

Multifaceted Rhizobacteria-Mediated Growth Augmentation in Chickpea

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Abstract Sixty-nine rhizobacteria isolated from chickpea rhizosphere were screened for their antagonistic potential against *Fusarium oxysporum* f. sp. *ciceris* under in vitro condition. Of these, 30 isolates inhibited growth of the pathogen and 13 potent antagonists were assessed for their functionality traits. Among the antagonistic traits exhibited by antagonists, production of diffusible antimetabolites insured its inhibitory effect on growth of test fungus as well as spore germination on solid media. Inhibition of fungus biomass in broth-based dual culture was revealed by 11 antagonists, while metabolic extracts of 5 isolates reduced the radial proliferation on solid media. Implication of biocidal volatiles in antagonism was demonstrated by 11 isolates; 3 produced cyanogenic volatiles, while all were ammonia producers. Investigation for hydrolytic activity demonstrated production of chitinase by 4 isolates and β -1,4-glucanase by 11, while all were protease and amylase producers. Revealing the dual potential of bioantagonists, all the isolates tested positive for IAA and salicylic acid production, 7 for siderophore production while 10 were P-solubilizers and 8 Zn-solubilizers. Under glass house condition, bacterization of chickpea seeds with potential isolate Ps14c alone and in consortium with *Mesorhizobium ciceris* reduced wilt incidence in pots (soil amended with *F. oxysporum*) to 48 and 28%, respectively, as compared to uninoculated control (74%) and also recorded appreciable increase in growth parameters. Present investigation clearly reveals the potential of rhizobacteria for use as bioinoculants.

Keywords Antagonism · Lytic enzymes · IAA · P-solubilization · Siderophore · Rhizobacteria · Bioinoculant · Soil amendment · Chickpea · *Fusarium oxysporum*

Introduction

Sustainable agriculture amalgamates environmental health, economic feasibility and social equity ensuring long-term productivity of natural resources and improved livelihood. It helps reduce the risks of complexities like climate

unpredictability and climate change. Conventional agriculture which involves injudicious and inappropriate use of agrochemicals has resulted not only in the deterioration of soil health but also has led to some major environmental problems, such as soil and water pollution and other health-related problems, besides leading to increased cost of inputs for crop production. The challenge of enhancing productivity while retaining environmental soundness requires urgent need to integrate alternatives to agrochemicals. In this context, bioagents share would be considered a viable option. Numerous soil bacterial species which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, and stimulate plant growth by a plethora of mechanisms are collectively known as plant growth-promoting rhizobacteria (PGPR) [28]. Insight into the mechanisms operative at different

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stages of plant growth include increased mineral nutrient, nitrogen fixation, repression of soil-borne pathogens (via production of hydrogen cyanide, siderophores, lytic enzymes, antibiotics, and/or competition for nutrients), improving plant tolerance to abiotic stresses by producing enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and producing phytohormones such as indole-3-acetic acid (IAA) and gibberellins [12].

Chickpea (*Cicer arietinum* L.) is the third largest produced food legume globally, India, being the largest producer [9]. Unprecedented use of agrochemicals (fertilizers and pesticides) to maximize its productivity has brought grave threats to the inborn soil fertility which has reached a plateau from where fertility status could not be increased even by increasing their dosage [15]. Moreover, the emerging demand for chemical-free food and necessity to achieve sustainability in agriculture within a holistic vision to focus on environmental protection has made possible exploitation of soil microorganisms for improving the crop health. Use of microorganisms presents an economically feasible and ecologically sound practice to enhance crop yield. In order to frame this practice into practical application, the present investigation was planned to isolate PGPR from rhizospheric soil, to elucidate different mechanisms implicated in plant growth promotion and antagonism and to study their effect on plant health.

Materials and Methods

Isolation and Screening of Rhizobacteria

Antagonistic to *Fusarium oxysporum* f. sp. *ciceris* (Foc)

A total of 36 soil samples were collected from rhizosphere of different chickpea fields [16]. Plants were cautiously excavated using a shovel, and the soil loosely attached to the root was collected as rhizosphere soil. The samples were then placed into prelabelled translucent ziplock bags and maintained at ambient temperature. The rhizobacteria were isolated on King's B medium by serial dilution technique. The isolated colonies on agar plates differing in colony morphology were selected, purified by subculturing and maintained on respective slants at 4–5 °C.

