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Selection of Entomopathogenic Nematodes to Control Nymphs of *Haplaxius crudus* (Van Duzee) (Hemiptera: Cixiidae)

Miriam Rosero Guerrero[1], Alex Enrique Bustillo Pardey[2]

[1]Colombian Oil Palm Research Center, Paratebueno, Colombia

[2]Colombian Oil Palm Research Center, Bogotá, Colombia

Email address:
mrosero@cenipalma.org (M. R. Guerrero), abustillo@cenipalma.org (A. E. B. Pardey)

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Abstract: *Haplaxius crudus* transmits the pathogen that causes Lethal wilt (ML for its name in Spanish), one of the main diseases that affects oil palms in Colombia. In its nymphal stage it feeds from grasses present at the plantations, and adults feed on oil palm leaves. With the objective of controlling the nymphal stage of *H. crudus*, the effectiveness of the following entomopathogenic nematodes was assessed: *Steinernema colombiense*, *S. websteri*, *Steinernema* sp. 1, *Steinernema* sp. 2, *Heterorhabditis bacteriophora*, *Heterorhabditis* sp. (Gua 31), *Heterorhabditis* sp. (Gua 236), *Heterorhabditis* sp. (CPHsp1301) and *Heterorhabditis* sp. (CPHsp1302). Pathogenicity was assessed under laboratory conditions using Petri dishes with roots of *Paspalum virgatum*. Virulence was assessed using mesh houses with PVC tubes and plastic trays with *P. virgatum*. Once the most virulent nematode was selected, three dosage levels were assessed under simulated field conditions, in order to select the most effective dosage. All assessed nematode species were pathogenic to nymphs of *H. crudus*, and nymph stage IV was the most susceptible stage, with death rates of over 80%. Regarding virulence, there were statistically significant differences among treatments ($P \leq 0.05$), producing nematode death rates between 28.3 and 88.2%. *Heterorhabditis* sp. (CPHsp1301), obtained from the soil of palm plantations, was selected because it caused a mortality rate of 78.4% with at a dosage of 1300 IJ/cm^2 in the sprayed area. The results are promising and further research should be performed under commercial oil palm plantation conditions.

Keywords: Oil Palm, Biological Control, *Steinernema*, *Heterorhabditis*

1. Introduction

Lethal wilt (ML for its name in Spanish), is one of the main diseases that affects oil palms (*Elaeis guineensis* Jacq.) in Colombia. It was recorded for the first time in the eastern region of the country in 1994, in the Bajo Upía area [1] and has forced the eradication of 670,562 palms between 2010 and 2017 in the Colombian eastern palm growing region [2].

The vector insect of the agent that causes ML is *Haplaxius crudus* (Van Duzee, 1907) (Hemiptera: Cixiidae) [3]. The nymphs of *H. crudus* grow in the roots of grasses located at superficial levels of the soil, often in decomposing fallen leaves at a depth of 3 cm. In the adult stage they feed on the leaves of different types of palms by inserting their stylet into the tissue of the leaf to suck the phloem [4-7], which implies that the adult stage is the only stage that is considered a vector for ML.

Infected palms display necrosis of the tips of the bracts of immature blossoms, the fruits are easily detached from the bunches and the leaflets begin to dry out from the tip to the base, causing the edges to roll in. This symptom is usually preceded by a yellow strip that becomes more diffused as the disease advances [7-8].

Numerous natural enemies of *H. crudus* have been reported in Mexico and USA (Florida), such as spiders, mites, hymenopterans and the entomopathogenic fungus *Hirsutella citriformis* [6, 9-12]. In Colombia, Cenipalma has been able to isolate in oil palm plantations *H. crudus* adults infected by *Metarhizium anisopliae*. Oil palm plantations attempt to control this insect by means of periodic application of insecticides, with a low success rate, while

incurring in higher production costs and affecting beneficial fauna in the plantations. The control measures for lethal wilt recommended by Cenipalma are to perform early detection of the disease, eradicate the affected palms, monitor adult *H. crudus* with yellow sticky traps, and eradicate grasses and sedges at plantations, replacing them for wide-leaf foliage [7, 13].

Because the nymphs of *H. crudus* are found in the roots of grasses and sedges in the ground of oil palm plantations [5], entomopathogenic nematodes are considered a viable control alternative. The entomopathogenic nematodes of the Steinernematidae and Heterorhabditidae families are considered important biological controllers for insects with in-soil reproductive habits [14, 15]. They display important features such as high virulence, the capacity to move about to search for, find and infect insects and the ability to survive for long periods of time in the soil with no host. They are also harmless to the environment and mammals and compatible with other entomopathogenic species [16-21].

