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Effect of endophytic bacterial consortium isolated from tea (*Camellia sinensis* (L.) O. Kuntze) roots on growth and yield of *Phaseolus vulgaris* L.

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ABSTRACT

This paper deals with the isolation of endophytic bacteria from roots of tea (*Camellia sinensis* (L.) O. Kuntze) shrubs of upper Assam, India, and evaluates their potential for promoting plant growth *in-vitro*. Out of forty bacterial isolates, *Bacillus cereus*, *Bacillus flexus*, *Pseudomonas* sp., and *Pseudomonas rhodesiae* were most effective in mineral solubilization and plant growth-promoting hormone production. *Pseudomonas* sp. showed the highest indole acetic acid (IAA, 16.75 ± 0.04 µg/m) production, gibberellic acid (GA_3, 4.12± 0.11 µg/ml) production, and potassium solubilization index (2.70 ± 0.05). *Bacillus cereu*s was the highest phosphate (174.33±2.0 µg/ml) and zinc solubilizer (solubilization index 2.35± 0.01and 2.53 ±0.02 for ZnO and ZnS respectively). Siderophore activity was the highest in *P. rhodesiae* (87.37 ± 0.73%). All the isolates mentioned above were active against test plant pathogens *Alternaria sp., Rhizoctonia solani, Sclerotinia sclerotiorum,* and *Fusarium solani*. Inoculation of *Bacillus cereus*, *Bacillus flexus*, *Pseudomonas* sp., and *Pseudomonas rhodesiae* to the soil in pot culture of *Phaseolus vulgaris* showed significant promotion of plant growth and yield.

Key words : *Endophytes, Plant growth promotion, Sustainable agriculture*

Introduction

Exponential population growth and rapid industrialization are the major causes of the decrease in agricultural land in third-world countries like India. To feed a huge population, the maximum production of food grains in a limited area of agricultural land was one of the vital problems of the last century. However, the impact of the green revolution and the introduction of high-yielding hybrid varieties led to a remarkable increase in food grain (Pingali and Prabhu, 2012; Wik *et al.,* 2008). To maintain the productivity of hybrids and high-yielding varieties, high input of chemical fertilizers, pesticides, herbicides, and chemically synthesized growth hormones are of utmost necessity. As a result, the present agriculture is mostly dependent on agrochemicals (Dhankera *et al.,* 2021)

In North-East India, most farmers are unaware of the adverse effects of agrochemicals and apply it beyond the prescribed limits to gain the maximum. Further, these agrochemicals contaminate soil and water affecting the environment in many ways (Kole *et al.,* 2002). Moreover, a high concentration of agrochemical residues in food affects human health (Grewal *et al.,* 2017). Excessive use of chemical fertil-

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izers alters the soil microbial flora resulting in degraded soil quality (Aktar *et al*., 2009).

Reduction of health and other environmental risks with a less negative impact on soil health, organic agriculture is considered one of the best concepts in the present day agriculture (Eva-Marie et al 2018). Application of biofertilizers, organic composts, organic pesticides, and herbicides leads to improvement of soil quality and high production of crops with less harmful impacts to nature (Durán-Lara *et al.,* 2020). The concept of manipulation of soil microbial flora with beneficial microbes is not new. Diverse kinds of microbial biofertilizers are now available in the market which are primarily microbial consortia isolated from soil (Jacoby *et al.,* 2017). However, the search for newer sources of microbes with high biofertilizer potential is still a subject of research.

Endophytic microorganisms are a group of microbes, mostly bacteria, and fungi, which colonize inside plant tissues without causing any harm to their hosts. Though they colonize all parts of the plant body, however, the density of endophytes is always higher in the root tissues in comparison to stem and leaves (Srivastava *et al.,* 2020). This may be due to the entry from the rhizosphere through natural pores and wounds of roots. However, some endophytes may enter plant tissues by making pores using different enzymes like endoglucanase (Rheinhold-Hurek *et al.,* 2006). Microbes may spend whole or a part of their life inside plant tissues without any visible effect (Stone *et al*., 2000) and most of them are beneficial to the hosts in diverse ways (Santoyoa *et al.,* 2006; , Lacava *et al*., 2013). Similar to that of rhizosphere microbes, endophytes induce plant growth directly by the production of plant growth hormones, mineral solubilization and mobilization, siderophore production, ammonia production, nitrogen fixation, antagonism against phytopathogens, and indirectly by induced systemic resistance (Fouda *et al*., 2021; Audipudi *et al.,* 2017; Glick, 2015; Sturz *et al.,* 1997; Duijff *et al.,* 1997; Krishnamurthy *et al.,* 1997). Though rhizosphere and endophytic microbes share similar mechanisms of plant growth promotion, however, after the establishment of their relationship, endophytes are less susceptible to the changing environment (Glick, 2012). Ryan *et al.* (2008) suggested that endophytic microbes may be applied to diverse sectors including industrial and medical (such as antibiotic production), plant yield and growth augmentation, protection of plants from various pathogenic agents, and as adjuncts in the phytoremediation (Fouda *et al*., 2021; Ahemad *et al.,* 2014). Endophytes with ACC deaminase activity can decrease plant ethylene levels and delay the leaf, flower and fruit senescence along with increasing salt tolerance in crops (Ali *et al.,* 2012; Karthikeyan *et al.,* 2012; Zhang *et al.,* 2011). Moreover, Singh *et al.* (2018) reported that various endophytic bacteria isolated from diverse plants have remarkable herbicide and pesticide activities.

