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Isolation and characterization of pea plant (*Pisum sativum* L.) growth-promoting Rhizobacteria

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Plant growth-promoting rhizobacteria (PGPR) are well-known to influence plant growth via a variety of mechanisms such as nitrogen fixation, production of volatile organic compounds and enzymes, and bioremediation contaminants from the environment. PGPR have been previously identified by other researchers using laboratory screening methods. It was hypothesized that relying on these routine laboratory tests, some PGPR species are being overlooked. These species could promote growth through genes that encode for the synthesis of specific growth stimuli or other growth-promoting traits such as vitamins, antibiotics, and secondary metabolites. To evaluate this hypothesis, PGPR (MA-7, ON-4, SP-7, and RA-9) and previously overlooked PGPR (SE-7, LE-26, SQ-7, and SQ-9) were tested both with sterilized and non-sterilized soil in pot and greenhouse experiments. The PGPR isolates significantly increased pea plant growth, albeit to different degrees based on isolate, in both types of soil. The increases were recorded in shoot and root length and fresh matter in non-sterilized soil whereas increases in root length and root fresh weight were observed in sterilized soil. Interestingly, strains SE-7 and SQ-7 of the four overlooked PGPR isolates tested were also able to promote pea plant growth similarly to the PGPR isolates under both pot and greenhouse conditions. Morphological and biochemical characterization of the four original PGPR isolates revealed that they were rod-shaped, gram-positive, and spore-forming. Sequencing of 16S ribosomal RNA showed that these strains were mostly similar to Bacillus sp. (99% similarity). Using the EzBioCloud 16S rRNA database, it was found that one strain was likely to be *Bacillus paramycoides* based on 100% similarity, two strains were *Bacillus wiedmannii* based on 99.05 and 100% similarity, and the remaining strain was *Bacillus amyloliquefaciens* based on 99.64% similarity.

Key words: Plant growth-promoting rhizobacteria (PGPR), pea, soil, 16S rRNA, Bacillus.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are bacteria which can directly or indirectly enhance plant growth (Joseph et al., 2007; Lugtenberg and Kamilova, 2009). PGPR promote growth directly by producing siderophores, phytohormones (such as auxins), solubilizing phosphate and indirectly by inducing systemic

resistance (Kumar et al., 2012; Spaepen et al., 2009).

Numerous bacterial species that promote plant growth have been identified, including *Azospirillium*, *Rhizobium*, *Serratia*, and *Enterobacter* strains. Furthermore, several bacterial genera, such as *Streptomyces*, *Pseudomonas*, and *Agrobacterium* have been studied and are increasingly marketed as biocontrol agents. These bacteria suppress plant disease by producing antibiotics and antifungal metabolites such as hydrogen cyanide and phenazines (Bhattacharyya andJha, 2012; Mahanty et al., 2017; Saharan and Nehra, 2011; Tilak et al., 2005).

PGPR increase the growth and yield of many important crops, including maize, banana, and Bt cotton (Agbodjato et al., 2016; Apastambh et al., 2016; Pindi et al., 2014). Furthermore, inoculation of pea and wheat plants with bacterial species of the genus *Pseudomonas* and *Bacillus* enhances plants shoot and root growth (Egamberdieva, 2008). Moreover, PGPR have contributed in regulating the growth promoting by a different functions and mechanisms such enhancement of crop production, protection from stresses, and bioremediation contaminants from the environment (Guo et al., 2015; Zhang et al., 2013; Zhuang et al., 2007).

Previous screening for PGPR has relied on routine laboratory tests. It was hypothesized that some PGPR have been overlooked using these method because promotion of plant growth may occur through genes involved in traits such as vitamins, antibiotics, and amino acids production (Babalola, 2010; Zhou et al., 2008). Alternatively, these overlooked PGPR may use quorum-sensing to secrete specific substances, where extracellular release of these substances improves plant growth (Lopes et al., 2017; Monnet and Gardan, 2015). The main objectives of the present study were to isolate PGPR, including some previously overlooked PGPR strains, and to evaluate their effects on pea plant growth under both pot and greenhouse conditions.

