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RESEARCH ARTICLE

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Isolation, Screening, and Identification of Actinomycetes with Antifungal and Enzyme Activity Assays against *Colletotrichum dematium* of *Sarcandra glabra*

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ABSTRACT

A serious leaf disease caused by *Colletotrichum dematium* was found during the cultivation of *Sarcandra glabra* in Jingxi, Rong'an, and Donglan Counties in Guangxi Province, which inflicted huge losses to plant productivity. Biological control gradually became an effective control method for plant pathogens. Many studies showed that the application of actinomycetes in biological control has been effective. Therefore, it may be of great significance to study the application of actinomycetes on controlling the diseases caused by *S. glabra*. Strains of antifungal actinomycetes capable of inhibiting *C. dematium* were identified, isolated and screened from healthy plants tissues and the rhizospheres in soils containing *S. glabra*. In this study, 15 actinomycetes strains were isolated and among these, strains JT-2F, DT-3F, and JJ-3F, appeared to show antagonistic effects against anthracnose of *S. glabra*. The strains JT-2F and DT-3F were isolated from soil, while JJ-3F was isolated from plant stems. The antagonism rate of strain JT-2F was 86.75%, which was the highest value among the three strains. Additionally, the JT-2F strain also had the strongest antagonistic activity when the antagonistic activities were tested against seven plant pathogens. Strain JT-2F is able to produce proteases and cellulase to degrade the protein and cellulose components of cell walls of *C. dematium*, respectively. This results in mycelia damage which leads to inhibition of the growth of *C. dematium*. Strain JT-2F was identified as *Streptomyces tsukiyonensis* based on morphological traits and 16S rDNA sequence analysis.

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1. Introduction

Sarcandra glabra (Thunb.) Nakai is a perennial herb of Chloranthaceae, mainly distributed in the provinces of South, East, and Southwest China, as well as in North Korea, Japan, Southeast Asia and India [1]. *Sarcandra glabra* is enrolled in the Chinese Pharmacopeia [2], and it is also an important member of the traditional "72 wind medicines" of the Yao ethnic minority in Guangxi Province [3]. It has heat-clearing and blood-cooling effects, promoting blood circulation, the dissolution of clots, expelling of wind and removal of gallbladder meridian obstructions, and is thus used for the treatment of blood-heat type purpura, rheumatoid arthralgia and traumatic injuries. Modern pharmacological and toxicological studies have shown that *S. glabra* has multiple functions, including antibacterial, anti-inflammatory and anti-tumor activities, inhibition of the influenza virus, promotion of fracture healing and analgesia. Acute toxicity tests reveal no major toxic effects of *S. glabra*, indicating its safety [4]. Preparations using *S. glabra* as the main active component include *S. glabra* lozenges, *S. glabra* tablets and injections, and Xuekang oral liquids [5]. A serious leaf disease caused by *Colletotrichum dematium* was found during the planting of *S. glabra* in Jingxi, Rong'an and Donglan Counties in Guangxi Province, and the average disease incidence was greater than 50% [6]. In particular, in the forest inter-planting pattern used in Rong'an County, the incidence of the disease reached 65% [6].

There are two main control methods for plant pathogens. However, the control of *C. dematium* of *S. glabra* by chemical agents may lead to various problems, such as pathogen resistance to drugs [7], rapid disease recurrence and pesticide residues. Application of bio-control bacteria is an important method for plant disease prevention and control owing to their environmental safety. Such methods are not only a research hotspot, but also represent a future trend in the development of "green" Chinese medicinal materials. Endophytic actinomycetes are actinomycetes that reside within healthy plant tissues and do not (at least in the short term) produce obvious symptoms of infection. Owing to their roles in promoting the growth of host plants, increasing

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plant disease resistance and enabling plants to establish stress as well as insect and weed resistance levels, endophytic actinomycetes have become a new and favorable type of biological resource [8–9]. However, the application of antagonistic microorganisms to control *C. dematium* of *S. glabra* has not been reported previously. In this study, different tissues and the rhizosphere soil of healthy *S. glabra* plants were screened, to isolate efficient bio-control actinomycetes that could control the *C. dematium* of *S. glabra* and thus provide a basis and reference for the biological control of *C. dematium*.

