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# Exploring Plant Growth Potential of Multifaceted Plant Growth Promoting Rhizobacteria Isolated from Chickpea

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**ABSTRACT**

Chickpea is one of the major legume crops of India. Although the area under cultivation has increased in last few years, still India is largest importer of chickpea. Plant Growth Promoting Rhizobacteria is part of rhizomicrobiome and plays an important role in growth enhancement by direct or indirect method. In the present study, Plant Growth Promoting Rhizobacteria was isolated from chickpea rhizosphere. They were screened for various *in vitro* plant growth promoting traits. A total of 17 isolates were found to show plant growth promoting traits and they were nominated as CPRB1-CPRB17. Phosphate solublization varied from 128 to 198 µg/ml. IAA productions was also shown by all the 17 isolates and varied from 0.564 to 0.692 µg/ml in the culture filtrates. None of the isolates was able to produce HCN but ammonia production was shown by all isolates. Siderophore production was shown by only two isolates. These isolates were also tested for antagonist activity against *Fusarium oxysporum*. Five isolates were found to show antagonist activity. Based on plant growth promoting traits and antagonist activity five isolates were selected and tested for their ability to promote plant growth singly and in consortia. The selected isolates CBRB1, CBRB8, CPRB10, CPRB12 and CPRB13 were identified by 16s rRNA sequencing as *Cytobacillus firmus, Bacillus cereus, Bacillus subtilis, Bacillus paralicheniformis* and *Bacillus licheniformis*.

**Key words:** Chickpea, plant growth promoting rhizobacteria, phosphate solubilization, IAA production, *Bacillus*

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the widely cultivated and third most important legume crop in the world. It is affordable protein source and sometimes referred to as the poor man's meat because of it high protein content that accounts for 40% of its weight which is equivalent to meat (Xing *et al.,* 2020). Additional health benefits of chickpea in reducing the risk of cancer, diabetes and heart diseases lead to an increase in its demand (Merga and Haji, 2019). From 2021 to 2022, India has increased the land harvested from 9995920 to 10740100 ha for chickpea but still yield increase was 11916 to 12610 g/ha when compared with Australia, where the production has increased from 14456 to 17251 g/ha with increase in area harvested from 606316 to 615750 (FAOSTAT, 2023). In many cases, it is apparent that increase in production in developing countries is primarily due to an area expansion although in case of developed countries it is due to yield improvements (Merga and Haji, 2019).

Various abiotic and biotic stresses like drought, salinity, fusarium wilt, ascochyta blight and botrylis grey mold are major reasons for yield loss (Mirchandani *et al.*, 2023). For combating biotic stress various resistant cultivars, chemical pesticides and biocontrol are used but due to changes in the pathogen resistance is lost. Chemical fungicides also lead to various environmental hazards and resistance in pests.

Plant Growth Promoting Rhizobacteria (PGPR) is a group of bacteria that actively colonizes plant roots and increases plant growth and yield. These microorganisms are part of rhizomicrobiome, known to promote plant growth by various mechanism like synthesis of phytohormones, facilitation of essential nutrients, producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme chitinase, bacteriocins, siderophores, inhibition of phytopathogens, ameliorating to both biotic and abiotic stress and increase plant immune system (de Andrade *et al.,* 2023). Array of PGPRs species have been reported to enhance growth in chickpea. These species include Stenotrophomonas, *Pseudomonas, Azospirillum, Acinetobacter, Enterobacter,*

*Burkholderia* sp., *Bacillus* and *Chryseobacterium* (Midekssa *et al.,* 2016, Gopalakrishnan *et al.,* 2017, Laranjeira *et al.,* 2022). Indigenous, PGPRs are used directly or in consortia with other PGPRs or fungi. PGPRs are also known in increasing chickpea-*Mesorhizobium* symbiosis. PGPRs having ability to produce in vitro of IAA and ACC deaminase lead to increase plant growth as compared to other PGPR (Alemneh *et al.,* 2021).

