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In vitro Inhibition of Fungal Root-Rot Pathogens of *Panax notoginseng* by Rhizobacteria

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The rhizobacteria of *Panax notoginseng* were isolated from six sites in Yanshan, Maguan and Wenshan Counties, Yunnan Province of China, and their antagonistic activity against *P. notoginseng* root-rot fungal pathogens was determined. Of the 574 rhizobacteria isolated, 5.8% isolates were antagonistic *in vitro* to at least one of the five pathogens, *Cylindrocarpon didynum*, *Fusarium solani*, *Phytophthora cactorum*, *Phoma herbarum*, and *Rhizoctonia solani*. The number of rhizo bacteria and the number that inhibited fungi differed depending on sampling sites and isolation methods. Rhizobacteria isolated from the site in Yanshan and Maguan showed more antagonistic effect than them in Wenshan. Heat treatment of rhizosphere soil at 80°C for 20 min scaled the antagonists up to 14.0%. Antagonistic bacteria in the roots proportioned 3.9% of the total isolates. The most antagonistic isolates 79-9 and 81-4 are *Bacillus subtilis* based on their 16S rDNA sequence and biochemical and physiological characteristics. Identification and evaluation of antagonistic bacteria against *P. notoginseng* root-rot pathogens in the main planting areas improved our understanding of their distribution in rhizosphere soil. Furthermore these results indicated that the interactions between biocontrol agent and soil microbes should be seriously considered for the successful survival and biocontrol efficacy of the agents in soil.

Keywords : antagonism, pathogenic fungi, *Panax notoginseng*, Rhizobacteria

Panax notoginseng (Burk.) F. H. Chen is an important Chinese medicinal herb, belonging to the same genus as *Panax ginseng*, and its production with high pharmacological quality requires special climatic conditions, geologic background and physicochemical properties of soil (Cui et al., 2005). The long growth period and the repeated cultivation of *P. notoginseng* in plantations, however, result in serious root-rot diseases affecting 5-20% of the plants (Dong et al., 1998). A recent survey demonstrated that *P. notoginseng* root rots always occurred in the presence of *Cylindrocarpon destructans* (Zinssmeister) Scholten, *C. didynum* (Hartig) Wollenw, *Fusarium solani* (Martius) Saccardo, *F. oxysporum* Schlechtendahl: Fries, *Phytophthora cactorum* (Lebert & Cohn) Schroeter, *Phoma herbarum* Westendorp, and *Rhizoctonia solani* Kühn (Miao et al., 2006). Because any disease control method must guarantee the pharmacological properties of medical herbs and introduce no chemical residues, the application of specific biocontrol agents is preferred as an alternative method to control *P. notoginseng* root rots (Emmert and Handelsman, 1999; Weller, 1988; Whipps 1997a, b). However, the interactions of soil-plant-microbe are complicated and specific. A soil that is suppressive to one pathogen is not necessarily suppressive to the others (Whipps, 2001). The reason might be the specific response of bacteria to the root exudates for their rapid growth on the seeds and in the rhizosphere (De La Fuente et al., 2006; Roberts et al., 2000). This specificity was thought to be one of most important features for bacterial colonization on the roots and propagation in soil to suppress pathogens. Based on the analysis of microbial community with molecular methods, some key organisms or groups of organisms responsible for disease suppression had been defined (Shiomi et al., 1999), and the impact of biological agents on soil microbial populations had been monitored (Natsch et al., 1998). Using specific primers associated with functional gene of biocontrol agent, the study of commercialized *Bacillus* and *Pseudomonas* gave a new view on antagonistic bacteria screening and population analysis (Gardener et al., 2005; Joshi and Gardener, 2006). But before using this method, the key antagonistic organisms from the specific plant rhizosphere must be isolated, identified and evaluated.

Biological agents, *Trichoderma* spp. (Hong et al., 2000; Kim et al., 1992) and *Bacillus cereus*-complex (GB10) (Li et al., 1997) can reduce *P. ginseng* root rot incidence caused

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by *C. destructans* or by *C. destructans* and other pathogenic fungi. However, there was no systemic study on the rhizobacteria against *P. pseudoginseng* pathogens. Only *Bacillus* has been screened against the major *P. pseudoginseng* pathogens in Korea (Kim et al., 1997) and *C. destructans* in China (Liu et al., 2004). Due to the complicated situation of *P. notoginseng* root rot in China (Miao et al., 2006) and the specific interaction among bacteria-plant-soil, biological control on this disease becomes more difficult than that caused by *C. destructans* alone. The objectives of the present study are: 1) to determine the distribution and frequency of antagonistic rhizobacteria in soil and roots of *P. notoginseng* in the plantation area; 2) to isolate rhizobacteria from *P. notoginseng* roots and rhizosphere soil and to determine their antagonistic potential against the five fungal root-rot pathogens *in vitro*.

