

RESEARCH LETTER – Pathogens & Pathogenicity

Isolation and identification of nematode-antagonistic compounds from the fungus *Aspergillus candidus*

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One sentence summary: A bioassay-guided fractionation of *Aspergillus candidus* culture medium yielded bioactive products that inhibit locomotion and hatching in two important species of plant-parasitic nematodes.

Editor: Stefan Olsson

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ABSTRACT

Culture medium from an isolate of the fungus *Aspergillus candidus* was extracted, fractionated and examined to discover compounds antagonistic to plant-parasitic nematodes that are important pathogens of agricultural crops. Column, thin layer and preparative chromatographies and spectral and elemental analyses, were used to isolate and identify two major constituents of an active fraction (Fraction F) obtained from the medium. Compound 1 was identified as 2-hydroxypropane-1, 2, 3-tricarboxylic acid (citric acid). Compound 2 was identified as 3-hydroxy-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid, an isomer of 1, 2-dimethyl citrate. Compound 1 and a citric acid standard, each tested at 50 mg mL⁻¹ in water, decreased hatch from eggs of the plant-parasitic nematode *Meloidogyne incognita* by more than 94%, and completely immobilized second-stage juveniles after 4–6 days exposure. Fraction F and Compounds 1 and 2 decreased the mobility of adults of the plant-parasitic nematode *Ditylenchus destructor* *in vitro*. Fraction F (25 mg mL⁻¹) inhibited mobility >99% at 72 hrs. Compounds 1 and 2 (50 mg mL⁻¹) each inhibited mobility more than 25% at 24 hr and more than 50% at 72 hr. This is the first assignment of nematode-antagonistic properties to specifically identified *A. candidus* metabolites.

Keywords: bio-based compounds; dimethyl citrate isomer; fungal toxins; natural products; nematotoxicity; spectral identification

INTRODUCTION

Plant-parasitic nematodes cause annual crop yield losses of more than $\text{€}100$ billion worldwide (Chitwood 2003), and af-

fect their damage through a variety of biological strategies. Two of the most important groups comprise *Meloidogyne* spp. and *Ditylenchus* spp. (Jones et al. 2013). *Ditylenchus*

Received: 24 September 2015; Accepted: 26 January 2016

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destructor is a pest of crops worldwide, but is particularly widespread in Kazakhstan on potato (CABI 2015). Nematodes gain entry through the lenticel, and complete the life cycle within the tuber in about three weeks. Consequently, reproduction is rapid (Palomares-Rius, Oliveira and Blok 2014). Infections easily spread within the crop through migration of nematodes to neighboring tubers. *Meloidogyne incognita* infects a wide variety of agricultural crops, and its life cycle occurs both inside and outside the root. The infective stage enters the host through the root wherein females mature and produce eggs for deposit in structures external to the root. Juveniles in the eggs develop, pass as the infective stage, and immediately seek new hosts (Perry, Moens and Starr 2009). This cycle is also measured in weeks.

Such sophisticated life cycles and rapid reproductive rates pose enormous challenges to the control of plant nematode pathogens. Despite concerted research and practical efforts, the \$100 billion loss level has remained rather steady for over a quarter century (Sasser and Freckman 1987; Nicol et al. 2011), illustrating the seriousness of the control problem and the difficulties involved. In addition, synthetic chemical nematicides widely used for nematode management can be highly toxic beyond their intended targets, and some are being removed from use (Nyczepir and Thomas 2009). Consequently, environmentally and economically acceptable alternatives are required. The examination of microorganisms for metabolites with nematotoxic properties can be useful in the search for novel control agents. In fact, fungal metabolites have long been recognized as natural products that may be exploited for medical and agricultural purposes (Nisa et al. 2015).

Fungi produce compounds that are active against nematodes (Chitwood 2003), and species from the genus *Aspergillus* are particularly attractive candidates for research studies. Nematicidal activities against *Bursaphelenchus xylophilus* (Hayashi et al. 2007), *Caenorhabditis elegans* (Singh et al. 1991; Zuckerman, Matheny and Acosta 1994), *M. incognita* (Zuckerman, Matheny and Acosta 1994), *Mononchus papillatus* (Bekmakhanova and Shemshura 1998; Bekmakhanova, Shemshura and Mazunina 2002) and *Pratylenchus penetrans* (Kimura, Nakahara and Fujioka 1996; Kusano et al. 2003; Kimura et al. 2007) have been reported for various *Aspergillus* spp.

Compounds from *Aspergillus candidus* (isolate 127) were found to be antagonistic to *D. destructor* (Shemshura 2006). Our goal in this work was to identify the most active components produced by this fungal isolate.

MATERIALS AND METHODS

Fungal culture and medium extraction

Aspergillus candidus was prepared from soil collected from the root zone of potato. A suspension (1:9; g soil: ml autoclaved distilled water, ADW) was serially diluted with ADW to a final dilution of 1:100 from the original suspension. This was used to initiate culture by inoculating Petri dishes containing nutrient wort-agar medium (Titan Biotech LTD, India). After 5 days incubation at 28°C, fungal colonies were picked and used to inoculate wort-agar tubes. Resulting isolates were examined and identified morphologically by the Laboratory of Physiologically Active Compositions, Institute of Microbiology and Virology, Republic of Kazakhstan. Isolate 127 of *A. candidus* was processed further to establish the laboratory culture and source of natural products.

