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Screening and identification of antagonistic bacterial strains against *Botrytis cinerea* in *Panax ginseng*

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Endophytic bacterial strains were isolated from healthy mountain-cultivated ginseng leaves, and *Botrytis cinerea* was responsible for the fungus disease. Antagonistic strain was identified based on morphological characteristic and 16S rRNA sequence analysis. The results showed that 56 endophytic bacteria were isolated from healthy mountain-cultivated ginseng leaves, and FS-1 strain has good inhibitory effect on *B. cinerea*. The antifungal effects of fermentation broth of FS-1 strain on eight plant pathogenic fungi were obvious. The strain FS-1 has very good nitrogen fixation ability and it can produce extracellular protease. The strain FS-1 was identified as *Bacillus cereus* according to the morphological, physiological and biochemical characteristics, and 16S rRNA sequence analysis. This study suggests that FS-1 strain has certain potential bio-control, and could be useful to effectively control *B. cinerea* that causes grey mold disease in ginseng.

Key words: Endophytic bacteria, antifungal, 16S rRNA sequence, bio-control.

INTRODUCTION

Ginseng grey mould is caused by *Botrytis cinerea*, which is one of the main diseases of ginseng plant, and it can cause serious economic losses (Li and Li, 2010). Ginseng mould is a kind of soil-borne disease; it causes the mycelium of pathogens to winter in infected plants and soil. The conidia of pathogens are infected directly by wound or ginseng plants in the second year. Ginseng mould causes harm to ginseng stems, leaves, flowers, fruit and root and ginseng production (Wang et al., 2011).

At present, a variety of chemical pesticides are widely used to prevent and control ginseng grey mould, such as carbendazim, iprodione, triadimefon, thiazole, difenoconazole, and pyrametostrobin (Zhao et al., 2015, Zhao et al., 2016 and Liu et al., 2017). However, with the application of chemical pesticides, plant pathogens develop resistance, and cause severe threat to the safety of the environment (Saito et al., 2016). Therefore, the biological control of ginseng grey mould is the most ideal method (Shi et al., 2017). Sun and Wang (2016) found that *Bacillus amyloliquefaciens* SZ-35 has a good inhibitory effect on gray mold disease in ginseng. Wang Wang et al. (2016)'s study showed that *B. amyloliquefaciens* screened from healthy ginseng root has certain antagonism effect against gray mold disease in ginseng. Chang-qing Chen et al. (2018) cleared that NJ13 strain has a good inhibition effect against grey

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mould in ginseng. However, there are no research reports on the use of antagonistic strain to control grey mould in ginseng plant. This study screened antagonistic strains isolated from the leaves of *Panax ginseng*. The morphological, molecular identification and biological functions of the antagonistic strains were studied, which laid a theoretical foundation for their further use in the production and provision of new bio-control bacteria resources for preventing ginseng mould.

MATERIALS AND METHODS

Strains

B. cinerea, *Fusarium graminearum*, *Fusarium oxysporum*, *Cylindrocarpon destructans*, *Pestalotiopsis paeoniicola*, *Trichothecium roseum*, *Fusarium proliferatum*, *Fusarium semitectum*, and *Alternaria panax* were provided by the Plant Pathology Laboratory, Institute of Special Animal and Plant Sciences of CAAS, China. Each pathogen was re-cultured in a Petri dish containing potato dextrose agar (PDA) and incubated at 28°C for seven days in the dark. After this, each sample was transferred to a PDA slant in a test tube and stored at 4°C until use.

Screening of antagonistic strains

The bacteria strains isolated from 20 mL LB culture medium were transferred to 50 mL triangle bottle and cultured under 28°C, 220 $r \cdot min^{-1}$ condition. The bacteria fermented in 1 mL of sterile water were poured into a PDA plate containing the grey mould. The bacterial spores were blown and placed in a bacterial suspension (10^5 $spores \cdot mL^{-1}$). It (tablet form) was mixed with PDA medium in a Petri dish containing *B. cinerea*. The tablet was uniformly punched with injection. It was placed in 50 µL fermented liquid, at 28°C and cultured for 3 days based on the inhibitory effect of bacteriostatic ring size of the strain (Zhou, 2021) (Figure 1).

