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Association of *Heterorhabditis amazonensis* and *Beauveria bassiana* aiming the control of *Hypothenemus hampei*

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ABSTRACT

Among the main problems for coffee production, it is possible to mention the occurrence of coffee borer, *Hypothenemus hampei*. In order to seek alternatives to control the pest, the proposed objective was to evaluate the interaction between *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01 on the control of coffee borer. Therefore, four experiments were set. In the first, *B. bassiana* UFU01, *H. amazonensis* MC01 and both entomopathogens associated were applied in bored fruits displaced in vermiculite. In the second experiment, the methodology was similar to the previous one; however, the fruits were immersed in the suspensions. In the third experiment was evaluated the transport of *B. bassiana* UFU01 conidia by *H. amazonensis*, checking the mortality of *Tenebrio molitor*. In the fourth experiment, the fungus and the nematode were added to Petri dishes containing agar-water simultaneously to observe if the nematodes could carry *B. bassiana* UFU01 conidia. The obtained data were subjected to analysis of variance and the Tukey test ($p < 0.05$), for comparison between means and regression analysis. It was found that, when entomopathogens were applied to vermiculite, treatments using *B. bassiana* UFU01 and *H. amazonensis* alone caused the highest mortality of *H. hampei* adults. For larvae, there was no difference among treatments. The same was observed when the fruits were dipped in the suspensions, with no difference among treatments. As for *T. molitor* mortality, it was observed that there was no difference among treatments. However, when applied in combination, there was a higher mortality caused by *B. bassiana* compared to *H. amazonensis*. In the last experiment, juveniles and conidia were found on the opposite side of the dish, and it can be inferred that the presence of conidia was due to transport by infective juveniles.

Key words: Biological control; *Coffea*; Entomopathogenic fungus; Entomopathogenic nematode.

1 INTRODUCTION

Brazil has gained great prominence in the coffee sector, with 2.16 million hectares of total area cultivated with coffee and the estimate for the year 2020, considering the positive period, is to reach between 57.2 million and 62.02 million processed bags (Companhia Nacional de Abastecimento - CONAB, 2020).

Among the obstacles to coffee production, the occurrence of coffee borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) can be highlighted. This insect is associated with annual losses of U$$ 500 million, affecting the income of more than 20 million rural producers in about 80 countries (Infante; Pérez; Vega, 2012). The losses are mainly associated with direct injuries caused to the fruits, which lead to weight loss and early fall (Oliveira et al., 2013). Adult *H. hampei* females make galleries in the endosperm of the coffee seed, reducing the production and quality of the final product, due to early fall, delay in development and fruit rotting (Parra; Reis, 2013). Thus, coffee borer causes the product to depreciate in terms of physical classification, as it compromises the sensory quality of the beverage (Souza et al., 2013).

Borer control through the chemical method is considered difficult due to the cryptic habit of the insect, remaining inside the fruit. In addition, due to poor harvest, many fruits are hanging on the plant or fallen on the ground, which serves as a shelter for the borer, intensifying infestation in the next harvest. The appropriate measures to avoid that post-harvest fruits remain in the field, in most cases, are not taken, thus causing an increase in pest incidence and making the reduction in population levels even more difficult (Carvalho; Souza, 2020).

The insecticide endosulfan used to be widely applied in the control of coffee borer. However, in 2013, its use was banned by the National Sanitary Surveillance Agency (ANVISA). Thus, after its prohibition, many efforts have been made in order to obtain a new product for this purpose, with a series of products being registered for the control of coffee borer (Brasil, 2020; Plata-Rueda et al., 2019; Souza et al., 2013), but it is understood that, for a better pest control, it is necessary to take joint control measures, adopting the concepts of integrated management.

Among other methods that can be applied in the control of coffee borer, the use of biological control can be considered potential, as long as crop management practices are taken into account. Entomopathogenic nematodes (Nematoda: Rhabditida) from the families Heterorhabditidae and Steinernematidae are potential pest control agents (Dolinski et al., 2017). According to Divya and Sankar (2009), the application of nematodes to bored fruits that fall on the soil

helps control the pest insect. The authors also indicate that the dispersion of *H. hampei* adults helps in the spread of the nematode in the field to other areas, allowing the colonization of other hosts. Allard and Moore (1989) considerate that *Heterorhabditis* sp. can cause coffee borer mortality of both larvae and adults, and can produce new generations of nematodes that will remain in the field in the search of new hosts.

