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Nematicidal Activity of Kojic Acid Produced by *Aspergillus oryzae* against *Meloidogyne incognita*

Tae Yoon Kim[1†], Ja Yeong Jang[1†], Sun Jeong Jeon[1], Hye Won Lee[1], Chang-Hwan Bae[2], Joo Hong Yeo[2], Hyang Burm Lee[1], In Seon Kim[1], Hae Woong Park[3], and Jin-Cheol Kim[1*]

[1]*Department of Agricultural Chemistry, Institute of Environmentally Friendly Agriculture, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Republic of Korea*

[2]*Biological and Genetic Resources Assessment Division, National Institute of Biological Resources, Incheon 22689, Republic of Korea*

[3]*World Institute of Kimchi, an Annex of Korea Food Research Institute, Gwangju 61755, Republic of Korea*

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*Corresponding author
Phone: +82-62-530-2132;
Fax: +82-62-530-2139;
E-mail: kjinc@jnu.ac.kr

†These authors contributed equally to this work.

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The fungal strain EML-DML3PNa1 isolated from leaf of white dogwood (*Cornus alba* L.) showed strong nematicidal activity with juvenile mortality of 87.6% at a concentration of 20% fermentation broth filtrate at 3 days after treatment. The active fungal strain was identified as *Aspergillus oryzae*, which belongs to section *Flavi*, based on the morphological characteristics and sequence analysis of the ITS rDNA, calmodulin (CaM), and β-tubulin (BenA) genes. The strain reduced the pH value to 5.62 after 7 days of incubation. Organic acid analysis revealed the presence of citric acid (515.0 mg/kg), malic acid (506.6 mg/kg), and fumaric acid (21.7 mg/kg). The three organic acids showed moderate nematicidal activities, but the mixture of citric acid, malic acid, and fumaric acid did not exhibit the full nematicidal activity of the culture filtrate of EML- DML3PNa1. Bioassay-guided fractionation coupled with ^{1}H- and ^{13}C-NMR and EI-MS analyses led to identification of kojic acid as the major nematicidal metabolite. Kojic acid exhibited dose-dependent mortality and inhibited the hatchability of *M. incognita*, showing EC_{50} values of 195.2 μg/ml and 238.3 μg/ml, respectively, at 72 h post-exposure. These results suggest that *A. oryzae* EML-DML3PNa1 and kojic acid have potential as a biological control agent against *M. incognita.*

Keywords: *Meloidogyne incognita*, nematicidal activity, kojic acid, *Aspergillus oryzae*

Introduction

Nematode disease is a serious threat to crop production worldwide. The loss of crop yield due to nematodes is ~14%, which represents over US $100 billion annually [27]. Root knot nematodes (RKNs; *Meloidogyne* spp.) are one of the most common and destructive nematode pathogens. RKNs cause root dysfunction by producing galls in the root of host plants. This leads to wilting and, at high nematode densities, death of the host plant. In addition, they can predispose the host plant to secondary infections. The control of RKNs is more difficult than that of other pathogens because they inhabit soil and have extremely wide host ranges. A number of synthetic nematicides have been used for nematode control, but several have been withdrawn or restricted owing to their deleterious effects on the environment and non-target organisms. Therefore, identifying safe substitutes has become increasingly important.

Biological control is one option, and is generally regarded as being target specific, with reduced persistence in the environment, low toxicity to non-target organisms, and safe use in organic agriculture [36]. Antagonistic microorganisms to nematodes and compounds with nematicidal activity could enable management of RKNs without exerting deleterious effects on the environment. Some commercial bionematicides are derived from microorganisms such as *Pasteuria* spp., *Pochonia chlamydosporia*, *Bacillus firmus*, *Paecilomyces lilacinus*, and *Trichoderma* sp. [30]. These organisms involve penetration of the nematode cuticle or

parasitization of female eggs, subsequently killing the nematodes [11, 25, 37, 41]. In addition, *Myrothecium verrucaria* produces toxin and it kills nematodes on contact by affecting their nervous system, disrupting sensory organs, and inhibiting egg hatching and development [46]. Furthermore, they lead to more vigorous and higher yielding crops.

Many fungi exhibit a variety of antagonistic activities, including production of nematicidal compounds [20, 24]. This facilitates development of novel nematicides or discovery of novel modes of action [13]. The fungus *Omphalotus olearius* produces omphalotin A, a toxic compound showing marked activity against *Meloidogyne incognita* [26]. Chaetoglobosin A was isolated as a nematicidal compound produced by *Chaetomium globosum* and it showed adverse effect on juvenile mortality (LC_{50} = 77.0 μg/ml) against *M. incognita* [18]. Linoleic acid was obtained as a nematicidal metabolite from *Arthrobotrys conoides* and *Arthrobotrys oligospora* [38], and *trans*-2-decenedioic acid produced by *Pleurotus ostreatus* causes irreversible immobility against *Panagrellus redivivus* [21].

