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Crude Aqueous Root Extracts of *Solanum* spp. Show Nematotoxic Activity Against *Meloidogyne incognita*

A. A. Ferreira[1,2], A. S. Ombredane[3], R. R. Pimentel[2,4], P. D. S. Ferreira[1,2], J. E. Cares[1], J. R. P. Santos[5], C. Furlanetto[1] & T. L. Rocha[2]

[1] Department of Plant Pathology, University of Brasilia, Brasilia, DF, Brazil

[2] Laboratory of Prospection of Bioatives Compounds, Embrapa Genetic Resources and Biotechnology, Brasilia, DF, Brazil

[3] Laboratory of Bioatives Compounds and Nanobiotechnology, University of Brasilia, Brasilia, DF, Brazil

[4] College of Agronomy and Veterinary Medicine, University of Brasilia, Brasilia, DF, Brazil

[5] Department of Phytosanitary Defense, Federal University of Santa Maria, Santa Maria, RS, Brazil

Correspondence: A. A. Ferreira, Department of Plant Pathology, University of Brasilia, Brasilia, DF, Brazil. Tel: 55-613-448-3270. E-mail: adrianabiomedica1@gmail.com

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Abstract

Brazilian crops are strongly affected by the root-knot nematode *Meloidogyne incognita*, which compromises food production and causes serious economic losses around the world. Synthetic nematicides are still some of the main control strategies for this pathogen, despite the high risk to human health and the environment. Therefore, sustainable biotechnology represents a viable alternative for nematode control. Plants of the Solanaceae family are present in several biomes and are a source of secondary metabolites of various chemical classes. In this context, the nematotoxic effect of crude aqueous root extracts (CAREs) of three *Solanum* species was investigated: *Solanum americanum*, *S. subinerme* and *S. lycocarpum.* CARE showed high nematicidal activity (94.6±9.2% mortality, $p \leq 0.001$) against second-stage juveniles (J_2) of *M. incognita*, even after heating to 50 °C. These CAREs also inhibited hatching of juveniles (98.4±0.7% of mortality, $p \leq 0.001$) in addition to presenting a mean lethal dose of 336 μg ml^{-1}. No toxic activity was observed against non-target organisms, such as fungi (*Trichoderma harzianum* and *Trichoderma asperellum*), yeast (*Candida maltosa*), nitrogen-fixing bacteria (*Bradyrhizobium diazoefficiaens, B. japonicum*), two strains of *Azospirillum brasilense*, AbV 5 and AbV 6 and *Bacillus* [2538 Ba]. Bioassay performed in a greenhouse demonstrated that CAREs of these species were effective in reducing the number of egg masses and galls (> 85%, $p \leq 0.0001$), and did not show a phytotoxic effect on soybean seedlings. Our findings indicate that CAREs from plants of the Solanaceae family, especially *S. lycocarpum*, demonstrated nematotoxic activity on J_2 and inhibited the hatching of *Meloidogyne incognita* eggs, representing an ecologically appropriate tool to control nematode infection in crops worldwide.

Keywords: control, natural products, nematotoxic extracts, root-knot nematode, Solanaceae

1. Introduction

Root-knot nematodes (*Meloidogyne* spp.) represent one of the most significant groups of plant pathogens worldwide (Jones et al., 2013) affecting more than 3,000 species of plants, including economically important crops (Przybylska & Obrępalska-Stęplowska, 2020). *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949, stands out for its wide geographic distribution, its wide variety of potential host plants, and its diverse evolutionary adaptations in relation to its parasitism (Bakhetia et al., 2005; Grigolli & Asmus, 2011; Jones et al., 2013). The main symptom observed in plants infected with *Meloidogyne* spp. is the formation of root galls. This

phenomenon leads to a decrease in the absorption of water and nutrients by plant roots, reducing their transport of nutrients throughout the plant, thus increasing the vulnerability of plants to infection by other pathogens, which can lead to death (Ritzinger & Fancelli, 2016).

Some strategies used to reduce damage caused by root-knot nematodes (PPN), include biological control, crop rotation, use of resistant cultivars and antagonistic plants (Abad et al., 2008; Kepenekci & Saglam, 2015; Medina-Canales et al., 2019). However, the main strategy used to control PPNs is based on the use of synthetic nematicides, which represent a high risk to human health and the environment (Chitwood, 2002; FAO, 2017; Rocha et al., 2017; Forghani & Hajihassani, 2020). The incorrect and indiscriminate use of pesticides leads to the accumulation of toxic residues in food, contamination of water and soil and toxic contamination of farmers. Besides, the development of resistant pests increased and biological control by natural enemies turned inviable (Corrêa & Salgado, 2011; Forghani & Hajihassani, 2020).

Currently, there is a trend in developed countries to ban the use of the most toxic pesticides. Over the past decade, many nematicides have been banned or restricted in Europe and the United States, among other countries (Kearn et al., 2014). This drastic reduction was the consequence of legislation, with the definition of new rules for the use of pesticides, aiming at reducing the risks and impacts caused by these chemicals (Kassim et al., 2013; Xiang et al., 2018). Thus, there is a need to search for new control strategies using sustainable compounds that effectively contribute to reducing the use of these chemicals worldwide (Dixon, 2001; Newman et al., 2003; Clardy, 2004; Forghani & Hajihassani, 2020).

Natural plant products are sources of bioactive compounds, and therefore may present an alternative in the control of plant parasitic nematodes, such as *M. incognita* (Makhubuet al., 2021). In the last 20 years, a large number of plant species have been described as presenting a wide variety of secondary metabolites with nematotoxic activities (nematicides and/or nematostatics) (Caboni et al., 2013; Rocha et al., 2017; Forghani & Hajihassani, 2020). According to the literature, extracts obtained from *Canavalia ensiformis* (L.) DC (López, 2012), and *Crotalaria spectabilis* Rhot seeds demonstrated both *in vitro* assays and, in the greenhouse, nematotoxic effect on second-stage (J_2) juveniles of *M. incognita* (Honório Júnior et al., 2010; Rocha et al., 2017). Nematicidal activity was also found in leaves of *Datura metel* L., *D. innoxia* Mill., and *Brugmansia suaveolens* (Humbold & Bonpland ex Willdenow) Bercht. & J. Presl, suggesting that the activity resulting from the presence of phytocompounds such as alkaloids, steroids, flavonoids, terpenoids, phenolic compounds, tannins, saponins and triterpenes (Nandakumar et al., 2017).

