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Streptomyces nobilis C51 Suppresses Gray Mold Caused by *Botrytis cinerea* in Tomato

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Authors' contributions

This work was carried out in collaboration between all authors. Author BJ performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors YH and SS managed design and analyses of the study. Author ZJ managed literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study sought to develop a biological control agent against gray mold disease caused by *Botrytis cinerea* in tomato using a strain of *Streptomyces*, which showed significant antagonistic activity against *B. cinerea*. Such strain (C51) was screened by dual culture method from a total of 78 actinomycetes and was identified as *Streptomyces nobilis* by morphological, biochemical and molecular analysis. The influence of different environmental conditions, such as temperature degree, pH range and medium type on the expression of the antifungal activity was thoroughly examined. The metabolites involved in the antagonistic action of C51 strain showed to be produced independently by the presence of *B. cinerea*. We also found that the culture filtrate of C51 potently inhibited the mycelial growth of *B. cinerea*, and morphological changes such as hyphal swelling and

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abnormal shapes were observed using culture filtrates. The antifungal activity of the culture broth was stable across a broad range of temperatures (28-121℃) and pH (pH 2-12), suggesting a wide range of applications of *Streptomyces nobilis* C51. The results of greenhouse test showed that *Streptomyces nobilis* C51 had the same disease inhibition effect as well as azoxystrobin against *B. cinere*a. The current observations could support the application of *Streptomyces nobilis* C51 as a promise biocontrol agent.

Keywords: Biocontrol; Botrytis cinerea; Streptomycetes nobilis; tomato.

1. INTRODUCTION

Since Micheli erected the genus *Botrytis* in 1729, it has become a widely known group of fungi causing diseases on economically important plants [1-4]. *B. cinerea* is one of the most destructive plant pathogens in this genus which attacks a wide variety of plants (over 200 plant species) and causes severe diseases worldwide. *B. cinerea* also spoils agricultural products after harvest since it can be active at very low temperatures [5-6]. This fungus infects flowers, fruits, and vegetative tissues in tomato and leads to severe loss in yield and quality.

Conventionally, *B. cinerea* are controlled by chemical fungicides such as benzimidazole and dicarboximide. However, this is becoming less acceptable because it conflicts with public concern for fungicide residues and also increases the potential for the build-up of resistance in *B. cinerea* to fungicides [7]. Consequently, development of high-efficient and environment friendly methods for preventing gray mold in tomato is an increasing demand from consumers and officials [8]. In this context, biological control through the use of antagonistic microorganisms has emerged [9].

Several antagonistic microbes including the genera *Bacillus subtilis* [10-11], non-pathogenic *Fusarium* [12], *Trichoderma* [13], *Pseudomonas* [14-15] and *Streptomyces* [16] are used as biocontrol agents. However, each antagonistic microbe possesses undesirable features for its use as biocontrol agent. *Pseudomonads* is unable to produce the resting spores which hinders their commercial use [17]. *Bacillus* strain has rich genes to produce a variety of antibiotics, but only a small part of genes can synthesize the antibiotics that can be secreted under natural conditions [18]. The non-pathogenic *Fusarium* strains suppress the pathogens depending on its competition of nutrients with pathogens so that a large amount of *Fusarium* need to be applied in practice.

Among various fungal and bacterial biocontrol agents, soil bacteria from genus *Streptomyces* are proved to be the most frequently used against various plant diseases. The antagonistic activity of *Streptomyces* is usually related to the production of antibiotics, secretion of extracellular hydrolytic enzymes, competition and hyperparasitism [19]. El-Abyad et al. [20] reported that the culture filtrate of either *S. pulcher* or *S. canescens* significantly inhibited spore germination, mycelial growth and sporulation of *Fusarium oxysporum* f. sp. *lycopersici* (the cause of Fusarium wilt), *Verticillium albo-atrum* (the cause of Verticillium wilt) and *Alternaria solani* (the cause of early blight) in tomato respectively. The *in vivo* studies demonstrated that coating tomato seeds with spores of the antagonist was the most effective in controlling the tomato diseases concerned [20]. Chemical fungicides have often been replaced with *Streptomyces* sp. to control pathogens [21]. These data indicated that *Streptomyces* species could be used as biological control agents effectively. However, the data related to the antagonistic ability of the *Streptomyces* metabolites against *B. cinerea* are limited.

In this study, a total of 78 actinomyces were screened using selective media from agricultural soils of Cangzhou, China. A *S. nobilis* strain named as C51 exhibiting the highest antagonistic activity against *B. cinerea* were isolated. The culture conditions for this strain were optimized and stability of the bioactive compounds at different temperature and pH was determined. Moreover, the antifungal activity of medium extracts from solid and broth culture of *S. nobilis* C51 were investigated. The antagonistic mechanism of the strain was discussed. *In vivo* experiments revealed that culture broth of *S. nobilis* C51 was capable to control gray mold disease in tomato effectively. This information will be useful to design strategies to enhance crop protection against *Botrytis* diseases.

