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

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*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°C 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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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°C 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\textsubscript{2}O) emissions and enhanced nitrogen deposition (Jiao et al. 2018; Reay et al. 2012).

The \textit{Paenibacillus} genus was first reclassified as a separate genus on the basis of the 16S rRNA gene sequences by Ash \textit{et al.} (Ash \textit{et al.} 1993). Since its creation, the \textit{Paenibacillus} genus embody more than 100 validly named species. Approximately 20 members of the \textit{Paenibacillus} genus had been reported to have the capacity of fixing nitrogen, such as: \textit{Paenibacillus polymyxa}, \textit{Paenibacillus macerans}, \textit{Paenibacillus azotofixans}, \textit{Paenibacillus sabinae}, \textit{Paenibacillus sonchi}, \textit{Paenibacillus forsythia}, \textit{Paenibacillus sophorae}, \textit{Paenibacillus taohuashanense} and \textit{Paenibacillus beijingensis} (Grau & Wilson 1962; Hong \textit{et al.} 2009; Jin \textit{et al.} 2011; Ma \textit{et al.} 2007; Ma & Chen 2008; Seldin \textit{et al.} 1984; Wang \textit{et al.} 2014; Witz \textit{et al.} 1967; Xie \textit{et al.} 2012). \textit{Paenibacillus} is a group of Gram-positive, aerobic or facultative anaerobic, rod-shaped, endospore-forming bacteria. The widely distributed \textit{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°C for 10 min. Three replicates per dilution were made. The nitrogen-free medium contained 20 g sucrose, 0.1 g K$_2$HPO$_4$, 0.4 g KH$_2$PO$_4$, 0.2 g MgSO$_4$·7H$_2$O, 0.01 g NaCl, 0.01 g FeCl$_3$, 0.002 g Na$_2$MoO$_4$ 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°C. 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)AAGGCGATC(CGA)ACCCG-3' and reverse 5'-CTG(GCA)GCCTTGTTCGCAGAT(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°C 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°C 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 amplification 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
was performed with 1000 cycles, and only bootstrap values greater than 50% were shown at the branch points.

**Nitrogenase activity assay**

For determination of the nitrogenase activity, strains were grown in 20 mL of LB broth medium in 50 mL flasks shaken at 200 rpm overnight at 37°C. The cultures were collected by centrifugation, precipitations were washed three times with sterilized water and then resuspended in nitrogen-limited medium (per liter: 26.3 g Na$_2$HPO$_4$·12H$_2$O, 3.4 g KH$_2$PO$_4$, 26 mg CaCl$_2$·2H$_2$O, 30 mg MgSO$_4$, 0.3 mg MnSO$_4$, 36 mg ferric citrate, 7.6 mg Na$_2$MoO$_4$·2H$_2$O, 10 μg p-aminobenzoic acid, 10 μg biotin, 0.4 % (w/v) glucose and 0.03 % (w/v) glutamic acid). The nitrogenase activity was determined using the acetylene reduction assay and expressed as nmol C$_2$H$_4$ · mg$^{-1}$ protein · h$^{-1}$ (Wang et al. 2013; Wang et al. 2018).

**Assessment of antagonistic activity against plant pathogens**

The assessment of the *Paenibacillus* strains isolated from the rhizospheres for antagonism against 6 plant pathogens including *Rhizoctonia cerealis* (ACCC 37393), *Fusarium graminearum* (ACCC 36249), *Gibberella zeae* (CGMCC 3.2873), *Fusarium solani* (CGMCC 3.17848), *Colletotrichum gossypii* (CGMCC 3.1859) and *Alternaria longipes* (CGMCC 3.2875), was performed in agar plate assay using potato dextrose agar (PDA). The fungal pathogens were inoculated in the center of the agar plate, and the *Paenibacillus* strains were placed at a distance of 3.5 cm from the center of the plate. After 3-7 days of incubation at 30°C, the plates were examined and measured for fungal pathogens growth inhibited zones around the *Paenibacillus* 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$_2$HPO$_4$, 1.15 g; MgSO$_4$•7H$_2$O, 1.5 g; glycerol, 10 g) supplemented with 100 μg·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$_2$SO$_4$ was mixed with 1 mL supernatant. Then, the mixture was stirred 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°C. After incubation, the cells were harvested by centrifugation at 6000 rpm for 5 min at
room temperature. The cell pellet was washed with sterile water and then adjusted to $10^8$ cells·mL$^{-1}$ with 0.9% saline solution.

