized P of TCP. In PSM, only two isolates of *Pseudomonas* (PPR4 and PPR8) solubilized phosphate. In PSM (ZP used as a sole source of insoluble inorganic P), only two *Pseudomonas* isolates (PPR5 and PPR8) solubilized phosphate as shown by the halo zone formation (Table 2).

To quantify phosphate solubilization, BPBM broth was used. When TCP was used as a substrate of insoluble inorganic P, the maximum phosphate solubilization was recorded in PPR8 (25 mg/mL). However, upon use of DCP as a substrate of insoluble inorganic P, the maximum phosphate was recorded in *Pseudomonas* sp. PPR8 (19 mg/mL). Similarly, the maximum phosphate solubilization from insoluble inorganic P as ZP (21 mg/mL) was recorded by *Pseudomonas* sp. PPR8 after a 7-day incubation (Fig. 1).

### Phytase production

Only two bacterial isolates of *Pseudomonas* spp. (PPR6 and PPR8) were capable of solubilizing calcium phytate by forming a halo zone around their

spots, confirming the release of free phytase; however, *Pseudomonas* sp. PPR8 also solubilized sodium phytate (Table 2).

### Organic acid production

All *Pseudomonas* isolates (except PPR1) were able to form clearing zones around their bacterial colonies with a shift from a pink/orange background to a white one, indicating a decline in the pH due to the production of organic acids. When DCP was used as a substrate, similar background changes were observed only in four isolates of *Pseudomonas* (PPR1, PPR4, PPR6, and PPR8). When ZP was used as a substrate, PPR3, PPR6 and PPR8 produced the shift in color from pink/orange to white around the bacterial spots, indicating the production of organic acids (Table 3).

### Potassium solubilization

Isolates of *Pseudomonas* spp. PPR2, PPR5, PPR7 and PPR8 were able to produce visible zones of potassium solubilization. Also these isolates showed the change in color along with an indication of the pH change, suggesting a decrease in the pH around the bacterial spots in AAM (Table 3).

**Table 3.** Plant growth promoting attributes of *Pseudomonas* spp. isolated from *P. vulgaris*.

| Isolates                 | ACCD | Potassium solubilization | Zinc solubilization |    | Organic acid production |     |    | Chitinase | $\beta$ -1,3-glucanase (laminarinase) | $\beta$ -1,4-glucanase | Oxalate-oxidase |
|--------------------------|------|--------------------------|---------------------|----|-------------------------|-----|----|-----------|---------------------------------------|------------------------|-----------------|
|                          |      |                          | ZP                  | ZC | TCP                     | DCP | ZP |           |                                       |                        |                 |
| PPR1                     | -    | -                        | -                   | -  | -                       | +   | -  | -         | +                                     | +                      | -               |
| PPR2                     | -    | +                        | -                   | -  | +                       | -   | -  | -         | +                                     | +                      | -               |
| PPR3                     | -    | -                        | -                   | -  | +                       | -   | +  | -         | +                                     | -                      | -               |
| PPR4                     | -    | -                        | +                   | -  | +                       | +   | -  | -         | +                                     | -                      | -               |
| PPR5                     | -    | +                        | -                   | -  | +                       | -   | -  | -         | +                                     | -                      | -               |
| PPR6                     | -    | -                        | -                   | -  | +                       | +   | +  | +         | -                                     | +                      | -               |
| PPR7                     | -    | +                        | -                   | -  | +                       | -   | -  | -         | -                                     | +                      | +               |
| PPR8                     | ++   | ++                       | +                   | -  | +                       | +   | +  | ++        | +                                     | +                      | +++             |
| <b>Standard cultures</b> |      |                          |                     |    |                         |     |    |           |                                       |                        |                 |
| MTCC 297                 | -    | -                        | -                   | -  | +                       | +   | +  | -         | -                                     | -                      | -               |
| MTCC 441                 | -    | -                        | +                   | -  | +                       | +   | +  | -         | -                                     | -                      | -               |
| MTCC 122                 | -    | -                        | -                   | -  | -                       | +   | +  | -         | -                                     | -                      | -               |

**Abbreviations:** -, negative, +, positive; -, absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0cm wide surrounding colonies. All experiments were done in triplicate with three independent trials.

### Zinc solubilization

Zn, an insoluble micronutrient, is also required by plants from the soil. Two isolates, PPR4 and PPR8, solubilized zinc phosphate with the visible parameter of pH change as confirmed by the color change around their respective inoculation spots on the agar medium. No isolate was capable of solubilizing zinc carbonate (Table 3).

