RESEARCH PAPER

Prospection and identification of nematotoxic compounds from *Canavalia ensiformis* seeds effective in the control of the root knot nematode *Meloidogyne incognita*

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Received 4 April 2017; accepted 21 October 2017
Available online 7 November 2017

KEYWORDS
Meloidogyne incognita;
Canavalia ensiformis;
Nematotoxic compounds

Abstract *Meloidogyne incognita* is responsible for significant crop losses worldwide. The main strategy to control this phytopathogen is still based on synthetic nematicides that are harmful to human health and the environment. In this context, aqueous seed extracts of antagonistic plants were screened for molecules effective toward the infective stage (*J*\textsubscript{2}) of *M. incognita*. The aqueous crude extract of *Canavalia ensiformis* (ACECe) showed the highest nematicidal activity (87 ± 3% mortality). ACECe dialysis fractionation allowed the collection of an external dialysate (EDCe) containing molecules smaller than 3.5 kDa effective against *J*\textsubscript{2} (96 ± 3.0% mortality); innocuous toward non targeted organisms as saprophytic nematodes, fungi, bacterium and insects larvae; active against *J*\textsubscript{2} (96 ± 2% mortality) after heating (50 °C); no cytotoxic for bovine red blood cells; reduction of *M. incognita* eggs masses by 82.5% in tomato plants at green house conditions. Fractionation of the EDCe by reversed-phase high-performance liquid chromatography (RP-HPLC) separated five nematotoxic fractions. Analyses of those fractions based

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https://doi.org/10.1016/j.biori.2017.10.003
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on gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) identified nine specific metabolites, follow-up testing of the individual authentic standards of each metabolite as the compounds: α-glucose, l-canavanine, xanthotoxin, cis-aconitic acid, trans-aconitic acid, malic acid, citric acid, palmitic acid and S-carboxymethylcysteine; all them confirmed to possess nematostatic properties. However, some of those metabolites were not described previously demonstrating biological action against M. incognita.

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Introduction

Phytopathogenic nematodes represent one of the major challenges faced by modern agriculture in optimizing yield/productivity. The potential host range of these phytopathogens encompasses more than 3000 plant species, including economically important crops (Abad, Favery, Rosso, & Castagnone-Sereno, 2003; Jatala & Bridge, 1990). From this perspective, the endoparasite root knot nematode Meloidogyne incognita has attracted significant attention. The parasitic cycle in roots attacked by M. incognita is characterized by the presence of galls containing giant cells (Williamson & Gleason, 2003). Moreover, the injury caused by M. incognita during the penetration process on roots facilitates the invasion of other deleterious microorganisms, such as bacteria and fungi (Potter, Olthof, Evans, Trudgill, & Webster, 1993; Williamson & Gleason, 2003). Hence, M. incognita is responsible for between 8 and 25% losses of yield for agricultural crops, representing an annual worldwide economic impact of millions of dollars (US) (McCarter, 2009; Reynolds et al., 2011; Sasser & Freckman, 1987).

Several strategies have been employed to control M. incognita induced crop damage, including biological control, crop rotation, chemical nematicides, the use of resistant cultivars, antagonistic plants, and integrated pest management. In this context, the application of synthetic nematicides has been the first choice for many years to control this phytoparasite (FAO, 2016; Ntalli & Caboni, 2012). According to literature, synthetic nematicides may promote human health disorders, the destruction of natural pest enemies, the transformation of formerly innocuous species into pests, harm to other non-target species, persistence in food chain and environmental pollution (Berny, 2007; Elbadri, Lee, Park, Yu, & Choo, 2008; Ntalli & Caboni, 2012, Rockström et al., 2016). As a result, the majority of conventional synthetic nematicides were banned or restricted in use, thereby narrowing the availability of this alternative (EC, 2013; EPA, 2009). Hence, an efficient and sustainable approach to control M. incognita is Straitaway required.

Plant natural products display some biological activity that could be associated with protection against a wide range of pests and pathogens, including root knot nematodes (Caboni et al., 2014; Naz et al., 2016; Prakash et al., 2014; Seiber, Coats, Duke, & Gross, 2014). It is estimated that there are approximately 500,000 plant species containing unknown molecules, including metabolites, with high potential use as biocides (Schultes, 1978). Indeed, more than 10,000 natural plant metabolites have been identified, although this still represents less than 10% of the estimated total (Oksman-Caldentey & Inzé, 2004; Wink, 1988). For instance, the plant nematotoxic (nematicidal and/or nematostatic) effects based on compounds, like isothiocyanates, thiophenics, glucosides, alkaloids, phenolics, tannins and others, have been identified and reviewed (Jang et al., 2014; Ntalli & Caboni, 2012).

The nematode-antagonistic plants have received substantial attention due to their remarkable capacity to either paralyze (nematostatic compounds) or to kill (nematicide compounds) nematodes inside plant tissue and in the surrounding soil, thus reducing population density (Sano, 2005). The well-documented nematode antagonistic plants comprise marigolds (Tagetes spp.), rattlesnep (Crotalaria spectabilis), chrysanthemums (Chrysanthemum spp.), castor bean (Ricinus communis), margosa (Azadirachta indica), velvet bean (Mucuna URerxina), Jack-bean (Canavalia ensiformis) and Abruzzi rye (Secale cereale) (Duke, 1990; Ferraz & Freitas, 2000 Ferraz & Freitas, 2004). Extracts obtained from different parts of these plants species, using a wide range of solvent polarities, were previously evaluated for nematotoxic activity in vitro as well under greenhouse conditions (Ferraz & Freitas, 2004). However, plant seeds have received relatively limited attention despite their tremendous potential as a source of nematicide and nematostatic compounds. As an example, studies using C. ensiformis seed powder as a soil additive lead to a dramatically reduced number of eggs of M. incognita and Meloidogyne javanica (Lopes, Ferraz, Dhingra, Ferreira, & Freitas, 2009; Silva, Souza, & Cutrim, 2002). Due to this previous report, the present work have been used the aqueous extract from C. ensiformis seed powder aiming to identify the potential compounds exhibiting nematotoxic activity toward second stage juvenile (J2) of M. incognita.