The family Solanaceae A. L. Jussieu is considered one of the largest groups of angiosperms present in the world and is represented by about 3,000 species distributed in 106 genera, being reported in several studies as plant species rich in bioactive secondary metabolites (Pereira et al., 2016). Among these genera, *Solanum* L. is the largest and most complex. Several species of these plants exhibit compounds with biotechnological potential to control PPN, such as alkaloids (hyoscine, nicotine, saponin), glycosides, glycoalkaloids, diterpenes, sesquisterpenes, flavonoids, amino acids and phenylpropanoid acids (Pereira et al., 2016; Resende et al., 2020). These compounds are produced through a secondary plant metabolism, playing an important role in the interaction with the environment as a system of plant defense. They are associated with cell differentiation, regulation of plant growth, mediation between plants and other organisms, and, above all, plant protection (Dobson, 2004; Kessler & Kalske, 2018).

In this context, the main objective of this study was to investigate the nematotoxic potential of crude aqueous root extracts (CAREs) of three plant species of the genus *Solanum* against the hatching of juveniles and juveniles of *M. incognita* in bioassays *in vitro* and in a greenhouse. *In vitro* bioassays were also carried out to determine the non-toxic effects on non-target organisms (bacteria, fungi and yeast), thermostability and phytotoxicity, in addition, eluates separated by solid phase chromatography (SPE-C18) were evaluated for their effect on second-stage juveniles of *M. incognita*. All *in vitro* bioassays were performed three at a time, and then repeated.

2. Materials and Methods

2.1 Obtaining Roots and Crude Aqueous Root Extracts of Plants

Seeds of three plant species of the Solanaceae family-*Solanum americanum* Mill., *Solanum subinerme* Jacq. and *Solanum lycocarpum* A. St. Hil., were previously acquired from the Germplasm Bank of Embrapa Hortaliças-DF, Brazil. The plants were grown in pots filled with commercial substrate Bioplant® in a greenhouse for 120-180 days**,** at temperatures ranging from 26 to 33 °C, at Embrapa Genetic Resources and Biotechnology-DF, Brazil. The process of obtaining crude aqueous root extract was an adaptation of methods previously described (Ferris & Zheng, 1999; Martins & Santos, 2016; Rocha et al., 2017). The roots were removed from the soil, washed, sectioned and macerated with the aid of a crucible (mortar) with a pistil using liquid nitrogen. Subsequently, the

ground-up material was homogenized, using distilled water (1:6 w:v) and continuously stirred (130 rpm) for 24 hours at 4 °C. The resulting material obtained from the suspension was filtered through gauze and filter paper, then lyophilized in a Savant-Super Modulyo lyophilizer (ThermoFisher, GA, USA) lyophilizer. The dried CAREs were weighed and stored at -4 °C. For bioassays using J_2 *M. incognita*, aliquots of 200mg/2mLwere stored at -20 °C.

2.2 Obtaining Eggs and Second Stage Juveniles (J_2) of Meloidogyne incognita

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) were grown in plastic pots with the commercial substrate Bioplant®, under greenhouse conditions. One plant per pot was inoculated with about 2,000 second stage juveniles (J_2) of *M. incognita*. After 90 days at 26-33 ^{o}C, tobacco roots were cut and ground for one minute in a sodium hypochlorite (NaOCl) solution containing 1% active chlorine, according to the method described by Hussey and Barker (1973), modified by Boneti and Ferraz (1981). For the recovery of eggs, overlapping granulometric sieves of 35, 45, 200 and 500 mesh were used (Coolen & D'Herde, 1972). To obtain the J_2, the eggs were collected in a 500 mesh sieve and transferred to a modified egg incubation chamber, using a glass beaker.

2.3 In vitro Nematotoxic Activity of CAREs on Meloidogyne incognita

The nematotoxic activity of each CARE was analyzed in accordance with (Dias et al., 2000). To perform the bioassays, 60±5 juveniles were transferred to 1.5 mL microcentrifuge tubes and incubated with the CAREs previously prepared at a final concentration of 1 mg mL^{-1} for 48 hours at 27±1 °C. Treatments were performed three at a time, and then repeated. Distilled water and 70% ethanol (EtOH) were used as negative and positive controls, respectively (Haseeb et al., 1996; Dias et al., 2000). After the exposure, the nematodes were observed and counted with the aid of an optical microscope, then separated into categories of mobile and paralyzed.

Paralyzed nematodes were then submitted to a recovery test. The J_2s with their bodies in a straight/dead posture (paralyzed/stretched) were transferred to 1.5 mL microcentrifuge tubes, centrifuged at 700 g for 10 min (Haseeb, Shukla, & Butool, 1996; Dias et al., 2000; Rocha et al., 2017), the supernatant was discarded and the J_2s were carefully re-suspended in 1.5 mL of distilled water. The procedure was repeated three times and after the last wash, the nematodes were left in 1.5 mL of distilled water for 24 hours. Juveniles were then counted, classifying those curled/unstretched as alive and the paralyzed/stretched as dead, thereby determining the nematotoxic activity of each CARE. The same centrifugation procedure (700 g for 10 min/three times each) was performed with juveniles in water as control, under the same conditions, where the juveniles remained alive.

2.4 Estimate of CARE's Thermostability

The thermostability evaluation was performed after CARE exposure (stock solution 50 mg mL^{-1}) at a temperature of 50 ºC for 24 hours (digital water bath, model Bunker NI 1229). Basically, 20 µl of each CARE, corresponding to 1 mg obtained after the heating process, were submitted to *in vitro* bioassays according to item 2.3, to check whether the nematotoxic properties remained (Rocha et al., 2017). Bioassay performed three at a time, and then repeated.

2.5 Hatching Test of Meloidogyne incognita Juveniles

The effect of *S. americanum*, *S. subinerme* and *S. lycocarpum* CAREs on the hatching of J_2 juveniles of *M. incognita* was investigated. About 100±5 eggs were transferred to 2 mL microcentrifuge tubes, incubated with 3 mg mL^{-1} of each CARE at 28 °C for a period of 15 days. Distilled water, and 0.06% active chlorine hypochlorite, were used as negative control and positive controls, respectively (internal positive control used in the laboratory). J_2 numbers were counted after intervals of 24 h, 48 h, 96 h, 7 days and 15 days of exposure to CARE (Adegbite, 2011; Kepenekci & Saglam, 2015; Nile et al., 2018). In addition, the morphology of the incubated and unincubated eggs was examined by optical microscopy (E100 Nikon Binocular). The tests were performed three at a time, and then repeated.

2.6 Determination of the Mean Lethal Dose (LD_{50})

The determination of the LD_{50} of CARE of *S. americanum* and *S. lycocarpum* was performed according to a dose-response curve. To this end, 60±5 J_2 *M. incognita* were exposed to CARE at 100, 250, 500 and 1,000 mg mL^{-1} for 48 hours at 27±1 °C (Caboni et al., 2013; Babaali et al., 2017; Khan et al., 2019). Afterwards, the nematodes were counted using an optical microscope. The nematotoxic effect was determined after the paralyzed nematodes were submitted to a recovery test, as mentioned in item 2.3. Bioassays were performed three at a time, and then repeated.

