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Full Length Article

Screening and Identification of Harmful and Beneficial Microorganisms Associated with Replanting Disease in Rhizosphere Soil of *Pseudostellariae heterophylla*

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Abstract

Pseudostellariae heterophylla, an important medicinal plant, has been shown to suffer from serious replanting disease that causes significant declines in both yield and quality. The objective of this study was to isolate the soil-born pathogen and antagonistic microorganism for investigation of the relationship between these microorganism and consecutive monoculture problems of *P. heterophylla*. In this study, we isolated the bacteria with different morphology from *P. heterophylla* rhizosphere soil, detected the pathogenicity of these bacteria to *P. heterophylla* plantlet and antagonistic ability to *Fusarium* of *P. heterophylla* biotype, identified their species and analyzed their abundance with qRT-PCR in rhizosphere soil of different planting years. We isolated *Enterobacter sp1* and *Burkholderia sp1* from *P. heterophylla* rhizosphere soil, and found *Enterobacter sp1* possessed the pathogenicity to *P. heterophylla* plantlet and *Burkholderia sp1* was antagonistic for *Fusarium* of *P. heterophylla* biotype. However, *Burkholderia sp1* was not the antagonistic microorganism to *Enterobacter sp1*. The growth of *Enterobacter sp1* was stimulated by phenolic acids at a certain concentration nearly closed to that in *P. heterophylla* rhizosphere soil, which increased as planting years of *P. heterophylla* increased. On the contrary, the phenolic acids had no promoting effect on the growth of *Burkholderia sp1*. And with the increase of planting years, the abundance of *Burkholderia sp1* decreased and abundance of *Enterobacter sp1* increased in the *P. heterophylla* rhizosphere soil. The results indicated that harmful microorganisms increase and beneficial microorganisms decrease, which were mediated by the accumulated autotoxins in rhizosphere soil of consecutively monocultured *P. heterophylla*, and hence leads to the imbalance of microbial community structure and the degradation of soil ecological function. So several abatement methods, such as crop rotation, specific microbial fertilizer and organic matter, could be used to improve the structure and functional diversity of microbial community, and overcome the obstacles of continuous cropping.

Keywords: *Pseudostellariae heterophylla*; Replanting disease; Rhizosphere soil; Soil-born pathogen; Antagonistic microorganism

Introduction

Pseudostellariae heterophylla, a member of the *Caryophyllaceae* family, is one of the most common and important medicinal herbal plants in China. It is perennial and its tuberous roots are widely used as a traditional Chinese medicinal ingredient for the treatment of splenic asthenia, anorexia, lassitude, weakness and palpitation (Lin *et al.*, 2011; Wang *et al.*, 2013). Nowadays, *P. heterophylla* is mainly cultivated in suitable areas within provinces of Fujian, Guizhou, Anhui and Jiangsu in China (Zeng *et al.*, 2012). High-quality *P. heterophylla* is produced predominantly in Ninde, Fujian Province, which has the best climatic and soil conditions for cultivation. However, the consecutively monocultured *P. heterophylla* plants are prone to replanting disease (Qu and Wang, 2008) and/or consecutive monoculture problems (Wu *et al.*, 2009), which results in declined yield and quality. To compensate for the production of *P. heterophylla*, farmers had to look for the less desirable farmland to produce and meet the market needs although the quality of the medicinal tuberous roots is lower.

Previous studies showed that rhizospheric microbial dynamics largely governed proper soil ecosystem function and had close interaction with consecutive monoculture problems (Qu and Wang, 2008; Qi *et al.*, 2009; Wu *et al.*, 2009; Wu *et al.*, 2011; Li *et al.*, 2012b; Wu *et al.*, 2013). Crop consecutive monoculture not only alters the physical and chemical properties of the soil, but also contributes to the development of diverse microbial groups in the

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rhizosphere (Minh, 2005; Lin *et al.*, 2007; Yoneyama and Natsume, 2010; Berendsen *et al.*, 2012; Ndabamenye *et al.*, 2013). The autotoxic compounds were often isolated from consecutive monoculture soil, such as phenolic acid and aliphatic acid compounds, which could selectively enhance specific microbial populations in the soil which then leads to a shift in microbial communities (Qu and Wang, 2008; Li *et al.*, 2012b). Wu *et al.* (2011) analyzed the rhizospheric biological properties of consecutively monocultured *R. glutinosa* using soil metaproteomics and found that consecutive monoculture can induce the changes of microbes in the expression of proteins.

