Appl Biochem Biotechnol (2015) 177:1338–1347
DOI 10.1007/s12010-015-1818-5

A Novel and Effective *Streptomyces* sp. N2 Against Various Phytopathogenic Fungi

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Received: 15 May 2015 / Accepted: 17 August 2015 /
Published online: 26 August 2015
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Abstract Phytopathogenic fungi would induce a variety of plant diseases, resulting in a severe reduction of agricultural output. However, the current plant disease control is mainly dependent on the environmentally and healthily hazardous chemical fungicides. Thus, the present work aimed to isolate an effective antagonistic microorganism against various soilborne phytopathogenic fungi. By dual culture with *Rhizoctonia solani*, a novel *Streptomyces* specie, *Streptomyces* sp. N2, was screened out from a total of 167 isolated actinomycetes, which displayed a strong inhibitory effect on *R. solani* (26.85± 1.35 mm of inhibition zone diameter). By means of macroporous resin and silica gel column chromatography coupled with preparative HPLC, an antifungal metabolite (3-methyl-3,5-amino-4-vinyl-2-pyrone, $C_6H_7O_2N$) was isolated and purified from *Streptomyces* sp. N2. The bioassay results showed that the purified antifungal metabolite could not only possess a broad-spectrum inhibitory effect on a range of plant pathogenic fungi in vitro (e.g., *R. solani*, *Pyricularia grisea*, *Fusarium oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, *Penicillium italicum*, and *Colletotrichum gloeosporioides*), but also had a significantly effective in vivo biocontrol efficacy on grape fruits anthracnose caused by *C. gloeosporioides*. Microscopic observation indicated that the antifungal metabolite from *Streptomyces* sp. N2 would exert its antimicrobial activity by disorganizing the cytoplasmic organelles of phytopathogenic fungi. The above results suggested that *Streptomyces* sp. N2 was one of promising fungicide for biocontrol of fungal plant diseases, especially due to its broad-spectrum and effective antagonist on various plant pathogens.

Keywords *Streptomyces* sp. N2 · Isolation · Metabolite · Antagonistic activity · Phytopathogenic fungi

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Introduction

The rhizosphere is a playground and battlefield for the tripartite interactions between soilborne pathogens, beneficial microorganisms, and the plant [1]. Once the soilborne pathogens, especially the fungal pathogens, can effectively escape the rhizosphere battle zone, they would reach and further infect the host tissue, resulting in a series of plant diseases [2, 3]. It is estimated that the plant diseases induced by soilborne pathogens would cause 10~16 % reduction of the global agricultural productivity [4].

Facing such a severe threat on global food security caused by plant diseases, it is extremely important to search and develop a highly effective fungicide against the soilborne plant pathogens. At present, chemical fungicides have been extensively used worldwide in current agriculture. However, the excessive use of chemical fungicides has led to many attendant problems, such as environmental pollution, deteriorating human health, development of pathogen resistance to fungicides, and phytotoxicity [5]. Due to being more environment-friendly, biological control is recognized as an alternative and promising approach to control the soilborne plant pathogens. To date, many antagonistic microbes have been developed as the biocontrol agents, like *Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp., *Trichoderma* spp., and nonpathogenic *Fusarium* spp. [6, 7]. For these antagonistic microbes, the mechanisms of pathogens suppression include production of antibiotics, cell wall-degrading enzymes, and parasitism (direct) and induction of host resistance (indirect) [8].

China is a large agricultural country with a great diversity of crops. Like other countries in the world, the outbreak of plant diseases is becoming an increasingly serious problem that affects the agricultural output. However, the control of plant diseases in China is mainly dependent on the chemical fungicides. Thus, the present work aimed to isolate an effective antagonistic microorganism, and a novel actinomycete strain, *Streptomyces* sp. N2, was screened out from the soil of Xishan National Forest Park of Kunming in Yunnan Province, which exerted a broad and efficient resistance to multiple plant pathogenic fungi.

Materials and Methods

Isolation and Screening of the Antifungal *Streptomyces* spp.

