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Diversity and antifungal activity of endophytes associated with *Spiranthes sinensis* (Orchidaceae, Magnoliophyta) in China

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ABSTRACT

***Spiranthes sinensis* (Pers) Ames is an important traditional medicinal plant under the Orchidaceae in China. This study investigated the diversity and antifungal activity of endophytes isolated from the leaves, stems, inflorescences and roots of *S. sinensis* collected in Chongqing, China. A total of 263 endophytes were obtained in this study. Endophytic fungi (57) and endophytic bacteria (48) were identified based on internal transcribed spacer rDNA regions (ITS) and 16S ribosomal RNA (16S rRNA) gene analyses, respectively. The results indicate that the endophytes were assigned to 25 genera of fungi and 11 genera of bacteria. The most frequently isolated endophytic fungi was *Fusarium* (20%) followed by *Alternaria* (8%) and *Penicillium* (8%). The dominant endophytic bacteria were *Burkholderia* (19%), *Bacillus* (18%) and *Pseudomonas* (17%). Antifungal activity of the endophytic strains (n=105) were evaluated against three agricultural plant pathogens (*Fusarium oxysporum*, *Botrytis cinerea, Alternaria solani*). Among them, 9 fungal and 8 bacterial isolates showed broad spectrum antifungal activity against at least one of the three pathogens. Especially, the endophytic bacteria of *Bacillus velezensis* and *B. siamensis* exhibited the best inhibitory activity against the three pathogens.**

INTRODUCTION

Endophyte is an endosymbiont that resides inside of living plant tissues, often isolated as fungi and bacteria, which do not show any symptom on the host plants (Wilson, 1995; Krishnapura and Belur 2016). It ubiquitously presents in all plant family and all kinds of climates (Santoyo et al., 2016). In a particular long-term interaction with plants, endophytes have played a very important role in affecting the growth of plants and possessed high diversity in function. In the past two decades, endophytes have been extensively investigated and found producing multiple biomolecules, biocatalysts and biological enzyme applied in medicine, agriculture, and industry (Golinska et al., 2015; Qadri et al., 2014; Zaferanloo et al., 2013). For some endangered medicinal plants, their endophytes may be an excellently substitute to reduce the stress of requirement, such as taxol and camptothecin(Zhou et al., 2010). In addition, many studies have published to demonstrate that endophytes are helpful in improving plant growth, relieving abiotic stress and protecting from pathogens (Deng and Cao, 2017; Hassan, 2017; Pandey et al., 2016).

Spiranthes sinensis is commonly known as the Chinese spiranthes, belong to the family of Orchidaceae,

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occurring in eastern Asia, west to the Himalayas, south and east to New Zealand, and north to Siberia (Surveswaran et al., 2017). The plant has valuable ornamental purpose because of the spiral inflorescence. Besides, *S. sinensis* has many medicinal properties as a folk medicine, such as anti-inflammatory, antiviral, antitumor, antidiabetic activity (Kovacs et al., 2008; Shie et al., 2015). It is popular to extract compounds from the whole plant (Li et al., 2013; Liang et al., 2014), ex. flavonoids, homocyclotriucallane, dihydrophenanthrenes and ferulic acid, which are highly effective antioxidants, biocontrol activity and less toxicity (Kovacs et al., 2008; Lin et al., 2001). Because of the environmental damage and over-excavation leading to a sharp decline, *S. sinensis* needs to be paid more attention. The study was aimed at investigating the endophytes diversity in *S. sinensis*, evaluating the antifungal activity of the endophytes, and finding good candidates for bio-control management of plant diseases.

MATERIALS AND METHODS

Plant sampling and endophytes isolation

In June of 2017, healthy plants of *S. sinensis* were collected from Hechuan, Chongqing China. Samples were washed under running water to clean the dust on the surface and then air dried. The roots, stems, leaves and inflorescences of the plants were separated and cut into 0.5 cm pieces. All the tissues were surface-sterilized by immersing in 95% ethanol for 1 min, 2% sodium hypochlorite for 4 min, and 95% ethanol for 30 s, followed by washing in sterile distilled water three times (Paul et al., 2013). After air-dried on sterilized filter paper, tissue pieces were placed onto potato dextrose agar (PDA) supplemented with the antibiotic gentamycin sulfate 0.4 mg/mL for endophytic fungal isolation and nutrient agar (NA) to isolate endophytic bacteria. To confirm the surface sterilization thoroughly on tissues, the third time washed water was spread on the media of isolation for 7 days. After the incubation on PDA at 25°C for 3, 6 and 10 days separately, fungal isolates were obtained by collecting mycelia at the edge of single colonies generated from the tissues. Pure cultures were stored on slants and deposited in the Culture Collection of Yangtze University (YZU). However, the endophytic bacteria were isolated after the incubation on NA at 27°C for 2, 5 and 7 days. Sub-cultured single colonies derived from the plant tissues were maintained in 20% glycerol stock solution at -80°C.

