Paenibacillus strains with nitrogen fixation and multiple beneficial properties for promoting plant growth

Xiaomeng Liu [1], **Qin Li** [1], **Yongbin Li** [1], **Guohua Guan** [1], **Sanfeng Chen** [Corresp. 1]

[1] State Key Laboratory for Agrobiotechnology and College of Biological Sciences, China Agricultural University, China, Beijing

Corresponding Author: Sanfeng Chen
Email address: chensf@cau.edu.cn

Paenibacillus is a large genus of Gram-positive, facultative anaerobic, endospore-forming bacteria. The genus *Paenibacillus* currently comprises more than 150 named species, approximately 20 of which have nitrogen-fixation ability. The N_2-fixing *Paenibacillus* strains have potential uses as a bacterial fertilizer in agriculture. In this study, 179 bacterial strains were isolated by using nitrogen-free medium after heating at 85℃ for 10 min from 69 soil samples collected from different plant rhizospheres in different areas. Of the 179 bacterial strains, 25 *Paenibacillus* strains had *nifH* gene encoding Fe protein of nitrogenase and showed nitrogenase activities. Of the 25 N_2-fixing *Paenibacillus* strains, 22 strains produced indole-3-acetic acid (IAA). 21 strains out of the 25 N_2-fixing *Paenibacillus* strains inhibited at least one of the 6 plant pathogens *Rhizoctonia cerealis*, *Fusarium graminearum*, *Gibberella zeae*, *Fusarium solani*, *Colletotrichum gossypii* and *Alternaria longipes*. 18 strains inhibited 5 plant pathogens and *Paenibacillus* sp. SZ-13b could inhibit the growth of all of the 6 plant pathogens. According to the nitrogenase activities, antibacterial capacities and IAA production, we chose 8 strains to inoculate wheat, cucumber and tomato. Our results showed that the 5 strains *Paenibacillus* sp. JS-4, *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. BJ-4 and *Paenibacillus* sp. SZ-15 significantly promoted plant growth and enhanced the dry weight of plants. Hence, the five strains have the greater potential to be used as good candidates for biofertilizer to facilitate sustainable development of agriculture.

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State Key Laboratory for Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing, 100193, P. R. China

* Correspondence: Sanfeng Chen

E-mail: chensf@cau.edu.cn

Tel.: +86-10-62731551

Abstract

Paenibacillus is a large genus of Gram-positive, facultative anaerobic, endospore-forming bacteria. The genus *Paenibacillus* currently comprises more than 150 named species, approximately 20 of which have nitrogen-fixation ability. The N_2-fixing *Paenibacillus* strains have potential uses as a bacterial fertilizer in agriculture. In this study, 179 bacterial strains were isolated by using nitrogen-free medium after heating at 85℃ for 10 min from 69 soil samples collected from different plant rhizospheres in different areas. Of the 179 bacterial strains, 25 *Paenibacillus* strains had *nifH* gene encoding Fe protein of nitrogenase and showed nitrogenase activities. Of the 25 N_2-fixing *Paenibacillus* strains, 22 strains produced indole-3-acetic acid (IAA). 21 strains out of the 25 N_2-fixing *Paenibacillus* strains inhibited at least one of the 6 plant pathogens *Rhizoctonia cerealis*, *Fusarium graminearum*, *Gibberella zeae*, *Fusarium solani*, *Colletotrichum gossypii* and *Alternaria longipes*. 18 strains inhibited 5 plant pathogens and *Paenibacillus* sp. SZ-13b could inhibit the growth of all of the 6 plant pathogens. According to the nitrogenase activities, antibacterial capacities and IAA production, we chose 8 strains to inoculate wheat, cucumber and tomato. Our results showed that the 5 strains *Paenibacillus* sp. JS-4, *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. BJ-4 and *Paenibacillus* sp. SZ-15 significantly promoted plant growth and enhanced the dry weight of plants. Hence, the five strains have the greater potential to be used as good candidates for biofertilizer to facilitate sustainable development of agriculture.

Introduction

Nitrogen is an essential element to affect the yields of crops by influencing leaf area development and photosynthetic efficiency (Fang et al. 2018). The application of chemical nitrogen fertilizer can improve soil fertility and thus agricultural production. High rates of nitrogen fertilizer might boost yields, but can reduce the quality of agricultural products. However, approximately 100 Tg chemical nitrogen is applied in agricultural products every year, while only 17 Tg nitrogen is accounted for in crops (Erisman et al. 2008). Excessive use of chemical fertilizer has resulted in seriously negative impacts, such as soil hardening and acidification, increased greenhouse gas (N_2O) emissions and enhanced nitrogen deposition (Jiao et al. 2018; Reay et al. 2012).

The *Paenibacillus* genus was first reclassified as a separate genus on the basis of the 16S rRNA gene sequences by Ash *et al*. (Ash et al. 1993). Since its creation, the *Paenibacillus* genus embody more than 100 validly named species. Approximately 20 members of the *Paenibacillus* genus had been reported to have the capacity of fixing nitrogen, such as: *Paenibacillus polymyxa*, *Paenibacillus macerans*, *Paenibacillus azotofixans*, *Paenibacillus sabinae*, *Paenibacillus sonchi*, *Paenibacillus forsythia*, *Paenibacillus sophorae*, *Paenibacillus taohuashanense* and *Paenibacillus beijingensis* (Grau & Wilson 1962; Hong et al. 2009; Jin et al. 2011; Ma et al. 2007; Ma & Chen 2008; Seldin et al. 1984; Wang et al. 2014; Witz et al. 1967; Xie et al. 2012). *Paenibacillus* is a group of Gram-positive, aerobic or facultative anaerobic, rod-shaped, endospore-forming bacteria. The widely distributed *Paenibacillus* bacteria could tolerate extreme environments and interact

with a variety of plants (Navarronoya et al. 2012). Currently, some *Paenibacillus* strains play a great role in agriculture and industry (Seldin 2011).

Plant rhizosphere is a habitat of functional microorganisms, which encompasses a complex and dynamic zone of interactions between networks of organisms and their plant hosts (Garcia & Kao-Kniffin 2018; Zhalnina et al. 2018). A large amount of strains isolated from plant rhizospheres are able to directly or indirectly promote plant growth, development and evolution, which are termed as plant growth-promoting rhizobacteria (PGPR) (Mohamed et al. 2019). PGPR can stimulate plant growth by a diversity of mechanisms including fixing nitrogen from atmosphere, solubilizing phosphorus, synthesizing siderophore, producing antimicrobial substances (antibiotics, bacteriocins and small peptides) and plant hormones such as indole, cytokinins or gibberellins (Graham et al. 2000; Neilands 1993). Given these advantages, PGPR are widely used in sustainable agriculture to promote plant growth and control fungal pathogens (Verma et al. 2018). Some of *Paenibacillus* species can influence plant growth by one or more of mechanisms mentioned above (Li et al. 2017; Weselowski et al. 2016; Xie et al. 2016). Nowadays, with the rapid growth of population, most regions have increased the cereals production by the overuse of fertilizers, which not only accounts for a larger percentage of farmers' expenses but also increase risks of negative effect on environment (Curatti & Rubio 2014; Ivleva et al. 2016; Tayefeh et al. 2018). It is the best choice to select the environmentally friendly *Paenibacillus* strains to substitute for chemical fertilizer due to its broad host range and its ability to secrete plant growth-enhancing substances and produce different kinds of antimicrobial substances (Cho et al. 2007; Da Mota & Seldin 2008; Fortes et al. 2008; Li et al. 2007; Timmusk et al. 2009).

