



Insights into non-symbiotic plant growth promotion bacteria associated with nodules of *Sphaerophysa salsula* growing in northwestern China

Zhen-Shan Deng¹ · Zhao-Yu Kong² · Bao-Cheng Zhang³ · Long-Fei Zhao⁴

Received: 7 July 2019 / Revised: 13 October 2019 / Accepted: 16 October 2019 / Published online: 4 November 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

In addition to rhizobia, other non-symbiotic endophytic bacteria also have been simultaneously isolated from the same root nodules. The existence of non-symbiotic endophytic bacteria in leguminous root nodules is a universal phenomenon. The vast majority of studies have detected endophytic bacteria in other plant tissues. In contrast, little systemic observation has been made on the non-symbiotic endophytic bacteria within leguminous root nodules. The present investigation was carried out to isolate plant growth-promoting endophytic non-symbiotic bacteria from indigenous leguminous *Sphaerophysa salsula* and their influence on plant growth. A total of 65 endophytic root nodule-associated bacteria were isolated from indigenous legume *S. salsula* growing in the northwestern arid regions of China. When combining our previous work with the current study, sequence analysis of the *nifH* gene revealed that the strain belonging to non-nodulating *Bacillus pumilus* Qtx-10 had genes similar to those of *Rhizobium leguminosarum* Qtx-10-1. The results indicated that horizontal gene transfer could have occurred between rhizobia and non-symbiotic endophytes. Under pot culture conditions, out of the 20 representative endophytic isolates, 15 with plant growth-promoting traits, such as IAA production, ACC deaminase, phosphate solubilization, chitinase, siderophore, and fungal inhibition activity showed plant growth-promoting activity with respect to various plant parameters such as chlorophyll content, fresh weight of plant, shoot length, nodule number per plant and average nodule weight per plant when co-inoculated with rhizobial bioinoculant *Mesorhizobium* sp. Zw-19 under N-free culture conditions. Among them, *Bacillus pumilus* Qtx-10 and *Streptomyces bottropensis* Gt-10 were excellent plant growth-promoting bacteria, which enhanced the seeding fresh weight by 87.5% and the shoot length by 89.4%, respectively. The number of nodules grew more than 31.89% under field conditions. Our findings indicate the frequent presence of these non-symbiotic endophytic bacteria within root nodules, and that they help to improve nodulation and nitrogen fixation in legume plants through synergistic interactions with rhizobia.

Keywords Root nodules · Plant growth promotion · Phylogeny · Non-symbiotic endophytes · *Sphaerophysa salsula*

Introduction

Plants of the legume family (*Leguminosae* or *Fabaceae*) are widespread all over the world. They are able to establish a nitrogen-fixing symbiosis with nodule-inducing soil

Communicated by Erko Stackebrandt.

✉ Zhen-Shan Deng
zhenshandeng214@163.com

Zhao-Yu Kong
kongzhaoyu@hotmail.com

Bao-Cheng Zhang
woshimiantian@126.com

Long-Fei Zhao
hnzhaolongfei@163.com

¹ College of Life Sciences, Yan'an University, 716000 Yan'an, China

² College of Life Sciences, Nanchang University, 330000 Nanchang, China

³ School of Biological and Agricultural Science Technology, Zunyi Normal College, 53602 Zunyi, China

⁴ College of Life Sciences, Shangqiu Normal University, 476000 Shangqiu, China

bacteria, here collectively called rhizobia. As well as the rhizobia, some non-symbiotic bacteria of other genera have also been isolated from legume nodules, *Rahnella* (Aserse et al. 2013), *Bacillus* (Bai et al. 2002, 2003; Deng et al. 2011; De Meyer et al. 2015; Saini et al. 2015; Zhao et al. 2018), *Enterobacter* (Benhizia et al. 2004; Ibáñez et al. 2009), *Agrobacterium* (De Lajudie et al. 1999), *Pseudomonas* (Shiraishi et al. 2010; Hoque et al. 2011), *Burkholderia* (Diouf et al. 2007), *Herbaspirillum* (Weiss et al. 2012), *Pantoea* (Kan et al. 2007), *Paenibacillus* (Deng et al. 2011; Zakhia et al. 2006; Li et al. 2008, 2012), *Klebsiella* (Ibáñez et al. 2009), *Endobacter* (Ramirez-Bahena et al. 2013), *Arthrobacter*, *Microbacterium*, *Curtobacterium* (Sturz et al. 1997; Palaniappan et al. 2010), *Xenophilus*, *Erwinia*, *Leclercia* (Xu et al. 2014), *Gluconacetobacter*, *Variovorax*, *Micromonospora* and *Hyphomicrobium* (Martínez-Hidalgo and Hirsch 2017; Xiao et al. 2017; Zhang et al. 2018).

These isolates are not able to infect the plant or induce nodules; neither do they fix nitrogen symbiotically and are termed non-symbiotic endophytic bacteria. The host plant provides endophytes with supply of nutrients and shelter from most abiotic stresses. In return, plants may receive benefits from microbial associations by the enhancement of plant growth or reduction of plant stress, e.g., through the ability of bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase to modulate the level of ethylene produced by plants under stress (Lodewyckx et al. 2002; Hardoim et al. 2008), production of indoleacetic acid (IAA), P solubilization, resistance to certain pathogens (Hardoim et al. 2002; Peix et al. 2015; Tariq et al. 2014), nitrogen fixation (Andrews et al. 2010) and siderophore production (Rajendran et al. 2008). Most legumes are good pioneer species, adapted to low nutrient soils and/or environments with heavy metals (Gonzalez-Andres et al. 2005; Vidal et al. 2009; Azcon et al. 2010). In this regard, previously mentioned studies have reported that other rhizobia such as non-symbiotic bacteria may inhabit legume root nodules and have proven benefit to their legume hosts. However, non-symbiotic bacteria may inhabit legume root nodules and visibly harm the host, and their biological role in the plant is largely unknown.

As previously found, the nodules have been proposed to form an ecological niche for survival of the bacteria (Muresu et al. 2008). Assistance in nodulation ability and improved nodulation and nitrogen fixation through synergistic interactions of rhizobia with legume plants have been proven by some earlier studies (Andrews et al. 2010; Rajendran et al. 2008). *Enterobacter* species were once reported to have infection ability and nodulation genes. Their mode of entry is probably associated with infecting rhizobia (Benhizia et al. 2004). Moreover, recent reports suggest increase in root wet weight and nodulation when co-inoculated with nodule endophytes, compared to inoculation with rhizobia

alone (Bai et al. 2002, 2003; Sturz et al. 1997; Barret and Parker 2006; Annapurna et al. 2013; Masciarelli et al. 2014). This is further supported by previous reports showing that significant increase in nodule activity and nitrogen content in soybean on co-inoculation of *Bacillus megaterium* LNL6 along with *Bradyrhizobium japonicum* MN110 (Subramanian et al. 2015).

The Leguminosae includes over 18,000 known species (<http://www.ildis.org/Leguminosae/>), but so far only a fraction of those have been investigated for nodulation (Sprent 2001) and even fewer for the occurrence of nodule endophytes. *Sphaerophysa salsula* is one of the wild herbaceous legumes commonly found in the northwestern arid regions of China. This deep-rooted legume has potential for vegetation of arid regions, but its bacterial partners are so far unknown. To obtain information needed to ensure good colonization and nitrogen fixation in future application, it was of interest to investigate the diversity of non-symbiotic bacteria inhabiting the root nodules of this plant. We recently isolated and characterized a number of inhabiting non-symbiotic bacteria from *Sphaerophysa salsula* root nodules (Deng et al. 2011). The present study is based on the same sampling sites from root nodules of *Sphaerophysa salsula*; however, it focuses on the non-symbiotic bacteria that are capable of atmospheric nitrogen fixation and those possessing the *nifH* gene. The aim of this study was to gain insights into their potential nitrogen fixation ability and to investigate the possible connections with the ecoregion and their properties in plants growing in diverse habitats. Additionally, the results on the non-symbiotic bacteria from our previous study (Deng et al. 2011) were applied and compared with the plant growth-promoting characteristics found in the present study. Thus, we hypothesized that they can enhance host and other plant growth.

