BIOLOGICAL CONTROL

Screening of Entomopathogenic Nematodes (Nemata: Rhabditida) and the Efficiency of *Heterorhabditis* sp. against the Sugarcane Root Spittlebug *Mahanarva fimbriolata* (Fabr.) (Hemiptera: Cercopidae)

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Avaliação de Nematóides Entomopatogênicos (Nemata: Rhabditida) e Eficiência de *Heterorhabditis* sp. Contra a Cigarrinha da Raiz da Cana-de-Açúcar *Mahanarva fimbriolata* (Fabr.) (Hemiptera: Cercopidae)

RESUMO - Este estudo teve por objetivo avaliar a patogenicidade de seis nematóides contra ninfas da cigarrinha da raiz da cana-de-açúcar *Mahanarva fimbriolata* (Fabr.), em condições de laboratório, e a eficiência do mais virulento no controle do inseto em condições de campo. No laboratório, foram avaliados sete tratamentos representados por dois espécimes do gênero *Heterorhabditis*, três de *Steinernema*, um espécime de *Steinernema glaseri* (Steiner), e a testemunha. No campo foram realizados dois experimentos. No primeiro foram avaliados nove tratamentos: *Heterorhabditis* sp. (CB-n5) aplicado sobre a palhada nas doses de $3,3 \times 10^9$, $6,6 \times 10^8$, $3,3 \times 10^8$ e $6,6 \times 10^7$ juvenis infectivos (JI)/ha; os nematóides aplicados no solo (embaixo da palhada) nas mesmas quatro doses; e a testemunha. No segundo experimento foram testados quatro tratamentos: *Heterorhabditis* sp. ($3,3 \times 10^8$ JI/ha); *Metarhizium anisopliae* ($2,6 \times 10^{12}$ conídios viáveis/ha); tiametoxam (Actara 200 WG) (1 kg/ha); e a testemunha. Em laboratório, *Heterorhabditis* sp. (CB-n5), *Steinernema* sp. (CB-n6) e *Heterorhabditis* sp. (CCA) foram os mais patogênicos, causando mortalidades de 100%, 98% e 96%, respectivamente. No primeiro experimento de campo, o nematóide *Heterorhabditis* sp. proporcionou até 70% de controle da cigarrinha, não havendo diferença significativa entre as doses e quanto à aplicação sobre a palhada ou sobre o solo. No segundo experimento, o inseticida proporcionou 67% de controle, não diferenciando significativamente do nematóide (56%) e do fungo (44%), em avaliação realizada sete dias após aplicação.

PALAVRAS-CHAVE: *Steinernema*, controle biológico, manejo integrado

ABSTRACT - The pathogenicity of six entomopathogenic nematodes was assessed against nymphs of the sugarcane root spittlebug *Mahanarva fimbriolata* (Fabr.), in the laboratory. The efficiency of the most virulent agent was tested in the field. Seven laboratory treatments were designed with two specimens of the genus *Heterorhabditis*, three of *Steinernema*, one specimen of *Steinernema glaseri* (Steiner), and a control group. In the field, two experiments were conducted. The first experiment involved nine treatments: the control, four treatments with *Heterorhabditis* sp. (CB-n5) applied to the straw mulch at doses 6.6×10^7, 3.3×10^8, 6.6×10^8 and 3.3×10^9 IJs/ha, and four treatments with the nematodes applied to the soil and beneath the straw mulch layer, at the same doses. The second experiment contained four treatments: the control and applications of *Heterorhabditis* sp. (3.3×10^8 IJs/ha), the fungus *Metarhizium anisopliae* (2.6×10^{12} viable conidia/ha), and the chemical insecticide thiamethoxan (Actara 200 WG) (1 kg/ha). *Heterorhabditis* sp. (CB-n5), *Steinernema* sp. (CB-n6), and *Heterorhabditis* sp. (CCA) were the three most virulent nematodes (100%, 98%, and 96% mortality, respectively). In the first field experiment, *Heterorhabditis* sp. controlled 74% of the insects, with no significant difference among doses or between application to the soil or mulch. In the second experiment, the chemical insecticide provided 67% control, not differing statistically from nematode (56%) or fungus (44%) control, seven days post-treatment.