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

**Statistical analysis**

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

**Ethics approval and consent to participate**
Results

The \textit{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 \textit{nifH} gene, and MoFe protein is encoded by \textit{nifD} and \textit{nifK} genes. The conserved \textit{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°C for 10 min from 69 soil samples collected from different plant rhizospheres in different areas. PCR amplification of \textit{nifH} gene (encoding Fe protein of nitrogenase) with universal primers was conducted using genomic DNA extracted from above bacteria. The results showed that a \textit{nifH} gene fragment of 323 nucleotides was detected in 25 isolates (Table 1). The PCR-amplified \textit{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 \textit{Paenibacillus} sp. HN-1 shared 84%-99% NifH sequence identity with other \textit{Paenibacillus} strains. The sequencing result of \textit{Paenibacillus} sp. HN-1 \textit{nifH} fragment displayed double peaks, which indicated that there were multiple \textit{nifH} genes in its genome.

As displayed in Table 1, all of the 25 strains with \textit{nifH} genes had nitrogenase activities with variation from 57.23 to 11868.65 nmol C$_2$H$_4$ · mg$^{-1}$ protein · h$^{-1}$. \textit{Paenibacillus} sp. SZ-1b presented the highest nitrogenase activity (11868.65 nmol C$_2$H$_4$ · mg$^{-1}$ protein · h$^{-1}$). \textit{Paenibacillus} sp. SZ-13a, \textit{Paenibacillus} sp. SZ-13b, \textit{Paenibacillus} sp. YN-3, \textit{Paenibacillus} sp. AH-4, \textit{Paenibacillus} sp. JS-4 and \textit{Paenibacillus} sp. CD-4b had higher nitrogenase activities (> 3000 nmol C$_2$H$_4$ · 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 database 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·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.
sp. BJ-6, Paenibacillus sp. YN-3, Paenibacillus sp. YB-3, Paenibacillus sp. JS-4) could yield relatively high amount of IAA (> 4 mg·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 N₂-fixing *Paenibacillus* strains could provide nitrogen for plants. Phylogenetic analysis showed that the 25 N₂-fixing *Paenibacillus* strains were divided into five clusters. 20 of the 25 N₂-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 N₂-fixing
*Paenibacillus* strains belonged to cluster III, cluster IV and cluster V (including *P. jilunlui*, *P. zanthoxyli*, and *P. stellifer* mainly).

In this study, 20 of the 25 N₂-fixing *Paenibacillus* strains had inhibitory effects against plant pathogenic fungi, with average inhibition zones varying from 15 to 35 mm on plate. Especially, *Paenibacillus* sp. SZ-13b could suppress 6 tested bacterial plant pathogens. Wherease, *Paenibacillus* sp. SZ-1b, *Paenibacillus* sp. SZ-15, and *Paenibacillus* sp. JS-4 could suppress 5 tested bacterial plant pathogens with strong inhibition activities. The 20 strains with inhibitory effects against plant pathogenic fungi belonged to cluster I and cluster II that were closely related to *P. polymyxa*, *P. jamilae*, *P. peoriae*, and *P. brasilensis*. Our results are consistent with the previous results that *P. polymyxa* have long been known for their great ability to produce peptide antibiotics to suppress the growth of plant pathogenic fungi (Deng et al. 2011; He et al. 2007; Helbig 2001; Raza et al. 2008). For examples, *P. polymyxa* M1 (HE577054), which was isolated from root tissues of wheat, was able to promote wheat growth and suppress several phytopathogens (Niu et al. 2011; Yao et al. 2008). *P. polymyxa* SQR-21 (CP006872) selected from the rhizosphere soil of watermelon could significantly inhibit *F. oxysporum* (Raza et al. 2009). *P. brasilensis* PB1 72 (NR025106) isolated from the maize rhizosphere was able to protect seeds and roots against phytopathogenic fungi (*Fusarium moniliforme* and *Diplodia macrospora*) (von der Weid et al. 2005; von der Weid et al. 2002).

