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Artigo Científico

Nematicidal activity *in vitro* and *in vivo* of neem oil on *Meloidogyne incognita*

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Abstract

Considering the losses caused by nematodes in the different crops, chemical substances with nematicidal effect have been investigated and found in various plants. The neem oil is presented as an alternative to manage this pathogen. The aim of this study was to evaluate *in vitro*, the action of different doses of neem oil on the hatching and immobility of *Meloidogyne incognita* juveniles and *in vivo* in order to verify the data about the efficacy of neem oil to control nematodes of galls in tomato plants. *In vitro* assays were installed in laboratory conditions and in a completely randomized design with four replications. The experiment consisted of the following treatments: 1. Distilled water (100%); 2. Furadan (5%); 3. Neem oil (1%); 4. Neem oil (5%); 5. Neem oil (10%). For the experiment done in greenhouse, the experimental design was completely randomized with five replicates. The experiment consisted of the following treatments: 1. Control; 2. Neem oil in the soil; 3. Neem oil foliar; 4. Neem oil in the soil + leaf. The use of neem oil *in vitro* was effective only in the immobility of *M. incognita* at concentrations of 1%. The neem oil reduces the number of galls of *M. incognita* when in foliar application + soil at concentrations of 1%.

Key words: *Azadirachta indica* A. Juss, immobility, nematode galls, hatching rate.

Atividade nematicida *in vitro* e *in vivo* do óleo de nim sobre *Meloidogyne incógnita*

Resumo

Considerando as perdas causadas por nematoides às diversas culturas, substâncias químicas com efeito nematicida têm sido pesquisadas e encontradas em várias plantas. O óleo de nim se apresenta como uma alternativa para manejar esse patógeno. O objetivo desta pesquisa foi avaliar *in vitro* a ação de diferentes doses do óleo de nim sobre a eclosão e imobilidade de juvenis de *Meloidogyne incognita* e *in vivo* com a finalidade de comprovar os dados relativos à eficácia do óleo de nim no controle de nematoides de galhas em plantas de tomate. Os ensaios *in vitro* foram instalados em condições de laboratório e em delineamento inteiramente casualizado com quatro repetições. O experimento constou dos seguintes tratamentos: 1. Água destilada (100%); 2. Furadan (5%); 3. Óleo de nim (1%); 4. Óleo de nim (5%); 5. Óleo de nim (10%). Para o experimento realizado em casa de vegetação, o delineamento experimental utilizado foi o inteiramente casualizado, com cinco repetições. O experimento constou dos seguintes tratamentos: 1. Testemunha; 2. Óleo de nim via solo; 3. Óleo de nim via foliar; 4. Óleo de nim via solo + foliar. A utilização do óleo de nim *in vitro* foi efetivo apenas na imobilidade de *M. incognita* nas concentrações de 1%. O óleo de nim reduz o número de galhas de *M. incognita* quando aplicado via foliar + solo nas concentrações de 1%.

Palavras-chave: *Azadirachta indica* A. Juss, imobilidade, nematoides das galhas, taxa de eclosão.

Actividad nematicida *in vitro* e *in vivo* de Aceite de Neem sobre *Meloidogyne incognita*

Resumen

Teniendo en cuenta las pérdidas causadas por nematodos en diferentes cultivos, distintas sustancias químicas con efecto nematicida han sido investigadas y encontradas en varias plantas. El aceite de Neem se presenta como una alternativa

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para gestionar este patógeno. El objetivo de este estudio fue evaluar *in vitro* la acción de diferentes dosis de aceite de Neem en la eclosión y inmovilidad de los juveniles de *Meloidogyne incognita* e *in vivo* con el fin de confirmar los datos sobre la eficacia del aceite de Neem para el control de nematodos de agallas en plantas de tomate. Los ensayos *in vitro* se instalaron en condiciones de laboratorio y en un diseño experimental completamente al azar con cuatro repeticiones. El experimento consistió en los siguientes tratamientos: 1. Agua destilada (100%); 2. Furadan (5%); 3. Aceite de Neem (1%); 4. Aceite de Neem (5%); 5. El aceite de Neem (10%). Para el experimento en invernadero, el diseño experimental fue completamente al azar con cinco repeticiones. Este experimento consistió en los siguientes tratamientos: 1. Testigo; 2. Aceite de Neem en el suelo; 3. Aceite de Neem foliar; 4. Aceite de Neem en el suelo + hoja. El uso del aceite de Neem *in vitro* fue eficaz sólo en la inmovilidad de *M. incognita* en concentraciones de 1%. El aceite de Neem reduce el número de agallas de *M. incognita* cuando se aplica foliar + suelo en concentraciones de 1%.

