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Assessing the Plant Growth-promoting Traits and Host Specificity of Endophytic Bacteria of Pulse Crops

R. Thamizh Vendan[1], D. Balachandar[2]

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ABSTRACT

Background: Symbiotic associations between legumes and Rhizobia are ancient and fundamental. However, the plant growth-promoting endophytes other than Rhizobia are not yet fully explored for pulses productivity. The present study was aimed to isolate efficient endophytic bacteria from pulses, assess their diversity, screen their plant growth-promoting activities and to test their potential as bio inoculants for pulses.

Methods: We have isolated several endophytic bacteria from pulse crops more specifically from blackgram (*Vigna mungo*) and greengram (*Vigna radiata*). After careful screening, 15 promising endophytic isolates were selected for this study. The identification of endophytic bacterial isolates was performed by 16S rRNA gene sequencing. The isolates were tested for their potential for the plant growth-promoting traits such as nitrogen fixation, phosphate solubilization, indole-3-acetic acid production, siderophore secretion and antifungal activity. Pot culture experiments were conducted with the screened potential endophytic cultures.

Result: The 16S rRNA gene sequencing revealed that species of *Enterobacter*, *Bacillus*, *Pantoea*, *Pseudomonas*, *Acromobacter*, *Ocrobacterium* were found as endophytes in blackgram and greengram. The *in vitro* screening identified *Bacillus pumilus* (BG-E6), *Pseudomonas fluorescens* (BG-E5) and *Bacillus licheniformis* (BG-E3) from blackgram and *Pseudomonas chlororaphis* (GG-E2) and *Bacillus thuringiensis* (GG-E7) from greengram as potential plant growth-promoting endophytes. These strains showed antagonism against plant pathogenic fungi. Upon inoculation of these endophytic PGPR strains, the blackgram and greengram growth and yield got increased. Among the strains, BG-E6 recorded 14.7% increased yield in blackgram and GG-E2 accounted for a 19.5% yield increase in greengram compared to respective uninoculated control. The experimental results showed that there was a host specificity found among the endophytic bacterial cultures with pulses. The cross inoculation of endophytic strains did not perform well to enhance the growth and yield of their alternate hosts.

Key words: Endophytic bacteria, Host specificity, Plant growth-promoting traits, Pulses.

INTRODUCTION

Pulses are generally grown under rainfed conditions in marginal lands and in soils of low fertility, which ultimately resulted in poor yield. Any amount of increase in the productivity of pulses would be useful to meet out the demand created by the ever-growing population. Symbiotic associations between legumes and Rhizobia are ancient and fundamental. Unfortunately, the nodulation and nitrogen fixation by *Rhizobium* is strongly influenced by various biotic and abiotic factors, which eventually hampered the effective symbiosis in pulses. At this juncture, endophytes are presumed to have an advantage over *Rhizobium*, in that they colonize the interior rather than the rhizosphere of the plants and hence, are better protected. There is a vast repertoire of literature on *Rhizobium* - legume symbiosis, while bacterial endophytes with legumes association are still yet to be explored.

Endophytes are the group of microorganisms that colonize the internal tissues of plants either symbiotically or in a mutualistic relationship (Dudeja *et al*., 2012). In legume crops, endophytic microbes are recovered from roots, nodules and different parts of the plant body (Narula *et al*., 2013; Saini *et al*., 2015). The internal tissues of plants are thought to provide a protective environment for endophytes than plant surfaces, where exposure to extreme environmental conditions, such as temperature, osmotic pressure and UV radiation are major limiting factors for bacterial survival. In plant-endophyte interactions, the plant host provides a protective niche for the endophytes while the endophytes in return produce useful metabolites and signals (Rosenblueth and Romero, 2006) which increase plant nutrient uptake (Ramos *et al*., 2011), modify plant growth, development of biomass (Compant *et al*., 2005), induce resistance to pathogens (Sturz and Matheson, 1996) and insects (Azevedo *et al*., 2000) and increase resistance to osmotic stress (Sziderics *et al*., 2007), heavy metals

[1]Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625 104, Tamil Nadu, India.
[2]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.

Corresponding Author: R. Thamizh Vendan, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625 104, Tamil Nadu, India. Email: rtvendan@tnau.ac.in

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(Rajkumar *et al*., 2009), contaminated chemicals (Siciliano *et al*., 2001) and other abiotic factors. Though numerous reports are available on plant-growth-promoting endophytes from a wide range of host plants (Firdous *et al*., 2019) and functional diversity (Santoyo *et al*., 2016), their host specificity is not yet fully understood, which is essential for inoculant development (Afzal *et al*., 2019). In the present work, we hypothesized that blackgram and greengram may harbor genetically and functionally diversified endophytic plant-growth-promoting bacteria and those endophytes with a broad host range can be used as a commercial inoculant. For this, we have isolated efficient endophytic bacteria from blackgram and greengram, assessed their diversity, screened their plant growth-promoting activities and tested their potential as bio inoculants for pulses.

