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Gibberellin Production by Newly Isolated Strain *Leifsonia soli* SE134 and Its Potential to Promote Plant Growth

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Very few plant growth-promoting rhizobacteria (PGPR) are known to produce gibberellins (GAs). The current study aimed to isolate a phytohormone-producing PGP rhizobacterium from soil and assess its potential to enhance plant growth. The newly isolated bacterium was identified as *Leifsonia soli* sp. SE134 on the basis of partial 16S ribosomal RNA gene sequence. Application of *L. soli* culture filtrate significantly increased the biomass, hypocotyl, and root lengths of cucumber seeds as compared with non-inoculated sole medium and distilled water treated controls. Furthermore, the PGPR culture was applied to the GA-deficient mutant rice cultivar *Waito-C*. Treatment with *L. soli* SE134 significantly increased the growth of *Waito-C* rice seedlings as compared with controls. Upon chromatographic analysis of *L. soli* culture, we isolated, detected and quantified different GAs; namely, GA_1 (0.61 ± 0.15), GA_4 (1.58 ± 0.26), GA_7 (0.54 ± 0.18), GA_8 (0.98 ± 0.15), GA_9 (0.45 ± 0.17), GA_{12} (0.64 ± 0.21), GA_{19} (0.18 ± 0.09), GA_{20} (0.78 ± 0.15), GA_{24} (0.38 ± 0.09), GA_{34} (0.35 ± 0.10), and GA_{53} (0.17 ± 0.05). Plant growth promotion in cucumber, tomato, and young radish plants further evidenced the potential of this strain as a PGP bacterium. The results suggest that GA secretion by *L. soli* SE134 might prove advantageous for its ameliorative role in crop growth. These findings can be extended for improving the productivity of different crops under diverse environmental conditions.

Key words: Gibberellins, plant growth promotion, PGPR, *Leifsonia soli*

Plants are continuously interacting with a large number of microorganisms in the rhizosphere. In fact, diverse studies have shown the usefulness of plant growth-promoting rhizobacteria (PGPR) in increasing the crop yield [12]. These rhizobacteria play a pivotal role in the supply of nutrients to plants, secrete bioactive plant growth regulators, mediate various biotic and abiotic stress conditions and improve soil texture [16]. With the growing human population, demands for higher crop production and a larger food supply have persuaded farmers to use artificial fertilizers in agricultural fields. To meet food demands, synthetic fertilizers are used, a practice that has not only affected the long-term fertility of agricultural lands but has also caused numerous environmental problems. Various biological approaches have been assessed and used to increase crop productivity and reduce the negative impacts of agrochemicals. The use of PGPR has a potential role in developing sustainable systems in crop production [37, 38]. It helps to improve crop plant growth and avoids various forms of agriculture pollution [40].

Diverse kinds of bacterial species such as *Acetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, and *Serratia* have been reported to increase plant growth by enhancing seed emergence, plant biomass, and crop yield [13, 21]. This improved crop growth is due to the potential of PGPR to produce various kinds of bioactive metabolites such as indole acetic acid and gibberellins (GAs) GAs are ubiquitous plant hormones that elicit diverse metabolic functions required during plant growth such as seed germination, stem elongation, sex

expression, flowering, formation of fruits, and senescence [17, 18]. GA production by PGPR promote the growth and yield of many crop plants by deconjugation of gibberellin-glucosyl in the root zone [31], causing 3β-hydroxylation of inactive 3-deoxy GAs to active forms such as GA_1, GA_3, and GA_4 [6, 7, 31] bacterial enzymes. GAs have been identified and isolated from higher plants, fungi, and bacteria. Until the present, 136 GAs from higher plants (128 species), 28 GAs from fungi (7 species), and only 4 GAs (GA_1, GA_3, GA_4, and GA_{20}) from bacteria (7 species) have been identified [29].

Studies have shown that various strains of PGPR such as *Rhizobium phaseoli* [4], *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* [5], *Bacillus pumilus* and *Bacillus licheniformis* [14], *Azospirillum brasilense* [20], *Bacillus macroides* CJ-29 and *Bacillus pumilus* CJ-69 [23], *Burkholderia* sp. KCTC 11096BP [24], and *Acinetobacter calcoaceticus* [25] can produce GAs in bacterial culture medium. However, until now, no report is available on gibberellin production in any species of *Leifsonia*. The present study aimed to assess the GAs and plant growth-promoting potential of *Leifsonia soli* SE134.

