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Full Length Research Paper

Nematicidal activity of *Gymnoascus reesii* against *Meloidogyne incognita*

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In the present investigation, antagonistic effect of culture filtrates of *Gymnoascus reesii* at different concentrations were tested on eggs and juveniles of root knot nematode *(Meloidogyne incoginta*), *in vitro*. The percentage mortality and inhibition of hatching of root-knot nematode were directly proportional to the concentration of culture filtrates of *G. reesii*. Compared with the nematicides treatments of Cadusafos and Avermectins, the effects of higher concentration of culture filtrates of *G. reesii* were in some cases not statistically different. To our knowledge, this is the first report of culture filtrates of *G. reesii* observed to have nematicidal activity toward root-knot nematode and a nematicidal metabolite (3E,5E)-2,5-dihydroxy-2,7-dihydrooxepine-3-carboxylic anhydride was isolated based on bioassay-guided fractionation from the extracts of the fungus *G. reessii*.

Key words: *Gymnoascus reesii*, nematicidal activity, *Meloidogyne incoginta.*

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) cause high levels of economic loss in a multitude of agricultural crops worldwide. They are capable of severely damaging a wide range of crops, in particular vegetables, causing dramatic yield losses mainly in tropical and sub-tropical agriculture (Sikora and Fernandez, 2005). For several decades the use of chemical nematicides is one of the primary means of control for root-knot nematodes. However, the potential negative impact on environment and ineffectiveness after prolonged use have led to a total ban or restricted use of most nematicides and an urgent need for safe and more effective options (Zuckerman and Esnard, 1994).

Biological control promises to be such an option. Application of microorganisms antagonistic to *Meloidogyne* spp. or compounds produced by these microbes could provide additional opportunity for managing the damage caused by root-knot nematodes. Research in this area has resulted in commercial biocontrol preparations reported to act against root-knot nematodes (Stirling, 1991; Fravel, 2000).

Over the last decades, research on root-knot nematode control was focused on proposing strategies for inhibition of egg hatch (Westcott and Kluepfel, 1993), degradation of hatching factor (Oostendrop and Sikora, 1989) or production of metabolites (Meadows et al., 1989). In particular, the search for nematode-antagonistic compounds from culture filtrates of fungi has greatly intensified in recent years because of the large number of toxins and potential new drugs among fungal metabolites. Number of fungi isolates from nematodes, soil and plants were proved to produce substances that inhibit nematode egg hatch or kill nematodes (Nitao et al., 1999). Some of these fungi produce toxic metabolites in culture filtrates (Khan and Saxena, 1997).

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For years, our group has been seeking nematode-antagonistic compounds from fungal metabolites. These studies have included screening the culture broths of 1,248 fungi isolated from soil for activity against *Meloidogyne hapla* (Liu et al., 2004). One fungal isolate that exhibited high nematicidal activity in these experiments was identified as *Gymnoascus reesii* Baran. Nematicidal potential of *G. reesii* Baran has remained unexplored except for few reports, which suggest that the culture filtrate of *G. reesii* displayed significant activity against the nematode *Haemonchus contortus*. However, there are no reports of nematicidal activity against *Meloidogyne* spp. in *G. reesii*.

The objective of this study was to evaluate the toxic activity of culture filtrate of our isolate of *G. reesii* against *M. incognita* eggs and second-stage juveniles (J2) *in vitro*.

MATERIALS AND METHODS

Fungal culture filtrates

The fungus was isolated using a soil dilution plate method directly from soil collected from the suburb of Beijing city, China and the isolate was identified to species as *G. reesii* Baran. A culture is maintained in the Institute of Microbiology, Chinese Academy of Sciences, China as isolate Za-130 (CGMCC No.2632). One-week-old *G. reesii* cultures on CMA plates (60 × 15 mm) were homogenized into Czapek-Dox broth medium (1 CMA plate/500 ml flask containing 125 ml Czapek-Dox broth) and incubated at 28°C on a shaker (180 rpm) for 5 days. After incubation, the culture broth was centrifuged at 13,700 g for 20 min, and the supernatant was sequentially passed through syringe filters designed for viscous samples containing particulates (GD/X series 1.0 µm GF/B and 0.45 µm GMF filters, Whatman, Clifton, NJ) and then sterile-filtered (GD/X sterile 0.2 µm PES filter, Whatman). The filtrates were used at concentrations of 1× solution (the original filtrate preparation) and diluted to -5× (1 part filtrate to 4 parts water), -10× (1 part filtrate to 9 parts water) by sterile water and condensed to +5× solution (a solution 5 times as concentrated as the 1x solution and +10× (a solution 10 times as concentrated as the 1 × solution) in vacuum at 40°C using a rotary evaporator.

