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# Diversity of Endophytic Bacteria in Ginseng and Their Potential for Plant Growth Promotion

**Regupathy Thamizh Vendan[1], Young Joon Yu[2], Sun Hee Lee[2], and Young Ha Rhee[2]***

*[1]Agricultural College & Research Institute, Tamil Nadu Agricultural University, Tiruchirappalli-620 009 Tamil Nadu, India*
*[2]Department of Microbiology, Chungnam National University, Daejeon 305-764, Republic of Korea*

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**Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and can form a range of different beneficial relationships. The diversity of bacterial endophytes associated with ginseng plants of varying age levels in Korea was investigated. Fifty-one colonies were isolated from the interior of ginseng stems. Although a mixed composition of endophyte communities was recovered from ginseng based on the results of 16S rDNA analysis, bacteria of the genus *Bacillus* and *Staphylococcus* dominated in 1-year-old and 4-year-old plants, respectively. Phylogenetic analysis revealed four clusters: *Firmicutes*, *Actinobacteria*, *α-Proteobacteria*, and *γ-Proteobacteria*, with *Firmicutes* being predominant. To evaluate the plant growth promoting activities, 18 representative isolates were selected. Amplification of *nifH* gene confirmed the presence of diazotrophy in only two isolates. Half of the isolates solubilized mineral phosphate. Except four, all the other endophytic isolates produced significant amounts of indole acetic acid in nutrient broth. Iron sequestering siderophore production was detected in seven isolates. Isolates E-I-3 (*Bacillus megaterium*), E-I-4 (*Micrococcus luteus*), E-I-8 (*B. cereus*), and E-I-20 (*Lysinibacillus fusiformis*) were positive for most of the plant growth promoting traits, indicating their role in growth promotion of ginseng.**

***Keywords***: endophytic bacteria, ginseng, diversity, plant growth promoting traits

Endophytic bacteria live in plant tissues without causing substantive harm to the host or gaining any benefit other than a non-competitive environment inside the host (Kobayashi and Palumbo, 2000). They ubiquitously inhabit most plant species and have been isolated from several types of tissues, seeds, roots, stems and leaves (Lodewyck *et al*., 2002). Significant variations in the populations of both indigenous and introduced endophytes have been reported. An assessment of the literature on endophytic bacteria shows a predominance of papers with the focus of interest mainly on their roles within the plant in relation to plant nutrition (Chi *et al*., 2005), pollutant catabolism (Siciliano *et al*., 2001), stress or defense responses (Cho *et al*., 2002), and invading pathogens (Ramesh *et al*., 2009). It has been reported that endophytic bacteria may promote plant growth and suppress plant diseases, probably by means similar to plant growth promoting rhizobacteria (Feng *et al*., 2006). Furthermore, plant growth promotion is often greater when it is induced by endophytes rather than by bacteria restricted to the rhizosphere and the root surface (Chanway *et al*., 2000). The potential of endophytic bacteria to fix nitrogen and promote plant growth has renewed interest in such associations. In view of the widespread application of endophytic bacteria in plants, human health and the environment, concerted efforts at identification of endophytic diversity coupled with exploitation of this diversity are necessary. To date, estimates of the diversity of endophytic bacterial species have been largely based on culture techniques. Culture-independent analysis of bacterial populations inside of citrus plants also suggests that bacterial endophytic populations are much more diverse than previously realized (Araújo *et al*., 2002).

Ginseng (*Panax ginseng* C.A. Meyer) has been regarded as one of the most important remedies in oriental medicine for more than 1,000 years (Yu *et al*., 2003). Ginseng is presently used as a health tonic and in adaptogenic, anti-aging, prophylactic and restorative remedies. In general, growth of high quality ginseng requires at least 4 years of cultivation in the shade. Although ginseng would be expected to harbor several endophytic populations capable of accelerating the growth of the plant, very few reports on the occurrence of endophytes in ginseng and their role in plant growth promotion and other functions are available (Cho *et al*., 2007; Qiu *et al*., 2007). Assessment of efficient beneficial endophytic microorganisms will be vital for improving the productivity of ginseng.