Additionally, 22 N₂-fixing *Paenibacillus* strains (except for *Paenibacillus* sp. AH-1, *Paenibacillus* sp. CD-4a and *Paenibacillus* sp. CD-4b) were capable of producing IAA, which is 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 N₂-fixing *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.
Figure 2: Qualitative analysis of IAA production by isolated strains. Data are means ± SE of three independent biological replicates. Bearing different alphabets are significantly different from each other according to the LSD test ($p < 0.05$).

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: 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 ($p < 0.05$).

Table captions

Table 1: Characterization and nitrogenase activity of isolates.

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

Ash C, Priest FG, and Collins MD. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. Antonie Van Leeuwenhoek 64:253.

Cho KM, Hong SY, Lee SM, Kim YH, Kahng GG, Lim YP, Kim H, and Yun HD. 2007. Endophytic bacterial communities in ginseng and their antifungal activity against pathogens. Microbial Ecology 54:341-351 DOI 10.1007/s00248-007-9208-3.

Curatti L, and Rubio LM. 2014. Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Science 225:130-137 DOI 10.1016/j.plantsci.2014.06.003.

Da Mota FFG, Eliane Aparecida, and Seldin L. 2008. Auxin production and detection of the gene coding for the Auxin Efflux Carrier (AEC) protein in Paenibacillus polymyxa. Journal of Microbiology 46:257-264 DOI 10.1007/s12275-007-0245-x.

Deng Y, Lu Z, Bi H, Lu F, Zhang C, and Bie X. 2011. Isolation and characterization of peptide antibiotics L1-F04 and polymyxin B produced by Paenibacillus polymyxa strain JSa-9.
Peptides 32:1917-1923 DOI 10.1016/j.peptides.2011.08.004.

Ding Y, Wang J, Liu Y, and Chen S. 2005. Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. Journal of Applied Microbiology 99:1271 DOI 10.1111/j.1365-2672.2005.02738.x.

Erisman JW, Sutton MA, Galloway J, Klimont Z, and Winiwarter W. 2008. How a century of ammonia synthesis changed the world. Nature Geoscience 1:636-639 DOI 10.1038/ngeo325.

Fang XM, Li YS, Nie J, Wang C, Huang KH, Zhang YK, Zhang YL, She HZ, Liu XB, and Ruan RW. 2018. Effects of nitrogen fertilizer and planting density on the leaf photosynthetic characteristics, agronomic traits and grain yield in common buckwheat (Fagopyrum esculentum M.). Field Crops Research 219:160-168 DOI 10.1016/j.fcr.2018.02.001.

Fortes TO, Alviano DS, Tupinambá G, Padrón TS, Antoniolli AR, Alviano CS, and Seldin L. 2008. Production of an antimicrobial substance against Cryptococcus neoformans by Paenibacillus brasilensis Sa3 isolated from the rhizosphere of Kalanchoe brasiliensis. Microbiological Research 163:200-207 DOI 10.1016/j.micres.2006.05.003.

García J, and Kao-Kniffin J. 2018. Microbial group dynamics in plant rhizospheres and their implications on nutrient cycling. Frontiers in Microbiology 9:1516 DOI 10.3389/fmicb.2018.01516.

Glickmann E, and Dessaux Y. 1995. A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Applied and Environmental Microbiology 61:793 DOI 10.0000/PMID16534942.

Graham PH, Vance CP, Graham PH, and Vance CP. 2000. Nitrogen fixation in perspective: an overview of research and extension needs. Field Crops Research 65:93-106 DOI 10.1016/S0378-4290(99)00080-5.

Grau FH, and Wilson PW. 1962. Physiology of nitrogen fixation by Bacillus polymyxa. Journal of Bacteriology 83:490-496.