Entomopathogenic fungi can also help in the control of coffee borer. The fungus *Beauveria bassiana* (Bals.) (Hypocreales: Cordycipitaceae) acts on several insect orders and is capable of causing epizootics in *H. hampei*. However, there are differences related to the fungus control potential in the field due to management practices adopted in each area, which can favor or hinder the occurrence of the fungus (Damon, 2000; Monzón; Guharay; Klingen, 2008).

Considering the possible application of entomopathogens directed to the soil in order to reach fallen bored fruits, the objective of this study was to verify the potential of *B. bassiana* UFU01 and the entomopathogenic nematode *Heterorhabditis amazonensis* MC01 applied alone and associated in the control of *H. hampei* under laboratory conditions.

2 MATERIAL AND METHODS

2.1 Association of *Beauveria bassiana* and *Heterorhabditis amazonensis*

The fungus and the entomopathogenic nematode used in the experiments were obtained from the bank of entomopathogens of Federal University of Uberlândia, MG, Brazil. The nematode was isolated from the soil using insect bait and the fungus was isolated from an *H. hampei* adult, both in the region of Monte Carmelo, MG, Brazil.

For the evaluation of *H. hampei* mortality caused by *B. bassiana* UFU01 and *H. amazonensis* MC01, two experiments were conducted under laboratory conditions. For each experiment, about 450 bored fruits were collected in a coffee field at approximately six years of age, located at the coordinates (18°44'46.2''S, 47°31'07.6''W), with a total of 5 ha of 'Rubi MG-1192' *Coffea arabica* L., spacing of 3.50 m between rows x 0.80 m between plants. The harvested fruits were taken to the laboratory where the material was sorted, selecting the fruits with injuries related to the presence of coffee borer.

The first experiment consisted of three treatments and the control, with 10 replications each. The treatments were: commercial product based on *B. bassiana* UFU01, applying 1 mL of suspension at a concentration of 1 x 10^9 conidia mL^{-1}; *H. amazonensis* MC01 at a concentration of 1,200 infective juveniles (IJs) mL^{-1} (150 IJ per fruit); and the association of *B. bassiana* UFU01 and *H. amazonensis* MC01, 0.5 mL of each suspension at the same previous concentrations. In the control, only 20 mL of water were applied. Both entomopathogens and water were added to vermiculite using a micropipette (Figure 1).

To set this experiment, which started on the same day as fruit collection, 0.09 m Petri dishes were used, containing 0.0045 kg of vermiculite in each dish, to which 19 mL of water were added in order to moisten the substrate. Eight bored fruits were placed per dish, so that they were covered by vermiculite, totaling 320 fruits.

The dishes were closed and sealed with Parafilm® and kept in a climatic chamber at 24 ± 1ºC, with a 12-hour photoperiod. Evaluations of larval and adult mortality of coffee borer started four days after the application of treatments for five days (totaling nine days of contact with the suspensions), with two replications per day being evaluated. The verification was carried out with the help of a utility knife, cutting the fruit in half and analyzing it. After the evaluation, the dead insects were placed in a humid chamber to confirm mortality by entomopathogens.

For the second experiment, the bored fruits were dipped into suspensions with the fungus and the nematode, alone or together. The experiment consisted of three treatments and a control, with five replications each. Ten fruits were used for replication. The suspensions were prepared at the same concentrations as the previous assay to obtain a final volume of 100 mL each. In the association of *B. bassiana* UFU01 and *H. amazonensis* MC01, 50 mL of suspension with the nematode and 50 mL of the suspension containing the fungus were used, keeping the concentration of the entomopathogens. In the control, only water was used.

To submerge the fruits, they were placed in measuring cylinders (200 mL) and the suspensions were then added, keeping the fruits submerged for 5 seconds. After contact with the products, they were placed in Petri dishes (0.09 m in diameter) lined with a sheet of filter paper. The dishes were closed with Parafilm® and kept in climatic chamber at 24ºC ± 1ºC with a 12-hour photoperiod.