Soil samples were collected from China's Anhui Province, Guangdong Province, Hubei Province, Jiangxi Province, and Yunnan Province. The suspensions of the 18 soil samples were prepared by mixing 10 g soil with 90 ml distilled water, and 0.1 ml aliquots of serially diluted samples were plated on Gause's No.1 medium (soluble starch 20 g, KNO_3 1 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, NaCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 20 g, distilled water 1000 ml, pH 7.2~7.4), respectively. After 7 days of incubation at 30 °C, the *Streptomyces* colonies on the agar plates were picked on the basis of their morphological characteristics and further purified on Gause's No.1 agar.

All the obtained isolates of *Streptomyces* spp. were tested and screened in vitro antagonistic activity against *Rhizoctonia solani* by using a dual culture assay, as described by Boukaew et al. [9]. Circular piece of agar (5 mm diameter) was excised from the 7-day-old *Streptomyces* spp. colony, which was placed on the center of potato dextrose agar (PDA) medium (leaching solution of potato 200 g, sucrose 20 g, agar 20 g, distilled water 1000 ml). Four mycelial plugs of 2-day-old *R. solani* were placed on the PDA plate, 5 cm away from the *Streptomyces* spp.

After incubation at 28 °C for 2 days, the strongest antifungal *Streptomyces* sp. was screened out according to its inhibition zones of dual culture plates.

Morphological Characteristics and Identification of Isolate N2

The morphological characteristics of isolate N2 was observed by using a scanning electron microscope (JSM, 6360LV, JEOL, Tokyo, Japan): isolate N2 was grown on Gause's No.1 agar at 28 °C for 7 days. The prepared samples were mounted on stubs, air-dried in a desiccator and splutter-coated with gold, and then and viewed on the scanning electron microscope at an accelerating voltage of 20 kV.

The identification of isolate N2 was according to its 16S rDNA gene sequencing and phylogenetic analysis: total genomic DNA was extracted using the Ezup DNA extraction kit (Sangon Biotech, Shanghai, China). The 16S rDNA was amplified using the primers, 27F (AGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCR products were gel purified with Gel purification Kit (Sangon Biotech, Shanghai, China). The obtained 16S rDNA sequence was analyzed by the NCBI database using BLAST algorithms at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using the Phylogeny.fr software available at: http://www.phylogeny.fr/.

Isolation and Purification of the Antifungal Metabolite Produced by *Streptomyces* sp. N2

The fermentation of *Streptomyces* sp. N2 was carried out in a 250-ml Erlenmeyer flask containing 40 ml of fermentation medium (sucrose, 40 g; corn starch, 20 g; corn steep liquor, 20 g; $(NH)_2SO_4$, 2 g; KH_2PO_4, 1 g; $MgSO_4$, 1 g; $MnSO_4 \cdot H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; distilled water, 1000 ml; pH 7.2 ~ 7.4), which was inoculated with a 2-cm^2 cell plug from the fresh slant, and cultivated at 28 °C on a rotary shaker at 200 rpm for 6 days.

The fermentation broth was centrifuged at 5000 rpm for 20 min, and then the mycelium was extracted with *n*-butyl alcohol for 24 h at room temperature. Then the extract was filtered, and the filtrate was evaporated with a rotary evaporator at 45 °C. The obtained aqueous concentration was successively subjected to column chromatography on SIPI-21 macroporous resin and silica gel, using the gradient mixtures of methanol-water (0:100, 20:80, 40:60, 80:20, and 100:0, *v*/*v*) and chloroform-methanol (100:0, 80:20, 70:30, 60:40, and 50:50, *v*/*v*) as elutions, respectively. By using thin layer chromatography (TLC) together with the oxford plate assay system, the fractions that had the same retention factor (Rf) value and antifungal activity were pooled and evaporated to dryness in vacuo. Final purification of the antifungal factions was performed via a preparative HPLC system (Shimadzu, LC-6AD): Shim-pack Prep-ODS column (20 mm × 250 mm, 10 μm); flow rate, 5 ml/min; mobile phase, 65:35 (*v*/*v*) of methanol-water; detection wavelength, 254 nm.