The investigation of plant metabolites is difficult due to their physiochemical diversity, disparity in abundance and stability, which require a range of different technologies and approaches for analysis. Methods of metabolites profiling have been used for decades. However, technical innovations and substantial improvements to these techniques are now enabling profiling experiments to be conducted in a high throughput manner (Kim, Choi, & Vervoort, 2010; Roessner & Bowne, 2009; Schripsema, 2010). These analyses are typically based on gas chromatography–mass spectrometry (GC–MS) (Fiehn, Kopka, Trethewey, & Willmitzer, 2000; Kopka et al., 2005; Roessner et al., 2001), liquid chromatography–mass spectrometry (LC–MS) (Schliemann, Ammer, & Strack, 2008), and the non-destructive
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Material and methods

Extraction of plant seed metabolites

Mature seeds of *C. ensiformis* (L.) DC (Fabaceae), *Crotalaria juncea* (L.) (Fabaceae), *Crotalaria paulina* (L.) (Fabaceae), *C. spectabilis* (L.) (Fabaceae), *Tagetes minuta* (L.) (Asteraceae), and *Mucuna pruriens* (L.) (Fabaceae) were kindly provided by Plant Germplasm Bank from Embrapa, Brasilia-DF, Brazil. Seeds were ground with a mortar and pestle using liquid nitrogen and subsequently pulverized applying 3 pulses of 30 s in a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO). Ten grams of each powdered seed material was suspended in 60 mL of distilled water (dH₂O) and gently agitated for 24 h at 4 °C. The resulting solutions were individually filtered through eight layers of cheesecloth, and the aqueous crude seed extracts were centrifuged, at 12,000 × g for 45 min at 4 °C. After this procedure it was observed a ring (lipids) in the extract surface that was carefully removed using a micropipette to remove the excess of lipids. The supernatants (aqueous crude extract = ACE, 50 mL) were collected, filter sterilized (0.22 μm pore size; Millipore Corp., Bedford, Mass.) and freeze-dried in a Savant-Super Modulyo (ThermoFisher, GA, USA) lyophilizer. ACE dried materials were subsequently weighed and stored at −80 °C.

Fractionation of *C. ensiformis* ACE

*C. ensiformis* ACE (ACECe) dried was resuspended in dH₂O to obtain a stock solution (250 μg/μL) exhibiting nematicidal activity was transferred to dialysis tubing Spectra/Por 3 with molecular weight cut off of 3.5 kDa (Spectrum Laboratories Inc.) and dialyzed against dH₂O for 24 h at 4 °C. The *C. ensiformis* internal dialysate (IDCe) containing molecules < 3.5 kDa and the external dialysate (EDCe) containing molecules > 3.5 kDa were lyophilized and stored at −80 °C.

Preparation of commercial plant compounds

Aqueous solutions of the commercial compounds l-canavanine, cis- and trans-acetonitril acids, l-malic acid, citric acid, l-tryptophan, l-methionine, α-pinitol, and S-carboxymethylcysteine were prepared at concentrations of 10 mg/mL and d-glucose at 100 mg/mL. In addition, solutions of palmid acid and xanthotoxin (10 mg/mL) were also prepared using (10 mg/mL) ethanol and acetone, respectively. All chemicals were purchased from Sigma–Aldrich with purity higher than 98.5%.

Extraction of *M. incognita* J2 and saprophytic nematodes

To recover eggs, 3-month-old infected tomato (*Lycopersicum esculentum*) roots were cut into 1–2 cm segments and blended for 1 min in a 0.5% sodium hypochlorite solution (Hussey & Barker, 1973). The eggs were collected and transferred to a modified Baermann funnel and maintained at 27 °C. After 48 h, the second stage juveniles (J₂) were counted using Peters slides and an Olympus BH2 B071 microscope. Saprophytic nematodes were obtained from 5-month-old tomato plants that had been previously infected with *M. incognita*. The roots containing several galls in a state of decomposition were cleaned with tap water to remove soil and the nematodes extracted using the methodologies described above.

In vitro nematode assays – *M. incognita* and free living saprophytic nematodes

ACECe (10 μg/μL), EDCE (5 μg/μL), IDCE (5 μg/μL), EDCe heated at 50 °C (EDCe heated) (5 μg/μL) and high-performance liquid chromatography (HPLC) from EDCe fractions (1.2 μg/μL) were diluted in dH₂O. Solutions of commercial chemicals, used as controls, were made up to a final concentration of 1 μg/μL using dH₂O, standards that were not immediately soluble in dH₂O were dissolved in either ethanol or acetone then diluted with dH₂O to final concentrations of 1% (ethanol) and 1% (acetone), respectively. The bioassays were conducted using the final concentration described above for each treatment. Sixty individuals of free living nematodes or J₂ of *M. incognita* were transferred to microcentrifuge tubes and incubated for 48 h at 27 ± 1 °C. Each treatment was replicated three times. Distilled water, ethyl alcohol 70% (EtOH), bovine serum albumin (BSA) and trifluoroacetic acid (TFA) (Sigma–Aldrich Pty Ltd.) were used as controls. After 48 h exposure, the nematodes were observed and counted microscopically with particular attention to their state as either coiled/nonlinear (alive) or paralyzed/straighted/dead. The paralyzed nematodes were submitted to a recovery assay. For nematode recovery assays, after 48 h treatment, J₂ nematodes exhibiting a typical dead posture (paralyzed and straighted form) were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 700 × g for 5 min. The supernatant was
discarded and the J₂ nematodes re-suspended in 1.5 mL dH₂O. This procedure was repeated three times. The nematodes were left suspended in dH₂O for a further 12 h. Nematodes were counted microscopically in order to determine either nematicidal or nematostatic activity of the extracts. The statistical analysis of dead and alive J₂’s after water treatment was evaluated by ANOVA and Tukey’s mean comparison tests, using SPSS software (SPSS Inc., Chicago, Illinois).