Studies have shown that symbiosis of PGPR along with arbuscular mycorrhizal fungi (AMF) enhance plant growth by plant hormone balance, nitrogen fixation translocation of water in soil and facilitation of nutrients absorption by solubilization (Chang *et al.,* 2017). Consortia of arbuscular mycorrhizal fungi (AMF) along with *Pseudomonas* sp., *Burkholderia* sp. and *Mesorhizobium* sp. have also shown to increase growth protein content under various irrigation conditions (Laranjeira *et al.*, 2022). Many PGPR increased tolerance under abiotic stresses (Khan *et al.,* 2020; El-Ballat *et al.*, 2023). Similarly, certain bacterial strains like *Bacillus, Enterobacter*, *Pseudomonas* and *Serratia* inhibit fussarium by producing enzymes including peroxidase, polyphenol oxidase and hydrogen cyanide, β-1,4-glucanase, chitinase phenylalanine ammonia lyase and prevent yield loss (Palmieri *et al.*, 2017; Fatima *et al.*, 2022).

In the present study, PGPRs were isolated from chickpea rhizosphere and screened for *in vitro* plant growth activities like phosphate solubilization, IAA production, HCN, ammonia production and antagonist activity against *Fusarium*. Based on their multifaceted plant growth activity and antagonist activity, five isolates were selected and used for seed priming of mungbean for plant growth parameters.

## MATERIALS AND METHODS

Rhizosphere soil of chickpea plants growing in fields was collected from Jagatpura area Jaipur (26° 51' 22.88" N, 75° 52' 45.90" E) and kept at 4°C until further processing. Bacteria were isolated by 10-fold serial dilution and spread plate method on nutrient agar medium and was incubated at 37°C for 24 h. Morphologically distinct colonies were selected and purified by repeated streaking and were used for further study.

Qualitative and quantitative estimation of phosphate solublization was done by using NBRIP medium amended with 0.4% bromophenol blue and tri calcium phosphate. Change in colour and pH was noted for phosphate solublization. Quantitative estimation of phosphate solubilization was done using NBRIP medium amended without bromophenol blue. After three days, an aliquot of 5 ml was withdrawn and centrifuged at 8000 rpm for 10 min and supernatant was collected. 0.5 ml of ammonium molybdate ($5.54\ 10^{-4}$ M) was added to 1 ml of supernatant and mixed well. 0.5 ml of sulfuric acid (0.25 N) was added to above preparation and mixed. One ml of thiourea (2 M) was added to above preparation and mixed. Absorbance was measured at 800 nm against blank A.

For qualitative and quantitative analysis of indole-3-acetic acid, bacterial culture was grown in nutrient broth amended with 1000 µg/ml L-tryptophane. After three days of incubation at 37°C one ml of supernatant was mixed with 2 ml of Salkowski´s reagent and left for 20 min at room temperature. Development of pink colour indicated the production of the indole-3-acetic acid which was quantified by measuring absorbance at 450 nm.

For HCN production nutrient broth medium was used amended with 4.4 g glycine/l. Bacterial isolates were inoculated and Whatmann filter paper No. 1 socked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were incubated at 28±2°C. Change in colour of filter paper from orange to brown was observed. Plates without inoculation served as control.

For ammonia production rhizobacterial isolates were grown in 1% peptone incubated for four days at 30°C. After four days, 0.5 ml of Nessler's reagent was added to the bacterial suspension. The development of brown to yellow colour indicated ammonia production.

Siderophore production was determined on Chrome Azurol S (CAS) agar plate assay. The 48 h old bacterial cultures were spotted separately on CAS agar (blue dye: 10 g/l; hexadecyltrimethylammonium (HDTMA) bromide: 73 mg/l. Minimal media 9 salt solution: 10 g/l, glucose: 0.2 g/l, casamino acid: 0.3 g/l, piperazine-N, N′-bis (2-ethanesulfonic acid): 3.2 g/l, and agar: 15 g/l and incubated at 37±1°C for 48 h. Colony with

orange yellow zone was considered positive for siderophore production. The antagonistic potential of PGPRs against *Fusarium oxysporum* was done by dual culture method. In which the fungi was inoculated in center and after growth bacterial strains were inoculated at both the sides. It was incubated at 25±2°C for 10 days. Foc discs inoculated PDA plates without bacterial culture served as a control. Distance between fungal colony and bacteria was considered as zone of inhibition and was measured in mm. Per cent inhibition was calculated as:

