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Impact of microbial consortium of *Rhizobium tropici* and *Rhizobium mayense* on the growth of *Phaseolus vulgaris* L.

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ABSTRACT

The main objectives of the present investigation was to characterize and recognize potential indigenous *Rhizobium* spp. associated with red clover nodules, as well as to evaluate the influence of prospective rhizobia inoculation (individual and consortia) on common bean plant growth. Total of 8 rhizobia like bacteria were isolated and were investigated for their specific PGP traits, production of hydrolytic enzymes. Results revealed, four isolates synthesize IAA (22.3 ± 1.45 to 88.6 ± 1.45 μg mL^{-1}), three isolates solubilized phosphate (40.69 ± 1.25 to 216.3 ± 1.31 μg mL^{-1}), three isolates solubilized potassium, produced siderophore, and showed chitinase production, two isolates possess ACC deaminase activity, one isolate was able to solubilize zinc, 5 isolates produced β- 1, 3-glucanase (0.38 ± 0.005 to 2.82 ± 0.011 % units) and four isolates produced cellulase. Two bacterial isolates found to exhibit most of the PGP activities, were selected and identified on the basis of phenotypic, biochemical tests and 16S rRNA sequencing, as *Rhizobium tropici* IHTF-1 and *Rhizobium mayense* IHTF-2. Interaction of these potential rhizobium strains individually and consortia with common bean plants under greenhouse conditions boosted root and shoot length, fresh and dry biomass compared to un-inoculated control plants. SEM study revealed that both the strains, IHTF-1 and IHTF-2 colonized common bean roots. These results imply that *Rhizobium* spp., identified in this work, can be used as bio-inoculants to increase common bean plant production in a sustainable way.

1. Introduction

In terms of important nutrients for human nutrition, common beans (*Phaseolus vulgaris* L.) are substantial reservoir of carbohydrates, proteins, essential minerals and vitamin B complex (which includes folic acid, thiamine, riboflavin and thiamine) (Wekesa et al., 2021). It belongs to the plant family *Fabaceae*. Due to its significant nutritional value, it is regarded as the legume with the highest direct human intake worldwide. The world's tropics, subtropics and temperate zones all have extensive common bean cultivation. Common beans, along with the majority of legumes, can derive advantages from symbiotic associations with diazotrophic bacteria, specifically rhizobium species (Sanyal et al., 2020; Fiori et al., 2021). Rhizobia are a group of soil bacteria that have ability to infect *Fabaceae* plants roots to form nodules, where the nitrogen fixation process happens, which is advantageous to the plants (De Meyer et al., 2015). Leguminous plants fix approximately 200 million tons of atmospheric nitrogen every year (Biswas and Gresshoff, 2014; Marzec-Grządziel et al., 2020). In legume-rhizobium symbiosis, rhizobia constantly supplies reduced nitrogen to the host plant, thereby reducing the host plant's reliance on detrimental nitrogenous fertilizers throughout its growth phase. The biological nitrogen fixation (BNF) that happens as a result of rhizobium-legume symbiosis benefits both the host plant and subsequent crops grown on that agricultural land. Apart from that, it also provides an easy way to improve soil fertility. Due to all these aspects, the rhizobium-legume relationship has received extensive attention as a paradigm of mutualistic associations and as a useful association for sustainable agriculture.

Numerous factors, including climatic conditions, susceptibility to fungal diseases, the bean cultivar's nodulation capacity, soil properties including clay and organic matter components, pH, and moisture levels, as well as agricultural management techniques including crop rotation and the use of agrochemicals, are known to affect the symbiosis in

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common beans (Fiori et al., 2021;Tabande and Naseri, 2020). The nodule occupancy by competing but ineffective rhizobia, which causes a poor contribution of nitrogen fixation to common beans and is regarded to be the lowest among grain legumes, is a major issue influencing the success of inoculation in common beans (Hungria et al., 2013). Thus, continuous efforts to choose bacterial strains that are more efficient, genetically stable, competitive, and adaptive can promote a more successful symbiosis, increasing growth and yield characteristics of common bean. The production of common beans is constrained in many regions of the world by inadequate soil fertility, notably nitrogen deficiency (Wekesa et al., 2021). N fertilizers are used by farmers, these fertilizers are not only expensive but also have negative environmental effects including groundwater contamination, a detrimental consequences on beneficial soil microorganisms, a decline in soil fertility, emergence of pest resistance, the enduring presence of pesticides in food grains and adverse effects on human health (Alori and Babalola, 2018; Igiehon and Babalola, 2018; Zheng et al., 2019). Microbial inoculants or bio-fertilizers have a potential to mitigate the adverse consequences of chemically synthesized fertilizers. An affordable and sustainable solution is rhizobial soil inoculation. Rhizobia are also members of the bacterial class known as plant growth promoting bacteria (PGPB), which can boost the growth and development of both leguminous and non-leguminous plants via variety of mechanisms (Gopalakrishnan et al., 2015b; Dent and Cocking, 2017). Such beneficial mechanisms include synthesis of compounds that stimulate plant growth like IAA (Ahemad and Kibret, 2014; Singha et al., 2018), mineral solubilization (particularly phosphorus, potassium) (Alori et al., 2017a; Khalid et al., 2020), siderophore production (Datta and Chakrabartty, 2014; Igiehon et al., 2019). Additionally, these PGPB enhance plant growth via indirect mechanism by suppressing phytopathogens through the production of hydrolyzing extracellular enzymes and volatile chemicals (like ammonia and hydrocyanic acid), antibiotics, etc., (Alori et al., 2017b; Das et al., 2017). Keeping in view the significance of PGPB, the current work aimed to identify rhizobia associated with red clover nodules evaluate them for multiple PGP activities and to analyze the effect of potential rhizobia inoculation under greenhouse conditions on the growth of *P. vulgaris* plants. According to the findings of this study, an appropriate formulation of these plant-growth-promoting rhizobia could be an effective method for growing common bean crop that is chemical fertilizer-free and nutritionally rich enough to meet the world's rising food need.

2. Materials and methods

2.1. Sampling and rhizobia isolation from nodules of red clover

Red clover (*Trifolium pratense* L.) was picked at the flowering stage from agricultural fields of Baramulla, Jammu & Kashmir, India (34.160°N 74.556°E). Plants were uprooted gently and the nodules located on the roots were carefully detached by using sterilized forceps and were surface sterilized first with 75 % ethanol solution for three minutes. Next the nodules were submerged in 0.1 % mercuric chloride solution for five minutes and finally washed several times with autoclaved distilled water (Singha et al., 2018). The disinfected nodules were then crushed aseptically in microfuge tubes containing 0.86 % sterile physiological saline. Resulting suspension was then streaked with the help of inoculating loop on CR-YEM (congo-red yeast extract mannitol) agar, and the plates were incubated in dark conditions for 72 h. Colonies of bacteria which showed no congo-red absorption, appear white, mucoid, were picked and were further purified and maintained on CR-YEM agar slopes.

