Volumen 31, Nº 1. Páginas 109-115
IDESIA (Chile) Enero-Abril, 2013

In vitro and foliar spray evaluation of *Verbena officinalis* (L.), *Erythrina mulungu* (Mart. ex Benth.), *Quassia amara* (L.), *Bidens pilosa* (L.) and *Plantago lanceolata* (L.), extracts on *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949

Evaluación in vitro *y aplicación foliar de* Verbena officinalis *(L.),* Erythrina mulungu *(Mart. ex Benth.),* Quassia amara *(L.),* Bidens pilosa *(L.) y* Plantago lanceolata *(L.), sobre* Meloidogyne incognita *(Kofoid & White, 1919) Chitwood, 1949*

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ABSTRACT

This study aimed to evaluate the efficiency of aqueous extracts of five species of medicinal plant, *Verbena officinalis* (L.), *Erythrina mulungu* (Mart. ex Benth.), *Quassia amara* (L.), *Bidens pilosa* (L.) and *Plantago lanceolata* (L.) *in vitro*, and sprayed on tomato plants for the control of *Meloidogyne incognita*. *In vitro* experiments were conducted in growth chambers in acrylic ELISA plates. The treatments were doses of 0, 10, 20, 30, 40 and 50 g · L^{-1} of different aqueous extracts. On the sixteenth day the number of second stage juveniles (J_2) hatched per treatment was evaluated. The results showed that aqueous extracts of *V. officinalis*, *E. mulungu*, *Q. amara*, *B. pilosa* and *P. lanceolata*, reduced the hatching of *M. incognita*, with the aqueous extract of *P. lanceolata* showing up to 100% reduction in hatching. The *in vivo* assay was conducted in a greenhouse. For this, four sprays of different extracts were applied to tomato shoots. According to the results of this test, none of the extracts were efficient at controlling *M. incognita* through foliar spraying. The efficiency of these extracts to control plant parasitic nematodes during *in vivo* tests should not be disregarded, however, further studies should be conducted to test different methods of application, doses or methods of extracting the active principles.

Key words: alternative control, *Meloidogyne incognita*, organic agriculture, nematicide.

RESUMEN

El objetivo del estudio fue evaluar la eficacia de los extractos acuosos de cinco especies de plantas medicinales, Verbena officinalis *(L.),* Erythrina mulungu *(Mart. ex Benth.),* Quassia amara *(L.),* Bidens pilosa *(L.) y* Plantago lanceolata *(L.)* in vitro*, y se pulverizaron en las plantas de tomates para el control de* Meloidogyne incognita*. Los experimentos* in vitro *fueron realizados en cámaras de crecimiento en placas de ELISA de tipo acrílico. Los tratamientos fueron dosis de 0, 10, 20, 30, 40 y 50 g · L^{-1} de diferentes extractos acuosos. En el día dieciséis se evaluó el número de juveniles segunda etapa (J_2) nacidos por tratamiento. Los resultados mostraron que los extractos acuosos de* V. officinalis*,* E. mulungu*,* Q. amara*,* B. pilosa *y* P. lanceolata *reducen la eclosión de los juveniles de* M. incognita*, y el extracto acuoso de* P. lanceolata *mostró hasta 100% de reducción de la tasa de eclosión. El ensayo* in vivo *se realizó en un invernadero, y para ello, había cuatro pulverizaciones de diferentes extractos en los brotes de tomate. Según los resultados de esta prueba, ninguno de los extractos por pulverización foliar era eficaz en el control de* M. incognita*. Sin embargo, no se debe descartar la eficiencia de estos extractos en el control de nematodos parásitos de plantas, pero más estudios se llevan a cabo utilizando diferentes métodos de aplicación, las dosis o métodos de extracción de los principios activos de los extractos.*

Palabras clave: *alternativa de control,* Meloidogyne incognita*, agricultura orgánica, nematicida.*