Per cent Inhibition (Inhibition %) = (Control – Treated)/ Control × 100

*In vitro* plant growth promotion was tested using mungbean (*Vigna radiata*) seeds. Based on multiple plant growth promoting traits, antagonist activity was conducted on 17 isolates. Five isolates were selected for further analysis. Mungbean seeds were bioprimed by selected isolates (CPRB1, CPRB8, CPRB10, CPRB12 and CPRB13). Seeds were surface-sterilized with 0.02% sodium hypochlorite for 2 min and rinsed thoroughly in sterile distilled water. For inoculation, seeds were coated with 20% gum Arabic as an adhesive, soaked separately with the selected bacteria (grown separately in nutrient broth, NB) for 40 min (108 CFU/ml). Seeds were incubated in growth chamber at 28°C. After seven days, number of germinated seeds was counted. Root and shoot length of individual seedlings was measured to determine the vigour index as:

Vigour index = (Mean root length + Mean shoot length) × % germination

Selected isolates were tested for biochemical tests (Gram reaction, catalase reactions, methyl red, Voges-Proskauer test, indole, citrate, urease, protease, amylase and gelatinase) using standard methods. Bacterial cultures were also molecularly characterized by 16S rDNA partial gene sequencing. For 16S rRNA genes isolates were sent to the sequencing company barcode biosciences. All the statistical analysis was performed by using SPSS Statistics Ver. 20 software. The statistical data were expressed as the mean of three independent replications±standard deviation (SD) of 13 replicates of each experiment along with thrice repetitions data of each replicate. The data were interpreted through analysis of variance (one-way ANOVA) followed by Duncan's multiple range test at the P≤0.001 significance level. All experiments were performed in triplicates and all the experiments repeated thrice.

## RESULTS AND DISCUSSION

A total of 17 morphological distinct microorganisms were obtained from the sampling site and they were nominated as CPRB1 to CPRB17. Out of 17 bacterial isolates, it was found that all the isolates were capable of differentially utilizing 5 g/l tri calcium phosphate in NBRIP medium. Significantly phosphate solublization activity was found highest in isolate no. CPRB13 (194 µg/ml) and lowest in isolate no. CPRB9 (136 µg/ml; Fig. 1) which was significantly higher than previous study on *Mesorhizobium* spp. which was estimated to release soluble phosphorus 125 - 150 P (µg/ml) by Muleta *et al.* (2021).

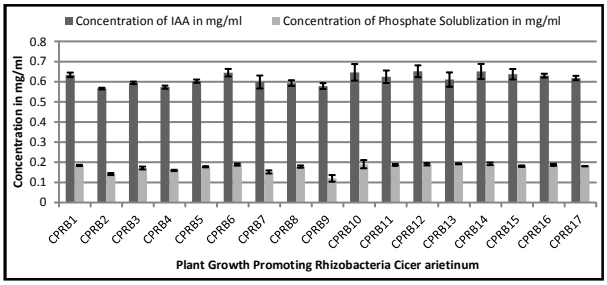


Fig. 1. Quantitative estimation of IAA produced and Phosphate Solubilization by bacterial isolate

In another study, other strains like *Enterobacter sakazaki, Bacillus flexus, Enterobacter* sp. *Pseudomonas fluorescence* and *Enterobacter* sp. Phosphate, *Bacillus* sp. strain AZ17 and *Pseudomonas* sp. strain AZ5 isolated from chickpea rhizosphere have been reported to solubilize phosphate in Pikovskaya (PK) medium. Quantitative estimation showed higher phosphate solublization by *Enterobacter* sp. (379 µg/ml) as compared to present study (Midekssa *et al.,* 2016). All the isolates were able to produce different concentrations of indole acetic acid (IAA) ranging from 0.562 to 0.652 mg/ml. Maximum IAA production was shown by CPRB12, whereas CPRB2 was weakest producer (Fig. 1). IAA production was significantly higher than recent study 0.45 µg/ml conducted on *Mesorhizobium* spp. by Laranjeira *et al.* (2022) and 58 µg/ml in

*Enterobacter* sp. by Midekssa *et al.* (2016). It may be due to the fact that indole-3-acetic acid (IAA) production is essential for rhizobacteria to stimulate plant growth and differ considerably between different species or strains of the same species (Lebrazi *et al.,* 2020). No isolate was found to produce HCN, while ammonia production was detected in 100% isolates. Some isolates were depicted as weak producers (CPRB2, 3, 5, 9, 11, 14 and 15) and some were strong producers (CPRB1, 4, 6, 7, 10, 12, 13, 16 and 17). Siderophore production was shown by two isolates (CPRB16 and CPRB13) through generation of orange halos surrounding the colonies.

