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Diversity of cultivable endophytic bacteria in mulberry and their potential for antimicrobial and plant growth-promoting activities

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ABSTRACT

Endophytic bacteria-based biocontrol is regarded as a potential plant disease management strategy. Present study analyzed the diversity of mulberry endophytic bacteria basing on a culture-dependent approach and further evaluated their antimicrobial and plant growth-promoting (PGP) activities. A total of 608 cultivable endophytic bacteria, belonging to 4 phyla and 36 genera, were isolated from four mulberry cultivars having different resistance to sclerotiniosis in three seasons. Taxonomic compositional analysis results showed that Proteobacteria, Firmicutes, and Actinobacteria were the three dominant bacterial phyla in all communities, with the representative genera *Pantoea*, *Bacillus*, *Pseudomonas*, *Curtobacterium*, and *Sphingomonas*. Diversity analysis results indicated that the diversity of winter community was higher than that of spring or autumn, and higher diversities were detected in the resistant cultivar communities compared with the susceptible cultivar. Antagonism assays results showed that 33 isolates exhibited strong and stable activity against three phytopathogens which are *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Colletotrichum gloeosporioide*. Eight endophytic bacteria were selected out from 33 antagonists based on the evaluation of antagonistic and PGP activities. Furthermore, pot experiment results revealed that all the 8 tested endophytes stimulated the growth of mulberry seedlings at different levels, and *Bacillus* sp. CW16-5 exhibited the highest promotion capacity, which the shoot length and the root fresh weight were increased by 83.37% and 217.70%, respectively. Altogether, present study revealed that mulberry harbors a large amount of diverse cultivable endophytic bacteria and they also serve as novel sources of beneficial bacteria and bioactive metabolites.

1. Introduction

Mulberry (*Morus* L.), as the irreplaceable food of silkworm (*Bombyx mori*), played a very important role in the Silk Trade of ancient China. Mulberry fruit, as the byproduct of sericulture in the past, has been proved to contain abundant vitamins, minerals, and bioactive substances that are beneficial for human health (Liang et al., 2011; Sultana and Kim, 2016). In recent years, the planting area of the mulberry tree has been remarkably increased due to the high demand for mulberry fruit for human use (Kuai and Wu, 2012; Sultana et al., 2013). However, mulberry fruit productivity is greatly threatened by the white fruit disease, a soil-borne fungal disease called 'mulberry fruit sclerotiniosis'. Four species in the family Sclerotiniaceae, namely *Ciboria shiraiana* (Hong et al., 2007; Hu et al., 2011), *Ciboria carunculoides* (Whetzel and Wolf, 1945; Sultana et al., 2013), *Sclerotinia sclerotiorum* (Lü et al., 2013), and *Scleromitrula shiraiana* (Hong et al., 2007), can infect mulberry flowers resulting in the occurrence of diseased mulberry fruit. To date, chemical and nonchemical managements have been used to control mulberry fruit sclerotiniosis. Effective chemical prevention methods are currently being used worldwide to control this disease (Lü et al., 2011; Ye et al., 2014; Ju et al., 2016), but long-term overuse of chemical fungicide is undoubtedly a threat to human health, with increasing environmental pollution and inducing the emergence of drug-resistant pathogens (Commare et al., 2002). Moreover, other approaches, such as breeding for resistance and traditional cultural control practices (rotation, soil management, and nonwoven fabric mulching) have failed to show significant effects against this fruit disease (Bae et al., 2010). In recent years, microbe-based biological control

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has been considered as a potential and sustainable alternative for combatting phytopathogens and promoting plant growth.

Endophytes are often defined as nonpathogenic bacteria and fungi that reside in the living tissue of healthy plants without causing apparent harm to their hosts (Bacon and White, 2000; Carroll, 1988). Endophytic bacteria, as important components of the plant microbiota, have been isolated from diverse plant species, and some have exhibited considerable potential for the application of plant protection areas. Some of these bacteria can accelerate seed germination and promote plant growth by several mechanisms, including nitrogen fixation, phosphate solubilization, siderophore production, and bioactive enzyme secretion (Liotti et al., 2018; Pereira et al., 2016). Some large populations of endophytic *Bacillus* (Zouari et al., 2016), *Pseudomonas* (Wicaksono et al., 2018), and *Pantoea* (Xie et al., 2017) species displayed strong antagonistic activity against phytopathogenic fungi by producing a variety of secondary metabolites with antimicrobial properties. In addition, part of endophytes showed good colonization ability of plant tissue, and this capacity can help them to confer beneficial effects on host growth, because colonization behavior is believed as a critical process for the interaction between beneficial microbes and plants (Compant et al., 2010; Sharma et al., 2008). Furthermore, analogous to the beneficial microbes in different environments, endophytic microorganisms also act as reservoirs of novel bioactive substances (Strobel, 2003) and thus have applications in medicine, agriculture, and industry. Due to their myriad of functions, endophytic bacteria can be regarded as good candidates for natural biological control antagonists (BCAs).

In fact, management of such biological control programs, using endophytic bacteria as BCAs, requires some understanding of the structure and dynamics of microbial endophytic communities (Liotti et al., 2018). Numerous studies have demonstrated that plant species, cultivars, and some environmental factors are key determinants of endophytic composition and function. Shen and Fulthorpe (2015) indicated that the diversity of endophytic bacteria in urban trees (*Acer negundo*, *Ulmus pumila*, and *Ulmus parvifolia*) was highly dependent on the season. Mocali et al. (2003) found that bacterial communities associated with elm were influenced by variations in seasons and organs. Additionally, significant differences in the densities and structures of endophytic microbial communities have also been detected in different cultivars or genotypes of plants (da Silva et al., 2014; Liotti et al., 2018; van Overbeek and van Elsas, 2008). Studies on the determinants of endophytic community structure have been reported for many crops, such as maize (da Silva et al., 2014), cotton (Adams and Kloepper, 2002), and potato (Marques et al., 2015), but information on the endophytic bacterial community of mulberry remains scarce.

In recent decades, some endophytic strains isolated from mulberry have exhibited strong antagonistic activity against various phytopathogens (Mu et al., 2008; Tan et al., 2012; Xie et al., 2017), but very few isolates have been used to effectively control the sclerotial disease of mulberry fruits. The aims of the present study were to (i) isolate cultivable endophytic bacteria and analyze the diversity of these communities from four mulberry varieties during winter, spring, and autumn in two consecutive years (2015 ~ 2016); (ii) screen for endophytic bacteria with antagonistic activity against *S. sclerotiorum* in vitro and further evaluate the inhibition activity of some antagonists toward other phytopathogens and their capability with production of plant growth-promoting (PGP) traits; and (iii) assess the effects of endophytes with potential biological control applications on the growth of mulberry seedlings under greenhouse conditions. Ultimately, the goal of present research was to establish a microbial collection of mulberry endophytes and further obtain resources of bioactive endophytes with potential applications in the biological control of mulberry fruit sclerotiniosis.

2. Materials and methods

2.1. Mulberry sample preparation

Healthy branches from four healthy mulberry cultivars with different degrees of resistance to sclerotiniosis (Changguo Sang = CGS, Chuan Sang No.7637 = CSQ, Xin Lunjiao = XLJ, and Hong Guo No.2 = HGE) were collected in this study: cultivars CGS and CSQ were resistant to sclerotiniosis, and cultivars XLJ and HGE were susceptible to the sclerotial disease (Huang et al., 2012). Among the four cultivars, CGS was collected at the Southwest University experimental farm (29° 49′ 1″ N, 106° 24′ 57″ E), and the remaining three were obtained from the Sericulture Science and Technology Institute experimental farm (29° 50′ 39″ N, 106° 25′ 55″ E). Both farms were located in an area near the north side of the Jialing River in Chongqing, China, and the two regions share same climate conditions. Two-year-old stems with approximately 1.5 ~ 2.0 cm in diameter were collected in January, April, and September, representing the seasons winter, spring, and autumn, respectively, for the two consecutive years 2015 ~ 2016. After removing leaves and small branches, the samples were immediately transported back to the laboratory and stored at 4 °C until further processing.