The consecutive monoculture disrupts the balance between pathogenic microorganisms and other rhizosphere microbes which is crucial for plant health (Chen *et al.*, 2008; Zhang *et al.*, 2009, 2011; Berendsen *et al.*, 2012). Raaijmakers *et al.* (2009) described the rhizosphere as both a playground and battlefield for beneficial microorganisms and soil borne pathogens. Beneficial microorganisms could promote plant growth and/or suppress plant diseases in rhizosphere soil via a variety of mechanisms including improved nutrient acquisition, production of growth regulators, and biosynthesis of pathogen-inhibiting compounds (Lugtenberg and Kamilova, 2009; Doornbos *et al.*, 2012; Berendsen *et al.*, 2012). So it is crucial to isolate probiotics and beneficial microorganisms antagonistic to detrimental microbes in consecutive monoculture soil. In this study, we isolated the bacteria with different morphology from *P. heterophylla* rhizosphere soil, detected the pathogenicity of these bacteria to *P. heterophylla* plantlet and antagonistic ability to *Fusarium* of *P. heterophylla* biotype, identified their species and analyzed their abundance in rhizosphere soil of different planting years. Our studies on consecutively monocultured *P. heterophylla* found that the diversity index of bacterial communities decreased significantly in two-year consecutive monocultured soil, the amount of the beneficial bacteria decreased and the reverse was true in the case of pathogenic bacteria. The results would aid in the development of specific microbial fertilizers or farming methods to improve the soil microbial community structure and ecosystem to promote the growth and yield of medicinal plants and other crops.

Materials and Methods

Experiment Design and Collection of Soil Samples

The experiment was conducted using GAP in an experimental field in Zherong County, Ninde Municipality, Fujian Province, P.R. China, during 2010–2012. The *P. heterophylla* cultivar 'Zheseng-2' used for this study was planted in December and harvested each year in July. The field trial was composed of three replicates of three treatments including one-year monoculture (OM), two-year consecutive monoculture (TM) and fallow treatment (CK) as a control. The experimental plots were 5 m × 5 m (25 m^2) for each treatment. Individual *P. heterophylla* tuberous roots were planted with a spacing of 5 cm × 10 cm (5 cm between each plant in a row and 10 cm between rows) between plants. The one-year monoculture *P. heterophylla* plots were planted on December 20, 2011. *P. heterophylla* was planted in the two-year consecutive monoculture plots on December 20, 2010. The *P. heterophylla* harvests from all the cropping systems took place in July 2012.

P. heterophylla germinated completely in March 2012. Soil samples were collected from five random locations in each plot in May 2012 during the growth peak period for *P. heterophylla*. Fresh plants were carefully uprooted from the soil with a forked spade. The roots were shaken to remove loosely attached soil. Rhizosphere soil samples (adhering to the roots and rhizomes after shaking) were sieved to remove impurities and only the portions that passed through a 80-mesh were retained for analysis.

Isolation and Purification of Bacteria

Fifty grams of fresh rhizosphere soil were soaked in 150 mL distilled water and rotated at 150 r min^{-1} for 2 h. After standing for half an hour, the suspension was transferred to a 250 mL flask. The suspension was respectively diluted by 10, 10^2, 10^3, 10^4 and 10^5 times. The 0.5 mL diluents of different concentrations were smeared on a plate containing 30 mL solid LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in 1 L distilled water). Each treatment was repeated 3 times. The plates were placed in culture incubator at 35℃ for 24 h. The plates containing about 100 microbial colonies were selected and used to picke single colony. The single colony was purified three times by the method of parallel crossed and cultured using liquid LB medium (10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water). The single colony was stored at -80℃ in liquid LB medium containing 30% glycerol.

Extraction and HPLC Analysis of Phenolic Acids in Rhizosphere Soil

Twenty five grams of soil was transferred into 50 mL centrifuge tube and added with 25 mL 1 M NaOH. The soil solution was evenly mixed and stood for 12 h. Then the soil solution was oscillated for 30 min and centrifuged at 5000 rpm for 5 min. The supernatant was removed into the 50 mL centrifuge tube and its pH value was adjusted to 2.5 with 12 M hydrochloric acid. After standing for 2 h, the solution was centrifuged at 5000 rpm for 5 min. The supernatant was filtered by 0.45 μm membrane which was used for HPLC analysis.

The concentration of phenolic acids in rhizosphere soil was determined using the Agilent HPLC system. The chromatographic system consisted of a Agilent Technologies 1260 Infinity system with a reversed-phase column ODS-C18（4.6 mm × 150 mm）and was used at a flow rate of 1.0 mL min^{-1}. The solvent system was 17%

solvent A (methanol) and 83% (1.3% v/v acetic acid in water). The injected volume was 5 µL of a water solution of the extracts. The column temperature was maintained at 30℃. The UV detector was performed at 280 nm. The data were recorded and processed with Agilent workstation.

Standards of phenolic acids, including hydroxybenzoic acid, vanillic acid, vanillin and ferulic acid, were purchased from Sigma Chemicals Co., U.S.A. Ten mg of every standard was dissolved in methanol and diluted into 100 µg mL^{-1}. Four kinds of phenolic acids were mixed and were respectively diluted into 10, 5, 1, 0.5 and 0.1 µg mL^{-1} of solution. The specific recovery rates (%) for the standards were hydroxybenzoic acid, 93.45±4.26; vanillic acid, 92.08±6.22; vanillin, 90.76±5.73; and ferulic acid, 91.67±4.89. Phenolic acids were found in the rhizosphere soil of *P. heterophylla* were identified by matching the retention time and their spectral characteristics against those of standards.