2.2. Assessment for PGP characteristics under in vitro conditions

Bacteria strains secluded from red clover nodules were assessed in vitro for a number of distinct PGP traits, such as the generation of indole-3-acetic acid (IAA), solubilization of minerals like phosphate, potassium and zinc, siderophore, ACC deaminase, and ammonia. Qualitative evaluation of bacterial isolates for IAA production was carried out as per the method outlined by Mir et al. (2022). For quantitative estimation of IAA, Hartmann et al. (1983), method was adapted. IAA produced by the bacterial isolates was measured by comparing the results to a standard graph constructed from standard IAA (HI-Media, India) and the obtained values were expressed in μg/ml. Qualitative estimation of phosphate solubilization was performed on NBRIP (National Botanical Research Institute's Phosphate) media in accordance with the methodology outlined by Mir et al. (2022). Phosphate solubilization was determined quantitatively by inoculating bacterial cultures into 50 ml of NBRIP broth and incubating them for 5–7 days under shaking conditions. After incubation, culture supernatant was evaluated for P solubilization as per the method outlined by Mehta and Nautiyal (2001), amount of soluble phosphate was calculated using the KH_2PO_4 standard curve (HI-Media, India) and represented as μg/ml. For siderophore production, CAS (Chrome Azurol S) agar medium plates were spot inoculated with actively growing bacterial strains and incubated in dark conditions at 30 °C for 72 h. Colour change of CAS agar medium from blue to orange/yellow around the point inoculated colonies indicated siderophore production (Schwyn and Neilands, 1987). ACC deaminase screening activity was carried out as per the method of Penrose and Glick (2003). Cell pellet of log phase culture of the isolated bacteria was spot inoculated on sterile minimal DF salt agar plates enriched with ACC (3 mM) as a nitrogen source. The capability of isolated bacteria to grow on plates is considered to exhibit ACC deaminase activity. DF salt minimal medium without ACC served as a negative control. Ability to solubilize potassium was evaluated by dipping the sterile filter paper disc into actively grown bacterial cultures and transferred onto the Aleksandrov agar plates. Halo zone development around the spots indicated the solubilization of potassium (Hu et al., 2006). Zinc solubilization ability was carried out in accordance with Saravanan et al. (2007) methodology. In brief, log phase culture of the isolated bacteria were spot inoculated on tris- mineral salt agar medium amended with zinc carbonate ($ZnCO_3$) at 1000 mg L^{-1} and the plates were incubated for seven days at 30 °C. Halo zone development around the spots was measured. Assay for ammonia production was performed in peptone water broth as per the procedure outlined by Mir et al. (2022).

2.3. Screening of isolates for chitinase, β- 1, 3-glucanase and cellulase (hydrolytic enzymes)

Screening for chitinase enzyme production was performed according to Hirano and Nagao (1988), method by spot inoculating log phase culture of the isolated bacteria on minimal medium supplemented with colloidal chitin (1 %), plates were incubated on a shaking incubator at 120 rpm speed for seven days. Formation of clearing zones surrounding inoculated sites indicated chitinase enzyme production. Quantitative estimation of β- 1, 3-glucanase was carried out as per the methodology outlined by Singh et al. (1999). Laminarin, a glucan polymer is cleaved by β-glucanase resulting in the release of glucose molecules. One unit of β-1, 3-glucanase activity was defined as the quantity of enzyme that liberated 1 μmol of glucose h^{-1}. *Rhizobium* spp., were inoculated in TSB and incubated on a shaking incubator at 120 rpm speed for three days. Cultures were centrifuged and to 1 ml of supernatant, 0.1 ml of laminarin (2 % w/v) was added, incubated at 40 °C for one hour. Following incubation, test tubes containing culture filtrates were added with 3 ml of dinitro salicylic acid (DNS) reagent and placed in a boiling water bath for ten minutes. A shift in colour from yellow to red signifies the presence of glucose, which in turn indicates β-glucanase activity. Glucose concentration was estimated by measuring the absorbance at 530 nm and OD values were plotted on standard graph. Cellulase production was carried

out on carboxymethylcellulose Congo red (CMC) media according to the methodology of Mir et al. (2022).

2.4. Identification of the bacterial strains

2.4.1. Identification based on morphological and biochemical characterization

Morphological characterization of the isolated bacteria was examined on CR-YEM agar medium. Using the methods outlined by Somasegaran and Hoben (1994), each colony was evaluated based on its colony-form, elevation, colour, margin and texture. Gram staining reaction was carried out as per the methodology of Vincent (1970). Isolated bacteria were also investigated for various biochemical tests namely starch hydrolysis, oxidase, catalase, gelatin liquefaction, citrate utilization, methyl red, indole test and nitrate reduction test (Somasegaran and Hoben, 1994). The methodology of Koskey et al. (2018) was adapted to determine whether the isolated bacteria were fast or slow growers. In brief YEMA medium incorporated with bromothymol blue (BTB) was streaked with log phase culture of the isolated bacteria, incubated for 72–96 h at 30 °C and noticed either for blue colour appearance due to alkali production (slow growers) or yellow colour due to acid production (fast growers). To differentiate rhizobia from other contaminating bacteria, keto-lactose test was performed. On the keto-lactose agar medium, actively growing rhizobial isolates were streaked and incubated for 72 h. Following incubation, Benedict's reagent was added to the plates, kept for few hours and then poured off. Development of bright yellow colour around the bacterial cultures confirms the presence of Agrobacterium and other contaminating bacteria (Bernaerts and De Ley, 1963). Finally, root nodule bacteria were streaked on GPA (glucose peptone agar) medium amended with indicator dye (bromocresol purple) to distinguish rhizobia, which usually exhibits no growth on the media without changing the pH, from Agrobacterium and other contaminants, which exhibits enormous growth on the GPA media with an apparent pH shift.

2.4.2. Identification of root nodule bacterial at molecular level

For the extraction of genomic DNA from root nodule bacteria, Dudeja and Singh (2008) methodology was used. Amplification of the 16S rRNA coding region, primers FGPS6 as forward and FGPS1509 as reverse were used (Mir et al., 2021). The 50 μL PCR reaction mixture and thermo cycling condition were followed as per the method of Mir et al. (2021). PCR amplified products were analyzed by running on 1.2 % agarose gel electrophoresis and were visualized with a UV transilluminator. QIAquick PCR purification kit (QIAGEN) was used to purify the amplified PCR products and sequenced at Eurofins Genomics Pvt. Ltd, Bangalore, India. The obtained 16S rRNA sequences were compared to existing gene sequences using the BLAST program and were deposited in NCBI GenBank, to get accession numbers. MEGA X (Molecular Evolutionary Genetics Analysis) was used for phylogenetic and molecular evolutionary analyses (Kumar et al., 2018).

2.5. Compatibility test

To develop microbial consortia that will effectively increase plant development, two bacterial strains must be compatible with one another. Bacterial cultures (IHTF-1+IHTF-2), were streaked in a straight line on YEM agar plates in such a way that each bacterial culture comes in contact with the other, i.e., radiating from the center. Petridishes were incubated in an incubator at room temperature for three days. Inhibition zone absence signified compatibility and the presence of inhibition zone demonstrated incompatibility with respective bacterial strains (Prasad and Babu, 2017).

2.6. Effects of potential isolated bacteria on common bean (P. vulgaris L.)

Under greenhouse conditions, plant growth promoting ability of two potential rhizobium isolates (IHTF-1 and IHTF-2) and their consortium (IHTF-1+IHTF-2), were evaluated using common bean seeds variety Akra Komal procured from Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, India (PJTSAU). Common bean seeds were surface sterilized as per the protocol mentioned by Gupta and Pandey (2019) and pre-germinated on Petridishes containing sterile wet filter paper for two days in dark conditions at room temperature. Plastic pots (15 × 12 cm) were filled with an optimum quantity of sterile soil (3/4th level), and two pre-germinated seedlings were sown in each pot. For the inoculation study, pure cultures of IHTF-1 and IHTF-2 were cultured in YEM broth for three days at 30 °C with shaking at 200 rpm in an incubated shaker. Following incubation, rhizobial isolates were centrifuged for 10 min, at 12,000 rpm to get the cell pellet. The acquired cell pellet was dissolved in sterile distilled water, and the final population of cells was adjusted to10^6 colony forming units per milliliter (CFU/mL). However for the development of consortium both the cultures IHTF-1 and IHTF-2 were mixed (50/50 v/v). Finally, rhizobial cultures were added to seven-day-old common bean seedlings that were being grown in pots. The pots were kept in a greenhouse where the highest and lowest temperatures were kept at 28 °C and 25 °C respectively, and the relative humidity (RH %) ranged from 70 to 80 %. A total of four treatments (IHTF-1, IHTF-2, consortium of IHTF-1 and IHTF-2 and uninoculated control) were maintained with three replications. Plants were uprooted at 28 DAS (days after sowing), and growth parameters like shoot and root length, fresh weight and total biomass (dry weight) were measured.

