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Assessment of the *Tephrosia toxicaria* essential oil on hatching and mortality of eggs and second-stage juvenile (J_2) root-knot nematode (*Meloidogyne enterolobii* and *M. javanica*)

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Abstract

The search for alternative biocontrol methods is necessary. Several molecules with nematicidal effect have already been identified from plant tissues. They can be considered as important alternative for nematode control. Therefore, the objective of this study was to assess the effect of essential oil of *Tephrosia toxicaria* on hatching and mortality of second-stage juvenile (J_2) root-knot nematode (RKN) and eggs of *Meloidogyne enterolobii* and *M. javanica*. For this purpose, 50 eggs/J_2 were incubated with the essential oil of *T. toxicaria* at seven concentrations (0.0, 50, 100, 200, 400, 600 and 800 µl.mL^{-1}) with six replicates in 48-well acrylic plates. The number of eggs and juveniles were counted with the aid of camera under a stereoscopic microscope. The analysis of the J_2 hatch was performed using the area below the hatching progress curve (ABHPC), calculated by the equation proposed by Campbell and Madden. The J_2 mortality rate was carried out concomitantly with the hatching observations, 48 hours and also 16 days after applying the treatments. In the evaluations, all the motionless J_2 were counted after 48 hours. To confirm the occurrence of mortality, the specimens were transferred to water, then examined in slides, under an optical microscope at a 40x magnification. The data were submitted to analysis of variance using the F-test and the averages were compared by the Skott-Knott test ($p \leq 0.01$). We confirmed that the essential oil of *T. toxicaria* has effectiveness to reduce juvenile hatching and cause J_2 mortality in all the assessed concentrations, demonstrating the nematicidal potential of the active principles present in *T. toxicaria.* The results of this study show that the *T. toxicaria* essential oil and its constituents can serve as an environmentally safe and a promising nematicide agent in the control of *Meloidogyne spp*.

Keywords: Sustainability, Root-knot nematode (RKN), Organic agriculture, Bionematicide, Bioassay, Timbó.
Abbreviations: ABHPC_area below the hatching progress curve; DMSO_dimethylthiosulphate; J_2_ juveniles of second-stage; RKN_root-knot nematodes.

Introduction

Root-knot nematodes (RKN) (Meloidogyne: Goeldi, 1887) (Tylenchida: Meloidogynidae), is currently represented by over 100 described species, infect more than 2,000 plant species worldwide and constitute a relatively small group. However, they are important plant pathogens, highly adapted to the most distinct environments such as tropical, subtropical and temperate zones (Ferraz and Brown, 2016). Due to their sedentary endoparasite way of infection, the root-knot nematodes gall modifies the plant physiology, interfering directly in the water and nutrients absorption and translocation, reducing the plant production and its quality, in order to live and feed themselves (Ferraz and Brown, 2016).

The infection occurs when second stage juveniles (J_2) of the root-knot nematodes come in contact with the root of the plant, attracted by the root exudates. They often penetrate immediately. After penetration, the J_2 migrates inter-cellularly to the cortex in the cell differentiation region. Susceptible plants react to J_2 feeding and undergo into pronounced morphological and physiological changes. In the feeding sites of these nematodes, the giant or nourishing cells are formed in the adjacent phloem or parenchyma. At the same time, the tissues around the nematode undergo hyperplasia and hypertrophy, resulting in root gall, typically

associated to *Meloidogyne spp*. infection (Ferraz and Brown, 2016).

One of the most used methods for the nematode control is the application of chemicals on the soil or seeds treatment. However, these products are ineffective in nematode control because they present high costs and can leave residues, jeopardizing food, human health and the environment (Oka et al., 2000). In this sense, control actions such as the use of resistant cultivars is the most effective and least expensive alternative to producers, however, there is no availability of resistant genotypes for most crops in the Brazilian market. In the meantime, efforts have been concentrated on integrating alternative actions aimed to greener agriculture through natural agricultural defenses against phytopathogens.

Many natural pesticides can be obtained from plants (Chitwood, 2002). These compounds can be derived from metabolic pathways that produce secondary compounds synthesized by plants with phytoprotective activity, pollinator attraction and environmental adaptation (Taiz and Zeigher, 2009).

