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Isolation of endophytic bacteria from *Rehmannia glutinosa* Libosch and their potential to promote plant growth

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In order to study the growth promoting potential of endophytic bacteria from *Rehmannia glutinosa* Libosch, a total of 25 different bacteria belonging to 7 genera were identified by 16S rRNA gene sequencing, including *Bacillus*, *Micrococcus*, *Lysinibacillus*, *Brevibacterium*, *Halomonas*, *Kocuria* and *Terribacillus*. In this study, thirteen bacterial strains were found to solubilize inorganic phosphate, with the isolate *Kocuria rosea* (EH15) having the highest phosphorus dissolution activity (3.70 μg/mL). Twelve isolates were positive for nitrogen fixation abilities. Twenty-two strains produced indole-3-acetic acid (IAA) in the presence of L-tryptophan, and eleven of the twenty-two isolates synthesized IAA in the absence of L-tryptophan. The strain *K. rosea* (EH15) was capable of producing the highest IAA amount (15.36 and 7.98 mg/L) in Luria Bertani (LB) broth containing 0.2% L-tryptophan and lacking L-tryptophan, respectively. Ten isolates had siderophore production abilities with *Bacillus amyloliquefacieus* EH10 (0.26) and *Brevibacterium frigoritolerans* EH13 (0.32) showing high siderophore production characteristics. Five bacteria endogenous were selected to evaluate the growth parameters of *Brassica napus* L. and all isolates exhibited a significantly greater increase in seedling height, root length, fresh weight and dry weight, than the control plants. The greatest improvement appeared in the case of co-inoculation of EH10 and EH15, except in dry weight, and the biggest enhancement in dry weight occurred in the strain EH15. In general, these endophytic bacteria indicate a potential as microbial fertilizers to promote the growth of *R. glutinosa* Libosch.

Key Words: indole-3-acetic acid; nitrogen fixation; phosphate solubilization; *Rehmannia glutinosa* Libosch; siderophore production

Introduction

Endophytes that reside in plant tissues without causing any harmful effects to their hosts play an important role in biogeochemical cycles (Singh et al., 2017). The application of endophytes as microbial fertilizers has resulted in the improvement of growth and an increase of yield in cereal crops, reduced the use of chemical fertilizers and pesticides, and effectively alleviated environmental pollution. Recently, they have been shown to have a considerable potential for agricultural use.

In recent years, the mechanisms of increasing plant growth by endogenous bacteria have been studied by many researchers. They can influence plant growth by producing phytohormones, such as indole acetic acid (Ferrara et al., 2012), cytokinins and gibberellins (Sandhya et al., 2017). In addition, their metabolic activities can help the production of phosphate solubilization (Prakash and Arora, 2019), nitrogen fixation (Xu et al., 2014), and 1-aminocyclopropane-1-carboxylate deaminase (Etesami et al., 2014). It has been reported that some endophytes can inhibit pathogenic microorganisms by producing siderophores (Wang et al., 2013) and antimicrobial metabolites (Liu et al., 2016). Due to the various benefits of endophytic bacteria, interest in endogenous bacteria has

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been growing in recent years and many results have been reported.

Rehmannia glutinosa Libosch has been cultivated for more than 1,000 years in China, mainly in Jiaozuo, Henan Province, known as Huai Radix *Rehmannia*, which is one of the four famous Huaiyao medicines. The fresh, dried or steamed roots of *R. glutinosa* Libosch are widely used as anti-senescence, hypoglycemic and antinflammatory agents (Xu et al., 2016). As a result of the continuous cultivation of *R. glutinosa* Libosch, the problems of soil hardening and heavy metal pollution are becoming more and more serious because of the excessive use of chemical fertilizers. In order to alleviate a similar problem in other plants, scientific researchers have turned their attention to the study of endophytic bacteria.

At present, research relating to *R. glutinosa* Libosch mainly focuses on quality evaluation, pharmacological action, active ingredients, and processing and cultivation (Chao et al., 2018; Feng et al., 2015; Ling and Liu, 2009; Teng et al., 2016). The research on endophytes of *R. glutinosa* Libosch is scarce and has mainly focused on the fungal community (Peng et al., 2013). As endophytic bacteria are a promising alternative for plant inoculation and have the potential to be used in environmental restoration and agriculture, it is of significance to study the growth promoting abilities of endophytic bacteria populations associated with *R. glutinosa* Libosch. In this study, endogenous bacteria were isolated and identified from *R. glutinosa* Libosch, the growth promoting properties of the isolates were studied, and the reasons for its growth promoting influence on *Brassica napus* L. seedlings were investigated.

Materials and Methods

Surface sterilization and isolation of bacterial endophytes. Bacterial endophytes were isolated from the roots, stems and leaves of *R. glutinosa* Libosch. The samples were thoroughly cleaned with tap water and macerated in soapy water for 30 min. This was followed by treatment with distilled water and the samples were cut into 1–2 cm fragments. Then the materials were dipped into 70% ethanol for 8 min, in 0.1% $HgCl_2$ for 20 s, before being finally rinsed 4–5 times with sterile distilled water. The final wash water was spread onto Luria Bertani (LB) medium as the control and was incubated on LB medium at 30°C for 10 days. The surface of the samples is considered to be completely disinfected if nothing grows on the plate. The roots, stems and leaves were then disinfected in the same way. After that, the surface-sterilized plant materials were incubated on LB medium at 30°C for 48h. Diverse colonies were chosen according to morphological differences in color, shape, size and texture, and then were purified by the streak plate method. Purified bacteria were preserved in 50% (v/v) glycerol solution at –80°C.