Yan *et al.,* (2018) isolated 274 endophytic bacterial isolates from two different tea cultivars of china and reported that most of the isolates were able to exhibit plant growth promoting activities both in the laboratory and natural conditions. Wenna et al., (2018) reported 46 Actinobacteria from tea leaves, stems and roots and found that most of them were promising agents against some plant pathogenic bacteria and fungi along with plant growth promoting hormone producing and mineral solubilizing activities. Win *et al.* (2018) studied the diversity of endophytic fungi of *Camellia sinensis* L. and reported *Colletotrichum gloeosporioides*, *Phomopsis* sp., *Phomopsis* sp., *Neurospora crassa, Pleosporales* sp., *Pestalotiopsis microspora*, *Trichoderma viride, Glomerella* sp., *Botryosphaeria* sp., *Penicillium sclerotiorum* and *Rosellinia* sp as most frequent endophytic fungi with remarkable plant growth promoting activities. As endophytes are native to the plant system and have a very less negative impact to plant health, they may be a good source of potential biofertilizers for sustainable agriculture.

Tea (*Camellia sinensis*) is one of the most popular refreshing beverages in the world. Assam alone produces one-sixth of total global tea production. However, excessive use of fertilizers and agrochemicals is considered one of the major causes of lowering the quality of Assam tea. Hence, the production of organic tea is now in demand. In the light of the facts narrated above, the present study aims to evaluate the endophytic bacteria of tea roots for their biofertilizer potential.

Materials and Methods

Sample collection

Collected young and growing root samples of mature tea shrubs from twelve different tea gardens of upper Assam, India between April to September. Root samples were packed in sterile polyethylene

bags and carried to the laboratory in a cooling box (4°C). Isolation of endophytic bacteria was done within 48 hours.

Five plots of 1600 sq. meters each with healthy tea shrubs were selected and roots were collected from 10 different shrubs of a single plot and mixed. Five subsamples were collected from each garden.

Isolation of endophytic bacteria

Roots were washed with tap water to remove the soil and debris adhering to the root surface. Kept in front of laminar airflow for 45 minutes to air dry the roots and then surface sterilization was carried out with the help of the following solutions: sterile distilled water for 5 min (repeated three times), 70% (v/v) ethanol for 5 min, sterile distilled water for 5 min, 0.1% $HgCl_2$ for 1 min and finally washed with sterile distilled water for ten times. The last washing water was plated onto nutrient agar (NA) and surface sterilization was confirmed by the absence of any microbial growth after 48 hours of incubation. 5grams of surface-sterilized root samples were crushed in 20 ml of sterilized distilled water to prepare the stock tissue homogenate and finally made the volume 50 ml. From the tissue homogenate, 10^{-3} dilution was made for inoculation in tryptic soy agar plates and incubated for 72 hours at 32 ^{0}C. The number of colony-forming units (CFU) was calculated with the help of a colony counter. To avoid unexpected contamination, the whole process was done in front of a laminar air hood (Bandara *et al.,* 2006; Hassan *et al.,* 2018; Fouda *et al.,* 2015). Pure culture of bacterial isolates was obtained by repeated streak plate method five times followed by microscopic observation. Colonies with identical cells and Gram's staining behaviour were confirmed as pure.

Screening and estimation of plant growth promoting activities of endophytes

Ammonia production

The ability of bacterial endophytes to produce ammonia in broth culture was detected by using Nessler's reagent. Isolates were grown in10 ml peptone water medium for 72 hrs at 32 °C. 0.5 ml of Nessler's reagent was added directly to the broth culture. Development of brown to yellow colour indicated ammonia production (Cappuccino *et al*., 1992).

HCN production

Screening of the bacterial isolates for HCN production was performed using the method of Lorck (1948). Isolated bacterial colonies were streaked on nutrient agar containing 4.4 g glycine/L. Placed a filter paper soaked with 0.5% picric acid over the media. Sealed with paraffin strips and incubated at 30±2 °C for 4 days. The development of orange or red colour indicated HCN production.

IAA production

Quantitative estimation of IAA was done according to the procedure by Brick *et al*. (1991). 100 ml of sterilized minimal salt (MS) medium was taken into conical flasks and added filter-sterilized L-tryptophan (1000 ìg/ml). 1 ml of 48hours old 0.6 OD bacterial cultures was inoculated to each flask and incubated at 32^{0}C for 8 days. 1 ml of 2, 4, 6, and 8 days old cultures were centrifuged at 6,000 rpm for 10 minutes. Supernatants were mixed with 2 ml of $FeCl_3$-perchloric acid reagent (50 ml 35% perchloric acid + 1 ml 0.5 M $FeCl_3$ solution) and 2 drops of ortho-phosphoric acid. After 25 minutes of incubation at room temperature, measured the development of pink colour at 530 nm wavelength by UV-VIS spectrophotometer. Took an uninoculated flask as reference.

GA_3 production

The amount of GA_3 produced by the bacterial endophytes was determined according to the standard method of Uthandi *et al*. (2010). Inoculated 1 ml of 48 hours old 0.6 OD culture into a conical flask containing 100 ml of minimal salt medium enriched with 1000 ìg/ml of tryptophan. Incubated the flasks in an incubator shaker at 32 °C. 30 ml of 2, 4, 6, and 8 days old culture broth from each flask was centrifuged at 6000 rpm to remove the bacterial cells. Added 2 ml of zinc acetate (1M) to 25 ml of culture supernatants from each flask and incubated for 2 minutes and then added 2 ml of potassium ferrocyanide. Centrifuged at 1000 rpm for 15 minutes. 5ml of 30% HCL was added slowly to 5 ml of supernatant, mixed well, and incubated at 20 °C for 75 minutes. An uninoculated flask was taken as a reference. Absorbance was measured by UV-VIS spectrophotometer at 254 nm wavelength.