MATERIALS AND METHODS

Isolation and screening of plant growth-promoting traits

Soil samples were collected from the rhizospheres (1-15 cm) of different crop plants including maize, onion, sweet potato, sesame, hyacinth, and radish at two sites located in the Jiangsu province, China. Isolation was done on nutrient agar by using a pour plate method and the plates were incubated at 37°C for 48 h. Based on morphology, eight bacterial isolates that showed different colonies morphology were picked up and purified many times. The eight bacterial isolates MA-7, ON- 4, SP-7, RA-9, SE-7, LE-26, SQ-7, and SQ-9 were screened for their ability to promote plant growth using routine laboratory methods, including production of indole-3-acetic acid (IAA), siderophores, ammonia, and solubilization of phosphate.

Indole acetic acid (IAA) production

IAA production was tested in tryptone broth medium. Freshly cultured isolates were inoculated into tubes containing 5 ml tryptone broth and incubated at 37°C for 7 days. Kovac's reagent (0.5 ml) was added and the formation of a red color in the alcohol layer was considered a positive result.

Siderophores production

Detection of siderophores was performed using king's B agar medium containing chrome azurol S as an indicator dye, $FeCl_3.6H_2O$ solution and hexadecyltrimethyl ammonium bromide. Five microliters of each fresh culture was inoculated onto a plate, and then was incubated at 28°C for 72 h. The presence of an orange halo around a colony indicated a positive result (Lacava et al., 2008).

Ammonia production

Detection of ammonia was assessed in peptone water medium. Bacterial isolates cultured for 24 h were inoculated into tubes containing 10 ml peptone water and incubated at 37°C for 48 h. After incubation, the culture was supplemented with Nessler's reagent (0.5 ml), and a positive result was recorded upon the development of a yellow color (Yadav et al., 2010).

Phosphate solubilizing activity

Phosphate solubilizing test was performed on Pikovaskaya's medium (PVK) supplemented with tricalcium phosphate. Freshly cultured isolates were inoculated onto plates containing PVK medium and the plates were incubated at 30°C for 7 days. A clear zone around colonies indicated a positive result.

Identification of PGPR strains

Identification according to Morphology, including cell shape, gram staining, and spore formation was characterized for PGPR isolates MA-7, ON-4, SP-7, and RA-9. Biochemical traits were assessed, including Voges-Proskauer test status, carbohydrates utilization, nitrate reduction, and hydrolysis of gelatin and starch. Growth at different pH (pH 5, 6, and 7), temperatures (5, 10, 20, 30, 40, 50, 55, and 60°C) and sodium chloride concentrations (2, 5, 7, and 10%) was also tested as previously described (De Vos et al., 2009).

Sequencing of 16S ribosomal RNA (rRNA) was performed for the PGPR isolates MA-7, ON-4, SP-7, and RA-9 by the Shanghai Sangon Biological Engineering Technology and Services CO., Ltd. The resulting sequences were assembled using the DNAMAN 6.0 software package, compared to the NCBI reference database, and submitted to NCBI Gene bank. A phylogenetic tree was generated using the MEGA 6.0 software package.

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Pot and greenhouse experiments

The eight bacterial isolates of interest, PGPR (MA-7, ON-4, SP-7, and RA-9) and overlooked PGPR (SE-7, LE-26, SQ-7, and SQ-9) were tested both in pot and greenhouse experiments with mock (sterile tap water) and *E. coli* treatments as controls. For pot experiment, the experiment was arranged in a single factorial analysis of variance with three replicates using sterilized and non-sterilized soil. Clay soil was collected from a farm located in the Jiangsu province, China. The soil was air-dried, milled, sieved through a 2 mm mesh, and then halved. The first half was sterilized, while the remaining half was left without sterilization.

Pea (*Pisum sativum* L.) seeds were sterilized for 3 min in 3% sodium hypochlorite then rinsed five times with sterilized distilled water, and placed on Petri dishes in the dark at 25°C for 2 to 4 days. The day before treatment, pots (10 cm, Diameter × 12.5 cm, Height) were divided into two groups and filled with the sterilized and non-sterilized soil and watered.