To obtain starting material for natural product preparation, fungal mycelia were transferred to soy medium (25 g soy,

30 g glucose, 2.5 g (NH₄)₂SO₄, 2.5 g NaCl, 5 g CaCO₃ in 1 L distilled water) and cultured for 5 days at 28°C with shaking. Four liters of culture medium were concentrated ca. 40× at 60°C under a vacuum, mixed with ethanol and centrifuged at 2500 × g (Fig. 1). The supernatant was concentrated by rotary evaporation and the residue brought up in water (ca. 100 mL). The aqueous preparation was partitioned in a progressive sequence starting with benzene followed by chloroform, butanol and ethyl acetate. The ethyl acetate fraction was dried, dissolved in ethanol and a slurry prepared (ca. 450 mL) by mixing with a 70–230 mesh silica gel (Fig. 1). The slurry was loaded into a 1 m × 2.5 cm glass column washed with a mixture of butyl alcohol:acetic acid:water (40:12.5:29). Bound material was eluted stepwise starting with 50 mL each of chloroform:ethyl acetate (7:3), then chloroform:ethyl acetate (5:5), chloroform:ethyl acetate (3:7), ethyl acetate and finally ethanol (Fig. 1). Each of these five solvent scheme fractions was screened for nematotoxicity against *D. destructor* (see below for bioassay). Chloroform:ethyl acetate (5:5) was the most biologically active, designated as Fraction F (Fig. 1), and subjected to further analysis.

Processing of Fraction F

Fraction F was evaporated to dryness and fractionated by analytical and preparative thin layer chromatography (TLC). Analytical Sorbfil TLC plates (Sorbpolimer, Krasnodar, RU) were developed using chloroform:methanol (8:3) and the separation visualized using a bromophenol blue solution (1 mg mL⁻¹ in ethanol) that produced spots indicating the presence of two compounds in Fraction F. These were designated as Compound 1 and Compound 2 (Fig. 2). Preparative TLC Sorbfil (Sorbpolimer) plates, developed as above, were used to process additional Fraction F to obtain higher yields of the two compounds for chemical identification and bioassays.

Chemical identification

The TLC-detected compounds were subjected to spectral and elemental analyses. Compound 1 was evaluated and compared with a citric acid standard for physical characteristics (infrared spectra (IR), nuclear magnetic resonance (NMR) and melting point) using standard testing instruments. For structural interpretation of Compounds 1 and 2, mass spectra were obtained using atmospheric pressure chemical ionization (APCI) with an LCQ Deca model ion trap mass spectrometer (Finnigan MAT, San Jose, CA). The samples were introduced as solutions using flow injection methods and ionized in positive and negative modes. Ionization spectra and fragmentation patterns were collected and analyzed using the Finnigan Xcalibur software.

Nematodes

Inoculum of *M. incognita* Race 1, originally isolated in Maryland USA, was reared on pepper 'PA-136' in greenhouse pots, with eggs and second-stage juveniles (J2) collected according to Meyer et al. (2006). Bioassays with *M. incognita* were conducted in Maryland. Adult *D. destructor* were collected fresh for each experiment from potatoes obtained from commercial growers in the Almaty region of Kazakhstan. Potato pieces were placed in sterile water in Petri dishes and the nematodes removed with forceps. Bioassays with *D. destructor* were done in Almaty.

