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Assessment of entomopathogenic nematodes and their symbiotic bacteria to control the stink bugs *Euschistus heros* and *Dichelops melacanthus* (Heteroptera: Pentatomidae) in the soybean-corn succession system

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Abstract: The Neotropical brown stink bug *Euschistus heros* and the green-belly stink bug *Dichelops melacanthus* are the most important pests today for the succession system of soy-corn in Brazil. *Dichelops melacanthus* attacks the emerging shoot (epicotyl) of corn plants at the seedling stage of their development, remaining and reproducing on the straw over generations. *Euschistus heros*, on the other hand, feeds directly on the grains and lodge under fallen leaves after the soybean harvest, due to the absence of another crop host. Entomopathogenic nematodes (EPNs) are capable of killing insects due to their ability to search for the host in the soil and to their associations with symbiotic bacteria that cause pathogenesis. This work aimed to assess strains of EPNs and their symbiotic bacteria regarding their potential to control adults of *E. heros* and *D. melacanthus*. Entomopathogenic nematodes could be used to kill the stink bugs adults remaining over the straws, while the symbiotic bacteria could be used to kill these insects above ground, and these bacteria are much easier to produce compared to the EPNs. To assess EPNs and their simbiotic bacteria, tests were conducted in the laboratory and under greenhouse conditions. The nematodes *S. diaprepesi* AM163, *S. carpocapsae* All and *S. carpocapsae* IP1 caused 100% mortality of *E. heros* on the sand substrate, at the rate of 140 IJs/cm² (1000 IJs/insect). The symbiotic bacteria tested topically provided low mortality of *E. heros* (<40.3%). In a test with *S. diaprepesi* AM163 applied on the sand substrate at five different rates, after 7 days, mortality of *E. heros* remained below 50%, except at the high rates of 24 IJs/cm² (50%) and 256 IJs/cm² (80%), respectively. Higher lethality rates also resulted in the increase of the number of IJs produced per insect, up to 88.4 IJs/cm² (5,400 IJs). The nematode *S. diaprepesi* AM163 applied to *E. heros* on two substrates (sand and straw) with two-layer thicknesses, at a rate of 88.4 IJs/cm², caused high insect mortality levels (≥69%), regardless of the substrate and its layer thickness. The nematode *S. diaprepesi* AM163 was twice more virulent to *E. heros* (69.1% mortality) than to *D. melacanthus* (28.8%). *Dichelops melacanthus* was equally resistant to both nematodes *S. diaprepesi* AM163 and *S. carpocapsae* IP1. In a greenhouse test, *S. diaprepesi* AM163 caused again a high mortality level to *E. heros* (72.5%) at the high rate of 88.4 IJs/cm². Thus, EPNs may turn up an alternative to control *E. heros* if high rates of production and use become profitable and viable.

Key words: Pest management, biological control, Pentatomidae, *Steinernema, Heterorhabditis*

1. Introduction

In Brazil, agriculture is one of the most important sectors for the country´s economy. Within this sector, soybean (*Glycine max* (L.) Merrill) is the crop with the highest production value (IBGE, 2017).[1] In Brazil, soybeans occupied an area of 36.9 million hectares, generating 120.8 million tons of grain (CONAB, 2020).[2] Innovations in the planting systems such as the no-till system, the succession of soybean-corn crops, and the use of super early cultivars have enabled greater supplies of food (Corrêa-Ferreira and Sosa-Gomez, 2017), but, on the other hand, they have provided better conditions for proliferation of several insect pests (Hoffmann-Campo et al., 2000).

In recent years there has been an increase in the populations of stink bugs due to their increasing resistance to chemical insecticides, the succession of susceptible crop hosts (Sosa-Gómez and Omoto, 2012), and the breaking of geographical barriers (Soares et al., 2018).

[1] IBGE (2017). Resultados definitivos: Brasil [online]. Website https://censos.ibge.gov.br/agro/2017/templates/censo_agro/resultadosagro/index.html [accessed 20 August 2020].

[2] CONAB (2020). Safra Brasileira de Grãos: Brasil [online]. Website https:// https://www.conab.gov.br/info-agro/safras/graos [accessed 16 August 2020].

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Among the stink bugs that attack soybean plants, the soybean Neotropical brown stink bug *Euschistus heros* (Fabricius, 1798) (Heteroptera: Pentatomidae) is the most important one (Corrêa-Ferreira, 2009; Panizzi et al., 2012; Bueno et al., 2015; Corrêa-Ferreira and Sosa-Gomez, 2017) as it feeds directly on the grains (Corrêa-Ferreira and Panizzi, 1999; Panizzi et al., 2012), causing up to 25% weight reduction and 20% death to the grains (Nunes and Corrêa-Ferreira, 2002).

The succession of soybean-corn crops has also leaded to an increase in populations of the green-bellied stink bug *Dichelops melacanthus* (Dallas, 1851) (Heteroptera: Pentatomidae), which is one of the most important pests of corn crops for damaging the early stages of the plants (seedlings). The great availability of food offered by the succession of both crops allows greater survival of *E. heros* and *D. melacanthus* (Gomez and Ávila, 2001; Corrêa-Ferreira and Sosa-Gomez, 2017). The life cycles of *E. heros* and *D. melacanthus* take 30–40 days, encompassing a stage of egg, five stages of nymph, and an adult stage (Corrêa-ferreira; Panizzi, 1999). The crop sucession system results in the accumulation of cultural remains like straw, over the ground, benefiting both *D. melacanthus* and *E. heros* proliferation. *Dichelops melacanthus* attacks the emerging shoot (epicotyl) of corn plants at the seedling stage of their development, remaining and reproducing on the straw over generations. *Euschitus heros*, on the other hand, can lodge under fallen leaves after the soybean harvest, due to the absence of another crop host (Chocorosqui, 2001). In cold climate regions, *E. heros* can also be found around soybean crops and under perennial plants such as mango, coffee and pigeon pea (Panizzi and Niva, 1994; Hoffmann-Campo et al., 2000), during winter seasons, remaining in diapause for up to 7 months (Hoffmann-Campo et al., 2000; Corrêa- Ferreira et al., 2009).

Biological control practices should be implemented to manage *E. heros* and *D. melacanthus* populations in the field, as an alternative to chemical insecticides (Panizzi and Slansky, 1985. Up to date, studies have focused mostly on the use of microhimenopteran parasitoids (Corrêa-Ferreira and Sosa-Gomez, 2017) and entomopathogenic fungi (Resquín-Romero et al., 2020).

The entomopathogenic nematodes (EPNs) *Steinernema* spp. and *Heterorhabditis* spp. (Nematoda: Rhabditida) have the ability to kill or cause pathogenesis in insects (Poinar, 1990), and remain active in the ground when they are not within the hosts (Kaya and Gaugler, 1993). These nematodes have a highly specific symbiotic relationship with the bacteria *Xenorhabdus* and *Photorhabdus* that cause quick death of the insect host. For both genera, the life cycle starts with an infective juvenile (IJ) that carries the entomopathogenic bacteria inside its intestine, in a special vesicule for steinernematids (Boemare 2002) and releases it into the hemocele of a suitable host. The nematodes and the symbiotic bacteria act together to overcome the insect's immune system, causing its death within 24 to 48 h (Dowds and Peters, 2002). Subsequently, the bacteria multiply inside the insect and the nematode reproduce by feeding on both. When food depletes, the nematode is induced to the form of IJ and emerge from the insect cadaver in search for a new host (Brown and Gaugler, 1997).