Palabras clave: *Azadirachta indica* A. Juss, la inmovilidad, nematodos de agallas, tasa de eclosión.

Introduction

Plant parasitic nematodes cause losses estimated in 12% of the agricultural production, being that approximately 9% occur in developed countries and 15% in developing countries, representing about 100 billion dollars in annual damage worldwide (SASSER and FRECKMAN, 1987).

In Brazil, the most significant damage is the result from the attack of nematode-of-galls, generally *M. incognita* and *M. javanica* which posses greater territorial distribution in the different Brazilian regions, although other species, such as the *M. hapla* and *M. arenaria* are observed in isolated areas of the country (SILVA et al. 2011)

The nematodes which belong to the genus *Meloidogyne Goeldi* (1887) are the most important phytopathogenic nematodes, because present a wide geographical distribution, a huge host range and cause great damage to crops (FREITAS et al., 2009).

In several approaches to the management of nematodes, is found the use of nematicidal, which, besides rising the costs of productions, presents risks to mean and to the environment (CAMPOS et al., 1998). For this reasons, alternative methods of control have been studied, for exemple the use of extracts of different species and parts of plants with nematicidal properties FERRIS and ZHENG, 1999; NEVES et al., 2005). In this sense, the applications of natural extracts can represent the substitution of conventional pesticides and become an alternative method to the nematodes control.

Another interesting characteristic in the use of botanical extracts is that there are different techniques of application. The extracts application via soil and leaf are among them. Application studies of these extracts via soil have presented good results in the control of phytonematodes, however, the application of such extracts, especially in the spray form over the shoot, has been little studied for the control of these pathogens (GARDIANO et al., 2008).

The plants fertilizers with effect of abiotic inducers of resistance, principally the silicated as example of the Rocksil and the industrialized based on natural products, such as the Neemseto, oil based of neem leaves (*Azadirachta indica* A. Juss.); the Chitosan, polysaccharide obtained from the shells of crustaceans and, the Biopirol, firewood extract, aqueous fraction obtained in the distillation of eucalyptus tar, has shown great potential in the control of plant diseases. Against the phytonematodes, the induced resistance in plants can vary according with the species and nutritional status of the host, type of inductor and pathogen involved (ASSUNÇÃO et al., 2010).

Some plants are more commonly studied as raw material for the preparation of extracts or for the extraction of essential oils with nematicidal properties as is the case of *Mucuna prurienses* L. (mucuna); *Tagetes* L. spp. (marigold); *Crotalaria* L. spp.; *Azadirachta indica* A. Juss. (neem); some grass-plot *Ricinus communis* L. (castor beans); brassica and medicinal and aromatic plants (FERRAZ et al., 2010).

The specie *Azadirachta indica* (A. Juss), Asteraceae family, popularly known as margosa or neem, calls the attention of researchers for containing compounds which possess chemical properties which can affect over 200 species of insects and also mites, nematodes, fungi, bacteria and even some phytovirus. The specific chemical basis for the nematicidal activity of the neem, remains obscure, even though the fractions containing steroidalterpenoids and glycosides seem to be toxic *in vitro* to *M. incognita* (CHITWOOD, 2002).

Through the foregoing, it is indispensable that further research are done, seeking to verify the potential of these plants, to be used in the control of phytonematodes, having in mind that the Brazilian

flora possess many species of native plants.

Thus, the objective of this study was to assess *in vitro* the effect of neem oil on the hatching and mortality rate of *Meloidogyne Incognita* juveniles and *in vivo* in tomato plants infested with nematode of galls.

