Nematicidal *Streptomyces* against *Meloidogyne incognita* by hydrosoluble macromolecules

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Abstract

Background: Although actinomycetes, which produce antibiotics, have been reported to produce nematicidal metabolites, macromolecules with nematicidal activities have rarely been described.

Results: The culture filtrates of *Streptomyces albogriseolus* strain DN41 and *S. fimicarius* strain D153 presented 82.13 % and 86.96 % nematicidal activity against *Meloidogyne incognita*, respectively, after 12 h. A pot experiment highlighted that these culture filtrates were characterised by significant efficacy. The biocontrol efficiency of strain DN41 reached 61.68 % and that of strain D153 68.33 % after 30 days at a dose of 100 mL pot⁻¹. The active metabolites produced by the two strains were hydrosoluble and sensitive to heat, with molecular weights above 8000 Da for DN41 and of 1000–8000 Da for D153. However, these metabolites were negative to protein reaction. A chemical characteristic test indicated that these active metabolites were polar polymers that contained a large amount of sugar and phenolic hydroxyl compounds.

Conclusions: These results revealed *S. albogriseolus* strain DN41 and *S. fimicarius* strain D153 as potential agents for the control of root-knot nematodes, as hydrosoluble macromolecules have good compatibility and are convenient for practical application.

Background

The root-knot nematode *Meloidogyne incognita* is distributed worldwide and causes increasing loss for vegetable crops annually [1, 2]. Among various control methods, the application of chemical nematicides is the most efficient and most widely used one [3]. However, most chemical agents have been regulated because they are considerably unfriendly to humans and the environment [4]. Therefore, safe and efficient biological control agents have increasingly been used as substitutes for chemical nematicides.

Actinomycete bacteria, especially the major group *Streptomyces*, produce a large amount of active secondary metabolites that can be used as biological control agents [5, 6]. For instance, many nematicidal secondary metabolites produced by *Streptomyces*, such as fervenulin-isolated from *Streptomyces* sp. CMU-MH021 [7], avermectin-isolated from *S. avermitilis* G8-17 [8], nemadectin-isolated from *S. microflavus* neau3 [9], actinomycins-isolated from *S. antibioticus* M7 [10], milbemycin-isolated from *S. bingchenggensis* BCJ60 [11], carbazomycins D and F-isolated from *Streptomyces* sp. CMU-JT005 [4], macrocyclic lactones-isolated from *S. avermitilis* NEAU1069 [12], angumycinones-isolated from *Streptomyces* sp. P294 [13], fungichromin B-isolated from *Streptoverticillium albireticuli* HA10002 [14], and SH2-isolated from *S. hydrogenans* DH16 [15], have been reported. Notably, majority of these metabolites have low molecular weights and are water-insoluble compounds, and hydrosoluble macromolecules with nematicidal activities have rarely been described.

In the present study, two actinomycete strains with high nematicidal activity against *Meloidogyne incognita*, namely *Streptomyces albogriseolus* strain DN41 and *S. fimicarius* strain D153, were isolated from soil samples. The preliminary isolation and structural determination of the active metabolites produced by these strains indicated that these nematicidal metabolites were hydrosoluble, heat-sensitive macromolecules, but not proteins. Furthermore, the active substances in the culture filtrates were characterised and the control effect of the filtrates on root-knot nematodes was assessed.

Results

Nematicidal activity of culture filtrates and extracts

The culture filtrates of the two *Streptomyces* strains displayed significant nematicidal activity against *M. incognita* after 12 h, 24 h, and 48 h. The mortality generated by strain DN41 reached 82.13 % and that by strain D153 86.96 % after 12 h. Notably, the highest effects exceeded 96 % mortality after 48 h. (Fig. 1A)

After the extraction of the organic solution, the aqueous phase was characterised by the strongest nematicidal activity and the respective mortality generated by strains DN41 and D153 surpassed 98.5 % after 12 h. In contrast, the extracts from the ethyl acetate and n-butanol phases did not display significant nematicidal activity. (Fig. 1 B, C)

Chemical characteristics of active metabolites

The active culture filtrates of strains DN41 and D153 were sensitive to heat and their activity were decreased with the increasing of temperature. Nematicidal activity was the highest at 37 °C, then decreased by 66 % for strain DN41 and 50 % for strain D153 at 47 °C, and was inhibited at 67 °C. (Fig. 2 A, B)

