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Influence of plants used in agricultural diversification on the nematode *Heterorhabditis amazonensis*

Influência de plantas utilizadas na diversificação agrícola sobre o nematoide *Heterorhabditis amazonensis*

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Highlights

Crotalaria spectabilis and *C. breviflora* do not affect the nematode.
Tagetes erecta causes suppression of the initial nematode population.
Plants and *Calosoma granulatum* did not interfere with the nematode displacement.

Abstract

In an agricultural system, to increase natural biological control, plants that attract natural enemies can be grown alongside the main crop. However, the effects of these plants on entomopathogenic nematodes (EPNs), important agents for controlling soil pests, and the action of their conservation are unknown. To assess the impact of these plants on EPNs, two experiments were carried out in a greenhouse. The first measured the effect of *Crotalaria spectabilis, Crotalaria breviflora,* and *Tagetes erecta* on the persistence and infectivity of *Heterorhabditis amazonensis* isolate RSC 5 for 27 days, compared to a control treatment without plants. The second trial evaluated the effect of *C. breviflora* and *T. erecta* on the displacement of the nematode. Additionally, the influence of predator *Calosoma granulatum* in this system was evaluated. The plants did not influence nematode behaviour in terms of persistence, infectivity, or displacement. However, *C. spectabilis* allowed the most significant persistence of nematodes in the substrate for a short time, and *T. erecta* caused the fastest suppression of the initial population of infectives juvenile. In the second experiment, neither the predator nor the plants affected the nematode's ability to move in the soil within 5 days. These results show that prior knowledge in agricultural diversification can help to control pests by inundative application of EPNs.

Key words: Conservative biological control. Conservation. Entomopathogenic nematodes inundative control. Persistence.

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Resumo

Em um sistema agrícola, a fim de aumentar o controle biológico natural, plantas que atraem inimigos naturais podem ser cultivadas junto à cultura principal. Porém, os efeitos destas plantas sobre nematoides-entomopatogênicos (NEPs), importante agentes de controle de pragas de solo, e sua ação na conservação destes, são desconhecidos. Com o objetivo de avaliar o efeito de algumas destas plantas sobre NEPs, foram montados dois experimentos em casa-de-vegetação. O primeiro observou o efeito das plantas *Crotalaria spectabilis, Crotalaria breviflora* e *Tagetes erecta* sobre a persistência e infectividade de *Heterorhabditis amazonensis* isolado RSC 5 durante 27 dias, comparadas a um tratamento-controle sem plantas. Já o segundo experimento avaliou o efeito de *C. breviflora* e *T. erecta,* com a presença ou não do adulto do predador *Calosoma granulatum* no deslocamento do nematoide. Os resultados indicaram que as plantas não influenciaram na persistência dos nematoides em longo prazo nem na infectividade e no deslocamento de *H. amazonensis.* Porém, a planta *C. spectabilis* possibilitou a maior persistência de nematoides no substrato em curto prazo, e *T. erecta* causou a supressão mais rápida da população inicial de JIs. No segundo experimento observou-se que nem a presença do predador e das plantas afetou a capacidade de deslocamento do nematoide no solo no período de 5 dias. Estes resultados mostram que o conhecimento prévio das plantas a serem utilizadas na diversificação agrícola pode auxiliar no controle inundativo de pragas por NEPs.

Palavras-chave: Controle biológico conservativo. Controle inundativo. Conservação. Nematoides entomopatogênicos. Persistência.

Introduction

Entomopathogenic nematodes (EPNs) of the Steinernematidae and Heterorhabditidae families are obligate parasites and natural enemies of several insects of economic importance (Lewis & Clarke, 2012). The life cycle of these EPNs is similar, with a single free-living resistant stage called an infective juvenile (IJ). These nematodes search for hosts and infect them, penetrating through natural openings or the cuticle. The IJs carry symbiotic bacteria (*Xenorhabdus* or *Photorhabdus* spp.) that is released into the host's hemocoel, which dies of sepsis after 24 to 72 hours of infection. Inside the host, nematodes feed, develop, mate, and reproduce, and this cycle is repeated until the food source is exhausted and a new generation of IJs is formed that will migrate to the soil and seek new hosts (Poinar & Grewal, 2012).