Heating test of the C. ensiformis ED

EDCe stock solution previously sterilized (250 μg/μL) was incubated in a water bath for 24 h at 50 °C. Then, an aliquot containing 500 μg was selected and submitted for assays against J₂ of M. incognita, as described previously. The temperature of 50 °C was chosen after literature search concerning the temperature recorded in the rhyzosphere of different plants (Hu & Feng, 2003).

Bacterial assays

The antibacterial activity assays of EDCe (50, 100 and 200 μg/μL) were evaluated using a procedure described by Boudart (1989) with modifications (see supplementary). The assays were performed using the bacterium species Bacillus subtilis NCTC 8236 kindly provided by the Microbiology Laboratory of Melbourne University.

Fungal assays

The antifungal activity assays of EDCe (50, 100 and 200 μg/μL) were conducted according to Abad et al. (1996), with modifications (see supplementary). Fungi (Colletotrichum gloeosporioides access 1915, C. gossypii var. cephalosporioides, Fusarium solani access 1624, Macrophomina phaseolina access 1496 and Phytophthora tabacum (Phytophthora nicotianae var. parasitica)) were kindly provided by the Microbiology Laboratory of Embrapa – Genetic Resources and Biotechnology, Brasília – DF, Brazil.

Insect assays

In vitro assay of EDCe (50, 100 and 200 μg/μL) against Anthonomus grandis larvae was carried out as described by Monnerat et al. (2000). For Spodoptera frugiperda, larval mortality was assessed again at day 5 (Prança, Martins, Batista, & Monnerat, 2004). Each treatment was repeated three times with dH₂O used as a negative control (see supplementary).

Hemolysis assay

Assays were conducted according the supplementary.

Semi-preparative HPLC fractionation

EDCe powder (100 mg) was transferred to a microcentrifuge tube containing buffer A (1 mL, dH₂O/TFA 0.1%) and vortexed three times for 30 s. The solution was centrifuged at 8000 × g for 10 min at 4 °C. The supernatant was filtered with a 0.2 μm Minisart RCA filter (Agilent Technologies) and stored at 4 °C.

The resulting sample was analyzed using an 1100 series High Performance Liquid Chromatography (HPLC) system equipped with a multi-wave length detector and Fraction Collector (Agilent Technologies, Santa Clara, US). EDCe (50 μL, 1 μg/μL) was injected onto a semi-preparative C₂₁ reversed-phase column (Phenomenex, Luna, C₂₁ (2), 250 mm × 10 mm, 10 μm) at room temperature. The mobile phase consisted of 0.1% aqueous TFA (buffer A) and 0.1% TFA in acetonitrile (buffer B). The sample was fractionated using a gradient elution (5% buffer B for 5 min; up to 50% buffer A:50% buffer B for 25 min; up to 100% buffer B for 5 min; 5% buffer B for 1 min; re-equilibration for 11 min) at a flow rate of 2.0 mL/min and collected into 22 individual fractions (F₁–F₂₂). Wavelengths of 216, 250 and 280 nm were monitored and fractions were collected automatically on time, using a threshold of 180–200 mL into a 24 well plate (Corning). The procedure was repeated several times and the corresponding fractions individually pooled, freeze dried and weighed then stored at −80 °C.

GC–MS analysis

Aliquots of EDCe (100 μL, 1 μg/μL), HPLC fractions (1 μg/μL) and commercial compounds (1 μg/μL) were analyzed as their TMS derivatives on an GC–MS system comprised of a Gerstel 2.5.2 autosampler, a 7890A GC and a 5973C quadrupole MS (Agilent Technologies, Santa Clara, US) using the methods of (Hill, Jha, Bacic, Tester, & Roessner, 2013), (see supplementary). Data obtained was processed using Chemstation software (Agilent Technologies, Santa Clara, US). C. ensiformis nematicide and nematostatic compounds were identified by comparison with mass spectra data from the libraries GC–MS Metabolomics RTL Library (Agilent Technologies, Santa Clara, US), NIST (http://www.nist.gov) and Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de, Kopka et al., 2005). The chromatograms and the corresponding mass spectra were deconvoluted using the Automated Mass spectral Deconvolution and Identification System (AMDIS, NIST, http://chemdata.nist.gov/mass-spc/amdis/) software. Quantification of individual compounds was carried out using external standards.

LC–MS analysis

Aliquots of EDCe (40 μL, 1 mg/mL) and HPLC fractions (0.5 mg/mL) were prepared as described previously and were diluted in dH₂O (460 μL), vortexed then transferred to LC vials (Agilent Technologies, Australia). Samples were analyzed using an 6520A Qq-TOF with attached 1200 series LC system comprised of degasser, binary pump, chilled auto-sampler and heated column compartment modules (Agilent Technologies, Santa Clara, US). Instrument conditions were set to: Nebulizer pressure 45 psi, gas flow rate 10 L/min, gas temperature 300 °C, capillary voltage 4000 V, fragmentor 150 V and skimmer 65 V. Both positive and negative ions were monitored in extended dynamic range mode with data collected in m/z 70–1700 amu. Aliquots
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NMR metabolite identification

Dried sample fractions (F1, F3, F6, F17, F18) (−10 mg) were re-suspended in d4-methanol (0.1 mL) and D2O (0.1 mL). Then, samples were diluted with sodium phosphate buffer (0.3 mL, pH 7 using DCl/NaOD titration/0.2%, w/v sodium azide). To each sample was added D2O (0.05 mL) containing 4,4-dimethyl-4-silapentane-1-sulfonic acid (5 mM, DSS). 1H NMR NMR spectra were collected on an 800-MHz Avance US2 spectrometer (Bruker, Bremen, Germany) then analyzed and identified using the Chenomx 5.1 NMR Suite software package (http://www.chenomx.com) (see supplementary).

