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An Assessment of *Steinernema rarum* as a Biocontrol Agent in Sugarcane with Focus on *Sphenophorus levis*, Host-Finding Ability, Compatibility with Vinasse and Field Efficacy

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Abstract: In Brazil, countless insect species attack and damage sugarcane (*Saccharum officinarum L.*), which is an extremely important crop since it is planted on more than 10 million hectares. Among these insects, the sugarcane billbug, *Sphenophorus levis* (Coleoptera: Curculionidae), is of great importance as the larvae open tunnels in the rhizome of the plant, causing high damage and losses. This insect is attracted mainly to vinasse, which is the liquid fraction generated from the alcohol production and discarded onto the sugarcane fields for fertigation. Toward a novel control method for *S. levis*, the native entomopathogenic nematode *Steinernema rarum* (Pam 25) was compared with *S. carpocapsae* (IL 1) and *Heterorhabditis bacteriophora* (HBEN01) in respect to their ability to search for larvae of two insect hosts (*Galleria mellonella* and *S. levis*) within the cane rhizome. The selected nematode *S. rarum* was also assessed for rate effects, its survival in vinasse and field efficacy to control sugarcane pests *S. levis, Hyponeuma taltula* (Lepidoptera, Erebidae) and *Leucothyreus alvarengai* (Coleoptera: Melolonthidae). *Steinernema rarum* exhibited superior virulence to *G. mellonella* and *S. levis* larvae inserted into the cane rhizomes (75–78% mortality) compared to *S. carpocapsae* (30–53%) and *H. bacteriophora* (18–28%). Vinasse affected *S. rarum* when infective juveniles were suspended in the liquid compost for more than 6 h but did not affect the nematode when kept on the straw and soil treated with the compost. *Steinernema rarum* tested at 1–3 × 10^8 infective juveniles/ha on the sugarcane field caused 74.1, 56.3 and 50.6% control of *S. levis*, *L. alvarengai* and *H. taltula*, respectively.

Keywords: sugarcane billbug; entomopathogenic nematodes; biological control; pest management

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1. Introduction

With more than 10 million cultivated hectares, sugarcane (*Saccharum officinarum* L.) is one of the main agricultural activities that drive the Brazilian economy, making Brazil the largest sugar producer and the second largest ethanol producer worldwide [1]. Among the producing states, São Paulo stands out with 55% of the total production in the country [1]. To reach this level, major investments have been made in technologies used in establishing and managing the crop, including strategies for integrated pest management. Even so, countless insects attack and damage sugarcane, and among the most important are the soil dwelling pests that feed on the root system.

The sugarcane billbug, *Sphenophorus levis* (Vaurie) (Coleoptera: Curculionidae), has assumed great importance in recent years, mainly because it occurs in areas of São Paulo

state and surrounding states [2]. The main damage to the crop is caused by the insect's immature phase, when the larvae open tunnels in the plant's rhizome, which can result in the death of 50 to 60% of tillers, losses in production of up to 30% and reduction in plant longevity [3]. This insect is attracted to and infests mainly the areas fertigated with vinasse, which is the liquid fraction generated from the alcohol production [4] and discarded onto the sugarcane fields.

To minimize losses, chemical insecticides have been used extensively in sugarcane pest management. However, the chemical insecticides tend to have low efficiency against *S. levis* because the larvae feed inside the roots and the adults remain buried or below the straw; therefore, the targets are hard to reach [3,5]. Thus, alternative strategies must be developed such as the use of entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* (Nematoda: Heterorhabditidae, Steinernematidae). These nematodes are effective in controlling various species of curculionid pests, including those in the genus *Sphenophorus* in the USA and Japan [6,7]. Both genera of nematodes exclusively attack insects and have similar life cycles, starting with an infective juvenile (IJ) that carries an entomopathogenic bacterium inside its intestine and releases the symbiont into the insect host's hemocoel [8]. The nematodes and symbiotic bacteria act together to overcome the insect's immune system, causing its death within 24 to 48 h [9]. Subsequently, the bacteria spread and multiply inside the insect and the nematode reproduces by feeding on both. When food is depleted, the nematode is induced into the IJ form and leaves the insect's cadaver in search of a new host [10].

The nematode *Steinernema brazilense* Nguyen et al. (isolate IBCB 06) was effective in controlling *S. levis* in greenhouse and field tests, causing 80% mortality of larvae within rhizomes and 60% mortality of adults in the field [2,11,12], and with gains in yield of up to 17 tons of cane per ha [3]. Despite these results, *S. brazilense* may not be the most suitable nematode for a biological control program as it is a relatively large species (1157.2 µm) [13] and therefore generates lower yields in commercial production processes compared to smaller nematodes, such as *S. rarum* (Doucet, 1986) Mamiya, 1988 (511 µm) [14]. In addition, little is known about the effect of vinasse on EPN populations after its application to the field for sugarcane fertigation, which is a very common practice in Brazil.

Thus, the present study aimed to assess the nematode *S. rarum* (isolate Pam 25), a native nematode found in South and North America [15–17], regarding its ability to search for insects within the cane rhizome, its performance at different application rates, survival in vinasse and field efficacy to control *S. levis* as well as the other sugarcane soil-inhabiting pests, *Hyponeuma taltula* Schaus (Lepidoptera, Erebidae, Herminiinae) and *Leucothyreus alvarengai* (Frey) (Coleoptera, Melolonthidae, Rutelinae).

2. Material and Methods

For all the experiments conducted under laboratory and greenhouse conditions, nematodes were produced in vivo using *Galleria mellonella* (L.) (Lepidoptera, Pyralidae) as a host [18]. For the field experiments, nematodes were produced in vitro by the sponge process [19].

2.1. Ability to Search for the Host

The nematode *S. rarum* (Pam 25), isolated from soil samples of the Pampa biome (31°42′1.5′′ S 54°14′9.8′′ W) located at Aceguá, RS, Brazil, was compared with *S. carpocapsae* (Weiser) (IL 1), isolated from a population of larvae and pupae of *S. levis* in a cane field (21°54′38″ S 46°54′34″ W) at São João da Boa Vista, SP, Brazil [20], and with *H. bacteriophora* Poinar (HBEN01), commercially available in Brazil, with respect to the nematodes' ability to search for larvae of two insect hosts (*G. mellonella* and *S. levis*) within the cane rhizome.

2.1.1. *Galleria Mellonella* as an Insect Host

For this study, *G. mellonella* larvae were used within the cane rhizome to simulate the behavior of *S. levis* larvae. The use of *G. mellonella* as an alternative host was due to the great

difficulty of rearing *S. levis* in the laboratory, or to obtain its larvae in sufficient quantities. There were eight treatments: the three nematodes applied to insect larvae inserted into the cane rhizome (partially buried in the soil), the three nematodes applied to the larvae just buried in the soil and the two controls consisting of larvae held inside the rhizome or larvae buried in the soil (without EPNs applied). Each treatment had 4 replications. Each replication was represented by a plastic pot (1.8 L, 14 cm diameter) containing 900 mL of fine sand (10% moisture) plus 4 insect larvae.