Therefore, EPNs could be an alternative to control *E. heros* and *D. melacanthus* when the stink bugs are in contact with the crop remains and straw covering the ground. In studies conducted by Marrero et al. (2015), *H. bacteriophora* (Poinar, 1975) caused 100% mortality of *P. guildinii* (Westwood) and *N. viridula* (Linnaeus), while, in study conducted by Guide et al. (2019), *H. amazonensis* (Andaló, Nguyen and Moino. 2006) caused 88% mortality of *D. melacanthus,* when both nematodes were tested in Petri dishes (9 cm) containing a filter paper lied on the bottom plate.

As for the symbiotic bacteria, several studies have highlighted their potential to control insect pests (Gotz et al., 1981; Abdel-Razek, 2003; Ansari et al., 2003; Mohan et al., 2003; Gerritsen et al., 2005; De Paula et al., 2006; Carneiro et al., 2008; Bussaman et al., 2009; Namsena et al., 2016), mites (Eroglu et al., 2019; Cevizci et al., 2020, Incedayi et al., 2021), plant diseases (Li et al., 1995; Chen et al.,1994; Chacon-Orozco et. al., 2020) and plant parasitic nematodes (Hu and Webster, 1995; Zakaria et al., 2013; El-Deen et al., 2014; Orozco et al., 2016). To control Southern green stink bugs, the bacterium *Chromobacterium subtsugae* caused 95% mortality of *N. viridula* when the sterilized (autoclaved) whole culture was tested (Martin et al., 2007), while the bacteria *Brevibacillus brevis, Bacillus thuringiensis kurstaki, Bacillus atrophaeus, Bacillus sphaericus, Bacillus cereus, Pantoea agglomerans, Vibrio hollisae* and *Pseudomonas flourescens* caused 75-100% mortalities of *Halyomorpha halys* (Tozlu et al., 2019). Thus, many bacteria have shown some virulence or toxicity to insects, highlighting the objective in the present study, to assess the virulence of some symbiotic bacteria against *E. heros*.

Up to date, no studies have assessed either the virulence of EPNs to *E. heros* and *D. melacanthus* under laboratory conditions that approximate to the field environment, held inside pots containing natural substrates; or the virulence of their symbiotic bacteria to *E. heros*; or the effect of substrate thicknesses on the nematode virulence; or the efficacy of *S. diaprepesi* in reducing *E. heros* population in greenhouse condition.

Thus, the current study aimed to investigate the potential of EPNs and some of their symbiotic bacteria to control *E. heros* and *D. melacanthus* in the soybean-corn succession system. Specific objectives are as follows: 1) to assess the virulence of EPNs and some of their symbiotic bacteria to

E. heros, 2) to assess *S. diaprepesi* AM163 at different rates on the mortality of *E. heros* and on the reproduction of the nematode in the insect, 3) to assess *S. diaprepesi* on two substrates, with two layer thicknesses, aiming to control *E. heros* just after the soybean harvesting, 4) to compare the virulence of *S. diaprepesi* to the stink bugs *E. heros* and *D. melacanthus*, 5) to compare the virulence of *S. diaprepesi* and *S. carpocapsae* to the stink bug *D. melacanthus*, and 6) to confirm the efficacy of *S. diaprepesi* in reducing the population of *E. heros* in greenhouse conditions.

2. Material and methods

2.1. Insects rearing

Euschistus heros and *Dichelops melacanthus* were reared in the Laboratory of Biological Control of the Instituto Biológico, Campinas, SP, Brazil. They were kept in plastic trays (20 × 30 cm) under 25°C, RH 60 ± 5% and 12-h photoperiod, and fed on bean pods (*Phaseolus vulgaris* L.), peanuts (*Arachis hypogaea* L.), sunflower seeds (*Helianthus annuus* L.) and filtered water. First instar nymphs remained on the chorion, with a gregarious behavior (Lockwood and Story, 1986). All tests were carried out with adult insects, one week after their emergency.

2.2. Entomopathogenic nematodes culture

Sixteen strains of EPNs, previously identified by their 28S ribosomal gene for the ITS (internal transcribed spacer) region (Joyce et al., 1994), were grown in *Galleria mellonella* (L.) larvae (3rd to 5th instar), and the IJs were harvested in White's trap (White, 1927), according to the procedures described by Kaya and Stock (1997). The IJs were kept suspended in water under 14 °C and used for the tests with a maximum of seven days after harvesting.

2.3. Entomopathogenic nematodes symbiotic bacteria culture

The symbiotic bacteria strains used in the essays were isolated from their respective EPN species maintained in the EPN collection of the Biological Control Laboratory of Instituto Biológico (Campinas- SP/Brazil). Larvae of *G. mellonella* were infected with these EPNs, and, following their death, punctured on the first abdomen to extract a drop of hemolymph on a petri dish containing NBTA selective growth medium (nutrient agar + 0.004% (w / v) triphenyl tetrazolium chloride + 0.025% (w / v) bromothymol) (Boemare and Akhurst, 1988).

The strains were grown in a 100 mL Erlenmeyer flask containing 20 mL of Tryptic Soy Broth (TSB), shaken at 150 rpm for 24 h under 27 °C. To confirm they remained at phase I (active phase), 100 µL of each strain were plated in NBTA medium. Colonies that were tinged blue had a sample taken and grown in Erlenmeyer flasks (500 mL) containing 300 mL of TSB culture medium shake at 150 rpm for 72 h, under 27 ° C.

Table 1. Nematodes strains of *Heterorhabdtis* and *Steinernema* tested for their virulence to *Euschistus heros* on sand substrate.

Nematode	Strain	Location/Origen
H. amazonensis	IBCB n10	Santa Fé do Sul- SP
H. amazonensis	IBCB n44	Santa Adélia – SP
H. bacteriophora	HB EN01	Germany*
S. brazilense	PONTO 2C	Porto Murtinho- MS
S. brazilense	IBCB n06	Porto Murtinho- MS
S. carpocapsae	All	USA
S. carpocapsae	IL1	São João da Boa Vista- SP
S. carpocapsae	IL2	São João da Boa Vista- SP
S. carpocapsae	IP1	São João da Boa Vista- SP
S. diaprepesi	AM163	Sinop-MT
S. feltiae	IBCB n47	Germany**
S. glaseri	CER21	Rio Verde- GO
Steinernema sp.	IBCBn20	Itirapina-SP
S. rarum	PAM 11	Bagé-RS
S. rarum	PAM 13	Bagé-RS
S. rarum	PAM 25	Bagé-RS

* Hybrid strain, originated from cross-breeding between *H. bacteriophora* HK1 (New Jersey) and HD01 (Germany) strains.
** Hybrid strain, originated from cross-breeding between 10 European isolates of *S. feltie* (Germany, Norway, Sweden, Finland, Italy and Holland).
S .: *Steinernema* and H .: *Heterorhabditis*.