Features such as easy application, compatibility with other control methods, and a wide range of hosts have caused EPNs to be marketed in several countries for short-term inundative or augmentative biological control programs (Campos-Herrera, El-Borai, Martín, & Duncan, 2016; Hodson, Siegel, & Lewis, 2012; Koppenhöfer, Shapiro-Ilan, & Hitpold, 2020; Mahmoud, 2016). These same features make EPNs ideal for conservative strategies of long-term biological control, which are more economical and sustainable (Stuart, Barbercheck, & Grewal, 2015). Changes in the agricultural environment that increase biodiversity become more attractive and /or favourable to natural enemies, thus reducing the negative impact of herbivores on plants of economic interest (Araj, Shields, & Wratten, 2019; Quispe, Mazón, & Rodríguez-Berrío, 2017).

For efficient conservation of natural enemies, it is necessary to identify which type of biodiversity is needed to maintain or increase biological control (Stuart et al., 2015). The plants used for increasing environmental heterogeneity may directly or indirectly affect the EPNs. The presence of roots can interfere with the search for insect hosts (Ennis, Dillon, & Griffin, 2010). The maintenance of soil moisture and temperature by plants can generate a microclimate favourable to EPNs (Campos-Herrera et al., 2016). Secondary compounds released by the roots can alter host susceptibility and post-infection IJ production (Gassmann, Stock, Tabashnik, & Singer, 2010; Hazir et al., 2016). Moreover, these compounds can be attractive, repellent (Jagodič, Ipavec, Trdan, & Laznik, 2017; Turlings, Hiltpold, & Rasmann, 2012), or toxic to EPNs (Santhi et al., 2019). Moreover, agricultural biodiversity also increases the diversity and insect availability that can serve as alternative hosts to the key pests (Helmberger, Shields, & Wickings, 2017) or as vehicles for the displacement of nematodes in the soil (Mertz, Agudelo, Sales, Rohde, & Moino, 2014).

Thus, this work aimed to evaluate the direct effect of plants used in the diversification of the agricultural environment on the persistence and infectivity of the entomopathogenic nematode *Heterorhabditis amazonensis.* By adding a predator (*Calosoma granulatum,* Coleoptera: Carabidae) to this system, the objective was to evaluate possible changes in the displacement of the nematode resulting from the increase in the system's biodiversity.

Materials and Methods

The experiments were conducted in a greenhouse and laboratory. The greenhouse was of the automatic type, with a cooling system that used evaporative air cooling by a porous medium exhaust. The insects used in the experiments were taken from creations kept in the Laboratory of Microbial Control, the Federal University of Lavras, Minas Gerais, Brazil.

Entomopathogenic nematode production

The nematode used in the experiments was the native Amazonian species *H. amazonensis* isolate RSC 5, produced by the in vivo method, adapted from Woodring and Kaya (1988), in caterpillars of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae). These were reared according to the methodology described by Dutky, Thompson and Cantwe (1964) using an artificial diet modified by Parra (1998). The viable IJs were counted and stored for a maximum of five days at 16±1°C in a climatic chamber until use in the experiment.

Calosoma granulatum cultivation

Cultivation conditions were adapted from the methodology proposed by Pasini (1995). Glass bottles (1.3 L, 12 cm in diameter, and 17 cm in height), externally wrapped in black plastic, filled up to 1/3 of the volume with Plantmax® commercial artificial substrate, sterilised, and moistened, were used for the maintenance of adult *C. granulatum.* These were fed daily with a piece of banana of the

variety 'Terra' (*Musa paradisiaca,* Musaceae), and water was offered in pieces of moistened cotton. When mated, the pairs were fed daily with two caterpillars of Spodoptera frugiperda (Lepidoptera: Noctuidae). Eggs were transferred to plastic containers filled with the same artificial substrate mentioned above. They were kept in a climate chamber at 27±1°C, with a photoperiod of 14 hours until the emergence of larvae, which were individualised in plastic containers containing substrate and artificially fed daily with caterpillars of *S. frugiperda* or larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) obtained from artificial laboratory conditions.

