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Co-culture development and bioformulation efficacy of psychrotrophic PGPRs to promote growth and development of Pea (*Pisum sativum*) plant

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Numerous microbes reside in the rhizosphere having plant growth promoting activity, and enhancing the property by increasing plant yield. Plant growth promoting rhizobacteria (PGPR) has gradually increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. Soil was collected from the rhizosphere of an agricultural farm and the psychrotrophic bacterial strains STA₃ (KY888133) and RM₂ (KY888134) were successfully isolated, and screened on the basis of phosphate solubilization. Further characterization was carried out by morphological, biochemical, and 16S rDNA characterization methods. The unique nature of psychrotrophic *Pentoea ananatis* and a suitable combination with *Pseudomonas fluorescens* regarding plant growth promotion activity has not been studied before to our knowledge. An assessment of various parameters of plant growth promoting activity, such as IAA, phosphate solubilization, bio-control activity, HCN and siderophore production, has been carried out. Both strains were found to be positive in various parameters except HCN and Biocontrol activity, which were positive only for the strain RM₂. Also, shelf life and their efficacy was determined before and after formulation. A great consistency was observed in all the cultures, even after 70 days of storage under bio-formulation at room temperature, while in the case of the co-culture CPP-2, the cfu ml⁻¹ was greater, followed by RM₂ and STA₃. Moreover, the growth indices of the pea plant were found to be better in the co-culture CPP-2 compared with individual strains, followed

by RM₂ and STA₃. Thus, the study suggests that the co-culture CPP-2 has a great potential for plant growth promotion as compared with individual strains followed by RM₂ and STA₃.

Key Words: bio-control; bio-formulation; *Pentoea* sp.; PGPRs; *Pseudomonas* sp.; Psychrotrophs; Siderophore

Introduction

Soil is saturated with microbes such as bacteria, fungi, actinomycetes, protozoa, and algae, with bacteria being by far the majority (i.e., 95%). It has been determined that soil hosts a huge number of bacteria (often around 10⁸–10⁹ cells per gram of soil) and that the number of culturable bacterial cells in soil is usually about 1% of the total number of cells present (Schoenborn et al., 2004). Soil conditions, viz. temperature, moisture, salt concentration, chemical composition, as well as the plant community, influences the bacterial community found in different soils. The bacteria that can promote plant growth are called plant growth promoting rhizobacteria (PGPR) (Verma et al., 2016). They endorse plant growth either directly by facilitating resource attainment of plant hormone levels, or indirectly by lessening the inhibitory property of various pathogenic agents on plant growth and development by acting as a biocontrol agent (Anwar et al., 2014). Psychrotrophic bacteria can grow over a wide range of temperatures. They can survive and thrive in a cold environment, at temperatures close to 0°C, and withstand mild temperatures of 15–30°C. The influence of PGPRs are relate to their pathogen growth suppression, nutrient avail-

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ability to plants, production of growth-promoting factors, enhancing abiotic stress tolerance mechanisms, and inducing systemic defence responses in plants (Kumar et al., 2017). The winter, or in a cold environment, promotes the growth of plant pathogens, and, as the temperature drops, the growth of antagonistic bacteria and microorganisms increases and nutrient availability slows down. Plants become vulnerable to disease, lodging, etc., and post fungal infection due to an insufficient supply of biogenic elements (Przemieniecki et al., 2014).

Furthermore, the study is required to explore the role and functions of psychrotrophic microorganisms that resist the temperature range of a mountainous region from 0 to 35°C and encompasses the great potential for various plant growth promoting activities (Mishra et al., 2008; Panwar et al., 2016; Selvakumar et al., 2009; Trivedi and Pandey, 2007). The present study describes the PGPR potential of psychrotrophic bacteria, residing in the rhizosphere of mustard and apple plants, and having a potential applicability to improving pea crop productivity in the changing temperatures of a mountainous region.