2.4. Virulence of entomopathogenic nematodes against *Euschistus heros*

Sixteen strains of EPNs (Table 1) were assessed for their virulence to *E. heros* adults. For each strain, there were three replications, each replication consisting of a 500 mL plastic pot (9.5 cm Ø) containing 100g of sand (14% humidity), a bean pod and 10 adult insects.

The nematodes were applied onto the substrate with a pipette, generating a rate of 140 IJs/cm² (1000 IJs/insect). The control treatment was inoculated with water. The pots were incubated under 25 °C, RH 60 ± 5% and 12-h photoperiod. Evaluation was carried out seven days after inoculation, based on insect mortality. The dead insects were transferred to White traps (White, 1927) in order to confirm the infection by the EPNs. The experiment was conducted twice in time.

2.5. Virulence of symbiotic bacteria to *Euschistus heros*

This experiment aimed to assess EPNs symbiotic bacteria for their virulence to the adults of *E. heros*. Treatments consisted of nine strains of symbiotic bacteria of the genera *Photorhabdus* and *Xenorhabdus* spp. (Table 2)

Table 2. Bacteria strains of *Photorhabdus* and *Xenorhabdus* (and their associated nematodes) tested for their virulence to *Euschistus heros* on filter paper.

Bacteria	Nematode	Strain	Location/Origen
P. luminescens	*H. amazonensis*	IBCBn10	Santa Fé do Sul- SP
P. luminescens	*H. bacteriophora*	HB EN01	Alemanha*
X. poinarii	*S. glaseri*	CER 21	Rio Verde – GO
Xenorhabdus sp.	*Steinernema* sp.	CER144	Rio Verde – GO
X. nematophila	*S. carpocapsae*	IL2	São João da Boa Vista- SP
Xenorhabdus sp.	*Steinernema* sp.	AM165	Sinop – MT
Xenorhabdus sp.	*S. braziliense*	Ponto 2C	Porto-Murtinho- MS
X. doucetiae	*S. diaprepesi*	AM163	Sinop – MT
X. szentirmaii	*S. rarum*	PAM 25	Bagé – RS

* Hybrid strains, originated from cross-breeding between *H. bacteriophora* strains HK1 (New Jersey) and HD01 (Germany).
S.: *Steienernema*; X.: *Xenorhabdus*; H.: *Heterorhabditis* and P.: *Photorhabdus*.

and a control. These bacteria were chosen because their associated nematodes provided varied results in the previous experiment. The experiment was carried out in a completely randomized design, with three replicates for each treatment. Each replication was represented by a 500 mL plastic pot (9.5 cm Ø) containing a filter paper lined on the bottom, a bean pod and 10 insects' adults.

The insects were immersed in the undiluted bacterial culture (with three days of growth) and, then, transferred to the pots. The control treatment was immersed in TSB medium without bacteria. If these bacteria did not cause insect mortality with the undiluted culture, they would not be candidates for being developed as bioinsecticides. The experiment was incubated at 25 °C, RH 60 ± 5% and 12-h photoperiod, and evaluation was carried out seven days after inoculation. The experiment was conducted twice in time.

2.6. Virulence of *Steinernema diaprepesi* at different concentrations against *Euschistus heros*

This experiment assessed different concentrations of *Steinernema diaprepesi* (Nguyen and Duncan, 2002) AM163 on the mortality of *E. heros* adults. Five treatments consisted of the concentrations 0.7, 3.5, 17.6, 88.4 and 442.4 IJs/cm² (≅1, 40, 200, 1000, 5000 IJs/insect), considering the area on the substrate surface (113 cm²) in the 1.5 L plastic pot (12.5 cm Ø). Each treatment had 4 replications, each replication represented by a 1.5 L plastic pot (12.5 cm Ø) containing 100g of sand (14% humidity), two bean pods, plus ten adult insects. The nematode was applied with a pipette, generating the different rates. The control treatment received only water. The pots were sealed with sheer fabric and elastic bands, and incubated at 25 °C, RH 60 ± 5% and 12-h photoperiod. Evaluation was carried out seven days later, based on insect mortality. The dead insects were transferred to White traps, using a trap for each replication in order to determine the number of IJs generated per insect. The experiment was conducted twice in time.

2.7. Virulence of *Steinernema diaprepesi* to *Euschistus heros* on two substrates with two-layer thicknesses

This study assessed the virulence of *Steinernema diaprepesi* AM163 to *E. heros* adults on two substrates with two-layer thicknesses, aiming its control on the bared ground or on the crop remains/straw left on the ground after the crop harvesting. The study consisted of four treatments: A) 200g of sand - 1 cm layer thickness, B) 1000g of sand - 5 cm layer thickness, C) 4g of straw - 1 cm layer thickness, D) 20g of straw - 5 cm layer thickness; each treatment had its respective control. The choice of sand and straw in two-layer thicknesses aimed to find out if the nematode would go deeper in greater layer thickness, resulting in lower rate on the surface of the substrate and, consequently, in lower levels of insect mortality.

The nematode *S. diaprepesi* was applied with a pipette, generating a rate of 88.4 IJs/cm² (1000 IJs/insect), which provided the highest insect mortality level (74.6%), with the highest reproduction rate of the nematode per insect (5400 IJs), in the previous experiment. Each treatment had three replications, each of which represented by a 1.5 L plastic pot (12.5 cm Ø) containing the substrate, two bean pods, plus ten adult insects. The sand was previously moistened to 14% humidity, while the straw remained

submerged in water for 24 h, before the water was drained and the moistened straw was transferred to the pots. Each treatment had its respective control with the application of only water.

The insects were released into the pots 24 h after the application of the nematode, in order to allow a previous distribution of the IJs in the substrates. The pots were sealed with sheer fabric and elastic bands, and incubated at 25 °C, RH 60 ± 5% and 12-h photoperiod. Evaluation was carried out seven days after the insects' release, based on their mortality. The dead insects were transferred to White traps to confirm the infection by the nematodes. The experiment was conducted twice in time.

2.8. Comparative susceptibility of *Dichelops melacanthus* and *Euschistus heros* to the nematode *Steinernema diaprepesi*

This study compared two species of stink bugs adults, *E. heros* and *D. melacanthus*, in respect to their susceptibility to the nematode *S. diaprepesi* AM163. Thus, the treatments consisted of the two insect species treated with the nematode and the two controls represented by the two insects treated with water. Each treatment had three replications; each replication represented by a 1.5 L plastic pot (12.5 cm Ø) containing 200 g of sand (14% humidity) (1 cm layer thickness) covered with 4 g of pre-moistened straw (soaked in water for 24 h before drainage) (1 cm layer thickness), two bean pods and 10 insect adults. The use of this double layer straw-over-sand substrate aimed to simulate the natural conditions in the field, since sand and straw allowed similar performance of *S. diaprepesi* against *E. heros* in the previous experiment.