2.7 CARE Evaluation in a Greenhouse

Tobacco seeds (*Nicotiana tabacum* cv. Xanthi) were sown in sterile soil supplemented with Bioplant® substrate (1:1, w/w) and kept for approximately 20 days for germination under greenhouse conditions. Subsequently, tobacco seedlings were transplanted into plastic pots of 300 ml each, containing sterilized soil, supplemented with Bioplant® substrate and washed sand (4:2:4, w/w/w). After 7 days, the plants were separated into five experimental groups: non-inoculated control plants, positive control (Aldicarb at 280 mg kg^{-1} of soil), negative control (distilled water), and treatments with CAREs from *S. subinerme* and *S. lycocarpum*, according to the methodology described by Nile et al. (2018).

Three thousand juveniles (J_2) of *M. incognita* were exposed to CAREs of *S. subinerme* and *S. lycocarpum* at 1, 2 and 3 mg mL^{-1} for 48 hours at room temperature. After this period, these nematodes were washed three times in distilled water, and a total of 1,000 J_2 were added to the tobacco plants via 1-2 cm holes in the soil around the stem of each plant. The tobacco plants were then incubated under greenhouse conditions at temperatures ranging from 26 to 33 °C. After 30 days after being inoculated with nematodes, the roots were removed from the shoots, washed and dyed using β-phloxin (Sigma, Aldrich, 0.015 mg/L) for 20 min. The number of egg masses and galls were counted.

In parallel, the reproduction factor (RF) was calculated according to Oostenbrink (1966) using the relationship between the final population and the initial population (RF = final population/initial population). The bioassays were carried out three at a time, and then repeated, using the randomized blocks model.

2.8 Soybean Phytotoxicity Assessment

For the phytotoxic evaluation, soybean seeds (*Glycine max*, BRS 7980) were superficially sterilized, incubated with 1 mg mL^{-1} of *S. americanum*, *S. subinerme* and *S. lycocarpum* CAREs in test tubes containing 20 mL of MS medium (Murashigie and Skoog, 1962). After two days of incubation, they remained for 12 days in a tissue culture room at 26±2 °C with a photoperiod of 16 hours, all seeds demonstrated an identical germination. The parameters evaluated were: total fresh weight, root fresh weight, stem fresh weight, root dry weight, total dry weight and length from the neck to the top. MS culture medium was used as a CARE-free control (MAPA, 2009). The experiment was carried out three at a time, and repeated five times each treatment.

2.9 Toxicity Against Non-target Bacteria

Antibacterial activity of CARE from *S. americanum*, *S. subinerme* and *S. lycocarpum* was evaluated against *Bradyrhizobium diazoefficiens* Delamuta SEMIA 5080 and *Bradyrhizobium japonicum* (Kirchner) Jordan SEMIA 5079, both isolated from soybean roots; two strains of *Azospirillum brasilense* Tarrand, Krieg & Döbereiner (AbV5 and AbV6) from the Collection of Multifunctional Microorganisms of Interest to Agriculture and Bio-industry of the Embrapa Soybean Unit, and one strain of *Bacillus* [2538Ba], (PGPB). Yeast Mannitol Agar with Congo Red (YMA) and Dextrose Yeast Glucose Sucrose (DYGS) medium were used for the growth of *Bradyrhyzobium* and *Azospirillum*, respectively. *Bacillus* [2538 Ba] was cultivated using Tryptic Soy Broth (TSB) medium.

Bacteria were continuously stirred (150 rpm) at 30 °C, (*Bacillus* strain) for 24 hours, 48 hours for (*Azospirillum* strains) and for 5-6 days (*Bradyrhyzobium* spp.). Then, 200 µL of each bacterial suspension were collected. With the aid of a Drigalski loop, equidistant deposits of bacterial suspensions were made in a Petri dish (90 mm) containing agar medium. Afterwards, the disc diffusion technique was performed using the standard method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (2003), with modifications, where basically 20 µL of each CARE (1 mg mL^{-1}) were deposited on discs placed on the surface on the agar medium (equidistant, at four points) in contact with the bacterial suspension. Distilled water was used as a negative control. Bioassays performed three at a time, then repeated. The Petri dishes were kept in a microbiological incubator at 30 ºC for 5 days. After the incubation period, the antibacterial activity was determined by presence or absence of a halo.

2.10 Toxicity of Crude Aqueous Root Extracts Against Non-target Filamentous Fungi

The antifungal activity of CAREs from *S. americanum*, *S. subinerme* and *S. lycocarpum* was evaluated with *Trichoderma harzianum* Rifai and *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg., provided by the Phytopathology Laboratory of Embrapa Genetic Resources and Biotechnology, Brasilia, DF. The fungal species were molecularly identified by the ITS-I and ITS-II regions, being preserved under the nomenclature CEN 766-*Trichoderma harzianum* and CEN 759-*Trichoderma asperellum*, respectively—both isolated from strawberry plants (*Fragaria ananassa* Duch.).

The test was performed three at a time, and then repeated. Basically 15 mg of each CARE were added to Petri dishes (90 mm) containing 25 mL of PDA medium, being homogenized with the aid of a Drigalski loop before the medium solidified. After the medium solidified, mycelial discs (7 mm) from both fungal species were removed from the mother plates and deposited in the center of each Petri dish previously prepared with PDA medium. As a control, a mycelial disk (7 mm) of each fungus species was placed in the center of each plate with PDA medium. The plates were kept at 25±2 ºC for three days, with a photoperiod of 16-hour/day. The mycelial growth analysis was qualitatively evaluated.

2.11 Toxicity of CAREs Against Non-target Yeast

The anti-yeast activity of CAREs from *S. americanum*, *S. subinerme* and *S. lycocarpum* was investigated against *Candida maltosa* Komag, Nakase & Katsuya CA 49, obtained from a revegetated area in the municipality of Brumadinho, state of Minas Gerais (Brazil) and grown in Yeast Extract-Peptone-Dextrose (YPD) medium. The disc diffusion technique was performed according to the technique recommended by the National Committee on Laboratory Standards (NCCLS) with modifications. CAREs were obtained from a stock solution (50 mg mL^{-1}). An aliquot of 20 µL of each CARE (1 mg) was deposited on filter paper discs, placed in contact with the yeast on the medium after it solidified, in 90 mm plates, at four equidistant points. Bioassays performed in triplicate, repeated twice in time. Distilled water was used as a negative control on each plate, being deposited at one of the points on the plate.