The nematicidal activity of dialysate DN41 reached 98.00 % 12 h after dialysis with a molecular weight cut-off at 8000–10 000 Da, whereas no activity was detected for dialysate D153. In contrast, when performing a dialysis with a molecular weight cut-off at 1000 Da the nematicidal activity of dialysate D153 reached 94.99 % after 12 h. (Fig. 2 C, D)

Notably, enzyme hydrolysis with proteinase-K did not significantly affect the dialysate of either strain. Furthermore, the reactions of ninhydrin biuret and ammonium sulphate precipitation were negative, which indicated that there were no amino acids, peptides, or proteins in the dialysates of both culture filtrates. Fehling's solution reacted with both dialysates and resulted in brick red precipitates, whereas Keller-Kiliani reactions were negative. This implied that the active substances contained reducing sugar but no α -deoxyglucose. Moreover, ferric chloride reactions were negative with the two dialysates, while potassium ferricyanide-ferric chloride tests were positive, which highlights that the dialysates contained aromatic amines and their reductive substances but no phenolics. Additionally, the production of lead acetate precipitates revealed there were tannins. However, the TLC detection of hydrolysed samples indicated there was no gallic acid nor catechinic acid. Likewise, phenolics, lactones, steroids, triterpenoids, and saponins were not detected in the two active dialysates. (Table 1)

Finally, the organic elemental analysis determined that the molecular formula of the active metabolite produced by strain DN41 was $(C_{34}H_{75}O_{63}N)_n$, and that of the active metabolite associated with strain D153 was $(C_7H_{13}O_9N)_n$ (Table 2).

Pot experiment

The culture filtrates of the two strains were significantly more efficient against *M. incognita* than the negative control was after 30 days of treatment. Notably, the inhibition ration in per 1g soil of the positive control reached 96.87 %, and the highest inhibition ration of the strain DN41 and D153 was of 81.54 % at the dose of 180 mL pot⁻¹ and the strain D153 was 97.56 % at the dose of 100 mL pot⁻¹, respectively (Fig. 3 A). The inhibition ration in per 1g root of the positive control reached 77.83 %, and the highest inhibition ration of the strain DN41 was of 66.42 % at the dose of 180 mL pot⁻¹ and the strain D153 was 77.79 % at the dose of 100 mL pot⁻¹, respectively (Fig. 3 B). The biocontrol efficiency of the positive control reached 68.33 %, and the highest biocontrol efficiency of strains DN41 and D153 was of 61.68 % and 68.33 %, respectively, at the dose of 100 mL pot⁻¹ (Fig. 4). Furthermore, the culture filtrates of both strains did not damage the plants, and, after 30 days, promoted greater plant height than the negative control did (Fig. 5).

Discussion

Natural products produced by actinomycetes, especially *Streptomyces* spp., are important biological resources to control root-knot nematodes [7]. In recent years, there have been multiple studies on the control of root-knot nematodes through the secondary metabolites produced by Streptomyces [16]. Notably, the culture filtrates of *S. avermitilis* Manp [17] and *S. hydrogenans* DH16 [15] have been reported to cause 89.54 % and 90.00 % mortality against *M. incognita*, respectively, after 72 h. In our study, *S. albogriseolus* strain DN41 and *S. fimicarius* strain D153 were newly reported to have effect on *M. incognita*. Notably, the mortality generated by the culture filtrates of *S. albogriseolus* ND41 and *S. fimicarius* D153 has

been reported to exceed 82 % after 12 h and 96 % after 48 h. This highlights that the two strains have a better effect on the control of *M. incognita* in vitro. In our pot experiment, the treatments with the culture filtrates of strains DN41 and D153 were characterised by a significant biocontrol efficiency, especially at the dose of 100 mL pot⁻¹, and their effects were close to the positive control avermectin.