He ZG, Kisla D, Zhang LW, Yuan CH, Green-Church KB, and Yousef AE. 2007. Isolation and identification of a Paenibacillus polymyxa strain that coproduces a novel lantibiotic and polymyxin. Applied and Environmental Microbiology 73:168-178 DOI 10.1128/AEM.02023-06.

Helbig J. 2001. Biological control of botrytis cinerea Pers. ex Fr. in strawberry by Paenibacillus polymyxa. Journal of Phytopathology 149:265-273 DOI 10.1046/j.1439-0434.2001.00371.x.

Hong YY, Ma YC, Zhou YG, Gao F, Liu HC, and Chen SF. 2009. Paenibacillus sonchi sp. nov., a nitrogen-fixing species isolated from the rhizosphere of Sonchus oleraceus. International Journal of Systematic & Evolutionary Microbiology 59:2656-2661 DOI 10.1099/ijs.0.009308-0.

Ivleva NB, Jeanna G, Staub JM, and Michael S. 2016. Expression of active subunit of nitrogenase via integration into plant organelle genome. PloS One 11:e0160951 DOI 10.1371/journal.pone.0160951 DOI 10.1371/journal.pone.0160951.

Jiao XQ, He G, Cui ZL, Shen JB, and Zhang FS. 2018. Agri-environment policy for grain
production in China: toward sustainable intensification. *China Agricultural Economic Review* **10**:00-00 DOI 10.1108/CAER-10-2017-0201.

Jin HJ, Lv J, and Chen SF. 2011. *Paenibacillus sophorae* sp. nov., a nitrogen-fixing species isolated from the rhizosphere of Sophora japonica. *International Journal of Systematic and Evolutionary Microbiology* **61**:767-771 DOI 10.1099/ijs.0.021709-0.

Khan AL, Waqas M, Kang SM, Alharrasi A, Hussain J, Alrawahi A, Alkhiziri S, Ullah I, Ali L, and Jung HY. 2014. Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. *Journal of Microbiology* **52**:689-695 DOI 10.1007/s12275-014-4002-7.

Li J, Beatty PK, Shah S, and Jensen SE. 2007. Use of PCR-targeted mutagenesis to disrupt production of fusaricidin-type antifungal antibiotics in *Paenibacillus polymyxa*. *Applied Environmental Microbiology* **73**:3480-3489 DOI 10.1128/AEM.02662-06.

Li Y, Li Y, Zhang H, Wang M, and Chen S. 2019. Diazotrophic *Paenibacillus beijingensis* BJ-18 provides nitrogen for plant and promotes plant growth, nitrogen uptake and metabolism. *Frontiers in Microbiology* **10**:1119 DOI 10.3389/fmicb.2019.01119.

Li Y, Liu X, Hao T, and Chen S. 2017. Colonization and maize growth promotion induced by phosphate solubilizing bacterial isolates. *International Journal of Molecular Sciences* **18** DOI 10.3390/ijms18071253.

Ma Y, Xia Z, Liu X, and Chen S. 2007. *Paenibacillus sabinae* sp. nov., a nitrogen-fixing species isolated from the rhizosphere soils of shrubs. *International Journal of Systematic & Evolutionary Microbiology* **57**:6-11 DOI 10.1099/ijs.0.64519-0.

Ma YC, and Chen SF. 2008. *Paenibacillus forsythiae* sp. nov., a nitrogen-fixing species isolated from rhizosphere soil of Forsythia mira. *International Journal of Systematic & Evolutionary Microbiology* **58**:319-323 DOI 10.1099/ijs.0.65238-0.

Mehta MP, Butterfield DA, and Baross JA. 2003. Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. *Applied Environmental Microbiology* **69**:960 DOI 10.1128/AEM.69.2.960-970.2003.

Mohamed I, Eid KE, Abbas MHH, Salem AA, Ahmed N, Ali M, Shah GM, and Fang C. 2019. Use of plant growth promoting Rhizobacteria (PGPR) and mycorrhizae to improve the growth and nutrient utilization of common bean in a soil infected with white rot fungi. *Ecotoxicology and Environmental Safety* **171**:539-548 DOI 10.1016/j.ecoenv.2018.12.100.