In Vitro Antagonism of the Antifungal Metabolite Against Plant Fungal Pathogens

The 5-mm-diameter inoculum plugs of *R. solani*, *Pyricularia grisea*, *Fusarium oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, *Penicillium italicum*, and *Colletotrichum gloeosporioides* were cut from the margin of 7-day-old colonies on PDA plates, respectively. Each plug was inoculated on the central of PDA pour plate mixed with 5 μg/ml antifungal

metabolite isolated and purified from *Streptomyces* sp. N2 broth, and then incubated at 28 °C. The growth inhibitory effects on plant fungal pathogens were represented as inhibition percentage, which was calculated according to the following equation: Inhibition (%)=[(Growth diameter in untreated control−Growth diameter in treatment)×100]/Growth diameter in untreated control [10].

In Vivo Biocontrol Capability of the Antifungal Metabolite

Grape fruits were used to test the in vivo biocontrol efficacy of the antifungal metabolite produced by *Streptomyces* sp. N2. Firstly, the grapes were surface disinfected in 70 % ethanol for 2 min, and followed by three rinses in sterile water. Next, each grape was cut into an approximately 8 mm length and 5 mm depth of triangular wound. The wound was artificially inoculated with a 5-mm-diameter inoculum plug of *C. gloeosporioides*, and then treated with 100 μl of the antifungal metabolite (10 μg/ml). After 5 days cultivation at 28 °C, the degree and size of the canker lesions were observed and recorded.

Effect of the Antifungal Metabolite on *R. solani* Mycelial Morphology

R. solani was inoculated on the PDA plate containing 5 μg/ml of the antifungal metabolite. After 5 days cultivation at 28 °C, a small tuft of *R. solani* mycelia was picked up and washed with 75 % (*v*/*v*) ethanol. The air-dry mycelium was put on a clean microscope slide and stained with a drop of Lacto-phenol cotton blue (phenol 10 g, cotton blue 0.5 g, glycerol 20 ml, lactic acid 10 ml, deionized water 10 ml). Then the stained mycelia was visualized by an optical microscope (Olympus CX31, Japan) equipped with camera.

Results

Isolation and Screening of Antifungal Actinomycetes

A total of 167 actinomycetes were isolated from the collected soil samples. According to the further screening with a dual culture assay, it was found that 12 isolates (serial number: N2, D8, E7, O3, I1, K3, JI, G8, P1, A10, F7, G1) exhibited the antagonistic activity against *R. solani*, and Fig. 1 showed the inhibition zone diameters of *R. solani* under the action of the above isolates.

From Fig. 1, it was found that strain N2 (isolated form Xishan National Forest Park of Kunming in Yunnan Province, China) was the most effective antagonist against *R. solani* among the 12 isolates, which inhibition zone diameter reached 26.85±1.35 mm. Therefore, isolate N2 was chosen as a potential biocontrol actinomycete for further study.

Characterization and Identification of Isolate N2

The morphological characteristics of isolate N2 was visualized by using scanning electron microscope. From Fig. 2, it was observed that isolate N2 formed an extensively branched substrate mycelium, aerial hyphae which carried elliptic, smooth-surfaced spores in rectiflexibiles spore chains. A phylogenetic tree of isolate N2 was constructed using 16S rDNA gene sequence (1450 bp), as shown in Fig. 3. With an alignment covering at least 90 %

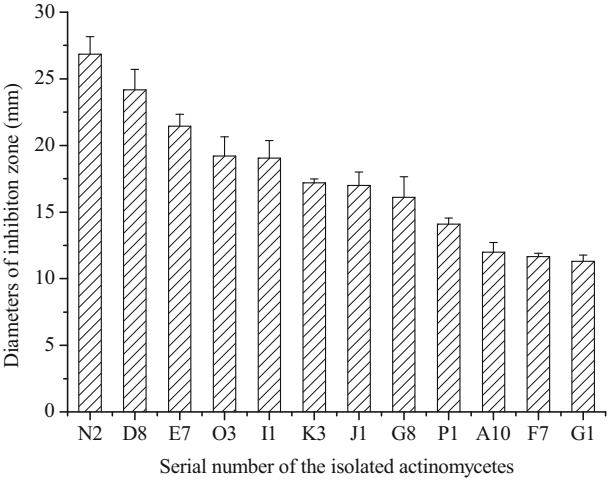

Fig. 1 The inhibition zone diameters of *R. solani* under the action of the 12 isolated actinomycetes (serial number: N2, D8, E7, O3, I1, K3, JI, G8, P1, A10, F7, G1). *Values* represent the averages of three measurements and *error bars* indicate standard deviation