Materials and Methods

Isolation, screening, and characterization of bacterial strains. Bacterial strains were isolated from the rhizosphere soil of mustard (*Brassica juncea*) and apple (*Mallus pumilus*) fields from the Ramgarh (29.45°N 79.55°E) and Satbunga (29.38°N to 79.46°E) regions of Uttarakhand, India, respectively. The soil samples were taken from a depth of 15–20 cm deep kept in plastic bags, and carried to the laboratory. Ten grams of soil were taken in a conical flask of 250 ml, and 90 ml of sterile distilled water was added to it. The flask was shaken for 10 minutes on a rotary shaker, 10-fold serial dilutions were prepared from the extract, and 0.1 ml of each dilution was spread on the plates of nutrient agar medium and the plates were incubated at 10°C for 2 days. Typical bacterial colonies were observed over the plate. A well, isolated single colony was picked up and re-streaked to a fresh nutrient agar plate and incubated in a similar manner. The technique was executed three times to obtain a pure culture. Subsequently, the identification of both the strains was made by comparing different biochemical properties of used specific biochemical test kits (KB013 HiMEDIA HiTM identification kit).

Determination of optimum pH and temperature for growth. The freshly grown cultures of bacterial isolates were inoculated on one set of nutrient broth of different pHs from pH 5.0–10.0 and incubated at 10°C to determine the optimum pH. Another set of tubes were inoculated in nutrient broth with the same fresh grown culture and incubated at different temperatures like 5–35°C to determine the optimum temperature. The change in growth was observed after 24 h of incubation and recorded in the form of O.D. at 600 nm.

Molecular characterization. Both strains were characterized on the basis of 16S rDNA sequencing. Polymerase chain reaction (PCR) amplification of the partial 16S rDNA gene region was carried out with the bacterial primer set

27 forward primer- 5'AGA GTT TGA TCA TGG CTC AG 3', 1492 reverse primer- 5'ACC TTG TTA CGA CTT 3'. The templates were purified by the Ethanol/EDTA precipitation method. The sequence was then analyzed by basic local alignment search tool (BLAST) at the National Center for Biotechnology Information database (NCBI, USA). Further, the isolates were identified based on similarity scores. The phylogenetic tree was constructed using the neighbour-joining method, and the phylogenetic data were obtained by aligning the different partial sequences of the 16S rDNA of closely-related strains. The culture information was submitted to the NCBI database and an accession number was obtained.

Production, quantification, and confirmation of indole-3-acetic acid (IAA). Both the strains were inoculated into 10 ml of nutrient broth supplemented with 2 mg ml⁻¹ of tryptophan and incubated at 10°C in a shaking incubator for 48 h. It was then centrifuged at 7500 rpm for 10 min. Then 1 ml of the supernatant was taken in another tube and 2 ml of Salkowski's reagent was added and incubated at 30°C for half an hour. The development of a dark pink to orange color indicates the production of IAA. Furthermore, to quantify the IAA produce in the tubes, the absorbance was measured at 530 nm. The concentration of IAA in each bacterial strain was determined and quantified by comparing with a standard curve of IAA (Verma et al., 2016).

The confirmation of IAA production is executed by means of thin layer chromatography (TLC), for which a single bacterial colony was inoculated to 10 ml of nutrient broth containing 2 mg ml⁻¹ of tryptophan and incubated at 10°C for 5 days on a shaker. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 minutes. The supernatant was acidified to pH 2.5–3.0 with 1 N HCl and extracted with ethyl acetate. The extracted ethyl acetate fraction was evaporated in a rotary evaporator at 40°C. The extract was dissolved in 1 ml of methanol and kept at –20°C. In a glass chamber, a mixture of benzene: n butanol: acetic acid in 4:5:1 proportion was prepared as the solvent system for the chromatogram. 1 µl of the extract was spotted and marked at the lower portion of the TLC plate. Similarly, a control spot of standard IAA (1 mg ml⁻¹) was also placed on the plate. The plate was placed inside the glass chamber and was covered. Once the solvent front reached the top layer of the plate, the plate was taken out from the chamber and dried naturally. After drying, IAA was identified by spraying the plates with Salkowski's reagent. The test and control samples were compared by spot size and Rf value.

Phosphate solubilization and its quantification. Each bacterial isolate was placed in the centre of separate Pikovskaya's agar plate and incubated at 10°C for 4 to 5 days. A clearing zone indicates the phosphate solubilization activity of the isolate. Further, phosphate solubilization potential was quantified by taking 1 ml of freshly-grown broth cultures of each strain and inoculated in 100 ml of Pikovskaya's broth (Pikovskaya, 1948), which were incubated at 10°C for 6 days. The amount of inorganic phosphate released in the broth was estimated by sampling broth culture after every 24 h. Each day, about 10 ml of

broth culture was centrifuged at 10,000 rpm for 10 min to separate the supernatant from the pellets. The available phosphate in the supernatant was estimated by a phosphomolybdic blue color method (Jackson, 1973).