Prior to inoculation, the eight bacterial isolates, PGPR (MA-7, ON-4, SP-7, and RA-9) with overlooked PGPR (SE-7, LE-26, SQ-7, and SQ-9) and *E. coli* were subcultured overnight on nutrient agar at 37°C. For the inoculation, a loopful of each isolate were put in 5 ml tubes of sterilized tap water. Subsequently, the germinated seeds with small visible roots were transferred into the bacterial suspensions, and soaked gently for 1 to 2 min. The soaked germinated seeds were sowed directly into the prepared pots (in total 48 pots), where each pot received six germinated seeds. The mock replicates were created by soaking germinated seeds in sterilized tap water prior to sowing into the pots.

After inoculation, the total number of viable bacteria was calculated for all isolates by serially diluting 1 mL of each bacterial suspension down to 10^{-7}. Quantification was performed using the pour plate method and the number of colony-forming units was recorded. The pots were incubated under controlled conditions in a small plastic house for 30 days and watered regularly.

The same eight bacterial isolates PGPR (MA-7, ON-4, SP-7, and RA-9) and overlooked PGPR (SE-7, LE-26, SQ-7, and SQ-9), together with the *E. coli* and sterile tap water (mock) controls were studied in the greenhouse based on their performance in the pot experiments. The greenhouse experiment was conducted in the greenhouse belongs to the college of Horticulture, Yangzhou University, China. The experiment was carried out in a completely randomized design with four replicates using the same inoculation method used for the pot experiments and grown for 21 days.

Harvesting and data analysis

For both pot and greenhouse experiments, the plants were removed from the soil pot for each replicate, washed gently, and put to loose surface moisture. Parameters included shoot length and root length was measured. The number of germinated seedlings and shoot and root fresh weights were also recorded. Data from both pot and greenhouse experiments were analyzed using IBM SPSS statistics software package version 19. Duncan's honest significant post-hoc test was used to identify statistically significant differences between means ($p < 0.05$) for both pot and greenhouse experiments.

RESULTS

Screening of plant-growth promoting traits

Based on the results of the laboratory tests for screening PGPR, the bacterial isolates MA-7, ON-4, SP-7, and RA-9 were identified as PGPR. Isolates ON-4, SP-7, and RA-9 solubilized phosphate and produced IAA, siderophores, and ammonia. MA-7 was capable of all this except the ammonia production. Bacterial isolates SE-7, LE-26, SQ-7, and SQ-9 were tested negative for all these traits (Table 1).

Strains identification

Based on morphological tests, MA-7, ON-4, SP-7, and RA-9 were determined to be rod-shaped, gram-positive, and spore-forming bacteria. Biochemical and physiological tests included carbohydrates utilization, growth at different temperature, pH values, and sodium chloride concentrations showed that the isolates belonging to the genus *Bacillus* (Table 2).16S rRNA genes sequences were performed, compared to NCBI reference database, and submitted to NCBI Gene bank (accession number for MA-7 was MG371983, ON-4 was MG371984, SP-7 was MG371985, and RA-9 was MG371986).

The four bacterial isolates were found to be closely related to *Bacillus* sp. (99% similarity). Using the EzBioCloud 16S rRNA database, MA-7 was found to most likely be *B. paramycoides*, ON-4 and SP-7, despite different morphologies, were *B. wiedmannii*, and RA-9 was *B. amyloliquefaciens*. A phylogenetic tree was constructed using neighbour-joining method based on 16S rRNA gene sequencing and the related sequences in EzBioCloud databases (Figure 1A, 1B, 1C, and ID).

Pot and greenhouse experiments

Overall, the PGPR isolates MA-7, ON-4, SP-7, and RA-9 successfully promoted pea plant growth. For the pot experiment, significant differences in shoot and root length and shoot fresh weight were observed between treatment cohorts. Significant increases in root fresh and dry weights were also recorded ($p \leq 0.05$ and $p \leq 0.001$). The PGPR isolate with the most growth-promoting potential was RA-9 which performed the highest for all growth parameters assessed. Interestingly, overlooked PGPR isolates SE-7 and SQ-7 performed similarly to the PGPR isolates in terms of promoting increases in shoot length and shoot and root fresh weights (Figure 2).

The greenhouse experiments were conducted according to the performance of the isolates in the pot experiment.