of the gene length at greater than 90 % nucleotide identity using BLAST against nr/nt database at the NCBI website, twenty-two 16S rDNA sequences were found from the strains of genus *Streptomyces*, such as *Streptomyces prasinopilosus*, *Streptomyces graminearus*, *Streptomyces phaeogriseichrom*, *Streptomyces griseofuscus*, and *Streptomyces costaricanus*. However, it was difficult to differentiate between N2 isolate and the species of genus *Streptomyces*, which indicated that the isolate N2 was a novel *Streptomyces* specie. Therefore, isolate N2 was named as *Streptomyces* sp. N2.

Production of the Antifungal Metabolite from *Streptomyces* sp. N2

After the sequential column chromatography on macroporous resin and silica gel, the collected antifungal fraction was further purified using preparative HPLC, and Fig. 4 showed its HPLC spectrum. It was observed that the collected antifungal fraction had three sub-fractions (named metabolite I, II, and III, respectively), which retention time was 29.9, 31.1, and 38.4 min, respectively. Among the three sub-fractions, metabolite I was the main component, occupying 54.47 % of the total peak area ratio. Bioactivity determination showed that metabolite I exhibited the strongest antifungal activity. By further spectroscopic analyses, it was found

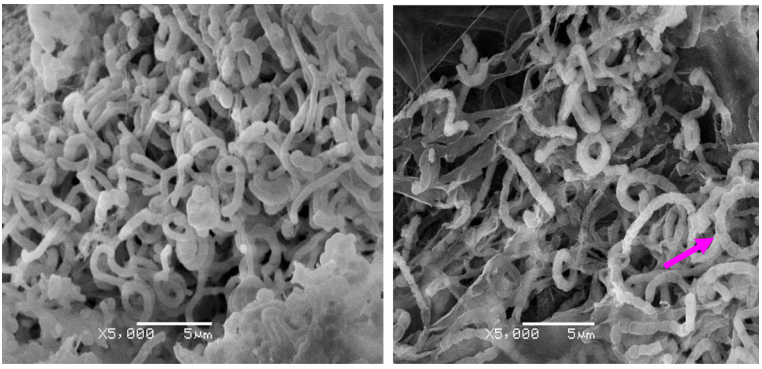

Fig. 2 Scanning electron micrograph of isolate N2 (the *arrow* indicated the spores chains)

Fig. 3 The phylogenetic tree of isolate N2 based on the 16S rDNA gene sequence

that the antifungal metabolite I was 3-methyl-3,5-amino-4-vinyl-2-pyrone (molecular formula, $C_6H_7O_2N$), which was validated as a novel structural biosubstance.

In Vitro and In Vivo Biocontrol Efficacy of the Antifungal Metabolite I

The antifungal metabolite I was prepared to assess the biocontrol efficacy of *Streptomyces* sp. N2. Firstly, the in vitro antifungal activity of metabolite I against phytopathogenic fungi was determined. It was observed visually that the antifungal metabolite I from *Streptomyces* sp. N2