To verify our speculation, we selected 20 plant growth-promoting representative bacteria, isolated from root nodules of *Sphaerophysa salsula*, and tested them for compatible co-inoculation with *Mesorhizobium* sp. Zw-19 to improve nodulation and nodule activity under pot culture conditions and field experiments, to determine their effect as PGPB on the growth of the *Sphaerophysa salsula*. Thus, the outcomes of this study could elucidate the relationship between them and improve their potential as a sustainable and inexpensive alternative to the use of agrochemicals.

Materials and methods

Isolation and characterization of nodule endophytes

Root nodules were collected and isolated. DNA extraction and sequence analysis of 16S rRNA gene were performed as previously reported (Deng et al. 2011).

Amplification and sequencing of the *nifH* gene

The primers 34F (5'-AAA GG(C/T) GG(A/T) ATC GG(C/T) AA(A/G) TCC ACCAC-3') and 491R (5'-TTG TT(G/C) GC(G/C) GC(A/G) TAC AT(G/C) GCC ATC AT-3'), and the procedure described by Van Berkum et al. (Van Berkum et al. 1996) were used for *nifH* gene-specific amplification by PCR. The bands corresponding to the expected size (460 bp) were cut, purified and sequenced. Sequences were assembled and aligned, and a phylogenetic tree was constructed as described for the 16S rRNA gene analysis.

Plant inoculation studies with endophytes

Sphaerophysa salsula seeds were surface sterilized with 96% sulfuric acid for 20 min, then washed ten times with sterile water to remove all traces of acid, immersed in 95% ethanol for 1 min, followed by 0.1% HgCl₂ for 2 min and rinsed eight times in sterile distilled water. Surface-sterilized seeds were allowed to germinate on moist filter paper kept in sterile Petri dishes containing ten seeds each. Seedlings were coated with overnight grown bacterial cultures by incubating them in a thick suspension (approximately, 10⁹ cfu mL⁻¹) of the bacteria for 8 h at 28 °C. Zw-19 was isolated from *Sphaerophysa salsula* root nodule in this work and 16S rRNA sequence confirmed it as *Mesorhizobium* sp. One milliliter of inoculum from the endophytic isolates alone and the mixture containing each isolate with *Mesorhizobium* sp. Zw-19 (500 + 500 µL) were inoculated on the surface of sterilized *Sphaerophysa salsula* seeds. Seeding without any inoculation as the negative control (NC) and with *Mesorhizobium* sp. Zw-19 alone as a positive control (PC), which received equal volumes of inoculum, was included for comparison, respectively. The plants were then transferred to glass tubes containing nitrogen-free plant nutrient solution (Vincent 1970) sealed with cotton plugs. All nodulation tests were performed in triplicate with non-inoculated control plants included. Roots were observed for nodule formation during 6–8 weeks after inoculation. The surface-sterilized roots and stems of seedlings inoculated with the endophytic bacteria alone were ground to estimate the colony-forming units (CFUs) of endophytic bacteria (Saini et al. 2015).

Chlorophyll estimation

The midrib of the leaves was removed and 1 g leaf tissue was crushed in 80% acetone; chlorophyll was measured spectrophotometrically using the specific absorption coefficients for chlorophyll a at 664 nm and chlorophyll b at 647 nm. The chlorophyll content was estimated by using the previous method (Graan and Ort 1984).

Characterization of the plant growth-promoting properties of endophytic bacteria

IAA production assay

Indoleacetic acid (IAA) production was analyzed according to the qualitative method (Glickmann and Dessaux 1995). Bacterial culture incubated in King B medium at 28 °C for 36 h was mixed with the Salkowski reagent (1:1 v/v) and incubated in darkness for 30 min. The production of IAA was recognized by the presence of red color.

Phosphate solubilization

An aliquot of 10 mL of fresh bacterial culture was spread onto TY medium supplied with 5 g L⁻¹ of Ca₃(PO₄)₂ and was incubated at 28 °C for 2–3 days. A clear halo around the bacterial colony indicated solubilization of mineral phosphate.

Chitinase production

The bacterial suspension was spot inoculated on chitin agar plates and the zone of clearance was recorded after incubation at 28 ± 2 °C for 48–72 h.

Siderophore detection

Bacterial cultures were multiplied in Ashby's Mannitol Broth (AMB) for 48 h and an equal volume of culture supernatant was added to chrome azurol S (CAS) assay solution (Schwyn and Neilands 1987). A color change from blue to pink was recorded.

ACC (1-aminocyclopropane-1-carboxylate) deaminase production

ACC deaminase enzyme production capability was assessed based on the ability to take ACC as a sole nitrogen source in a minimal medium (Duan et al. 2009). Cultures were spot inoculated on plates containing minimal medium supplemented with 3 mM ACC substrate. Plates containing minimal medium without ACC served as negative control, and those with (NH₄)₂SO₄ (2.0 g mL⁻¹) as a nitrogen source served as positive control. The plates were incubated for 4 days at 28 ± 2 °C and the growth of the isolates on ACC-supplemented plates was compared with that of the positive and negative control plates.

Fungal inhibition growth assay

Spores of fungal cultures (*Fusarium oxysporum*, *Alternaria burnsii* and *Rhizoctonia solani*) were grown on potato

dextrose agar (PDA) plates. An agar plug (5 mm diameter) taken from an actively growing fungal culture was placed on one side of the surface of the PDA plate. Test bacteria were streaked perpendicular to the agar plug on the opposite side toward the edge of the plates. The plate inoculated with fungal agar plugs alone was used as the control. The plates were incubated at 28 ± 2 °C until fungal mycelia completely covered the agar surface in the control plate, and the zone of inhibition was recorded. A test was considered positive when bacteria interfered with the normal spread of the fungus and presented inhibition zone in three replications.

Root hair deformation assay

Bacterial cultures were grown in 50 ml liquid media incubated at 30 °C on a shaker. After 48 h incubation, 5 ml of the culture was transferred to 95 ml fresh liquid medium and allowed to grow for another 12 h. The inoculation of bacterial culture (0.5 ml) was done by making a suspension in *N*-free Fahraeus medium at a density of approximately 10^9 cfu/ml. *Sphaerophysa salsula* seeds were surface sterilized with 96% sulfuric acid for 20 min. Seeds were then washed ten times with sterile water to remove all traces of acid, immersed in 70% ethanol for 1 min, followed by 0.1% HgCl₂ for 2 min, and rinsed eight times in sterile distilled water. Sterilized seeds were germinated on 0.8% (w/v) agar contained in 9 cm Petri dishes for 2 days in the dark at 25 °C. Two-day-old seedlings were transferred aseptically to sterilized glass slides (one per slide) containing nitrogen-free Fahraeus medium semi-solidified with 0.8% (w/v) agar. Before applying the bacterial cultures, the roots of all the plants were examined microscopically and Fahraeus slides containing plants with deformed hairs were discarded. After 1 day, seedlings were inoculated with mid log phase cells of bacterial cultures. The inoculation of sterilized water and the sterilized medium served as control. Seedlings were grown under day-light fluorescent tubes in a plant growth chamber with a 16 h photoperiod for 10 days at 25 °C (day) and 22 °C (night). The morphological features of primary root

infection were examined by bright field microscopy at day 2 after inoculation.