Phosphate solubilization

Screening of bacterial isolates for their phosphate solubilizing activity was done by using Pikovskya's agar (glucose,10 g/l; $Ca_3(PO_4)_2$, 5 g/l; $(NH_4)_2 SO_4$, 0.5 g/l; NaCl, 0.2 g/l; $MgSO_4.7H_2O$, 0.1 g/l; KCl, 0.2 g/

l; yeast extract, 0.5 g/l; $MnSO_4H_2O$, 0.002 g/l; and $FeSO_4.7H_2O$, 0.002 g/l, agar 15 g/l) medium. Pure culture of endophytic bacteria was inoculated on Pikovskya's agar plates and incubated at 32 °C for 72 hrs. Colonies with clear zones around it referred to as positive for phosphate solubilization (Gour, 1990).

Estimation of phosphate solubilization by bacterial endophytes was performed according to the procedure of Jackson (1973). 250 ml of Pikovskya's broth was inoculated with 1 ml of 48-hour-old 0.6 OD bacterial cultures. Kept at 32 ^{0}C in incubator shaker for eight days at 250 rpm. 5 ml of 2, 4, 6, and 8 days cultures from each culture were centrifuged for 10 minutes at 5000 rpm. 1 ml of culture supernatant was mixed with 10 ml of chloromolybdic acid and adjusted the volume up to 40 ml with distilled water. Added 1 ml of chlorostannous acid and made the final volume 50 ml. The absorbance of the developing blue colour was measured at 600 nm wavelength. The amount of soluble phosphate was calculated from the standard curve of KH_2PO_4. Change of culture pH was recorded with the help of a digital pH meter.

Zinc solubilization

Periodic Zinc solubilization activity of bacterial endophytes was screened by using the halo zone formation method in Basal medium plates containing 0.1% insoluble ZnO or ZnS (Venkatakrishnan *et al.*, 2003). Zinc solubilization was measured as follows:

$$\text{Zinc solubilization index} = \frac{\text{Total diameter of the halo zone}}{\text{Colony diameter}}$$

Potassium solubilization

Periodic Potassium solubilization activity of endophytes was screened by the halo zone forming method in Aleksandrov's agar medium containing 0.2% insoluble mica (Hu *et al.*, 2006). Clear zones around the colonies were considered positive for potassium solubilization activity. Potassium solubilization was measured as per the standard formula.

$$\text{Potassium solubilization index} = \frac{\text{Total diameter the halo zone}}{\text{Diameter of the colony}}$$

Siderophore production

Screening of siderophore production endophytes was carried out in chrome azurol S (CAS) agar medium (Alexender and Zuberer, 1991). The CAS agar medium is a mixture of different solutions prepared and sterilized separately before mixing. Solution I (Fe-CAS indicator solution): $FeCl_3.6H_2O$ (in 10 mM HCl), 10 ml, 1mM aqueous solution of CAS (1.12 mg/ml), 50 ml and aqueous solution of hexadecyl trimethyl ammonium bromide (1.82 mg/ml), 40 ml.

Solution II (buffer solution): 30.24 g of PIPES (piperazine-N-N-bis-2-ethane sulphonic acid) was dissolved in 750 ml of a salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl, and 1.0 g NH_4Cl. pH was adjusted to 6.8 by adding 50% KOH and making the final volume 800 ml. Solution II was autoclaved after adding 15 g of agar.

Solution III: 2 g glucose, 2 g mannitol and trace elements dissolved in 70 ml distilled water. Solution IV: 30 ml 10% casamino acid sterilized by syringe filter. All four solutions were autoclaved separately and then solution III and IV were added to the buffer solution (solution II). Mixed well Added solution I (indicator) to the above mixture with sufficient stirring and poured into Petri plates. Actively growing bacterial cultures were spotted on CAS agar medium plates and incubated for one week at 32 °C. Orange haloes around colonies were considered as positive for siderophore production.

The amount of siderophores produced by the endophytes was determined by using CAS- shuttle assay. Bacterial endophytes were cultured in iron-starved minimal salt broth up to day 6. 2 ml of bacterial broth from each culture was centrifuged at 3000 rpm for 10 minutes and mixed 0.5 ml of supernatant was mixed with 0.5 ml of CAS reagent (Solution I). Measured the absorbance at 630 nm against a reference containing 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in the aliquot was determined by using the following formula

$$\% \text{ of siderophore units} = \frac{Ar - As}{Ar} \times 100$$

Here, Ar = absorbance of reference at 630 nm and As= absorbance of samples at 630 nm.

Antagonistic activity

Antifungal activity of endophytic bacteria was evaluated using the dual culture method against 4 (four) broad-spectrum pathogenic fungi viz; *Alternaria* sp. *Rhizoctonia solani*; *Sclerotinia sclerotiorum* and *Fusarium solani* were collected from the Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam. Dual culture plates were incubated

at 28 ºC for 7 days. Antifungal activity was indicative as mycelial growth inhibited in the direction of the active bacterial colonies. Inhibition percentage was calculated by the following formula (Paul, 2012).

$$\% \text{ of inhibition} = \frac{A1 - A2}{A1} \times 100$$

Where A1= radial growth of pathogenic mycelia on PDA media without inoculation of test bacteria. A2= radial growth of pathogenic mycelia on PDA media towards the test bacterial line on the medium.