Mus F, Alleman AB, Pence N, Seefeldt LC, and Peters JW. 2018. Exploring the alternatives of biological nitrogen fixation. *Metallomics* **10** DOI 10.1039/C8MT00038G.

Navarronoya YE, Hernándezmendoza E, Moralesjiménez J, Janroblero J, Martínezromero E, and Hernándezrodríguez C. 2012. Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings. *Applied Soil Ecology* **62**:52-60 DOI 10.1016/j.apsoil.2012.07.011.

Neilands JB. 1993. Siderophores. *Archives of Biochemistry & Biophysics* **302**:1-3 DOI 10.1006/abbi.1993.1172.

Niu B, Rueckert C, Blom J, Wang Q, and Borriss R. 2011. The genome of the plant growth-promoting rhizobacterium *Paenibacillus polymyxa* M-1 contains nine sites dedicated to
nonribosomal synthesis of lipopeptides and polyketides. *Journal of Bacteriology* **193**:5862-5863 DOI 10.1128/JB.05806-11.

Raza W, Yang W, and Shen QR. 2008. *Paenibacillus polymyxa*: antibiotics, hydrolytic enzymes and hazard assessment *Journal of Plant Pathology* **90**:419-430 DOI 10.2307/41998534.

Raza W, Yang XM, Wu HS, Wang Y, Xu YC, and Shen QR. 2009. Isolation and characterisation of fusaricidin-type compound-producing strain of *Paenibacillus polymyxa* SQR-21 active against Fusarium oxysporum f.sp. nevium. *European Journal of Plant Pathology* **125**:471-483 DOI 10.1007/s10658-009-9496-1.

Reay DS, Davidson EA, Smith KA, Smith P, Melillo JM, Dentener F, and Crutzen PJ. 2012. Global agriculture and nitrous oxide emissions. *Nature Climate Change* **2**:410-416 DOI 10.1038/nclimate1458.

Roller C, Wagner M, Amann R, Ludwig W, and Schleifer KH. 1994. *In situ* probing of Gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**:2849-2858 DOI 10.1099/00221287-140-10-2849.

Seldin L. 2011. Paenibacillus, nitrogen fixation and soil fertility. N.A. Logan and P. De Vos (eds.), *Endospore-forming Soil Bacteria*, *Soil Biology* **27** DOI 10.1007/978-3-642-19577-8_15.

Seldin L, Elsas JDV, and Penido EGC. 1984. *Bacillus azotofixans* sp. nov. a nitrogen-fixing species from Brazilian soils and grass roots. *International Journal of Systematic Bacteriology* **34**:451-456.

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology & Evolution* **30**:2725-2729 DOI 10.1093/molbev/mst197.

Tayefeh M, Sadeghi SM, Noorhosseini SA, Bacenetti J, and Damalas CA. 2018. Environmental impact of rice production based on nitrogen fertilizer use. *Environmental Science Pollution Research* **25**:1-11 DOI 10.1007/s11356-018-1788-6.

Timmusk S, Van West P, Gow NA, and Huffstutler RP. 2009. *Paenibacillus polymyxa* antagonizes oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*. *Journal of Applied Microbiology* **136**:S265-S266 DOI 10.1111/j.1365-2672.2009.04123.x.

Vandamme P, Pot B, Gillis M, Vos P, De, Kersters K, and Swings J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* **60**:407 DOI 10.1006/mpat.1996.0035.

Verma RK, Sachan M, Vishwakarma K, Upadhyay N, Mishra RK, Tripathi DK, and Sharma S. 2018. Role of PGPR in sustainable agriculture: molecular approach toward disease suppression and growth promotion. DOI 10.1007/978-981-13-0044-8_9.

von der Weid I, Artursson V, Seldin L, and Jansson JK. 2005. Antifungal and root surface colonization properties of GFP-tagged *Paenibacillus brasiliensis* PB177. *World Journal of Microbiology & Biotechnology* **21**:1591-1597 DOI 10.1007/s11274-005-8123-3.

von der Weid I, Duarte GF, van Elsas JD, and Seldin L. 2002. *Paenibacillus brasiliensis* sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. *International Journal of Systematic & Evolutionary Microbiology* **52**:2147-2153 DOI 10.1099/ijs.0.02979-0.
Wang LY, Li J, Li QX, and Chen SF. 2014. *Paenibacillus beijingensis* sp. nov., a nitrogen-fixing species isolated from wheat rhizosphere soil. *Antonie Van Leeuwenhoek* 104:675-683. DOI 10.1007/s10482-013-9974-5.