2.2. Isolation of endophytes

Surface sterilization of the mulberry stems was performed according to a previously described procedure (Strobel et al., 2000), and the endophytic bacteria were isolated using the fragmentation technique (Liotti et al., 2018). In brief, samples were washed with tap water to remove soil and other debris before being cut into pieces with 3.5 ~ 5.0 cm in length. The samples were then thoroughly soaked in a 70% ethanol/water (*v/v*) solution and rapidly flame-sterilized. Then, the stems were peeled to obtain smaller fragments and placed on water agar (WA), Gause's agar (GA), and potato dextrose agar (PDA) medium, respectively. The plates were incubated at 28 °C for 20 days and examined daily for the presence of colonies at the edge of the stem piece. Colonies with different morphological characteristics were chosen from each plate and purified using a streak plate on PDA medium. All the purified isolates were stored with 30% glycerol at −80 °C.

2.3. Classification of endophytic bacteria

Classification of the bacteria was based on the analysis of 16S rRNA gene sequencing using the universal primers 27 F/1492R (Bredow et al., 2015). The total DNA of the strain was extracted with PrepMan Ultra Sample Preparation Reagent kit (Applied Biosystems, Palo Alto, CA, USA) according to the manufacturer's instructions. The DNA from all the purified isolates was used for PCR amplification of the 16S rRNA gene, and this reaction was carried out in a 25-μL volume with the following conditions: one cycle of 95 °C for 4 min; followed by 30 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 8 min. The PCR-amplified products were purified with the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) and then sequenced by the Sanger method at Sangon Biotechnology Co., Ltd., Shanghai, China. The generated sequences were aligned using BioEdit software version 7.0 and then subjected to analysis by the Basic Local Alignment Search Tool (BLAST) search program of the NCBI database (National Center for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the sequence homology with closely related organisms (Altschul et al., 1997). In present study, the microorganisms with high level of identity (97–100%) were selected as the closest match, and all bacterial isolates were respectively classified to the genus level according to the information of the closest microbes. In addition, the taxonomic database of NCBI was used to classify all the endophytic bacterial strains, including the level of phylum, class, order, and family. All 16S rRNA gene sequences obtained in this study were submitted to GenBank under the accession numbers MH768978-

MH769584 and MF375212 (Fang et al., 2018).

2.4. Endophytic bacterial community analysis

To understand the impacts of seasons and mulberry cultivars on the distribution of endophytic bacteria, 7 endophytic bacterial communities were constructed. Three communities were based on seasons: (1) winter, community isolated in the winters of 2015 and 2016; (2) spring, community isolated in the springs of 2015 and 2016; and (3) autumn, community isolated in the autumns of 2015 and 2016. In addition, four communities were based on mulberry cultivars: (1) CSQ, community isolated from cultivar CSQ; (2) CGS, community isolated from cultivar CGS; (3) XLJ, community isolated from cultivar XLJ; and (4) HGE, community isolated from cultivar HGE.

The diversity of different endophytic bacterial communities was compared at genus level using the following indices (Qadri et al., 2014): 1) Isolation frequency (IF), which was calculated as the number of isolates from a certain genus divided by the total number of isolates from all samples; 2) Simpson's diversity index (*D*), which was calculated as $D = 1 - \Sigma pi^2$, where $pi = Ni/N$, *Ni* was the number of isolates of the genus *i* and *N* was the total number of isolates from a certain community; 3) Shannon-Weaver diversity index (*H'*), which was calculated as $H' = -\Sigma pi\ (\mathbf{ln}\ pi)$; and 4) Pielou's evenness (*E*), which was calculated as $E = H'/Hmax$, where $Hmax = \mathbf{ln}\ (S)$, with *S* as the total number of genera in a certain community. In addition, Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were constructed for all classified genera (Liotti et al., 2018).

2.5. Screening of mulberry endophytic bacteria as potential biocontrol agents controlling mulberry fruit sclerotiniosis

The following procedure for screening the bioactive endophytes was summarized in Fig. S1 (Liotti et al., 2018; Passari et al., 2016).

2.5.1. Determination of antimicrobial activity of endophytic bacteria

S. sclerotiorum, the fungal pathogen that causes mulberry fruit sclerotiniosis, was used as a target to assay the biocontrol potential of all the endophytic isolates. Each purified endophytic bacteria was grown on a LB plate at 30 °C for 24 h and one colony for each strain was inoculated into LB medium and incubated at 30 °C for 96 h at 180 rpm. The cultures were then centrifuged at 10,000 × *g* for 30 min to discard the cells, and the cell-free supernatant was obtained by passing the supernatant through a 0.22-μm filtration membrane. The antagonistic activity of all the endophytic bacteria was qualitatively assayed by the well diffusion technique (Zouari et al., 2016). In brief, *S. sclerotiorum* agar discs (5.0 mm) were placed at the centers of PDA plates, and 100 μL of cell-free supernatant was added into wells that were 3.0 cm from the center. The plates were incubated at 25 °C for 5 days, and the diameters (Di) of the fungal inhibition zones were measured. Only freshly transferred cultures were used for all the tests, and all treatments were performed in triplicate.

To make antagonistic strains wider application in biological control of common plant diseases, the isolates that exhibited strong antagonistic activity against *S. sclerotiorum* in a qualitative bioassay were selected for further quantitative testing of their activity against *S. sclerotiorum* and two other fungal pathogens *Botrytis cinerea* and *Colletotrichum gloeosporioide*, of which the former can cause gray mold of multiple fruits (Jurick et al., 2017; Williamson et al., 2007) and the latter can infect a wide range of plant species by a specialized infection structure called appressorium (Huang et al., 2016; Priyatno et al., 2012). The antifungal bioactivities were evaluated on PDA medium by the dual culture technique (Passari et al., 2016). Briefly, a 5-mm pathogenic mycelial disc was placed in the center of a PDA plate, and then the tested bacterial culture in logarithmic phase (10^8 colony forming units per microliter, (CFU/mL)) was streaked on opposite sides of the same plate with 3.0 cm from the center and incubated at 25 °C for 5 days. Plates that were inoculated with a pathogen plug of the same size were tested in the absence of endophytic bacterial cells as a control. The inhibition rate (I) was calculated using the following formula: $I(\%) = [(C–T)/(C\text{-}C_0)] \times 100$, where C represented the growth diameter of the fungal pathogen in the control, T represented the growth diameter of the pathogen in the dual culture plate, and C_0 represented the diameter of the test fungal agar discs (5.0 mm). All experiments were carried out in triplicate.

2.5.2. PCR detection of genes related to antibiotic biosynthesis

Direct antagonism of phytopathogens is a key biocontrol mechanism for most BCAs, and this mechanism is dependent on efficient antibiotics production, mainly secondary metabolites with antimicrobial activity. Among these compounds, polyketides and lipopeptides were the two major representatives, especially nonribosomally synthesized cyclic lipopetides (surfactin, iturin, and fengycin) (Cawoy et al., 2015). To investigate the putative antifungal mechanism of the antagonists, the functional genes associated with biosynthesis of antimicrobial substances were determined by a PCR assay (Gond et al., 2015; Wang et al., 2016). Primers were synthesized according to sequences chosen from the coding regions of *PKSI* (polyketide synthase), *NRPS* (nonribosomal peptide synthetase), *sfp* (surfactin biosynthesis), *srfC* (surfactin synthase), *ItuD* (iturin A biosynthesis), and *FenD* (fengycin biosynthesis). The six primers used for amplification of the functional genes are listed in Table S1.