The use of essential oils has been reported in the literature as a promising alternative in the nematodes control (Oka et al., 2000). There are many studies that demonstrate the efficiency of essential oils such as the following: *Eucalyptus camaldulensis*, *E. saligma* and *E. urophylla* on mortality and hatching of *Meloidogyne exigua* J_2 juveniles (Salgado et al., 2003), the essential oil of *Eucaliptus sp*. on J_2 *M. incognita* (Ibrahim et al., 2006), the effect of the essential oils of *Lippia sidoides*, *Ocimum gratissimum*, *Cymbopogon winterianus*, *Cymbopogon citratus*, *Lippia alba* and *Eucalyptus terenticornis* on the hatch of the second stage juveniles (J_2) of *M. incognita* race 2 (Moreira et al., 2009), the essential oil of *Kadsura heteroclite* against *M. incognita* (Li et al., 2011), the essential oil of *L. alba* in the control of *M. incognita* race 1 (Marino et al., 2012), the essential oil of *Vigna radiata* in the control of gall nematodes, *Meloidogyne* sp., (Wani and Bhat, 2012), *Myrtus communis* essential oil on the mortality of J_2 of *M. incognita* (Ardakani et al., 2013), the effect of the essential oil of *Rosmarinus officinalis* in the control of the *M. javanica* (Mattei et al., 2014), the effect of the essential oil of *L. alba* (Verbenaceae) on hatching and mortality of juveniles of *M. incognita* (Gonçalves et al., 2016), the rhizome essential oil *Alpinia galanga* on hatching and mortality of J_2 of *M. hapla* (Nematoda: Tylenchida) (Jeon et al., 2016).

It has been observed that the constituent compounds of the essential oils can act directly, interfering in the hatching and mortality of the nematodes in the soil (Bosenbecker, 2006), or, if they are absorbed by the plants, they may modify the composition the root exudates, interfering in the location of the roots by the nematodes, making them less attractive to the pathogen. I also may change the plant physiology, preventing the formation of special feeding cells, or even activating mechanisms of induced resistance in the plant (Schwan-Estrada et al., 2003).

Several benefits may result from identifying the phytochemicals involved in these interactions. These compounds can be directly used as nematicides or may serve as a model for synthetic nematicides development, generating more efficient and possibly less toxic products (Chitwood, 2002).

In this context, *Tephrosia* is presented as an extensive genus distributed in tropical and subtropical regions of the world, with about 680 species being reported, more than 350 just in South America. From these, 62 species were investigated chemically, from which chalcones, flavanones, flavones, flavanoids, pterocarpans, isoflavones, rotenoids, biflavanones (Vasconcelos, 2010; Touqeer et al., 2013), steroids were isolated among others. Many of the isolated metabolites have biological and /or pharmacological activities such as antitumor, antiviral, insecticide, antimicrobial and ichthyotoxic (Al-Hazimi et al., 2006; Touqeer et al., 2013).

The *Tephrosia toxicaria* (Papilionaceae) is popularly known as timbó and anil bravo. They are herbaceous plants, sub-shrub or shrub, under xerophytic conditions with roots modified in a well-developed xylopodium. That has leaves imparipinnates with opposite, linear or elliptical leaflets with parallel and numerous lateral veins; terminal or axillary inflorescence, arranged in axillary or terminal racemes. Its fruits are pods, varying from five to seven centimeters in length, with 10-12 seeds, curved or straight, fertile and with small seeds (Leitão Filho, 2009). The whole plant is ichthyotoxic, especially the roots and seeds.

The plants of this genus have been used in herbal preparations, insecticides, poison to rats and fish by several indigenous peoples. They have low toxicity in mammals and the value of its LD_{50} in rats is 132 mg/kg (Yu, 2008; Vasconcelos, 2010; Silva, 2013). The phytochemical study of the Tephrosia genus started in 1907 on the species *T. vogelli*, in which rothenoid tephrosin was isolated and ichthyotoxic activity (associated with the ability to paralyze fish) was observed, a widespread activity in artisanal fisheries in dams at northeastern Brazil (Lima, 2010).

The objective of this bioassay was to evaluate the nematicidal effect of the *in vitro* essential oil of *Tephrosia toxicaria,* at seven concentrations, on egg hatching and mortality of juvenile J_2 in root-knot nematodes (*M. enterolobii* and *M. javanica*).

Results and Discussion

Table 1 presents the summarized results of the mean squares and variance analysis coefficients (ANOVA) for egg hatching (%), area below the hatching progression curve (AACPE), and J_2 mortality (%) data. According to the analysis of these results, it can be verified that all the treatments were significant ($P \leq 0.01$), both for the main effects and for the interaction, except for hatching when the nematode species were evaluated.

