Pesticide Biochemistry and Physiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Nematicidal activity of verrucarin A and roridin A isolated from *Myrothecium verrucaria* against *Meloidogyne incognita*

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ARTICLE INFO

Keywords:
Hatched juvenile mortality
Macrocyclic trichothecenes
Meloidogyne incognita
Myrothecium verrucaria
Nematicidal activity
Root-knot nematode

ABSTRACT

The widespread use of synthetic nematicides has caused significant problems to the environment as well as human health. To address this issue, eco-friendly control measures, such as microbial nematicides, are being developed. During the screening of *Myrothecium* strains with nematicidal activity against the root-knot nematode (RKN) *Meloidogyne incognita*, we found that the acetone extract of *Myrothecium* sp. KACC 40321 was highly effective against hatched juveniles of *M. incognita* at 7 days after exposure. The fungus was identified as *Meloidogyne verrucaria*. Two macrocyclic trichothecenes verrucarin A and roridin A were isolated and identified as major active metabolites by bioassay-guided fractionation and instrumental analysis. When the second-stage juveniles were treated with the chemicals, no juvenile mortality was observed. However, they effectively killed juveniles from treated eggs. The hatched juvenile mortality was used to evaluate the *in vitro* nematicidal activity of the compounds against *M. incognita*. The median effective concentrations were 1.88 μg/mL for verrucarin A and 1.50 μg/mL for roridin A. Among various liquid media, commercial malt extract broth (cMEB) was found to be the best for the production of verrucarin A and roridin A, followed by potato dextrose broth. The cMEB culture filtrate effectively reduced the formation of galls and egg masses on tomato roots in a pot experiment. In addition, the culture filtrate reduced the formation of galls on the roots of melon plants and the number of RKNs in the soils under field conditions. These results suggest that *M. verrucaria* KACC 40321 can be used as a biocontrol agent against RKNs in various crops. To the best of our knowledge, this is the first study to report the effectiveness of verrucarin A and rorridin A against hatched juveniles of *M. incognita*.

1. Introduction

Root-knot nematodes (RKNs) are part of the genus *Meloidogyne*, which is found across the world, particularly in tropical or subtropical areas [1]. They attack > 2000 species of plants, including almost all crops [2]. Owing to these nematode pests, the world economy loses over US $100 billion annually [3]. *Meloidogyne incognita* (Kofoid and White) growing on chitwood is one of the most widespread RKNs [4]. RKNs damage plants by devitalizing root tips, resulting in the formation of giant cells on the infected root. The symptoms may be similar to those of nutrient deficiency, such as stunting, lack of vigor, and wilting under moisture stress, because *Meloidogyne* infection affects water and nutrient uptake and upward translocation by the root system. Moreover, secondary infection by other pathogens often results in extensive decay of nematode-infected tissues. Therefore, RKNs may not only reduce crop yield but also reduce product quality by disrupting the host

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https://doi.org/10.1016/j.pestbp.2018.04.012
Received 13 November 2017; Received in revised form 20 March 2018; Accepted 23 April 2018

plant physiology [2].

RKNs can be controlled using sole or integrated various strategies including steam sterilization of the soil, soil fumigation with nematicides, several cultural practices, such as crop rotation, fallow soil, soil solarnization, and certain soil amendments, or use of resistant varieties. In the field, root knot disease is widely controlled by fumigating the soil with approved chemical nematicides such as the organophosphates or carbamates [3,5]. The indiscriminate use of synthetic nematicides has caused significant problems to the environment and human health and has generated a considerable demand for environmentally friendly alternative strategies.

Biological agents, which include living organisms and their metabolic products, are a potentially effective alternative for the management of nematode pests. Nematophagous fungi, a group of natural enemies of nematodes, have become the major target organisms from which biological agents are being developed to control parasitic nematodes. Fungi produce a huge diversity of secondary metabolites; several large classes of commercial compounds are derived from them. Therefore, secondary metabolites in fungi could have great potential owing to their novel structures and nematicidal activities [6].

Myrothecium species are soil-borne or weak plant pathogens and prolific producers of bioactive secondary metabolites. *Meloidogyne verrucaria* and its metabolites can control plant damage from nematodes by reducing egg hatching, inhibiting development, or even killing the nematodes. Although 30 metabolites of *M. verrucaria* have been reported [7,8], the nematicidal molecules in *M. verrucaria* have not been identified yet [3]. Therefore, the purpose of this study was to isolate and identify nematicidal compounds from *M. verrucaria* and evaluate their activities against *M. incognita in vitro*, *in vivo*, and in the field.

2. Materials and methods

2.1. Fungal strain

Myrothecium sp. KACC 40321 was obtained from Korean Agricultural Culture Collection. A stock culture of fungal strain was stored in 8% dimethyl sulfoxide (DMSO) suspension at −80°C deep freezer. The strain was incubated on potato dextrose agar (PDA; Difco, USA) for 10 days and agar plugs containing fresh mycelia were then inoculated into 500 mL Erlenmeyer flasks containing 200 mL of potato dextrose broth (PDB; Difco, USA). The fungus was grown at 25°C and 150 rpm for 14 days for bioassay and isolation of active metabolites.