Identification of the endophytic bacteria by 16S rRNA sequencing. Genomic DNA extraction and 16S rRNA region amplification were carried out according to the method reported by Ward (1992). The amplification of the 16S rRNA gene was performed using two primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR procedure was conducted as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and primer extension at 72°C for 30 s, and a final extension at 4°C for 30 min. The amplified products were purified and sequenced by Sangon Biotech (Shang Hai). The sequences obtained were compared with the sequences retrieved from the NCBI website using BLAST (Schmidt et al., 2018). The phylogenetic tree was constructed using MEGA 7.0 software according to the neighbor-joining method.

Phosphate solubilization. The isolates were screened for phosphate solubilization abilities according to the method of Surange (1997). The bacterial isolates were inoculated on the Pikovskaya medium (10.0 g glucose L^{-1}, 5.0 g $Ca_3(PO_4)_2$ L^{-1}, 0.5 g $(NH_4)_2SO_4$ L^{-1}, 0.3 g NaCl L^{-1}, 0.1 g $MgSO_4 \cdot 7H_2O$ L^{-1}, 0.3 g KCl L^{-1}, 0.03 g $FeSO_4 \cdot 7H_2O$ L^{-1}, 0.5 g yeast extract L^{-1}, 0.03 g $MnSO_4 \cdot 2H_2O$ L^{-1}, 15 g agar L^{-1}, 1 L of distilled water) and the medium was incubated at 30°C for 3 days. The tests were carried out in a randomized order with 25 treatments and 3 replicates. A halo zone around the bacterial colony was formed as an indication of phosphate solubilization. The diameters of the halo and colony were measured using digital calipers and the activity of phosphate solubilizing was described as the phosphorus solubilizing index (SI), and the SI were recorded based on the definition: SI = (diameter of halo zone + colony diameter)/colony diameter (Liu et al., 2017).

To quantify the activity of producing phosphate, 0.1 mL of bacterial solution cultured in LB liquid medium was added into the PKO liquid medium supplemented with 5 g $Ca_3(PO_4)_2$ L^{-1} and was incubated at 30°C in a shaking incubator at 130 rpm for 3 days. The tests were carried out in a randomized order with 25 treatments and three replicates. A liquid medium without inoculum was the control. After incubation, the culture supernatant was harvested (10,000 rpm for 5 min) and was subsequently used for measuring the phosphate amount produced by isolates, using the ascorbic acid method at 700 nm in a spectrophotometer.

Nitrogen fixation. The N-fixing abilities of the isolates were determined based on the method of Andrade et al. (2014). The isolates were inoculated on an Nfb semi-solid medium (5.0 g malic acid L^{-1}, 0.5 g K_2HPO_4 L^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ L^{-1}, 0.1 g NaCl L^{-1}, 0.02 g $CaCl_2 \cdot 2H_2O$ L^{-1}, 2.0 mL of trace element solution (0.2 g $Na_2MoO_4 \cdot 2H_2O$ L^{-1}, 0.235 g $MnSO_4 \cdot H_2O$ L^{-1}, 0.28 g H_3BO_3 L^{-1}, 0.008 g $CuSO_4 \cdot 5H_2O$ L^{-1}, 0.024 g $ZnSO_4 \cdot 7H_2O$ L^{-1}, 1 L of distilled water), 2 mL of bromthymol blue (0.5% aqueous solution (dissolved in 0.2 N KOH)), 4 mL of 1.64% EDTA-Fe solution, 1 mL of vitamin solution (0.01 g biotin L^{-1}, 0.02 g pyridoxine L^{-1}, 1 L of distilled water), 4.0 g KOH L^{-1}, PH adjusted to 6.8 with KOH, 1.75 g agar L^{-1}). The isolates were incubated on the Nfb medium at 30°C for 5 days and the formation of pellicle indicated an ability of nitrogen fixation.

Indole-3-acetic acid (IAA) production. The IAA production capacities of the strains were determined according to the method of Patten and Glick (2002). For screening out the isolates with the ability of producing IAA, the bac-

Table 1. Identification of the endophytic bacteria by 16S rRNA similarity search.

Isolates	Submitted 16S rRNA sequence to NCBIunder GenBank accession number	Closest NCBI match/species with GenBank accession number	Percentage of identity (%)
EH1	MN750756	*Bacillus subtilis* (MK860021.1)	100
EH2	MN750757	*Micrococcus luteus* (MH045862.1)	100
EH3	MN750758	*Bacillus licheniformis* (MH842141.1)	99.93
EH4	MN750759	*Lysinibacillus parviboronicapiens* (NR_114213.1)	99.65
EH5	MN750760	*Bacillus sonorensis* (MK860002.1)	100
EH6	MN750761	*Bacillus thuringiensis* (KJ769222.1)	99.51
EH7	MN750762	*Bacillus velezensis* (MG996871.1)	100
EH8	MN750763	*Bacillus safensis* (MK859936.1)	100
EH9	MN750764	*Bacillus methylotrophicus* (KC790266.1)	99.93
EH10	MN750765	*Bacillus amyloliquefaciens* (KU291367.1)	100
EH11	MN750766	*Bacillus cereus* (FJ501984.1)	100
EH12	MN750767	*Bacillus simplex* (KF818647.1)	99.86
EH13	MN750768	*Brevibacterium frigoritolerans* (KY753242.1)	99.86
EH14	MN750769	*Halomonas hydrothermalis* (CP023656.1)	99.78
EH15	MN750770	*Kocuria rosea* (MK696247.1)	100
EH16	MN750771	*Bacillus firmus* (KP165527.1)	99.86
EH17	MN750772	*Terribacillus goriensis* (CP008876.1)	100
EH18	MN750773	*Bacillus altitudinis* (KU663665.1)	100
EH19	MN750774	*Bacillus flexus* (KR999917.1)	100
EH20	MN750775	*Bacillus wiedmannii* (MK088275.1)	100
EH21	MN750776	*Bacillus horikoshii* (CP020880.1)	99.93
EH22	MN750777	*Bacillus tequilensis* (MH762888.1)	100
EH23	MN750778	*Bacillus aryabhattai* (MK859946.1)	100
EH24	MN750779	*Bacillus pseudomycoides* (MH578628.1)	99.59
EH25	MN750780	*Bacillus mycoides* (JN215520.1)	99.59