Endophytes identification

Fungal mycelia were scraped from PDA cultures for DNA extraction using the method described by Cenis (1992). The ITS region was amplified with primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Partial sequence of the 16S rRNA gene was amplified using 27F (5'-AGAGTTTGATCCTGGCTCA-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primer pairs (Lane, 1991). Total volumes of 25 µL PCR reaction mixtures consisted of 2 µL genomic DNA,1.25 µL each primer, 12.5 µL 2×Taq PCR StarMix with loading dye (Genstar, Beijing, China) and 8 µL distilled sterile water. The PCR products were examined and successful products were directly sent to BGI Company (Beijing, China) for sequencing. Each resulting fungal sequence was executed with a BLASTN search in NCBI database (http://blast.ncbi.nlm.nih.gov/). Bacterial sequences were also searched in the EzBioCloud (http://eztaxon-e.ezbiocloud.net/) database.

Screening antifungal activity

The antifungal activity of fungal and bacterial endophytes was screened against three important agriculture pathogens- *F. oxysporum*, *B. cinerea* and *A. solani* by dual culture method (Paul et al., 2012). These three pathogens were collected from cotton, tomato and potato, respectively, and had been deposited in the Culture Collection of Yangtze University. Mycelial blocks (6 mm) of the fungal pathogen were placed in the center of PDA plate. The same size endophytic fungal blocks were placed at 2.5 cm distance from the pathogenic fungal block. For bacterial samples, the distance between the two was for 1.5 cm. After incubated for 7 days at 25°C, the inhibition zones between the pathogen and endophytes were measured. The growth inhibition was evaluated by the following formula: Growth Inhibition % (MIC)= [(growth in the control-growth in the sample) / growth in the control)] ×100 (Mccalley and Torresgrifol, 1992).

Data analysis

To characterize the diversity of endophytes, diversity index were analyzed. Isolation rate (IR) and isolation frequently (IF) of endophytes were calculated using the following formula:

$$\mathrm{IR} = \frac{\mathrm{Ni}}{\mathrm{Nt}} \text{ and } \mathrm{IF} = \frac{\mathrm{Ni}}{\mathrm{Ne}}$$

Where Ni is the number of endophytes isolated, Nt is the total number of tissue segments, and Ne is the total number of endophytes.
Species richness (*S*) was used as the number of taxa contained. Shannon index of diversity (*H'*) was calculated

using the following formula:

H'=-∑pi (ln pi) (Shannon and Weaver, 1949) to exhibit the diversity of the endophytes species.

RESULTS

Diversity of endophytes

The plant stems, leaves, inflorescences or roots were independently cut into 210 segments which are 7 points per plate on PDA and NA media to isolate endophytic fungi and bacteria, respectively. A total of 106 endophytic fungi and 157 bacteria were obtained from 840 tissue segments (420 segments on PDA, the same number of segments on NA). The number of endophytic fungi from stems, leaves, inflorescences and roots were 18, 40, 7 and 41, and the number of endophytic bacteria was 60, 49, 26 and 22, respectively. The isolation rate (IR) of all the endophytes was 0.31 (Table 1). The highest number of isolates was obtained from leaf samples which contributed 89 strains with the IR of 0.42, followed by stem (78, 0.37), root (63, 0.3), and inflorescence (33, 0.15). The isolation frequently (IF) showed the richness of endophytes from tissues in the total isolates (Table 1), while the population of endophytes from leaf (IF=0.34) and stem (0.30) were more than those from inflorescence (0.12) and root (0.24). Endophytic fungi obtained from all the tissues excluding the root were more than endophytic bacteria.

Based on colony characteristics (size, color, shape and texture), 57 endophytic fungi strains and 48 endophytic bacterial strains were selected for further study. All the endophytic fungi showed 97-100% sequence similarity with the published sequences in NCBI database, based on ITS gene sequences. The strains were resulted into 25 different genera, except unidentified 4 strains (Table 2). Among them, 22 genera are belonging to Ascomycota, including *Alternaria*, *Ascochyta*, *Cadophora*, *Cercospora*, *Diaporthe*, *Fusarium*, *Leptodontidium*, *Leptosphaerulina*, *Macrophomina*, *Paraboeremia*, *Penicillium*, *Peniophora*, *Phoma*, *Phyllosticta*, *Pilidium*, *Pleosporales*, *Preussia*, *Rhizopycnis*, *Talaromyces*, *Thozetella*, *Trichoderma* and *Xylaria*; two strains (*Ceratobasidium* sp. YZU 172002, *Tulasnella* sp. YZU 172048) were Basidiomycota and one strain (*Mucor* sp. YZU 172009) were Zygomycota. Among all endophytic fungi, *Fusarium* was the most frequently isolated fungal genus (20 %), followed by *Penicillium* (8%) and *Alternaria* (8%) (Figure 1A).