2.5.3. Determination of plant growth-promoting (PGP) traits of antagonists

The PGP traits of the antagonists, including phosphate solubilization, siderophore production, nitrogen fixation, and hydrolytic enzymes activity (cellulose, protease, and chitinase) were qualitatively determined by following standard procedures. A cell suspension of each antagonist was prepared as described above and 10 μL of each culture was spotted on different agar medium plates, respectively. Pikovskaya's (PVK) agar medium containing tricalcium phosphate ($Ca_3[PO_4]_2$) (Vyas et al., 2007), chrome azurol-s (CAS) agar medium (Jasim et al., 2013), and nitrogen-free (NFM) agar medium (Ben Abdallah et al., 2018) was used for evaluation of phosphate solubilization, siderophore production, and nitrogen fixation, respectively. The cellulose, protease, and chitinase activities was detected on carboxymethyl cellulose (CMC) agar medium containing 0.2% (*w/v*) Congo red, skim milk agar (SMA) medium, and half strength tryptic soya agar (TSA) medium supplemented with 0.6% (*w/v*) colloidal chitin, respectively (Afzal et al., 2017; Patagundi et al., 2014). These activities were qualitatively evaluated by the presence of a transparent zone or hydrolysis-induced halo around the bacterial colony after 7 days of incubation at 30 °C, whereas nitrogen fixation activity was indicated by positive growth on the medium after 2 days of incubation at 30 °C. All experiments were performed in triplicate.

2.5.4. Effects of the antagonistic endophytes on the growth of mulberry seedlings

The antagonistic isolates, exhibiting good antifungal ability and high PGP potential, were selected from the dominant groups of mulberry endophytic bacteria and subjected to further evaluation of their effects on mulberry seedling growth. Ten milliliters of bacterial culture, which was prepared in King's medium (Glickmann and Dessaux, 1995) and adjusted to a density of 1.0×10^7 CFU/mL with sterilized distilled water, was applied to each pot in which 25-day mulberry seedlings were planted. Seedlings inoculated with water served as a control. Five pots with three plants per pot were used in each treatment. After inoculation forty-five days, five seedlings were randomly selected from each treatment to measure parameters associated with plant growth, including the root and shoot length, and fresh weight of root and shoot (Xie et al., 2017).

Table 1
Diversity profile of the endophytic bacterial communities in mulberry.

	Seasons			Mulberry cultivars			
	winter	spring	autumn	CSQ	CGS	HGE	XLJ
Number of isolates	127	188	293	147	169	143	149
Number of genera	18	22	23	23	22	15	13
Shannon-Weaver (*H'*)	2.18	2.04	1.75	2.21	2.04	1.67	1.82
Simpson's index (*D*)	0.83	0.81	0.72	0.84	0.78	0.70	0.78
Pielou's evenness (*E*)	0.75	0.66	0.56	0.71	0.66	0.62	0.71

Diversity indices were calculated at the genus level. Winter, spring, and autumn represent the community isolated in the winters, springs, and autumns of 2015 and 2016, respectively; CSQ, CGS, HGE, and XLJ represent community isolated from Chuan Sang No. 7637, Changguo Sang, Hong Guo No. 2, and Xin Lunjiao, respectively.

2.5.5. Statistical analysis

Data for bacterial promoting plant growth were analyzed by a one-way analysis of variance (ANOVA) with a least significant difference (LSD) test, and the means were compared at a significance level of $P < 0.05$. The program SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Isolation of endophytic bacteria and bacterial community analysis

A total of 608 endophytic bacteria were isolated from all samples (127 from the winter community, 188 from the spring community, 293 from the autumn community, 147 from the CSQ community, 169 from the CGS community, 143 from the HGE community, and 149 from the XLJ community) (Table 1). Based on the 16S rRNA gene sequencing results, culturable bacterial isolates were divided into 36 genera, which were distributed into 4 phyla, 7 classes, 13 orders, and 20 families as shown in Table 2.

All of the isolates belonged to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, and the first three were the predominant phyla among the endophytic bacterial communities of mulberry (Table 2). Under the most abundant phylum Proteobacteria (382 of the 608 isolates, accounting for 62.83%), bacteria belonging to the classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria were detected, and a majority of the isolates were obtained from the class Gammaproteobacteria (337 of the 382 isolates, accounting for 88.22%). As the second most dominant bacterial phylum, Firmicutes only contained the class Bacilli (163 isolates), consisting of *Bacillus* spp. (158 of the 163 isolates, accounting for 96.93%) and *Paenibacillus* spp. (5 of the 163 isolates, accounting for 3.07%). Isolates from the phylum Actinobacteria (60 isolates), including representatives of *Curtobacterium* (50 of the 60 isolates, accounting for 83.33%), were also major bacterial groups, but only 3 isolates were from the phylum Bacteroidetes (Table 2). At the genus level, the most common bacterial genera in the collection were *Pantoea* (31.25% of 608 isolates), *Bacillus* (25.99% of 608 isolates), and *Pseudomonas* (13.32% of 608 isolates) (Table S2 and Table S3). Species of the genus *Curtobacterium* (8.22% of 608 isolates) have also been identified as frequently occurring endophytic bacteria in mulberry. Several genera, such as *Sphingobacterium*, *Klebsiella,* and *Streptomyces*, only contained one isolate (Table 2).

Table 2
Cumulative list of cultivable endophytic bacteria in mulberry and their taxonomic information.

Phyla	Classes	Orders	Families	Genera
Actinobacteria (60)	Actinobacteria	Micrococcales	Microbacteriaceae	*Curtobacterium* spp. (50)
				Frigoribacterium spp. (2)
				Microbacterium spp. (4)
				Schumannella sp. (1)
		Geodermatophilales	Geodermatophilaceae	*Geodermatophilus* sp. (1)
		Corynebacteriales	Nocardiaceae	*Rhodococcus* sp. (1)
		Streptomycetales	Streptomycetaceae	*Streptomyces* sp. (1)
Proteobacteria (382)	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium* spp. (5)
				Ensifer sp. (1)
				Rhizobium spp. (15)
			Methylobacteriaceae	*Methylobacterium* spp. (3)
			Brucellaceae	*Ochrobactrum* sp. (1)
		Sphingomonadales	Sphingomonadaceae	*Sphingomonas* spp. (11)
				Sphingobium sp. (1)
				Novosphingobium spp. (2)
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter* spp. (2)
			Comamonadaceae	*Comamonas* sp. (1)
				Delftia spp. (3)
	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Atlantibacter* spp. (19)
				Buttiauxella sp. (1)
				Cronobacter sp. (1)
				Enterobacter spp. (2)
				Klebsiella sp. (1)
				Kluyvera spp. (14)
				Leclercia spp. (2)
				Pantoea spp. (190)
			Erwiniaceae	*Erwinia* spp. (7)
			Yersiniaceae	*Rouxiella* sp. (1)
		Pseudomonadales	Moraxellaceae	*Acinetobacter* spp. (2)
			Pseudomonadaceae	*Pseudomonas* spp. (81)
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas* spp. (8)
				Xanthomonas spp. (8)
Bacteroidetes (3)	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium* sp. (1)
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium* spp. (2)
Firmicutes (163)	Bacilli	Bacillales	Bacillaceae	*Bacillus* spp. (158)
			Paenibacillaceae	*Paenibacillus* spp. (5)