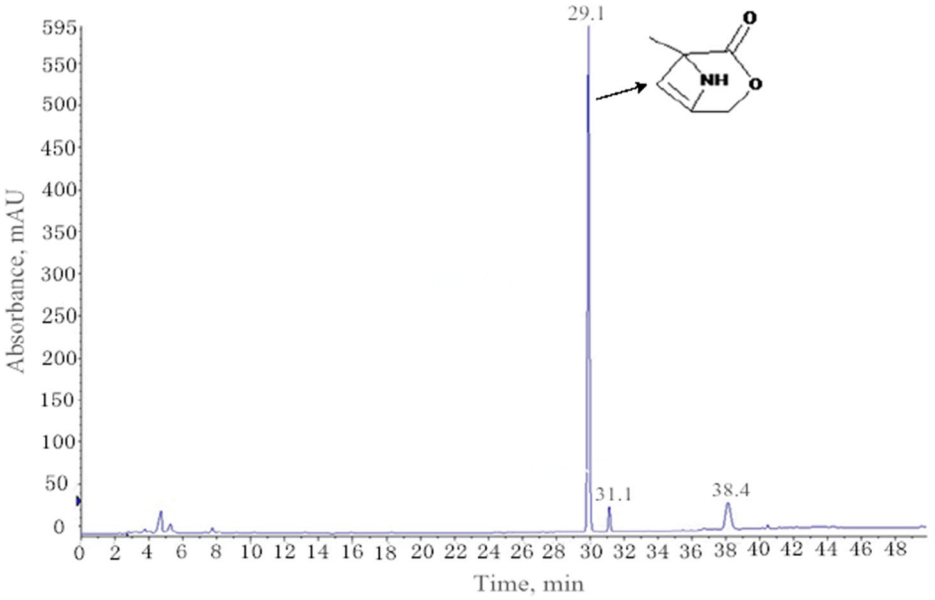

Fig. 4 Preparative HPLC spectrum of the collected antifungal fraction through the sequential column chromatography on macroporous resin and silica gel

displayed a broad-spectrum inhibitory effect on a range of plant pathogenic fungi, such as *R. solani*, *Pyricularia grisea*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, *Penicillium italicum*, and *C. gloeosporioides*, as shown in Fig. 5. By further calculating the growth diameters under metabolite I treatment and the untreated control, the inhibition percentage of the above phytopathogenic fungi was 78.07, 71.59, 81.48, 59.12, 88.60, and 48.65 %, respectively, as shown in Table 1.

Moreover, the in vivo biocontrol efficacy of the antifungal metabolite I was tested in controlling decays of grape fruits caused by *C. gloeosporioides*. When the grapes were treated with the antifungal metabolite I from *Streptomyces* sp. N2, the degree and size of the canker lesions were significantly smaller than those untreated, as shown in Fig. 6. Compared to the untreated grapes with a 2.56±0.07 cm^2 of lesion area, the treated grapes had almost no symptoms of lesions, suggesting that the antifungal metabolite I had an effective biocontrol efficacy in grapes.

Interaction Mechanism of the Antifungal Metabolite I on Phytopathogenic Fungi

The biocontrol mechanism of the antifungal metabolite I on phytopathogenic fungi was investigated by using *R. solani*. Microscopic observations showed that the antifungal metabolite I from *Streptomyces* sp. N2 would cause significant morphological changes and structural alterations in *R. solani* mycelium, as shown in Fig. 7. The untreated mycelium had regular hyphae with a smooth surface and integrated cell organelles. However, after treated with the antifungal metabolite I, *R. solani* hypha became clearly bending, short swollen, and irregularly branching. Furthermore, the treated *R. solani* showed a significant cellular disorganization, in which the