Based on the 16S rRNA gene sequence analysis, all the endophytic bacteria showed 98-100% similarity with sequences of the type strains in EZtaxon database. A total of 48 bacterial endophytes were classified into 11 different genera (Table 3), which were *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Luteibacter*, *Paenibacillus*, *Pantoea*, *Paraburkholderia*, *Pseudomonas*, *Rhizobium* and *Sphingomonas*. *Burkholderia* was the dominant bacteria (19 %) found in *S. sinensis*. The other frequently isolated bacteria were *Bacillus* (18%), *Pseudomonas* (17%) and *Acinetobacer* (11%) (Figure 1B). The diversity indices showed the species richness of endophytic fungi (*S*=33) and bacteria (*S*=24). The Shannon diversity index of endophytic fungi and bacteria were 2.882 and 2.203, respectively.

Antifungal activity of the endophytes

In this study, nine endophytic fungal strains showed antifungal activity against at least one pathogenic fungus, which were belonging to *Fusarium*, *Leptosphaerulina*, *Paraboeremia*, *Phyllosticta*, *Pleosporales* and *Talaromyces* (Table 4). Isolates of YZU 172042 (*Pleosporales* sp.), YZU 172047 (*Fusarium* sp.) and YZU 172049 (*Fusarium* sp.) could inhibit all three tested pathogens with clear inhibition zone. Isolates of YZU 172042 and YZU 172052 showed broad-spectrum inhibition (the inhibited rate were>50%). Eight endophytic bacterial isolates displayed broadly antifungal activity against three pathogens, comprising the genera of *Bacillus*, *Burkholderia*, *Pantoea*, *Pseudomonas* and *Sphingomonas* (Table 5). Strains of YZU 173017 (*B. velezensis*) and YZU 173039 (*B. siamensis*) exhibited notable activity higher than other isolates, which inhibitory rate was up to 80% (Figure 2).

DISCUSSION

In this study, endophytic fungi and bacteria were isolated from *Spiranthes sinensis* and their diversity and antifungal activity were investigated. *Fusarium*, *Alternaria*, *Rhizopycnis*, *Phoma* and *Penicillium* were frequently isolated endophytic fungal genera in this study, which resembled similar results with previous studies (Ning, 2009). Indeed, *Fusarium* and *Alternaria* as the most frequently isolated species were reported in almost all host plant studied, such as *Glycine max* (Fernandes et al., 2015), *Rhizophora mucronata* (Hamzah et al., 2018), *Taxus baccata* L. (Ashkezari et al., 2017) and many plants from desert areas (Sun et al., 2012). Some other endophytic fungi were occasionally found, such as *Cerato basidium*, *Leptosphae rulina*, *Paraboeremia* and *Tulasnella*, which may correlate with plant origin and plant characters.

For Orchidaceae plants, mycorrhizal fungi are very important microorganism present in their growth processes. The plants established symbiosis with their corresponding fungal mycorrhiza and critically depended on them for completion of their life cycle (Jacquemyn et

Table 1. The number of endophytes isolated from *S. sinensis* in China.

Tissue	Endophytic fungi	Endophytic bacteria	Total	IR	IF
Inflorescence	7	26	33	0.15	0.12
Leaf	40	49	89	0.42	0.34
Stem	18	60	78	0.37	0.3
Root	41	22	63	0.3	0.24
Total	106	157	263	0.31	

IR, The number of endophytes isolated/the total number of tissue segments; IF, the number of endophytes isolated/the total number of isolated.

Table 2. Endophytic fungi from *S. sinensis* identified based on the ITS region.