2. Materials and methods

2.1. Materials

The test pathogens included, *Colletotrichum* of *S. glabra*, *C. dematium* Z1, which were isolated and preserved by our laboratory. The antagonistic fungi spectrum of the endophytic fungi with high antagonistic activity against *C. dematium* was tested against 7 fungal phyto-pathogens. The phyto-pathogenic species selected were as follows: (i) *Botrytis cinerea* of *Nicotiana tabacum*, (ii) *Fusarium oxysporum* of *Musa nana*, (iii) *F. oxysporum* of *Citrullus lanatus*, (iv) *Stagonosporopsis cucurbitacearum* of *Siraitia grosvenorii*, and (v) *Alternaria panax*, *C. gloeosporioides*, and *F. solani* of *Panax notoginseng* (Table 2).

Test samples consisted of five healthy plants (including roots, stems, and leaves) and the rhizosphere soils of *S. glabra* that were collected from Jingxi and Donglan Counties, respectively. The samples were numbered and to maintain freshness, were stored in bags, which were then delivered to the laboratory. The samples were used for actinomycetes isolation within 24 h.

The culture medium used for growing plant pathogenic fungi was potato dextrose agar (HKM, Guangdong Huankai Microbial Sci. & Tech. Co., LTD., Guangzhou, China) which consisted of 200.0 g potato dextrose, 20.0 g glucose, 15.0–20.0 g agar and 1 L distilled water. This medium was also used to test their antagonistic activities. Gauze's Medium No.1 (HKM, Guangdong Huankai Microbial Sci. & Tech. Co., LTD., Guangzhou, China) was used for growing actinomycetes.

2.2. Isolation of endophytic actinomycetes

The endophytic actinomycetes were isolated using the dilution method according to a previous study. 10 g of sieved soil sample was placed in a crucible dish and heated in an oven at 45 °C for 24 h until dried. The samples were suspended in 100 mL distilled water in 200 mL triangular bottles, oscillated on the shaking table for 30 min and left to stand for 30 min, and subsequently serially diluted ranging from 10^{-1} up to 10^{-7}. From each dilution, 0.1 mL of suspension was spread evenly onto the surface of Gauze's Medium No.1 agar media and the plates were incubated at 30 °C for 2 weeks. The colonies produced from each serially diluted plate were purified using potato dextrose agar and incubated at 30 °C for 2 weeks. Purified isolates presumed to be *Streptomyces* spp. were selected based on their morphologies [10–12].

2.3. Screening of antagonistic actinomycetes

Antagonistic actinomycetes were screened using the confrontation method according to previous publications [13–15]. The same procedure was used for the second round of screening. Briefly, one colony plug (6 mm in diameter) of 2 weeks actinomycetes growth was divided into two and placed equidistant on both sides of the center of a dish containing approximately 15 mL of PDA and a depth of 2 mm. Two colony plugs (6 mm in diameter) from the actinomycetes were symmetrically placed 3 cm from one 5-day-old fungal phyto-pathogen inoculant in order to establish a the co-culture treatment. Without the actinomycetes, one colony plug of the fungal phyto-pathogen was placed on another PDA plate using the same method, and this was used as the growth control. All treatments and controls were ran in duplicate and incubated at 30 °C. When the fungal phyto-pathogen colony had completely reached the center of the petri dishes in the growth control, the radius of the relative fungal phyto-pathogen colony in the treatment dishes was measured. The average radius of each fungal phyto-pathogen in the treatment was recorded as R1, and that in the growth control was recorded as R2. The growth inhibition percentage of the fungal phyto-pathogen in the actinomycetes, i.e., the phyto-pathogen antagonism was calculated with the following formula:

$$\text{Inhibition percentage } (\%) = \frac{R_2 - R_1}{R_2} \times 100.$$

2.4. Antibacterial spectra of antagonistic actinomycetes

This was described previously [13–15] and in detail in Section 2.3. With the co-culture treatment, the antagonistic spectrum of the actinomycetes with high antagonistic activity against *C. dematium* was tested against the other seven fungal phyto-pathogens.