For the treatments with the cane rhizome, *G. mellonella* larvae were held in small metal cages (3 cm long) and introduced individually into 10 cm long sugarcane rhizomes, through a 5 cm deep and 1.5 cm wide hole drilled at one of the sectioned ends of the rhizome. One larva was inserted per rhizome. After the insertion of the larvae, the holes were filled with cane fibers (simulating the frass left by *S. levis* larvae) and the rhizomes were buried 5 cm deep in the sand (10% moisture) with the holes facing the soil (Figure 9). Four rhizomes were buried per pot. This methodology was devised considering that *S. levis* penetrate into the cane rhizome, which allows EPNs to enter the tunnels caused by the insect's larvae and, consequently, reach the host [20].

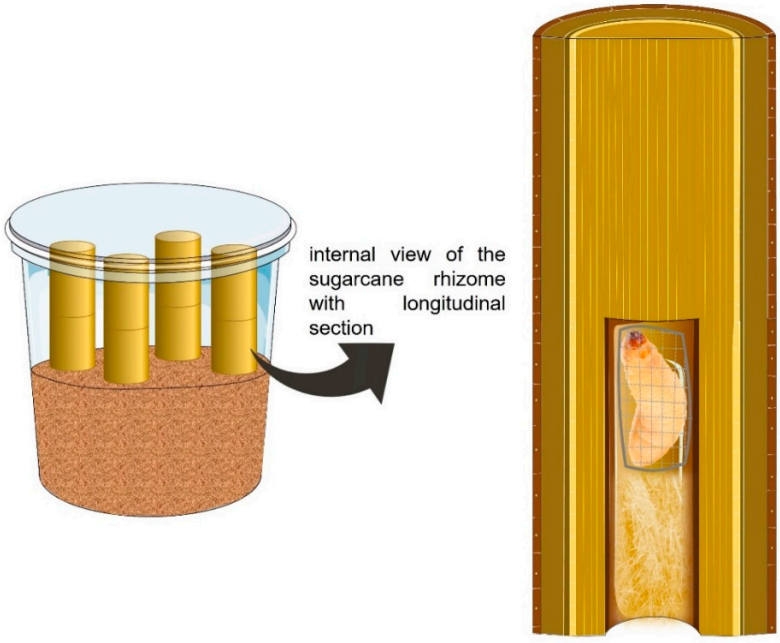

Figure 1. *Galleria mellonella* larvae held inside a metal cage and inserted inside a cane rhizome partially buried in the soil.

To verify the natural ability of the three EPN species to find the host in the soil, *G. mellonella* larvae were individually held in the cages and buried superficially. For both situations (larvae inserted in the rhizome and buried in the soil), the nematodes were tested at a rate of 100 IJs/insect (2.6 IJs/cm^2), which was applied with the aid of a pipette at a volume of 5 mL/pot. The pots were incubated at 25 °C and with a 12-h light–dark period, and larval mortality was assessed one week after application. Dead larvae were transferred to Petri dishes (9 cm) over a filter paper slightly moisturized with water to confirm the cause of death, if by the nematodes, characterized by nematode reproduction. The experiment was conducted twice, maintaining the same conditions for each trial.

2.1.2. *Sphenophorus levis* Larvae as a Host

For this study, *S. levis* larvae collected from the sugarcane field, at 2°/3° instar, were used as the host. The methodology used was the same as mentioned above using *G. mellonella*, except that the *S. levis* larvae were held freely inside the rhizome, not

inside a cage as used for *G. mellonella*, in order to allow the insect to feed on the inner tissue of the rhizome.

There were eight treatments: the three nematodes applied to insect larvae inserted into the cane rhizome (partially buried in the soil), the three nematodes applied to the larvae just buried in the soil and the two controls consisting of larvae held inside the rhizome or larvae buried in the soil (without EPNs applied). Each treatment had four replications. Each replication was represented by a plastic pot (1.8 L, 14 cm diameter) containing 900 mL of fine sand (10% moisture) plus four insect larvae

For the larvae buried in the soil, insects were held individually inside cages containing a small piece of stem for feeding. The experiment was conducted twice, maintaining the same conditions for each trial.

2.2. Performance at Different Application Rates

This study assessed the ability of *S. rarum* (Pam 25) to search for the host inside the rhizome at five different rates. The experiment was conducted under greenhouse conditions with the goal of estimating the best rate to control *S. levis* populations in sugarcane fields. For this study, as for the previous one, *G. mellonella* larvae were used as a model host. The experiments were conducted in basins with 40 cm diameters, which represented 40 cm of cane row.

Two experiments were conducted. One with larvae of *G. mellonella* held individually in metal cages and inserted into the cane rhizome partially buried in the soil; and another with larvae of the insect held individually in the cages and buried in the soil. For each experiment, there were five treatments represented by the five nematode rates: 125, 500, 2000, 8000 and 32,000 IJs/40 cm of row; therefore, rates were represented as number of IJs per 40 cm of cane row. A non-treated control was also included. Each treatment had four replications, with each replication consisting of a plastic basin (40 cm diameter × 20 cm depth) containing 8 kg of fine sand (10% moisture) plus five insect larvae. Thus, five rhizomes were partially buried per basin (Figure 2).

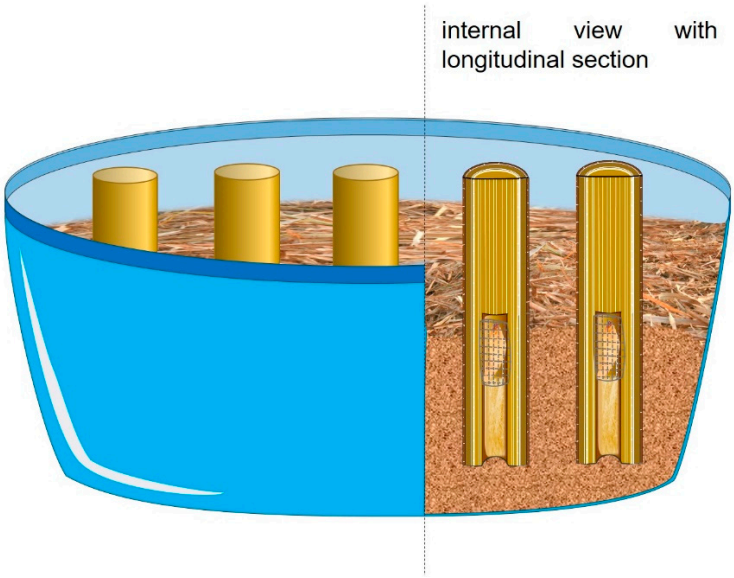

Figure 2. Basin containing 5 rhizomes with *Galleria mellonella* larvae held inside, partially buried in the soil.