MATERIALS AND METHODS

Sampling

The healthy and disease symptomLess pulse crops [Blackgram (*Vigna mungo*) and Greengram (*Vigna radiata*)] were collected from the seven districts (Tiruchirappalli, Thanjavur, Nagapattinam, Pudukkottai, Karur, Perambalur and Coimbatore) of Tamil Nadu state, India.

Isolation of endophytic bacteria

Bacterial endophytes were isolated from stem tissues of pulses, more specifically *V. mungo* and *V. radiata*, which were grown in the above districts of Tamil Nadu. The stems were washed in running tap water to remove soil particles and other impurities. They were split into longitudinal sections and excised to 1-2 cm pieces with a sterile surgical blade and further they were placed in a beaker, soaked in distilled water and drained. These pieces were surface-sterilized with 70% ethanol for 30 sec followed by sterile distilled water thrice. After that, they were sterilized with 0.1% mercuric chloride for 5 min and again rinsed with sterile distilled water thrice. The bark of surface disinfected stems were removed with a sterilized razor blade and the stems were cut into 4-6 mm long pieces, which were placed on tryptic soy (TS) agar medium amended with benomyl (50 µg/mL) to inhibit fungal growth. Plates were incubated at 28°C for 1-10 days to allow the growth of endophytic bacteria from the cut pieces (Araújo *et al*. 2002).

In another way, stem fragments were homogenized in 5 mL of sterile phosphate buffer saline (containing 8 g/L of NaCl, 0.2 g/L of KCl, 1.4 g/L of Na_2HPO_4 and 0.24 g/L of KH_2PO_4) by using a blender and serial dilutions were plated onto TS agar. The plates were incubated at 28°C for 1-10 days or until growth was observed. After the period of incubation bacterial colonies from each stem fragment/ homogenized sample were selected at random and purified.

Molecular identification of endophytic isolates

The identification of endophytic bacterial isolates was performed by 16S rRNA gene sequencing. For this total DNA of the endophytes was isolated using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method (Clark, 2013) and dissolved in distilled water to a final concentration of 20 ng/µl and stored at 4°C. Nearly full-length of the 16S rRNA gene was amplified from elite isolates using universal eubacterial primers, FD1 and RP2 (Weisburg *et al*., 1991). The gene amplification was performed in the thermocycler (Eppendorf Master cycler, Germany) with a 25 µL reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 µM of each primer, 2.5 mM of $MgCl_2$ and 2.5 µM of Taq DNA polymerase and the buffer supplied with the enzyme (Thermo Scientific). The conditions of the polymerase chain reaction were initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final elongation at 72°C for 10 min. The amplified products were resolved on a 1.5% agarose gel in 1X TBE buffer and documented in Alpha imager TM 1200 documentation and analysis system. The band of the expected size was gel-purified using spin columns according to the manufacture's instructions (Thermo Scientific) and cloned using PTZ57R/T vector supplied with the T/A cloning kit (Fermentas) before sequencing. Sequencing reactions were performed using an ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The phylogenetic tree was constructed with existing 16S rRNA gene sequences from related eubacteria obtained from the NCBI Gene Bank database.

Determination of nitrogenase activity

Acetylene reduction assay (ARA) was used to determine the nitrogenase activity of the endophytic isolates (Hardy *et al*. 1968). Each bacterial isolate was grown in a 20-mL test tube containing 10 mL of nitrogen-free semi-solid medium for 72 hr at 30°C. Each test tube was sealed with a rubber stopper and 1 mL of acetylene gas was replaced to the air in the headspace (10 mL). The test tubes were incubated at 30°C for 24 h. One mL of each gas sample from the headspace was assayed for ethylene production by gas chromatography (GOW MAC series 750, New Jersey, USA) equipped with a hydrogen flame ionization detector (FID) and a Porapack N column. Nitrogenase activity was calculated as nmol of ethylene produced per mg of protein per h.

Indole-3-acetic acid (IAA) production

Indole-3-acetic acid production of endophytic isolates was estimated by the method developed by Gorden and Paleg (1957). The cultures were grown in yeast extract glucose broth in the flasks with tryptophan at 100 mg^{-1} level, which is wrapped with black paper during incubation to avoid photo-inactivation. Twenty-five mL of the sample was withdrawn at the desired interval of time and the cells were spun down at 5000 rpm for 15 minutes in a centrifuge (Kubota, Japan). The pH of the cell-free filtrate was adjusted to 2.8 with 1 N

HCl. The acidified supernatant was extracted with ethyl acetate. At 4 hr interval, two more extractions were done and organic phases were pooled and evaporated to dryness in dark. The residue was dissolved in 2.0 mL of methanol for the analysis. A quantity of 0.5 mL of the sample was taken in the test tube and to this 1.5 mL of distilled water followed by 4.0 mL of Salper's reagent (0.5 M ferric chloride in 50 mL of 35% perchloric acid) were added and incubated in darkness for 1 hr at 28°C. The intensity of the pink color developed was read in Systronics-UV-Vis Spectrophotometer-108 at 535 nm and IAA content was quantified by referring standard graph.