2. MATERIALS AND METHODS

2.1 Fungal Strains and Culture Conditions

B. cinerea was used as the tested fungal pathogen which was cultured on Potato Dextrose Agar (PDA) at 22℃ for 10 days. The spores were harvested by flooding the surface of 10-day-old cultures with distilled water and the inoculum concentration used was 1×10^6 spores /mL.

2.2 Soil Sampling and Isolation of Actinomycetes

Samples of soil were collected from orchards and agricultural fields in different parts of Cangzhou, China. 5 g soil sample was mixed with 45 mL of sterile water and shaked vigorously for 10 min. The soil mixture was serially diluted in sterile distilled water. 100 µL of 10^4 dilution was spread onto Gause's Synthetic Agar Medium (soluble starch 20.0 g/L; KNO_3 1.0 g/L; NaCl 0.5 g/L; $MgSO_4\cdot7H_2O$ 0.5 g/L; $FeSO_4\cdot7H_2O$ 0.01 g/L; KH_2PO_4 0.5 g/L; agar 18.0 g/L), which was supplemented with 50 µg/mL of filter-sterilized $K_2Cr_2O_4$ to inhibit bacteria and fungus growth. Plates were incubated at 28℃ for 10~14 days. Subsequently, colonies with different morphological appearances were selected from the countable plates and re-streaked onto a new plate of the same media to obtain pure colonies. For long term storage, the spores' suspensions of actinomycetes were maintained in 30% (w/v) glycerol at -20℃.

2.3 Dual Culture Bioassay

The antagonistic activity of the actinomycetes strains were determined using agar plates [17]. Nine centimetres Petri plates containing potato dextrose agar (PDA) were inoculated with 6 mm mycelial disks of either *B. cinerea* (4-day-old culture) and actinomycetes strain (7-day-old culture) 3.0 cm apart from each other. Plates inoculated with *B. cinerea* alone served as control. The plates were incubated at 22℃ for 4 days, then diameter of inhibition zones was measured. The antifungal activity was calculated according to Kanini et al. [22]. The experiment was performed three times with five replicate PDA plates.

2.4 Phenotypic Characteristics of C51 Strain

To determine cultural characteristics of C51 strain, we have grown it on various ISP medium [23] at 28℃ for 14 days. The color of aerial and substrate mycelia including soluble pigment were determined. The growth situation at different medium were determined by the following standard:

$$\text{Relative value of growth (R)} = \pi R_z^2 \Big/ \pi R_{str}^2$$

(R_z= radius of streptomycete colony on various medium and R_{str} = radius of streptomycete colony on Gause's Synthetic Agar Medium).

R = ~0.5 Poor; R = 0.5~0.8 Moderate; R = 0.8~1.2 Good; R = 1.2~ Excellent.

Biochemical tests including starch and gelatin hydrolysis, voges-proskauer, citrate utilization, indole, methyl red, gelatin and nitrate reduction were determined by standard methods [24]. Utilization of different carbon sources such as glucose, starch, mannitol, fructose, soluble corn starch and maltose, and nitrogen sources namely arginine, tyrosine, glutamic acid, aspartic acid, $NaNO_3$ and $(NH_4)_2SO_4$ were also tested.

2.5 Taxonomy of C51 Strain

For molecular identification, the 16S rDNA gene of C51 strain was amplified using the primers 27F and 1492R [25]. PCR amplifications were performed as following: initial denaturation at 94℃ for 2 min, 35 cycles of 94℃ (60 sec), 50℃ (60 sec), and 72℃ (2 min) followed by final extension at 72℃ for 10 min. Amplified PCR products were separated by gel electrophoresis on 1% (w/v) agarose gel and then purified using Extract PCR kit (TaKaRa Biotechnology Co., Dalian, China). Purified PCR product of approximately 1,400 bp was sequenced at Life Technologies Biotechnology Company, Shanghai, China. For the detection of closest relatives, the 16S rDNA gene sequences of C51 strain of were identified using the BLAST program (http://www.ncbi.nlm.nih.gov/Blast/). Sequence data was compiled using the MEGA 6.05 software and aligned with sequences obtained from the GenBank (http://www.ncbi.nlm.nih.gov/) databases using the DNAMAN aligning utility. Phylogenetic analysis was performed using the neighbour joining method implemented in MEGA 6.05.

2.6 Effects of Culture Conditions on Antagonistic Activity of *S. nobilis* C51

The agar plate antagonism bioassay of C51 strain was performed on five different solid media

including PDA, Arginine Glycerol Salts (AGS), Nutrient Agar (NA), Czapek Agar (CzA) and Yeast extract-malt extract medium (YEME). The antagonistic activity of C51 at different temperature degrees (22, 24, 26, 28 and 30℃) and different pH (5, 6, 7, 8, 9 and 10) was tested using YEME medium. The C51 strain was inoculated and incubated at different culture conditions for 4 days. The antifungal activity following exposure to various temperatures and pH conditions was measured as above.