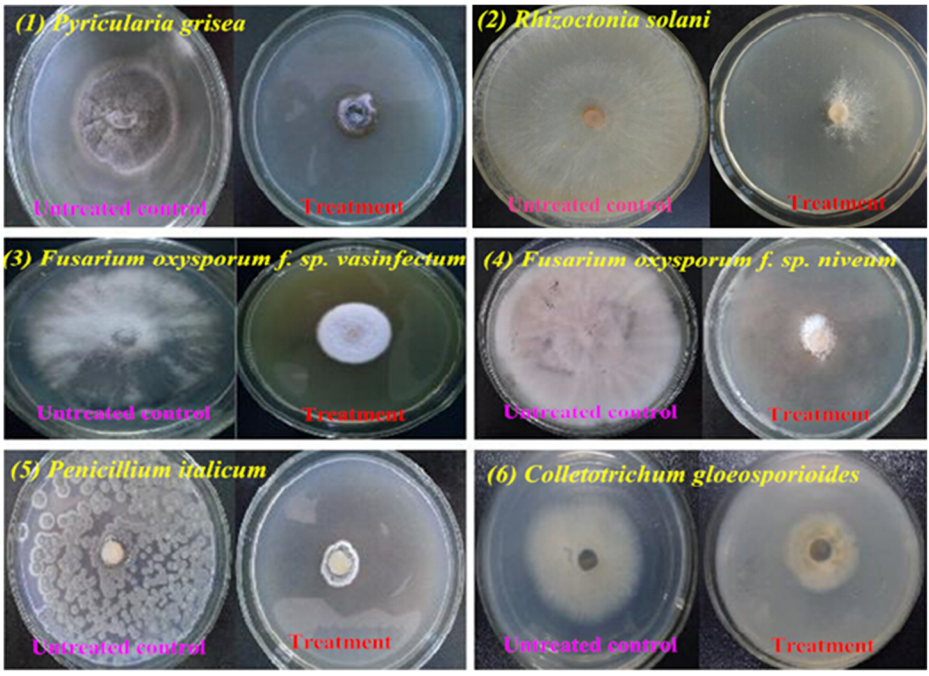

Fig. 5 The mycelia growth of *R. solani*, *Pyricularia grisea*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, *Penicillium italicum*, and *C. gloeosporioides* under untreated control and the treatment with the antifungal metabolite purified from *Streptomyces* sp. N2

Table 1 Inhibition percentage of the phytopathogenic fungi under the action of *Streptomyces* sp. N2

Phytopathogenic fungi	Growth diameter (mm)[a]		Inhibition percentage (%)
	Untreated control	Treatment	
Rhizoctonia solani	90.10±2.10	26.00±1.95	78.07
Pyricularia grisea	64.70±2.75	24.11±2.40	71.59
Fusarium oxysporum f. sp. *niveum*	90.00±0.58	23.19±0.50	81.48
Fusarium oxysporum f. sp. *vasinfectum*	82.70±0.15	38.54±0.80	59.12
Penicillium italicum	89.60±0.39	17.30±0.36	88.60
Colletotrichum gloeosporioides	71.50±0.35	40.60±0.30	48.65

[a] The results were means±SD (standard deviation) of triplicate determinations

damaged and disintegrated cytoplasmic organelles were visually observed. According to the morphological changes and structural alterations in *R. solani* mycelium, it could be inferred that the interaction mechanism of the antifungal metabolite I on phytopathogenic fungi was via changing the structure of cell membranes and further disorganizing the cytoplasmic organelles.

Discussion

During the whole growth processes, plants are under continuous attack by a vast number of soilborne pathogens, especially the fungal pathogens. For example, the outbreak of the destructive rice blast disease and rice sheath blight disease is a serious and recurrent problem in the rice-growing regions of the world, which were caused by *Magnaporthe grisea* (anamorph. *Pyricularia grisea*) and *R. solani*, respectively [10, 11]. Fusarium wilt is known to be one of the most severe and prevalent soilborne diseases that result in serious economic loss [12], such as the fusarium wilt of watermelon caused by *F. oxysporum f.* sp. *niveum* [13] and the fusarium wilt of cotton caused by *F. oxysporum* f. sp. *vasinfectum* [14]. The predominant and destructive plant disease anthracnose caused by fungi in the genus *Colletotrichum* would affect the yield and quality of many plants, including vegetables, fruits,