Field layout

The field experiments were conducted in Yan'an University, Shaanxi, China, and designs were randomized complete blocks under field conditions. The experiment was designed as a $3 \times 2 \times 2$ factorial organized in a randomized complete block split plot with three replications, and three plant samples comprised one replicate. The main plot units consisted of PGPR (plant growth-promoting rhizobacteria) strain inoculation (the mixture of endophytic isolates including *Bacillus pumilus* Qtx-10 and *Streptomyces bottropensis* Gt-10 with *Mesorhizobium* sp. Zw-19 by adding 100 mL of bacterial suspension 10^8 CFU mL⁻¹ per plant (no-PGPR inoculation as a control). Each sub-plot was 5×2 m and consisted of three rows of plants with 40 cm between rows. The space between plots was 80 cm with 1 m between replications. The experiments started in early May and ended in mid-July in 2018.

Results

Isolation and characterization of nodule endophytes

Sixty-five endophytic bacteria (Deng et al. 2011) that grew on PDA, KingB or NA medium which could not induce nodules were isolated from six different samples (Table 1), and 20 representative endophytes were selected and tested for their ability to nodulate *Sphaerophysa salsula* and plant growth-promoting assays (Table 2). In the nodulation tests under aseptic conditions, no nodules were induced by the 20 representative endophytic bacteria. The amount of CFU, from the surface-sterilized roots and stems of the inoculated seedlings varying from 1.13×10^4 to 6.52×10^5 CFU per gram of fresh root and 3.21×10^4 to 8.54×10^5 per gram of fresh stem, also fits the range of endophytic bacteria (Table 1).

Table 1 Physicochemical characteristics of the studied soils

No. sample	Sample location	Altitude	Latitude and longitude	N %	C %	C/N	K %	P %	pH
Gt	Gaotai	1355.84 m	39° 03' 50" N, 98° 57' 27" E	0.087	1.00	11.5	1.00	0.072	8.54
Gz	Guazhoun	1164 m	39° 42' 36" N, 98° 30' E	0.028	0.29	10.4	1.23	0.042	8.74
M	Minqin	1325.75 m	38° 37' 12" N, 103° 48' E	0.052	0.55	10.6	2.10	0.059	8.11
Q	Qingtongxia	1125.5 m	38° 1' 12" N, 106° 4' 12" E	0.071	0.78	11	2.02	0.064	8.40
Zy	Zhangye	1649.74 m	38° 55' 48" N, 100° 27' 36" E	0.061	0.74	12.1	2.01	0.059	8.16
Zw	Zhongwei	1240 m	39° 30' 36" N, 105° 10' 48" E	0.051	0.59	11.6	2.14	0.064	8.58

Total nitrogen Kjeldahl method, Soil organic carbon K₂Cr₂O₇ volumetric analysis, Total P acid-soluble-molybdenum antimony colorimetry resistance, Potassium extraction-flame photometer method, Soil pH value potentiometry

Table 2 Results of comparative growth profile of inoculation tests

Strains	Seeding fresh weight (g/plant)	Number of nodules (per plant)	Nodule fresh weight (mg/plant)	Chlorophyll content ($\mu\text{M/g}$ leaf fresh weight)	Seeding shoot length (cm)	Bacterial UFC/g (fresh root)	Bacterial UFC/g (fresh stem)
NC	0.07 ± 0.01b	0	0	25.77 ± 3.22d	6.38 ± 1.06f	0	0
PC (Zw-19 alone)	0.08 ± 0.01b	1.57 ± 0.79d	1.63 ± 0.66f	28.20 ± 2.35c	6.87 ± 0.81e	ND	ND
Mq-10	0.08 ± 0.02b	0	0	28.21 ± 2.50c	8.74 ± 1.91bc	8.62 × 10 ⁵	7.32 × 10 ⁵
Mq-10 + Zw-19	0.11 ± 0.01a	1.33 ± 0.58d	2.77 ± 0.32de	33.23 ± 5.04b	10.85 ± 1.26ab	ND	ND
Gt-1	0.10 ± 0.04a	0	0	31.23 ± 3.30b	9.22 ± 1.40b	4.54 × 10 ⁴	8.82 × 10 ⁴
Gt-1 + Zw-19	0.11 ± 0.02a	3.60 ± 2.55b	5.22 ± 3.34ab	32.17 ± 1.27b	10.80 ± 0.48ab	ND	ND
Gaoshi-1	0.06 ± 0.03b	0	0	27.82 ± 6.69c	8.10 ± 0.53c	6.30 × 10 ⁴	7.41 × 10 ⁴
Gaoshi-1 + Zw-19	0.10 ± 0.03a	3.33 ± 1.51b	4.00 ± 2.02c	33.40 ± 6.67b	9.91 ± 0.28b	ND	ND
Qtx-10	0.11 ± 0.03a	0	0	28.60 ± 1.99c	8.52 ± 0.87c	5.35 × 10 ⁵	4.11 × 10 ⁵
Qtx-10 + Zw-19	0.15 ± 0.02a	3.33 ± 1.53b	6.47 ± 2.08a	33.23 ± 5.48b	9.98 ± 0.12b	ND	ND
Zw-22	0.07 ± 0.01b	0	0	28.06 ± 2.87c	8.12 ± 1.48c	1.15 × 10 ⁴	3.27 × 10 ⁴
Zw-22 + Zw-19	0.14 ± 0.04a	3.43 ± 1.62b	6.20 ± 1.75a	38.6 ± 1.78a	11.10 ± 1.27a	ND	ND
Zw-13-3	0.10 ± 0.04a	0	0	28.65 ± 1.97c	9.64 ± 1.16b	5.40 × 10 ⁵	7.53 × 10 ⁵
Zw-13-3 + Zw-19	0.10 ± 0.02a	3.67 ± 2.08b	6.75 ± 1.58a	34.6 ± 4.29b	11.30 ± 0.66a	ND	ND
Gt-20	0.10 ± 0.01a	0	0	28.60 ± 1.99c	8.52 ± 0.87c	6.36 × 10 ⁴	5.45 × 10 ⁴
Gt-20 + Zw-19	0.09 ± 0.02ab	3.40 ± 2.07b	3.72 ± 1.72c	33.75 ± 3.66b	9.83 ± 1.93b	ND	ND
Zw-22-2	0.08 ± 0.08b	0	0	27.35 ± 2.90c	7.90 ± 0.99d	3.65 × 10 ⁴	1.05 × 10 ⁵
Zw-22-2 + Zw-19	0.10 ± 0.03a	3.67 ± 1.63b	2.78 ± 1.52de	29.03 ± 2.66bc	10.42 ± 1.15ab	ND	ND
Zy-3	0.07 ± 0.01b	0	0	26.67 ± 2.28c	8.41 ± 1.86c	4.53 × 10 ⁵	5.42 × 10 ⁵
Zy-3 + Zw-19	0.09 ± 0.02ab	3.67 ± 2.08b	2.82 ± 0.61de	33.57 ± 6.57b	10.08 ± 2.19ab	ND	ND
Qtx-12	0.07 ± 0.01b	0	0	28.33 ± 4.31c	8.90 ± 0.97bc	5.63 × 10 ⁴	5.72 × 10 ⁴
Qtx-12 + Zw-19	0.10 ± 0.02a	2.50 ± 0.71c	2.37 ± 0.58e	35.57 ± 2.40ab	9.90 ± 0.80b	ND	ND
Gzn-9-1	0.08 ± 0.02b	0	0	31.96 ± 4.63b	9.47 ± 1.40b	5.54 × 10 ⁵	6.43 × 10 ⁵
Gzn-9-1 + Zw-19	0.12 ± 0.04a	2.50 ± 0.71c	2.93 ± 1.26de	35.97 ± 6.11a	10.95 ± 0.79ab	ND	ND
Gt-10	0.08 ± 0.03b	0	0	28.86 ± 2.81c	7.88 ± 2.72d	6.52 × 10 ⁵	3.53 × 10 ⁵
Gt-10 + Zw-19	0.13 ± 0.03a	4.33 ± 1.75a	4.39 ± 1.34b	37.13 ± 3.8a	13.01 ± 3.47a	ND	ND
Gt-25	0.08 ± 0.03b	0	0	29.16 ± 3.78bc	8.48 ± 1.61c	9.42 × 10 ⁴	4.05 × 10 ⁵
Gt-25 + Zw-19	0.09 ± 0.02ab	2.50 ± 0.71c	1.10 ± 0.04 g	30.23 ± 3.38b	9.90 ± 1.13b	ND	ND
Qtx-19	0.08 ± 0.01b	0	0	31.32 ± 6.20b	8.48 ± 1.40c	6.61 × 10 ⁴	5.20 × 10 ⁴
Qtx-19 + Zw-19	0.11 ± 0.04a	3.33 ± 0.52b	3.16 ± 1.80d	32.07 ± 2.27b	11.24 ± 3.17a	ND	ND
Zw-11	0.11 ± 0.03a	0	0	26.60 ± 2.97c	8.42 ± 0.79c	6.56 × 10 ⁴	8.93 × 10 ⁴
Zw-11 + Zw-19	0.12 ± 0.03a	4.25 ± 1.49a	3.89 ± 1.07c	34.40 ± 0.89b	10.98 ± 0.78ab	ND	ND
Qtx-11	0.08 ± 0.01b	0	0	26.13 ± 2.27c	8.81 ± 1.45bc	3.55 × 10 ⁵	8.54 × 10 ⁵
Qtx-11 + Zw-19	0.10 ± 0.01a	2.00 ± 1.00c	2.38 ± 1.08e	28.20 ± 4.66bc	9.20 ± 0.67b	ND	ND
Gaoshi-7	0.11 ± 0.03a	0	0	26.05 ± 1.78c	9.82 ± 0.36b	4.61 × 10 ⁴	6.77 × 10 ⁴
Gaoshi-7 + Zw-19	0.14 ± 0.04a	3.50 ± 1.31b	6.03 ± 1.92a	38.13 ± 6.99a	11.08 ± 1.34a	ND	ND
Zy-2-1	0.10 ± 0.03a	0	0	28.87 ± 1.46c	8.90 ± 2.18bc	3.31 × 10 ⁴	5.45 × 10 ⁴
Zy-2-1 + Zw-19	0.09 ± 0.01ab	1.83 ± 0.98 cd	1.83 ± 0.98f	32.80 ± 4.92b	8.93 ± 0.82bc	ND	ND
Mq-17	0.06 ± 0.02bc	0	0	26.52 ± 2.30c	7.36 ± 0.93d	3.41 × 10 ⁴	4.55 × 10 ⁴
Mq-17 + Zw-19	0.13 ± 0.03a	3.00 ± 1.93b	4.76 ± 2.54b	32.68 ± 3.10b	11.36 ± 3.18a	ND	ND
Mq-2-1	0.07 ± 0.03b	0	0	30.26 ± 5.80b	8.61 ± 1.45bc	5.31 × 10 ⁴	5.60 × 10 ⁴
Mq-2-1 + Zw-19	0.09 ± 0.01ab	3.00 ± 1.50b	3.33 ± 1.99d	34.10 ± 4.01ab	10.68 ± 0.70ab	ND	ND