The *Paenibacillus* strains have the potential to increase agricultural productivity, including weight of crops and root growth. The main purpose of this research was to isolate and identify

Paenibacillus strains, to study the effect of these isolates on plant growth, and then to select the potential bacterial strains to be used in sustainable development of agricultural production.

Materials & Methods

Sample collection，isolation procedures and culture conditions

Sixty-nine soil samples were collected from various plant rhizospheres in different areas of China, which were described in Table 1 in detail. The soil samples were diluted gradiently by 0.9% saline solution (up to 10^{-5}) and then screened on nitrogen-free medium after heating at 85℃ for 10 min. Three replicates per dilution were made. The nitrogen-free medium contained 20 g sucrose, 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g NaCl, 0.01g $FeCl_3$, 0.002 g Na_2MoO_4 and 1.2-1.4 g agar per litre of water. Single colony for each possible species was selected after cultivation for 3-5 days at 30℃. To reduce the influence of nitrogen from the soils and purify the strains, the isolates were transferred to the fresh nitrogen-free medium. The strains isolated in this study and their sources were listed in Table 1. All isolates are stored in our lab, and 16S rRNA sequences are available in database of GenBank.

Amplification, cloning and sequencing of *nifH* gene

PCR amplification of *nifH* gene was carried out using the following primers: forward 5'-GGCTGCGATCC(CGA)AAGGCCGATC(CGA)ACCCG-3' and reverse 5'-CTG(GCA)GCCTTGTTTCGCGGAT(CG)GGCATGGC-3' as described by Ding *et al.* (Ding et al. 2005). The *nifH* gene fragments were purified using TIANgel Midi Purification Kit (Tiangen Biotech Co., LTD. Cat. #DP210) and ligated to vector pGEM-T (Promega Co., Cat. #R6881) at

16℃ overnight. Recombinant plasmids were transformed into *Escherichia coli* JM109 and transformants were selected by blue/white screening procedure. Plasmids containing *nifH* gene were extracted and purified. Purified plasmids were then sequenced using the M13F and M13R primers by Shanghai Majorbio Bio-pharm Technology Co., LTD.

Morphological characterization of strains

For observation of colony morphology, the bacterial strains were spread on Luria-Bertani (LB) agar. After incubation at 37℃ overnight, single colony was observed. Cell morphology was viewed by optical microscopy (Olympus CX22LED, Japan).

Sequence analysis and construction of the phylogenetic trees

All strains were cultured in LB broth medium overnight. After collection of bacteria by centrifugation, genomic DNA of isolates was extracted and purified using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., LTD. Cat. #DP302) according to the manufacturer's instructions. The amplication of 16S rRNA genes was performed with the universal primers: 27F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1492R (5'-CGG(CT)TACCTTGTTACGACTT-3') as described by Khan *et al*. (Khan et al. 2014). Then the 16S rRNA gene fragments were ligated into vector pGEM-T (Promega Co., Cat. #R6881) and sequenced by Shanghai Majorbio Bio-pharm Technology Co., LTD. The sequences of 16S rRNA gene were submitted to nucleotide database of GenBank and the accession numbers were displayed in Table 1. And the sequences were aligned with BLAST software from NCBI (http://www.ncbi.nlm.nih.gov/Blast/).

The phylogenetic tree was constructed from evolutionary distance matrices using the neighbor-joining method with MEGA6 software package (Tamura et al. 2013). Bootstrap analysis

129 was performed with 1000 cycles, and only bootstrap values greater than 50% were shown at the
130 branch points.

131 **Nitrogenase activity assay**

132 For determination of the nitrogenase activity, strains were grown in 20 mL of LB broth
133 medium in 50 mL flasks shaken at 200 rpm overnight at 37℃. The cultures were collected by
134 centrifugation, precipitations were washed three times with sterilized water and then resuspended
135 in nitrogen-limited medium (per liter: 26.3 g Na_2HPO_4**.**$12H_2O$, 3.4 g KH_2PO_4, 26 mg $CaCl_2 \cdot 2H_2O$,
136 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg ferric citrate, 7.6 mg $Na_2MoO_4 \cdot 2H_2O$, 10 μg *p*-
137 aminobenzoic acid, 10 μg biotin, 0.4 % (w/v) glucose and 0.03 % (w/v) glutamic acid). The
138 nitrogenase activity was determined using the acetylene reduction assay and expressed as nmol
139 $C_2H_4 \cdot mg^{-1}$ protein $\cdot h^{-1}$(Wang et al. 2013; Wang et al. 2018).

140 **Assessment of antagonistic activity against plant pathogens**

141 The assessment of the *Paenibacillus* strains isolated from the rhizospheres for antagonism
142 against 6 plant pathogens including *Rhizoctonia cerealis* (ACCC 37393), *Fusarium graminearum*
143 (ACCC 36249), *Gibberella zeae* (CGMCC 3.2873), *Fusarium solani* (CGMCC 3.17848),
144 *Colletotrichum gossypii* (CGMCC 3.1859) and *Alternaria longipes* (CGMCC 3.2875), was
145 performed in agar plate assay using potato dextrose agar (PDA). The fungal pathogens were
146 inoculated in the center of the agar plate, and the *Paenibacillus* strains were placed at a distance
147 of 3.5 cm from the center of the plate. After 3-7 days of incubation at 30℃， the plates were
148 examined and measured for fungal pathogens growth inhibited zones around the *Paenibacillus*
149 strains. All tests were carried out in three duplicates.

Measurement of indole-3-acetic acid (IAA) production

The ability of producing IAA was assessed by colorimetric analysis. For the measurement of IAA production, the tested strains were grown in 20 mL King B broth medium (per liter: peptone, 20 g; K_2HPO_4, 1.15 g; $MgSO_4.7H_2O$, 1.5 g; glycerol, 10 g) supplemented with 100 $\mu g \cdot mL^{-1}$ Trp (IAA precursor). The non-cultured medium was used as the negative control and *Azospirillum brasilense* SP7 was selected as the positive control. The culture supernatants were obtained by centrifuging at 12000 rpm for 10 min. The test strains were measured by colorimetric assay according to the method described by Glickmann *et al*. (Glickmann & Dessaux 1995). Briefly, 2 mL Salkowski reagent containing 4.5 g/L $FeCl_3$ in 10.8 M H_2SO_4 was mixed with 1 mL supernatant. Then, the mixture was stired evenly and left in the darkness for 30 min at room temperature. The production of IAA was measured using spectrophotometer (Shimadzu UVmini-1240, Japan) at 530 nm. Each treatment had three biological replicates.

Evaluation of plant growth-promoting effect

The tested strains were evaluated for their potential to promote plant growth on wheat cultivar Jimai 22 (Shandong Runfeng Seed Industry Co., Ltd), cucumber Zhongnong 8 (Beijing Shengfeng Garden Agricultural Technology Co., Ltd) and tomato Jiafen 15 (Tianjin Xingke Seed Co., Ltd) seedlings in the greenhouse of China Agricultural University, Beijing, China. The lengths and dry weights of three plants inoculated with strains were determined by the procedure described by Li *et al*. (Li et al. 2017).

For preparing the bacterial cultures, each isolate was grown 150 mL LB broth medium for 24 h at 30℃. After incubation, the cells were harvested by centrifugation at 6000 rpm for 5 min at

171 room temperature. The cell pellet was washed with sterile water and then adjusted to 10^8
172 cells·mL^{-1} with 0.9% saline solution.