Plant cultivation

The plants used in the experiments were *Tagetes erecta* L. (Astraceae), *Crotalaria breviflora* L., and Crotalaria spectabilis Roth (Fabaceae), and seedlings were grown in a commercial substrate for planting (Plantmax®) from commercially-obtained seeds. The seedlings were transferred to plastic vases with a capacity of 2 L, with one seedling per vase filled with 1 kg of the substrate (soil, sand, and organic fertiliser, in the proportion of 2:1:1). The pots were kept in a greenhouse at a temperature of 29±4°C and humidity of around 80%. Irrigation was performed twice a day.

Persistence and infectivity experiment

Seedlings (15 cm tall, about 25 days after emergence) were transferred to plastic pots (one seedling per pot) and additionally assembled pots like the others (with the same dimensions and the same amount of substrate, as mentioned in the previous section) but kept without plants. All pots, with or without plants, were placed in a greenhouse and watered daily.

The experiment consisted of 40 pots with *T. erecta, C. breviflora,* and *C. spectabilis* plants (plants in the flowering stage at 70 days after emergence and about 40 cm in length) and 40 pots without plants. Each plant species was considered a treatment, and pots without plants were considered the control treatment. Under the substrate in each pot, after watering, a 30 ml distilled water suspension containing 15,000 IJs of nematode *H. amazonensis* was applied homogeneously. Then, the vases were covered and wrapped with a piece of voile-type fabric, the ends of which were tied to protect the substrate from any arthropods that could inhabit the soil and keep the plant's stem free. Plants were watered twice a day by wetting the fabric.

Insect-trap methodology was used to assess infectivity. The percentage of dead insects in the trap was used to monitor changes in nematode infectivity over time (Hass, Griffin, & Downes, 1999). To assess persistence, the Baermann funnel extraction methodology was used (Baermann, 1917). This methodology made it possible to extract viable nematodes and quantify them since it depends on the movement of EPNs and the action of gravity.

At 3, 11, 19, and 27 days after suspension application, ten pots of each treatment and control were separated, and the plant was discarded when present. The substrate was carefully separated and homogenised to separate two aliquots of 150 mL. One aliquot was transferred to a plastic pot with a lid (10×5×5cm), where three *G. mellonella* caterpillars were buried as trap

insects for the nematode. The pots were kept in the laboratory for five days. Subsequently, the caterpillars were removed from the substrate, and the dead ones were dissected to confirm nematode presence.

The other aliquot of the substrate was used for the extraction of nematodes using a Baermann funnel. Glass funnels (200 mL capacity) containing an aluminium mesh at the bottom (as a support for the sample) and a piece of malleable silicone rubber pipe connected to its stem, closed with a Mohr clamp, were used. The substrate aliquot was placed under a paper towel, positioned at the bottom of the funnel on the aluminium mesh, and the sample was covered with distilled water. After 24 hours, the Mohr clamp was opened, freeing the passage of the suspension and allowing its collection in a Becker-type flask. After 24 hours of decanting the extracted solutions, the extra water was discarded, standardising a volume of 10 mL for all extracted samples. The entire process was carried out in an acclimatised room at 26±2°C. Ten aliquots of 10 µL of the suspension of each sample were inoculated in ten wells of a microtiter plate. The IJs present in each well were quantified under a stereoscopic microscope. Thus, the number of IJs in 1 mL of the suspension was determined.

Each treatment and control had ten repetitions evaluated on four consecutive dates, with an interval of eight days between them. The pots were arranged randomly on benches in the greenhouse, and the experimental design was completely randomised. The greenhouse was kept at a temperature of 29±4°C and humidity of about 80%. Irrigation was carried out directly onto the soil (about 300 mL of water) twice a day.