The plates were kept at 28 °C for 24 hours in an incubator chamber with a 12-hour photoperiod. Data were assessed with presence or absence of halo.

2.12 Fractionation of Crude Aqueous Root Extracts by Solid Phase Extraction (SPE-C18)

Fractionation and pre-concentration of CAREs from *S. americanum*, *S. subinerme* and *S. lycocarpum* were performed by solid phase extraction (SPE-C18). This method allows the separation of extracts into four different eluates through four column phases (C-18) (Szewczyk et al., 2015). The first eluate (1) was obtained by vacuum filtration using ultrapure water + 0.1% trifluoroacetic acid (TFA) and (999 mL ultrapure water + 1 mL TFA). The second eluate (2) was obtained by vacuum filtration containing 75% ultrapure water + 0.1% TFA + 25% acetonitrile (749 mL ultrapure water + 250 mL acetonitrile + 1 mL TFA). The third eluate (3) was obtained by vacuum filtration containing 50% ultrapure water + 0.1% TFA + 50% acetonitrile, being (499 mL water + 500 mL acetonitrile + 1 mL TFA). Finally, the fourth eluate (4) was obtained by vacuum filtration containing 100% acetonitrile + 0.1% TFA, (999 mL acetonitrile + 1 mL TFA). After the separation process, the materials were reduced in a rotaevaporator (BUCHI Rotavapor R210) until total solvents were released, and then transferred to falcon tubes and dried in a speed vac device (CentriVap-Labconco) for 24 hours. The dehydrated material was kept away from light and heat in plastic tubes. Then, the activity of these different eluates was evaluated against *M. incognita* J_2 as described in item 2.3.

2.13 Statistical Analysis

After verifying the normality of the data, the statistical differences between the experimental groups were evaluated, using analysis of variance (ANOVA) and Tukey's test for post-hoc analysis with a significance level of 0.05. Half-maximal inhibitory concentration (LD_{50}) values were determined by non-linear regression with 95% confidence limits. All values were expressed as mean±standard error of the mean (SEM) and a p-value < 0.05 was considered statistically significant. These analyzes were performed using Graph Pad Prism 5.03 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Nematicidal Effect of CAREs From Solanum spp. Before and After Heating

After the exposure period, all CAREs paralyzed 100% of the nematodes (Figure 1A). The recovery bioassay showed that the CAREs of all *Solanum* species tested had a nematicidal effect, with nematode mortality above 70% ($p \leq 0.05$) (Figure 1B). In addition, J_2 exposed to CAREs of *S. americanum*, *S. subinerme* and *S. lycocarpum* exhibited tensioned and fully stretched bodies with the presence of vacuoles throughout the intestinal region.

Furthermore, all CAREs, heated at 50 °C for 24 hours, were able to paralyze 100% of the nematodes after 48 hours of exposure to 1 mg mL^{-1} (Figure 1C). CAREs of all species maintained nematicidal activity above 80% ($p \leq 0.0001$) (Figure 1D).

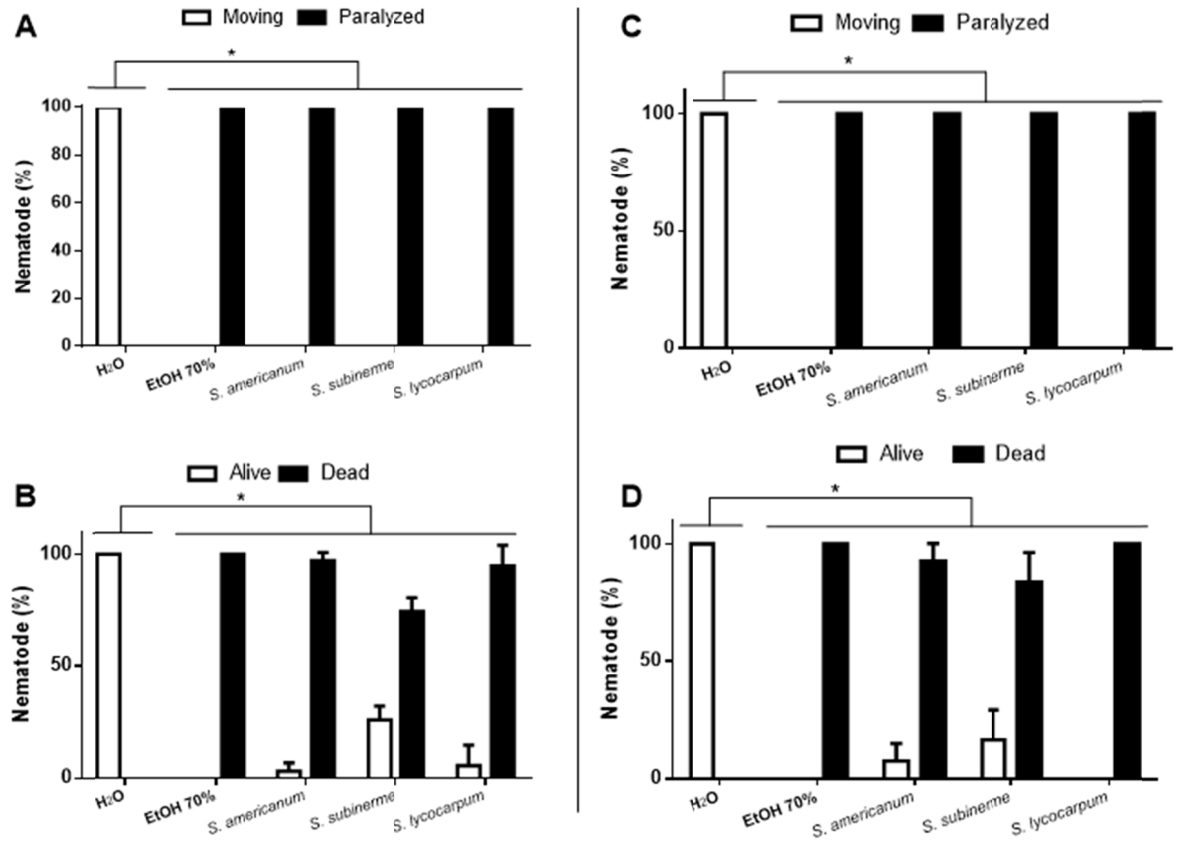

Figure 1. Effect of CAREs from *Solanum* species on J_2 *Meloidogyne incognita*. (A-B) Bioassay of J_2. Nematotoxic activity (A) and recovery bioassay (B) after exposure of J_2 to CAREs for 48 hours at 1 mg mL^{-1}. (C-D) Thermostability test: CAREs heated to 50 °C for 24 hours. Nematotoxic activity (C) and bioassay of recovery (D) of J_2 exposed to CARE heated for 48 hours at 1 mg mL^{-1}. Two-factor ANOVA with Tukey's post-hoc test. There is a significant difference when compared to the H_2O negative control: (*: $p \leq 0.0001$)