Steinernema diaprepesi was applied with a pipette, generating a rate of 88.4 IJs/cm² (1000 IJs/insect), which provided the highest insect mortality level (74.6%), with the highest reproduction rate of the nematode per insect (5400 IJs) in the previous experiment. The pots were sealed with sheer fabric and elastic band, and incubated at 25 °C, RH 60 ± 5% and 12-h photoperiod. Evaluation was carried out seven days after inoculation, based on insect mortality. The experiment was conducted twice in time.

2.9. Comparative virulence of *Steinernema diaprepesi* and *Steinernema carpocapsae* to *Dichelops melacanthus*

This study compared the nematodes *S. diaprepesi* AM163 and *S. carpocapsae* (Weiser, 1955) IP1 in respect to their virulence to the bug *D. melacanthus*, which showed greater resistance to *S. diaprepesi* when compared to the bug *E. heros*. These two nematodes were selected because they caused 100% mortality of *E. heros* in the first test and because they have different foraging behaviors. While *S. diaprepesi* displays a "cruise" behavior (Del Valle et al., 2014), seeking its target insects, the nematode *S. carpocapsae* displays an "ambush" behavior (Koppenhöfer and Kaya, 1996), staying in lurk waiting for the host to approach.

The study consisted of three treatments represented by the insect treated with the two nematodes and the control (insects treated with water). Each treatment had three replications, each of which represented by a 1.5 L plastic pot (12.5 cm Ø) containing 200 g of sand (14% humidity) covered with 4 g of pre-moistened straw (soaked in water for 24 h before drainage), two bean pods and 10 adult insects.

The two nematodes were applied with a pipette, generating the rate of 88.4 IJs/cm² (1000 IJs/insect), which provided the highest insect mortality level (74.6%), with the highest reproduction rate of the nematode per insect (5400 IJs), in the previous experiment. The pots were sealed with sheer fabric and elastic band and incubated at 25 °C, RH 60 ± 5% and 12-h photoperiod. Evaluation was carried out 7 days after inoculation, based on the insect mortality. The experiment was conducted twice in time.

2.10. Efficacy of *Steinernema diaprepesi* to reduce *Euschistus heros* population in greenhouse

The study assessed *S. diaprepesi* AM163 in respect to its efficacy to reduce *E. heros* population under greenhouse conditions, expecting to predict its efficacy in the field since both greenhouse and field have similar conditions. Thus, the study consisted of only two treatments: application of the nematode and control.

Each treatment had four replications distributed randomly in blocks, each consisting of a cement box (63 x 83 x 53 cm) filled with soil, containing two plots (treatments). Each replication was represented by a PVC tube (25 cm in diameter × 21 cm in height), deepened 2 cm in the soil of the box, containing 8g of pre-moistened straw (24 h soaked in water before drainage) covering the soil (pre-moistened with 12 L of water/cement box), two bean pods and 10 adult insects. The nematode *S. diaprepesi* was applied over the straw inside the tubes with a Becker, generating a rate of 88.4 IJs/cm² (4332 IJs/insect), which provided the highest insect mortality level (74.6%), with the highest reproduction rate of the nematode per insect (5400 IJs), in the previous experiment. For the control tubes, only water was applied.

The experiment was irrigated daily by micro sprinklers. During the experiment, the temperature ranged between 18 to 25 °C, and evaluation was carried out seven days after the release of the insects into the tubes, based on insect mortality. The dead insects were transferred to White traps in order to confirm the infection by the nematode. The experiment was conducted twice in time.

2.11. Statistical analysis

The laboratory experiments were carried out in a completely randomized design, while the greenhouse experiment was carried out in random blocks. For all tests, except for the "virulence of EPNs", "virulence of bacteria"

and "efficacy of *S. diaprepesi*", the data were corrected using the Abbott formula ([survivors in the control - survivors in the treatment] / survivors in the control) × 100) (Abbott, 1925) considering the insect mortalities in the respective controls. Then, the corrected data were subjected to normality tests, while the homogeneity of variances were subjected to Shapiro–Wilk and Barlett tests. For the tests that followed the assumptions, Student's t test was performed when there were only two variables, while analysis of variance (ANOVA) was performed when there were more than two variables. Subsequently, the Tukey 5% test was performed.

For the experiment with "*S. diaprepesi* at different rates against *E. heros*", data were corrected by Abbott formula and analyzed by logarithm regression between nematode rates and insect mortalities. To analyze the number of IJs generated per insect, generalized linear model (GLM) was used with Quasipoisson distribution and "log" connection. Subsequently, analysis of variance was performed, followed by comparisons of means using Tukey test at 5%.

All percentage data (frequencies) were transformed into arcsine $\sqrt{x}$ / 100 before analysis (Southwood, 1978). Analyzes were performed using the software SPSS 16.0 (SPSSInc., Chicago, IL, USA) as well as the R 3.6 (R Core Team 2019), through the packages lme4 (Bates et al., 2015), emmeans (Searle et al., 1980), drc (Ritz et al., 2015) and hnp (Moral et al., 2017).

3. Results

3.1. Virulence of entomopathogenic nematodes to *Euschistus heros*

The nematodes *Steinernema diaprepesi* AM163, *S. carpocapsae* All and *S. carpocapsae* IP1 caused 100% mortality of *E. heros* (Figure 1), but they did not differ significantly from the other six nematodes tested, which caused mortality above 88% ($F_{16, 101}$ = 27.57, p = 0.71). Regarding the least virulent nematodes, three of them caused mortalities below 42%, similar to the control (p = 0.2) (Figure 1).

The dead insects were observed under a stereomicroscope, showing IJs emerging from the cadavers (Figure 2).

3.2. Virulence of symbiotic bacteria to *Euschistus heros*

All symbiotic bacteria caused low mortality levels of *E. heros* (21.7% to 40.3%), with only *X. doucetiae* AM163 and *P. luminescens* IBCBn10 (associated with *S. diaprepesi* and *H. amazonensis*, respectively) differing significantly from the control ($F_{9, 59}$ = 2.58, P = 0.016) (Figure 3).

3.3. Virulence of *Steinernema diaprepesi* at different rates to *Euschistus heros*

The logarithmic curve generated by the correlation of the different rates of *S. diaprepesi* AM163 with the *E. heros* mortality was highly significant (R = 0.96) (Figure 4). Based on the equation, LC50 was determined as 24 IJs/cm², and the LC80 as 256 IJs/cm².