Eggs and J2

Meloidogyne incognita was cultured in the greenhouse on tomato plants (New L-402, nematode susceptible) inoculated with a single nematode egg mass (Khan et al., 2005). After 55 days, egg masses were handpicked from galls of tomato roots and surface sterilized in 0.5% sodium hypochlorite for 3 min and washed with sterile water 3 times. J2 were hatched from the egg masses, collected daily and stored at 4°C. Eggs were extracted by the NaOCl technique (Coolen and D' Herde, 1972). Eggs were poured on a 75 µm-pore sieve and collected on 5 µm-pore sieve. The extracted eggs were gently washed with tap water to remove sodium hypochlorite (Oka and Yermiyahu, 2002; Nico et al., 2004).

Effect on egg mass hatch

Two sterilized healthy egg masses of nearly uniform size were transferred to a 6 cm-diameter autoclaved Petri dishes containing 3 ml filtrate of different dilutions. Egg masses placed in sterilized distilled water and 10 µl/ml Avermectins (1.8% avermectins emulsifiable manufactured by Hebei Veyong Bio-chemical Co. LTD, China) and 10 µg/ml Cadusafos (10% cadusafos granular manufactured by FMC Corporation, USA) served as three controls; three replicates of each treatment and control were included. Plate lids were sealed with parafilm and the plates were kept at 25°C. After 7 days hatched J2 were counted with the use of an inverted microscope and percentages of cumulative hatch and relative suppression of hatch rate were calculated.

Effect on individual egg hatch

One milliliter of egg suspension (containing about 250 eggs/ml) was combined with 2 ml of filtrate of different concentraions in 6 cm-diameter autoclaved Petri dishes. Eggs in sterilized distilled water and 10 µl/ml Avermectins and 10 µg/ml Cadusafos served as three controls. Treatments and controls were 3 replicates. Plate lids were sealed with Parafilm and the plates were kept at 25°C. After 7 days hatched J2 were counted with the use of an inverted microscope and percentages of cumulative hatch and relative suppression hatch rate were calculated.

Effect on J2

To determine the effects of culture filtrates on J2 of *M. incognita* 1 ml filtrates of different concentrations were transferred to wells of 24-well tissue culture plates to which about 100 J2 was added. Sterilized distilled water and 10 µl/ml Avermectins and 10 µg/ml Cadusafos again served as three controls. Three replicates were used per treatment. After 24 h at 25°C, the dead J2 were counted under an inverted microscope and the corrected mortality was calculated. Nematode mortality was confirmed after lack of movement to distilled water and observation after an additional 72 h (San Martin and Magnunacelaya, 2005). Mortality data was corrected for corrected mortality mortality following Abbot (1925). The experiment was repeated, and the results of the repeated trial were combined for analysis.

Extraction, isolation and identification of secondary metabolites of *G. reesii*

After incubation, the cultures were centrifuged, and the broth was filtered through a glass-filter funnel. The broth filtrate was extracted by mixing for 2 h with Amberlite XAD-16 resin equilibrated in H_2O (1 volume gel-6 volumes broth). In latter study, bioassay-guide was used throughout the isolation process. The resin was removed from the broth, washed with 3 bed volumes H_2O, and eluted with 3 bed volumes methanol (MeOH).

The MeOH eluate was evaporated under vacuum, and the residue was partitioned in 30% MeOH in H_2O and chloroform ($CHCl_3$).The chloroform fraction was evaporated to dryness and fractionated by Silica gel CC using chloroform-MeOH gradient elution to yield fraction A_1-A_5 according to thin layer chromatography (TLC) analysis. The active fraction A_2, obtained on elution with chloroform/ MeOH 90%, was further purified by recycling preparative high-performance liquid chromatography (HPLC) (Japan Analytical Industry Co., Ltd) eluted with MeOH/ H_2O (3:7,v/v) to furnish compound.

