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Identification of antagonistic bacterium strain and biocontrol effects on ginseng root rot disease

Chunyuan Zhou[1,2], Chengci Piao[3] and Hao Zhang[1,2]*

[1]Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun 130112, China.
[2]Jilin Provincial Key Laboratory of Traditional Chinese Medicinal Materials Cultivation and Propagation, Changchun 130112, China.
[3]Institute of Ji An Ginseng Research, Ji An 134200, China.

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From healthy mountain-cultivated ginseng leaves, 45 endophytic bacteria strains were isolated. Antagonistic bacterium strain FS17 was screened with the confront culture method, and identified as *Bacillus subtilis* based on morphological characteristic and 16S rRNA sequence analysis. Growth rate method was used to determine that the fermentation broth of FS17 had inhibitory effect on 10 plant pathogenic fungi, and the inhibition rate ranged from 46.83 to 93.25%. The fermentation broth of strain FS17 with lower disease incidence and lesion diameter had better control effects with no significant difference compared with that of carbendazim. This study suggests that strain FS17 showed strong inhibition effect and wide antagonistic spectrum, and could be useful to effectively control *Cylindrocarpon destructans* causing ginseng root rot.

Key words: Ginseng root rot, antagonistic bacterium, *Bacillus subtilis*, biocontrol.

INTRODUCTION

Ginseng root rot is caused by *Cylindrocarpon destructans*, this disease is a destructive disease in the process of ginseng planting, the mortality rate of more than half (Li et al., 2022). Ginseng root rot is a kind of soil-borne diseases, severely impacts the quality of ginseng. Most infections occur during the rainy and winter season, because these seasons are highly ideal for fungal mycelial growth, which leads to spore invasion of the plants with initial indications of leaf loss and decreased root development in the plant (Kim et al., 2017). Ginseng seedlings is invaded, which symptoms are not obvious in the early stage, then the leaves turn yellow slowly in the late, finally lead to wilt, which take is greatly harmful to the ginseng production (Park et al., 2017).

At present, a variety of chemical pesticide such as carbendazim, thiophanate-methyl, fludioxonil, myclobutanil, hymexazol and pyrametostrobin and so on are widely used in the prevention and control of ginseng root rot (Sun et al., 2020). However, with the application of chemical pesticides, plant pathogens will develop resistance, and cause severe threat to the environment security (Cameron and Sarojini, 2014). Therefore, the biological control of ginseng root rot is the most ideal method. Gou et al. (2015) screened *Bacillus amyloliquefaciens* HB - 3 from ginseng rhizosphere soil; it can significantly inhibit the growth of ginseng root rot

*Corresponding author. E-mail: zhanghao@163.com.

fungus; Wang et al. (2019) understand that *B. amyloliquefaciens* FS6 can stable colonize ginseng plant body and conduction, and can to a certain extent induced disease resistance of ginseng. The endophytic bacteria mainly live in plant tissues, they can be colonized long-term in the plant body, and it is not susceptible to the influence of the external environment condition. Endophytic bacteria and pathogens is the relationship of mutual benefit, which is a kind of important bio-control resources (Kim et al., 2020). This study was based on isolating endophytic bacteria from the leaves of *Panax ginseng* and screening antagonistic bacteria strains against ginseng root rot. Strain morphological and molecular identification were completed. The strain FS17 was identified as *Bacillus subtilis,* which had the very good biological control effect on ginseng root rot disease.

MATERIALS AND METHODS

Strains

Cylindrocarpon destructans, Fusarium solani, Fusarium graminearum, Fusarium semitectum, Pestalotiopsis paeoniicola, Trichothecium roseum, Fusarium proliferatum, Fusarium oxysporum, Alternaria panax, Fusarium equiseti and *Cladosporium cladosporioides*, were obtained from the plant pathology lab, Institute of Special Animal and Plant Sciences of CAAS, China. Each pathogen was recultured in a Petri dish containing potato dextrose agar (PDA) and incubated at 25°C for 5 day stored at 4°C until use.

Screening of antagonistic bacteria

The antagonist activity of bacterial isolates was tested against *C. destructans*, using the dual culture technique described by Zhou et al. (2021). Bacterial strains were streaked at the edges of Petri plates containing Potato Dextrose Agar medium and, after 48 h of incubation at 28°C, a 6mm mycelial plug of pathogenic fungus was placed in the centre of each plate. The plates were then incubated at 28°C for 5 days. All experiments were performed in triplicate and repeated three times based on the inhibitory effect of bacteriostatic ring size screening of strain (Zhou et al., 2021).