2.7 Extraction of *S. nobilis* C51 Metabolites from Solid Cultures

S. nobilis C51 was grown on YEME with pH 7 and incubated at 28℃ since these were selected as the best conditions for high antifungal activity expression. After 7 days, the medium around *S. nobilis* colony in a radius of 2 cm was cut off and crushed into slurry. The slurry was centrifuged at 6,000 g for 30 min and the supernatant was collected by tips. To determine the antifungal activity, 50 µL of the extracted material from medium were placed into wells on PDA plates, formed using a cork borer 6×5 mm (diameter×depth) that were inoculated with the fungus.

In addition, the inhibition zones of co-cultures of the selected *S. nobilis* and *B. cinerea* on YEME plates were removed and mixed. The supernatant was obtained from the slurry by centrifugation as described above. After filtration, 50 µL of the supernatant was placed into wells on PDA agar plates which were inoculated with the fungus.

2.8 Fermentation of *S. nobilis* C51

S. nobilis C51 was cultured in a 250 mL Erlenmeyer flask containing 50 mL of YEME at 28℃ with 180 rpm for 10 days. The culture broth of *S. nobilis* C51 was centrifuged at 6,000 g at 4℃ for 20 min and the supernatant were sterilized by filtration using the membrane of 0.22 µm in pore size. The resulted crude extracellular metabolites were stored at 4℃.

2.9 Antifungal Activity of the Culture Broth of *S. nobilis* C51

6 mm mycelial disks was taken from a freshly growing colony of *B. cinerea* and placed on PDA plates (9 cm) which contained the indicated dilution of the cell-free suspension of *S. nobilis* C51. They were kept in incubator at 22℃ for 3-4 days. Diameters of the fungal colonies were measured and recorded. Inhibition rate was calculated by formula described as Paul [26].

To further evaluate the antifungal effect of the extracellular metabolites, fungal hyphal morphology which incubated on the PDA plate with and without cell-free culture broth of *S. nobilis* C51 was observed under a light microscope (*Leica*, Germany). Abnormalities in the morphologies of hyphae was observed and photographed with a digital camera attached to the photoport of the microscope.

2.10 Assay for the Temperature and pH Stabilities of Antifungal Activity of *S. nobilis* C51 Culture Broth

For the thermal stability test, the culture broth was exposed to 28, 40, 50, 60, 70, 80, 90, 100 and 121℃ for 20 min and cooled down on ice. For the pH stability test, the culture broth was adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 for 24 hours and adjusted to the original pH. The treated culture filtrates (1 mL) were mixed with 19 mL warm molten PDA and their antifungal activities against *B. cinerea* was assessed by radial growth inhibition assay. Fungal growth was measured after 4 days of incubation and the percentage of growth inhibition was calculated. Residual activities against *B. cinerea* were measured as relative to the activity of 28℃.

2.11 Greenhouse Studies

To prove the inhibitory effects of *S. nobilis* C51 under greenhouse conditions (photo period of 12 h of light,12 h of darkness, light intensity of 100 $\mu mol \cdot m^{2} \cdot s^{-1}$ and temperature of 22 ± 2℃), a greenhouse experiment was undertaken. The suspension of *S. nobilis* C51 was diluted 0, 5, 10 and 20 time(s) with sterilized water and sprayed onto whole tomato plants (> 15 cm in height). The chemical fungicides azoxystrobin and water were used as positive and negative controls respectively. The solution of *B. cinerea* spores (1×10^{6} spores/mL) were sprayed onto whole plants 1 day before treatments with 0, 5, 10 and 20 time(s) diluted suspension of *S. nobilis* C51. Each agent was tested in triplicate. All treated plants were covered with transparent plastic bags to prevent accidental dryness. 7 days after inoculation, the disease severity of gray mold was recorded. The disease incidence of the gray mold infected plants was divided into 5 scales. The gray mold development on each plant was

recorded using 1-5 scale developed, where 0 = no visible gray mold development, 1 = 1-5%, 3 = 6-15%, 5 = 16-25%, 7 = 26-50%, and 9 =≥51% of the leaf surface covered with gray mold. A, B, C, D and E are the number of leaves corresponding to the scale 9, 7, 5, 3, and 1, respectively, and F is the total number of assessed leaves. Disease severity was calculated using the following formula [27].

Disease severity (%) =

$$\frac{9A+7B+5C+3D+E}{9F}\times 100$$

Disease inhibition rate (%) = (Untreated disease severity –Treated disease severity) / Untreated disease severity×100.

2.12 Statistical Analysis

Antagonistic effects of *S. nobilis* C51 against *B. cinerea* were analyzed based on completely randomized design (CRD) with three replications. Statistical analysis was performed with SPSS version 17.0 software. The various data sets were analyzed by one-way analysis of variance (ANOVA).