In the search for fungal strains with nematicidal activity, a fungal strain showed strong activity against *M. incognita*. Therefore, the aims of this study were (i) to identify a fungal strain antagonistic towards *M. incognita*, (ii) to investigate the effect of the culture filtrate on juveniles and eggs of *M. incognita*, (iii) to identify the active compound produced by the strain, and (iv) to evaluate its nematicidal activity on *M. incognita* second-stage juvenile (J2) viability and egg hatching.

Materials and Methods

Isolation of Fungi with Nematicidal Activity

For the isolation of fungi, leaves were sampled from various plants washed in running tap water and then air-dried. The tissue was cut into small pieces of ~1 cm length and 0.5 cm width and surface sterilized with 95% ethanol for 1 min, then in 4% sodium hypochlorite for 4 min, and again in 95% ethanol for 30 sec. Finally, the tissue pieces were rinsed three times in sterilized water and dried with a sterilized paper towel in a laminar air-flow chamber. The segments were transferred to PDA plates supplemented with streptomycin (0.4 mg/ml) and incubated at 25°C for 5–15 days. Individual hyphal tips of the developing fungal colonies were placed onto fresh PDA plates without antibiotics and incubated for 5–10 days to isolate pure cultures. All pure isolates were maintained in PDA slant tubes and stored in 20% glycerol at −80°C. For screening of isolates with nematicidal activity, the fungi were incubated in 500 ml Erlenmeyer flasks containing 200 ml of potato dextrose broth at 25°C for 7 days with shaking at 150 rpm. The culture filtrates obtained by filtration using a sterile gauze were used for the assessment of nematicidal activity against *M. incognita*.

Identification of the Antagonistic Fungal Strain

The fungal strain EML-DML3PNa1 was selected from the primary screening and cultured on PDA medium in the dark at 27°C for 7 days and then preserved in 20% glycerol stock in a deep freezer (−80°C) at the Environmental Microbiology Laboratory (EML) culture collection, Chonnam National University, Gwangju, Korea. The strain was also deposited as ex-type (NIBRFGC4108) at the culture collection of the National Institute of Biological Resources (NIBR), Incheon, Korea.

Fine structures of the fungus were observed using scanning electron microscopy. Samples were fixed in 2.5% paraformaldehyde-glutaraldehyde buffer with 0.05 M phosphate (pH 7.2) for 2 h and washed in cacodylate buffer. The samples were then fixed in 1% osmium tetroxide for 1 h, washed again in cacodylate buffer, dehydrated in graded ethanol and isoamyl acetate, and dried under a fume hood. Finally, the samples were covered with gold in a sputter coater and observed using a Hitachi S4700 field-emission scanning electron microscope (Hitachi, Japan) at the Korea Basic Science Institute, Gwangju, Korea.

DNA Extraction, PCR Amplication, and Sequencing

The isolate was cultured on PDA overlaid with cellophane at 27°C for 3–5 days. Total genomic DNA was directly extracted from fungal mycelia of the EML-DML3PNa1 isolate using the HiGene Genomic DNA Prep Kit for fungi (Biofact Corp., Korea). The internal transcribed spacers (ITS1 and ITS2) and 5.8S region of the ribosomal DNA were amplified using the universal primer pair ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [47] in a 20 μl reaction mixture comprising 2 μl of genomic DNA, 1.5 μl of each primer (5 pM), 1 μl Accupower PCR premix (Bioneer Corp., Korea) containing Taq DNA polymerase, dNTPs, buffer, and tracking dye, and 14 μl of sterile distilled water. The PCR was carried out with the following parameters: 2 min at 95°C for initial denaturation, followed by 30 cycles of 1 min at 94°C for denaturation, 30 sec at 54°C for annealing, and 1 min at 72°C for extension, with 10 min at 72°C for terminal extension. To amplify β-tubulin (BenA) and calmodulin (CaM), the primer pairs Cmd5 (5′-CCGAGTACA AGGARGCCTTC-3′) and Cmd6 (5′-CCGATRGAGGTCATRACGTGG-3′), and Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) [12, 17] were used. The PCR was conducted using the following conditions: 5 min at 95°C for initial denaturation, followed by 35 cycles of 1 min at 95°C for denaturation, 1 min at 61°C for annealing, and 2 min at 72°C for extension, with 8 min at 72°C for terminal extension. The PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer Corp.), according to the manufacturer's instructions. DNA sequencing was performed in an ABI 3700

Automated DNA sequencer (Applied Biosystems Inc., CA, USA).