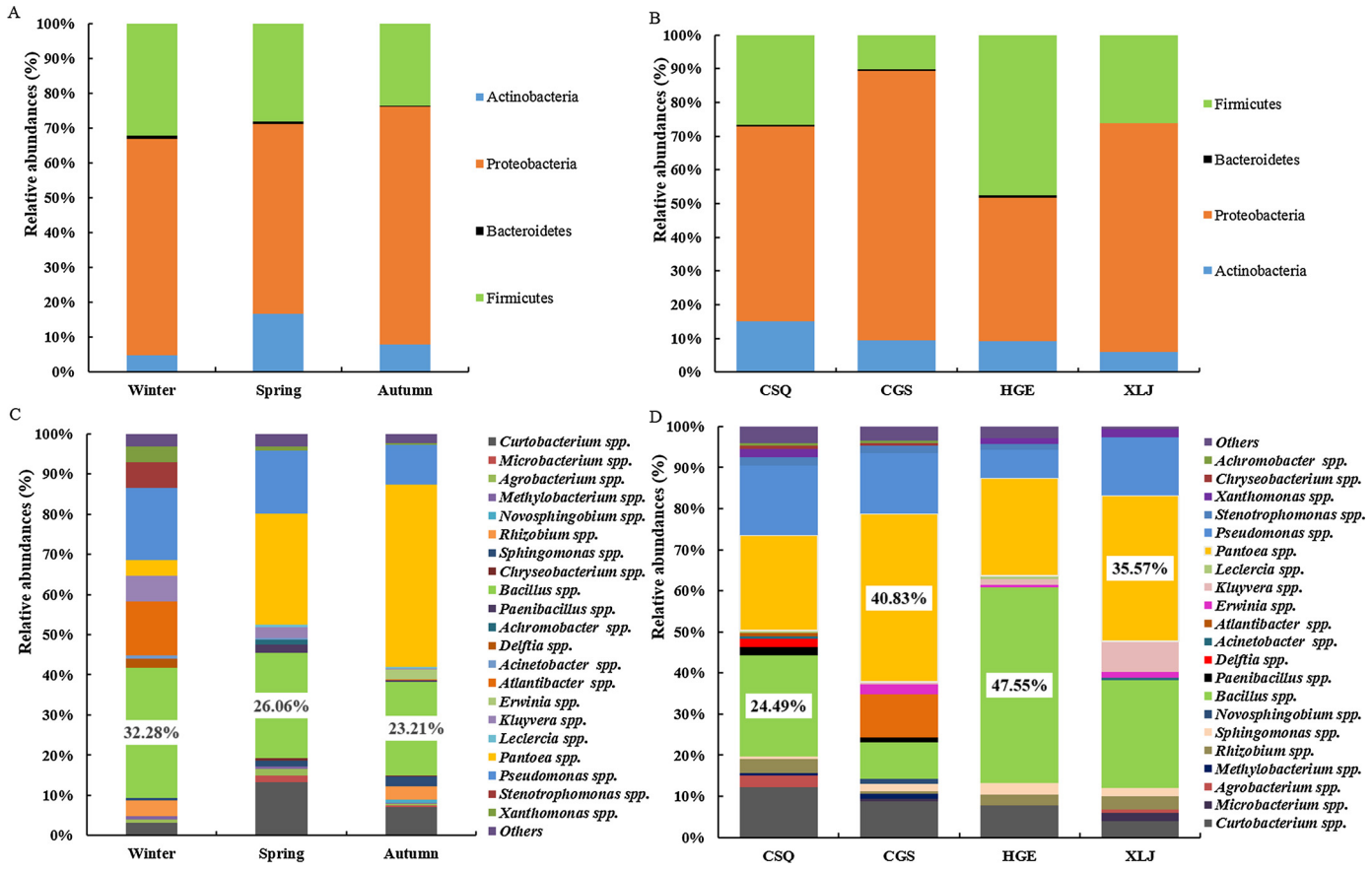

Fig. 1. Relative abundances (%) of cultivable endophytic bacteria in different communities isolated from mulberry at the phylum (A and B) and genus (C and D) levels. (A) and (C), communities isolated in different seasons, winter, spring, and autumn represent the community isolated in the winters, springs, and autumns of 2015 and 2016, respectively; (B) and (D), communities isolated from different mulberry cultivars, CSQ, CGS, HGE, and XLJ represent community isolated from Chuan Sang No. 7637, Changguo Sang, Hong Guo No. 2, and Xin Lunjiao, respectively.

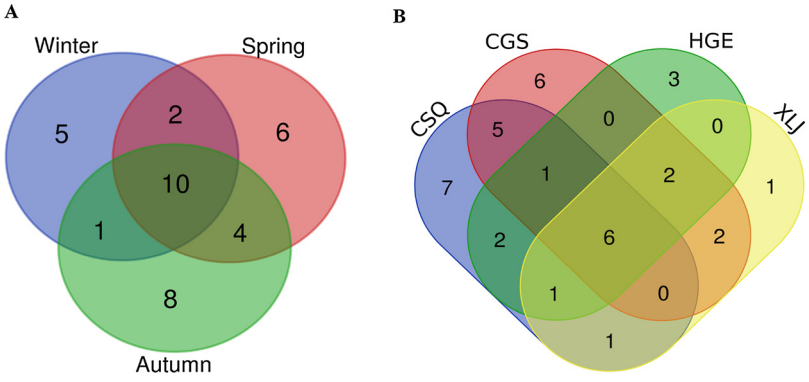

Fig. 2. Venn diagram of endophytic bacteria isolated from mulberry. (A) grouping by season, winter, spring, and autumn represent the community isolated in the winters, springs, and autumns of 2015 and 2016, respectively; (B) grouping by cultivar, CSQ, CGS, HGE, and XLJ represent community isolated from Chuan Sang No. 7637, Changguo Sang, Hong Guo No. 2, and Xin Lunjiao, respectively. Values represent the number of genera.

3.2. Composition and diversity of endophytic bacteria in different communities

Seasonal communities differed with respect to the richness of class and genus, although there was no obvious difference in bacterial community variations at the phylum level: the winter community harbored 4 phyla, 6 classes, and 18 genera; the spring community harbored 4 phyla, 6 classes, and 22 genera; and the autumn community harbored 4 phyla, 5 classes, and 23 genera (Fig. 1A, C and Table S2). Gammaproteobacteria was the dominant class for each seasonal community, accounting for 52.76, 48.94, and 60.75% of the total bacteria isolated from the winter, spring, and autumn community, respectively. However, within the class Gammaproteobacteria, seasonal variations resulted in a higher abundance of *Pseudomonas* spp. in the winter community (34.33% of 67 isolates), whereas a higher abundance of *Pantoea* spp. in the spring community (56.52% of 92 isolates) and autumn community (74.72% of 178 isolates). Furthermore, parts of isolates were seasonally specific: *Stenotrophomonas* spp., *Delftia* spp., and *Ensifer* sp. were only detected in the winter community; *Rhizobium* spp. were not included in the spring community; and *Paenibacillus* spp. occurred in the spring and autumn communities. Interestingly, noticeable changes were not observed in the seasonal endophytes of the genera *Bacillus* and *Curtobacterium*, especially *Bacillus* spp. with a generally stable relative abundance (surpasses 23% in each season) (Fig. 1C, Table S2).