2.7. Root colonization assay by SEM

The colonization of common bean roots by rhizobium isolates (IHTF-1 and IHTF-2) was investigated using scanning electron microscopy (SEM). To summarize, the common bean seeds, variety Akra Komal were disinfected by dipping them for one minute in 70 % ethanol, then soaking them for ten minutes in a 1 % sodium hypochlorite solution, and finally rinsing several times with sterilized water. After being surface cleaned, the seeds were placed for an hour in the corresponding test cell pellet suspension (IHTF-1, IHTF-2) before being planted in pots filled with sterilized fine sand (Gopalakrishnan et al., 2015a). Seeds dipped in autoclaved distilled water (unprimed) were maintained as control. Three seeds were sown in each pot and the pots were incubated for two weeks in a greenhouse where the highest and lowest temperatures were kept at 28 °C and 25 °C respectively, and the relative humidity (RH %) ranged from 70 to 80 %. Following incubation, plants were taken out of the sand pots and their roots were washed with PSB. Root tissue samples from inoculated and un-inoculated plants were then fixed in 2.5 % glutaraldehyde for 24 h in the refrigerator. After two PSB washes, the roots were post-fixed for four hrs in 2 % OsO4. The samples were then dehydrated in an ethanol gradient series, and later in an automated sputter coater (HITACHI E-1010) samples were coated with gold-palladium and observed for bacterial colonization employing SEM (HITACHI S-3700N).

2.8. Statistical analysis

Every experiment was run in triplicate, statistical analysis was done by ANOVA and means, ranking, standard deviation, and standard errors were calculated by using SPSS version 26. The comparison between treatment means was made using the Duncan's multiple range test (DMRT) at $p < 0.05$. Means with the same letter do not differ significantly at $p < 0.05$.

3. Results

3.1. Isolation of nodule associated bacteria

Total eight morphologically different bacterial strains were secluded from root nodules of red clover on CR-YEMA medium (a selective medium used for isolation of *Rhizobium* spp.) using standard plating methods. The isolates were labelled as IHTF-1, IHTF-2, IHTF-3, IHTF-4, IHTF-5, IHTF-6, IHTF-7 and IHTF-8 (Fig. 1).

3.2. Root nodule bacteria PGP activities

The results of the qualitative test of IAA synthesis demonstrated that four of the eight bacterial isolates significantly changed the colour of the nitrocellulose membrane to pink after being applied with Salkowski's reagent (Fig. 2a). Concentration of IAA production from these isolates in quantitative assay varied from 22.3 ± 1.45 to 88.6 ± 1.45 μg/ml. Bacterial isolate IHTF-1 produced highest concentration of IAA (88.6 ± 1.45 μg/ml), followed by IHTF-2 (52.3 ± 0.88 μg/ml), IHTF-4 (26.3 ± 1.20 μg/ml) and IHTF-6 (22.3 ± 1.45 μg/ml) (Table 1). Results of the qualitative test of phosphate solubilization revealed that out of eight bacterial isolates, only three isolates showed phosphate solubilization ability which was demonstrated by the development of clearing zones on NBRIP medium after 5 days of incubation period (Fig. 2b). In quantitative estimation, the bacterial isolate IHTF-1 demonstrated the highest phosphate solubilization with a value of 216.3 ± 1.31 μg/ml, followed by IHTF-2 (152.8 ± 1.15 μg/ml), IHTF-7 (40.69 ± 1.25 μg/ml). The medium's pH was also found to be lower, which is a result of the bacterial isolates secreting different organic acids. pH dropped from 7.0 to a minimum of 4.6, 5.2, and 6.1 in the cases of bacterial isolates IHTF-1, IHTF-2, and IHTF-7 (Table 1). Three of the eight bacterial isolates (IHTF-1, IHTF-2, and IHTF-5) developed orange halos zones surrounding the colonies on CAS agar medium, indicated siderophore production activity (Fig. 2c). Out of eight isolates, only two isolates (IHTF-1 and IHTF-2) grew on DF minimal medium supplemented with ACC, indicated that bacteria possessed ACC deaminase activity (Fig. 2d). Three of the eight bacterial isolates had cleared zones ranging from 6 to 16 mm around their respective colonies on Aleksandrov media which suggested their ability to solubilize insoluble form of potassium. Bacterial isolate IHTF-1 exhibited the highest solubilization zone (16 mm), followed by IHTF-2 (14 mm). Bacterial isolate IHTF-8 showed least zone of solubilization (6 mm) (Fig. 2e). Among the eight bacterial isolates, isolate IHTF-1 generated a cleared zone on tris-mineral salt medium amended with $ZnCO_3$. All the 8 bacterial isolates were able to produce ammonia in peptone water broth, furthermore among eight isolates, bacterial isolates IHTF-1, IHTF-2 and IHTF-3 displayed highest (+++) ammonia production as demonstrated by a shift in colour from yellow to deep brown, when Nessler's reagent was added to tubes containing cultures. While, other isolates exhibited moderate (++) and weak (+) ammonia production as demonstrated by a shift in colour to dark yellow and light yellow, respectively (Fig. 3a). In vitro PGP activities of root nodule bacteria are represented in Table 1.

3.3. Production of hydrolyzing extracellular enzymes

Chitinase production by bacterial isolates was confirmed by the generation of clearing zones on chitin minimal medium. Results revealed that only three isolates were positive for chitinase production. Furthermore out of three isolates, bacterial isolate IHTF-1 exhibited maximum hydrolysis zone of (16 mm), followed by IHTF-2 (12 mm) and IHTF-6 (9 mm). Five of the eight bacterial isolates produced β- 1, 3-glucanase in the range of 0.38 ± 0.005 to 2.82 ± 0.011 % units. Highest generation of β- 1, 3-glucanase enzyme was shown by bacterial isolate IHTF-2 (2.82 ± 0.011 % units), followed by IHTF-1 (1.78 ± 0.011 % units), IHTF-8 (1.69 ± 0.008 % units), IHTF-7 (1.35 ± 0.010 % units) and IHTF-5 (0.38 ± 0.005 % units) (Fig. 3b). Four of the eight bacterial isolates were positive for cellulase production, which was evident by the generation of clear zones on CMC agar plates. Furthermore out of 4 isolates, bacterial isolate IHTF-1 exhibited maximum hydrolysis zone of (22 mm), followed by IHTF-2 (12 mm), IHTF-4 (8 mm), and IHTF-8 (6 mm) (Fig. 3c). In vitro production of hydrolytic enzymes by bacterial isolates is represented in Table 2.

3.4. Identification of isolates

3.4.1. Morphological and biochemical characterization

Two potential isolates (IHTF-1 and IHTF-2) were chosen for morphological and biochemical characterization based on their in-vitro PGP activities. When cultured on CR-YEM agar medium, both isolates did not absorb Congo red dye and upon microscopic observation, both isolates were found Gram negative and rod-shaped (Fig. 4a–c). Both the isolates showed white/creamy, raised, round, entire and showed mucoid texture due to the production of exopolysaccharides. Both the isolates turned the colour of the medium from deep green to yellow, when streaked on YEM enriched with bromothymol blue (BTB), which indicated the isolates are fast growers and acid producers (Fig. 4d). Both the isolates showed positive results with nitrate reduction, starch hydrolysis, catalase, oxidase, and negative results for gelatin, indole, methyl red, citrate utilization and ketolactose test. Rhizobia can be distinguished from other contaminating bacteria using the keto-lactose test. No yellow colour was observed around the colonies, when

Fig. 1. (A) *Trifolium pratense* (red clover) plants; (B) Root nodules of red clover.