Culturing the Bacteria with Aqueous Extract of *P. heterophylla* Roots and Phenolic Acid

The aqueous extract from *P. heterophylla* roots and phenolic acid were used to detect their effect on the selected bacterial colonies. Ten grams of fresh *P. heterophylla* roots were ground into the slurry and diluted to 100 mL solution with distilled water. The solution was autoclaved at 120℃ for 20 min, then filtered. The 5 mL aqueous extract of *P. heterophylla* roots was added in 45 mL of 1/2 liquid LB medium (5 g tryptone, 2.5 g yeast extract and 5 g NaCl in 1 L distilled water), in which 0.1 mL of selected bacterial colonies was cultured at 120 rpm, 30℃. The control treatment was 5 mL distilled water and 45 mL of 1/2 liquid LB medium. The OD value was detected at 600 nm by using spectrophotometer when the selected bacterial colonies were cultured respectively for 6, 7 and 8 h.

Hydroxybenzoic acid, vanillin and ferulic acid were used to culture the selected bacterial colonies. These phenolic acids were respectively diluted into six concentrations, which were 9 mmol L^{-1}, 3 mmol L^{-1}, 1 mmol L^{-1}, 0.33 mmol L^{-1}, 0.11 mmol L^{-1} and 0 mmol L^{-1}. The 5 mL of phenolic acid solution was added into 45 mL of 1/4 liquid LB medium (2.5 g tryptone, 1.25 g yeast extract and 2.5 g NaCl in 1 L distilled water), in which 0.1 mL of selected bacterial colonies was cultured at 120 rpm, 30℃. The control treatment was 5 mL distilled water and 45 mL of 1/4 liquid LB medium. The OD value was detected at 600 nm when the selected bacterial colonies were cultured for 4 h, respectively.

The mixed solution of hydroxybenzoic acid, vanillin and ferulic acid was diluted into 1 mmol L^{-1}, 0.33 mmol L^{-1} and 0 mmol L^{-1}. And the culture method was the same as the above-mentioned. The OD value was detected at 600 nm when the selected bacterial colonies were cultured respectively for 3, 4, 5, 6 and 7 h.

Bacteria -*P. heterophylla* Co-culture and Bacteria-*Fusarium* Confrontation Culture

The plantlet of *P. heterophylla* was co-cultured with selected bacterial colonies. The plantlets were planted in the culture flask containing 25 mL solid MS medium containing 1.25 mg L^{-1} 6-BA and 0.5 mg L^{-1} NAA. The selected bacterial colonies were cultured in 1/2 liquid LB medium. And when the OD value of the culture fluid was 0.4, 0.1 mL of fluid was added in culture flask and co-cultured with plantlets of *P. heterophylla*. The control treatment was LB medium. The growth of plantlets was observed every day.

The selected bacterial colonies were inoculated on solid LB medium and co-cultured with *Fusarium*. The growth of selected bacterial colonies and *Fusarium* was observed every day.

Molecular Identification of Bacterial Species and Quantitative Analysis by qRT-PCR

The total soil DNA was extracted according to the high salt/SDS method of Zhou *et al.* (1996). Soil samples of 5 g were mixed with 13.5 mL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 mL of proteinase K (10 mg/ml) in 50-mL centrifuge tubes by shaking at 225 rpm for 30 min at 37℃. After the shaking, 1.5 mL of 20% SDS was added, and the samples were incubated in a 65℃ water bath for 2 h with gentle inversions every 15 min. The supernatants were collected after centrifugation at 6,000 rpm for 10 min. The soil pellets were extracted two more times by adding 4.5 mL of the extraction buffer and 0.5 mL of 20% SDS, vortexing for 10 s, incubating at 65℃ for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroformisoamyl alcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 13,000 rpm for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 µL.

The universal bacterial primers were 8-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 926-907R (5'-CCG TCA ATT CMT TTR AGT TT-3'), and used to amplify 16S rDNA fragments of bacteria. The primers of 1405f (5-TGY ACA CAC CGC CCG T-3 and 456r (5- CCT TTC CCT CAC GGT ACT G -3) were used to amplify 16S-23S rDNA. PCR reactions and purification of PCR products followed the method of Wang *et al.* (2004). The reaction conditions were as follows: denaturation at 95°C for 5 min, 35 cycles consisting of 94°C for 35 s, 55°C for 30 s, and 72°C for 1.5 min, and one additional 10-min cycle for chain elongation.

