



RESEARCH ARTICLE

Plant growth promoting and antagonistic *Enterobacter* sp. EPR4 from common bean rhizosphere of Garhwal Himalayan inhibits a soil-borne pathogen *Sclerotinia sclerotiorum*

Pankaj Kumar^{1,2*}, Ramesh Chandra Dubey² & Ashutosh Kumar Rai³

¹Department of Microbiology, Dolphin (PG) Institute of Biomedical and Natural Sciences, Dehradun – 248 007, Uttarakhand, India (Present address)

²Department of Botany and Microbiology, Gurukul Kangri Vishwavidyalaya, Haridwar – 249 404, Uttarakhand, India

³Department of Biochemistry, College of Medicine Imam Abdulrahman Bin Faisal University, Dammam, 31441 Saudi Arabia

*Email: guptapankaj23@gmail.com



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Abstract

Plant growth stimulating and antagonistic properties of 7 bacterial isolates of beneficial *Enterobacter* spp. (EPR1- EPR7) screened from the rhizospheric soil of *Phaseolus vulgaris* plants growing in Garhwal Himalaya, Uttarakhand, India was studied against soil borne phytopathogen *Sclerotinia sclerotiorum* causes root rots in various crops. Among the isolates, EPR4 showed 64.8% reduction in colony growth of the fungal pathogen in dual culture. All seven isolates are capable of producing Indole Acetic Acid (IAA), but EPR4 also produced cyanogens, solubilized inorganic and organic phosphate, siderophore, ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, and extracellular enzymes like chitinase which inhibited the phytopathogen. For the EPR4 strain, 16S rRNA gene sequencing was followed by NCBI - BLAST similarity showed the maximum sequence similarity (100%) with the species of *Enterobacter* (available on NCBI data base), and recognized as *Enterobacter* sp. EPR4 (GenBank accession number JN225424). The *Enterobacter* sp. EPR4 has the potential to be used as a biocontrol agent against *S. sclerotiorum* as well as a good plant growth promoter for common bean and other crops grown in India's Garhwal Himalaya.

Keywords

Enterobacter sp., Biocontrol, PGPR, *Phaseolus vulgaris*, *Sclerotinia sclerotiorum*

Introduction

The average growth rate of worldwide populations is 1% per year, while a few number of countries having more growth rates (1). Food supply has also increased parallel to population growth, but concern has been expressed as to whether this parallel increase can be continuing with current agricultural practice (2). This demand can be met by a sustainable agricultural method that not only maintains crop output to fulfill the needs of growing populations, but also avoids ecological disruption without depleting natural resources. Thus, agricultural scientists become more attentive towards the beneficial soil bacteria as a better alternative of chemical or synthetic fertilizers to facilitate eco-friendly biological control of soil and seed borne phytopathogens.

Generally, only 2-5% of rhizospheric bacteria are beneficial for plant and are known as Plant Growth Promoting Rhizobacteria (PGPR) which stimulate plant growth either by direct and/or indirect methods (3). Direct mechanisms can be demonstrated in absence of plant pathogen or other

rhizosphere microorganisms, which includes atmospheric N₂ fixation, siderophore production, phosphorus mineralisation, biosynthesis of phytohormones (IAA, cytokinin, and Gibberellic acid (GAs) increasing the nutrient level of soil (4). While indirect mechanisms involve the ability of PGPR to reduce the harmful effect of pathogenic microorganisms on the crop and suppress phytopathogens by multi-dimensional actions, such as production of siderophore which chelate Fe³⁺ iron making it unavailable to pathogens, production of cyanogens, synthesis of antimicrobial metabolites (antibiotics), fungal cell-wall degrading enzymes, competition for nutrients and specific niche on the roots (5, 6).

Phaseolus vulgaris L. (common bean) is a popular member of subfamily Papilionaceae of Fabaceae. It is an important pulse crop of India, cultivated in Maharashtra, Himachal Pradesh, Uttarakhand and Jammu & Kashmir, covering 80-85 ha of land (7). Among the legumes, common beans are most edible pulse in the world, second only to soybeans.

