

Allelopathic Interactions of *Solanum stramonifolium* Jacq. Might Be Used for *Meloidogyne incognita* Management

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Abstract

The root-knot nematode (RKN) *Meloidogyne incognita* infects a large host range causing enormous agricultural losses. Although the control of this pest is mainly based on the use of chemical pesticides, natural alternatives are currently being largely considered, especially the use of plant-derived molecules, as allelochemicals and plant extracts. *Solanum stramonifolium* is a plant of the Solanaceae family and has been studied for its potential to control RKN, either by the use aqueous extracts of its seeds, or due to its immunity to nematode penetration. Our studies demonstrate that *S. stramonifolium* has an efficient defense mechanism against RKN completely preventing its penetration into its root system. Additionally, the compounds exuded by this plant in the soil can affect nematode infection in nearby grown *Nicotiana benthamiana* (-89% biomass for treatment I respectively, and -61% and +57% of galls for the treatments and *Solanum lycopersicum* (-79% biomass for treatment I, and -83% of galls for the treatments I and II). Herein, external (ED, < 3.5 kDa) and internal (ID, > 3.5 kDa) dialysates were prepared from the extract of *S. stramonifolium* seeds. *Arabidopsis thaliana* Col-0 inoculated with RKN treated with the dialysates showed a reduced gall diameter in 45% and 35% for ED and ID respectively. Additionally, a significant reduction in biomass was observed in germinated seeds after treatment with ID. In addition, *Arabidopsis* roots showed changes in their ploidy levels when treated with ED and ID, compared to the untreated control, indicating its effect on endoreduplicating cells. Finally, a partial identification of glycoalkaloids present in the ED and root extracts may help to explain plant immunity to root-knot nematode infection. Results obtained shed light on the defense mechanisms of *S. stramonifolium* against *M. incognita*, its allelopathic potential in co-cultivated plants and its biotechnological potential of dialysates obtained from its seeds, representing a strong alternative to the management of RKN in conventional agriculture.

Keywords: biomass, dialysates, plant extracts, root-knot nematode, *Arabidopsis thaliana*

1. Introduction

Nematodes can adversely affect agricultural production, causing estimated losses of US\$157 billion a year in commodities. The root-knot nematode (RKN), known for the damage caused to plant roots, leads to metabolic disorders in the plant, loss of vigor, defoliation and consequently decreased productivity (Abad et al., 2008).

Although the main way to control this group of nematodes is through synthetic chemicals, several products based on methyl bromide, organophosphate and carbamate-based were recently banned in Europe or had their use

restricted in other parts of the world due to the risks to human health and environment (Kearn, Ludlow, Dillon, O'Connor & Holden-Dye, 2014).

A different alternative for biological control of parasitic nematodes, makes use of other organisms as predators, parasites, or pathogens, acting as antagonist agents. However, this method is commercially insufficiently exploited (Viaene, Coyne, & Davies, 2013; Timper, 2011). In this context, to use natural compounds from plants can figure as alternative, mitigating the effects on environment and human health (Campos, de Oliveira, Pascoli, de Lima, & Fraceto, 2016).

Allelopathy can be defined as a process caused by allelochemicals, secondary metabolites from plants, fungi, animals and microorganisms, that can modify or interfere with the development of other organisms and thereby affecting biological process, as reproduction, survival, growth and development (F. Cheng & Z. Cheng, 2015).

Allelochemicals can be useful to control soil microorganisms and several studies have shown that these compounds can decrease the nematode population in the soil (Odeyemi, Afolami, & Adingun, 2013; Gavande, K. Jain, B. Jain, & Mehta, 2015; Mashela & Dube, 2014; Mashela, 2014). Some groups can be specially found in Solanaceae species, as alkaloids, phenols, carbohydrates and proteinaceous compounds (Gavande, K. Jain, B. Jain, & Mehta, 2015). Additionally, phytochemical studies of the genus *Solanum* indicate the presence of alkaloids and flavonoids, and some genera possess natural resistance towards nematodes (Silva, Nascimento, Batista, Agra, & Camara, 2003).

Methanolic extracts of the aerial parts of *Solanum paniculatum* showed biological activity towards larvae of *Artemia salina* (Silva, Nascimento, Batista, Agra, & Camara, 2007). Additionally, several studies to evaluate the resistance of *Solanum torvum* against nematodes were performed (Bagnaresi et al., 2013; Rahman et al., 2002; Uehara, Tateishi, Kadota, & Iwahori, 2017; Dhivya, Sadasakthi, & Sivakumar, 2016; Karumari, Sumathi, Vijayalakshimi, & Ezhilarasi, 2014).

The species *Solanum stramonifolium* Jacq. can be found worldwide and is known for its potential to be resistant to RKN nematode infection and other pests, as fungi and bacteria (Gousset et al., 2005; Mendonça, Santana, Mattos, & Pinheiro, 2010). The biotechnological potential of extracts from *S. stramonifolium* seeds against RKN has already been revealed, including as for potential product development, as well the request for intellectual property protection (Costa et al., 2022). Thus, we investigated here the allelopathic effects of *S. stramonifolium* seed extracts on the model plant *Arabidopsis thaliana*. As well performed morphological analysis of *S. stramonifolium* roots infected by *M. incognita* finally investigating how nematode infections evolved in susceptible plant species like tobacco and tomato grown at the proximity of *S. stramonifolium* plants.

2. Method

2.1 Nematode Material, Egg Collection and Hatching

To obtain second stage juveniles (J2s), the method described by Hussey (1973) was used here. Tobacco (*Nicotiana benthamiana*) plants grown in plastic pots with soil (60% ordinary soil, 30% sand and 10% commercial substrate) were inoculated with 1500 J2 in the greenhouse (26-33 °C). Three months old plant roots were carefully washed in water and triturated in a blender with a 0.5% aqueous sodium hypochlorite solution. The homogenate was filtered by passing through a 100, 300 and 500 mesh size and washed with tap water. The eggs were put in a hatching chamber with distilled water for 48 h and nematodes were counted using a Peters slide under a microscope (Dickson & Struble, 1965).

2.2 Microscopic Evaluation of Nematode Infection of *Solanum stramonifolium*

Solanum stramonifolium seeds were germinated in soil (commercial substrate/sand 3:1), kept for 30 days in a growth chamber (16 h/8 h photoperiod at 21 °C/18 °C, respectively), and each plant was inoculated with 2000 J2. At different time points (10, 20 and 35 days after inoculation-DAI), roots were collected and stained with acid fuchsin according to Bybd, Kirkpatrick and Barker (1983). Roots were then cleared with a 5.25% aqueous sodium hypochlorite solution for 5 min under agitation at room temperature (RT). Plant material was washed in tap water to remove the sodium hypochlorite for 30 min at RT, and transferred to acid fuchsin solution (1.4% in acetic acid) diluted in water (1:1) for 5 min. The solution was heated until boiling and roots were left at RT overnight (ON). Subsequently, roots were carefully washed with tap water and transferred to glycerol acidified solution (dH₂O, glycerol and lactic acid 1:1:1) with occasional agitation at RT until excess of fuchsin stain was removed. Sectioned roots tips containing the root elongation zones were used for observations. This region is where RKN normally infects, therefore these root apices were collected, placed in a microscope slide in glycerol 90% and observed under a microscope (Axionplan II®, Zeiss) and imaged using software ZEN 2 Blue® (Zeiss). At least two biological replications were performed where at least 20 root segments were observed.

2.3 Allelopathic Resistance Induction in *Nicotiana benthamiana* L. and *Solanum lycopersicum* Mill

Three-weeks-old *N. benthamiana*, *S. lycopersicum* and *S. stramonifolium* plants were grown in soil (commercial substrate/sand 3:1) in a grown chamber (16 h light/8 h photoperiod at 21 °C/18 °C, respectively) and used to evaluate the allelopathic resistant-induction before and after infection, as described below.

Thirty-five DAI, plants roots were carefully washed with water to remove remaining soil, preserving eventual egg masses. The galls were counted under a binocular and gg masses were subsequently stained with fuchsin as previous described. The data collected was used to determine the number of eggs per root gram (NERG) (Boneti & Ferraz, 1981), egg mass index (EMI) (Huang, Miranda & Maluf, 1986), gall index (GI) (Charchar & Aragão, 2005) and reproduction factor (RF) (Oostenbrink, 1966). Results were analyzed by ANOVA and Dunnett test ($p < 0.05$) using R-Studio 2022 v12.0. Plants of each species in isolated pots were used as control (Figure 1).