Morphological identification

The morphological characteristics of antagonistic strain were observed after incubation on BPA (The reagent company of Shiao, China) at 28°C for 3 day, and identified using light microscopy according to the morphological characteristics.

16 s rRNA sequence analysis

Using 50 µL amplification reaction system: Taq mixture 25 µL, universal primers 27F(5′-AGAGTTTGATCCTGGCTCAG-3′) 2 µL and 1492R(5′-GGTTACCTTGTTACGACTT-3′) 2 µL, DNA template 2 2 µL, ddH_2O 19 2 µL. Amplification conditions for: 95°C in advance 4 min 55°C 94°C modified 30 s, annealing 45 s, 72°C 1 min, 30 cycle; finally extended under 72°C 8 min, eventually check response (Kim et al., 2019). Amplified PCR products were cloned and sequenced according to the Pan's protocol, and nucleic acid identified was determined using NCBI BLAST (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was conducted using maximum likelihood in MEGA 5.10 and then the topology of the phylogenetic tree was evaluated by 1,000 resamplings.

Bacteriostatic spectrum determination of strain FS17

The antagonist bacteria strains were inoculated in LB medium under the condition of 28°C, 220 r·min^{-1} for 24 h. Liquid was fermented at 4°C, 10000 r·min^{-1} after centrifugation for 10 min, using 0.45 µm membrane filtration. Sterile fermentation liquor made from fermented liquid of 1, 3, 5, 7 and 9% concentrations was cooled to 40 to 45°C, respectively with melt blending, PDA medium in tablet, with PDA medium without sterile fermentation liquor as a control. Six kinds of pathogenic fungi cake (5 mm in diameter), was developed in 7 day in the middle plate at 25°C, and mycelium under the condition of constant temperature was cultured for 7 day. Computation formula of inhibition rate is as follows:

Inhibition rate (%) = (controlled colony diameter - processing colony diameter) / (controlled colony diameter - 5) ×100.

Control effect of strain FS17 against *F. solani*

Health ginseng roots were washed with tap water, reoccupy 2% NaClO soaking treatment 3 min, and then to dry after washing three times with sterile water. The wound (size 2 × 2 mm) was made using sterilized needle on the root surface. The roots were inoculated with 20 µL strain FS17 fermented liquid (10^8 CFU/mL). After 4 h, the spore suspension of *C. destructans* were shaken on the roots, with water as a control, carbendazim (1000 µg/mL) as the reagent control, each dealing with a repeat 3 times. At 25°C, relative humidity of 95% in the constant temperature incubator, respectively in the 3 and 5 days after statistical disease incidence, and to measure disease spot diameter (Wang et al., 2017).

Data statistics and analysis

Data were compiled using EXCEL (Microsoft). At least three independent experiments were performed in each case. The values were represented as mean ± SD of three replicates for each treatment. Statistical significance was analysed with Student's *t*-test and analysis of variance followed by Tukey's post hoc test (SPSS v. 13.0; SPSS Inc).

RESULTS

Screening of antagonistic bacteria strains

A total of 45 endophytic bacteria strains were isolated from the leaves of forest ginseng. Eight of these showed prominent antagonistic activities against *C. destructans in vitro.* Among them, 17 strains had bacteriostasis effect of ginseng root rot; strain FS-17 had an 21 mm of inhibition zone against *C. destructans* in the dual-culture test (Figure 1).

Morphological identification of antagonistic bacteria

The colonies of FS-17 strain was white, rough surface

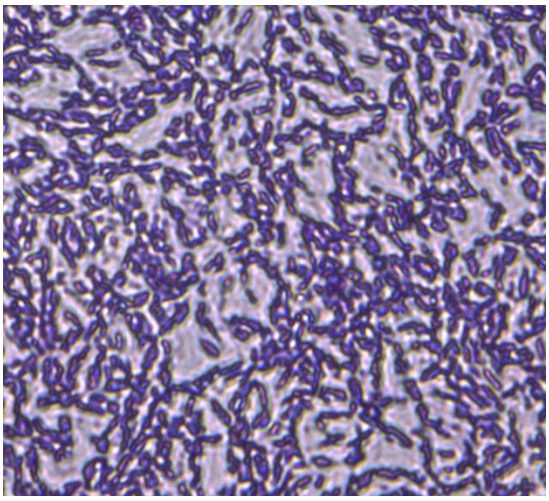

Figure 1. Morphological characteristics of strain FS17. A: Morphological characteristics of strain 17 on BPA culture medium B: Morphological characteristics of strain 17 under electron microscope.