173 Wheat, cucumber and tomato seeds were sterilized with 10% sodium hypochlorite for 10 min
174 and washed with sterilized water three times. Then the seeds germinated on sterile wet filter in
175 Petri dishes in the dark at 25℃ for 5-7 days. After germination, seedlings were soaked in bacterial
176 suspensions (10^8 cells·mL^{-1}) for 15 min. Then three seedlings of different plants were transplanted
177 into 12-cm-diam pots containing in the medium of turfy soil (Beijing Jixiang Feiyun Garden
178 Engineering Co., Ltd. Cat. #101G) : vermiculite (Beijing Jixiang Feiyun Garden Engineering Co.,
179 Ltd. Cat. #GM010108) of 1:1, and grown in the greenhouse (16 h day/8 h night and 22°C/10°C
180 day/night temperature). Each treatment had three pots. Two weeks later, each of the seedlings was
181 watered with 15 mL bacterial suspensions (10^8 cells·mL^{-1}) again. The un-inoculated seedlings
182 were used as negative controls, while the un-inoculated seedlings watered with nitrogen fertilizer
183 (83 mg N·kg^{-1} soil) were set as positive controls (Li et al. 2019). After five-week growth, the plants
184 were harvested and the roots were washed carefully with running water to remove the adherent
185 soil. The lengths of the shoot and root and dry weights of the shoot and root were recorded and
186 statistically analyzed, respectively.

187 **Statistical analysis**

188 Each treatment had three replicates. Statistical analysis was performed using SPSS 20.0
189 (SPSS, Chicago, IL, USA). Means of different treatments were compared using the least
190 significant difference (LSD) at 0.05 level of probability.

191 **Ethics approval and consent to participate**

Not applicable.

Results

The *nifH* gene analysis and nitrogenase activity assay

Nitrogenase is comprised of two component proteins: Fe protein and MoFe protein (Mus et al. 2018). The Fe protein is encoded by *nifH* gene, and MoFe protein is encoded by *nifD* and *nifK* genes. The conserved *nifH* gene has been exploited to screen the genetic potential for nitrogen-fixing bacteria in the environment (Ding et al. 2005; Mehta et al. 2003).

In this study, 179 strains were isolated by using nitrogen-free medium after heating at at 85℃ for 10 min from 69 soil samples collected from different plant rhizospheres in different areas. PCR amplification of *nifH* gene (encoding Fe protein of nitrogenase) with universal primers was conducted using genomic DNA extracted from above bacteria. The results showed that a *nifH* gene fragment of 323 nucleotides was detected in 25 isolates (Table 1). The PCR-amplified *nifH* gene fragments from 25 isolates were sequenced and their predicted amino acid sequences of NifH were aligned with the NifH sequences from other diazotrophs. The results showed that all of them except for *Paenibacillus* sp. HN-1 shared 84%-99% NifH sequence identity with other *Paenibacillus* strains. The sequencing result of *Paenibacillus* sp. HN-1 *nifH* fragment displayed double peaks, which indicated that there were multiple *nifH* genes in its genome.

As displayed in Table 1, all of the 25 strains with *nifH* genes had nitrogenase activities with variation from 57.23 to 11868.65 nmol $C_2H_4 \cdot mg^{-1}$ protein $\cdot h^{-1}$. *Paenibacillus* sp. SZ-1b presented the highest nitrogenase activity (11868.65 nmol $C_2H_4 \cdot mg^{-1}$ protein $\cdot h^{-1}$). *Paenibacillus* sp. SZ-13a, *Paenibacillus* sp. SZ-13b, *Paenibacillus* sp. YN-3, *Paenibacillus* sp. AH-4, *Paenibacillus* sp. JS-4 and *Paenibacillus* sp. CD-4b had higher nitrogenase activities (> 3000 nmol $C_2H_4 \cdot mg^{-1}$

protein · h^{-1}). The nitrogenase activity, cell morphology, colony morphology, GenBank accession number and origin/location were listed in Table 1.

Sequencing and phylogeny of 16S rRNA

The 16S rRNA gene sequence is named as the evolution clock of bacterial phylogeny because of high conservation and slow evolution, which is widely used in identification of bacteria (Roller et al. 1994; Vandamme et al. 1996). The 16S rRNA gene sequences of the 25 strains were compared with the datebase reserved in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The alignment results indicated all of the isolates were *Paenibacillus*. The GenBank accession numbers of them after the bacterial names were shown in Table 1.

A phylogenetic tree was constructed based on 16S rRNA sequence, which branched into 5 clusters on the basis of the distance data. The cluster I totally including 17 isolates formed a larger cluster with *P. polymyxa*, *Paenibacillus jamilae* and *Paenibacillus peoriae*. Among the 17 isolates, 6 isolates exhibited 99.2%-99.6% 16S rRNA sequence similarities with *P. polymyxa*. 7 isolates had the highest similarities with *P. jamilae*, and 4 isolates showed particularly high homologies with *P. peoriae* (>99.5%). The cluster II contained 3 isolates, which displayed the highest similarity with *Paenibacillus brasilensis*, ranging from 99% to 99.2%. The cluster III only included *Paenibacillus* sp. CD-4a, which had highest 16S rRNA sequence similarity with *Paenibacillus jilunlli* (99.6%). The cluster IV which consisted of 2 strains clustered with *Paenibacillus zanthoxyli* showing 99.3% to 99.6% 16S rRNA sequence similarities with *P. zanthoxyli*. The cluster V covering 2 isolates formed a monophyletic cluster with *Paenibacillus stellifer* bacteria, and their 16S rRNA sequences similarities with *P. stellifer* were above 99%.

Antibacterial capacity determination

In the study, all 25 *Paenibacillus* strains were tested against 6 plant pathogens. The results (Table 2) showed that 21 bacteria presented antibiosis, inhibiting at least one of the 6 indicator phytopathogens. Out of them, 18 bacteria could inhibit 5 plant pathogens (*R. cerealis*, *F. graminearum*, *G. zeae*, *C. gossypii* and *A. longipes*). Furthermore, *Paenibacillus* sp. SZ-13b exhibited an extremely good antibiotic activity, which was able to inhibit the growth of all indicator phytopathogens. The growth of *F. graminearum* was strongly inhibited, showing the average inhibition zones larger than 25 mm. While the growth of *F. solani* was weakly inhibited, which was only inhibited by two strains (*Paenibacillus* sp. SZ-13b and *Paenibacillus* sp. BJ-6) with the inhibition zones around 5 and 15 mm. In addition, *Paenibacillus* sp. AH-3, *Paenibacillus* sp. HN-1, *Paenibacillus* sp. CD-4a and *Paenibacillus* sp. CD-4b could not exhibit any antibiotic effect on 6 indicator fungi.

In general, out of the 25 tested strains, 80% strains presented antimicrobial activity against plant pathogens, with average inhibition zones varying from 15 to 35 mm. Combination with their phylogeny of 16S rRNA, the isolates with inhibition flocked together, which were particularly close to *P. polymyxa* and its highly close species (Fig. 1).

Assessment of IAA production and plant growth promoting traits

IAA is an essential plant hormone regulating the growth and development of plants. In this study, we determined the ability of producing IAA for all strains. Fig. 2 showed that besides *Paenibacillus* sp. AH-1, *Paenibacillus* sp. CD-4a and *Paenibacillus* sp. CD-4b, the rest of tested strains were capable of producing IAA. Out of them, *Paenibacillus* sp. WF-6 produced the highest yield of IAA (7.19 $mg \cdot L^{-1}$). In addition, the other 9 bacteria (*Paenibacillus* sp. BJ-2, *Paenibacillus* sp. SZ-1a, *Paenibacillus* sp. SZ-1b, *Paenibacillus* sp. BJ-4, *Paenibacillus* sp. BJ-5, *Paenibacillus*

sp. BJ-6, *Paenibacillus* sp. YN-3, *Paenibacillus* sp. YB-3, *Paenibacillus* sp. JS-4) could yield relatively high amount of IAA (> 4 $mg \cdot L^{-1}$).