Phosphate solubilization

Twenty four hours old cultures of endophytic isolates were inoculated in the center of Pikovaskaya agar plates supplemented with 0.5% tricalcium phosphate, incubated at 28±1°C for 4 days. The phosphate solubilization (mm) zone formed around colonies was recorded after 48 hr of inoculation (Pikovaskaya, 1948).

Siderophore production

The capacity of the endophytic bacteria for the production of siderophore was assessed by the Chrome azurol S (CAS) method (Schwyn and Neilands, 1987). Siderophore production was estimated qualitatively by the spot inoculation of the cultures on the plates containing CAS medium and incubated at 28±2°C for 2-3 days. The appearance of orange/reddish-brown color indicates positive for siderophore production.

Antagonistic activity

All the endophytes were primarily screened for the production of antimicrobial substances following the cross-streak assay method (Williston *et al*. 1947) against plant fungal pathogens *viz.*, *Rhizoctonia solani, Cercospora canescens, Macrophomina phaseolina* and *Uromyces phaseoli* (obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore). Nutrient agar plates were inoculated with bacterial endophytes as a single streak at the center of the Petri plate and incubated for 5 days at 30°C. Overnight grown cultures of the test organisms were streaked at the right angle to the producer endophyte and observed for its growth/inhibition after 24 - 48 hr of incubation at 30°C. The length of the inhibition zone was measured to the nearest mm.

Pot culture study to assess the bio-efficacy of endophytic bacterial isolates

Among the 15 endophytic bacterial isolates studied for plant growth-promoting traits, only five isolates (GG-E2, GG-E7, BG-E5, BG-E6, BG-E3) showed their potential for the above characters and they were selected to assess the influence on the blackgram and greengram, through pot culture experiments. These five isolates were considered as 5 treatments and in addition to that, a control (uninoculated) was also kept. Two sets of experiments were conducted with similar treatments for *V. mungo* and *V. radiata* separately. Briefly, bacterial culture suspensions were incubated for 3 d at 30°C in a shaking incubator at 200 rpm to an estimated cell density of 10^8 cfu/mL. *V. mungo* [Variety: VBN (Bg) 5] and *V. radiata* [variety: VBN (Gg) 3] seeds were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with autoclaved distilled water. The seeds were mixed with the inoculants separately at 50 mL /kg and allowed for shade drying for 30 min. Earthen pots of sizes 20 × 30 cm were prepared with sterilized soil and farmyard manure in a ratio of 2:1. The soil of the experiment was sandy clay loam (sand - 56%, silt - 15% and clay - 28%) in texture, acidic in soil reaction (pH 4.7-6.5), low in organic carbon (0.36%); nitrogen (105.3 kg/ha), medium in phosphorous (38.40 kg/ha) and high in potassium (203.64 kg/ha). These pots were filled with 10 kg of above soil. Each pot was supplied with 330 mg of nitrogen as urea, 1875 mg of phosphorus as single super phosphate and 250 mg of potassium as muriate of potash as a basal dose. Inoculated seeds were sown in the pots and six plants per pot were allowed to grow under controlled greenhouse conditions at 28±2°C. Pots were watered with an equal amount of tap water at regular intervals. The experiments were set out as a completely randomized block design with four replications. The yield attributes *viz.*, root length, shoot length, nodule number, nodule dry weight, plant fresh weight, plant dry weight were observed 45 days after sowing. Grain yield was recorded at the time of harvest.

Statistical analysis

The data obtained were analyzed with XLSTAT (version 2010.5.05 add-in with Windows Excel) by analysis of variance to determine significance ($P<0.05$) among the treatments. Duncan's multiple range test was performed between individual means to reveal the significant difference. The data collected from the field experiments were assessed for principal component analysis (PCA) using XLSTAT to reveal the similarities and differences between treatments (endophytic isolates) and to evaluate the relationship between endophyte strains and assessed growth and yield attributes of blackgram and greengram (Wold *et al*. 1987). The plant-growth-promoting traits of the endophytic isolates were used to generate a double dendrogram and heatmap using comparative functions and multivariate hierarchical clustering methods in NCSS 2020 (NCSS, Kaysville, Utah). The quantitative relative abundance of each plant-growth-promoting trait of the endophytic strains was included in the double dendrogram with clustering based on Ward's minimum variance and utilizing Manhattan distance calculation with no scaling. The treatment samples (endophytic isolates) with more similarity (less distance) based on the plant-growth-promoting traits are more closely related and clustered. Similarly, those attributes that have similar proportions across all strains are clustered to each other.

