

Effect of entomopathogenic nematodes on nymphs of *Aeneolamia albofasciata* and its persistence in sugarcane soils of Veracruz

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Abstract

The objective of this study was to evaluate the pathogenicity of nematodes, *Heterorhabditis* sp. (CPVG13) and *Steinernema* spp. (CPVC12, CPVC13) on the spittlebug, *Aeneolamia albofasciata* and determine its persistence in the soil of three sugar mills Potrero, Constanca and Motzorongo, of the state of Veracruz. In the first experiment the pathogenicity of the entomopathogenic nematodes was evaluated in nymphs of spittlebug, *A. albofasciata* of 3rd and 4th instar, 120 (infective juvenile) JI/nymph were applied on boxes with six cavities, previously covered with filter paper, each cavity contained a grass stolon (*Cynodon* sp.) and a nymph per cavity. In the second experiment, the persistence of entomopathogenic nematodes (NEPs) in soil was evaluated; the experiment lasted 70 days, with independent weekly evaluations, of 32 experimental units per week. The pathogenicity experiment revealed that both nematodes were able to cross the saliva that protects the nymphs, kill and develop in the corpses producing new progeny of JI. The highest mortality (62.5%) was observed with *Heterorhabditis* (CPVG13), also producing the highest concentration of JI per nymph (3500JI/nymph). Significant differences were found between the persistence profiles between *Heterorhabditis* (CPVG13) and *Steinernema* (CPVC12). A significant effect of the soil on the survival of both nematodes was found. The isolation that persisted most in soil was the *Steinernema* compared with that of *Heterorhabditis* (CPVG13). Deepening these studies, will allow to determine the impact of the interaction between spittlebug-NEPs and the soil.

Keywords: entomopathogenic nematodes, spittlebug, sugarcane.

Reception date: February 2019

Acceptance date: April 2019

Introduction

Spittlebug, *Aeneolamia albofasciata* (Lallemand) (Hemiptera Cercopidae), is one of the most damaging pests of sugarcane. It occurs in large areas of the Gulf of Mexico and coastal area of the Pacific Ocean (Flores-Caceres, 1994; Peck, 2001; Gómez, 2007; López-Collado *et al.*, 2013, Alatorre-Rosas and Hernández-Rosas, 2015). Alatorre-Rosas *et al.* (2013); Alatorre-Rosas and Hernández-Rosas (2015) mention that *A. albofasciata* has an altitudinal distribution ranging from 10 to 1 700 meters above sea level. The effects generated by feeding nymphs and adults cause water stress, delaying the growth of the plant, reducing biomass production (Dinardo-Miranda *et al.*, 2000; Valerio *et al.*, 2001; Alatorre-Rosas and Hernández-Rosas, 2015). *A. albofasciata* causes losses of up to 9 t ha⁻¹ of sugarcane (De la Cruz *et al.*, 2005; Alatorre-Rosas and Hernández-Rosas, 2015).

The nymphs appear a few days after the onset of the rains and last throughout the rainy season, with population peaks of nymphs and adults appearing staggered and overlapping. The newly hatched nymphs begin the production of saliva, which covers them completely, gives them shelter, serves as a defense against their natural enemies and at the same time protects against adverse weather conditions (Marshall, 1966; Whittaker, 1970; Bodegas, 1973; Alatorre-Rosas and Hernández-Rosas, 2015).

For the control of adults, it is recommended the use of insecticides in severe infestations and of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) (Torriello *et al.*, 2008; Hernández-Rosas *et al.*, 2013; Alatorre-Rosas and Hernández-Rosas, 2015). The use of *M. anisopliae* as a biological insecticide has been generalized in different cane areas, mainly for the regulation of adults (Flores-Caceres, 1994; Berlanga *et al.*, 1997; Badilla, 2002; Carballo and Falguni, 2004; Castillo-Zeno 2006; Bustillo and Castro, 2011; Alatorre-Rosas and Hernández-Rosas, 2015). To date, formulated in inert wettable powder with spores of *M. anisopliae* and native isolates of entomopathogenic nematodes for the regulation of populations of the nymph or spittlebug, because saliva is an effective barrier against natural enemies and other control strategies (Alatorre-Rosas and Hernández-Rosas, 2015; Grifaldo-Alcántara *et al.*, 2017).

The entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* have generated interest as a strategy of biological control of nymphs of different spittlebug species, in this sense the species *Steinernema* spp. and *Heterorhabditis* spp. against spittlebug, *Mahanarva fimbriolata* have achieved mortalities between 96 and 100% under laboratory conditions (Leite *et al.*, 2002). Field studies showed that *Heterorhabditis* spp. in doses of 5 x 10¹⁰ JI/h, it caused mortalities of 42.3% and with *H. bacteriophora* in doses of 1.5 x 10¹¹ JI/ha, mortality on nymphs of *A. varia* was 76% (Moreno *et al.*, 2012).

Abiotic factors can have a positive or negative effect on the performance, effectiveness and level of success of entomopathogenic nematodes in the control of insect pests. That is why the application of entomopathogenic nematodes can be unpredictable in natural conditions. In this sense (Kaya, 1990; Kaya and Koppenhöfer, 1996; Smith, 1996) mention the importance of taking into account the intrinsic factors: behavior and physiological, as well as temperature, humidity, soil texture and radiation (extrinsic), in addition to biotic factors such as competition, which affect its persistence.

For this reason, it is important to carry out this study with some of the indicators that allow to evaluate the type of soils, the existing biological diversity and determine the effect on the entomopathogenic nematode's permanence. The objective of this investigation was to evaluate the pathogenicity of native isolates of *Steinernema* spp. (CPVC12; CPVC13) and *Heterorhabditis* sp. (CPVG13) and its persistence in soil from three sugar mills in the state of Veracruz.

Material and methods

Susceptibility of nymphs of *A. albofasciata* towards entomopathogenic nematodes nymphs of *A. albofasciata*

The third and fourth instar nymphs of *A. albofasciata* were collected from field with cane cultivated in Atoyaquillo of sugarmill El Potrero, coordinates 18° 59' 38.74 North latitude and 96° 46' 50 West longitude. The nymphs were collected with the support of stainless-steel spatulas, were separated by gender and placed individually in plastic plates with cavities, which contained moist moistened paper toweling and stolons of star grass (*Cynodon* sp.). A preliminary test with grass stolons showed that rootlets of different lengths are formed in the area of the nodes, which remain active for several days (10-12). It was considered that if the nymphs could adhere to the rootlets and stay at least 4-5 days producing the excretion of saliva, it would constitute a favorable period for the trials proposed in this investigation.

The nymphs collected were transferred to the laboratory and kept under observation for 24 h, those that survived the management were selected. Subsequently, each nymph was confined individually in boxes Costar® (Corning Inc. NY USA) with 6 cavities, covered with moistened filter paper and a grass stolon (as food). During the experiment, the nymphs were kept at room temperature. The nymphs that survived and continued to produce saliva were used in the experiment, due to their adaptation to space and food exchange.

Entomopathogenic nematodes

The nematode isolates used for this research were obtained from larvae of *G. mellonella* that were used as bait insects in soil samples from three sugar mills in the state of Veracruz, Mexico (Central Progreso, Constancia and La Gloria). The experiments were carried out with two isolates of *Steinernema* sp. (CPVC12, CPVC13) and one of *Heterorhabditis* sp. (CPVG13) (Table 1). The morphological and molecular taxonomic determination of the isolates is currently in process and will be reported in a later article.

Table 1. Nematodes used for the pathogenicity test.

Treatment	UE (a plate)	Reply (plate)	Nymphs totals (Reply)	Repetitions in time	Total nymph/UEs	Total plates
1. <i>Steinernema</i> (CPV12)	1	4	24	2	48	8
2. <i>Steinernema</i> sp. (CPVC13)	1	4	24	2	48	8
3. <i>Heterorhabditis</i> (CPVG13)	1	4	24	2	48	8
4. Control (without nematodes)	1	4	24	2	48	8
Totals		16	96		192	32

The nematodes were propagated on last instar larvae of *G. mellonella* (L.) and harvested as infective juvenile (JI) by the White trap method (Kaya and Stock, 1997), sterilized with 0.01% benzalkonium chloride and two changes of sterile distilled water. The suspension of infective juvenile (JI) was centrifuged at 500 rpm for 5 min, in order to eliminate dead nematodes by decanting the supernatant. The nematodes were kept at room temperature in distilled water, oxygenating them with a fish tank pump continuously to reduce mortality. The concentration of JI in suspension was estimated using aliquots of 20 μL , using the average of 7 counts, adjusting the concentration required in the experiment.