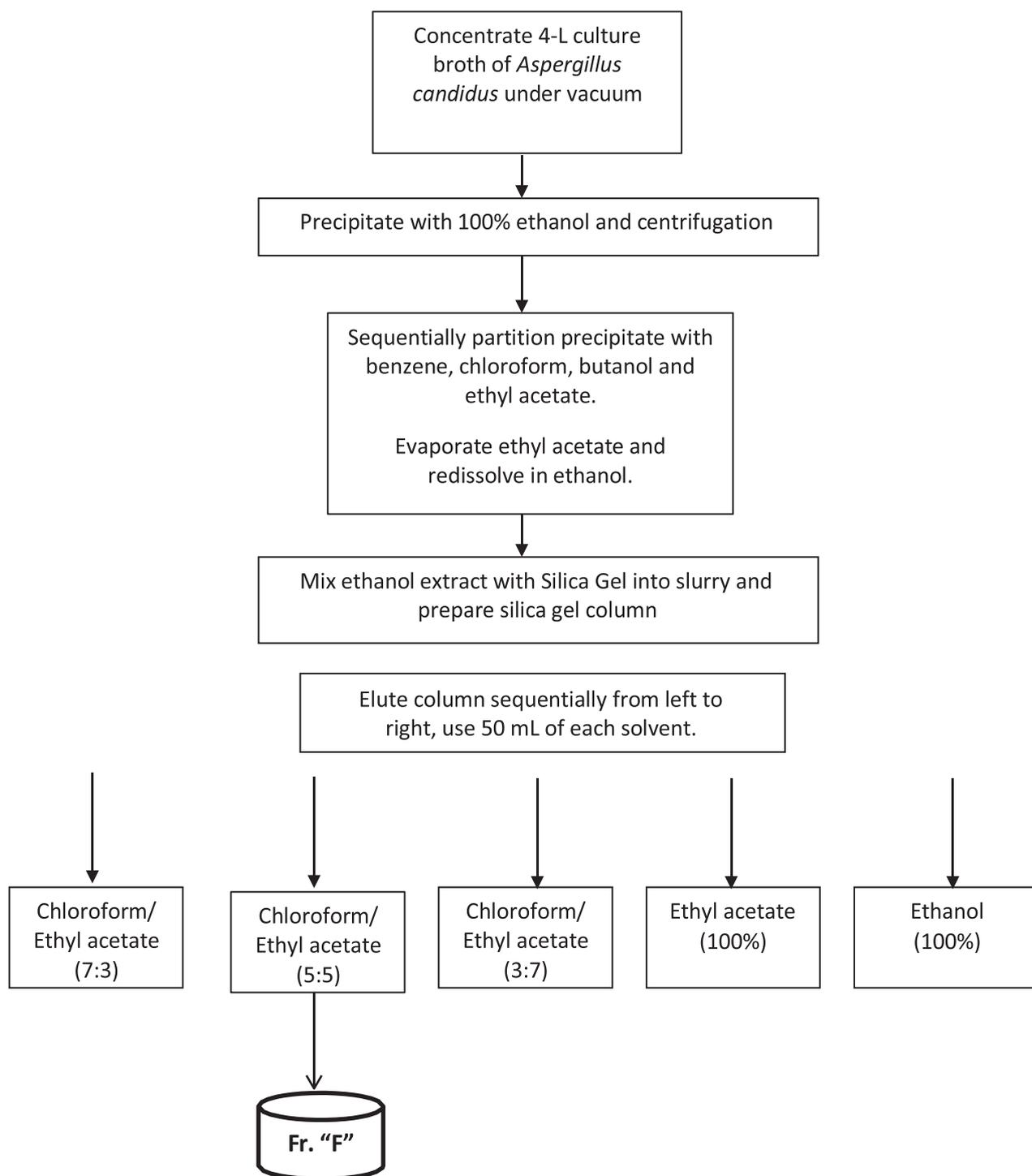


Figure 1. Procedure for obtaining Fraction F from *Aspergillus candidus* culture.

Bioassays

Meloidogyne incognita

The effects of selected compounds on hatching were assessed with *M. incognita* in 96-well microtiter plates. Eggs (ca. 50 per well) were incubated at 26°C in 45 mg mL⁻¹ in ADW of Fraction F, Compound 1, or citric acid standard. Control eggs were incubated in ADW. Each treatment plus control was replicated 5 times in each of two experiments except for Compound 1

(limited quantity allowed use in experiment 1 only, 4 replicates). After 8 days (experiment 1) or 7 days (experiment 2), all J2 that hatched during the incubation periods were counted and scored for mobility (see below). Data were pooled within treatment across both experiments (N = 10 for all treatments except Compound 1, N = 4). Hatch levels were estimated by using the mean number of J2 across all control wells as a benchmark value. Percent hatch within each treatment well replicate was calculated as $[(J_{2T}/J_{2CM}) \times 100]$, where T = treatment and CM = control mean.

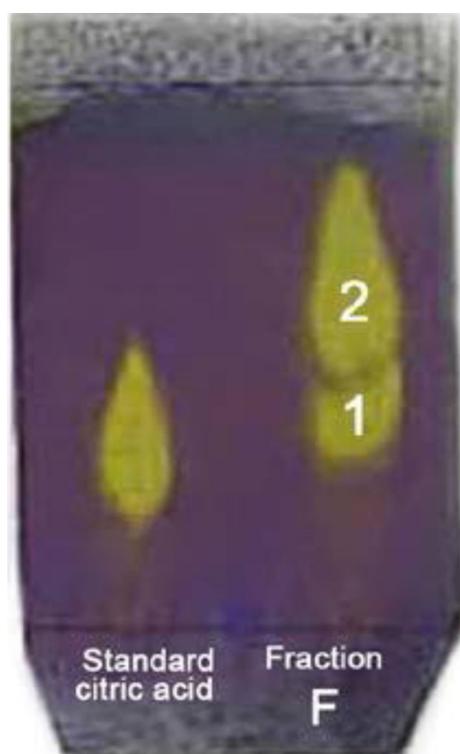


Figure 2. Thin layer chromatographic separation of *Aspergillus candidus* Fraction F components. Sorbifil plates were developed with chloroform:methanol (8:3). The two spots in Fraction F correspond to citric acid (Compound 1, slower moving) and 1,2-dimethyl citrate (Compound 2, faster moving). See text for details.

Means of percent hatch were averaged across all treatment wells to provide mean percent hatch for each treatment.

Behavior effects were quantified by scoring J2 as mobile or immobile at the end of the two hatch experiments, calculating percent immobile for each well, and averaging across all treatment or control wells. In a separate assay, J2 that had hatched in ADW were subsequently transferred to wells (ca. 40 J2 per well) containing Fraction F or citric acid control (45 mg mL⁻¹ in ADW) or ADW control. After 6 days (for experiment 1) or 4 days (for experiment 2), J2 immobility in these treatments was measured as above.

Ditylenchus destructor

The behavioral effects of Fraction F, Compound 1, Compound 2 and citric acid standard on *D. destructor* were assessed on adult nematodes incubated in 96-well microtiter plates at 26°C. 10 adult nematodes per well were exposed to test compounds at doses of 1, 2.5, 5, 10, 25 and 50 mg mL⁻¹ in ADW. Controls were incubated in ADW. Within each experiment, treatments were replicated 5 times per each dose and ADW control. Experiments were replicated 3 times each for nematode exposures of 24 hr and 72 hr. All nematodes in each well were scored as mobile or immobile, and then transferred to ADW for an additional 24 hr for recovery and final scoring. Percent recovery was calculated as [(initial % immobile - post ADW % immobile) / initial % immobile] × 100. All treatment means were compared across 3 replicate experiments within the 24 hr and 72 hr groups (N = 15 for each treatment dose and for each exposure time).