Wang LY, Zhang LH, Liu ZZ, Zhao DH, Liu XM, Zhang B, Xie JB, Hong YY, Li PF, Chen SF, Dixon R, Li JL. 2013. A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli*. *PLoS Genetics* 9:e1003865 DOI 10.1371/journal.pgen.1003865.

Wang TS, Zhao XY, Shi HW, Sun L, Li YB, Li Q, Zhang HW, Chen SF, and Li JL. 2018. Positive and negative regulation of transferred *nif* genes mediated by indigenous GlnR in Gram-positive *Paenibacillus polymyxa*. *PLoS Genetics* 14:e1007629 DOI 10.1371/journal.pgen.1007629.

Weselowski B, Nathoo N, Eastman AW, Macdonald J, and Yuan ZC. 2016. Isolation, identification and characterization of *Paenibacillus polymyxa* CR1 with potentials for biopesticide, biofertilization, biomass degradation and biofuel production. *BMC Microbiology* 16:244 DOI 10.1186/s12866-016-0860-y.

Witz DF, Detroy RW, and Wilson PW. 1967. Nitrogen fixation by growing cells and cell-free extracts of the *Bacillaceae*. *Archives of Microbiology* 55:369-381.

Xie J, Shi H, Du Z, Wang T, Liu X, and Chen S. 2016. Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in *Paenibacillus polymyxa* and its closely related species. *Scientific Reports* 6:21329 DOI 10.1038/srep21329.

Xie JB, Zhang LH, Zhou YG, Liu HC, and Chen SF. 2012. *Paenibacillus taohuashanense* sp. nov., a nitrogen-fixing species isolated from rhizosphere soil of the root of Caragana kansuensis Pojark. *Antonie Van Leeuwenhoek* 102:735-741 DOI 10.1007/s10482-012-9773-4.

Yao LJ, Wang Q, Xue-Chi FU, and Mei RH. 2008. Isolation and identification of endophytic bacteria antagonistic to wheat sharp eyespot disease. *Chinese Journal of Biological Control*.

Zhalnina K, Louie KB, Hao Z, Mansoori N, da Rocha UN, Shi S, Cho H, Karaoz U, Loqué D, Bowen BP, Firestone MK, Northen TR, Brodie EL. 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nature Microbiology* 3:470-480 DOI 10.1038/s41564-018-0129-3.
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.
Figure 2

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

Data are means ± SE of three independent 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

Figure 4: 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 ($p < 0.05$).
Table 1 (on next page)

Characterization and nitrogenase activity of isolates.
### Table 1. Characterization and nitrogenase activity of isolates.