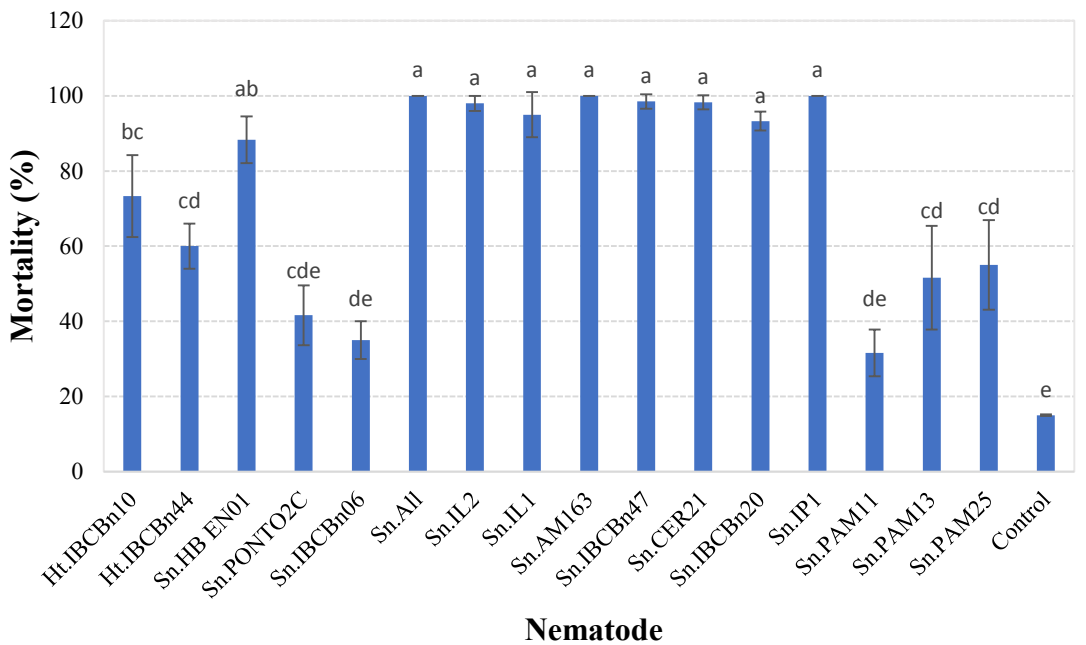

Figure 1. Mortality (%) of *Euschistus heros* seven days after their exposition to 16 EPNs strains applied on sand substrate, at the rate of 140 IJs/cm² (1000 IJs/insect). Sn.: *Steinernema*. Ht.: *Heterorhabditis*. Averages followed by the same letter do not differ significantly according to the Tukey test 5%.

Figure 2. a) *Euschistus heros* adults killed by *Steinernema diaprepesi*, b) nematodes emerging from the insect's cadaver.

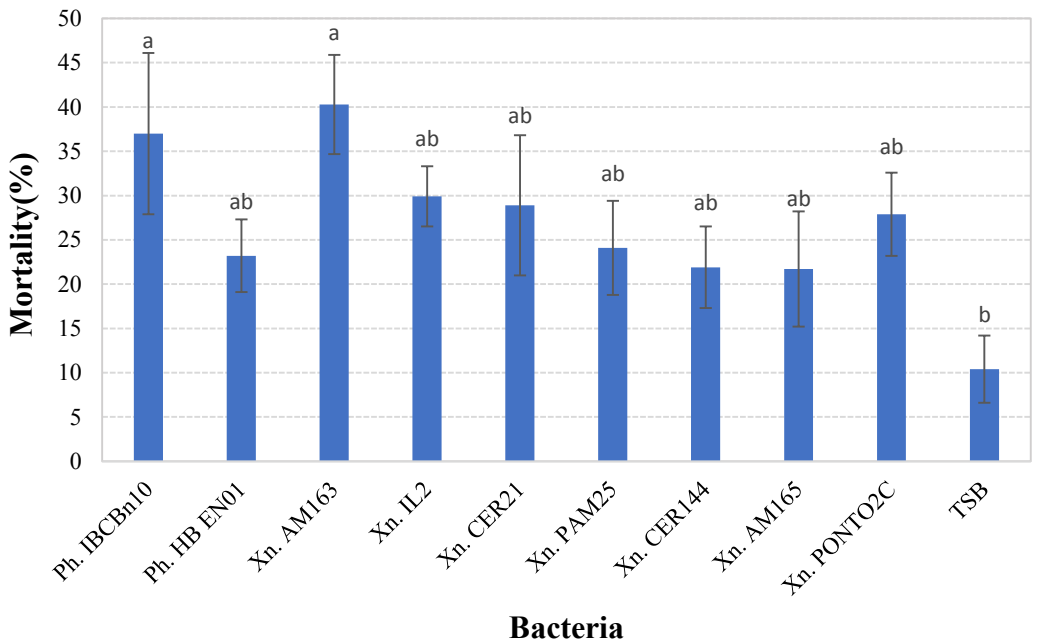

Figure 3. Mortality (%) of *Euschistus heros* adults 7 days after their inoculation with symbiotic bacteria cultures. Xn.: *Xenorhabdus*. Ph.: *Photorhabdus*. Averages followed by the same letter do not differ significantly according to the Tukey test 5%.

The number of IJs generated per insects also increased along with the nematode rates, but up to 88.4 IJs/cm² in which the IJs generated per insect reached 5400 IJs, with a significant difference only in relation to the two lowest rates of 0.7 IJs/cm² and 3.5 IJs/cm². (GLM; $F = 17.2144$; $p < 0.0001$ / Figure 5).

3.4. Virulence of *Steinernema diaprepesi* to *Euschistus heros* on two substrates with two-layer thicknesses

Steinernema diaprepesi AM163 was tested on sand with layers having thicknesses of 1 and 5 cm caused 83% and 80% mortality of *E. heros*, respectively; levels did not differ from those obtained by its application on the straw

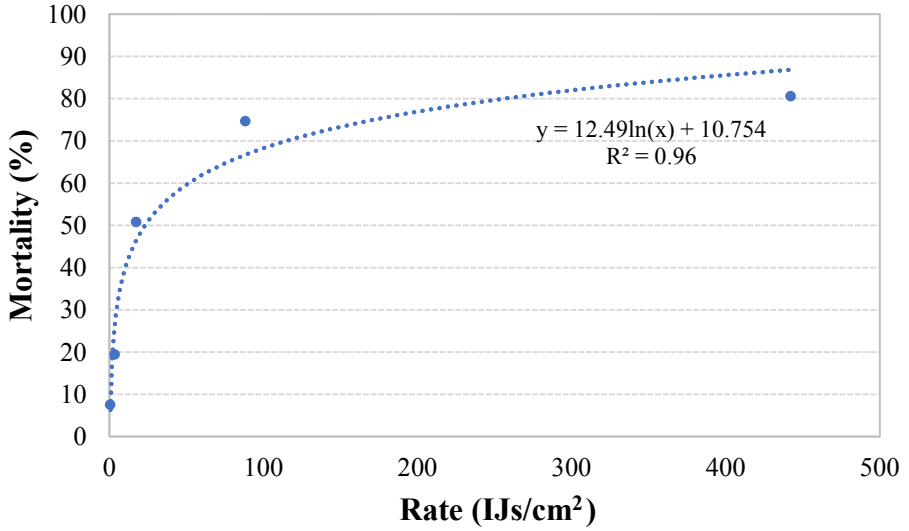

Figure 4. Corrected mortality of *Euschistus heros* (Abbott formula) 7 days after its exposition to *Steinernema diaprepesi* AM163 at the rates of 0.7, 3.5, 17.6, 88.4 and 442.32 IJs/cm^2. Curve generated by logarithm regression between rates and mortalities.