RESULTS AND DISCUSSION

Endophytic bacterial diversity in pulses

We have isolated several endophytic bacteria from pulse crops more specifically from blackgram (*V. mungo*) and greengram (*V. radiata*). After careful screening, 15 promising endophytic isolates were selected for this study. Out of 15, eight bacterial endophytes were isolated from *V. mungo* and they were designated as BG-E1 to BG-E8. Seven *V. radiata* isolates were selected and named as GG - E1 to GG-E7. Based on the nucleotide sequence of the 16S rRNA gene, all the fifteen isolates were determined and aligned with reference strains in Gene Bank (Table 1). All the isolates showed high similarities (≥ 98%) with their closest related species. The phylogenetic tree showing the relationships between the isolates and related reference species is depicted in Fig 1. The phylogenetic tree could discriminate against the endophytic bacterial isolates of pulses and be arranged into four different clusters: F*irmicutes*, α-*Proteobacteria*, β-*Proteobacteria* and γ-*Proteobacteria*. The cluster *Firmicutes*, which encompasses gram-positive

Table 1: Phylogenetic affiliation of different endophytic bacteria isolated from different pulses.

Isolate	Host crop	16S rRNA gene homology	Similarity (%)
BG-E1	Blackgram	*Enterobacter cloacae*	99
BG-E2	(*Vigna mungo*)	*Bacillus thuringiensis*	99
BG-E3		*Bacillus licheniformis*	98
BG-E4		*Pantoea agglomerans*	99
BG-E5		*Pseudomonas fluorescens*	99
BG-E6		*Bacillus pumilus*	99
BG-E7		*Enterobacter cloacae*	100
BG-E8		*Achromobacter xylosoxidans*	98
GG-E1	Greengram	*Enterobacter gergoviae*	99
GG-E2	(*Vigna radiata*)	*Pseudomonas chlororaphis*	99
GG-E3		*Achromobacter piechaudii*	99
GG-E4		*Ochrobacterium sp.*	98
GG-E5		*Bacillus pumilus*	99
GG-E6		*Bacillus subtilis*	99
GG-E7		*Bacillus thuringiensis*	99

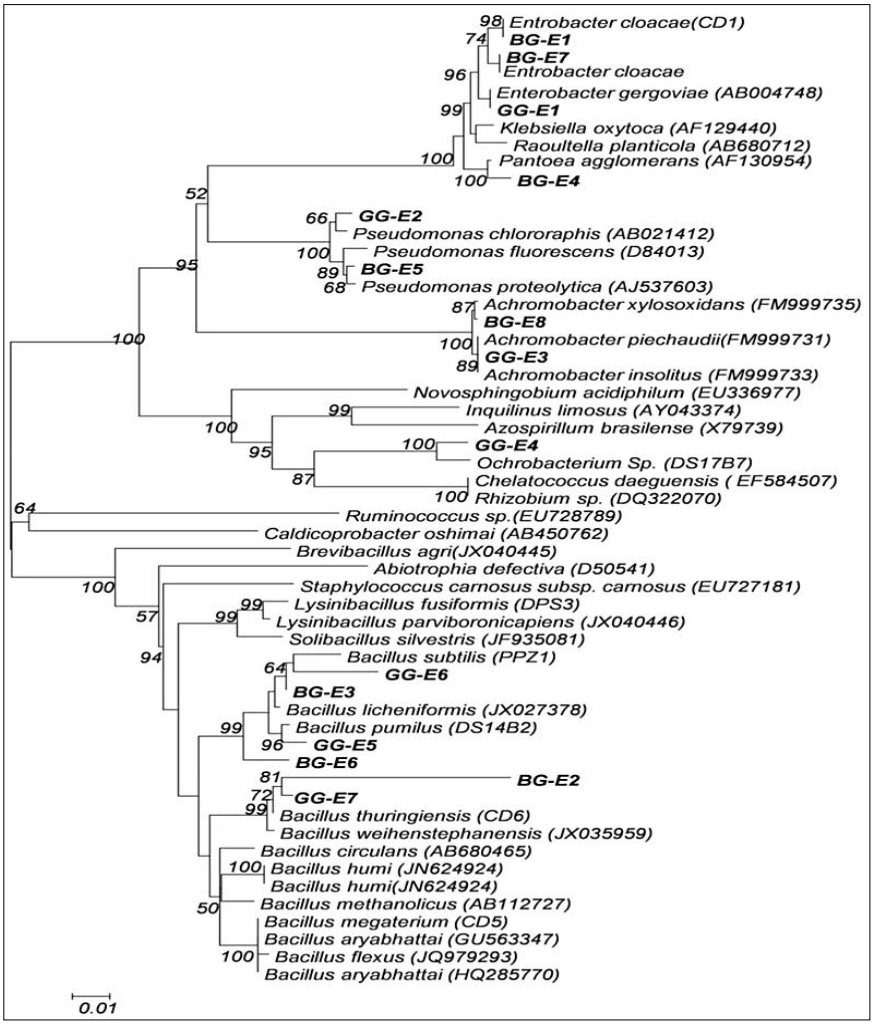


Fig 1: Phylogenetic tree based on the 16S rRNA gene sequences of endophytic bacterial isolates from blackgram (*V. mungo* - BG-E) and greengram (*V. radiata* - GG-E) with other related species using the Neighbor-joining method.

bacteria with low G+C content, was the most predominant group among the isolates, which consisted of the genus *Bacillus*. Next to *Firmicutes*, more number of isolates were found in the *γ-Proteobacteria* cluster. It contains three members from *Enterobacter*, two members from *Pseudomonas* and one isolate from *Pantoea*. Two members in the cluster *β-Proteobacteria* belonged to the genus *Achromobacter* and one isolate, *Ochrobacterium* from *α-Proteobacteria* was also found.