teria were inoculated into LB liquid medium supplemented with 0.2% L-tryptophan and were incubated with 130 rpm at 30°C for 24 h. After incubation, the bacterial cultures were centrifuged with 10,000 rpm at 4°C and 1 mL of the culture supernatant was mixed with 2 mL of Salkowski's reagent, and the mixture was incubated in the dark for 30 min at room temperature. Then the formation of pink color was observed, which indicated that the isolates had the ability of producing IAA.

In order to measure the ability of the isolates selected to produce IAA, the bacterial strains were incubated into 50 mL of LB broth containing 0.2% of L-tryptophan and lacking L-tryptophan, respectively. The media were incubated at 30°C for seven days. The tests were performed in a randomized way with 22 treatments and three replicates. The non-inoculated medium was used as the control. After incubation, 1 mL of the collected bacterial supernatant (10,000 rpm for 5 min) was mixed with 2 mL of Salkowski's reagent and incubated in the dark for 30 min. The mixture was then used to determine the amount of IAA production at 530 nm in a spectrophotometer. The IAA concentration was calculated from a calibration curve of pure IAA ranging from 0 to 20 mg/L.

Siderophore production. According to the method of Schwyn and Neilands (1987), the blue agar chrome azurol-S (CAS) medium was used to screen out the isolates with

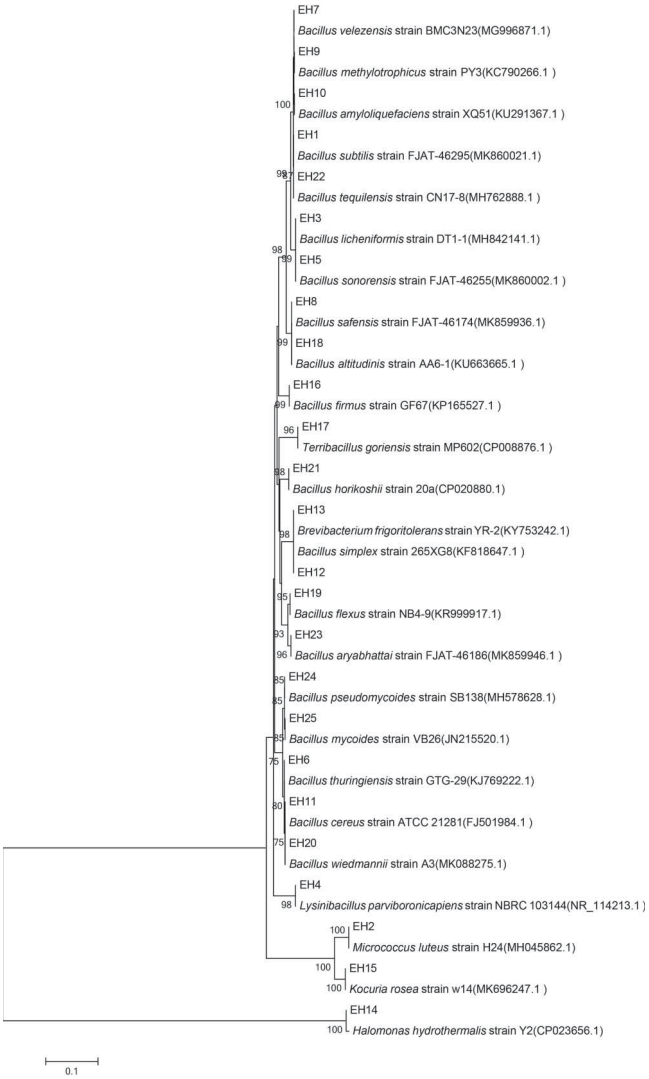

Fig. 1. Phylogenetic relationships of the partial 16S rRNA sequence of the isolates along with representative sequences retrieved from NCBI.

Bootstrap values, expressed as a percentage of 1000 replications, are shown at the branching points. Only bootstrap values greater than 70% are shown in the tree. The scale bar represents ten nucleotide substitutions per 100 nucleotides.

siderophore production potential. The pure colonies of the isolates were inoculated on CAS plates and the agar plates were incubated at 30°C for 3 days. The experiments were carried out in a randomized order with 25 treatments and three replicates. Then the bacterial cultures were observed for the formation of yellowish orange halos around the colonies, which showed the production of siderophores.

The isolates with a yellowish orange halo were further incubated into liquid MKB medium (Koh et al., 1995) and were incubated at 30°C for 3 days. Then the cell-free supernatant was collected (10,000 rpm for 5 min), and 3 mL of the bacterial supernatant was added into the same amount of CAS solution, and incubated in a dark environment for 1 h. The uninoculated supernatant was used as the control. All tests were carried out in a randomized order with 10 treatments and three triplicates. The absorbance of the isolates was recorded at a length wave of 630 nm using a spectrophotometer. The A/Ar (OD_{630} of the isolates/OD_{630} of the reference) represented the capacity of producing siderophores.