2.2. Identification of antagonistic fungal strain

Myrothecium sp. KACC 40321 was cultured on PDA at 25°C for 4 days. Total genomic DNA was directly extracted from fungal mycelia using the NucleoSpin®Plant II (Macherey-nagel, Düren, Germany), according to the manufacturer's instructions. The partial 18S rRNA-ITS1–5.8S rRNA-ITS2-parital 28S rRNA (ITS-rRNA) region was amplified with the universal primer pair ITS1 (5′-CTTGG TCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATA TGC-3′) in a 20-μL reaction mixture [9]. The PCR product sequencing was performed (Genotech, Taejeon, Korea) and was edited using ATGC software.

The ITS-rRNA region sequence was compared using BLAST search with the sequence available in the GenBank databases. Sequences from this study and those retrieved from GenBank were aligned and edited, and phylogenetic analyses were performed using MEGA 6 with the default settings [10]. Phylogenetic trees were constructed from the data obtained by neighbor-joining (NJ) analysis with the number of bootstrap trials set to 1000. The Kimura 2-parameter model was selected as the best model to construct trees for NJ. The bar indicates the number of substitutions per position.

2.3. Nematodes

RKN *M. incognita*, which was isolated and identified by Hwang et al. [11], was maintained on tomato (*Lycopersicones culentum* Mill. Cv. Seokwang) in a greenhouse (27 ± 5°C) at Chonnam National University. The nematode eggs were extracted from the roots of tomato plants infected with *M. incognita* by 1% sodium hypochlorite solution. The eggs were collected by passage through a series of sieves with pore sizes of 45 and 25 μm. Collected eggs were rinsed with distilled water and used for egg hatching inhibition and hatched juvenile mortality experiments. Second-stage juveniles (J2 s) were obtained using modified Baermann funnels at 28°C within 5 days [12].

2.4. Nematode bioassay

During incubation in PDB medium, the fungus produces polymers, which renders the fermentation broth semisolid, and thus difficult to obtain the culture filtrate containing active metabolites. Therefore, the semisolid culture broths were directly extracted with acetone at 1:1 (v/v) and filtered through four layers of sterile cheese cloth to obtain an acetone extract, which was evaporated using a rotary vacuum evaporator at 40°C until only water remained in the solution to obtain the culture filtrate (N-1200BV, Eyela, Japan).

The effects of *Myrothecium* sp. KACC 40321 culture filtrate on *M. incognita* J2 s and eggs were evaluated in the range of 1.25 to 20%. Approximately 50 J2 s and 150 eggs per well were used for the bioassay using 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). Sterile distilled water (SDW) containing 1% acetone or methanol, PDB, or one malt extract broth medium (cMEB) purchased from Kisan Bio Co., Ltd. (Seoul, Korea), were was used as the negative control. The plates were gently shaken and incubated in a dark plastic box with 100% humidity at room temperature. All experiments were conducted with three replicates and repeated twice. J2 s were judged at 1 and 3 days after treatment. The J2 s and eggs were observed under an optical microscope (Leica DM IL LED; Leica Microsystems CMS GmbH, Wetzlar, Germany). Three days after treatment, J2 s were considered dead if they did not move upon probing with a fine needle [13].

J2 and hatched juvenile mortality rates were calculated using Abbott formula reported in 1925 [14]: Mortality (%) = [(mortality percentage in treatment − mortality percentage in untreated control)/(100 − mortality percentage in untreated control)] × 100. The inhibition percentage of *M. incognita* egg hatching was determined at 7, 10, and 14 days after treatment. The percentage hatch inhibition was calculated according to the following formula [15]: Hatch inhibition (%) = [(percentage egg hatch in untreated control − percentage egg hatch in treatment)/percentage egg hatch in untreated control] × 100. The percentage egg hatching was calculated as follows: percentage egg hatching = [number of juveniles/number of (eggs + juveniles)] × 100.

Stock solutions of roridin A and verrucarin A (Sigma-Aldrich, St. Louis, MO, USA) were prepared in acetone at 100-fold higher concentration than that used in the test. The hatched juvenile mortality and egg-hatching inhibition effects of the active compounds were tested at 0.1–100 μg/mL. Acetone at 1% was used as an untreated control. One microliter of each stock solution was added to each well of a 96-well tissue culture plate containing 99 μL of egg suspension. The experiment was conducted twice with three replicates.

2.5. Extraction and isolation of nematicidal metabolites

The culture broth (1.6 L) was extracted with acetone at 1:1 (v/v) and filtered through four layers of sterile gauze. The acetone was removed using a rotary vacuum evaporator to obtain the culture filtrate. The culture filtrate (1.2 L) was partitioned twice, with ethyl