The aim of the present study was to examine the diversity of endophytic bacteria in ginseng plants of varying age levels. The plant growth promoting ability *viz*., nitrogen fixation, indole acetic acid (IAA) production, mineral phosphate solubilization and siderophore (iron sequestering compounds) production of some of these selected endophytes was assessed.

## Materials and Methods

### Plant material and surface sterilization of stems

The diversity of endophytic bacteria was estimated in stems of ginseng

* For correspondence. E-mail: yhrhee@cnu.ac.kr; Tel: +82-42-821-6413; Fax: +82-42-822-7367

plants. Healthy (uninfected) stems were collected from variously aged ginseng plants maintained at Okcheon, Korea. Since, 4-year-old ginseng plants are economically important, we analyzed the endophytic diversity from 1~4-year-old plants. Stems were washed in running tap water and graded by surface appearance to exclude samples that showed superficial damage. Surface disinfection was done by stepwise washing in 70% ethanol for 5 min, sodium hypochlorite solution (2% available Cl) for 5 min and 70% ethanol for 30 sec, followed by two rinses in sterile distilled water. To confirm that the disinfection process was successful, the stems were pressed onto tryptic soy (TS) agar and the plates were examined for growth after incubation at 28°C for 3 days.

**Isolation of endophytic bacteria**

The bark of surface-disinfected stems was removed with a sterilized razor blade and the stems were cut into 4-6 mm long pieces, which were placed on TS agar 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 a further experiment, ginseng stem fragments were homogenized in 5 ml of sterile phosphate-buffered 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. Following incubation, bacteria recovered from each stem fragment and/or homogenized sample were selected at random and purified. They were grouped on the basis of phenotypic characteristics, e.g., colony morphology, colony color, cell shape, motility and Gram reaction by using VITEK® 2 Compact (bioMérieux, France). Fifty-one isolates were selected for further identification and they were designated as E I-E IV according to the age of the plant source.

**DNA extraction and 16S rRNA gene analysis**

The isolated endophytic bacteria were cultured in TS broth at 28°C for 24 h and centrifuged at 14,000×g for 5 min at 4°C. The genomic DNA was extracted from the pellet by using the G-spin™ genomic DNA extraction kit (iNtRON Biotechnology, Korea). To amplify the 16S rRNA genes, the polymerase chain reaction (PCR) was performed with the universal primers 27F and 1492R (Bai *et al*., 2002). The PCR consisted of a 95°C hold for 5 min, followed by 35 cycles of 45 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C, and a final extension for 15 min at 72°C. The amplification was performed using a PE2400 thermal cycler (Perkin Elmer, USA). The PCR fragment was purified by using a QIAquick PCR Purification kit (QIAGEN, USA). Sequencing was performed on an ABI Prism 3730 DNA analyzer (Applied Biosystems, USA) by using an ABI BigDye cycle sequencing kit (Applied Biosystems) with EF-Taq DNA polymerase (Solgent, Korea). All reference sequences were obtained from the National Centre for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases. The 16S rRNA similarity sequences searches were performed using the BLASTN tool in the NCBI website (McGinnis and Madden, 2004). Sequences were aligned by using the multiple sequence alignment program, CLUSTAL W (Tompson *et al*., 1994). Phylogenetic analysis was performed using MEGA 4.0 software (Tamura *et al*., 2007). Neighbor-joining method was employed to infer the tree topology. The reliability of the trees was tested by bootstrapping 1,000 replicates generated with a random seed. The matrix distances were calculated based on p-distance.

**Plant growth promoting activities of endophytic bacteria**

Diazotrophy of bacterial endophytes was screened by PCR analysis of partial *nifH* gene sequences. The sequences of the amplification primers were *nifH*-b1 (5′-GGC TGC GAT CCC AAG GCT GA-3′) (Burgmann *et al*., 2004) and CDHP*nif*723R (5′-GAT GTT CGC GCG GCA CGA ADT RNA TSA-3′) (Steward *et al*., 2004). Amplification was carried out in a 30 μl final volume containing 10 ng of genomic DNA from each isolate with each primer at a concentration of 0.5 μM, each deoxynucleoside triphosphate at a concentration of 250 μM, 1.0% bovine serum albumin fraction V (Fisher Scientific, USA), 0.05% Tween 20, 2.5 mM $MgCl_2$, 5 U of Super-Therm DNA polymerase (JMR, UK), and 1× PCR buffer (supplied with *Taq* enzyme). Positive (*Rhizobium leguminosarum*) and negative (DW) controls were also maintained. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and documented.