Lima (2010) observed the predominant presence of sesquiterpenes in the leaves and stems of *T. egregia*. In addition, they observed monoterpenes and *trisnor*-sesquiterpenes geijereno and pregeijereno and β-caryophyllene as major constituents in the essential oils of both plant parts, besides *trisnor* -squickiterpene dictamnol (Arriaga et al., 2005). In *T. toxicaria* Ribeiro et al. (2006) reported β-caryophyllene, germacreno D, as major constituents, in addition to α-humulene and bicyclogermacrene. Arriaga et al. (2008) reported that the most important constituents in *T. cinerea* are β-cariophilene, germacrene D, caryophyllene, humulene epoxide II, which are species with the characterized composition.

Table 1. Summary of analysis of variance with mean squares and coefficients of variation (CV) for egg hatching (%), ACCPE and mortality (%) of juvenile of second stage (J_2) of *M. enterolobii* and *M. javanica*, in function of the application of seven concentrations of *T. toxicaria* essential oil. IFCE - Campus Sobral, Sobral-CE, Brazil, 2016.

Source of Variance	DL	Mean Squares		
		Hatching	ACCPE*	Mortality
Nematodes (A)	1	0.42857^{ns}	2242.33333**	58.33333**
Concentrations (B)	6	13025.19048**	9891.19048**	13300,07937**
Interaction (A x B)	13	0.42857**	2012.7353**	41.66667**
Residue	70	-	-	-
CV (%)	-	6.70	4.56	3.19

*Area below the hatch progress curve; ^{ns} non-significant value; (nematodes) ** Significant value an 1,0% by the F test. (Concentrations of extracts) ** significant value for the 4th degree polynomial regression.

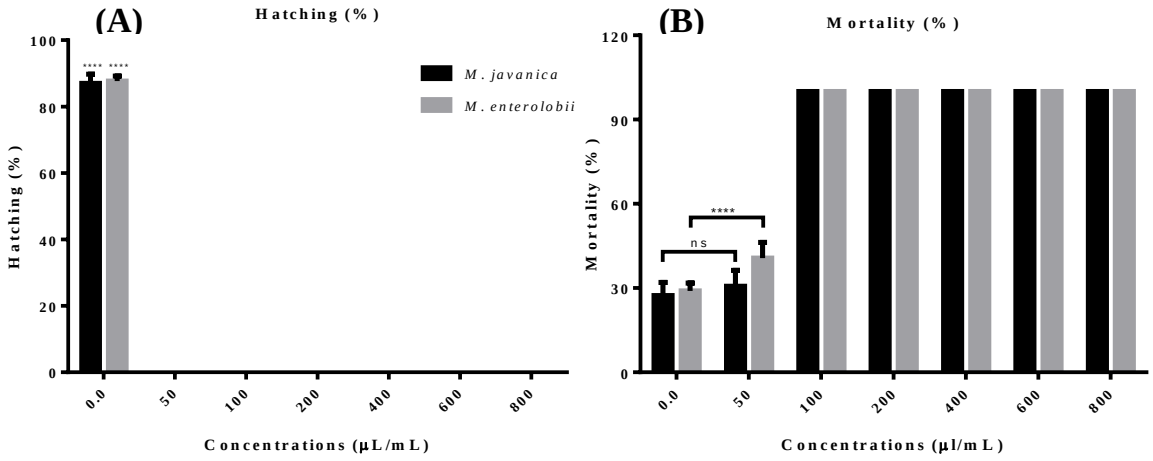

Fig 1. Mean of hatchability of eggs (%) - A and mortality (%) - B of juvenile of second stage (J_2) of *M. enterolobii* and *M. javanica*, as a function of the application of seven concentrations of *T. toxicaria* essential oil. IFCE - Campus Sobral, Sobral-CE, Brazil, 2016.