The rest of the methodology was the same as used in the previous study, except the insects inside and outside the rhizomes were arranged in a row along the diameter of the basin, 5 cm apart; the nematodes were applied in a volume of 10 mL next to both sides of the row; and, after application, the soil was covered with cane straw (10 cm thick) to simulate conditions of sugarcane fields. The experiment was conducted twice, maintaining the same conditions for each trial, with temperature ranging from 20 to 28 °C.

2.3. Survival in Vinasse

This study aimed to assess the compatibility of vinasse with the nematode *S. rarum* (Pam 25). There were six treatments: liquid vinasse, cane straw moistened with vinasse, sand moistened with vinasse, cane straw moistened with water, sand moistened with water and just water. Vinasse was obtained at the Company Abengoa, São João da Boa Vista, SP.

Each treatment had three replications, each replication consisting of a cylindrical plastic pot (500 mL) containing the nematode plus the substrate. For straw treatments, 20 g of straw was dipped in 500 mL of water or vinasse until the liquid was absorbed (80 mL). Subsequently, the excess liquid was discarded, and the moisturized straw was transferred into the pots. For sand treatments, 80 g of sand was moistened with water or vinasse until reaching field capacity. The sand with a final moisture content of 17% was then transferred to the pot. For treatments without straw and sand, 50 mL of water or vinasse was added to the pots.

S. rarum was applied to each substrate in a 2 mL suspension containing 10,000 IJs. The pots were capped and incubated in a chamber with controlled temperature (25 °C). Evaluations were carried out after 3, 6, 12, 24 and 48 h, as well as after 4, 8, 15 and 30 days, based on 3 samples of about 0.5 to 1 g, taken at random from each pot. Each sample was transferred to a Petri dish containing 10 mL of water, and the IJs found were evaluated whether alive or dead to determine viability. For straw and sand substrates, the samples were slightly shaken/stirred with the help of a spatula to force the release of the IJs into the water and allow their visualization separately from their substrates. At least 100 IJs were counted randomly per sample.

2.4. Field Test I—Efficacy of Nematodes

This study aimed to assess combinations between *S. brazilense* (IBCB 06) (isolated from soil samples collected in Porto Murtinho, MT, Brazil, 22°10′16″ S, 57°07′ W) and *S. rarum* (Pam 25) for the control of *S. levis* and *H. taltula* populations in a sugarcane field. The experiment was carried out in a sugarcane field (variety CTC 20) (21°53′56.53″ S, 46°57′35.48″ W) fertigated with vinasse, with high levels of *S. levis* and *H. taltula* infestations in the previous ratoon. Fertigation began on 1 September 2017, and nematodes were applied three days after fertigation when the cane plants were 0.3 m high, after the second ratoon.

There were three treatments including *S. brazilense* alone, *S. rarum* alone, a mixture of the two nematodes and a non-treated control group. Each treatment and control had six replications with each replication consisting of a 100 m long plot with three rows of cane (spaced 1.5 m apart). The plots were spaced laterally from each other by another 3 rows of cane, thus being 4.5 m apart. Therefore, each plot was 450 m^2, comprising a total experimental area of 10,800 m^2.

The nematodes were applied at 1–2 cm depth in the soil of the cane rows, with the aid of three-row disc cutters, coupled to a tractor, covering the entire plot in each pass of the tractor. The nematodes were applied at a rate of 2×10^8 IJs/ha, and for the mixture of the two species, each was used at half the rate. This rate was chosen based on a previous study with *S. brazilense* [3] and considering that rates higher than 3×10^8 IJs/ha are cost-prohibitive for sugarcane farmers. Evaluation was carried out 21 days after the application by removing 10 cane clumps per plot, each spaced 10 m apart. To remove clumps, trenches of 0.5 × 0.5 × 0.5 m were made. After the removal of each clump, live specimens of *S. levis* and *H. taltula* found within the cane rhizomes or in the soil around the roots of the plants (larvae, pupae and adults) were counted.

2.5. Field Test II—Rate Effects

The goal of this study was to assess the effect of two rates of *S. rarum* on the control of *S. levis* and *L. alvarengai* in a sugarcane field. Two identical experiments were carried out in two closed areas of a sugarcane field (variety CTC 20), 400 m apart (21°55′17.24″ S, 47°1′20.75″ W), with high levels of *S. levis* and *L. alvarengai* infestations in the previous ratoon. The first experiment was set up on 25 September and the second on 26 September

2019, when the cane was about 0.3 m high, after the third ratoon, three months after the last harvest.

For each experiment, there were three treatments which consisted of two rates of *S. rarum* (1×10^8 IJs/ha and 3×10^8 IJs/ha), and the insecticide fipronil (Regent 800WG) at 1.2 L/ha; a non-treated control was also included. Each treatment and the control, in each experiment, had 4 replications, with each replication consisting of a 100 m long plot with 3 rows of cane (1.5 m between rows). The plots were spaced laterally from each other by another 3 rows of cane; thus, rows were 4.5 m apart from each other. Therefore, each plot was 450 m^2, comprising a total experimental area of 14,400 m^2.

The nematode and the chemical insecticide were applied at 1–2 cm deep in the soil of the cane rows, with the aid of three-row disc cutters, coupled to a tractor, covering the entire parcel in each pass of the tractor. Evaluations were carried out 21 days after each application by removing six cane clumps per plot, spaced 15 m apart. For the removal of clumps, trenches of 0.5 × 0.5 × 0.5 m were made. After the removal of each clump, the live insects of *S. levis* (larvae, pupae and adults) found inside the rhizomes of the cane or in the soil around the roots of the plants, and larvae of *L. alvarengai* found in the soil, were counted.

2.6. Statistical Analyses

The first two experiments with *G. mellonella* and *S. levis* larvae (effect of nematode species and insect placement) were conducted as a factorial with the effects of nematode species and insect placement as the main factors. If the effects of nematode species and insect placement were found to act independently (no interaction detected between them), then the analysis focused solely on these main effects, and simple effects were not elucidated further [21].

For the experiment in the greenhouse (testing different rates of *S. rarum* on *G. mellonella* larvae buried in the soil as well as those held inside the rhizome), data were analyzed by logarithmic regression between rate and mortality.

For all experiments, except the one in the greenhouse (testing different rates of *S. rarum* on *G. mellonella* larvae buried in the soil as well as those held inside rhizome), treatment effects were detected using analysis of variance (ANOVA). If the F value was significant, then treatment differences were further elucidated with Tukey's test ($p < 0.05$). Numerical data (insects' populations) were square root transformed prior to analysis, and percentage data were arcsine transformed (arcsine of the square root) [22]; nontransformed means are presented in the Section 3.