Greenhouse evaluation

Five samples containing 1 kg of soil were collected from different places of a farm situated around Brasilia-DF – Brazil. Natural soils were prepared according to Jenkins (1964) and analyzed by optical microscopy to certify the absence of M. incognita using a conventional taxonomy. Four hundred grams (400 g) of the soil that lacked M. incognita were placed into 500 g plastic pots. Tomato plant seedlings (L. esculentum Mill, Santa Clara), 15 days post-germination, were transplanted into each pot. Plants were allowed to grow for more than 5 days and then separated into negative control, positive control and EDCE treated. The nematocide Aldicarb (280 mg/kg of soil) was used as positive control and dH2O was used as negative control. Experiments were performed in triplicate (n=3). EDCE solution containing 20 mL (1 mg/mL, final concentration 50 mg/kg of soil) was applied to the soil around each plant. Five hundred (500) M. incognita J2’s were added to each plant, 1–2 cm the soil surface. Tomato plants were then incubated under greenhouse conditions at temperatures varying from 26 to 33 °C. After 45 days of nematode inoculation, the roots were washed, weighed and labeled with β-phenoxin (Sigma–Aldrich, 0.015 mg/L) for 20 min. Samples were rated for egg mass index (e.m.i.) using scale of: 0 = No galls or egg masses; 1 = 1–2 galls or egg masses; 2 = 3–10 galls or egg masses; 3 = 11–30 galls or egg masses; 4 = 31–100 galls or egg masses and 5 = more than 100 galls or egg masses (Taylor & Sasser, 1978). Data were submitted to analysis of variance and treatment means were compared by Tukey’s test (P = 0.05).

Results

Antagonistic plant seed extract screening assay

In vitro assays using ACE (10 µg/µL) from T. minuta, C. ensiformis, M. pruriens, C. juncea, C. spectabilis and C. paulina seeds were performed against second stage juveniles (J2) of M. incognita. The ACECe elicited the highest level of J2 mortality, killing 87 ± 3% of the nematodes after 48 h exposure (Fig. 1). In order to characterize the nematotoxic molecules present in the ACECe, a dialysis procedure was carried out allowing molecular size separation of the plant extract into two different fractions, external dialysate (ED; <3.5 kDa) and internal dialysate (ID; >3.5 kDa).

A second assay against J2 of M. incognita demonstrated that molecules from EDCE (5 µg/µL) produced 96 ± 3% J2 larvae mortality, while IDCe compounds showed no lethal activity (Fig. 2). These results indicated that the toxic effect of C. ensiformis was due to molecules with low molecular weight, found mainly in the EDCE.

Heating tests using EDCE solution (5 µg/µL) that had been heated at 50 °C for 24 h demonstrated a high level of toxicity retained against J2 of M. incognita with 96 ± 2% of the J2 larvae killed after 48 h of exposure (Fig. 2).

Non-target organism assays – saprophytic nematode, bacteria, fungi and insects assays

In vitro assay for free living saprophytic nematodes using 5 µg/µL of EDCE demonstrated a mortality rate of only 6 ± 3%, compared with 96 ± 3% for J2 of M. incognita (Fig. 3 and Table 1). Assays using EDCE against the agriculturally relevant fungi C. gloeosporioides (access 1915), F. solani (access 1624), M. phaseolina (access 1496) and P. tabacum var. Parasitica did not show any growth inhibition halos around discs containing 250 µg/5 µL, 500 µg/10 µL and 1000 µg/20 µL of EDCE. Indeed, all fungi grew normally, covering the full Petri dish after 96 h exposure, except around discs where the antibiotic nystatin was used as a positive control (Table 1). The same concentrations of EDCe also failed to inhibit the growth of the bacteria, B. subtilis, after 16 h incubation. Similar to fungus inhibition assays, the rings of clearing (halos) were only observed around discs containing the antibiotic sirodesmin that was used as a positive control (Table 1).

Furthermore, no insecticidal effect was observed when the agricultural pests A. grandis and S. frugiperda insect larvae were grown on an artificial diet containing 200 µg/µL of EDCe. After 7 days incubation, the larvae of both insects exhibited the same physiological characteristics (weight, size, color and mobility) and were indistinguishable from those used in the control (Table 1); longer term effects were not measured.

Cytotoxic evaluation

EDCe did not show any hemolytic activity when in the presence of either total bovine blood or bovine red blood cells at concentrations varying from 0.125 to 16 mg/mL (see supplementary Fig. 51).
**Figure 1**  Nematode assay showing the effect of aqueous crude extracts (ACE) of antagonistic plant seeds (10 μg/μL) on second stage juveniles (J2) of *M. incognita* after 48 h exposure with dH2O and ethyl alcohol 70% (EtOH) used as controls. Bars indicate the percentage of dead nematodes; error bars indicate standard deviation.