3.2 Inhibition of Hatching of Juveniles of Meloidogyne incognita Exposed to CAREs of Solanum americanum, S. subinerme and S. lycocarpum

The effect of CAREs of *Solanum* species on the hatching of *M. incognita* juveniles was observed after 7 and 15 days of exposure to 3 mg mL^{-1} (for *S. americanum*) and 2 mg mL^{-1} (for *S. subinerme*) under identical conditions. A high rate of inhibited hatching-approximately 99%-was observed ($p \leq 0.0001$) when compared to the negative control (Figure 2A). The positive control (0.06% NaOCl) prevented hatching as expected. No juveniles of *M. incognita* were detected after 24, 48 and 96 hours. Figure 2B illustrates representative light microscopy images of normal egg development (A-D) and eggs after 15 days of exposure to CAREs (A1-D1). It is possible to observe that exposure to CARE leads to the presence of vacuoles and rupture of membranes (indicated by the black arrows) showing the unviability of first-stage juveniles (J1).

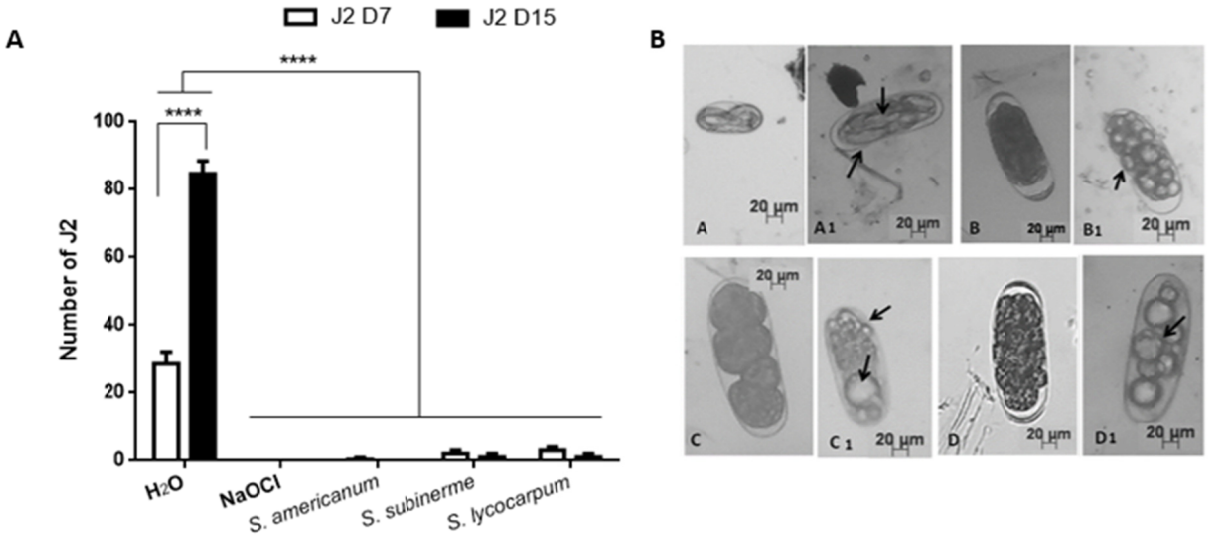

Figure 2. Effect of CAREs from *Solanum* spp. against *Meloidogyne incognita* eggs after 7 and 15 days of exposure. (A) Effect on the total number of J_2 after exposure. H_2O and NaOCl were used as negative and positive controls, respectively. (B) Micrographs of *Meloidogyne incognita* eggs before (A-D) and after (A1-D1) exposure to CAREs of *Solanum* species for 15 days. Egg with J_2 inside, before exposure (A); egg with J_2 inside, with breaking of the egg shell after exposure (A1); egg before cell division (B); egg with vacuoles after exposure (B1); egg division into four-cell stage (C); four-cell stage egg with vacuoles after exposure (C1); egg in blastula-stage (D); blastula-stage egg with vacuoles after exposure (D1). Arrows indicate eggshell rupture and presence of vacuoles, showing the impossibility of incubation after exposure. (A) Two-factor ANOVA with **** $p \leq 0.0001$ (Tukey's post hoc test)

3.3 Median Lethal Dose (LD50) of CAREs

After 48 hours of exposure to *S. lycocarpum* CARE led to 90% paralyzation of nematodes at 100 µg mL^{-1} and 100% at 250, 500 and 1000 µg mL^{-1}. In parallel, the CARE from *S. americanum* only showed activity at higher concentrations (500 and 1,000 µg mL^{-1}), also with 100% paralyzation of nematodes. Nematodes exposed to *S. americanum* CARE remained mostly alive (> 80% viability) at lower concentrations (100 and 250 µg mL^{-1}). On the other hand, CARE from *S. subinerme* led to more than 80% of nematodes paralyzed at higher concentrations (250, 500 and 1,000 µg mL^{-1}), while most nematodes remained alive after exposure to a lower concentration (100 µg mL^{-1}) (Figure 3A).

The recovery assay demonstrated nematicidal activity at 1,000 µg mL^{-1} for *S. americanum* CARE, and at 500 and 1,000 µg mL^{-1} for *S. subinerme* and *S. lycocarpum*, with more than 90% of nematodes killed ($p \leq 0.001$). Lower concentrations of CARE from *S. subinerme* and *S. lycocarpum* (100 and 250 µg mL^{-1}) and from *S. americanum* (100 µg mL^{-1}) had a nematostatic effect in which more than 80% of the nematodes regained their motility (Figure 3B). According to the dose-response curve, the LD_{50} of the CAREs of *S. americanum*, *S. subinerme* and S. *lycocarpum* are 715.9, 387.1 and 336.6 µg/mL, respectively.