All statistical comparisons were conducted using SPSS version 16.0 software, with a p-value of ≤ 0.05 indicating significance.

3. Results

3.1. Ability to Search for the Host

The three nematodes caused significantly higher mortality of *G. mellonella* and *S. levis* larvae for the insects buried in the soil than those held inside the rhizomes (*Galleria*: $F_{1,56}$ =68.64; $p < 0.001$) (*Sphenophorus*: $F_{1,56} = 55.71$; $p < 0.001$). For *G. mellonella* and *S. levis* larvae buried in the soil (outside the rhizomes), the three nematodes provided more than 90% mortality, not differing significantly from each other, but differing from the buried control (*Galleria*: $F_{3,56} = 55.32$; $p < 0.001$) (*Sphenophorus*: $F_{3,56} = 59.56$; $p < 0.001$). For *G. mellonella* larvae held inside the rhizome, *S. rarum* exhibited the highest virulence, providing 75% insect mortality, which differed from the other nematodes tested on the insects inside the rhizome and from the control ($F_{3,56} = 21.21$; $p < 0.001$) (Figure 3).

For *S. levis* larvae held inside the rhizome, *S. rarum* also exhibited the highest virulence (78.1%), though not differing from *S. carpocapsae* (56.2%), but differing from *H. bacteriophora* (28.1%) and the control (3.1%) ($F_{3,56} = 28.96$; $p < 0.001$) (Figure 4).

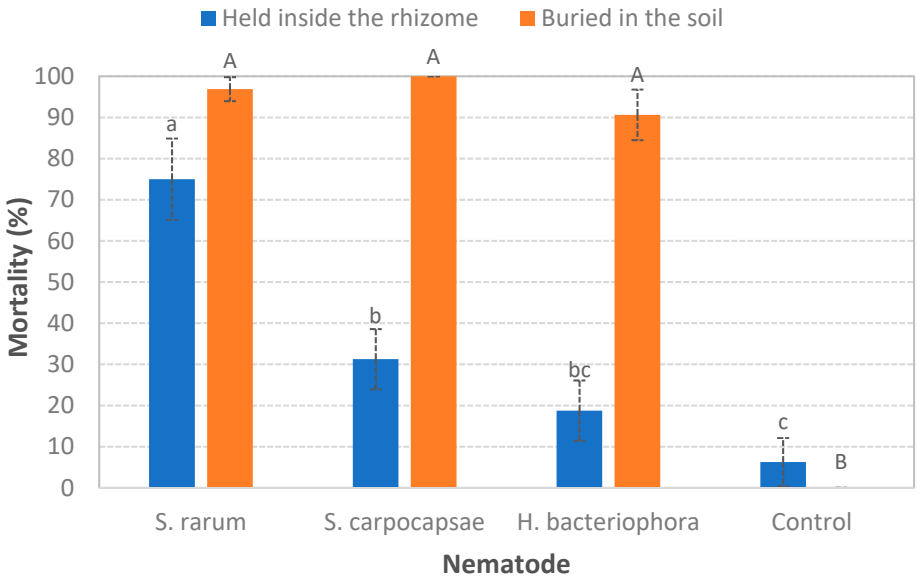

Figure 3. Mortality of *Galleria mellonella* larvae caused by entomopathogenic nematodes seven days after application. Held inside the rhizome = larvae held inside the cane rhizome. Buried in the soil = larvae buried in the soil, outside the cane rhizome. Means followed by different upper- or lower-case letters differ significantly within categories according to Tukey's test ($p < 0.05$).

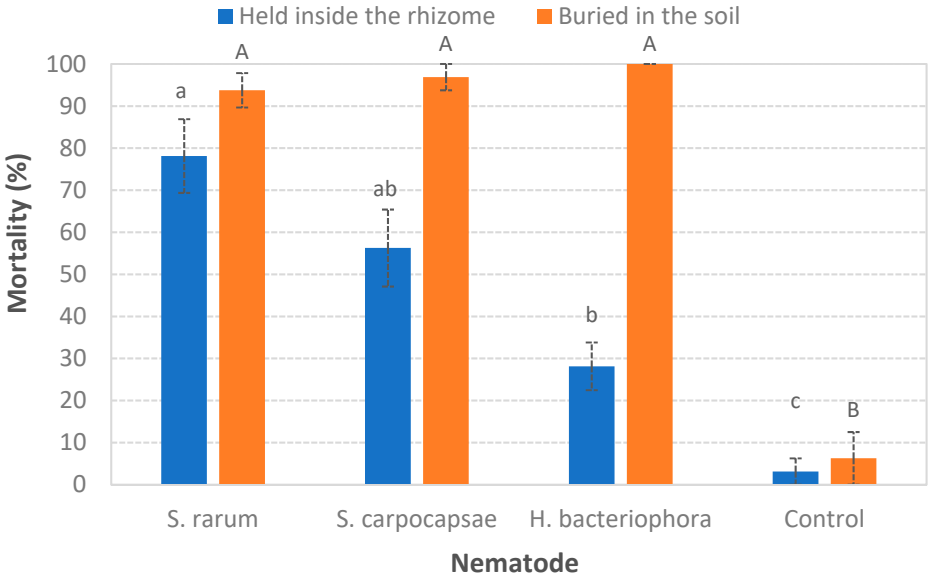

Figure 4. Mortality of *Sphenophorus levis* larvae caused by entomopathogenic nematodes seven days after application. Held inside the rhizome = larvae held inside the cane rhizome. Buried in the soil = larvae buried in the soil, outside the cane rhizome. Means followed by different upper- or lower-case letters differ significantly within categories according to Tukey's test ($p < 0.05$).

3.2. Performance at Different Application Rates

For *S. rarum* (Pam 25), the rates of 125 to 32153 IJs/40 cm of row generated larval mortality of *G. mellonella* from 30 to 95% for the insects buried in the soil (Figure 5), and from 12 to 72% for those inserted in the rhizome hole (Figure 6). These results indicate a significant logarithmic trend in both situations (R = 0.9796 and 0.9473, respectively).

3.3. Survival in Vinasse

After thirty days of exposure to different treatments, *S. rarum* had viability above 80%, except when suspended in pure vinasse, where viability dropped to less than 80% after

6 h of exposure, and to 0% after 4 days. The reduced viability in pure vinasse differed significantly from the other treatments ($F_{14, 385} = 5.53$; $p < 0.001$) (Figure 7).