KEY WORDS: *Steinernema*, biological control, integrated pest management

The sugarcane root spittlebug, *Mahanarva fimbriolata* (Fabr.), became an important sugarcane pest after 1995, with the expansion of the crop mechanical harvesting, which discards the cane leaves. The leaves cover the soil and provide a perfect environment for the development of *M. fimbriolata* nymphs that feed on root sap; this leads to a 40% decrease in sugar production (Dinardo-Miranda *et al*. 1999).

To control this insect, chemical insecticides and the fungus *Metarhizium anisopliae* (Metsch) Sorokin have been used and results have been varied. Both techniques have disadvantages: chemicals contaminate the environment and the unformulated fungus depends on weather conditions (Almeida *et al*. 2003, Dinardo-Miranda *et al*. 2003).

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been assessed as control of spittlebug pests of sugarcane since 1981 (Hunt 1981), but they were never evaluated under field conditions in Brazil. El-Kadi (1977) reported on a rhabditid nematode pathogenic to *M. fimbriolata*; however, this nematode was later identified as either *Protorhabditis* or *Caenorhabditis*, neither of which are pathogenic (Allard 1987).

Leite *et al.* (2002) were the first ones to assess the effectiveness of *Steinernema* sp. and *Heterorhabditis* sp. in controlling sugarcane root spittlebugs in Brazil; they reported 80% spittlebug mortality when working with a strain (CCA) of *Heterorhabditis,* in the laboratory.

The aims of this study were to evaluate the pathogenicity of five entomopathogenic nematodes against nymphs of spittlebugs in the laboratory and to test the efficiency of the most virulent agents in the field.

Material and Methods

Screening Trial. The seven treatments in this experiment included *Heterorhabditis* sp. (strain CB-n5), *Heterorhabditis* sp. (strain CCA), *Steinernema* sp. (strains CB-n6, CB-n7, and CB-n8), *Steinernema glaseri* (Steiner) (strain CCA), and a control group. The origin of each nematode is in Table 1. The nematodes are stored in the Coleção de Entomopatógenos of the Laboratório de Controle Biológico of the Centro Experimental do Instituto Biológico, Campinas, SP. Except for *S. glaseri,* species confirmed by Dr. Khuong Ba Nguyen (University of Florida, Gainesville, FLA), the nematodes were not identified yet.

The nematodes were reared in larvae of *Galleria mellonella* (L.) and were harvested as infective juveniles (IJs) by using the White trap method (White 1927). Each treatment had five replicates, and each replicate had ten 4-5th-instar nymphs of *M. fimbriolata* in a petri dish (10 cm diameter). The inoculum consisted of 2-ml nematode suspension containing 4,000 IJs applied to the filter paper lining at the bottom of the dish. The insects fed on a 5 cm-long cane stem placed in the petri dish. Nymphs obtained from a sugarcane field were placed in the dishes, which were sealed and incubated in the dark, at 24 ± 1°C.

The dishes were observed every two days and dead insects were counted. The dead insects from each petri dish were transferred to a different White trap, to determine the average number of IJs produced per insect cadaver for each nematode strain. The number of IJs was determined using a Peter counting slide, 30 days after preparing the White traps.

Field Trial. The nematode *Heterorhabditis* sp. (CB-n5), chosen for the field trial, was produced *in vitro,* in sponges soaked in liquid medium as described by Bedding (1984). The nematodes were harvested as IJs and then they were separated from the substrate by sieving the nematode culture through a 500-mesh sieve. The nematodes were washed, suspended in tap water, and counted, using a Peter slide.