**Figure 2**  Nematode assay exhibiting the effect of *C. ensiformis* ED fraction (EDCe) (final concentration 5 μg/μL) and ID fraction (IDCe) (final concentration 5 μg/μL) against J2 of *M. incognita*. dH2O, ethyl alcohol 70% (EtOH), bovine serum albumin (BSA) with the aqueous crude extract of *C. ensiformis* (ACECe) used as controls. ED solution 5 μg/μL heated at 50 °C. Bars represent the percentage of dead nematodes; error bars indicate standard deviation. Assays were performed in triplicate with nematodes exposed for a period of 48 h.

**Figure 3**  Nematode assay showing the effect of *C. ensiformis* ED fraction (ED Ce) (5 μg/μL) against saprophytic nematode (SN) and *Meloidogyne incognita* (MI). Distilled water was used as control for both nematode. Error bars indicate standard deviation. Assays were performed in triplicate with nematodes exposed for a period of 48 h.

**Greenhouse evaluation**

To measure the effectiveness of treating soil with EDCe to inhibit J2 infection and progression to later life stages tomato plants were infected. Status of the nematodes was evaluated by comparing the effect of a commercial nematicide (Aldicarb, used as positive control) and dH2O (used as negative control) with EDCe under simulated conditions in a greenhouse. The treatment with EDCe showed a remarkable reduction in egg mass number. Egg masses decreased by 82% for the EDCe treatment and 94.2% for the positive control employing the commercial nematicide in comparison to the negative control using dH2O (Fig. 5). On average, only 18 egg masses (egg mass index (e.m.i.) = 3) were observed with the treatment of EDCe against 6 egg masses for the chemical nematicide (e.m.i. = 2) and 103 for dH2O (e.m.i. = 5) (Fig. 5).

**Light microscopy**

The anatomy of J2 of *M. incognita* exposed to EDCe was examined by light microscopy and showed disruption of the normal anatomy (Fig. 4A, B) with the formation of structures similar to vacuoles along abnormally straighted J2 body (Fig. 4C, D).

**Purification and analysis of bioactive fractions**

The purification and fractionation of EDCe was performed using a semi-preparative C18-RP/HPLC column with a linear gradient of 5–95% (TFA/ACN) at a flow rate of 2 mL/min. Fractionation was repeated 40 times with a total of 87 mg recovered from 200 mg of crude EDCe in 22 chromatographic
Table 1  Summary table of in vitro non-target organisms assays using different concentrations of C. ensiformis ED (EDCe) against fungi, insect larvae, nematodes and bacteria. All assays were conducted in triplicate.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>µg/µL of C. ensiformis ED/assay</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td>50, 100, 200</td>
<td>None = normal growth</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>50, 100, 200</td>
<td>None = normal growth</td>
</tr>
<tr>
<td>Macrophomina phaseolina</td>
<td>50, 100, 200</td>
<td>None = normal growth</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>50, 100, 200</td>
<td>None = normal growth</td>
</tr>
<tr>
<td>Insect larva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthonomus grandis (larvae)</td>
<td>200</td>
<td>None = normal development</td>
</tr>
<tr>
<td>Spodoptera frugiperda (larvae)</td>
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<tr>
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<tr>
<td>Meloidogyne incognita</td>
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</table>

Figure 4  Light microscopy illustrating the effect caused by C. ensiformis ED (EDCe) on M. incognita J2. (A) J2 before addition of (EDCe) showing typical curled shape. (B) J2 intestinal region before addition of (EDCe), arrow indicates normal morphology. (C) J2 48h after addition of (EDCe), showing linear and straightened profile, arrow indicates vacuoles. (D) J2 intestinal region 48h after addition of (EDCe), arrow indicates vacuoles and breakdown of normal morphology.

![Light microscopy](image)

Figure 5  Evaluation C. ensiformis ED (EDCe) soil treatment (50mg/kg of soil) to limit M. incognita second stage juvenile (J2) infection of tomato plants and ability to progress to later life stages under greenhouse conditions. Bars represent the number of egg masses observed for tomato roots after the period of treatment. The experiment was conducted in triplicate with dH2O and the commercial nematicidal Aldicarb (280mg/kg of soil) used as positive and negative controls, respectively (n = 3). The egg mass index was used to evaluate the effectiveness of C. ensiformis external dialyzate.

![Graph](image)

fractions (F1–F22), representing a mass recovery of 43%. Each fraction (500 µg) was individually tested against M. incognita J2’s (Fig. 6A). Only fractions F1, which comprised 11% by mass of the total composition of the EDCe, F3 (14% of EDCe), F6 (0.35% of EDCe), F17 (0.27% of EDCe) and F18 (0.05% of EDCe) demonstrated the ability to paralyze and linearly straightened above 95 ± 3% the nematodes from a typical coiled or non-linear shape after 48h exposure (Fig. 6B). Paralyzed nematodes were then transferred to water for a further 12h (recovery assay).
Results showed that 90% of J₂’s in fractions F1, F3 and F18 remained paralyzed, confirming the nematicidal activity. For F6 and F17 around 87% of J₂ exposed to the treatments recovered their motility, indicating a nematostatic activity (Fig. 6C).

Metabolite profiling and identification

GC–MS analyses were performed using the EDCe unfractoned and also the identified nematicide/nematostatic fractions (F1, F3, F6, F17 and F18) after HPLC separation.
of the ED. Greater than 170 individual peaks were readily identified in the resulting chromatograms of ED Ce revealing the presence of a large number of metabolites eluting over the temperature gradient. Chromatograms of the active fractions were superimposed and invariably exhibited the occurrence of more than one peak that overlapped with those found in the ED Ce (see Fig. S2 supplementary).