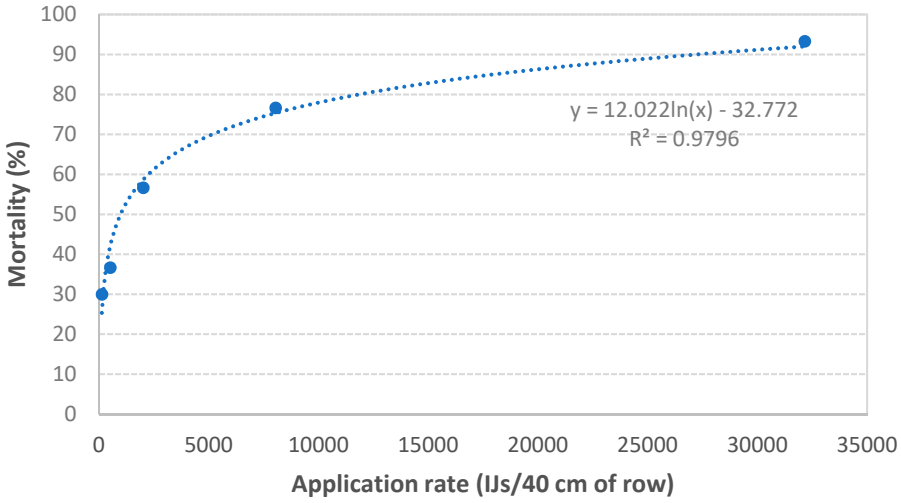

Figure 5. Mortality of *Galleria mellonella* larvae buried in the soil, by *Steinernema rarum* (Pam 25) at rates of 125, 502, 2009, 8038 and 32,153 IJs/40 cm of row, two weeks after the application.

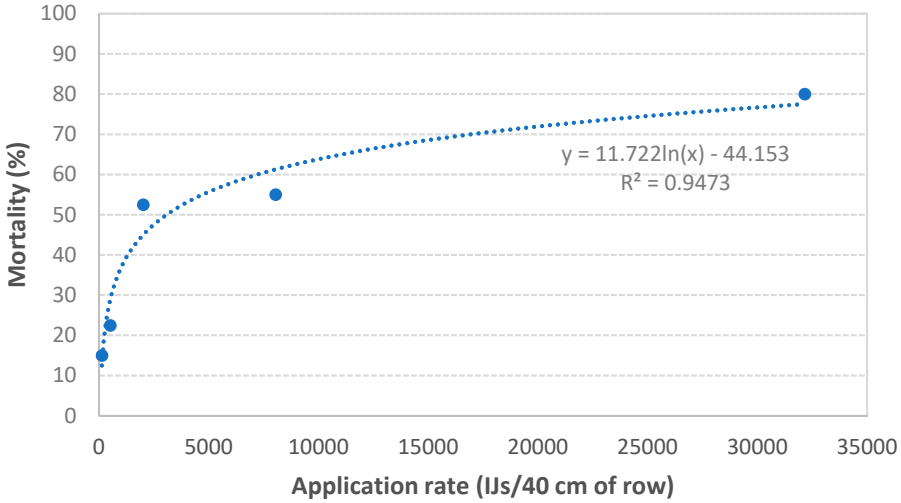

Figure 6. Mortality of *Galleria mellonella* larvae within the cane rhizome, by *Steinernema rarum* (Pam 25) at rates of 125, 502, 2009, 8038 and 32,153 IJs/40 cm of row, two weeks after the application.

3.4. Field Test I—Efficacy of Nematodes

The nematode *S. brazilense* provided 55.6% control of both *S. levis* and *H. taltula*, while *S. rarum* provided 74.1% and 50.6%, respectively, with all the insect populations of all treatments differing significantly from the respective controls (*Sphenophorus*: $F_{3,23} = 8.145$; $p < 0.001$) (*Hyponeuma*: $F_{3,23} = 5.754$; $p = 0.005$) (Figure 8). The mixture of both nematodes caused 48.1% and 54.3% control of *S. levis* and *H. taltula*, respectively, with their insect populations differing only from their respective controls, but not from the nematodes tested alone.

3.5. Field test II—Rate effects

Twenty-one days after the application of *S. rarum* against *S. levis* and *L. alvarengai*, the nematode provided 60.1% and 56.3% control, respectively, at the lowest rate (1×10^8 IJs/ha), and 71.5% and 58.4%, respectively, at the highest rate (3×10^8 IJs/ha), with no significant difference between the rates for each insect population, but all nematode treatments were different from the controls (*Sphenophorus*: $F_{3,47} = 9.715$; $p < 0.001$) (*Leucothyreus*: $F_{3,47} = 8.29$;

$p < 0.001$), except for *L. alvarengai*, whose lowest dose did not differ from the control. The insecticide fipronil, on the other hand, was not effective and did not differ from the controls ($p = 0.063$) (Figure 9).

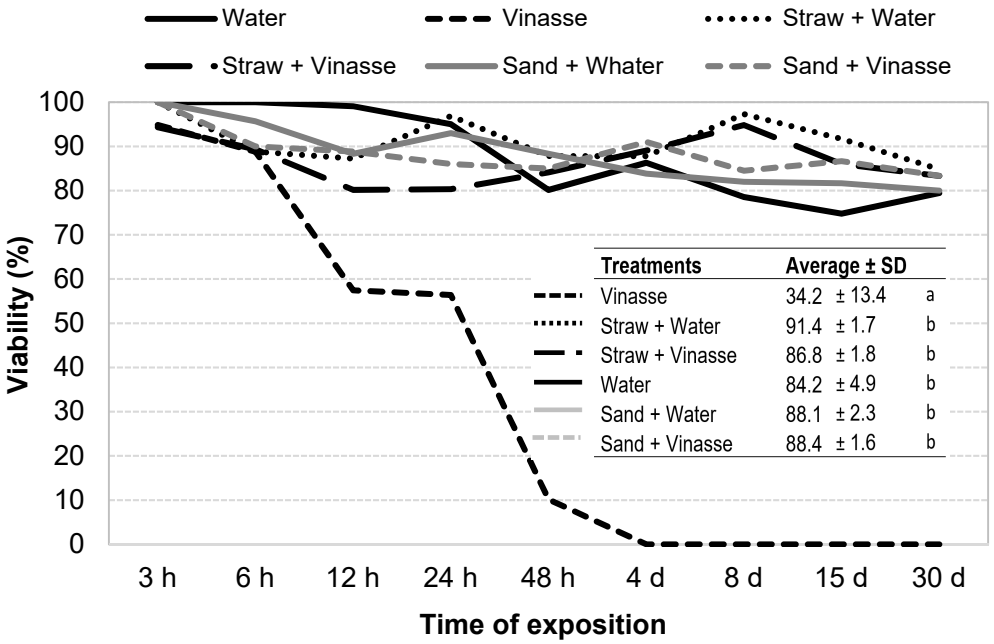

Figure 7. Viability of infective juveniles of *Steinernema rarum* (Pam 25) exposed to liquid vinasse; straw + vinasse; soil + vinasse; water; straw + water; and soil + water, during periods in hours (h) and days (d). The table shows means for viability over the entire experimental period (repeated measures) (n ± standard error). Means followed by different letters within columns differ significantly based on Tukey's test ($p < 0.005$).