Materials and Methods

Isolation of PGPR and Effect on Crop Seed Germination

Soil samples were collected from various agricultural fields in the Daegu area (Republic of Korea). About 10 g of soil samples was transferred to 250 ml flasks containing 100 ml sterile Amies solution [3]. The resulting suspensions were serially diluted (10^{-4}) and 0.1 ml aliquots were grown on tryptic soy/agar (TSA; Merck Co., Germany) for isolation of rhizobacteria. Bacterial cultures were incubated for 48 h at 30°C, and bacterial colonies differentiated by their morphology, pigmentation, and growth rate were selected, counted, and restreaked on fresh TSA medium. For long-term preservation, bacteria were stored in 50% glycerol at −80°C.

In order to investigate the effect of bacterial culture on seed germination, cucumber seeds were purchased from Seminis Korea Co. (Korea), surface sterilized with NaOCl (5%) for 10 min, and thoroughly rinsed with distilled water (DW). Seeds were kept in a petri dish at 25 ± 2°C in a culture room. The seeds were treated with ten dilutions of bacterial culture broth (30°C, 200 rpm and 3 days). DW and nutrient broth (NB) were used as controls for this experiment.

Bioassay of Gibberellin Mutant *Waito-C* Rice

Production of plant growth-promoting metabolites in PGPR was analyzed by performing a screening bioassay on gibberellin biosynthesis deficient mutant *Waito-C* rice. *Waito-C* rice seeds were surface sterilized with 2.5% sodium hypochlorite for 30 min, rinsed with autoclaved DW, and incubated for 24 h with 20 ppm uniconazol [19] to obtained equally germinated seeds. The germinated seeds were grown on agar medium (0.8%) in a growth chamber (day/night cycle: 14 h; 28°C/10 h; 18°C; relative humidity 60–70%; light intensity 1,000 μmm-2s natrium lamps) for 10 days. After the two-leaves stage was attained, 10 μl of culture filtrate solution of isolated bacteria was applied at the apex of *Waito-C* rice seedlings. One week after treatment, the shoot length, chlorophyll content, and shoot fresh and dry weights were recorded and compared with those of DW and NB controls. The experiment was repeated twice and each treatment had three replications comprising nine plants per replica.

Identification and Phylogenetic Analysis

The isolated bacterial strain was identified on the basis of its partial 16S ribosomal RNA gene (rDNA) sequence. The chromosomal DNA was isolated through standard procedures [35]. The almost complete 16S rDNAs were PCR amplified using primers 27F (5′-AGAGTTTGATC (AC) TGGCTCAG-3′) and 1492R (5′-CGG (CT) TACCTTGTTACGACTT-3′), which are complementary to the 5′ end and 3′ end of the prokaryotic 16S rDNA, respectively. The amplification reaction was performed as previously described [1]. The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to look for nucleotide sequence homology of this bacterial isolate. Closely related sequences were aligned by ClustalW using MEGA ver. 5.0 software, and the neighbor-joining tree was generated using the same software. Bootstrap replication (1000 replications) was used for statistical support for the nodes in the phylogenetic tree.

Plant Growth Promotion Capacity of *Leifsonia soli*

Seeds of cucumber, tomato, and young radish purchased from Seminis Korea Co. were surface sterilized with NaOCl (5%) for 10 min, rinsed with distilled water, and treated with 20 ppm uniconazole. Seeds were washed again and sown in plastic pots under controlled greenhouse conditions at 30 ± 2°C. Plant seedlings were treated with 5 ml of bacterial suspension 14 days after sowing, and the growth attributes of shoot length, plant fresh weight, and chlorophyll content, were recorded after 14 days of treatment. The bacterial culture suspension was incubated for 3 days at 30°C on a shaking incubator at 200 rpm. DW and NB (5 ml) were used as controls for this experiment. The experiment was repeated twice, while the individual treatment was repeated three times containing nine plants per treatment.