### ACC deaminase production

*Pseudomonas* sp. PPR8 produced ACC deaminase (71 nM  $\alpha$ -ketobutyrate/mg/h). The other strains failed to utilize ACC as a sole source of N in the minimal medium, reflecting their inability to produce ACC deaminase (Table 3).

### HCN production

Five isolates of *Pseudomonas* spp. (PPR2, PPR3, PPR4, PPR7 and PPR8) were able to produce HCN as seen by the color change of filter paper from yellow to moderate and reddish brown. When glycine was present in the medium, the color of the filter paper turned to deep brown, clearly indicating the production of HCN by bacterial isolates (Table 2).

### Siderophore production

All the isolates of *Pseudomonas* spp. were screened for siderophore production on CAS agar and found to be positive, as confirmed by the forming of orange halo zones around their respective spots (Table 2). Quantitative analysis revealed that *Pseudomonas* sp. PPR8 produced 32  $\mu$ g/mL of siderophore in the CAS medium.

### Cell wall-degrading enzymes

*Pseudomonas* spp. PPR6 and PPR8 showed chitinase activity. All the isolates of *Pseudomonas* spp., except PPR6 and PPR7, grew on laminarin azure-amended minimal medium, indicating the production of  $\beta$ -1,3-glucanase. Most of the *Pseudomonas* spp. utilized cellulose

in the minimal medium, indicating the production of  $\beta$ -1,4-glucanase. *Pseudomonas* sp. PPR8 showed the maximum chitinase activity (3.4 U/mL/h) (Table 3).

### Production of the OO enzyme

All the isolates were tested for their ability to degrade oxalic acid by producing OO. *Pseudomonas* spp. PPR7 and PPR8 were found to degrade oxalic acid as indicated by the formation of a clear halo around the bacterial spots on the plates containing oxalic acid degradation selective medium (Table 3).

### Antagonistic activities of *Pseudomonas* isolates using dual cultures and cell-free culture filtrates

The increase in the growth inhibition of fungal pathogens corresponded to the incubation period. *Pseudomonas* sp. PPR8 showed the maximum inhibition (%) against *M. phaseolina* (64.98%, 48.41%) followed by *F. oxysporum* (66.58%, 49.85%), *F. solani* (63.69%, 45.39%), *S. sclerotiorum* (52.37%, 44.54%), *R. solani* (53.28%, 42.96%) and *Colletotrichum* sp. (54.48%, 45.59%) in dual culture and CFCF, respectively (Table 4). Colony growth inhibition was more pronounced in the dual culture in comparison to that of cell-free culture filtrate.

## DISCUSSION

The rhizosphere is a dynamic root region creating the ecological environment for plant-microbe interaction

**Table 4.** Antagonistic activities of *Pseudomonas* sp. PPR8 isolated from *Phaseolus vulgaris* against fungal phytopathogens.

| Fungal phytopathogens     | Growth inhibition (%) by <i>Pseudomonas</i> sp. PPR8 |                 |
|---------------------------|--|-----------------|
|                           | Dual culture   | CFCF            |
| <i>M. phaseolina</i>      | 64.98 $\pm$ 0.4                                      | 48.41 $\pm$ 0.4 |
| <i>F. oxysporum</i>       | 66.58 $\pm$ 0.7                                      | 49.85 $\pm$ 0.6 |
| <i>F. solani</i>          | 63.69 $\pm$ 0.4                                      | 45.39 $\pm$ 0.3 |
| <i>S. sclerotiorum</i>    | 52.37 $\pm$ 0.2                                      | 44.54 $\pm$ 0.3 |
| <i>R. solani</i>          | 53.28 $\pm$ 0.4                                      | 42.96 $\pm$ 0.4 |
| <i>Colletotrichum</i> sp. | 54.48 $\pm$ 0.3                                      | 45.59 $\pm$ 0.4 |

CFCF – cell-free culture filtrate.

and provision of essential macro- and micronutrients from a limited nutrient pool [37]. Isolation of *Pseudomonas* species from the rhizosphere of different crops has previously been widely studied. *Pseudomonas* spp. are one of the most promising groups of inhabitants of the rhizosphere that enhance the growth of crop plants and control soil-borne phytopathogens. They are known to suppress several phytopathogens such as *Fusarium udum* [38], *M. phaseolina* [4], *Pythium* spp. [39], and *Rhizoctonia* spp. [40]. Among the PGP traits, IAA production by the PGPR has a cascading effect on plant development due to its ability to influence root growth, which in turn affects nutrient uptake and plant productivity [41]. The capability to increase plant growth parameters in germinating seed bioassays is highly related to the IAA level produced by *Pseudomonas* spp. The other complex interactions between plants and bacteria producing IAA may be due to the promotion in root growth, directly by stimulating plant cell elongation or cell division, or indirectly by influencing bacterial ACC deaminase activity [41].

Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality and resistance to plant diseases are associated with phosphorus nutrition [11]. Different bacterial species are capable of solubilizing complex inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, rock phosphate and zinc phosphate, which increase the availability of phosphorus in the soil [10]. The above findings support that *Pseudomonas* sp. PPR8 solubilized inorganic phosphate in a significant amount, which may be beneficial to better plant growth. HCN production is a common trait of *Pseudomonas* (80%) and *Bacillus* (50%) in the rhizosphere [42]. A glycine-amended culture medium of *Pseudomonas* sp. PPR8 has been reported to enhance HCN production [20]. Siderophore production is very common among pseudomonads [1]. Sharma and Johri [43] reported the growth-promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize under iron limiting conditions. Iron-siderophore binding also prevents the proliferation of plant pathogens due to the sequestration of iron

from the environment. The use of PGPR, including phosphate potassium and zinc solubilizing bacteria, as biofertilizers was suggested as a sustainable solution to improve plant nutrient and production [9,44]. PPR8 strongly solubilized potassium and zinc by the production of organic acid, supporting earlier findings. In our study, *Pseudomonas* sp. PPR8 produced a significant amount of ACC deaminase, which may improve the growth and yield of common bean indirectly. Shaharoon et al. [45] reported a significant increase in the growth and yield of wheat by the ACC deaminase-producing *Pseudomonas* spp. and *Burkholderia caryophylli*.

Hyphal lysis by cell wall-degrading enzymes excreted by microorganisms is a well-known feature of mycoparasitism. Chitinase,  $\beta$ -1,3-glucanase and  $\beta$ -1,4-glucanase (cellulose) are especially important fungus-specific enzymes that degrade fungal cell-wall components such as chitin,  $\beta$ -1,3 glucan and glucosidic bonds [46]. The lytic action of chitinase and  $\beta$ -1,3-glucanase are major antifungal enzyme activities. We observed that *Pseudomonas* sp. PPR8 produced the extracellular chitinase,  $\beta$ -1,3-glucanase and  $\beta$ -1,4-glucanase enzymes. The action of *Pseudomonas* sp. PPR8 was quite effective on all six fungal pathogens because chitin is a cell-wall components. Therefore, it is likely that cell-wall lysis could have been caused by the concerted action of both chitinase and  $\beta$ -1,3-glucanase. Fridlender et al. [47] reported that  $\beta$ -1,3-glucanase produced by *P. cepacia* decreased the incidence of the diseases caused by *R. solani*, *S. rolfsii* and *P. ultimum*. Gupta et al. [48] reported the chitinase-mediated destructive antagonistic potential of *Pseudomonas aeruginosa* GRC1 against *Sclerotinia sclerotiorum* causing stem rot of peanut. From our observations, it is suggested that the oxalate oxidase produced by *Pseudomonas* sp. PPR8 may be one of the inhibitory factors of the fungal pathogens. Similar observations were also reported previously by Nagarajkumar et al. [40]; also, biological control of sheath blight of rice was observed under greenhouse conditions using a PGPR strain, *Pseudomonas fluorescens* PfMDU2 [40]. In the present study, *in vitro* reduction in radial growth of *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *R. solani* and *Colletotrichum* sp. was detected under the dual cul-

ture and CFCF of *Pseudomonas* sp. PPR8. The formation of inhibition zones might be due to the action of biologically active components, including chitinolytic enzymes, laminarinase, cellulase, HCN, antibiotics and siderophore [12,13,16,48].

Based on the above, it can be concluded that *Pseudomonas* sp. PPR8 can be considered as an efficient rhizospheric agent possessing various plant growth-promoting properties. These activities of *Pseudomonas* sp. PPR8 authenticate it as a good bioinoculant and biocontrol agent for the sustainable agro-productivity of common bean.

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