Phylogenetic Analysis

The ITS rDNA, calmodulin (CaM), and β-tubulin (BenA) gene sequences of strain EML- DML3PNa1 were compared by BLASTn search with sequences available in the GenBank databases. Sequences from this study and those retrieved from GenBank were initially aligned using ClustalX ver. 1.83 [43], and then edited with BioEdit ver. 5.0.9.1 [14]. Phylogenetic analyses were performed using MEGA 6 [40] with the default settings. Phylogenetic trees were constructed from the data using the neighbor-joining (NJ) method. The Kimura two-parameter model was selected as the best model to construct NJ trees. The fungus *Talaromyces flavus* was used as the outgroup. Bootstrap support values >50% are indicated at the nodes. The bar indicates the number of substitutions per position.

Bioassay

M. incognita was isolated and identified by Hwang *et al*. [19] and multiplied on tomato (*Lycopersicum esculentum* Mill. cv. Seokwang) plants. Eggs were extracted using a blender (HM-2100S; Hanil, Korea) in 1% sodium hypochlorite (NaOCl) solution for 1 min from infected roots of tomato. The eggs were collected on a 25 μm sieve after passing through a 45 μm sieve. The eggs were gently washed with distilled water and then hatched using the modified Baermann funnel method [45]. The collected eggs and J2s of *M. incognita* were used in subsequent experiments.

To investigate the effect of culture filtrate of the EML-DML3PNa1 strain on *M. incognita*, culture filtrates (5, 10, or 20 μl) were transferred into wells of a 96-well tissue culture plate to which approximately 50 J2s and 200 egg suspensions were added. The final volume of each well was 100 μl. Sterile water was used as a negative control. After treatment, the plates were sealed with plastic film to prevent evaporation and then incubated at room temperature (25°C). Each treatment was replicated three times. After 3 days of treatment, the killing of J2s and inhibition of egg hatching of *M. incognita* were evaluated. Nematodes were considered as dead if their body did not move together with a stiff and straight body shape on probing with a fine needle [9]. Juvenile mortality was calculated according to the Abbott formula [1]: Mortality (%) = [(mortality percentage in treatment – mortality percentage in control)/(100 – mortality percentage in control)] × 100. The rate of inhibition of nematode egg hatching was calculated using the following formula: HI (%) = [(C - T)/C] × 100, where C and T are the percentages of eggs hatched in the control and treatment, respectively. The rate of egg hatching was calculated as follows: percentage of eggs hatched = 100 × juveniles/[eggs + juveniles].

To evaluate the effect of organic acid produced by EML-DML3PNa1 on J2s of *M. incognita*, stock solutions of citric acid, malic acid, fumaric acid, and a mixture of the three organic acids (51.5:54.5:2.2 (w/w/w)) were prepared at a concentration 10-fold higher than the test concentrations in distilled water. The samples were applied at a concentration range of 50–800 μg/ml. The experiment was performed with three replicates and nematicidal activity was assessed after 3 days of treatment.

The juvenile killing and inhibition of egg hatching of a purified compound isolated from the culture filtrate of EML-DML3PNa1 were tested at a concentration range of 1.4–1,000 μg/ml, and the effect was compared with that of *trans*-cinnamaldehyde (cinnamaldehyde; Sigma-Aldrich, Germany). Nematicidal metabolite and cinnamaldehyde were dissolved in methanol and ethanol, respectively. Stock solutions 100-fold higher than the test concentrations were prepared. The test concentration of organic solvent did not exceed 1% of the volume. The experiments were performed twice with three replicates per treatment. Juvenile mortality and the rate of inhibition of egg hatching were determined after 3 days of incubation.

Organic Acid Analysis of Culture Filtrate of EML-DML3PNa1

To identify the nematicidal compounds produced by the EML-DML3PNa1 strain, organic acid analysis was conducted because the culture filtrate was weakly acidic (pH 5.6). The organic acid produced by EML-DML3PNa1 was identified by high-performance liquid chromatography (HPLC) using an Agilent 1100 series HPLC (USA) equipped with a UV detector at 210 nm (G1314A, Agilent HPLC 1100 series). The culture filtrate of EML-DML3PNa1 was diluted 5-fold, passed through a membrane filter (0.45 μm), and injected onto an Aminex HPX-87C column (4.6 × 250 mm; Bio-Rad, France). The mobile phase was 5 M sulfuric acid with a flow rate of 0.6 ml/min at 28°C. Quantitative analysis was performed using the analytic reagents oxalic acid, citric acid, tartaric acid, malic acid, succinic acid, lactic acid, fumaric acid, and acetic acid to generate calibration curves.