Meanwhile, the structures of the bacterial communities in mulberry were also influenced by the plant cultivars (Fig. 1B and D). At the phylum level, Bacteroidetes occurred in all the mulberry cultivar

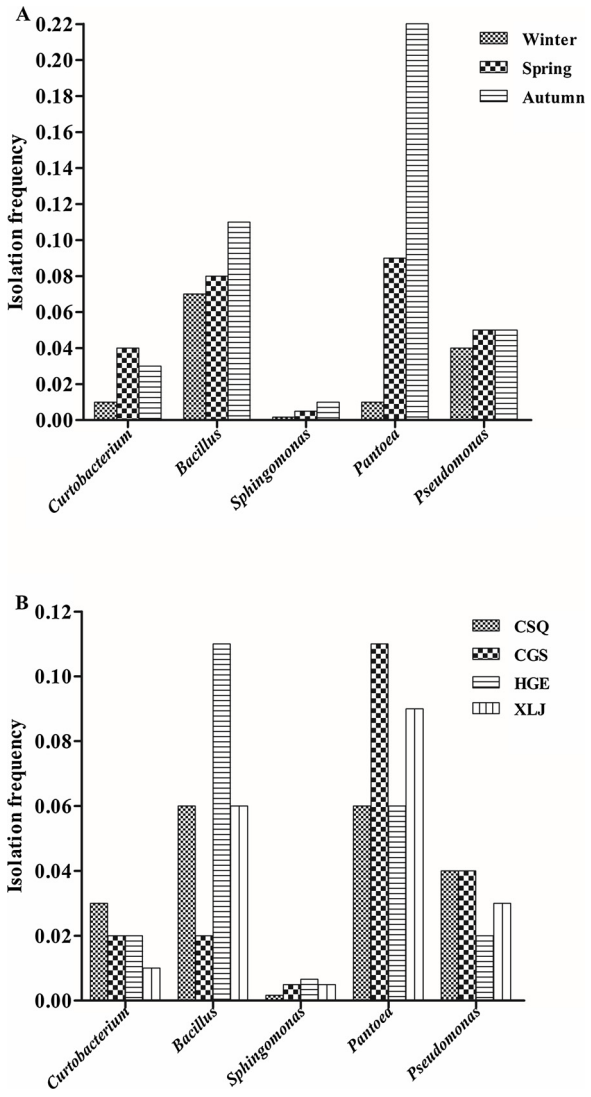

Fig. 3. Isolation frequency of the core mulberry endophytic bacteria in the communities isolated from different seasons (A) and different cultivars (B). Winter, spring, and autumn represent the community isolated in the winters, springs, and autumns of 2015 and 2016, respectively; CSQ, CGS, HGE, and XLJ represent community isolated from Chuan Sang No. 7637, Changguo Sang, Hong Guo No. 2, and Xin Lunjiao, respectively.

communities except the XLJ community, and the other three phyla (Actinobacteria, Firmicutes, and Proteobacteria) were detected in all mulberry cultivar communities (Fig. 1B). At the genus level, the number of genera in the communities of the resistant cultivars (22 genera in CGS and 23 genera in CSQ) was higher than that in the susceptible cultivars (15 genera in HGE and 13 genera in XLJ) (Table 1). Additionally, although the isolates belonging to the genera *Pantoea*, *Bacillus*, *Pseudomonas*, *Curtobacterium*, *Rhizobium,* and *Sphingomonas* were present in all the mulberry cultivars, but the highest relative abundance of *Bacillus* spp. were detected in the CSQ community (24.49%) and the HGE community (47.55%), respectively, whereas the highest relative abundance of *Pantoea* spp. were detected in the CGS community (40.83%) and the XLJ community (35.57%), respectively. Among the isolates with low abundance, *Agrobacterium* spp. were only detected in the CSQ and XLJ communities, *Kluyvera* spp. occurred in all the mulberry cultivar communities except the CSQ community, and *Paenibacillus* spp. were only observed in the CSQ and CGS communities (Fig. 1D and Table S3).

The common and unique bacterial genera in different communities are shown in the Venn diagram (Fig. 2). In all groupings, the number of shared genera (n = 10, Fig. 2A) among different seasonal communities was higher than that (n = 6, Fig. 2B) among different plant cultivar communities. Additionally, the number of shared genera was higher than the number of unique genera in a majority of the communities, except the CGS and CSQ communities. Among the shared genera, *Pantoea*, *Bacillus*, *Pseudomonas*, *Curtobacterium*, and *Sphingomonas* occurred in all communities, regardless of the season or cultivar community, suggesting that the bacteria of these five genera are the core members of mulberry endophytes (Fig. 3).

The biodiversity profiles of the 7 endophytic bacterial communities in mulberry were compared based on the diversity indices, including α-diversity indices (Shannon-Wiener index and Simpson's diversity index) and Pielou's evenness. For the seasonal communities, all the diversity indices for the winter community (*H′* 2.18, *D* 0.83, *E* 0.75) were highest compared with that of the spring community (*H′* 2.04, *D* 0.81, *E* 0.66) and autumn community (*H′* 1.75, *D* 0.72, *E* 0.56) (Table 1). The diversity of endophytic bacteria in mulberry markedly differed at different sampling time, suggesting that season was an important factor for endophytic bacterial community variation. Regarding the mulberry cultivar communities, the Shannon-Wiener diversity index in the resistant cultivar communities (*H′* 2.21 in CSQ and *H′* 2.04 in CGS) was higher than that in the susceptible cultivar communities (*H′* 1.67 in HGE and *H′* 1.82 in XLJ). Moreover, the Simpson's diversity index was highest in the CSQ community (*D* 0.84) and lowest in the HGE community (*D* 0.70). Pielou's evenness was mostly uniform in all the mulberry cultivars and ranged between 0.62 and 0.71 (Table 1). The above observations indicated that resistant mulberry cultivars (CGS and CSQ) harbored more diverse endophytes than susceptible cultivars (XLJ and HGE).

3.3. Screening of antagonistic endophytes

A total of 100 isolates (16.45% of 608) exhibited antimicrobial activity against *S. sclerotiorum*, as revealed by the presence of an inhibition zone (Fig. S2), and 68% of the antagonists belonged to the genus *Bacillus* (data not shown). Among the 100 antagonistic isolates, 33 strains showed stable and strong antifungal activity (Di > 1.0 cm). The quantitative assay results also indicated that these strains exhibited varying degrees of antagonistic potential against the three target pathogens, with inhibition rates ranging from 38.18% to 73.94% against *S. sclerotiorum*, 33.20% to 69.50% against *B. cinerea*, and 19.44% to 72.21% against *C. gloeosporioides* (Table 3 and Fig. S2). Seven *Bacillus* strains (CW16-5, XW15-6, XS16-10, XS16-5, XA15-9, XP-27, and HA15-19) efficiently antagonized *S. sclerotiorum*, and the inhibition rates were higher than 68%, of which the isolate CW16-5 presented the highest antagonism (73.94%). *Bacillus* sp. XP-27, *Pseudomonas* sp. XA15-33, and *Curtobacterium* sp. XA15-35 exhibited outstanding inhibitory activity against all the tested phytopathogens, with inhibition rates greater than 51%, although the antagonistic activity of isolate XA15-35 against *C. gloeosporioides* was slightly weak (40.61%) (Table 3).

The preliminary analysis results of antifungal mechanism showed that at least one antibiotic biosynthesis-related gene could be amplified in all 33 target strains, and all positive amplification products were consistent with the expected size (Wang et al., 2016; Gond et al., 2015). Fourteen antagonists (42.42% of 33) had the potential to produce polyketides, and all 26 antagonistic *Bacillus* strains were found to harbor genes involved in lipopeptides biosynthesis (Table 3). The strain *Bacillus* sp. CW16-5 with marked antagonistic activity, tested positive for the genes *PKSI*, *NRPS*, *sfp*, *srfC,* and *ItuD*, suggesting that the beneficial effects of CW16-5 might be due to the direct antagonism toward phytopathogens through the efficient production of polyketide and two families of lipopeptides (surfactin and iturin).

Table 3
Determination of antimicrobial activity and functional genes associated with antibiotic biosynthesis for 33 antagonists.