Evaluations of larval and adult mortality of coffee borer started 11 days after the application of entomopathogens, and all replications were analyzed on the same day. The dead insects were kept in a humid chamber to confirm mortality. A General Linear Model was adjusted for the data of the first experiment with a binomial distribution and a logit link function. Analysis of deviance was performed to detect significant difference in the factors using Chi-Squared test. For the second experiment, with normal distribution and homocesdasticity, data was submitted to Analysis of Variance (ANOVA). If the the means were considered different, Tukey's test ($p < 0.05$) was applied for the treatments, and regression analysis was performed for the time of evaluation.

2.2 Phoresis of *Beauveria bassiana* by *Heterorhabditis amazonensis*

To verify the possible transport of *B. bassiana* UFU01 conidia by *H. amazonensis* MC01, two experiments were carried out. The first experiment was conducted using *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae, created according to the methodology of Potrich et al. (2007), arranged in Petri dishes (0.09 m in diameter) containing 0.0045 kg of vermiculite. A steel screen (0.01 m high, 0.09 m wide) was placed in the center of the dish in order to divide the dish into two sides and keep the larvae on only one side of the dish.

Five *T. molitor* larvae were placed on one side of each dish. In the control and in the treatment with *B. bassiana*, water and the fungus, respectively, were applied on the entire dish, while in the treatments with the nematode and the association of *B. bassiana* with the nematode, entomopathogens were added on only one side of the dish, opposite to the presence of *T. molitor*. Three treatments were used: *B. bassiana*, *H. amazonensis* and the association of the two entomopathogens. Each treatment consisted of 10 replications.

Vermiculite was first moistened with 14 mL of water and the suspensions were then applied. For *B. bassiana*, 1 mL of suspension was used at a concentration of 1 x 10^9 conidia. *Heterorhabditis amazonensis* MC01 was added to vermiculite at a concentration of 1,200 IJ mL^{-1}. For the association between *B. bassiana* and *H. amazonensis*, 0.5 mL of each suspension was added at the same previous concentrations. In the control, 15 mL of water were added. No type of diet was provided for the insects inside the dishes.

The dishes were properly closed and sealed using Parafilm® and kept in climatic chamber at 24 ± 1ºC with a 12-hour photoperiod. The mortality of *T. molitor* larvae was assessed after 12 days, evaluating insect mortality; thus, it was verified if there were conidia on the side of the dish where it was not applied. In the dead larvae, the characteristic symptoms of death by the entomopathogen were verified and, when necessary, they were kept in a humid chamber or dissected.

The second experiment was set in order to observe the possible transport of *B. bassiana* conidia by *H. amazonensis* IJs when inoculated together in Petri dishes containing agar-water. Therefore, 20 Petri dishes (0.09 m in diameter) containing 20 mL of water-agar per dish at a concentration of 1% were used.

Three treatments and a control were used, with five replications, arranged in a completely randomized design, as follows: *B. bassiana* UFU01, at a concentration of 1 x 10^9 conidia mL^{-1}; *H. amazonensis* MC01 at a concentration of 1,200 IJ mL^{-1}; and the association of *B. bassiana* UFU01 and *H. amazonensis* MC01 at the same previous concentrations and in equal volumes. In the control, only water was used.

For each treatment, 0.25 mL of the suspension of one side of the dish were added, subsequently closed with Parafilm® and kept in climatic chamber at 24 ± 1ºC for 24 hours darkness; the evaluation was performed after seven days. The evaluations were performed in a stereomicroscope, verifying the presence of IJs and conidia on both sides of the dish, in order to observe if there was conidia transport by the nematode. The data obtained were submitted to analysis of variance and the Tukey test ($p < 0.05$) to compare the treatment means.

The statistical analysis was performed in R software version 3.5.0 and the graphical artwork in SigmaPlot version 11.

3 RESULTS

3.1 Association of *Beauveria bassiana* and *Heterorhabditis amazonensis*

Regarding the mortality of *H. hampei* adults when entomopathogens were applied to vermiculite, it was found that there was no interaction between treatments and times of evaluation ($p = 0.628$), using the data obtained from final coffee borer mortality. There was no difference between the treatments using *B. bassiana* UFU01 and *H. amazonensis* MC01 alone, but they caused higher mortality than the treatment with the pathogens applied together and the control ($p = 0.003$) (Table 1).