Optimization of IAA production and phosphate solubilization at different pH and temperature values. The effect of temperature on IAA production and phosphate solubilization were determined by inoculating the freshly-grown culture in the NB medium supplemented with 2 mg ml⁻¹ tryptophan, and Pikovskaya's broth, respectively, and incubating them for 24 h at different temperatures, ranging from 5–35°C. Similarly, to check the effect of pH on IAA production and phosphate solubilization, freshly-grown bacterial cultures were inoculated in the above broth media at different pH values ranging from 5.0–9.0, in the case of IAA production, and 4.0–9.0, in the case of phosphate solubilization, respectively, and incubating them for 24 h at 10°C. IAA production was quantified with the above described IAA quantification method. Similarly, the available phosphate in the supernatant was estimated by the phosphomolybdic blue color method (Jackson, 1973).

Hydrogen cyanide production. The production of HCN was assessed on King's B medium containing 4.4 g l⁻¹ of glycine. Freshly-grown broth cultures of both strains were spread on King's B medium containing glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (2% sodium carbonate) was placed inside the lid of the plate. The plates were sealed with parafilm and incubated at 10°C for 4–5 days. The development of orange to red color indicates HCN production.

Siderophore production and biocontrol activity. For siderophore production, both strains were grown in a minimal medium for 24 h on a rotary shaker at 10°C. The assay used to detect siderophores was Chrome Azurol S (CAS) assay. The CAS assay is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, which releasing the dyes free from the complexes of iron. The blue color of the medium is due to the dye complexes with iron. The presence of siderophores is confirmed by a color transformation from blue to orange due to ferric ion binding and the release of free dye.

Co-culture development and talc based formulation. For the development of the co-culture CPP-2 (RM₂ + STA₃), a single colony from each culture was inoculated into the nutrient broth and incubated at 10°C for 24 h with continuous shaking (150 rpm). The absorbance was recorded at 600 nm wavelength. Further, 300 µl of the active culture (A₆₀₀ = 0.6) of both strains (150 µl from RM₂ + 150 µl from STA₃) were mixed to prepare the particular co-culture. Active co-culture and individual cultures were inoculated into 200 ml of nutrient broth separately, 24 h incubated cultures of each broth was separated into four parts, 50 ml each in centrifuge tubes, and spun at 5000 rpm for 10 min. Later, the supernatant was partially decanted and the tubes were vortexed for 15 minutes. Then, 2.5 gm of autoclaved talc (HiMedia Lab Pvt. Ltd., Mumbai) was added to each tube with pellets under sterile conditions. It was composed of talcum steatite, talc fine

powder, and hydrous magnesium silicate. The tubes were vortexed again for 45 min to achieve a homogeneous mixing of talc with the bacterial suspension. With a sterile spatula, the mixture was then emptied into glass petri plates. The plates were left at the optimum temperature of the respective culture till completely dry.

The viability of bacterial isolates and the co-culture CPP-2 in the formulation were ascertained by a serial dilution method. In each case, 50 mg of the talc based formulation was dissolved in 1 ml of sterile distilled water in an Eppendorf tube. Later, 10 µl of the suspension was dissolved in 990 µl sterile distilled water and the dilution plating was done in a nutrient agar medium. The plates were incubated at an optimum temperature and their viability was checked initially after two and four days. Thereafter, the cfu ml⁻¹ count was determined at a regular interval of 7 days for 3 weeks, followed by a 15 day interval until 70 days. The above pattern was followed keeping in view in the rapidity of changes in viable counts. The plate count was carried out in triplicate and the final cfu ml⁻¹ values were the average of the three readings.