To select the antagonistic bacteria, a 5-mm disk of test fungus, *F. oxysporum* (Procured from Department of Plant Breeding and Genetics, PAU, Ludhiana) was placed at the center of agar plate containing PDA and Kings B in 2:1 ratio, while rhizobacterial isolates were streaked 3 cm apart from the fungal inoculum and plates were incubated at 25 °C for 7 days. Radial growth of the test fungus was measured, and percent growth inhibition was calculated. A control plate with fungus alone was used as control. The

growth of pathogen was measured in terms of diameter, and percent inhibition was calculated as under:

$$\% \text{ Inhibition} = (R - r) / R \times 100$$

where r is radial growth of fungus in dual plate and R radial growth of fungus in control plate.

Assessment of antagonistic potential of Rhizobacteria

Fungal Biomass Inhibition in Liquid Medium

One milliliter of overnight grown bacterial culture and a 5-mm disk of test fungus were inoculated in 50 ml of potato dextrose broth and incubated at 25 °C. Broth inoculated only with fungus disk served as control. The difference in dry weights of the fungus from dual and the control cultures was recorded after 5 days of incubation by passing through preweighed filter paper (Whatman no. 1) which was dried for 24 h at 70 °C and weighed. The percent reduction in weight of the test fungus was calculated.

Production of Diffusible Antimetabolites

Diffusible antimetabolites production by rhizobacteria was assayed by method of Montealegre et al. [21]. PDA plates covered with cellophane membrane were overlaid with nutrient agar and inoculated with 100 µl of the potential bioantagonists. After incubation for 72 h at 25 °C, the membrane along with the bacterial growth was removed and a disk of pure culture of pathogen was placed at the center of the plate and the growth was measured after 72 h. Effect of diffusible metabolites on spore germination was studied by spreading 1 mL conidial suspension (10^4 conidia ml^{-1}) of *F. oxysporum* on PDA following removal of cellophane membrane. Petri dishes were incubated at 24 °C for 6 d, and germination was evaluated visually.

Production of Antibiotic Compounds

The antimetabolites from 72-h-old culture supernatants of rhizobacterial antagonists were extracted using ethyl acetate as solvent. After evaporating ethyl acetate at 40 °C, extracts were dissolved in 1 ml of 50% methanol and tested for growth inhibition of *F. oxysporum* in agar wells made in PDA medium; 50% methanol alone served as control.

Production of Volatile Antifungal Compounds

The production of volatile antifungal compounds by the isolates was assayed by sealed plate method [10]. Overnight grown bacterial culture was spread on nutrient agar medium, and a second petri dish containing PDA was

inoculated with a 5-mm plug of the test fungus and placed over the bacterial culture. The two plates were sealed with parafilm and incubated at 25 °C. Control consisted of a petri plate containing PDA medium inoculated with 5-mm plug of the test fungus placed over nutrient medium without bacteria. Radial growth of the test fungus was measured over 24-h intervals for a period of 5 days.

Assessment of Inhibitory traits of Rhizobacteria

Production of HCN

Petri plates containing 10% Trypticase soya agar (TSA) supplemented with 0.44% of glycine were inoculated with bacteria, and lid was roofed with filter paper wetted with 0.5% picric acid in 2% sodium carbonate. The plates were incubated at 28 °C for 3–5 days. Change in color of the filter paper from yellow to orange-brown indicated cyanide production [5].

Production of Ammonia

Ammonia production was detected by adding Nessler's reagent (0.5 ml) to 10 ml of bacterial cultures grown in peptone water for 48–72 h at 30 °C. Development of brown to yellow color indicated ammonia production.

Detection of Lytic Enzymes

β-1,4-Glucanase

Plates containing minimum salts medium with (1% w/v) carboxy methyl cellulose (CMC) were spot inoculated with the rhizobacterial isolates. After incubating for 48 h at 30 °C, the agar medium was flooded with an aqueous solution of Congo red for 15 min, washed and visualized for zones of hydrolysis detecting β-1,4-glucanase.

Chitinase and Protease

The rhizobacteria were inoculated on plates with minimum salts medium containing (1% w/v) colloidal chitin and (1% w/v) casein, respectively, incubated for 48 h at 30 °C, and formation of zones of hydrolysis indicated chitinase and protease production.

Amylase

The log-phase cultures were spotted on the plates containing starch agar medium and incubated at 28 °C for 48 h. The petri plates were then flooded with Gram's iodine. The hydrolysis of starch was observed as a colorless zone surrounding the colonies against purple background.

Assessment of Plant Growth Promotion Traits

Siderophore Production

Siderophore production was assayed using chromeazurol S agar (CAS) [24]. Formation of yellow halo around bacterial colonies on purple background was considered as affirmative result. Siderophore index was calculated as the sum of colony diameter and the halo zone diameter/the colony diameter.