Based on the above, the objective of this study was to assess the effectiveness of the entomopathogenic nematode species in controlling nymphs of *H. crudus*.

2. Materials and Methods

2.1. Location

The study was carried out in laboratory conditions (25 ± 3 °C and 63 ± 13% RH), and in a mesh house (26.76 ± 3.56 °C and 78.77 ± 13.79% RH) at the facilities of Cenipalma in Villanueva, Casanare and at the plantations of the Experimental Field Palmar de las Corocoras, in Paratebueno, Cundinamarca (26.2 °C, 73.4% RH and 2451 mm of rainfall).

2.2. Biological Materials

Nine species of entomopathogenic nematodes were used for the pathogenicity experiments (Table 1), reproduced from larvae in the last stage of *Galleria mellonella* L. (Lepidoptera: Pyralidae), following the procedure established in references [22, 23]. The nematodes used in all the experiments had emerged 24 hours earlier from *G. mellonella* larvae. The nymphs of *H. crudus* were obtained from the colony of Cenipalma established at the plantation of Guaicaramo S. A., in Barranca de Upía, Meta.

Table 1. *Entomopathogenic nematodes assessed for control of nymphs of Haplaxius crudus.*

Nematode	Origin
Heterorhabditis bacteriophora	Fresno, Tolima
Heterorhabditis sp. (CPHsp1301)	Tumaco, Nariño
Heterorhabditis sp. (CPHsp1302)	Villanueva, Casanare
Heterorhabditis sp. (Gua 31)	Guática, Risaralda
Heterorhabditis sp. (Gua 236)	Guática, Risaralda
Steinernema colombiense	Quimbaya, Quindío
Steinernema websteri	Chinchiná, Caldas
Steinernema sp. 1.	Buenavista, Quindío
Steinernema sp. 2.	Chinchiná, Caldas

2.3. Pathogenicity Experiments

The pathogenicity of the nematodes was assessed in laboratory conditions in two experiments, the first using stage III nymphs of *H. crudus* and the second with stage IV nymphs. The experimental unit consisted of one 9 cm Petri dish with filter paper, in which roots of the grass *Paspalum virgatum* L. were placed, and three nymphs of *H. crudus*. The nymphs used in the experiments were put in contact with 100 IJ (infective juveniles)/cm^2 of the nematodes applied by spraying, in 2 ml of sterile water. Only sterile water was applied to the control treatment. The experiments were performed using fully random sampling with 10 treatments (nine species of entomopathogenic nematodes plus the control group) (Table 1). Each treatment had 10 repetitions for a total of 30 nymphs per treatment.

The assessments of mortality of the nymphs of *H. crudus* were performed daily over eight days. Observations were also made on the production of nematodes in 50% of the dead individuals using a modified "White" camera [24]; the remaining 50% of dead individuals were analyzed through dissection to establish the number of nematodes that had managed to become parasites of each specimen 24 hours after death. The data were analyzed using analysis of variance and the differences of treatment were assessed using the Duncan test at 5% by means of the SAS 9.3 statistical package.

2.4. Virulence Experiments

The virulence of the nematodes was assessed under mesh house conditions with the nematode species selected in the previous experiment that had caused a mortality rate of over 80% in the nymphs. The experiments were divided into two groups; first, an assessment was performed for the species of Steinernematidae (*S. colombiense, S. websteri, Steinernema* sp. 1 and *Steinernema* sp. 2), and afterwards of the species of Heterorhabditidae (*H. bacteriophora, Heterorhabditis* sp. (CPHsp1301), *Heterorhabditis* sp. (Gua 31) and *Heterorhabditis* sp. (CPHsp1302)), due to the availability of nymphs of *H. crudus* at the Breeding Unit for this insect.

The experimental unit consisted of *P. virgatum* planted in containers (tubes) of polyvinyl chloride (PVC) of 5 cm in diameter and 7 cm long infested with 10 stage IV nymphs of *H. crudus*. The nymphs were put in contact with 100 IJ/cm^2 applied by spraying in suspension in 10 ml of sterile water. The experiments were performed based on a fully random design with five treatments, four Heterorhabditidae species plus a control group (first experiment) and four Steinernematidae species plus a control group (second experiment). Each treatment had six repetitions. The assessments on mortality of *H. crudus* were performed every 48 hours, until the adults emerged.