Morphological identification

The morphological characteristics of the antagonistic strains were observed after incubating them on BPA (The Reagent Company of Shiao, China) at 28°C for 3 days. They were identified using light microscopy according to the morphological characteristics of their color.

Analysis of 16 s rRNA sequence

The 16 s rRNA sequence was amplified using polymerase chain reaction (PCR); the PCR was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The amplified PCR products were cloned and sequenced according to the Pan's protocol, and nucleic acid was determined using NCBI BLAST (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was conducted using maximum likelihood in MEGA 5.10. The topology of the phylogenetic tree was evaluated by 1,000 re-samplings.

Bacteriostatic spectrum determination

The antagonist bacteria strains were inoculated in LB medium at 28°C, 220 $r \cdot min^{-1}$ for 24 h. They were fermented in liquid at 4°C, 10000 $r \cdot min^{-1}$ after centrifuging for 10 min. The fermented liquid was sterilized, using 0.45 µm microporous filtration membrane. The liquid was fermented at 1, 3, 5, 7 and 9% concentrations, and cooled to 40 ~ 45°C, respectively with melt blending. The PDA medium containing the tablet, and the PDA medium without sterilized and fermented liquid (control) developed 6 kinds of pathogenic fungi cakes (5 mm in diameter) for 7 days. The mycelium was placed upside down in the medium plate, at a constant temperature of 25°C and cultured for 7 days. The diameter of the colonies was measured using cross over method. The antibacterial rate of the pathogenic bacteria was calculated. Each of these pathogens was set at three repetitions.

Bacteriostatic rate (%) = (controlled colony diameter - processing colony diameter) /(controlled colony diameter - 5) by 100.

Determination of nitrogen fixation ability

The antagonistic bacteria were inoculated on Ashby's medium (Cui et al., 2016), liquid medium, sterile water (control) 3 times. They were cultured at a constant temperature of 28°C in an incubator, and liquid medium at 120 r/min. The culture was shaken after 7 days. The tablet on the colony and liquid medium is cloudy and positive.

Extracellular protease qualitative detection

The antagonistic bacteria strains were inoculated on casein medium at a constant temperature of 28°C in an incubator. They were cultivated for 48 h, and observed whether they produced a transparent circle.

RESULTS

Screening of antagonistic bacterial strains

A total of 56 endophytic bacteria strains were isolated through two rounds of leaf sample screening, according to the morphological characteristics of the colonies. Fifteen of these showed prominent antagonistic activities against *B. cinerea in vitro.* Strain FS-1 had 18 cm inhibition zone against *B. cinerea* in the dual-culture test (Table 1), which was the largest inhibitory activity among the isolates.

Morphological identification of antagonistic bacteria

The colonies of FS-1 strains were like candle. They had the shape and quality of a candle; no pigment, and their diameter was 5 mm (Figure 2A). They were placed on the BPA medium at a constant temperature of 37°C, for 24 h and then they were cultured. The FS-1 strains had rod shape with size of 0. 5 to 0.8 µm × 1.0 to 1.5 µm. They were Gram positive (Figure 2B). FS - 1 strains needed oxygen to grow at temperature ranges between 30 and 38°C, pH 5.6 - 7.2, maltose, sucrose and salicin, enzyme, determination of V - P, oxidation of glucose fermentation, gelatin liquefaction, nitrate reduction, malonic acid, urea enzyme, hydrogen sulfide, egg yolk lecithin enzyme,