Salicylic Acid (SA) Production

For SA assay, bacterial cells grown in succinate medium were harvested by centrifugation at 8000 rpm for 5 min and resuspended in 1 ml of 0.1 M phosphate buffer. Four milliliters of cell free culture filtrate was acidified with 1 N HCl to pH 2.0, and SA was extracted in CHCl₃. Four milliliters of water and 5 μl of 2 M FeCl₂ were added to the pooled CHCl₃ phases. The absorbance of the purple iron–SA complex was read at 527 nm. A standard curve was prepared with SA in succinate [19].

IAA Production

For qualitative assay of IAA, petri dishes containing Luria agar supplemented with 5 Mm L-Trp were inoculated with bacterial inoculums, then overlaid with an 82-mm-diameter disk of Whatman no. 1 filter paper and incubated at 27 °C for 2–4 days. Formation of pink coloration on addition of Salkowski's reagent over filter paper indicated IAA production. The results were analyzed visually on a three-point scale (+ low; ++ medium, +++ high), and diameter of pink halo was measured [6].

P-Solubilization

The P-solubilization ability of rhizobacterial isolates was tested by spot inoculating cultures on Pikovskaya agar medium (glucose, 10 g; Ca₃(PO₄)₂, 5 g; (NH₄)₂SO₄, 0.5 g; NaCl, 0.2 g; MgSO₄·7H₂O, 0.1 g; KCl, 0.1 g; yeast extract 0.5 g; MnSO₄ and FeSO₄ trace; D/W, 1 l; agar, 15 g; pH 7). The isolates forming halo zone around the colony were considered as phosphate solubilizers. Diameter of halo was recorded after 4 days of incubation.

Zn-Solubilization

The Zn-solubilization ability of rhizobacterial isolates was confirmed by formation of halo zone around colonies inoculated on modified Pikovskaya medium containing 1% insoluble ZnO. Diameter of halo was measured after 4 days of incubation.

Morphological and Biochemical Characterization of Rhizobacterial Isolates

The bacterial isolates were characterized based on colony morphology and biochemically by following Bergey's Manual of Systematic Bacteriology [26].

Molecular Characterization as *Pseudomonas* sp.

DNA Extraction

For bacterial DNA extraction, 5 ml of overnight grown culture was centrifuged (10 min at 10,000 rpm). Pellet suspended in 650 μ l of extraction buffer (consisting of 250 mM NaCl, 100 mM Tris-HCl, 100 mM EDTA, 10% SDS and 10% PVP; pH 8.0) was incubated at 65 °C for 30 min followed by addition of 100 μ l of potassium acetate buffer (pH 4.5). Genomic DNA was extracted twice with 700–800 of chloroform isoamyl alcohol (24:1). 0.6 volume isopropanol was added to precipitate the nucleic acids and centrifuged at 10,000 rpm for 10 min, and supernatant was carefully discarded by aspirating the isopropanol. Pellet was washed by adding 0.5 ml ice cold 70% ethanol, air-dried and dissolved in 100 μ l of sterile distilled water. Biotometer was used to quantify the extracted DNA.

PCR Amplification

Polymerase chain reaction (PCR) was carried out to amplify 16S rRNA using *Pseudomonas* genus-specific primers. Reaction mixture consisted of Taq DNA polymerase 2 units; Mg⁺⁺ of 1.5 mM; 10 \times buffer; 0.2 mM each of the four dNTPs and 30 ng template DNA, 25 pmol each of the primers. Thirty-five amplification cycles were performed in an automated DNA thermocycler with the following parameters: denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min, elongation at 72 °C for 1 min and final extension for 10 min. Primer sequences were as follows:

Oligo1 Forward sequence—5'-GACGGGTGAGTAA TGCCTA-3'.

Oligo2 Reverse sequence—5'-CACTGGTGTCC TTCCTATA-3'.

Five microliters of the PCR amplifications was run on 1.2% (wt/vol) agarose gel electrophoresis in Tris-borate-EDTA buffer (pH 8.3) (1.5 h at 40 V), stained with ethidium bromide and photographed on a UV transilluminator.

Glasshouse Experiment

Glasshouse experiment was conducted during the year 2013–2014 in Schools of Biotechnology, Punjab