2.3.1 Allelopathic Resistance Induction in *Nicotiana benthamiana* and *Solanum lycopersicum* Before *Meloidogyne incognita* Inoculation

Three-weeks-old *N. benthamiana*, *S. lycopersicum* and *S. stramonifolium* plants grown in soil (commercial substrate/sand 3:1) in a growth chamber (16 h light/8 h photoperiod at 21 °C/18 °C, respectively) were transferred to rectangular soil pots (33 × 14 × 6 cm) with 10 cm between each plant. *Solanum stramonifolium* were placed between *N. benthamiana* and *S. lycopersicum*. All plants were inoculated with 2000 J2 simultaneously.

2.3.2 Allelopathic Resistance Induction in *Nicotiana benthamiana* and *Solanum lycopersicum* After *Meloidogyne incognita* Inoculation

Nicotiana benthamiana and *S. lycopersicum* were cultivated in pair with 20 cm of distance between each-other for 20 days, and after that inoculated with 2000 J2. After 48 h, *S. stramonifolium* plants were planted between *N. benthamiana* and *S. lycopersicum* (10 cm each plant).

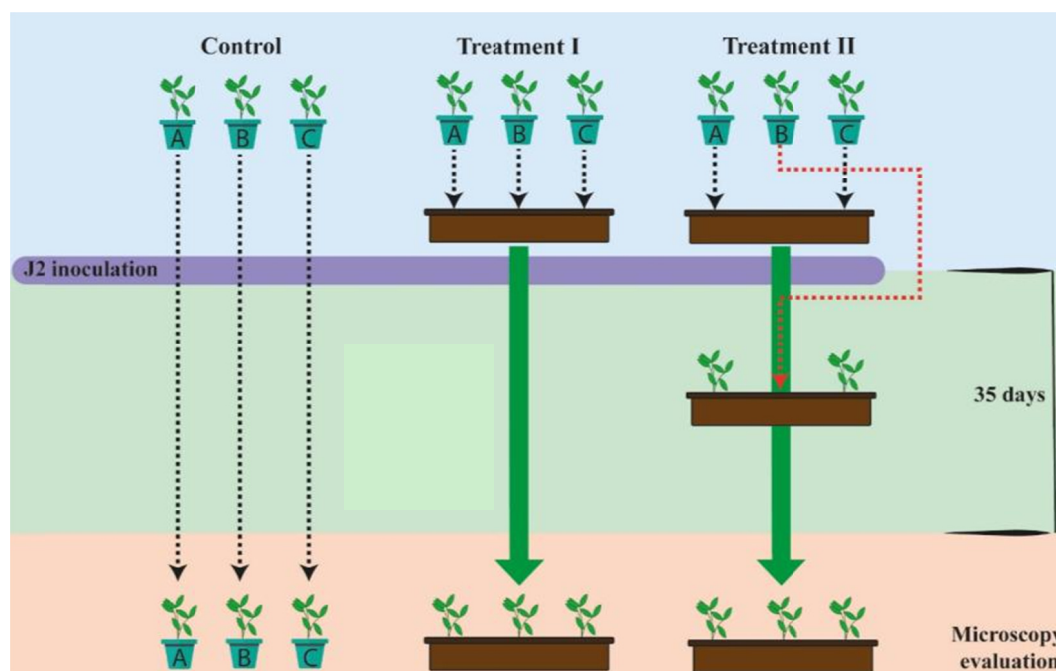


Figure 1. Flowchart of the allelopathic response induction experiment. A: *Nicotiana benthamiana*; B: *Solanum stramonifolium* and C: *Solanum lycopersicum*

2.4 Seeds Extract Preparation

The aqueous extract was obtained as described by Rocha et al. (2017). *Solanum stramonifolium* CNPH 19 (Kindly provided by Plant Germplasm Bank from Embrapa Hortaliças-DF/Brazil.) seeds were grinded and mixed with distilled water (1:6 w/v). The solution was left under magnetic agitation at 6 °C for 12 h and centrifuged at 10000 g at 5 °C for 30 min. The supernatant was dialyzed using a dialysis membrane with cutoff 3.5 kDa with distilled water for 12 h at 6 °C. The inner and outer membrane solutions were freeze-dried in a

Savant-Super Modulyo (Thermo Fisher, GA, USA) lyophilizer and labeled as: (ID) internal dialysate and (ED) external dialysate.

2.5 Morphological Alterations in Galls of *Arabidopsis thaliana*

Three-week-old *Arabidopsis thaliana* (L.) Heynh. genotype Columbia (Col-0) seedlings were inoculated in soil pots (previously treated with larvicides Vectobac® and Steinernema®, following manufacturer's instructions), containing five plants per pot and three replicas per treatment line. Seedlings were grown vertically with a 16 h light/8 h dark photoperiod at 21 °C/18 °C, respectively. Plants were treated in 2 different conditions: 1) with prior treatments (pre-treatment) with ED solution (300 µg per plant) for 24 h, soil change after prior treatment and then irrigation using ED solution (300 µg per plant) once per week after J2 inoculation; 2) treatment with ED solution (300 µg mL⁻¹) once per week after J2 inoculation, as described in Table 1. Plants without ED treatments were used as control. The experiment was performed in three replica and with three biological repetitions.

Table 1. Plant treatments to determine the activity of ED *in vivo* against J2

Treatment	Pre-treatment (ED)	Soil change	J ₂ inoculation	Irrigation (ED)
IT	+	+	+	+
CT	-	-	-	+
Control	-	-	+	-

Note. IT: Initial treatment; CT: Continuous treatment.

Ten galls of initial treatment (IT), continuously treatment (CT) and controls were dissected and fixed in PIPES (Piperazine-N,N-bis(2-ethanesulfonic acid)) buffer 50 mM pH 6.9 containing 2% glutaraldehyde. Plant material was gradually dehydrated with ethanol as described by Almeida-Engler et al. (2012) and embedded in Technovit® 7100 according to manufacturer protocol (Heraeus Kulzer, Wehrheim, Germany). Tissue sections of galls were obtained using a microtome (HM360, Micron) with a thickness of 3 µm and placed floating on distilled water on slides (Menzel-GläserSuperfrost®, ThermoScientific) to dry on a hot plate at 45 °C. Sections were stained with toluidine blue 0.05% for 30 sec, washed with distilled water and mounted with DEPEX (Sigma-Aldrich). Observations were done with an inverted transmission microscope (Axionplan II®, Zeiss) equipped with the ZEN 2 Blue® software (Zeiss). The diameter of galls was obtained from measurement of the largest transversal line of each gall and the surface of 2 largest giant cells per gall were measured. Results were analyzed by ANOVA *post-hoc* Dunnett or Kruskal-Wallis *post-hoc* Dunn's comparison tests ($p \leq 0.05$) using R-Studio 2022 v12.0.

2.6 Test of Germination and Biomass Assays of *Arabidopsis thaliana* Treated With the ED and ID

Arabidopsis thaliana Col-0 seeds were surface sterilized (Almeida-Engler et al., 1999) and were treated with 300 µg mL⁻¹ of ED in sterile water and 100 µg mL⁻¹ for ID for 2 h at RT. The solution was removed, the seeds were washed with distilled water and dried ON at RT. They were transferred to plates with MS sterile medium (Murashige & Skoog, 1962) and placed ON at 4 °C to synchronize germination. Plates were then placed for 10 days in a growth chamber following a regime of 16 h light/8 h photoperiod at 21 °C/18 °C, respectively. Germinated plants were counted and weighted. Non-treated sterile seeds were used as negative controls. Sixty seeds were used for each treatment, in replica and with 3 biological repetitions. Results were analyzed by ANOVA and Dunnett test ($p < 0.05$) using R-Studio 2022 v12.0.

2.7 Flow Cytometry Analysis

Arabidopsis thaliana Col-0 sterile seeds were surface sterilized as described by Almeida-Engler et al. (1999) and germinated *in vitro* in MS medium in a germination chamber following a regime of 16 h light/8 h photoperiod at 21 °C/18 °C, respectively, and treated with ED (300 µg mL⁻¹) and ID (100 µg mL⁻¹). After 7 days, the roots were hand-chopped with a razor blade in 400 mL of 45 mM magnesium dichloride, 30 mM sodium citrate, 20 mM MOPS, pH 7.0, and 0.1% Triton X-100 (Galbraith et al., 1991), and filtered in a 22 µm mesh size. Nuclei of samples were stained with 1 µg mL⁻¹ 4',6-diamidino-2-phenylindole in distilled water. Analysis of the nuclei was performed with CyFlow flow cytometer, FloMax software (Partec, Münster, Germany). Data were collected from ~8000 nuclei per run. The fractions of nuclei with ploidy levels ranging from 2C to 64C were expressed as percentage of the total number of nuclei measured. Two replicates and two biological repetitions were performed for each treatment.