Genus	Strain	Closest species in NCBI	Similarity (%)	Accession No.
Alternaria	YZU 172036, YZU 172021	*A. alternata*	100	MF614038
	YZU 172029, YZU 172030	*Alternaria* sp.	100	MH399363
Ascochyta	YZU 172025	*A. viciae-pannonicae*	98	EU167559
Cadophora	YZU 172051	*Cadophora* sp.	100	KT268419
Ceratobasidium	YZU 172002	*Ceratobasidium* sp.	97	DQ102430
Cercospora	YZU 172046	*C. asparagi*	100	KY549098
Diaporthe	YZU 172003, YZU 172004	*D. longicolla*	100	MF125057
Fusarium	YZU 172038	*F. acuminatum*	100	KY910870
	YZU 172016, YZU 172011	*F. oxysporum*	100	KY910858
	YZU 172014, YZU 172039			
	YZU 172015, YZU 172017, YZU 172027			
	YZU 172012,YZU 172033	*Fusarium* sp.	100	KY582097
Leptodontidium	YZU 172047, YZU 172049, YZU 172054, YZU 172055, YZU 172056	*Leptodontidium* sp.	100	KY031672
Leptosphaerulina	YZU 172022	*L. chartarum*	99	KJ796400
Macrophomina	YZU 172006, YZU 172005	*M. phaseolina*	100	KF951634
Mucor	YZU 172009	*M. irregularis*.	98	KX148754
Paraboeremia	YZU 172042	*Paraboeremia* sp.	99	LC310980
Penicillium	YZU 172007	*P. brasilianum*	100	JQ781748
	YZU 172008	*P. citrinum*	99	KY921954
	YZU 172034, YZU 172041	*Penicillium* sp.	100	GU566206
	YZU 172028			
	YZU 172041			
Peniophora	YZU 172053	*Peniophora* sp.	97	HQ608147
Phoma	YZU 172043	*P. herbarum*	99	KM513613
	YZU 172013, YZU 172023	*Phoma* sp.	100	HQ631000
Phyllosticta	YZU 172032	*Phyllosticta* sp.	100	KY964333
Pilidium	YZU 172018, YZU 172019, YZU 172040	*Pilidium* sp.	99	KF367478
Pleosporales	YZU 172020	*Pleosporales* sp.	100	KT268393
	YZU 172052	*Pleosporales* sp.	99	KX100397
Preussia	YZU 172045	*Preussia* sp.	99	JN225886
Rhizopycnis	YZU 172035	*Rhizopycnis* sp.	100	DQ682600
Talaromyces	YZU 172044	*T. assiutensis*	99	JN899320
	YZU 172024	*T. verruculosus*	100	HQ608025
Thozetella	YZU 172050	*Thozetella* sp.	100	KY582136
Trichoderma	YZU 172001	*Trichoderma* sp.	99	KF367487
Tulasnella	YZU 172048	*Tulasnella* sp.	99	KF537664
Xylariaceae	YZU 172026	*Xylariaceae* sp.	99	KM513576