3. RESULTS

3.1 Actinomycete Isolation, Identification and Antifungal Bioassay

A total of 78 actinomycetes were isolated using selective media. Judged by the radius of inhibition, 18 isolates with antifungal activity against *B. cinerea* were screened (Table 1). The radius of inhibition of the 18 isolates were from 1.57 ± 0.26 mm to 11.07 ± 0.25 mm. The total percentage of antagonistic isolates was 23%. Among 18 isolates the strain C51 exhibited the highest antifungal activity with 11.07 ± 0.25 mm radius of inhibition (Fig. 1).

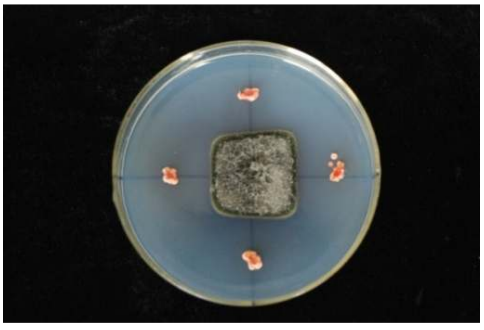

Fig. 1. The antifungal activity against *B. cinerea* of C51 for 7 days

Table 1. Actinomycetes strains with antifungal activity originated from Cangzhou

Sample code	Antifungal activity against *B. cinerea**
C04	7.90±0.08
C08	9.67±0.23
C11	3.06±0.89
C12	4.87±1.01
C13	6.7±0.15
C15	2.37±1.23
C23	2.88±0.45
C24	3.77±0.39
C31	2.91±0.05
C32	2.17±1.83
C33	3.37±2.03
C34	3.45±1.42
C41	3.76±0.49
C42	1.57±0.26
C43	6.17±0.72
C44	1.97±0.16
C51	11.07±0.25
C54	2.81±1.62

**Antagonistic activity levels as expressed by the quotient of the inhibition zone area around actinomycetes colony area (see section 2.3)*

It was observed that the upper colonial appearance of C51 strain on ISP 2 medium was purplish white without soluble pigment production (Table 2). The biochemical identification revealed that the C51 strain was positive for citrate, H_2S, and starch hydrolysis but negative for Voges-proskauer, indole and nitrate reduction, and utilized glucose and mannitol as carbon sources (Table 3, Fig. 2). The data obtained from morphological and biochemical analysis demonstrated that C51 strain might be *Streptomyces* species. To further identify this strain, we performed 16S rRNA gene sequence analysis (GenBank accession Number KM025197; Fig. 3). The results grouped the C51 strain to the species of *S. nobilis* (99% identity with the 16S rRNA sequence of the closest phylogenetic relative) (Fig. 4).

3.2 The Effect of Different Artificial Condition on Antagonistic Activity of *S. nobilis* C51

Giving that the culture medium plays a crucial role in regulating the antagonistic feature of microbes, we used five kinds of medium to cultivate C51 strain and evaluated the effects of medium type on antagonistic activity of *S. nobilis* C51. The highest antagonistic activity was obtained when *S. nobilis* C51 was grown on

Table 2. Cultural characteristics of *S. nobilis* C51 on different media

Type of medium	Aerial mycelium	Substrate mycelium	Growth situation
ISP - 1 / Casein enzymichydrolysate-yeast extract	Light yellow	Dark yellow	Moderate
ISP - 2/ Yeast – Malt extract agar	Purplish white	Reddish orange	Good
ISP - 3 / Oatmel agar	Red	Reddish orange	Excellent
ISP - 4 / Inorganic salts agar	Purplish Red	Red maroon	Excellent
ISP - 5 / Glycerol Asparagine agar	Pinkish white	Orange	Moderate
ISP - 6 / Peptone-yeast extract iron agar	Light grey	White	Poor
ISP – 7/ Tyrosine agar	Pink	Red maroon	Excellent
PDA agar	Orange	Orange-red	Excellent
Sucrose-nitrate agar	Pinkish white	Orange-red	Excellent
Gause's synthetic agar	Orange-red	Dark red	Good

YEME medium. The antagonistic activity of the strain grown on NA medium was slightly less than that grown on YEME medium (Fig. 5a). Notably, no antagonistic activity was detected when cells were grown on CzA medium (data not shown).

Subsequently, the optimal temperature and pH were determined. The results showed that the maximal antagonistic activity was obtained when C51 strain was cultivated in YEME medium at 28℃ with pH 7.0 (Figs. 5b and 5c).

3.3 The Antagonistic Activity of Extract of *S. nobilis* C51 Metabolites from Solid Cultures

To investigate whether the antagonistic activity of *S. nobilis* C51 is induced by the presence of pathogen, we extracted the metabolites from solid medium of C51 strain in presence or absence of *B. cinerea* and compared the antifungal activity of the extracts. The results revealed that the extract from the agar inhibition zones of *S. nobilis* C51 with the presence of *B. cinerea* could strongly suppress the growth of *B. cinerea* (Fig. 6). Likewise the same antifungal activity was observed from the extract of the solid culture of C51 isolate without the presence of *B. cinerea*. These data implicated that the presence of target fungus might not be the requirement for the induction of antifungal activity of *S. nobilis* C51.