Pot trials and bacterial coating to check pea plant growth promotion. Pot trials of the selected strains were carried out on pea (*Pisum sativum*) plants. For the preparation and sterilization of the potting mixture, nutrient deficient and fertilizer free soil was dried, passed 2 mm sieve and mixed with an equal volume of sand to prepare soil and sand mixture in the ratio of 1:1 (w/w). About 4–5 kg of the potting, as mixture prepared above, was taken in an autoclaving bag and sterilized in an autoclave for 3 alternate days at 121 psi for 1 h. Thermocot pots of 8 cm height and 1 cm spout diameter were used for the preparation of pots: 12 pots were used, 9 for bacterial strains and their respective co-culture, and three were used as a control. Pots were then three quarter filled with potting mixtures. For seed sterilization, autoclaved distilled water (2–3 litres), absolute ethyl alcohol, mercury chloride (0.1%), and an autoclaved filter paper was required. First, seeds were washed two times with distilled water, and then washed with HgCl₂ for 5 minutes. Washing steps were repeated thrice with distilled water for 5 minutes and then washed with ethyl alcohol for 5 min. washing was again repeated three times with distilled water for 5 min, and then the seeds were dried using the filter paper.

For the bacterial coating, carboxyl methyl cellulose (CMC), log phase of the broth culture of bacterial strains were used, about 1 gm of CMC was mixed with 0.5 ml of the respective log phase of the broth culture of bacterial isolates in a sterile glass bowl and about 3 to 4 seeds were coated with CMC mixed bacterial culture in such a way that the seeds were properly coated with the mixture. The sowing of the seeds in the pots was performed by taking about 3 to 4 coated seeds in each pot. In the control, sterile seeds without any coating were sown. The pots were then labelled with the name of their respective bacterial strains and kept in the polyhouse, where the environmental temperature was about 20–25°C and moistened periodically with a booster dose of the fresh culture of selected strains. Plants were harvested after 80 days and analyzed for different parameters like root and shoot fresh weight, dry weight, and their respective heights.

Table 1. Biochemical characterization of bacterial strains.

S. No.	Biochemical test	RM ₂	STA ₃
1	VP	- ve	- ve
2	Indole	+ ve	+ ve
3	MR	+ ve	- ve
4	Citrate	+ ve	+ ve
5	Glucose	+ ve	+ ve
6	Adonitol	- ve	- ve
7	Arabinose	+ ve	+ ve
8	Lactose	- ve	- ve
9	Sorbitol	+ ve	+ ve
10	Mannitol	+ ve	+ ve
11	Rhamnose	+ ve	- ve
12	Sucrose	- ve	+ ve
13	Nitrate Reductase	+ ve	- ve
14	Gelatinase	- ve	+ ve
15	Starch hydrolysis	+ ve	+ ve
16	Catalase	+ ve	+ ve
17	Oxidase test	+ ve	+ ve
18	H ₂ S Test	- ve	+ ve
19	Gluconate	+ ve	+ ve
20	Casein hydrolysis	- ve	+ ve
21	Deamination of Amino acid	+ ve	+ ve
22	Urease	+ ve	- ve

Results

Isolation, screening, and characterization of bacterial strains

A total of seven bacterial strains were isolated: four from the Ramgarh soil sample and three from the Satbunga soil sample. Two strains were screened out, and named as RM₂ for the Ramgarh, and STA₃ for the Satbunga, soil samples. Both strains were Gram negative, rod shaped, with a smooth surface and raised colonies. The STA₃ had a yellow color while RM₂ had a white color. The screening was done on the basis of their ability to solubilize insoluble inorganic phosphate by spotting 24-h-grown cultures on Pikovskaya's agar plates. The optimum temperature was found to be 10°C and the optimum pH was found 7.0. Both the strains were biochemically characterized by using specific HiMedia test-kits (Table 1). Both the strains RM₂ and STA₃ responded differently in all the biochemical tests, suggesting unique metabolic potentials and provisional categorization into the group of *Pentoea* sp. and *Pseudomonas* sp. respectively. Further, the partial sequence data of RM₂ and STA₃ were analyzed by BLAST search that showed an unambiguous similarity (97–99%) with *Pentoea ananatis* and *Pseudomonas fluorescens*, respectively. The sequences were submitted to the NCBI database under accession numbers KY888134 and KY888133 respectively. The phylogenetic tree was constructed using MEGA4 software by the neighbor-joining (NJ) method (Fig. 1).

Siderophore, HCN production, and biocontrol activity

The variation in color changes on the CAS agar plate in the case of both the strains indicates the production of siderophores. The production of HCN was detected for both strains. The strain RM₂ was found positive while strain STA₃ was negative. The results of HCN production revealed a remarkable change in color from yellow to brown against the control, suggestive of HCN production.

Fig. 1. Phylogenetic construction for the 16S rDNA sequences of the bacterial strains using MEGA4 software by the neighbor-joining method.