Significant differences were observed between treatments cohorts in terms of number of germinated seedlings and shoot and root fresh weights ($p \leq 0.05$ and $p \leq 0.001$). There were also significant increases in shoot and root dry weights using non-sterilized soil (Table 3).

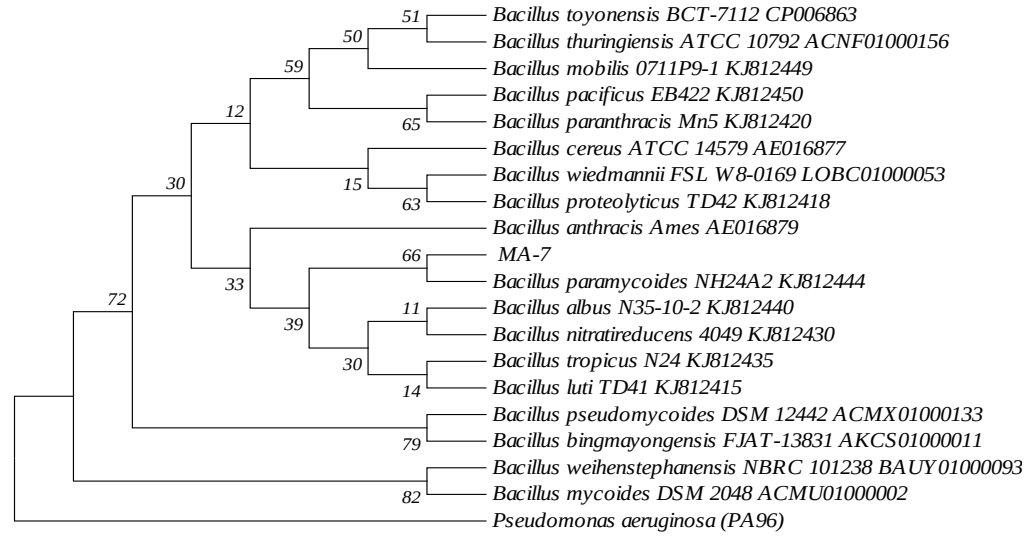
Table 1. Laboratory PGPR screening tests.

Isolate	Phosphate solubilization	Siderophores production	IAA production	Ammonia production
MA-7	+	+	+	-
ON-4	+	+	+	+
SP-7	+	+	+	+
RA-9	+	+	+	+
SE-7	-	-	-	-
LE-26	-	-	-	-
SQ-7	-	-	-	-
SQ-9	-	-	-	-

Table 2. Biochemical and physiological tests.

Characteristics	MA-7	ON-4	SP-7	RA-9
Gram stain	+	+	+	+
Endospore stain	Ellipsoidal	Cylindrical	Ellipsoidal	Ellipsoidal
Aerobic growth	+	+	+	+
Anerobic growth	+	+	+	-
Voges-Proskauer	+	-	+	+
Acid from:				
D-Glucose	+	+	+	+
D-Mannitol	-	-	-	+
Hydroysisof starch	+	+	+	+
Hydrolysisof gelatin	+	-	+	+
Nitrate reduction	-	-	+	+
Growth at pH				
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
Growth in NaCl				
0%	+	+	+	+
2%	+	+	+	+
5%	+	+	+	+
7%	+	+	+	+
10%	-	+	+	+
Growth at				
5°C	-	-	-	-
10°C	+	+	+	+
20°C	+	+	+	+
30°C	+	+	+	+
40°C	+	+	+	+
50°C	-	-	-	+
55°C	-	-	-	-
60°C	-	-	-	-