3.4 Antifungal Activity of the Culture Broth of *S. nobilis* C51

Based on the results described above, it has been known that *S. nobilis* C51 could secrete the bioactive component extracellularly. Thus, we further assess the effects of cell-free culture broth of C51 strain on the growth of *B. cinerea.* Plant pathogenic fungi was inoculated on the plate containing culture broth of *S. nobilis* C51 with different dilutions. *B. cinerea* was inhibited on the PDA with 5-fold and 10-fold dilutions of culture broth of *S. nobilis* C51 completely (Fig. 7). The inhibition rates with 20-fold and 80-fold dilutions were 70.8 ± 0.41% and 14.5 ± 0.95%, respectively. The swollen balloon-like structure was found when *B. cinerea* was cultivated for 3 days on PDA plate with 20-fold dilutions cell-free culture filtrate(Fig. 8b), while the hypha of *B. cinerea* exhibited a normal morphology on PDA plate (Fig. 8a).

3.5 Thermal and pH Stability of *S. nobilis* C51 Culture Broth

To explore the environmental adaptation of the bioactive components, the thermal and pH stability of culture broth of strain C51 was investigated. The culture broth of *S. nobilis* C51 was exposed to various temperatures for 20 min, and the remaining activity was measured. The antifungal activity of heat-treated culture broth against *B. cinerea* was highest at 28℃ (100%), and more than 80% of the activity was maintained at 40, 50, 60, and 70℃. Notably, after treatment with 121^{o}C for 20 min, 76.4 ± 0.4% of activity remained (Table 4).

The antifungal activity of pH-adjusted culture broth against *B. cinerea* was highest at pH 6 (100%), and more than 85% of the activity was maintained at pH 2, 3, 4, 5, 7 and 8. At pH 12, 48.6±1.43% of activity still remained. These results demonstrated that the antifungal activity of the culture broth of strain C51 was stable at a wide range of temperature and pH (Table 5).

Table 3. Biochemical characteristics of the selected *S. nobilis* C51

Test type	Activity result
Starch hydrolysis	+
Gelatin liquefaction	-
Nitrate reduction	-
H_2S production test	+
Citrate utilization	+
Voges-proskauer	-
Indole	-
Malonate utilization	-
Cellulose utilization	+
Melanin production	-
Tyrosinase production	+
Milk coagulation and peptonization	+
C-source utilization	
Glucose	+
Maltose	+
Arabinose	-
Fructose	-
Sucrose	+
Xylose	+
Inositole	+
Mannitol	+
Rhamnose	-
N-source utilization	
$NaNO_3$	+
$(NH_4)_2SO_4$	-
Arginine	+
Tyrosine	-
Asparagine	+
Histidine	+
Cysteine	-
Glutamate	-
Phenylalanine	-

+, positive; -, negative

Table 4. Antifungal activity of heat-treated culture broth of *S. nobilis* C51 against *B. cinerea*

Temperature (℃)	Relative remaining activity (%)
28	100
40	92.1±2.0
50	85.6±3.9
60	85.0±1.3
70	80.3±1.7
80	78.4±1.8
90	78.0±1.7
100	76.5±1.4
121*	76.4±0.4

**Autoclaved at 121℃ for 20 min*

Table 5. Antifungal activity of culture broth of *S. nobilis* C51 against *B. cinerea* at various pHs

pH*	Relative remaining activity (%)
2	85.5±0.8
3	91.1±0.4
4	91.6±1.0
5	92.9±4.5
6	100
7	88.0±6.8
8	85.5±6.2
9	79.3±3.1
10	75. 5±2.0
11	70.5±2.7
12	48.6±1.4

**pH of the culture broth was adjusted, and residual activities against B. cinerea were measured as relative to the activity at pH 6*

3.6 Greenhouse Experiment

To determine if this inhibitory efficacy of *S. nobilis* C51 works well under greenhouse conditions, a greenhouse experiment was done. When 0, 5, 10 and 20 time(s) diluted culture broth were used, the disease severity were 27.9 ± 1.07%, 40.49 ± 1.83%, 43.09 ± 2.78% and 49.51 ± 5.27% respectively. All treatments could control the disease efficiently and there were less lesion on the leaves of treatment than that of water control. The percentage of disease inhibition rate for the stock solution of *S. nobilis* C51 was 72.63±0.33%, and azoxystrobin was 72.51±0.80% (Table 6). The results suggested that *S. nobilis* C51 can be considered as an efficient agent as semilar as chemical fungicide for *B. cinerea* treatment.