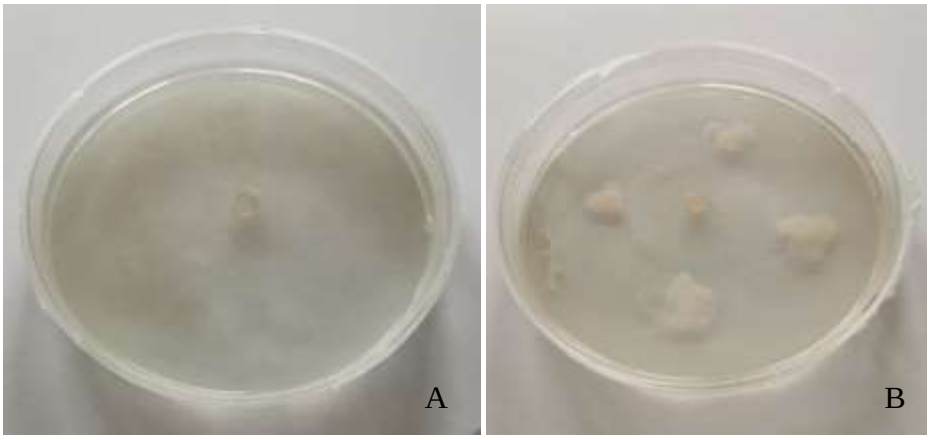

Figure 1. Antagonistic effects of strain FS-1 against *Botrytis cinerea.* A: *Botrytis cinerea* on PDA medium B: Confrontation culture.
Source: Zhou et al. (2021)

Table 1. Physiological and biochemical characteristics of strain FS-1

Item	Result
Motility	-
Lipase	-
Gelatin hydrolysis	+
Nitrate reduction	+
Methyl red	-
Lipase	-
Hydrogen sulfide	+
Lecithinase	+
Maltose	+
Sucrose	+
Salicin	+
Voges-Proskauer test	+
Urease	-
Starch hydrolysis	-
Citrate utilization	-
Malonate utilization	+
Catalase	+
Oxidation-fermentation of glucose	+

Source: Wang et al. (2011)

motility, hydrolyzed starch, citrate utilization, and methyl red (Table 1). The strain FS - 1 was identified as *Bacillus* spp. according to the morphological characteristics combined with the physiological and biochemical test results.

Analysis of 16S rRNA sequence

PCR products of 1472-bp were obtained by amplifying the 16S rRNA of the genomic RNA of FS-1 strain. Sequence analysis showed that FS-1 strain shared 100% identity with a number of *B. cereus* in the NCBI database (Accession No. MN746190). Phylogenetic tree was constructed by using 16S rRNA sequences, and it clearly showed that FS-1 strain was clustered with members of the genus *Bacillus* (Figure 3). FS-1 strain was identified as *B. cereus* based on the results of the 16S rRNA sequence analysis and the morphological characterization.

Bacteriostatic spectrum

The bacteriostatic spectrum test results showed that different concentrations of liquid fermented FS-1 strains (8 kinds of plant pathogenic fungi) have certain inhibitory effect. Their bacteriostatic spectrum ranges changed with the fermented liquid concentration; the inhibition rate of the pathogens changed. When the fermented liquid concentration was 9%, the bacteriostatic rate of *T. roseum* was highest; at 91.47%, the bacteriostatic rate of *F. oxysporum* was the lowest; at 47.91%, the bacteriostatic rate of the other 6 kinds of pathogens was between 49.26 and 90.02% (Table 2).

Nitrogen fixation ability

The colony of FS-1 strain formed was inoculated on the solid Ashby's medium, at a constant temperature of 28°C. It was placed in an incubator for 3 days. The FS-1 strain was inoculated on the liquid Ashby's medium at a constant temperature of 28°C and cultured in an incubator for 5 days. It became turbid. The experimental results showed that FS-1 strain had ability to fix nitrogen.