The isolate most effective at promoting growth was MA-7, which had the greatest positive effect on growth, resulting in the highest number of germinated seedlings and fresh and dry matter (Table 3). Interestingly, the overlooked PGPR isolates SE-7 and SQ-7 had effects on plant growth similar to those of PGPR isolates, where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Bacillus proteolyticus TD42 KJ812418
Bacillus anthracis Ames AE016879
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Bacillus albus N35-10-2 KJ812440
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Bacillus luti TD41 KJ812415
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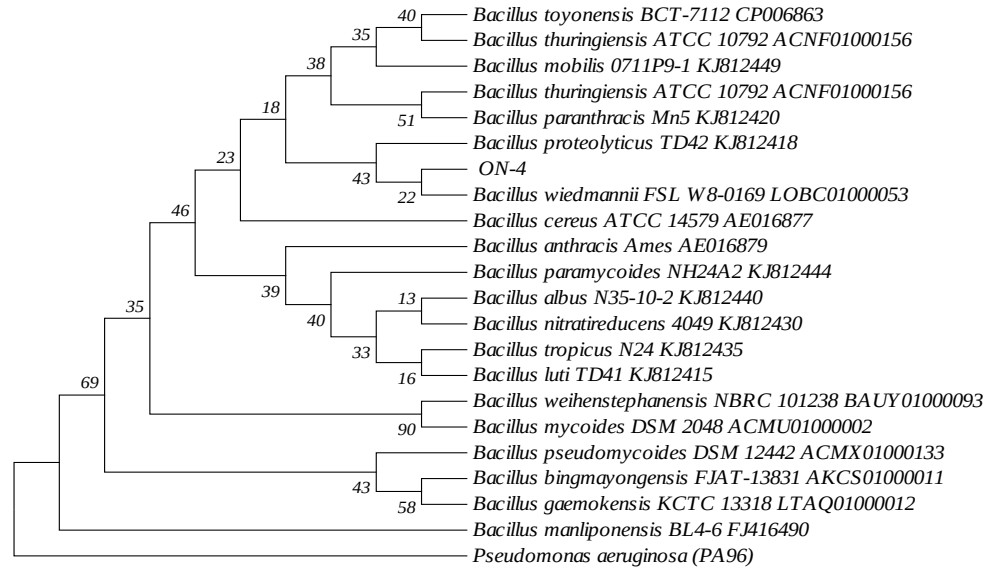
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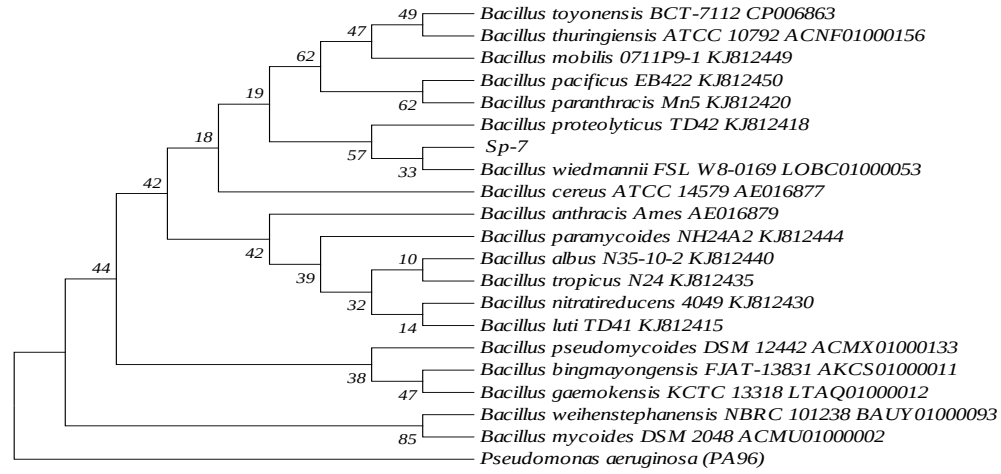
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Pseudomonas aeruginosa (PA96)