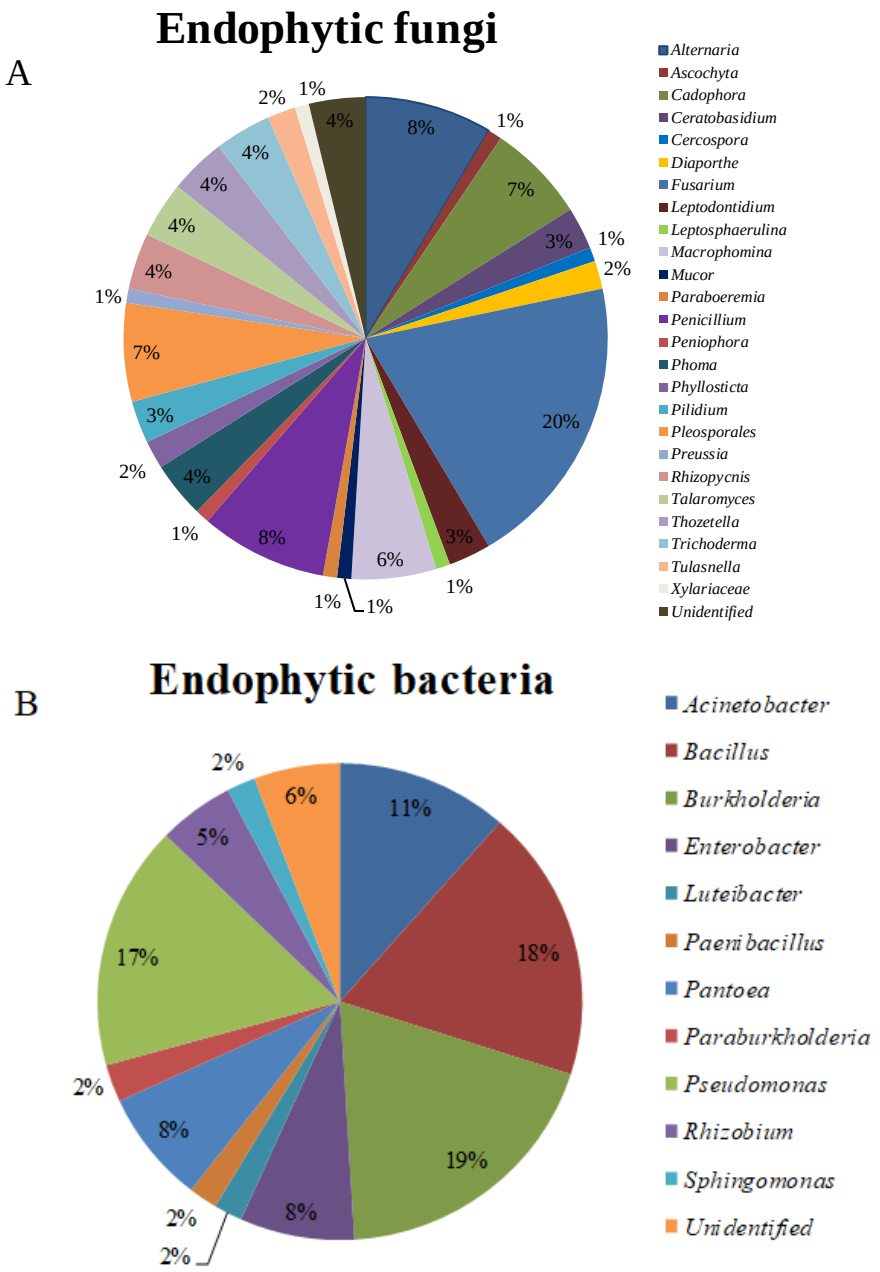

Figure 1. Taxonomic groups of endophytic fungi (A) and bacteria (B) from *S. sinensis*.

Table 3. Endophytic bacteria from *S. sinensis* identified based on their 16S RNA sequences.

Genus	Strain	Closest type strain	Similarity (%)	Accession No.
Acinetobacter	YZU 173037	*A. calcoaceticus* DSM 30006 (T)	100	AIEC01000170
	YZU 173005, YZU 173014	*A. oleivorans* DR1 (T)	99.93	CP002080
	YZU 173018, YZU 173021, YZU 173025, YZU 173032, YZU 173036	*A. oleivorans* DR1 (T)	100	CP002080

Table 3. Contd.

	YZU 173035	*B. acidiceler* CBD 119 (T)	99.85	DQ374637
	YZU 173022, YZU 173046	*B. aryabhattai* B8W22 (T)	99.9	EF114313
	YZU 173030, YZU 173038			
Bacillus	YZU 173031	*B. cereus* ATCC 14579 (T)	99.43	AE016877
	YZU 173047	*B. oleronius* DSM 9356 (T)	99.79	X82492
	YZU 173039	*B. siamensis* KCTC 13613 (T)	99.93	AJVF01000043
	YZU 173017	*B. velezensis* CR-502 (T)	99.78	AY603658
	YZU 173029	*B. velezensis* CR-502 (T)	100	AY603658
	YZU 173002	*B. ambifaria* AMMD (T)	99.77	CP000442
	YZU 173006	*B. contaminans* LMG 23361 (T)	99.86	JJOA01000042
	YZU 173008	*B. contaminans* LMG 23361 (T)	99.93	LASD01000006
Burkholderia	YZU 173012	*B. contaminans* LMG 23361 (T)	99.86	LASD01000006
	YZU 173040	*B. contaminans* LMG 23361 (T)	99.93	JJOA01000042
	YZU 173007	*B. territorii* LMG 28158 (T)	99.93	LK023503
	YZU 173026	*B. territorii* LMG 28158 (T)	99.79	LK023503
	YZU 173048	*E. asburiae* JCM 6051 (T)	99.42	BBED01000197
Enterobacter	YZU 173028	*E. asburiae* JCM 6051 (T)	99.93	BBED01000197
	YZU 173044	*E. ludwigii* EN-119 (T)	99.64	JTLO01000001
	YZU 173034	*E. r tabaci* YIM Hb-3 (T)	99.53	KP990658
Luteibacter	YZU 173011	*L. anthropi* CCUG 25036 (T)	98.79	FM212561
Paenibacillus	YZU 173024	*P. cineris* LMG 18439 (T)	100	AJ575658
	YZU 173013	*P. dispersa* LMG 2603 (T)	100	DQ504305
Pantoea	YZU 173041	*P. dispersa* LMG 2603 (T)	100	DQ504305
	YZU 173043	*P. eucalypti* LMG 24198 (T)	99.48	EF688009
P. burkholderia	YZU 173020	*P. metalliresistens* D414 (T)	99.51	KF601211
	YZU 173003	*P. koreensis* Ps 9-14 (T)	99.64	AF468452
	YZU 173027	*P. koreensis* Ps 9-14 (T)	99.93	AF468452
Pseudomonas	YZU 173009	*P. moorei* RW10 (T)	98.68	AM293566
	YZU 173033	*P. moorei* RW10 (T)	98.68	AM293566
	YZU 173010	*P. parafulva* NBRC 16636 (T)	98.56	BBIU01000051
	YZU 173004	*P. protegens* CHA0 (T)	100	CP003190
Rhizobium	YZU 173001	*R. rhizogenes* NBRC 13257 (T)	99.85	BAYX01000035
Sphingomonas	YZU 173016	*S. yabuuchiae* GTC 868 (T)	100	AB071955

T: Type strain.

