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IDENTIFICATION AND CHARACTERIZATION OF THE ENDOPHYTIC PLANT GROWTH PROMPTER *BACILLUS CEREUS* STRAIN MQ23 ISOLATED FROM *SOPHORA ALOPECUROIDES* ROOT NODULES

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ABSTRACT

Endophytes MQ23 and MQ23R isolated from *Sophora alopecuroides* root nodules were characterized by observing their ability to promote plant growth and employing molecular analysis techniques. Results showed that MQ23 and MQ23R are potential N_2-fixing endophytes and belong to the same species as *Bacillus cereus*. MQ23 was shown to be able to produce siderophores, IAA, and demonstrate certain antifungal activity to plant pathogenic fungi. Co-inoculation with MQ23+MQ23II showed a more significant effect than inoculation alone *in vitro* for most of positive actions suggesting they have a cooperative interaction. Results of plant inoculation with endophytes indicated that the growth indexes of co-inoculated MQ23+MQ23II were higher than those of inoculated alone ($p<0.05$) (the exception being for root fresh weight) when compared to negative control. There have been little of any studies of nonrhizobial putative endophytes with growth-promotion attributes in *S. alopecuroides* root nodules. This could be exploited as potential bio-inoculants and biocontrol agents in agriculture.

Key words: Plant growth promoting characterization; *Sophora alopecuroides*; Root nodules; Endophytic bacteria; *Bacillus cereus*

INTRODUCTION

In the last few decades, endophytic bacteria have attracted more and more attention as novel resources as a method of biocontrol for plant diseases and promoters of plant growth (18, 22). Generally, the endophytic bacteria live inside the plant tissues and do not cause visible damage or morphological change on their hosts. They can benefit the host plants by the production of IAA (indoleacetic acid, phytohormones), siderophores, and antibiotic compounds, through nitrogen fixation, by phosphate solubilization, and with the suppression of phytopathogens through competition (16, 27). Additionally, they may help the symbiotic rhizobia form nodules with non-specific hosts, further aiding plant growth (20). The occurrence of *Bacillus* species as endophytes has been reported from different plants such as pigeon pea (8), wheat, kudzu (31), and soybean nodules (26). They have been shown to benefit to their hosts by promoting nodulation and

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growth. Moreover, plant studies have shown that the beneficial effects of plant growth promoting microorganisms can be enhanced by co-inoculation with other microorganisms. Co-inoculation frequently increased growth and yield compared to single inoculation (4, 8, 10). However, up to now, nodule endophytes of *S. alopecuroides* have not yet been specifically studied.

In a recent study, we collected and characterized nodule endophytic bacteria from the legume *S. alopecuroides* (41). The aims of this experiment were (i) to identify the phylogenetic position of *Bacillus cereus* MQ23 and MQ23R, and (ii) to determine their promoting plant growth characterization via plant inoculation tests.

MATERIALS AND METHODS

Isolation of nodule endophytes

Healthy *S. alopecuroides* plants were collected from Northwestern China. Three nodules were randomly selected from each plant, washed with sterile distilled water to remove soil particles, and surface sterilized with 95% alcohol for 30s and with 0.1% (w/v) $HgCl_2$ for 2 min, rinsed 6-8 times to eliminate thoroughly $HgCl_2$ with sterile distilled water. The surface sterilized nodules were crushed and streaked on yeast-extract-mannitol agar (YMA) plates for the isolation of endophytic bacteria with the standard methods described previously (36). The plates were incubated at 28°C for 5 days and single colonies were further purified by repeatedly streaking on the same medium. In order to verify surface sterilization, aliquots of water from final rinse were spread onto the Nutrient Agar (NA) medium and incubated. Plates without any contaminants were considered effectively surface sterilized and their corresponding YMA plates were used for the isolation of endophytes. Nodulation capability was verified for nodule isolates by inoculating on surface sterilized and pre-germinated seeds. The inoculated *S. alopecuroides* seedlings were grown for six weeks under greenhouse conditions and nodules were checked and isolated its endophtes.