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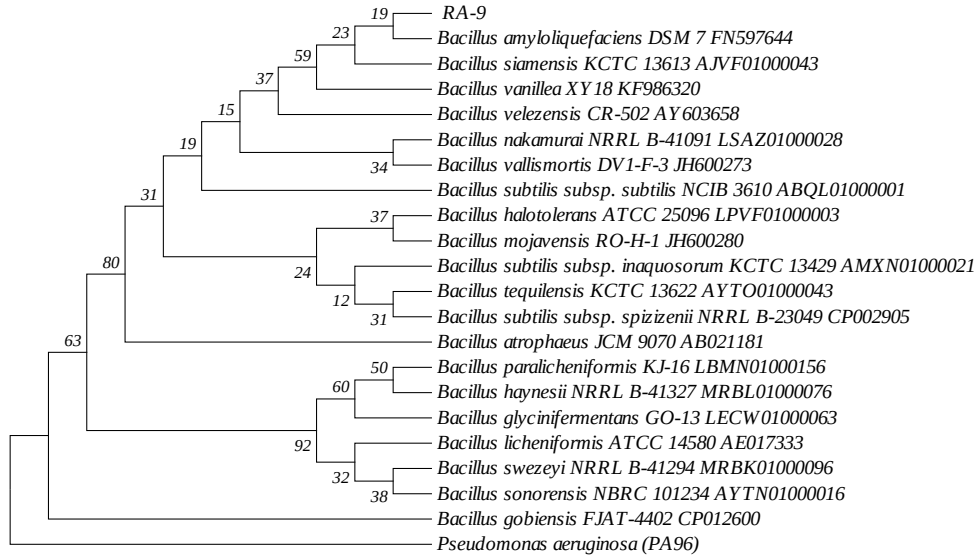

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Figure 1. A. Phylogenetic tree based on 16S rRNA sequencing of MA-7 and those of related bacteria and out-group species.B.Phylogenetic tree based on 16S rRNA sequencing of ON-4 and those of related bacteria and out-group species. **C.** Phylogenetic tree based on 16S rRNA sequencing of SP-7 and those of related bacteria and out-group species. **D.** Phylogenetic tree based on 16S rRNA sequencing of RA-9 and those of related bacteria and out-group species.

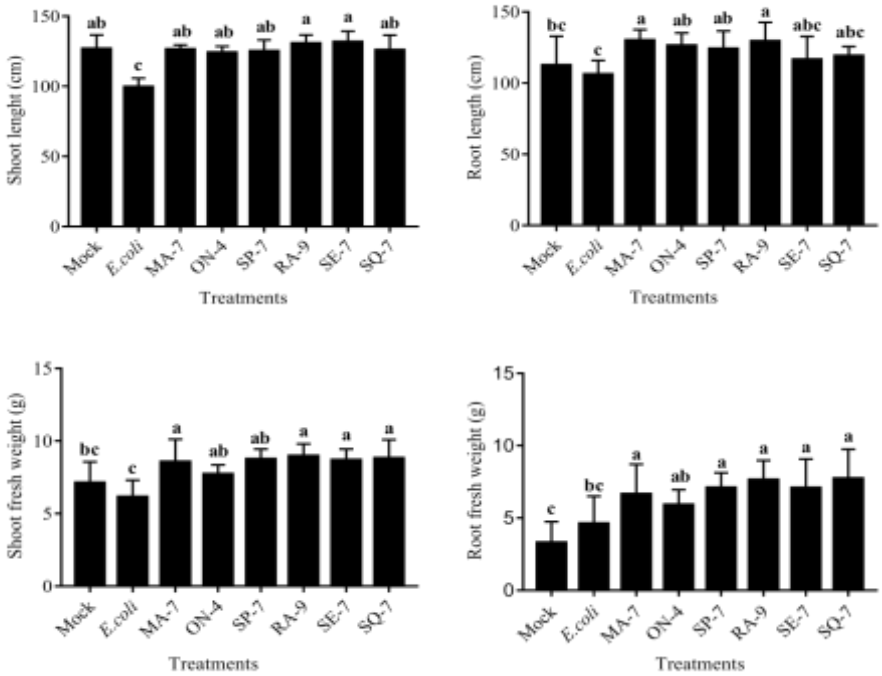

Figure 2. The effect of PGPR and overlooked PGPR isolates on pea plant growth in pot experiment using non-sterilized soil. PGPR: MA-7, ON-4, SP-7, and RA-9. Overlooked PGPR: SE-7and SQ-7, Controls: Mock treatment (Sterile tap water) and *E. coli*. Means having the same letter(s) and not significantly different; from one another according to Duncan's honest significant difference post-hoc test ($p \leq 0.05$).

Table 3. Effects of PGPR and Overlooked PGPR isolates on pea plant growth in greenhouse experiments using non-sterilized soil.