Table 4. Antifungal activity of endophytic fungi from *S. sinensis* against *B. cinerea*, *F. oxysporum* and *A. solani*.

Strain No.	Endophytic fungi	Antagonistic activity		
		B. cinerea	***F. oxysporum***	***A. solani***
YZU172020	*Pleosporales* sp.	++	-	++
YZU172022	*Leptosphaerulina chartarum*	++	-	-
YZU172032	*Phyllosticta* sp.	++	-	++
YZU172033	*Fusarium* sp.	++	-	-
YZU172042	*Paraboeremia* sp.	++	++	++
YZU172044	*Talaromyces assiutensis*	+	-	++
YZU172047	*Fusarium* sp.	++	++	+
YZU172049	*Fusarium* sp.	++	+	+
YZU172052	*Pleosporales* sp.	++	++	++

-, no inhibition; +, inhibition rate 0- 40%; ++, inhibition rate 40-70%.

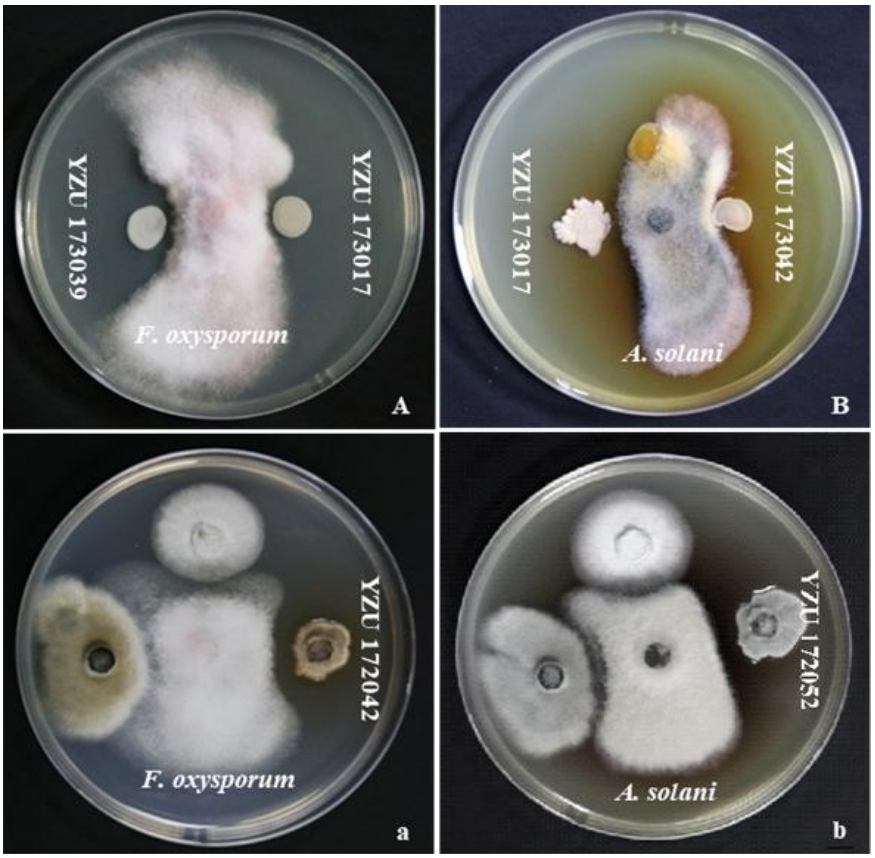

Figure 2. Endophytic fungi and bacteria from *S. sinensis* showing antifungal activity in dual culture against pathogens *F. oxysporum* (A, a) and *A. solani* (B, b).

Table 5. Antifungal activity of endophytic bacteria from *S. sinensis* against *B. cinerea*, *F. oxysporum* and *A. solani*.

Isolate	Endophytic bacteria	Antagonistic activity		
		B. cinerea	*F. oxysporum*	*A. solani*
YZU 173004	*Pseudomonas protegens*	+++	++	++
YZU 173012	*Burkholderia contaminans*	++	++	++
YZU 173016	*Sphingomonas yabuuchiae*	+	-	++
YZU 173017	*Bacillus velezensis*	+++	+++	+++
YZU 173026	*Burkholderia territorii*	++	++	++
YZU 173039	*Bacillus siamensis*	+++	+++	+++
YZU 173040	*Burkholderia contaminans*	+++	++	+++
YZU 173041	*Pantoea dispersa*	-	-	+++

-, no inhibition; +, inhibition rate 0- 40%; ++, inhibition rate 40-70%; +++, inhibition rate 70-100%.

al., 2016). In the present study, *Ceratobasidium* sp. (YZU 172002) and *Tulasnella* sp. (YZU 172048) were isolated from root which were reported as mycorrhizal fungi from *Dendrobium nobile* (Orchidaceae) (Chen et al., 2012; Jacquemyn et al., 2016).