Purification and Identification of the Nematicidal Metabolite

The culture filtrate was successively partitioned twice with equal volumes (700 ml) of ethyl acetate (EtOAc) and butanol (BuOH). The evaporation of organic extracts yielded 0.6 g for the EtOAc layer, 1.3 g for the BuOH layer, and 4.3 g for the aqueous layer. The three layers were tested for nematicidal activity against J2s of *M. incognita.* The EtOAc layer resulted in marked mortality of *M. incognita.* Therefore, this layer was subjected to further purification. A portion of the EtOAc layer (100 mg) was applied to a preparative thin-layer chromatography (prep-TLC) plate (20 × 20 cm; Kieselgel 60GF 254 with 0.5 mm layer thickness; E-Merck) and a chloroform: methanol (7:1 (v/v)) developing solvent was applied. The bands were scraped off of the plates and extracted with acetone:metanol (1:3 (v/v)). The extracts were tested for nematicidal activity against J2s of *M. incognita*. The active spot was observed by ultraviolet (UV) light (254 nm) at an R*f* value of 0.2. The isolated active compound was subjected to HPLC (Waters, Corp., USA) equipped with a Waters 515 HPLC pump and a Waters 996 detector. The column was a C18 reversed-phase column (Atlantis T3, 5 μm, OBD 19 × 250 mm; Waters, Co., Ireland). The mobile phase was 0.1% trifluoroacetic acid (TFA) in water

(eluent A) and 0.1% TFA in acetonitrile (eluent B). The gradient applied was linear from 0% to 50% B in 20 min and from 50% to 100% B in 5 min, at a flow rate of 1 ml/min. The prep-HPLC yielded one pure compound (10 mg).

The chemical structure of the purified metabolite was determined using electron ionization (EI) mass spectrometry and nuclear magnetic resonance (NMR) analyses. EI-mass analysis was performed using a double-focusing high-resolution mass spectrophotometer (JMS-AX505; JEOL Ltd., Japan). ^{1}H-NMR and ^{13}C-NMR spectra were acquired in 650 µl of methanol-d4 using a Bruker Avance III HD 500 MHz instrument (Bruker Biospin GmbH, Germany) at 500 MHz for ^{1}H-NMR spectra and 125 MHz for ^{13}C-NMR spectra. Chemical shifts were calculated using tetramethylsilane as the internal standard.

Statistical Analysis

Data were subjected to analysis of variance using the SPSS Statistics software (SPSS, ver. 21.0 for Windows; USA) with Duncan's multiple range test to compare the treatment means ($p < 0.05$). The 50% effective concentration (EC_{50}) was obtained by probit analysis (95% confidence limits) using the SPSS software.

Results

Identification of the Fungal Strain with Nematicidal Activity

One-hundred fungal strains isolated from various plant leaves were tested for nematicidal activity against J2s of *M. incognita*. Among them, strain EML-DML3PNa1 isolated from the leaf of white dogwood (*Cornus alba* L.) collected at Mt. Deogyu showed the strongest mortality of *M. incognita*. Exposure to 5%, 10%, and 20% culture filtrate of this strain resulted in 49%, 67%, and 87.6% mortality rates of *M. incognita* J2s, respectively, after 3 days of exposure (Fig. 1). Similarly, egg hatching of *M. incognita* was reduced by 45%, 55%, and 79%, respectively, as compared with negative control after 3 days of inoculation.

The EML-DML3PNa1 isolate grew rapidly on Czapeck yeast autolysate medium, and colonies attained a diameter of 30–35 mm after 5 days at 35°C. Colonies were grayish yellow in the center, with a lighter margin. Conidia were globose to ellipsoidal. Conidia formed in chains (Fig. 2). The ITS rDNA, CaM, and BenA gene sequences of strain EML-DML3PNa1 were deposited in GenBank under accession numbers KT921994, KT921995, and KT921993, respectively. A Basic Local Alignment Search Tool search of ITS sequences via the NCBI database indicated that EML-DML3PNa1 most closely resembled the *A. oryzae* TUHT88 (Accession No. LN82484) and *A. flavus* TUHT116 (Accession No. LN482512) strains with 96.2% (429/446 bp) and 95.8% (429/448 bp) homology, respectively. The CaM gene sequences of EML-DML3PNa1 showed identity values of 99.1% (526/531 bp) and 100% (513/513 bp) with *A. oryzae* CBS100925 (Accession No. EF202055) and *A. flavus* NRRL 506 (Accession No. JN185447), respectively. The BenA gene sequence of EML-DML3PNa1 shared 99.6% (490/492 bp) and 99.8% (486/487 bp) identities with *A. oryzae* USME09 (Accession No. KF669493) and *A. flavus* CBS 816.96 (Accession No. GQ850380), respectively. Phylogenetic analysis of multiple genes showed that EML-DML3PNa1