No.	Strain	Inhibition rate ± SD (%)			Functional genes					
		Ss [a]	*Bc* [a]	*Cg* [a]	*PKSI*	*NRPS*	*Sfp*	*srfC*	*ituD*	*FenD*
1	*Bacillus* sp. CW15-1	50.55 ± 2.62	34.82 ± 1.27	43.59 ± 1.41	+[b]	+	-[b]	+	+	+
2	*Bacillus* sp. CW15-2	57.15 ± 1.17	49.19 ± 2.23	29.03 ± 1.74	+	+	–	+	+	+
3	*Bacillus* sp. CW15-3	56.36 ± 0.55	48.81 ± 1.65	31.10 ± 3.73	+	+	–	+	+	+
4	*Bacillus* sp. CW15-4	50.97 ± 0.90	34.30 ± 1.26	36.31 ± 1.69	+	+	–	+	+	+
5	*Pantoea* sp. CA15-44	39.21 ± 0.92	49.32 ± 1.35	44.25 ± 1.65	–	+	+	–	–	–
6	*Pantoea* sp. CA15-43	39.64 ± 0.55	57.77 ± 1.32	42.85 ± 0.38	+	–	–	–	–	–
7	*Bacillus* sp. CW16-5	73.94 ± 0.82	43.39 ± 1.46	37.39 ± 1.65	+	+	+	+	+	–
8	*Pantoea* sp. CA15-30	38.18 ± 0.18	52.93 ± 0.59	38.88 ± 1.65	–	–	+	–	–	–
9	*Bacillus* sp. XW15-3	58.00 ± 0.36	43.26 ± 1.24	56.24 ± 2.58	+	+	–	+	+	+
10	*Bacillus* sp. XW15-6	70.67 ± 1.75	43.07 ± 0.73	50.79 ± 1.61	+	+	–	+	+	–
11	*Bacillus* sp. XS16-9	50.24 ± 1.17	35.10 ± 0.39	31.18 ± 3.72	+	+	+	+	–	+
12	*Bacillus* sp. XS16-10	71.64 ± 1.11	57.96 ± 1.37	51.03 ± 1.00	–	+	+	+	–	–
13	*Bacillus* sp. XS16-4	61.94 ± 0.64	50.55 ± 1.84	39.70 ± 3.02	+	+	+	+	–	+
14	*Bacillus* sp. XS16-5	68.97 ± 0.38	57.58 ± 1.13	68.98 ± 1.49	+	+	–	+	+	–
15	*Bacillus* sp. XA15-9	68.91 ± 0.36	48.48 ± 1.94	19.52 ± 4.09	–	+	+	+	+	–
16	*Bacillus* sp. XA15-10	62.18 ± 0.83	56.93 ± 1.18	21.92 ± 1.22	–	–	+	+	+	+
17	*Curtobacterium* sp. XA15-35	59.58 ± 1.94	55.64 ± 1.46	40.61 ± 2.25	–	–	+	+	+	–
18	*Pantoea* sp. XA15-46	56.48 ± 0.46	50.03 ± 2.07	26.14 ± 1.52	–	+	+	+	+	–
19	*Pseudomonas* sp. XA15-33	51.76 ± 1.18	52.10 ± 1.07	68.49 ± 2.21	+	+	–	–	–	–
20	*Bacillus* sp. XA15-11	64.61 ± 0.86	49.97 ± 1.24	56.82 ± 1.14	–	+	+	+	+	–
21	*Bacillus* sp. XP-27	69.94 ± 1.00	69.50 ± 1.10	72.21 ± 2.23	+	+	–	+	+	+
22	*Bacillus* sp. QW16-9	54.67 ± 1.91	59.77 ± 1.02	63.94 ± 1.83	–	+	+	+	+	–
23	*Bacillus* sp. QW16-12	54.67 ± 0.28	41.46 ± 0.87	59.39 ± 1.25	–	+	+	+	+	+
24	*Bacillus* sp. QW16-13	48.30 ± 1.11	61.64 ± 1.13	56.24 ± 1.17	–	+	+	–	+	–
25	*Rhizobium* sp. QW16-15	57.27 ± 1.01	43.33 ± 1.18	19.44 ± 1.41	–	+	+	+	+	+
26	*Bacillus* sp. QW16-16	47.21 ± 0.46	49.13 ± 2.86	58.15 ± 2.24	–	+	+	–	+	–
27	*Bacillus* sp. QW16-17	45.39 ± 0.47	46.42 ± 1.18	29.20 ± 2.11	–	+	+	+	+	+
28	*Bacillus* sp. HW16-12	59.45 ± 2.07	48.81 ± 1.46	57.65 ± 2.38	+	+	+	+	+	–
29	*Bacillus* sp. HA15-7	51.45 ± 1.11	35.98 ± 1.51	54.51 ± 5.23	–	+	+	+	–	–
30	*Bacillus* sp. HA15-19	73.03 ± 1.51	33.20 ± 0.40	46.48 ± 2.65	–	+	+	+	–	–
31	*Bacillus* sp. HA15-23	50.36 ± 0.96	54.93 ± 0.58	52.44 ± 0.76	–	+	+	–	+	+
32	*Bacillus* sp. HA15-6	52.18 ± 0.83	50.93 ± 2.23	37.14 ± 1.45	–	+	+	+	+	–
33	*Bacillus* sp. HA15-34	55.88 ± 1.34	57.83 ± 1.35	67.00 ± 2.27	–	–	+	–	–	–

Percentage of fungal growth inhibition compared to the growth obtained in control plates. Data represent the mean ± standard deviation (SD) from three replicates.
[a] (*Ss*) *Sclerotinia sclerotiorum*, (*Bc*) *Botrytis cinerea*, (*Cg*) *Colletotrichum gloeosporioide*.
[b] (–) negative, (+) positive.

3.4. PGP traits and plant growth promotion assay of antagonistic endophytes

Among the 33 antagonists with stable and strong antifungal activities, the isolates that tested positive for siderophore production up to 90.9%, followed by cellulase (78.8%), and protease production (72.7%) (Table 4 and Fig. S3). Twenty one isolates (63.6%) possessed nitrogen fixation activity, and phosphate solubilization was detected in 16 isolates (48.5%) (Table 4). In addition, the *Pantoea* spp. CA15-44 and CA15-30 presented distinct phosphate solubilization as well as both siderophore production and nitrogen fixation. The isolates *Bacillus* spp. QW16-12 and HW16-12 could produce siderophores, cellulase, and protease. This phenomenon revealed that mulberry endophytic antagonists also possess high PGP potential and might be good candidate strains as biofertilizers.

Eight strains with prominent antagonistic activity and high PGP potential, were selected from the dominant genera of endophytic bacteria, and their effects on the growth of mulberry seedlings were further evaluated in pot experiments (Fig. 4). The results showed that all the tested endophytes stimulated the growth of mulberry seedlings at different levels compared with the water-treated control, and the strain *Bacillus* sp. CW16-5 exhibited the highest PGP activities. When the seedlings were treated with CW16-5, all the growth parameters, including root length, shoot length, and fresh weight of root and shoot, were significantly ($P < 0.05$) higher than those of the water-treated control, especially with shoot length and root fresh weight increasing by 83.37% and 217.70%, respectively. In addition, isolates *Bacillus* sp. HW16-12 and *Pseudomonas* sp. XA15-33 also greatly facilitated mulberry seedling growth in comparison with the water-treated control, as the shoot length of these seedlings increased by 75.06% and 21.48%, respectively, and the root fresh weight of these seedlings increased by 158.06% and 103.88%, respectively. Furthermore, the strain *Pantoea* sp. CA15-44 increased both the root (43.64%) and shoot biomass (206.14%) (Fig. 4).