Extracellular protease

FS-1 strain can form a transparent circle around the

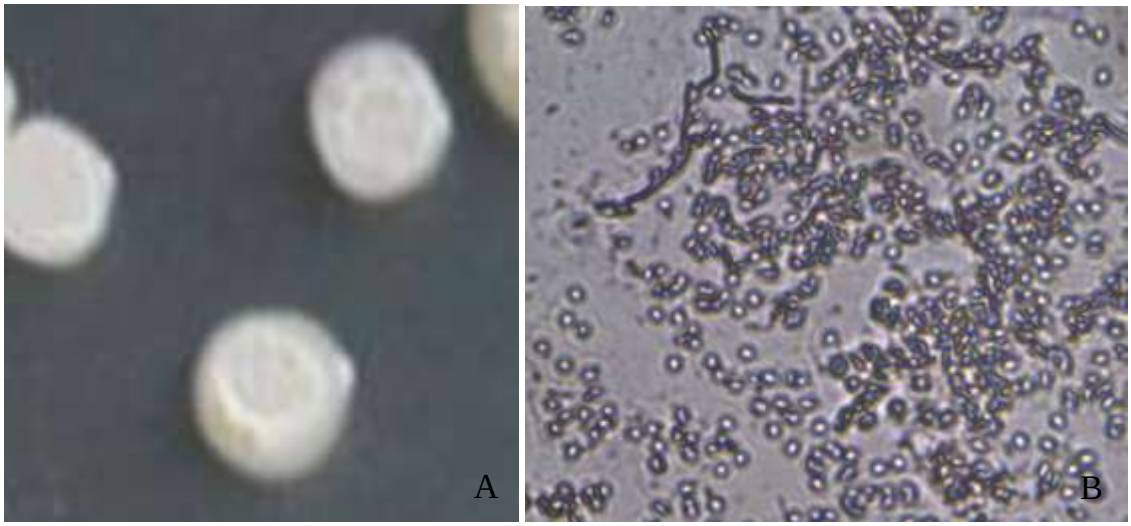

Figure 2. Antagonistic effects of strain FS-1 against *Botrytis cinerea*. A: Morphological characteristics of strain FS-1 on BPA culture medium B: Morphological characteristics of strain FS-1 under electron microscope.
Source: Authors

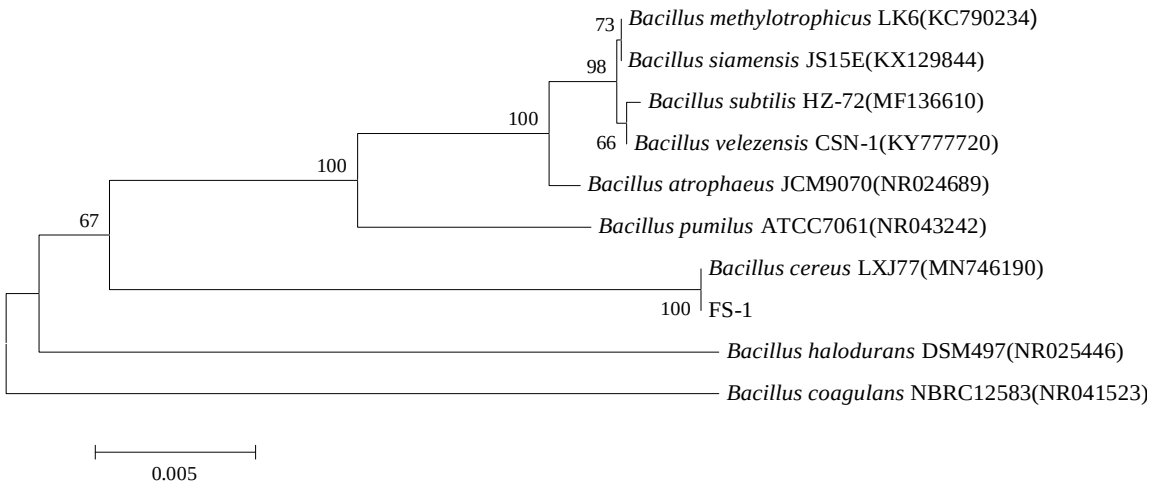

Figure 3. Phylogenetic tree of strain FS-1 based on 16S rRNA.
Source: Zhou et al. (2021)

Table 2. Inhibition effects of fermentation broth of FS-1 strain on plant pathogenic fungi.