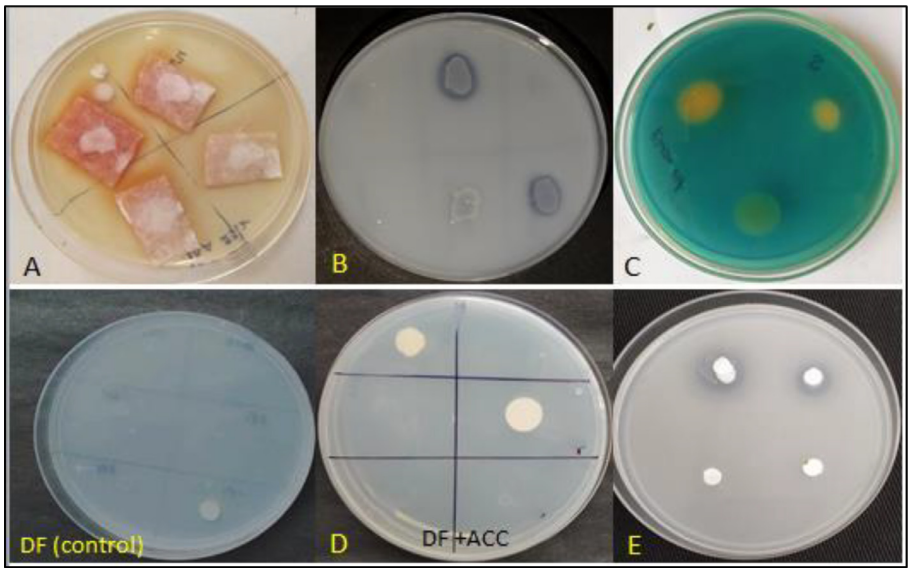

Fig. 2. (A) Pink colour of NCM disc indicates IAA production by bacterial isolates; (B) Formation of halo zones on NBRIP agar plates indicated phosphate solubilization; (C) CAS agar plates showing orange halo zones indicated siderophore production; (D) Growth of bacterial isolates on DF medium enriched with ACC indicates ACC deaminase activity; (E) Aleksandrov agar plates showing halo zones indicated potassium solubilization.

Table 1
PGP (in vitro) traits of the bacteria isolated from red clover nodules.

Isolate	IAA (μg/ml)	P-solubilization (μg/ml)	Siderophore	ACCD	KS zone (mm)	Zn solubilization zone (mm)	NH_3
IHTF-1	88.6 ± 1.45	216.3 ± 1.31 (4.6)	+	+	16 mm	12 mm	+++
IHTF-2	52.3 ± 0.88	152.8 ± 1.15 (5.2)	+	+	14 mm	–	+++
IHTF-3	–	–	–	–	–	–	+++
IHTF-4	26.3 ± 1.20	–	–	–	–	–	++
IHTF-5	–	–	+	–	–	–	++
IHTF-6	22.3 ± 1.45	–	–	–	–	–	++
IHTF-7	–	40.69 ± 1.25 (6.1)	–	–	–	–	++
IHTF-8	–	–	–	–	6 mm	–	++

–, +, ++ and +++ denotes no activity, mild activity, medium activity and strong activity. ACCD, denotes 1-aminocyclopropane-1- carboxylate deaminase. KS, denotes potassium solubilization. Values in the parentheses indicate the pH decreased from initial 7.0. Numerical values are mean ± Standard Error (SE).

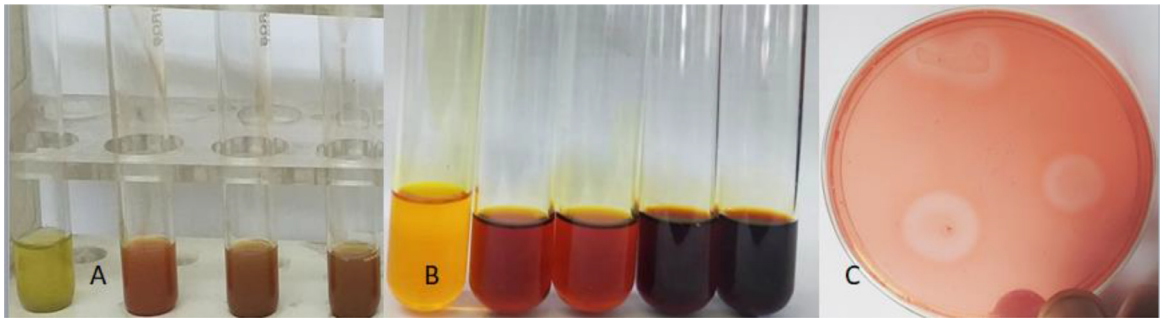

Fig. 3. (A) Brown colour of tubes indicated ammonia production; (B) Yellow to red colour change indicates β-1, 3-glucanase production; (C) CMC agar plates showing halo zones indicated cellulase production.

Benedict's reagent was added to the keto-lactose agar plates which are the characteristics feature of *Rhizobium* spp. (Fig. 4e). Both the isolates (IHTF-1 and IHTF-2) showed no growth when streaked on glucose peptone agar medium that indicated the features of rhizobia. Based on their phenotypic and biochemical activities, the isolated bacterial strains were tentatively identified as *Rhizobium* spp., (Table 3).

3.4.2. Identification of the selected PGP bacterial isolates at molecular level

Two promising isolates, designated as IHTF-1 and IHTF-2, showed significant PGP properties were further chosen for identification at molecular level. PCR amplification of the 16S rDNA region yielded approximately amplicon size of 1500-base pairs (Fig. 5). The 16 S rRNA gene sequences obtained (602 base pairs for IHTF-1 and 1154 base pairs for IHTF-2) were matched with other closely related recognized species from GenBank databases, aligned and the phylogenetic dendrograms was generated (Fig. 6A, B). Bacterial isolates IHTF-1 and IHTF-2 were found to be 98 % and 99 % similar to *Rhizobium tropici* and *Rhizobium mayense*, respectively. 16S rRNA sequences of *R. tropici* and *R. mayense* were deposited in NCBI GenBank and assigned the following accession numbers: IHTF-1; OL823119, IHTF-2; MW846221.

Table 2
Hydrolytic enzymes production by isolated bacteria.

Isolate	Chitinase (mm)	β- 1, 3-glucanase (% units)	Cellulase (mm)
IHTF-1	16	1.78 ± 0.011	22
IHTF-2	12	2.82 ± 0.011	12
IHTF-3	NA	NA	NA
IHTF-4	NA	NA	8
IHTF-5	NA	0.38 ± 0.005	NA
IHTF-6	9	NA	NA
IHTF-7	NA	1.35 ± 0.010	NA
IHTF-8	NA	1.69 ± 0.008	6

- denotes no activity. Data interpreted from three replicates of two independent experiments. Numerical values are mean ± Standard Error (SE).

Table 3
Morphological and biochemical characteristics of the selected bacterial isolates.

Characters	Isolates	
Morphological	IHTF-1	IHTF-2
Colony- shape	Circular	Circular
Colony margin	Entire	Entire
Colony elevation	Raised	Raised
Colony colour	White/Creamy	White/Creamy
Texture	Mucoid	Mucoid
Gram Reaction	Gram −ve, rods	Gram −ve, rods
Biochemical		
Nitrate reduction test	+ve	+ve
Starch hydrolysis test	+ve	+ve
Catalase test	+ve	+ve
Oxidase test	+ve	+ve
Gelatin liquefaction test	−ve	−ve
Indole test	−ve	−ve
Methyl red test	−ve	−ve
Citrate utilization test	−ve	−ve
Ketolactose test	No yellow zone	No yellow zone
Acid alkaline production	Acid producers/fast growers	Acid producers/fast growers
Glucose peptone agar	No growth observed	No growth observed
Identification of the isolate	*Rhizobium* spp.	*Rhizobium* spp.