Table 1. Physiological and biochemical characteristics of strain FS17.

Item	Result
Arabinose	-
Gelatin hydrolysis	+
Nitrate reduction	+
Methyl red	+
Glycerinum	+
Benzpyrole	+
氧化酶试剂	+
Fructose	+
Sucrose	+
Galactose	-
Voges-Proskauer test	+
Dulcitol	-
Mannitol	-
Citrate utilization	+
Malonate utilization	+
Catalase	+
Glucose	+

was opaque on BPA medium, 24 h, 37°C constant temperature culture. The FS-17 strain was rod, produced spores, born in or close to, size 0.7- 0.8 μm×2.0-2. 5 μm, Gram positive reaction (Figure 1B). The physiological and biochemical characters of FS-17 strain are shown in Table 1. A preliminary test showed that the strain FS-17 was identified as *Bacillus* species according to morphological characteristics combined with the physiological and biochemical test results.

Molecular identification of antagonistic bacteria

PCR products of 1500 bp were obtained from amplification of the 16S rRNA of the genomic RNA of strain FS-17. Sequence analysis showed that strain FS-

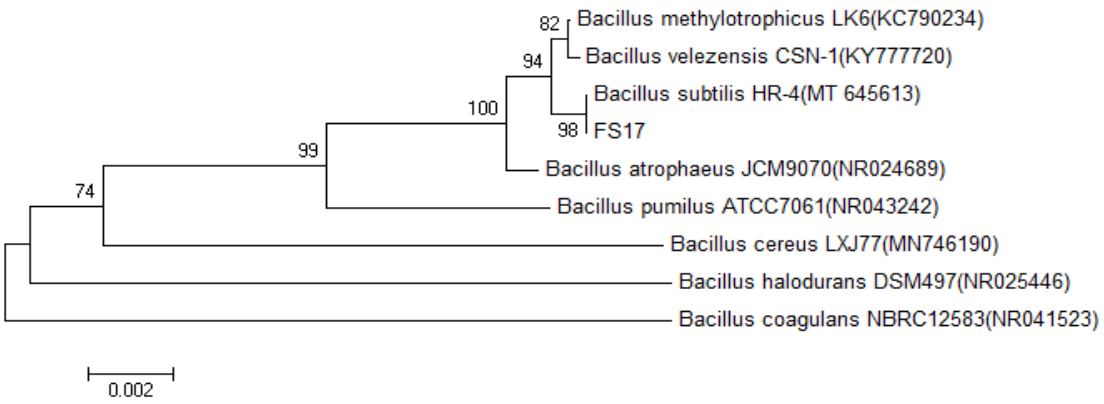

Figure 2. Phylogenetic tree of strain FS17 based on the 16S rRNA.

Table 2. Inhibition effects of fermentation broth from strain FS17 on plant pathogenic fungi.

Pathogen	Inhibition rate of different concentration (%)				
	1%	3%	5%	7%	9%
F. graminearum	5.17±3.12^{ab}	7.35±1.08^{c}	26.17±1.43^{d}	45.37±3.94	48.02±2.97^{d}
F. semitectum	3.82±0.53^{ab}	22.17±5.12^{abc}	25.79±3.16^{d}	35.24±5.24^{d}	46.83±2.58^{d}
F. solani	5.74±4.01^{ab}	11.32±9.14^{c}	34.51±8.53^{cd}	44.15±3.64^{d}	49.48±6.62^{d}
P. paeoniicola	0.61±1.02^{b}	6.35±7.61^{c}	52.42±9.36^{bc}	90.27±5.37^{a}	90.27±5.58^{a}
T. roseum	12.37±16.22^{a}	33.72±2.28^{c}	78.47±5.27^{a}	90.58±2.37^{a}	91.55±3.82^{a}
F. proliferatum	1.73±0.97^{ab}	4.58±2.21^{c}	2.94±1.53^{d}	72.14±4.02^{bc}	79.52±1.05^{ab}
F. oxysporum	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}
F.equiseti	7.35±2.38^{ab}	12.97±2.47^{ab}	25.53±1.58^{c}	46.42±3.19^{d}	56.48±2.58^{d}
C. cladosporioides	13.27±1.49^{a}	43.84±2.91^{c}	80.32±1.74^{a}	91.23±2.41^{a}	93.25±3.04^{a}