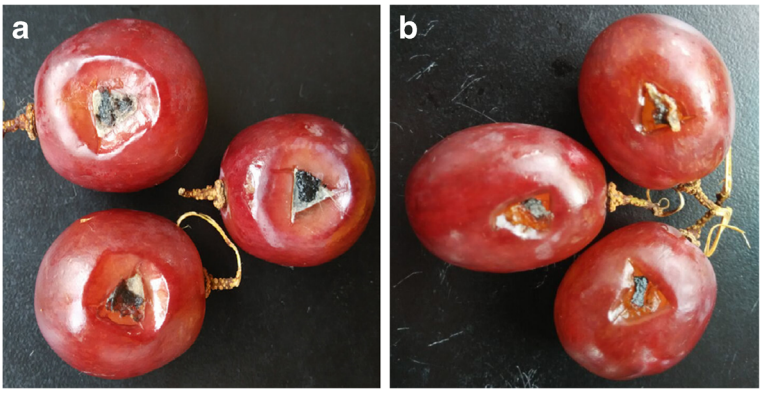

Fig. 6 The in vivo biocontrol efficacy of *Streptomyces* sp. N2 in controlling decays of grape fruits caused by *C. gloeosporioides*. **a** Untreated control group; **b** treated with the antifungal metabolite purified from *Streptomyces* sp. N2

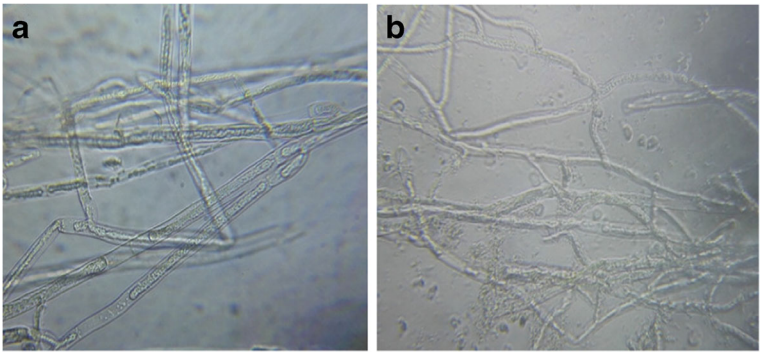

Fig. 7 The morphological changes of *R. solani* under light microscope (×400). **a** Untreated control group; **b** treated with the antifungal metabolite purified from *Streptomyces* sp. N2

and trees [15]. Additionally, frequent occurrence of postharvest diseases would result in a significant loss to fruit, such as the green and blue mold decay of caused by *Penicillium digitatum* and *Penicillium italicum* in citrus fruit [16].

However, plant pathogens are difficult to control because their populations are variable in time, space, genotype, and even self-evolution [17]. The current plant disease control is mainly dependent on the environmentally and healthily hazardous chemical fungicides. Therefore, it is urgent to search and develop a highly effective biofungicide. It is well known that actinomycetes have the ability to produce a variety of bioactive secondary metabolites. According to statistics, approximately 60 % of agriculturally useful antibiotics are produced by different *Streptomyces* species [18]. For example, many antifungal metabolite compounds have been successfully explored for commercial use in the fungal diseases control for rice, vegetables, and fruits, such as Blasticidin S of *Streptomyces griseochromogenes*, Kasugamycin of *Streptomyces kasugaensis* and *Streptomyces kasugaspinus*, Polyoxins of *Streptomyces cacaoi* var. *asoensis*, and Validamycin A of *Streptomyces hygroscopicus* var. *limoneus* [19].

In the present work, a novel actinomycete, *Streptomyces* sp. N2, was successfully isolated and screened out from Xishan National Forest Park of Kunming in Yunnan Province, China. One antimicrobial metabolite (3-methyl-3,5-amino-4-vinyl-2-pyrone, $C_6H_7O_2N$) was separated and purified from *Streptomyces* sp. N2, which exerted its antifungal activity by disorganizing the cytoplasmic organelles of phytopathogenic fungi. Interestingly, this antifungal component exhibited a broad-spectrum and effective antagonistic activity on a range of phytopathogenic fungi, suggesting that *Streptomyces* sp. N2 had a potential ability to be developed as an efficient biocontrol agent against various fungal plant diseases. However, it should be pointed out that further studies were needed to identify the chemical structure of the purified antifungal metabolite and assess its biocontrol efficacy in planta.

Acknowledgments This work was financially supported by the Training Program for Young Scientists of Jiangxi Provincial Department of Science and Technology (20142BCB23025), International Scientific and Technological Cooperation Projects of Jiangxi Provincial Department of Science and Technology (20141BDH80033), and Jiangxi Undergraduate Training Programs for Innovation and Entrepreneurship (201410410008).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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