Extraction and Quantification of Gibberellins

Bacterial gibberellins were extracted from culture filtrates after 3 days of incubation according to an established protocol [27]. Extracted GAs were subjected to reverse-phase C18-HPLC. The GAs were chromatographed on a 3.9 × 300 m Bondapak C18 column (Waters Corp., USA) and eluted at 1.5 ml/min with the following gradient: 0 to 5 min, isocratic 28% methanol (MeOH) in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28% to 86% MeOH; 35 to 36 min, 86% to 100% MeOH; 36 to 40 min, isocratic 100% MeOH. Up to 48 fractions of 1.5 ml each were collected. The fractions were then prepared for injection into a gas

Table 1. Effect of bioactive *L. soli* SE134 culture on seed germination in cucumber.

Treatment	Hypocotyl (cm)	Root (cm)	Fresh weight (mg)	Hypocotyl diameter (mm)
Control (DW)	4.42 ± 0.49a	5.48 ± 0.54a	0.28 ± 0.05a	1 ± 0.16a
Medium (NB)	5.16 ± 0.55a	4.72 ± 0.52a	0.31 ± 0.03a	1.06 ± 0.13a
L. soli SE134	7.86 ± 0.92b	6.44 ± 0.59b	0.52 ± 0.04b	1.32 ± 0.13b

DW is distilled water and NB is nutrient broth. For each set of treatments, the different letters indicate significant differences ($p < 0.05$) between PGPR and controls as evaluated by Duncan's multiple range test. ± refers to SD of the mean of nine plants per treatment.

chromatograph/mass spectrometer (GC/MS) with selective ion monitoring mode (SIM) (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, USA). For each GA type, 1 µl of sample was injected into a DB-1 capillary column with a 30 m × 0.25 mm i.d. and 0.25 µm film thickness (J & W Scientific Co., USA). The GC oven temperature was programmed for a 1 min hold at 60°C, then to rise at 15°C/min to 200°C followed by 5°C/min to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a mass selective detector with an interface and source temperature of 280°C, an ionizing voltage of 70 eV, and a dwell time of 100 msec. Full-scan mode (the first trial) and three major ions of the supplemented [$^2H_2$] GA internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia) and bacterial gibberellins were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI (Kovats Retention Index) value, and GA quantification was based on peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Statistical Analysis

The data were analyzed for standard error using Sigma Plot Software (10.0) and Student's t-test was used to identify significant values. The mean values were compared using Duncan's multiple range test at $p \leq 0.05$ (ANOVA SAS release 9.1; SAS, Cary, NC, USA).

Results

Isolation of PGPR and Effect on Crop Seed Germination

Among 891 bacterial strains isolated from agricultural fields, SE134 was selected on the basis of its role in improving seed germination of cucumber. An SE134 culture was applied to the cucumber seeds. The bacteria-free culture medium (NB) and DW were applied to the seeds of cucumber as a control. The results showed that SE134 application significantly increased seed germination as compared with controls (Table 1). Fresh biomass and hypocotyl and root lengths were significantly higher in SE134-treated cucumber as compared with control applications.

Gibberellin Mutant *Waito-C* Rice Bioassay

Waito-C rice is dwarf mutant rice with a deficient GA biosynthesis pathway. The purpose of this assay was to determine whether the rhizobacteria secrete any growth regulating substances that can overcome the growth deficiency of *Waito-C* rice. The results show that when SE134 culture filtrate was applied, *Waito-C* rice growth was significantly higher than that of the control plants (Table 2). The growth-promoting effect of SE134 was more highly significant than that of the rest of the bacterial strains as well as the negative control (data not shown).

Identification of Isolated Bacterium

A BLASTn search of the 16S rDNA sequence of SE134 showed 100% similarity with *Leifsonia soli* (Fig. 1). In the phylogenetic analysis, the sequence of the isolate showed 99% sequence homology with *L. soli*, indicating it to be a strain of this species. The 16S rDNA sequence of this strain was submitted to GenBank and was given the accession number KC819804.

Plant Growth Promotion Capacity of *Leifsonia soli* SE134

The growth-promoting capacity of isolate *L. soli* SE134 was bioassayed on cucumber, tomato, and young radish

Table 2. Effect of *L. soli* SE134 on the growth of GA-deficient mutant rice *Waito-C*.