The *nifH* gene amplification

The *nifH* gene encoding the Fe protein subunit of nitrogenase was used a molecular markers to estimate the symbiotic gene diversity and phylogeny (7). The forward primer nifH40F (5'-GGNATCGGCAAGTCSACSAC-3'), reverse primer nifH817R (5'-TCRAMCAGCATGTCCTCSAGCTC-3') and procedure described by Vinuesa *et al*. (37) were used for the *nifH* gene specific PCR assay. The PCR products were separated by horizontal electrophoresis in 1% (w/v) agarose gels and the patterns were visualized as described (38).

SDS-PAGE of whole cell protein

Solubilized proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (4% acrylamide stacking and 12.5% acrylamide resolving gels) as described by Laemmli (17). Electrophoresis was performed in a Mini Protean II apparatus (Bio-Rad) with a discontinuous buffer system. The gel was run at 30 mA until the bromophenol blue marker had reached the bottom of the gel. Gels were then stained with Coomassie Brillant Blue R-250 0.125% (w/v) in methanol/acetic acid/water (5:1:4 v/v) and de-stained in the same solvent mixture before being swollen to their original sizes in acetic acid 7% (v/v). Molecular weight marker polypeptides (prestained SDS-PAGE Standards-Low Range, Bio-Rad) were run in the same gels to allow the estimation of molecular weights. Visual comparisons of band patterns were made on the wet gels with transillumination.

PCR-RFLP based on 16S rRNA gene

The total genomic DNA was extracted from each of the nodule isolates outlined previously (24). In the present study, the 16S rRNA gene was selectively amplified from the genomic DNA with the universal forward primer P1 (5'-CGGGATCCAGAGTTTGATCCTGGCTCAGAACGAACGCT-3') and reverse primer P6 (5'-CgggATCCTACggCTACCTT

gTTACgACTTCACCCC-3') (34) as described (33). An aliquot of PCR product was digested separately with restriction endonucleases *Hha*I, *Hae*III, *Msp*I, *Hinf*I according to the producer's guide. The restricted bands were separated by horizontal electrophoresis in 2% (w/v) agarose gels and the patterns were visualized as described previously (38).

Sequencing and phylogenetic analysis

Aliquots of PCR products of isolate MQ23 and MQ23R were directly sequenced using the same primers for PCR-RFLP analyses as described by van Berkum *et al.* (34). The acquired and related sequences were matched with ClustalX1.81 software, imported into Bioedit 4.8.4 and manually corrected. Phylogenetic tree were constructed using the Jukes–Cantor model and neighbor-joining (NJ) method (28) in TREECON package, and computation of the similarity of each strain tested was done with the DNAMAN application (version 6.0.3.40, lynnon corporation). The 16S rRNA gene sequences obtained were submitted to NCBI GenBank (http://www.nvbi.nlm.nih.gov/) under the accession numbers HM241939, HM241940, and HM241941.

Plant growth promoting characteristics of isolates

Siderophore examination

Bacterial cultures were multiplied in Lysogeny broth (LB, 10 g NaCl/L) for 48 h and aliquots of pure bacterial culture were inoculated in plates containing agar Chrome Azurol S (CAS) and incubated at 30 °C. Each plate was observed daily for 7 days to detect the appearance of orange color around the colony (30). Experiments were performed in triplicate.

Phosphate solubilization

Log phase LB pure bacterial cultures were spot inoculated on Pikovasky's inorganic and Mongina organic culture plates, incubated at 30 °C, and observed daily for 7 days for appearance of transparent halos (14). Experiments were performed in triplicate.

Antifungal activity

Spores of fungal cultures (*Fusarium oxysporum*, *Magnaporthe grisea*, *Botrytis cinere* Pers., *Valsa mali* Miyabe et Yamada, *Alternaria alternate*) were grown on Potato Dextrose Agar (PDA) plates and a small block of agar with fungal growth was cut using sterile puncher (Ø=4 mm) and placed in the centre of a fresh PDA plate. Tested strains were spot inoculated on the PDA plate's edge about 25 mm from the centre, incubated at 30 °C for 7 days, and observed for zones of clearance. Fungal mycelia cultivated for 7 days without spot inoculation were used as control.

Organic acid production

Bacterial cultures were spot inoculated in MM9 (29) agar medium and observed for a drop in pH using methyl red as an indicator dye, which changes from yellow to pink below pH 5.0. Isolates having the ability to produce organic acid displayed a pink zone around the colony.