Introduction

The nematodes of the genus *Meloidogyne* Goeldi (1887), are some of the most important plant parasites, they have a wide geographical distribution, a huge host range and cause major damage to crops (Freitas *et al.*, 2007). In the literature there are many methods described for the control of

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Fecha de Recepción: 21 Marzo, 2012.
Fecha de Aceptación: 18 Noviembre, 2012.

nematodes (Ferraz *et al.*, 2010). However, control of these plant parasites is still a difficult task, mainly due to the inherent limitations of these control methods (Neves *et al.*, 2008). For example, the use of chemical nematicides increase production costs, and cause human and environmental risks (Campos *et al.* 1998). With this in mind many researchers over recent decades have been studying alternative methods of nematode control, such as the use of botanical extracts that have nematicidal properties (Ferris and Zheng, 1999; Oka *et al.*, 2000; Neves *et al.*, 2005).

Among the various plants studied for the preparation of nematicidal extracts, such as essential oils, *Mucuna pruriense*s, *Tagetes* spp., *Crotalaria* spp., *Azadirachta indica*, *Ricinus communis*, the genus *Brassica*, several species of medicinal aromatic plants and many grasses have been the most commonly investigated (Ferraz *et al.*, 2010).

An interesting feature in the use of botanical extracts for the control of parasitic plant nematodes, is that they can be applied via soil or foliar spray. Moreover, in several studies it has been found that the ground application of aqueous botanical extracts have shown positive results for the control of root-knot nematodes, as reported by Franzener *et al.* (2007); Javed *et al.* (2008); Gardiano *et al.* (2009) and Gardiano *et al.* (2010). When Franzener *et al.* (2007) applied aqueous extract of *Tagetes patula* flowers to the soil, at the concentration 0.05 g • mL^{-1}, they found that the aqueous extract effected populations of *M. incognita* by reducing the number of galls, eggs in the tomato roots and second-stage juveniles (J_2) in soil, by 62.2%, 61.5% and 52.8%, respectively. Javed *et al.* (2008) also observed reductions in the gall and egg numbers of *M. javanica*, when aqueous extracts of neem leaves where applied to the soil at doses of 1.5 to 3.0%. The aqueous extract of lemon grass (*Cymbopogon citratus* (DC) Stapf) and leaves of *Crotalaria mucronata* L., when applied to the soil at doses of and 0.2 g • mL^{-1}, respectively, also showed the same nematicidal effect (Gardiano *et al.*, 2009; Gardiano *et al.*, 2010).

However, research on the application of plant extracts, mainly in the spray form to the shoot for nematode control is scarce (Gardiano *et al.*, 2008a). In this context, the study aimed to assess the nematicide potential, *in vitro*, of *Verbena officinalis* (L.), *Erythrina mulungu* (Mart. Ex Benth.), *Quassia amara* (L.), *Bidens pilosa* (L.) and *Plantago lanceolata* (L.) aqueous extracts, on the hatching of *M. incognita* and *in vivo*, on tomato plants with extracts applied through a foliar spray in the greenhouse also against *M. incognita*.

Material and Methods

***In vitro* test:** We tested the aqueous extracts of *V. officinalis*, *E. mulungu*, *Q. amara*, *B. pilosa* and *P. lanceolata*, at concentrations of 0, 10, 20, 30, 40 or 50 g • L^{-1}. For the extract preparation we used the methodology described by Ferris and Zeng (1999). For this, 0.5 g of each dried herb, chopped to approximately 1 cm in length, was added separately to its own 600 mL capacity beaker , 10 mL of tap water was added to each, and allowed to rest for 24 hours in the dark at 25 °C. After this period the aqueous extracts of the plant species were filtered separately through double layers of cheesecloth. The assay was mounted on a ELISA polystyrene microplate. In each well of the plate was placed separately 100 mL of an aqueous suspension containing 50 eggs of *M. incognita* and 100 mL of the respective extracts. To the control treatment was added only tap water. The plates were kept in growth chambers for 15 days at 25 ± 1 °C in the dark. On the sixteenth day the number of second-stage juveniles (J_2) hatched in each treatment was evaluated. The experiment was conducted in a completely randomized design with six repetitions. The experimental unit was represented by a well of ELISA microplate. Data were subjected to analysis of variance and when significant were performed the analysis regression at 5% probability using the statistical program Sisvar 5.0.