IAA production was estimated by growing the isolates in nutrient broth supplemented with 200 μg/ml of tryptophan at 30°C with shaking for 48 h in dark. Twenty-five mililiters of the sample was withdrawn and the cells were spun down at 5,000 rpm for 15 min. The filtrate was adjusted to pH 3.0 using 2 N HCl and evaporated in a vacuum evaporator. The residue was dissolved in 2 ml methanol and added to 1.5 ml of distilled water in a test tube. Four ml of fresh Salper reagent was rapidly added and kept in complete darkness for 1 h prior to reading of the absorbance at 535 nm (Hartmann *et al*., 1983).

The capacity of the endophytes for the production of siderophore was assessed by the Chrome azurol S (CAS) method (Schwyn and Neilands, 1987). Siderophore production of the ginseng endophytic isolates was observed through the color change in the CAS blue medium. Mineral phosphate solubilization activity was qualitatively assayed on dicalcium phosphate plates (Goldstein, 1986) by the observation of a distinct zone of clarification around the colonies after 48 h of incubation at 30°C.

**Nucleotide sequence accession numbers**

The nucleotide sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers from FJ613531 to FJ613581.

## Results and Discussion

### Isolation of culturable endophytic bacteria from ginseng stems

We have isolated several endophytic bacteria from differently aged ginseng plants. The nucleotide sequences of 16S rRNA genes of a total of 51 isolates were determined and aligned with those of reference strains in GenBank. As shown in Table 1, all isolates showed high similarities (≥98%) with their closest related species. At least sixteen Gram-positive bacterial species in six genera and three Gram-negative bacterial species in three genera were found, indicating the complexity of endophytic population present in the stem portion of ginseng plants. Particularly, the results revealed that the E-I isolates were dominated by the genus *Bacillus*. E-II isolates belonged to *Bacillus*, *Pseudomonas*, *Agrobacterium*, and *Stenotrophomonas*, whereas E-III isolates belonged to *Bacillus*, *Microbacterium*, *Agrobacterium*, and *Paenibacillus*. The mature, 4-year-old plants (E-IV) predominantly harboured the bacterial genus, *Staphylococcus.*