Agricultural University, India, to study the influence of selected rhizobacteria exhibiting both plant growth promotion and antagonistic traits as seed bioinoculants alone and in consortium with *Mesorhizobium ciceris* on seedling emergence, growth parameters, and wilt incidence in chickpea. Medium black clayey soil collected from chickpea field, Punjab Agricultural University, Ludhiana, was autoclaved 3 \times (1 h, 121 °C) at 24-h intervals, and 1 kg sterilized soil amended with microconidial suspension (1000 conidia mL⁻¹) of *F. oxysporum* @ 50 ml/kg was filled per pot. Susceptible variety JG-62 of chickpea was procured from the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. Following surface sterilization with 0.1% mercuric chloride for 30 s, seeds were washed 5–6 times with sterile distilled water and dried under stream of sterile air. Seeds were then soaked in 10 ml of bacterial inoculum containing 3 \times 10⁸ cfu mL⁻¹ for 3–4 h, thereafter dried and sown @ 10 seeds/bag. Each treatment was replicated 7 times. For dual inoculation, PGPR and *Mesorhizobium* inoculum was mixed in 1:1 ratio. Wilt incidence and growth parameters including seed germination, plant fresh and dry weight, plant height and chlorophyll content were recorded. While plant weight and height were recorded at the time of harvest, chlorophyll content was determined on 25th day of germination as it shows drastic decline at the time of harvest.

Statistical Analysis

Experimental data were analyzed using standard analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using CPCS1 software. Differences were considered significant at the $p \leq 0.05$ level. Standard errors were calculated for all mean values.

Results and Discussion

The plant rhizosphere represents a versatile and dynamic ecological environment for notable microbes. These microorganisms not only interact with plants permitting their better growth through production of growth substances and enhanced mineral nutrition but also interact with other detrimental pathogens which hinder the normal development of plants. Exploiting these soil inhabiting bacteria as promising bioinoculants framed the basis of present investigation. With an aim to explore the diverse microflora, we collected the soil samples from different chickpea growing rhizosphere. From these, a total of 69 rhizobacterial isolates showing rapid growth on Kings B medium were isolated and subjected to preliminary screening for antagonism against *F. oxysporum* f. sp. *ciceris*. The in vitro dual plate-based assay carried out to test inhibitory action of 69 bacterial isolates

revealed that bacterial antagonists were the natural resident of chickpea rhizosphere. However, this inhibitory trait was exhibited only by 30 isolates that too in the varying range of 4.0–48%. As the main motive of present investigation was to develop bioinoculants with both bioantagonistic and biofertilizing traits, we selected the 13 best antagonists that inhibited growth of the test pathogen in the range of 23.3–48% (Table 1) for the evaluation of antagonistic and plant growth promotion traits.

Assessment of Inhibitory Traits

The assessment of bacterial antagonists for fungal biomass inhibition under broth-based dual culture revealed that 11 out of 13 isolates inhibited growth of the pathogen as indicated by reduction in dry weight of the fungal biomass compared to control. Reduction in biomass of the test fungi was recorded in the range of 17.4–71.8% (Table 1). The remark to conclude here was that most of the isolates (except Ps15a, Ps19d and Ps24d) recorded much higher reduction in biomass of test fungus than under dual plate assay. This may be attributed to the fact that broth-based environment allowed a synergistic expression of all the mechanisms involved in biocontrol from all the interacting sites. Concomitantly, variable responses in reduction in fungal biomass in broth culture as compared to dual plate assay have been reported as certain antifungal metabolites are induced or repressed by the absence or presence of pathogen [18]. In a similar study, Kumari and Khanna [16] reported 11.6–62.97% reduction in proliferation of *F. oxysporum* by *Bacillus* sp. isolates from chickpea rhizosphere under broth-based technique.

In another set of experiment implicating the role of diffusible metabolites in growth inhibition of the test fungus, it was observed that precultures of 8 out of 13 rhizobacterial antagonists reduced the mycelial proliferation of test fungus in the range of 13.33–76.6% (Table 1), maximum being exhibited with Ps14c (76.6%) followed by Ps29c (66.6%). Likewise, when we spread the spore suspension of *F. oxysporum* over media containing diffusible bioactive metabolites of precultures of bacterial antagonists, conidial germination of the pathogen was inhibited as indicated by scanty fungal mycelial growth from spores compared to control plates where ample growth of fungus was observed. This clearly suggested that antibiosis is an important contributor to the antagonistic effect of these bacteria through the diffusion of antifungal compounds into the medium. In confrontation with the present finding, Martinez et al. [18] reported that plates containing exudates of precultured *A. piechaudii*, *P. chlororaphis* and *S. griseus* inhibited both mycelial growth and conidiation of *Helminthosporium solani*. Moreover, implication of diffusible bioactive metabolites such as phenazine, pyoluteorin, pyrrolnitrin and cyclic lipopeptides in antagonism is well documented [14].