***In vivo* test:** This test was conducted from January to March of 2011 during which the average temperature in the greenhouse was 24.5 °C, while the mean minimum and maximum were 18.6 °C and 30.5 °C, respectively. The test was mounted using polypropylene pots of 1.0 liter capacity, containing a mixture of soil and sand at a 2:1 (v:v) ratio, previously autoclaved for one hour at 120 °C at 1 atm. In each pot one tomato seedling cv. Santa Clara ® (Isla ®, Brazil) was planted for 25 days. After which each plant was inoculated with an aqueous suspension containing 3000 eggs of *M. incognita*. After inoculation of the plants with the nematode, the tomato plants were sprayed with the aqueous extracts tested in the *in vitro* assay at a concentration of 100 g • L^{-1}, for this 100 g of shoots of each tested plant were added separately to 1000 mL of tap water at ambient temperature. Using a hand sprayer (Brasutil mark Disma ® PD350, Brazil) the extracts were applied to tomato plants until the runoff point was reached. For the preparation of aqueous extract, the same methodology described in the *in vitro* assay was used. However, in this study, the amount of each plant material used was 100 g • L^{-1}. To avoid the extracts coming in contact with the soil, each pot was sealed with a transparent plastic. The spraying was carried out in the late afternoon, according to the methodology described by Bala and Sukul (1987). During the experimental period four sprays were conducted, with a two week interval between each. The statistical design was completely randomized with seven repetitions per treatment. Two treatments one where only water was added and one which was treated with the nematicide carbofuran / Furadan ® (FMC químca, California / USA). The nematicide was applied in the soil, only at the time of planting, as in agronomic recommendations set by the manufacturer.

Sixty days after the inoculation of tomato plants with the nematode, the height of the shoot, the fresh mass of the shoots and roots, gall numbers, number of egg masses and eggs per root system were evaluated. For the assessment of the number of egg masses, the methodology described by Rocha *et al.* (2005) was used.

Data were subjected to variance analysis and when significant, the means were compared using Duncan's test at 5% probability, using the statistical package "Statistica" (Statsoft).

Results and Discussion

All doses of the aqueous extracts of *V. officinalis*, *E. mulungu*, *Q. amara*, *B. pilosa* and *P. lanceolata*, tested *in vitro*, reduced the hatching percentage of J_2 *M. incognita* when compared to the control (Figures 1 A, B, C, D and E).

The aqueous extract of *V. officinalis* reduced the hatching percentage of J_2 *M. incognita* by 17.5%, 51.3%, 63.7%, 92.1% and 93.5% at the doses 10, 20, 30, 40 and 50 g • L^{-1}, respectively, when compared with the control treatment (Figure 1A). The aqueous extract of *Q. amara*, at doses of 10, 20, 30, 40 and 50 g • L^{-1} reduced the hatching of the nematode J_2 by 8.80%, 22.6%, 33.20%, 44.31% and 49.5%, respectively (Figure 1B). The aqueous extract of *B. pilosa* reduced hatching by 27.5%, 31.8%, 73.2%, 76.7% and 99.7% at the doses 10, 20, 30, 40 and 50 g • L^{-1}, respectively (Figure 1C). The aqueous extract of *P. lanceolata* at a dose of 10 g • L^{-1}, showed a 68.6% reduction in hatching of J_2 of *M. incognita* when compared with the control. While, the doses of 20, 30, 40 and 50 g • L^{-1} reduced hatching by more than 92.7%. The aqueous extract of *E. mulungu* at doses of 10 and 20 g • L^{-1} decreased hatching by 21.1% and 35.5%. While, at doses of 30, 40 or 50 g L^{-1} a reduction of 65.1%, 80.0% and 78.8%, were found respectively, compared to the control treatment.