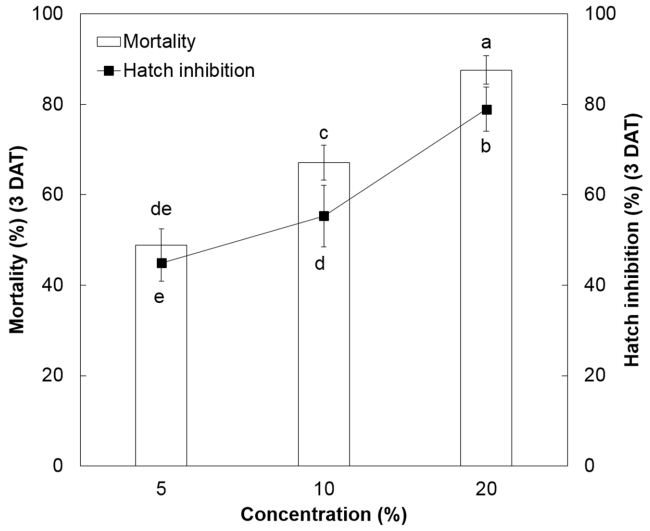

Fig. 1. Effect of EML-DML3Na1 culture filtrate on second-stage juveniles and eggs of *M. incognita*.
Values are means ± standard deviation of three replicates.

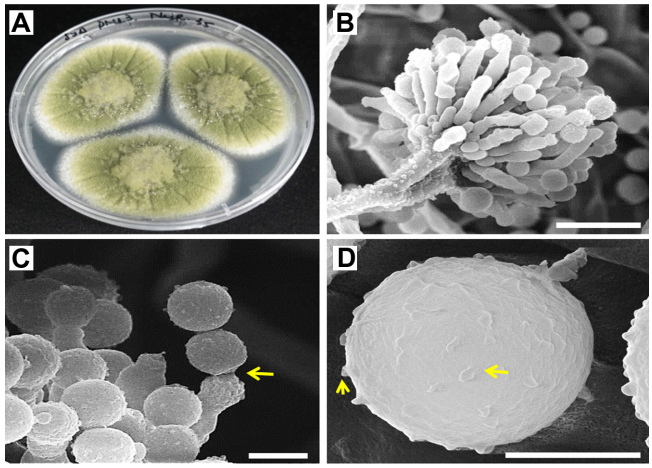

Fig. 2. Colony and conidial shapes of EML-DML3Na1.
(**A**) Colony on Czapeck yeast autolysate agar at 35°C; (**B**) Conidia on phialides; (**C**) Conidia in chains (yellow arrow); (**D**) Conidial surface covered with numerous scales. Scale bars: (**B**) 10 µm; (**C**) 2.5 µm; (**D**) 2 µm.