Strain	SL (cm)	CC (SPAD)	SFW (g)	SDW (g)
Control (DW)	8.41 ± 0.21b	27.9 ± 1.5b	0.75 ± 0.09b	0.10 ± 0.12b
Medium (NB)	9.12 ± 0.45b	28.4 ± 1.8b	0.79 ± 0.11b	0.12 ± 0.22b
L. soli SE134	13.45 ± 0.28a	30.6 ± 1.15a	0.89 ± 0.81a	0.29 ± 0.09a

DW is distilled water and NB is nutrient broth. SL = shoot length; SFW = shoot fresh weight; SDW = shoot dry weight; CC = chlorophyll. For each set of treatments, the different letters indicate significant differences ($p < 0.05$) between PGPR and controls as evaluated by Duncan's multiple range test. ± refers to SE of the mean of nine plants per treatment.

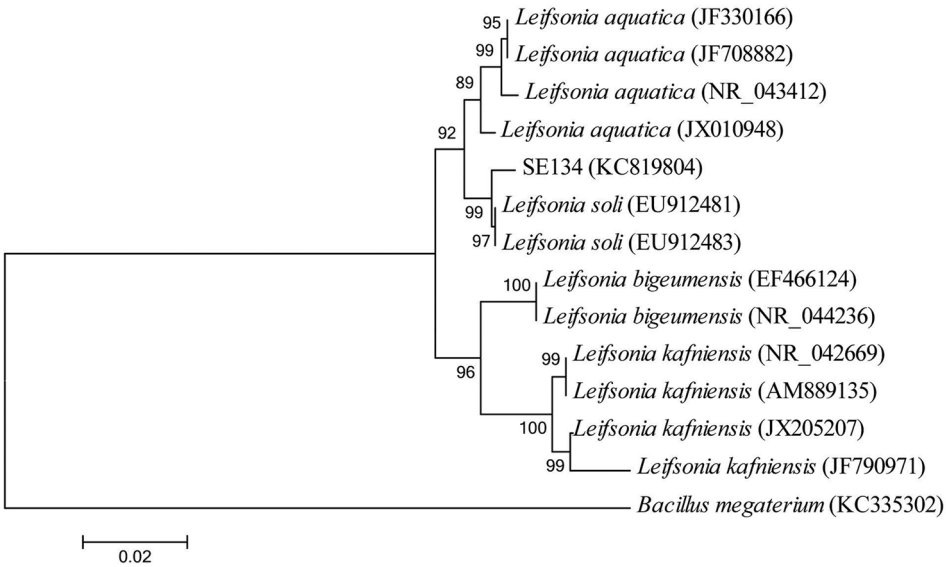

Fig. 1. Identification of bacterium *L. soli* SE134 by 16S rRNA gene-based phylogenetic analysis.

plants. The control included both DW and NB treatments (Table 3). *L. soli* SE134 bacterial suspension significantly promoted the growth of all seedlings. Shoot length, plant fresh weight, and chlorophyll were significantly higher as compared with control treatments.

Extraction and Quantification of Gibberellins

L. soli SE134 was grown for 3 days in NB culture medium (30°C; 200 rpm) and centrifuged (4°C; 6000 rpm) to obtain a clear supernatant (100 ml). The supernatant was extracted and chromatographed to detect GAs. GC/MS-SIM analysis revealed that various physiologically active and inactive GAs were present in different quantities (Fig. 2). Physiologically bioactive GAs included GA_1 (0.61 ± 0.15 ng/100 ml), GA_4 (1.58 ± 0.26 ng/100 ml), and GA_7 (0.54 ± 0.18 ng/100 ml). Physiologically inactive GAs were GA_8 (0.98 ± 0.15 ng/100 ml), GA_9 (0.45 ± 0.17 ng/100 ml), GA_{12} (0.64 ± 0.21 ng/100 ml), GA_{19} (0.78 ± 0.15 ng/100 ml), GA_{20} (0.18 ± 0.09 ng/100 ml), GA_{24} (0.38 ± 0.09 ng/100 ml), GA_{34} (0.35 ± 0.10 ng/100 ml), and GA_{53} (0.17 ± 0.05 ng/100 ml). The quantity of physiologically bioactive GA_4 was significantly higher as compared with other GAs.

Discussion

The use of PGPR has a potential role in the development of sustainable systems in crop production [37, 38]. It not only helps to improve crop plant growth and yield but also

Table 3. Effect of *Leifsonia soli* SE134 on growth of cucumber, tomato, and young radish.