Experimental procedure

The process of inoculation of the different isolates was the same (Table 1). Costar[®] plates with 6 cavities were used. Each cavity was covered with two layers of filter paper previously sterilized and moistened. In each cavity, a stolon (3.5 cm) of star grass (*Cynodon* sp.) where the formation of rootlets was induced, on these a nymph of *A. albofasciata* was placed. The inoculation was carried out applying a single concentration of infective juvenile, 120 JI cm^{-2} according to Alves *et al.* (2005). The application was made on the filter paper, allowing the displacement of the JI and search for the host insect.

The treatments were incubated at 25 ± 2 °C and relative humidity (74% min and 84% max) and photoperiod of 9 and 15 h dark-light. In the control, the nymphs were treated only with sterile water. The nymphs were reviewed every 48 h and mortality was determined by plaque taking into account the symptoms (red or beige color, flaccidity) of the nymphs. Each dead nymph was removed from the plate and placed in a humid chamber, which consisted of a Petri dish with moistened filter paper, the emergence of JI was determined by infected nymph. The JI were collected during a period of 4 to 5 days and the total JI was determined in 10 ml of the suspension collected by nymph. This value was taken as indicative of the reproductive capacity of the nematodes. Each treatment had four repetitions (four plates/24 nymphs) and the whole experiment was repeated on three different occasions. The experiment lasted 7 days after the application of infective juvenile. The entire laboratory phase was carried out under aseptic conditions.

Effect of soil with the persistence of entomopathogenic nematodes

Soil samples from 8 sites were selected, two belonging to the Constancia sugarmill, two to Central Motzorongo and four to El Potrero. The soil samples were taken in an area 30 x 30 cm long by wide and approximately 10 cm deep using a shovel. From each site thirteen samples (a total of 5 kg) were obtained and were stored at 4 °C for no more than two weeks. Composite samples were prepared, combining the 13 sub samples of each site by ingenuity.

To avoid the effect of native nematodes in the persistence experiment, soil sampling was carried out using the bait insect method with *G. mellonella* (Zimmerman, 1986). Each soil sample was moistened with sterile distilled water at field capacity (60%). Samples of 500 g of each locality were taken by placing them in plastic containers of 500 mL of capacity (experimental unit), seven larvae of *G. mellonella* of the last instar were individually confined in metal mesh cages and introduced in the containers with soil and distributed randomly. Each container was covered with

a lid with perforations to allow constant aeration, the vessels were inverted (Miduturi and Moens, 1997) and incubated in a breeding chamber at 25 ± 2 °C for 7 days, to confirm the presence of native nematodes. This procedure was repeated twice.

Cultivable microbial populations

Within the first week of collection, the natural populations of bacteria, fungi and actinomycetes of each soil sample were estimated. Samples of 10 g of soil from each location, sieved in No. 10 mesh, were placed in a 250 mL Erlenmeyer flask containing 90 mL of sterile distilled water. The samples were shaken for 18 minutes in an orbital shaker (Thermolyne Cimarec® 3), to subsequently prepare serial dilutions (Dhingra and Sinclair, 1985). A final dilution factor of 10^{-3} and 10^{-5} was considered adequate for fungal isolation, 10^{-4} and 10^{-6} for bacteria and actinomycetes 10^{-3} and 10^{-5} .

Planting was done by plaque extension in Petri dishes with nutritive agar (NA) for bacteria, potato dextrose agar (PDA) for fungi and Czapeck Dox agar with pH 8.0 for actinomycetes with three replications by dilution. Plates were incubated at 27 °C/5d, 7d and 10d for bacteria, fungi and actinomycetes, respectively. After the incubation period, the number of colony forming units (UFC) was estimated by selecting the dilution in each medium that favored the proper enumeration (10^{-3} for bacteria and fungi and 10^{-6} for actinomycetes). The UFC/gram of dry soil (air) was calculated by multiplying the arithmetic average of the number of UFC per plate by the dilution factor. The result was divided by the estimated dry soil weight. The physical and chemical characteristics of the soil were carried out by the Soil Fertility Laboratory of the Edaphology Program, Postgraduate School.