Material and Methods

For an attestation of the effectiveness of this study, were done two experiments, being one *in vitro* in Nematology Laboratory and *in vivo* in a greenhouse. Both experiments were conducted in the Catholic University of Tocantins, Agricultural and Environmental Sciences Campus, located in the city of Palmas – TO, whose coordinates are 48°17'31.77"W and 10°17'2.80"S being in an altitude of 230m.

Initially the experiment were done *in vitro*, during the period from August, 09th to 25th of 2012, whose objective was to assess the inhibition of juveniles hatching in the second stage of *M. incognita* on neem oil. For the set up of the test, were used populations of *M. incognita*, kept in tomato plants in a greenhouse. The nematodes eggs were extracted from the tomatoes plants roots by the Hussey and Barker method, modified by BONETI and FERRAZ (1981). The eggs suspension was calibrated in counts chamber of Peters with the aid of a stereoscopic microscope.

The experiment consisted of the following treatments: 1. Distilled water (100%); 2. Furadan (5%); 3. Neem oil (1%); 4. Neem oil (5%); T5. Neem oil (10%), with four replications. The solutions dosage were diluted in 1 L of distilled water

In each Petri dish of 5 cm of diameter, were placed, separately, 10 mL of each solution and after added 150 eggs of *M. incognita* and taken into incubation at 26 °C, in a B.O.D. chamber. The number of juveniles hatched and remaining eggs were assessed with the aid of a stereoscopic microscope, 16 days after the incubation. Afterwards, we calculated the percentage of hatch of juveniles by the formula: Hatching percentage = [number of hatched juveniles / (number of hatched juveniles + number of remaining eggs)] x 100.

In a second moment, was done the assessment of the immobility of juveniles of the second stage of *M. incognita* on the neem oil. In this test, the juveniles of second stage (J2) used were obtained by the eggs extraction from the tomato plant roots infected with the *M. incognita.*

The experimental unit consisted of a Petri dish with 5 cm of diameter. In each dish were put 10 mL of the extract solution with neem oil containing approximately 100 J2 of *M. incognita* and incubated at 26 °C. In the control treatments was used only water.

Twenty four hours after, each suspension of extract or only water, contained in the dishes, containing juveniles, was poured separately into a sieve of 0.025 mm of opening (500 meshes) and the nematodes were washed carefully with running water.

It was assessed the percentage of moving juveniles and after was determined the percentage of inactive or active nematodes. Both the tests *in vitro* were installed in a completely randomized design, with four replications per treatment.

The data obtained after the tests *in vitro*, were submitted to the variance analysis and the averages compared through the Scott-Knot test at 5% of probability.

After the realization of the test *in vitro*, was conducted the experiment *in vivo* in a greenhouse, with the purpose of verify the data relative to the efficacy of the neem oil in the control of nematodes of galls. The experimental design was completely randomized, with four treatments and five replications. The experiment consisted of the following treatments: 1. Control; 2. Neem oil via soil; 3. Neem oil via leaf; 4. Neem oil via soil + leaf.

In September, 01st of 2012, was done the sowing of the tomato seeds var. Santa Cruz in polyethylene trays in order to obtain seedlings. After 20 days of the sowing, was done the transplantation of seedlings for pots of 5 L containing autoclaved soil and sand in the proportion 2:1, using two plants / pot.

After one week of the transplantation, each plant was inoculated with 1500 eggs + juveniles of second stage (J2), extracted according to the methodology of Jenkins (1964).

For the preparing the solutions, it was decided to use in all treatments the concentration of 1% of neem oil, which was pulverized weekly using 20 mL per port, except for the treatment 4. Neem oil via soil + leaf where was used 20 mL in the soil and 20 mL in the shoot, totalizing 40 mL / pot.

The first application of the solution with neem oil occurred at September, 22nd of 2012 and the last application at November, 24th of 2012, when it equaled 10 applications. Passed four days of the last pulverization, was done the assessment of tomato roots, where was obtained the number of galls in approximately 10g of roots per treatment.

The data of the experiment *in vivo* was submitted to variance analysis using the statistic program ASSISTAT® (version 7.6. beta) being the averages of the treatments compared by the Tukey test at 5% of probality.