Pathogen	Inhibition rate of different concentration (%)				
	1	3	5	7	9
F. graminearum	5.67±3.12^{ab}	7.15±1.08^{c}	28.17±1.43^{d}	47.37±3.94	50.02±2.97^{d}
F. oxysporum	1.82±0.53^{ab}	24.68±5.12^{abc}	27.79±4.16^{d}	37.89±5.24^{d}	47.91±2.98^{d}
C. destructans	5.24±4.01^{ab}	11.69±9.14^{c}	34.64±8.52^{cd}	44.87±3.24^{d}	49.26±6.12^{d}
P. paeoniicola	0.51±1.02^{b}	6.15±7.24^{c}	52.53±9.84^{bc}	90.13±5.91^{a}	90.02±5.36^{a}
T. roseum	12.15±16.47^{a}	3.89±2.98^{c}	78.17±5.12^{a}	90.25±2.46^{a}	91.47±3.28^{a}
F. proliferatum	1.34±0.97^{ab}	4.23±2.89^{c}	2.94±1.53^{d}	72.14±4.02^{bc}	79.52±1.05^{ab}
F. semitectum	3.97±3.58^{ab}	51.59±8.13^{a}	63.27±16.48^{ab}	67.49±4.13^{bc}	76.14±5.94^{b}
A. panax	6.93±6.52^{ab}	41.46±11.57^{ab}	73.25±7.98^{a}	80.23±3.12^{ab}	80.59±3.12^{ab}

Source: Zhou et al. (2021)

Figure 4. Production of extracellular proteases in agar plates by FS-1 strain.
Source: Zhou et al. (2021)

colonies (Figure 4) inoculated on the casein medium plate at a constant temperature of 28°C in an incubator for 2 days. Its diameter was 18.53 mm. Extracellular protease qualitative test results showed that FS-1 strain can produce extracellular protease.

DISCUSSION

At present, these chemical pesticides were used to prevent and control ginseng gray mold disease such as difenoconazole, propiconazole, mancozeb, carbendazim, kresoxim-methyl, cyprodinil, azoxystrobin, polyoxins, fluazinam, hymexazol, copper hydroxide, etc. But studies have shown that with long-term use of chemical pesticides, pathogens will develop resistance (Saito et al., 2016). Therefore, there is need to seek new methods to prevent and control ginseng grey mould to slow down the drug resistance of pathogenic bacteria. Endophytic bacteria in plant body can form stable colonization for long, and they are not susceptible to the influence of external environment. Endogenous bacteria and pathogens have mutual reciprocity and benefit; they are a kind of important resources (Aly et al., 2011). It has been reported that *B. amyloliquefaciens*, *Bacillus methylotrophicus, Paenibacillus polymyxa*, and *Enterobacter cloacae* can be used to prevent and control ginseng disease (Wang et a., 2016, Jiang et al., 2013, Li et al., 2013 and Jiang et al., 2019), but it has not been reported that *B. cereus* can be used to prevent and treat ginseng grey mould.

Endophytic bacillus is a kind of non-pathogenic bacteria that can produce spores, a variety of antibacterial substances, such as antibiotics and antimicrobial proteins. Bacillus has strong resistance, reproduces fast, can survive in an environment, forms colonization fast, is an important resource of bio-control bacteria, has wide application in the prevention and control of green plant disease. In this study, a strain isolated from the leaves of *P. ginseng* was found to have very good inhibitory effect against ginseng gray mould bacterium. The endophytic bacterium identified is *B. cereus* strain; its bacteriostatic spectrum is more extensive. FS - 1 to 8 kinds of common plant pathogenic fungi are highly bacteriostasis. Zhou et al. (2017)'s studies showed that *B. cereus* metabolites can effectively restrain the growth of the pathogen of cotton yellow dwarf. *B. cereus* is a kind of bio-control bacterium. This study found that FS - 1 strain can fix nitrogen in plants. Extracellular protease test results showed that the strain can produce extracellular protease, and degrade the cell wall of enzymes. This has laid a foundation for its further use and application in the production of new bio-control bacteria resources for preventing ginseng mould.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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