2.5. Identification of antagonistic strains

Actinomycetes strains were streaked on agar Gauze's Medium No.1. Plates were then cultured at 30 °C for 10 days and then colony morphology was observed under a light microscope (Phenix Optical

Technology Co., Ltd, Shangrao, China), to observe their morphological characteristics [16–18].

The antagonistic actinomycetes that had been cultured for 5 days were inoculated into a 100-mL flask filled with 40 mL of liquid Gauze's Medium No.1. The suspension was cultured at 30 °C and 180 r/min for 48 h, and the actinomycetes were collected by centrifugation. An improved SDS method was used to extract genomic DNA from actinomycetes using a DNA extraction kit (Sangon Biotech Co., Shanghai, China). The extracted DNA was amplified by PCR using the universal primer pair 27 F (5′-AGAGTTTCCTGCTCAG-3′) and 1492 R (5′-GGTTACCTTTACGACTT-3′), which was synthesized by Sangon Biotech Co. The PCR amplification was carried out in a 25-μL system, which contained 0.5 μL template DNA (20–50 ng/μL), 2.5 μL 10× Buffer (including Mg^{2+}), 1 μL dNTPs (2.5 mmol/L), 0.2 μL enzyme, 0.5 μL each primer and double-distilled H_2O to the final volume. The thermal cycling reaction was as follows: pre-denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and elongation at 72 °C for 60 s, followed by a final repair and extension at 72 °C for 10 min. Water was used as the blank control.

The PCR results were detected by gel electrophoresis. Briefly, 4 μL PCR products were loaded onto 1% (w/v) agarose gels that were placed in 1× TBE buffer (0.9 mol/L Tris-Borate and 0.01 mol/L EDTA, pH = 8.3). The electrophoresis was performed at 110 V for 40 min. The amplified DNA products were purified and sequenced by Sangon Biotech Co., and the sequencing results were queried using a BLAST algorithm-based search against GenBank databases. A phylogenetic tree was constructed by using the Neighbor-Joining method of MEGA 4.0 (http://www.megasoftware.net/mega4/mega.html) based on the similarity levels of the 16 s rDNA sequences.

The classification status of fungal species and genera was determined. After the target sequence and reference sequence BLAST, the homology was ≥95%, and the genus was identified. The similarity ≥99%, a species was identified [19].

For the microscopic observation of fungal mycelia, a colony was placed directly on the object table of an inverted microscope. The morphological changes in mycelia under-treated and control conditions were observed, recorded, and photographed.

2.6. Determination of enzyme production and the indole acetic acid (IAA) content of antagonistic strains

A single colony of antagonistic actinomycetes was inoculated into 50 mL of potato dextrose broth medium and cultured at 30 °C and 180 r/min for 3 days. Then, the fermentation broth was filtered using an aseptic gauze and filter paper, and centrifuged at 10,000 r/min for 15 min. The final broth was acquired after passing through a 0.22-μm filter membrane.

The protease, cellulase and chitinase activities in the fermentation broth (prepared above) were determined using ELISA enzyme activity kits (Andy Gene Biotechnology Company, Beijing, China) according to the manufacturer's instructions.

2.7. Statistical analysis

The results of the statistical analysis are expressed as means ± standard deviations. Data from different groups were compared using a one-way ANOVA with SPSS 19.0 software (http://www.spss.com), and the LSD test was used for multiple comparison analyses and testing for variance homogeneity between the groups, respectively. Differences were considered significant at $p < 0.05$. The 16S rRNA gene sequences were deposited in the Genbank and an accession number was allocated (MK368447).

3. Results

3.1. Isolation and screening of antagonistic actinomycetes

A total of 15 actinomycetes strains were isolated from healthy tissues (root, stem, and leaf) and rhizosphere soils of *S. glabra*. Among them, 2 strains had antagonism rates of less than 60%, 10 strains had antagonism rates of 60%–80%, and 3 strains had antagonism rates of more than 80% (Table 1). Strains JT-2F and DT-3F were isolated from rhizosphere soils of *S. glabra*, while strain JJ-3F was isolated from the stems. The antagonism rates of JT-2F, DT-3F, and JJ-3F on *C. dematium* were 86.75%, 85.54%, and 80.72%, respectively, with JT-2F having the greatest antagonism rate and JJ-3F having the lowest rate (Table 1).