Table 1: Mortality of *Hypothenemus hampei* adults caused by *Beauveria bassiana* and *Heterorhabditis amazonensis* MC01 after five days of evaluation. Temperature of 24 ± 1ºC and 12-hour photoperiod.

Treatment	Mortality (%)*
Beauveria bassiana	28.16 ± 8.73 a
Heterorhabditis amazonensis MC01	27.97 ± 8.74 a
H. amazonensis MC01 + *B. bassiana*	0.76 ± 0.87 b
Control	0.01 ± 1.11 b

* Means ± standard deviation followed by the same letter did not differ significantly by the Tukey's test at 5% significance.

Considering the mortality of adults throughout the evaluation periods, it is possible to observe that there was an increase in insect mortality, reaching more than 40% of dead adults (Figure 1).

When analyzing the mortality of coffee borer larvae, an interaction was observed between evaluation periods and treatments ($p = 0.043$). From the 3rd day of evaluation, there was a significant difference between treatments and the control; the same was observed until the 5th day of evaluation (Table 2).

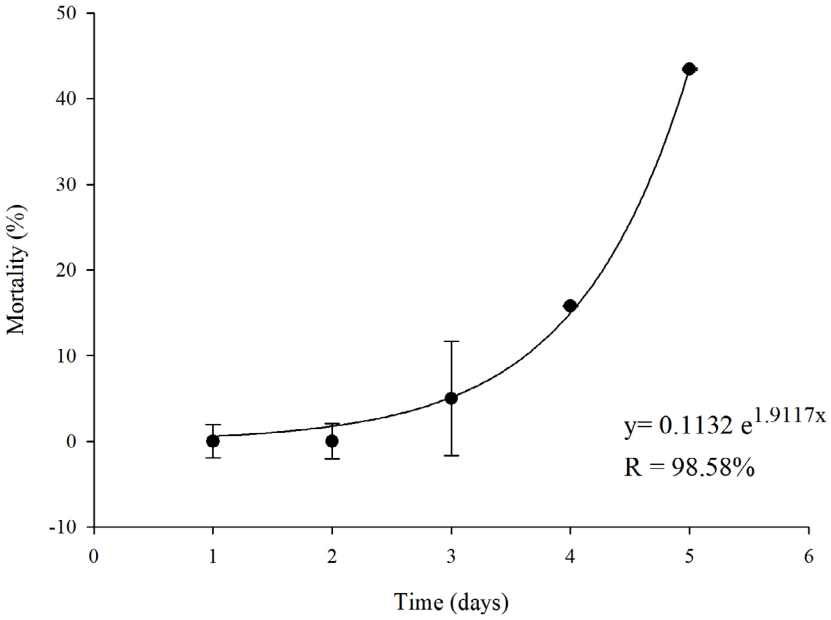

Figure 1: Total mortality of *Hypothenemus hampei* adults (%) caused by the entomopathogens in five days of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Table 2: Mortality of *Hypothenemus hampei* larvae (%) caused by *Beauveria bassiana* UFU01 (1 x 10^9 conidia mL^{-1}) and *Heterorhabditis amazonensis* MC01 (1,200 IJ mL^{-1}) kept in dishes with vermiculite for each day of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Treatment	Mortality (%)*				
	Evaluation period (days)				
	1st	2nd	3rd	4th	5th
B. bassiana	38.46 ± 13.49a	76.47± 10.29a	68.42 ± 10.66a	81.25 ± 9.76a	75.00 ± 12.50a
H. amazonensis	52.94 ± 12.11a	72.22 ± 10.56a	75.00 ± 10.83a	76.47 ± 10.29a	92.30 ± 7.39a
B. bassiana + *H. amazonensis*	48.57 ± 8.45a	25.00 ±10.83b	69.23 ± 12.80a	66.67 ± 9.62a	93.33 ± 6.44a
Control	29.41 ± 11.05a	20.00 ± 8.94b	23.07 ± 11.69b	11.11 ± 7.41b	18.75 ± 9.76b

*Means ± standard deviation followed by the same letter did not differ significantly by the Tukey's test at 5% significance.