According to above results of nitrogenase activities, antibacterial capacities and IAA production, we chose 8 strains (*Paenibacillus* sp. SZ-1b, *Paenibacillus* sp. BJ-4, *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-13b, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. YB-3, *Paenibacillus* sp. WF-6, *Paenibacillus* sp. JS-4) to assess their capabilities of promoting growth of plants (wheat, cucumber and tomato). Inoculation of plants with some *Paenibacillus* isolates appeared to promote plant growth including plant height and dry weight (Fig. 3 and Fig. 4). As shown in Fig. 4A, wheat seedlings inoculated with *Paenibacillus* sp. JS-4 led to a maximum increase (30.9%) in shoot length, followed by *Paenibacillus* sp. SZ-1b (23.3%) and *Paenibacillus* sp. BJ-4 (22.3%). While inoculation with *Paenibacillus* sp. SZ-14 yielded a maximum increase (54.2%) in root length, followed by *Paenibacillus* sp. JS-4 (18.2%). Inoculation of wheat plants with *Paenibacillus* sp. JS-4 showed a greatly significant increase in shoot and root dry weights. Besides, *Paenibacillus* sp. BJ-4 and *Paenibacillus* sp. SZ-10 had higher dry weights of shoot and root as compared to the controls (Fig. 4B). The effects of these two bacteria on wheat seedlings were equal to the positive control with chemical nitrogen fertilizer. In Fig. 4C, cucumber seedlings inoculated with *Paenibacillus* sp. SZ-10 resulted in the highest heights both in shoot (50.0%) and in root (94.4%), followed by *Paenibacillus* sp. SZ-14 (33.7% and 38.7%, respectively) and *Paenibacillus* sp. WF-6 (18.4% and 62.4%, respectively). In addition, inoculation with *Paenibacillus* sp. SZ-10 presented the highest increase in dry weights of shoot and root of eight selected isolates, which showed more significant effect on cucumber seedlings than the positive control. And inoculation with *Paenibacillus* sp. SZ-14 had the second highest increase in total dry weight (Fig. 4D), which was the same as the positive control with chemical nitrogen fertilizer. Overall, *Paenibacillus* sp. SZ-

10 showed significant growth-promoting effects on the cucumber plants. As shown in Fig. 4E and F, most isolates could promote growth of tomato. Out of them, inoculation with *Paenibacillus* sp. BJ-4 presented to enhance development of tomato length, both in shoot (64.6%) and in root (55.2%) (Fig. 4E). And inoculation with *Paenibacillus* sp. SZ-15 displayed maximum increases in shoot and root dry weights (Fig. 4F), which showed more promotive effect on shoot dry weight of tomato than the positive control.

Discussion

Paenibacillus species are ubiquitous in nature, and they are capable to form resistant endospores to allow them surviving in a wide range of environmental variables and to enhance plant growth by several mechanisms (Bloemberg & Lugtenberg 2001). In this study, 179 bacterial strains were isolated by their growth on nitrogen-free medium from plant rhizosphere all over China. 16S rRNA sequence analysis showed that 25 of 179 bacteria belong to *Paenibacillus* genus.

We revealed that 25 *Paenibacillus* strains had the *nifH* gene encoding the Fe protein of Mo-nitrogenase. Also, the 25 *Paenibacillus* strains exhibited nitrogenase activities. These results demonstrated that the 25 N_2-fixing *Paenibacillus* strains could provide nitrogen for plants. Phylogenetic analysis showed that the 25 N_2-fixing *Paenibacillus* strains were divided into five clusters. 20 of the 25 N_2-fixing *Paenibacillus* strains were in cluster I and cluster II that were closely related to *P. polymyxa*, *P. jamilae*, *P. peoriae*, and *P. brasilensis*. The other five N_2-fixing

300 *Paenibacillus* strains belonged to cluster III, cluster IV and cluster V (including *P. jilunlii*, *P.*
301 *zanthoxyli*, and *P. stellifer* mainly).

302 In this study, 20 of the 25 N_2-fixing *Paenibacillus* strains had inhibitory effects against plant
303 pathogenic fungi, with average inhibition zones varying from 15 to 35 mm on plate. Especially,
304 *Paenibacillus* sp. SZ-13b could suppress 6 tested bacterial plant pathogens. Wherease,
305 *Paenibacillus* sp. SZ-1b, *Paenibacillus* sp. SZ-15, and *Paenibacillus* sp. JS-4 could suppress 5
306 tested bacterial plant pathogens with strong inhibition activities. The 20 strains with inhibitory
307 effects against plant pathogenic fungi belonged to cluster I and cluster Ⅱ that were closely related
308 to *P. polymyxa*, *P. jamilae*, *P. peoriae*, and *P. brasilensis*. Our results are consistent with the
309 previous results that *P. polymyxa* have long been known for their great ability to produce peptide
310 antibiotics to suppress the growth of plant pathogenic fungi (Deng et al. 2011; He et al. 2007;
311 Helbig 2001; Raza et al. 2008). For examples, *P. polymyxa* M1 (HE577054), which was isolated
312 from root tissues of wheat, was able to promote wheat growth and suppress several phytopathogens
313 (Niu et al. 2011; Yao et al. 2008). *P. polymyxa* SQR-21 (CP006872) selected from the rhizosphere
314 soil of watermelon could significantly inhibit *F. oxysporum* (Raza et al. 2009). *P. brasilensis* PB1
315 72 (NR025106) isolated from the maize rhizosphere was able to protect seeds and roots against
316 phytopathogenic fungi (*Fusarium moniliforme* and *Diplodia macrospora*) (von der Weid et al.
317 2005; von der Weid et al. 2002).

318 Additionally, 22 N_2-fixing *Paenibacillus* strains (except for *Paenibacillus* sp. AH-1,
319 *Paenibacillus* sp. CD-4a and *Paenibacillus* sp. CD-4b) were capable of producing IAA, which is
320 a primary plant hormone regulating plant growth and development. Among them, *Paenibacillus*

sp. WF-6, *Paenibacillus* sp. SZ-1a, *Paenibacillus* sp. SZ-1b, *Paenibacillus* sp. BJ-5, *Paenibacillus* sp. YB-3 generated higher yield of IAA.

According to the results of nitrogenase activity, IAA level and inhibitory effect against plant pathogens, 8 strains were chosen to inoculate wheat seedlings, cucumber seedlings and tomato seedlings to analyse their plant promotion effects. We found that *Paenibacillus* sp. JS-4 and *Paenibacillus* sp. BJ-4 promoted wheat growth as well as the chemical nitrogen fertilizer did. While *Paenibacillus* sp. SZ-10 and *Paenibacillus* sp. SZ-14 promoted cucumber growth as well as the chemical nitrogen fertilizer did. The 2 strains *Paenibacillus* sp. SZ-15 and *Paenibacillus* sp. BJ-4 significantly promoted tomato growth. Moreover, the 4 strains including *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. YB-10, and *Paenibacillus* sp. WF-6 could promote tomato growth. From these results, we found that the plant promotion effects exhibited by a *Paenibacillus* strain varied among plants. At present, we do not know why a same *Paenibacillus* strain had different promotion effects on different plants.