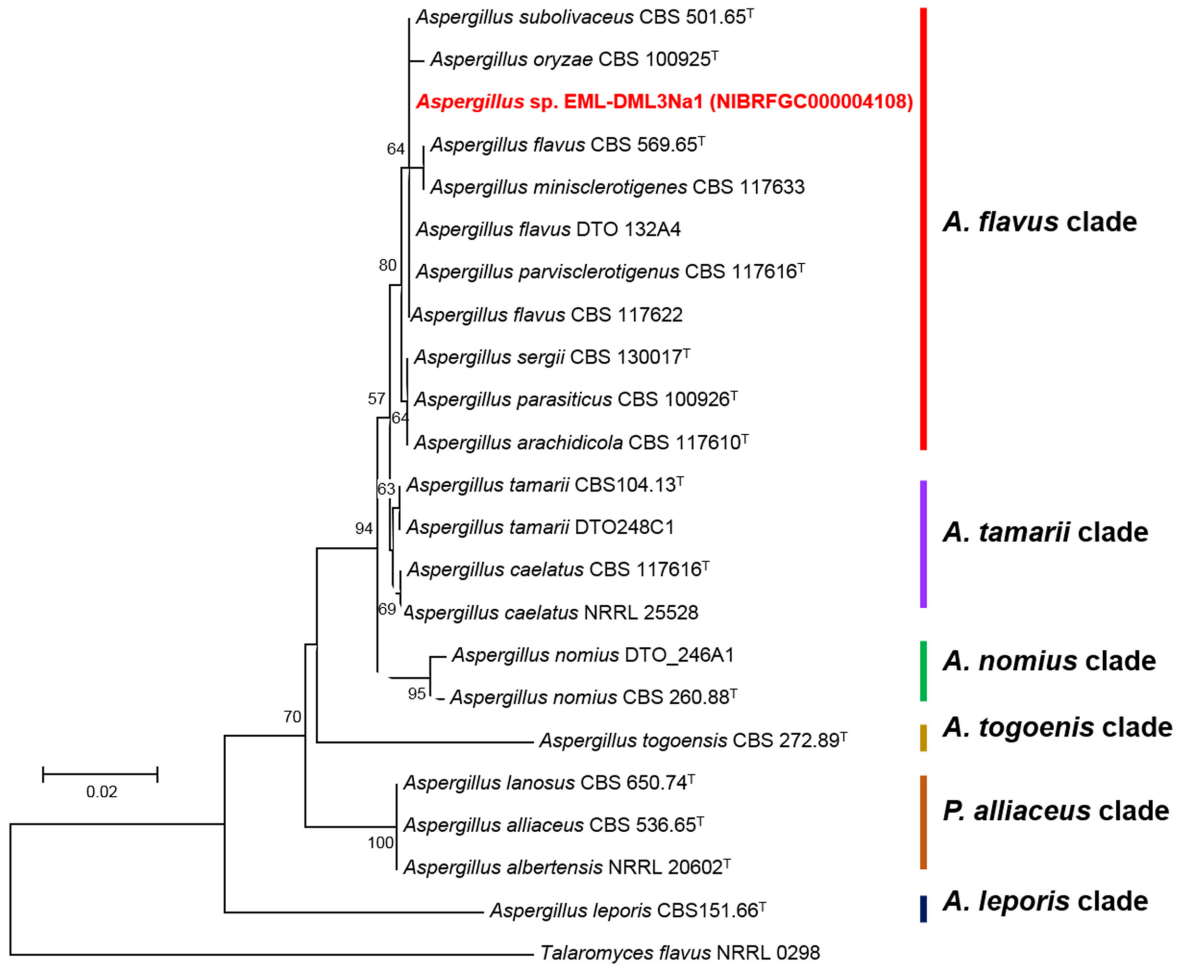

Fig. 3. Phylogenetic tree showing the relationships between the EML-DML3Na1 isolate with known species.
The tree was generated by neighbor-joining analysis using the combination of internal transcribed spacer rDNA, calmodulin, and β-tubulin gene sequences using MEGA6. *Talaromyces flavus* was used as the outgroup. Bootstrap support values ≥50% are indicated at nodes. The tree was based on the classification system constructed by Varga *et al*. [44].

belongs to the *flavus* clade, which includes *A. oryzae*, *A. subolivaveus*, *A. flavus*, *A. minisclerotigenes* and *A. sergei*, within the *Flavi* section (Fig. 3) as reported previously by Varga *et al*. [44].

Characterization of Nematicidal Compounds Produced by EML-DML3PNa1

Organic acid analysis revealed the presence of citric acid (515.0 mg/kg), malic acid (506.6 mg/kg), and fumaric acid (21.7 mg/kg) in culture filtrate of *A. oryzae* EML-DML3PNa1 (Table 1). The three organic acids showed nematicidal activity in a dose-dependent manner against J2 of *M. incognita* (Fig. 4). Among the three organic acids, malic acid exhibited the greatest nematicidal activity, followed by citric acid and fumaric acid. The J2 mortality rate induced by the mixture of the three organic acids at a 51.5:54.5:2.2 (w/w/w) ratio was almost identical to that of malic acid, indicating a synergistic effect among the organic acids. However, the mixture of the three organic acids did not show the full nematicidal activity of culture filtrate of *A. oryzae* EML-DML3PNa1. Therefore, we predicted the existence of another nematicidal compound in the culture filtrate. To identify this second nematicidal metabolite produced by *A. oryzae* EML-DML3PNa1, the EtOAc extract was successively separated by prep-TLC and prep-HPLC. The nematicidal metabolite was obtained as an off-white solid. EI-mass analysis of the isolated metabolite displayed a strong molecular ion at *m/z* 142 and fragment ions at *m/z* 113, 97,

Table 1. Yield of organic acids produced by *A. oryzae* EML-DML3Na1 in potato dextrose broth medium.