Zw-19 singly and in combination with endophytic bacterial isolates. Each value in columns represents mean ± S.D. of three replicates per treatment. In the same column, significant differences according to LSD at $P=0.05$ levels are indicated by different letters and each set consisted of N number of plant. $N=9$

NC negative control (without any treatment), PC positive control (treated with only Zw-19), ND not determinate

Sequence analysis of *nifH* gene

Amplified fragments of *nifH* corresponding to the expected size of 460 bp were produced in PCR from the 19 tested rhizobial DNA and from that of one single endophytic strain. The phylogenetic analysis of the sequenced fragments (Fig. 1) showed that this *nifH* sequence was related to that of *M. amorphae*, *M. gobiense*, *M. septentrionale* and *M. albiziae*. The *nifH* sequence amplified from *Bacillus pumilus* Qtx-10 was 100% similar to that of *Rhizobium leguminosarum* Qtx-10-1 and 96.14% similar to that of *M. albiziae*. Interestingly, on collecting them from the same sampling site, we found that *Bacillus pumilus* Qtx-10 co-occurred with *Rhizobium leguminosarum* Qtx-10-1 in the same nodule. This close relationship of the same ecological niche between them is speculated to cause the co-evolution of symbiotic genes. However, lateral gene transfer between non-symbiotic and symbiotic bacteria is rare. The positions of some isolates in this study showed aberrant features in the phylogenetic trees. Gene transfer somehow drives genetic diversity and adaptability, and lateral symbiotic gene transfer may have significant effects at the species or genus level.

Root hair deformation assay

The root hair deformation assay performed in *Sphaerophysa salsula* revealed the presence of modified root hair structures in bacterial treatments. The deformation was induced only in a small zone of the root containing root hairs that had almost

stopped growing. Out of 20 representative endophytic isolates, only treatment with Qtx-10, Gzn-9-1 and Zw-13-3 inoculation resulted in deformed root hairs with bulging at the root tip. Examination of the root segments under bright field microscopy showed hemispherical bulbous structures and root structures resembling the initial stages of nodule or tumor outgrowths that could not be observed in uninoculated control seedlings (Fig. 2a–d).

Plant growth promoting assays

Salkowski reaction of culture supernatants revealed that of the strains Qtx-10, Gzn-9-1 and Zw-13-3 were able to synthesize IAA from tryptophan. Based on the sequencing of the 16S rRNA gene, Qtx-10 was identified as *Bacillus pumilus*, Gzn-9-1 was designated as *Bacillus licheniformis* and Zw-13-3 belonged to *Lysinibacillus fusiformis*. All the isolates were chitinase negative, only Zw-12-2 belonging to *Bacillus safensis* showed phosphate solubilization activity, having a clear halo around the colony. Out of 20 representative endophytic isolates, 11 strains gave a positive CAS assay test showing that they produced siderophores, belonging to genera *Staphylococcus*, *Lysinibacillus*, *Bacillus*, *Paracoccus*, *Streptomyces*, *Nocardia*, *Mycobacterium*, *Paenibacillus* and *Pseudomonas*, respectively. The capability to inhibit plant pathogenic fungi was analyzed by using *Fusarium oxysporum*, *Alternaria burnsii* and *Rhizoctonia solani* as target organisms, and six strains showed antifungal activity. The capability of inhibiting fungal growth was

Fig. 1 Phylogenetic tree of representative strains isolated from *Sphaerophysa salsula* (labeled in bold) and reference strains generated by the neighbor-joining method based on nearly full length (460 bp) *nifH* sequences. Bootstrap values (1000 replicates) are indicated as percentages (>70%) above the branches. Scale bar indicates 2% substitution of nucleotide