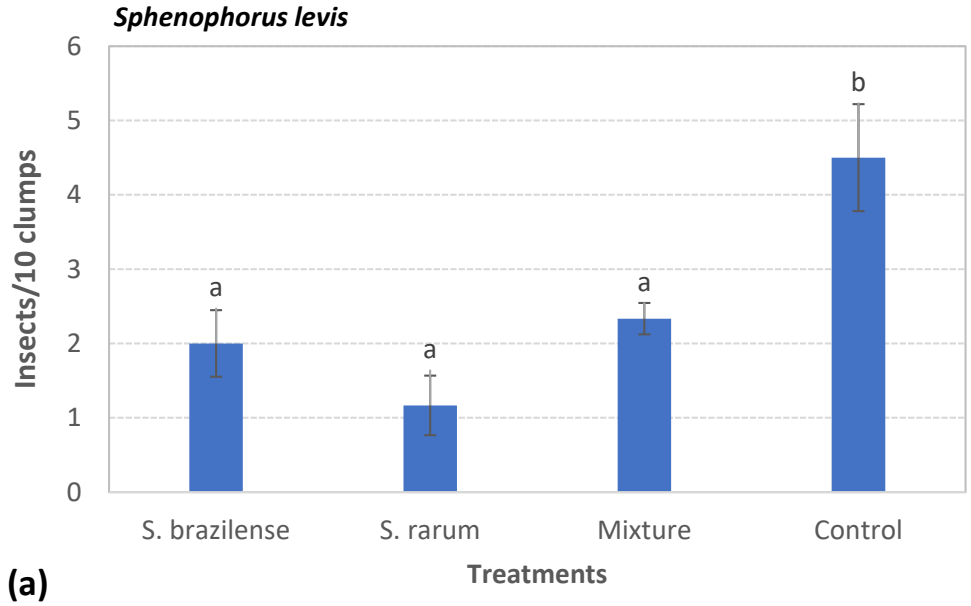

Figure 8. *Cont.*

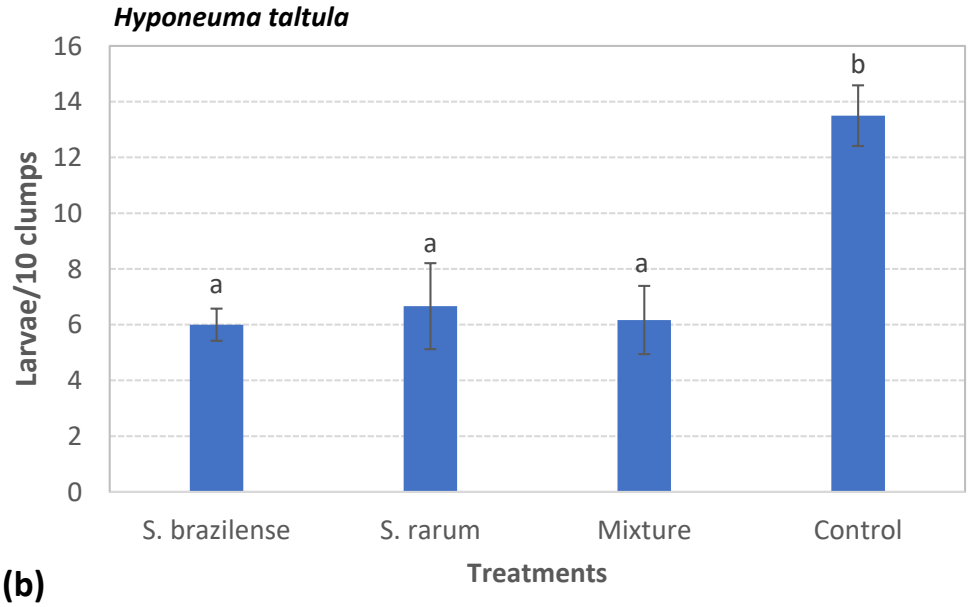

Figure 8. Populations of *Sphenophorus levis* (larvae + pupae + adults) (**a**) and *Hyponeuma taltula* (larvae) (**b**) in a sugarcane field, 21 days after the application of *Steinernema rarum* (2×10^8 IJs/ha), *S. brazilense* (2×10^8 IJs/ha), a mixture of both nematodes (total of 2×10^8 IJs/ha with each nematode contributing half) and control. Means followed by different letters in the columns differ significantly by the Tukey test ($p < 0.005$).

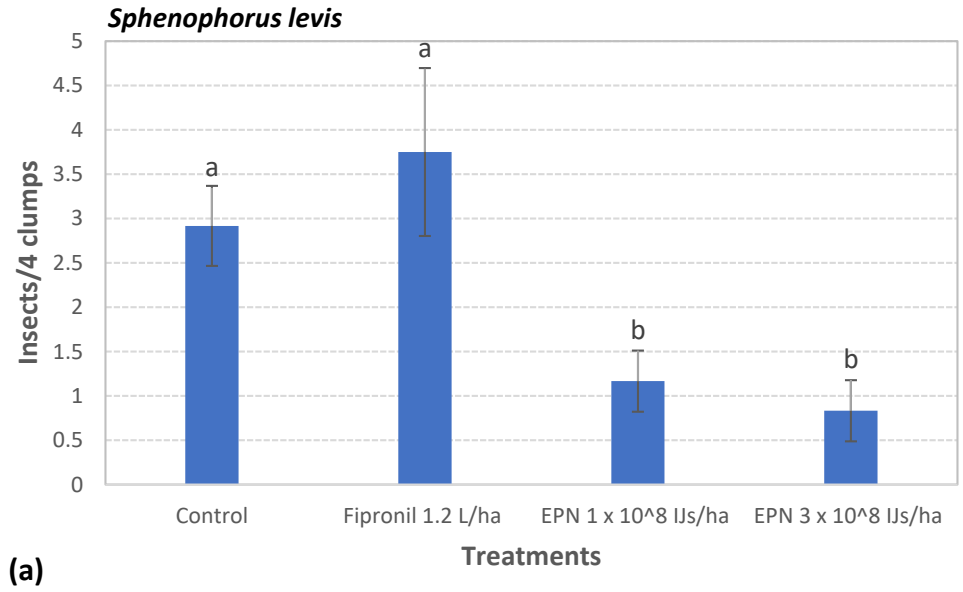

Figure 9. *Cont*.