**Table 1.** Similarity values of 16S rDNA sequences from the endophytic bacteria of the ginseng stems

| Isolate[a] | Nearest relative with accession number[b] | Similarity[c] |
|---|---|---|
| E-I-1 | *Bacillus pseudomycoides* (AM747227) | 99 |
| E-I- 3, 5, 9, 16 | *Bacillus megaterium* strain EJH-7 (DQ093582) | 99 |
| E-I-4 | *Micrococcus luteus* strain 164 (EU730941) | 99 |
| E-I-6 | *Bacillus megaterium* (AB244481) | 99 |
| E-I-8 | *Bacillus cereus* strain DS16 (EU834245) | 99 |
| E-I-10, 17 | *Bacillus thuringiensis* serovar kurstaki (EU153549) | 98 |
| E-I-11 | *Bacillus subtilis* strain SC2-4-1 (EF488171) | 99 |
| E-I-12 | *Bacillus thuringiensis* isolate LDC-391 (EU625359) | 99 |
| E-I-13 | *Bacillus subtilis* strain QD434 (EF472262) | 99 |
| E-I-14 | *Bacillus pumilus* strain CT13 (EU660365) | 99 |
| E-I-15 | *Lysinibacillus sphaericus* C3-41(CP000817) | 99 |
| E-I-18 | *Bacillus thuringiensis* (AM747225) | 99 |
| E-I-19 | *Bacillus acidiceler* strain CBD 119(DQ374637) | 99 |
| E-I-20 | *Lysinibacillus fusiformis* strain X-9 (EU187493) | 99 |
| E-II-1 | *Bacillus pumilus* strain HN005 (EU596537) | 99 |
| E-II-2 | *Pseudomonas marginalis* strain ATCC 10844T(AB021401) | 100 |
| E-II-3 | *Stenotrophomonas maltophilia* strain LMG 20578(AY040357) | 100 |
| E-II-4 | *Bacillus flexus* strain L2S2 (EU221413) | 99 |
| E-II-5 | *Bacillus amyloliquefaciens* (AB301002) | 99 |
| E-II-7 | *Agrobacterium tumefaciens* strain C58 (AE007870) | 99 |
| E-III-1 | *Bacillus thuringiensis* isolate LDC-415 (EU625360) | 99 |
| E-III-2 | *Microbacterium phyllosphaerae* (AJ277840) | 99 |
| E-III-3 | *Agrobacterium tumefaciens* strain ISSDS-106(EF620475) | 99 |
| E-III-4 | *Paenibacillus glucanolyticus* (AB073189) | 99 |
| E-IV-1 | *Staphylococcus epidermidis* strain DS14(EU834244) | 99 |
| E-IV-2, 21 | *Staphylococcus pasteuri* isolate CV5(AJ717376) | 99 |
| E-IV-3, 4, 6, 8, 10, 11, 14, 17, 20 | *Staphylococcus epidermidis* strain RW35(EU419922) | 99 |
| E-IV-5, 7, 9, 13, 19 | *Staphylococcus epidermidis* strain S09 (AY741152) | 99 |
| E-IV-12 | *Staphylococcus epidermidis* strain KL-096 (AY030342) | 99 |
| E-IV-15 | *Staphylococcus epidermidis* strain TMPSB-D10(EU513396) | 99 |
| E-IV-16 | *Stenotrophomonas maltophilia* isolate HK40(AJ011332) | 100 |
| E-IV-18 | *Staphylococcus sp*. RP22 (EU375136) | 99 |
| E-IV-22 | *Bacillus subtilis* strain GH38(AB301009) | 99 |
| E-IV-23 | *Bacillus subtilis* (EU256502) | 99 |

[a] E-I to E-IV denotes the age of the plant (1 to 4 years) followed by the number indicating the representative isolate; [b] Closest relative species and its accession number in the 16S rDNA sequence database; [c] Per cent similarity of the sequence in BLAST result

## Phylogenetic analyses of the isolates

The phylogenetic tree showing the relationships between the isolates and related reference species is depicted in Fig. 1. The phylogenetic tree could discriminate the endophytic bacterial isolates of ginseng and was arranged into four different clusters: *Firmicutes*, *Actinobacteria*, *α-Proteobacteria*, and *γ-Proteobacteria*. The cluster *Firmicutes*, encompasses gram-positive bacteria with low G+C content, was the most dominant group among the isolates (44 of 51 isolates) which included four different genera, with *Bacillus* and *Staphylococcus* being predominant. Two isolates in the cluster *Actinobacteria* (a group of Gram-positive bacteria with high G+C content) were related to *Micrococcus luteus* and *Microbacterium phyllosphaerae*. All the two isolates in the cluster *α-Proteobacteria* belonged to *Agrobacterium tumefacience*. Three members in the cluster *γ-Proteobacteria* belonged to the genera *Pseudomonas* and *Stenotrophomonas*. Recently, Cho *et al*. (2007) isolated 13 different genera in 63 endophytic isolates from the interior of ginseng roots cultivated in three different areas and demonstrated marked regional differences in microbial community: the respective dominant species were high G+C Gram-positive bacteria, low G+C Gram-positive bacteria and *Proteobacteria*.

Our results show the predominant existence and wide distribution of the genus *Bacillus* in all the age groups. 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). Surette *et al*. (2003) have reported the isolation of up to 360 endophytic microorganism strains from *Daucus*


**Fig. 1.** Phylogenetic tree based on 16S rDNA sequences of the endophytic bacteria of the ginseng stems and other related genera using neighbor-joining method. Bootstrap values of 500 or more (from 1,000 replicates) are indicated at the node. Scale bar, 0.05 substitutions per base position.