The samples were stored at 4°C. The 16S-23S rDNA fragments were sequenced. The bioinformatics analysis of rDNA fragments was performed with the BLASTX and BLASTP programs on the website of the National Centre for Biotechnology Information (NCBI). The construction of Phylogenetic trees were performed in the NCBI website http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn &PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

The abundance of *Enterobacter sp1* and *Burkholderia sp1* in *P. heterophylla* rhizosphere soil was analyzed by quantitative PCR. The primers for *Enterobacter sp1* were p1 (5-GGT GTA GCG GTG AAA TGG-3) and p2 (5-GCA TTT CAC CGC TAC ACC-3). The primers for *Burkholderia sp* were glu1 (5-CTC TGC AAC TCG AGT GCA TGA GC-3) and glu2 (5-CGG TTA GAC TAG CCA CTT CTG GTA AA-3). For quantitative PCR, 8 ng DNA were used as templates. Quantitative PCR reactions were performed using an Applied Biosystems-Step One platform and SYBR Green I Kits. Forty cycles of PCR were performed at 95°C (20 s), 60°C (20 s), and 72°C (35 s), within the linear range of reaction. The relative levels of target gene expression in each sample were calculated using the $2^{-\Delta\Delta^{CT}}$ method.

Statistical Analysis

All data were subject to an analysis of variance using the Statistical Analysis System Program (SPSS). Each value was expressed as the mean of three replicates ±standard error (SE).

Results

Screening the Harmful and Beneficial Bacteria from *P. heterophylla* Rhizosphere Soil

From the rhizosphere soil of *P. heterophylla*, fifteen bacterial colonies were isolated and had obvious morphological differences from others. The selected bacterial colonies were respectively co-cultured with *P. heterophylla* plantlets and *Fusarium* (Fig. 1). The results showed most of fifteen bacterial colonies had no pathogenicity to *P. heterophylla* plantlets. Only one of bacterial colony had pathogenicity to *P. heterophylla* plantlets, and was named *Strain 1*. The co-culture between *Fusarium* and selected bacterial colonies showed that one strain can limit the growth of *Fusarium* (Fig. 1), and was named *Strain 2*.

Morphological and Molecular Identification of the Harmful and Beneficial Bacteria

The *Strain 1* and *2* were identified by morphological and molecular method (Fig. 2 and Fig. 3). The color of *Strain 1* was white, and *Strain 2* was light yellow (Fig. 2). Two strains were all gram-negative bacteria and their morphology was rod or short rod. The length of amplified 16S rDNA fragment was 870 bp from *Strain 1*, and the length of 16S-23S rDNA fragment was 590 bp from *Strain 2*. Analysis of the sequences using the BLAST algorithm demonstrated that *Strain 1* was belong to *Enterobacter* named *Enterobacter sp1*, and *Strain 2* was belong to *Burkholderia* named *Burkholderia sp1* (Fig. 3).

Content of Phenolic Acid in *P. heterophylla* Rhizosphere Soil

In *P. heterophylla* rhizosphere soil, the content of 4 phenolic acids was detected by HPLC (Fig. 4). Four kinds of phenolic acids were the most abundant in rhizosphere soil of two-year consecutive monoculture, and the least abundant in rhizosphere soil of one-year monoculture. The total content of 4 phenolic acids amounted to 2.98 mg per kg soil in rhizosphere soil of two-year consecutive monoculture, which were 1.6 times that of fallow soil and 3.5 times that of one-year monoculture (Table 1).

Effects of *P. heterophylla* Root Extract and Phenolic Acid on *Enterobacter sp1* and *Burkholderia sp1*

The aqueous extract of *P. heterophylla* root had the effect on promoting the growth of *Enterobacter sp1*, but no effect on the growth of *Burkholderia sp1* (Table 2). When *Enterobacter sp1* was cultured with aqueous extract of *P. heterophylla* root for 6 h, the OD value of bacterial fluid were higher than that of control. And there was significant difference between treatment and control when *Enterobacter sp1* was cultured for 7 h.

The Table 3 showed effects of different phenolic acid on the growth of *Enterobacter sp1* and *Burkholderia sp1*. Three kinds of phenolic acid had the greatest effect on the growth of *Enterobacter sp1* at a concentration of 33 µmol L^{-1}, and the effect reduced with increasing concentrations of different phenolic acids. The phenolic acids had no effect on the growth of *Burkholderia sp1*, but had an inhititory effect with increasing concentrations. The mix of hydroxybenzoic acid, vanillin and ferulic acid had the same effect on the growth of *Enterobacter sp1* and *Burkholderia sp1* as single phenolic acid (Table 4).