2.8 Centrifugal Partition Chromatography of External Dialysate

Centrifugal Partition Chromatography (CPC) separation was performed on an Armen Instrument apparatus (SCPC-50-L) coupled with a Spot CPC system, an integrated binary pump, a Flash DAD 600 UV/vis detector and a LS-5600 fraction collector (Armen Instrument). All modules were operated by the Armen Glider CPC software.

The best biphasic solvent system to our sample was determined by coefficient partition (K) of each phase for compounds of interest (Ito, 2005). Several biphasic solvent systems were tested. K was calculated as follow: biphasic solvent system was prepared in a 4 mL vial with 10 mg of crude extract. Each tube was vigorously shaken and allowed to equilibrate. Upper and lower phases of each tube were then analyzed by HPLC-PDA to calculate K basing on peak area.

Ethyl acetate, n-butanol and distilled water (1:4:5, v/v/v) were added in a separatory funnel, vigorously shaken and allowed to equilibrate. Upper and lower phases were collected to constitute mobile/stationary phases and ED was dissolved in lower phase for injection.

CPC separation was carried out in ascending mode, at 5 mL/min, 2000 rpm and monitored at 254 nm. The lower phase was first loaded into the CPC column to constitute the stationary phase. The upper phase was then pumped through the stationary phase until equilibrium of the system was reached. The extrusion step was performed after 45 min with 100% lower phase.

All collection tubes (5 mL) with similar chromatographic profiles were combined in 4 fractions, lyophilized, and submitted to nematocidal test to determinate the active fraction.

2.9 Semi-preparative HPLC

The active fraction obtained from CPC fractions tested against *M. incognita* was then submitted to semi-preparative High-Performance Liquid Chromatography-HPLC, using Agilent LC 1200 Series linked an Agilent LT-ELSD (Evaporative Light-Scattering Detector). The C-18 semi-preparative column (250 × 10 mm, 5µm, Phenomenex®) was equilibrated with methanol (MeOH) 2% and water both acidified with 1% formic acid. The column temperature was adjusted to 25 °C. Separation was carried on as follow: isocratic 5% MeOH and then a linear gradient until 95% MeOH in 55 min was applied. The detector was adjusted to 254 nm and flow rate at 4 mL/min. The sensor of ELSD was used only in first chromatography of each fraction. The fractions were collected, lyophilized, and tested in nematocidal assay.

2.10 Ultra-performance Liquid Chromatography—Ultra-resolution Mass Spectrometry Analysis of External Dialysate of Seeds and Aqueous/Ethanol Roots Extracts

Solanum stramonifolium roots grown for 15 days in sterile MS medium were ground using liquid nitrogen and homogenized in distilled water and 100% ethanol for 12 h at 6 °C, and subsequently lyophilized for analysis.

UPLC-HRMS analyses were performed on an UPLC Acquity system (Waters, Milford, MA, USA) coupled with a XEVOG2 QTOF instrument (Waters). All separations were achieved on an Acquity UPLC BEH C18 column (100 mm × 2.1 mm I.D., 1.7 µm) at 25 °C with a flow rate of 0.400 mL/min. A guard column (5 mm × 2.1 mm, 1.7 µm) with the same stationary phase was used. The mobile phase consisted of water + 0.1% FA (solvent A) and ACN + 0.1% FA (solvent B) was used in multistep gradient mode. The gradient was operated as follow: isocratic 5% B for 0.5 min, 5 to 100% B for 17.5 min, and a final isocratic step for 5 min at 100% B.

Ethanol/aqueous root extracts and external dialysate of seed extract were diluted in MeOH at 0.5 mg/mL and thermostated at 10 °C before injection. The injection loop was set at 0.5 µL. The HRMS data were acquired with a mass range of 100–1500 m/z; ESI conditions operated in negative mode were as follow: source temperature 120 °C, desolvation temperature 500 °C; capillary voltage 1.5 KV, cone voltage 10 V. Nitrogen was used as a cone (10 L/h) and desolvation gases (1000 L/h). Lockspray flow rate was set at 20 µL/min and lockspray capillary at 2.5 KV. For the HRMS/MS acquisitions, a method including the detection (full scan) and fragmentation of the most intense peaks per scan was used. Collision energy was varying from 10 to 35 V.

2.11 Nematotoxic Activity of CPC and HPLC Fractions

The nematotoxic activity was evaluated as described by Rocha et al. (2017), using 50 µg mL⁻¹ of each fraction. The statistical analysis of dead and alive J2 after water treatment was evaluated using ANOVA and Tukey's mean comparison tests, using software PAST v3 (p < 0.05).

3. Results

No infection could be detected in *S. stramonifolium* roots inoculated with *M. incognita* for the different time-points analyzed (Figure 2). Fuchsin stained roots illustrated the absence of nematodes within the roots especially in the root elongation zone where normally nematodes penetrate. The complete absence of galls or egg masses was also observed in *S. stramonifolium* roots 10, 20 and 30 DAI. As a control, nematodes of the same batch used in this experiment were tested for viability by inoculating tomato (*S. lycopersicum*) and tobacco (*N. benthamiana*) plants.

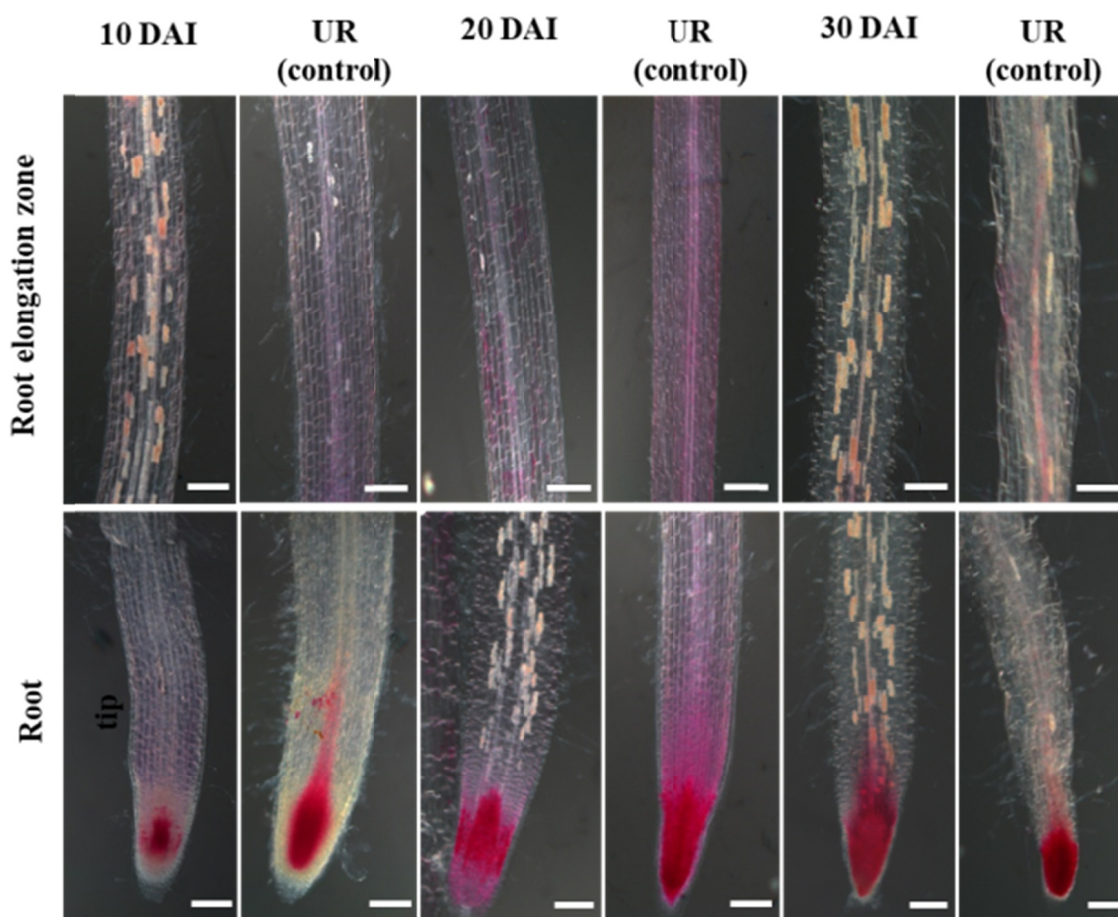


Figure 2. Morphological analysis and fuchsin stained roots of *S. stramonifolium* inoculated with 2000 juvenile nematodes at different time points after inoculation (10, 20 and 30 DAI) with respective controls. The red color is meristematic tissues stained with fuchsin. UR, uninfected roots; DAI, days after inoculation. Bars = 150 μ m

Allelopathic response induced before *M. incognita* inoculation in soil has been evaluated and are shown in Figure 3. The biological assay to evaluate the effect of *S. stramonifolium* on model plants biomass development before J2 inoculation (Treatment I), a root biomass reduction was observed for *N. benthamiana* (Figure 3A) and *S. lycopersicum* (Figure 3C).