Taken together, 25 N_2-fixning *Paenibacillus* strains were isolated from plant rhizospheres. The 5 strains including *Paenibacillus* sp. JS-4, *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. BJ-4 and *Paenibacillus* sp. SZ-15 with the significant effects of promoting plant growth have great potential as bio-fertilizer.

Microbial fertilizers are widely used in plantation of vegetables in China. The members of *Bacillus* genus, such as *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, are usually used in biofertilizers. The *Paenibacillus* strains with nitrogen fixation and multiple bacterial properties for promoting plant growth obtained in this study have great potential to be developed as biofertilizers.

Conclusion

In conclusion, 25 N_2-fixing *Paenibacillus* strains were isolated from plant rhizospheres. Most of them possessed multiple beneficial properties and characteristics of PGPR. They could fix atmospheric nitrogen, produce the profitable phytohormone IAA, control against a wide set of plant pathogens, and enhance growth of diverse important plants. Especially, the 5 strains including *Paenibacillus* sp. JS-4, *Paenibacillus* sp. SZ-10, *Paenibacillus* sp. SZ-14, *Paenibacillus* sp. BJ-4 and *Paenibacillus* sp. SZ-15 with the significant effects of promoting plant growth could be developed and commercially formulated to substitute for environmentally harmful chemical fertilizer and pesticides in field experiments.

Figure captions

Figure 1: Neighbour-joining phylogenetic tree based on 16S rRNA sequence showing the position of isolated strains with other closely related strains of the genus *Paenibacillus* in GenBank. The tree was structured using neighbor joining method, with the bootstrap percentage values obtained from 1000 cycles. Only bootstrap values greater than 50% are shown at the branching points. Bar, 0.005 substitutions per nucleotide position. Isolated strains in this study are underlined with the bold letters.

363 **Figure 2**: Qualitative analysis of IAA production by isolated strains. Data are means ± SE of
364 three independent biological replicates. Bearing different alphabets are significantly different
365 from each other according to the LSD test ($p < 0.05$).

366 **Figure 3**: Plant growth promotion by some *Paenibacillus* strains. (A) Wheat seedlings
367 inoculated with *Paenibacillus* sp. JS-4; (B) Cucumber seedlings inoculated with *Paenibacillus*
368 sp. SZ-10; (C) Tomato seedlings inoculated with *Paenibacillus* sp. SZ-15.

369 **Figure 4**: Effects of eight selected strains inoculation on shoot and root length of wheat (A),
370 dry weight of wheat (B), on shoot and root length of cucumber (C), dry weight of cucumber
371 (D), on shoot and root length of tomato (E), dry weight of tomato (F). Control: un-inoculated
372 seedlings. Date represent the means ± SE of 3 independent biological replicates. In the root
373 group or shoot group, bearing different alphabets are significantly different from each other
374 according to the LSD test ($p < 0.05$).

375 **Table captions**

376 **Table 1**: Characterization and nitrogenase activity of isolates.

377 **Table 2**: Antimicrobial activity of *Paenibacillus* isolates, which inhibit 6 indicator bacteria.

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Figure 1

Figure 1:Neighbour-joining phylogenetic tree based on 16S rRNA sequence showing the position of isolated strains with other closely related strains of the genus *Paenibacillus* in GenBank.

The tree was structured using neighbor joining method, with the bootstrap percentage values obtained from 1000 cycles. Only bootstrap values greater than 50% are shown at the branching points. Bar, 0.005 substitutions per nucleotide position. Isolated strains in this study are underlined with the bold letters.

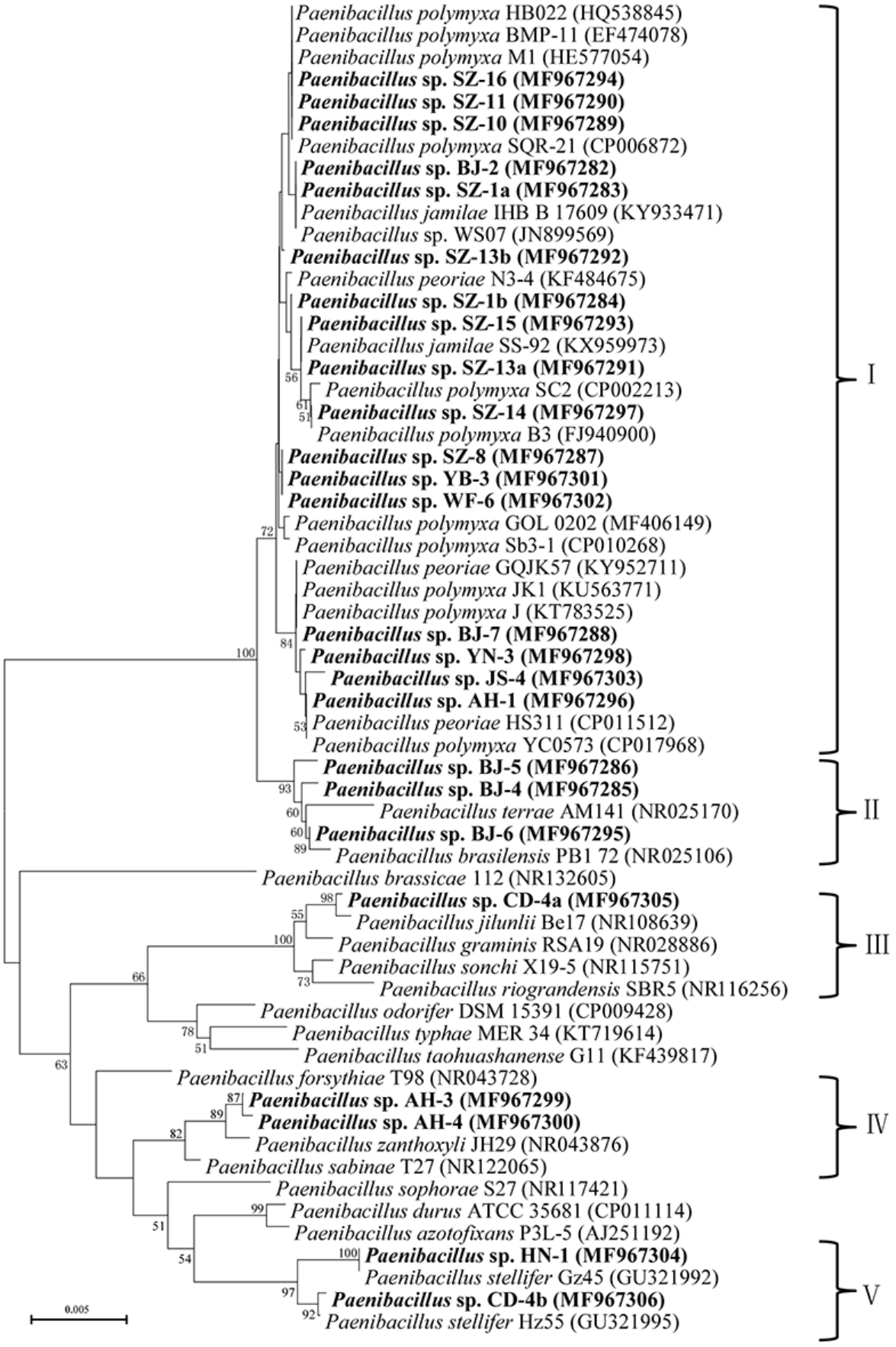
Paenibacillus polymyxa HB022 (HQ538845)
Paenibacillus polymyxa BMP-11 (EF474078)
Paenibacillus polymyxa M1 (HE577054)
***Paenibacillus* sp. SZ-16 (MF967294)**
***Paenibacillus* sp. SZ-11 (MF967290)**
***Paenibacillus* sp. SZ-10 (MF967289)**
Paenibacillus polymyxa SQR-21 (CP006872)
***Paenibacillus* sp. BJ-2 (MF967282)**
***Paenibacillus* sp. SZ-1a (MF967283)**
Paenibacillus jamilae IHB B 17609 (KY933471)
Paenibacillus sp. WS07 (JN899569)
***Paenibacillus* sp. SZ-13b (MF967292)**
Paenibacillus peoriae N3-4 (KF484675)
***Paenibacillus* sp. SZ-1b (MF967284)**
***Paenibacillus* sp. SZ-15 (MF967293)**
Paenibacillus jamilae SS-92 (KX959973)
***Paenibacillus* sp. SZ-13a (MF967291)**
Paenibacillus polymyxa SC2 (CP002213)
***Paenibacillus* sp. SZ-14 (MF967297)**
Paenibacillus polymyxa B3 (FJ940900)
***Paenibacillus* sp. SZ-8 (MF967287)**
***Paenibacillus* sp. YB-3 (MF967301)**
***Paenibacillus* sp. WF-6 (MF967302)**
Paenibacillus polymyxa GOL 0202 (MF406149)
Paenibacillus polymyxa Sb3-1 (CP010268)
Paenibacillus peoriae GQJK57 (KY952711)
Paenibacillus polymyxa JK1 (KU563771)
Paenibacillus polymyxa J (KT783525)
***Paenibacillus* sp. BJ-7 (MF967288)**
***Paenibacillus* sp. YN-3 (MF967298)**
***Paenibacillus* sp. JS-4 (MF967303)**
***Paenibacillus* sp. AH-1 (MF967296)**
Paenibacillus peoriae HS311 (CP011512)
Paenibacillus polymyxa YC0573 (CP017968)
***Paenibacillus* sp. BJ-5 (MF967286)**
***Paenibacillus* sp. BJ-4 (MF967285)**
Paenibacillus terrae AM141 (NR025170)
***Paenibacillus* sp. BJ-6 (MF967295)**
Paenibacillus brasilensis PB1 72 (NR025106)
Paenibacillus brassicae 112 (NR132605)
***Paenibacillus* sp. CD-4a (MF967305)**
Paenibacillus jilunlii Be17 (NR108639)
Paenibacillus graminis RSA19 (NR028886)
Paenibacillus sonchi X19-5 (NR115751)
Paenibacillus riograndensis SBR5 (NR116256)
Paenibacillus odorifer DSM 15391 (CP009428)
Paenibacillus typhae MER 34 (KT719614)
Paenibacillus taohuashanense G11 (KF439817)
Paenibacillus forsythiae T98 (NR043728)
***Paenibacillus* sp. AH-3 (MF967299)**
***Paenibacillus* sp. AH-4 (MF967300)**
Paenibacillus zanthoxyli JH29 (NR043876)
Paenibacillus sabinae T27 (NR122065)
Paenibacillus sophorae S27 (NR117421)
Paenibacillus durus ATCC 35681 (CP011114)
Paenibacillus azotofixans P3L-5 (AJ251192)
***Paenibacillus* sp. HN-1 (MF967304)**
Paenibacillus stellifer Gz45 (GU321992)
***Paenibacillus* sp. CD-4b (MF967306)**
Paenibacillus stellifer Hz55 (GU321995)
I
II
III
IV
V
0.005