3.5. Compatibility studies

In order to assess *R. tropici* strain IHTF-1 and *R. mayense* strain IHTF-2 as consortia for plant growth investigations, their compatibility was examined. The results revealed that both strains were compatible to each other and there was no inhibition zone at the point of contact between the two strains (Fig. 7).

3.6. Impact of Rhizobium sp., on plant growth parameters of common bean

Based on PGP activities in-vitro, two promising isolates *R. tropici* strain IHTF-1 and *R. mayense* strain IHTF-2 exhibiting highest results were selected and studied for plant growth promotion under greenhouse conditions (pot trials) on common bean. Results showed that common bean plants treated with rhizobial isolate IHTF-1 and IHTF-2 individually and consortium (IHTF-1 + IHTF-2) has shown promising effect on shoot and root length, fresh and dry weight respectively at 30 days after sowing over the control. Common bean plants inoculated with consortium recorded the maximum improvement in shoot length (41 %), followed by those treated with IHTF-1 (29 %) and IHTF-2 (11 %). The root length was enhanced by 47 % when plants were treated with a consortium of IHTF-1+IHTF-2, followed by IHTF-1 (37 %) and IHTF-2 (16 %). Plants treated with consortium showed maximum increase in fresh weight (52 %), followed by IHTF-1 (41 %) and IHTF-2 (14 %). Similar findings were also observed with total dry weight. Treatment of common bean plants with consortia of *R. tropici* strain IHTF-1 and *R. mayense* IHTF-2 resulted in maximum dry weight of 48 %, followed by *R. tropici* IHTF-1 (31 %) and *R. mayense* IHTF-2 (20 %) compared to un-inoculated plants (Figs. 8 and 9).

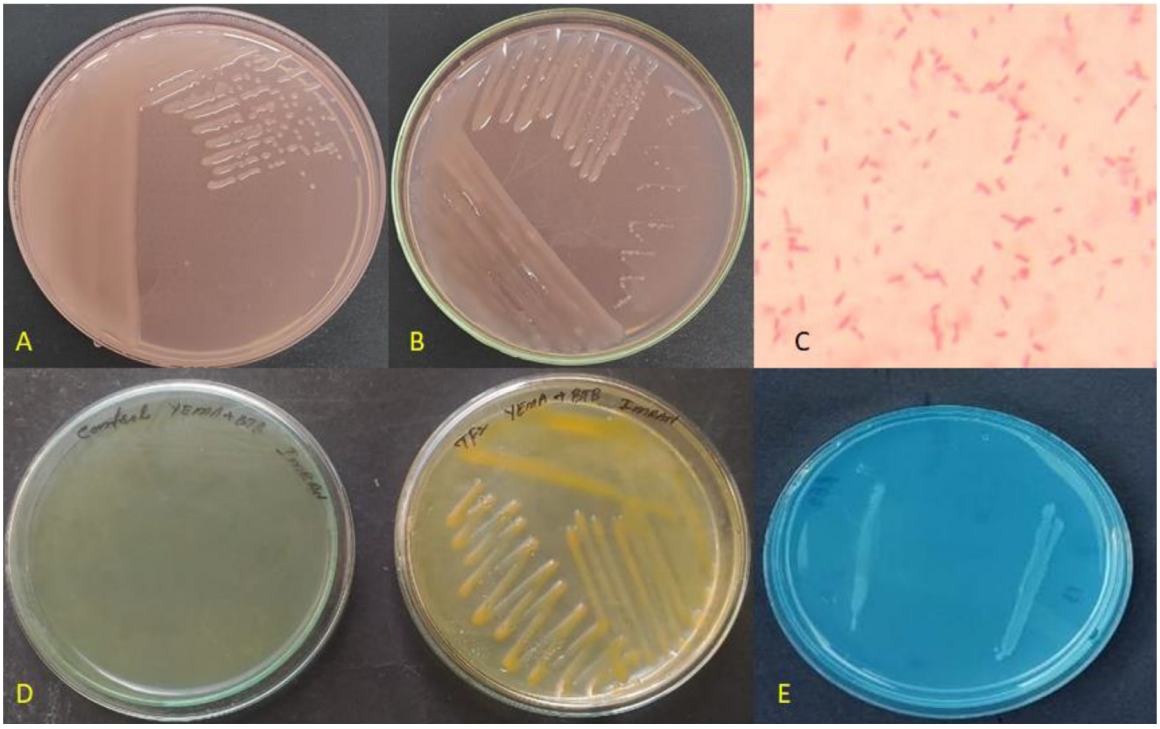

Fig. 4. (A, B) Growth of bacterial isolate IHTF-1 and IHTF-2 on YEM media; (C) Gram staining reaction; (D) Yellow colour observed on YEM-BTB media; (E) Negative keto-lactose test.

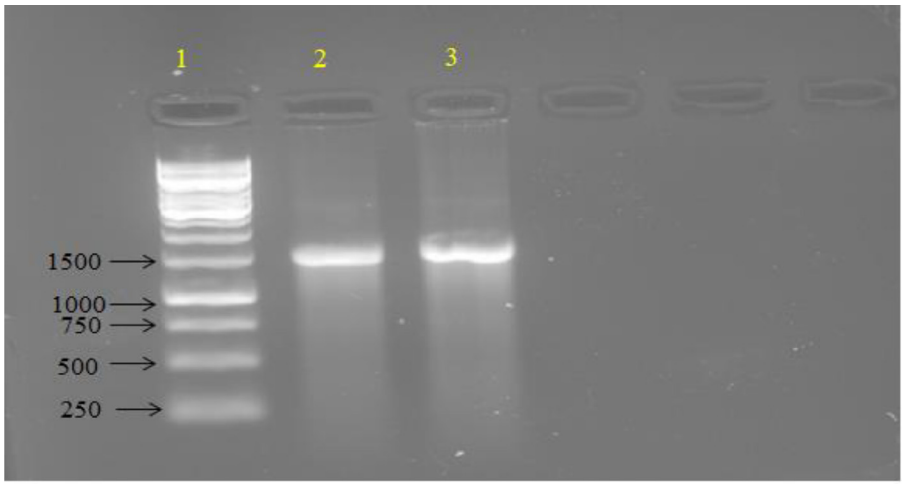

Fig. 5. PCR amplification of 16S rDNA gene. Lane 1, Ladder (1-kb); Lane 2, IHTF-1; Lane 3, IHTF-2.

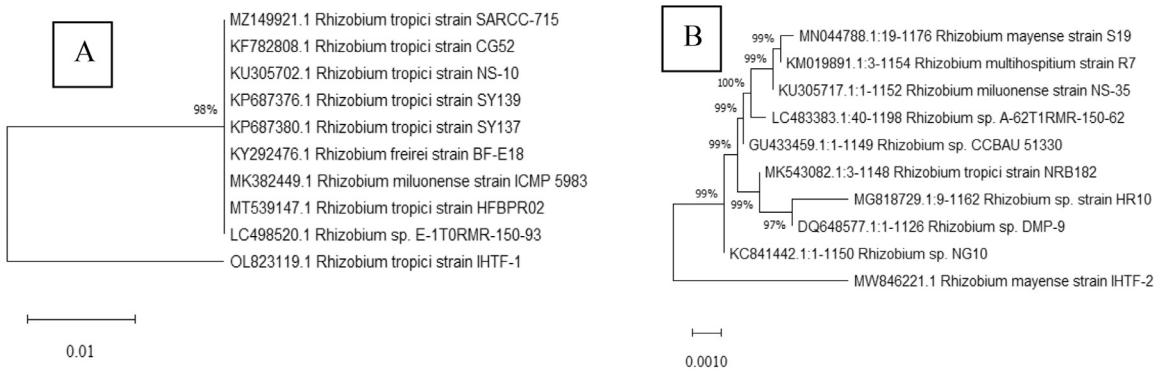

Fig. 6. (A) Phylogenetic tree of *Rhizobium tropici* strain IHTF-1; (B) Phylogenetic tree of *Rhizobium mayense* strain IHTF-2. MEGA X software was used to create phylogenetic trees.