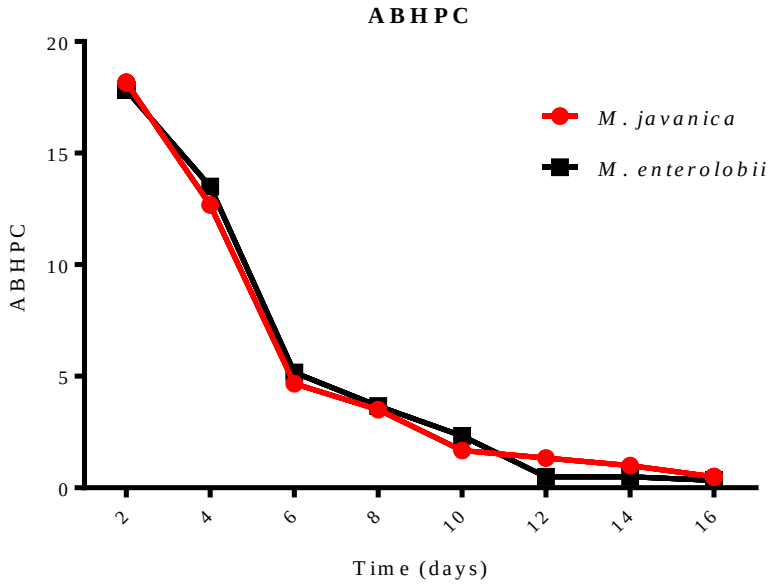

Fig 2. Progress curves of juvenile hatching of second stage (J_2) of *M. enterolobii* and *M. javanica*, as a function of the application of seven concentrations of *T. toxicaria* essential oil, during 16 days.

On the other hand, this result differs from Lima (2010), which did not observe a nematicidal effect of the essential oil of leaves and stems of *T. egregia.* In this species, there is predominance of sesquiterpenes, besides monoterpenes and *trisnor*-sesquiterpenes geijereno and pregeijereno as major constituents, in addition to *trisnor*-sesquiterpene dictamnol. Thus, this result is probably due to the constituents present in *T. toxicaria* which are β-caryophyllene and germacrene D, α-humulene and bicyclogermacrene (Ribeiro et al., 2006).

These results are in accordance with those observed by Yu (2008), who studied the toxicology and biochemistry of botanical insecticides, reporting that plants of this genus have been used as insecticide, rat and fish poison due to the high concentration of rotenone.

The Figure 1 shows the mean hatch percentage (%) - A and mortality (%) - B of J_2 of *M. enterolobii* and *M. javanica*, as a function of the application of seven concentrations of essential oil of *T. toxicaria*.

It is verified that the hatching of J_2 *M. enterolobii* and *M. javanica* were occurred more accentuated in the first eight days of egg incubation that possibly already contained the developed juvenile. From the eighth day, there was a reduction in hatching until a total inhibition on day 12th for the *M. enterolobii* and at day 14th for the *M. javanica*. It is possibly because of the toxic effect of *T. toxicaria* essential oil which is more harmful to the embryonic development than to the already formed juvenile.

It is known that eggs with cells and embryos at various development stages are commonly found in the egg masses and possibly in the eggs suspension extracted from these masses, causing great variation in hatching over time (Salgado and Campos, 2003).

Among the fixed constituents reported for *Tephrosia* species, the predominance of flavonoid compounds was observed. Many flavonoids have biological activities, such as: antioxidant, antiprotozoal, cytotoxic, hepatoprotective, antimicrobial, antifungal, among others (Ganapaty et al., 2009; Amou et al., 2009; Touqeer et al., 2013).

Following the phytochemical study of the genus Tephrosia species, the authors reported the chemical composition and larvicidal activity of *T. toxicaria*, and the flavonoids obovatine, 6a, 12a-dehydro-α-toxicarol and α-toxicarol were isolated, being tested against *A. aegypti* larvae (Lima, 2010; Vasconcelos, 2010). The abundance of phenolic substances of the flavonoid type in *T. toxicaria* may justify the nematicidal activity.

The study carried out by Ardakani et al. (2013), analyzed the effects of *Myrtus communis* essential oil on the mortality of *Meloidogyne incognita.* The authors observed the presence of thirty-eight major compounds identified in the essential oil of *M. communis*. The major chemical components found were 1,8-cineol, α-pinene, linalool, α-terpineol, linalyl acetate. This is responsible for the inhibitory activity of hatching and mortality of *M. incognita* juveniles.

Gonçalves et al. (2016) analyzed the activity of the *Lippia alba* (Verbenaceae) essential oil on *Meloidogyne sp.*, they verified that this oil contains compounds with significant effects on the mortality of juveniles of second stage of *M. incognita*. The three *L. alba* essential oil chemotypes show a similar effect at the concentration of 1,000 ppm, causing mortality above 99% of *M. incognita* juveniles (J_2). In the present study, it was possible to observe the presence of the compounds myrcene, linalool and carvone, as a result of the proven nematicidal activity of this plant species, which have been investigated in previous studies (Moreira et al., 2009; Ntalli et al., 2011).