Figure 2

Figure 2: Qualitative analysis of IAA production by isolated strains.

Data are means ± SE of threeindependent biological replicates. Bearing different alphabets are significantly different from each other according to the LSD test ($p < 0.05$).

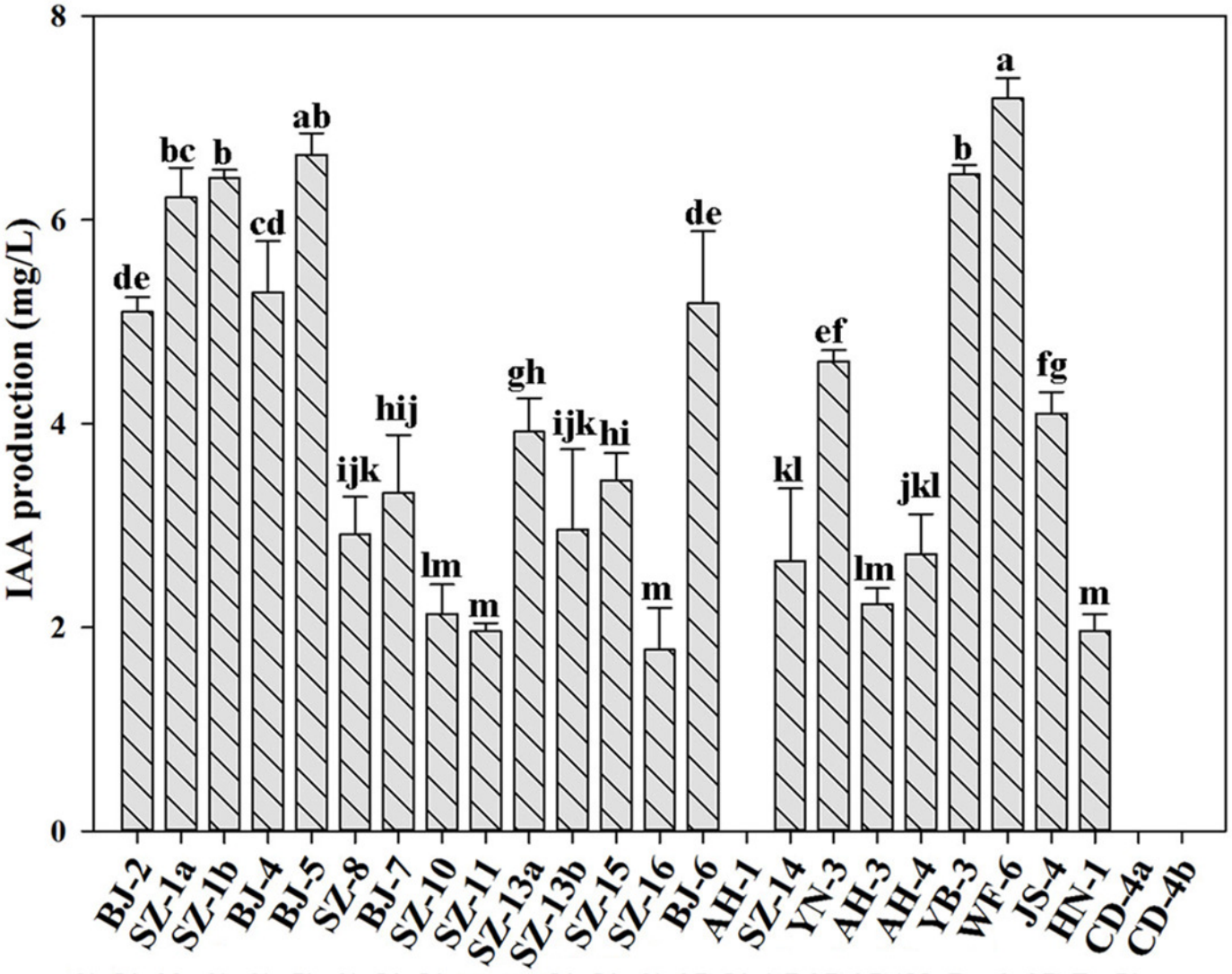

Figure 3

Figure 3: Plant growth promotion by some *Paenibacillus* strains.