In the first experiment, we evaluated the effect of different doses of *Heterorhabditis* sp. (CB-n5) and the importance of sugarcane mulch as a barrier to nematode action. The experiment was carried out on a sugarcane crop, variety RB 5486 (average plant height 2 m and rows 1.5 m apart), located in the municipality of Catanduva, SP, Brazil, from December 17, 2002 to March 01, 2003, one year after the second harvest. The sugarcane had been machine-harvested and a 10-15 cm layer of mulch (discarded leaves) was left on the soil. The experiment was conducted

Nine treatments were tested. Treatments were: the control group (no nematodes applied); four treatments with *Heterorhabditis* sp. applied to the straw mulch at the doses 6.6×10^7, 3.3×10^8, 6.6×10^8 , and 3.3×10^9 IJs/ha; and four other treatments, with the nematodes applied to the soil, beneath the straw mulch layer, at the same doses as above. Each treatment block consisted of a row of sugarcane divided into plots. Each plot was 1-m long and 2 m from adjacent plots. Each treatment was replicated seven times and each replicate was randomly distributed in a different row of

Table 1. Brazilian nematodes isolates used in the laboratory experiment.

Nematode	Collected from	Origin
Heterorhabditis sp. (CB-n5)	Soil from a citrus grove	Itapetininga, SP
Steinernema sp. (CB-n6)	Forest soil	Rio Claro, MS
Heterorhabditis sp. (CCA)	Soil from a corn field	Araras, SP
Steinernema sp. (CB-n7)	Forest soil	Rio Claro, MS
S. glaseri (CCA)	*Mygdolus fryanus* (Westwood) egg	Araras, SP
Steinernema sp. (CB-n8)	Soil from a sugarcane field	Catanduva, SP

sugarcane. The treated rows were 3-m apart and alternated with non-treated rows.

The nematode suspension (200 ml) was poured over the mulch and along the center of a 1-m sugarcane row. The volume corresponded to an output of 2,000 L/ha. The mulch was temporarily removed when the suspension was applied directly to the soil, following the same procedures as over mulch.

At the beginning of the experiment the spittlebug population was 27 nymphs per meter of sugarcane row, on average. Evaluations conducted 6, 12, 30, and 82 days after nematode application involved counting the nymphs on soil surface (sucking the root) along the 1-m sugarcane row and in the 30 cm-wide strip on either side of the row. The mulch was moved temporarily and carefully before counting the nymphs.

The second experiment was designed to assess the efficiency of *Heterorhabidits* sp. (CB-n5) and compare the nematode with the fungus *M. anisopliae* and the chemical insecticide thiamethoxan (Actara 200 WG). This experiment was conducted in the same region and conditions as the first experiment, from January 18 to 30, 2003, one year after the first harvest. The crop soil was covered with a thinner layer of mulch (5-10 cm) compared to the layer in the first experiment.

The four treatments in the second experiment consisted of *Heterorhabditis* sp. (3.3×10^8 IJs/ha), *M. anisopliae* (2.6×10^{12} viable conidia/ha - 3 kg of fungus plus rice substrate/ha), Actara 200 WG (1 kg/ha), and the control group (no treatment). The fungus strain CB-348, previously selected to control this insect, was isolated from *M. fimbriolata* nymphs found in Sertãozinho, SP. The experimental design was the same as for the first field experiment, although with nine replicates.

The nematode and the fungus were applied using a hand sprayer; the chemical insecticide was applied with a backpack sprayer. For all preparations, 200 ml (output of 2.000 L/ha) were sprayed on each side of the plot. The experiment started when the spittlebug population reached 25 nymphs/m of sugarcane row, on average. The evaluations were conducted seven days after the applications, using the same method as in the first field experiment.