IAA production

Indole acetic acid (IAA) production was estimated by inoculating a bacterial suspension (3×10^7 cfu ml^{-1}) in 10 ml (LB) broth containing L-tryptophan (100 µg ml^{-1}), and shaken in an incubator for 72 h. IAA concentration in the culture supernatant was estimated using Sackowski´s reagent (9).

Plant inoculation experiment under greenhouse conditions

S. alopecuroides seeds were treated with 98% sulphuric acid for 60 min, rinsed with sterile water to remove residual sulphuric acid, surface sterilized with absolute alcohol for 1 min and 0.1% (w/v) $HgCl_2$ for 2 min, finally rinsed 6 times with sterile distilled water. Surfaced sterile seeds were then allowed to germinate axenically in Petri dishes filled with moist filter paper at 28 °C, inoculated by immersing in the liquid bacterial culture with a thick suspension (approx.10^9-10^{10} cfu ml^{-1}) for 3 h, and grown in pots filled with sterilized vermiculate moistened with nitrogen-free plant nutrient

solution as described by Vincent *et al* (36). Each isolate cultured in 5 ml of YM broth to the exponential phase was used as inoculant. The inoculated seedlings were cultured under greenhouse conditions and programmed for a 14 h/d photoperiod at a constant temperature of 28 °C during the day and 20 °C during the night with about 50% relative humidity. *Mesorhizobium* sp. MQ23II was acquired from our laboratory, and 16S rRNA sequence confirmed it as *Mesorhizobium* sp. All pots experiments were performed in ten repetitions with five seedlings per pot; variables used were seedlings with *Mesorhizobium* sp. MQ23II alone as positive control (PC), seedlings without any bacteria alone as negative control (NC), seedlings with isolate MQ23, and seedlings co-inoculated with *Mesorhizobium* sp. MQ23II and isolate MQ23 (1:1 v/v). The plants were harvested after six weeks when well developed nodules could be detected. Parameters such as plant biomass and dry weight, shoot and root length, and nodule number per plant were estimated compared with control plants that were not inoculated.

RESULTS

Isolation and determination of endophytes

Twenty-eight endophytes were isolated from *S. alopecuroides* root nodules. Physiological and biochemical tests were conducted (data not shown), including temperature, pH values and salt tolerance, antibiotic sensitivity, metal susceptibility, and dye resistance. On the basis of these characteristics, a selected strain was termed MQ23, and strain reisolated from *S. alopecuroides* nodule in plant inoculation test was termed MQ23R.

The *nifH* gene amplification

The results of *nifH* gene PCR amplification showed that isolates MQ23 and MQ23R both could produce single product bands on agarose gel by electrophoresis at the expected *nifH* gene fragment size, about 785bp (Figure 1), with *nifH* PCR primers nifH40F and nifH817R. This indicated these two isolates share common *nifH* gene. Since *nifH* encodes the highly conserved Fe protein of nitrogenase and has been used as a marker gene for nitrogenase, the presence of same *nifH* gene fragments in MQ23 and MQ23R strains under different environment in this study provided genetic evidence of nitrogen fixation capabilities, and a way to analyze the nitrogen fixation potential. But, nif-DNA only shows the strains that contain nitrogenase genes and does not indicate whether or not these nitrogenase genes are expressed and these strains are active and fix nitrogen. Therefore, the fact may play a significant role in nitrogen fixation under field and greenhouse environment. Further work will be necessary to address this question.

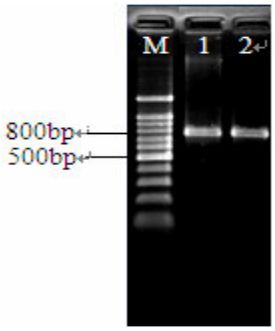

Figure 1. The *nifH* gene amplification of inoculated and reisolated strains (M: 100bp marker; 1:MQ23; 2:MQ23R)

Identification and phylogenetic analysis of endophytes MQ23 and MQ23R

There were some differences between MQ23 and MQ23R in its host growth environment. For example, strain MQ23 was isolated from healthy *S. alopecuroides* plants growing in Northwestern China. Where there have a dry climate, an alkaline soil profile, host *S. alopecuroides* shows the excellent performance of drought and alkaline tolerance and anti-sandstorm for its developed root system under field environment. However, strain MQ23R was isolated from nodules of healthy *S. alopecuroides* inoculated with MQ23

under greenhouse conditions. Though its host growth environment existed differences, our experiment results of SDS-PAGE (Figure 2) showed that whole-cell protein profiles generated by endophytes MQ23 and MQ23R were very similar or identical, indicated exist close phylogenetic relationship between them, perhaps they have same origins. However, compared with profiles of strain MQ23II, there were many differences among them, whether the number or density of bandings. On the other side, the analysis of PCR-RFLP based on 16S rRNA gene (Figure 3) in present study indicated that restriction patterns of MQ23 and MQ23R are identical, while strain MQ23II show distinct banding patterns from them.

