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Effects of some insecticides on the neutral lipid percentage, survival and infectivity of *Steinernema carpocapsae* ALL and *Heterorhabditis amazonensis* JPM 4

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HIGHLIGHTS

- We studied the compatibility of entomopathogenic nematodes (EPNs) with three phytosanitary products used for tomato crops and the effects of these products on the infectivity of these nematodes against *Galleria mellonella* larvae.
- The percentage of neutral lipids was evaluated in two species of EPNs after exposure to phytosanitary products.
- The insecticides abamectin and chlorpyrifos maintained IJs viable, but reduced their infectivity capacity after exposure to insecticides.
- These insecticides reduced the amount of lipid in IJs and consequently their ability to infect a host.

Abstract: Lipids are an important energy source for entomopathogenic nematodes (EPNs) and directly influence their infectivity in the host. Some insecticides reduce the infectivity of infective juveniles (IJs) while keeping them viable after exposure. Thus, the objective of this study was to correlate the amounts of lipid reserves in the EPN *Heterorhabditis amazonensis* JPM 4 and *Steinernema carpocapsae* ALL with their survival and infectivity when exposed to insecticides that keep the nematodes viable but reduced their infective capacity against *Galleria mellonella*. Among the tested insecticides, Vertimec® and Klorpan® were incompatible (class 2) with the two EPN species because they reduced infectivity. The insecticides Vertimec® and Klorpan® maintained the viability of the IJs but reduced their infectivity and their lipid amounts after insecticide exposure.

Keywords: abamectin, chlorpyrifos, triflumuron, compatibility.

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INTRODUCTION

Entomopathogenic nematodes (EPNs) are utilized as biological control agents of various insect pests because of their different forms of action, specificity, and strong capacity to adapt to new environments; additionally, EPNs can occur in synergy with other entomopathogenic agents[1]. Among EPNs, the species in the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are of great relevance due to their symbiotic association with bacteria of the genera *Xenorhabdus* Thomas & Poinar, and *Photorhabdus* Boemare, Louis & Kuhl, respectively, causing rapid insect death[2].

These nematodes are used in areas where different agricultural inputs are applied, such as chemicals, fertilizers and soil amendments[3]. However, this treatment can cause large problems if used indiscriminately. For instance, the treatment can cause detrimental effects to the environment and animals, including humans and natural enemies of the insect pests[4]. Various studies on the

compatibility of insecticides with EPNs have been performed, and in some cases, the authors conclude that the agrochemical products keep the nematodes viable but reduce their infectivity capacity[5, 6]. One factor related to the viability and infectivity of nematodes is the lipid amounts present in infective juveniles (IJs)[7]. The IJs are dependent on lipids as their energy source during their search for a host[8, 9], being able to survive and remain infective for weeks or even months until they find a susceptible host[10]. Thus, the controlled manipulation of lipid amounts in IJs has a considerable potential to increase EPN viability and infectivity[7].

Thus, the objective of this study was to correlate the amounts of lipid reserves in the EPNs *Heterorhabditis amazonensis* JPM 4 and *Steinernema carpocapsae* ALL with their survival and infectivity when exposed to insecticides that keep the nematodes viable but reduce their infective capacity against *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae.

MATERIALS AND METHODS

The nematodes used for this bioassay were *Steinernema carpocapsae* ALL (Weiser) Wouts, Mrácek, Gerdin & Bedding and *Heterorhabditis amazonensis* JPM 4, Andaló, Nguyen & Moino Jr., (obtained from soil samples from Lavras, Minas Gerais, Brazil).

The nematodes were reared in late instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae)[11]. Rearing of *G. mellonella* was performed according to the method of Dutky[12] using a modified artificial diet[13].

The nematodes obtained from *G. mellonella* larvae were kept in aqueous suspension at 16 ± 1 °C and were stored for up to one week before the experiment.