The diversity of endophytic bacteria from *S. sinensis* was firstly investigated in the present study, 157 endophytic bacterial strains under 11 genera, except unidentified strains. *Burkholderia* and *Bacillus* were found to be the prevailing species, which was also reported as the dominant endophytic bacteria in *Dendrobium sinense*, some Orchidaceae plants and agriculture crops (Song et

al., 2015; Bredow et al.,2015; Esposito-Polesi et al., 2017; Yu et al., 2013). Previous studies showed that these species could promote the growth of host plants and protect against pathogen attacks through various modes of action (Haque et al., 2016). Root is considered as the primary tissue for existing endophytic bacteria due to numerous microorganisms in soil possible entry (Bulgarelli et al., 2012), but analyzing the distribution of endophytes *S. sinensis*, the total number of endophytic bacteria obtained from stem samples was more than those from root. Besides, the number of endophytes from inflorescence is less than from other tissues, which might be due to the plant in flowering stage, not fully bloomed (Jia et al., 2016).

Endophytic fungi showing antifungal activity against three important plant pathogens (*F. oxysporum*, *B. cinerea, A. solani*) were from the genera *Fusarium*, *Pleosporales*, *Phyllosticta*, *Phoma* and *Aromyces*. But, all of them had not displayed outstanding activities. Comparing with fungi, the endophytic bacteria exhibited higher activity against the pathogens. Particularly, YZU 173017 (*Bacillus velenzensis*) and YZU 173039 (*B. siamensis*) stood out as the more effective strains against all the tested pathogens. Cao (2018) reported that the plant-growth promoting of *B. velezensis* isolated from the tomato rhizosphere soil possessed strong antagonistic activity against *Ralstonia solanacearum* and *F. oxysporum*. Genome sequence of *B. siamensis* KCTC 13613 was analyzed and found strong antibacterial activity (Jeong, 2015).

The culturable endophytes showed the richness of endophytes in *S. sinensis* according to diversity index. However, many endophytic microorganisms were still missing and unable to grow on normal medium because of unknown optimum growth conditions and symbiosis with hosts (Tholozan et al., 1999). Endophyte is important for the endangered plants in the future to explore their function for plant growth. This study isolated culturable endophytes from *S. sinensis* and revealed the diversity of endophytic community. Further study will be conducted on promoting plant growth and antimicrobial compounds with mechanism of the two bacterial strains to gain knowledge of its potential as bio-control agents.