According to the regression analysis, different behaviors were observed in the linear and polynomial models of the tested treatments. For *B. bassiana*, it was found that, until the 4th day of evaluation, a mortality of 81.25% was obtained and, from this day, there was a reduction in larval death, while for the nematode and the association between the nematode and the fungus, a growth until the 5th day of evaluation was verified, obtaining, respectively, mortalities of 92.30% and 93.33% (Table 2 and Figure 2). Thus, the data obtained in this experiment demonstrate the potential of the entomopathogens in causing mortality to *H. hampei* larvae.

Considering the results obtained when bored fruits were dipped in suspensions with entomopathogens, for *H. hampei* adults, no significant difference was observed between treatments, differing only from the control (Table 3). The indexes found can be considered satisfactory, reaching 50% of adult mortality.

Related to larval mortality, it was observed that there was no difference between the treatments tested, but they differed from the control. In addition, high insect mortality rates were obtained, reaching values of up to 98% of dead larvae (Table 4).

3.2 Phoresis of *Beauveria bassiana* by *Heterorhabditis amazonensis*

When analyzing the mortality of *T. molitor* after 12 days of entomopathogen release, it was observed that there was no difference between the treatments tested, but they differed from the control. In addition, high insect mortality rates were obtained, reaching values of up to 96% of dead larvae (Table 5).