Similar results in nematodes control were also found by other authors when using the same family of *V. officinalis* (Verbenaceae) plants. Elbadri *et al.* (2008), studying the medicinal plant *Lantana camara* (L.), family Verbenaceae, found that the methanol extract of this plant, showed a nematicidal effect against *M. incognita* causing 73.3% mortality of juveniles. This fact probably occurs because *V. officinalis* has anthelmintic activity (Lorenzi and Matos, 2008).

Other plants of the same family such as *E. mulungu* (Fabaceae) have shown nematicidal activity, as is the case of *Mucuna* spp. which contains compounds such as L-Dopa (3,4-dihydroxyphenylalanine), which is a substance with strong nematicidal effects (Ferraz *et al.*, 2010). Other compounds with nematicidal activity were isolated from aerial parts of plants of *Mucuna aterrima* (Pipper and Tracy), such as triacontanyl tetracosanoate tiacontil tetracosanate and triacontanol, which showed nematicidal activity against *M. incognita* race 3 (Nogueira *et al.*, 1996). Perhaps because *E. mulungu* also belongs to Fabaceae family, some of these substances occur in the plant and provide the nematicidal properties presented in this study, which could explain the reduction in percentage of *M. incognita* J_2 hatchings.

In studies performed *in vitro* using the medicinal plant *Trigonella foenum-graecum* (L.), that also belongs to the Fabaceae family, Elbadri *et al.* (2008) found that the aqueous extract of this plant, showed a nematicidal effect toward *M. incognita* causing 73% mortality when juveniles were exposed to extractsfor 72 hours.

While investigating other species of genus *Plantago,* Insunza *et al.* (2001), found that the aqueous extract at 0.2 g • mL^{-1}, obtained from roots of *Plantago major* (L.), caused 85.4% immobility in juvenile *Xiphinema americanum*. The nematicidal effect of plants belonging to the genus *Plantago*