Fig. 2. IAA quantification by both strains in the presence and absence of tryptophan.

The biocontrol potential of both the strains RM₂ and STA₃ were assessed against the common phytopathogenic fungi *Trichoderma longibrahaitum* and *Fusarium oxysporum*, based on the zone of the inhibition bioassay method. The antagonistic effect of the RM₂ strain was found to be positive, while the strain STA₃ showed negative, in both cases.

IAA production, quantification, and its confirmation

The filtrate of both the strains RM₂, and STA₃ and the co-culture CPP-2 at different times confirmed a straight and time-dependent increase in IAA formation. The IAA production was found to be high (33 $\mu\text{g ml}^{-1}$) with regular consistency from 3–7 days in the case of the co-culture CPP-2 compared with both the strains individually. Improved production of IAA was found for strain RM₂ (26 $\mu\text{g ml}^{-1}$) and STA₃ (31 $\mu\text{g ml}^{-1}$) by adding 200 $\mu\text{g ml}^{-1}$ of tryptophan concentration, compared with 12 $\mu\text{g ml}^{-1}$ and 11 $\mu\text{g ml}^{-1}$ respectively in the absence of tryptophan (Fig. 2). Furthermore, the confirmation of IAA was carried out by the TLC method, in which the culture filtrates of both the strains were used to extract IAA for characterization. Chromatograms show almost the same R_f value as the standard IAA concentration, 0.31 for RM₂ and 0.37 for STA₃, respectively, which validates the intrinsic aptitude of isolated strains to produce phytohormone.

Quantitative measurement and optimization of phosphate solubilization

Solubilized phosphate were found to be high in the case of co-culture CPP-2 (275–850 $\mu\text{g ml}^{-1}$) followed by RM₂ (261–820 $\mu\text{g ml}^{-1}$) and STA₃ (270–801 $\mu\text{g ml}^{-1}$). The statistics revealed a time-dependent increase of solubilized phosphate. Intermittent examination of pH of the culture

Fig. 3. Quantification of phosphate solubilization of both the strains and simultaneous reduction of pH during incubation.

Fig. 4. Optimization of phosphate solubilization. A. at different temperatures. B. at different pH.

filtrate showed a notable decrease from the pH 7.0 to 3.13, which synchronized with the amount of solubilized phosphate, which is found to be more in the case of CPP-2 (7.0–3.13) followed by RM₂ (7.0–3.4) and STA₃ (7.0–3.6) (Fig. 3).

The optimum temperature of the strains RM₂ and STA₃ for phosphate solubilization were obtained at 10°C (260, 269 µg ml⁻¹), which was followed by 5°C (211, 210 µg ml⁻¹) and 15°C (194, 131 µg ml⁻¹), respectively (Fig. 4A). The pH optima of the strains RM₂ and STA₃ for phosphate solubilization was found to be pH 7.0 (279, 280 µg ml⁻¹) followed by pH 8.0 (213, 279 µg ml⁻¹), respectively (Fig. 4B).

Co-culture development, shelf life, and functional characterization of carrier-based formulation

For the development of co-culture CPP-2 (RM₂ + STA₃), a single colony from each strain was inoculated into the nutrient broth and incubated at 10°C for 24 h with continuous shaking (150 rpm). Absorbance was recorded at

Fig. 5. Comparative analysis of bioformulation. A. IAA production. B. phosphate solubilization.

Fig. 6. Comparative growth parameters of Pea plant. A indicates measurements of height. B indicates measurements of weight.

600 nm wavelength. Further, 300 µl of the active culture ($A_{600} = 0.6$) of both strains (150 µl from RM₂ + 150 µl from STA₃) were mixed to prepare the particular co-culture CPP-2. The viability of bacterial isolates and the co-culture in the formulation were ascertained by a serial dilution method, the cfu ml⁻¹ count was determined after regular intervals of 7 days for the subsequent 3 weeks, followed by a 15 days interval for 70 days. The above pattern was followed keeping in view in the rapidity of

Table 2. Total viable count of bacterial strains and their respective consortia under bioformulation.