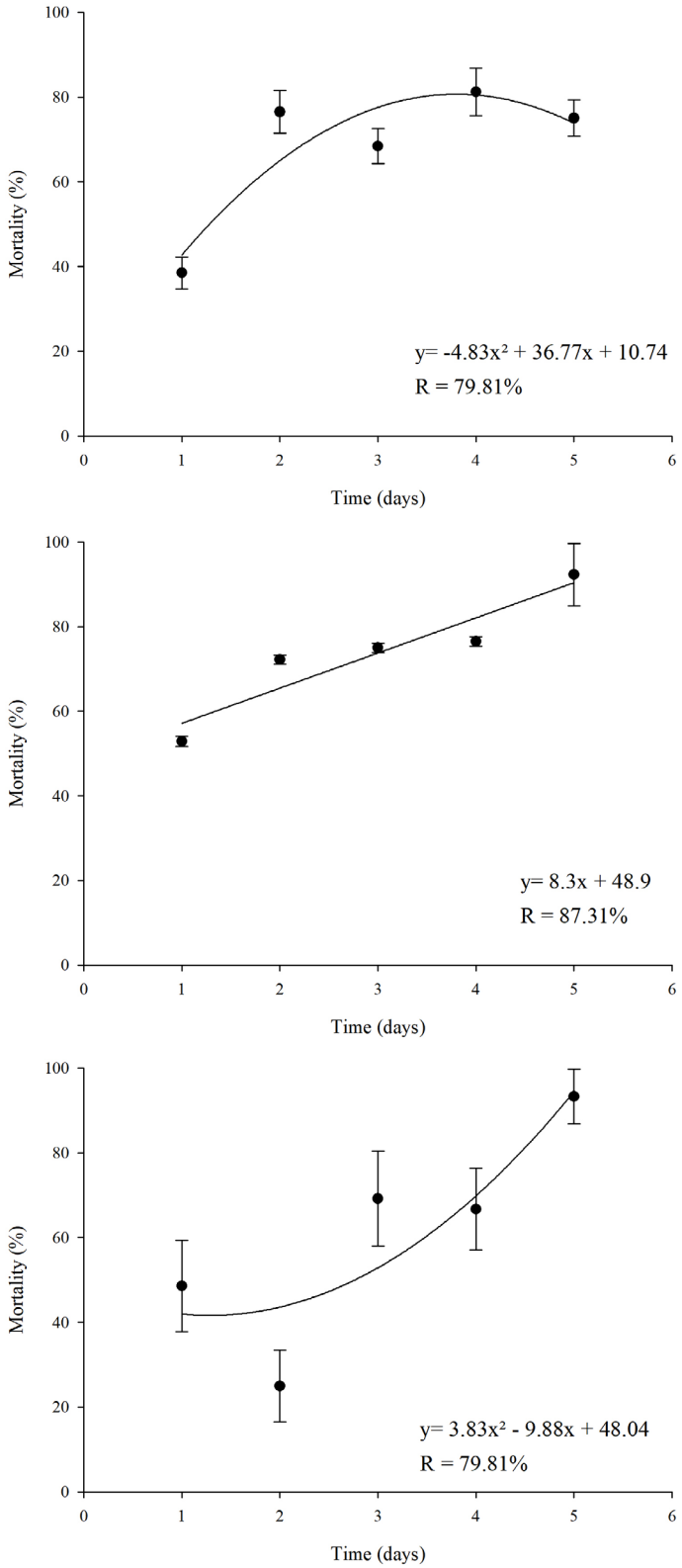

Figure 2: Mortality of *Hypothenemus hampei* larvae caused by *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01 and association between entomopathogens during five days of evaluation. A. *Beauveria bassiana*. B. *Heterorhabditis amazonensis* MC01. C. *Beauveria bassiana* + *Heterorhabditis amazonensis* MC01. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Table 3: Mortality of *Hypothenemus hampei* adults (%) when immersed in *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01 suspensions after 11 days of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Treatment	Mortality (%)*
Heterorhabditis amazonensis MC01	52.1 ± 22.6 a
H. amazonensis MC01 + *B. bassiana*	52.0 ± 12.6 a
Beauveria bassiana	44.6 ± 15.0 a
Control	0.0 ± 0.0 b
CV (%)	37.16

*Means ± standard deviation followed by the same letter do not differ significantly by the Tukey test at 5% significance.

Table 4: Mortality of *Hypothenemus hampei* larvae when immersed in *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01 suspensions after 11 days of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Treatment	Mortality (%)*
H. amazonensis MC01 + *B. bassiana*	98.0 ± 4.5 a
Heterorhabditis amazonensis MC01	92.2 ± 10.8 a
Beauveria bassiana	86.6 ± 12.4 a
Control	0.0 ± 0.0 b
CV (%)	12.33

*Means ± standard deviation followed by the same letter do not differ significantly by the Tukey test at 5% significance.

Table 5: Mortality of *Tenebrio molitor* larvae (%) caused by *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01 after 12 days of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Treatment	Mortality (%)*
Beauveria bassiana	96.0 ± 8.4 a
H. amazonensis MC01 + *B. bassiana*	96.0 ± 8.4 a
Heterorhabditis amazonensis MC01	88.0 ± 10.3 a
Control	0.0 ± 0.0 b
CV (%)	11.27

*Means ± standard deviation followed by the same letter do not differ significantly by the Tukey test at 5% significance.

When comparing the mortality caused by *B. bassiana* and *H. amazonensis* MC01 applied together, it can be observed that there was a significant difference between the amount of insects died by entomopathogens, verifying that *B. bassiana* yielded an increase of 44% in mortality in relation to the nematode (Table 6). As *B. bassiana* was applied only on the side opposite to the presence of *T. molitor* larvae, it can be inferred that the nematode helped in the dispersion in fungal conidia, with a phoretic association.

Table 6: Mortality of *Tenebrio molitor* larvae (%) caused by each entomopathogen, *Beauveria bassiana* UFU01 and *Heterorhabditis amazonensis* MC01, when applied together after 12 days of evaluation. Temperature of 24 ± 1 ºC and 12-hour photoperiod.

Treatment	Mortality (%)*
Beauveria bassiana	70.0 ± 14.1 a
Heterorhabditis amazonensis MC01	26.0 ± 16.4 b
CV (%)	31.97

*Means ± standard deviation followed by the same letter do not differ significantly by the Tukey test at 5% significance.

Regarding the evaluation of *B. bassiana* conidia transport applied together with *H. amazonensis* MC01 infective juveniles in agar plates, it was found that in the control and in the treatment with the fungus alone, no entomopathogenic propagules were found on the opposite side of the dish. In the treatment with the nematode, infective juveniles were found on the opposite side of the dish. In the treatment with the association of fungus and nematode, juveniles and conidia were found on the opposite side of the dish, and it can be inferred that the presence of conidia was due to the transport made by infectve juveniles (Table 7).

Table 7: Number of individual and associated *Beauveria bassiana* UFU01 conidia and/or *Heterorhabditis amazonensis* MC01 infective juveniles found on the opposite side of the Petri dish application. Temperature of 24 ± 1 ºC and 24-hour darkness.