Further revealing the involvement of antibiosis in antagonism through secretion of antibiotic like compounds by bacterial antagonists, metabolic extracts from the culture filtrate of the bacterial isolates dissolved in 50% methanol restricted the growth of test fungus in the range of 31.8–50.0% as observed by the inhibition zone between the well containing extract and fungal growth. The antimicrobial compounds has been reported to induce fungistasis in phytopathogens, inhibit spore germination, lyse fungal mycelia or exert fungicidal effect by impairing electron transport chain [14]. Inhibition of fungal growth by only 5 isolates that too in lower range as compared to diffusible metabolites clearly indicated that production of antimicrobial compounds was largely influenced by the type of medium used. Martinez et al. [18] in their studies against *H. solani* have reported similar observations. Séveno et al. [25] reported that production of the antibiotic phenazine by *Pseudomonas aureofaciens* was six times higher in solid culture than with liquid culture.

Unveiling the role of biocidal volatiles in antagonism, 11 isolates inhibited the fungal radial proliferation under sealed plate assay, albeit with different effectiveness, maximum being recorded with isolate Ps-14c (39.5%). A similar antifungal activity of volatile organic compounds by rhizobacteria has been reported as an antagonistic armor against phytopathogens by Giorgio et al. [11]. They confirmed the strong inhibitory activity of 6 rhizobacteria isolated from common bean via volatiles on fungal mycelium and sclerotia of *Sclerotinia sclerotiorum*. When tested for ammonia production which is reported to play a significant role in bioantagonism, all the isolates exhibited affirmative results (Table 2). The role of ammonia producing *B. subtilis* MA-2 and *Pseudomonas fluorescens* MA-4 in augmentation of biomass of medicinal plant *Geranium* has been reported by Mishra et al. [20]. In similar studies Samuel and Muthukkaruppan [22] reported ammonia production in 95% of strains isolated from the rhizosphere of rice, mangroves and soils contaminated by effluent. Assessment of rhizobacterial isolates for production of hydrogen cyanide (HCN) showed confirmatory results with only 3 isolates, Ps24d being strongest producer as indicated by change in color of filter paper from yellow to orange (Table 2). The difference observed in fungal growth inhibition under sealed technique and production of different volatile compounds clearly suggests involvement of some more volatile compounds in antagonism. Ammonia and HCN along with other volatiles like dimethyl disulfide, dimethyl trisulfide methyl and ketone 2-propanone present an integrative part of the pool of biologically active volatiles attributable to antagonistic trait of rhizobacteria [11].

Supplementing our study on antagonistic behavior of rhizobacterial isolates, antagonists were assessed for the production of lytic enzymes. The rationale behind this investigation was that cell wall of fungi provides both protective and aggressive functions, and if it is removed or weakened, the

Table 1 Evaluation of antagonistic potential against *Fusarium oxysporum* by rhizobacterial isolates

Isolates	% growth inhibition of <i>Fusarium oxysporum</i>				
	Under dual plate technique	Under broth-based technique	Due to diffusible metabolites	Due to ethyl acetate extracts	Due to fungicidal volatiles
Ps 4b1	30.70 ± 0.40	–	13.33 ± 0.52	40.00 ± 1.15	30.70 ± 0.61
Ps7a	33.30 ± 0.57	59.30 ± 0.34	–	–	33.30 ± 0.34
Ps13b	32.50 ± 0.28	55.40 ± 0.52	–	50.00 ± 1.70	32.50 ± 0.46
Ps14c	48.00 ± 0.52	66.5 ± 0.46	76.60 ± 1.5	43.30 ± 0.57	39.50 ± 0.40
Ps14d	30.70 ± 0.52	57.10 ± 0.57	15.0 ± 0.28	–	30.70 ± 0.34
Ps15a	26.15 ± 0.61	17.46 ± 0.23	16.66 ± 0.57	–	6.15 ± 0.57
Ps16a	29.23 ± 0.57	47.11 ± 0.51	–	–	9.23 ± 0.28
Ps19d	33.30 ± 0.46	18.14 ± 0.57	–	–	–
Ps20b	30.71 ± 0.40	–	13.33 ± 0.42	–	10.70 ± 0.40
Ps24d	33.30 ± 0.23	29.16 ± 0.61	30.00 ± 0.57	31.80 ± 0.80	33.30 ± 0.23
Ps28c	23.30 ± 0.34	32.17 ± 0.28	–	33.30 ± 0.17	23.30 ± 0.52
Ps29c	33.30 ± 0.40	71.87 ± 0.52	66.60 ± 0.61	–	–
P-I	35.50 ± 1.50	20.75 ± 0.40	16.66 ± 0.34	–	33.30 ± 1.50