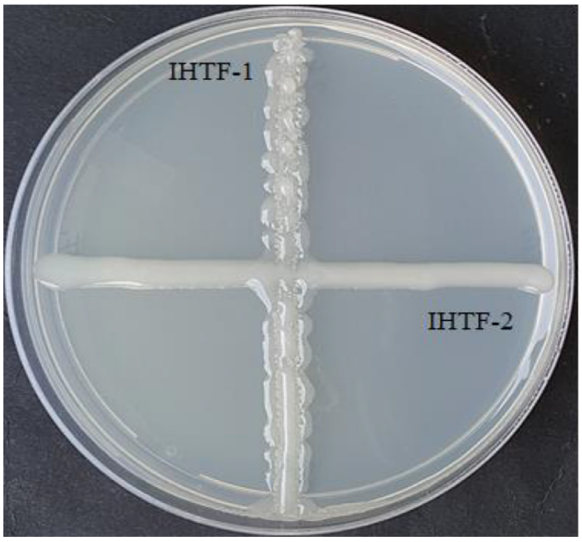

Fig. 7. Compatibility of *Rhizobium tropici* and *Rhizobium mayense*, with no zone of inhibition at their intersection.

3.7. Colonization of common bean roots by selected potential PGP Rhizobium *sp.*

A proficient PGPB ought to adhere to the root surface in order to establish itself in the rhizosphere, promote plant growth, and increase yield. SEM was used to investigate the ability of bacterial isolates, *R. tropici* strain IHTF-1 and *R. mayense* strain IHTF-2, to colonize root tissues of common bean seedlings (14 days old). The results of root colonization assay clearly showed that both the bacterial strains successfully adhered to the root surfaces, but were not present in the control seedlings (Fig. 10).

4. Discussion

Farmers are growing increasingly conscious of the use of beneficial bacteria as biocontrol agents and biofertilizers to preserve soil health and increase crop yield and quality. These beneficial bacteria promote plant growth and development via various PGP traits like nutrient assimilation, phytohormone production and systemic resistance to abiotic and biotic stresses (Alori et al., 2017a, 2017b; Mir et al., 2022). They discourage the practice of utilizing hazardous chemicals among farmers which in turn tend to equilibrate agro ecological systems (Alori and Babalola, 2018; Mir et al., 2022). The present study aimed to shed light on the rhizobia inhabiting red clover root nodules and to evaluate them for multiple PGP traits, hydrolytic enzyme production and investigate their bio-stimulation effects on common bean (*P. vulgaris* L.) plants. Eight morphologically distinct bacterial strains were isolated from red clover root nodules and tested in vitro for particular PGP characteristics.

Because they are endophytic and have the capacity to fix nitrogen, rhizobia are thought to be a better option for promoting plant growth than rhizobacteria (Bhattacharyya and Jha, 2012). Apart from various plant beneficial features, rhizobia are also known to produce plant growth hormones like indole-3-acetic acid (IAA), which promote the growth and development of plants by accelerating the germination

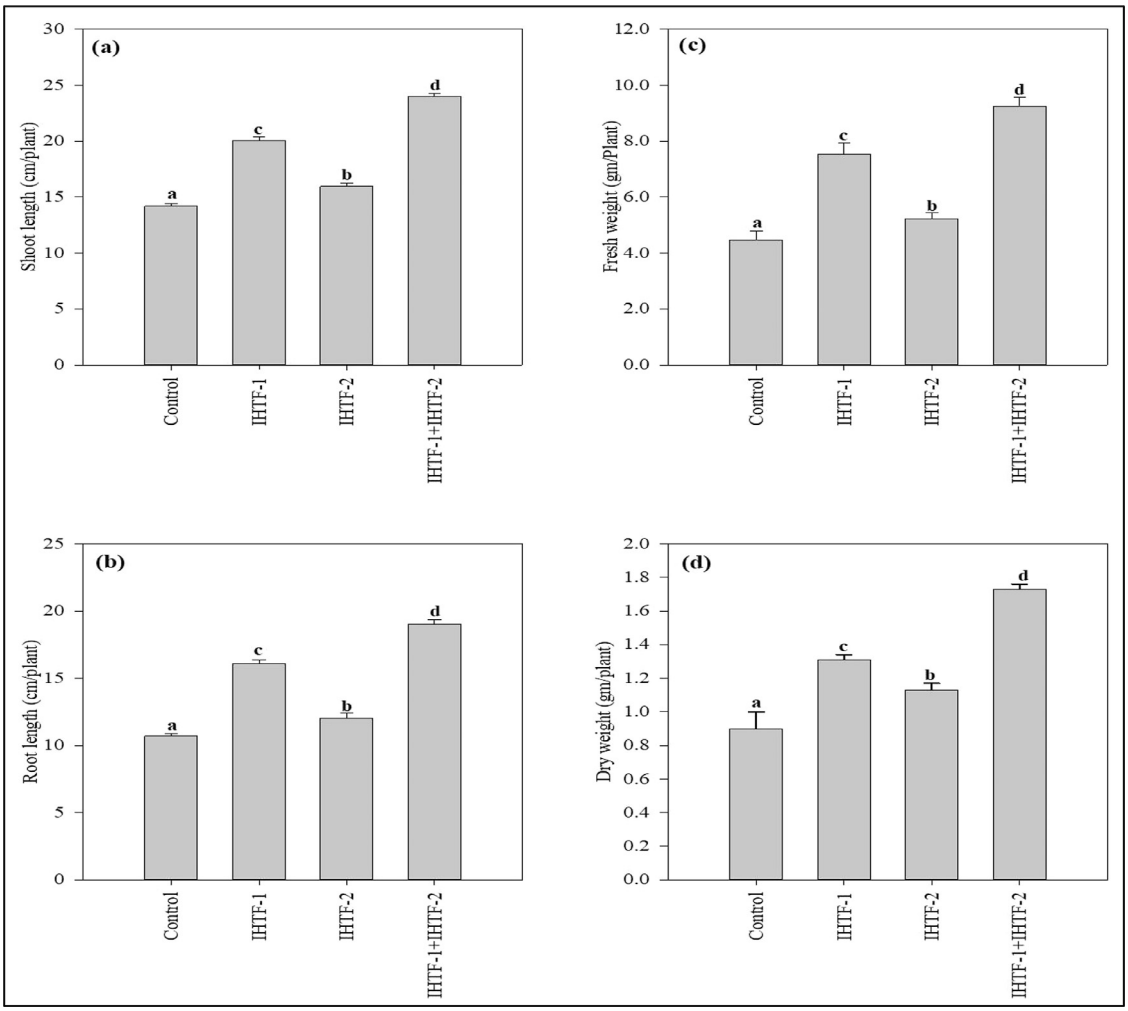

Fig. 8. Impact of bacterial strains IHTF-1, IHTF-2 individually and consortium (IHTF-1 + IHTF-2) on common bean plants grown under greenhouse conditions (a) Effect of isolates on shoot length; (b) Effect of isolates on root length; (c) Effect of isolates on Fresh weight; (d) Effect of isolates on dry weight. Data represented as mean ± SD of 3 replicates. Letters are statistically significant at $p < 0.05$. Comparison between treatment mean values was made using DMRT.

of seedlings and the growth of roots and shoots. IAA is a hormone that is important in the development of nodules in leguminous plants and is linked to the formation of vascular bundles, cell division, and differentiation (Rasool et al., 2021; Mir et al., 2022). Present investigation revealed the synthesis of IAA by four isolates. Highest IAA production was shown by *R. tropici* IHTF-1 (88.6 ± 1.45 μg/ml), followed by *R. mayense* IHTF-2 (52.3 ± 0.88 μg/ml) (Table 1). These results have been found greater compared to the previous investigations of IAA synthesis by diverse species of rhizobium secluded from rhizopsphere and nodules of *Cajanus cajan, Cicer arietinum* and *Arachis hypogaea* (1.49 to 77 μg/ml) (Singha et al., 2018; Gopalakrishnan et al., 2018; Igiehon et al., 2019; Khalid et al., 2020).