| Isolates          | Cell morphology | Colony morphology | Nitrogenase activity<sup>a</sup> | GenBank accession number | Origin and Location                                                                 |
|-------------------|-----------------|-------------------|----------------------------------|--------------------------|-------------------------------------------------------------------------------------|
| *Paenibacillus*   | Rods            | Moist, milky      | 1085.61±75.64<sup>ghi</sup>     | MF967282                 | Jujube, mountain in Huairou, Beijing 40°32’ N, 116°62’ E                          |
| sp. BJ-2          |                 |                   |                                  |                          | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| *Paenibacillus*   | Rods            | Moist, milky      | 118.65±3.97<sup>k</sup>         | MF967283                 | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| sp. SZ-1a         |                 |                   |                                  |                          | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| *Paenibacillus*   | Rods            | Moist, milky      | 11868.65±1740.55<sup>a</sup>    | MF967284                 | Apple, orchard in Shunyi, Beijing 40°13’ N, 116°65’ E                              |
| sp. SZ-1b         |                 |                   |                                  |                          | Persimmon, mountain in Shunyi, Beijing 40°13’ N, 116°65’ E                           |
| *Paenibacillus*   | Rods            | Dry, white        | 468.63±42.20<sup>ijk</sup>      | MF967286                 | Maize, field in Changping, Beijing 40°22’ N, 116°20’ E                              |
| sp. BJ-4          |                 |                   |                                  |                          | Wheat, farmland in Miyun, Beijing 40°37’ N, 116°85’ E                               |
| *Paenibacillus*   | Rods            | Moist, milky      | 1131.54±15.92<sup>gh</sup>     | MF967287                 | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| sp. SZ-8          |                 |                   |                                  |                          | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| *Paenibacillus*   | Rods            | Moist, milky      | 314.60±19.18<sup>jk</sup>      | MF967288                 | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| sp. BJ-7          |                 |                   |                                  |                          | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| *Paenibacillus*   | Short rods      | Moist, milky      | 371.28±7.67<sup>ijk</sup>      | MF967289                 | Maize, farmland in Changping, Beijing 40°22’ N, 116°20’ E                           |
| **Paenibacillus** | Shape | Growth Habit | Number | Country and Location |
|------------------|-------|--------------|--------|---------------------|
| sp. SZ-11        | Rods  | Moist, milky | 857.47±114.89ghij | Pepper, herbary in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. SZ-13a       | Rods  | Dry, milky   | 9731.36±259.71b  | Medicinal plant, farmland in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. SZ-13b       | Rods  | Dry, milky   | 3131.89±100.61c  | Medicinal plant, farmland in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. SZ-15        | Rods  | Moist, milky | 1316.19±36.64g   | Wheat, farmland in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. SZ-16        | Rods  | Moist, milky | 444.73±119.11hijk| Spinach, herbary in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. BJ-6         | Short rods | Dry, milky | 176.7±29.43jk   | Bamboo, mountain in Huairou, Beijing 40°32’ N, 116°62’ E |
| sp. AH-1         | Short rods | Moist, milky | 192.43±73.08jk | Grape, orchard in Hefei, Anhui 31°86’ N, 117°27’ E |
| sp. SZ-14        | Rods  | Moist, milky | 331.95±22.73jk | Rice, farmland in Changping, Beijing 40°22’ N, 116°20’ E |
| sp. YN-3         | Short rods | Moist, white | 3201.92±104.96c | Sugarcane, farmland in Pu’er, Yunnan 23°07’ N, 110°03’ E |
| sp. AH-3         | Short rods | Moist, white | 57.23±14.44k  | Arbor, natural forest in Wuhu, Anhui 31°95’ N, 118°73’ E |
| sp. YN-3         | Short rods | Moist, white | 6514.37±997.12c | Arbor, natural forest in |
| Strain          | Type     | Habitat                | Location                  | Code   |
|----------------|----------|------------------------|---------------------------|--------|
| *Paenibacillus* sp. AH-4 | Rods     | Moist, milky           | Hefei, Anhui              | 31°95’ N, 118°73’ E |
| *Paenibacillus* sp. YB-3 | Rods     | Moist, milky           | Fruit, mountain in Yibin, Sichuan | 28°77’ N, 104°62’ E |
| *Paenibacillus* sp. WF-6 | Rods     | Moist, milky           | Wheat, field in Weifang, Shandong | 36°62’ N, 119°10’ E |
| *Paenibacillus* sp. JS-4 | Rods     | Moist, milky           | Reed, countryside in Suzhou, Jiangsu | 31°32’ N, 120°62’ E |
| *Paenibacillus* sp. HN-1 | Short rods | Moist, milky         | Rice, farmland in Xiangtan, Hunan | 27°52’ N, 112°53’ E |
| *Paenibacillus* sp. CD-4a | Rods     | Moist, milky           | Rape, field in Chengdu, Sichuan | 30°67’ N, 104°07’ E |
| *Paenibacillus* sp. CD-4b | Short rods | Moist, milky         | Fruit, mountain in Chengdu, Sichuan | 30°67’ N, 104°07’ E |

a: The unit of nitrogenase activity is nmol C₂H₄ ⋅ mg⁻¹ protein ⋅ 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: 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.