Values represent mean ± SE of three replications

fungi die unless they are osmotically protected. When assessed for chitinase production, only 4 isolates tested positive for it, Ps-20b being the strongest producer. Among 13 bacterial antagonists, 11 produced β -1,4-glucanase, while all the isolates tested positive for production of protease and amylase (Table 2). Not surprisingly, isolate Ps14c which exhibited maximum fungal growth inhibition tested positive for all the lytic enzymes. The role of hydrolytic enzymes produced by rhizobacteria for their biocontrol activity is well documented [15]. In their report, Kumar et al. [15] confirmed the antagonistic behavior of *Bacillus* sp. BPR7 against all six test pathogens, viz. *Macrophomina phaseolina*, *F. oxysporum*, *F. solani*, *Sclerotium sclerotiarum*, *Rhizoctonia solani* and *Colletotrichum* sp. due to production of mycolytic enzymes, viz. chitinase, β -1,3-glucanase and β -1,4-glucanase that degraded the components of fungal cell wall such as chitin, β -1,3-glucan and glucosidic bonds. Compant et al. [8] reported that the production of allelochemicals such as lytic enzymes and antibiotics by rhizobacteria favors colonization and antagonistic activity in the environment. The amylase has been reported to have only a discrete effect on phytopathogens cell wall as there is no report of the presence of starch in fungal cell wall. However, this enzyme plays a key role in the early phase of antagonistic association [4] and assists easy survival of microorganisms by mediating hydrolysis of most widely distributed starch or any other readily available related α -glucan elsewhere [17].

Assessment of Plant Growth Promotion Traits of Rhizobacterial Isolates

In perspective of the present requirement of attaining sustainability in agriculture, the next part of experiment aimed at

screening the bacterial antagonists for plant growth-stimulating traits. Of 13 isolates tested for siderophore production, 7 produced siderophore as evidenced by the formation of orange halo on CAS agar (Table 2). Isolate Ps14c maintained its dual potential with second highest siderophore index (2.82), first being observed with Ps20b (2.94). In support of the present finding, Akhtar and Siddiqui [2] reported siderophore production by *Pseudomonas* sp. isolated from chickpea rhizosphere in the range of 1.6–1.7 cm. The twin role of siderophores in stimulating plant growth through enhanced host nutrition and by disease suppression by scavenging limited iron depriving phytopathogens of iron nutrition is well documented [12]. Chakraborty et al. [7] reported that *B. megatherium* from tea rhizosphere produced siderophores which contributed to plant growth promotion and disease reduction. All the isolates in the present study also produced salicylic acid in the range of 5.75–58.75 μ g/ml (Table 2). Isolate Ps14c produced substantial amount of salicylic acid (28.74 μ g/ml) which in combination with other antagonistic and plant growth-promoting traits could benefit plants under biotic and abiotic stress. Role of salicylic acid in enhancement of drought and salt stress tolerance in plants and inducing host resistance against phytopathogens is well documented [27].

Rhizobacterial isolates confirmed their potential to solubilize phosphates and zinc by formation of hydrolytic halo zone around the colonies on solid medium supplemented with $\text{Ca}_3(\text{PO}_4)_2$ and ZnO, respectively. Of 13 isolates, 10 exhibited phosphate-solubilizing potential, while 8 were Zn-solubilizers (Table 2). Highest P-solubilization potential was exhibited by isolate Ps19d with solubilization area of 4.91 cm^2 , while P-I showed highest Zn-solubilization potential (3.14 cm^2). The varied solubilization potential of

rhizobacterial isolates has been reported to be related to the location from which they are isolated [23]. Saravanan et al. [23] reported that zinc-solubilizing *Bacillus* sp. (ZSB-O-1) isolated from the sphalerite ore material showed higher solubilizing ability in the ZnS (sphalerite) ore containing medium due to their adherence with the ore particles. Moreover, the inability of P-solubilizing isolates Ps14c and Ps7a to solubilize Zn showed their low Zn tolerance potential. Zinc although is an important nutrient at low concentration but is toxic at higher concentration, and its solubilization might limit the growth of the bacteria at higher level [23]. In spite of this, data in the literature have cited Zn and phosphate solubilization ability of rhizobacterial isolates. Eleven out of twelve isolates of *Bacillus* sp. from soybean rhizosphere showed phosphate-solubilizing ability [30], while only 3 of total 48 bacteria isolated from the rhizosphere of rice plants showed zones of clearance of Zn-solubilization on media supplemented with insoluble Zn as ZnO or Zn₃(PO₃)₂ [29]. This shows that although there occurs a plenty of microorganisms in soil but not all of them have biofertilizing traits, screening them for solubilization of inorganic and insoluble minerals will certainly help to reduce the load of harmful chemicals which are being used at an unprecedented rate to meet the nutrition of plants.