Effects of isolates on the growth promotion of B. napus L. seedlings. Endophytic bacteria were selected according to their various plant promoting growth (PGP) traits, such as phosphate solubilization, nitrogen fixation, IAA and siderophore production. Cell suspensions of the selected strains were used for the growth promotion studies of *B. napus* L. seedlings.

Seeds of *B. napus* L. were disinfected with 70% ethanol for 30 s and 1% sodium hypochlorite for 5 min. Finally, the seeds were rinsed 4–5 times with sterile distilled water. To obtain cell suspensions, the isolates were inoculated in LB liquid medium at 30°C for 48 h. Each bacterial culture was then centrifuged with 10,000 rpm for 5 min and the bacterial supernatant was discarded. The collected bacteria were then diluted to 10^8 cell/mL using phosphate buffer solution (8 g NaCl L^{-1}, 2.9 g Na_2HPO_4 L^{-1}, 0.2 g KCl L^{-1}, 0.2 g KH_2PO_4 L^{-1}, 1 L of distilled water, pH 7.4).

In pot experiments, each pot was packed with 50 g of sterile soil and five sterilized surface seeds were sown in each pot containing the sterile soil. Then 1 mL of the cell suspension prepared was inoculated into each pot and each treatment was conducted in triplicate. The treatment of inoculating with a phosphate buffer solution was used as the control. The pots were placed in the greenhouse. Every day, 1 mL of phosphate buffer solution was applied to each of the seedlings for 15 consecutive days. After that, the seedling height, root length, and fresh and dry weight were measured. The seedling height and root length were measured with a tape measure. The fresh weight was measured using an electronic balance, and the dry weight was determined in the same way after drying at 70°C for 48 h to a constant weight.

Statistical analysis. Data were expressed as the average value ± standard deviation and were analyzed by variance (ANOVA) analysis. Tukey's test was carried out to identify the significant differences ($P < 0.05$) among the processed data using SPSS 17.0 software (IBM, USA).

Results

Isolation and identification of endophytic bacteria

A total of 25 different endophytic bacteria were isolated from diverse tissues of *R. glutinosa* Libosch. 16S rRNA sequencing indicated that they belonged to seven genera, including *Bacillus*, *Micrococcus*, *Lysinibacillus*, *Brevibacterium*, *Halomonas*, *Kocuria* sp. and *Terribacillus*. Most (76% of the species) of the isolates belonged to the genus *Bacillus*. The sequence similarity of bacterial strains analyzed by 16S rRNA was 99.51–100% (Table 1). Details of BLAST analysis have been listed in Table 1. Analysis of the phylogenetic tree indicated that all strains identified were on the same branch with the representative strains having the highest similarity in the 16S rRNA sequence alignment (Fig. 1). The strains EH12 and EH13, belonging to the genera *Bacillus* sp. and *Brevibacterium* sp., respectively, showed a high consistency, which indicated that the two different genera

Table 2. Evaluation of the mechanisms of promotion of *R. glutinosa* Libosch growth.

Isolates	Most closely related species	Phosphate solubilization	Nitrogen fixation	IAA production		Siderophore production
				Trp	No Trp	
EH1	*Bacillus subtilis*	+	+	+	+	+
EH2	*Micrococcus letus*	–	–	+	–	–
EH3	*Bacillus licheniformis*	+	+	+	+	+
EH4	*Lysinibacillus parvibpronicapiens*	–	+	+	–	–
EH5	*Bacillus sonerensis*	+	–	+	–	+
EH6	*Bacillus thuringiensis*	–	–	+	–	–
EH7	*Bacillus velezensis*	–	–	+	+	+
EH8	*Bacillus safensis*	–	–	+	–	–
EH9	*Bacillus methylotrophicus*	–	–	+	+	–
EH10	*Bacillus amyloliquefacieus*	+	+	+	–	+
EH11	*Bacillus cereus*	+	+	+	–	–
EH12	*Bacillus simplex*	+	+	+	+	–
EH13	*Brevibacterium frigoritolerans*	–	–	+	+	+
EH14	*Halomonas hydrothermalis*	–	+	+	–	+
EH15	*Kocuria rosea*	+	+	+	+	–
EH16	*Bacillus firmus*	–	–	+	+	–
EH17	*Terribacillus goriensis*	–	+	+	+	–
EH18	*Bacillus altitudinis*	–	–	–	–	+
EH19	*Bacillus flexus*	–	+	–	–	–
EH20	*Bacillus wiedmannii*	+	–	+	–	–
EH21	*Bacillus horikoshii*	–	–	+	–	+
EH22	*Bacillus tequilensis*	–	–	+	–	–
EH23	*Bacillus aryabhattai*	+	+	+	+	+
EH24	*Bacillus pseudomycoides*	+	–	–	–	–
EH25	*Bacillus mycoides*	–	+	+	+	–

Trp: LB with 0.2% L-tryptophan; No Trp: LB without L-tryptophan; IAA: Indole-3-acetic acid; (–) not detected; (+) detected.

probably come from close groups. The isolates EH2 identified as *Micrococcus* sp., and EH15 belonging to *Kocuria* sp., showed a high degree of uniformity, which suggested that they may originate from close groups. The isolate EH14 being from *Halomonas* sp. was in a separate branch compared with the other genera, which indicated that they had a relatively distant relationship.