Essential oils obtained from several plants have demonstrated formidable potential as nematicidal bioactive sources. Most of these plants are herbs and spices that contain nematicides such as the compounds carvacrol and thymol (Oka et al., 2000). The substances present in the essential oils of spices such as cumin, fennel, mint, Syrian oregano and oregano showed high nematicidal activity.

Plants are known to be a source of naturally occurring pesticides, such as synthetic pyrethroids that have been developed based on structures of naturally occurring pyrethrins and are used commercially for insect control. Several compounds with nematicidal action were isolated from plants, mainly from members of the Asteraceae family. The α-terthienyl and its analogues have been isolated from *Tagetes* spp. and are considered highly effective nematicides *in vitro* and *in vivo*. Other non-volatile plant compounds, such as polyacetylenes and benzofuran derivatives, also have nematicidal activity. However, none of them have been developed yet as large-scale commercial nematicides.

In Figure 2, the mean data of the area below the hatching progression curve (ABHPC) of *M. enterolobii* and *M. javanica*, as a function of the application of seven concentrations of *Tephrosia toxicaria* essential oil, are presented.

The progress of *M. enterolobii* and *M. javanica* juvenile hatching in control treatment (water) was compared with several concentrations of essential oil. The results showed that even applying the lowest (50 µL) concentration of essential oil, the toxic effect was occurred from the first few days of inoculation. It is also observed that there was variation in hatching among the nematodes, with *M. javanica* dying faster than *M. enterolobii*. However, in both nematodes, hatching was ceased completely at 12 days after incubation. In water, the hatching was observed up to the 16th day.

Jeon et al. (2016) examined the chemical composition and nematicidal activity of some essential oils in *Meloidogyne hapla* (Nematoda: Tylenchida) under laboratory conditions and verified that rhizome essential oil of *Alpinia galanga* has a nematicidal effect against eggs and *M. hapla* (100%) juveniles, followed by Caraway (22.3%), *Eugenia caryophyllata* (9.4%), *Cinnamonum zeylanicum* (7.2%), *Mentha pulegium* (2.4%) and *Foeniculum vulgare* (2.1%). The identified constituents of *A. galanga* were methyl cinnamate, 1,8-cineol, β-pinene, α-pinene and p-cymene, and the nematicidal action was attributed to them. In comparison with other studies, there is an incongruity when confronting the chemical compositions of the essential oils of some species. However, it is known that the quantitative and chemical composition differences of extracts and essential oils of plants are attributed to the geographical origin, extraction methods, plant parts, chemo-types and the harvest conditions and times.

While Li et al. (2011) evaluated the chemical composition and toxicity of the essential oil generated from the stem of *Kadsura heteroclite* against *Sitophilus zeamais* and *Meloidogyne incognita*. The authors discovered that it has a strong nematicidal action against *M. incognita* compared to a synthetic insecticide/nematicide, Carbofuran®. The

essential oil had the same level of toxicity against *M. incognita* and showed potential to be developed as a possible natural nematicide for the control of root-knot nematodes. The δ-cadinene, δ-cadinol and calarene compounds are considered the nematicidal agents of the essential oil studied.

Batist et al. (2008) evaluated the use of Eucalyptus spp. essential oil as a natural pesticide. They verified that it has high nematicidal activity. Pandey et al. (2000) showed that the essential oil (250 ppm) of *E. citriodora* and *E. hybrida* was highly toxic to *M. incognita* and inhibited the presence of galls reducing the number of eggs. Salgado et al. (2003) reported that the essential oils of *E. camaldulensis*, *E. saligma* and *E. urophylla* caused mortality and inhibited the hatching of J_2 juveniles of *Meloidogyne exigua* in coffee, concluding that these essential oils contain nematicidal compounds. Recently, Ibrahim et al. (2006) reported that the essential oil of *Eucalyptus sp*. is toxic to the second stage juveniles (J_2) of *M. incognita*. These authors suggest that this action was due to the presence of chemical constituents β-pinene, 1,8-cineol, isopulegol, citronellal, citronellol, which have proven larvicidal and insecticidal activities. Due to the broad spectrum of biological activity, the *Eucalyptus* spp. essential oils are considered safe compounds.