3.2. Inhibitory effects of antagonistic actinomycetes on C. dematium of S. glabra

In confrontation culture plates containing the *C. dematium* pathogen and endophytic actinomycetes, the two colonies were not in direct contact but showed antagonistic effects. As shown in Figure 1(A–D), the growth of pathogen colonies near the endophytic actinomycetes was significantly inhibited. The mycelia of the *C. dematium* of *S. glabra* growing on potato dextrose agar-containing plates with or without antagonistic actinomycetes were isolated after 7 days of culture and observed

under a microscope. The mycelia of the treatment group (Figure 1(D)) were distorted and twisted, and some mycelia were darker in color and larger in size, with distorted conidiophores and decreased numbers of spores (Figure 1). Figure 1(C) is normal mycelia, which is smooth with no change in color.

3.3. Antibacterial spectra of antagonistic actinomycetes

The antibacterial activities of the antagonistic actinomycetes JT-2F, DT-3F, and JJ-3F against seven pathogens of crops and medicinal plants were determined. The order of the average antagonism rates among them was as follows: JT-2F > DT-3F > JJ-3F (Table 2), but their effects were not significant. The antagonistic actinomycetes JT-2F, DT-3F, and JJ-3F provided a broad-spectrum antibacterial resistance to seven plant pathogens, while the strongest antagonistic effects of JT-2F, DT-3F, and JJ-3F were against *A. panax* of *P. notoginseng*, *F. oxysporum* of *M. nana* and *S. cucurbitacearum* of *S. grosvenorii*, respectively. This would suggest that all three strains can be used for future biological control (Supplementary Figure).

3.4. Identification of antagonistic strains

3.4.1. Morphological identification

The colony of the JT-2F strain on agar Gauze's Medium No.1 was round and gray, with a milky white edge and wrinkled surface, the spores were ovoid, the air hyphae were not obvious, the colony grew slowly and had a strong earthy smell. For other specific physiological and biochemical characteristics as well as culture features and reference strains of related species, Bergey's Manual of Determinative Bacteriology was consulted [18].

Table 1. Actinomycetes strains with antifungal activities against *Colletotrichum dematium* of *Sarcandra glabra*.

Strain	Inhibitory rate[1]
JT-2F	(86.75 ± 1.20)[a]
DT-3F	(85.54 ± 1.54)[a]
JJ-3F	(80.72 ± 2.72)[b]

[1]The values are shown as mean ± SD. Different letters indicate significant differences at the 0.05 level.

3.4.2. Molecular identification

A 1,404-bp fragment was obtained by PCR amplification from the genomic DNA of strain JT-2F using universal primers for 16 s rDNA. Sequences were subjected to BLASTn analysis against the NCBI nucleotide database (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). The sequence from the JT-2F strain had a high homology with the16s rDNA of *Streptomyces* sp. (Table 3), sharing 100% identity. The 16 s rDNA sequences of strains sharing 100% identity were used to construct a phylogenetic tree with MEGA 4.0 software. The JT-2F strain was on the same branch of the phylogenetic tree as *Streptomyces tsukiyonensis* AB184594 (Figure 2). Therefore, based on the morphological characteristics and the 16 s rDNA sequence, the JT-2F strain was preliminarily identified as *S. tsukiyonensis*. The sequence data produced in this study were deposited with NCBI (accession number MK368447).