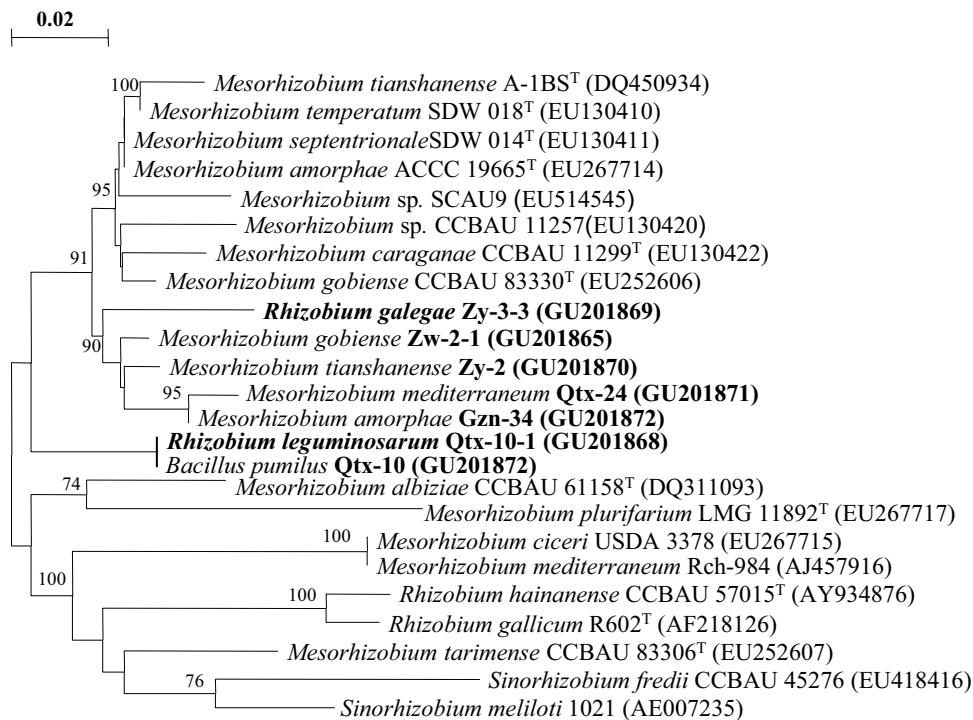


Fig. 2 Root hair deformation bacterial cultures-inoculated *Sphaerophysa salsula* seedlings: **a** uninoculated control with normal root hairs, **b** microscopic view of infected root hairs in *Bacillus pumilus* Qtx-10 inoculated *Sphaerophysa salsula* seedlings, **c** microscopic view of infected root hairs in *Bacillus licheniformis* Gzn-9-1 inoculated *Sphaerophysa salsula* seedlings, **d** microscopic view of infected root hairs in *Bacillus safensis* Zw-13-3 inoculated *Sphaerophysa salsula* seedlings. Scale bars = 200 μ m in (a); 300 μ m in (b, c); 200 μ m in (d)

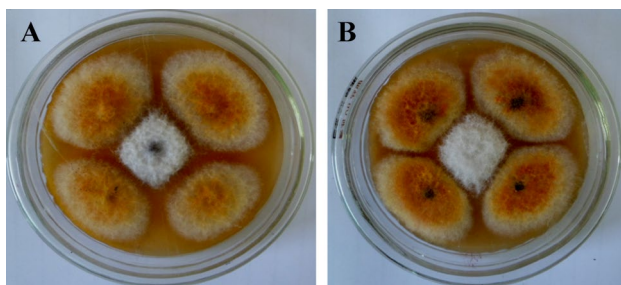
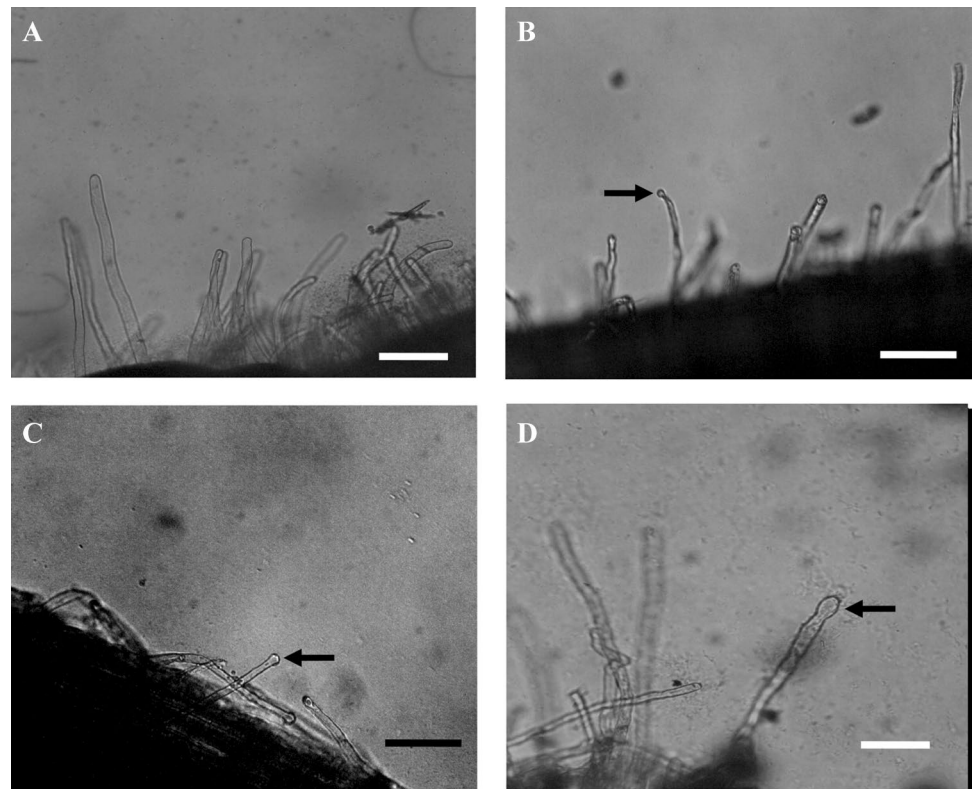


Fig. 3 Antifungal activity of endophytes test. **a** Inhibition zones of strain Gt-1 against *Valsa mali* Miyabe et Yamada; **b** Inhibition zones of strain Qtx-10 against *Valsa mali* Miyabe et Yamada

widely spread in the different genera belonging to *Paracoccus*, *Paenibacillus*, *Bacillus pumilus*, *Inquilinus* and *Mycobacterium*, respectively. Antifungal activity of Zy-3, Gt-1, Qtx-10, Gzn-9-1, Mq-10 and Mq-2-1 was checked against *Fusarium oxysporum* using potato dextrose agar (PDA) medium. The antifungal activity of strains tested varied with inhibition zones in diameter from 18.20 to 36.00 mm, strains Gt-1 and Qtx-10 induced larger inhibition zones compared to the other strains (inhibition zone more than 30.00 mm) (Fig. 3), Gt-1, Qtx-10 and Gzn-9-1 could also exhibit broad-spectrum activities against test fungi. Production of IAA, siderophore and antifungal activity was simultaneously exhibited by isolates of Qtx-10 and Gzn-9-1.

Under pot culture conditions, 15 out of the 20 strains showed plant growth-promoting activity with respect to various plant parameters. Among the isolates, all of the 15 isolates promoted the fresh weight and shoot length of *Sphaerophysa salsula* seedlings when inoculated alone and showed similar effects on the fresh weight and shoot length as the rhizobial bioinoculant strain Zw-19. While Gaoshi-1, Zw-22, Zy-3, Qtx-12 and Mq-17 showed decrease in seeding fresh weight when inoculated alone, all five isolates promoted plant growth when applied as a mixture with Zw-19 (Table 2). Qtx-10 inoculation enhanced the seeding fresh weight by 87.5%, and the isolate Gt-10 enhanced the shoot length by 89.4%. Over the positive control, Gt-25 and Zy-2-1 when co-inoculated with Zw-19 showed decrease in seeding fresh weight compared to its individual application. The chlorophyll content was improved in plants inoculated with all of the 20 representative endophytic isolates, over the noninoculate control, and their chlorophyll values were also improved when co-inoculated with Zw-19. Except Gt-25, there was an increase in the average nodule fresh weight per plant when those 19 strains were inoculated in combination with Zw-19.