The results showed the predominant existence and wide distribution of *Bacillus, Pseudomonas* and *Enterobacter* in the pulses. Bacteria belonging to the genera *Bacillus* and *Pseudomonas* are easy to culture and cultivation-dependent studies have identified them as frequently occurring endophytes (Seghers *et al*., 2004). Several reports concerning the presence of bacteria belonging to *Bacillus* and *Pseudomonas* genera inside the various parts of plants as endophytic bacteria exist already (Vendan *et al*., 2010; Etminani and Harighi, 2018). The previous studies include *Bacillus*, *Pseudomonas* and *Enterobacter* isolated from many different plant species, suggesting that these bacteria have developed an evolutionary niche within plants.

Plant growth-promoting traits of endophytes

The ability to fix N_2, in other words, the presence of nitrogenase enzyme, is only limited to certain bacteria and archaea. The endophytic bacteria assimilate atmospheric nitrogen and convert it into ammonia, transferring this molecule to the plant metabolism (Gaiero *et al*., 2013). The N_2-fixing ability of bacterial endophytes was screened in this study by acetylene reduction assay (Table 2). All the fifteen isolates showed nitrogen-fixing activity, however, the isolate BG-E6 (*Bacillus pumilus*) recorded higher acetylene reduction activity of 29.4 nmoles C_2H_4 mg protein^{-1} hr^{-1} followed by GG-E2 (*Pseudomonas chlororaphis*) with 27.2 nmoles C_2H_4 mg protein^{-1} hr^{-1}. Our results were in line with the earlier study, in which Yan *et al*. (2018) reported the nitrogenase activities of five endophytic nitrogen-fixing isolates determined by acetylene reduction assay, ranging from 28.5 to 38.0 nmol C_2H_4 mg protein^{-1} hr^{-1}.

Phytohormones are versatile low molecular weight natural signaling molecules that act even at micromolar concentration and regulate all physiological and developmental processes of plants. The best-known phytohormones that are produced by endophytic microbiota are IAA, which is synthesized via the indole-3-pyruvate (IPyA) pathway (Singh *et al*., 2017). The capacity to synthesize IAA is widespread among soil and plant-associated bacteria. In this study, the endophytic bacterial isolates were found to produce IAA in the amount ranging from 0.74 to 3.12 µg/mL (Table 2). Maximum IAA production was detected in isolate BG-E6 and minimum by the isolate GG- E5. Previous reports indicated that many endophytic bacteria including, *Pseudomonas*, *Serratia* and *Bacillus* can synthesize IAA (Bhutani *et al*., 2018: Liu *et al*., 2010). Similarly, Pandya *et al*. (2015) reported the highest IAA production of 10.80 µg/mL by endophytic bacteria isolated from *V. radiata*.

The solubilization of insoluble P and making it available to plants are yet other important traits of endophytes (Oteino *et al*., 2015). In our study, we examined all the selected endophytic bacterial isolates for their phosphate solubilizing ability by detecting extracellular solubilization of precipitated tricalcium phosphate. Out of 15 isolates, only 11 endophytic isolates showed notable phosphate solubilization activity (Table 2). Based on the solubilization zone, the isolate BG-E6 (*Bacillus pumilus*) recorded higher solubilization of mineral phosphate (0.61 mm) followed by GG-E2 (*Pseudomonas chlororaphis*) with a solubilization zone of 0.58 mm. The endophytes release organic acids like 2-ketogluconic acid, gluconic acid (Oteino *et al*., 2015) and

Table 2: Nitrogen fixation, IAA production, phosphate solubilization and siderophore production of endophytic bacterial isolates.