The distribution of bacterial endophytes in different tissues varied in the study. 18 species (representing 72% of all species obtained) occurred in roots, 13 species (representing 52% of all species) were isolated from stems and 10 species (representing 40% of all species) appeared in leaves. *Bacillus subtilis* and *Bacillus licheniformis* occurred in roots, stems and leaves.

Phosphate solubilization

Of 25 isolates analyzed, 10 bacterial isolates were observed to be able to form clear halo zones around the colonies (Table 2). Nine species forming clear halo zones belonged to *Bacillus*, including *B. subtilis* (EH1), *B. licheniformis* (EH3), *Bacillus sonerensis* (EH5), *Bacillus amyloliquefacieus* (EH10), *Bacillus cereus* (EH11), *Bacillus simplex* (EH12), *Bacillus wiedmannii* (EH20), *Bacillus aryabhattai* (EH23), and *Bacillus pseudomycoides* (EH24), and one isolate belonged to *Kocuria* sp., which was identified as *Kocuria rosea* (EH15).

The phosphate solubilization index (SI) showed significant differences ($P < 0.05$) among the isolates (Table 3). The isolate *B. simplex* (EH12) showed the highest SI (3.42), followed by the strain *B. subtilis* (EH1) with a good SI (3.20). The lowest SI (1.16) was achieved by *K. rosea* (EH15).

There was variability in the production of phosphorus in PKO liquid medium (Table 3). The amount of phosphorus production ranged from 0.23 μg/mL (EH17) to 3.70 μg/mL (EH15). Our study found that there was no positive correlation between the SI and the potential of phosphate produced by certain isolates (Table 3). For instance, *Halomonas hydrothermalis* (EH14) and *Bacillus horikoshii* (EH21) which were non-halo-forming were detected to produce a phosphate amount of 0.74 and 0.57 μg/mL, respectively (Table 3). In addition, the strain *B. simplex* (EH12) with the highest SI (3.42) produced 1.93 μg/mL of phosphate and the isolate *K. rosea* (EH15) with the lowest SI (1.16) produced the highest amount of phosphate (3.70 μg/mL).

Nitrogen fixation

Of 25 endophytic bacteria tested for nitrogen fixation capacities, 12 (48%) isolates were capable of growing and forming pellicles in the Nfb semi-solid medium (Table 2; Fig. 2). Of these, 8 (66.7%) isolates belonging to *Bacillus* were positive for forming pellicles, including *B. subtilis* (EH1), *B. licheniformis* (EH3), *B. amyloliquefacieus* (EH10), *B. cereus* (EH11), *B. simplex* (EH12), *Bacillus flexus* (EH19), *B. aryabhattai* (EH23), and *Bacillus mycoides* (EH25). Other isolates forming pellicles belonged to *Lysinibacillus*, *Halomonas*, *Kocuria* and *Terribacillus*.

Indole-3-acetic acid (IAA) production

All bacterial endophytes were preliminarily screened for their capacity to produce IAA. A bacterial supernatant of 22 isolates changed to a pink color after mixing with

Table 3. Promoting growth activities of the endophytic bacteria isolated from *R. glutinosa* Libosch.

Isolates	Most closely related species	Phosphate solubilization		IAA production (mg/L)		Siderophore production
		SI	P concentration (ug/mL)	Trp	No Trp	
EH1	*Bacillus subtilis*	3.20±0.07^{b}	1.61±0.04^{c}	14.23±0.32^{b}	2.23±0.20^{fg}	0.48±0.04^{d}
EH2	*Micrococcus letus*	—	—	2.68±0.16^{o}	—	—
EH3	*Bacillus licheniformis*	2.12±0.02^{c}	1.19±0.08^{d}	3.64±0.10^{no}	2.33±0.12^{efg}	0.44±0.03^{cd}
EH4	*Lysinibacillus parvibpronicapies*	—	—	6.01±0.14^{fghij}	—	—
EH5	*Bacillus sonerensis*	1.30±0.01^{fg}	0.32±0.07^{g}	5.00±0.25^{jklm}	—	0.44±0.04^{cd}
EH6	*Bacillus thuringiensis*	—	—	5.25±0.31^{ijkl}	—	—
EH7	*Bacillus velezensis*	—	—	3.99±0.21^{mn}	1.86±0.22^{g}	0.66±0.01^{e}
EH8	*Bacillus safensis*	—	—	4.48±0.13^{klmn}	—	—
EH9	*Bacillus methylotrophicus*	—	—	7.84±0.27^{e}	3.30±0.40^{cd}	—
EH10	*Bacillus amyloliquefacieus*	1.40±0.06^{ef}	0.26±0.08^{g}	6.26±0.13^{fghi}	—	0.26±0.06^{a}
EH11	*Bacillus cereus*	2.17±0.09^{c}	2.19±0.02^{b}	4.23±0.13^{lmn}	—	—
EH12	*Bacillus simplex*	3.42±0.05^{a}	1.93±0.07^{b}	6.45±0.06^{fg}	1.90±0.14^{g}	–
EH13	*Brevibacterium frigoritolerans*	—	—	14.25±0.39^{b}	3.87±0.23^{bc}	0.32±0.08^{ab}
EH14	*Halomonas hydrothermalis*	—	0.74±0.09^{ef}	10.76±0.19^{d}	—	0.65±0.01e
EH15	*Kocuria rosea*	1.16±0.09^{g}	3.70±0.07^{a}	15.36±0.28^{a}	7.98±0.17^{a}	—
EH16	*Bacillus.firmus*	—	—	6.78±0.53^{f}	2.85±0.26^{de}	—
EH17	*Terribacillus goriensis*	—	0.23±0.02^{g}	5.40±0.50^{hijk}	2.75±0.08^{def}	—
EH18	*Bacillus altitudinis*	—	—	—	—	0.42±0.03^{bcd}
EH19	*Bacillus flexus*	—	—	—	—	—
EH20	*Bacillus wiedmannii*	1.28±0.02^{fg}	1.27±0.04^{d}	6.40±0.45^{fgh}	—	—
EH21	*Bacillus horikoshii*	—	0.57±0.03^{f}	5.54±0.11^{ghij}	—	0.35±0.03^{abc}
EH22	*Bacillus tequilensis*	—	—	3.86±0.28^{n}	—	—
EH23	*Bacillus aryabhattai*	1.56±0.05^{e}	0.79±0.05^{e}	12.81±0.82^{c}	3.01±0.09^{d}	0.34±0.02^{abc}
EH24	*Bacillus pseudomycoides*	1.74±0.10^{d}	2.10±0.06^{b}	—	—	—
EH25	*Bacillus mycoides*	—	—	6.86±0.34^{ef}	4.23±0.10^{b}	—