Afterwards, a new experiment was conducted under simulated field conditions using the nymphs of *H. crudus*. The experimental unit consisted of plastic trays, 30 cm long by 23 cm wide, planted with *P. virgatum* plants and infested with 17 stage IV nymphs. The nymphs were put in contact with 1300 IJ/cm^2 applied by spraying in 150 ml of sterile water. Each tray was covered with hollow cylinder made

from wire and covered with tulle cloth. The experiments used a fully random statistical design. Each treatment had six repetitions for a total of 102 nymphs per treatment. Nymph mortality and the emergence of *H. crudus* adults were recorded every 48 hours over 15 days.

2.5. Assessment of the Most Effective Dosage

Once the most virulent nematodes on nymphs of *H. crudus* were selected, experiments were carried out to establish the most effective dosage for controlling the nymphs of this insect under mesh house and field conditions. The experimental unit consisted of *P. virgatum* plants planted in plastic trays, 30 cm long by 23 cm wide (area: 690cm^2) by 12 cm tall, infested with 17 nymphs of *H. crudus*. The experiment in the mesh house was performed under a fully random design, with four treatments and six repetitions. Three dosages of the selected nematodes were assessed: 100, 500 and 1300 IJ/cm^2 of the experimental unit.

2.6. Field Assessment

In field conditions at an oil palm plantation, the nematode selected in the above experiment was assessed in three dosages, 500, 1000 and 1300 IJ/cm^2 under a full random block design. The experimental unit consisted of *P. virgatum* planted in plastic trays, 30 cm long by 23 cm wide (area: 690cm^2) by 12 cm tall, infested with 17 nymphs of *H. crudus.* Each experimental unit was placed under an oil palm and covered with a cylinder formed by a wire structure covered with tulle cloth. The IJs were sprayed using a hand-held spray gun in the planned dosages. Nymph mortality and the emergence of adults of *H. crudus* were assessed every 48 hours over 15 days.

The data were analyzed by means of analysis of variance and the differences between treatments were analyzed using the Duncan test at 5%, using the SAS 9.3 statistical package.

3. Results and Discussion

3.1. Experiments of Pathogenicity

All the assessed nematode species were pathogenic for nymphs of *H. crudus* and there were statistically significant differences in the mortality rates they produced on the assessed species (Table 2). In the laboratory, most species of entomopathogenic nematodes infect a wide variety of insects because they come into direct contact with their hosts, the environmental conditions are propitious and there are no ecological barriers to infection [14, 25].

It was also found that susceptibility varies depending on the insect's age: the nematodes are more pathogenic on more developed nymphs, finding that in stage IV nymphs the mortality rates were over 80%, with the exception of one species, whereas in stage III mortality rates were below 57% (Table 2). Similar results were found in a study with *Aeneolamia varia* Fabricius (Hemiptera: Cercopidae), which also found that nematode susceptibility increases with age: stage IV nymphs were more susceptible to the nematodes [26]. Other studies found that second instars of *Bradysia odoriphaga* Yang & Zhang (Diptera: Sciaridae) were less susceptible to infection by nematodes as compared with the older stages (third and fourth instar and pupae) [27]. This susceptibility may be attributed to the size of the natural cavities (mouth, anus and spiracles) that are the entry point for the entomopathogenic nematodes [28].

Table 2. *Mortality (%) of stage III and IV nymphs of Haplaxius crudus caused by entomopathogenic nematodes under laboratory conditions.*

Entomopathogenic nematodes	% mortality nymphs of *H. crudus*	
	Stage III	Stage IV
Heterorhabditis bacteriophora	45.2 a*	93.3 a
Heterorhabditis sp. (CPHsp1301)	-	90.0 a
Heterorhabditis sp. (Gua 31)	47.6 a	90.0 a
Steinernema colombiense	57.1 a	86.7 a
Steinernema websteri	50.0 a	93.3 a
Steinernema sp. 1.	45.2 a	96.7 a
Steinernema sp. 2.	45.2 a	100.0 a
Heterorhabditis sp. (Gua 236)	-	53.3 b
Control	14.3 b	10.0 c

*Data in the same column followed by the same letter are not significantly different, according to the Duncan test ($P \geq 0.05$).