The experiment to evaluate the same effect after J2 inoculation (Treatment II) did not reduce the biomass of *N. benthamiana* or *S. lycopersicum* (Figures 3A and 3C),

The number of galls showed a decreasing for experiment on *N. benthamiana* before J2 inoculation of 61% (Figure 3A-Treatment I), and an increasing after inoculation of 57% (Figure 3A-Treatment II). *Solanum lycopersicum* showed a significant decreasing (83%) of galls for treatment I, when compared to control (Figure 3C).

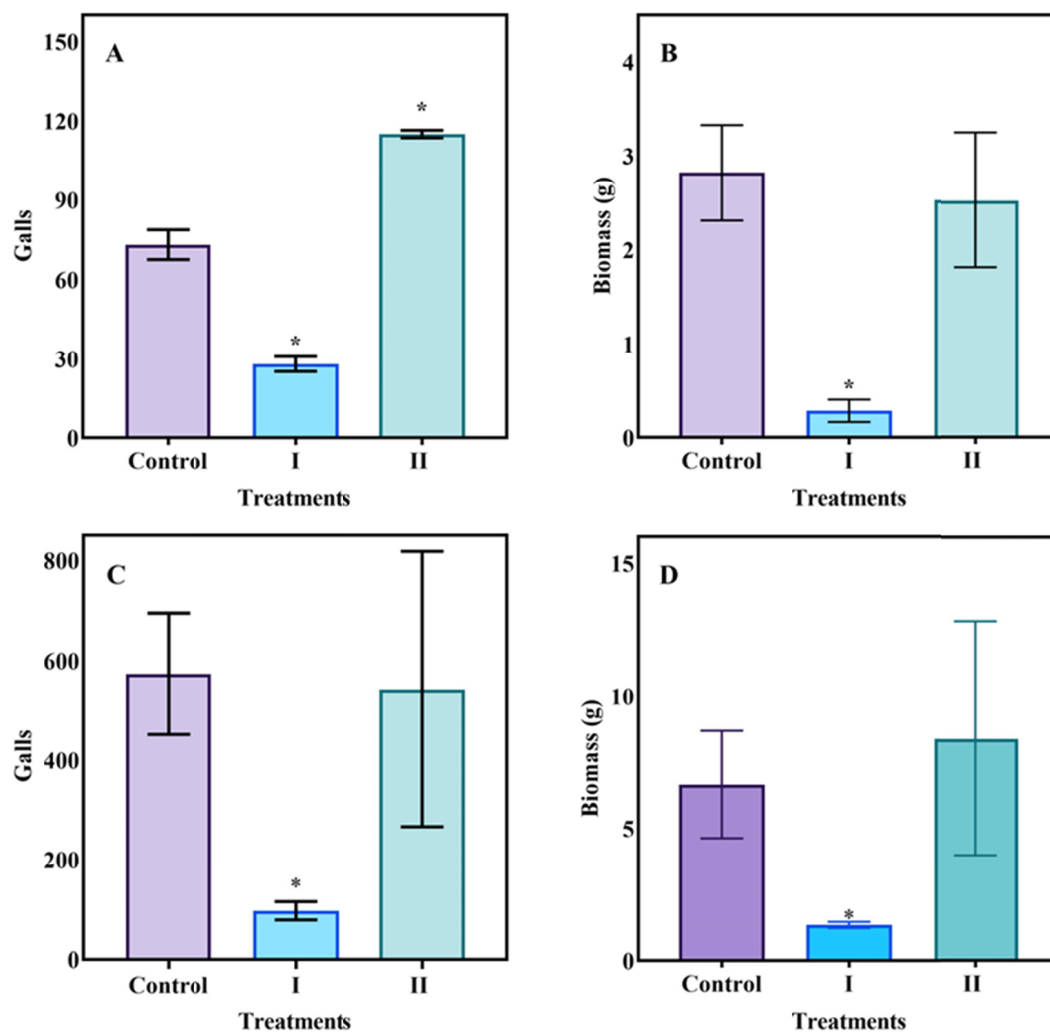


Figure 3. Allelopathic effect of *S. stramonifolium* on *N. benthamiana* (A and B) and *S. lycopersicum* (C and D) over galls and biomass development before (I) and after (II) *J2 M. incognita* inoculation. Pair wise comparisons were performed by using ANOVA *post-hoc* Dunnett comparison tests ($p \leq 0.05$). Bars represent standard deviation. * shows significant difference to the control

Gall diameter and giant cell surface were measured and illustrated in Figure 4 and 5. Measurements showed a significant reduction in gall diameter: from 400 μm (CT) and 480 μm (IT) when compared to control untreated plant (Figure 5A). On the other hand, no significant changes were observed when evaluated the surface of giant cells of treated and untreated plants (Figure 5B).

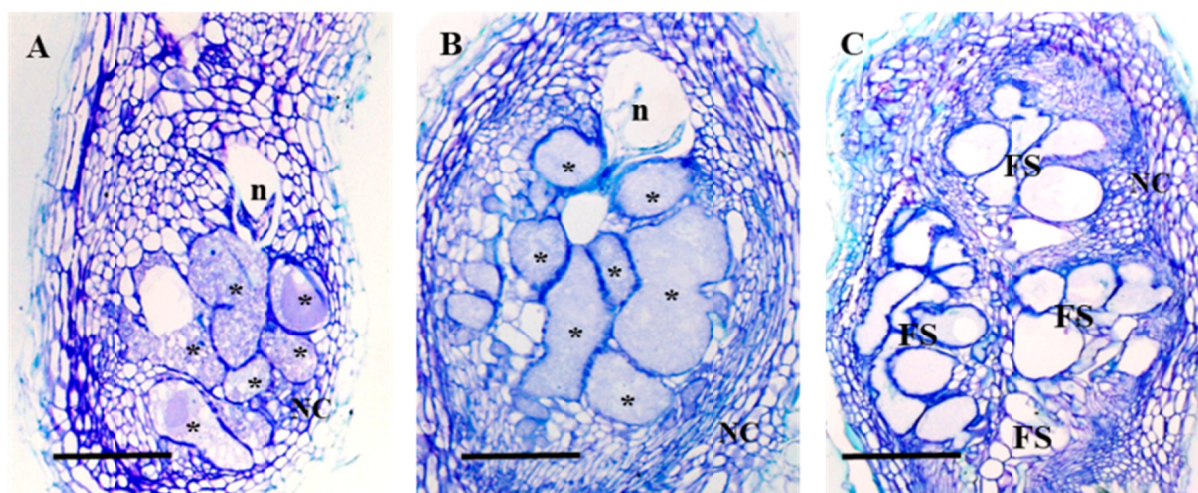


Figure 4. Morphological analysis of *Meloidogyne incognita*-induced galls in *Arabidopsis thaliana* treated with ED. Bright-field micrographs of toluidine blue stained section of ED treatment before infection (A); ED treatment before and continuous after inoculation (B); and no treatment (C). FS, feeding site; *, giant cells; NC, neighboring cells; n, nematode. Bars = 50μm