SI: the phosphate solubilization index; (–) not detected.
Data are expressed as average value ± standard deviation; All the experiments were carried out in triplicate; Treatments with the diverse letter show significant difference in Tukey's test ($P < 0.05$).

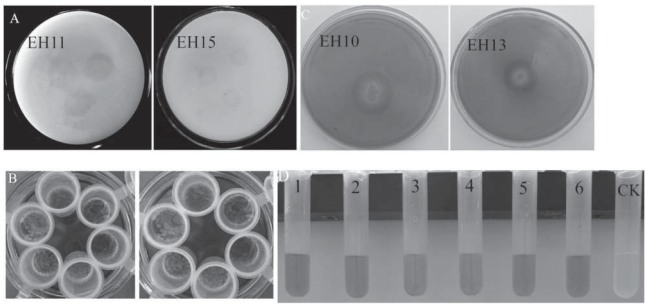

Fig. 2. PGP traits of some representative isolates identified from *R. glutinosa* Libosch.
A. phosphate solubilization; B. nitrogen fixation; C. siderophore production; D: IAA (1, *K. rosea* (EH15); 2, *B. frigoritolerans* (EH13); 3, *B. subtilis* (EH1); 4, *B. aryabhattai* (EH23) 5, *H. hydrothermalis* (EH14); 6, *Bacillus methylotrophicus* (EH9); ck, control).

Salkowski's reagent. The isolates *K. rosea* (EH15) and *Brevibacterium frigoritolerans* (EH13) displayed deeper pink colors compared with the rest of the strains (Fig. 2), whereas the bacteria *Bacillus altitudinis* (EH18), *B. flexus* (EH19) and *B. pseudomycoides* (EH24) did not display any pink colors.

In the quantitative analysis of IAA production among the 22 isolates inoculated in the medium containing L-tryptophan, the amount of IAA production ranged from 2.68 mg/L (EH2) to 15.36 mg/L (EH15). The isolates *B. altitudinis* (EH18), *B. flexus* (EH19) and *B. pseudomycoides* (EH24) were not observed to produce IAA. Of the 22 isolates, 11 strains were found to synthesize IAA in the absence of L-tryptophan (Table 3) and the range of IAA production was from 1.86 mg/L (EH7) to 7.98 mg/L (EH15). The 11 isolates belonged to the genera *Bacillus* (EH1, EH3, EH7, EH9, EH12, EH16, EH23 and EH25), *Brevibacterium* sp. (EH13), *Kocuria* sp. (EH15) and *Terribacillus* sp. (EH17). As shown in Table 3, the amount of IAA produced by the 11 isolates in the presence of L-tryptophan was, in all cases, greater than in the absence of L-tryptophan, which indicated that L-tryptophan can contribute to the production of IAA.

Siderophore production

The strains with the ability of siderophore production were screened using chrome azurol S (CAS) agar medium. Ten isolates were found to form yellow halos around the colonies, which indicated that they had the potential of producing siderophores (Table 2; Fig. 2).

According to the efficiency scale proposed by Payne (1994), the size of A/Ar reflects the capability of siderophore production. In this study, two isolates exhibited a low A/Ar (0.6–0.8), four isolates showed a moderate A/Ar (0.4–0.6), and four bacterial strains showed a high A/Ar (0.2–0.4) (Table 3). As shown in Table 3, higher siderophore activities were indicated by *B. amyloliquefacieus* (EH10), *B. frigoritolerans* (EH13), *B. aryabhattai* (EH23) and *B. horikoshii* (EH21) with an A/Ar of 0.26, 0.32, 0.34 and 0.35, respectively.

Table 4. Effect of cell suspensions of endophytic bacteria selected on growth promotion of *B. napus* L. seedlings.

Isolates	Seedling height (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Control	5.59 ± 0.26^{f}	2.94 ± 0.03^{f}	0.3261 ± 0.0021^{f}	0.0115 ± 0.0002^{d}
EH1	9.44 ± 0.04^{b}	4.53 ± 0.1^{c}	0.5134 ± 0.0098^{c}	0.0216 ± 0.0003^{a}
EH10	7.77 ± 0.13^{d}	3.57 ± 0.1^{e}	0.4819 ± 0.0049^{d}	0.0192 ± 0.0005^{c}
EH11	6.65 ± 0.08^{e}	4.07 ± 0.08^{d}	0.5667 ± 0.0022^{b}	0.0205 ± 0.0006^{b}
EH13	8.53 ± 0.09^{c}	3.45 ± 0.02^{e}	0.4016 ± 0.0017^{e}	0.0187 ± 0.0002^{c}
EH15	9.86 ± 0.07^{a}	4.86 ± 0.03^{b}	0.4114 ± 0.0101^{e}	0.0230 ± 0.0016^{a}
EH10+EH15	10.24 ± 0.21^{a}	5.13 ± 0.04^{a}	0.5917 ± 0.0082^{a}	0.0206 ± 0.0004^{b}

Data are means ± standard deviation of three replicates. Treatments with the different letters show significant difference in Tukey's test ($P < 0.05$); Control: The plants inoculated with phosphate buffer solution.