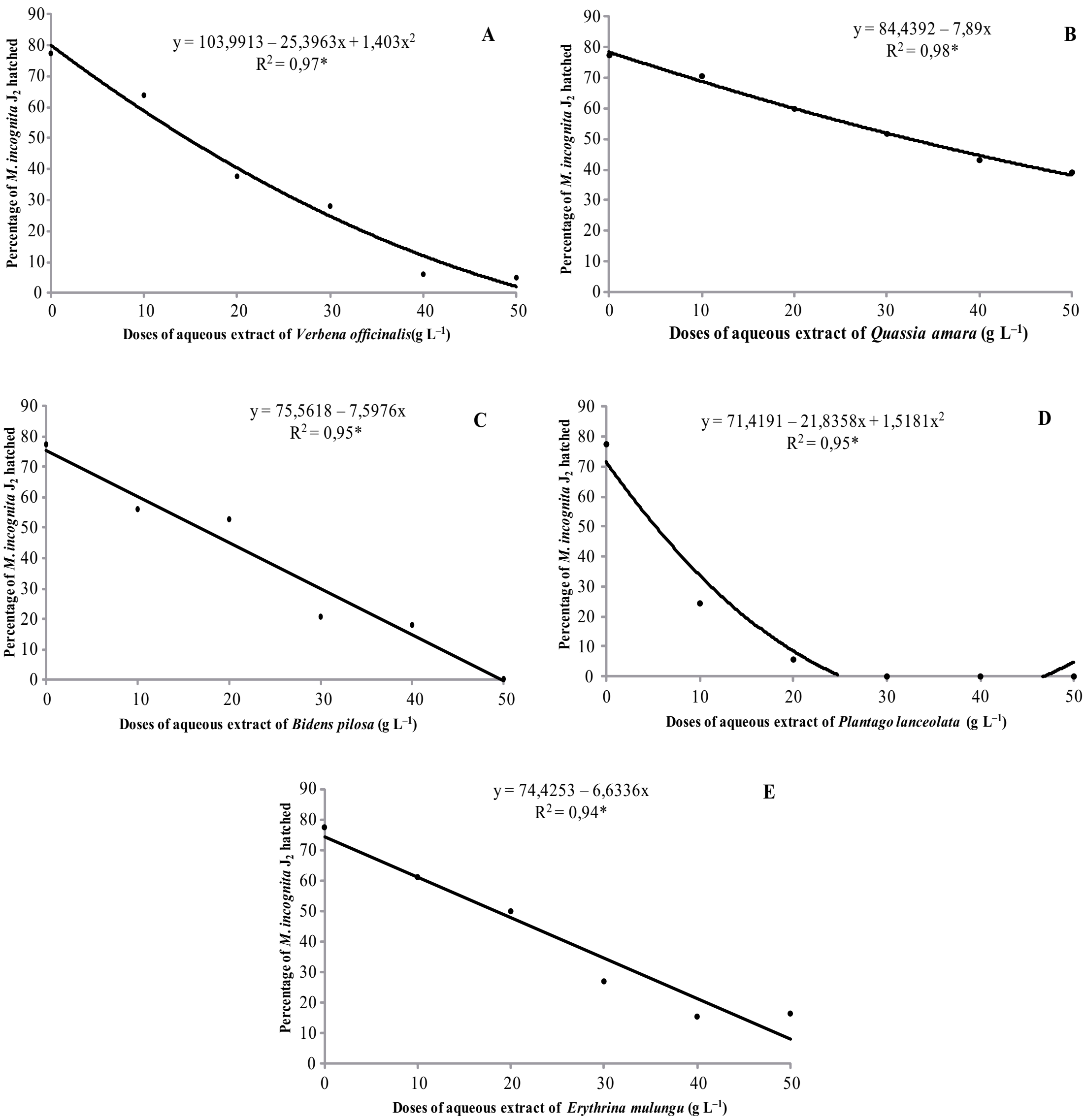

Figure 1. *In vitro* effect of aqueous extracts of *Verbena officinalis* (A), *Quassia amara* (B), *Bidens pilosa* (C), *Plantago lanceolata* (D) and *Erythrina mulungu* (E), on hatching of J_2 *Meloidogyne incognita*. Guarapuava, Paraná, Brazil, 2011.

was also demonstrated by Meyer *et al.* (2006), by using a methanol extract of *Plantago rugelii* (L.) they observed a 64% reduction in the hatching of *M. incognita* juveniles. It should be noted that in this test, the aqueous extract of *P. lanceolata* presented a more toxic extraction of active ingredients when compared to the results obtained with the use of methanol extract of *P. rugelii* presented earlier. Further to this according Silva *et al.* (2008), the use of aqueous extracts in field conditions, for environmental reasons, are more suitable than those of methanol.

Bidens pilosa is a plant belonging to the Asteraceae family, a number of plants belonging to this family, have a broad spectrum of polyacetylenes with broad biological activity, including nematicidal activity, according Chitwood *et al.* (2002). It is possible that the results obtained in this assay may be due to the presence of these compounds in the *B. pilosa extract*.

Table 1. Fresh shoot mass, shoot height of tomato plants, fresh root mass, number of galls, number of eggs and number of egg masses of *Meloidogyne incognita* after the spraying of tomato plant shoots with aqueous extracts of *Verbena officinalis*, *Quassia amara*, *Bidens pilosa*, *Plantago lanceolata* and *Erythrina mulungu*. Guarapuava, Paraná, Brazil, 2011.