Displacement experiment

The displacement experiment evaluated the effect of *T. erecta* and *C. breviflora* plants and the ground predator *C. granulatum* on the displacement of the nematode towards a host. Before the plants entered the flowering stage (about 70 days after sowing), one of each species was transferred to the centre of plastic pots (40 cm long, 20 cm wide, and 20 cm deep), and the substrate to which they were planted was distributed at the bottom of this new pot. On top of the substrate, 4 L of soil (red latosol) was added to fill the pot to 12 cm. The plants were maintained under these conditions until they started to bloom. This experiment also included pots without plants, which were considered the control treatment, that were set up in the same way as the others. In these, 2 L of the substrate (same mixture used in pots with plants) was added, and 4 L of soil (red latossol) were spread on the bottom of the pot.

Styrofoam screens (mesh with a 1 mm opening) were used to assemble envelopes (7×7 cm). Ten *G. mellonella* caterpillars (3 cm long) were placed in each envelope, and then, it was closed with paper clips. At 10 cm away from the plant stem and 10 cm from the end, a trench was made where the envelope containing the caterpillars was buried vertically. On the opposite side, also 10 cm away from the plant's stem and 10 cm from the end of the pot, a small hole was made, 3-5 cm deep, where a 30 mL suspension containing 20,000 IJs of the nematode *H. amazonensis* was applied.

In 15 of the 30 pots of each treatment (both plant species and control), a *C. granulatum* adult (± 40 days of age) was released to freely move about the vessel. Half of the pots in each

treatment and control had a beetle, and the other half did not. A 5 mm thick slice of banana (variety Terra) and water-soaked cotton in a plastic cup were placed randomly in the pot to feed the predator.

Thus, each plant species in the presence or absence of an adult predator was considered a treatment, and the control was considered the pot without a plant with the presence or absence of the insect. Evaluations were performed five days after IJ application and consisted of removing envelopes containing *G. mellonella* larvae. The dead nematodes were dissected and observed under a stereoscopic microscope to confirm nematode mortality. The confirmed mortality of each replicate was counted.

Plants were watered carefully, twice a day, using a small amount of water (about 100 mL) to avoid soil drench. The temperature in the greenhouse was 29±4°C, and the humidity was approximately 80%. The pots were arranged randomly on the benches, and the experiment was in a completely randomised design.

Data analysis

Data were tested for normality by the Shapiro-Wilk test. Those obtained in the persistence and infectivity experiments were submitted to the non-parametric Kruskal-Wallis analysis (P≤0.05), and the treatments were compared to each other by the Simes-Hochberg test. The data obtained in the displacement experiment were paired and compared to each other by the Wilcoxon test (P≤0.05) using Action Stat software.

Result and Discussion

The EPNs infected and killed G. mellonella larvae (Figure 1) and were detected using Baermann funnel extraction (Figure 2) until 27 days after inoculation. The proportion of infected caterpillars and the number of extracted nematodes decreased throughout the evaluations, indicating a reduction in nematode survival over time in all treatments (Figures 1 and 2).

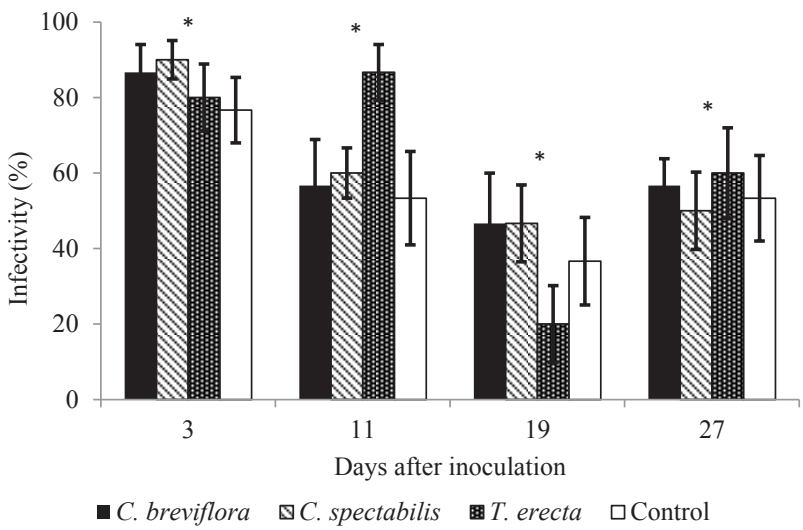

Figure 1. Infectivity of *Heterorhabditis amazonensis* (mean mortality of *Galleria mellonella* caterpillars) in the control (substrate only), *Crotalaria breviflora, Crotalaria spectabilis,* and *Tagetes erecta* treatments, after 3, 11, 19, and 27 days of inoculation in a greenhouse experiment (29±4°C, 80% relative humidity). *non-significant difference (P ≤ 0.05).