The results of field experiments indicated that the inoculum of strain Zw-19 combined with two kinds of bacteria (Qtx-10 and Gt-10) has a significant role in growth promoting than the single inoculum Zw-19. Under the conditions of field, the growth rate of the plant height of *Sphaerophysa*

salsula was more than 15.99% higher than that of Zw-19. Moreover, the number of nodules was 31.89% higher than that under field conditions. The growth-promoting effect of combined Zw-19 with endophytic bacteria on to *S. salsula* has a significant role in various plant growth promotion parameters (Table 3).

Discussion

Nodules can be colonized internally by several bacterial genera unrelated to rhizobial symbiotic nitrogen fixation. Benhizia et al. (Benhizia et al. 2004) reported *Pseudomonas* strains to be associated with legume nodules. Actinobacteria such as *streptomyces lydicus* have been reported to colonize pea nodules (Tokala et al. 2002), and *Bacillus thuringiensis* can naturally coinhabit soybean nodules along with *Bradyrhizobium japonicum*. *Bacillus cepacia* was also recorded as a plant endophyte (Balandreau et al. 2001) and as a nodule occupant (Vandamme et al. 2002), and some other species in the genera *Bacillus* have been found in nodules (Bai et al. 2002; Barret and Parker 2006). Our finding (Deng et al. 2011) is consistent with previous studies showing *Bacillus* to be enriched in the nodules (Zakhia et al. 2006), and *Bacillus* and *Pseudomonas* were mainly endophytic bacteria in the nodules (Kan et al. 2007). As De Meyer et al. (2015) mentioned, the majority of the investigated nodules contained *Bacillus* (17.9%), *Paenibacillus* (12.5%) and *Pseudomonas* (15.9%) found in legume root nodules from native legume species in Flanders (De Meyer et al. 2015), which indicates that certain legume species prefer certain non-symbiotic endophytic bacteria in their root nodules. The implications of the discovery would be significant, especially if it is confirmed that the association between legume nodules and Gram-positive bacteria is common in nature and that this interaction is beneficial for plant growth.

In this study, an evidence of the high similarity (99%) sequences homologous to the *nifH* gene of *Mesorhizobium* was detected within strain Qtx-10 belonging to the genera *Bacillus*, and it offered strong evidence to suggest that lateral gene transfer of *nifH* might have happened between the symbiotic and endophytic bacteria. The frequent lateral

gene transfer might help *S. salsula* to attract the most adapted bacteria, subsequently, and improve the diversity of bacteria within the root nodule of *S. salsula*. This result is similar to the previously reported sequences of *nifH* amplified from *Bacillus* spp. CCBAU15524 (EF471734) and CCBAU15518 (EF471735) were 99% similar to that of *B. japonicum* (AJ563961) (Li et al. 2008). Sequence analysis of the *nifH* gene revealed that the strains belonging to *Xenophilus*, *Acinetobacter*, *Phyllobacterium*, and *Rhizobium* had genes similar to those of *Mesorhizobium* and *Sinorhizobium* (Xu et al. 2014). Seven endophytic non-rhizobial bacteria, which belong to *Enterobacter cloacae*, *Chryseobacterium indologenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter ludwigii* and *Klebsiella variicola* were isolated possessing the *nifH* gene (Dhole et al. 2016). Zhao et al. (2011) demonstrated that the *nifH* gene was amplified from representative strains *Bacillus cereus* and *Bacillus amyloliquefaciens* which were isolated from the root nodules of soybean (*Glycine max* L.) and had nitrogen fixation activity (Zhao et al. 2011). In the present study, *nifH* genes of strain Qtx-10 and other rhizobia were more close than between the 16S rRNA genes of *Bacillus* and rhizobia, which was consistent with the results of Zhao et al. (2011). This suggests that the presence of *nifH* gene in both *Bacillus* and rhizobia probably occurred through horizontal gene transfer. All previous studies and our study demonstrated that horizontal gene transfer could have occurred between rhizobia and non-rhizobial endophytes. They may have co-evolved. Therefore, the non-symbiotic endophytic bacteria in nodules may serve potential receptors of symbiotic genes and may be resources of novel symbiotic bacteria. Although rhizobia have been studied for more than 120 years, symbionts of less than 10% of the 750 legume genera have been fully characterized. Our work suggests that characterization of the symbionts of the yet unexplored legumes may reveal the rhizobial nature of additional members of possibly other taxonomic classes. Such a study may contribute significantly to the understanding of the origin and the evolution of the legume–rhizobia symbioses, showing that the ability to establish a symbiosis with legumes is more widespread in bacteria than anticipated to date.

Table 3 Results of comparative growth under field conditions

Strains	Plant height (cm)	Fresh weight (g)	Dry weight (g)	Root length (cm)	Number of nodules (per plant)	SPAD
PC(Zw-19 alone)	59.48 ± 2.91b	16.08 ± 0.12b	9.81 ± 0.02b	31.00 ± 0.69b	5.21 ± 1.02b	50.56 ± 0.22c
Qtx-10+GT-10	62.50 ± 2.24b	16.40 ± 0.19b	9.84 ± 0.03b	31.70 ± 2.24b	2.21 ± 2.01c	51.65 ± 0.45b
Qtx-10+GT-10+Zw-19	70.80 ± 1.47a	17.48 ± 0.08a	11.01 ± 0.02a	34.82 ± 1.03a	7.66 ± 1.67a	52.97 ± 0.62a

Each value in columns represents mean ± S.D. of three replicates per treatment, and three plant samples were consisted in one replicate. In the same column, significant differences according to LSD at $P=0.05$ levels are indicated by different letters

Based on its importance, both the horizontal transfer and the function of *nifH* in nodule endophytic *Bacillus* need to be studied further.

In the present work, we describe here non-symbiotic bacteria that are closely associated with nodules of *S. salsula* and their interaction with *S. salsula* nodulating rhizobial strain Zw-19 in planta. Our results parallel other studies, showing that the legume root nodules are known to accommodate several eubacterial genera apart from rhizobia, and their population densities are reported to be in the range of 10^4 viable bacteria per gram of fresh nodule tissue (Sturz et al. 1997). Although the role of such bacteria is not clear, it has been hypothesized that IAA of bacterial origin from the nodules is transported to other plant parts (Basu and Ghosh 1998) and use IAA as a part of their colonization strategy, involving phyto-stimulation and circumvention of plant defense mechanisms. Moreover, IAA might act as a signal molecule in bacteria–bacteria communication (Spaepen et al. 2007). The cooperative interactions between rhizobia and other plant root-colonizing bacteria are of relevance in the improvement of nodulation and N_2 fixation in legume plants (Barea et al. 2005). Besides cellulase, protease produced by many *Bacillus* and *Paenibacillus* strains was proposed to degrade fungal cell walls, thereby inhibiting plant pathogens (Lodewyckx et al. 2002). Bacteria that produce siderophores can inhibit plant pathogens by competing with them for iron in the rhizosphere. Some plants can also bind, transport and exploit bacterial iron–siderophore complexes (Lodewyckx et al. 2002). Perhaps, some of these functions explain why the production of siderophores was common among our non-symbiotic strains. Furthermore, the re-isolation of these bacteria from stem, roots and nodules of the inoculated host plants of *Sphaerophysa salsula* evidenced their endophytic properties according to the definition of endophytic bacteria (Hallmann et al. 1997).