Statistical analyses

Individual means were compared using Student's t-test. Differences among multiple means were determined by ANOVA and compared using Tukey Kramer's adjustment for multiple comparisons (GraphPad Prism, GraphPad Software, LaJolla CA; JMP Software, SAS Institute, Cary, NC).

RESULTS

Isolation and identification of nematode-antagonistic compounds

Fraction F included two compounds separated by TLC as two isolated spots (Fig. 2). The main compound found (Compound 1) was acidic in nature with a retention factor (Rf) value of 0.03 to 0.11, while Compound 2 had an Rf value of 0.23 to 0.35. These zones were cut out, eluted with methanol and the resulting extracts evaporated to dryness. Based on the tests outlined in the paragraph below, the slower moving spot (Compound 1) was identified as citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid), and the upper spot (Compound 2) was identified as 1,2-dimethyl citrate (3-hydroxy-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid).

Physical and chemical properties were used to identify the compounds. The melting point of Compound 1 was determined to be 153°C which matched the melting point of citric acid. The IR, NMR and APCI negative ion spectra for Compound 1 all agreed with the values measured for citric acid. Thus, we feel confident this compound is citric acid. Compound 2 was characterized using mass spectrometry and mass spectrally compared against a chemically synthesized form of dimethyl citrate (below). Spectra developed from Compound 2 were equally well detected both in positive and negative ion monitoring mode, and produced the positive molecular ion of 221 and negative ion of 219 *m/z*, respectively. These masses correspond with the respective ionization spectra of dimethyl citrate under these ionization modes. The fragmentation pattern for the negative ion spectra yielded an ion with 173 *m/z* mass units (Fig. 3A).

A synthetic 1,3-dimethyl citrate (2-hydroxy-4-methoxy-2-(2-methoxy-2-oxoethyl)-4-oxobutanoic acid) was produced by reacting citric acid with the reagent Methyl-8® (N,N-Dimethylformamide dimethyl acetal) as described by Saleem *et al.* (2004). A product from this reaction was a compound with a parent mass similar to that of Compound 2 (e.g., 220-negative APCI); however, it fragmented to an ion fragment different from Compound 2 (e.g., 143 *m/z*, Fig. 3B). Saleem *et al.* (2004) also reported that ions resulting from an electron impact (EI) mass spectrum of this dimethyl compound were dominated by an *m/z* ion 143, which caused us to suspect that this synthetic product was the same compound.

Interpretation of the spectra of these two compounds (Compound 2 and the synthetic dimethyl citrate) are provided in Fig. 4. Based on careful spectral analyses it appears that for both compounds the fragments are formed by cleaving the bond between the α carbon and the carbonyl groups of the more distant carbonyl pairs in the molecular structure depicted in Fig. 4. Thus, fragmentation of Compound 2 yielded a mass of 174.05 with loss of the non-esterified carboxylic group (Fig. 4A) while the other compound produced a larger leaving group (e.g. only esterified groups exist at these two carbonyl positions). The leaving group in this case was 76 *m/z* units and produced the 144.04 *m/z* fragment (Fig. 4B), which corresponded to 1,3-dimethyl citrate (2-hydroxy-4-methoxy-2-(2-methoxy-2-