Mattei et al. (2014) investigated the effect of *Rosmarinus officinalis* essential oil on the control of *Meloidogyne javanica* and *Pratylenchus brachyurus* in soybean and verified that the population of *M. javanica* was reduced by the application of *R. officinalis* essential oil. However, none of the studied concentrations affected the population of *P. brachyurus*. For these reasons, the authors conclude that rosemary essential oil was effective in reduction of the *M. javanica* population when compared to control; however, it had no effect on *P. brachyurus* reproduction. This factor can also be found among species of the same genus or physiological specie. Moreira et al. (2015) found that *M. javanica* responded better than *M. enterolobii* to the treatment with antagonistic plants, showing that it is less susceptible to the presence of compounds in neem (*Azadirachta indica*) and sorghum (*Sorghum bicolor*).

Marino et al. (2012), tested control of *M. incognita* race 1 with essential oil of *Lippia alba* and verified that it has in vitro nematicidal effect, by reducing the hatching rate of *M. incognita* race 1 and causing the juvenile stage J_2 hatched mortality. It is suggested that this action is due to the presence of the citral, geraniol, linalool and limonene constituents, with proven activity (Moreira et al., 2009; Oka et al., 2000; Echeverrigaray et al., 2010; Salgado and Campos, 2003).

The reduction of the hatch rate and high mortality of J_2 of this nematode may be associated with the chemical composition of the essential oils. Oka et al. (2000) reported that the essential oils are formed as several compounds, which can interact and act in vital processes of the nematode metabolism, in the embryonic phase, as well as in the mechanisms of movement, due to a possible disruption of the nervous system.

According to Andres et al. (2012), the compounds present in the essential oils interact with the cytoplasmic membrane may cause changes and damage to the structure of polysaccharides, lipids and phospholipids to be disrupted, promoting membrane depolarization, such as those of the mitochondria, resulting in the release of calcium ions and proteins. It is also believed that nematicidal action of the essential oils must be attributed to the presence of phenols, aldehydes and alcohols that cause the oxidation of these membranes (Bruni et al., 2004).

Ntalli et al. (2011), examined the synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven species native to Greece. They observed that active synergistic mixtures exhibit high nematicidal activity and can be used in mixtures as promising agents for the management of these phytopathogens. Further research on the dependence of interactions on terpene concentration and the relationship of terpene in mixtures, as well as on the underlying biological mechanisms of terpenes responsible for J_2 paralysis, are in progress.

The synergistic activity of trans-anethole/geraniol was the most potent combination, followed by trans-anethole/eugenol, carvacrol/eugenol and geraniol/carvacrol. Evidence of component interactions in essential oils (EO) has been observed by previous studies of the present authors. The nematicidal activity of individual terpenes measured at the same concentrations as expected in the OE by their EC_{50} value was not as high as the activity of the corresponding EO. The isolated EO of *Foeniculum vulgare*, among the components of which there is synergism, is probably an example. This can be corroborated by the EC_{50} value of the 24-hour oil, which is lower than expected, according to the amount of trans-anetol present in the essential oil (Ntalli et al., 2011).

Echeverrigaray et al. (2010) investigated nematicidal activity of monoterpenoids against the nematodes of the *Meloidogyne incognita* galls and observed that monoterpenoids are the main chemical components of the essential oils currently found with anti-hatching action and mortality against *M. incognita* and with larvicide activity. The nematicidal activity depends on the terpenoids in their reactive groups and chemical structure. Five monoterpenoids (borneol, carveol, citral, geraniol and α-terpineol) incubated individually inhibited egg-clumping and affected the mobility of J_2, indicating that the essential oils with high content of these compounds may be useful as nematicides for the natural control of *M incognita*. However, the practical, large-scale field use of these isolated terpenoids or essential oils require further studies on formulation, phytotoxicity, mode of action and application.

Moreira et al. (2009) examined the effect of six essential oils on hatching and mortality *Meloidogyne incognita* race 2, and found that all the tested essential oils are effective in the inhibition of hatching of J_2 from *M. incognita* race 2 at concentrations of 5, 0 and 10.0 ml L^{-1}, being those of rosemary pepper (*Lippia sidoides* Cham.) and citronella grass (*Cymbopogon citratus* (DC) Stapf.) were the most efficient. It is believed that the observed nematicidal action may have occurred due to the presence of substances such as thymol, cravacrol (rosemary pepper) and citronellal (citronella grass), which present antimicrobial action against well-known fungi and bacteria.