Diverse group of plant growth enhancing microorganism such as *Bacillus*, *Pseudomonas*, *Azotobacter* and *Enterobacter* have been reported by various workers from different plants (8-11). Due to the presence of several plant growth promoting qualities, *Enterobacter* spp. has received special attention as a plant growth enhancer and has been isolated from various plants such as maize, soybean, citrus etc. (12-15). The promising PGPR *Enterobacter* sp. NRRU-N13 having ability to produce IAA and solubilize phosphate, as well as increase root and shoot lengths, dry weights and biomass of rice efficiently when used as bio-inoculant (10). It was reported that *E. harmaechei* subsp. *steigerwaltii* has a significant protective potential against fungal pathogen leading to better tomato growth (16). Recently, some other researchers have isolated promising *Enterobacter* sp. having numerous plant growth promoting properties and significantly enhanced growth of tomato and sugarcane (17, 18). In this present investigation we aimed to find out growth promoting *Enterobacter* spp. associated with rhizosphere of a legume crop, *P. vulgaris* as well as their antagonistic properties against *Sclerotinia sclerotiorum*. *S. sclerotiorum* is one of the most destructive and widely distributed fungal pathogens of plants, causing white mold disease in over 400 plant species all over the world (19).

Materials and Methods

Microorganisms (Isolation and procurement)

To isolate beneficial rhizospheric bacteria, young and healthy plants of *P. vulgaris* were uprooted carefully and transferred to the laboratory in sterile plastic bags. Seven pure bacterial isolates were selected following the standard serial dilution technique. Isolates were purified by streaking three times on fresh NAM plate, there was single type colony found after third streaking. All the isolates were phenotypically (morphologically, physiologically and biochemically) characterized following the Bergey's Manual of Determinative Bacteriology (20).

The fungal phytopathogen *S. sclerotiorum* has been procured from the Department of Forest Pathology, Forest Research Institute (FRI), Dehradun, Uttarkhand, India.

Molecular characterization (16S rRNA genes sequencing) of potential isolate

Based on outcomes of plant growth promoting activities the isolate EPR4 was found as most promising PGPR and hence, this isolate was subjected to 16S rRNA gene sequencing (21). Similar 16S-rRNA gene sequences were obtained from NCBI GeneBank database and constructed phylogenetic tree using MEGA 10 software.

Plant growth promoting attributes

IAA Production

The log phase cultures of isolates were inoculated separately on nutrient broth medium and incubated at 30 °C for 24 h and the supernatant was recovered by centrifuging the broth for 15 min at 4 °C at 10000 rpm. A mixture of 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski's reagent was added in fresh tube having 2 ml of supernatant. The mixture was left undisturbed at 25 °C for 25 min and the appearance of pink color indicates IAA production (2).

Phosphate Solubilization

Phosphate solubilization ability was detected on two types of inorganic and organic phosphate containing medium having tri-calcium phosphate and sodium phytate respectively by spotting the culture (24 h old) separately on respective agar plates. For the establishment of a clean zone around the bacterial colonies, the inoculated plates were incubated at 30 °C for 24-72 h (either due to organic acids or enzymes produced by isolates) (22).

Cyanogen (HCN) Production

Exponentially grown cultures of each isolate were examined for putative HCN production using the standard methodology (23).

Siderophore Production

On chrome-azuroil S (CAS) medium, siderophore production was measured using standard procedure (24). The overnight cultures were spot inoculated individually on CAS medium and incubated at 30 °C for 24-72 h. The development of siderophores is indicated by the appearance of an orange to yellow halo around bacterial growth.

ACC deaminase Production

Log phase cultures were harvested as pellets, washed twice in distilled water before being re-suspended in saline and spotted on DF (Dworkin and Foster) minimal medium consisting of three different composition viz. (a) ACC as sole source of nitrogen, (b) ammonium sulphate (positive control) and (c) without nitrogen source (negative control) (25).

In vitro antifungal activities of isolates

To evaluate the antagonistic activities, the dual culture technique was performed for all isolates against *S. sclerotiorum* using the standard methodology (26). Agar block (5 mm diameter) from 5 days old culture of *S. sclerotiorum*

was transferred at the center of the assay plate. One loopful log phase culture of each isolates was streaked straight apart 2 cm away from the pathogen. Plate having only fungal pathogen at center served as control. All the plates of isolates were incubated at 28 ± 1 °C for 5-7 days. The inhibition of fungal growth was measured by using a formula: Inhibition (%) = $100 \times \frac{C-T}{C}$, where, C- fungal radial growth in control, T- fungal radial growth in dual culture.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) for antagonism study was carried out following the method (27). Fungal mycelia were collected from the interaction zone and was fixed in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) for overnight at 4 °C and washed three times (10 min each) in phosphate buffer followed by rinsing it (thrice) for 10 min in sterile distilled water. Samples were dehydrated by using gradual increasing concentration of ethanol *i.e.* 70, 80, 90 and 100% (5 min in each stage) and three changes in 100% ethanol. Ethanol was replaced by liquid CO₂. The air dried samples were mounted on stubs followed by bombardment with gold particles and observed at 15 kV in a LEO 485 VP SEM.