The structure of compound isolated from the cultures of *G. reesii* was determined by spectroscopic analysis. Infrared (IR) spectra was obtained in KBr pellets with a ATR, TENSOR27 spectrophotometer (ATR, Bruker, Karlsruhe, German). MS was performed on a Bruker APEX IV FTMS mass spectrometer (APEX, Bruker, Karlsruhe, German) spectrometers. The nuclear magnetic resonance (NMR) spectra were recorded on DRX-600 NMR

(Bruker, Karlsruhe, Germany) with TMS as an internal standard and coupling constants were represented in Hertz.

Statistical analyses

The experiment was repeated. A completely randomized design was used. Stat.10 for Windows (SPSS Inc. 2000: SPSS Base 10.1 User's Guide: SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Duncan's New multiple range test was employed to test for significant difference between treatments at P = 0.05 and means were compared with the Least Significant Differences (LSD) test (P =0.05).

RESULTS

Effect on egg mass hatch

The hatchability test (Table 1) indicated that culture filtrates of *G. reesii* strongly suppressed hatching of *M. incognita* egg masses. The percentage reduction ranged from 22.19 to 99.45%. The different concentrations of the culture filtrate exhibited significant differences compared to the water control; the culture filtrates at the original, +5× and +10× concentrations had no significant difference when compared to the Cadusafos and Avermectins controls (Table 1).

Effect on individual egg hatch

The culture filtrate of *G. reesii* also strongly decreased individual egg hatching of *M. incognita* (Table 1); hatching was suppressed by 8.02 to 97.18%. The difference between all filtrate concentrations except the -10× solution and the water control was significant; the relative suppression rate of the Cadusafos and Avemectins controls significantly differed from those of the filtrate treatments except at the +5× and +10× concentrations.

Effect on J2

All *G. reesii* filtrate concentrations exhibited nematicidal effects of various degrees on J2 of *M. incognita* (Table 1). The percentage mortality was directly proportional to the filtrate concentration. The corrected mortality of all treatments showed significance compared with the water control. The effect of culture filtrates at +10× concentration on percentage mortality significantly differed from the effects of the Cadusafos and Avermectins.

Extraction, isolation and identification of secondary metabolites of *G. reesii*

Based on bioassay-guided isolation, an active compound was obtained from the extracts of the fungus *G. reesii*. The EIMS of compound showed a molecular ion peak at m/z (100) 349.05248 $[M+Na]^+$, and the molecular formula was determined to be $C_{14}H_{14}O_9$ by MS and elemental analysis. The IR spectrum showed the absorption for carboxyl and hydroxyl (3360 cm^{-1}), unsaturation hydrocarbon (3124, 3096, 3067 cm^{-1}), saturation hydrocarbon (3956, 2910, 2868 cm^{-1}), carboxyl (1743 cm^{-1}), C=C double bond (1674, 1627 cm^{-1}), saturation hydrogen carbonide (1449, 1424, 1366 cm^{-1}). The NMR spectral data of the compound are presented in Tables 2 and the assignation was based in correlation experiments. Thus, the compound was identified as (3E,5E)-2,5-dihydroxy-2,7-dihydrooxepine-3-carboxylic anhydride.

DISCUSSION

Fungal natural products are very promising potential sources of new chemicals to manage plant-parasitic nematodes (Anke and Sterner, 1997). Culture filtrates of many fungi possess activity against nematodes, and the nematicidal action of these culture filtrates may involve the production of toxic metabolites by the fungi (Caroppo et al., 1990; Singh et al., 1991; Hallmann, 1996; Nitao et al., 1999; Miyako et al., 2000, 2003; Satoshi et al., 2004; Yoshinori et al., 2004; Dong et al., 2005, Heydari et al., 2006; Guohong et al., 2007; Asami et al., 2007; Ting et al., 2008; Lin et al., 2009).