Organic acid	Yield (mg/kg)
Acetic acid	ND [a]
Citric acid	515.0
Fumaric acid	21.7
Lactic acid	ND
Malic acid	545.6
Oxalic acid	ND
Succinic acid	ND
Tartaric acid	ND

[a]ND: not detected.

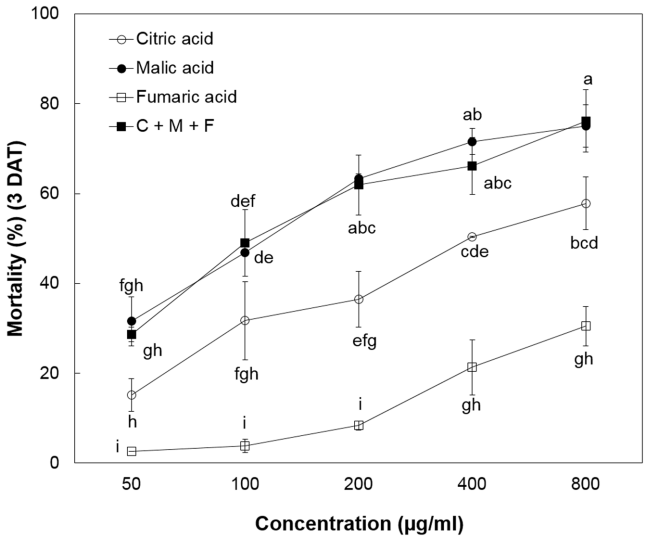

Fig. 4. Nematicidal activity of citric acid, fumaric acid, malic acid, fumaric acid, and a mixture of citric acid:malic acid:fumaric acid (51.5:54.5:2.2 (v/v/v)) at various concentrations against *M. incognita*.

85, 68, and 57. The ^{1}H-NMR spectra exhibited signals at δ 4.42 (2H, singlet) δ 6.51 (1H, singlet), and δ 7.95 (1H, singlet). The ^{13}C-NMR spectra showed signals at δ 61.33, 110.84, 141.12, 147.71, 170.46, and 177.16 ppm. These data were in agreement with those of kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone) reported previously [32, 33].

Effect of Kojic Acid on *M. incognita* J2s and Eggs

The EC_{50} value of kojic acid against J2s of *M. incognita* was 195.2 μg/ml after 3 days of treatment (Fig. 5). The EC_{50} value of cinnamaldehyde (used as a positive control) was 19.3 μg/ml. The EC_{50} values of kojic acid and cinnamaldehyde for inhibition of egg hatching were 238.26 and 75.46 μg/ml,

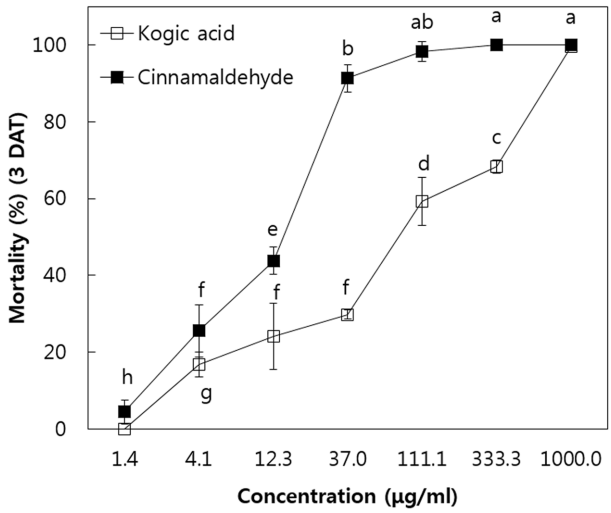

Fig. 5. Nematicidal activity of kojic acid and *trans*-cinnamaldehyde against *M. incognita*.
Values are means ± standard deviation of three replicates.

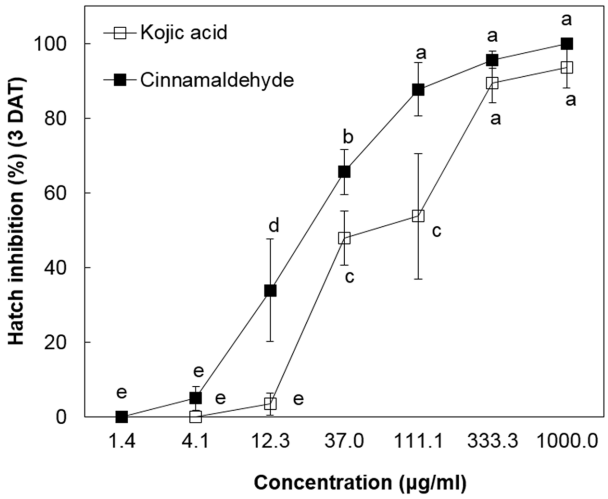

Fig. 6. Inhibition of *M. incognita* egg hatching by various concentrations of kojic acid and *trans*-cinnamaldehyde.
Values are means ± standard deviation of five replicates.

respectively, after 3 days of treatment (Fig. 6).