Plant	Treatment	Shoot length (cm plant^{-1})	Plant fresh weight(g plant^{-1})	Chlorophyll (SPAD)
Cucumber	Control (DW)	17.54 ± 1.45b	2.42 ± 0.43b	29.3 ± 4.36b
	DW + NB medium	16.72 ± 2.03b	2.18 ± 0.33a	28.7 ± 3.98b
	L. soli SE134	20.42 ± 0.72a	2.78 ± 0.34c	33.5 ± 2.45a
Tomato	Control (DW)	16.98 ± 1.21b	1.12 ± 0.57b	28.7 ± 3.58b
	DW + NB medium	17.02 ± 1.17b	1.45 ± 0.47b	29.2 ± 3.12b
	L. soli SE134	19.38 ± 1.85a	2.15 ± 0.44a	32.7 ± 1.98a
Young radish	Control (DW)	16.26 ± 1.01b	1.76 ± 0.35b	26.7 ± 1.45b
	DW + NB medium	16.52 ± 1.14b	1.82 ± 0.25b	26.9 ± 1.15b
	L. soli SE134	17.56 ± 1.21a	2.05 ± 0.35a	29.3 ± 1.95a

DW is distilled water and NB is nutrient broth. For each set of treatments, the different letters indicate significant differences ($p < 0.05$) between PGPR and controls as evaluated by Duncan's multiple range test. ± refers to SD of the mean of nine plants per treatment.

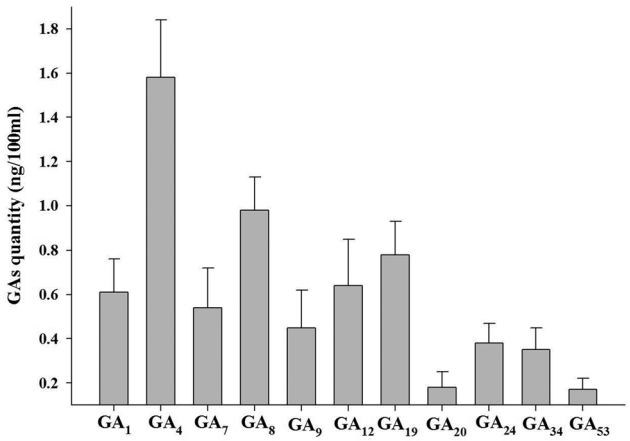

Fig. 2. Gibberellin production by *L. soli* SE134.
The bacterial culture was centrifuged and 100 ml of culture filtrate was analyzed for the presence of GAs using a GA extraction protocol [27].

avoids various forms of agricultural pollution [40]. Different strains of *Alcaligenes*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Beijerinckia*, *Bacillus*, *Enterobacter*, *Burkholderia*, *Acinetobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium*, and *Serratia* [33, 34, 39] have been identified as PGPR. These studies have suggested using PGPR as biofertilizers [2, 28]. Crop plants are protected by PGPR that may produce secondary metabolites, such as alkaloids, antibiotics, and toxins [9, 36]. Very little work has been performed on the synthesis and secretion of plant growth regulators from bacteria. In the present study, we assayed *L. soli* SE134 culture on cucumber and *Waito*-C rice seedlings. Among tested strains, *L. soli* SE134 culture filtrate significantly promoted the growth of cucumber and *Waito*-C rice seedlings, suggesting the presence of active plant growth metabolites. *Waito*-C is a dwarf and bioactive GA-deficient rice cultivar, with a blocked C13-hydroxylation pathway for GA biosynthesis [19]. To observe the sole effect of *L. soli* SE134 as a GA producer, *Waito*-C seeds were treated with uniconazol to further suppress endogenous GA production by blocking its biosynthesis pathway [19]. Using rice seedlings to elucidate the effect in a controlled environment is beneficial and easy to monitor, while using *Waito*-C rice can help to analyze the effect of GA-producing strains.