Statistical Analysis. The data were analyzed by one-way analysis of variance (ANOVA) and the means compared by the Tukey´s studentized range test ($P < 0.05$), except for the last evaluation of the first field experiment (82nd day). In this case, the comparisons were done using Duncan´s multiple range test ($P < 0.05$). Infection rates were arcsine-transformed prior to analysis. All means were transformed back to the original units for presentation.

Results and Discussion

Screening Trial. *Steinernema* sp. (strain CB-n6), *Heterorhabditis* sp. (CB-n5), *Heterorhabditis* sp. (CCA), *Steinernema* sp. (CB-n7), and *S. glaseri* (CCA) did not differ significantly in their virulence to *M. fimbriolata* on the 5th evaluation day ($F = 14,8$; $P = 0,248$); the first three nematodes had mortality levels > 95% (Table 2). The five nematodes did not differ on the 2nd evaluation day ($F = 5.5$; $P = 0,059$); *Steinernema* sp. (strain CB-n6) killed the insects faster (54% mortality), and was followed by *Heterorhabditis* sp. (CB-n5) (40%) and *Heterorhabditis* sp. (CCA) (38%).

The nematodes reproduced in the host and the number of juvenile progeny from each cadaver was positively correlated with agents' virulence. The highest production by *Steinernema* sp. (CB-n6) (4,867 juveniles per cadaver) was not significantly different from production by *Heterorhabditis* sp. (CB-n5) and *Heterorhabditis* sp. (CCA) (4,844 and 3,928, respectively) ($F = 12.4$; $P = 0,915$).

The nematodes were highly virulent to spittlebug nymphs in the laboratory (Table 2); mortality reached 100% on the fifth day, as reported by Leite *et al.* (2002). The positive correlation between nematode virulence and number of juvenile progeny can reflect the higher number of IJs among the most virulent nematodes that penetrate hosts, resulting

Table 2. Mortality (± standard error) of *M. fimbriolata* nymphs on the 2nd and 5th days after insect inoculation with different entomopathogenic nematodes; and number (± standard error) of juvenile progeny generated by each nematode; (temperature: 24 ± 1°C).

Nematode	Mortality (%)		Number of juvenile progenies/cadaver
	2nd day	5th day	
Heterorhabditis sp. (CB-n5)	40.0 ± 8.36 ab	100.0 ± 0.00 a	4844.4 ± 1078.69 a
Steinernema sp. (CB-n6)	54.0 ± 5.09 a	98.0 ± 2.00 a	4866.8 ± 511.84 a
Heterorhabditis sp. (CCA)	38.0 ± 12.40 ab	96.0 ± 4.00 a	3928.3 ± 564.48 ab
Steinernema sp. (CB-n7)	22.0 ± 4.89 ab	92.0 ± 3.74 a	1533.3 ± 672.02 bc
S. glaseri (CCA)	26.0 ± 5.09 ab	84.0 ± 7.48 ab	1305.2 ± 491.56 bc
Steinernema sp. (CB-n8)	6.0 ± 4.00 b	50.0 ± 10.00 bc	438.5 ± 71.72 bc
Control	8.0 ± 5.83 b	22.0 ± 14.96 c	0.0 c

Means in a column followed by different letters are significantly different ($P \leq 0.05$; Tukey´s studentized range test).

in higher reproduction. Leite *et al.* (2002) observed a positive correlation between virulence and number of IJs that penetrate *M. fimbriolata* nymphs and argued that reproduction within the host was also influenced by nematode size, with larger individuals occupying more space and, consequently, producing fewer offspring. This can explain why *S. glaseri,* the largest species we studied, had fewer descendents.

The nematodes probably penetrated the host through the anus and spiracle because the spittlebug is a sucking insect. Aguillera (2002) found that the genus *Heterorhabidits* could also penetrate the host through the cuticle, by perforating it with its small teeth. Brown *et al.* (1992) tested *Steinernema carpocapsae* (Weiser) against edaphic populations of the woolly apple aphid, *Eriosoma lanigerum* (Hausmann), in the laboratory and found nematodes within the body cavity of several aphids. The nematodes may have entered through the anus, via a droplet of honeydew.