Bioassay of compatibility between entomopathogenic nematodes and insecticides

To determine the compatibility of the EPNs with the insecticides, the methodology modified by Negrisoli Jr et al.[4] was used. For this bioassay, three insecticides registered for tomato crops were used: Vertimec 18® as abamectin, toxicological classification (T.C.) III, 1.2 L/ha; Certero® as triflumuron, T.C. II, 300 mL/ha; and Klorpan 480® as chlorpyrifos, T.C. I, 800 mL/ha. One liter of each insecticide was prepared with twice the dosage recommended for application to one hectare: 400 L. From this solution, a 1-mL aliquot was transferred to each glass vial (one replicate), with a total of five vials per treatment. Then, 1 mL of the suspension containing 2,500 IJs was added to each vial, and they were manually agitated. The vials were kept in a climate-controlled chamber at 27 ± 1 °C.

Nematode survival was evaluated after 48 hours of exposure to the insecticides. A 0.1-mL aliquot (approximately 125 IJs) was observed under a stereoscopic microscope for assessing the effects of the insecticides. The nematodes that did not react to stimulation by a bamboo pick were considered dead. For the infectivity assay, IJs that survived in the presence of insecticide exposure were washed in 3 mL of distilled water. The solution was then allowed to decant for 30 minutes in the climate-controlled chamber at 27 ± 1 °C. The supernatant (3 mL) was then discarded, and the washing process was repeated three times. After the last wash, a 0.2-mL aliquot (approximately 250 IJs) was removed from the bottom of each vial and pipetted into five Petri dishes (9 cm of diameter) per treatment. Each Petri dish contained a filter paper that had been previously soaked in 1.8 mL of distilled water. Ten *G. mellonella* larvae were placed on each Petri dish, and the dishes were then kept in a climate-controlled chamber for three days under the same conditions described above. After the three day period, the killed larvae were transferred to a dry chamber, mounted in Petri dishes (9 cm diameter) with filter paper, and kept in a climate-controlled chamber at 27 ± 1 °C at a relative humidity (RH) 70 ± 10% for three more days. After that period, the larvae were observed under a stereoscopic microscope and dissected to determine the presence of nematodes.

The experimental design used was a completely randomized one with four treatments, namely, a control (distilled water), Vertimec®, Certero® and Klorpan®, with five replicates per treatment; the experiment was conducted two times. An analysis of variance was performed on the mortalities of nematodes and larvae. The differences in viability and infectivity for the EPN species were analyzed using Tukey's test ($p < 0.05$) in the SISVAR software program[14]. The effects of the treatments on the EPNs' infectivity in *G. mellonella* were classified according to Peters & Poullot[15] based on the guidelines of the International Organization for Biological Control (IOBC) and the following formula:

E% = 100 – (100 – % corrected mortality) × (100 – Red)

in which Red is percentage reduction in infectivity in the treatment.

The corrected mortality was equal to zero for all treatments, and was thus not considered in the calculation of E%.

The percentage reduction in EPN infectivity was calculated by the formula:

$$Red = (1 - It / Ic) \times 100$$

In which:
It = mortality of *G. mellonella* in each treatment
Ic = mortality of *G. mellonella* in control treatment

Based on the value of E% the products were classified as: 1 – non-toxic (< 30%), 2 – slightly toxic (30-79%), 3 – moderately toxic (80-99%) and 4 – toxic (> 99%).

For the treatments that presented a percentage of dead larvae that was greater than in the control treatment, E% was considered equal to zero and the product was considered non-toxic.

Analysis of lipid percentage after insecticide exposure

To analyze the neutral lipid percentage, the IJs were exposed to the insecticides that maintained nematode viability but reduced their infectivity according to the results of the previous bioassay. The control group was exposed to distilled water. Following the period of exposure, 2 mL of IJ suspension (2,500 IJs/mL) was concentrated to a volume of 0.5 mL by decantation. Using the suspension, the lipid amounts in the IJs' bodies were analyzed using the histological colorimetric method with "Oil Red O" dye[16].