Experimental procedure

For this experiment, soil from El Potrero, Central Motzorongo and Constanica sugarmills were used, these were selected because the presence of nematodes was not recorded. Two isolates, CPVC12 (*Steinernema* spp.) and CPVG13 (*Heterorhabditis* sp.) were used. The procedure was the same for each type of soil and nematode evaluated. For each soil and nematode combination, 22 containers with 500 g of soil each were prepared, in the center of 11 of the containers, 5 000 infective juveniles were suspended, suspended in 2.5 mL of sterile distilled water (ADE), in the 11 remaining containers only 2.5 mL of ADE was deposited, these being the control treatment.

The survival of the nematodes was quantified by the proportion of larvae of *G. mellonella* infected when in contact with soil inoculated by nematodes. For this, in two containers, with and without nematodes, they were introduced in seven cages made of metal mesh in each container. Cages 1 cm in diameter by 4 cm in length contained a larva of *G. mellonella*. Seven days later, this procedure was repeated using two different containers (with and without nematodes) and so on at 14, 21, 28, 35, 42, 49, 56, 63 and 70d after inoculation of the nematodes. The experiment was incubated at 25 °C in total darkness. The larvae of *G. mellonella* were left in contact with the soil for 6 d and then they were removed from the soil and the number of dead larvae was quantified.

Each larva was placed in a cavity of a plate of six cavities containing moist filter paper, and incubated to the same experimental conditions. The presence of infective juvenile (JI) was determined by observations between 10 a 15d. The experiment was carried out under a completely randomized design, where each soil/nematode combination had two replicates, and the experiment was repeated twice (four repetitions in total).

The data were analyzed using logistic regression (assuming that the data had a binomial distribution), where each number of dead larvae was a proportion of the total larvae evaluated. When it was necessary, the presence of greater dispersion of the data under the assumption of the binomial distribution was allowed, comparing the ratio of the mean deviation of the treatments and the residual mean deviation against a F distribution, instead of comparing the deviation of the treatments with a distribution of χ^2 . Before comparing between treatments, the results obtained between repetitions in time were compared to be combined. With the combined repetition data, survival between nematode isolates, between the 4 soil types, between the evaluation times and finally the interaction between these factors was first compared. The analysis was carried out in the GenStat v. 8 (Payne *et al.*, 2005).

Results

Susceptibility of nymphs of *A. albofasciata* against *Heterorhabditis* sp. and *Steinernema* spp.

In general, differences were observed in mortality caused by *Steinernema* spp. (CPVC12; CPVC13) and *Heterorhabditis* sp. (CPVG13). The percentage of mortality (62.5%) was higher for *Heterorhabditis* sp. at 25 °C, compared to *Steinernema* (CPVC12) (56.25%) and *Steinernema* sp. (CPVC13) (33.33%). Although mortality was observed in the treatments, it was not possible to perform a statistical analysis, since the control treatment showed high mortality from other causes. However, this experiment showed that the isolates of both *Steinernema* and *Heterorhabditis* had the ability to cross the saliva produced by the nymphs, penetrate the insect cavity and cause infection (Figure 1b, 1c).

The dissections of the corpses of infected nymphs allowed to verify the reproductive capacity of the entomopathogenic nematodes (Figure 1d, 1e). The nymphs exposed to *Heterorhabditis* (CPVG13) and *Steinernema* sp. (CPVC12) showed differences in abundance of infective juvenile (Table 2). In the case of *Heterorhabditis*, the highest proportion corresponded to 3 500 JI per nymph (Table 2).

Table 2. Estimation of the number of infected nymphs and JI found in nymphs of *A. albofasciata*.