Screening of Chitinase secretion ability

Extracellular chitinase production of isolates was evaluated by using Chitin Minimal Medium (CMM) having colloidal chitin as sole source of carbon. All the isolates were spot inoculated on CMM plates and incubated at 30 ± 2 °C for 4-5 days. The appearance of clear zone in the vicinity of bacterial colonies confirms the secretion of chitinase by isolates (28).

Preparation of colloidal chitin

Colloidal chitin was made using 10g of crab shell chitin (Sigma) by dissolving slowly in one liter of concentrated HCl under stirring conditions at 4 °C for 7 days. Resulting viscous mixture was incubated in water bath at 37 °C until the viscosity of the mixture is decreased. A total of 4 lit. of sterile distilled water was added to the mixture and left overnight at 4 °C. The precipitate was collected in a filter paper by decanting supernatant. To achieve a natural pH, the precipitate was rinsed three times with sterile distilled water. The saturated colloidal chitin was air dried and was dissolved in 250 ml sterile distilled water prior to use (29).

Screening of Oxalate oxidase producing ability

Over night grown culture of bacterial isolates was spot inoculated on agar plate having oxalic acid and incubated under dark condition for 7 days at 30 ± 1 °C. The appearance of semi transparent zone around bacterial growth indicates the production of oxalate oxidase (30).

Results

Isolation and Characterization of beneficial Microbes

Seven efficient isolates were chosen for further study and finally selected for evaluation of their plant growth promoting attributes. All seven isolates were found positive for one or the other characteristics. All the isolates are Gram-negative and non-spore forming, extremely fast

growing with average mean doubling time of 30 min. All isolates failed to grow on GPA but able to grow on HAB and tolerate 8% KNO₃ (Table 1).

Table 1. Morphological, physiological and Biochemical characters of Rhizospheric bacteria isolated from *Phaseolus vulgaris*.

Characteristics	EPR 1	EPR 2	EPR 3	EPR 4	EPR 5	EPR 6	EPR 7
Gram reaction	-	-	-	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Endospore	-	-	-	-	-	-	-
Growth on GPA	-	-	-	-	-	-	-
Growth on HAB	+	+	+	+	+	+	+
8% KNO ₃ tolerance	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Ca- glyc	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	+	+	-	-
ONPG ^K	-	+	+	-	-	-	+
Lysine decarboxylase ^K	+	+	+	+	+	+	+
Ornithine decarboxylase ^K	+	+	+	+	+	+	+
Urease	-	-	+	+	+	+	-
Phenylalanine deamination ^K	-	-	-	-	-	-	-
Nitrate reduction ^K	-	+	-	-	+	+	+
H ₂ S Production ^K	-	-	-	+	-	-	-
Citrate utilization ^K	-	-	+	+	+	+	+
Voges Proskaur's ^K	-	+	+	+	+	+	-
Methyl red ^K	-	-	-	-	-	-	-
Indole ^K	-	+	-	-	+	-	-
Malonate ^K	-	+	-	+	+	+	-
Esculin hydrolysis ^K	+	+	+	+	+	+	+
Oxidase ^K	+	+	+	+	+	+	+

+ positive; - negative; GPA- glucose peptone agar; HAB- Hoffer's alkaline broth; Ca- glyc, precipitation in Calcium glycerophosphate; ONPG- *o*-nitrophenyl-β-D-galactoside; K- Performed using Hi25™ Hi-Media Enterobacteriaceae Identification Kit alongwith standard phenotypic test following Bergey's Manual of Determinative Bacteriology.

Molecular characterization (16S rRNA genes Sequencing) of potential isolate EPR 4

Since the isolate EPR 4 has better PGP activities as compared to all other isolates. So, 16S rRNA gene sequence of EPR4 isolate was sequenced and furthermore analyzed for its characterization. Using the EPR4 sequence and typical sequences from NCBI databases, a neighbor-joining dendrogram was created. Phylogenetic study of the EPR4 strain's 16S rRNA sequence revealed that the strain had the highest sequence similarity (100%) with the species of *Enterobacter*. It occupied the same phylogenetic branch as the *Enterobacter* group and named as *Enterobacter* sp. EPR4 (NCBI GenBank accession number JN225424 was received from NCBI in the year 2011) (Fig. 1).