Molecular approaches for microbial identification

Extraction of bacterial genomic DNA

Bacterial cultures were grown for 24 hours in an incubator shaker at 32 ^{0}C. 1.5 ml of culture was taken and centrifuged. Discarded the supernatant, added lysis buffer (300 µl) to the pallet, and mixed well. Kept in a hot water bath at 65 ºC for 30 minutes and then transferred to -20 ºC for 10 minutes. After that 100 µl of 5 M sodium chloride (NaCl) solution was added and mixed gently. Centrifuged the tube for 10 minutes at 12000 rpm. The aqueous phase was transferred to another tube and added an equal volume of chloroform: isoamyl alcohol (24: 1) and mixed. The upper aqueous phase was transferred to another tube and mixed with 2.5 volumes of absolute chilled isopropyl alcohol and incubated at -20 ^{0}C for 24 hours. Centrifuged at room temperature and discarded the supernatants. Rinsed the pallet with 70% ethanol, air-dried, and mixed with 30 µl tris- EDTA buffer at pH 6.8. after treating with RNAse, DNA extraction was confirmed by gel electrophoresis.

Bacterial 16s rDNA amplification

16s rDNA of bacterial endophytes was done using universal 16s primer sequences (US16F8/20 5′AGA GTT TGA TCC TGG CTG AG3' and US16R154/20 5′AAG GAG GTG ATC CAG CCG CA3') for 35 cycles in 25 µl PCR reaction mixture. Amplification was confirmed by agarose gel electrophoresis and nanodrop quantifier.

Sequencing of 16 S rDNA was done for isolates with remarkable plant growth promoting activities and sequence homology was studied using BLAST. Sequences of identified bacterial isolates were submitted to Genbank.

Assessment of endophytes for their biofertilizer activity in pot culture

To evaluate the plant growth-promoting activities of endophytic bacteria, a pot culture experiment was carried out on French beans (*Phaseolus vulgaris*) in an outdoor environment in a green net shade house. Enhancement of plant growth was measured in terms of shoot height, the number of leaves, and the width of leaves. In addition, the number of pods and weight of pods was also taken into consideration to evaluate the crop yield.

Vermicompost used in the experiment was sterilized to remove already existing microbes. A mixture of *Bacillus cereus*, *Bacillus flexus*, *Pseudomonas* sp., and *Pseudomonas rhodesiae* with a cell load of 3 x 10^7 cells/ml was added and mixed to pre-sterilized vermicompost (50 ml/kg) and termed as Bio-verm. Potting mixtures (soil treatments) for this experiment are given in Table 1.

Results

A total of 40 (forty) endophytic bacterial isolates were isolated from all the root samples collected from twelve different tea gardens of Assam and were screened for plant growth promoting activities *in-vitro*. *Pseudomonas rhodesiae, Bacillus flexus, Pseudomonas* sp., and *Bacillus cereus* were found to be efficient during the qualitative screening and hence were subjected to quantitative estimation of plant growth promotion *in-vitro*.

Microbial population, Ammonia, and HCN production

During the study, it was observed that the endophytic bacterial population ranged between 6.3 x 10^3 to 10.3 x10^3 cfu/g of root samples. The highest cfu was observed in sample ORG- B (Organic garden B), collected from Baghmora, Jorhat, and lowest in the sample DOR (Digboi Oil Refinery) collected from a garden nearby Digboi Oil Refinery, Tinsukia.

All four endophytic bacteria *Pseudomonas rhodesiae*, *Bacillus flexus*, Pseudomonas sp. and *Bacillus cereus* were found to be positive in ammonia (Fig. 1) and HCN production.

IAA production

Quantitative estimation of IAA and GA 3 in the bacterial culture supernatant was done up to 96 hours of incubation at 24 hours intervals and observed that

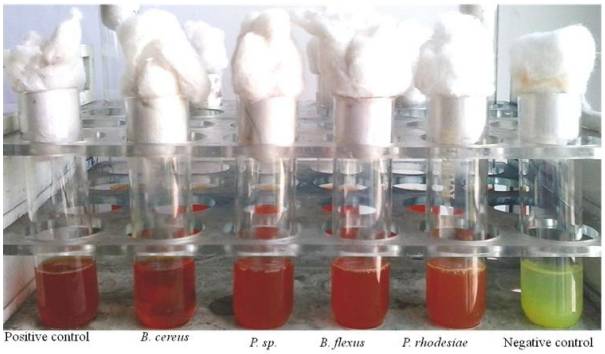

Fig. 1. Production of ammonia by bacterial endophytes.

Table 1. Soil treatments in pot culture.

Treatments	Potting mix
Control (C)	Garden soil
Treatment I (T1)	Garden soil +Vermicompost (3:1)
Treatment IV (T2)	Garden soil +Bio-verm(3:1)
Treatment V (T3)	Garden soil + Bio-verm(1:1)
Treatment VII (T4)	Garden soil +5 ml of liquid culture
Treatment VIII (T5)	Garden soil +10 ml of liquid culture
Treatment IX (T6)	Garden soil +15 ml of liquid culture

the IAA activity increased up to 72 hours and then decreased. The IAA activity ranged between 5.04 ± 0.05 and 8.46 ± 0.14 µg/ml after 24 hours; 9.19 ± 0.35 and 11.46 ± 0.25 µg/ml after 48 hours; 14.80 ± 0.20 and 16.75 ± 0.04 µg/ml after 72 hours, and 11.26± 0.26 and 14.24± 0.26 µg/ml after 96 hours of incubation. The highest IAA activity was shown by *Pseudomonas* sp. (16.75 ± 0.04 µg/ml) followed by *Bacillus cereus* (15.54 ± 1.32 µg/ml) after 72 hours of incubation (Table 2, Fig. 2).