Among 17 bacterial isolates, only five isolates were found to be capable of inhibiting *F. oxysporum* by CPRB1: 52%, CPRB8: 60%, CPRB10: 57%, CPRB12: 57%, and CPRB13: 58%. Recently, *Mesorhizobium* in combination with *Bacillus* sp. and *Pseudomonas* sp. showed antagonism against Foc through the production of various compounds (Kumari and Khanna, 2020). Biocontrol properties of PGPRs were recently evaluated by Sankar *et al.* (2019) against Fusarium wilt of chickpea and found that *Pseudomonas chlororaphis* reduced disease by 80.7% in glasshouse and 70.18% in the field condition.

Based on above results, five isolates were selected and mungbean seeds were bioprimed with single isolates and their consortia. It was found that consortia CPRB12, 10, 1, CPRB8, 12, 13, CPRB1, 8, 12, CPRB1, 8, 10 showed significant increase in root, shoot length and vigour index as compared to control and also as compared to single inoculant. Mean root length was found to be increased from 0.94 to 6.10 cm. Highest increment was shown with consortia CPRB12, 10, 1; CPRB1, 8, 12 and CPRB8, 12, 13 which was 6.10±1.03, 6.01±0.79 and 5.68±0.70 cm, respectively (Table 1). There was 1.72, 1.75 and 1.66 folds increase in mean root length with these three observed strains when compared with control. Maximum shoot length was observed with CPRB12, 10, 1 (7.20±1.16 cm) followed by CPRB8, 12, 13 (4.96±0.62) and CPRB10 (4.10±0.54 cm) which was 1.85, 1.45 and 1.76 fold increase in shoot length. Per cent germination values ranged from 60 to 100. CPRB10 and CPRB1, 8, 13 showed 100% germination, whereas only 60% germination was observed with CPRB1 and CPRB1, 12, 13 followed by CPRB8, 12, 13 where only 70% germination was observed. CPRB12, 10, 1 and CPRB1, 8, 12 showed 1.62 and 1.20 folds increase in vigour index, respectively. Many studies have reported increase in yields and plant growth in chickpea inoculated with a PGPR consortium (Baliyan *et al.*, 2018; Abdiev *et al.,* 2019). *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus megaterium* in combination with salicylic acid and putrescine also showed increase in carotenoids, sugar and protein content in chickpea (Khan *et al.*, 2020). Biochemical test was performed and the results are shown in Table 2. For selected isolates and phylogenetic relationship was determined by comparing its 16SrDNA sequence with closely related neighbour sequences retrieved from the GenBank database of the National Center for

**Table 1.** Effect of selected PGPR's on seedling growth parameters (vigour index and germination %) of chickpea plant with respect to control plant

| Isolates | Mean root length (cm) | Mean shoot length (cm) | Germination (%) | Vigour index |
|---|---|---|---|---|
| Control | $3.48\pm0.43^{e}$ | $3.89\pm0.27^{d}$ | $93.33\pm3.33^{b}$ | $737.00\pm24.56^{e}$ |
| CPRB1 | $1.23\pm0.14^{h}$ | $0.96\pm0.45^{i}$ | $60.00\pm5.77^{e}$ | $131.60\pm12.66^{m}$ |
| CPRB8 | $0.94\pm0.11^{i}$ | $1.67\pm0.60^{h}$ | $70.00\pm3.33^{d}$ | $182.90\pm8.70^{m}$ |
| CPRB10 | $3.72\pm0.41^{d}$ | $4.10\pm0.54^{c}$ | $100.00\pm3.33^{a}$ | $782.00\pm26.06^{c}$ |
| CPRB12 | $3.37\pm0.66^{f}$ | $2.12\pm0.73^{g}$ | $80.00\pm3.33^{c}$ | $439.60\pm18.31^{k}$ |
| CPRB13 | $4.83\pm0.74^{c}$ | $3.92\pm0.68^{d}$ | $80.00\pm3.33^{c}$ | $700.60\pm29.19^{f}$ |
| CPRB8, 10, 12 | $3.33\pm0.64^{f}$ | $2.88\pm0.30^{e}$ | $80.00\pm5.77^{c}$ | $497.40\pm35.89^{g}$ |
| CPRB10, 12, 13 | $1.41\pm0.24^{h}$ | $0.96\pm0.95^{i}$ | $80.00\pm5.77^{c}$ | $189.80\pm13.69^{k}$ |
| CPRB1, 8, 12 | $6.01\pm0.79^{a}$ | $3.91\pm0.47^{d}$ | $90.00\pm3.33^{b}$ | $891.90\pm33.03^{b}$ |
| CPRB1, 8, 13 | $2.03\pm0.36^{g}$ | $2.57\pm0.26^{f}$ | $100.00\pm3.33^{a}$ | $460.33\pm15.34^{i}$ |
| CPRB1, 12, 13 | $0.95\pm0.20^{i}$ | $0.70\pm0.90^{j}$ | $60.00\pm5.77^{e}$ | $99.00\pm9.52^{o}$ |
| CPRB12, 10, 1 | $6.10\pm1.03^{a}$ | $7.20\pm1.16^{a}$ | $90.00\pm3.33^{b}$ | $1197.00\pm44.33^{a}$ |
| CPRB8, 12, 13 | $5.68\pm0.70^{b}$ | $4.96\pm0.62^{b}$ | $70.00\pm3.33^{d}$ | $745.20\pm35.48^{d}$ |
| CPRB1, 8, 10 | $2.20\pm0.59^{g}$ | $3.5\pm0.62^{d}$ | $80.00\pm3.33^{c}$ | $568.80\pm23.7^{h}$ |