The phylogeny of 16S rRNA genes has been used as one of the main criteria for the differentiation of species, genera and higher taxa in current bacterial taxonomy. In this work, upon constructed phylogenetic relationship of 16S rRNA genes sequences (Figure 4) and rRNA sequence analysis we could see that isolates MQ23 and MQ23R had 100% sequence homology, belonged to the same species. They were most related to *Bacillus cereus* ATCC14579^T and *Bacillus thuringiensis* ATCC10792^T (with 100% and 99.9% similarity, respectively), they formed a *Bacillus* sub-clade. Therefore, the nodule endophytes were designated as *Bacillus cereus* MQ23 and *Bacillus cereus* MQ23R.

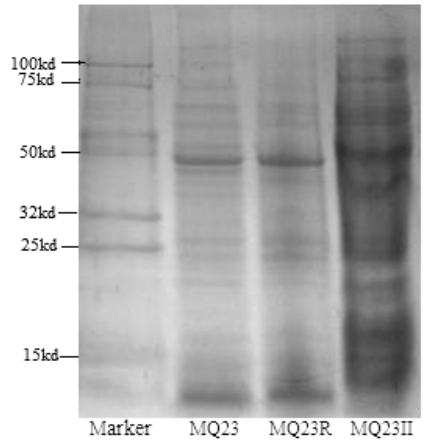

Figure 2. Protein electrophoresis pattern of SDS-PAGE. Lane's order from left to right: marker, MQ23, MQ23R, MQ23II. MQ23 was original strain, and MQ23R was reisolated from *S. alopecuroides* root nodule of plant reinoculation. MQ23II was a *Mesorhizobium* sp.

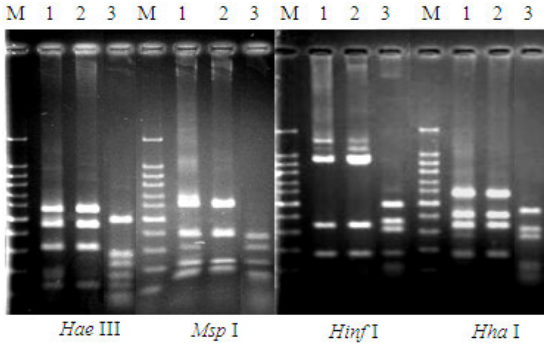

Figure 3. Electrophoretic pattern of restriction endonuclease (*Hae*III, *Msp*I, *Hinf*I, *Hha*I) to strain MQ23, MQ23R and MQ23II. Lane's order from left to right: M: 100bp marker; 1: MQ23; 2: MQ23R; 3: MQ23II.

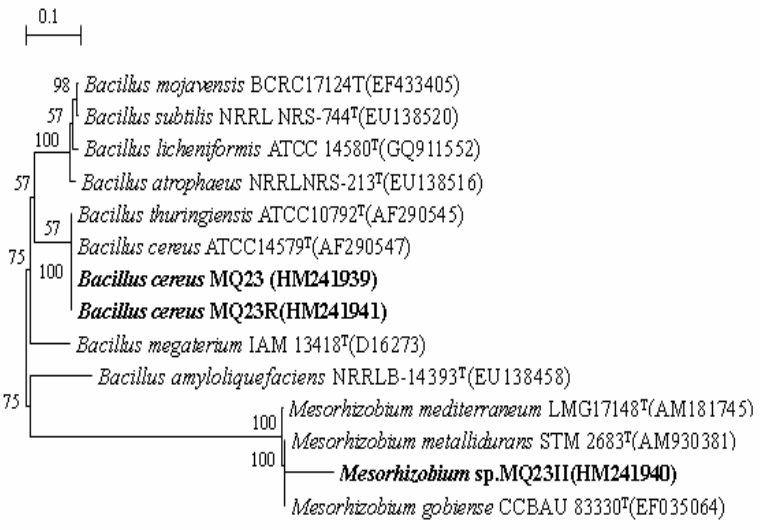

Figure 4. Neighbor joining tree based on alignment of nucleotide sequences of the 16S rRNA gene from tested strains (shown in bold) and reference strains. GenBank accession numbers were placed in parentheses. Bootstrap values greater than 50% were indicated. Scale bar represents the number of substitutions per site.