GA_3 production

Periodic estimation of GA3 production activity was carried out up to 96 hours of incubation in an interval of 24 hours. It was observed that the amount of GA_3 in the culture supernatant increased up to 96 hours of incubation and then decreased gradually. The amount of GA_3 in the culture supernatant

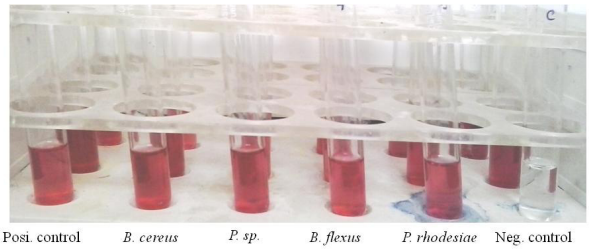

Fig. 2. Estimation of IAA content in bacterial culture supernatants.

ranged between 0.44± 0.02 and 0.73± 0.01µg/ml after 24 hours, 1.07± 0.21 and 1.67± 0.06 µg/ml after 48 hours, 2.91± 0.17 and 4.12± 0.11 µg/ml after 72 hours and 4.43 ± 0.19 and 6.46 ± 0.49 µg/ml after 96 hours of incubation. The highest GA3 production was recorded for *Pseudomonas* sp. (6.46 ± 0.49 µg/ml) followed by *Bacillus cereus* (5.56 ± 0.15 µg/ml) (Table 2).

Phosphate solubilization

The amount of soluble phosphate in the culture supernatant was measured up to day 8 in an interval of 48 hours. A gradual increase in the concentration of soluble phosphate was noticed from day 2 to day 8. The concentration of phosphate in the supernatant ranged between 65.3±2.1 and 80.71±1.6 µg/ml after 2 days; 86.5±1.6 and 122.46±0.9 µg/ml after 4 days and 101.36±1.3 and 156.23±2.1 µg/ml after 6 days of incubation. The concentration of soluble phosphate in the culture supernatant was found to be the highest in *B. Cereus* (174.33±2.0 µg/ml) followed by *B. flexus* (166.57±0.7 µg/ml); P. sp. (143.56±2.9µg/ml); and *P. rhodesiae* (134.4±0.8µg/ml) after 8 days of incubation (Table 2, Fig. 3).

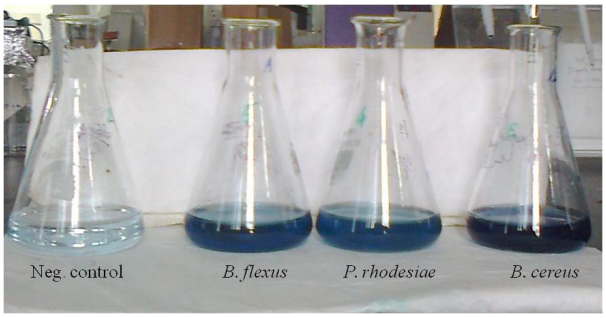

Fig. 3. Estimation of available phosphate content in culture supernatants.

Potassium solubilization

Qualitative estimation of potassium solubilization ability of the endophytic bacterial isolates was carried out by a simple halo zone formation method. The potassium solubilization index was recorded up to day 8 in an interval of 2 days. A gradual increase in potassium solubilization was observed up to day 6 of incubation and then gradually decreased. The potassium solubilization index ranged between 1.27 ± 0.04 and 1.94 ± 0.02 after 2 days, 1.58 ± 0.03 and 2.13 ± 0.09 after 4 days, 1.79 ± 0.02 and 2.70 ± 0.05 after 6 days and 1.50 ± 0.02 and 2.25 ± 0.1 after 8 days of incubation. The highest potassium solubili-

Table 2. *In-vitro* plant growth promoting activity of bacterial endophytes of tea