Expanding the horizon of biofertilizing traits of rhizobacteria, production of phytohormones presents another contributory factor in stimulating plant growth. The present investigation revealed the biosynthesis of IAA as one of the major growth-promoting attribute associated with all the rhizobacterial isolates; however, a great variation in quantity was observed as indicated by difference in the intensity of pink color developed (Table 2). The discrepancy in IAA producing ability of rhizobacteria depending upon species, strains, culture condition, growth stage and substrate availability is well documented [3]. In spite of such variation, IAA biosynthesis by rhizobacteria has been reported to be associated with rapid establishment of roots in young seedlings which increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, therefore enhancing their likelihood for survival [3].

Characterization of Rhizobacterial Isolates

Bacterial antagonists were identified based on morphological observation and biochemical characterization using Bergey's manual of determinative bacteriology as reference. Isolates Ps15a, Ps16a, Ps19d, Ps29c and P-I showed the characteristic fluorescent green pigmentation on Kings B medium which has been proposed as general medium for the non-selective isolation and pigment production of *Pseudomonas* species. The pigments such as fluorescein,

pyorubin and non-fluorescent blue pigment, pyocyanin are readily produced by culturing on Kings medium B. However, production of fluorescence by pseudomonads is not constant during incubation and not common to all species of this group. Biochemical characterization of the isolates showed that all were indole, methyl red, Voges–Proskauer negative and citrate and catalase positive. They also hydrolyzed starch and reduced nitrates. Based on these characteristics, isolates were tentatively classified as *Pseudomonas* sp. However, molecular characterization using *Pseudomonas* genus-specific primers showed amplification only in 4 out of 13 isolates and yielded a product of about 618 bp confirming their identity as *Pseudomonas* sp. Negative PCR results with nine tentatively assigned *Pseudomonas* isolates clearly show that great difference exists between the designed PCR method and phenotypical identification that proved them as *Pseudomonas* sp. Among PGPR, fluorescent pseudomonads occur commonly in the rhizosphere of plants and they have immense potential in agriculture for use as biofertilizer and biocontrol agent [1]. The investigation further concludes the existence of a wide range of microbial diversity in soil with enormous prospective to emerge as bioinoculant.

Pot Experiment

The potential of isolate Ps14c, superior in terms of both in vitro-assayed plant growth-promoting and bioantagonistic attributes, was tested under controlled condition as single inoculant and in consortium with chickpea nodulating *Mesorhizobium ciceris* in stimulating plant growth and managing wilt in *F. oxysporum* challenged chickpea plants. Corroborating our earlier results, it was observed that yellowing symptoms in non-bacterized pathogen inoculated pots appeared early in contrary to bacterized and fungicide-treated plants where symptoms of wilting was delayed. The initial protection offered by antagonist Ps14c could be attributed to production of lytic enzymes, different diffusible metabolites and antibiotics which might have inhibited the pathogen growth. The effect of seed bacterization with bioantagonistic agent was very much evident in terms of final wilt incidence which was reduced to 48% compared to 74% in uninoculated control and was comparable to treatment with fungicide captan where 46% of plants showed wilting. Consortium administration of bacteria with *M. ciceris* recorded maximum protection (72%) against the disease which reflects the synergistic interaction between the bacterial inoculants (Table 3). The same kind of interaction has been observed by Hahm et al. [13] who reported that the degree of disease suppression was significantly increased where dual culture treatment was used. The reflection of seed bacterization on plant growth was also observed. Inoculation with isolate Ps14c alone and in consortium with rhizobia