4. Discussion

Plants harbor an abundance of endophyte, and some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth and enhancement of disease resistance (Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Porras-Alfaro and Bayman, 2011). The genetic diversity of endophytic bacteria is determined by environmental conditions and host plant characteristics. The former include climate, temperature, moisture, and drought stress, while the latter include various plant properties, such as genotype, period of growth, and plant tissue (da Silva et al., 2014; Liotti et al., 2018; Mocali et al., 2003; van Overbeek and van Elsas, 2008). Elucidation of the diversity of endophytic bacteria is beneficial for understanding the function and potential role of these bioactive bacteria in the micro-ecosystems of host plants. Currently, culture-dependent methods and culture-independent approaches have been successfully used for bacterial community analysis in diverse environments (Shen and Fulthorpe, 2015; Mocali et al., 2003). With the development and implementation of next-generation sequencing (NGS) technologies, culture-independent methods based on 16S rDNA amplicon sequencing in the Illumina MiSeq and HiSeq systems have played an important role

Table 4
Characterization of antagonists for plant growth-promoting (PGP) traits and extracellular enzymes.

No.	Strain	PGP properties			Extracellular enzymes		
		P-solubilization	Siderophores	Nitrogenase	Celluase	Chitinase	Protease
1	*Bacillus* sp. CW15-1	–	+ + +	–	+ +	–	+ +
2	*Bacillus* sp. CW15-2	+	+ + +	+	+ +	–	+ +
3	*Bacillus* sp. CW15-3	+	+ + +	–	+ +	+	+
4	*Bacillus* sp. CW15-4	–	+ + +	–	+ + +	–	+ + +
5	*Pantoea* sp. CA15-44	+ + +	+	+	–	–	–
6	*Pantoea* sp. CA15-43	+ + +	+	+	–	–	–
7	*Bacillus* sp. CW16-5	+ + +	–	+	–	–	–
8	*Pantoea* sp. CA15-30	+ + +	+	+	–	–	–
9	*Bacillus* sp. XW15-3	–	+ + +	–	+ + +	–	+ +
10	*Bacillus* sp. XW15-6	+	+ + +	–	+ + +	–	+ +
11	*Bacillus* sp. XS16-9	–	+	–	+	–	+ +
12	*Bacillus* sp. XS16-10	–	+ +	–	+ +	+	+
13	*Bacillus* sp. XS16-4	+	+ +	+	+	+	+
14	*Bacillus* sp. XS16-5	–	+ + +	+	+ +	–	+
15	*Bacillus* sp. XA15-9	–	+ +	+	+	–	+ +
16	*Bacillus* sp. XA15-10	–	+ +	+	+ +	–	+ + +
17	*Curtobacterium* sp. XA15-35	+ + +	–	+	–	+	+
18	*Pantoea* sp. XA15-46	–	+ +	+	+	–	–
19	*Pseudomonas* sp. XA15-33	+	–	+	–	+	–
20	*Bacillus* sp. XA15-11	+ +	+	–	+	–	+
21	*Bacillus* sp. XP-27	–	+ + +	–	+	+	–
22	*Bacillus* sp. QW16-9	+	+	+	+	–	+
23	*Bacillus* sp. QW16-12	–	+ +	+	+ +	–	+
24	*Bacillus* sp. QW16-13	–	+ +	–	+	–	+ + +
25	*Rhizobium* sp. QW16-15	–	+ +	+	+ +	–	–
26	*Bacillus* sp. QW16-16	–	+ +	–	+ + +	–	+ + +
27	*Bacillus* sp. QW16-17	+	+	–	–	–	+
28	*Bacillus* sp. HW16-12	+	+ + +	+	+ +	–	+
29	*Bacillus* sp. HA15-7	+	+	+	+	–	+ +
30	*Bacillus* sp. HA15-19	–	+	+	+	–	+
31	*Bacillus* sp. HA15-23	–	+ +	+	+	+	–
32	*Bacillus* sp. HA15-6	+	+ +	+	+ +	–	+
33	*Bacillus* sp. HA15-34	–	+ +	+	+	–	+

For phosphate solubilization, + indicates diameters < 4 mm, + + indicates diameters of 4 ~ 8 mm, and + + + indicates diameters > 8 mm. For siderophore production, + indicates diameters < 2 mm, + + indicates diameters of 2 ~ 5 mm, and + + + indicates diameters > 5 mm. For extracellular enzyme activities (cellulase, chitinase, and protease), + indicates diameters < 5 mm, + + indicates diameters of 5 ~ 10 mm, and + + + indicates diameters > 10 mm. For nitrogenase activity, + indicates positive activity of the corresponding strain and – indicates no activity. Diameters represent the means of three replicates.

in the analysis of complex microbial communities, but most information regarding microbial diversity has been obtained using conventional cultivation techniques.

In the present study, we adopted a culture-dependent approach to analyze seasonal fluctuations in the cultivable endophytic bacterial communities isolated from mulberry stems. To better understand the relationship between microbial community and host cultivar, four healthy mulberry cultivars with different disease resistance properties were applied. A total of 608 bacterial isolates and 36 bacterial genera were collected from 24 mulberry samples (Table 2). In all analyzed samples, the genera *Pantoea*, *Bacillus*, and *Pseudomonas* were the major groups in the mulberry endophytic bacterial communities, and both the season and plant cultivar could affect the characteristics of these endophytic communities (Fig. 1 and Table 2). To the extent of our knowledge, this study is the first to characterize and compare the endophytic bacterial communities in different mulberry cultivars over a relatively long period. Although the diversity of endophytic bacteria was not investigated by the culture-independent technique, the culture-dependent method remains a valid tool for understanding the characteristics of cultivable microorganisms and further obtaining functional isolates with good bioactive capacities for use in plant protection areas.

Notably, *Bacillus* was an extremely common mulberry endophytic group in our study, regardless of season (Fig. 1A, C and Table S2) or cultivar communities (Fig. 1B, D and Table S3). This finding was consistent with observations for elm trees and other plants (Leifert et al., 1989; Mocali et al., 2003; Shen and Fulthorpe, 2015). The dominant status of *Bacillus* species throughout the two years might be due to their inherent capacity with production of stable endospores, which persist for long periods in a number of microenvironments associated with plants (Chen et al., 2014; Hu et al., 2014; Zhao et al., 2014). Additionally, it has been verified that different plant hosts can attract specific bacteria through the release of certain compounds via their roots, making these microorganisms successful endosphere colonizers (de Weert et al., 2002; Compant et al., 2005), and the *Bacillus* group with strong environmental adaptability is a typical colonizer of various crops (Compant et al., 2010; Liu et al., 2006). *Bacillus* strains were also detected in all the mulberry cultivars used in our experiments, in other words, the presence of this group was independent of the mulberry host genotype. The data from this study revealed *Bacillus* was the preponderant bacterial endophyte among all bacterial forms appearing in mulberry host, and it might be hinted in the importance of *Bacillus* genus for this plant genus. This speculation was consistent with the functional description of mulberry endophytic *B. subtilis* 7PJ-16 in our previous study, and strain 7PJ-16 was able to control mulberry fruit sclerotiniosis in the field and stimulate the mulberry seed germination as well as mulberry seedling growth under greenhouse conditions (Xu et al., 2019).