(A) Wheat seedlings inoculated with *Paenibacillus* sp. JS-4; (B) Cucumber seedlings inoculated with *Paenibacillus* sp. SZ-10; (C) Tomato seedlings inoculated with *Paenibacillus* sp. SZ-15.

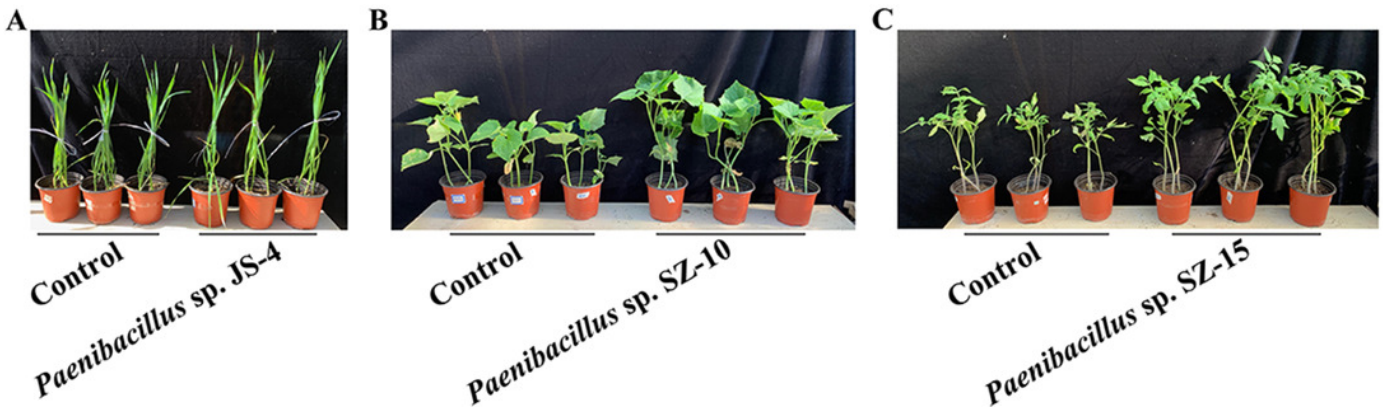

Figure 4

Figure4: Effects of eight selected strains inoculation on shoot and root length of wheat (A), dry weight of wheat (B), on shoot and root length of cucumber (C), dry weight of cucumber (D), on shoot and root length of tomato (E), dry weight of tomato (F).

Control: un-inoculated seedlings. Date represent the means ± SE of 3 independent biological replicates. In the root group or shoot group, bearing different alphabets are significantly different from each other according to the LSD test ($p < 0.05$).

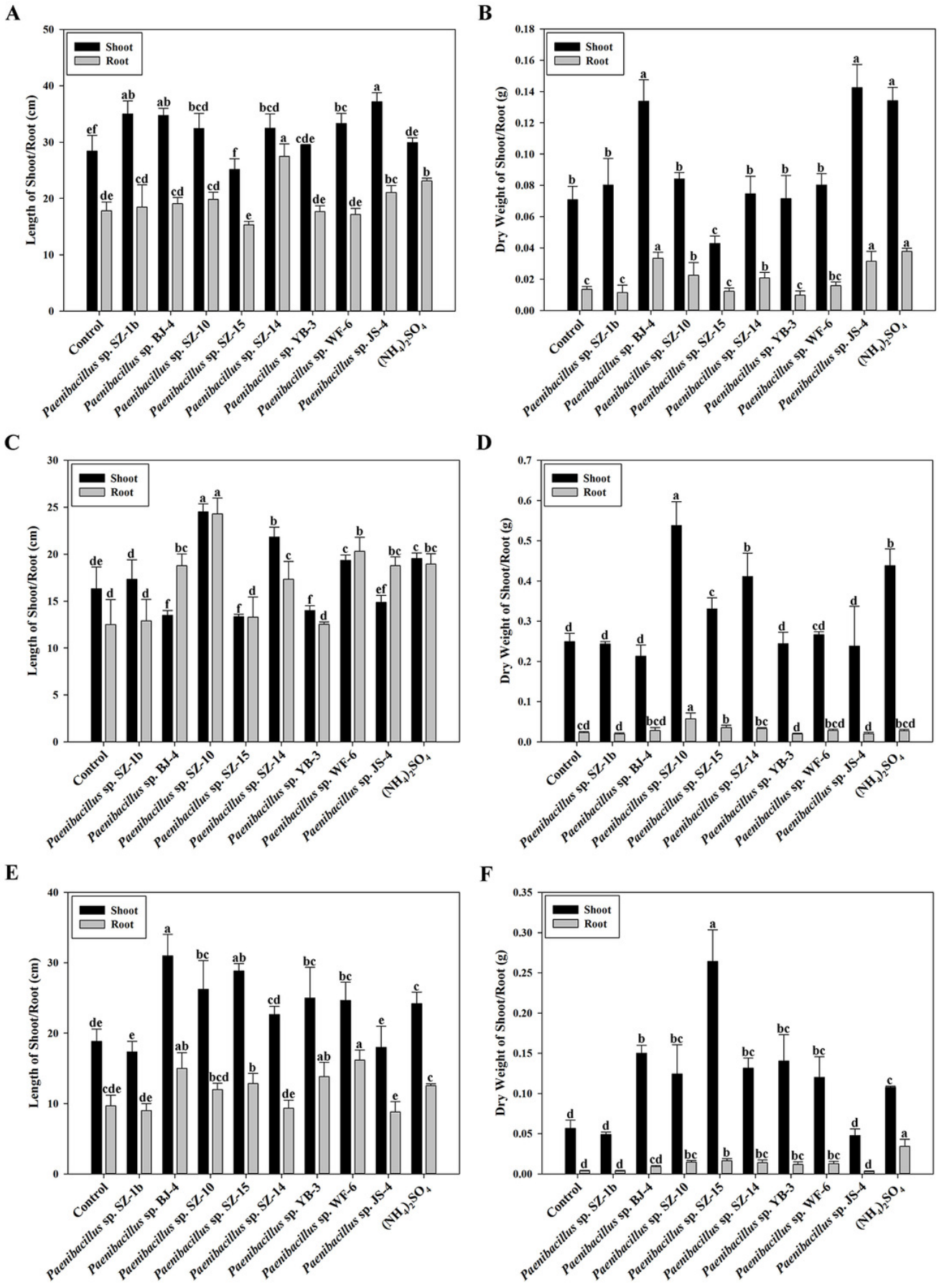
A
Length of Shoot/Root (cm)
Shoot
Root
B
Dry Weight of Shoot/Root (g)
C
Length of Shoot/Root (cm)
D
Dry Weight of Shoot/Root (g)
E
Length of Shoot/Root (cm)
F
Dry Weight of Shoot/Root (g)
Control
Paenibacillus sp. SZ-1b
Paenibacillus sp. BJ-4
Paenibacillus sp. SZ-10
Paenibacillus sp. SZ-15
Paenibacillus sp. SZ-14
Paenibacillus sp. YB-3
Paenibacillus sp. WF-6
Paenibacillus sp. JS-4
(NH4)2SO4

Table 1(on next page)

Characterization and nitrogenase activity of isolates.

1 **Table1.** Characterization and nitrogenase activity of isolates.