3.5. Determination of cell wall hydrolase levels and IAA content of antagonistic strains

The fermentation broths of JT-2F, DT-3F, and JJ-3F were used to detect protease, cellulase and chitinase levels, as well as the IAA content of the organisms. As shown in Table 4 the JT-2F strain had the greatest protease and chitinase levels at 22.90 pg/mL and 77.95 pg/mL, respectively, as well as the greatest IAA content at 5.21 pmol/L. However, the cellulase level of the JT-2F strain was the lowest (35.56 pg/mL), while the cellulase level of the DT-3F strain was the greatest (73.51 pg/mL), followed by

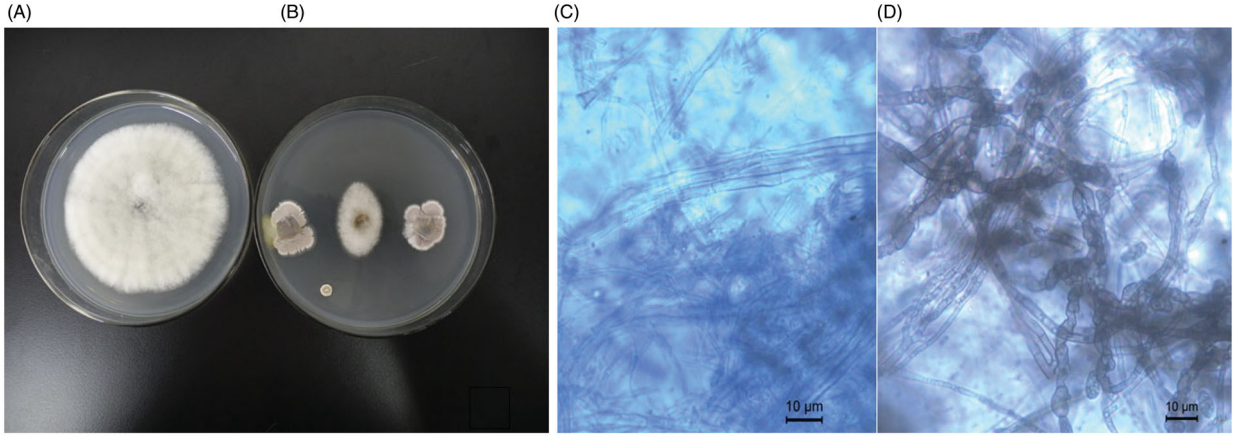

Figure 1. Antagonistic effects of the actinomycetes strain JT-2F against *Colletotrichum dematium* of *Sarcandra glabra*. (A) Control; (B) Inhibition of antagonistic strain JT-2F; (C) Normal mycelia; (D) The mycelia of *C. dematium* of *S. glabra* after confrontation.

Table 2. Antifungal spectra of actinomycetes.

Type	Host plant	Pathogen/strain numbers	Strength of inhibition/%		
			JT-2F	DT-3F	JJ-3F
Crops	*Nicotiana tabacum*	*Botrytis cinerea*/YC-1	83.33	76.19	78.57
	Citrullus lanatus	*Fusarium oxysporum*/XG-1	81.18	82.35	74.12
	Musa nana	*F. oxysporum*/ XJ-3	82.35	88.24	78.82
Medicinal plants	*Panax notoginseng*	*Alternaria panax*/SQ-1	87.06	83.53	81.18
	P. notoginseng	*Colletotrichum gloeosporioides*/ SQ-3	81.18	82.35	81.18
	P. notoginseng	*F. solani*/ SQ-4	76.47	82.35	75.29
	Siraitia grosvenorii	*Stagonosporopsis cucurbitacearum*/LHG-1	81.18	70.59	82.35
Average inhibition rate[1]			$(81.82 \pm 1.20)^a$	$(80.80 \pm 1.60)^a$	$(78.79 \pm 2.00)^a$

[1]The different lowercase letters indicate significant differences at the 0.05 level.

Table 3. Sequences of the actinomycetes strains used in the phylogeny analysis.

Species	Strain no.	GenBank accession numbers 16S rDNA region
Nocardiopsis synnemataformans	DSM44143	Y13593
Streptomyces aureofaciens	NBRC12843	AB184189
S. avellaneus	NBRC13451	AB184413
S. candidus	NRRLISP-5141	DQ026663
S. cinereorectus	JCM6916	AY999764
S. herbaricolor	NBRC12876	AB184212
S. indigoferus	NBRC12878	AB184214
S. mauvecolor	LMG 20100	AJ781358
S. netropsis	NBRC3723	AB184792
S. olivoverticillatus	NBRC15273	AB184636
S. pratensis	AS 4.1868	AY999894
S. purpeofuscus	NBRC12905	AB184234
S. sannanensis	NBRC 14239	AB184579
S. scopuliridis	RB72	EF657884
S. stramineus	NBRC16131	AB184720
S. termitum	NBRC13087	AB184302
S. tsukiyonensis	NBRC14353	AB184594
S. turgidiscabies	ATCC700248	AB026221