Compared with chemical control, biological control through the use of natural antagonistic microorganisms has emerged as a promising strategy due to the rare environment contaminants and low health risks. As many literatures have verified, the development of new BCAs against plant diseases requires the screening of high numbers of candidate antagonists (Du et al., 2017; Liotti et al., 2018). Among these

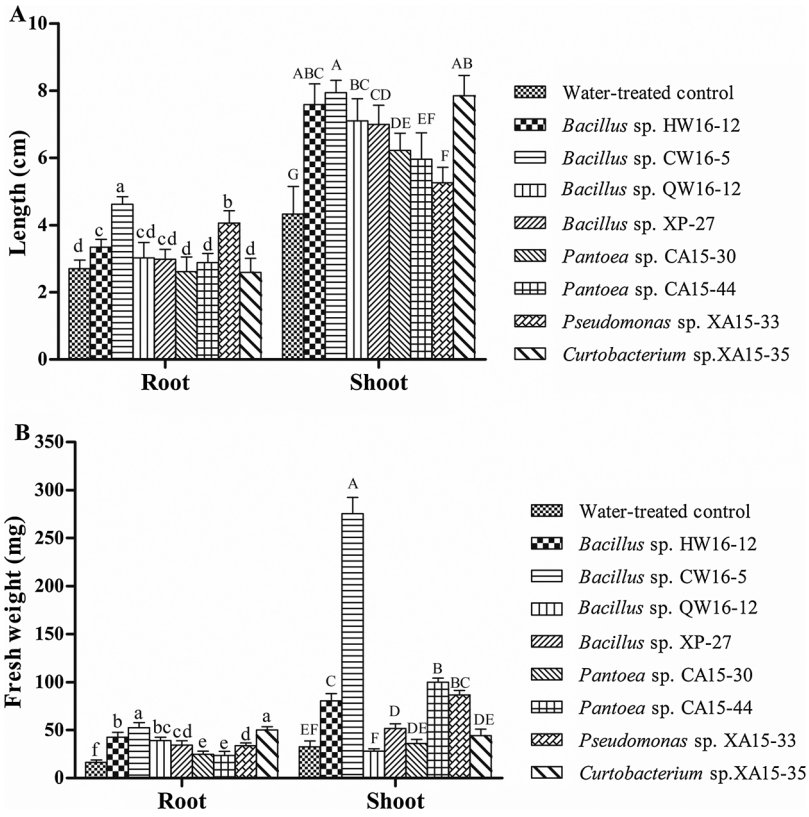

Fig. 4. Effects of the antagonists on the growth of mulberry seedlings under greenhouse conditions. (A) root and shoot lengths; (B) root and shoot fresh weights. Data are given as the mean ± SD of five replicates, and the different letters on the bars define groups of treatments that showed significant differences at the $P < 0.05$ level of confidence by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test.

antagonists, members of the genus *Bacillus* (Chen et al., 2014; Hu et al., 2014; Zhao et al., 2014; Sun et al., 2017a), *Pseudomonas* (Lee et al., 2017; Sun et al., 2017b; Wicaksono et al., 2018), and *Pantoea* (Xie et al., 2017) can be considered ideal candidates. In recent years, *Bacillus* spp. have received much attention because they are well-known antibiotic producers, and various *Bacillus* species have been found to control diverse phytopathogenic fungi and bacteria, such as *Fusarium graminearum* (Dunlap et al., 2013; Zhao et al., 2014), *S. sclerotiorum* (Chen et al., 2014; Hu et al., 2014; Sun et al., 2017a), and *Xanthomonas oryzae* (Lin et al., 2001). In this study, 100 out of 608 strains were selected from the mulberry endophytic bacterial community, of which 33 exhibited strong antagonistic activities against *S. sclerotiorum* (a pathogen that causes mulberry fruit sclerotiniosis). In addition, four strains of *Pantoea* spp. (CA15-30, CA15-43, CA15-44, and XA15-46), one strain of *Pseudomonas* spp. (XA15-33) and 26 isolates of *Bacillus* spp. also exhibited broad-spectrum antagonism toward different phytopathogens (Table 3). Using PCR assay, at least one functional gene associated with antibiotic biosynthesis was detected in each antagonist. A majority of the antagonists were positive for the presence of the lipopeptide genes, including *Sfp*, *srfC*, *ItuD*, and *FenD,* and these microorganisms mainly originated from the group of *Bacillus* spp. with excellent antagonistic effects. Our study demonstrated that the antifungal effect of antagonistic *Bacillus* strains was closely associated with lipopeptide biosynthesis. Similar results have been observed in numerous studies (Zhao et al., 2014; Gond et al., 2015; Zouari et al., 2016).

Apart from controlling diseases, endophytic bacteria have also attracted considerable attention for their capacity to promote plant growth via direct or indirect mechanisms: 1) direct PGP mechanisms include nitrogen fixation, siderophore biosynthesis, phosphate solubilization, and phytohormone production such as indol-3-acetic acid (IAA), the former three of which are associated with plant nutrient acquisition; and 2) indirect PGP mechanisms include antifungal compound production and bioactive enzyme secretion, such as cellulases, chitinases, proteases, and these hydrolytic enzymes can promote the lysis of fungal cell walls (Jasim et al., 2013; Reinhold-Hurek and Hurek, 2011; Santoyo et al., 2016). Our study concerning functional PGP traits and pot experiments found that many antagonists were positive for several enzymatic activities and PGP traits, and some greatly stimulated the development of mulberry seedlings (Table 4 and Fig. 4). In particular, *Bacillus* sp. CW16-5 showed the highest promotion activity in terms of all the growth parameters when compared with the water-treated control, including the root length, shoot length and fresh weight of the root and shoot. The roles and mechanisms of these mulberry endophytic bacteria with antifungal and PGP functions remain to be further characterized.

Additionally, bacteria of the genus *Curtobacterium*, a high G + C gram-positive group, is worth mentioning because they are also common endophytic inhabitants of mulberry (Fig. 3, Table S2, and Table S3). This observation was consistent with the presence of *Curtobacterium* species in many plants, including rice (Elbeltagy et al., 2000), citrus (Araújo et al., 2001), and prairie plants (Zinniel et al., 2002). Several reports have indicated that *Curtobacterium* isolates can function as biological control agents against many pathogens by inducing systemic resistance (Raupach and Kloepper, 1998; Lacava et al., 2007) or producing antibiosis (Sturz and Matheson, 1996). Also, Sturz et al. (1997) suggested that *Curtobacterium luteum* can stimulate intensive plant growth for red clover (*Trifolium pratense* L.) when applied individually or in mixtures with *Rhizobium* strains. In our work, both cell suspensions and cell-free filtrates of *Curtobacterium* sp. XA15-35 exhibited obvious antifungal activity toward *S. sclerotiorum* (Table 3), and the application of this strain had positive effects on the growth of mulberry seedlings (Fig. 4). The function of XA15-35 might be closely associated with the high phosphate solubilization as well as the production of hydrolysis enzymes (chitinase and protease) (Table 4). Therefore, *Curtobacterium* sp. XA15-35 might also be a potential biological resource for the biocontrol of mulberry fruit sclerotiniosis.

5. Conclusions

The present research elucidated the structures, diversity, and

functions of cultivable endophytic bacterial communities in mulberry. Overall, 608 isolates were obtained from all the mulberry samples, and these strains belonged to four bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes), including representatives of *Pantoea*, *Bacillus*, *Pseudomonas*, *Curtobacterium*, and *Sphingomonas*. Based on the comparative analysis of the bacterial communities, seasonal and mulberry cultivar differences could lead to the structural alteration of the endophytic bacterial communities, and it was noted that higher diversities of endophytic bacteria were found in the winter and also in the resistant cultivar communities. In addition, a total of 100 bacterial endophytes among all the isolates exhibited antagonistic activity against *S. sclerotiorum*, and 33 antagonists showed strong and stable activity. Furthermore, some antagonistic strains also possessed high PGP potential and 8 tested strains promoted mulberry seedling growth at different levels in the pot experiments. Our findings indicated that mulberry harbors rich endophytic bacteria and some isolates exhibit great antagonistic activity as well as PGP properties, which could facilitate the further application of these benificial endophytes as biocontrol and plant growth-promoting agents in plant protection areas.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126328.

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