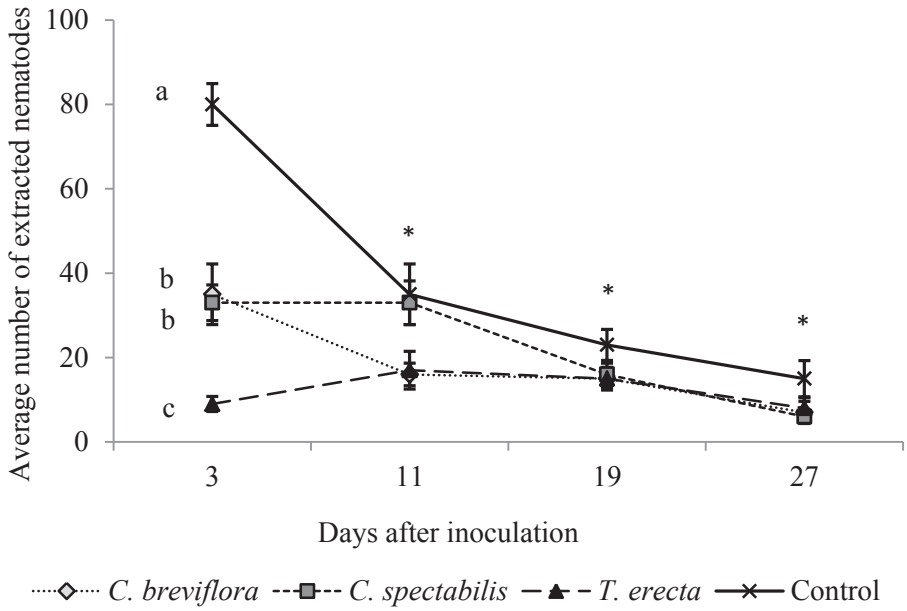

Figure 2. Persistence of *Heterorhabditis amazonensis* nematodes extracted via Baermann funnel from the control (substrate only), *Crotalaria breviflora, Crotalaria spectabilis,* and *Tagetes erecta* treatments in a greenhouse experiment. Different letters next to symbols indicate significant differences between the means on the same evaluation date (Kruskal-Wallis). *non-significant difference (P ≤ 0.05).

Using the insect-trap methodology (Figure 1), after three days of inoculation, the mean infectivity between treatments was 83%, and at 27 days after inoculation, it was 55%. The Kruskal-Wallis test (P ≤ 0.05) showed no significant difference between the treatments in any of the assessments.

The persistence of IJs for 27 days, causing mortality of more than half of the trap insects, indicates its high resistance to the high temperatures of the greenhouse (average of 29°C, with peaks of 33°C). The decrease observed over time was probably related to the decline in the nematode population. Calabuche-Gómez et al. (2019) observed that, among the abiotic factors, temperature most negatively affects *H. amazonensis* over time, with temperatures equal to and above 25°C, drastically reducing nematode viability and infectivity after seven days of exposure in the laboratory.

The lowest average mortality of *G. mellonella* larvae was observed 19 days after inoculation, with 38% dead insects (Figure 1). This low mean value was mainly due to the *T. erecta* treatment, which in this evaluation, had the lowest mortality among all observations. This plant demonstrated high variation in mortality over time.

Regarding the quantification of nematodes via Baermann funnel extraction, there was a significant difference between treatments only three days after inoculation (Figure 2). In this evaluation, the largest number of nematodes was found in the control treatment (pot without plants), with twice as many nematodes as in *C. breviflora* and *C. spectabilis* (Figure 2). The least nematodes

were accounted for in the treatment with *T. erecta,* about four times less than others. In the other evaluations, there was a reduction in the number of nematodes in all treatments, with no significant difference between them.