Different superscripts indicate significant differences at $P \leq 0.001$.

**Table 2.** Biochemical test for selected bacterial isolates

| Bacterial isolates | Gram's reaction | MR | VP | Catalase test | Citrate utilisation | Protease activity | Geletin hydrolysis | Urea hydrolysis | Indole test | Starch hydrolysis |
|---|---|---|---|---|---|---|---|---|---|---|
| CPRB1 | + | + | - | + | - | - | + | - | - | + |
| CPRB8 | + | - | + | + | + | + | - | - | - | + |
| CPRB10 | + | + | - | + | + | + | + | - | - | + |
| CPRB12 | + | - | + | + | + | - | + | - | - | - |
| CPRB 13 | + | - | + | + | + | - | + | + | - | + |

Biotechnology Information via BLAST search (*http://www.ncbi.nlm.nih.gov/BLAST*). Based on maximum identity score, first 10 sequences were selected and aligned using multiple alignment software program Clustal W. *Bacillus cereus*: Accession no. PP823819 (Fig. 2A), *Bacillus subtilis*: Accession no PP823820 (Fig. 2B), *Bacillus paralicheniformis*: Accession no. PP823821 (Fig. 2C) and *Bacillus licheniformis*: Accession no. PP823822 (Fig. 2D). *Cytobacillus firmus*: Accession no. PP818449 (Fig. 2E).