Table 2 Functionality traits of rhizobacterial isolates

Isolates	Growth-stimulating traits					Antagonistic traits ^b						
	Siderophore index	IAA production in cm ³ ^a	P-solubilization		Zn-solubilization		Salicylic acid production (µg/ml)	HCN production	Ammonia production	Lytic enzymes production		
			Halo dia (cm)	Area (cm ²)	Halo dia (cm)	Area (cm ²)				Chitinase	Cellulase	Protease
Ps 4b1	–	1.9 ± 0.11 (++)	–	–	–	–	9.20 ± 0.57	–	++++	–	+	+
Ps7a	1.34 ± 0.43	1.7 ± 0.05 (+)	1.4 ± 0.03	1.53	–	–	10.81 ± 0.34	–	++++	–	+	+
Ps13b	2.33 ± 0.57	2.1 ± 0.11 (++)	–	–	–	–	6.21 ± 0.12	–	++++	–	+	+
Ps14c	2.82 ± 0.40	2.3 ± 0.11 (++++)	1.9 ± 0.03	2.83	–	–	28.74 ± 0.73	–	++++	+	+	+
Ps14d	–	0.7 ± 0.008 (+)	2.0 ± 0.04	3.14	1.3 ± 0.02	1.32	23.46 ± 0.26	–	++++	–	–	+
Ps15a	–	1.6 ± 0.06 (++)	1.5 ± 0.02	1.76	1.4 ± 0.03	1.53	37.39 ± 0.22	++	++++	–	–	+
Ps16a	–	1.4 ± 0.03 (++)	1.4 ± 0.03	1.53	1.3 ± 0.01	1.32	58.42 ± 0.24	–	++++	–	+	+
Ps19d	–	2.5 ± 0.11 (++++)	2.3 ± 0.04	4.15	1.6 ± 0.04	2.01	31.05 ± 0.60	–	++++	+	+	+
Ps20b	2.94 ± 0.26	1.5 ± 0.02 (++)	2.5 ± 0.02	4.91	1.4 ± 0.03	1.53	31.97 ± 0.53	–	++++	+	+	+
Ps24d	1.81 ± 0.31	2.5 ± 0.08 (++++)	–	–	–	–	9.89 ± 0.51	+++	++++	–	+	+
Ps28c	–	1.6 ± 0.17 (+)	1.6 ± 0.01	2.01	1.8 ± 0.04	2.54	42.55 ± 0.31	++	++++	–	+	+
Ps29c	1.86 ± 0.34	0.9 ± 0.006 (+)	0.9 ± 0.02	0.63	1.1 ± 0.01	0.94	5.75 ± 0.43	–	++++	–	+	+
P-J	1.60 ± 0.12	3.1 ± 0.28 (++)	1.0 ± 0.03	0.78	2.0 ± 0.02	3.14	34.70 ± 0.40	–	+++	+	+	+

Values represent mean ± SE of three replications

^a (+) sign in bracket represents intensity of pink color developed^b – and + signs represent absence and presence of character and for HCN and ammonia production, number of + signs indicate intensity of color developed

Table 3 Effect of seed treatment with bioagents on wilt incidence in chickpea

Treatments	Wilt incidence (%)			
	20th day	25th day	30th day	35th day
Ps14c + <i>Rhizobium</i> + Foc	5.7	8.6	17.2	28.0
Ps14c + Foc	15.7	22.8	37.2	48.0
Foc + fungicide	11.4	22.8	38.6	46.0
Foc (control)	18.6	28.6	50.0	74.0

Table 4 Effect of seed bacterization on growth parameters in chickpea

Treatments	No. of seeds germinated ^a	Plant fresh weight (g)	Plant dry weight (g)	Plant height (cm)	Chl content (mg/g)
Ps14c + <i>Rhizobium</i> + Foc	68 (9.71)	6.27 ^a	1.37 ^a	20.33 ^a	0.83 ^a
Ps14c + Foc	66 (9.57)	5.90 ^a	1.26 ^{ab}	18.45 ^b	0.74 ^{ab}
Foc + fungicide	63 (9.00)	5.75 ^a	1.18 ^b	17.4 ^b	0.66 ^{bc}
Foc (control)	62 (8.85)	4.65 ^b	0.97 ^c	15.2 ^c	0.63 ^c
C.D (5%)	NS	1.05	0.13	1.4	0.11

Value in bracket presents average number of seeds germinated

Each value represents mean \pm SE of three replications. In the same column, significant differences at $p < 0.05$ levels are indicated by different letters. Data followed by same letter in the same column are not significantly different from each other according to analysis of variance (ANOVA)

^a A total of 70 seeds were sown for each treatment @ 10 seeds/replication

recorded 95 and 98% seed emergence, respectively, as compared to 89% in control. Higher seed germination recorded in bacterized plants could be associated with auxin secretion by rhizobacteria which helped in root elongation and better survival of seedlings in the initial stage of growth. Plants also showed better growth in terms of plant height, plant fresh weight and dry weight. Chlorophyll content recorded on 25th day of germination showed significantly higher values in dually inoculated plants as compared to uninoculated and fungicide-treated plants (Table 4). The benefit of using bioagent was clearly depicted in present investigation as fungicide treatment no doubt reduced wilt incidence in plants, but seed bacterization with isolate Ps14c offered dual benefit of plant growth stimulation and protection from wilt pathogen. This dual benefit was further stimulated on dual inoculation of bacterium and rhizobia resulting in enhanced shoot length, plant weight and photosynthetic ability of plants. This undoubtedly indicated active role of rhizobia in secretion of some plant growth-promoting hormones such as IAA and cytokinins. The similar synergistic effect in use of dual cultures is well documented in the literature [13].

Conclusion

Plant rhizosphere is rich depository of microorganisms; exploring this micro-biome to unravel an elite culture, having both biofertilizing and bioantagonistic traits, would

offer sustainable agricultural benefits. This study presents a scrupulous approach to develop efficient bioagents for enhanced plant growth. However, further research on soil–plant system is necessary to gain their commercial viability and worldwide acceptability.

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