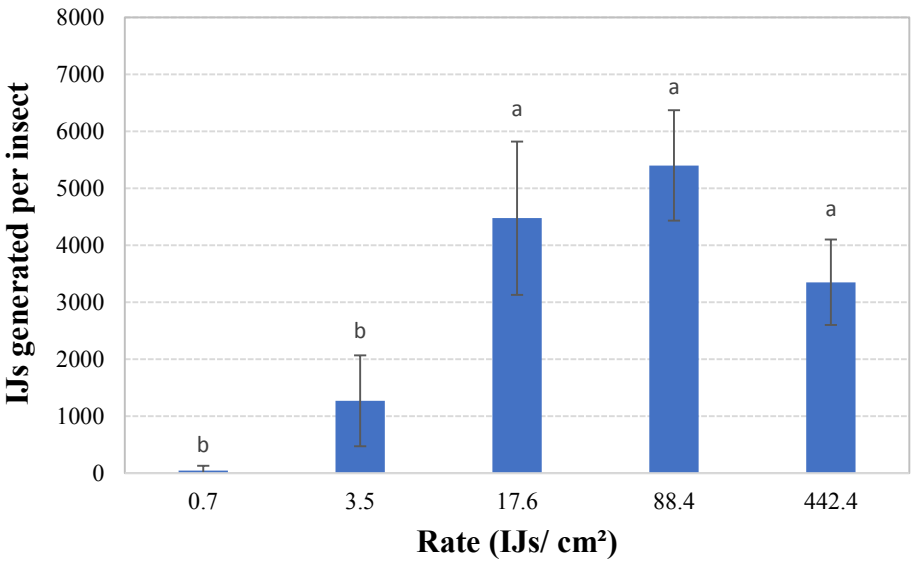

Figure 5. Reproduction of *Steinernema diaprepesi* AM163 in *Euschistus heros* exposed to different rates of the nematode. Averages followed by the same letter do not differ significantly according to the Tukey test 5%.

substrate with the same layers having thicknesses of 1 cm (70%) and 5 cm (69%) (Figure 6) (One-way ANOVA; F = 1.3632; p = 0.2826).

3.5. Comparative susceptibility of *Dichelops melacanthus* and *Euschistus heros* adults to the nematode *Steinernema diaprepesi*

Steinernema diaprepesi AM163 was significantly more virulent to *E. heros* than to *D. melacanthus* (Figure 7), causing 69.1% and 28.8% mortality of adults, respectively, seven days after its application (T test; t = 3.5162; d.f.= 9.4968; p = 0.006).

3.6. Comparative virulence of *S. diaprepesi* and *S. carpocapsae* to *Dichelops melacanthus*

Comparing *S. diaprepesi* AM163 and *S. carpocapsae* IP1 in respect to their virulence to *D. melacanthus* (more resistant in the previous experiment), both species caused low mortality rates (<25%) (Figure 8), not differing significantly from each other (T test; t = –0.6811; df =

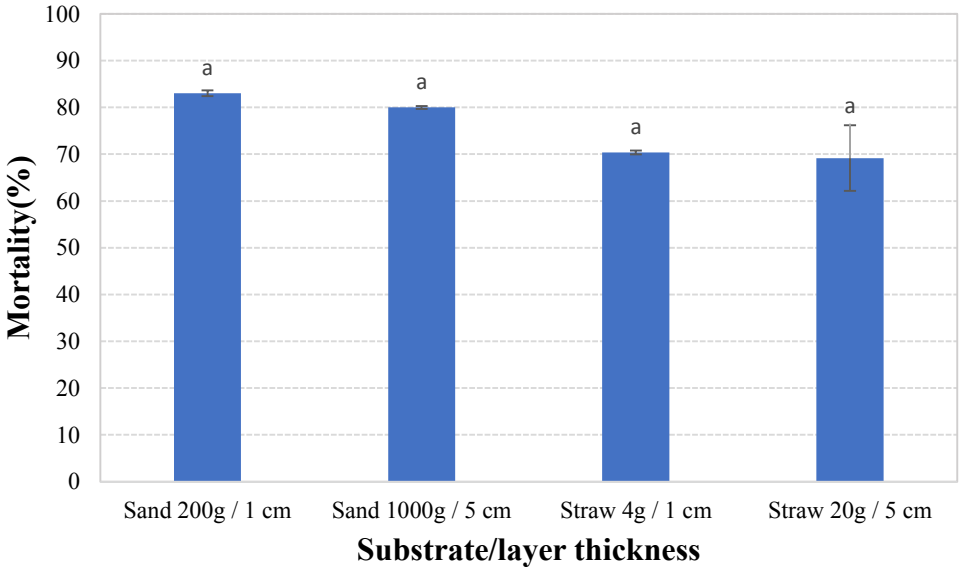

Figure 6. Corrected mortality (%) of *Euschistus heros* adults (Abbott formula) seven days after their exposition to *Steinernema diaprepesi* AM163 applied on two substrates with two-layer thicknesses, at the rate of 88.4 IJs/cm² (1000 IJs/insect). Averages followed by the same letter in the column do not differ significantly according to the Tukey test 5%.

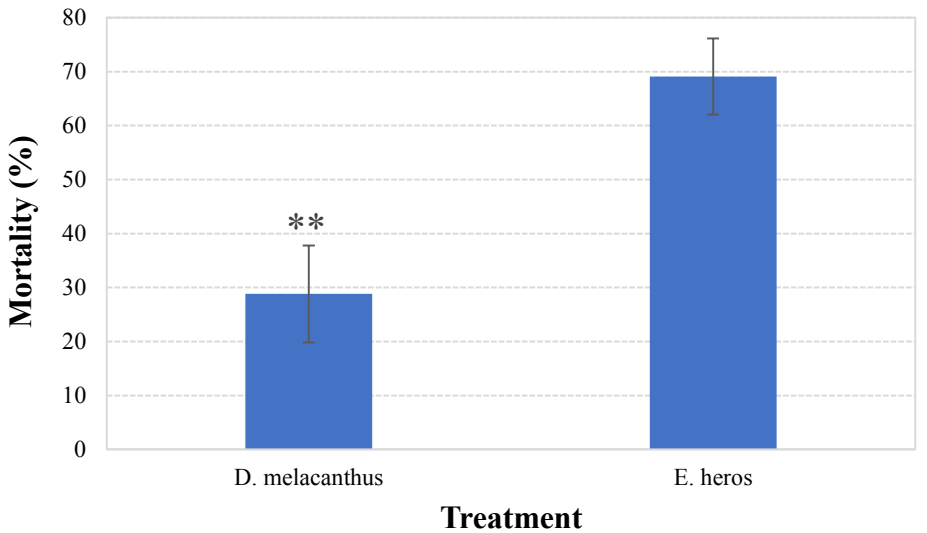

Figure 7. Corrected mortality (%) of *Euschistus heros* and *Dichelops melacanthus* (Abbott's formula) seven days after their exposition to *Steinernema diaprepesi* AM163 applied on a double layer straw-over-sand substrate, at the rate of 88.4 IJs/cm² (1000 IJs/insect) (**p < 0.01; Student's t test).