The Abundance Analysis of *Enterobacter sp* and *Burkholderia sp* in *P. heterophylla* Rhizosphere Soil

Before analysis of *Enterobacter sp1* and *Burkholderia sp1*, linear relationship between the number of copies and Ct value was performed by quantitative PCR (Fig. 5C). Every copy represented a bacterium. Quantitative PCR could detect at least 2.3×10^2 copies. The formula of linear relationship between *Enterobacter sp1* copies and Ct value was $y= 10^{(36.638-x)/3.0379}$ (Fig. 5A). And it was $y= 10^{(36.522-x)/2.623}$ for *Burkholderia sp1* (Fig. 5B). In the formula, y represented the number of copies in a tube and x represented

Table 1: Contents of phenolic acids in the different soil samples of *P.heterophlla*

Cropping patterns	Hydroxybenzoic acid (mg per kg soil)	Vanillic acid (mg per kg soil)	Ferulic acid (mg per kg soil)	Vanillin (mg per kg soil)
Fallow	0.36±0.01b	0.64±0.02b	0.57±0.01b	0.32±0.03b
One–year monoculture	0.25±0.01c	0.31±0.02c	0.18±0.01c	0.11±0.01c
Two–year consecutive monoculture	0.54±0.08a	0.91±0.09a	1.03±0.28a	0.50±0.04a

Note: The lowercase letters behind data represented significant differences (P<0.05)

Table 2: The effects of aqueous extract on the growth of *Enterobacter sp1* and *Burkholderia sp1*

Microorganisms	Treatments	OD value in 6 h	OD value in 7 h	OD value in 8 h
Enterobacter sp1	Control	0.1927±0.0064a	0.4737±0.0135b	0.9987±0.0243a
	Aqueous extract	0.2227±0.0098a	0.5385±0.0140a	1.0720±0.0115a
Burkholderia sp1	Control	0.1013±0.0122a	0.3112±0.0051a	0.7743±0.0161a
	Aqueous extract	0.1209±0.0064a	0.3240±0.0062a	0.8205±0.0120a

Note: The lowercase letters behind data represented significant differences (P<0.05)

Table 3: The effects of phenolic acids in different concentration on the growth of *Enterobacter sp1* and *Burkholderia sp1*

Microorganisms	Phenolic acids	0 umol•L^{-1}	11 umol•L^{-1}	33 umol•L^{-1}	100 umol•L^{-1}	300 umol•L^{-1}	900 umol•L^{-1}
Enterobacter sp1	Hydroxybenzoic acid	0.9566±0.0083d	1.0118±0.0050c	1.0723±0.0012a	1.0481±0.0033b	1.0184±0.0012c	0.9403±0.0014e
	Vanillin	0.9366±0.0037b	0.9486±0.0025a	0.9430±0.0028ab	0.8889±0.0013c	0.7325±0.0007d	0.6198±0.0007e
	Ferulic acid	0.9566±0.0083d	0.9908±0.0046c	1.0890±0.0073a	1.0168±0.0030b	0.8949±0.0053e	0.9404±0.0036d
Burkholderia sp1	Hydroxybenzoic acid	0.2395±0.0142a	0.2408±0.0231a	0.2442±0.0026a	0.2411±0.0048a	0.2425±0.0084a	0.2071±0.0175b
	Vanillin	0.2517±0.0061a	0.2489±0.0155a	0.2484±0.0069a	0.2414±0.0098a	0.2265±0.0043b	0.1789±0.0247c
	Ferulic acid	0.2479±0.0045a	0.2493±0.0217a	0.2476±0.0012a	0.2582±0.0023a	0.2316±0.0047b	0.2019±0.0155c

Note: The lowercase letters behind data represented significant differences (P<0.05)

Table 4: The effects of mixing phenolic acids in different concentration on the growth of *Enterobacter sp1* and *Burkholderia sp1*

Microorganisms	Concentration	OD value in 3 h	OD value in 4 h	OD value in 5 h
Enterobacter sp1	0 umol•L^{-1}	0.1815±0.0007b	0.5608±0.0056b	0.9533±0.0057b
	33 umol•L^{-1}	0.2034±0.0006a	0.5954±0.0013a	0.9805±0.0023a
	100 umol•L^{-1}	0.1740±0.0008c	0.5281±0.0054c	0.9215±0.0061c
Burkholderia sp1	0 umol•L^{-1}	0.0707±0.0006a	0.1965±0.0014a	0.6327±0.0032a
	33 umol•L^{-1}	0.0656±0.0010b	0.1806±0.0014b	0.5646±0.0019b
	100 umol•L^{-1}	0.0627±0.0003c	0.1861±0.0030b	0.5707±0.0032b

Note: The lowercase letters behind data represented significant differences (P<0.05)

Table 5: Number of *Enterobacter sp1* and *Burkholderia sp1* in different soil samples of *P. heterophylla*

Cropping patterns	Ct value of *Enterobacter sp1*	*Enterobacter sp1* ($\times 10^4$ g^{-1} fresh soil)	Ct value of *Burkholderia sp1*	*Burkholderia sp1* ($\times 10^4$ g^{-1} fresh soil)
Fallow	25.72±0.17a	5.9±0.7b	27.51±0.19a	4.2±0.7b
One–year monoculture	25.70±0.37a	7.3±1.7b	26.19±0.19c	15±2.3a
Two–year consecutive monoculture	24.97±0.23b	14.5±2.5a	27.38±0.11b	6.2±0.6b

Note: The lowercase letters behind data represented significant differences (P<0.05)

Ct value. The abundance of *Enterobacter sp1* and *Burkholderia sp1* in *P. heterophylla* rhizosphere soil was calculated with the formula N=y × c ×V/ (8×2), in which N represent the number of bacteria in 1 gram of soil, c represented the concentration of soil DNA (unit was ng DNA per μL), V represented the volume of soil DNA, 8 represented every PCR tube contained 8 ng of soil DNA, and 2 represented 2 g of soil.