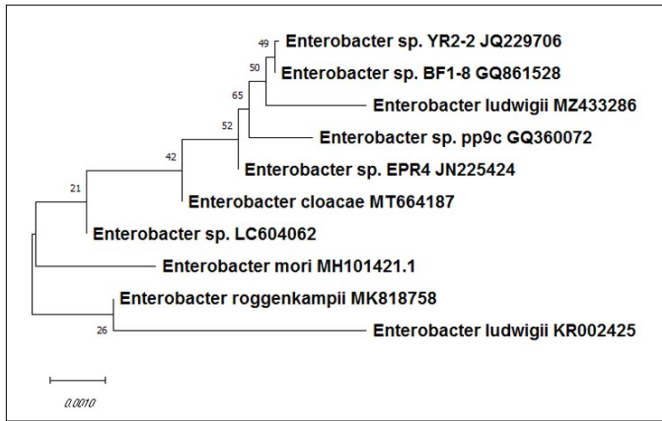


Fig. 1. Phylogenetic analysis of root nodulating bacteria *Enterobacter* sp. EPR 4 (GenBank accession number JN225424) isolated from *P. vulgaris* based on 16S rRNA gene sequences available online from National center for biotechnology information (NCBI). The tree was constructed after multiple alignments of sequence data by Clustal W. Distance and clustering with the neighbor-joining method was performed by using the Mega 10. Bootstrap values based on 1000 replications are listed as percentage at the branching points.

Plant growth promoting activities

IAA production

Except EPR 2, all isolates were able to produce IAA. The formation of pink color in their culture filtrate was seen with and without tryptophan (Table 2).

Table 2. Plant Growth-Promoting and antagonistic attributes of rhizobacteria (EPR1-7) isolated from *Phaseolus vulgaris*.

Isolates	IAA ^A	Pikovskaya's medium (TCP as Inorganic Phosphate) ^B	Phosphate solubilizing medium (Sodium phytate as Organic phosphate) ^C	HCN ^D	Siderophore ^E	ACCD ^F	Chitinase ^G	Oxalate-oxidase ^H	Antagonisms against <i>S. sclerotiorum</i> ^I
EPR1	+	-	-	-	-	-	-	-	-
EPR2	-	+	-	-	+	-	-	-	-
EPR3	+	++	-	-	+	-	-	-	-
EPR4	+++	+++	++	+	++	+	+++	++	+++
EPR5	+	+	-	-	-	-	++	-	++
EPR6	++	+	-	+	+	-	++	-	++
EPR7	+	+	-	-	+	-	-	-	-

A -, IAA negative, +, IAA positive; **B** -, 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 >1.0cm wide surrounding colonies; **C**, Organic phosphate; **D**-, HCN negative, +, HCN positive; **E** -, Siderophore production negative; +, Siderophore Production positive; **F** -, ACCD negative, + ACCD positive; **B** - Potassium solubilization negative, **G** -, Chitinase negative, +, chitinase positive; **H** -, Absence of clearing zone around bacterial, + Presence of clearing zone around bacterial spot on oxalic acid degrading agar media; **I** -, no inhibition of *S. sclerotiorum*, +, inhibition of *S. sclerotiorum*; (+++, ++, + decreasing order of inhibition). All experiments were done in triplicate with three independent trials

Phosphate solubilization

On the Pikovskaya's agar plate with inorganic phosphate, all seven isolates produced distinct halos around the growth, but only EPR4 were able to solubilize organic phosphate as indicated by formation of clear halo zone (Table 2). Such clearing around the bacteria colonies showed their phosphate solubilization ability.

Cynogen (HCN) production

HCN production is indicated by a change in filter paper color from yellow to brown or deep brown. Except EPR4 and EPR6, none of the isolates produced HCN as there was color change in the filter paper soaked with Na₂CO₃ and picric acid (Table 2).

Siderophore production

All the isolates were evaluated for siderophore production on CAS agar. Remarkably, five isolates, except EPR1 and EPR5, showed siderophore production forming orange halo around their colonies on CAS agar (Table 2). The discharge of siderophore by all five isolates started after 16 h of incubation and it continuously increased up to 3rd days.