Various agricultural practices involving plant extracts and organic waste, alone or in combination, have proven to be effective against weeds, microorganisms and insect control, and some commercial natural oils are available for organic farming. The protection of plants against phytonematoids

has been obtained by the application of essential oils or organic changes of plants rich in essential oil.
The nematode-soil complex becomes an obstacle to the control of this phytopathogen, due to all the complexity offered by the microbiota and soil structure. This may interfere in the action of the bioactive compounds of the nematoid, since these compounds can be degraded by the microorganisms, adsorbed to the clay particles and volatilized or even leached during irrigations or larger rains. It is known that eugenol, one of the major constituents present in the essential oils of *Cinnamomum zeylanicume*, *Mentha x piperita* and *Syzygium aromaticum*, has nematicidal action and can be transformed into common organic acids by the action of soil microorganisms (Rabenhorst, 1996).
In general, the approaches for the sustainable management of phytopathogens have been studied in several ways. In this study the tested plant-sourced nematicides are believed to be promising tools in the control of *Meloidogyne sp*. However, the design of the targets or mechanisms of action together with the practical application forms in the field are the main challenges to be overcome. New knowledge mainly about secondary metabolites is required for the discovery of new compounds with nematicidal activity. It may play an important role in the management, and such compounds can be synthesized on a large scale. The metabolomic approach of plants that allows studying the metabolites of the plant as the final products of cellular processes is another powerful tool for this scope.
The biochemical understanding of the interaction between these semiochemicals and the gall nematodes, as well as the plant-parasite interactions, are crucial to the development of new environmental friendly and sustainable control strategies for these nematodes.

Materials and methods

This study was developed in the Phytosanitary and Seeds Laboratory of the Federal Institute of Education, Science and Technology of Ceará, IFCE - *Campus* Sobral, from April to May 2015. The Campus is located at the city of Sobral, which has a tropical climate with a dry season (Köppen-Geiger: Aw climatic classification), at an altitude of 70 meters and geographical coordinates: Latitude 03°40'58'' South and Longitude, 40°21'4'' West.

Obtaining and preparing concentrations of essential oils

The plant material was collected in the district of São José, municipality of Ibiapina-CE, in March 2015. The identification of the botanical material was carried out in the Herbarium of the Federal University of Rio Grande do Norte (HUFRN), where the exsiccated plant was incorporated under the record number UFRN022960. The collected material was taken to the Phytosanitary and Seeds Laboratory, where it was separated, the leaves were dried at room temperature for one week, and then submitted to hydrodistillation in a Clevenger® apparatus.
Due to the viscosity of the essential oil, it was necessary to solubilize it before the preparation of each sample. Therefore, equal parts of essential oil and dimethylsulphate (DMSO) were placed in a recipient, forming the standard solution after mixing. The highest concentration (800 µl.mL^{-1}, oil: water) was the first to be prepared from that mixture using distilled water as diluent, the other concentrations (0, 50, 100, 200, 400, 600 and 800 µl.mL^{-1}) were obtained by dilution in equal parts of water and aliquots of the previous concentration, according to (Moreira et al., 2009). The assay control consisted of eggs incubated in distilled water (0.0 µl.mL^{-1}).

Multiplication of the* Meloidogyne enterolobii *and* M. javanica *inoculum

The inoculum was collected from the commercial 'Paluma' guava plantations on the Acaraú Low Irrigated Perimeter in the municipality of Marco, CE, in February of 2014. This inoculum was then maintained and multiplied from individual egg masses obtained from the monospecific population of *M. enterolobii* and *M. javanica* in tomato plants (*Solanum lycopersicum* Mill.) 'Santa Clara' used for the continuous multiplication of pathogens in recipients of 26.46 Ib. The substrate was a mixture of soil and bovine manure dried in the proportion of 1: 1 (v / v), which were maintained under Agricultural Toll conditions, at the IFCE - *Campus* Sobral, at an average temperature of 28±3 ºC. To identify the studied nematode species, a technique developed by Carneiro and Almeida (2001) was used, in which the esterase pattern is used, confirming the exact and safe identification of the phytopathogens.