Regarding the penetration of the assessed nematodes in *H. crudus*, they managed to enter the body of the nymphs and multiplied inside it. The greatest production of IJs was found with *S. colombiense,* with an average of 4810 JI/nymph (Table 3). The multiplication of the nematode inside the insect would not only affect the susceptible state, but the nematodes that emerge from the corpse could also cause secondary infections in other development stages and increase inoculation in the field [29]. In this regard, in the references [30, 31] mention that applications of *Heterorhabditis* sp. in the field on *Popillia japonica* Newman (Coleoptera: Scarabaeidae) and *Leucopholis lepidophora* Blanchard (Coleoptera: Scarabaeidae) enabled the nematode to reproduce inside the insect, to emerge and to produce secondary infections and reference.

Table 3. *Average number of nematodes that parasitized and emerged in stage III and IV nymphs of Haplaxius crudus.*

Entomopathogenic nematodes	Juveniles that parasitized		Emerging infective juveniles	
	Stage III	Stage IV	Stage III	Stage IV
Heterorhabditis bacteriophora	5.0	29.0	102.5	306.0
Heterorhabditis sp. (CPHsp1301)	-	8.0	-	3697.2
Heterorhabditis sp. (Gua 31)	24.0	22.0	2010.0	562.5
Heterorhabditis sp. (Gua 236)	-	7.0	-	725.0
Steinernema colombiense	5.0	6.0	115.6	4810.0
Steinernema websteri	15.0	17.0	152.3	565.2
Steinernema sp. 1.	13.0	15.0	384.0	609.3
Steinernema sp. 2.	4.0	33.0	4.0	1311.4

The symptomatology observed in the dead nymphs of *H. crudus* were flaccidity, incapability of developing on to the adult stage and reddish color in the case of the Heterorhabditidae species (Figure 1).

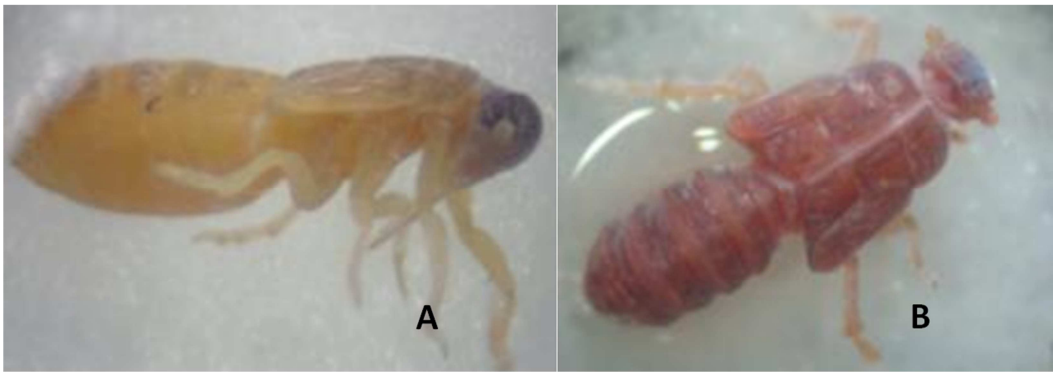

Figure 1. *Symptomatology of nymphs of Haplaxius crudus infected with entomopathogenic nematodes. A. nymph infected with Steinernema sp. B. Change of color caused by Heterorhabditis sp.*

3.2. Experiments of Virulence

The virulence experiments found that all the assessed nematode species were virulent on nymphs of *H. crudus* and there were statistically significant differences in the mortality rates they produced on the assessed species (Table 4). In the experiment with plastic trays, that were most similar to field conditions, there were statistically significant differences among the species of *Heterorhabditis* ($F = 17.33$; $df = 4$; $P < 0.0001$) and the mortality rates were above 60%. Meanwhile, the *Steinernema* species produced mortality rates above 80% and there were no differences among them ($F = 55.60$; $df = 4$; $P < 0.0001$) (Table 4).

The nematode species *Steinernema* sp1. and *Heterorhabditis* sp. (CPHsp1301) were the most virulent of each family and were selected for further use in the dosage assessment study, and subsequently for assessment in the field. *Heterorhabditis* sp. (CPHsp1301) has an additional attribute, in that it is native in isolation at an oil palm plantation in Tumaco, Nariño.