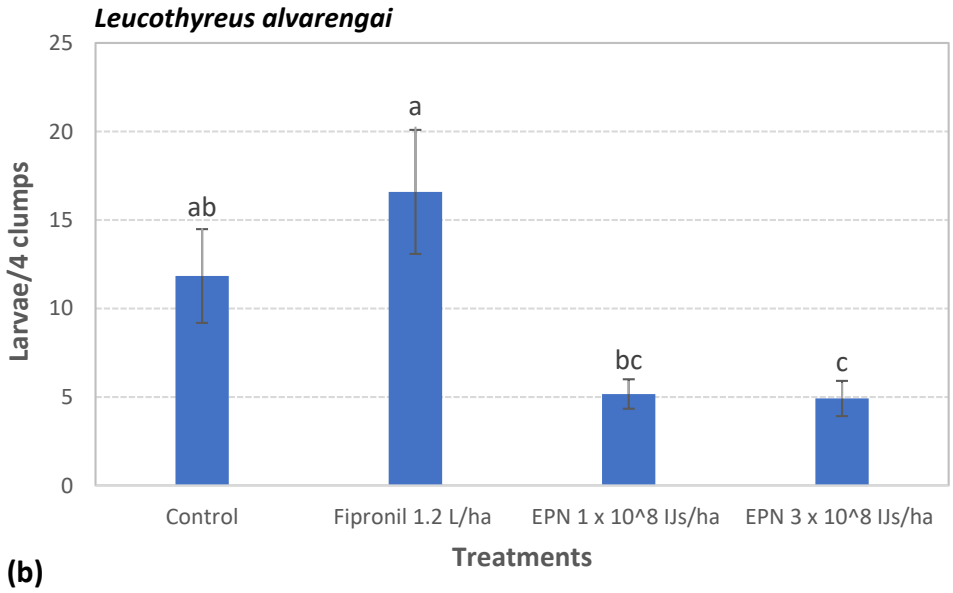

Figure 9. Population of *Sphenophorus levis* (larvae + pupa + adults) (**a**) and *Leucothyreus alvarengai* (larvae) (**b**) in a sugarcane field, 21 days after the application of *Steinernema rarum* (1×10^8 IJs/ha and 3×10^8 IJs/ha) and fipronil (1.2 L/ha). Means followed by different letters differ significantly by the Tukey test ($p < 0.005$).

4. Discussion

The entomopathogenic nematodes *S. rarum*, *S. carpocapsae* and *H. bacteriophora* caused >90% mortality of *G. mellonella* and *S. levis* larvae buried outside the rhizome, indicating that they were equally attracted and virulent to the insects. The differential results obtained with *G. mellonella* larvae inside the rhizomes indicate that *S. carpocapsae* and *H. bacteriophora* were probably less able to enter the rhizomes and infect the targets. Moreover, the similar trends of performances for the three nematodes tested on larvae of *G. mellonella* and of *S. levis* inside the rhizome validate the use of *G. mellonella* as an alternative host model to select EPNs for the control of *S. levis*.

The native EPN species *S. rarum* (Pam 25) which originated from soils of the Pampa biome, RS, Brazil, showed the best performance to reach and kill larvae of *G. mellonella* within the cane rhizome but did not differ from *S. carpocapsae* (IL1) when larvae of *S. levis* were used as a target within the rhizome. *Steinernema carpocapsae* (IL 1) was found causing natural infections in the population of *S. levis* within the cane rhizome [20] but has not been able to keep the insect population below the threshold in the sugarcane area, which suggests the need for an inundative application with this or another more efficacious nematode. Comparing *S. brazilense* with *H. indica* against larvae of *S. levis* inside the rhizome, the former EPN showed significantly better performance (80% mortality) compared to the latter (35%) at the lowest dose tested (150 IJs/insect or 2.4 IJs/cm^2) [11].

In the present study, the holes in the rhizome were filled with cane fibers to simulate the frass left by *S. levis* in the field. Thus, another aspect that may have contributed to the better performance of *S. rarum* is its smaller size (511 µm) when compared to *S. carpocapsae* (558 µm) and *H. indica* (588 µm) [23], allowing the nematode to enter narrower openings in the rhizome to reach the insect, and in the host to reach its hemocoel [24–27]. Their performances were assessed just one week post-inoculation; thus, additional research is needed to assess the efficacy of three nematode species in different substrates with different organic contents [28], and over a longer period in laboratory, greenhouse and field conditions. It is not clear at this point whether the superior activity of *S. rarum* against *S. levis* may have been due solely to higher efficiency in penetrating the host material, or whether *S. rarum* may possess higher levels of innate virulence, or a better host-seeking ability to the target pest; additional research is needed to elucidate the basis for the increased virulence observed in our study.

Steinernema rarum exhibits an intermediate behavior between ambush and cruiser when *G. mellonella* larvae are the host, with a preferential searching site at the depth of 0–2 cm below the soil surface [29]. This characteristic is of great importance to the control of *S. levis* because most of the holes left by this insect in the cane rhizome are located below the soil surface (90 %), mainly at the depth of 0–2 cm (35.23%) [20].

Steinernema rarum has been studied for more than three decades, and its potential to control insects of different orders, including curculionids, is well known [30]. Comparing the virulence of *S. rarum, S. feltiae* and *H. bacteriophora* on 33 different species of insects distributed in the orders Anoplura, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera, with a rate of 100 IJs/insect [31], *Steinernema rarum* stood out for causing mortalities above 90% for most tested insects, considered of agricultural importance: *Eriopis connexa* (Germar), *Hippodamia convergens* (Guerin-Meneville), *Naupactus cinereidorsum* Hustache, *Epicauta adspersa* (Klug), *Tenebrio molitor* L. and *Chrysodina* sp. *Steinernema rarum* also stood out for its successful development in adult and immature insects, while the other two species of nematodes developed only in immature insects.

Sugarcane vinasse is a residue from the sugar–ethanol industry, and it has been mostly used in fertigation practices, i.e., utilizing it as a liquid fertilizer for crops, reducing the water input for plant growth [32]. However, vinasse was toxic to *S. rarum* when IJs were suspended in the compost and kept for more than 6 h, but not when in contact with the straw and soil moistened with the vinasse. Vinasse is already known to affect the life cycle and reproduction of phytoparasitic nematodes. Two species of phytopathogenic nematodes, *Meloidogyne incognita* (Kofoid & White) and *Meloidogyne javanica* (Treub), were exposed to soils containing different concentrations of vinasse [33]. After 90 days, there was a drastic decrease in reproductive activity and in the number of eggs for both due to the increased concentrations, especially for *M. incognita*, which was almost completely inhibited. The nematicidal effect that resulted from the application of vinasse was due to the increase in organic matter provided by this by-product in the soil, generating compounds that stimulate the reproduction of organisms antagonistic to nematodes, such as fungi, bacteria and other microorganisms. In addition, the decomposition of organic matter promotes the release of organic acids that may negatively affect nematodes [34].