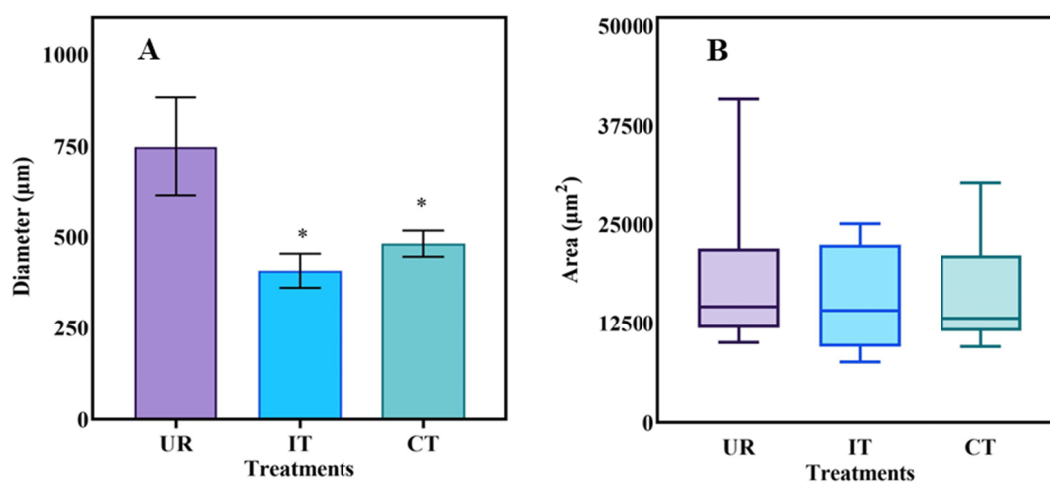


Figure 5. Analysis of *M. incognita*-induced galls in *A. thaliana* during ED treatment. Diameter measurements (μm) of galls induced by *M. incognita* in *Arabidopsis* (Col-0) treated with ED compared to untreated roots (UR).

IT: Treatment with ED before *M. incognita* inoculation; CT: ED treatment before and continuous after inoculation after *M. incognita* inoculation. Bars represent SD. Pair wise comparisons were performed by using ANOVA *post-hoc* Dunnett or Kruskal-Wallis *post-hoc* Dunn's comparison tests ($p \leq 0.05$). Bars represent standard deviation. * shows significant difference to the control (UR)

The results obtained from seeds of *Arabidopsis* treated with the ID showed biomass reduction of 91% when compared to (Figure 6D). To evaluate the effect of ED and ID on the nuclear ploidy levels, flow cytometric measurements were performed on *Arabidopsis* roots untreated and treated with ED and ID (Figure 7). Ploidy levels in control and treated roots was ranged from 2C to 16C. Treatments apparently led to slight increase in 2C ploidy levels. Higher ploidy levels (8C and 16C) slightly decreased upon ID and ED treatments.

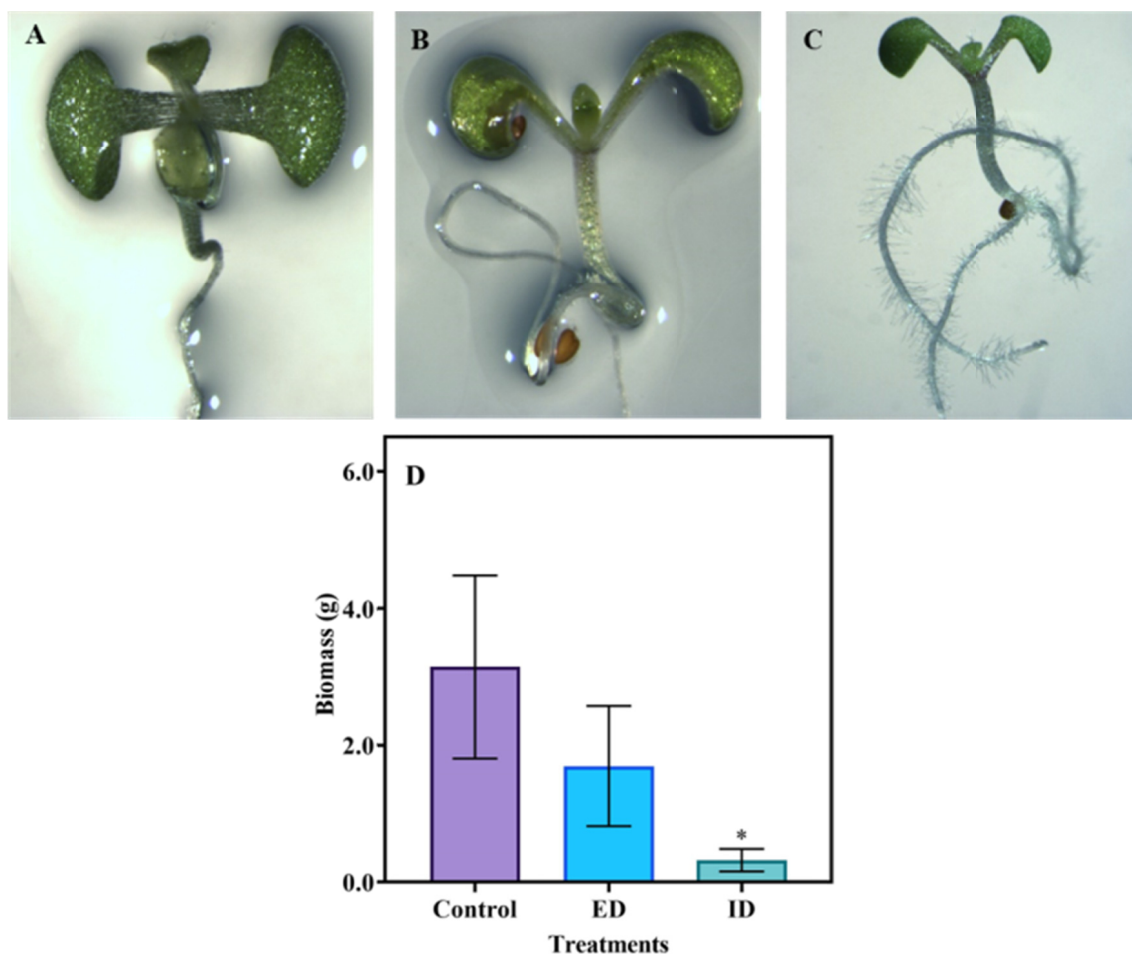


Figure 6. Biomass and germination effects of ED and ID on *A. thaliana* (10 days after germination) grown in MS medium. (A) Untreated plant; (B) plant treated with ED (300 µg mL⁻¹) with decreasing of biomass; (C) plant treated with ID (100 µg mL⁻¹) with decreasing of biomass; (D) Biomass measurements using seeds treated with ED and ID in MS medium 10 after germination. Pair wise comparisons were performed by using ANOVA *post-hoc* Dunnett mean comparison tests ($p \leq 0.05$). Bars represent standard deviation ($p \leq 0.05$)

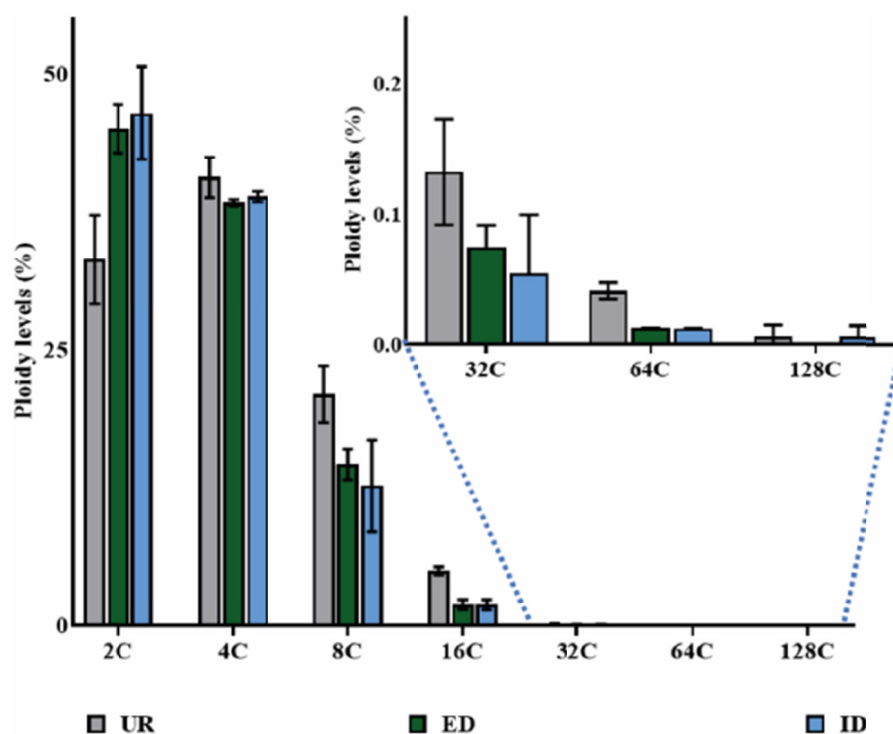


Figure 7. Flow cytometry analysis of Arabidopsis (Col-0) treated with ED, ID, and distilled water (negative control-UR), showing increased 2C levels in nuclei of treated roots. Endoreduplication index of ± 7000 nuclei of roots of Arabidopsis (Col-0). Error bars represent standard deviation

To purify the nematotoxic compounds, the ED was submitted to Centrifugal Partition Chromatography (CPC) as initial purification step (Figure 8), minimizing the presence of compounds that could interfere in future purification steps. The obtained fractions were separated in four groups (F1-4). The nematotoxic activity was concentrated in F4, showing 100% of lethality against *M. incognita* in nematotoxic assay and, the same results showed in recovery assays (Figure 9).