Treatment	Germinated seedlings	Shoot fresh weight/*P(g)	Shoot dry weight/P (g)	Root fresh weight/P (g)	Root dry weight/P (g)
Mock	3.00 d	1.93 c	0.26 d	1.02 de	0.17 b
E. coli	6.50 b†	3.04 abc	0.41 bcd	0.75 f	0.28 ab
MA-7	8.00 a	6.71 a	0.83 a	3.84 a	0.39 a
ON-4	7.50 ab	6.09 ab	0.73 abc	3.62 ab	0.38 a
SP-7	6.75 b	4.63 abc	0.60 abcd	2.81 abcd	0.30 ab
RA-9	4.00 cd	2.40 bc	0.31 d	1.40 cde	0.15 b
SE-7	7.25 ab	6.39 ab	0.75 abc	3.70 a	0.35 ab
LE-26	4.25 c	2.99 abc	0.37 cd	1.66 bcde	0.16 b
SQ-7	7.00 ab	6.48 a	0.80 ab	4.11 a	0.35 ab
SQ-9	6.50 b	4.15 abc	0.52 abcd	3.26 abc	0.28 ab

PGPR isolates: MA-7, ON-4, SP-7, and RA-9. Previously overlooked PGPR isolates: SE-7, LE-26, SQ-7, and SQ-9. Controls: Mock treatment (water) and *E. coli*. Means having the same letter(s) and not significantly different from one another according to Duncan's honest significant difference post-hoc test ($p \leq 0.05$); P, Plant.

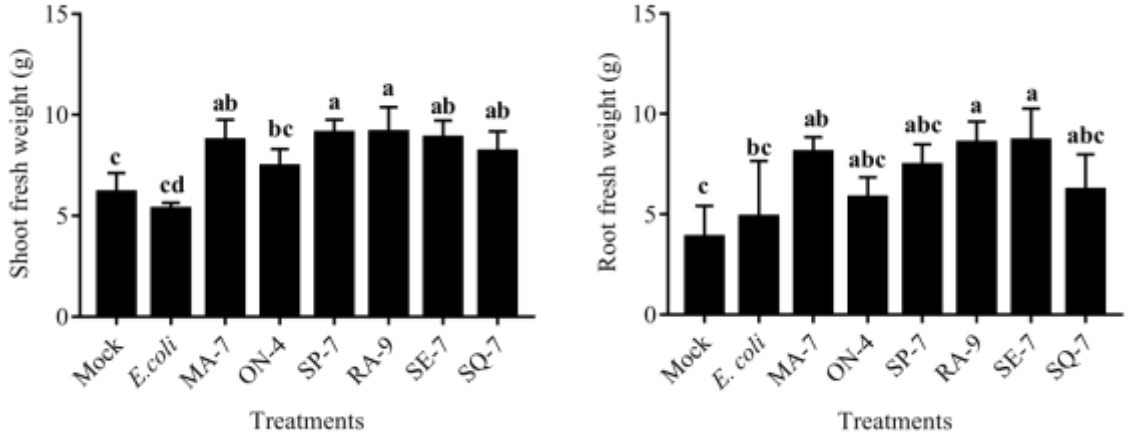

Figure 3. The effect of PGPR and overlooked PGPR isolates on pea plant growth in pot experiment using sterilized soil. PGPR: MA-7, ON-4, SP-7, and RA-9. Overlooked PGPR: SE-7and SQ-7, Controls: Mock treatment (Sterile tap water) and *E. coli*. Means having the same letter(s) and not significantly different; from one another according to Duncan's honest significant difference post-hoc test ($p \leq 0.05$).

promoted increases in shoot and root fresh weights (Table 3). Significant increases in shoot and root fresh weights were also recorded in sterilized soil ($p \leq 0.001$and $p \leq 0.05$ respectively) (Figure 3).

DISCUSSION

In the present study, it was shown that PGPR isolates overlooked in the previous screens may perform well in terms of improving plant growth. The previously overlooked PGPR isolates SE-7and SQ-7 were found to be good promoters of pea plant growth. Specifically, they significantly increased shoot fresh and root fresh weights. Overall, these results support that routine laboratory used to screen for PGPR traits may overlook beneficial isolates. These overlooked isolates may promote growth through genes encoding for certain growth-promoting traits such as vitamins, antibiotics, and secondary metabolites or specific secreted substances related to quorum-sensing.