Strains	Dilution factor	2 nd	4 th	11 th	18 th	25 th	40 th	55 th	70 th
RM ₂	10 ⁻⁶	284 ± 2	274 ± 2	248 ± 2	240 ± 2	190 ± 2	155 ± 2	132 ± 2	62 ± 2
STA ₃	10 ⁻⁶	278 ± 2	271 ± 2	245 ± 2	240 ± 2	147 ± 2	145 ± 2	80 ± 2	56 ± 2
CPP-2	10 ⁻⁶	290 ± 2	274 ± 2	270 ± 2	250 ± 2	193 ± 2	175 ± 2	147 ± 2	67 ± 2

changes in the viable counts. The plate count was carried out in triplicate and the final cfu ml⁻¹ were the average of the three readings. Initially, the colony number observation of both strains are approximately equal up to 18 days, but, later on, the strain RM₂ showed a little greater colony number compared to strain STA₃. With the progression of storage, the co-culture CPP-2 showed a resembling sustained viability, with the counts dropping marginally from 290 ± 2 × 10⁶ to 147 ± 2 × 10⁶ after 55 days of storage and afterward reduced to 67 ± 2 × 10⁶ after 70 days. However, in the case of the individual bacterium RM₂, the cell viability faintly decreased (from 284 ± 2 × 10⁶ to 190 ± 2 × 10⁶) up to the 25th day, with a remarkable decrease recorded after 55 days (132 ± 2 × 10⁶) and a subsequent reduction to 62 ± 2 × 10⁶ after 70 days. In the case of STA₃, the cell viability decreased in a similar fashion (from 278 ± 2 × 10⁶ to 147 ± 2 × 10⁶) up to the 25th day, to (80 ± 2 × 10⁶) after 55 days and a subsequent reduction to 56 ± 2 × 10⁶ CFU ml⁻¹ (Table 2).

The efficiencies of IAA production (Fig. 5A) and phosphate solubilization (Fig. 5B) of co-culture CPP-2 and the individual strains were analyzed before, and after, retrieval from the talc-based formulation. In all the cases of PGP studies, the efficiency of the individual strains and their co-culture CPP-2, were found to be more or less the same after retrieval as compared with that before the formulation. Thus, the growth phases were not affected; the co-culture reiterates the same growth pattern as before.

Pot trial experiments

The experimental trials of the psychrotrophic strains RM₂, STA₃ and the co-culture CPP-2 were performed on pea seeds for 80 days by supplying sterile soil and a booster dose of the respective culture. Utmost effects of all the respective strains were observed on both the shoot and root lengths in comparison with the control plant (Fig. 6A). Consequently, the overall weights of the bacterial-strain coated plants were also found to be positively affected with regards to increasing their fresh and dry shoot/root weights (Fig. 6B).

Discussions

The Ramgarh and Satbunga regions are a hot spot of biodiversity and STA₃ and RM₂ psychrotrophic bacterial species have been isolated and screened from this region. STA₃ and RM₂ show a close similarity with *Pseudomonas* sp. and *Pentoea* sp., on the basis of biochemical and molecular characterization. Considering the fact that these strains demonstrated plant growth promoting traits at temperatures ranging from 5–35°C, we label them psychrotrophic plant growth-promoting bacterium, rather

than psychrophilic (cold loving) ones. Among the PGP traits, IAA production by a bacterial strain has a cascading effect on the plant development due to its ability to influence root growth, which, in turn, affects the nutrient uptake and ultimately the plant productivity. These IAA production data suggest that the isolated strains act as effective bioinoculants. These trends agree with previous reports indicating IAA formation in the stationary stage of culture (Park et al., 2017; Sahasrabudhe, 2011; Verma et al., 2016; Walpola et al., 2013). The confirmation of IAA with the TLC findings are in agreement with previous reports (Sahasrabudhe, 2011).