From the Table 5, the results showed that the abundance of *Enterobacter sp1* in rhizosphere soil of two-year consecutive monoculture was significantly higher than that in rhizosphere soil of one-year monoculture and fallow (Table 5). There was no significant difference between one-year monoculture and fallow. In contrast, the abundance of *Burkholderia sp1* in rhizosphere soil of one-year monoculture was significantly higher than that in rhizosphere soil of two-year consecutive monoculture and fallow. The abundance of *Burkholderia sp1* had no significant difference between rhizosphere soil of two-year consecutive monoculture and fallow.

Discussion

The rhizosphere soil is the major ecological environment

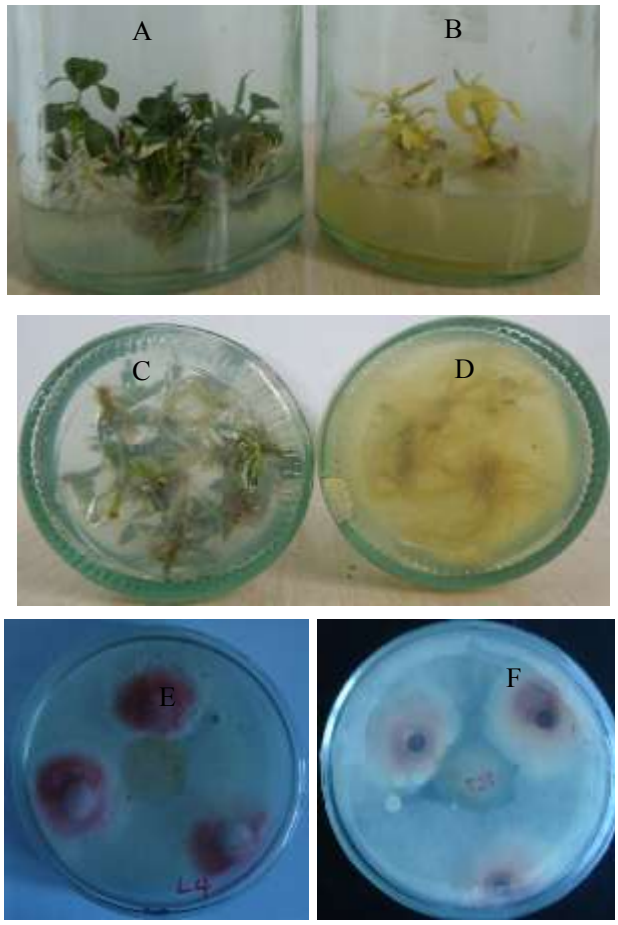

Fig. 1: Bacteria -*P. heterophylla* co-culture and bacteria - *Fusarium* confrontation culture

Note: Fig. A and C showed that *P. heterophylla* plantlets were co-cultured with the bacteria which had no pathogenicity to *P. heterophylla*. The *P. heterophylla* plantlets nomally growed in Fig A and C. Fig. B and D showed that *Strain 1* had pathogenicity to *P. heterophylla* plantlets. Fig. E showed that the bacteria isolated from *P. heterophylla* soil had no inhibitory effect on the growth of *Fusarium*. Fig. F showed that *Strain 2* had inhibitory effect on the growth of *Fusarium*

for plant-microbe interactions including associative, symbiotic, neutralistic, or parasitic interactions, which depend on soil environment, plant nutrient status in soil, plant defence mechanism, and the proliferating type of microorganism in the rhizosphere zone (Hayat *et al.*, 2010). Plant growth-promoting rhizosphere microorganisms (PGRPs) possess the important functions of accelerating nutrient availability and assimilation, improving plant productivity and suppressing disease-causing microbes and nematodes (Tyler *et al.*, 2008; Munees and Mulugeta, 2014). PGRPs are frequently used as biofertilizers and compensate for the stress and reduction in plant growth caused by weed infestation, drought, heavy metals, salt and other unfavorable environmental (Amico *et al.*, 2005; Robert *et al*., 2006; Groppa *et al.*, 2012; Babu *et al.*, 2014). These bacteria are of beneficial agricultural importance and belong