ACC deaminase production

The ACC deaminase activities of all seven isolates were tested on DF minimal media. Except EPR4, none of the other isolates were able to use ACC as their sole supply of nitrogen (Table 2).

In vitro antifungal activities of isolates

Dual culture interaction studies in *in vitro* condition revealed that strains EPR4, EPR5 and ERR6 inhibited the growth of *S. sclerotiorum* by 64.8%, 55% and 52% respectively, but the other isolates failed to inhibit the growth of pathogen (Table 2; Fig. 2 A, B). Under a scanning electron microscope, fragmentation and deterioration of mycelia were plainly visible (Fig. 2 C). The morphological defects caused by structural changes and mycelia lysis eventually led to fungal mortality.

Chitinase and Oxalate Oxidase enzyme production

Only three isolates EPR 4, EPR 5 and EPR 6 were able to produce chitinase but the production of enzyme oxalate oxidase was observed only in EPR 4 (Table 2).

Discussion

In this study we have isolated different rhizospheric bacteria from common bean, and screened primarily based on their phenotypic characteristics. Among them only seven isolates were selected and screened for the further study. Based on plant growth promoting properties of all seven isolates (EPR1 to EPR7), the isolate EPR4 was found as most promising having best PGP activities and therefore its genomic characterization was performed. EPR4 was

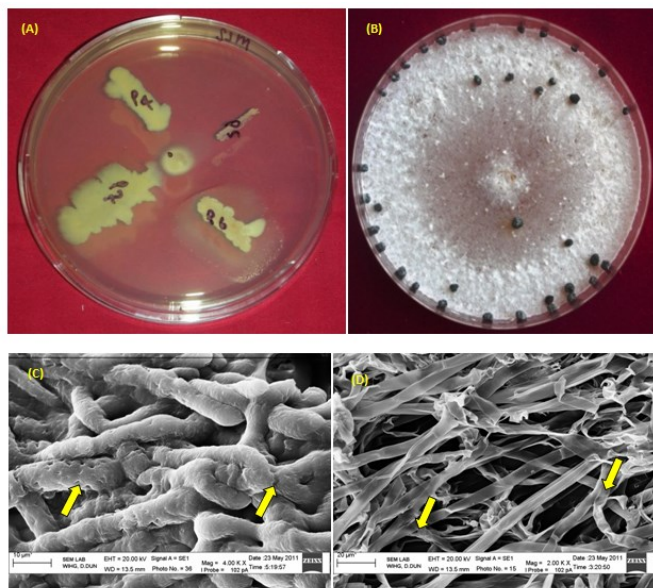


Fig. 2. *In vitro* inhibition of *S. sclerotiorum* by PGPR *Enterobacter* sp. EPR4 (A), Control (only *S. sclerotiorum*) (B), Scanning electron microscopic photographs of dual culture showing deformities (arrow) due to *Enterobacter* sp. EPR4 in *S. sclerotiorum* (C), Control (only *S. sclerotiorum*) (D)

recognized as *Enterobacter* sp. EPR4 (GenBank accession number JN225424).

All the seven isolates have been evaluated for their PGP activities and found that EPR4 having abilities to produce IAA, solubilize organic and inorganic phosphate, produce siderophore, HCN and enzyme like ACC deaminase. Besides these growth promoting activities, some enzymes like chitinase and oxalate oxidase having ability to degrade fungal cell wall have been detected in *Enterobacter* sp. EPR4. Previously, several researchers have reported comparable findings for *Enterobacter* isolated from crops growing in different climatic condition. Reports are on the Isolation of 4 *Enterobacter* spp. capable of producing IAA and siderophore and to solubilize phosphate and effectively promoted plant growth (12). Saengsanga (10) isolated several *Enterobacter* spp. from rice but *Enterobacter* sp. NRRU-N13 was most promising PGPR because of good IAA producer and solubilizer of tri-calcium phosphate. Similar findings were also observed (15).

In this study, the strain EPR4 was the good IAA producer followed by EPR6 among all seven isolates. Indole acetic acid is a phytohormone that is produced by a large number of soil bacteria and EPR4 and EPR6 are no exception (31, 32). IAA not only regulate plant cell division and root elongation but also serves as signalling molecules in various plant-microbe interactions (33).