A staining solution was prepared with 0.5 g of "Oil Red O" dye in 100 mL of 100% absolute alcohol. The solution was agitated for 15 minutes and then filtered with Whatman nº. 1 filter paper and kept in the dark at 5 °C in a glass container. All glassware used to prepare the staining solution was previously cleaned and sterilized. Three milliliters of the "Oil Red O" staining solution was added to each IJ suspension concentrated to a volume of 0.5 mL, and the mixture was then heated in a water bath at 60 °C for 20 minutes. After cooling down to room temperature, the IJs, with the neutral lipids in their bodies stained red, were concentrated in 0.5 mL of staining solution by centrifugation at 2,000 rpm for 1 minute. Then, 3 mL of distilled water and pure glycerin (1:1) was added, and the solutions were stored at room temperature. The experimental design used was completely randomized with three treatments, namely, a control, Vertimec® and Klorpan®, with five replicates per treatment; the experiment was conducted two times. From each replicate, 2 IJs were randomly chosen for use, totaling 10 IJs per treatment.

Following the previous procedure, glass slides with IJs were obtained for each treatment and photographed using an Olympus Cx 31 trinocular microscope. Using the photographs, the total area of the IJs bodies was estimated, and the red stained area corresponded to the lipid area. For the area estimation, Image Tool Software version 3.0 for Windows was used. Thus, based on the red stained area, the percentage of neutral lipids in relation to the total IJ body area was calculated.

RESULTS AND DISCUSSION

The mortality of IJs from the two species of nematodes after exposure to insecticides was low (Table 1). The mortality rates of *S. carpocapsae* ALL IJs after exposure to the insecticides Vertimec® (13.6%), Certero® (18.6%), and Klorpan® (12.8%) were significantly different than the mortality rate in

Table 1. Percentage of mortality (mean ± SE) of *Heterorhabditis amazonensis* JPM4 and *Steinernema carpocapsae* ALL exposure to the insecticides used on the tomato crop (27 ± 1 °C, RH of 70 ± 10%).

Heterorabditis amazonensis		*Steinernema carpocapsae*	
Treatment	Mortality	Treatment	Mortality
Control	9.0 ± 0.20 a[a]	Control	2.0 ± 0.20 c
Vertimec	13.2 ± 0.19 a	Vertimec	13.6 ± 0.17 ab
Certero	9.2 ± 0.20 a	Certero	18.2 ± 0.64 a
Klorpan	16.2 ± 0.29 a	Klorpan	12.8 ± 0.20 b

[a]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.

the control (2.0%) (F = 6.94, df = 3, p < 0.05, CV = 6.59) (Table 1). For *H. amazonensis* JPM 4, the mortality rates caused by the treatments Vertimec® (13.2%), Certero® (9.2%), and Klorpan® (16.2%) did not differ from the mortality rate of the control (9.0%) (Table 1) (F = 1.89; df = 3; p > 0.05, CV = 6.38), and the treatments did not reduce the IJs' viability.

The nematode *S. carpocapsae* ALL had the highest mean percentage of infectivity against *G. mellonella* larvae in comparison to *H. amazonensis* JPM 4. Vertimec® and Klorpan® reduced the infectivity of *H. amazonensis* JPM 4, causing 30 and 26% mortality of *G. mellonella* larvae, respectively (Table 2). These values were significantly different from the control (86%) (F = 36.06, df = 3, p < 0.05, CV = 21.12). The insecticide Certero® did not reduce IJ infectivity, resulting in 76% mortality of *G. mellonella* larvae. Similar results were observed for *S. carpocapsae* ALL, with Vertimec® and Klorpan® significantly differing from the control (96%), causing 20 and 50% mortality of *G. mellonella* larvae (F = 35.89, df = 3, p < 0.05, CV = 20.45), respectively, and reducing the infectivity of this nematode. The Certero® treatment did not reduce IJ infectivity and resulted in 82% mortality of *G. mellonella* larvae (Table 2).

The insecticides Vertimec® and Klorpan® were considered mildly toxic (class II – slightly harmful) for the two EPN species. There was a correlation in the toxicological classification of the product Klorpan® (class II) and the reduction of IJ infectivity. There was no correlation in the toxicological classification of the product Vertimec® (class III), which caused a loss in the IJ infectivity when compared to the control treatment. However, the toxicity of an *in vitro* insecticide may not always be related to its toxicity in the field[17] because in the laboratory, the contact is extreme and guaranteed for 48 hours. One possible way to use nematodes and incompatible insecticides on the same crop would be to time the application of the nematodes after the insecticide persistence period or vice-versa[18]. The insecticide Certero® was considered compatible (class 1 – harmless) with both nematode species.