Field Trial. *Heterorhbditis* sp. (CB-n5) was selected for being the most virulent to spittlebug nymphs, along with *Steinernema* sp. (CB-n6) and *Heterorhabditis* sp. (CCA), and for being the only nematode grown *in vitro* in our laboratory, at the time of this study.

In the first field experiment, *Heterorhbditis* sp. provided 43-74% control on the 6th evaluation day; 56-73% on the 12th day; 52-63% on the 30th day; and 31-69% on the 82nd day (Figs. 1 and 2). There were no significant differences among doses or between the applications to soil or mulch at each interval ($F_{6th\ day} = 4.7$, $P = 0.302$; $F_{12th\ day} = 6.4$, $P = 0.935$; $F_{30th\ day} = 3.7$, $P = 0.85$; $F_{82nd\ day} = 1.6$, $P = 0.178$); all doses were different from the control in the two first evaluations ($F_{6th\ day} = 4.7$, $P < 0.001$; $F_{12th\ day} = 6.4$, $P < 0.001$).

In the second field experiment (Fig. 3), the nematode performed the second most efficient insect control (56%) after the chemical insecticide (67%), and was better than the fungus (44%); the differences, however, were not significant ($F = 5.5$, $P = 0.592$). All treatments, except for that with the fungus, differed from the control ($F = 5.5$, $P = 0.004$).

In the field trials, the rainfall immediately after the applications probably improved nematode conditions to react against the insect. In the first trial (Fig. 1), all tested doses provided similar levels of control, regardless of their interval. By the 12th day, the nymph population decreased by 56-67% when the nematodes were applied to the straw mulch; when applied to the soil beneath the straw layer, nymph population decreased by 66-73%. Therefore, the straw mulch did not affect nematode ability to reach the soil and find the insects. As reported in a previous study, straw mulch does not affect *H. marelatus* (Liu & Berry 1996) efficiency in controlling black vine weevil on strawberries (Wilson *et al*. 1999). *S. carpocapsae* can move 3.5 cm/day on bare soil and 7.5 cm in rye mulch-covered soil (Hsiao & All 1998); therefore, mulch can enhance the movement of some entomopathogenic nematodes in agricultural systems.

The nematodes applied to straw mulch led to higher control (48-69%) than when applied to soil (31-59%), on the 82nd evaluation day. Straw mulch seems to retain part of the nematode population and to provide appropriate