Effect of endophytic bacteria on plant growth promotion of B. napus L.

Based on PGP indications, the isolates *B. subtilis* (EH1), *B. amyloliquefacieus* (EH10), *B. cereus* (EH11), *B. frigoritolerans* (EH13) and *K. rosea* (EH15) were selected for evaluating the growth promotion of *B. napus* L. by individual inoculation and co-inoculation in pot experiments.

Inoculated plants exhibited a significantly greater ($P < 0.05$) seedling height, root length, and fresh and dry weight, than the control treatment after 15 days (Table 4; Fig. 3). As shown in Table 4, after 15 days of inoculation, the larger improvement in seedling height and root length was caused by the co-inoculation of EH10 and EH15 compared with the control, with a 0.83-fold and 0.75-fold increase, respectively. Another notable bacterial strain was EH15: seedling height (9.86 cm) was increased 0.76-fold and root length (4.86 cm) was enhanced 0.65-fold. However there was no significant difference in seedling height between EH15 and (EH10 + EH15) (Table 4). The greater fresh weight (0.5917 g) also appeared in the co-inoculation of EH10 and EH15, with a 0.81-fold increase greater than the control, and this was followed by the EH11 isolate, which showed a 0.74-fold greater increase. Other treatments EH15, EH1 and (EH10 + EH15), dry weight were increased by 1.00-fold, 0.87-fold and 0.78-fold, respectively, than the control. In conclusion, the co-inoculation of EH15 or (EH10+EH15) should be considered as promising growth promoters in the production of *R. glutinosa* Libosch.

Fig. 3. Influence of cell suspensions of endophytic bacteria selected on the growth promotion of *B. napus* L. seedlings.

CK (from the right) represents the control with inoculated phosphate buffer solution. The co-inoculation of EH10 (*B. amyloliquefacieus*) and EH15 (*K. rosea*), EH15 (*K. rosea*), EH1 (*B. subtilis*), EH13 (*B. frigoritolerans*), EH10 (*B. amyloliquefacieus*), EH11 (*B. cereus*) showed greater growth compared with that of the control.

Discussion

Bacterial endophytes have been reported to be a new type of bio-fertilizer and bio-protectants to reduce environmental pollution caused by chemical fertilizers and pesticides (Hong and Park, 2016). Therefore, the study of the potential of endophytes has attracted the attention of microbiologists. Studying the promotion growth abilities of endophytic bacteria is one aspect of this research. Although bacterial endophytes with the capacities of enhancing plant growth have been isolated from different medicinal and crop plants (Liu et al., 2017), this is the first report to research the PGP traits of endogenous bacteria from *R. glutinosa* Libosch.

In the present study, the method of surface disinfection for isolating endophytic bacteria was considered to be viable because nothing grew on the control plate. So it could be considered that the bacterial colony isolated in the study are true bacterial endophytes of *R. glutinosa* Libosch.

In earlier reports, the genera *Bacillus*, *Sphingopyxis*, *Virgibacillus*, *Enterobacter*, *Pseudomonas* and *Klebsiella* as common endogenous bacteria have been isolated from other plants (Costacurta et al., 1995; Dias et al., 2009; Lata et al., 2006). In our study, the species compositions of endophytic bacteria isolated from *R. glutinosa* Libosch were different from the plants previously reported. It may be caused by the different host and the diverse environments in which endophytic bacteria live, and differences in the culture media for isolation.

The distribution of endogenous bacteria isolated from diverse tissues varied. Root tissues were found to be a more suitable host for endophytic bacteria as compared with stems and leaves. Similar results were also found in the *Ferula sinkiangensis* (Liu et al., 2017). The reason is that the soil is a chief inoculum of microorganisms, and, therefore, roots, being in contact with the soil, contain more diverse endophytic bacterial populations (Ullah et al., 2017).

The phosphate solubilization properties of isolates can be preliminarily selected by the formation of clear halo zones around the colonies on a PKO medium, but it should

not be the only way. Previous studies have shown that some isolates with a relatively low SI also showed efficient phosphate solubilizing activities in a liquid medium (Andrade et al., 2014; De Abreu et al., 2017). This result was verified in our study. For instance, the isolates *H. hydrothermalis* (EH14), *Terribacillus goriensis* (EH17) and *B. horikoshii* (EH21) with SI(1) were detected to produce phosphate in the liquid medium (Table 2; Table 3), the isolate *K. rosea* (EH15) with the lowest SI (1.16) was found to produce the highest phosphate concentration. The reason may be that organic acids secreted by endophytic bacteria can reduce the pH of the medium and chelate with a calcium ion, converting insoluble inorganic phosphorus into organic phosphorus (De Abreu et al., 2017; Marra et al., 2012). This suggests, in further research, we should study the relationship between the SI, the organic acid, and the phosphate solubilizing produced by endogenous bacteria.