These findings indicate that the viability and infectivity of the two studied species of EPNs, *S. carpocapsae* ALL and *H. amazonensis* JPM 4, were not affected by exposure to the chitin inhibitor insecticide Certero®. This result could be due to the absence of chitin in the nematodes' cuticle, which primarily consists of collagens, cuticulins and other proteins[18]. In addition, the insecticide with an action similar to that of Certero®, the chitin inhibitor diflubenzuron, did not provoke any inhibition in the reproduction and development of *S. carpocapsae* in *in vitro* tests[19]. Chitin inhibitor insecticides are known for not having an effect on the viability of *Heterorhabditis bacteriophora* Poinar[20], supporting the results of Rovesti & Deseö[21] for *S. carpocapsae* and *Steinernema feltiae* (Filipjev), Wouts, Mrácek, Gerdin & Bedding and those of De Nardo & Grewal[22] for *S. feltiae* using the Adept IGR® in addition to the results obtained in the present study.

Head[23] studied the compatibility of five insecticides with *S. feltiae* and found that Dynamec® caused low IJ infectivity (0.1%) in *G. mellonella* larvae. The same result was observed in the present study. Other studies have also shown low mortality of *S. carpocapsae* IJs when exposed to chlorpyrifos[24, 25, 26]. A possible explanation for the insensitivity of EPNs to this insecticide is the presence of butyrylcholinesterase in the synapses of parasite nematodes, which protects the acetylcholinesterase from the insecticidal action, acting as a first line of defense[27]. Negrisoli Jr et al.[4] observed that Pyrinex® caused low mortality rates in *H. bacteriophora* (2.8%) and *S. carpocapsae* (2.2%) but reduced the IJs' infectivity in *G. mellonella* larvae, similar to the results of the present study.

The insecticide Certero® did not affect EPN infectivity and therefore was not used for the lipid colorimetric assay. The neutral lipid percentages in the IJs were reduced in both species after insecticide

Table 2. Mortality of *Galleria mellonella* larvae (mean ± SE) for *Heterorhabditis amazonensis* JPM4 and *Steinernema carpocapsae* ALL exposure to the insecticides used on the tomato crop (27 ± 1 °C, RH of 70 ± 10%).

	Heterorabditis amazonensis			*Steinernema carpocapsae*		
Treatment	Infectivity (%)[a]	E%[c]	C[d]	Infectivity (%)	E%[c]	C[d]
Control	86.0 ± 0.60 a[b]	–		96.0 ± 0.40 a	–	
Vertimec	30.0 ± 0.40 b	65.1	2	20.0 ± 0.54 c	79.1	2
Certero	76.0 ± 0.50 a	11.6	1	82.0 ± 0.50 a	14.5	1
Klorpan	26.0 ± 0.40 b	69.7	2	50.0 ± 0.54 b	47.9	2

[a]Dead *Galleria mellonella* larvae.
[b]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.
[c]Treatment effect: E % = 100 – (100 – % corrected mortality) × (100 – Red). % of corrected mortality was null in all treatments and therefore not considered for calculating E.
[d]IOBC toxicological classification of the insecticides: 1—harmless (< 30%), 2—slightly harmful (30-79%).

exposure (Figure 1). For *H. amazonensis* JPM 4, the lipid percentages were 72.6% after exposure to Klorpan® and 63% after exposure to Vertimec®. The lipid percentage after exposure to Vertimec® was significantly different from that of the control (80.48%) ($F = 5.47$, $df = 2$, $p < 0.05$; $CV = 16.34$). Regarding *S. carpocapsae* ALL, the lipid percentages were 41.82% after exposure to Klorpan® and 45.14% after exposure to Vertimec®, both of which were significantly different from the control (74.7%) ($F = 37.86$, $df = 2$, $p < 0.05$, $CV = 17.25$).