The nematocidal metabolites of the two strains of actinomycetes evaluated in the present study both are compounds with a large polarity and are soluble in water but only slightly soluble in organic solvents. Their active substances are sensitive to heat and, hence, are inhibited at high temperatures. Through dialysis, we determined that the molecular weight of the active substance of strain A is above 8000–10 000 Da, while that of the active substance of strain B is relatively small, namely between 1000 and 8000 Da. We first speculated that the active substances were proteins. However, after the addition of Protease-K, the activity of these substances was not affected. Furthermore, the reactions of biuret and ninhydrin, as well as the precipitation of ammonium sulphate, were all negative, which indicates that the active substances of the two strains of Actinomyces were heat-sensitive macromolecular compounds, but not proteins. Notably, these compounds contained a large amount of reducing sugars, but no α -deoxyglucose, lactone ring structure, steroids, nor triterpenes. Additionally, the metabolites did not contain phenolic compounds, but included aromatic amines and reductive substances. Based on the hydrolysis with potassium ferricyanide-ferrous chloride reaction and lead acetate precipitation reaction, we inferred that the samples might be tannins, and gallic acid, catechin, and the hydrolysed samples would all be positive to the same chromogenic reaction. However, no gallic acid nor catechin was detected with TLC after hydrolysis. A possible explanation is that the large amount of phenolic hydroxyl in the structure of the active substance induced the positive result of the colour reaction of potassium ferricyanide-ferrous chloride. Therefore, the nematocidal compounds may be polymers containing number of phenolic hydroxyl groups and sugars. Based on the elemental analysis, the molecular formula of the active substance of strain DN41 is (C₃₄H₇₅O₆₃N)_n, and that of the active substance of strain D153 is (C₇H₁₃O₉N)_n. Nonetheless, the structures of these macromolecular compounds are complex, and further investigation is required to clarify the bonding between the groups in these compounds.

To date, few hydrosoluble macromolecules with nematocidal activity have been reported. The water-soluble nucleoside antibiotic 1100-50, which was isolated from *S. lavendulae* SANK 64297, partly inhibited *M. hapla* J2s after 24 h [18]. Moreover, the actinomycete strain Snea253 produced amino oligosaccharides with a 98.21 % nematocidal activity against *M. incognita* 24 h after treatment with 1 mL of crude extracts [19]. In the present study, we discovered new hydrosoluble macromolecule polymers with a strong nematocidal activity, which contain number of sugars and phenolic hydroxyl compounds.

Conclusions

In conclusion, in our study, the two strains of *S. albogriseolus* ND41 and *S. fimicarius* D153 showed significant nematocidal activity against *M. incognita*, and significant biocontrol potentials in pot experiment, especially at the dose of 100 mL pot⁻¹.

Moreover, the two strains showed no damage on the plant growth. We discovered new hydrosoluble macromolecule polymers with a strong nematocidal activity, which contain number of sugars and phenolic hydroxyl compounds. As hydrosoluble compounds have good compatibility and are convenient for application, the results of our study provide a baseline for the development of novel nematocides against root-knot nematodes.

Methods

Preparation of nematodes and actinomycetes

M. incognita were obtained from infected tomato plants based on Kihika et al. [20]. Root pieces with galls were uprooted and washed with running water to remove the soil. Nematode eggs were selected, under a dissecting microscope, with a 300-mesh sieve in a culture plate containing sterile water, then placed in dark conditions at $27\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 2–5 days. Hatched juveniles (J2s) were collected for subsequent nematicidal activity assay and pot experiment.

The actinomycetes *S. albogriseolus* DN41 and *S. fimicarius* D153 were isolated from soil samples obtained from tomato roots infested with *M. incognita* in Yunnan Province, China. The strains DN41 and D153 were cultured in sterile liquid MB medium at $28\text{ }^{\circ}\text{C}$ and 180 rpm for 7 days [21]. The cultures were then filtered with a $0.22\text{-}\mu\text{m}$ membrane and the filtrates were stored at $4\text{ }^{\circ}\text{C}$ for subsequent use.

Nematicidal activity assay

The nematicidal activity of culture filtrates, extracts, and dialysates against *M. incognita* were measured according to the modified method of Park et al. [22]. Freshly hatched J2s were added to a 16-well plate (200 J2s in $20\text{ }\mu\text{L}$) and treated with 1 mL test samples. Sterile water served as control. Every treatment had three replicates and was repeated three times. Plates were placed in an incubator at $25\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$. The number of dead juveniles was counted after 12, 24, and 48 h. Nematodes were considered dead if their bodies were straightened and could not move upon contact with a fine needle, as observed under a microscope. Correction mortality was calculated as follows: Correction mortality (%) = $(D_{\text{tr}} - D_{\text{c}}) / (D_{\text{T}} - D_{\text{c}}) \times 100$, where D_{tr} is the death number in the treatment group, D_{c} is the death number under control conditions, and D_{T} is total death number.