The growth and development of plants need phosphorus, but the efficiency of phosphorus that can be directly utilized by plants in the soil is low. As has been reported, the growth parameters of maize, such as plant height, root length, fresh and dry weight have been increased greatly after inoculation with phosphate solubilizing bacteria (Hussain et al., 2013). In this study, the isolate *K. rosea* (EH15) with the highest phosphate solubilization activity (3.70 μg/mL) was found to enhance the seedling height, root length, and fresh and dry weight. The result can be explained by the fact that phosphatase, as an enzyme produced by phosphate dissolving endophytes, contributes to phosphate absorption by plants (Jog et al., 2014).

The appearance of endogenous bacteria with nitrogen fixing abilities was shown more than 30 years ago (Cavalcante and Dobereiner, 1988). Since then, some researchers have isolated and identified these microorganisms and illustrated the mechanisms of nitrogen fixing from different plants (James, 2000; Stoltzfus et al., 1997). As a new type of bio-fertilizer, endogenous nitrogen-fixing bacteria are environmentally friendly and can effectively promote plant growth (Singh et al., 2011). As far as we know, our paper is the first to report on the nitrogen fixing ability of endophytic bacteria from *R. glutinosa* Libosch. In our study, the genus *Bacillus* was a predominant population with an N-fixing capability. This result is similar to that reported by Cocking (2003). Our results have indicated that it is possible to have several diazotrophic bacteria present in *R. glutinosa* Libosch, so it is necessary to conduct more specific tests in order to confirm the presence of endophytic bacteria providing a nitrogen supply for *R. glutinosa* Libosch.

IAA was considered as a production of tryptophan metabolism in microorganisms (Ahmad et al., 2005). So it can be considered that the presence of tryptophan in the culture medium can contribute to increase IAA production by endophytic bacteria (Table 3). However, in recent years, some scholars have proved that some isolates could also synthesize IAA in the absence of tryptophan (Ilić et al., 1999). In our study, the genera *Bacillus* (EH1, EH3, EH7, EH9, EH12, EH16, EH23 and EH25), *Brevibacterium* (EH13), *Kocuria* (EH15) and *Terribacillus* (EH17) synthesized IAA in the absence of L-tryptophan, which illustrates that an L-tryptophan pathway is not the only way to synthesize IAA, and that IAA can also be released from IAA conjugates (IAA-methylester, IAA-sugarand and IAA-aminoacids) through hydrolytic cleavage (Zhao, 2010).

The ability of endophytic bacteria to produce iron carries have been reported in rice, *Glycyrrhiza uralensis* F and sugarcane plants (Li et al., 2018; Loaces et al., 2011; Silveira et al., 2019). Siderophore production by the isolates is of great significance for the absorption of nutrients, such as iron, effective utilization to plants and improving competence for the nutrients with the pathogens in their ecological niche, indirectly inhibiting disease-causing organisms (Ali et al., 2018; Rungin et al., 2012). Thus, siderophore production by the isolate can promote the growth of plants. In our study, *B. napus* L. inoculated with EH10 with the highest siderophore production ability resulted in a greater increase in seedling height (39.04%), root length (21.40%), and fresh and dry weight (47.75, 66.76%) than un-inoculated plants, promoting the suggestion that it is of great interest to study the siderophore production of endophytes as bacterial fertilizers.

In previous reports, endophytic bacteria with the potential of increasing plant growth had the abilities of producing IAA (Khan and Doty, 2009; Khan et al., 2014; Rangjaroen et al., 2015). In our study, we also found that all isolates promoting the growth of *B. napus* L. showed IAA production activity. For instance, EH15 with the highest activity of producing IAA resulted in a significant improvement in seedling height and root length, which illustrates that IAA can affect cell division, extension and differentiation, root formation, and the control process of nutrition growth (Borah et al., 2019). Besides, the most significant enhancement in seedling height, root length and fresh weight was given by the co-inoculation of *B. amyloliquefacieus* (EH10) and *K. rosea* (EH15), which may be caused by the fact that the combined effect of high phosphate solubilization (3.70 μg/ml), IAA production (15.36 mg/L), nitrogen fixation and siderophore production (0.26) can supply a more balanced nutrition for plants and facilitate their growth (Kruasuwan and Thamchaipenet, 2016).

As the seeds of *B. napus* L. germinate more quickly and the plant seedlings grow faster than *R. glutinosa* Libosch, endophytic bacteria with the ability of phosphate solubilization, nitrogen-fixing, IAA production and siderophore production were applied to the growth of *B. napus* L. If the growth of *B. napus* L. is promoted, it can be inferred that these endophytic bacteria can also promote the growth of *R. glutinosa* Libosch. Therefore, in a further experiment, these endophytic bacteria will be used as plant growth promoters in the case of *R. glutinosa* Libosch.

In previous reports, although *B. amyloliquefacieus* and *K. rosea* were found to have the ability of promoting the growth of plants (Mohammadzadeh et al., 2014; Soares et al., 2016), to our knowledge, their co-inoculation has not been previously reported. This is the first report of the co-inoculation of *B. amyloliquefacieus* and *K. rosea* being applied to increase the growth of *B. napus* L.. The co-inoculation of EH10 and EH15 has been shown to have a better influence on the the growth of *B. napus* L. than other

treatments. Thus, this indicates that the co-inoculation of (EH10 + EH15) should be used as a biofertilizer to promote the growth of *R. glutinosa* Libosch

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Author's Contributions

Shanshan Wang performed experiments and wrote the initial draft; Hanwei Li and Chengming Dong designed the study; Yunhao Zhu completed the analysis of molecular data; Suiqing Chen and Weisheng Feng provided help in the analysis of data; Xiuhong Su and Baoyu Ji provided important revisions to this article. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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