The production of organic acid, phosphatase enzyme and its role in phosphate solubilization has been the subject of previous report (Anwar et al., 2014). Phosphate solubilization was more pronounced in the pH ranges from pH 7.0–8.0 for both strains. Phosphate solubilization showed a decline in activity as pH range changes in any direction from pH 7.0, suggesting that it is a neutral phosphate solubilizer, as reported earlier by Jena and Chandi (2013). The phosphate solubilization results showed a sporadic decline in activity with increasing temperature, but worked over a wide range of temperature from 5–35°C, suggesting that it is a psychrotroph. The phosphate solubilization aptitude of the bacterial strains could have played an imperative role in plant growth promotion (Sagervanshi et al., 2012). The availability of iron for microbial assimilation, in environments such as the rhizosphere, is extremely limited. In highly oxidized and aerated soils, the predominant form of iron is the ferric form, which is soluble in water (pH 7.4) at about 10⁻¹⁸ M (Neilands et al., 1987). Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganisms, the biosynthesis of which is carefully regulated by iron (Neilands, 1995). Siderophore production directly related to plant growth promotion and the biological control of plant pathogenic fungi (Katiyar and Goel, 2004; Sakthivel and Karthikeyan, 2012). Bacterial antagonism or biocontrol activity towards phytopathogenic fungi is known to be mediated by a variety of compounds of microbial origin, viz., bacteriocins, enzymes, toxic substances, volatiles, and others. In the present study, the bacterial strain RM₂ showed inhibition of the test phytopathogenic fungi *Fusarium oxysporum* and *Trichoderma longibrachiatum*. The most widespread bacterial mechanisms responsible for the inhibition of pathogen proliferation include the ability to colonize the root zone, production of antibiotics and enzymes that degrade pathogen cell walls, production of siderophores and hydrogen cyanide production (Ahmad and Kibret, 2014; Kumar et al., 2017; Shen et al., 2013). Under the bioformulation study,

co-culture CPP-2 was found to be slightly more stable and viable as compared with individual bacteria strains. Talc-based PGPR formulation has been utilized for storage and management of various plant pathogens (Shanmugam et al., 2011). For commercialization, the viability of bioinoculants in a prescribed formulation for a certain period of storage is desirable (Bazilah et al., 2011). These findings help to conclude that both strains sustained their plant growth promoting activities even after storage in the talc-based formulation for 70 days. The efficiency parameters after bioformulation towards PGP activities were found to be more or less the same as compared with those before formulation in all the cases. Similar results of the efficiency parameters on the bacterial consortium-II before and after bioformulation have been reported earlier in the case of the biodegradation of LDPE and HDPE (Sah et al., 2011).

Additionally, both the strains and their co-culture reported in this study seem to be ideal candidates for promotion as a bioinoculant, due to their cold tolerance and multiple abilities of plant growth promotion traits. The co-culture CPP-2 has a more positive effect on root and shoots elongation, followed by the individual culture of RM₂ and STA₃, in comparison with the control. The results also indicate that all the cultures have the potential to improve the plant growth development; this is based on a preliminary study under poly-house conditions in the specific range of temperature of about 20–25°C, so these strains were considered to be an effective bioinoculant. The results of PGPR traits indicated that both the strains and their respective co-culture CPP-2 are very useful for the enhancement of plant growth by some direct mechanism approximating phytohormone and phosphate solubilizing activity that provides nutrition to the plants, and some indirect mechanism similar to inhibiting the growth of pathogens by producing siderophore, an inherent property of biocontrol activity (Bharucha et al., 2013; Mia et al., 2012; Sivakumar et al., 2012). There are many reports about psychrotrophic *Pseudomonas* related to PGPR (Selvakumar et al., 2009), while a few reports are available concerning psychrotolerant *Pantoea* sp. (Panwar et al., 2016). The present paper is, however, the first report concerning psychrotrophic *Pantoea* sp. In a pot experiment, *Pseudomonas* sp. OG enhanced the growth of chickpeas (*Cicer arietinum* L.) and mung beans (*Vigna radiata*) by increasing the size of the parts of the plant above ground, the roots and the plant biomass (Thakker et al., 2013).

Conclusion

PGPRs have quite a lot characteristics viz. phytohormone production, a symbiotic nitrogen fixation, siderophore production, antibiotics synthesis, etc. In the present study, two bacterial strains were isolated and screened on the basis of one of the PGPR activities. Furthermore, in-vitro PGP activities were analysed and a co-culture CPP-2 was prepared by mixing an appropriate volume of the two strains. Subsequently, all cultures were treated for pea plant growth promotion in a pot trial experiment and the pea plant growth parameters were found

to be enhanced, in the case of co-culture CPP-2 in compared with the individual bacterial strain. Simultaneously, investigation of their shelf life under bioformulation, their efficiency, before and after bioformulation, found persistent. The shelf life and their efficacy were found healthy in case of co-culture CPP-2 compare to individual bacterial strain, that suggest, both the strains and their co-culture have the most promising shelf life at normal temperatures. Hence, the present strains can act as potential candidates for the growth and development of pea crop plants under bioformulation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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