The next most crucial nutrient for plant growth after nitrogen is phosphorus (P). Even though the soil has a large reservoir of P, the proportion of forms that are accessible to plants is usually low. The limited availability of P to plants is partly due to the fact that the majority of P in soils is found in insoluble forms (Gopalakrishnan et al., 2015b; Mir et al., 2022). Although chemically synthesized P fertilizers offer plants with readily accessible sources of P, but they are not only costly, the overuse of them causes eutrophication and affects the ecosystem (Alori et al., 2017a). Phosphate solubilizing PGPB as an inoculant is one of the alternative ways utilized in environmentally friendly farming to meet plant phosphate requirements. Phosphate solubilization results from the PGPB's discharge of various acids, including gluconic acid and citric acid etc., which reduces the pH of the culture media (Alori et al., 2017a). In our study, three isolates solubilized tri-calcium phosphate, lowering the pH of the NBRIP broth medium from initial 7.0 to a minimum of 4.6. *R. tropici* IHTF-1 demonstrated the highest phosphate solubilization (216.3 ± 1.31 μg/ml), while *R. mayense* IHTF-2 demonstrated the second-highest solubility (152.8 ± 1.15 μg/ml) (Table 1). The results of the current investigation are consistent with those of Kumar et al. (2011); Singha et al. (2018); Igiehon et al. (2019) and Khalid et al. (2020), stated P solubilization by diverse species of rhizobium secluded from nodules of many leguminous plants.

After phosphorus and nitrogen, potassium (K) is recognized as the 3rd most significant macronutrient for plant growth and development. Over 90 % of potassium exists in insoluble forms such as illite, micas and orthoclases and soil soluble potassium levels are usually low (Sattar et al., 2019). Under potassium deficient conditions, crops will have slow growth, under-developed roots, produce small seeds and reduction in yield (Kumar et al., 2022). In the current study, 3 bacterial isolates demonstrated cleared zones on Aleksandrov medium with the maximum zone of solubilization showed by *R. tropici* IHTF-1 (16 mm) followed by *R. mayense* IHTF-2 (14 mm) (Table 1). There is currently little understanding of the mechanism by which potassium solubilizing bacteria (KSB) can solubilize K-containing minerals. The main method by which KSB solubilizes potassium is by reducing the pH of the medium. Lowering the medium's pH is the primary way that KSB solubilizes potassium. Reducing the pH of the

Fig. 9. Enhancement of plant growth in common bean on treatment with PGP *Rhizobium* sp., as individual strains and consortium. (a) Control, un-inoculated common bean plants; (b) Plants treated with *Rhizobium tropici* IHTF-1; (c) Plants treated with *Rhizobium mayense* IHTF-2; (d) Plants treated with consortium of IHTF-1 + IHTF-2.

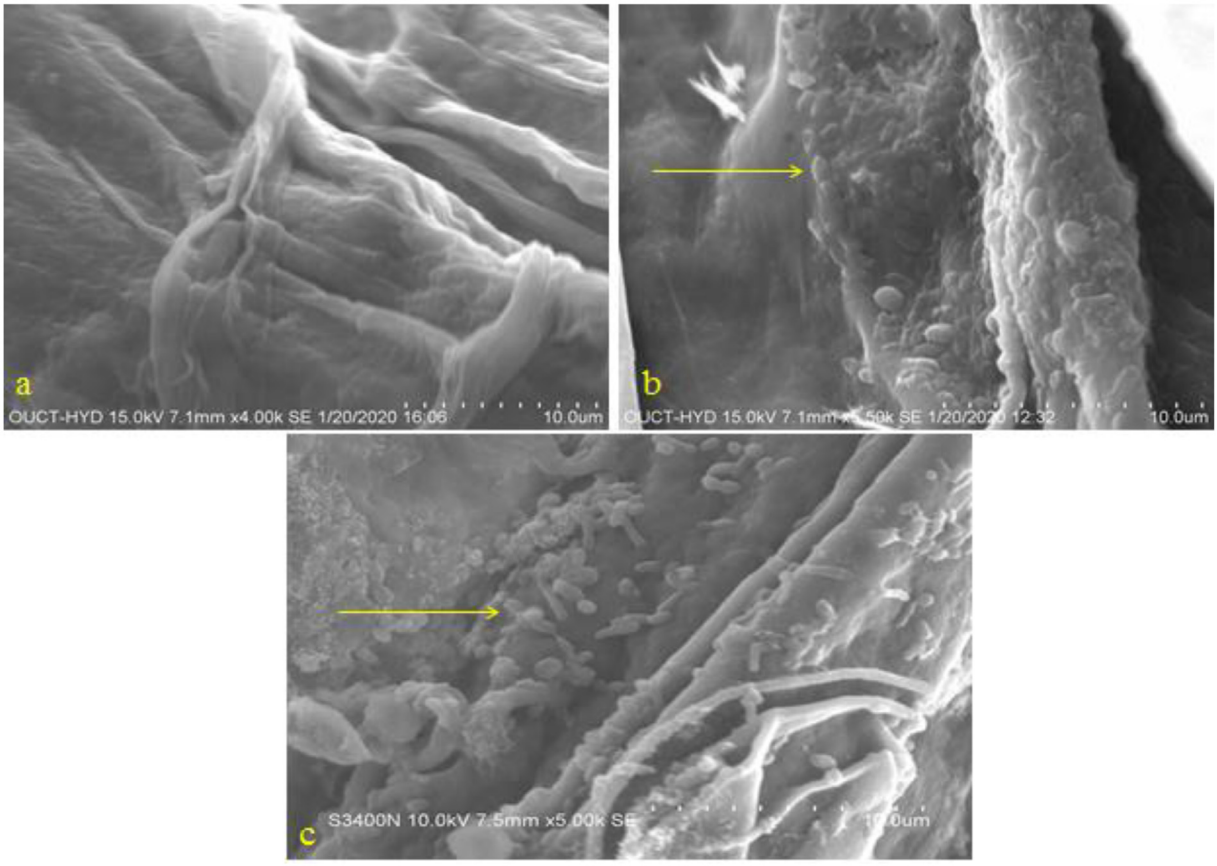

Fig. 10. SEM images showing colonization on the common bean root surface (a) Un-inoculated (control) root surface of common bean plants (b) Root colonization of common bean by *Rhizobium tropici* strain IHTF-1 (c) Root colonization of common bean by *Rhizobium mayense* strain IHTF-2.

medium suggest the release of organic acids, which are in charge of transforming insoluble potassium into soluble forms and making it available to plants. These acids include succinic acid, gluconic acid, citric acid, oxalic acid, malic acid and tartaric acid (Meena et al., 2014). Our findings are comparable with those of Meena et al. (2015), reported potassium solubilization by *Rhizobium pusense, Agrobacterium tumefaciens, Rhizobium rosettiformans*.