9.7665; p = 0.5116), confirming the greater resistance of *D. melacanthus* to entomopathogenic nematodes.

3.7. Efficacy of *Steinernema diaprepesi* to reduce *Euschistus heros* population in greenhouse

The nematode *S. diaprepesi* AM163 caused 72.5% mortality of *E. heros* adults, differing significantly from the control (5%) (T test, t = 10.42; df = 9.7558; p < 0. 0001) (Figure 9).

4. Discussion

The nematodes *S. diaprepesi* AM163, *S. carpocapsae* All and *S. carpocapsae* IP1 caused 100% mortality of *E. heros* on the sand substrate. *S. diaprepesi* AM163 was selected as model for the next tests as it was isolated from a warmer region of Brazil (Sinop – MT), where large soybean growth areas are located, thus, probably being a more adapted species to hot climates.

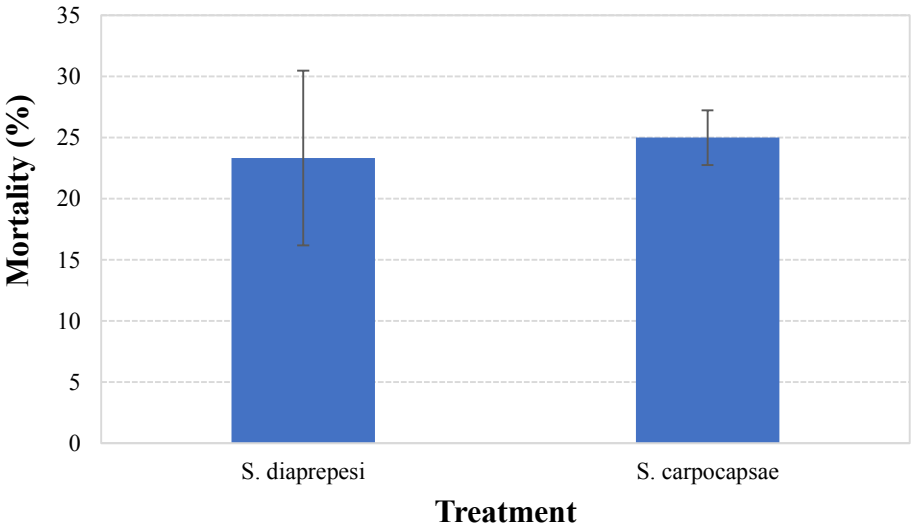

Figure 8. Corrected mortality (%) of *Dichelops melacanthus* (Abbott's formula) seven days after its exposition to *Steinernema diaprepesi* AM163 and *Steinernema carpocapsae* IP1 applied on a double layer straw-over-sand substrate, at the rate of 88.4 IJs/cm² (1000 IJs/insect) ($p > 0.05$; Student's t test).

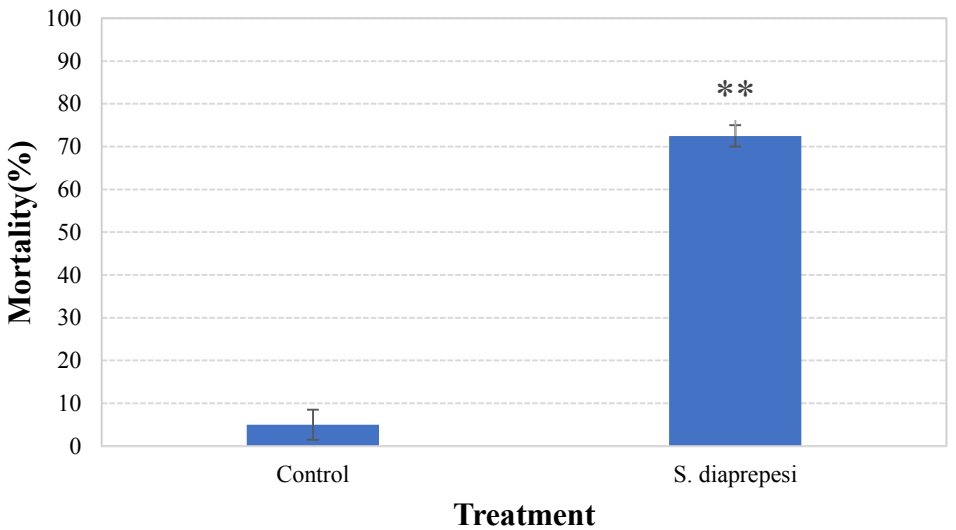

Figure 9. Mortality of *Euschistus heros* adults seven days after their exposition to the nematode *Steinernema diaprepesi* AM163 applied on the straw at the rate of 88.4 IJs/cm² (1000 IJs/insect), inside greenhouse (**$p < 0.01$; Student's t test).

There have been some reports of EPNs serving as biological control agents against Hemiptera species on the leaves (Martínez-Córdoba et al., 2011; Naranjo et al., 2013; Niekerk and Malan, 2013; Platt et al., 2020), trunks/stems/shoots (Shapiro-Ilan and Mizell, 2012; Marrero et al., 2015; Guide et al., 2015; Guide et al., 2019) and soil (Leite et al., 2005; Melo et al. 2006; Batista and Auad, 2010; Vieux and Malan, 2013; Mbata and Shapiro-Ilan, 2013; Batista et al., 2014; Alves et al., 2009; Guerrero & Pardey, 2019; Moussa et al., 2021; Zart et al., 2021). Susceptibilities of these insects range widely, with some species being more resistant than other usually tested insect hosts, requiring high rate of EPNs (100 IJs/cm²) to provide > 70% mortality (Guide et al., 2015; Guide et al., 2019). The present study is the first to assess EPNs and their symbiotic bacteria against.

The symbiotic bacteria suspended in the undiluted bacterial culture produced low mortality of *E. heros* (<40.3%). Three factors can contribute to the low virulence displayed by the symbionts. The first refers to

the infection process, as the bacteria depend on the EPNs to be introduced into the insect's hemocele (Poinar, 1990; Dolinski, 2006). The second, bacteria can be more virulent in association with EPNs, as they may not be recognized by the insect host immunity systems (Balasubramanian et al., 2009; Toubarro et al., 2013; Ono and Yoshiga, 2018). The third factor is related to characteristics of the stink bugs that may hinder infections by microorganisms, such as the chemical composition of the cuticle (Sosa-Gómez et al., 1997; Corrêa-Ferreira and Panizzi, 1999; Oliveira, 2014), volatiles produced by metathoracic glands (Lopes et al., 2015), oral apparatus and digestive physiology (Lomate and Bonning, 2016).