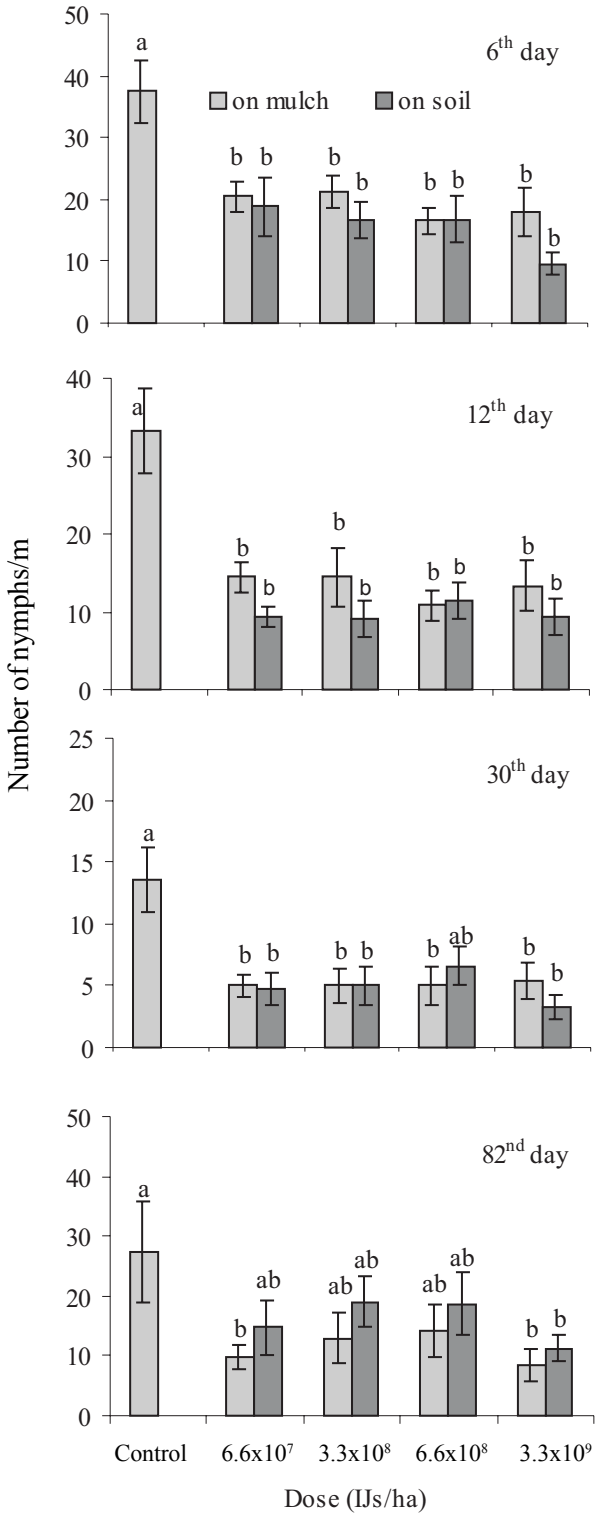

Figure 1. Population (± standard error) of *M. fimbriolata* nymphs in sugarcane plots treated with different doses of *Heterorhabditis* sp. (CB-n5), at different days after treatment. The same letters above the bars indicates no significant differences ($P \leq 0.05$, Tukey´s studentized range test).

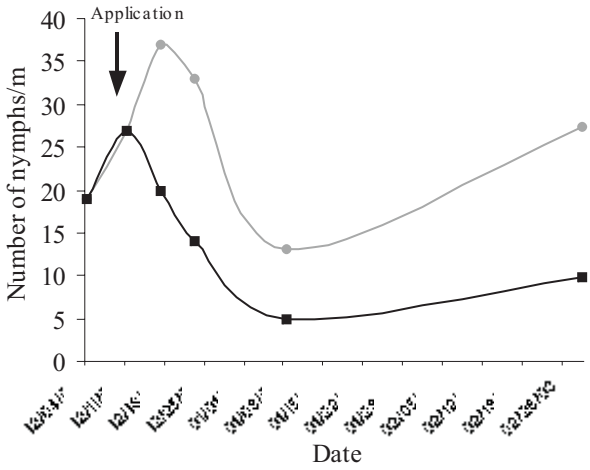

Figure 2. Population fluctuation of *M. fimbriolata* nymphs in sugarcane plots treated with 6.6 x 10^7 IJs/ha dose of *Heterorhabditis* sp. (CB-n5) (■). Control (♦).

conditions for nematodes to act against nymphs feeding on the soil-litter interface and on soil surface. Peat mulch increased the percentage of spruce cone maggot (*Strobilomyia neanthracina* Michelsen) larvae infected with *S. feltiae* (Filipjev 1934) (=*bibionis*) (strain 27), *S. feltiae* (strain Umea) and *S. carpocapsae* (strain All) when the maggots were placed on nematode-treated soil beneath the peat layer (Sweeney *et al.* 1998).