Extraction of active metabolites

The culture filtrates of the two *Streptomyces* strains were extracted with ethyl acetate firstly and raffinate phase were extracted with n-butanol sequentially. The ethyl acetate, n-butanol, and aqueous phase extracts were dissolved in methanol and diluted to a 5 % methanol aqueous solution. The final concentration of each sample was adjusted to 1 mg mL^{-1} for the nematicidal activity test.

Culture filtrates (1 mL) with nematicidal activity were added to 2 mL centrifugal tubes, then heated 1 h in a water bath at $37\text{ }^{\circ}\text{C}$, $47\text{ }^{\circ}\text{C}$, $57\text{ }^{\circ}\text{C}$, $67\text{ }^{\circ}\text{C}$, $77\text{ }^{\circ}\text{C}$, $87\text{ }^{\circ}\text{C}$, and $97\text{ }^{\circ}\text{C}$, respectively. The nematicidal activity of each sample was tested after heating.

The culture filtrates were eluted with XAD-16 resin (Amberlite, USA) to remove non-polar compounds. Eluates were placed into pretreated dialysis bags (Spectrumlabs, USA) with a molecular weight cut-off at 1000 Da and $8000\text{--}10\text{ }000\text{ Da}$, respectively, and dialyzed for 24–48 h. The dialysis buffer was replaced every 3–6 h. The dialysates were freeze-dried. Parts of the dialysates were diluted with deionized water to a concentration of 1 mg mL^{-1} for the nematicidal activity test.

Determination of chemical characteristics

Each active dialysate solution (1 mL) was transferred to a 2-mL centrifugal tube, to which $1\text{ }\mu\text{L}$ of 20 mg mL^{-1} proteinase-K was added for hydrolysis, 2 h at $37\text{ }^{\circ}\text{C}$. The respective nematicidal activity of hydrolysates was then tested.

To identify the chemical components and characteristics of active dialysates, the following reactions were conducted according to the methods from Kong [23]: the ninhydrin reaction to identify amino acids, biuret reaction and ammonium sulfate precipitation test to identify peptides and proteins, Fehling's solution and Keller-Kiliani reaction to identify sugar, ferric chloride reaction to identify phenols, potassium ferricyanide-ferric chloride reaction to identify aromatic amine, lead acetate precipitation to identify tannins, hydroxamic acid iron test to identify lactones, thin layer chromatography (TLC) to compare the standards of gallic acid and catechinic acid, and with heated chloroform to identify carcass as well as

triterpenoids and their glycosides. An organic elemental analyser (Vario EL, Germany) was used to determinate the molecular formula of active metabolite substances.

Pot experiment

The pot experiment was performed in a controlled greenhouse environment following the modified method of Hu [24]. Each plastic pot was filled with 150 g of soil and 3-week-old Zhongza9 tomato seedlings (Jingdian Seeds Company, Yunnan, China) were transplanted. After a week, 500 newly hatched J2s were inoculated per pot. The respective culture filtrates of the two strains were inoculated to pots at different doses, namely 30 mL pot⁻¹, 100 mL pot⁻¹, and 180 mL pot⁻¹. The negative control consisted in plants grown in untreated soil, while the positive control corresponded to soil with 2.5 g pot⁻¹ of 0.5 % avermectin. Each treatment had five replicates and was repeated three times. Pots were cultured in a greenhouse at 27 ± 2 °C and watered daily.

After 30 days, plants were uprooted and 10g rhizosphere soil samples were collected from each pot. Juveniles were extracted by modified Baermann funnel technique from soil samples, roots with galls were rinsed with sterilized water and cut 5g root from each plant and placed in a culture plate containing sterile water. The nematode populations in per 1g soil and root were calculated under a dissecting microscope after setting aside for 24h at room temperature. The inhibition ration in per 1g soil and root, biocontrol efficiency and growth condition of plant were used to measure the biocontrol potentials of two strains.

Inhibition ration was estimated with the following formula: Inhibition ration (%) = $(NP_{tr} - NP_c) / NP_c \times 100\%$, where NP_{tr} is the nematode populations in per 1g soil or root of the treatment group, NP_c is the nematode populations in per 1g soil under control conditions. Biocontrol efficiency was estimated with the following formula: Biocontrol efficiency (%) = $(GI_c - GI_t) / GI_c \times 100$, where GI_c is the gall index under negative control conditions, and GI_t is the gall index in the treatment group [25]. The gall index was measured according to a ten-point scale [26]. Moreover, the growth condition of plant was measured with plant height (cm), shoot weight (g), and root weight (g).