	Endophytes	*P. rhodesiae*				*B. flexus*				*P.* Sp.				*B. cereus*			
Plant growth promoting hormone producing activity	Incubation	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr
	IAA production (in µg/ml)	7.42 ± 0.20	11.46 ± 0.25	14.97 ± 0.20	11.26 ± 0.26	5.04 ± 0.05	9.19 ± 0.35	14.80 ± 0.20	12.61 ± 0.01	8.46 ± 0.14	13.38 ± 0.89	16.75 ± 0.04	14.24 ± 0.26	5.87 ± 0.29	11.36 ± 0.64	15.54 ± 1.32	13.64 ± 0.22
	GA_3 production (in µg/ml)	0.54 ± 0.03	1.43 ± 0.09	3.27 ± 0.22	5.44 ± 0.23	0.44 ± 0.02	1.67 ± 0.06	2.92 ± 0.10	4.43 ± 0.19	0.64 ± 0.05	1.29 ± 0.08	4.12 ± 0.11	6.46 ± 0.49	0.73 ± 0.01	1.07 ± 0.21	2.91 ± 0.17	5.56 ± 0.15
	Incubation period	Day 2	Day 4	Day 6	Day 8	Day 2	Day 4	Day 6	Day 8	Day 2	Day 4	Day 6	Day 8	Day 2	Day 4	Day 6	Day 8
Mineral solubilization or mobilization activity	Amount of soluble P (in µg/ml)	73.53 ±1.7	92.33 ±2.0	111.83 ±1.4	134.4 ±0.8	72.5 ±0.9	86.56 ±0.2	116.06 ±1.9	166.57 ±0.7	65.3 ±2.1	86.5 ±1.6	101.36 ±1.3	143.56 ±2.9	80.71 ±1.6	122.46 ±0.9	156.23 ±2.1	174.33 ±2.0
	Potassium solubilization index	1.87 ± 0.15	2.13 ± 0.09	2.49 ± 0.02	2.20 ± 0.03	1.27 ± 0.04	1.58 ± 0.03	1.68 ± 0.01	1.50 ± 0.02	1.94 ± 0.02	2.13 ± 0.08	2.70 ± 0.05	2.25 ± 0.1	1.5 ± 0.1	1.62 ± 0.07	1.79 ± 0.02	1.58 ± 0.05
	ZnO solubilization index	1.03 ± 0.05	1.24 ± 0.02	1.76 ± 0.02	1.55 ± 0.02	1.26 ± 0.01	1.54 ± 0.02	1.92 ± 0.02	1.75 ± 0.01	1.10 ± 0.01	1.61 ± 0.01	2.23 ± 0.02	1.88 ± 0.02	1.35 ± 0.01	1.73 ± 0.01	2.35 ± 0.01	1.93 ± 0.02
	ZnS solubilization index	1.19 ± 0.03	1.49 ± 0.01	1.74 ± 0.02	1.56 ± 0.01	1.33 ± 0.02	1.62 ± 0.02	2.11 ± 0.02	1.87 ± 0.01	1.31 ± 0.02	1.61 ± 0.01	2.34 ± 0.02	2.08 ± 0.01	1.56 ± 0.02	1.97 ± 0.01	2.53 ± 0.02	2.0 ± 0.01
	Siderophore %	33.36 ± 1.78	87.37 ±0.73	71.09 ±0.75		32.41 ± 1.67	68.46 ±0.79	54.33 ±0.55		43.52 ± 2.27	82.64 ± 1.12	66.59 ± 0.34		28.48 ± 0.7	72.39 ± 0.66	63.78 ± 0.54	

Values are average of three replicates ±

zation was noticed in *Pseudomona*s sp. (2.70 ± 0.05) followed by *P. rhodesiae* (2.49 ± 0.02) after 6 days of incubation, Table 2.

Zinc solubilization

To observe the zinc solubilization ability of all four test endophytic bacterial species both ZnO and ZnS were used separately. The zinc solubilization index was found to be the highest in *B. cereus* (2.35± 0.01and 2.53 ±0.02 for ZnO and ZnS respectively) followed by *Pseudomonas* sp. (2.23± 0.02 and 2.34 ± 0.02 for ZnO and ZnS respectively) after 6 days of incubation. A gradual increase in zinc solubilization index was observed up to day 6 and then decreased, Table 2.

Siderophore production

After screening in a solid medium, periodic estimation of siderophore percentage in CAS broth culture supernatant was carried out up to day 6 in an interval of 2 days. It was observed that the siderophore percentage in the culture supernatant increased gradually up to day 4 and then decreased. The siderophore activity ranged between 28.48 ± 0.7 and 43.52 ± 2.27% after 2 days, 68.46 ± 0.79 and 87.37 ± 0.73% after 4 days and 54.33 ± 0.55 and 71.09 ± 0.75% after 6 days of incubation. The highest siderophore activity was recorded for *P. rhodesiae* (87.37 ± 0.73%) followed by *Pseudomonas* sp. (82.64 ± 1.12%) after 4 days of incubation (Table 2, Fig. 4).

Antagonistic activity

The selected bacterial endophytes were tested against *Alternaria* sp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Fusarium solani* to evaluate their antagonistic activity. *P. rhodesiae* exhibited 28.4±1.05, 31.8±0.95, 45.5±1.22, and 40.3±1.37% of inhibition on the growth of *Alternaria* sp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Fusarium solani* respectively. *B. flexus* inhibited the growth of *Alternaria* sp., *Rhizoctonia*

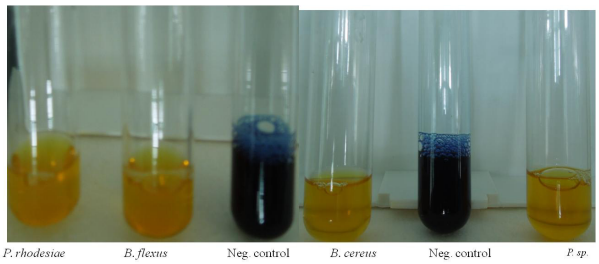

Fig. 4. Siderophore activity of bacterial endophytes of tea plant.endophytes.endophytes.

solani, *Sclerotinia sclerotiorum*, and *Fusarium solani* by 52.6±1.19, 67.2±1.17, 61.6±2.02, and 22.5±1.07% respectively. Similarly, *Pseudomonas* sp. and *Bacillus cereus* showed 61.5±1.77, 54.4±1.22, 50.3±1.71, and 26.4±1.23%; and 57.0±1.21, 41.2±1.34, 42.5±1.07, and 51.5±1.09% against the same pathogens respectively (Table 3).