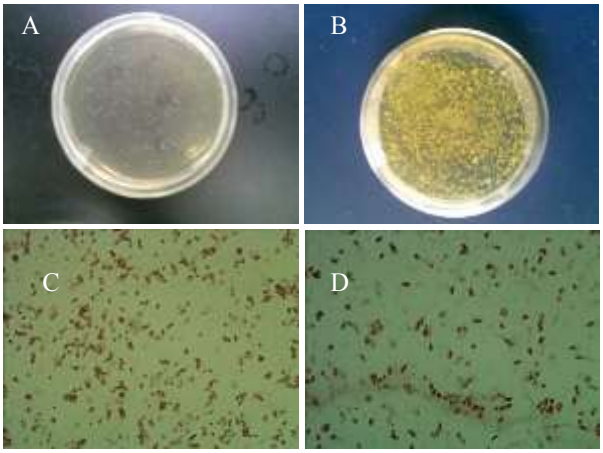

Fig. 2: The microexamination of *Enterobacter.sp1 and Burkholderia.sp1*

Note: Fig. A and B showed that *Enterobacter. sp1* and *Burkholderia. sp1* were respectively cultured on the solid LB medium. Fig. C and D showed that *Enterobacter. sp1* and *Burkholderia. sp1* were respectively observed under the microscope

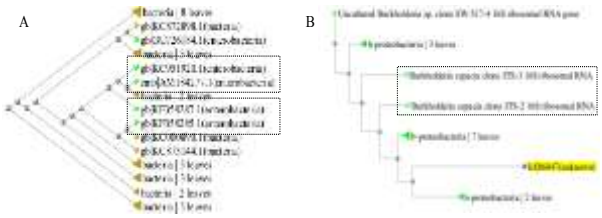

Fig. 3: Phylogenetic tree based on 16S rDNA sequence of *Strain 1 and Strain2* with other reference sequences

Note: Fig. A was the phylogenetic tree of *Strain 1*; Fig. B was the phylogenetic tree of *Strain2*. The dashed boxes showed the sequences of *Enterobacter.sp1 and Burkholderia.sp1*. in the genbank of NCBI had higher homology with *Strain 1 and Strain 2*

to the genera *Acetobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Derxia*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Ochrobactrum*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Zoogloea*, etc (Singh *et al.*, 2011). In this study, we isolated a strain of bacterium from *P. heterophylla* rhizosphere soil which belonged to genera *Burkholderia* by molecular identification. *Burkholderia sp1* had antagonistic ability to *Fusarium* isolated from sick plants of *P. heterophylla*, and no pathogenicity to plantlet of *P. heterophylla*. Various antagonistic microorganisms have different biological control mechanisms, and someone has several mechanisms already known (Mccullagh *et al.*, 1996; De Curtis *et al.*, 2010; Nally *et al.*, 2013). The mechanisms involve antibiotic effect, space and nutrition competition, parasitism and inducing systemic resistance of plant, etc (Inbar *et al.*, 1996; Boopathi and Sankara, 1999; Orwa *et al.*, 2002; Gong *et al.*, 2006). The mechanism of *Burkholderia sp1* antagonizing to *Fusarium* may belong to antibiotic effect

which would be verified in our future work.

The results of abundance analysis showed the amount of *Burkholderia sp1* decreased in *P. heterophylla* rhizosphere soil with planting years increased. On the contrary, the amount of *Enterobacter sp1* increased with planting years increased, which was isolated from *P. heterophylla* rhizosphere soil and had pathogenicity to plantlet of *P. heterophylla*. This phenomenon has been subject of extensive research for decades. Plant root exudates are believed to a major influence on the diversity of plant rhizosphere microorganisms (Hawes *et al.*, 2003; Yang, 2009; Berendsen *et al.*, 2012). It's reported that small molecule metabolites secreted by plant roots were terpenoids, phenolics, steroids, alkaloids and cyanogenic glycosides, which not only inhibited the growth of plant, but also induced the dynamic changes of microbial communities (Vokou *et al.*, 2006; Saraf *et al.*, 2014). Ma *et al.* (2005) reported that phenolic acids not only affected the amount and diversity of microbial communities in soil, but also promoted growth of soil-borne pathogens such as *Fusarium oxysporum* and *Phytophthora*. It is reported that phenolic acids were isolated from fibrous roots and rhizosphere soil of *R. glutinosa* and might contribute to the autotoxicity of *R. glutinosa* (Li *et al.*, 2012b). Qu and Wang (2008) found that phenolic acids selectively enhance specific microbial population in soil, and lead to the development of shifted communities with differing qualitative and quantitative compositions. In this study, the amount of phenolic acids including hydroxybenzoic acid, vanillic acid, vanillin and ferulic acid increased with planting years of *P. heterophylla* increased. The phenolic acids stimulated the growth of *Burkholderia sp1* at a certain concentration which was close to the concentration of phenolic acids in *P. heterophylla* rhizosphere soil.