Statistical analysis

Data from the experiments were tested using a one-way analysis of variance, and significant differences were analyzed using SPSS software 26.0, the means of the treatments were compared with Duncan's multiple-range test ($p < 0.05$).

Declarations

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Authors' contributions

All authors contributed to the study and conducted experiments. Minmin Yang carried out the main experiments and data collection. Tingting Bo, Yuxin Li, Caimi Wu participated in the pot experiment. Qianru Hu analysed data and wrote a manuscript draft. Minghe Mo participated in experimental design. Yajun Liu guided experimental design and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

ND41: *Streptomyces albogriseolus* ND41; D153: *S. fimicarius* D153; J2s: second-stage juveniles.

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Tables

Table 1 Chemical Characteristics of active metabolites

Characteristics	Experiment	ND41	D153
Dialysis	Dialysis bag	MwCO $\geq$ 8000Da	MwCO1000-8000Da
Protein	Proteinase-K	+	+
Amino acids	Ninhydrin test	+	+
Peptides, Proteins	Biuret test	+	+
	Ammonium sulfate precipitation test	+	+
Sugar	Fehling test	+	+
	Keller-kiliani test	+	+
Phenolics	Ferric chloride test	+	+
Aromatic amine	Potassium ferricyanide - ferric chloride test	+	+
Tannins	Lead acetate precipitation test	+	+
Lactones	Hydroxamic acid iron test	+	+
Gallic acid	TLC after hydrolysis	+	+
Catechinic acid	TLC after hydrolysis	+	+
Steroids, saponins, and terpenoids	Chloroform test	+	+

“ + ” : Positive reaction; “ - ” : Negative reaction.

Table 2 The elementary analysis of active samples

Strain	Molecular formula	C/N Ratio	Content (%)	Peak Area
ND41	(C ₃₄ H ₇₅ O ₆₃ N) _n	37.18	N:0.957	N: 421
			C:35.56	C:12526
			H:5.106	H:7811
D153	(C ₇ H ₁₃ O ₉ N) _n	6.451	N:5.168	N:490
			C:33.34	C:2301
			H:5.130	H:1668

Figures

Figure 1

Nematicidal activity of culture filtrates and extracts of two strains against *M. incognita*. A. nematicidal activity of culture filtrates, DN41: *S. albogriseolus* DN41; D153: *S. fimicarius* D153; B. nematicidal activity of extracts of the strain ND41; C. nematicidal activity of extracts of the strain D153. The experiment was performed with three replicates and data was the average of replicates. The bar means standard deviation.

Figure 2

The effect of different temperature and dialysis on the nematocidal activity of two strains against *M. incognita*. A. response of *S. albogriseolus* ND41 to temperature; B. response of *S. fimicarius* D153 to temperature; C. nematocidal activity of dialysate from *S. albogriseolus* ND41; D. nematocidal activity of dialysate from *S. fimicarius* D153.

Figure 3

The inhibition ratio of two strains against *M. incognita* in soil and root at different doses. A. the inhibition ratio against *M. incognita* in soil.; B. the inhibition ratio against *M. incognita* in root. ND41-30, ND41-100 and ND41-180 were treated with culture filtrates of *S. albogriseolus* ND41 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. D153-30, D153-100 and D153-180 were treated with culture filtrates of *S. fimicarius* D153 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. PC were treated 2.5 g pot-1 of 0.5 % avermectin. The experiments were performed with three replicates and data was the average of replicates. The bar means standard deviation. * present the significant difference ($p < 0.05$).

Figure 4

Biocontrol efficiency of two strains against *M. incognita* at different doses. ND41-30, ND41-100 and ND41-180 were treated with culture filtrates of *S. albogriseolus* ND41 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. D153-30, D153-100 and D153-180 were treated with culture filtrates of *S. fimicarius* D153 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. PC were treated 2.5 g pot-1 of 0.5 % avermectin. The experiment was performed with five replicates and data was the average of replicates. The bar means standard deviation.

Figure 5

Plant growth treated by two strains at different doses. ND41-30, ND41-100 and ND41-180 were treated with culture filtrates of *S. albogriseolus* ND41 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. D153-30, D153-100 and D153-180 were treated with culture filtrates of *S. fimicarius* D153 at the dose of 30mL pot-1, 100 mL pot-1 and 180 mL pot-1. PC were treated 2.5 g pot-1 of 0.5 % avermectin. The experiment was performed with five replicates and data was the average of replicates. The bar means standard deviation.