Treatment	Fresh shoot mass (g)	Shoot height of tomato plants (cm)	Fresh root mass (g)	Number of galls per root	Number of eggs per root	Number of egg mass per root
Verbena officinalis	5.37 b	24.46 ab	5.43 ab	395 a	236,364 ^{ns}	123 ^{ns}
Plantago lanceolata	5.97 ab	25.31 ab	4.34 bcd	372 a	207,42	142
Erythrina mulungu	3.60 bc	22.66 b	3.36 de	238 b	184,117	119
Quassia amara	4.13 bc	22.37 b	3.67 cd	198 b	193,721	137
Bidens pilosa	2.37 c	17.51 c	1.99 e	198 b	151,414	88
Control (water)	7.78 a	27.59 a	6.67 a	400 a	220,244	124
Control (Carbofuran)	5.12 b	26.01 ab	5.09 abc	143 c	139,87	103
CV (%)	40.71	16.12	31.94	30.89	37.10	34.60

The means followed by the same letter do not differ by Duncan test at 5% probability. ^{ns} Not significant by F test at 5% probability.

Many authors have found nematicidal activity during *in vitro* experiments, by plants of the genus *Tagetes* spp. (Asteraceae). Bharadwaj and Sharma (2007), found that during *in vitro* experiments, that the doses of 6.6%, 10%, 13.3%, 16.6% and 20% of the aqueous extract derived from leaves of *T. patula*, inhibited the hatching of *M. incognita* J_2 by 100% after 48 hours exposure.

Insunza *et al.* (2001), observed an *in vitro* nematicidal effect of aqueous extracts from the aerial parts and roots of *T. erecta* and *T. patula* at a dose of 0.25g • mL^{-1}, on the nematode *Xiphinema americanum* and verified the presence of nematostatic or nematicidal effect after 24 hours exposure. Assessing the *in vitro* activity of aqueous extract obtained from the leaves of the plant *T. erecta* at doses of 5, 25 and 50 g • L^{-1}, Hasabo and Noweer (2005), found 67%, 72% and 100% mortality of J_2 *M. incognita*, respectively.

The nematicidal effect observed in the medicinal plant *Q. amara*, may be due to the presence of compounds termed quassinoids, which are degraded tetracyclic or pentacyclic triterpenoids. The chaparrinona quassinoids, Klaineanone, glaucarubolone found in the seeds of the plant *Hannoa undulata* (Simaroubaceae) inhibited the penetration of J_2 *M. javanica* into tomato plants at a concentration of 5.1 mg • mL $^{-1}$ (Chitwood 2002).

The spraying of different aqueous extracts *in vivo*, on tomato plants affected negatively the vegetative growth of the plants during this assay (Table 1). Foliar spray of aqueous extracts of *V. officinalis, E. mulungu*, *Q. amara*, *B. pilosa* and the control (carbofuran) decreased by 31.0%, 53.7%, 47.0%, 70.0% and 34.2% respectively, the fresh mass of tomato shoots. The extracts of *E. mulungu*, *Q. amara*, *B. pilosa* reduced the growth of tomato plants, when compared with the control treatment.

Furthermore, this study found reductions of 18.0%, 19.0% and 36.5%, of the height of tomato plants when sprayed with the aqueous extracts of the *E. mulungu*, *Q. amara*, *B. pilosa* respectively. The fresh mass of roots was reduced by 35.0%, 49.6%, 45.0% and 70.2%, compared with the control (water), when the tomato plants were sprayed with the aqueous extracts *E. mulungu*, *Q. amara*, *B. pilosa* e *P. lanceolata*, respectively. It can be seen therefore in this assay, the occurrence of phytotoxicity in tomato plants with a spray of aqueous extracts of the *E. mulungu*, *Q. amara*, *B. pilosa*.

Similar results were found by Lopes *et al.* (2005); Gardiano *et al.* (2008a) and Gardiano *et al.* (2008b), while researching different extracts, and their effects on vegetative growth of tomato plants infected with *Meloidogyne* spp.