The lowest dose (6.6 x 10^7 IJs /ha) controlled the sugarcane root spittlebug more efficiently when we consider its lower cost and the non-significant differences from the other concentrations (Fig. 2). This is a very low dose compared to others used to control pests with entomopathogenic nematodes (Georgis 1990). The nematode suspension was poured along the center of a 1-m row, and then probably spread over a 20-30-cm wide strip on either side of each sugarcane row (150 cm), where most spittlebug

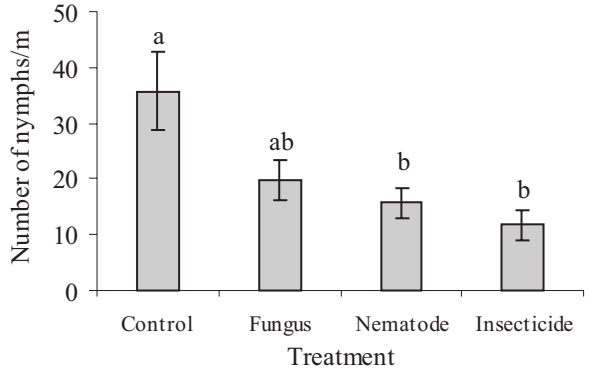

Figure 3. Population (± standard error) of *M. fimbriolata* nymphs in sugarcane plots treated with *Heterorhabditis* sp. (3 x 10^8 IJs/ha), *M. anisopliae* (2.6 x 10^{12} conidia /ha), and thiametoxan 200 WG (1 kg/ha), seven days after treatment. The same letters above the bars indicates no significant differences (P≤ 0.05, Tukey´s studentized range test).

nymphs were located. According to Kaya (1990), *H. bacteriophora* can disperse to 30-45 cm from the application site. Thus, each dose/ha provided a nematode concentration that was three to four-fold higher in the areas most infested with the insect. This implied that the lowest dose (6.6 x 10^7 IJs/ha) provided a concentration of 2-3 IJs/cm^2 (equivalent to 2-3 x 10^8 IJs/ha), which was lower than that of *H. indica* (11 IJs/cm^2), as recommended to control citrus root weevils in Florida (McCoy *et al*. 2002).

The narrow range of control rates obtained by means of the wide variety of doses in our research was also found in other studies with entomopathogenic nematodes. The nematode *H. bacteriophora* was evaluated against the burrowing bug, *Cyrtomenus bergi* Froeshner (Hemiptera: Cydnidae), and provided 40% to 70% infection at doses 200-4,000 nematodes/ml, respectively (Barberena & Bellotti 1998). The reason for the weak dose response is unknown and may be related to the fact that entomopathogenic nematodes are more strongly attracted by insects after becoming infected by conspecific nematodes. The poorly understood interactions between IJs parasites and the potential host make the interpretation of the dynamics of entomopathogenic nematodes' infection difficult (Lewis 2002).

The second field experiment (Fig. 3) confirmed the effect of *Heterorhabditis* sp. (CB-n5) in controlling the sugarcane root spittlebug with the dose 3.3 x 10^8 IJs/ha; the control (56%) was similar to that with the chemical insecticide (67%). The fungus *M. anisopliae*, which has been used as an alternative to control this insect, reduced the nymph population by 44%, similarly to the treatments above and the control. Indeed, this fungus controlled *M. fimbriolata* more efficiently in sugarcane crops 15-30 days after application (Loureiro 2004), with a small effect every seven days (the same time interval as used here).

None of the treatments reduced the insect population below the economic threshold (five nymphs/m of sugarcane row). Before we can decide on the most suitable of the three treatments, their cost and long-term, post-treatment performance, as well as their persistence in the environment need to be assessed. Spittlebug nymphs are usually found sucking the sugarcane root on soil surface and underground. In these habitats, the nymphs are easy targets for entomopathogenic nematodes, especially the genus *Heterorhabditis*, which has a tendency to penetrate the soil (Georgis & Poinar 1983).

Our promissing results are from two trials in which entomopathogenic nematodes were tested against the sugarcane root spittlebug under field conditions. Additional studies are needed to evaluate other nematodes and to screen new strains for finding new means to control the sugarcane root spittlebug.

Acknowledgments

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