Other previous studies reported that plant growth promotion when rhizobia are co-inoculated with *Bacillus* spp. include studies on *Rhizobium leguminosarum* by *trifolii* with either *B. insolitus* or *B. brevis* (Sturz et al. 1997) and with *Bacillus* spp. and the soybean endosymbiont, *Bradyrhizobium japonicum* (Bai et al. 2003; Liu and Sinclare 1993). Our results are in agreement with the above reports. To the best of our knowledge, this study is the first report to show that the effects of a *Streptomyces bottropensis* endophyte with *Mesorhizobium* spp. on *Sphaerophysa salsula* growth. Our results revealed that the association between legume nodules and endophytic bacteria is common in nature and that this interaction is beneficial for plant growth.

Many different mechanisms have been proposed as the basis of nodulation enhancement by epiphytic or endophytic root-associated bacteria (Barea et al. 2005). Using cell-free supernatants of bacterial cultures, it has been demonstrated that plant growth-regulating substances produced by rhizobacteria

affected nodulation and nitrogen fixation, (Hardoim et al. 2002; Peix et al. 2015; Tariq et al. 2014; Andrews et al. 2010; Rajendran et al. 2008; Gonzalez-Andres et al. 2005; Vidal et al. 2009; Azcon et al. 2010; Muresu et al. 2008; Barret and Parker 2006; Annapurna et al. 2013; Masciarelli et al. 2014; Subramanian et al. 2015; Sprent 2001; Van Berkum et al. 1996; Vincent 1970; Graan and Ort 1984; Glickmann and Dessaux 1995; Schwyn and Neilands 1987; Duan et al. 2009; Tokala et al. 2002; Balandreau et al. 2001; Vandamme et al. 2002; Dhole et al. 2016; Zhao et al. 2011; Basu and Ghosh 1998; Spaepen et al. 2007; Barea et al. 2005; Hallmann et al. 1997; Liu and Sinclare 1993; Azcón-Aguilar and Barea 1978; Manero et al. 2003). Further work is needed in this regard.

In previous studies, endophytic bacteria contribute to plant growth, ecological performance or reduction of plant stress (Lodewyckx et al. 2002). Our work revealed that a wide variety of rhizobial and non-symbiotic endophytic bacteria can colonize *S. salsula* nodules; moreover, they can assist the symbiotic interaction between rhizobia and the host plant.

The previous report (Bai et al. 2003) showed that *Bacillus subtilis* and *Bacillus thuringiensis* can naturally coinhabit soybean nodules along with *Bradyrhizobium japonicum*, and that these Gram-positive bacteria can enhance plant productivity in co-inoculation experiments. A more recent report (Zakhia et al. 2006) described the association of 14 bacterial genera with wild legume nodules in Tunisia. In the present study, most of the root nodules of *S. salsula* harbor prevalently rhizobia. Meantime, these nodules are also colonized internally by nonrhizobial endophytes. We plan to examine if undomesticated legumes differ from those cultivated in agriculture in their production of metabolites. Investigating these aspects will provide a better insight into the microbial interactions occurring in native and introduced wild legume plants and will lead to a better understanding of their nitrogen-fixing symbioses in further studies.

In sum, nodulation mainly occurred at the junction of the primary and secondary roots. The Qtx-10, Gzn-9-1 and Zw-13-3 inoculation resulted in root hair deformation in *Sphaerophysa salsula*. Furthermore, more lateral root formation can provide more potential rhizobial invasion sites, and thus enhance nodulation. But the tip bulging and deformation were not prominent and also uniformly distributed throughout the emerging root hairs, implying that the root infection process is a random event (Ibáñez et al. 2009; Rolfe et al. 1997).

Conclusions

Conclusively, the representative nodule endophytes improved nodulation and nodule activity in *Sphaerophysa salsula* on co-inoculation with *Mesorhizobium* sp. Zw-19.

These results demonstrated the diverse non-symbiotic bacteria associated with this plant grown promotion in northwestern China and the universal existence in its nodules. These results provide valuable information about the interactions among the symbiotic bacteria, non-symbiotic bacteria and their habitats.

Acknowledgements This work was supported by the National Natural Science Foundation Project (31660106); Special Project of Service for Local Area Foundation of Education Department of Shaanxi Province of China (16JF029); Innovation Program of Shaanxi Province of China (2012CGX7; 2012KTZB03-02-03; 2016TTC-N-3-1); National Natural Science Foundation of China (U1204301) and Research Fund for County key technology project of Shanxi Province of China (2018XY-14); The Special Fund of Technology Innovation Team for the Development and Utilization of Biological Resources of Yan'an City; The Eighth Batch of Provincial Agricultural Standardization Demonstration Area Projects in Shaanxi Province in 2019.

Author contributions Z-YK: formal analysis; B-CZ: methodology; L-FZ: software; Z-SD: supervision; Z-SD: visualization; Z-YK: writing—original draft

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

- Andrews M, Hodge S, Raven J (2010) Positive plant microbial interactions. *Ann Appl Biol* 157:317–320
- Annapurna K, Ramadoss D, Bose P, VithalKumar L (2013) In situ localization of *Paenibacillus polymyxa* HKA-15 in roots and root nodules of soybean (*Glycine max* L.). *Plant Soil* 373:641–648
- Aserse AA, Räsänen LA, Assefa F, Hailemariam A, Lindström K (2013) Diversity of sporadic symbionts and nonsymbiotic endophytic bacteria isolated from nodules of woody, shrub, and food legumes in Ethiopia. *Appl Microbiol Biotechnol* 97:10117–10134
- Azcon R, Peralvarez MD, Roldan A, Barea JM (2010) Arbuscular mycorrhizal fungi, *Bacillus cereus*, and *Candida parapsilosis* from a multicontaminated soil alleviate metal toxicity in plants. *Microb Ecol* 59:668–677
- Azcón-Aguilar C, Barea JM (1978) Effect of interactions between different culture fractions of Phosphobacteria and *Rhizobium* on mycorrhizal infection, growth and nodulation of *Medicago sativa*. *Can J Microbiol* 24:520–524
- Bai YM, Daoust F, Smith DL, Driscoll BT (2002) Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Can J Microbiol* 48:230–238
- Bai Y, Zhou X, Smit D (2003) Enhanced soybean plant growth due to coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. *Crop Sci* 43:1774–1781
- Balandreau J, Viillard V, Cournoyer B, Coenye T, Laevens S, Vandamme P (2001) *Burkholderia cepacia* genomovar III is a common plant-associated bacterium. *Appl Environ Microbiol* 67:982–985
- Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. *J Exp Bot* 56:1761–1778
- Barret CF, Parker MA (2006) Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in *Costa Rica*. *Appl Environ Microb* 72:1198–1206
- Basu PS, Ghosh AC (1998) Indole acetic acid and its metabolism in root nodules of a monocotyledonous tree *Roystonea regia*. *Curr Microbiol* 37:137–140
- Benhizia Y, Benhizia H, Benguedoua A, Muresu R, Giacomini A, Squartini A (2004) Gamma proteobacteria can nodulate legumes of the genus *Hedysarum* Syst Appl Microbiol 1 27:462–468
- De Lajudie P, Willems A, Nick G, Mohamed TS, Torck U, Filai-Maltouf A, Kersters K, Dreyfus B, Lindström K, Gillis M (1999) *Agrobacterium* bv. 1 strains isolated from nodules of tropical legumes. *Syst Appl Microbiol* 22:119–132
- De Meyer SE, De Beuf K, Vekema B, Willems A (2015) A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). *Soil Biol Biochem* 83:1–11
- Deng ZS, Zhao LF, Kong ZY, Yang WQ, Lindström K, Wang ET, Wei GH (2011) Diversity of endophytic bacteria within nodules of the *Sphaerophysa salsula* in different regions of Loess Plateau in China. *FEMS Microbiol Ecol* 76:463–475
- Dhole A, Shelat H, Vyas R, Jhala Y, Bhange M (2016) Endophytic occupation of legume root nodules by *nifH*-positive non-rhizobial bacteria, and their efficacy in the groundnut (*Arachis hypogaea*). *Ann Microbiol* 66:1–11
- Diouf D, Samba-Mbaye R, Lesueur D, Ba AT, Dreyfus B, de Lajudie P (2007) Genetic diversity of *Acacia seyal* Del. rhizobial populations indigenous to Senegalese soils in relation to salinity and pH of the sampling sites. *Microb Ecol* 54:553–566
- Duan J, Müller KM, Charles TC, Vesely S, Glick BR (2009) 1-aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from southern Saskatchewan. *Microb Ecol* 57:423–436
- Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl Environ Microb* 61:793–796
- Gonzalez-Andres F, Alegre J, Ceresuela JL (2005) The rhizobia nodulating shrubs for revegetation of arid lands: Isolation of native strains and specificity of the plant-rhizobia interaction by cross inoculation tests. *Arid Land Res Manag* 19:307–326
- Graan T, Ort DR (1984) Quantitation of the rapid electron donors to P700 the functional plastoquinone pool, and the ratio of the photosystems in spinach chloroplasts. *J Biol Chem* 259:14003–14010
- Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* 43:895–914
- Hardoim PR, van Overbeek LS, van Elsas JD (2002) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 10:463–471
- Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 16:463–471
- Hoque MS, Broadhurst LM, Thrall PH (2011) Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia. *Int J Syst Evol Microbiol* 61:299–309
- Ibáñez F, Angelini J, Taurian T, Laura Tonelli M, Fabra A (2009) Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. *Syst Appl Microbiol* 32:49–55
- Kan FL, Chen ZY, Wang ET, Tian CF, Sui XH, Chen WX (2007) Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai-Tibet Plateau and in other zones of China. *Arch Microbiol* 188:103–115