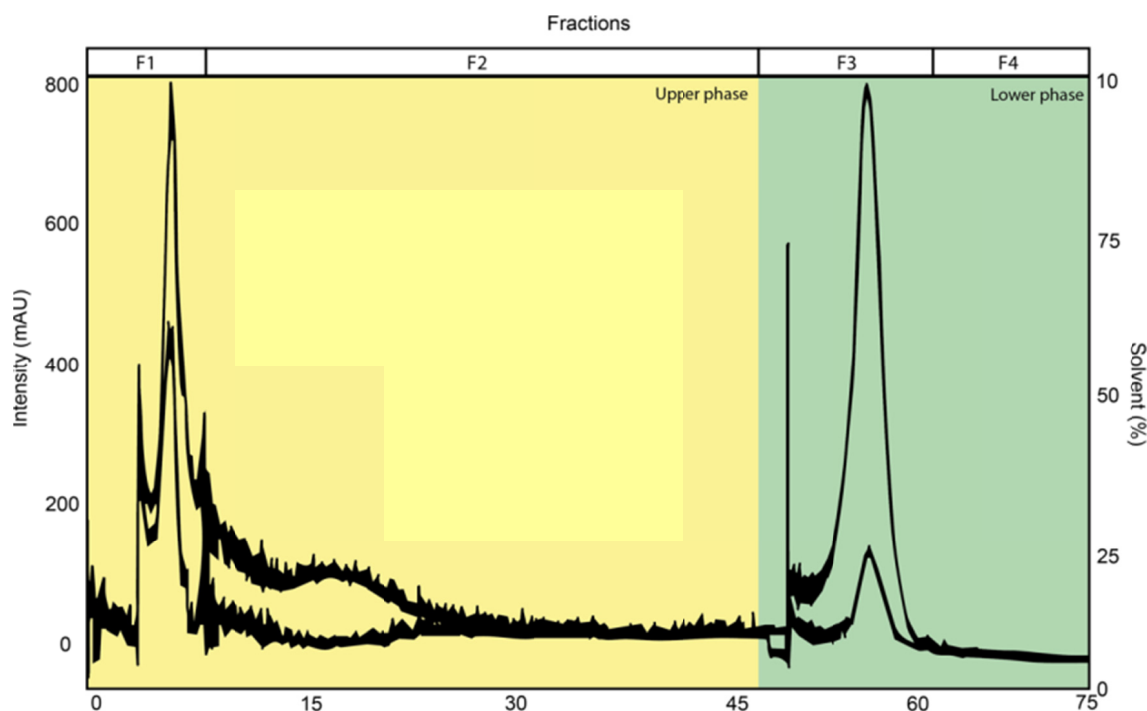


Figure 8. CPC of ED of *S. stramonifolium*. It was used ethyl acetate, n-butanol and distilled water (1:4:5, v/v/v) with flow rate of 5 mL·min⁻¹. Absorbance was monitored at 254 nm. After the absorbance stabilizes, 100% of lower phase was used as eluant. F1-4 indicates the fraction collected

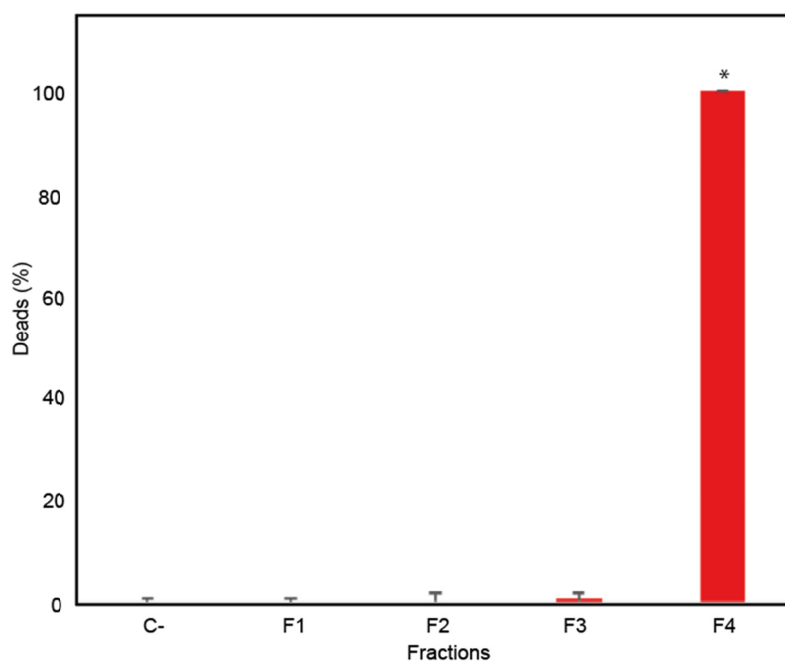


Figure 9. Recovery assay of F1-F4 against *M. incognita* using 50 µg of each fraction. Pair wise comparisons were performed by using Kruskal-Wallis *post-hoc* Dunn's comparison tests ($p \leq 0.05$). Bars represent standard deviation. * shows significant difference to the control (C-)

Furthermore, in F4 fraction, the three glycoalkaloids were characterized by MS/MS fragmentations as previously described, corresponding to [Solasodine + deoxyHex + deoxyHex + Glu + H]⁺ for solamargine, and solanine corresponding to [Solasodine + deoxyHex + Glu + Glu + H]⁺. The new glycoalkaloid corresponding to [Solasodine + Glu + deoxyHex + deoxyHex + Xyl + H]⁺.

Further fractionation of F4 by semi-preparative HPLC led to 13 sub-fractions (P1-13) (Figure 10). The bioassays showed that the nematocidal activity was concentrated in sub-fraction P3 with 100% lethality (Figure 11).

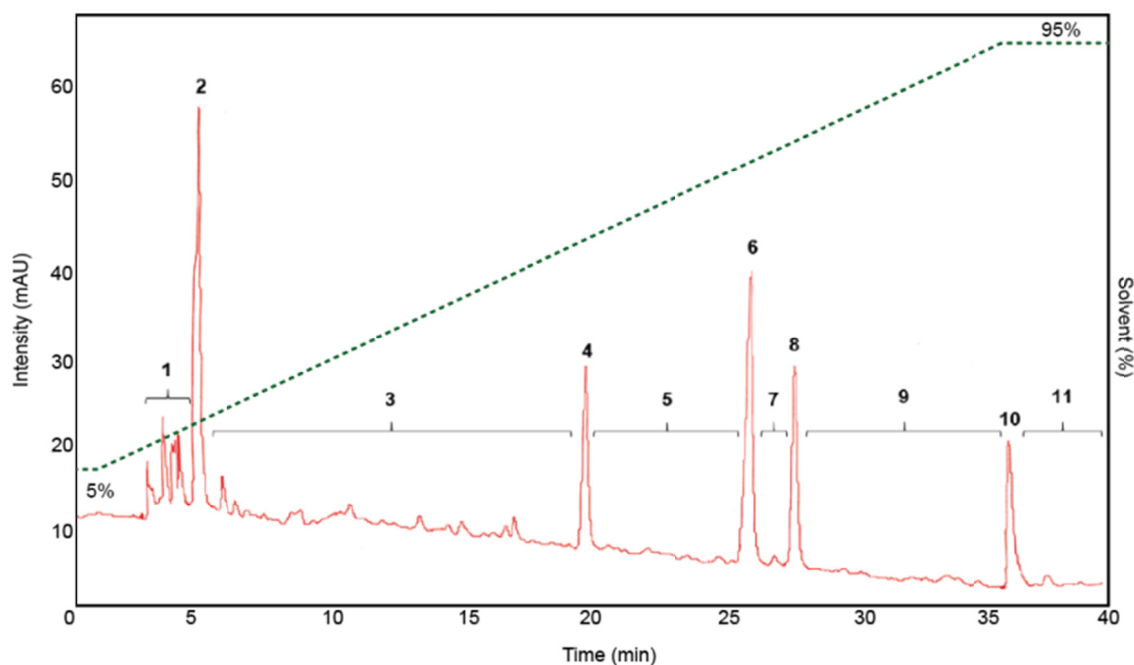


Figure 10. C18 HPLC of F4 obtained from CPC ED analysis. The green line indicates the linear gradient from 5-95% eluent (MeOH+ 0.1% FA). Absorbance was monitored at 254 nm, with a flow rate of 4 mL min⁻¹