Endophytic bacteria	Acetylene reduction activity (nmol C_2H_4 mg protein^{-1} hr^{-1})	IAA production (µg/mL)	Phosphate solubilization (Diameter of the clearing zone in mm)	Siderophore production
BG-E1	16.2	0.86	0.16	-
BG-E2	18.4	1.43	0.00	-
BG-E3	21.2	2.38	0.36	+
BG-E4	24.4	1.38	0.18	-
BG-E5	25.8	2.52	0.48	+
BG-E6	29.4	3.12	0.61	+
BG-E7	18.8	1.44	0.18	-
BG-E8	0.0	0.72	0.00	-
GG-E1	22.0	0.94	0.00	+
GG-E2	27.2	2.88	0.58	+
GG-E3	15.6	1.36	0.14	+
GG-E4	18.4	1.26	0.21	+
GG-E5	0.0	0.74	0.00	-
GG-E6	21.4	1.12	0.22	+
GG-E7	22.4	2.18	0.38	+

(+) presence; (-) absence.

others that lower the pH and ultimately solubilize the insoluble phosphate. Several researchers reported that endophytic bacterial species *viz.*, *Bacillus* and *Pseudomonas* have shown the potential to solubilize the insoluble phosphate (Grover *et al*., 2011; Naveed *et al*., 2014).

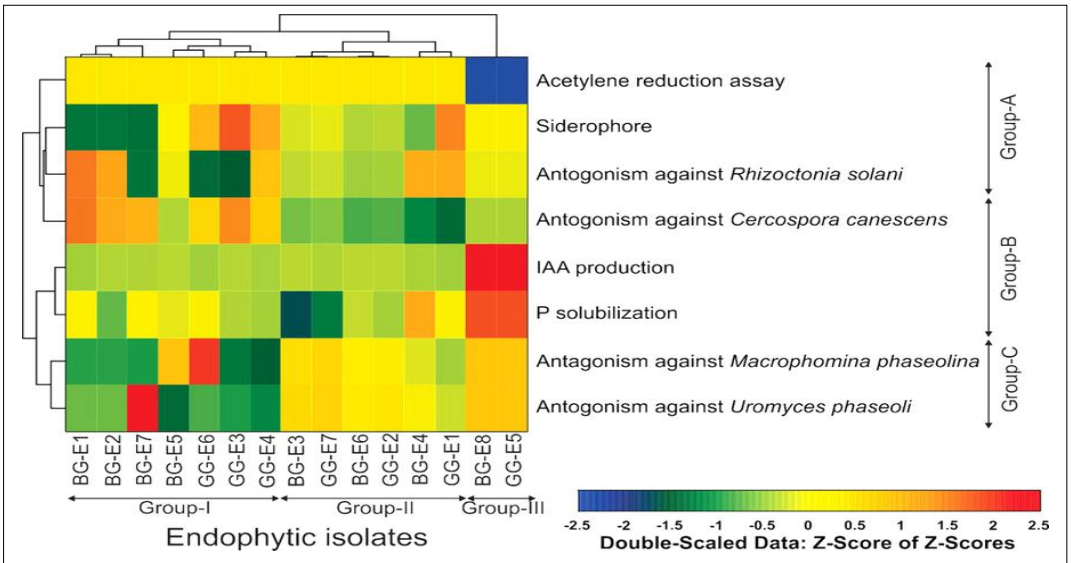
Bacterial endophytes are also known to liberate iron-chelating molecules (siderophores), which increase the accessibility of iron to the plants in iron-limiting conditions (Szilagyi-Zecchin *et al*., 2014). In our study, out of 15 isolates, only 9 isolates produced siderophore, as evidenced by the change of color in the CAS blue medium from bluish-green to orange (Table 2). This is in line with the work of Liaqat and Eltem (2016) who reported the production of siderophore by only 2 out of 7 endophytic bacteria isolated from the peach rootstock.

Biocontrol is a mechanism, wherein microorganisms are used to promote the growth of plants indirectly by inhibiting the growth of pathogens. In the present study, only 4 endophytic isolates (BG-E3, BG-E6, GG-E2 and GG-E7) showed antifungal activity against all the four test organisms *viz.*, *Rhizoctonia solani, Cercospora canescens, Macrophomina phaseolina* and *Uromyces phaseoli* (Table 3). Plant growth-promoting endophytic bacteria can antagonize soil-borne pathogens through various mechanisms such as competition, antibiosis and/or parasitism (Le Cocq *et al*., 2016). The ability of endophytic bacteria colonizing internal plant tissues to protect host plants from soil-borne pathogens was well-reviewed by Eljounaidi *et al*. (2016).

The hierarchical clustering analysis of PGP traits of endophytic isolates revealed three major clusters. Cluster I consists of seven endophytic isolates *viz.*, BG-E1, BG-E2, BG-E7, BG-E5, GG-E6, GG-E3, GG-E4 with a moderate level of PGP traits; cluster II with BG-E3, BG-E7, BG-E6, BG-E2, BG-E4, GG-E1 with high PGP traits; cluster III with BG-E8 and GG-E5 with least PGP activities (Fig 2). The trait-based clustering also had three groups *viz.*, group-A with ARA, siderophore and *Rhizoctonia* antagonism; group-B with IAA, P solubilization and *Cercospora* antagonism; group-C with antagonism against *Macrophomina* and *Uromyces*. Based on the double clustering method, the best endophytic strains *viz.*, BG-E3, BG-E5, BG-E6, GG-E2, GG-E7 were selected for pot culture experiments.