4. DISCUSSION

In this study, an antagonistic strain against *B. cinerea* was isolated from soil and was named as *S. nobilis* C51. *In vitro* and greenhouse experiments indicated that *S. nobilis* C51 could be used as a good candidate for the biocontrol of gray mold in tomato caused by *B. cinerea.* In nature, plant coexist and interact with naturally colonized microflora including plant pathogens and beneficial microbes, which make the natural environment become the best source to screen antagonistic microorganisms. In this work, we collected soil samples from the farmland where the soil-borne and air-borne disease in crops occurred seriously and screened the microbial strains which have the ability to control *B. cinerea.* We focused on screening the

Streptomyces genus by using the selective medium since several strains of this genus have been used as biocontrol agents for a wide variety of fungal pathogens [28-29]. We obtained a strain encoded C51 showing the highest antagonistic activity against *B. cinerea* in the dual culture experiment. The morphological, physiological and molecular analysis assigned this isolate to be *S. nobilis*. Our results suggested a role of sampling area for screening the targeted antagonistic microorganism. Recently, Kanini et al. screened the indigenous *Streptomyces* with antifungal activity with high percentages from the area which is characterized by its high temperature and low humidity [22,30]. This environment may exert the stress on the indigenous populations of *Streptomyces* and possibly leads to the prevalence of microorganisms with antagonistic properties. As like, the situations of frequently occurrence of soil- and air-borne disease impose the selective pressure on the local population of *Streptomyces* and might confer them the ability to suppress the growth of the fungal pathogens.

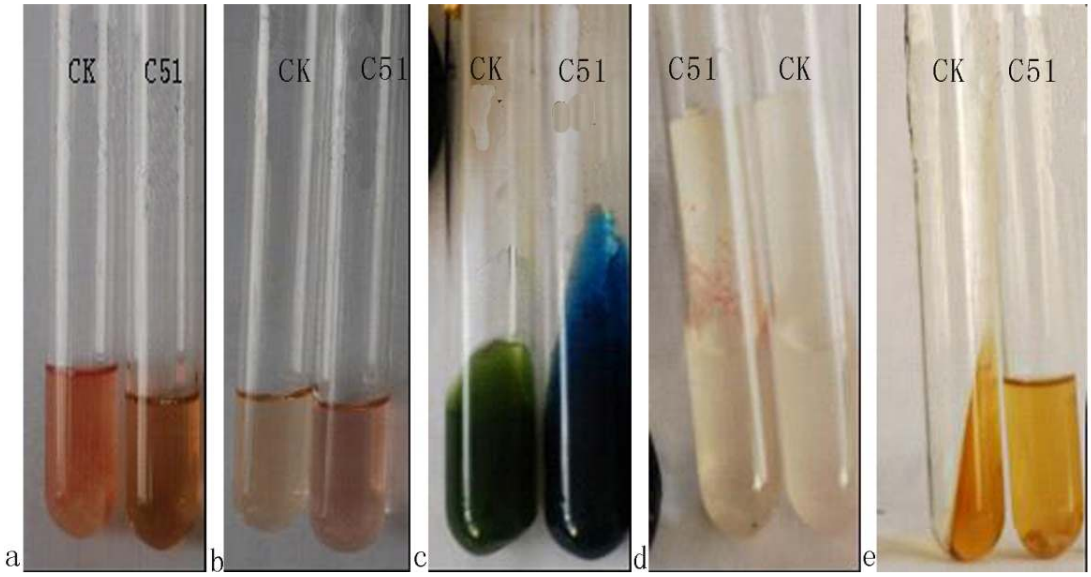

Fig. 2. The biochemical characterization of C51 strain. (a) Nitrate reduction; (b) MR reaction; (c) Citrate utilization; (d) Cellulose decomposition; (e) Gelatin liquefaction

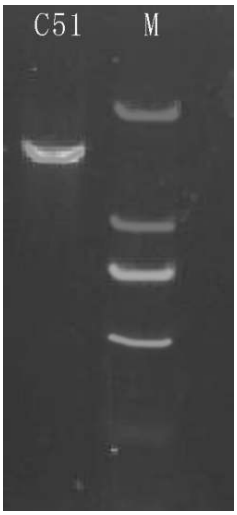

Fig. 3. The 16S rRNA electrophoretogram of C51 strain. M is the DL2000 marker

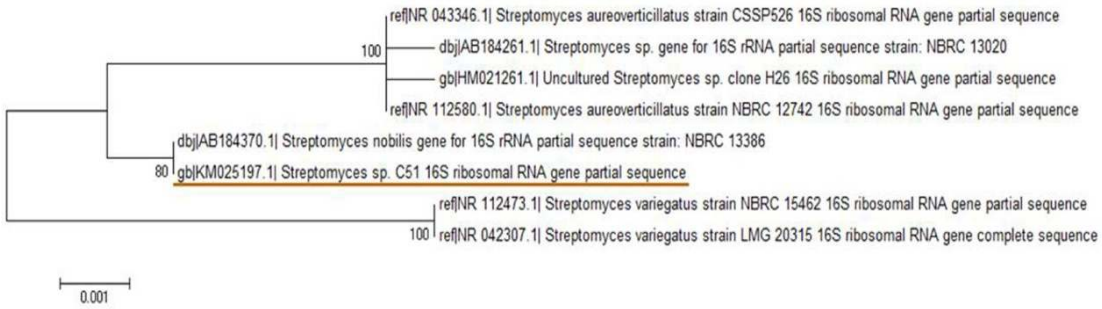

Fig. 4. Phylogenetic tree of the 16S rRNA based on the neighbour-joining method, showing the position of the *S. nobilis* C51 (GenBank accession number KM025197.1)