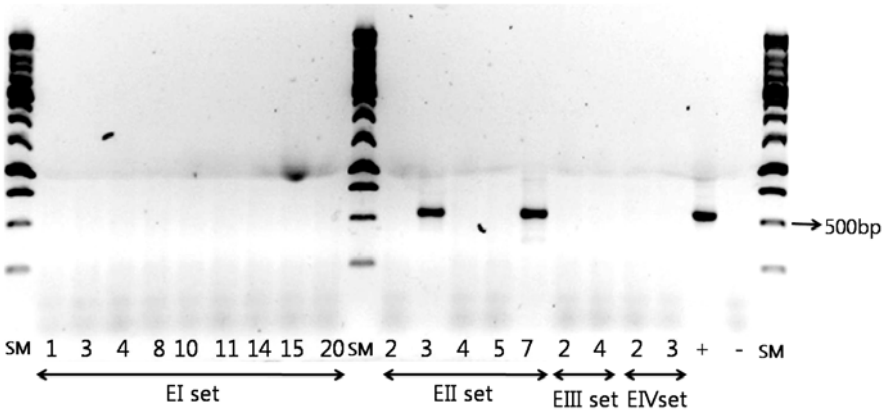


**Fig. 2.** Screening of endophytic bacteria for diazotrophy using partial amplification of *nifH* gene. Lanes: SM, 1 kb DNA size marker; 2-10, Endophytic bacterial isolates of E-I; 12-16, Isolates of E-II; 17 & 18, Isolates of E-III; 19 & 20, Isolates of E-IV; 21, (+) Positive control (*Rhizobium leguminosarum*); 22, (-) Negative control (DW)

*carota*, which were classified into 28 genera, with *Pseudomonas*, *Staphylococcus*, and *Agrobacterium* being predominant. Some *Bacillus* and *Staphylococcus* strains have also been reported as major endophytic bacteria in food crops like citrus (Araújo *et al*., 2002) and sugarcane (Velazquez *et al*., 2008). The variations in the endophytic bacterial communities were attributed to plant age, plant source, tissue type, time of sampling, and environment condition (Kobayashi and Palumbo, 2000). However, our present study clearly showed that the age of the plant could largely influence the variation in the endophytic community of ginseng plants. Also, variation in endophytic diversity might be a function of the different maturation stages specific to each plant, which might influence the different types and amounts of root exudates (Ferreira *et al*., 2008) that should be addressed in further studies.

## Assessment of plant growth promoting traits of endophytic bacteria

Although the analysis of bacterial diversity by using culture techniques has its own limitation, its main advantage is that a number of bacterial strains can be isolated and characterized for further study (Park *et al*., 2005a). In this study, eighteen different isolates were selected based on their richness in the endophytic population of four different aged ginseng plants and screened for plant growth promoting activities as listed in Table 2. The $N_2$-fixing ability of bacterial endophytes of ginseng was screened by partial amplification of the *nifH* gene. Out of 18 isolates, only two isolates – E-II-3 (*Stenotrophomonas maltophilia*) and E-II-7 (*Agrobacterium tumefaciens*) – showed amplification of 590 bp of the *nifH* gene (Fig. 2). Much evidence exists for significant $N_2$ fixation by endophytic diazotrophs such as *Gluconacetobacter*, *Azoarcus*, and *Herbaspirillum* (Reinhold-Hurek and Hurek, 1998). *A. tumefaciens* is capable of fixing nitrogen in a free-living condition (Kanvinde and Sastry, 1990) and *S. maltophilia* isolated from various Korean agricultural crops can display nitrogenase activity above 150 nmol $h^{-1}$ $mg^{-1}$ protein (Park *et al*., 2005b). Previous studies employing different *nifH* primers have also shown successful and specific amplification of *nifH* from a variety of bacteria and natural samples (Potrich *et al*., 2003). However, positive amplification of *nifH* in only two isolates in this study suggests the presence of host specificity or preference of diazotrophic endophytes similar to those in microbe-plant mutualisms found with *Rhizobium* and legumes.