Endophytic bacterial inoculation on growth of blackgram and greengram

The results of the pot culture experiment revealed that the inoculation of endophytic bacteria increased the growth and yield parameters of blackgram when compared to uninoculated control (Table 4). Among the five selected cultures, BG-E6 (*Bacillus pumilus)* registered higher yield attributes *viz.*, root length, shoot length, nodule number, nodule dry weight, plant fresh weight, plant dry weight and yield than other treatments. The above treatment recorded a higher grain yield of 3.04 g/plant, which was a 14.7 per cent increased yield over uninoculated control. Similarly, in greengram, the isolate GG-E2 (*Pseudomonas chlororaphis*) recorded higher yield attributes and yield than other treatments. It registered a higher grain yield of 2.32 g/plant, which was a 19.5 per cent increased yield over control. The endophytes can promote legume growth and yield due to their specific beneficial traits (Naveed *et al*., 2017). When applied as a microbial inoculant, the endophyte has been found to promote plant growth *via* different mechanisms such as hormone production, P solubilization, siderophores and



The heat map indicates the relative ratio of each trait after Z-transformation. The clustering of both PGP traits and endophytic strains is based on Ward minimum variance and Manhattan distance methods. BG - Blackgram endophytic isolates; GG - Greengram endophytic isolates.

Fig 2: Double dendrogram and heat map relating the endophytic isolates and their plant growth-promoting traits based on the Ward minimum variance clustering method.

Table 3: Screening of endophytic bacterial cultures for antimicrobial activity against certain fungal pathogens.

Endophytic bacteria	*Rhizoctonia solani*	*Cercospora canescens*	*Macrophomina phaseolina*	*Uromyces phaseoli*
BG-E1	+	+	-	-
BG-E2	+	+	-	-
BG-E3	+	+	+	+
BG-E4	+	-	-	-
BG-E5	+	+	+	-
BG-E6	+	+	+	+
BG-E7	-	+	-	+
BG-E8	-	-	-	-
GG-E1	+	-	-	-
GG-E2	+	+	+	+
GG-E3	-	+	-	-
GG-E4	+	+	-	-
GG-E5	-	-	-	-
GG-E6	-	+	+	-
GG-E7	+	+	+	+

(+) The cultures that show antifungal activity. (-) The cultures that do not show antifungal activity.

Table 4: Impact of plant-growth-promoting endophytic strains on nodulation and yield of the blackgram and greengram.

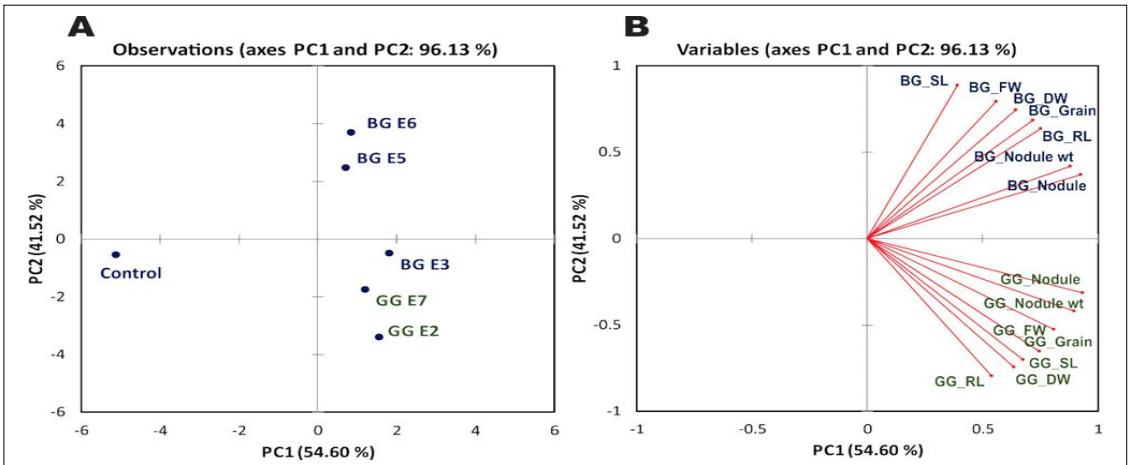
Endophytic strains	Blackgram			Greengram		
	Nodule number	Nodules dry weight (gm)	Grain yield (g/plant)	Nodule number	Nodules dry weight (gm)	Grain yield (g/plant)
GG-E2	11.34	12.15	2.81	12.10	13.62	2.32
GG-E7	11.66	12.35	2.87	11.67	12.38	2.26
BG-E5	12.35	13.50	3.00	10.80	11.32	2.08
BG-E6	12.67	13.93	3.04	9.53	10.55	2.05
BG-E3	11.96	14.15	2.88	11.57	12.29	2.18
Control	7.96	9.15	2.65	6.53	7.84	1.94
SEm±	0.21	0.45	0.07	0.21	0.42	0.07
CD (P=0.05)	0.64	1.37	0.23	0.64	1.29	0.22

production of organic acids (Khalifa *et al*., 2016). In this present study also, the above isolates showed their potential in all the plant growth-promoting traits studied, which eventually resulted in the higher growth and yield of pulses.