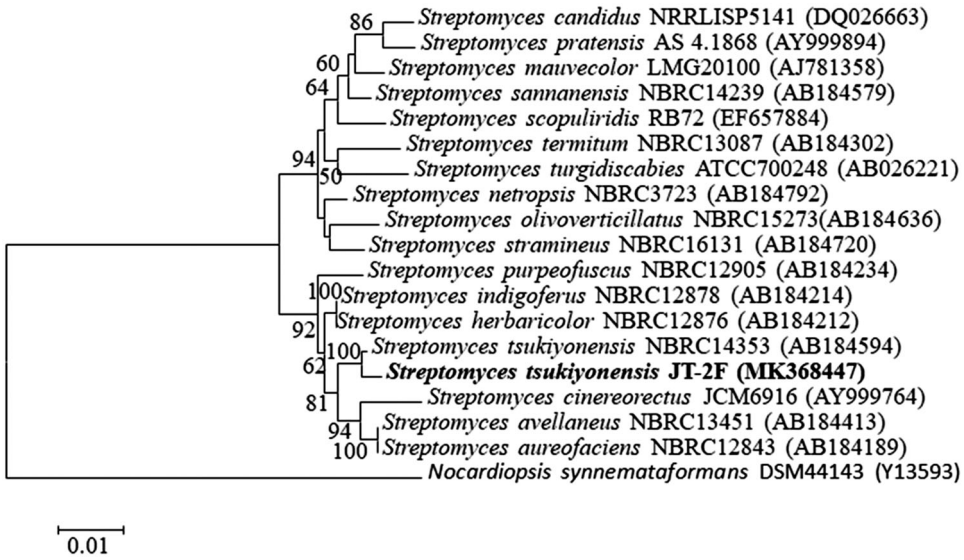

Figure 2. Phylogenetic tree of actinomycetes based on the homology of the 16S rDNA region of the JT-2F strain.

that of the JJ-3F strain (58.21 pg/mL). Protease, cellulose, and chitinase are the main hydrolases of fungal cell walls. Thus, strain JT-2F produces an antagonistic effect on the pathogen by secreting cell wall hydrolases. IAA is an effective hormone that promotes plant growth. After analyzing the results of the confrontation testing and the hydrolase activities, the antagonistic actinomycetes strain JT-2F was used for further studies.

4. Discussion

A general disease survey of *S. glabra* in the main production area (Jingxi) in Guangxi Province showed that the major disease was anthracnose, which had a long onset time and was widespread, resulting in the decreased production and quality of traditional Chinese medicinal materials. At present, chemical agents are mainly used to control anthracnose, which involves alternate applications of agents,

Table 4. The cell wall hydrolase and indole acetic acid contents in the antagonistic actinomycetes strains (means ± standard deviations).[1]

Strain	Protease (pg/mL)	Cellulase (pg/mL)	Chitinase (pg/mL)	Indole acetic acid (pmol/L)
JT-2F	$(22.90 \pm 2.90)^{a}$	$(35.56 \pm 2.49)^{c}$	$(77.95 \pm 1.21)^{f}$	$(5.21 \pm 1.10)^{h}$
DT-3F	$(21.15 \pm 1.19)^{a,b}$	$(73.51 \pm 3.22)^{d}$	$(70.70 \pm 0.19)^{d}$	$(3.02 \pm 0.08)^{h}$
JJ-3F	$(19.34 \pm 1.30)^{b}$	$(58.21 \pm 1.90)^{e}$	$(50.70 \pm 1.28)^{g}$	$(2.44 \pm 0.26)^{h}$

[1]The different letters indicate significant differences at the 0.05 level.

such as carbendazol and thiophonate-methyl, whenever *S. glabra* plants suffer from anthracnose [19]. However, long-term applications of chemical pesticides not only result in negative environmental effects but can also easily lead to drug-resistant pathogens, resulting in disease recurrence.