Gibberellin production by PGPR promotes the growth and yield of many crop plants. The plant's endogenous gibberellin-glucosyl conjugates are released *via* root exudation by a process of deconjugation [32], while bacterial enzymes 3β-hydroxylize the inactive 3-deoxy gibberellins to active forms such as GA_1, GA_3, and GA_4 [6, 7, 31]. The first report of gibberellin characterization in bacteria using physico-chemical methods was by Atzorn *et al*. [4], who demonstrated the presence of GA_1, GA_4, GA_9, and GA_{20} in *Rhizobium meliloti* culture solution. Similarly, the current study confirms the previous findings of Joo *et al*. [22] on 3β-hydroxylated GAs in *B. cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69. The authors also showed the usefulness of HPLC coupled with GC-MS-SIM while suggesting an efficient way to isolate, detect, and quantify GA.

Bastian *et al*. [5] detected the phytohormones GA_1 and GA_3 in chemically defined cultures of *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*. Both bacteria are associated with Gramineae species in the endophytic mode of life and were found to promote plant growth and yield. Gutierrez-Manero *et al*. [14] isolated the PGPR *Bacillus pumilus* and *Bacillus licheniformis* from the rhizosphere of alder (*Alnus glutinosa* [L.]). Full-scan gas chromatography-mass spectrometry analyses on extracts of culture media showed the presence of GA_1, GA_3, GA_4, and GA_{20} in addition to the isomers 3-epi-GA_1 and iso-GA_3.

Another PGP rhizobacterium, *Acinetobacter calcoaceticus* SE370, was found to be a novel GA producer, as it secretes 10 different GAs in its growth media [25], including physiologically active GA_1, GA_3, and GA_4 [25]. The bioactive GA_1, GA_3, and GA_4 content was 0.45, 6.25, and 2.83 ng per 100 ml, respectively. Similarly, another PGP strain from the rhizosphere, *Promicromonospora* sp. SE188, was found to produce physiologically active GA_1 (0.99 ± 0.03 ng/ml) and GA_4 (1.58 ± 0.11 ng/ml) and inactive GA_9 (0.2 ± 0.04 ng/ml), GA_{12} (2.38 ± 0.11 ng/ml), GA_{19} (0.85 ± 0.07 ng/ml), GA_{20} (0.17 ± 0.08 ng/ml), GA_{24} (0.57 ± 0.07 ng/ml), GA_{34} (0.24 ± 0.03 ng/ml), and GA_{53} (0.29 ± 0.02 ng/ml) [26]. The GAs were analyzed through GC/MS in selected ion monitoring (SIM) mode, which provides a more reliable GA quantification technique as compared with TLC, bioassays, or HPLC-UV, which give poor resolution and the least degree of reliability. The major advantage of GC/MS is its unbiased character. In comparison with non-MS detection-based chromatographic techniques (HPLCDAD,GC-FID), where only compounds targeted by a special analytical protocol are found, GC-MS analysis can result in interesting and unexpected new information about a particular extract [11].

In the current study, bacterial culture suspensions significantly promoted the growth of cucumber, tomato, and young radish, which may be due to the GA production capacity of newly isolated *L. soli* SE134. However, this isolate had not been previously reported to produce gibberellins. *L. soli* SE134 was found to produce eleven GAs, including bioactive GA_1, GA_4, and GA_7. Although the

quantities of the GAs were lower than in *Promicromonospora* sp. SE188 and *A. calcoaceticus* SE370, GA secretion was higher in *L. soli* SE134. Additionally, we did not detect GA_3 in *L. soli* SE134 culture, which suggests a different pathway of GA biosynthesis as compared with that of *Bacillus pumilus*, *Bacillus licheniformis*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, and *A. calcoaceticus* SE370. However, the GA profile of *L. soli* SE134 is similar to that of *Promicromonospora* sp. SE188 and *Rhizobium meliloti*. The genus *Leifsonia* is classified in Actinobacteria [10], which contains twelve species and two subspecies, with *L. aquatica* as the type species [10, 30]. Members of the genus *Leifsonia* have also been isolated from roots, soil, Himalayan glaciers, cyanobacterial mats, and water samples [8, 10, 15]. The present study is the first to report that a species of *Leifsonia* can also produce GAs.

In conclusion, the GA-secreting bacterium *L. soli* SE134 might be advantageous for plant growth promotion and metabolism. Its positive role in this regard was demonstrated by the measurement of plant growth attributes in cucumber and *Waito-C* rice. Understanding the interactions between PGPR and plants can improve the quality and quantity of crops. Such studies can be extended for improving agricultural productivity under the appropriate extreme environmental conditions.

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