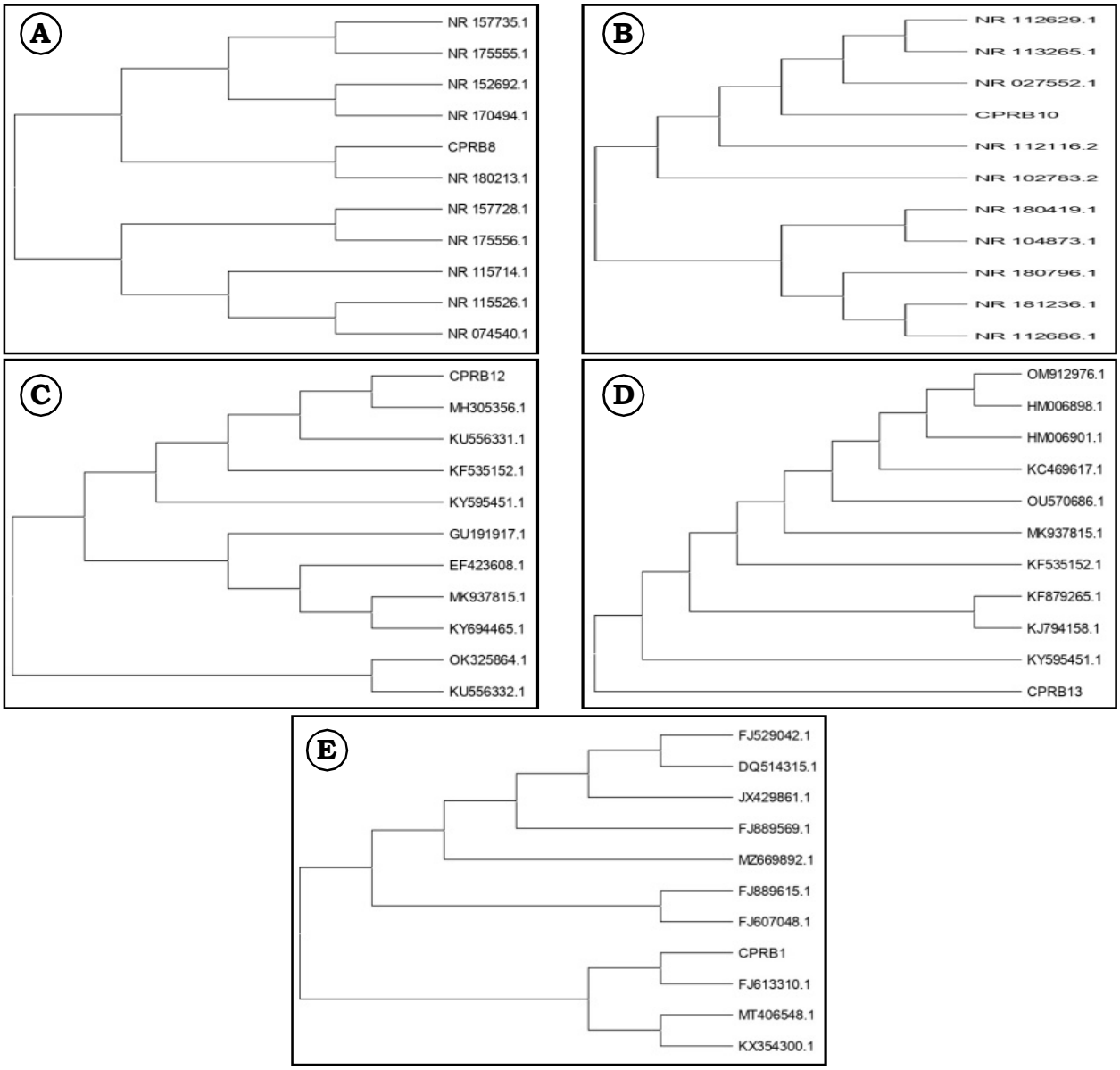


Fig. 2. Phylogenetic tree of 16 rDNA gene sequence of CPRB8 (A), CPRB10 (B), CPRB12 (C), CPRB13 (D) and CPRB1 (E) using MEGA 10 (Numbers represent accession numbers).

The results are in agreement with the previous reports where most of the potential bacterial species as a biocontrol agent were identified as *Bacillus* irrespective of the crops like corn wheat and soybean (Cherif-Silini *et al.*, 2016; Radhakrishnan *et al.*, 2017; Akinrinlola *et al.*, 2018). In a recent study, *Bacillus licheniformis* isolated from chickpea from marine water Gujarat, India did not produce IAA, HCN and siderophore but these srains were able to solubilize phosphate and found to increase chickpea height by 26.23% and increase in biomass by 33.85% in pot trials and several other enzymes which increased plant growth (Rathod *et al.*, 2023). Even three different strains of endophytic *Bacillus subtilis* were also reported to produce IAA, ammonia production and siderophore production though they were not able to solublize phosphate and were found to show anatgonism against Fusarium inhibition percentage (70.9) (Vellaichamy *et al.*, 2022). Negative results on chickpea biomass due to bacterial inoculation were also reported by Mnalku and Mitiku (2019), who observed lower biomass production in plants inoculated with CP-5 rhizobium strains compared to the treatments without inoculation. It may be due to failure of forming symbiosis.

## CONCLUSION

This study highlighted the antagonistic activity and comparative plant growth promoting activity of different bacterial isolates from Jagatpura area Jaipur. A total of 17 morphologically different bacterial isolates were obtained. All of the bacterial isolates were capable of producing IAA ranging from 0.562 to 0.652 mg/ml and also showed positive result for phosphate solubilization. However, isolate CPRB6 gave the best result. Five isolates, namely, CPRB1, CPRB8, CPRB10, CPRB12 and CPRB13 showing antagonism were screened for *in vitro* plant growth activity and showed significant increase in plant growth. For better results, one can use mixed cultures with different combinations of the isolates. Combinations showing better results can be bioformulated and can be applied in the field as an alternative of chemical pesticides for sustainable environment.

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