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Supplementary Figure 1

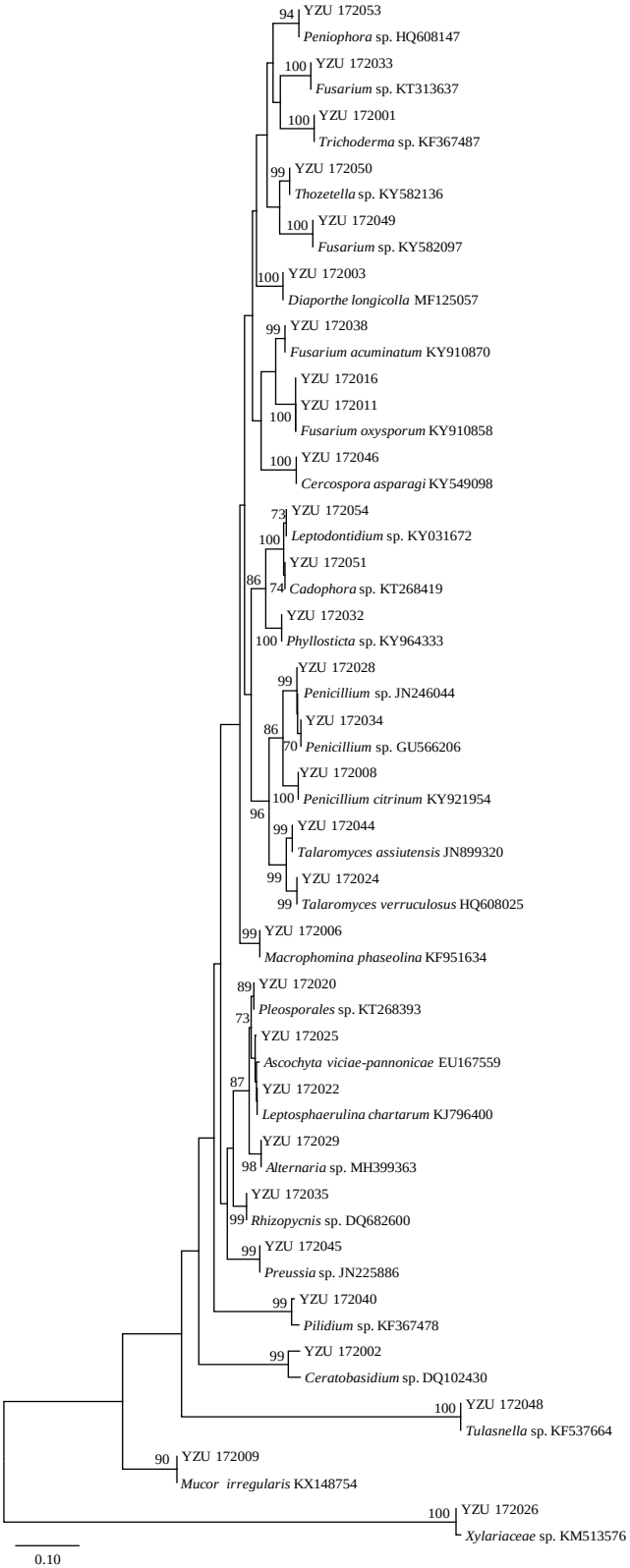

Figure S1. Phylogenetic tree generated from parsimonious analysis of endophytic fungi isolated from *S. sinensis* in China based on ITS sequences.

Supplementary Figure 2

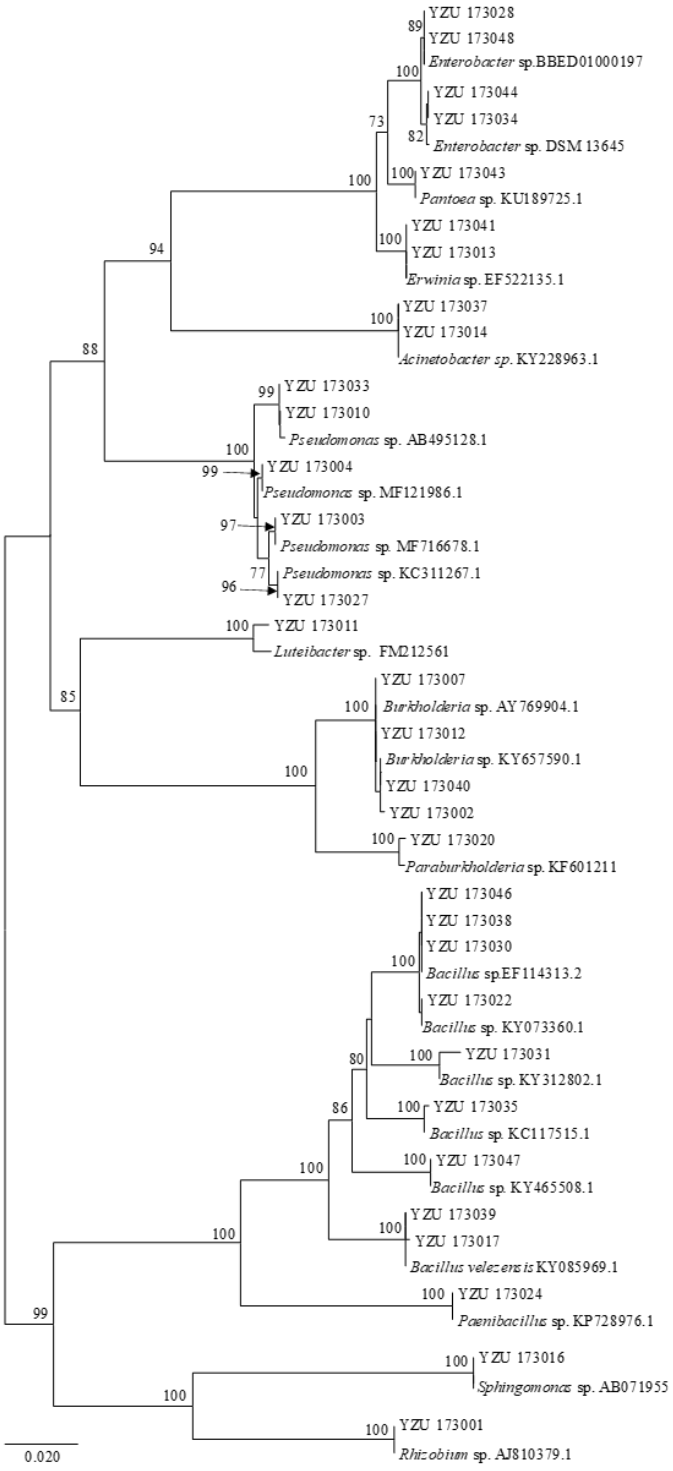

Figure S2. Phylogenetic tree generated from parsimonious analysis of endophytic bacteria isolated from *Spiranthes sinensis* based on 16S rDNA sequences.