The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists, as it can enhance the availability of phosphorus for microbial and/or plant growth. We examined all the selected endophytic bacterial isolates for their phosphate solubilizing ability by detecting extracellular solubilization of precipitated tricalcium phosphate with glucose as sole source of carbon (Table 2). Half of the endophytic isolates showed phosphate solubilizing activity. Based on the solubilization zone, the isolate E-I-20

**Table 2.** Phosphate solubilization, IAA production and Siderophore production ability of ginseng endophytic bacterial isolates

| Isolate No. | Diameter of P solubilization zone (mm) | IAA Produced (μg/ml) | Siderophore Production - Color Change |
|---|---|---|---|
| E-I-1 | 0.00 | 0.00 | - |
| E-I-3 | 0.35 | 1.78 | + |
| E-I-4 | 0.32 | 13.93 | + |
| E-I-8 | 0.38 | 4.61 | + |
| E-I-10 | 0.00 | 0.00 | - |
| E-I-11 | 0.29 | 0.52 | - |
| E-I-14 | 0.23 | 0.52 | - |
| E-I-15 | 0.00 | 2.30 | + |
| E-I-20 | 0.39 | 7.23 | + |
| E-II-2 | 0.00 | 0.00 | - |
| E-II-3 | 0.00 | 0.00 | - |
| E-II-4 | 0.29 | 2.04 | + |
| E-II-5 | 0.25 | 0.31 | - |
| E-II-7 | 0.00 | 0.31 | - |
| E-III-2 | 0.31 | 2.46 | + |
| E-III-4 | 0.00 | 2.04 | - |
| E-IV-2 | 0.00 | 1.57 | - |
| E-IV-3 | 0.00 | 0.84 | - |

+, indicates siderophore production
-, indicates siderophore non production

(*Lysinibacillus fusiformis*) recorded higher solubilization of mineral phosphate (0.39 mm). Isolates, E-I-8 (*Bacillus cereus*) and E-I-3 (*B. megaterium*) also showed notable solubilization activity. Gluconic acid mediated solubilization of calcium phosphate has been shown in the endophytes *viz.*, *Erwinia herbicola* and *Burkholderia cepacia* (Goldstein *et al*., 1993). The bacterial endophytes presently studied might also use mechanisms similar to those organisms for phosphate solubilization.

Except four, all the other endophytic isolates produced significant amounts of IAA in nutrient broth supplemented with tryptophane as precursor (Table 2). The isolate E-I-4 (*Micrococcus luteus*) produced higher amounts of IAA (13.93 μg/ml) followed by the isolates E-I-20 (*L. fusiformis*, 7.23 μg/ml) and E-I-8 (*B. cereus*, 4.61 μg/ml). Our results were in line with earlier study, in which seven of 10 endophytic isolates were positive for IAA production (Jha and Kumar, 2007). The capacity to synthesize IAA is widespread among soil- and plant-associated bacteria. The ability to produce IAA is considered responsible for plant growth promotion by beneficial bacteria such as *Azospirillum* spp., *Alcaligens faecalis*, *Klebsiella*, *Enterobacter*, *Acetobacter diazotrophicus*, and *Herbaspirillum seropedicae* (Costacurta and Vanderleyden, 1995).

Microorganisms produce and secrete siderophores to sequester iron. The production of siderophores by micro-organisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma and Johri, 2003). Only seven isolates (E-I-3, E-I-4, E-I-8, E-I-15, E-I-20, E-II-4, and E-III-2) produced siderophore, as evidenced by the change of color in the CAS blue medium from bluish-green to orange (Table 2). Siderophore production may be a common phenotype among endophytes. A study of siderophore production of endophytic *Methylobacterium* spp. associated with citrus plants suggested that there is a correlation between the area of isolation and their production of siderophores (Lacava *et al*., 2008). Likewise, in this study, more 1-year-old isolates displayed siderophore production than older plants.

In this study, the potential of endophytic isolates for plant growth promotion was determined by assessing the factors such as $N_2$ fixation, phosphorus solubilization, IAA production, and siderophore secretion. Although isolates exhibiting all the plant growth-promoting features simultaneously were rare, isolates E-I-3 (*B. megaterium*), E-I-4 (*M. luteus*), E-I-8 (*B. cereus*), and E-I-20 (*L. fusiformis*) were positive for most of these characteristics, indicating their role in promotion of growth of ginseng plants. Their potential as growth-promoting microbial inoculants for ginseng warrants further study.

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