A total of 158 individual peaks were found within the ED Ce, of which a total of 52 compounds belonging to different chemical classes were identified by library matching, with the further 106 peaks remaining unknowns in the summary of peaks in each fraction after common contaminants and background ions were removed. Analysis of nematicidal and nematostatic fractions found a total of 16 unknowns and 18 identified sugars and amino acids in F1; F3: 13 unknowns and 15 identified amino acids and sugars; F18: 13 unknowns and 7 identified fatty acids, aromatic amino acids and xanthotoxin; F6: 14 identified amino acids and organic acids, with 16 unknowns; F17: 17 unknowns and 6 identified fatty acids, aromatic amino acids and S-carboxymethylcysteine (see supplementary Table S1). Identified compounds obtained from comparison of GC–MS mass spectra of ED Ce as well as HPLC separation fractions are described in the supplementary section (Tables S2 and S3).

LC–MS analyses were also performed on the same fractions. Mass spectra generated from these fractions, in both negative and positive ion mode were compared with the METLIN mass spectral library (see Table S4 supplementary). Several compounds of distinct chemical classes were detected, but only those showing matches above 70% were considered for further comparison; within F1: 3 sugars and asparagine were identified; F3: glutamic acid and N-acetylsereine; F6: valine, cis/trans-aconitic acid, and citric acid; F17: phenylalanine; F18: tryptophan (see Table S4 supplementary).

Furthermore, profiling of nematotoxic fractions was undertaken using NMR spectroscopy. The resulting spectra were compared against the Chenomx NMR metabolite database to further confirm the presence of the identified metabolites from GC–MS and LC–MS analyses. Each fraction displayed a complex signal demonstrating the presence of many metabolites. Within F1: included common amino acids - glycine, asparagine and a variety of sugars; F3: further amino acids, sugars and L-canavanine; F6: organic acids and valine; F17: phenylalanine and S-carboxymethylcysteine; F18: some amino acids, xanthotoxin (supplementary Table S5).

After the compounds identification by GC–MS, LC–MS and 1H NMR analyses an extensive literature was performed in order to identify any known biological function of the compounds detected and identified. Furthermore some of the compounds chosen were obtained commercially wit high purity degree and they were used to validate the nematotoxic activity against M. incognita J2.

Activity testing for commercial chemical compounds identified

To verify the effectiveness of the isolated compounds previously identified in ED Ce fractions on J2 of M. incognita, assays were carried out using commercially sourced, highly purified materials. Hence, D-glucose at a final concentration of 0.26 μg/μL paralyzed the majority of nematodes after 48 h exposure (Fig. 7A). In the recovery assay, 98% of J2 recovered their motility confirming the nematostatic activity for glucose at the applied concentration (Fig. 7B). However, D-glucose at a concentration of 0.5 μg/μL exhibited a nematocidal effect killing more than 90% of J2 after 48 h exposure (Fig. 7B). D-Glucose was completely ineffective against M. incognita J2 juveniles after 48 h (Fig. 7A). Similarly, L-canavanine did not show any nematotoxic activity at a concentration of 0.26 μg/μL after 48 h exposure (Fig. 7A). In contrast, at a concentration of 0.5 μg/μL, 50% of J2 remained paralyzed after the recovery assay, confirming a discrete nematicidal activity and a potential toxic threshold (Fig. 7B). Among the organic acids, malic, citric, cis-aconitic, trans-aconitic and the fatty palmitic acid only the trans-aconitic and palmitic acid were able to paralyze 98% and 90% of J2 at concentrations of 0.5 μg/μL after 48 h exposure (Fig. 7A). The remaining organic acids identified only paralyzed a very low percentage of J2 (Fig. 7A). Trans-aconitic acid at concentration of 0.5 μg/μL demonstrated a nematicide activity of 97% while malic, citric and cis-aconitic acids exhibited only a nematicide effect of 55%, 68% and 65%, after the recovery assay (Fig. 7B). Palmitic acid showed a nematostatic effect for both concentrations evaluated with above 90% of J2 recuperating their mobility after the recovery assay (Fig. 7B). The amino acids L-phenylalanine, L-methionine and L-tryptophan were completely innocuous for both concentrations (Fig. 7A). Inversely, the compound S-carboxymethylcysteine paralyzed around 90% of J2 for both concentrations tested after 48 h exposure (Fig. 7A). Following recovery assay the majority of J2 recuperated their mobility confirming the nematostatic effect (Fig. 7B). Approximately 87% of J2 treated with xanthotoxin were paralyzed after 48 h exposure (Fig. 7A). The paralyzed J2 did not recover their mobility after the recovery assay confirming the nematicide activity (Fig. 7B). The control, 50 mM Tris/HCl pH 8, exhibited a nematostatic activity while 50 mM acetic acid pH 5 showed a nematicidal effect after recovery assay and have been used to certify the pH influence on the plant material activity (ACE, ED, ID, HPLC fractions and commercial compounds) (Fig. 7B).

Discussion

This work explored an alternative strategy to be used in conjunction with the other current nematode control methods. A traditional bioprospecting approach coupled to modern high throughput metabolomics methods was adopted aiming the identification of nematotoxic metabolites from antagonistic plants.

It is known that natural products target a wide variety of proteins and other biological components with specific purposes, like some leguminous nematode-antagonistic plant seeds that are a rich source of natural nematicidal and nematostatic compounds. The screening test demonstrated that the ACE Ce has an effective nematicide activity against second stage juveniles (J2) of M. incognita. A similar result with other plant species was reported by (Khurma & Mangotra, 2004) where aqueous extracts of Trigonella foenum
Figure 7  (A) Nematode assay exhibiting the ability of commercial compounds to paralyze second stage juveniles (J2) of *M. incognita* after 48 h exposure (*n* = 3). 60 J2 were used per replicate, error bars indicate standard deviation. (B) Recovery assays showing control (white fill), nematicidal (dark grey fill) and nematostatic (light grey fill) effects on J2 (*n* = 3), error bars indicate standard deviation.