The large population reduction observed in the control treatment between 3 and 11 days after inoculation may be related to the effect of high temperature (Calabuche-Gómez et al., 2019). The competition of EPNs with other microorganisms may have been another factor. When counting under a microscope, a great diversity of nematodes and other organisms were observed in the control treatment compared to the other treatments (observational data, not quantified).

Extraction also indicated that the control treatment and *C. breviflora* caused the most considerable reduction in population between the 3rd and 11th day after inoculation, when the population dropped by half between the two evaluations. In the *C. spectabilis* treatment, the most significant reduction occurred between the 11th and 19th day after inoculation since the number of nematodes was stable between the 3rd and 11th. The *T. erecta* treatment was the only one that did not follow the same pattern as that of other plants because the number of nematodes in it was low on the 3rd and 27th day after inoculation (about ten nematodes).

Plants of the *Crotalaria* genus are widely used as cover crops or in partnership with other cultures having a large biomass capacity and biological nitrogen fixation (by the symbiosis that they perform with bacteria of the *Rhizobium* genus) (Berriel, Monza, & Perdomo, 2020). Furthermore, they attract a great diversity of insects and natural enemies (Supriyadi, Wijayanti, Arniputri, Puspitarini, & Dwiyatno, 2019; Tavares et al., 2010). They are also able to control phytonematodes, benefiting antagonists and producing secondary compounds that are toxic or inhibitory to them, such as the monocrotaline pyrrolizidine alkaloids, which reduce the hatching of juveniles and motility (Galbieri, Fuzattoii, Ciaii, Welteri, & Fanan, 2011; Osei, Gowen, Pembroke, Brandenburg, & Jordan, 2010; Ratnadass, Fernandes, Avelino, & Habib, 2012; Santana et al., 2012). However, despite the literature demonstrating its nematicidal effect, the results presented here show that *C. spectabilis* maintained the highest and most stable number of viable EPNs on the 3rd day after inoculation, in relation to the other plants evaluated (Figure 2), with no effect on their infectivity (Figure 1). Therefore, *C. spectabilis* can be safely used as a green manure to increase environmental heterogeneity, favouring natural enemies, in addition to the control of phytonematodes, without causing significant adverse effects on the population of the nematode *H. amazonensis* when flooding is applied.

Although *C. breviflora* did not affect infectivity (Figure 1), it reduced the EPN population faster than *C. spectabilis.* In both, pyrrolizidine alkaloids were found. These are secondary metabolites that affect various parameters of phytopathogenic nematodes in vitro. However, these effects vary between plant and nematode species (Gardiano et al., 2010; Thoden & Boppré, 2010).

T. erecta caused a rapid population reduction of nematodes in an inundative application to the soil. Plants of this genus also control phytopathogenic nematodes when used as a cover crop or in rotation with other crops. In field experiments, Adekunle (2011) observed that the incorporation of *T. erecta* and *C. juncea* plants caused a reduction in phytonematode damage in legumes infected with *Meloidogyne incognita.* Plants of the

genus *Tagetes* act as non-host plants to phytonematodes, benefit antagonists, and are a source of nematicidal extracts (Adekunle, 2011).

One of the main nematicide compounds produced by *T. erecta* is α-terthienyl (Hooks, Wang, Ploeg, & Mcsorley, 2010). However, little is known about the effect of this compound on EPNs. Kanagy and Kaya (1996) conducted studies with the EPN *Steinernema glaseri* and demonstrated that high concentrations of α-tertienil reduce the number of IJs that infect hosts. However, it is still unclear how the plant releases this compound and how its toxic effect is activated since EPNs do not have better or worse performance in the presence of *Tagetes* roots. This indicates that perhaps the natural concentrations of this compound suppress phytonematodes but not EPNs (Grubišić, Uroić, Ivošević, & Grdiša, 2018; Hooks et al., 2010; Kanagy & Kaya, 1996).

The results presented here show that *H. amazonensis* was initially harmed by the action of *T. erecta* in the persistence experiment (Figure 2). In addition, the infectivity results (Figure 1) showed that, despite not having differed, the infectivity pattern varied over time, not following the same pattern as the other plants.