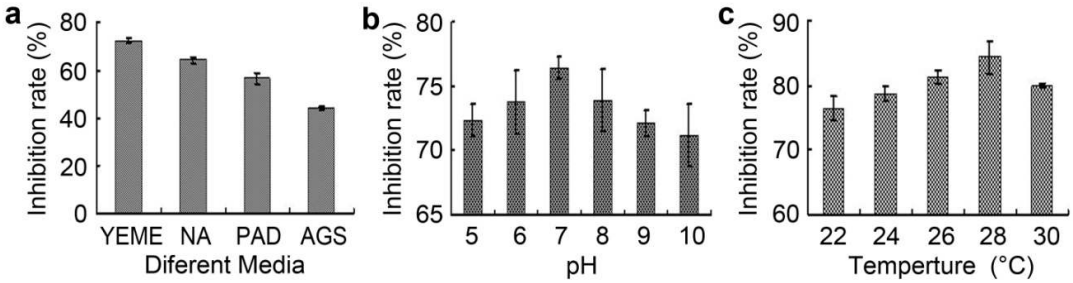

Fig. 5. Effects of culture conditions on antagonistic activity of C51 strain culture broth. (a) Medium, (b) pH, and (c) Temperature effect

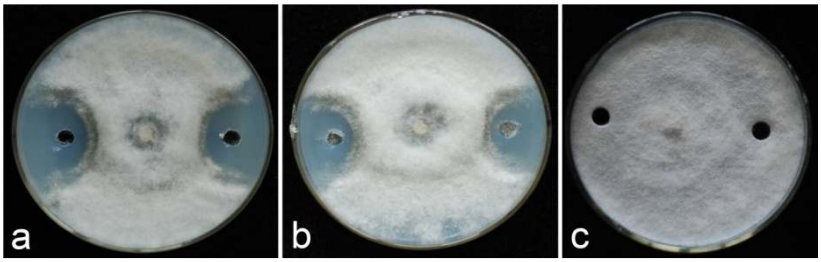

Fig. 6. Antifungal activity of the extract of *S. nobilis* C51 from the solid medium with or without *B. cinerea.* Inhibition zone caused by (a) Extract of *S. nobilis* C51 from the solid medium with *B. cinerea,* (b) Extract of *S. nobilis* C51 from the solid medium without *B. cinerea,* (c) Negative control (no *S. nobilis* C51 extract added)

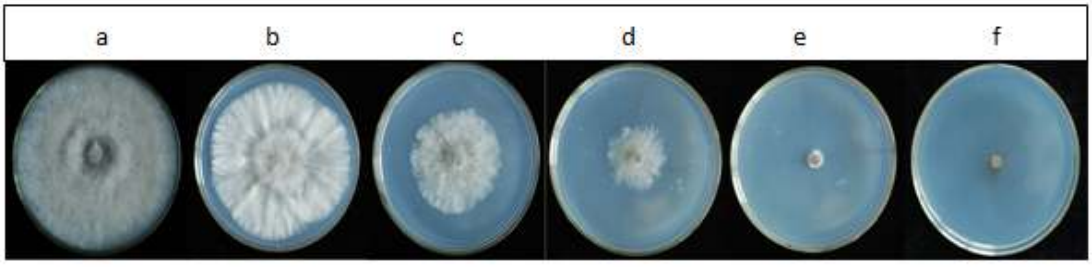

Fig. 7. The effects of cell-free culture broth of *S. nobilis* C51 on the growth of *B. cinerea*. *B. cinerea* was cultured on PDA (a), PDA with 80 (b), 40 (c), 20 (d), 10 (e), and 5 (f) folds dilution of cell-free culture broth of *S. nobilis* C51

Table 6. Inhibition rate of *B. cinerea* by *S. nobilis* C51 and other treatments under greenhouse conditions*

Treatment	Disease severity (%)	Disease inhibition rate (%)
The stock solution	27.90±1.07	72.63±0.33
5-fold dilutions	40.49±1.83	65.73±1.22
10-fold dilutions	43.09±2.78	56.90±0.67
20-fold dilutions	49.51±5.27	52.30±1.00
Azoxystrobin	27.04±1.28	72.51±0.80
spray with *B. cinerea* spores	96.54±1.55	