In the present study, vinasse affected the nematode only in liquid form, probably due to the high concentration of salts (potassium, sulfate, nitrogen, magnesium, calcium and others) and organic matter [35,36], not its low pH value (4.2). Nematodes tolerate a wide pH range, from 4 to 8, but not an increase in the concentration of salts [37]. Organic matter, on the other hand, reduces the availability of oxygen in the liquid vinasse [38], also affecting the suspended IJs. Oxygen becomes a limiting factor in clay soils, water-saturated soils or soils with a high organic content [39]. Sugarcane vinasse is reported to be a nitrogen-deficient medium, which is mostly composed of acid-insoluble nitrogen [40]. Vinasse is also characterized as a feedstock rich in phenolic compounds and melanoidins [41].

In straw and soil, vinasse did not affect the nematode, probably due to the lower moisture content, dilution of nitrogen and salts and increased oxygenation. Potassium, phosphorus, calcium, nitrate and other salts are adsorbed on solid materials of the soil when the vinasse is added in moderate doses [35]. Although the liquid vinasse caused high mortality for *S. rarum*, viability remained high (> 80%) for up to 6 h, which would allow for EPN application in the field together with vinasse during fertigation (as long as the vinasse is at an adequate temperature).

In the first field test, *S. rarum* showed the same efficacy as *S. brazilense* to control *S. levis* and *H. taltula*, with no improvement observed when applying the mixture of both species. In a similar study, mixtures of *S. carpocapsae* and *H. bacteriophora* were less effective against larvae of *Diabrotica speciosa* compared to these species tested alone [42]. On the other hand, application of two EPN species with different foraging strategies was superior to single-species application when targeting two insect hosts that occupy different soil niches [43].

In the second field test, the results obtained to control *S. levis* coincided with those obtained in greenhouse tests, where five rates of *S. rarum* were tested on *G. mellonella* larvae inserted within the cane rhizome and buried in the soil. Based on the linear equation (Figure 6), the dose to kill 50% of *G. mellonella* inserted in the rhizome was 3082 JIs/40 cm of row, which represents 5.1×10^7 JIs/ha considering that 1 ha has 6666 m of row (1.5 m apart). Thus, by the equations obtained in the greenhouse tests, the doses of 1×10^8 IJs/ha and 3×10^8 IJs/ha would result in 57% and 70.7% mortality, respectively, levels very close to those obtained in the field, 60% and 71%, respectively. These data highlight the methodologies used in the laboratory and greenhouse tests as valid for the selection of species and doses of EPNs aiming at the control of *S. levis*.

Entomopathogenic nematodes have been used in the USA and Japan to control *Sphenophorus* spp. in lawns, pastures and maize [6,44–46]. The control levels of these insects with EPNs are usually above 70%, but with a dose (2.5×10^9 IJs/ha) well above that tested in the present study ($1–3 \times 10^8$ IJs/ha). In the present study, the satisfactory results (>60% of control) obtained with lower doses/ha ($1–3 \times 10^8$ IJs/ha) were due to the high susceptibility of the *S. levis* larvae, the use of the previously selected *S. rarum* and its application directed to the rows of sugarcane planting. Considering the cane rows are 1.5 cm apart, the applied nematode reached the concentration of 7.5 IJs/cm^2 in the 20 cm wide strip of each row, where larvae, pupae and recently emerged adults of *S. levis* were located. For most insects, EPNs are effective only if high rates are applied (e.g., a minimum of 25 IJs/cm^2) [6]. For *S. levis*, remaining inside the rhizome as larvae and pupa probably led these stages to evolve toward higher susceptibility to EPNs because they remain protected from the entomopathogens in their cryptic habitat and thus do not develop resistance. In contrast, *Sphenophorus* species that occur in the USA feed on the root but may stay outside, buried in the soil [6]. Conceivably, insects tend to be more resistant to EPNs if they are naturally exposed to the soil during their life cycle [43,47,48].

Steinernema rarum caused 56.3–58.4% control of *L. alvarengai*, levels which are slightly above those already obtained with *S. brazilense* for the control of this insect (50%) [3]. These results can be considered satisfactory in the biological control of *S. levis*, *S. alvarengai* and *H. taltula*, especially if compared to those obtained with fipronil. It appears that application of *S. rarum* for control of *S. levis* will provide additional benefits in controlling other sugarcane pests such as *L. alvarengai* and *H. taltula*, but additional trials to measure efficacy against these two pests are needed. Moreover, the experiments conducted in laboratory and field conditions proved vinasse is compatible with infective juveniles of *S. rarum* when applied to the soil or to the straw. However, further studies should also be carried out in order to assess the persistence of *S. rarum* in a sugarcane field with and without fertigation using vinasse.

This study is the first to assess *S. rarum* in a sugarcane field and highlight its potential to control *S. levis* in the crop. Prior studies also indicated that EPNs are able to reach *S. levis* within the cane root, and that larvae, pupae and newly emerged adults are quite susceptible to EPNs [7,10,20]. The use of *S. rarum* presents itself as a viable alternative to the control of *S. levis* and other sugarcane soil insect pests and can be recommended at concentrations of $1–3 \times 10^8$ IJs/ha, with satisfactory results and without adversely affecting the environment. Application should be conducted immediately after the cane harvest or at the beginning of the plant's development when the tractor can still enter the field. In Brazil, this period comprises April to December when the populations of larvae and pupae reach the highest peaks [5]. In drier seasons, soil moisture may be preserved by the thick layer of straw covering the ground or by the frequent application of vinasse, favoring a better performance by EPNs; thus, application of vinasse may be recommended along with the nematode application. Expanded field tests are needed to further optimize application of *S. rarum* for control of *S. levis* and other sugarcane pests.

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M.S.O.S., J.F.M.C., M.E.P.F., F.B.B., R.S.A.S., J.G.C.-O., D.I.S.-I., S.H., C.J.B. and L.G.L.; formal analysis, L.G.L., M.S.O.S., J.F.M.C., J.G.C.-O., D.I.S.-I., S.H. and C.J.B.; investigation, M.S.O.S., J.F.M.C., M.E.P.F., F.B.B., R.S.A.S. and J.G.C.-O.; resources, J.G.C.-O., L.G.L., F.B.B. and R.S.A.S.; data curation, M.S.O.S., J.F.M.C., J.G.C.-O., D.I.S.-I., S.H., C.J.B. and L.G.L.; writing—original draft preparation, M.S.O.S. and L.G.L.; writing—review and editing, D.I.S.-I., S.H., L.G.L. and J.F.M.C.; visualization, M.S.O.S., J.G.C.-O., D.I.S.-I., S.H. and L.G.L.; supervision, L.G.L., J.G.C.-O., D.I.S.-I. and S.H.; project administration, L.G.L., J.G.C.-O., F.B.B. and R.S.A.S.; funding acquisition, L.G.L., J.G.C.-O., F.B.B. and R.S.A.S. All authors have read and agreed to the published version of the manuscript.

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