Treatment	Total number of entomopathogenic propagules*
H. amazonensis MC01 + *B. bassiana*	5.4 ± 1.5 a
Heterorhabditis amazonensis MC01	5.0 ± 1.2 a
Beauveria bassiana	0.0 ± 0.0 b
Control	0.0 ± 0.0 b
CV (%)	37.49

*Means ± standard deviation followed by the same letter in the column do not differ significantly by the Tukey test at 5% significance.

4 DISCUSSION

The nematode *H. amazonensis* MC01 was used since it is a population found in the region where the experiments were carried out, thus being better adapted to local conditions. In addition, according to Guide et al. (2018), high larval mortality rates were obtained with *H. amazonensis* isolates RSC5 and GL, 100% and 97%, respectively, and *H. amazonensis* GL was one of the nematodes that caused the highest adult mortality, 54%.

The greater potential of both entomopathogens to kill larvae than adults, observed in both experiments, may be associated with the difficulty in penetrating in the rigid integument of the adult insect. Benavides-Machado, Quintero and López (2010) argue that variations in the susceptibility of *H. hampei* larvae and adults to entomopathogenic nematodes, for example, may be associated with morphological and behavioral differences at each stage of insect development. The larvae has a less chitinized cuticle, with more exposed spiracles and lower mobility, facilitating IJ penetration, while adults, in addition to presenting a more rigid integument, have spiracles protected by elytra, besides well-developed jaws.

Cuthbertson and Audsley (2016) evaluated fungi and entomopathogenic nematodes for the control of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), an insect in which the larvae, as well as those of *H. hampei*, remain inside the fruit, in an environment of difficult access, which makes control difficult. The authors observed that the nematode *Heterorhabditis bacteriophora* and the fungus *Isaria fumosorosea* acted mainly in the larval stage of the insect, causing up to 95% mortality. However, they found that the nematodes did not work well when applied to the pending fruits on the plant, but when sprayed directed to the soil, via drench.

Lara, Lopez and Bustillo (2004) applied *Heterorhabditis* sp. and *Steinernema* sp. directed to the soil in order to reach the borer present in the fallen fruits, and verified that the nematodes caused mortality of up to 82% and 88% of insects, respectively, concluding that infective juveniles were able to penetrate the fruit and that they caused mortality mainly in larvae.

Acevedo and Núñez (2009) found that *H. bacteriophora* is capable of causing an average mortality of 46% *H. hampei*, confirming the ability of IJ to penetrate coffee fruits and cause insect mortality of larvae, pupae and adults. However, this result differs from that found by Guide et al. (2018) who, despite having observed 100% mortality of *H. hampei* larvae when *Heterorhabditis* spp. and *S. feltiae* were applied directly to the insects, found that when they were applied to bored fruits, infective juveniles were not able to penetrate the fruit.

Sánchez and Rodríguez (2007) applied *H. bacteriophora* directly to the insect in a laboratory test and obtained mortality results of 100% for larvae and pupae. When the bored fruits were placed in trays containing soil, simulating a field situation, 60% mortality rates were obtained, reaching 93% after 17 days.

In this study, as observed by Acevedo and Núñez (2009), it was found that infective juveniles were able to kill larvae and adults within the fruit since, in one of the experiments, nematode and fungus were applied to vermiculite, having to penetrate the fruits to find the host.

Results presented by Manton, Hollingsworth and Cabos (2012) demonstrate that *S. carpocapsae* was able to cause mortality at the different development stages of coffee borer when applied to the soil directed at fallen fruits, causing mortality of 26.6% in adults and 23.7% in larvae. Guide et al. (2018) obtained similar values when testing *S. carpocapsae* for adults, 22%. However, the values found for larvae were higher, 87.5%. Benavides-Machado, Quintero and López (2010) obtained adult mortality rates of up to 24.6% when using *H. bacteriophora*. These values were similar to that found in this study for adults, when *H. amazonensis* was applied to vermiculite, 27.97% (Table 1).