Pot culture experiment

After 30 days of inoculation of the endophytic bacterial consortium (*Bacillus cereus*, *Bacillus flexus*, *Pseudomonas* sp., and *Pseudomonas rhodesiae*) to the potting mix of *P. vulgaris*, the average shoot height was highest in T3(37.59±2.79 cm) followed by T2 (35.62±1.94 cm), T6 (35.37±2.30), T5 (34.42±2.01 cm), T4 (34.0±0.97 cm), T1 (32.68±3.08 cm) and control (C; 29.974 cm). The average width of leaves (3.7±0.2 cm) and the average number of leaves (15.2±1.30 nos.) were found to be the highest in T6 followed by T 3 (leaf width-3.7±0.18 cm; the number of leaves was (15±2.0 nos) and T5 (leaf width-3.5±0.27 cm; the number of leaves 13.4±1.51 nos). The average number of bean pods per plant and the average yield per plant were found to be the highest in T3 (Pods-15.4±2.6 nos per plant; Average yield per plant-216.81±7.64 g). It was observed that though, the number of bean pods per plant was higher in T6 (14.2±1.3 nos./plant) than T4 (10.2±0.8 nos./plant), still, the yield was higher in T4 (162.52±2.97 g/plant) in comparison to T6 (139.35±9.28 g/plant) (Table 4).

Molecular approaches for microbial identification (Table 5 Fig. 5.)

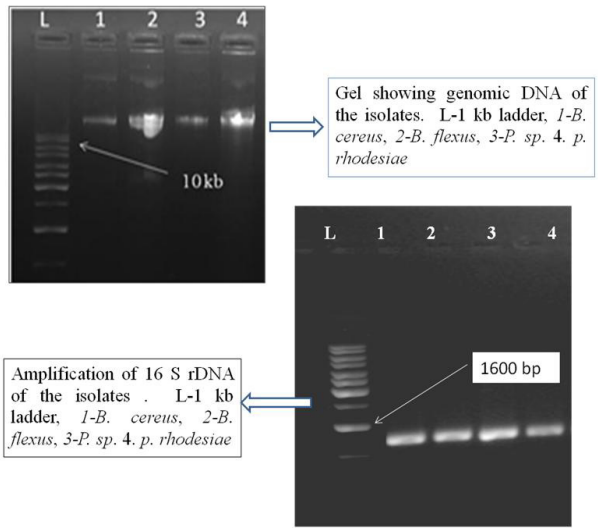

Fig. 5. Isolation of genomic DNA from bacterial isolates and their 16s rDNA amplification gel.

Table 3. Antagonistic activity of bacterial endophytes

Bacterial endophytes	% of inhibition against			
	Alternaria sp.	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*	*Fusarium solani*
P. rhodesiae	28.4±1.05	31.8±0.95	45.5±1.22	40.3±1.37
B. flexus	52.6±1.19	67.2±1.17	61.6±2.02	22.5±1.07
P. sp.	61.5±1.77	54.4±1.22	50.3±1.71	26.4±1.23
B. cereus	57.0±1.21	41.2±1.34	42.5±1.07	51.5±1.09

Values are average of three replicates ± SD

Table 4. Evaluation of growth of *P. vulgaris* by the bacterial consortium

Treatments	C	T1	T2	T3	T4	T5	T6
Shoot height (in cm)	29.97±1.98	32.68±3.08	35.62±1.94	**37.59±2.79**	34.0±0.97	34.42±2.01	35.37±2.30
Width of leaves (in cm)	2.9±0.20	3.2±0.05	3.4±0.15	3.7±0.18	3.2±0.07	3.5±0.27	**3.7±0.2**
Number of leaves per plant	11.6±1.14	13±1.58	13.2±1.92	15±2.0	12.4±1.14	13.4±1.51	**15.2±1.30**
Number pods per plant	8.8±1.3	8.8±1.3	10.2±0.8	**15.4±2.6**	9.6±1.6	16.4±1.3	14.2±1.3
Average Yield of bean in g/plant (up to 50 days)	89.35±4.39	112.06±9.10	162.52±2.97	**216.81±7.64**	108.24±5.13	126.71±7.15	139.35±9.28

Values are average of five replications ± SD

Table 5. Bacterial endophytes and their accession numbers

Endophytic bacteria	Genbank accession no.
Pseudomonas rhodesiae	KF225790
Bacillus flexus	KF225789
Pseudomonas sp.	KF225787
Bacillus cereus	KF225785

Discussion

Soil is the main source of microorganisms, from which microbes migrate to all parts of our environment. The density of microorganisms is much higher in the rhizosphere region compared to other parts of the soil (Meena *et al.,* 2020). The higher concentration of microbes in the rhizosphere is mainly due to high organic materials around the root system. These organic compounds are mainly secreted from the plant body in the form of root exudates (Jacoby *et al.,* 2017). From the rhizosphere, some best compatible microbes migrate towards the root internal system through natural wounds and cracks, through the sites of lateral root emergence, and directly by secreting some enzymes in a controlled amount (Rheinhold-Hurek *et al*., 2006) and then migrate to the aerial parts through the vascular system (Kennedy, 2005). Such microorganisms residing inside the plant tissues are called endophytes (Srivastava *et al.,* 2020) and most of these endophytes are efficient plant growth promoter (Fouda *et al.,* 2021; Audipudi *et al.,* 2017; Glick, 2015; Sturz *et al.,* 1997; Duijff *et al.,* 1997, Zachow *et al*., 2010). It is now well known that endophytes are not only well compatible with the internal environment of the plant but also have higher PGP effects in comparison to the rhizosphere (Tsavkelova *et al*., 2007). Therefore, several researchers have reported them as a promising source of biofertilizer and biocontrolling agents (Kumar *et al.,* 2017.