Species NEP/repetition	Infected larvae	Population of JI/nymph	
		Minimum	Maximum
<i>Steinernema</i> R1	13	4	1 500
<i>Steinernema</i> R2	14	7	1 500
<i>Heterorhabditis</i> 1	12	1 000	3 500
<i>Heterorhabditis</i> 2	18	10	3 500
S. (Constancy) 1	6	9	1 000
S. (Constancy) 2	10	4	1 000
Control	0	-	-

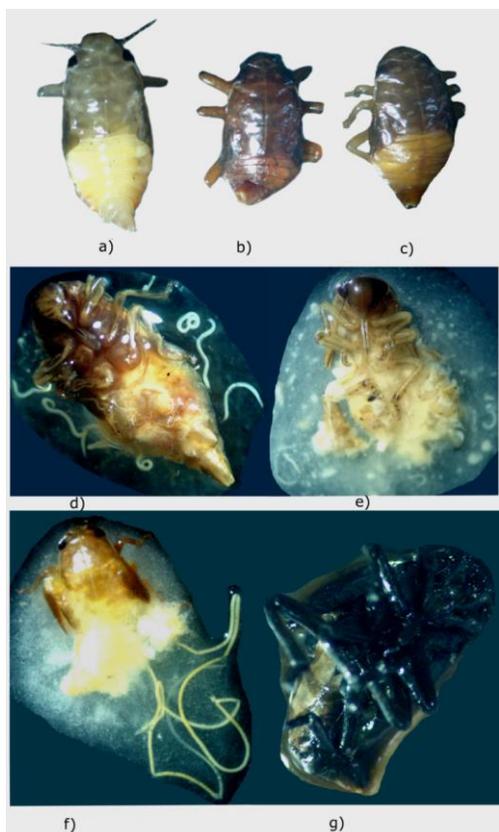


Figure 1. Spittlebug nymphs: a) healthy nymph; b) nymph infected by *Heterorhabditis*. (CPVG13); c) infected by *Steinernema* (CPV12). Dissections made to parasitized nymphs; d) adult nematodes of (CPVG13) *Heterorhabditis*; e) adult nematodes of (CPV12) *Steinernema*; f) meristemated nematodes; and g) nymphs with presence of necrosis, possible presence of bacteria.

Cultivable microbial populations

The largest population of bacteria and fungi was present in Constancia soil (3×10^9 UFC g^{-1} of soil and 1.5×10^4 UFC g^{-1} of soil for bacteria and fungi, respectively) (Figure 2a) while in Central Motzorongo the proportion of bacteria (3×10^8 UFC) and fungi (4×10^4 UFC g^{-1} of soil) was considered low (Figure 2b). The largest actinomycete population was found in site 2 of El Potrero plantation (7×10^5 UFC g^{-1} of soil) and the lowest population was obtained in Constancia (8.6×10^4 UFC g^{-1} of soil) and Motzorongo (1.7×10^5 UFC g^{-1} of soil) (Figure 2c). The physical and chemical characteristics of the soil (Table 3).

Table 3. Physical-chemical characteristics of the soil of three sugar mills.

ID	pH	CE	MO (%)	N*	P	K	Texture Bouyoucos
PotreroS1	4.8	0.32	1.7	0.09	41	0.4	Clay
PotreroS2	5.1	0.28	5.4	0.27	64	0.7	Clay
PotreroS3	6.4	0.26	3.3	0.16	12	0.3	Clay loam
PotreroS4	5.3	0.14	5.3	0.26	15	0.3	Clay

ID	pH	CE	MO (%)	N*	P	K	Texture Bouyoucos
ConstanciaS1	5.9	0.21	3.7	0.19	39	0.3	Clay loam
ConstanciaS2	6.5	0.16	5.3	0.26	31	0.4	Clay
MotzorongoS1	5.2	0.21	2.2	0.11	23	0.6	Clay loam
MotzorongoS2	6	0.23	1.4	0.07	18	1	Sandy clay loam

CE= electrical conductivity; MO= organic material; N= nitrogen; P= phosphorus; K= potassium.