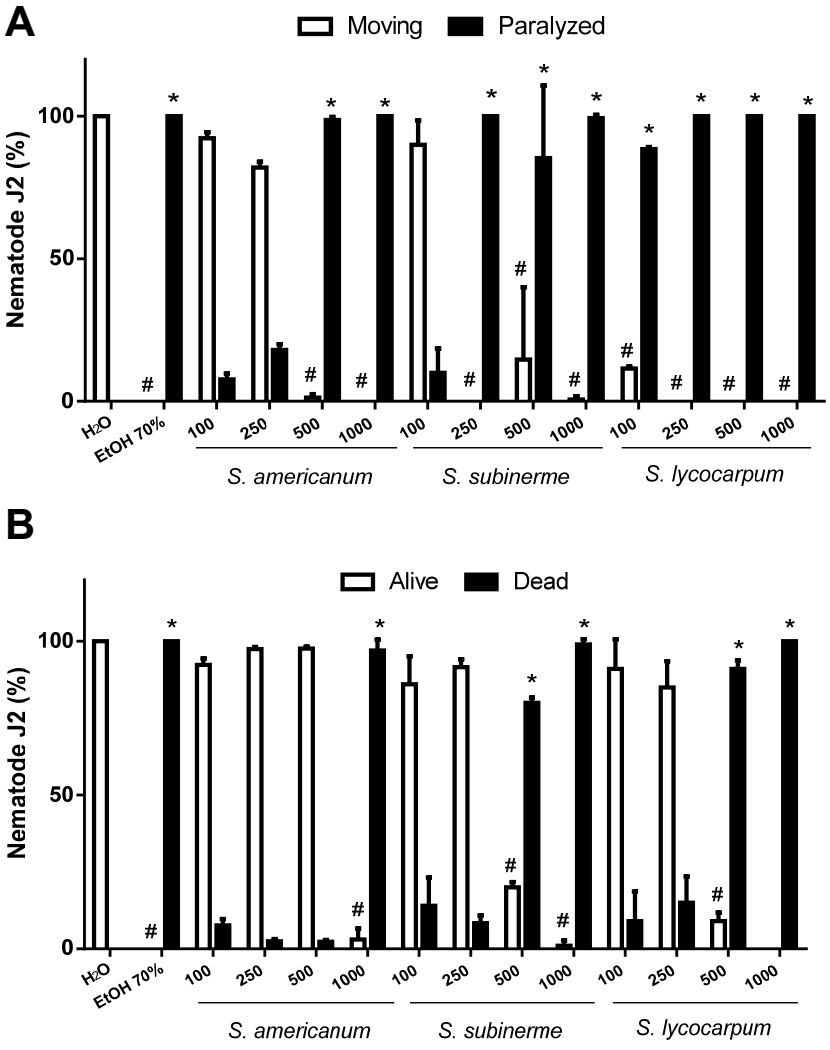

Figure 3. Determination of the median lethal dose (LD_{50}) of CAREs. (A) J_2 Bioassay of *Meloidogyne incognita* incubated with CAREs of *Solanum* spp. at concentrations of 100, 250, 500 and 1,000 µg mL^{-1} for 48 hours. (B) Recovery test after washing process and 24 hours in dH_2O. Two-way ANOVA**,** with test Tukey post-hoc test. Significant differences between # experimental groups (live) vs H_2O control group (live) and* experimental groups (dead) vs H_2O control group (dead): $p \leq 0.0001$

3.4 CARE Evaluation in a Greenhouse

The CAREs of *S. subinerme* and *S. lycocarpum* demonstrated dose-dependent activity with a significant reduction in the number of eggs layed (NEM), number of galls (NG), reproduction factor (RF) and number of eggs±juveniles (J_2) per gram of root (NEJ_2/GR) when compared to the negative control (dH_2O) ($p \leq 0.001$) (Table 1). The highest concentration tested for CAREs (3 mg mL^{-1}) led to an effect similar to the positive control using the commercial nematicide Aldicarb.

In addition, CARE-treated plants of *Solanum* species did not show significant change in stem length when compared to the negative control (dH_2O) or uninoculated control plants, having not increased the phytotoxic effect. The positive control led to a significant increase in stem length after exposure time ($p \leq 0.01$) (Table 1).

Table 1. Effect of CAREs from *Solanum* spp. on tobacco (*Nicotiana tabacum* cv. Xanthi) infected with *Meloidogyne incognita* after 30 days of exposure under greenhouse conditions. Evaluation of mean stem length (Stem length), number of egg masses (NEM), number of galls (NG), reproduction factor (RF) and number of eggs + second stage juveniles (J_2) per gram of root (NEJ_2/GR)

Treatments		Stem length CM	NEM	NG	RF	NEJ_2/GR
Control (nematode free)		13.1±8.6	0	0	0	0
Positive control (Aldicarb)		21.5±2.7**	0±0****	0±0 ****	0.01±0.01****	2.66±2.30**
Negative control (dH_2O)		11.9±0.7	149.0±0	120.0±0	2.41±0.42	645.88±158.23
S. subinerme	0.5 mg mL^{-1}	11.9±5.8	226.6±40.5*	184.3±40.2*	6.72±3.69	1755.8±849.3
	1 mg mL^{-1}	11.5±7.0	183.6±52.2	161.0±26.6	3.95±2.54	1276.8±960.8
	2 mg mL^{-1}	9.2±3.9	61.6±39.1*	58.0±36.8	0.70±0.04***	235.14±77.14
S. lycocarpum	1 mg mL^{-1}	16.9±3.9	153.3±16.5	143.3±24.6	3.37±0.71	697.20±262.5
	2 mg mL^{-1}	13.7±4.8	14.0±2.0***	13.3±1.1***	0.25±0.10 ****	48.45±15.56 **
	3mg mL^{-1}	16,0±7.2	0.3±0.5****	0.3± 0.5****	0.01±0.00 ****	2.36±2.37 **

Note. One-way ANOVA- Significant differences between experimental groups vs negative control (dH_2O) for each parameter (NEM, NG, RF and NEJ_2/GR) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and ** ** $p \leq 0.0001$ (Tukey post hoc test).

3.5 In vitro Phytotoxicity Assessment

The CAREs of *S. americanum*, *S. subinerme* and *S. lycocarpum*, which showed high activity against nematodes, did not lead to changes in soybean seedling weight (fresh root weight, dryroot weight and total freshand dried seedling weight) after 12 days of exposure (Table 2). A trend towards increased length from the base of the stem to the apex (N-A-M) was observed after exposure to all CAREs, but no significant differences were reported.

Table 2. Phytotoxicity assessment on soybean seedlings after 12 days of exposure to crude aqueous root extracts of *Solanum* spp.

Treatments	N-A-M (mm)	FW/Total (g)	DW/Total (g)	FW/Root (g)	DW/Root (g)
Negative control (dH_2O)	58.1±29.0	1.28±0.23	0.20±0.01	0.54±0.08	0.05±0.00
S. americanum	114.69±31.4	1.67±0.62	0.20±0.04	0.59±0.38	0.06±0.03
S. subinerme	117.5±61.3	1.81±0.58	0.22±0.58	0.72±0.19	0.07±0.02
S. lycocarpum	129.02±30.7	1.62±0.32	0.20±0.02	0.59±0.11	0.07±0.01

Note. N-A-M: Length from neck to apex, FW: fresh-weight of seedlings, DW: dry-weight of seedlings. One-way ANOVA-Tukey post hoc test. No significant difference observed.