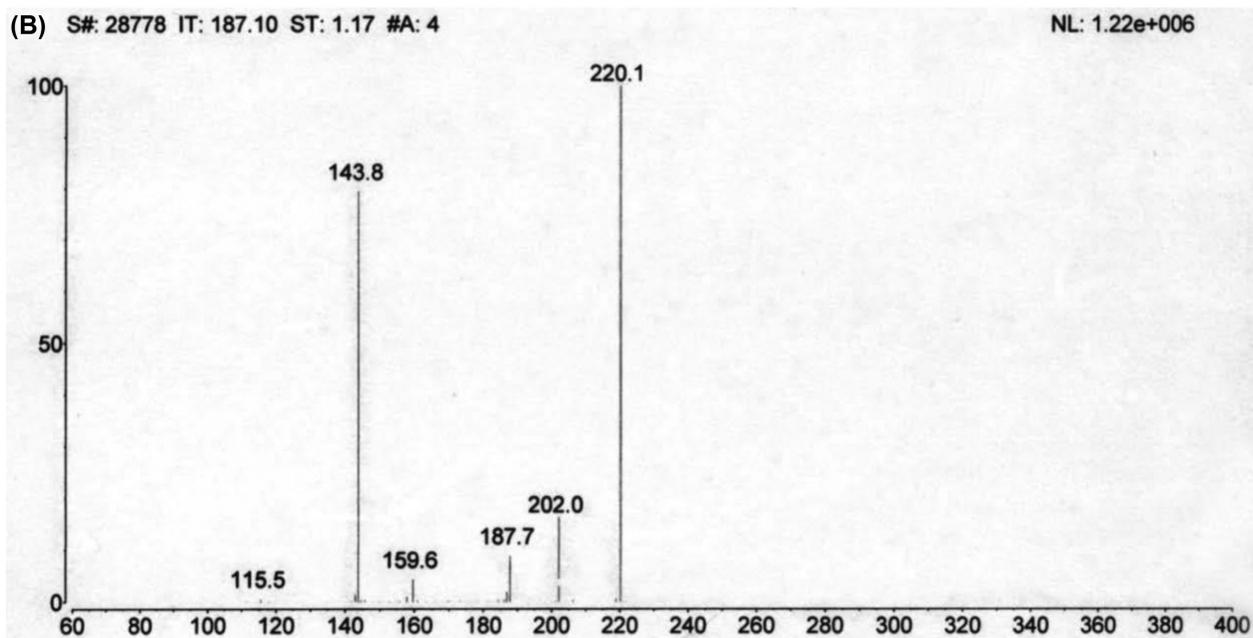
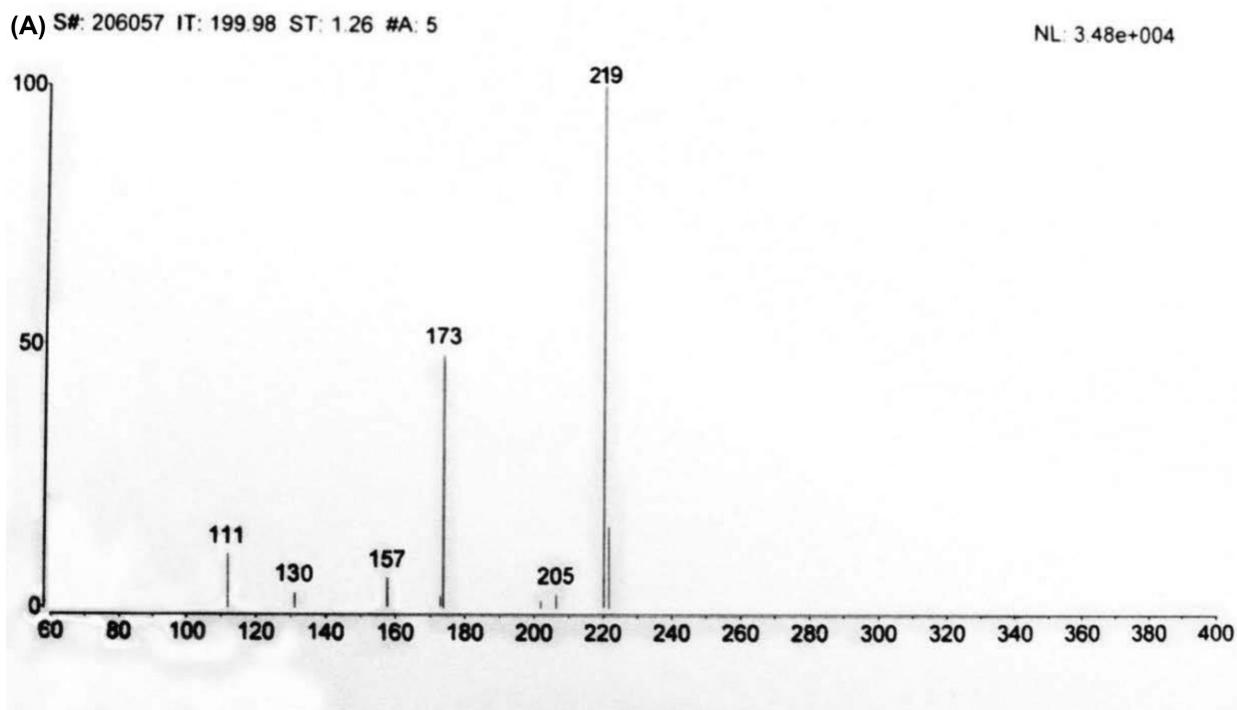


Figure 3. Negative APCI-spectra for: (A) *Aspergillus candidus* Compound 2 (3-hydroxy-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid), and (B) synthetic dimethyl citrate (2-hydroxy-4-methoxy-2-(2-methoxy-2-oxoethyl)-4-oxobutanoic acid).

oxoethyl)-4-oxobutanoic acid) for the structure of this chemically synthesized compound.

Antagonistic effects on nematodes

Adult, egg and juvenile stages were each affected by material prepared from *A. candidus* culture medium. Percent hatch of *M. incognita* at 7–8 days exposure to 45 mg mL⁻¹ Fraction F or

Compound 1 was significantly ($P < 0.05$) lower than in water controls (Table 1). The decreases in hatch (>94%) were similar to that effected by citric acid standard. The J2 that did hatch directly into the 3 treatment solutions became completely immobile, whereas immobile J2 only accounted for 24% of all J2 in the water controls. Previously hatched and mobile J2 that were transferred from water to either Fraction F or citric acid were completely immobilized after 4–6 days (Table 1).