Streptomyces is the largest genus of Actinobacteria. *Streptomyces* spp. have disease preventive activities, and acts as a broad-spectrum antibacterial agent that can be used to produce a variety of antibiotics [20]. These organisms have potential application values in the development of biological pesticides and several different preparations. In this study, the antagonistic effects of *S. tsukiyonensis* against *C. dematium* of *S. glabra*, as well as seven tested pathogens, including pathogenic fungi of three kinds of crops and also including *M. nana*, *N. tabacum* and *C. lanatus* and two kinds of medicinal plants, as well as including *P. notoginseng* and *S. grosvenorii*, were identified for the first time. Owing to the prevalence and serious effects of anthracnose disease of *S. glabra*, the identification of efficient bio-control strains is of great significance to the development of the *S. glabra* industry.

Yang et al. [21] isolated two antagonistic actinomycetes, *S. celluloflavus* and *S. yatensis*, from *Calendula officinalis*, which had inhibition rates against *F. oxysporum* of 70.12% and 54.46%, respectively. Ma et al. [22] isolated an antagonistic actinomycete, Lj20, from the roots of pepper plants. It had a strong inhibitory effect on tomato cinerea and was identified as *S. rochei*. Jiao et al. [23] isolated an actinomycetes strain from the rhizosphere soil of tropical medicinal plants, and it was identified as *S. violaceoniger*. This organism was found to have a large antagonistic effect against anthracnose of Chinese yam. From the soil of Wudang mountain, Zou et al. [24] isolated a *Streptomyces* sp. with a broad-spectrum antibacterial activity and a strong antibacterial activity against *Candida albicaus* CMCC 98001 and *Mycobacterium smegmatis* MC2. This was identified as *S. xanthocidicus*. From more than 2,200 marine actinomycetes strains, Pu et al. [25] isolated a strain with a strong antagonistic effect against *Ralstonia solanacearum* of peanut, and it was identified as *S. zhapoensis*, a new species related to *S. aureus*. Wang et al. [26] isolated and screened the actinomycetes strain *S. griseinus*, which had a good inhibitory effect on white-rot fungus of grape obtained from soil. From rhizospheres in strawberry growing soil, Shen et al. [27], using a combination of morphology and molecular biology techniques, isolated an actinomycetes strain which was identified as *S. xanthophaeus*. Shi et al. [28] isolated actinomycetes strain Ac16, which had strong inhibitory activities against most common plant pathogens, including those causing cucumber wilt, capsicum wilt, watermelon wilt, wheat scab, maize swarms, apple spot defoliation, tomato early blight and cotton verticillium wilt, and was subsequently identified as *S. coeruleofuscus* var.

In this study, *S. tsukiyonensis* produced a variety of active antibacterial substances, such as chitinase, cellulase, and proteases, which are antibacterial peptides and therefore can inhibit pathogen growth either by themselves or synergistically with other cell wall-degrading enzymes [29–30]. Cell wall lysozyme is a complex hydrolase that can hydrolyze the cell walls of fungi and, therefore, inhibit the growth of pathogens, resulting in antibacterial and disease preventive functions. *S. tsukiyonensis* can also produce IAA, which is an effective hormone that promotes plant growth. However, the activity of IAA produced by *S. tsukiyonensis* needs to be confirmed in further studies. Here, the antagonistic effects of the isolated *S. tsukiyonensis* strain against pathogens were determined in the laboratory, while the effects of *S. tsukiyonensis* in the field are still unknown. In addition, whether *S. tsukiyonensis* can promote *S. glabra* growth, and how to apply it as a biological pesticide, require further study.

5. Conclusions

Based on the morphological characteristics and 16S rDNA sequence analysis, strain JT-2F was identified as *S. tsukiyonensis*. The antibiosis mechanism indicated that strain JT-2F could produce proteases and cellulase to degrade the protein and cellulose components of cell walls, respectively, and thus damage *C. dematium*'s mycelia leading to growth inhibition of *C. dematium*.

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Disclosure statement

The authors have no conflict of interest.

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