Results and Discussion

Results that refer to the tests *in vitro*, in which were quantified the hatching and immobility rates of the juveniles of *M. incognita* are found in the Tables 1 and 2. It is observed in Table 1 that there was no significant difference with relation to the hatching rate of juveniles, knowing that the control did not statistically differ from the other treatments using the neem oil for the immersion of the galls nematodes. In general, all the treatments presented low rate of hatching. Possibly, the temperature in which the eggs were put was not ideal to allow the hatching.

The toxicity of the plant extract used in the first days of incubation is of extreme importance, which can contribute for the reduction of the nematode infectivity in the root system of the plants.

The results obtained by SILVA et al. (2008) show that occured a reduction of 90% in the number of eggs and 84% in the number of cysts caused in soybean by the nematode *H. glycines* (ICHINOHE, 1952), when used the aqueous extract and methanolic, obtained from neem plants.

In the second experiment, it is observed that there was effect of the neem oil on the J2 of *M. incognita* as for immobility (Table 2). The different concentrations with neem oil differed statistically between themselves, besides presenting lesser percentage of active juveniles than the control. The use of Furadan (5%) provided a lower reduction of active juveniles between the other treatments, being that did not differed from the 1% and 5% doses of neem oil, being these understood in 11% and 15.4%. On the other hand, the concentration of oil at 10% presented higher percentage in relation to the number of active juveniles, with 30.7%.

RAO et al. (1997), reported the rate of gall and the final population of *M. incognita* decrease significantly in eggplant seedlings (*Solanum melongena* L.) which had the roots immersed in suspension of neem leaves

Among the concentrations of neem oil used in the test *in vitro*, it is noted that the treatment with the dosage of 1% was effective in the control of active J_2 with percentage of 11% and different from the control with 84.8%, besides this result being statistically smaller than the other treatments.

Table 1. Effect of neem oil on hatching of second stage juveniles of *M. incognita* after 16 days B.O.D

Treatments	J_2 hatched (%)	Reduction of hatching(%)
Witness	6.7 a	86.6[1]
Furadan (5%)	0.0 a	100
Neem oil (1%)	11.6 a	88.3
Neem oil (5%)	18.3 a	81.6
Neem oil (10%)	5 a	95

1 Averages followed by the same letter do not differ, by Scott-Knott test at the 5% level of significance.

Table 2. Percentage of second stage juveniles of *M. incognita* active after exposure for 24 hours in neem oil

Treatments	J_2 assets(%)[1]
Control	84.8 a
Furadan (5%)	4.9 c
Neem oil (1%)	11.0 c
Neem oil (5%)	15.4 bc
Neem oil (10%)	30.7 b

1 Averages followed by the same letter do not differ, by Scott-Knott test at the 5% level of significance.

In the greenhouse experiment, aiming to assess the action of neem derivatives on populations of *M. incognita* e *Pratylenchus goodeyi* Sherand Allen in banana trees, MUSABYIMANA and SAXENA (1999) obtained significant population reductions.

In the experiment done *in vitro* in tomato plants, it is observed by Table 3 that the treatments that received neem oil, there was a reduction of the number of galls of *M. incognita* in comparison to the control, presenting significant differences at the level of 5% of probability by the Tukey test.

Table 3. Number of galls on tomato roots infested with *Meloidogyne incognita* after applications of neem oil

Treatments	Number of Galls[1]
Control	385.6 a
Neem oil in the soil	323.4 ab
Neem oil foliar	203.2 ab
Neem oil in the soil + foliar	188.0 b

[1] Averages followed by the same letter do not differ, by Scott-Knott test at the 5% level of significance.

However, it is verified that upon the different conditions of application of neem oil, the roots presented lower rate of galls when applied via soil + leaf; although did not statistically differ when applied only to the soil or leaf. Studies done by RODRIGUES et al. (2008), show that the neem oil when applied via leaf or soil, separately, does not significantly changes the number of galls. In this sense, it is stated that the neem oil when applied via leaf + soil can reduce the infestation of nematodes of galls.

Conclusions

The use of neem oil *in vitro* was effective only in the immobility of the *M. incognita* at concentrations of 1%.

The neem oil reduces the number of galls of *M. incognita* when applied via leaf + soil at concentrations of 1%.

The results obtained here indicated that the neem oil can be used in the control of nematodes of galls.

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