*graceum*, *Sesbania sesban*, *Albizia lebbeck*, *Cassia fistula* and *Milettia pinnata* were responsible for the highest *M. incognita* J2 mortality (90–97%). In addition, we found that small molecules extracted from *C. ensiformis* seeds were related to the observed toxic effects as pointed by the EDCe mortality of about 96 ± 3%. Previous reports indicated that a variety of common metabolites were toxic to several nematodes species, including the non-protein amino acid L-canavanine that is naturally abundant in seeds of *C. ensiformis* (Birch, Fellows, Geoghegan, Robertson, & Watson, 1992; Rosenthal, 1990); the L-3,4-dihydroxyphenylalanine which is active against *M. incognita* and *Heterodera glycines* (Barbosa, Barcelos, Demuner, & Santos, 1999; Osman, 1993); and L-arginine and L-glutamic acid against *M. javanica*. Talavera and Mizukubo (2005) revealed the action of L-methionine against *M. incognita*, while Oliveira et al. (2009) demonstrated the activity of cysteine on *M. exigua in vitro* and in vivo.

J2 larvae from *M. incognita* showed remarkable anatomic modifications with the development of anomalous features in the form of large vacuoles after 48 h of exposure to the EDCe (see Fig. 4). This alteration is potentially related to the presence of toxic compounds that are able to cross the nematode cuticle, interfering in vital processes, which may include enzymatic, nervous and respiratory systems. Alternatively the juveniles’ death might be linked to dehydration through the existence of an osmotic potential caused by high sugar concentration in the EDCe. It was demonstrated that solutions containing 0.26–0.5 μg/mL sucrose reduced larvee motility, leading to morphological alterations and cell death of *M. javanica* and *M. incognita* juveniles (Santiago, Homechin, Montalvan, & Krzyzanowski, 2005).

EDCe was also tested against fungi and bacterium species (Table 1). A range of concentrations up to 50 μg/μL of EDCe could not inhibit the growth of either *C. gloeosporioides*, *F. solani*, *M. phaseolina*, *P. tabacum* or *B. subtilis*. This absence of activity could be associated with a concentration of a specific compound present in the EDCe. Similar results were observed for extracts of *Plantago lanceolata* and *P. rugellii*, which were able to kill *M. incognita* larvae, but had no toxicity toward *Enterobacter cloacae*, *Pseudomonas fluorescens*, *Trichoderma virens*, *Fusarium oxysporum*,
Identification of metabolites from plant antagonistic seeds toward phytonematode

*Phytophthora capsici*, *Pythium ultimum* and *Rhizoctonia solani* (Meyer et al., 2006). Besides no insecticidal effects were observed when *A. grandis* and *S. frugiperda* larvae, both significant agricultural pests, were grown on an artificial diet containing 200 μg/μL of EDCe. Concanaclavin A (lectin), vicilin, canatoxin and urease present in seeds of *C. ensiformis* are found to be toxic against different classes of insects (Cartini & Grossi-de-Sá, 2002). However, these polypeptides were removed during the dialysis procedure. Since only 200 μg/μL of EDCe were used in our bioassays, the concentration of L-canavanine was too low to affect the growth of both larval insects. In addition, an in vitro assay using EDCe at a concentration of 100 μg/μL killed only 5% of free living nematodes compared to the 90% mortality of *M. incognita*. The low mortality rate of saprophytic nematodes may be associated with their cuticles. The cuticle is a complex structure of plates, fibers, and several layers of proteins. It can differ dramatically between species and also at their stage of development (Bird & Bird, 1991).

**In vitro** cytotoxic evaluation using a hemolysis assay demonstrated that the EDCe has no hemolytic activity against bovine blood or isolated bovine red blood cells indicating that these molecules possibly are not risky to non-targeted mammalian organisms (see supplementary Fig. S2). Moreover, it was also shown that the EDCe after being incubated at 50°C for 24 h was able to kill 96% of the phytoparasites. The thermostability of any potential nematicidal compounds is an essential physical characteristic to be determined as any compound must be stable in the prevailing environment. This result reflects the thermostability of the active compounds present in the sample. Moreover, it is important to highlight that the experiment was conducted at a higher temperature than the average temperatures of soil layers where roots of different cultures, including cotton, soy, coffee, sugar-cane and other important crops are grown (Hu & Feng, 2003).

The EDCe metabolites and their ability to inhibit the infective J2 stage were evaluated by simulating field conditions at greenhouse conditions. The egg mass index (Taylor & Sasser, 1978) was used to measure the ability of the J2 stage of *M. incognita* to infect tomato plant roots and progress to later life stages. Results showed a remarkable 82% reduction in the number of egg masses from *M. incognita* infection of tomato plants harvested 45 days after inoculation to the treatment using EDCe against 94.2% to the commercial nematicide Aldicarb. Furthermore, it was not observed visual modifications to the tomato plant along the experiment.

With effectiveness toward J2 of *M. incognita* determined, attention turned to identification of the active metabolites. To achieve that an active bioassay-guided HPLC purification and fractionation strategy was employed using EDCe. Metabolites were separated into 22 fractions which were submitted to in vitro assays against *M. incognita* J2. Significant activity was observed for the fractions F1, F3, F6, F17 and F18. Two effects were observed: a nematicide effect for F1, F3 and F18 and a nemostatic effect was demonstrated for F6 and F17 fractions against *M. incognita* J2. The variation in efficacy detected for each fraction could either be due to the presence of different compounds in each fraction.