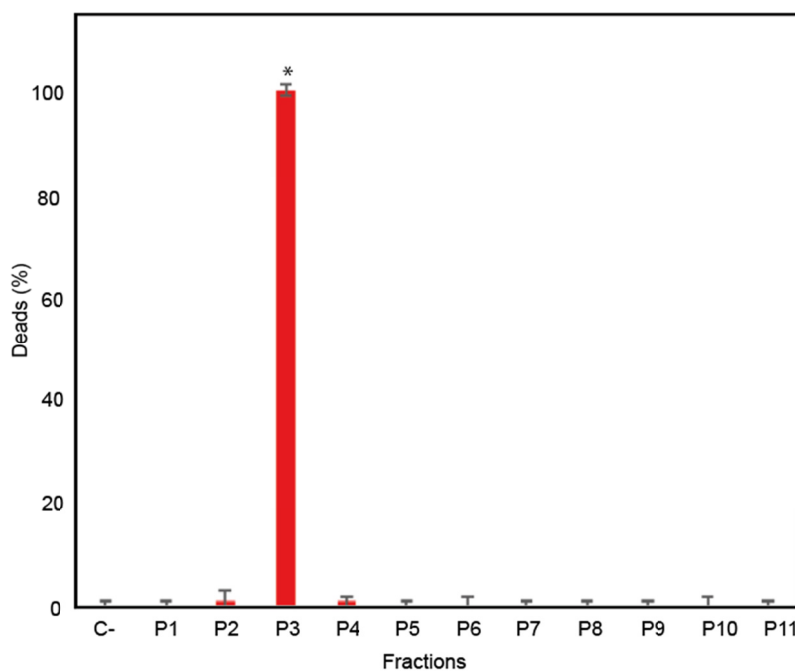


Figure 11. Recovery assay of P1-P11 against *M. incognita* using 50 µg of each fraction. Pair wise comparisons were performed by using Kruskal-Wallis *post-hoc* Dunn's comparison tests ($p \leq 0.05$). Bars represent standard deviation. * shows significant difference to the control (C-)

In fraction P3 obtained from HPLC analysis, mass spectrometry identified the presence of glycoalkaloids in subfraction A (Figure 12), such as Tomatidine [α -Solanidine + Glu + Rha + Gal + H]⁺; Solamargine [Solasodine + Glu + Gal + Rha + H]⁺; Solanine [Solasodine + Glu + Glu + Gal + H]⁺; and Tomatidine [α -Solasonine + Gal + Rha + Glu + H]⁺.

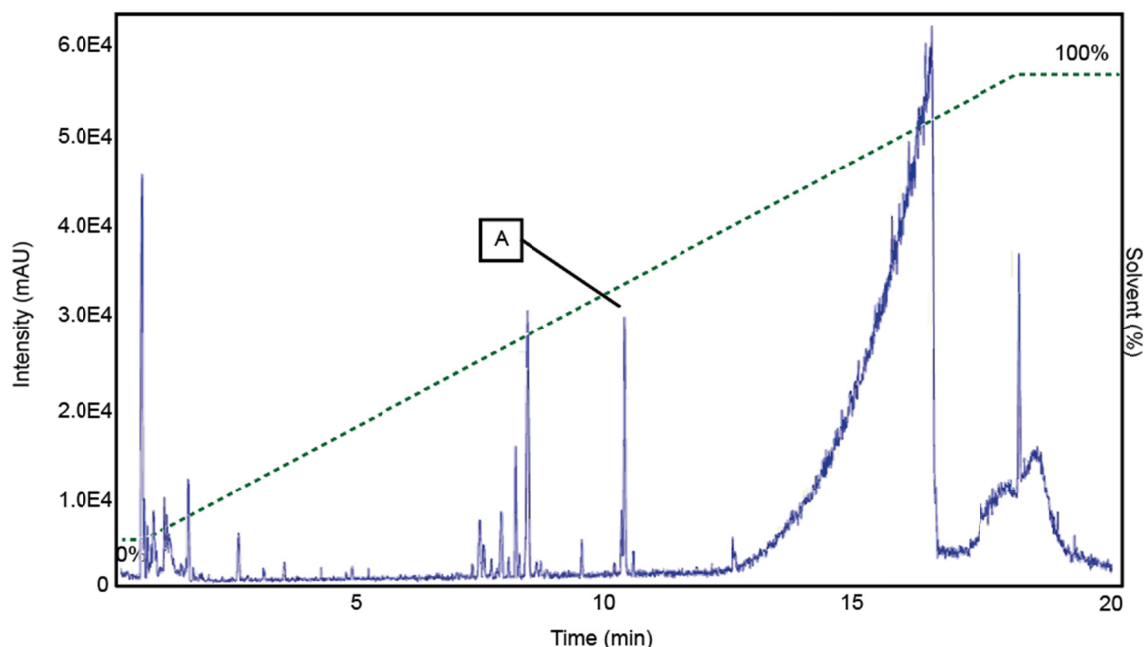


Figure 12. Chromatogram of the P3 fraction obtained by UPLC-URMS using semi-preparative C18 column coupled to analytical C18. A indicates the peak selected to illustrate the identification of molecules as described below. The green line indicates the linear gradient from 5-100% eluent (ACN + 0.1% FA). Absorbance was monitored at 254 nm, with a flow rate of 1 mL min⁻¹.

Additionally, ethanolic and aqueous extract from two-weeks-old *S. stramonifolium* roots were analyzed by UPLC-URMS and compared each other, showing similar chromatographic profile, with three preeminent peaks (A, B and C) from 8 to 10 min retention time (RT) (Figure 13).

The MS/MS analysis of peaks A, B and C showed a three steroidal glycoalkaloid. Solamargine and solanine were observed at m/z 884.5017 and 868.5038 respectively to peaks A and C. A new glycoalkaloid were observed in peak B with m/z 1000.5480.