Extraction and quantification of the* Meloidogyne enterolobii *and* M. javanica *inoculum

The eggs of the two species of nematodes used in the assays were extracted from infested 'Santa Clara' tomato plants using the Hussey and Barker (1973) technique. In order to improve the procedure and the visualization of the extracted eggs and juveniles J_2, the obtained suspension was submitted to the method of Flotation and Centrifugation, proposed by Jenkins (1964) and complemented with kaolin (Coolen and D'Herde, 1972). The number of eggs and juveniles J_2 was counted with the aid of a Peters chamber under a stereoscopic microscope (Tihohod, 1993).

Evaluation of essential oil on J_2 hatching of Meloidogyne enterolobii and M. javanica

A total of 48-well acrylic plates with a capacity of 1.5 ml each were used as hatching chambers, including 50 eggs of *M. enterolobii* and *M. javanica* placed in 1.5 ml of essential oil solution in each concentration tested. The hatching chambers were placed in polyethylene trays and maintained in a BOD incubator through 16 days. This time is required to the formation and hatching of J_2 while they are still in the beginning of embryonic development (Campos et al., 2003; Moreira et al., 2009).
The J_2 hatch was counted using a stereoscopic magnifying glass (Miotic SMZ-161), starting 24 hours after the assay was set up and continued for 16 days after incubation, at 48-hour intervals between evaluations, totaling eight counts. In each evaluation, the number of J_2 hatched on the plates was recorded, obtaining the total quantity by the sum of the J_2 at the 16 days of observation.
The analysis of the J_2 hatching was done by means of the area below the hatching progress curve (AACPE), calculated

by the equation proposed by Campbell and Madden (1990) *apud* Salgado et al. (2003):

$$AACPE = \sum_{i=1}^{n-1}\left(\frac{Yi + Yi+1}{2}\right)\times(Ti+1 + Ti) \quad \text{Equation 1}$$

The percentage values of J_2 hatched were applied in equation (1), considering: Yi = percentage of hatching in the i^{th} evaluation; Ti = time in days in the i^{th} evaluation; n = number of evaluations.

Mortality evaluation of J_2 of Meloidogyne enterolobii and M. javanica in essential oil

The J_2 mortality assessment was performed concomitantly with hatching observations, starting 24 hours after assay setup and performed until the day 16. During the evaluation, all motionless J_2 were counted in each period of 48 hours. To confirm the occurrence of mortality, the specimens were transferred to water and then examined in slides, under an optical microscope at a 40x magnification to verify some minimal activity in the specimens. The final number of J_2 dead in the test was obtained by the sum of the eight counts until the 16^{th} day of observation.

Statistical design

The experiment was organized in a Completely Randomized Design (CRD) planned in a 2 x 7 factorial scheme with two species of gall nematodes (*M. enterolobii* and *M. javanica*) and seven oil concentrations (0, 50, 100, 200, 400, 600 and 800 $\mu l.mL^{-1}$). Each treatment consisted of six replicates consisting of 50 eggs and juveniles J_2, placed in 96 well acrylic plates (hatching chambers), totaling 300 eggs per treatment. Each well with 50 eggs or juveniles J_2 was included in experimental unit.

The data obtained in this test were submitted to verification of the variances homogeneity by the Hartlet test, according to Banzato and Kronka (2006). After the data normality were observed, analysis of variance was performed using the statistical program Assistat®, version 7.7 Beta, and the variances were compared by the F test at the level of 1.0% of probability. The comparison of means between treatments with nematodes was performed by the Skott-Knott test at the 1.0% probability level. For the treatments of essential oil concentrations, the regression analysis was used to fit the most appropriate model to describe the biological phenomenon.

Conclusion

The essential oil of *Tephrosia toxicaria* was effective on mortality of juvenile J_2 and inhibited juvenile hatching at all evaluated concentrations, demonstrating the nematicidal potential of essential oils and their compounds. The two evaluated root-knot nematodes (*M. enterolobii* and *M. javanica*) showed similar behavior in response to treatment with essential oil at different concentrations. The results of this study show that the *T. toxicaria* essential oil and its constituents can serve as an environmentally safe and promising nematode agent in the control of *Meloidogyne* spp. In addition, it will be important to seek application forms that can potentiate and optimize the effect and persistence of these bioactive compounds in the soil for a sufficient period to reduce populations of these pathogens to acceptable levels. In this sense, the next step will be the chemical characterization of this oil, followed by the study of their active principles in order to verify which one or how many are responsible for the nematicidal effects observed in this bioassay.

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