Based on partial 16S rRNA sequencing and microbiological tests, PGPR isolates MA-7, ON-4, SP-7, and RA-9 were found to be different *Bacillus* species. Phylogenetic tree was constructed using neighbour-

joining method based on 16S rRNA genes sequences of the isolates and those of related bacteria in the EzBioCloud 16S rRNA databases and out-group species in the NCBI database. It was found that, MA-7 was most likely *B. paramycoides*, ON-4 and SP-7, despite different morphologies, were *B. wiedmannii*, and RA-9 was *B. amyloliquefaciens*. Strains MA-7, ON-4, SP-7, and RA-9 improved pea plant growth under both pot and greenhouse conditions, potentially by producing IAA, siderophores, and ammonia, and/or solubilizing phosphate. Typically, the major mechanisms underlying direct promotion of growth by PGPR involve phytohormone and siderophore production and solubiliztion of phosphate (Bhattacharyya andJha, 2012). Furthermore, numerous PGPR species are able to chelate calcium irons or exudate organic acid and, thus, solubilized phosphate through metabolic activity (Saharan andNehra, 2011).

It was also found that strain RA-9 (*B*. *amyloliquefaciens*) was the best promoter of growth of the PGPR strains tested under pot conditions. Idriss et al. (2002) and Idris et al. (2007) reported that diluted culture filtrates or growing cells of *B*. *amyloliquefaciens* strains enhanced the growth of maize seedlings and duck weed. Other researchers have reported that *B*. *amyloliquefaciens* and *B*. *subtilis* promote plant growth by secreting extracellular phytases and releasing volatile components (Ramírez andKloepper, 2010; Ryu et al., 2003). Studies on biocontrol of plant pathogens, such as Fusarium (*Fusarium oxysporum*) and Ralstonia (*Ralstonia solanacearum*), found that *B*. *amyloliquefaciens* strains release antifungal compounds, which suppress these diseases and, thus, improve plants growth (Huang et al., 2013; Li et al., 2017; Wei et al., 2011; Yuan et al., 2013).

For greenhouse experiments, the highest number of germinated seedlings and most fresh and dry matter occurred in the presence of MA-7. This is corroborated by the work by Penrose et al. (2001), who reported that bacterial-secreted IAA stimulates cell division and promotes root elongation in seedlings. Similar result reported by Ambrosini et al. (2015) reported that *B. mycoides* strain B38V isolated from the rhizospheres of sunflower (*Helianthus annuus* L.) was shown to improve plant growth. Other researchers have identified *Bacillus* species that solubilized phosphate, produce antimicrobial peptides, and promote growth (Jouzani et al., 2017; Lee et al., 2009; Raddadi et al., 2008). Based on EzBioCloud 16S rRNA database, it was found that strains ON-4 and SP-7, despite having different morphologies were both most likely to be *B. wiedmannii*. These two strains significantly improved pea plant growth in terms of increasing shoot and root fresh weights. Liu et al. (2017) identified novel bacillus strains with more than 97% similarity to *B. cereus* strains that could be further separated into branches. These strains included *Bacillus Para mycoides*, *B. wiedmannii*, and *B. proteolyticus*

These findings corroborate the findings of this present study, where *Bacillus* species can be PGPR. In addition, it was expected that these strains also promote pea plant growth by additional mechanisms such as secreting of metabolites, production of vitamins, and facilitation of amino acids production uptake (Babalola, 2010). Furthermore, *Bacillus* species are considered an important source of bio active substances and their ability to form pores allows them to survive in a wide range of environments and increases their longevity in commercial formulation (Ongena andJacques, 2008; Pérez-García et al., 2011).

In this study, some previously overlooked PGPR and original PGPR significantly improved pea plant growth under pot and greenhouse conditions. Therefore, additional research is needed to study the mechanisms by which previously overlooked PGPR strains promote growth. In addition, further screening is required to identify previously overlooked PGPR strains for different crops under different conditions. Furthermore, sterile tap water could be a good resource to prepare and store bacterial suspensions until further work can be done.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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