In the present study, *X. doucetiae* AM163 and *P. luminescens* IBCBn10 were the most virulent bacteria for *E. heros*, differing significantly from the control. These mortalities might have been caused by the toxic effect produced by the metabolites or by the penetration of the bacteria cells through the natural openings such as spiracle and anus. Other studies have also shown some effects of symbiotic bacteria cultures on eggs, larva and pupa of Lepidoptera, as well as on Coleoptera, Thysanoptera and mites (Abdel-Razek, 2003; Mohan et al., 2003; Gerritsen et al., 2005; Bussaman et al., 2009; Namsena et al., 2016; Eroglu et al., 2019; Cevizci et al., 2020), highlighting the toxic effect of their metabolites to the former two groups of arthropods. More recently, Incedayi et al. (2021) identified the compounds produced by *X. nematophila* and proved that Xenocoumacin is the most toxic compound. As for the non-symbiotic bacteria *Chromobacterium subtsugae*, also produced metabolites that caused mortality of *N. viridula* (Martin et al., 2007). Thus, new studies should be conducted in order to screen symbiotic and non-symbiotic bacteria against *E. heros*, as well as to identify the compounds produced by the bacteria that caused mortality to the insect.

In the test with *S. diaprepesi* AM163 applied on the sand substrate, at different rates, mortality of *E. heros* surpassed 50% only at high concentration levels of the nematode, above 24 IJs/cm². In order to obtain 80% mortality, the rate of 256 IJs/cm² was required, something not consistent with the products currently available in the market, and beyond the usual rate of 25 IJs/cm², considered as effective for most soil inhabiting insect pests (Shapiro-Ilan et al., 2002). The need for such high rates might be more related to the behavioral aspects of the *E. heros* than to its intrinsic susceptibility. *E. heros* is an active insect that locomotes over the straw or the organic matter, reducing the chance for the nematode to reach the host. Up to date, no study was conducted in order to test EPNs on stink bug held below ground, but some studies testing nematodes against low active spittlebugs such as nymphs of *Mahanarva* species living over the soil surface our belowground have shown the need of much lower rates (< 10 IJs/insect) to provide >50% mortalities.

Interestingly, the increase in concentration of applied EPN also resulted in augmented levels of nematodes recovered per insect. This was observed up to the concentration of 88.4 IJs/cm² since, at the higher rate, there was a slightly drop in IJs generated per insect. Thus, very high rates can probably lead to a greater competition of nematodes for food or to a greater contamination in the insect's cadaver, affecting the reproduction of the nematode. Other studies have also demonstrated this reduction in nematode reproduction at the highest rates of *Steinernema* sp. (640 IJs/insect), of *S. feltiae* (Filipjev, 1934) (200–400 IJs/insect), and of *S. thermophilum* (100 IJs/insect) for larva of *Tenebrio molitor* (L.) (Baliadi et al. 2011), *G. mellonella* (Rahoo et al. 2017), and *Athalia lugens proxima* (Yadav and Lalramliana 2012), respectively. According to these studies, the rate that start causing a decrease in nematode reproduction depend on the susceptibility of the host.

The nematode *S. diaprepesi* AM163 tested on *E. heros* on two substrates with two-layer thicknesses, at the rate of 88.4 IJs/cm², caused high insect mortality levels, regardless of the substrate and its layer thickness, being slightly higher on the soil (83 % for 1 cm and 80% for 5 cm) than on the straw (70% for 1 cm and 69% for 5 cm) but with no statistical difference. The similarity in mortalities caused to *E. heros* on the two layers thicknesses (1 and 5 cm) of each substrate may suggest an equal attraction of the IJs to the surface in an attempt to reach the adults moving there. *S. diaprepesi* is considered to display a cruiser behavior (Del Valle et al., 2014), which can generate better results against buried insects in relation to those on the substrate surface (Banu et al., 2017). According to the present study, *S. diaprepesi* may also forage on the surface of the substrate as this is where the brown stink bug remains. Wilson et al. (2012) stated that EPNs can have both ambush and cruiser behaviors in each species, with a predominance of one or the other depending on the type of substrate.

The nematode *S. diaprepesi* AM163 was twice more virulent to *E. heros* compared to *D. melacanthus*, even though both insects belong to the same Pentatomidae family. The difference of susceptibility for these two bugs can be related to their differences in behavior. *E. heros* usually remains in the aerial part of the soybean plants during the reproductive phase of the crop, dropping to the straw only after the harvesting or during diapause period (Corrêa-Ferreira et al., 2009). On the other hand, *D. melacanthus* remains in contact with the soil surface or straw at the initial stages of the corn germination, causing damages to the emerging plantlets (Corrêa-Ferreira and Panizzi, 1999). These facts might have conducted to differential selection, making the bug that attacks only the aerial part of soybean more susceptible to EPNs. It is conceivable that insects

tend to be more resistant to EPNs if they remain naturally more exposed to the soil during their life cycle (Kaya et al., 1993; Jackson 1996; 1999).

In fact, *D. melacanthus* was equally more resistant to both nematodes *S. diaprepesi* AM163 and *S. carpocapsae* IP1, which differ in their "cruiser" and "ambusher" behaviors, respectively, according to Koppenhöfer and Kaya (1996) and Del Valle et al. (2014). Testing *S. diaprepesi* and *S. carpocapsae* on *D. melacanthus*, Guide et al. (2019) obtained higher mortality rates (78 and 64% respectively); however, using higher rate (100 IJs/cm^2) and holding the insects inside Petri dishes (9 cm) containing filter paper lied on the bottom. The nematode *S. feltiae* selected by Guide et al. (2019) as one of the most virulent, caused 72% mortality inside the Petri dishes (over filter paper), and less than 40% in the greenhouse (over straw), with more than 25% mortality in the control. In the present study, the use of straw to screen EPNs against *E. heros* allowed the selection of species that may display a better performance over this substrate, explaining the similar results (> 70%) obtained in both lab and greenhouse tests, where the same substrate was used, treated with the rate of 88.4 IJs/cm^2. This rate is very high, especially when compared to rates used in other tests against soil inhabitant bugs, such as those tested against spittlebugs on the genus *Mahanarva*, > 1 IJs/cm^2 (Leite et al., 2005; Batista and Auad, 2010; Batista et al., 2014).

5. Conclusion

Elevated concentration rates of *S. diaprepesi* (> 24 IJs/cm^2) were required to cause mortalities levels higher than 50% to *E. heros*. Thus, if high rates become economically acceptable for the adoption of the technology, EPNs might present themselves as potential agents to control *E. heros* soon after the soybean crop be harvested, when the insect is forced to go down to the straw, or during the diapause period in the colder regions, when the insect is found in the crop remains. New studies are needed to select more virulent nematodes to ensure their persistence in the field and to develop attractants or baits for association with EPNs. Stink bugs are among the main pests of soybeans in Brazil and in many other countries. Up to date, there is no effective microbial product to control these insects, which are recognized as resistant to various microorganisms. The current study demonstrates the possibility of breaking this resistance with high rates of nematodes.

Acknowledgments

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