Another typical property via which rhizobacteria can significantly improve plant development is phosphate solubility. Unlike IAA, phosphate solubilisation is highly variable features of bacteria. Except EPR1, our all isolates solubilized inorganic phosphate, whereas only EPR4 solubilized organic phosphate. This finding indicates that these phosphate solubilizing strains secrete several organic acids, such as gluconic acid etc. and enzymes like phosphatases, phytases etc. and make available the essential nutrient phosphorus to sustain plant growth (34). Reports

are on a phosphate solubilising *E. cloacae* (B1) efficiently enhanced growth characteristics, physiological characteristics as well as yield characteristics of wheat plant that strongly support our findings (35).

In our findings, the strain EPR4 has the ability to produce ACC deaminase. ACC, the precursor of ethylene, is cleaved by the microbial ACC deaminase in plants and diminished the ACC level in stressed plants and finally limits the ethylene synthesis preventing damage to plants due to stress. In natural condition, plants face various types of stresses and produce ethylene (36). Reports are on 2 ACC deaminase producing (>1500 nmol α -ketobutyrate mg protein $^{-1}$ h $^{-1}$) PGPR which significantly declined the ethylene levels (37). IAA secreted by soil bacteria not only enhance the growth of roots by stimulating cell division/elongation but also influence bacterial ACC deaminase activity (38).

Siderophore are able to sequester the majority of the accessible Fe $^{3+}$ in the rhizosphere and provide it to plants and directly assist plant growth while inhibit the growth of other pathogens in the vicinity of the root system due to lack of iron (5, 34). HCN, chitinase and oxalate oxidase produced by EPR4 also help in suppressing *S. sclerotiorum* and indirectly promote growth of common bean. In the present study, only EPR4 and EPR6 isolates were able to produce cyanogens. HCN is a good inhibitor of cytochrome c oxidase and protects plants from various fungal phytopathogens (32). Chitinases efficiently hydrolyze the chitin (a linear homopolymer of p-1,4-linked N-acetyl-D-glucosamine (GlcNAc)). In our study, *Enterobacter* sp. EPR4 was found as a good producer of chitinase that inhibited the growth of *S. sclerotiorum in vitro* (39). Similarly, the chitinase producing *Enterobacter* sp. NRG4 stalled the growth of 4 fungal phyto-pathogens viz., *Fusarium moniliforme*, *A. niger*, *Mucor rouxi* and *Rhizopus nigricans* (40). The use of various beneficial microbes or their products to protect plants from several phytopathogenes and insect pests is an eco-friendly approach for sustainable agriculture.

Enterobacter sp. EPR4 produces oxalate oxidases recorded during present study. Obviously, the beneficial soil microbes producing oxalate oxidases are good for management of *Sclerotinia* pathogen by converting oxalic acid to CO $_2$ and H $_2$ O $_2$. H $_2$ O $_2$ support the thickening process of plant cell wall which arrest the pathogens and protect plants from their deleterious effect.

During present study the promising strain *Enterobacter* sp. EPR4 strongly suppressed the growth of *S. sclerotiorum* indicating a close association between the production of siderophore, HCN and chitinase. The synthesis of siderophore, HCN and chitinase enzymes may be responsible for this possible PGPR's antagonistic activity against *S. sclerotiorum* (41).

In fungal mycelia, abnormal hyphal enlargement, fungal cell wall digestion, cytoplasm coagulation, hyphal perforation, hyphal tip disintegration and halo cell formation were caused by *Enterobacter* sp. EPR4 that probably may be mediated by HCN, siderophore, cell wall degrading lytic enzymes, and oxalate oxidase. Losses of

structural integrity of fungus were also observed in the SEM studies.

It has also been suggested that siderophores, HCN, lytic enzymes and other metabolites produced by various potent PGPR effectively freeze the growth of mycopathogens like *F. oxysporum*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *S. sclerotiorum* (5). Further, studies on the recital of *Enterobacter* sp. EPR4 and its mutant variant for their plant growth promotion will reveal the details mechanism of this strain.

Conclusion

It can be inferred that *Enterobacter* sp. EPR4 has a strong ability to promote plant growth. It strongly inhibits the *in vitro* growth of phytopathogen *S. sclerotiorum*. Since this strain has dual properties *i.e.* growth enhancing properties as well as biocontrol of phytopathogens, EPR4 can be utilized to prepare good-quality bio-fertilizers for common bean as well as other growing in Garhwal Himalaya. Further, to evaluate the effect of this strain in natural field condition is under study.

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Authors contributions

PK carried out the experimental works and wrote the paper. RCD designed the experiments and improved the manuscript. AKR participated in data analysis and editing of the manuscript and all authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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