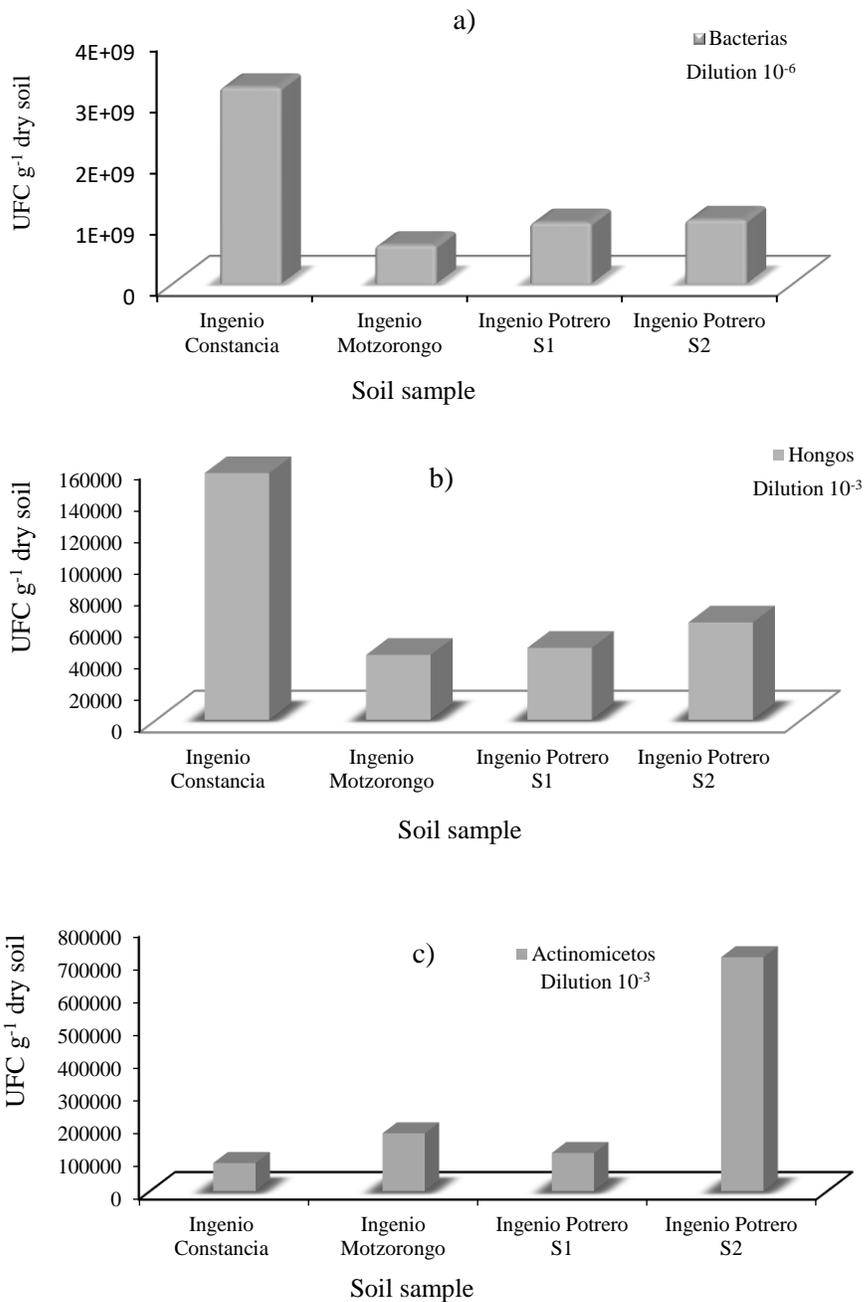


Figure 2. Colony forming units. a) bacteria; b) fungi; and c) actinomycetes, obtained from cane soil cultivated in the Constancia, Motzorongo plant and El Potrero sugarmills.

Effect of soil with the persistence of entomopathogenic nematodes

There were no significant differences between the data of the four repetitions ($\chi^2_3 = 1.3215$, $p = 0.427$), which allowed the data to be combined to make a joint analysis and see the effect of treatments. Significant differences were found between the persistence profiles between *Heterorhabditis* (CPVG13) and *Steinernema* (CPVC12) ($\chi^2_1 = 64.3496$ $p < 0.001$). The isolation that persisted the most was the (CPVC12) of *Steinernema* (Figure 3B) compared to the isolation (CPVG 13) of *Heterorhabditis*. A significant soil effect was found in the survival of both nematodes ($\chi^2_3 = 11.2522$, $p = 0.01$); however, this effect was different for each nematode isolation ($\chi^2_3 = 17.3337$, $p < 0.001$), where *Steinernema* at 8 weeks showed high proportion of dead insects indicating high persistence of the JI, while in the inoculations with *Heterorhabditis* the persistence of JI began to decline from the 5th week after the inoculation occurred. The time that the nematodes spent in the soil had a significant effect on their survival ($\chi^2_9 = 462.3188$, $p < 0.001$) and this effect was similar for the two species of nematodes ($\chi^2_9 = 13.9253$, $p = 0.125$).