3.6 Assessment of CAREs Activity Against Non-target Organisms

The *in vitro* bioassay of CAREs from three *Solanum* species against non-target organisms was performed on three bacterial species, two fungal species and one yeast species. After the exposure period, no halo of growth inhibition around the discs containing 1 mg of CAREs from *S. americanum*, *S. subinerme* and *S. lycocarpum* was observed against bacterial and yeast species (Table 3). Furthermore, no growth changes in fungi were observed after incubation in all CAREs at 15 mg/25 mL^{-1} (Table 3).

Table 3. Summary table of *in vitro* assays of non-target organisms against bacteria, fungi and yeast exposed to CAREs from *Solanum americanum*, *S. subinerme* and *S. lycocarpum*

Group	*Microorganism*	CAREs Concentrations	Activity
Bacteria	*Bradyrhizobium diazoefficiaens* *Bradyrhizobium japonicum* *Azospirillum AbV5* *Azospirillum AbV6* *Bacillus* [2538 Ba]*	1 mg 20 μL^{-1}	None-normal growth
Fungi	*Trichoderma harzianum* *Trichoderma asperellum*	15 mg 25 mL^{-1}	None-normal growth
Yeast	*Candida maltosa* CA 49	1 mg 20 μL^{-1}	None-normal growth

Note. * Only treated with CARE from *Solanum lycocarpum*.

3.7 Effect of SPE-C18 Eluates on Juveniles of Meloidogyne incognita

According to the results of the *in vitro* bioassay, the SPE-C18 eluates were able to paralyze 100% of J_2 after 48 hours of exposure.

The recovery bioassay showed that the most effective eluates were those of *S. americanum* and *S. subinerme*, with nematicidal activity of 86 and 66% ($p < 0.001$), respectively, and eluate 1 of *S. lycocarpum*, with a 96% ($p < 0.0001$) mortality rate for J_2 when compared to the negative control (Table 4).

Table 4. Summary of the *in vitro* bioassay and recovery assay with different eluates of CAREs from *Solanum* spp. obtained from SPE-C18 on juveniles of *Meloidogyne incognita*

Treatments		Bioassay (%)		Recovery assay (%)	
		Moving	**Paralyzed**	**Alive**	**Dead**
Negative control (dH_2O)		100.0±0.0	0.0±0.0	100.0±0.0	0.0±0.0
DMSO		100.0±0.0	0.0±0.0	100.0±0.0	0.0±0.0
Positive control (EtOH 70%)		0.0±0.0****	100.0±0.0****	0.0±0.0	100.0±0.0***
S. americanum	**Eluate 1**	0.0±0.0****	100.0±0.0****	32.6±24.8**	67.3±24.8***
	Eluate 2	0.0±0.0****	100.0±0.0****	13.6±2.0***	86.6±2.3***
	Eluate 3	0.0±0.0****	100.0±0.0****	69.3±14.5	30.6±14.5
S. subinerme	**Eluate 1**	0.0±0.0****	100.0±0.0****	56.6±11.0*	45.3±11.0*
	Eluate 2	0.0±0.0****	100.0±0.0****	34.0±24.8***	66.0±24.8***
	Eluate 3	0.0±0.0****	100.0±0.0****	54.0±17.6*	46.0±17.7*
S. lycocarpum	**Eluate 1**	0.0±0.0****	100.0±0.0****	3.6±2.0***	96.3±2.0***
	Eluate 2	0.0±0.0****	100.0±0.0****	20.3±18.8***	79.6±18.8***
	Eluate 3	0.0±0.0****	100.0±0.0****	50.0±24.9*	50.0±24.9*

Note. Two-way ANOVA-Tukey post-hoc test. Experimental groups vs negative control (dH_2O) for each column (moving/paralyzed/alive/dead): *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$.

4. Discussion

The use of natural products has been increasingly investigated in agriculture thanks to a great range of biodiversity, and the still, little known secondary metabolites it encompasses; some which may have nematicidal activity. In this work, CAREs from three *Solanum* species demonstrated dose-dependent toxicity on juveniles of *M. incognita* (J_2) with nematicidal effect after exposure to higher concentrations, and a nematostatic effect after exposure to lower concentrations. The nematicidal and/or nematostatic activity may be directly related to the high concentration of an active compound, or synergism between different secondary metabolites present in each CARE. The time and dose dependent nematicidal activity of aqueous extracts of mixed plant leaves, roots and parts of *Datura stramonium*, *D. innoxia* and *D. talula* L. on J_2 of *M. incognita* was reported by Babaali et al. (2017). According to the *in vitro* bioassays, the LD_{50} of these aqueous extracts ranged between 75.1 and 486.8 mg mL^{-1} (Babaali et al., 2017) which is much higher than the results obtained in the present study, with LD_{50} of 715.9, 387.1 and 336.6 µg mL^{-1} for CARE of *S. americanum*, *S. subinerme* and *S. lycocarpum*, respectively, after 48 hours of exposure.

Additionally, under *in vitro* conditions, the aqueous extract of *Phyllanthus amarus* Schum & Thonn leaves showed nematicidal activity against *M. incognita* J_2 at 5,000 ppm (corresponding to about 5 mg mL^{-1}) with a mortality rate greater than 90 % and LD_{50} of 2,084.49 ppm (corresponding to about 2,084 mg mL^{-1}) (Khan et al., 2019), superior to the LD_{50} of the CAREs tested in this study. *In vitro* bioassays by Caboni et al. (2013) demonstrated the nematicidal effect of the aqueous extract of *Mentha* × *piperita* L., *M. spicata* L. and *M. pulegium* L. against *M. incognita* with an LD_{50} of 1,005, 745 and 300 μg mL^{-1} after 72 hours of exposure. Taken together, the data suggest that our extracts have great nematicidal potential with a lower LD_{50} than other aqueous plant extracts.

The thermostability of the plant extract is an important physical characteristic rarely reported in studies investigating nematicidal compounds. Here, we observed that all CAREs maintained nematicidal activity greater than 80% after heating at 50 °C for 24 hours, suggesting stability of the active compounds present in these extracts. Rocha et al. (2017), also reported thermostability of the aqueous extract of *Canavalia ensiformis* seeds under the same heating conditions, maintaining a nematicidal effect of 96% of J_2 mortality. It is important to emphasize that we tested at temperatures higher than the average soil temperature as measured worldwide. Hu and Feng (2003) reported that the average soil temperature in the United States ranged from 7.85 to 23.85 °C. In Brazil, the average soil temperature reached higher levels of 31.02 and 30.85 °C at 0.02 and 0.06 m depth, respectively, in the Caatinga dry forest (Dantas et al., 2019), and 42.6 °C at 0.05 m depth in São Gabriel (Bahia, Brazil) (de Farias et al., 2017). The thermostability of CAREs represents an essential property for storage and applications in the control of plant parasitic nematodes.