Table 4. *Mortality (%) of stage IV nymphs of Haplaxius crudus caused by entomopathogenic nematodes under mesh house conditions.*

Entomopathogenic nematodes	% mortality nymphs of *H. crudus*	
	PVC	Plastic trays
	Experiment 1	
Heterorhabditis bacteriophora	36.7 a*	71.6 a
Heterorhabditis sp. (CPHsp1301)	38.3 a	74.5 a
Heterorhabditis sp. (Gua 31)	28.3 a	74.5 a
Heterorhabditis sp. (CPHsp1302)	60.0 b	63.7 a
Control	6.6 c	14.7 b
	Experiment 2	
Steinernema colombiense	53.3 a	84.3 a
Steinernema websteri	51.7 a	88.2 a
Steinernema sp. 1.	71.7 b	88.2 a
Steinernema sp. 2.	70, 0 b	84.3 a
Control	8.3 c	14.7 b

*Data in the same column followed by the same letter are not significantly different, according to the Duncan test ($P \geq 0.05$).

3.3. Assessment of Dosage Effectiveness

The assessment of dosage in the case of *Steinernema* sp. 1 displays statistically significant differences ($F = 126.96$; $df = 4$; $P < 0.0001$). The dosage of 1300 IJ/cm^2 produced the highest mortality rate of 75.4% (Table 5). In the second experiment to assess the dosage of *Heterorhabditis* sp. (CPHsp1301), there were statistically significant differences ($F = 43.8$; $df = 3$; $P < 0.0001$) between the two assessed dosages. The dosage of 1300 IJ/cm^2 produced the highest mortality rate, at 82.3% (Table 5). Based on these results and taking into consideration that it is a nematode isolated from the soil at an oil palm plantation, *Heterorhabditis* sp. (CPHsp1301) was selected for assessment in field conditions in three dosages. There are some differences known in terms of survival, pathogenicity and host range between indigenous and non-indigenous nematodes species. Indigenous species of nematodes may be more successful in biocontrol because of compatibility to native habitats [14, 32].

Table 5. *Mortality (%) of stage IV nymphs of Haplaxius crudus caused by different dosages of Steinernema sp.1 and Heterorhabditis sp. (CPHsp1301) under mesh house conditions.*

Dosage	% mortality nymphs of *H. crudus*	
	Steinernema sp. 1	*Heterorhabditis* sp. (CPHsp1301)
100 IJ/cm^2	42.1 b*	47.0 b
500 IJ/cm^2	73.5 a	75.4 a
1300 IJ/cm^2	75.4 a	82.3 a
Control	4.9 c	0 c

*Data in the same column followed by the same letter are not significantly different according to the Duncan test ($P < 0.05$).

3.4. Field Assessment

The mortality of nymphs of *H. crudus* produced by *Heterorhabditis* sp. (CPHsp1301) with the evaluation of three dosages under simulated field conditions displayed statistically significant differences ($F = 19.6$; $df = 3$; $P < 0.0001$) among the assessed dosages, of which the dosage of 1300 IJ/cm^2 produced the greatest mortality rate (78.4%) (Table 6).

It should be noted that increasing the dosage from 1000 to 1300 IJ/cm^2 did not significantly increase the mortality rate, which indicates that after reaching a certain threshold an

increase in nematodes does not imply a higher mortality rate [33, 34]. It should also be pointed out that the dosages assessed in this study are high, but consistent with other experiments that recommend high dosages to control insects, such as those performed in reference [35] using a dosage of 1.2 x 10^{10} IJ/ha of *S. riobrave* to control *Diaprepes abbreviates* L. (Coleoptera: Curculionidae), producing mortality rates of over 90%, and those performed in reference [36] using a dosage of 1.5 x 10^{11} IJ/ha of *Heterorhabditis* sp. to control the spittle *A. varia,* producing a mortality rate of 76%.

Table 6. *Mortality (%) of nymphs of Haplaxius crudus caused by different dosages of the entomopathogenic nematode Heterorhabditis sp. (CPHsp1301) under simulated field conditions.*

Dosage of *Heterorhabditis* sp. (CPHsp1301)	**% mortality nymphs of *H. crudus***
500 IJ/cm^2	58.8 a
1000 IJ/cm^2	71.5 b
1300 IJ/cm^2	78.4 b
Control	25.5 c

*Data in the same column followed by the same letter are not significantly different according to the Duncan test ($P < 0.05$).

4. Conclusions

Although this is the first report that studies the efficacy of entomopathogenic nematodes against *H. crudus,* the results of this study demonstrate the potential of *Heterorhabditis* sp. (CPHsp1301) to reduce *H. crudus* nymphs populations, reproduce inside the insect, to emerge and to produce secondary infections. Further research is needed, to validate these results to control *H. crudus* under commercial oil palm plantations, so this strategy can be incorporated in an integrated pest management program against this insect.

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