Plant growth promoting characteristics of isolates

All of three treatments MQ23, MQ23II and MQ23+MQ23II (Table1) gave a positive CAS assay showing that they all produced siderophores. Compared between of them, MQ23 showed the strongest capability of producing siderophores. As for phosphate solubilization (organic phosphate and inorganic

phosphate), isolate MQ23 showed negative while coinoculation MQ23+ MQ23II were positive. All treatments were negative for organic acid product, positive for IAA product, and the amount of IAA produced by three treatments was 0.368 mg/l, 22.385 mg/l and 23.068 mg/l, respectively. Another, three treatments showed certain antifungal activity to plant pathogenic fungi, and inhibition ratios was given in Table 1. As a whole, for positive action coinoculation MQ23+MQ23II showed better effect than inoculation alone in vitro. Therefore, *Bacillus cereus* MQ23 and *Mesorhizobium* sp. MQ23II have better cooperative interaction. But definite mechanism of their interaction is further work.

Table 1. Plant growth promoting characteristics of endophytes isolated from root nodules

Treatments	Plant growth promoting characteristics									
	Sid. (D/d)	Phosphate solubilization		Org. acid	IAA (mg/L)	Antifungal activity(colony diameter)				
		Org.P (D/d)	Inorg. P (D/d)			*F. o* (cm)	*M. g* (cm)	*B. c* (cm)	*V. m* (cm)	*A. a* (cm)
MQ23	1.65aA[§]	-	-	-	0.368aA	-	-	+3.5 (56.25*)	-	+5.5 (19.12)
MQ23II	1.11bB	1.46aA	1.2aA	-	22.385bB	-	-	-	+4.5 (40)	+4.2 (38.24)
MQ23+ MQ23II	1.48aAB	2.01bB	2.0bB	-	23.068cC	-	-	+3.3 (58.75)	+3.9 (48)	+4.0 (41.18)
Control	/	/	/	/	-	5.8	5.5	8.0	7.5	6.8

Sid. -Siderophore; Org.P-Organic Phosphate; Inorg. P-Inorganic Phosphate; Org.acid-Organic acid; *F. o- Fusarium oxysporum*; *M. g-Magnaporthe grisea; B. c- Botrytis cinere* Pers.; *V. m- Valsa mali Miyabe et Yamada*; *A. a- Alternaria alternate.*

D/d means the ability to produce siderophores. D-Diameter of Colony and halo; d- Colony diameter.

+ positive action; - negative action; / blank; Control for IAA assay was LB(10g NaCl/L) without inoculated bacterial suspension under same incubation condition; Control for antifungal activity assays were fungal mycelia cultivated for 7 days on PDA plates without tested strains under the same incubation condition.

[§]The same letter means no significant difference between treatments, the capital letter indicates significant level at 0.01 while lowercase letter indicates significant level at 0.05. The data in columns is average values of five repetitions.

*Inhibition ratio=(Control colony diameter-treatment colony diameter)100% / Control colony diameter .

Plant inoculation assay

To further confirm plant growth promoting characteristics, we performed plant inoculation assay. Results in Table 2 showed that each growth indexes of inoculated MQ23 except root fresh weight, slightly higher than those of negative control (NC), but only root fresh weight and root dry weight showed significant difference ($p<0.05$). Compared with MQ23II (PC), plant inoculated MQ23 only presented significant difference in nodule numbers. However, growth indexes of coinoculated MQ23+MQ23II were higher than those of inoculated alone (MQ23, PC, NC), except for root fresh weight, and showed significant difference ($p<0.05$) compare with NC.