Lopes *et al.* (2005) also found reductions of 32.5%, 26.5% and 29.7%, when they applied foliar sprays of aqueous extracts of *Ocimum basilicum* leaves and leaves and seeds of *Mucuna* spp., respectively. According to Gardiano *et al.* (2008a), when tomato plants infected with *M. javanica* were sprayed with 10% aqueous extracts of either *Chrysanthemum parthenium*, *Arctium lappa*, *Calopogonium mucunoides*, *Cymbopogon citratus*, *Baccharis trimera*, *Equisetum* sp., *Mentha* spp. and *Ricinus communis*, a reduction was observed in fresh root mass compared with the control treatment, which was sprayed only with water.

Researching the effect of spray dyes obtained from *Azadirachta indica*, *Petiveria alliacea*, *Melia azedarach*, *Plectranthus barbatus*, *Canavalia ensiformis* or *Ricinus communis* on tomato plants infected with *M. javanica*, Gardiano *et al.* (2008b) found that these treatments did not significantly influence the mass of the root systems of the plants.

Spraying tomato plants with aqueous extracts of the *E. mulungu*, *Q. amara*, *B. pilosa*. affected the infectivity of nematodes compared with the control (water) (Table 1), however, showed less effect than was observed with the use of the synthetic nematicide. The application of aqueous extracts of *E. mulungu*, *Q. amara*, *B. pilosa* decreased the number galls observed when compared with the control (water) by 40.5%, 50.5% and 50.5%, respectively. While carbofuran reduced gall numbers by 64. 2%. As for the number of eggs and egg masses of *M. incognita*, it was found that there was no statistical difference between treatments (Table 1).

Gardiano *et al.* (2008b) found no satisfactory results for reducing gall and egg numbers of *M. javanica*, when tomato plants were sprayed with dyes of *Azadirachta indica*, *Petiveria alliacea*, *Melia azedarach*, *Canavalia ensiformis*, *Plectranthus barbatus* and *Ricinus communis*.

Gardiano *et al.* (2008a) also found no significant results in reducing galls and reproduction of *M. javanica*, when tomato plants were sprayed with aqueous extracts, at a dose of 10%, obtained from the aerial parts of *Arctium lappa*, *Calopogonium mucunoides*, *Cymbopogon citratus*, *Baccharis trimera*, *Equisetum* sp., *Melia azedarach*, *Mentha* spp., *Ricinus communis*, *Ocimum basilicum*, *Momordica charantia* and *Chrysanthemum parthenium*. According to Franzener *et al.* (2007), spraying tomatoes with the aqueous extract obtained from the root of*Tagetes patula* at dilutions of 1:1, 1:2, 1:3, 1:4 (v:v) (extract:water), barely reduced the number of galls, with results similar to those of untreated plants.

These results indicate that the aqueous extracts of *V. officinalis*, *E. mulungu*, *Q. amara*, *B. pilosa* and *P. lanceolata* sprayed on tomato shoots, do not control *M. incognita*. This may suggest that the mode of application was not suitable for this test or that the active principle of the plants were not released to the mode of extraction used (Gardiano *et al.* 2010).

Moreover, according to Chitwood (2002), the nematicidal effect of plant extracts, when applied to the shoots, become dependent on the absorption of bioactive compounds through the leaves, translocation of these substances and the subsequent release of these compounds by root exudation, and the possibility of the activation of induced resistance in plants. No effect of aerial spraying of tomato plants was observed in this study, may also have occurred by non-systemic activity of the compounds present in the extracts or non-activation of the protective mechanisms of the plant. However, further studies should be conducted to confirm these hypothesis.

Conclusions

These results showed that under laboratory conditions the aqueous extracts of the plants evaluated at different concentrations reduce hatching of J_2 *M. incognita*. Under greenhouse conditions, the aqueous extracts of *V. officinalis*, *E. mulungu*, *Q. amara*, *B. pilosa* and *P. lanceolata*, used as foliar sprays showed no effectiveness in controlling *M. incognita*. The efficiency of these extracts to control plant parasitic nematodes during *in vitro* tests should not be disregarded, however, further studies should be conducted to test different methods of application, doses or methods of extracting the active principles.

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