Isolates	Cell morphology	Colony morphology	Nitrogenase activity[a]	GenBank accession number	Origin and Location
Paenibacillus sp. BJ-2	Rods	Moist, milky	1085.61±75.64^{ghi}	MF967282	Jujube, mountain in Huairou, Beijing 40°32′ N, 116°62′ E
Paenibacillus sp. SZ-1a	Rods	Moist, milky	118.65±3.97^{k}	MF967283	Maize, farmland in Changping, Beijing 40°22′ N, 116°20′ E
Paenibacillus sp. SZ-1b	Rods	Moist, milky	11868.65±1740.55^{a}	MF967284	Maize, farmland in Changping, Beijing 40°22′ N, 116°20′ E
Paenibacillus sp. BJ-4	Rods	Dry, milky	1296.94±439.17^{g}	MF967285	Apple, orchard in Shunyi, Beijing 40°13′ N, 116°65′ E
Paenibacillus sp. BJ-5	Rods	Dry, white	468.63±42.20^{hijk}	MF967286	Persimmon, mountain in Shunyi, Beijing 40°13′ N, 116°65′ E
Paenibacillus sp SZ-8	Rods	Moist, milky	1131.54±15.92^{gh}	MF967287	Maize, field in Changping, Beijing 40°22′ N, 116°20′ E
Paenibacillus sp. BJ-7	Rods	Moist, milky	314.60±19.18^{jk}	MF967288	Wheat, farmland in Miyun, Beijing 40°37′ N, 116°85′ E
Paenibacillus sp. SZ-10	Short rods	Moist, milky	371.28±7.67^{ijk}	MF967289	Maize, farmland in Changping, Beijing 40°22′ N, 116°20′ E

Paenibacillus sp. SZ-11	Rods	Moist, milky	857.47±114.89^{ghij}	MF967290	Pepper, herbary in Changping, Beijing
Paenibacillus sp. SZ-13a	Rods	Dry, milky	9731.36±259.71^{b}	MF967291	40°22′ N, 116°20′ E Medicinal plant, farmland in Changping, Beijing
Paenibacillus sp. SZ-13b	Rods	Dry, milky	3131.89±100.61^{e}	MF967292	40°22′ N, 116°20′ E Medicinal plant, farmland in Changping, Beijing
Paenibacillus sp. SZ-15	Rods	Moist, milky	1316.19±36.64^{g}	MF967293	40°22′ N, 116°20′ E Wheat, farmland in Changping, Beijing
Paenibacillus sp. SZ-16	Rods	Moist, milky	444.73±119.11^{hijk}	MF967294	40°22′ N, 116°20′ E Spinach, herbary in Changping, Beijing
Paenibacillus sp. BJ-6	Short rods	Dry, milky	176.7±29.43^{jk}	MF967295	40°22′ N, 116°20′ E Bamboo, mountain in Huairou, Beijing
Paenibacillus sp. AH-1	Short rods	Moist, milky	192.43±73.08^{jk}	MF967296	40°32′ N, 116°62′ E Grape, orchard in Hefei, Anhui
Paenibacillus sp. SZ-14	Rods	Moist, milky	331.95±22.73^{jk}	MF967297	31°86′ N, 117°27′ E Rice, farmland in Changping, Beijing
Paenibacillus sp. YN-3	Short rods	Moist, white	3201.92±104.96^{e}	MF967298	40°22′ N, 116°20′ E Sugarcane, farmland in Pu’er, Yunnan
Paenibacillus sp. AH-3	Short rods	Moist, white	57.23±14.44^{k}	MF967299	23°07′ N, 110°03′ E Arbor, natural forest in Wuhu, Anhui
Paenibacillus	Short rods	Moist, white	6514.37±997.12^{c}	MF967300	31°95′ N, 118°73′ E Arbor, natural forest in

sp. AH-4					Hefei, Anhui
Paenibacillus sp. YB-3	Rods	Moist, milky	733.92±49.28^{ghijk}	MF967301	31°95′ N, 118°73′ E Fruit, mountain in Yibin, Sichuan
Paenibacillus sp. WF-6	Rods	Moist, milky	2081.30±340.66^{f}	MF967302	28°77′ N, 104°62′ E Wheat, field in Weifang, Shandong
Paenibacillus sp. JS-4	Rods	Moist, milky	6843.56±365.69^{c}	MF967303	36°62′ N, 119°10′ E Reed, countryside in Suzhou, Jiangsu
Paenibacillus sp. HN-1	Short rods	Moist, milky	4476.80±306.64^{d}	MF967304	31°32′ N, 120°62′ E Rice, farmland in Xiangtan, Hunan
Paenibacillus sp. CD-4a	Rods	Moist, milky	272.67±14.24^{jk}	MF967305	27°52′ N, 112°53′ E Rape, field in Chengdu, Sichuan
Paenibacillus sp. CD-4b	Short rods	Moist, milky	5174.69±478.7^{d}	MF967306	30°67′ N, 104°07′ E Fruit, mountain in Chengdu, Sichuan
					30°67′ N, 104°07′ E

a: The unit of nitrogenase activity is nmol $C_2H_4 \cdot mg^{-1}$ protein $\cdot h^{-1}$.

Results are means ± SE of 3 independent biological replicates. Different letters are significantly different from each other according to the least significant differences (LSD) test (P<0.05).

Table 2(on next page)

Table 2: Antimicrobial activity of the *Paenibacillus* strains, which inhibit 6 indicator bacteria.

Table 2. Antimicrobial activity of the *Paenibacillus* strains, which inhibit 6 indicator bacteria

Strains	*R. cer*	*F. gra*	*G. zeae*	*F. sol*	*C. gos*	*A. lon*
Paenibacillus sp. BJ-2	++	++	++	-	+++	+
Paenibacillus sp. SZ-1a	++	++	++	-	++	++
Paenibacillus sp. SZ-1b	+++	+++	++	-	++	+++
Paenibacillus sp. BJ-4	++	+++	++	-	++	+++
Paenibacillus sp. BJ-5	-	+++	+	-	++	++
Paenibacillus sp. SZ-8	++	+++	++	-	+++	++
Paenibacillus sp. BJ-7	++	+++	+++	-	++	+
Paenibacillus sp. SZ-10	++	+++	+++	-	++	++
Paenibacillus sp. SZ-11	++	++	++	-	++	++
Paenibacillus sp. SZ-13a	+++	++	++	-	++	++
Paenibacillus sp. SZ-13b	++	++	++	+	++	++
Paenibacillus sp. SZ-15	+++	++	+++	-	+++	++
Paenibacillus sp. SZ-16	++	++	++	-	++	++
Paenibacillus sp. BJ-6	++	++	-	+	++	-
Paenibacillus sp. AH-1	+++	+++	+++	-	++	++
Paenibacillus sp. SZ-14	++	+++	++	-	+++	++
Paenibacillus sp. YN-3	++	++	++	-	++	++
Paenibacillus sp. AH-3	-	-	-	-	-	-
Paenibacillus sp. AH-4	+	-	-	-	-	-
Paenibacillus sp. YB-3	++	+++	++	-	++	+++
Paenibacillus sp. WF-6	++	++	++	-	++	++
Paenibacillus sp. JS-4	++	+++	++	-	+++	+++
Paenibacillus sp. HN-1	-	-	-	-	-	-
Paenibacillus sp. CD-4a	-	-	-	-	-	-
Paenibacillus sp. CD-4b	-	-	-	-	-	-

R. cer, *R. cerealis*; *F. gra*, *F. graminearum*; *G. zeae*, *G. zeae*; *F. sol*, *F. solani*; *C. gos*, *C. gossypii*; *A. lon*, *A. longipes*. (-), no inhibition; (+), inhibition zone diameters from 5 to 15 mm; (++), inhibition zone diameters from 15 to 25 mm; (+++), inhibition zone diameters from 25 to 35 mm.