Discussion

RKNs have been reduced by use of chemical synthetic nematicides. However, the withdrawal of methyl bromide, the most effective and frequently used fumigant for control of nematodes, and restricted use of organophosphate- and carbamate-based nematicides have made management of RKNs problematic [29]. Thus, alternative environmentally

friendly control measures are required. Fungi isolated from plants are a major source of novel secondary metabolites, some of which have useful biological activities [31, 34]. Nematicidal activity was reported in the culture filtrate of *Fusarium oxysporum* isolated from tomato roots [15]. Similarly, culture filtrate of *Acremonium implicatum* isolated from tomato root galls killed 96% of J2s of *M. incognita* [42]. *Geotrichum* sp. AL4 isolated from *Azadirachta indica* produced two novel nematicidal metabolites, chlorinated and epimeric 1,3-oxazinane derivatives [23]. The metabolites showed similar nematicidal activity, both killing 60% of *Bursaphelenchus xylophilus* at a concentration of 100 μg/ml. In addition, a nematicidal metabolite, 3-hydroxypropionic acid, was isolated from *Phomopsis phaseoli*; its LD_{50} values against *M. incognita* were 12.5–15 μg/ml [35].

Our results indicated that treatment with the culture filtrate of *A. oryzae* EML- DML3PNa1 killed *M. incognita* J2s and suppressed egg hatching. The nematicidal factors were identified as organic acids; namely, citric acid, malic acid, fumaric acid, and kojic acid. As the results of HPLC analysis, kojic acid was detected in the fermentation broth at a concentration of 414 μg/ml (data not shown). Considering the mortality and the contents of the acids, the nematicidal activity of kojic acid and malic acid predominated. Recently, the nematicidal activity of kojic acid against juveniles of *Caenorhabditis elegans* was reported [28]. However, its nematicidal activity has not been reported against plant parasitic nematodes yet. Thus, the killing of *M. incognita* J2s and inhibition of egg hatching of kojic acid are reported for the first time in this study.

Nematicidal activities of organic acids have been reported previously. Zukerman *et al*. [49] reported that *A. niger* PD-42, an antagonistic fungus to nematodes, produced citric acid (0.9 g/l) and oxalic acid (6.1 g/l) as nematicidal metabolites. In addition, Aissani [3] reported the effect on *M. incognita* J2s of malic acid; its EC_{50} value was >1,000 mg/l. Acetic acid was identified as a nematicidal substance produced by *Paecilomyces lilacinus* and *Trichoderma longibrachiatum* [10]. Additionally, nematicidal activities of several other organic acids—such as lactic acid, tartaric acid, butyric acid, propionic acid, valeric acid, and caprylic acid—have been reported [5, 7, 22, 39].

Regarding the mode of action of kojic acid, Abdel-Rahman *et al*. [2] reported the nematicidal activity of organic acids as determined by log P and Henry's Law constant (K_H) values. They revealed that among the tested organic acids, most of the highly active nematicidal compounds possessed log *p* values of −0.65 to 2.42 and mean K_H values of 2.6×10^{-7} atm.m^3/mole. Because kojic acid has a log *p* value of −0.9 and K_H value of 2.4×10^{-7} atm.m^3/mole, it likely has strong nematicidal activity compared with other organic acids.

Kojic acid is produced by several *Aspergillus* spp., and it and its derivatives recently have received attention owing to their antimicrobial, antiviral, anti-inflammatory, antitumor, antidiabetic, and skin-whitening activities [4, 6, 8, 16, 48]. However, no report on the nematicidal activity of kojic acid against plant parasitic nematodes has been published to date. Kojic acid has been isolated from many oriental foods—such as miso, sake, and shouy—and is generally regarded as safe. It can be used to control RKNs in an environmentally friendly manner. Our research suggests that the culture filtrate of *A. oryzae* EML- DML3PNa1 and its compound kojic acid exhibit potent nematicidal activities against *M. incognita*. Thus, *A. oryzae* EML-DML3PNa1 and kojic acid could be used as microbial nematicides for the control of RKNs without unfavorable effects on the environment. Further studies are needed to evaluate the efficacy in pot and field experiments, to examine the spectrum of applicable nematode species, and to establish the optimal conditions for production of kojic acid.

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