Species of *Aspergillus, Penicillium, Trichoderma, Fusarium, Paecilomyces* and *Alternaria* are known to produce toxins and antibiotics like aflatoxin, penicillin virdin, fusaric acid, lilacin, and phyto-alternarin (Nafe-Roth, 1972; Arai et al., 1973; Wheeler, 1975; Ghewande et al., 1984). Adverse effect of the culture filtrates of several fungi on hatching and mortality of root-knot nematodes has been reported by others also (Mankau, 1969; Shukla and Swarup, 1971; Khan et al., 1984; Mani and Sethi, 1984; James et al., 1999, 2001; Meyer et al., 2004; Man-Hong et al., 2006; Sahebani, 2008).

In vitro results indicated that exposure to the culture filtrate of *G. reesii* reduced the ability of *M. incognita* eggs to hatch and induce mortality in J2 to a varying degree depending on the culture filtrate concentration. Our results indicated that nematicidal potential of the culture filtrate of *G. reesii* at higher concentrations than lower. The filtrate inhibited egg mass hatching greater than individual eggs; for example, the inhibition of egg masses and individual egg hatching of the 1× concentration were 97.74 and 73.94%, respectively. Therefore, this concentration could provide good control at the egg mass stage. Our results also indicated that the +5× and +10× concentrations showed great antagonistic activity towards J2 of *M. incognita* and that the relativity mortality rate of the +5× concentration (85.6%) and +10× concentration (98.2%) were both higher than those of the Cadusafos and Avermectins controls', indicating the possible implication of this biocontrol fungus as a new tool for an management program for the root-knot nematode.

Table 1. Effect of culture filtrate of *G. reessii* at different concentrations on hatching of eggs in egg masses, individual eggs and juveniles of of *M. incognita.*

Treatments	Mortality of juveniles (%)	Corrected mortality (%)	The number of hatched juveniles of individual eggs	Relative suppression rate (%)	The number of hatched juveniles of eggs in egg masses	Relative suppression rate (%)
-10×	38.6fF	35.6	65.3aA	8.02	182.3bB	22.19
-5×	60.4eE	58.5	47bB	33.80	154.7bB	33.97
Original filtrate	74.3dD	73.1	18.7cC	73.94	5.3cC	97.74
+5×	85.6bB	84.9	5dD	92.96	2.0cC	99.15
+10×	98.2aA	98.1	2dD	97.18	1.3cC	99.45
Water (ck1)	4.6gG	——	71aA	——	234.3aA	——
Cadusafos (ck2)	82.3bcBC	81.4	3.5dD	95.07	2.3cC	99.02
Avermectins (ck3)	77.4cdCD	76.3	3.3dD	95.35	2.7cC	98.85
LSD(p=0.05)	7.28		3.00		33.66	

Table 2. The ^{13}C, ^{1}H and HMBC spectrometry number of compound isolated from *G. reessii*.

Position	δ_C	δ_H	HMBC
1	59.06(s)	4.29,d,4.55,d	(5.96)
2	88.58(s)	5.88,d	4.29, 4.55, 5.96
3	108.58(d)	5.95,s	(4.29), 4.55
4	109.78(d)	5.96,s	(5.88)
5	146.45(d)		4.29, 4.55, 5.88, 5.96
6	152.08(d)		5.96
7	169.64(s)		5.95

Fungal antibiotics and other toxic compound present in metabolites might be responsible for the inhibition of egg hatch and J2 mortality. As *G. reesii* produces secondary metabolites such as Polyenylpyrroles and Polyenylfurans (Benjamin et al., 2006), aromatic butenolides (Ben et al., 2005) and Roquefortine E (Ben et al., 2005).

According to Benjamin et al. (2005, 2009), *G. reesii* displayed significant growth inhibitory activity against the bacterium *Bacillus subtilis*, the nematode *Haemoncs contortus*, the plant fungal pathogen *Septoria nodorum*, and a tumor cell line (murine NS-1). However, there is no report of nematicidal activity against the plant-parasitic nematode by *G. reesii.*

In summary, this is the first study of the nematicidal activity of culture filtrate of *G. reesii* on *M. incognita* and a compound was also isolated according to its nematicidal activity. We believe *G. reesii* is a new biological control factor that may be a potentially good source of a microbial nematicide that can be harnessed for successful nematode control.

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