Vera et al. (2011) found that *B. bassiana* was able to decrease *H. hampei* infestation by up to 50%, with mortality of 40% of insects, including larvae, pupae and adults, similar to the value found in this study for *H. hampei* adults. In addition, the authors observed a decrease in the population of *H. hampei* when the fungus is applied to bored fruits that fall on the soil, reducing future generations of the pest. These results reinforce the data presented in this study, where values of up to 86% larval mortality and 44% adult mortality were obtained, demonstrating the potential use of *B. bassiana*, mainly in the control of coffee borer.

Studies associating entomopathogens have been developed in order to verify the synergistic effect on the control of Curculionidae considered agricultural pests. Ansari, Shah and Butt (2008) obtained mortality rates of up to 100% when associating *Metarhizium robertsii* with *S. kraussei* or *H. bacteriophora* in the control of *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae). The association of *B. bassiana* and *S. carpocapsae* was considered beneficial in the control of *Hylobius abietis* L. (Coleoptera: Curculionidae), with a smaller number of adults emerging when using the entomopathogens together (Williams et al., 2013).

Simi et al. (2018) studied the combined action of *Steinernema brazilense* and *B. bassiana* in the control of *Conotrachelus humeropictusos* (Fiedler) (Coleoptera: Dryophthoridae), and found that there was a synergistic interaction in the joint application of the two entomopathogens, causing mortality of up to 65.6% insects, which corresponded to a 15.6% increase in mortality when compared to the application of the nematode alone. In addition, the authors also observed that infective juveniles were able to transport *B. bassiana* conidia through the soil at a depth between 0.07 and 0.10 m. Conidial transport was observed in this study when it was found that *B. bassiana* was capable of causing 70% mortality of *T. molitor* larvae when applied together with *H. amazonensis* on the side opposite to the presence of the larvae (Table 6), as well as when fungal conidia were visualized together with infective juveniles on the opposite side of their release in Petri dishes with agar (Table 7).

The increase in insect mortality when associated with different entomopathogens can be associated with stress caused by one of the control agents, which can weaken the insect and increase susceptibility to the other pathogen, increasing insect mortality or even reducing lethal time (Shaohui et al., 2014).

However, antagonistic effects have also been observed. Shapiro-Ilan et al. (2004) found synergism in the association of entomopathogens in the control of *Curculio caryae* (Horn) (Coleoptera: Curculionidae) larvae only when *H. indica* and *M. anisopliae* were tested together. The other associations tested involving *B. bassiana*, *M. anisopliae*, *Isaria fumosorosea* and the bacterium *Serratia marcescens* were considered antagonistic. In this study, an antagonistic effect was observed only when the joint application was made to vermiculite to control adults (Table 1), obtaining less than 1% of insect mortality. According to Tarasco et al. (2011), antagonistic effects may be related to compounds released both by fungi and by the symbiotic bacteria of entomopathogenic nematodes.

Although no additive effect was observed between the fungus and the nematode under the tested conditions, it is possible to observe the action potential of these organisms, even if in isolation, on coffee borer due to the mortality rates found at two stages of insect development. In addition, it must be considered that, in the laboratory, it is easier for the pathogen to find and contact the host, which may have reflected in the non-observation of an additive effect in the associated application.

Even though synergism was not observed when *H. amazonensis* MC01 and *B. bassiana* UFU01 were applied together in order to cause mortality in *H. hampei* larvae and adults, tests under field conditions should be carried out to verify if the nematode ability to search for the host in the field and to transport fungal conidia can increase insect mortality rates, by providing greater reach to the fungus to the insects located inside the fruit when fallen in the soil, thus being one more possible strategy to increase pest mortality factors.

5 CONCLUSIONS

Beauveria bassiana UFU01 and *H. amazonensis* MC01 were considered pathogenic to *H. hampei*, causing larval and adult mortality when applied alone and also associated. When applied to vermiculite, the association between nematode and *B. bassiana* UFU01 was not able to cause adult mortality.

Heterorhabditis amazonensis MC01 is capable of transporting *B. bassiana* UFU01 conidia, which was observed due to the mortality of *T. molitor* larvae. When associated with fungus and the nematode, *B. bassiana* caused higher mortality of *T. molitor* larvae than infective juveniles.

Infective juveniles transported *B. bassiana* UFU01 conidia adhered to their cuticle to the opposite side of the Petri dish, thus highlighting the occurrence of phoresis between *H. amazonensis* MC01 and *B. bassiana* UFU01.

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