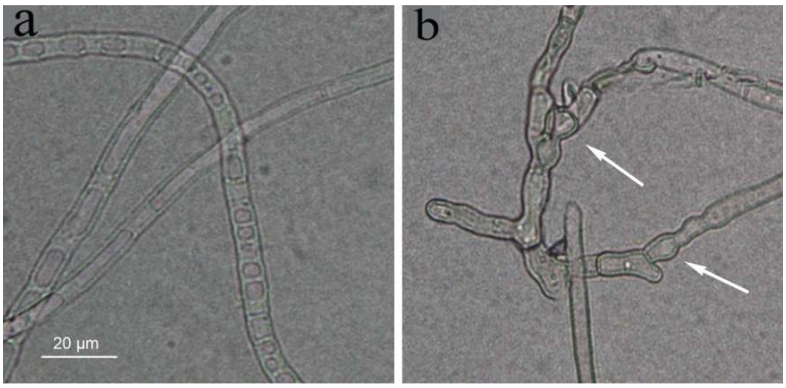

Fig. 8. Microscopic images of mycelium of *B. cinerea* grown on PDA plates with and without cell-free culture broth of *S. nobilis* C51. (a) The mycelia on the PDA plates, (b) The mycelia on PDA plates with cell-free culture broth of *S. nobilis* C51. The arrows indicated the abnomal swollen balloon-like structure of mycelium. (Scale bar=20 μm)

Recently, Kanini et al. [30] found that the extracts from the agar inhibition zone of the isolated *S. rochei* ACTA1551 could strongly suppress the growth of pathogen *Fusarium oxysporum*, meanwhile the same antifungal activity was observed from the extracts of the solid agar around the colony of *S. rochei* ACTA1551 without the presence of *F. oxysporum*. They, therefore, speculate that the antifungal activity of *Streptomyces* is fungus independent. Our results showed that the production of antifungal compounds by *S. nobilis* C51 does not require the target fungus and the expression of antifungal activity of *S. nobilis* C51 might be constitutive. The cell-free culture broth of *S. nobilis* C51 has strong ability to suppress the growth of *B. cinerea* further supports this conclusion. However, the induction pattern of antifungal activity for the *Streptomyces* might be fungal pathogen specific. For instance, Kanini et al. observed the antifungal activity only in the solid culture extracts but not in the liquid culture supernatant of *S. rochei* ACTA1551 when another soil-borne fungal plant pathogen *Rhizoctonia solani* was used as tested pathogenic fungus [30]. In addition, the intrinsic difference between solid and liquid culture including oxygen availability, the nutrient limitation and metabolitic intense may contribute to biosynthesis of antifungal substance in *Streptomyces* [31-32].

The antifungal activity of *Streptomyces* is usually related to the production of antifungal compounds and the extracellular hydrolytic enzyme such as chitinases, β 1-3 glucanases [33-34]. *S. violaceusniger* YCDE9 was reported not only to produce the extracellular hydrolytic enzymes but also to excrete three antifungal compounds [35]. Prapagdee et al. found that the extracellular hydrolytic enzymes in the exponential culture filtrate and secondary thermostable compounds in the stationary culture filtrate of *S. hygroscopicus* SRA14 play an important role in the inhibition of the growth of *Colletotriculum gloeosporioides* and *Sclerotium rolfsii* [36]. In the present study, the cell-free culture broth of *S. nobilis* C51 still retained 76.4% of antifungal activity even after treatment with autoclave at 121℃ for 20 min. We speculated that the antifungal substance produced by *S. nobilis* C51 is probably not a protein or an

extracellular enzyme, but rather a secondary metabolite. Thus, the further separation and identification of the bioactive substance from *S. nobilis* C51 are required and under investigation in our laboratory. Moreover, we found that the antifungal activity in the culture broth of *S. nobilis* C51 had broad thermal and pH stability. These features could make it possible for the bioactive components of this strain to be used as biocontrol agent under the diverse environmental conditions.

Previous study by light microscopic analysis revealed that the culture filtrates of *S. hygroscopicus* SRA14 caused the cellular changes in hyphal morphology including hyphal swelling, distortion and cytoplasm aggregation of fungal pathogens [36]. Joo also reported that the extracellular chitinase produced by *S. halstedii* AJ-7 is responsible for the abnormal hyphal morphology [37]. On the other hand, abnormal hyphal structure such as thickness and bulbous roundness of the inhibited fungal hyphae resulting from diffusible secondary compounds including antibiotics and metabolites has been previously reported [38]. In accordance with these observations, we monitored the abnormal changes in hyphal structure of *B. cinerea* after treatment with the culture broth of *S. nobilis* C51. Taken the nature of the thermal and pH stability of antifungal activity into account, the pathogenic fungal growth inhibition by the strain C51 is likely to be due to presence of extracellular metabolites which cause growth aberration, hyphal swelling and suppress the fungal growth.

5. CONCLUSION

In this investigation, *B. cinerea* has been shown to be inhibited by diluted C51 culture broth. The disease severity was lower when higher concentrations of C51 culture suspension were used to control gray mold in tomato. It means that the application of *S. nobilis* C51 may offer farmers an opportunity to limit the use of chemical fungicides and permit biological measures for the control of gray mold. Future research will be aimed at developing technology for the application of biocontrol agents in largescale and investigating the mechanisms of strain C51 for the control of gray mold in plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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