The nematode *S. carpocapsae* has the capacity to survive for long periods of time in water by remaining quiescent in a J-shaped posture, thus saving energy[28]. Because of this behavior, it can be inferred that IJ locomotion may spend more energy from its reserves and may consequently lead to a decrease in infectivity[29]. In addition, the IJ's size and its initial lipid amounts appear to be related to their survival and infectivity[30]. Furthermore, the *Steinernema* isolates can survive longer periods than *Heterorhabditis,* as the lipid content is depleted more rapidly in the latter[31, 32].

According to Menti et al.[33], the lipid contents of the three EPNs *S. feltiae, Heterorhabditis megidis* GR and *H. megidis* UK 211 Menti, Wright & Perry were 38, 49 and 45%, respectively. These results are similar to the results of Fitters et al.[31]. Moreover, EPNs reared in *G. mellonella* (*in vivo*) exhibit higher initial lipid contents than those reared *in vitro*[7]. Andaló et al.[29] observed neutral lipid mean values of 97.4% for *Steinernema riobrave* Cabanillas, Poinar & Raulston, 90.3% for *S. carpocapsae* and 90.7% for *Heterorhabditis* sp. JPM 4. The same authors observed that *Heterorhabditis* isolates were less tolerant than *Steinernema* isolates at both low (8 °C) and high temperatures (24 and 28 °C).

According to Wright et al.[34], the infectivity of *S. carpocapsae* IJs declines rapidly after 90 days of storage. In *Heterorhabditis*, there is also a direct correlation between neutral lipids and infectivity[35]. Wright et al.[34] observed high levels of infectivity after 180 days of storage in water for *S. feltiae*, whereas for *S. carpocapsae,* the infectivity was stable up to 60 days. However, after this period, there was a decline in infectivity, and only a few individuals were infective after 120 days[34].

All the above observations clearly demonstrate that the quality and quantity of IJ lipids are particularly important for the use of EPNs as pest control agents, as these factors critically influence nematode viability and infectivity. The possibility of using EPNs in integrated pest management programs (IPMs) depends on careful study of the selection of adapted species or populations to provide information on their physiological and biochemical attributes, and so to allow their efficiency to be increased for commercial use[36].

Based on these results, it is possible to observe that the use of some insecticides in association with EPNs reduces the amounts of lipids they store. Lipids are an essential resource for IJs when searching for hosts. The formulation of insecticides compatible with EPNs that are capable of maintaining adequate amounts of lipids is of critical importance. Other factors may also be involved in the reduction of IJ infectivity in the present work, such as alteration of metabolic activity, inhibition of symbiotic bacteria and damage in sensorial organs; these should be studied further. In the present work, the nematodes *H. amazonensis* JPM 4 and *S. carpocapsae* ALL were considered incompatible with the insecticides Vertimec® and Klorpan®. These two products reduce the infective capacity of IJs and their lipid amounts.

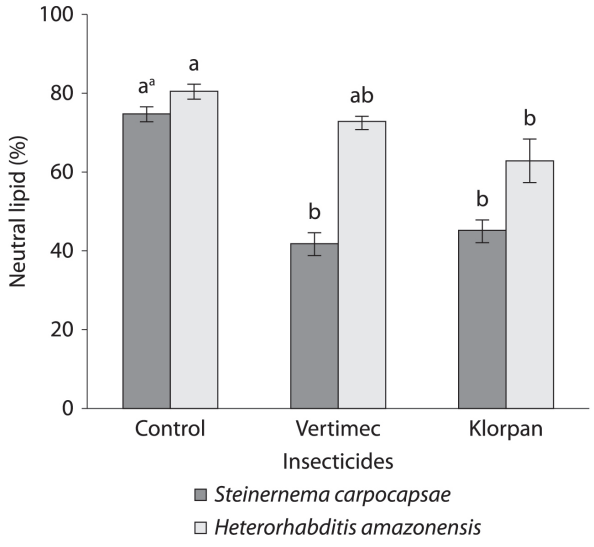

Figure 1. Effect of the insecticides on the neutral lipid percentage in infective juveniles after exposure. [a]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.

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