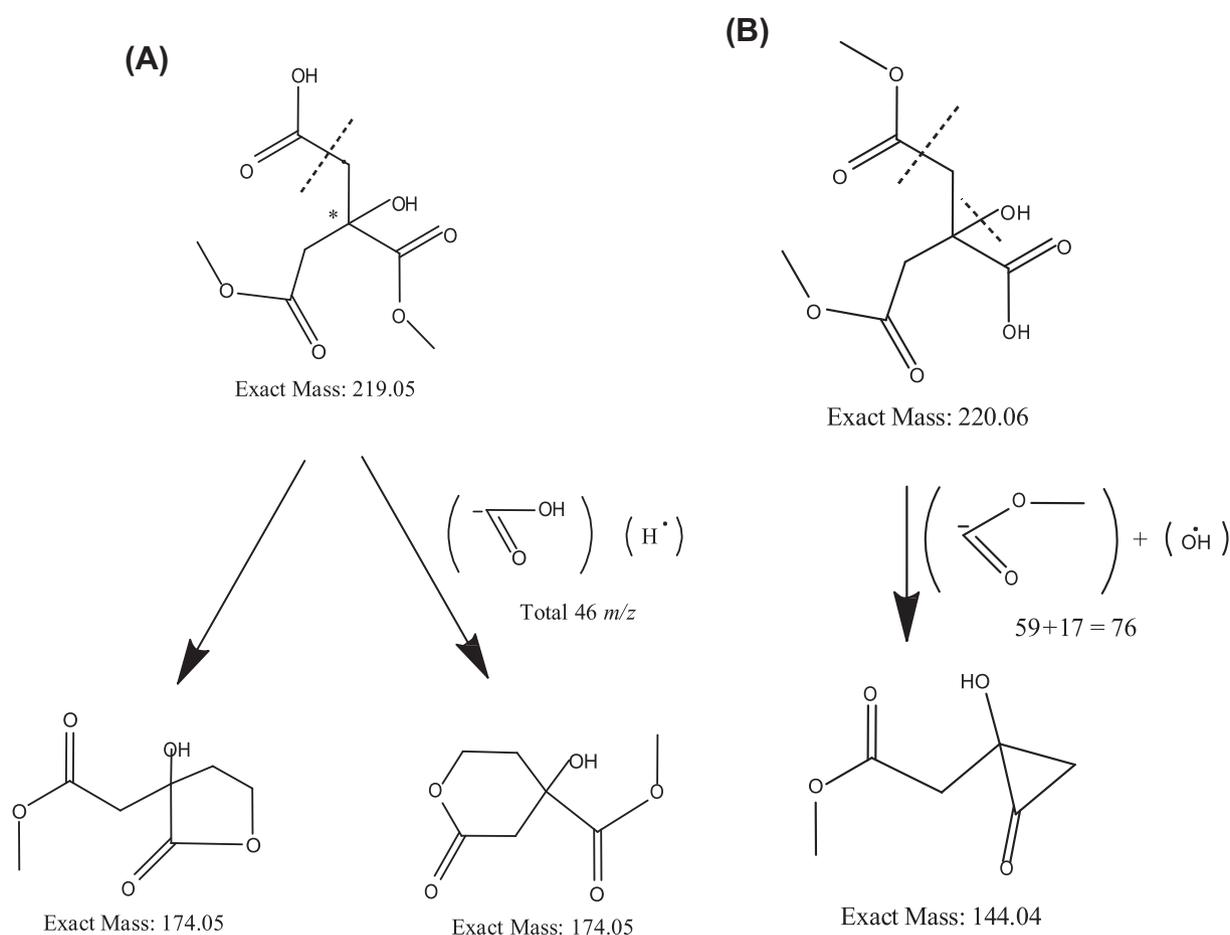


Figure 4. Mass spectral interpretation and presumed APCI MS-ionization fragments from: (A) *Aspergillus candidus* Compound 2, and (B) synthetic dimethyl citrate.

Table 1. Effect of *Aspergillus candidus* metabolites on hatching and mobility of second-stage juveniles (J2) of the plant-parasitic nematode *Meloidogyne incognita*.

Treatment ^b	Relative % hatch ^a	Percent J2 immobility	
		Hatched directly into treatments ^c	Transferred from water ^d
Fraction F	5.9 ± 2.1 ¹	100 ²	100 ²
Compound 1	2.0 ± 0.8 ¹	100 ²	nd
Citric acid standard	4.9 ± 1.4 ¹	100 ²	100 ²
Water control	100 ²	24.4 ± 1.6 ¹	9.4 ± 2.1 ¹

Data are expressed as mean ± SE of 10 replicates for each treatment (except Compound 1; N = 4). Means were compared across treatments. Within a column, means followed by different numbers are significantly different ($P < 0.05$; Tukey's adjustment for multiple comparisons). ^aRelative percent hatch within each treatment well replicate was calculated as $[(J_{2T}/J_{2CM}) \times 100]$, where T = treatment and CM = control mean. For purposes of calculation, the control mean was considered the 100% benchmark (see text). ^bFinal concentration of each treatment in the wells was 45 mg mL⁻¹ in ADW. ^cJuveniles hatched into treatment solution directly from eggs immersed in the treatment solution. ^dJuveniles hatched from eggs immersed in water and then transferred to treatment solutions.