Materials and Methods

Collection of plant roots and soil samples. The roots and rhizosphere soil of healthy *P. notoginseng* plants were collected from 17 fields in Wenshan, Yanshan, and Maguan Counties, Yunnan Province of China. The soil texture was determined to be red loam, yellow loam, and yellow clay (Table 1). Two or three *P. notoginseng* roots and adhering soil were collected from each field as one sample, and stored at 4°C and processed within 30 days after collection.

Isolation of rhizobacteria. For each sample, about 10 g of *P. notoginseng* roots with the adhering soil was placed into a 500-ml flask containing 90-ml of sterile phosphate buffer (PBS, pH 6.8) and 0.25 μg L^{-1} Tween-20. The flasks were agitated at 150 rpm for 30 min on a gyratory shaker (HZQ-Q; Donglian Electronic Technology). A serial ten-fold dilution was performed. An aliquot of 0.1 ml of each dilution was spread, in 9-cm diameter plates, over the surface of Nutrient agar (NA) to isolate general bacteria and King's B medium (KMB) to isolate *Pseudomonas* spp. (Schaad et al., 2001). To isolate *Bacillus* spp., 5 ml of soil suspension was heated at 80°C for 20 min and serially diluted (Kloepper and Bowen, 1991); 0.1 ml from each dilution was spread onto NA plate in 9-cm Petri dishes.

For isolation of bacteria within roots, the roots were transferred from PBS to a 50-ml plastic centrifuge tube for washing vigorously with water. After surface sterilization with 1% sodium hypochlorite for 5 min, the roots was rinsed four to five times with sterile distilled water and blotted dry with sterilized Whatman paper. About 1 g of lateral roots was cut into 2 to 4-mm pieces and ground in a sterilized pestle with 10 ml PBS. The suspension was ten-fold serially diluted with PBS, and 0.1 ml of each dilution was spread on NA plate in 9-cm Petri dishes and incubated at 28°C for 2 days. Colonies with different appearance were transferred to new agar plates for purification. The purified isolates were stored on NA or King's B slants at 4°C.

Pathogenic fungi of *P. notoginseng*. One of the following pathogens was selected for *in vitro* screening of rhizobacteria for antagonistic activity: *C. didynum*, *P. herbarum*, *P. cactorum*, *R. solani*, and *F. solani*. These fungi were isolated and identified by Miao et al. (2006) and maintained on potato dextrose agar (PDA; Oxoid) plates at 25°C in our laboratory.

***In vitro* screening of bacteria for antagonistic activity.** All isolated rhizobacteria were tested at first for their inhibition against *P. cactorum*, *R. solani*, and *F. solani* on PDA plates. Antagonists were further tested for the antagonism against *C. didynum* and *P. herbarum*. Rhizobacteria were inoculated at three locations (60, 180, and 300°) between the center and edge of the plate (triple-culture technique) and incubated at 28°C. One day later, a 5-mm diameter plug of a fresh culture of fungi was placed in the center and incubated at 26°C. The slowly growing fungi, *C. didynum* and *P. herbarum*, were pre-cultured for 5-7 days on the PDA plates at 26°C before the bacteria were inocu-

Table 1. Characteristics of sampling sites in this study

Sites		No. of samples[a]	Plant age (Years)	Soil type	Previous crop	Condition of plants
County	Village					
Wenshan	Panzhihua	2	1	Red loam	*Panax notoginseng*	Healthy
	Panzhihua	3	2	Red loam	*P. notoginseng*	Healthy
	Jiangbing	1	2	Yellow Clay	Sugarcane	Healthy
	Pingba	1	2	Yellow Clay	Barren mountain	Healthy
Yanshan	Panlong	4	2	Yellow Clay	Corn	Healthy in infested field
Maguan	Maanshan	3	2	Yellow loam	Corn	Healthy
	Tangzibian	3	2	Yellow loam	Corn	Healthy in infested field

[a]Two or three *P. notoginseng* roots and adhering soil were collected from each field as one sample, stored at 4°C and processed within 30 days after collection.

lated. Distance between colonies of fungus and bacterium (so called "inhibition zone") was measured after 4-5 days incubation. The antagonistic activities were categorized into three levels according to the width of inhibition zone: zone width ≤ 5 mm, between 5 and 10 mm, and ≥ 10 mm.