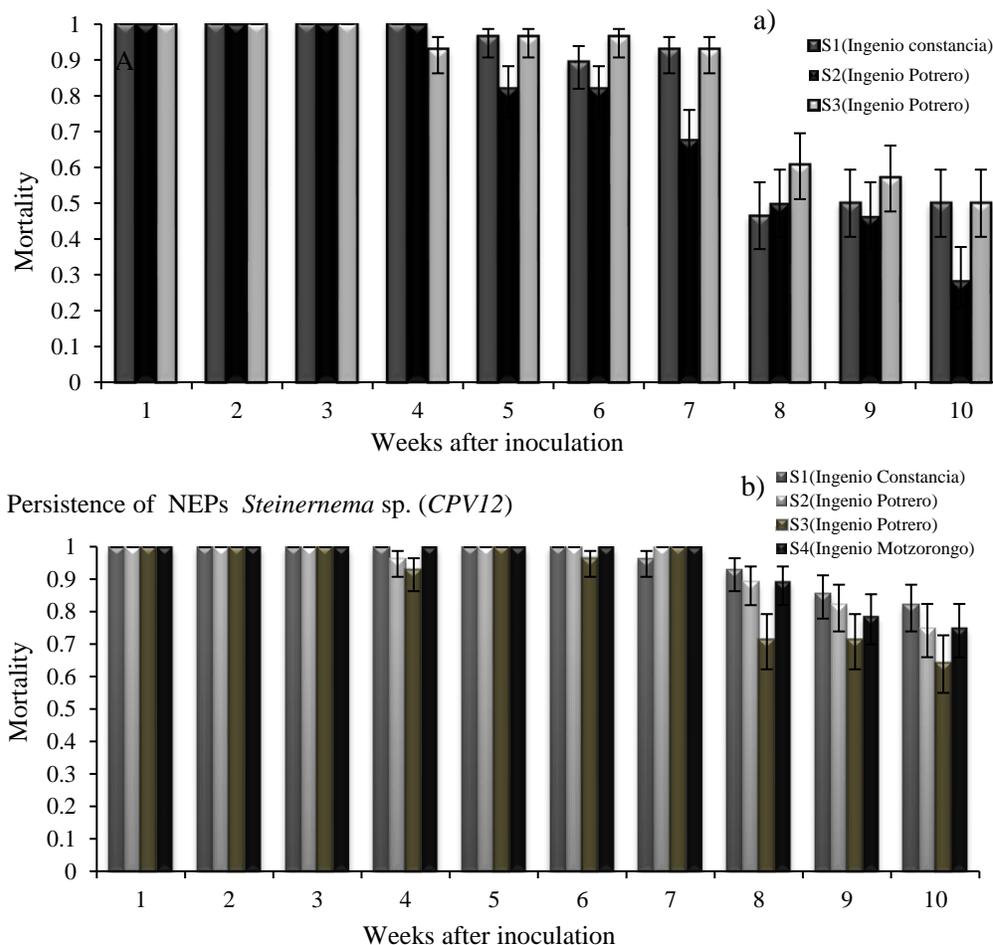


Figure 3. Proportions of mortality a) *Heterorhabditis* sp.; and b) *Steinernema* sp. in larvae of *G. mellonella*. The error bars represent confidence limits of 95%, transformed from the logistic scale. The persistence of JI constitutes the average value of dead larvae of *G. mellonella* by date of evaluation at 25 °C.