In addition to J_2, egg masses and hatching processes are important targets for nematode control in crops worldwide (Das et al., 2021). Extracts obtained from *S. americanum*, *S. subinerme* and *S. lycocarpum* were effective in inhibiting the hatching of *M. incognita* eggs (99%) after the exposure period of the 72 hours. The effect of plant extracts on the incubation process of *M. incognita* has been widely reported. Aqueous extracts from the leaves of *Azadirachta indica* A. Juss. (neem), *Chromolaena odorata* (L.) RM King & H. Rob., *Nicotiana tabacum* L., *Carica papaya* L., *Cannabis sativa* L., *Cassia alata* L. and *Vernonia amygdalina* Coll., have demonstrated significant inhibition (60%) of the hatching process (Adgebite, 2011). Furthermore, aqueous extracts of *A. indica* leaves, bark, flowers and seeds were able to completely inhibit (100%) the hatching process (Nile et al., 2018). Inhibition of J_2 hatching was also observed with other plants of the Solanaceae family, such as *Hyoscyamus niger* L. against *M. incognita* and *M. javanica* (Treub, 1885) Chitwood 1949 (Kepenekci & Saglam, 2015). In addition, greenhouse experiments showed a significant dose-dependent reduction in the number of *M. incognita* laid (> 85%) and in the number of galls (85%) on tobacco roots after J_2 exposure to CAREs from *S. subinerme* and *S. lycocarpum*, suggesting that the presence of CAREs in the soil affected the ability of J_2 to infect plant roots *in vivo*. This effect was also reported by Nile et al. (2018) with a reduction of up to 80% in the number of galls, and 89% in the number of egg masses, after exposure of tomato plants infected with juveniles of *M. incognita* to aqueous extracts of leaves, bark, flowers and *A. indica* seeds (Nile et al., 2018).

The use of plants of the Solanaceae family as biofumigants also led to an effective reduction (> 70%) in the number of galls on potato plants infected with *M. incognita* J_2 (Sari et al., 2018). Several other studies using extracts from different plant species, such as papaya leaves and seeds (*Carica papaya*), garlic cloves (*Allium sativum* L.), mint leaf extracts (*Mentha piperita*), menthol (*Ageratum conyzoides* L.), bitter melon (*Mormodica charantia* L.), rosemary (*Rosmarinus officinalis* L.), oregano (*Origanum vulgare* L.), showed satisfactory results on the control of PPNs in the greenhouse, demonstrating the great potential of plant extracts (Dias et al., 2000; Gardiano et al., 2011; Martins and Santos, 2016). Furthermore, the decrease in the number of egg masses (NEM) and galls (NG) is directly associated with a lower reproduction factor (RF) of nematodes. CARE from *S. lycocarpum* showed a greater decrease in NEM, NG and RF when compared to *S. subinerme* extract at the same concentration. The results so far suggest that *S. lycocarpum* represents the best candidate for nematode control among the species evaluated in the present study.

Furthermore, the CAREs that showed nematicidal activity did not induce phytotoxicity in soybean seedlings after 12 days of incubation, representing a satisfactory result that points to the potential of its use in the development of nematotoxic products. Furthermore, it was shown that the CAREs of *S. subinerme* and *S. lycocarpum* led to a trend of increasing length from the base of the stem to the apex of plants, having measured twice the size of the negative control group. This phenomenon may be associated with the presence of important classes of phytohormones that induce growth, such as gibberellins, auxins, cytokinins and ethylene (Taiz & Zeiger, 2004). It is noteworthy that the CAREs evaluated in this study did not inhibit the growth of non-target microorganisms, such as nitrogen-fixing bacteria *A. brasilense* (AbV5 and AbV6), *B. japonicum* and *B. diazoefficiens* that are widely used in commodities of great economic importance such as maize and soybeans (Reis Júnior, 2008; Hungary, 2011; Lucca Braccini et al., 2016; Quadros et al., 2020; Bonatelli et al., 2021). These are nitrogen-fixing bacteria that act in symbiosis with

plant roots (Quadros et al., 2020; Bonatelli et al., 2021). Likewise, no change in the growth of *T. harzianum* and *T. asperellum*, filamentous fungi used as biological control agents (Newman, 2003; Caboni et al., 2013; Newman & Cragg, 2016) was observed after exposure to CAREs. These results suggest that the CAREs evaluated in this study had important nematicidal effects and were specific for nematodes without interfering with beneficial microorganisms present in the environment.

Finally, the nematicidal activity of the CAREs of the three solanaceous species herein evaluated may be related to the presence of secondary metabolites of different chemical classes such as alkaloids, tannins, terpenes, triterpenes, flavonoids, coumarins, among other compounds previously mentioned in the literature for plants of the family Solanaceae (Pereira et al., 2016). In this context, the technique called solid phase extraction (SPE) has been used to conduct molecular investigation of CAREs. This technique eliminated a large part of the problems found in liquid-liquid extraction (Barrionuevo & Lanças, 2001) and also reduced the steps in the handling of samples, facilitating the analysis of compounds present in the extracts (Zygmunt & Namiésnik, 2003). Each eluate of CAREs from *S. americanum*, *S. subinerme* and *S. lycocarpum* showed different nematotoxic effects against *M. incognita* J_2, suggesting an efficient separation of classes of biocompounds**,** with distinct biological activity. Despite the need for additional chromatographic purification steps, as well as the identification analysis of compounds, the results obtained here clearly create opportunities for the development of new nematotoxic products based on green technology. We emphasize the need to continue the process of identifying these compounds, as well as conducting *in vitro* and *in vivo* tests and studies to reveal the synergism between molecules that enhance the nematicidal effect.

In conclusion, crude aqueous root extracts of the three species of *Solanum* tested showed nematotoxic activity against juveniles of *M. incognita*. The crude aqueous root extract of *S. lycocarpum* root showed the highest nematicidal activity among the *Solanum* species tested in this study, without showing toxicity against bacteria, fungi and yeasts and non-phytotoxic effect for soybean. Our results indicate that plants of the Solanaceae family, and especially *Solanum lycocarpum*, have the potential to be used as a new sustainable biotechnology resource for products that aim to prevent crop infection by nematodes, and that are safe for human health and environmentally correct. Other investigations for the prospecting and identification of nematotoxic compounds of these *Solanum* species are in progress.

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