The observation plot showing the positions of PGPR strains as treatments and loading plot presenting the growth and yield variables of blackgram and greengram explained by the first two components (PC1 and PC2) are presented as Fig 3. The PC1 showed 54.6% variability and PC2 adds 41.5% variability to the total cumulative variability (96.13%). All the five endophytic strains had differences in their performance and were positioned in a different quadrant of the plot. The BG-E5 and BG-E6 were positioned in both PCs positive quadrant (top left-hand quadrant); while, BG-E2, GG-E7 and GG-E2 positioned in PC1 positive and PC2 negative quadrant (bottom left-hand quadrant). The control was positioned in the PC1 and PC2 negative quadrant (Fig 3A). In the loading plot, all the variables of blackgram positioned the quadrant where orthogonally similar to scoring plot PGPR strains (BG-E6 and BG-E5). Likewise, all traits of greengram were orthogonally positioned with BG-E6, GG-E7 and GG-E2. The nodule number (11.4%), nodule weight (10.6%), grain yield (8.5%) of blackgram and greengram contribute to PC1 and root length, shoot length and biomass contribute equal and significant contribution (10-13%) to PC2.

Host specificity of endophytic bacteria

The experimental results revealed that the inoculation of endophytic bacteria isolated from blackgram (BG-E5, BG-E6 and BG-E3) recorded higher growth and yield parameters in the same host (blackgram), with poor response in greengram. Similarly, the endophytes isolated from greengram (GG-E2 and GG-E7) have less bio-efficacy influence with blackgram. Hence, it clearly showed that there was a host specificity found among the endophytic bacterial cultures like rhizobia. Plant genotype is an important determinant in the development of positive plant-endophyte association (Afzal *et al*. 2019). The central role of phytohormone signaling in plant-endophyte interactions suggests that once recruited by a particular host, endophytes undergo host-specific adaptations; the upshot is a highly specialized, finely tuned mutualism. Such mutualisms may make plants better able to tolerate the endophyte and the endophyte in turn more responsive to the plant's metabolism



(A) Scoring plot showing the positions of PGPR treatments; (B) Loading plot showing orthogonal positions of growth and yield attributes of blackgram and greengram. The % variance explained by each component (PC1 and PC2) is given in parentheses in axes. In both the plots, GG- Greengram; BG- Blackgram; RL- Root length; SL- Shoot length; FW - fresh weight of the plant; DW - Dry weight of the plant; Grain- Grain yield. BG-E6, BG-E5, BG-E3, GG-E7, GG-E2 - Endophytic PGPR strains; Control- Uninoculated control.

Fig 3: Principal component analysis plots relating the endophytic bacterial isolates and their impact on growth and yield of blackgram and greengram.

(Schulz and Boyle, 2005). The plant growth-promoting ability of endophytic bacteria can be influenced by the genotype of the plant host. Kim *et al*. (2012) reported that the growth promotion of switchgrass by *Burkholderia phytofirmans* PsJN is plant genotype-dependent. The findings of Long *et al*. (2008) were in agreement with this study, wherein they observed that plant growth-promoting endophytic bacteria of *Solanum nigrum* were highly host-specific, where these bacteria were unable to produce growth enhancement in *Nicotiana attenuata*, a non-host plant.

Similarly, Dastogeer *et al*. (2018) observed an obvious clustering of endophytic communities associated with different *Nicotiana* species and they implied that endophyte community structure can be highly influenced by host genotypes. They revealed that plant growth-promoting effects of natural endophytic bacteria on their host and non-host plant species are not the same. In our study also, the endophytes showed more pronounced plant growth-promoting effects with the host crop than in non-host crops. Besides, endophytes may have evolved from parasites and may still have parasitic tendencies (Kogel *et al*., 2006) potentially contributing to incompatible interactions with non-hosts. Due to these incompatible interactions, the endophytes may elicit inappropriate responses in a non-host plant which ultimately resulted in lower growth and yield. These findings demonstrate that endophytic bacteria were host specific and if they inoculated with non-host plants, despite their plant growth-promoting properties, they displayed inappropriate response and poor yield.

CONCLUSION

From the above investigation, we conclude that the plant-growth-promoting endophytes of blackgram and greengram are genetically and functionally diversified. From the *in vitro* screening experiments, five potential strains (GG-E2, GG-E7, BG-E5, BG-E6, BG-E3) with nitrogen-fixing, P solubilizing, growth hormone and siderophore producing strains, antagonist against plant pathogenic fungi were identified. Further, we also confirmed that these strains could increase the growth and yield of blackgram and greengram, significantly. The results also confirmed that these potential endophytes have host specificity for effective colonization and plant growth promotion.

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Conflict of interest

All authors declared that there is no conflict of interest.

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