Discussion

These results show that isolates of entomopathogenic nematodes CPVC13; CPVC12 (*Steinernema*) and CPVG13 (*Heterorhabditis*) can infect nymphs of *Aeneolamia albofasciata*, being able to cross the physical barriers of the insect, mainly the foamy mass of saliva that serves them as protection. The infection capacity of the nematode isolates used can be confirmed by the reproduction of these in their host, especially by the CPVG13 isolation of *Heterorhabditis* (Table 2), as well as by previous reports of infection in other species of hemiptera under conditions of laboratory, greenhouse and field (Ferrer *et al.*, 2004; Leite *et al.*, 2005; Rosero- Guerrero, 2011; Morero *et al.*, 2012). There was a greater infection caused by *Heterorhabditis* compared to *Steinernema*, which could be due to the fact that Heterorhabditides have the ability to penetrate through the cuticle of the host with the help of a small tooth they possess (Bedding and Molyneux, 1982; Aguilera, 2001). The high mortality of *A. albofasciata* nymphs in the control was probably caused by pathogens brought from the field, as well as by the manipulation at the time of collection.

One of the factors of natural mortality observed in this investigation was the presence of mermitid nematodes of the genus *Hexamermis* sp., a phenomenon that has already been previously reported in *A. varia* (Poinar and Linares, 1985) and *Manhanarva fimbriolata* (Bennett, 1984). For this reason, it is considered important that, in future experiments, the biological material comes from colonies of insects kept under controlled conditions, thus avoiding high mortality during the bioassay and obtaining statistically reliable results.

In the experiment of persistence of entomopathogenic nematodes, significant differences were observed between treatments and nematode strains, which means that there was a decrease in the initial population of nematodes over time. In both *Steinernema* and *Heterorhabditis* the persistence of JI measured as the ability to kill bait insects over time, showed slight variations from the fourth week, in the case of *Steinernema* the mortality remained close to 100% until the seventh week; however, in the eighth week, the mortality ratio showed variations of 0.65 to 0.9 depending on the soil where it was incubated (Figure 3B). In the case of *Heterorhabditis*, the persistence of JI showed more evident variations in the following weeks. Just like *Steinernema*, from the eighth week there was a reduction in the persistence of JI, but in this case the fall was more drastic (0.25) (Figure 3B).

Heterorhabditis showed problems of persistence in the different types of soil (Figure 3A), in clayey soil, clay loam and sandy loam, with acidic to neutral pH (Table 3) *Heterorhabditis* showed a drastic drop in the mortality curve, which means that soil represents an important factor for the persistence of this entomopathogenic nematode (Kung *et al.*, 1990; Portillo-Aguilar *et al.*, 1999).

According to (Kaya, 1990; Kaya and Koppenhöfer, 1996; Epsky *et al.*, 1998) the interaction between soil microorganisms and entomopathogenic nematodes can affect the survival of the JI, affect the establishment and development of the symbiont and affect the insect once invaded by the nematode. However, in this study differences were found in the concentration of bacteria, fungi and actinomycetes present in the soil, which may be related to the persistence of entomopathogenic nematodes. The nematode vs microbiota interaction could affect the nematode strain differently,

causing greater mortality in *Heterorhabditis* (CPVG13). However, more in-depth studies are needed to corroborate the effect of soil-microbiota on the persistence and parasitic activity of entomopathogenic nematodes.

Conclusions

Isolates of entomopathogenic nematodes of the genus *Steinernema* and *Heterorhabditis* had the ability to infect nymphs (spittlebug) of *Aeneolamia albofasciata*, for their ability to cross the frothy saliva barrier that covers the nymph. In addition to showing biological effectiveness for its virulence to develop and reproduce in the host. In particular, by the genus *Heterorhabditis* (CPVG13).

The persistence of the isolates of *Steinernema* sp., as of *Heterorhabditis* sp., regarding JI in both cases was maintained, in the case of *Steinernema* at the seventh week the mortality was almost 100%, only that at the eighth week there was variations between 0.65 to 0.9 depending on the type of soil where it was maintained, however *Heterorhabditis* the persistence of JI was similar only that its sudden drop was highlighted by the mortality that was more pronounced and this was observed in both clayey soil, clay loam, and loam. sandy as in acidic to neutral pH.

Acknowledgments

To the National Council of Science and Technology (CONACYT) for the scholarship to Oscar Parada Dominguez. This study was funded by the Foundation Produce Veracruz AC, a national project: Design of a contemporary management program Integrated spittlebug in sugarcane (2012-2013). It was supported by Projects of Scientific Research and Technological Development 2013; through, of the trust No. 167304, Postgraduates College.

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