Iron (Fe) is an essential plant micronutrient found in soils. Iron exists as insoluble forms (hydroxides and oxyhydroxides forms) under aerobic conditions, which is generally inaccessible to plants. Plant growth promoting rhizobia have the capability to synthesize high affinity iron chelating compounds called siderophores, which can directly increase plant development by delivering iron ions to plants and indirectly inhibit the growth of pathogens in rhizosphere by reducing the accessibility of iron to pathogens (Olanrewaju et al., 2017; Igiehon et al., 2019). In the current study, among 8 bacterial isolates, three isolates (IHTF-1, IHTF-2 and IHTF-5) produced siderophore. The results of this study align with earlier research conducted by Gopalakrishnan et al. (2018);Igiehon et al., 2019; Mir et al. (2021); Kumar et al. (2022), who reported that siderophores are produced by

variety of rhizobium species, including *R. pusense, Rhizobium leguminosarum* bv. *viciae, R. tropici, R. meliloti, Sinorhizobium meliloti, R. leguminosarum* bv. *Trifolii, Bradyrhizobium* sp., *Rhizobium cellulosilyticum, Rhizobium taibaishanense* and *Rhizobium multihospitium*.

Under stressful situations such as pathogen invasion, salinity, water logging, drought and heavy metals, endogenous ethylene levels in plants are dramatically enhanced, which can negatively impact the growth and development of the plant (Chandwani and Amaresan, 2022). Many rhizobium species possess the ability to generate 1-aminocyclopropane-1- carboxylate (ACC) deaminase, which breaks down ACC (ethylene precursor) into ammonia and α-ketobutyrate. This process lowers the levels of ethylene in plants and shields them from various stressful conditions (Gopalakrishnan et al., 2015b; Olanrewaju et al., 2017). In our present investigation, *R. tropici* IHTF-1 and *R. mayense* IHTF-2 showed ACC deaminase activity (Fig. 2D). The results of this study align with earlier research conducted by Gopalakrishnan et al. (2015b); Gopalakrishnan et al. (2018); Igiehon et al. (2019) who reported production of ACC deaminase by variety of rhizobium species, including *R. tropici*, *R. cellulosilyticum*, *R. taibaishanense*, *R. pusense, R. hedysari, R. gallicum, Mesorhizobium loti* and *Bradyrhizobium japonicum*. Rhizobia which possess the enzyme ACC deaminase decreases the negative impacts of ethylene on the nodulation process (Nascimento et al., 2018a). According to Nascimento et al. (2018a), rhizobia which possess the enzyme ACC deaminase lessen the detrimental effects of ethylene on the process of nodulation. Better nodulation and increased N_2 fixation were seen in legumes when PGPB with ACC deaminase activity was applied (Glick, 2014). Similarly, Cedeño-García et al. (2018), reported alfalfa plants inoculated with *Ensifer meliloti* and ACC producing PGPB improved root length, nodule number and dry weight under controlled conditions (pot culture studies).

A wide variety of polymeric components found in phytopathogens, such as cellulose, hemicellulose, proteins, chitin, and DNA, can be hydrolyzed by extracellular hydrolytic enzymes, also known as mycolytic enzymes, produced by a variety of PGP bacteria. These hydrolytic enzymes compromise the structural integrity of the phytopathogens cell walls (Olanrewaju et al., 2017; Kumar et al., 2022). Their potential to suppress the growth of phytopathogens renders them more valuable in the bio-control process. In addition to biocontrol activity, the production of hydrolytic enzymes such as esterases, lipases, cellulases, proteases glucosidases, amylases etc., influences plant cell development and organization, facilitating bacterial entry, colonization, and maintenance (Nascimento et al., 2018b). Rhizobia thrive as free-living organisms, but they can also induce and colonize root nodules in legume plants. A significant event in the establishment of the rhizobium-legume symbiosis is the localized disruption of a cellulosic plant cell wall by cellulase enzyme produced rhizobium species, by which the bacterial symbiont passes to establish a nitrogen-fixing, intracellular endosymbiotic state within its legume host (Menéndez et al., 2019). In our study, bacterial isolate IHTF-1 and IHTF-2, were able to produce all the three (chitinase, cellulase and β- 1, 3-glucanase) hydrolytic enzymes (Table 2). The findings of this study coincide with the earlier research conducted by Sridevi and Mallaiah (2008); Mir et al. (2021); Kumar et al. (2022), which documented the existence of protease, lipase, β-1, 3-glucanase, chitinase, cellulase enzymes in different rhizobium strains, including *R. tropici*, *R. meliloti*; *R. multihospitium* and *Mesorhizobium* spp. According to Kumar et al. (2011), *R. leguminosarum* and *E. meliloti* isolated from fenugreek root nodules showed excellent chitinase and β-1, 3-glucanase activity. Similarly, Gopalakrishnan et al. (2018); Chaudhary et al. (2021), reported *R. pusense* secluded from chickpea and mung bean nodules exhibited protease, β- 1, 3-glucanase and cellulase activity.

Earlier, rhizobial diversity was researched using phenotypic and biochemical features and it was believed symbiotic nodulating bacteria belong to only α- proteobacteria viz. *Rhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium* and *Sinorhizobium*. In recent times, advanced PCR-based genotyping methods have revealed the presence of multiple native rhizobial strains, including β- and γ-proteobacteria, linked to the nodules of numerous *Fabaceae* plants (Martínez-Hidalgo and Hirsch, 2017; Mir et al., 2021). These bacteria can thrive in a variety of climatic situations and soil types. 16S rRNA which is coded by 16S rDNA is considered as the most authentic marker for the identification of bacteria (Singha et al., 2018). In the present investigation, bacterial strains IHTF-1 and IHTF-2 exhibiting most of the PGP traits were identified by 16S rRNA sequence analysis as *R. tropici* and *R. mayense*.

In present investigation, treatment of common bean seedlings with *R. tropici* strain IHTF-1 and *R. mayense* strain IHTF-2 individually substantially boosted shoot and root length as well as fresh and dry weight at 30 days after sowing compared to control plants (un-inoculated) (Figs. 8 and 9). The findings of in-vivo greenhouse studies also demonstrated that applying both strains (IHTF-1 + IHTF-2) as consortium improved plant growth of common bean plants compared to application of individual strains. The results of the compatibility test and greenhouse study showed that both strains were compatible to each other and that using them together had a cumulative growth-promoting effect. Korir et al. (2017), reported treatment of common bean plants with *R. tropici* individually and co-inoculation of *R. tropici* CIAT 899 with PGPR such as *Paenibacillus polymyxa* and *Bacillus megaterium* increased, nodulation, root and shoot dry weights, compared to un-inoculated plants. Similarly the findings of this study coincide with the earlier research conducted by Mostasso et al. (2002); (Vargas et al. (2000); Fiori et al. (2021); Wekesa et al. (2021) reported plants of common bean inoculated with *R. tropici* enhanced bean yield. The synthesis of IAA, phosphate and potassium solubilization, siderophore production and other PGP traits that our bacterial isolates possess may be the cause of the stimulation of common bean plant growth parameters in comparison to control plants.

5. Conclusion

Present study concludes that two rhizobial strains (*R. tropici* IHTF-1 and *R. mayense* IHTF-2) secluded from the root nodules of red clover possessed excellent PGP traits and increased growth parameters of *P. vulgaris* L. This study further clarifies that IHTF-1 and IHTF-2 co-inoculation (consortium) may be an effective bioinoculant for common beans. These results imply that *Rhizobium* spp., identified in this work, may be used as bio-inoculants to increase common bean plant production in a sustainable way. In future, these rhizobial strains could be tested in the field to see how stable they are under various environmental circumstances and developed into promising green biofertilizers.

Declaration of competing interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Mohammad Imran Mir: Conceptualization, Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Nagaraju Mukkamula:** Data curation, Formal analysis, Supervision, Visualization. **B.Kiran Kumar:** Conceptualization, Investigation, Methodology, Supervision, Visualization. **Javid A. Parray:** Writing – review & editing, Software, Supervision, Visualization. **Ira Khan:** Data curation, Formal analysis, Software, Visualization. **Bee Hameeda:** Conceptualization, Data curation, Methodology, Resources, Supervision, Writing – review & editing. **Olubukola Oluranti Babalola:** Conceptualization, Investigation, Methodology, Writing – review & editing.

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