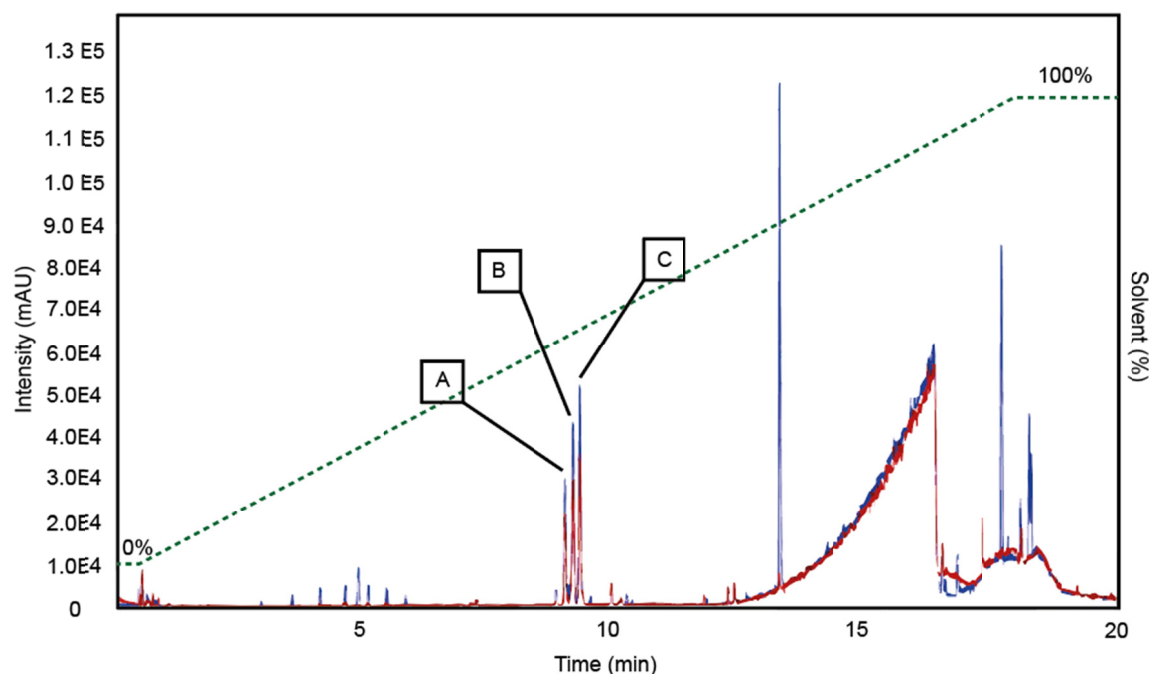


Figure 13. Chromatogram of ethanolic (red) and aqueous (blue) extracts obtained by UPLC-URMS using semi-preparative C18 column coupled to analytical C18. Peaks A, B and C were selected to illustrate the identification methodology used. The green line indicates the linear gradient from 5-100% eluent (ACN + 0.1% FA). Absorbance was monitored at 254 nm, with a flow rate of 1 mL min⁻¹

4. Discussion

In our experiments, the total absence of galls, egg mass or nematodes in *S. stramonifolium* inoculated with J2 suggests a strong and effective defense plant system that can act as repellent, nematostatic or nematicide factor against *M. incognita*. These results corroborate with previous studies that showed that *S. stramonifolium* is immune to infection by the root-knot nematode specie (Costa et al., 2022). Second stage juvenile nematodes (J2) are attracted towards the roots by chemical compounds, as metabolites, sugars, CO₂ and amino acids detected by amphidial glands (Robinson, 2002). Antagonistically, compounds secreted in soil by roots can act as a repellent factor, affecting nematode metabolism (Hiltpold, Jaffuel, & Turlings, 2015).

Allelochemicals exudated on soil can affect nematodes, as reported in *Pisum sativa* root cap exudate, that reduces the motility of *M. incognita* and attracts other entomopathogenic nematodes. Also, other roots compounds may act immediately after nematodes penetrate in the root and killing them due to a constitutive plant defense mechanism (Hiltpold, Jaffuel, & Turlings, 2015).

Several plants show resistance to nematodes infection due the allelochemical release on soil. Examples are *Solanum torvum*, showing resistance to *M. incognita* (Bagnaresi et al., 2013). Mendonça, Santana, Mattos, and Pinheiro (2010) suggest that *S. stramonifolium* can be classified as resistant plant against *M. incognita*, however our results suggest that the plant tested is immune to RKN. Possibly, the compounds exudate on soil by *S. stramonifolium* acts as plant defense against RKN. Those molecules can be isolated on future and applied directly over the soil or used in transgenic plants.

In our experiment using galls treated with ED in initial treatment (IT) and continuous treatment (CT), the IT showed a galls diameter reduction slightly bigger than CT. The IT could remain in soil and affect nematode migration towards the roots, decreasing J2 infectivity.

Nematodes enter the root in the elongation zone and migrate until the root tip, where they reposition to penetrate the vascular system and advance inducing galls in susceptible plant roots progressing in their lifecycle (Wyss, Grundler, & Munch, 1992). In this step, the initial treatment using ED may act on J2, increasing plant resistance to nematodes infection.

Our results showed the formation of multiple feeding sites per gall only occurred in control experiments, suggesting that compounds on ED prevents multiple nematode root penetrations. The observed reduction in gall

diameter upon ED treatment could be the result of apparent reduced giant cells (GC) size even when GC measurements were not statically significant likely due to their irregular sizes.

Meloidogyne incognita establishes permanent feeding sites on roots, inducing giant-feeding cells that undergo multiple nuclear divisions without cytokinesis, showing increased metabolic activity, which will nourish the nematodes until the end of their life cycle. Once the produced eggs hatch, nematodes migrate few days in soil until penetrating the roots of the host plant (Williamson & Hussey, 1996). Also in our results, gall diameter reduction seems related to the presence of the different allelochemicals, as well as the presence of phytohormones in plant seed extracts.

The germination and biomass assays of wild-type Col-0 seeds treated with ED and ID were performed to evaluate the inhibition of dialysates on plant development. The results suggest a slight effect of dialysates, especially of ID in the biomass during *A. thaliana* development. The biomass reduction shown by ID and ED suggested to be caused by plant compounds already isolated from other plants. Several research tested the effects of allelochemicals on root development. BOA (2(3H)-benzoxazolinone) and DIBOA (2,4-dihydroxy-1,4(2H)-benzoxazin-3-one) obtained from *Secale cereale* affected the root development of *Cucumis sativa*, especially root elongation. Intracellularly, BOA and DIBOA reduced the amount of starch granules in amyloplasts, ribosomes density, the regeneration of root cap cells, the number of mitochondria and the lipid catabolism (Burgos, Talbert, Kim, & Kuk, 2004). *Echinochloa crus-galli* and *Cassia obtusifolia* seeds were tested to determine the effects of volatile cineoles (1,4-cineole and 1,8-cineoles). The results showed a decrease in root growth and germination rates. The 1,8-cineole decrease all mitotic stages of mitosis, while the 1,4-cineole only decrease the length only of prophase stage (Romagni, Allen, & Dayan, 2000).

Flow cytometry analysis suggested the effects of allelochemicals on ploidy levels in uninfected roots. High 2C levels and decreased 8C and 16C levels suggests that the ED and ID slightly decrease the endocycle likely keeping the cells in a mitotic state. Nematode feeding site induction is dependent of cell cycle activation since giant-feeding cells undergo multiple divisions without cytokinesis (Williamson & Hussey, 1996) followed by activation of the endocycle (Almeida-Engler et al., 2012). The change in ploidy levels detected in our experiments might be due to the presence of hormones in the seeds (e.g., Auxin and gibberellin) which may control the endocycle switch from mitosis (Heldt & Piechulla, 2011; Joubes & Chevalier, 2000). Other seed compounds such as monoterpene analogs and essential oils can also interfere in the cell cycle delaying the endocycle in root plants, as *Citrus unchiu* (Romagni et al., 2000; Shimada, Endo, Fujii, Hara, & Omura, 2005). These hormones may up- or down regulate the endocycle, switching this event to mitosis or endocycle (Joubes & Chevalier, 2000). Other compounds also can interfere and decrease a specific phase of the cell cycle. Monoterpene analogs compounds isolated from essential oil of many species can affect and delaying the cell cycle in root plants (Romagni et al., 2000).

In summary, the data reported here show allelopathic effects from ED and ID extracts from *S. stramonifolium* seeds on gall development in *A. thaliana* as well as on the ploidy level in uninfected roots from the same species. Additionally, *S. stramonifolium* showed complete immunity to J2 infection; besides, of a strong biomass decrease was observed in commercial species like tobacco.

The presence of glycoalkaloids in the P3 fraction and in the ethanolic and aqueous extracts of *S. stramonifolium* roots can provide information that relates plant immunity to root-knot nematode infection with the nematocidal activity present in the external dialysate. New studies and advances in the purification of nematocidal compounds from the tested material may elucidate the biotechnological potential present in *S. stramonifolium*, enabling the development of green technologies to control *M. incognita*.

5. Concluding Remarks

The data obtained here provide a potential source of knowledge to better understand the allelochemical interaction between different plants species and nematodes. Further studies in this field will undoubtedly contribute to discover novel alternatives to control nematodes pests in the agriculture.

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Authors contributions

Dr. Tiago Gonçalves Costa was responsible for study design, data collection and drafted the manuscript. Dr. Thales L. Rocha and Prof. Janice A. Engler were involved with the study design, manuscript insights and revising. Dr. Thomas Michel contributed with purification and identification of molecules. Dr. Simoni Campos Dias, helped with the manuscript writing organization, Athamy S. de P. Cruz and Thaís C. de Sousa contribute with data collection and analysis. All the authors read approved and contributed equally to the study.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Obtained.

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Data sharing statement

No additional data are available.

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