Finally, metabolite profiling of plant extracts integrating GC–MS, LC–MS, and NMR technologies combined with a thorough literature search represented a powerful and reliable approach providing substantial information of the chemical composition of *C. ensiformis* fractions. In this way, the HPLC nematicidal fractions of *C. ensiformis* allowed the identification of components belonging to distinct chemical classes. The nematicide activity observed for F1 on *M. incognita* juveniles might be correlated with the osmotic effect inflicted by the high content of different sugars, such as d-glucose and D-pinitol. Literature searching indicated the sugar d-glucose present in F1 and F3 to be nematostatic or nematocidal (Santiago et al., 2005). D-Pinitol, a bioactive carbohydrate located in F1 has been reported as a larvicidal molecule (Chaubat et al., 2005). Despite the high effectiveness of D-pinitol against different insect larvae classes, no effect was noted when its commercial form was tested against *M. incognita* juveniles using a higher concentration than the active F1, excluding D-pinitol as the active compound responsible for causing nematodes death. However, the *in vitro* assay using 0.26 μg/μL of commercial d-glucose against *M. incognita* juveniles demonstrated the same killing effect observed for the crude F1. Moreover, similar results published by Santiago et al. (2005) also demonstrated that solutions containing 0.26–0.5 μg/μL d-glucose decreased the motility and ultimately killed *M. javanica* and *M. incognita* juveniles in experiments conducted *in vitro*, corroborating our results. A nematicide activity was also observed for F3 after 48 h exposure. Both d-glucose and L-canavanine were confirmed in F3 by NMR, with L-canavanine by far the most abundant compound found within the fraction. L-canavanine is reported as a remarkable insecticide and at low concentrations and this non-proteinogenic amino acid can also kill nematodes from different genera (Birch et al., 1992). Intriguingly, the commercial form of this compound failed completely at a concentration of 0.26 μg/μL and killed only 50% of *M. incognita* juveniles at higher concentrations (0.5–1.0 μg/μL). The nematicostatic activity caused by F6 is possibly associated to the high content of the organic acids, cis and trans-aconitic acids, malic acid and mainly citric acid. The organic acids found in F6: cis and trans-aconitic acid as leishmanicides (Kar, Kar, Bhattacharya, & Ghosh, 1993) while citric acid is reported to be weakly nematicotoxic (Shemshura et al., 2016). The mixture of these compounds is able to dramatically decrease the pH making the environment very acidic and consequently affecting the nematodes behavior. In this sense, two internal controls were used to evaluate the action of acidic and alkaline pH on J2 of *M. incognita*. In the case of alkaline pH (50 mM Tris/HCl pH 8) a nematicostatic activity was observed while for acidic pH (50 mM acetic acid pH 5) a nematicide activity. In *in vitro* assay results using these compounds individually at 0.26 μg/μL showed exactly the same result observed for F6. Nevertheless, when the concentration of the organic acid trans-aconitic acid and fatty acid palmitic acid was increased the juveniles were not able to recover any of their motility, confirming a nematicidal activity. It is important to note that nematodes belonging to distinct species behave differently depending upon the pH environment. Yet, the action of these acids appears to be dose dependent as demonstrated by *in vitro* assay. Fraction 17 exhibited a nematicostatic activity. A high content of phenylalanine and S-carboxymethylcysteine with lower concentrations of palmitic acid were found in this fraction.
Some amino acids and palmatic acid are known to be very toxic to different nematode species (Oliveira et al., 2009). In vitro assays using commercial phenylalanine and palmatic acid were innocuous against M. incognita J2. Paradoxically, commercial S-carboxymethylcysteine demonstrated a nematostatic activity paralyzing about 100% of the juveniles at 0.5 µg/µL concentration. This result indicated that this cysteine derivative is the compound involved with the nematostatic effect exhibited by F17. Similarly, Oliveira et al. (2009) demonstrated the activity of cysteine on Meloidogyne exigua in experiments conducted in vitro and in greenhouse. The nematocide activity showed by F18 against juveniles of M. incognita could be associated with xanthotoxin, a plant toxin present in this fraction. Xanthotoxin exhibits a noteworthy action on insects and mollusks and also acts as an antifendat. In vitro assay using commercial xanthotoxin was able to demonstrate a 95% kill of juveniles to 0.5 µg/µL. This result indicated that the activity observed for F18 may be related to the action of this compound.

Conclusion

Amongst the antagonistic plants used aqueous crude extract, C. ensiformis exhibited the highest nematocide activity. Its EDCe also demonstrated to be very effective against M. incognita (Jj), but did not adversely affect beneficial saprophytic nematodes, fungi, bacterium and insect pests. Hemolysis tests showed that EDCe was not able to disrupt bovine red blood cells and when heated at 50°C retained a high level of toxicity against M. incognita. Moreover, the EDCe decreased the number of M. incognita eggs mass by 82% at experiments conducted at greenhouse conditions. The metabolite profiling of C. ensiformis seeds using a comprehensive approach based on GC/MS, LC/MS, and 1H NMR technologies and a thorough literature search provided a consistent and substantial amount of information concerning the nematotoxic compounds present in this leguminous plant. The nematocide compound α-glucose, l-canavanine and xanthotoxin, as well as the nematostatic compounds trans aconitic acid, citric acid, palmatic acid and S-carboxymethylcysteine used against M. incognita larvae, were identified using this integrated approach. These results corroborate the potential of ACEcE as a source of nematotoxic compounds.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are thankful to José C.C. Magalhães and Warley da Silva Almeida for their help with nematode assay, and to Nirupama Jayasinghe, Terra Stark, Daniel Dias, Nazanin Amini and Jairus Bowne for GC/MS support on technical and statistical analyses. This work was supported by the Brazilian Agricultural Research Corporation – (Embrapa), National Council for Scientific and Technological Development – (CNPq). GC-MS, LC-MS and NMR were conducted at Metabolomics Australia which is supported by funds from the Australian Government’s National Collaborative Research Infrastructure Scheme (NCRIS) administered through Bioplates Australia (BPA) Ltd.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bior.2017.10.003.

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