Displacement experiment

There was no difference in *G. mellonella* larvae mortality between treatments by the Wilcoxon test ($P \leq 0.05$). This indicated that a similar number of nematodes managed to move 20 cm away towards the host in five days, regardless of the presence of the plant or the *C. granulatum* adult. Thus, caterpillar mortality was 30 to 40% in these treatments (Figure 3).

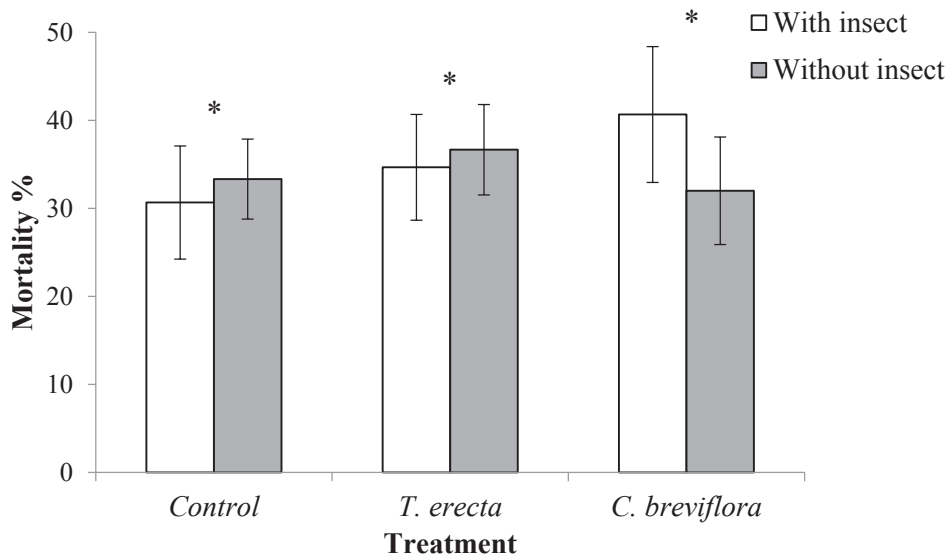

Figure 3. Mortality percentage of *Galleria mellonella* caterpillars due to the action of the nematode *Heterorhabditis amazonensis* applied in the substrate (control) or where the species *Crotalaria breviflora* or *Tagetes erecta* were placed and an adult of *C. granulatum* was released or not. Nematodes (20000 IJs) applied 20 cm away from the host, five days before evaluation, in a greenhouse experiment (29±4°C, 80% RH). *non-significant difference ($P \leq 0.05$).

Both larvae and adults of the *C. granulatum* predator are considered good agents for phoretic dispersion of the nematode in laboratory tests (Mertz et al., 2014). However, due to the testing distance (20 cm) and the time available to cover this distance (5 days), it can be deduced that the distance and time were sufficient for the nematodes to overcome any factor imposed by the plants that could limit its movement toward the host. In the experiment carried out by Andaló et al. (2012), in six days, the nematode *H. amazonensis* caused 80% mortality of *G. mellonella* caterpillars that were 40 cm away from the inoculation point in a sand column, indicating that this nematode has great horizontal displacement capacity in search of the host.

Plants used to beneficially increase the diversity of the agricultural system may have effects on some EPN parameters. The conservative biological control of EPNs is only possible after identifying the environmental factors that affect these nematodes. While the direct effects of soil physical properties, such as water potential, porosity, and temperature, have been studied in detail on EPNs, little attention is given to understanding the importance of biological interactions and how they vary in different habitats (Campos-Herrera, Stuart, El-Borai, Gutierrez, & Duncan, 2010).

Conclusions

Plants can interfere in the number of newly inoculated nematodes in the soil, without affecting their infectivity or displacement towards a host. Cultivating plants such as *C. spectabilis* together with those of economic interest, which in the case of the adoption of inundative biological control with nematodes, will keep the newly inoculated population viable in the soil for a period, increased control efficiency. In addition, *T. erecta* had an immediate antagonistic effect on the persistence of *H. amazonensis* without affecting its infectivity.

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