Enterobacter sp1 belonged to genera *Enterobacter* which could degrade MCPA (Jiang *et al.*, 2012) and carbendazim (Li *et al.*, 2012a), and use chlorobenzene as the sole carbon source (Xie *et al.*, 2012). Few study reported that *Enterobacter* was a phytopathogen. The main bacterial phytopathogens known to us are *Agrobacterium*, *Burkholderia cepacia*, *Ralstonia*, *Erwinia*, *Pectobacterium athrosepticum*, *Pseudomonas syringae*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xanthomonas oryzae*, *Xylella fastidiosa*, *Clavibacter michiganensis*, *Leifsonia xyli*, *Spiroplasma kunkelii* and *Phytoplasma* groups, and *Coryneform* (Robert, 2011). In our study, *Enterobacter sp1* may be a harmful microorganism which was stimulated to grow by phenolic acids in *P. heterophylla* rhizosphere soil, and *Burkholderia sp1* had no antagonistic ability to *Enterobacter sp1*. So it's important to further study the pathogenic mechanism of *Enterobacter sp1* and isolate related antagonistic microorganism in future study.

In plant rhizosphere soil, apart from beneficial microorganism and pathogen, there were opportunistic microbes, which were unlike the neutralistic ones. Opportunistic microbes converted into beneficial

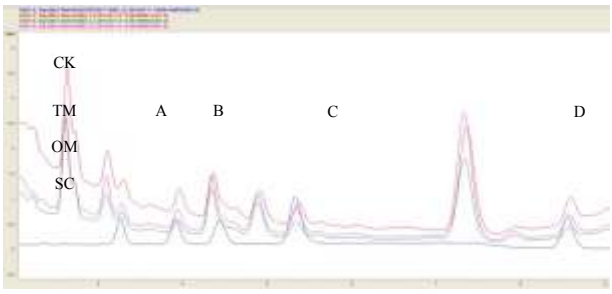

Fig. 4: HPLC chromatograms of phenolic acids in the different soil samples of *P. heterophlla*

Note: CK was fallow soil; TM was two–year consecutive monoculture; OM was one–year monoculture; SC was the standard curve of phenolic acids; A was hydroxybenzoic acid and its retention time was 3.270 min; B was vanillic acid and its retention time was 3.930 min; C was vanillin and its retention time was 5.340 min; D was ferulic acid and its retention time was 8.560 min

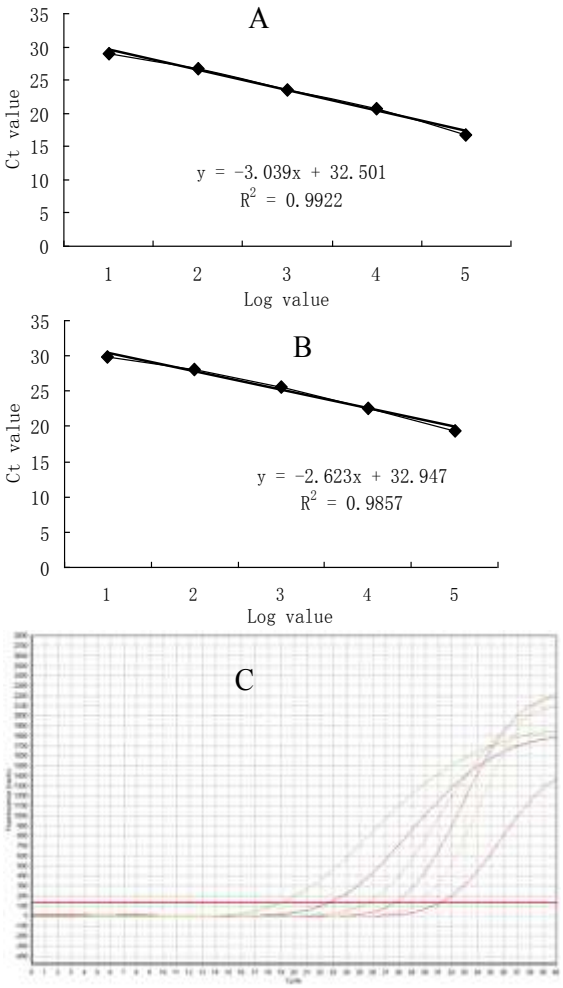

Fig. 5: Curves of real-time PCR detection and the standard curves of real-time PCR

Note: Fig. A was the standard curve of real-time PCR for *Enterobacter sp1*. Fig. B was the standard curve of real-time PCR for *Burkholderia sp1*. Fig. C was the curves of real-time PCR detection for standard curve

microorganism or pathogen when the environment factors of rhizosphere changed (involving soil type, mineral composition, water content, ionic strength, pH, redox potential, clay and nutrients content, physical factors, plant type, livestock grazing, presence of bacteriophages) and the balance was broken in microbial diversity. This shift either caused soil disaster, or improved soil environment. It's important to isolate more beneficial microorganism antagonistic to detrimental microbes, and use diverse methods to restore benign soil health preventing the shift of opportunistic microbes into detrimental ones and accelerating the progress of soil remediation. In addition, specific microbial fertilizer and organic matter such as soybean meal, fish meal, and rapeseed meal could be used in the cultivation of *P. heterophylla* or other medicinal plants to restore soil microbial diversity, reduce the accumulation of autotoxic substances, improve the functional diversity of the microbial community, and control soil diseases.

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