Fraction F and citric acid each exhibited effects on *D. destructor* mobility by 24 hr exposure to 2.5 mg mL⁻¹ (Fig. 5A), and all treatments were inhibitory at 5 mg mL⁻¹ at both 24 and 72 hr (Fig. 5A and B). Percent immobility at 24 hr by Fraction F, Compound 1, or citric acid increased continuously ($P < 0.05$) from 5 to 50 mg mL⁻¹, and from 10 to 50 mg mL⁻¹ for Compound 2. Fraction F and citric acid were equally inhibitory through 25 mg mL⁻¹, but Fraction F was more inhibitory (56.7 ± 1.3%; $P < 0.05$) at 50 mg mL⁻¹ than citric acid (48.6 ± 1.9%). At 24 and 72 hr, Compounds 1 and 2 were equally inhibitory at all doses, and were always less inhibitory than either Fraction F or citric acid

(Fig. 5A and B). At 72 hr, Fraction F inhibition increased between each dose ($P < 0.05$) from 5 to 25 mg mL⁻¹, and citric acid inhibition increased between each dose ($P < 0.05$) from 10 to 50 mg mL⁻¹ (Fig. 5B). In contrast to the 24 hr exposures, at 72 hr Fraction F was significantly more inhibitory than citric acid from 10 to 50 mg mL⁻¹ ($P < 0.05$), and only Fraction F caused complete inhibition (50 mg mL⁻¹, 72 hr). Compounds 1 and 2 only exceeded 50% inhibition at 50 mg mL⁻¹ and 72 hr (Fig. 5B), and due to their weak inhibition at lower doses, benefitted the most from increased concentrations. Inhibition increases from exposure to 5 mg mL⁻¹ vs. 50 mg mL⁻¹ were 7.7-fold and 5.6-fold

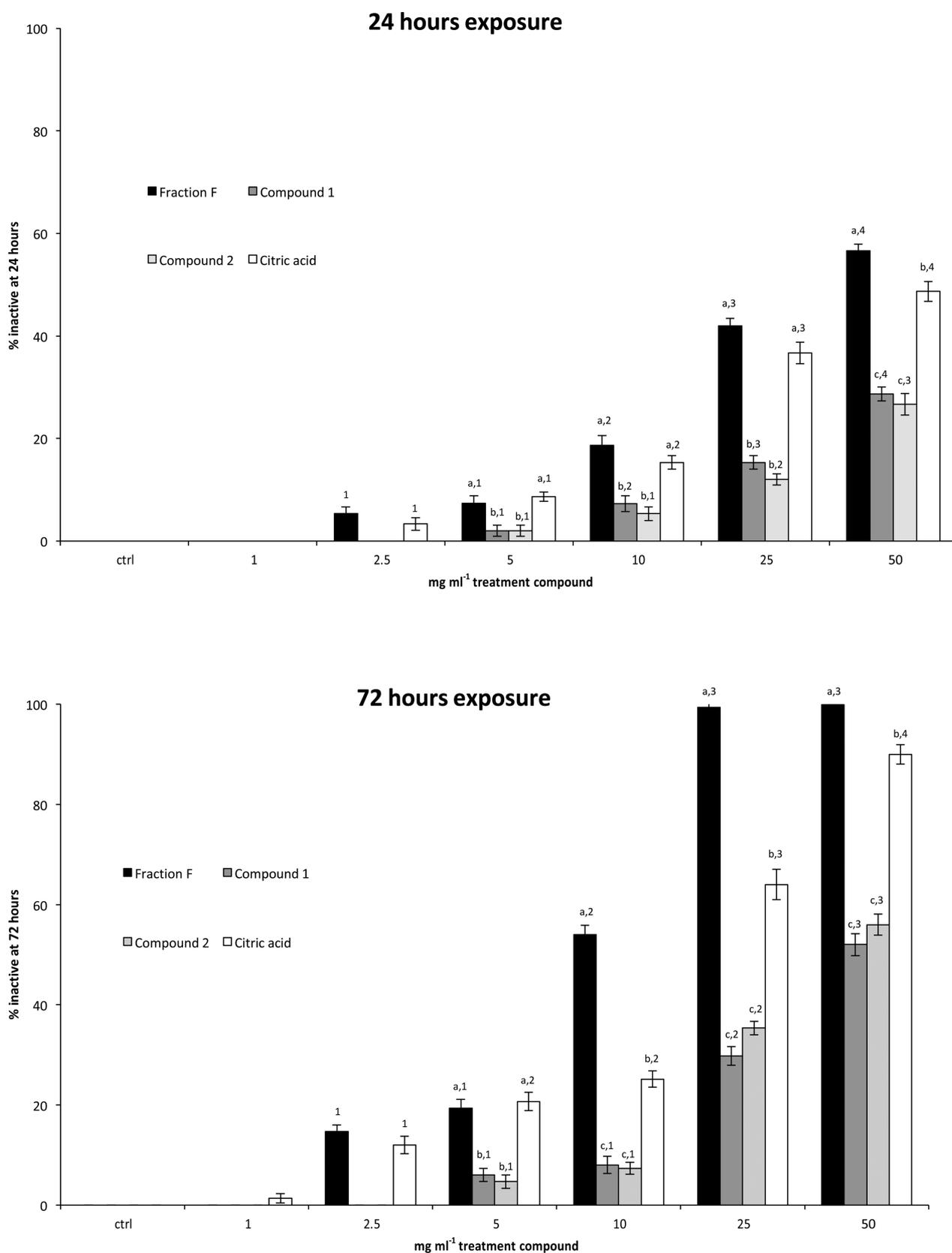


Figure 5. Nematotoxicity of *Aspergillus candidus* Fraction F (black bars), Compound 1 (citric acid; dark grey), Compound 2 (dimethyl citrate, light grey), and citric acid standard (white), against the plant-parasitic nematode *Ditylenchus destructor*. Nematodes were exposed to treatment solutions *in vitro* for 24 hr (A) or 72 hr (B) and immobility determined as described in the text. Percent immobility means ($N = 15$; \pm SE) were compared by 1-way ANOVA. Within doses and between treatments, means labeled with different letters are significantly different ($P < 0.05$). Within treatments and between doses, means labeled with different numbers are significantly different ($P < 0.05$).