17 shared 100% identity with a number of *B. subtilis* in the NCBI database (Accession No. MT645613). Phylogenetic tree was constructed by using 16S rRNA sequences, and it clearly showed that strain FS-17 is clustered with members of the genus *Bacillus* (Figure 2). Strain FS-17 was identified as *B. subtilis* based on the results of the 16S rRNA sequence analysis and the morphological characterization.

Bacteriostatic spectrum determination of strain FS17

Bacteriostatic spectrum test results showed that different concentrations of strains fermented liquid of FS-17 of 10 kinds of plant pathogenic fungi have certain inhibitory effect. Its bacteriostatic spectrum ranges with the change of the fermented liquid concentration, the inhibition rate of pathogens was changing. Among them, when the fermented liquid concentration was 9%, bacteriostatic rate of *F. solani* was highest, at 93.25%, bacteriostatic rate of *F. semitectum* was the lowest, at 46.83%, bacteriostatic rate of the other 8 kinds of pathogens was between 48.02 and 91.55% (Table 2).

Control effect of strain FS17 against *C. destructans*

The test results showed that the incidence rate and disease spot diameter of strain FS17 fermented liquid and carbendazim were significantly than water control. The control effect of strain FS17 fermented liquid against *C. destructans* was 40.15 and 65.18%, respectively after dealing with 3 and 5 days. And carbendazim processing control effect was 48.32 and 67.29%, respectively. The control effect of strain FS17 fermented liquid against *C. destructans* was slightly lower than contrast agents carbendazim (Table 3).

DISCUSSION

At present, carbendazim, thiophanate-methyl, fludioxonil, myclobutanil, hymexazol, pyrametostrobin, etc., chemical pesticides were used to prevent and control ginseng root

Table 3. Control effects of strain FS17 on ginseng root rot.

Treatment	3 days		5 days	
	Colony diameter (mm)	Control effect (%)	Colony Diameter (mm)	Control effect (%)
Water	5.21±0.43[a]	-	11.63±1.43[a]	-
Strain FS17	3.48±0.29[b]	40.15[a]	4.02±0.61[b]	65.18[a]
Carbendazim	3.12±0.94[b]	48.32[a]	3.47±0.53[b]	67.29[a]

rot disease. But studies have shown that with long-term use of chemical pesticides, pathogen will develop resistance (Saito et al., 2016). Therefore, there is need to seek new methods of prevention and control of ginseng root rot, to slow down the occurrence of the pathogenic bacteria drug resistance and its life cycle in plant tissues, endophytic bacteria in plant body can colonize in long-term stability, and is not susceptible to the influence of the external environment condition. Endogenous bacteria and pathogen have mutual reciprocity and benefit; they are a kind of important resources (Aly et al., 2011). Report has it that *B. amyloliquefaciens*, *Bacillus methylotrophicus, Paenibacillus polymyxa* and *Enterobacter cloacae* can be used for ginseng disease prevention and control (Olamide et al., 2019; Durairaj et al., 2018; Wang et al., 2016; Jiang et al., 2013), and *B. subtilis* can be used for the prevention, and treatment of ginseng root rot has not been reported.

Endophytic bacillus is a kind of non-pathogenic bacteria that can produce spores and a variety of antibacterial substances, such as antibiotics and antimicrobial proteins. Bacillus resistance is strong, fast in reproduction, very easy to survive in the environment, colonization is a kind of important resources of bio-control bacteria, it has wide application in the green plant disease prevention and control. In this study, a strain isolated from the leaves of *P. ginseng* has a very good inhibitory effect to ginseng mould bacterium, FS17 endophytic bacteria identified the strain for *B. subtilis*, strain bacteriostatic spectrum is more extensive, FS17 to 10 kinds of common plant pathogenic fungi are highly bacteriostasis. The control the effect of FS17 strain against ginseng root rot was remarkable. Disease spot diameter was much smaller than control, comparative with the control effect of carbendazim. Therefore, the FS17 strain can be used for prevention and control ginseng root rot.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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