The present study aims to isolate and evaluate the plant growth promotion and ability to control some common broad-spectrum plant pathogens (fungal) by endophytic bacteria of tea roots collected from upper Assam, India. It was observed that the bacterial population was highest in the root sample ORB-B (6.3 x 10^3cfu/g of roots) which was collected from a garden maintained organically. As the microbial population in the rhizosphere of organically maintained crops is higher in comparison to conventional agrochemical-based crops, it might be assumed that more microbial cells invaded and colonized inside the roots of tea shrubs maintained organically. Moreover, different agrochemicals may have an adverse impact on the endophytic and rhizosphere microbial population which may also be a cause of depleted endophytic bacterial population in root samples of conventional agrochemical-based tea gardens of Assam (Wang *et al.,* 2006; Lally *et al.,* 2017). In our study, it is observed that all four selected endophytic bacteria, i.e. *Bacillus cereus*, *Bacillus flexus, Pseudomonas* sp., and *Pseudomonas rhodesiae*; have shown very high IAA and GA_3 production; phosphate, potassium, and zinc solubilization activity in-vitro. The mineral solubilization ability of *Bacillus* and *Psedomonas* isolates is very well studied. Several species of the genus Bacillus like *B. megaterium, B. circulans, B. coagulans, B. subtilis, B. azotofixans, B. macerans, B. velezensis*, etc. are reported as (Goswami *et al.,* 2016; Fira *et al.,* 2018). The various direct and indirect mechanisms of plant growth promotion by *Bacillus* spp. are nitrogen fixation, solubilization and mineralization of phosphorus and other nutrients, phytohormone production, production of siderophores, antimicrobial compounds and hydrolytic enzymes, induced systemic resistance (ISR) and tolerance to abiotic stresses (Mazylyte *et al.,* 2022). *Pseudomonads* are a group of gram-negative rod-shaped bacteria with a high potentiality for mineral solubilization, hormone production, and secretion of compounds having biocontrol activity (Sah *et al.,* 2021). Many reports have revealed that *Pseudomonads fluorescent* are very much efficient in plant growth promotion and controlling phytopathogens (Suresh *et al.,* 2021; Suresh *et al*., 2010; Garrido-Sanz *et al.,* 2016; Shanahan *et al.,* 1992; Redondo-Nieto *et al*., 2013). Lally *et al.* (2017) reported that endophytic *Pseudomonas* consortia increase the growth of *Brassica napus* both *in vitro* and in field conditions. Several other researchers also reported that endophytic *Pseudomonads* were efficient in plant growth promoting hormone production as well as mineral solubilizing activities (Otieno *et al.,* 2015; Kushwaha *et al.,* 2019; Salehin *et al.,* 2021). These results completely support the results of our present study.

Before going to the pot culture experiment, the compatibility of the bacterial endophytes was tested by co-inoculating them in nutrient agar medium and observed that all four isolates grew together and the colonies overlapped each other. As tea is a very slow growing plant, it takes a very long time to evaluate

the effect of these microbes on its growth. Hence, the French bean (*Phaseolus vulgaris*) was taken as the study material for the evaluation of the plant growth-promoting activity of the isolates in terms of stem height, number of leaves, number of bean pods, and average yield per plant. In one set of pot culture experiments, we applied the mixture of bacterial isolates and sterilized vermicompost to the soil in different ratios, and in the other set direct application of mixed bacterial culture broh to soil was done. Statistical analysis through ANOVA revealed that the pots inoculated with the endophytic bacterial inoculants (Solid and liquid form) significantly enhanced the growth of Phaseolus vulgaris. In our study, it was observed that the average yield of bean pods was almost equal in T3 and T6, however total yield in terms of weight was remarkably higher in T3 than T6. Hence, application of the bacterial consortia isolated from tea roots mixing with vermicompost is more effective than direct application of the culture.

Conclusion

Increasing pollution of the environment is one of the major challenges to twenty-first-century human civilization. Pollution of agricultural soil by the overuse of chemical fertilizers, herbicides, pesticides, etc. leads to major damage to the soil quality and fertility. These agrochemicals have an adverse effect on the normal flora of the soil, thereby decreasing the density of the normal soil microbial population which further affects on normal biogeochemical cycling of mineral nutrients. A major part of chemical fertilizers applied to the soil with a depleted microbial population is converted into insoluble forms causing low availability of such nutrients. Similarly, the injudicial use of bactericides, fungicides, pesticides, and herbicides increases the concentration of residues of such agrochemicals, which is one of the main causes of health issues. As tea is one of the dominant crops of India, frequent and excessive use of chemical fertilizers and other agrochemicals is very prevalent.

The present study, therefore, targeted to isolate some native endophytic bacteria having a high potential to promote plant growth from the roots of tea bushes available in upper Assam and identified Bacillus cereus, *Bacillus flexus, Pseudomonas* sp., and *Pseudomonas rhodesiae* as potent plant growth promoting and biocontrolling agents for Phaseolus vulgaris and assumes that more detailed investigation and proper application of these microbes in consortium form may lead to a new formulation of biofertilizer and biocontrol agents for tea and other crops.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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