($P < 0.05$) for Fraction F and citric acid, respectively, at 24 hr. Corresponding values at 72 hr were 5.2-fold and 4.4-fold. For Compound 1, increases ($P < 0.05$) were 14.3-fold at 24 hr and 8.7-fold at 72 hr. Corresponding values for Compound 2 were 13.3 and 12.0.

At all doses of 5 mg mL⁻¹ and above, there were significant increases ($P < 0.05$) from 24 to 72 hr in the inhibition of mobility by each of the four treatments. The mean increase for all treatments with all concentrations ($n = 16$) was 2.1 ± 0.1 fold. The mean fold increase across all doses for individual treatments ($n = 4$) was 2.4 ± 0.2 for Fraction F and 2.0 ± 0.4 , 2.2 ± 0.3 , and 1.9 ± 0.2 for Compound 1, Compound 2 and citric acid, respectively. None of these means were significantly different from each other, or from the total mean. Likewise, means of fold increases across all treatments at 5 mg mL⁻¹ (2.6 ± 0.2), 10 mg mL⁻¹ (1.8 ± 0.4), 25 mg mL⁻¹ (2.2 ± 0.3) and 50 mg mL⁻¹ (1.9 ± 0.1) were the same. Thus, the relative increases in immobility with time were not factors of either dose or compound.

Some recovery of mobility following the post-treatment 24 hr incubation in ADW was observed for all treatments except for Fraction F, 50 mg mL⁻¹, 72 hr. This revealed nematostatic effects of many of the treatments. However, any recovery of less than 100% indicates nematotoxicity, which varied among treatments. At the highest dose tested, there was partial recovery from all 24 hr exposures. Percent recovery was 22.2 ± 6.6 or less for citric acid, Fraction F and Compound 1. Recovery from Compound 2 treatment was 35.4 ± 3.3 percent, which was significantly greater ($P < 0.05$) than from any other treatment. Recovery from 72 hr exposures was less than 1% for citric acid and 0% for Fraction F. Percent recoveries from Compound 1 (22.9 ± 2.1) and Compound 2 (27.2 ± 2.0) were each significantly greater than from citric acid and Fraction F 72 hr exposures.

DISCUSSION

Antagonism of the fungus *A. candidus* against two plant-parasitic nematodes is primarily due to the production of two major compounds that we have identified as citric acid (Compound 1) and 1,2-dimethyl citrate (3-hydroxy-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid) (Compound 2). We believe this is the first report of the nematode-antagonistic effects of this 1,2-dimethyl citrate. However, the actual toxic isomeric form still remains to be identified since there are two chiral forms, R and S, with the chiral center at the carbon where the hydroxyl group is attached (note the asterisk in Fig. 4A). Hawranik and Sorensen (2010) characterized 1,3-dimethyl citrate from *A. niger* and noted that it is also a constituent of numerous higher plants. The most common organic acids isolated from *Aspergillus* spp. are carboxylic acids such as citric acid (Lesniak, Pietkiewicz and Podgorski 2002; Zhang and Roehr 2002).

We observed that Compounds 1 and 2 each caused immobility of both *D. destructor* and *M. incognita*. Pinkerton and Kitner (2006) inhibited mobility of the nematode *P. penetrans* using a biologically derived citric acid mixture, but it was in the presence of detergent. With both *D. destructor* and *P. penetrans*, however, immobility could be reversed by transfer to water. Riga et al. (1997) reported that juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* were repelled by citric acid, whereas Wang, Bruening and Williamson (2009) reported that citric acid and other carboxylic acids were attractants for *M. hapla*. At the maximum dose tested, we observed some mobility recovery with *D. destructor*, but it was consistently low, es-

pecially with Fraction F, indicating that all treatments were nematotoxic.

The results of the mobility experiments with *D. destructor* suggest that the effects of Compounds 1 and 2 are not simply additive relative to Fraction F, from which they are derived. It is possible that Fraction F contains additional, unidentified antagonistic factors that contribute to its effectiveness at comparatively low doses. Compounds 1 and 2 may have synergistic effects that contribute to the potency of Fraction F. Given these observations, an expanded exploration of *A. candidus* for additional nematode suppressive compounds is warranted.

Fungal metabolites such as citric acid and oxalic acid may bind metal ions (Gadd 1993) including Zn²⁺, that is possibly involved with nematode hatch (Nitao, Meyer and Chitwood 1999). This suggests a mode of action for these compounds in *M. incognita*. Further analyses of the chemical properties of *A. candidus* metabolites, including additional organic acids (Magnuson and Lasure 2004), and their modes of action in plant-parasitic nematodes should provide important discoveries for crop protection.

ACKNOWLEDGEMENTS

We thank Mikhail Arinbasarov, Boris Baskunov and Natalia Vinokurova for their assistance in the chemical identifications, Paula Crowley and Shannon Rupprecht for assistance with *M. incognita* bioassays and data analysis.

FUNDING

We thank the ISTC for financial support to NEB and ONS.

Conflict of interest. None declared.

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