The antagonistic bacteria in a sample that inhibits at least one fungus *in vitro* were counted, and their percentage was determined as the formula (number of antagonistic bacteria ×100/the total number of rhizobacteria). The percentage of bacteria antagonistic toward each pathogenic fungus was also calculated.

Identification of bacterial antagonists. Isolates 79-9 and 81-4 with strong antagonistic activity were identified according to their 16S rDNA sequences, biochemical and physiological characteristics. Genomic DNA was extracted following the standard procedure (Sambrook and Rusell, 1998). Oligonucleotide primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1494R (5'-CTACGGCTACCTTGTTACGA-3') (Weisburg et al., 1991) were used for 16S rDNA amplification. PCR reactions and 16S rDNA cloning were carried out following Lei et al.'s description (2007). The positive clones were sequenced at Invitrogen, China. ClustalX program (version 1.8) (Thompson et al., 1997) was used to align the 16S rDNA sequence and reference sequences from the GenBank database. A phylogenetic tree was constructed with Mega 3.1 based on the 16S rDNA sequences of seven strains related to 79-9 and 81-4 and three other strains from GenBank (Kumar et al., 2004). Biochemical and physiological characteristics were determined according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Results and Discussion

Screening and identification of antagonistic bacterial strains. In this study, a total of 445 bacterial isolates were obtained from the rhizosphere soil and 129 from inside the roots. All the isolates were tested against three rapid growing fungi, *P. cactorum, R. solani*, and *F. solani* on PDA plates. Those showed antagonism against at least one of the three pathogenic fungi were further tested for their antagonism against *C. didynum* and *P. herbarum*. Among all the strains isolated, 5.8% isolates showed antagonism to at least one fungal pathogen (Table 2). Most antagonistic bacteria produced an inhibition zone of 5 to 10 mm in width.

In the recent years, some molecular techniques have been used to define the key organisms responsible for disease suppression (Shiomi et al., 1999) or to screen and monitor some antagonistic bacteria in the rhizosphere of specific crop (Gardener et al., 2005; Joshi and Gardener, 2006). For example, specific primers were designed to detect several groups of well-known successful biocontrol agents. However, this method is not appropriate in the case of *P. notoginseng* because: 1. *P. notoginseng* is a kind of medical herbs with special pharmacological properties and produces different root exudates from other crops, which might play an important role in rhizobacterial community (De La Fuente et al., 2006; Roberts et al., 2000); 2. The composition of microbes in *P. notoginseng* rhizosphere has not been analyzed and the key antagonists might have some special characteristics. Thus the regular method, including isolation, identification and biological control test, is more suitable to achieve our objectives instead of the molecular method.

Strains 79-9 and 81-4 isolated from Wenshan and Maguan Counties, respectively, were most antagonistic to the pathogenic fungi, and were identified. The 16S rDNA sequences of strain 79-9 (1432 bp) and 81-4 (1419 bp) were deposited in GenBank under accession number EU624322 and EU624321, respectively, and were compared with 10 sequences of bacterial strains from GenBank. High similarity

Table 2. Antagonistic rhizobacteria isolated from rhizosphere soil and roots of *Panax notoginseng* in Yunnan Province of China

Sampling sites	Total no. of bacteria	% antagonistic[a]	From rhizosphere						From roots	
			King's B[b]		NA[b]		NA-H[c]		NA-E[d]	
			No. of bacteria	% antagonistic	No. of bacteria	% antagonistic	No. of bacteria	% antagonistic	No. of bacteria	% antagonistic
Wenshan	212	3.3	36	5.6	63	1.6	51	3.9	62	3.2
Yanshan	164	7.9	42	2.4	39	0.0	53	22.6	30	0.0
Maguan	198	6.6	39	2.6	62	0.0	60	15.0	37	4.1
Total	574	5.8	117	3.4	164	0.6	164	14.0	129	3.9

[a]% antagonistic=Number of antagonistic bacteria × 100/Number of total rhizobacteria isolated from different medium, different parts or different treatment methods.
[b]Bacteria isolated from samples heated at 80°C for 20 minutes.
[c]Bacteria isolated from roots of *P. notoginseng*.
[d]King's B and NA represent King's B medium and nutrient agar usually used to isolate *Pseudomonas* spp. and bacteria in the soil, respectively.