Table 2. Effect of *Bacillus cereus* MQ23 on growth and nodulation of *S. alopecuroides*

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Shoot dry Weight (g/plant)	Root dry weight (g/plant)	Nodule number /plant
MQ23	8.6666±0.6118ab*	9.9977±0.9636ab	0.0797±0.0378ab	0.0722±0.0119b	0.0078±0.0041ab	0.0137±0.0037a	0b
MQ23II (PC)	8.2317±1.4739ab	10.011±0.7859ab	0.069±0.0178ab	0.1159±0.0307ab	0.0092±0.0025ab	0.0131±0.001ab	2.333±0.5774a
MQ23II+ MQ23	9.9833±0.2021a	11.9613±1.6113a	0.1276±0. 0314a	0.087±0.0116b	0.0123±0.0012a	0.0159±0.0015a	4.333±1.5275a
Native control (NC)	6.38±1.0868b	9.171±0.5688b	0.0519±0.0029b	0.1542±0.0057a	0.0036±0.0009b	0.0079±0.0015b	0b

*The letters a and b indicate different Tukey grouping. The same letter means no difference among treatments, while different letters means significant difference ($p<0.05$).

The value in the column is the averages of ten repetitions.

PC-Seedlings inoculated with *Mesorhizobium* sp. MQ23II alone as positive control; NC-Seedlings inoculated without any bacteria as negative control.

MQ23II+MQ23 indicate co-inoculated treatments with MQ23 and MQ23R (1:1 v/v).

DISCUSSION

Endophytic *Bacillus cereuses* are potential N_2-fixing strains

The results of *nifH* gene PCR amplification (Figure 1) indicated these two isolates MQ23 and MQ23R share common *nifH* gene under different environment. The fact may play a significant role in nitrogen fixation under field and greenhouse environment. Ding *et al*. (6) reported that bacteria belonging to *Bacillus* sp. have been identified as plant-growth-promoting rhizobacteria, and *nifH* genes have been detected in some species of *Bacillus cereus*. Zehr *et al*. (40) showed that molecular tools for detection and characterization of the nitrogenase gene (*nifH*) can provide new information on activity of diverse nitrogen fixing organisms (12). Another, the coexistence of endophytic *Bacillus cereus* and symbiotic rhizobia in root nodules is an important ecological event because the coexisting bacteria may have more opportunities to exchange their core genomic and symbiont information, as revealed in other report (39).

Identification of endophytes MQ23 and MQ23R

Based on whole-cell protein profiles (Figure 2) generated by endophytes MQ23 and MQ23R, our experiment results further supported this viewpoint that combination of protein SDS-PAGE with computerized analysis of cellular protein profiles provide an effective relatively simple and reproducible approach to investigate of taxonomic relationships among many bacterial species (5,15, 23), such as *Bacillus* (13). Previous reports also showed protein profiles of SDS-PAGE as having a high degree of correlation with DNA PCR-RFLP analysis, rRNA sequence analysis, and DNA-DNA hybridization for several bacterial species (3, 11, 25, 35).

In this work, analysis of 16S rRNA gene PCR-RFLP, gene sequencing and phylogeny further confirmed that defining a bacterial species based upon the consensus of combined results of several analyses can overcome the potential prejudicial grouping by each of the single analysis, supported previous studies (19).

Co-inoculation enhanced plant growth

Previous reports informed that the cooperative interactions between rhizobia and other plant root colonizing bacteria play a role in the improvement in nodulation and N_2 fixation in legume plants (2); other such examples include when rhizobia are coinoculated with *Rhizobium leguminosarum* bv *trifolii* and either *B. insolitus* or *B. brevis* (32), and with *Bacillus* spp. and the soybean endosymbiont *Bradyrhizobium japonicum* (1, 21). Geetha *et al*. (8) reported that co-inoculation enhanced growth and nodulation of the pigeon pea with *Bacillus* strains and *Rhizobium* spp. Similarly, Selvakumar *et al*. (31) showed that the non-rhizobial plant growth promoting bacteria *Bacillus thuringiensis* KR-1 from the nodules of Kudzu promoted growth and positively influenced nutrient uptake in wheat seedlings. Therefore, this report extends similar observations to another legume-rhizobium system that of *S. alopecuroides*.

To the best of our knowledge, this is the first report about the presence of putative non-rhizobial endophytes having growth-promotion attributes in *S. alopecuroides* root nodules. Further studies are required to prove the endophytic nature of *Bacillus cereus* MQ23 and to harness their potential as bio-inoculants and biocontrol agents in agriculture.

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