- Li JH, Wang ET, Chen WF, Chen WX (2008) Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biol Biochem* 40:238–246
- Li Li, Hanna S, Leone M, Wei GH, Kristina L, Räsänen LA (2012) Biogeography of symbiotic and other endophytic bacteria isolated from medicinal *Glycyrrhiza* species in China. *FEMS Microbiol Ecol* 79:46–68
- Liu ZL, Sinclair JB (1993) Colonization of soybean roots by *Bacillus megaterium* B153-2-2. *Soil Biol Biochem* 25:849–855
- Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgey M, van der Lelie D (2002) Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 21:583–606
- Manero FJ, Probanza A, Ramos B, Flores JJ, García-Lucas JA (2003) Effects of culture filtrates of rhizobacteria isolated from wild lupin on germination, growth, and biological nitrogen fixation of lupin seedlings. *J Plant Nutr* 26:1101–1115
- Martínez-Hidalgo P, Hirsch AM (2017) The nodule microbiome: N₂-fixing rhizobia do not live alone. *Phytobiomes* 1:70–82
- Masciarelli O, Llanes A, Luna V (2014) A new PGPR co-inoculated with *Bradyrhizobium japonicum* enhances soybean nodulation. *Microbiol Res* 169:609–615
- Muresu R, Polone E, Sulas L (2008) Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. *FEMS Microbiol Ecol* 63:383–400
- Palaniappan P, Chauhan PS, Saravanan VS, Anandham R, Sa TM (2010) Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. *Biol Fert Soils* 46:807–816
- Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ (2015) Bacterial associations with legumes. *Crit Rev Plant Sci* 34:17–42
- Rajendran G, Sing F, Desai AJ, Archana G (2008) Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. *Bioresour Technol* 99:4544–4550
- Ramirez-Bahena MH, Tejedor C, Martin I, Velazquez E, Peix A (2013) *Endobacter medicaginis* gen. nov., sp. nov., isolated from alfalfa nodules in an acidic soil. *Int J Syst Evol Microbiol* 63:1760–1765
- Rolfé RW, Djordjevic MA, Weinman JJ, Mathesius U, Pittock C, Garter E, Ride KM, Dong Z, McCull M, McIver J (1997) Root morphogenesis in legumes and cereals and the effect of bacterial inoculation on root development. *Plant Soil* 194:131–144
- Saini R, Dudeja SS, Giri R, Kumar V (2015) Isolation, characterization, and evaluation of bacterial root and nodule endophytes from chickpea cultivated in Northern India. *J Basic Microbiol* 55:74–81
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47–56
- Shiraishi A, Matsushita N, Hougetsu T (2010) Nodulation in black locust by the Gamma-proteobacteria *Pseudom* sp. and the Betaproteobacteria *Burkholderia* sp. *Syst Appl Microbiol* 33:269–274
- Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism–plant signaling. *FEMS Microbiol Rev* 31:425–448
- Sprenst JI (2001) Nodulation in Legumes. Royal Botanic Gardens, Kew
- Sturz AV, Christie BR, Matheson BG (1997) Nowak J Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on growth. *Biol Fert Soils* 25:13–19
- Subramanian P, Kim K, Krishnamoorthy R, Sundaram S, Sa T (2015) Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. *Plant Growth Regul* 76:327–332
- Tariq M, Hameed S, Yasmeen T, Zahid M, Zafar M (2014) Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). *World J Microbiol Biotechnol* 30:719–725
- Tokala RK, Strap JL, Jung CM, Crawford DL, Hamby Salove M, Deobald LA, Bailey F, Morra MJ (2002) Novel plant–microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl Environ Microbiol* 68:2161–2171
- Van Berkum P, Beyene B, Eardly BD (1996) Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). *Int J Syst Bacteriol* 46:240–244
- Vandamme P, Goris J, Chen WM, Devos P, Willems A (2002) *Burkholderia tuberum* sp. nov. And *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Syst Appl Microbiol* 25:507–512
- Vidal C, Chantreuil C, Berge O, Maure L, Escarre J, Bena G, Brunel B, Cleyet-Marel JC (2009) *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of *Anthyllis vulneraria* growing on metalcolous soil in Languedoc France. *Int J Syst Evol Microbiol* 59:850–855
- Vincent JM (1970) A manual for the practical study of root-nodule bacteria, IBP handbook, 15th edn. Blackwell Scientific Publications, Oxford
- Weiss VA, Faoro H, Tadra-Sfeir MZ, Raittz RT, de Souza EM, Monteiro RA (2012) Draft genome sequence of *Herbaspirillum lusitanum* P6-12, an endophyte isolated from root nodules of *Phaseolus vulgaris*. *J Bacteriol* 194:4136–4137
- Xiao X, Chen WM, Zong L, Yang J, Jiao S, Lin YB, Wang ET, Wei GH (2017) Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. *Mol Ecol* 26:1641–1651
- Xu L, Zhang Y, Wang L, Chen WM, Wei GH (2014) Diversity of endophytic bacteria associated with nodules of two indigenous legumes at different altitudes of the Qilian Mountains in China. *Syst Appl Microbiol* 37:457–465
- Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, de Lajudie P (2006) Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*-like gene within the genera *Microbacterium* and *Starkeya*. *Microb Ecol* 51:375–393
- Zhang BG, Du NN, Li J, Shi P, Wei GH (2018) Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across China. *Sci Total Environ* 643:569–578
- Zhao LF, Xu YJ, Sun R, Deng ZS, Yang WQ, Wei GH (2011) Identification and characterization of the endophytic plant growth promoter *Bacillus cereus* strain mq23 isolated from *Sophora alopecuroides* root nodules. *Braz J Microbiol* 42:567–575
- Zhao LF, Xu YJ, Lai XH (2018) Antagonistic endophytic bacteria associated with nodules of soybean (*Glycine max* L.) and plant growth-promotin. *Braz J Microbiol* 49:269–278

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.