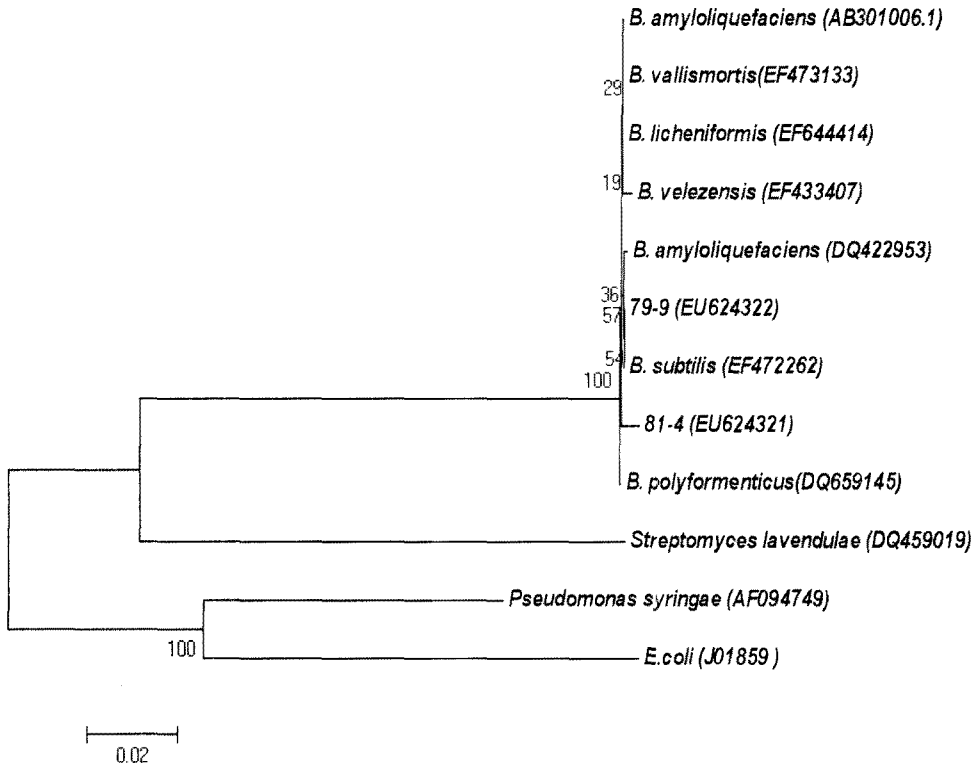

Fig. 1. Phylogenetic tree based on the 16S rDNA sequence data, showing the relationship of strain 79-9 and 81-4 to the most closely related bacteria. The GenBank accession numbers are provided in parentheses. The dendrogram was generated by the neighbour-joining method. The numbers at the branch points are bootstrap values based on 1000 replications. The *Escherichia coli* sequence was used as an outgroup. Bar indicates 5 nucleotide substitutions per 1000 nucleotide positions.

was identified in the sequences among strain 79-9, 81-4, and *Bacillus* spp. (Fig. 1). Homology of their 16S rDNA sequence was 99% with *Bacillus subtilis*, *B. licheniformis*, *B. velesensis*, and *B. amyloliquefaciens*. Regarding biochemical and physiological characteristics, strain 79-9 and 81-4 are Gram positive, produce endospores, consume glucose under aerobic conditions, express catalase enzyme activity, and optimally grow at pH 5.7. They are positive to Voges-Proskauer reaction, nitrate reduction, starch hydrolization, but they can't use calcium propionate as its carbon source. Based on their 16S rDNA sequences, biochemical, and physiological characteristics, strains 79-9 and 81-4 were identified as *B. subtilis*, and were deposited in Chinese Agricultural Culture Collection of China (ACCC) under the numbers ACCC 10719 and ACCC 10720.

Though our isolates 79-9 and 81-4 were identified to be *B. subtilis*, a species which has been reported to inhibit the growth of *C. destructans* (Kim et al., 1997; Liu et al., 2004), strains 79-9 and 81-4 were more efficient as potential biocontrol agents since they showed inhibitory ability to all of the five pathogenic fungi associated with *P. notoginseng* root rot disease.

Effect of the isolation method and the sampling site on antagonistic rhizobacteria. In order to improve the understanding on the composition of the functional microbes with antagonistic activity and improve the screening efficacy, two isolation methods, selective King's B medium and sample heat-treatment before isolation, were used. King's B medium and sample heat-treatment are usually used to isolate *Pseudomonas* (Burr et al., 1978) and *Bacillus* strains (Walker et al., 1998), respectively, but little is known about the relationship between antagonistic percentage and screening methods or locations. The number of rhizosphere bacteria isolated and the antagonistic percentage was varied a lot with different isolation media and sample treatments. The percentage of antagonistic rhizobacteria was 3.4% when bacteria were isolated with King's B medium, much higher than with NA medium. Heating treatment increased the percentage of antagonistic bacteria in the soil suspension from 0.6% to 14%. The percentage of antagonistic bacteria isolated from *P. notoginseng* roots was 3.9%, higher than that from rhizosphere.

In Wenshan, the highest percentage of antagonistic bacteria was isolated from King's B medium, reached 5.6%. It was 3.9% when isolated by sample heat treatment and 3.2% from inside roots. In Yanshan and Maguan, the antagonistic percentage isolated by soil heat treatment was

Table 3. Numbers of rhizosphere bacteria isolated from each site and the percentage of bacteria antagonistic to the five pathogenic fungi

Site	No. of rhizobacteria isolated	Percentage of rhizobacteria antagonistic to five pathogenic fungi[a]				
		C. didynum	*P. herbarum*	*P. cactorum*	*R. solani*	*F. solani*
Wenshan County	212	1.9	1.9	2.8	1.9	1.9
Yanshan County	164	0.0	1.8	7.3	0.6	3.7
Maguan County	198	1.5	0.5	5.6	0.5	2.5
Total	574	1.2	1.4	5.1	1.1	2.6

[a]All of the pathogenic fungi were described by Miao et al. (2006).

22.6 and 15.0%, respectively, far higher than from King's medium (Table 2). These results indicated that different functional microbes with antagonistic activity survived in three locations. Both *Pseudomonas* and *Bacillus* might play important antagonistic roles in Wenshan, whereas *Bacillus* was more important than *Pseudomonas* in Yanshan and Maguan. We speculated that soil texture and tillage practices might account for this difference, since these sites differed in soil texture and tillage practices under the same climate conditions. Soils in Yanshan were yellow clay, mainly consisted of montmorillonite and had been rotated with corn for one year, but five out of seven soil samples in Wenshan were red loam and replanted in *P. notoginseng*. Crop rotation affected not only the composition of the soil microbes, but also the number of the antagonistic bacteria in soil. Peters et al. (2003) found rhizobacteria recovered from potato root tissues after a 3-year rotation showed greater antibiotic activity than that after a 2-year rotation under minimum tillage management. In the other aspect, antagonistic activity of the soil was also affected by soil texture. Amir and Alabouvette (1993) demonstrated that sandy soil appeared more conducive and clay soil was more suppressive to Fusarium wilt. The amendment of montmorillonite made the soil more suppressive than the non-amended control. Rosenzweig and Stotzky (1979) also found that soil added with montmorillonite increased the bacterial activity, and the antagonism between bacteria and fungi in soil. Therefore, to control *P. notoginseng* root-rot disease efficiently, rotation with corn and supplementation of montmorillonite in biocontrol agents might be the best choice.

Distribution of antagonistic rhizobacteria at different locations. The percentages of rhizobacteria inhibitive to each pathogenic fungus *in vitro* were different from each other and varied among sampling sites (Table 3). Most of the antagonistic bacteria were isolated from Yanshan and Maguan (Table 2). Among them, 7.3 and 5.6% of inhibitive bacteria are against *P. cactorum* in Yanshan and Maguan, respectively. Against *F. solani*, the antagonistic percentage was 3.7% in Yanshan and 2.5% in Maguan (Table 3), respectively. These results indicated that *P. cactorum* and *F. solani* could be suppressed more easily than the other three fungi when tested *in vitro*. Rhizobacteria isolated from Wenshan showed higher constant antagonistic activities than Yanshan and Maguan against the test fungi except for *P. cactorum*. No bacteria could inhibit the growth of *C. didynum* in Yanshan. It could explain why we got the strong antagonistic bacteria strain 79-9 from Wenshan and strain 81-4 from Maguan.

During the development of commercial microbial products, the complex interactions between biocontrol agents and indigenous microbiota should be considered seriously (Whipps, 2001). The distribution of inhibitive bacteria against different fungi at different locations gave us an understanding of the antagonistic characters of these bacteria, and will benefit our further research on application of these antagonists in field. For example, when we introduce antagonist 79-9 into the field, its biocontrol efficacy in Wenshan County might be better than in Yanshan and Maguan counties since strain 79-9 was more antagonistic against *P. notoginseng* root-rot pathogens in Wenshan County. Further research about the interaction between the antagonistic bacillus and the soil microbes in vivo should be considered.

Conclusion

This study evaluated the potential of antagonistic organisms as biocontrol agent against *P. notoginseng* root rot disease *in vitro*. The number of rhizosphere bacteria and the number that inhibited root rot fungal pathogens differed depending on sampling sites and isolation methods. Of the 5.8% of all the isolated bacteria that inhibited the growth of at least one root rot pathogen, 18 bacteria were active against at least two fungal pathogens. More effort should be conducted on the interaction research between antagonistic organisms and soil microbes to successfully develop a biological control agent.

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