POTENTIAL OF ENTOMOPATHOGENIC NEMATODES FOR CONTROL OF THE ERVA-MATE PEST *Hedypathes betulinus* (KLUG, 1825) (COLEOPTERA: CERAMBYCIDAE)

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Abstract

The intensive exploitation of "erva mate" (*Ilex paraguariensis* St. Hil.) (Aquifoliaceae) has favored the emergence of pests, including *Hedypathes betulinus*. We evaluated 18 isolates of entomopathogenic nematodes *Steinernema* and *Heterorhabditis* genera for control of *H. betulinus* adults, and tested nematode in-vivo replication capacity in *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae. We also evaluated the effect of adjuvants for foliar application, and the efficiency of nematode isolates selected in erva mate seedlings in the greenhouse via application to soil and plant shoots. The PI and CB40 isolates showed the hgh virulence (92.5% insect mortality by both). The PI isolate showed low productivity in *G. mellonella* larvae, thus only CB40 was used in subsequent tests. Only emulsified vegetable oil adjuvant was compatible with nematodes. Soil application of nematodes proved ineffective; however, shoot application on erva mate seedlings showed significant mortality (82.5%) after exposure to the CB40 isolate without adjuvant.
Keywords: Steinernema, Heterorhabditis, Ilex paraguaiensis, biological control.

Resumo

A exploração intensiva da erva-mate (*Ilex paraguariensis* St. Hil.) (Aquifoliaceae), tem favorecido o surgimento de pragas incluindo a broca-da-erva-mate (*Hedypathes betulinus*). Visando seu controle avaliou-se a virulência de 18 isolados de nematoides entomopatogênicos dos gêneros *Steinernema* e *Heterorhabditis* sobre adultos do inseto e a capacidade de multiplicação *in vivo* dos nematoides em larvas de *Galleria mellonella* L. (Lepidoptera: Pyralidae). Foi avaliado também o efeito de adjuvantes foliares e a eficiência dos nematoides selecionados sobre o inseto, em mudas de erva-mate, em casa de vegetação, com aplicação no solo e na parte aérea das plantas. Os isolados PI e CB40 foram os de maior virulência sobre o inseto (92,5% de mortalidade, para ambos), porém o isolado PI mostrou-se pouco produtivo em larvas de *G. mellonella* e apenas o isolado CB40 foi usado nos testes subsequentes. Dentre os adjuvantes avaliados, somente o óleo vegetal foi compatível com o nematoide. No teste em casa-de-vegetação com aplicação no solo não foi observada mortalidade dos insetos. A aplicação na parte aérea das mudas de erva-mate apresentou efeito significativo, em relação à testemunha, sendo que com o isolado CB40 aplicado sem adjuvante obteve-se mortalidade de 82,5%.
Palavras-chave: Steinernema, Heterorhabditis, Ilex paraguaiensis, Controle Biológico.

INTRODUCTION

Erva mate *Ilex paraguariensis* Saint Hil. (Aqüifoliaceae) plants are native to southern and midwestern Brazil, Paraguay, and Argentina. Changing in erva mate to monoculture production has increased the productivity, and in Brazil erva mate annual production is estimated approximately in 252,700 tons, concentrated in the states of Brazilian south region (Paraná, Santa Catarina and Rio Grande do Sul states) (IBGE, 2013). Monoculture production in Brazil also favors the occurrence of pests, especially *Hedypathes betulinus* (Klug, 1825) (Coleoptera: Cerambycidae), named "broca da erva-mate" (PENTEADO et al., 2000; BORGES et al., 2003). Adults live in the shoot, and mate and lay eggs near the base of the plant, on the branches and in bark crevices (CASSANELLO, 1993; GALILEO et al., 1993; D'AVILA et al., 2006). Resident larvae are responsible for the damage, by building galleries on branches and longitudinally along the trunk, preventing sap movement

and weakening the plant. In the case of severe infestations or successive pest generations on the plant, death may occur (SOARES, 1998; GUEDES et al., 2000).

Entomopathogenic fungi have been found in natural epizootics association with *H. betulinus* (SOARES et al., 1995; SOARES and IEDE, 1997; LEITE et al., 2000; LEITE et al., 2006). Also, many studies were carried out with isolates selection in the laboratory and in field evaluation (SOARES; IEDE, 1997 MILK et al., 2000 BORGES, 2007; GOMM, 2010, FANTI; ALVES, 2013). These studies resulted in a product based on the fungus *Beauveria bassiana* (Bovemax® EC) that has since been registered for commercial use in integrated pest management (AGROFIT, 2015). Entomopathogenic nematodes (EPNs) Mermithidae have also been found in association with the pest in the field. However, due to some biological characteristics of this nematode family, there is little potential for their use in controlling *H. betulinus*.

Alves et al. (2009a) in a pioneer study demonstrated the susceptibility of *H. betulinus* to the nematodes genus *Steinernema*, in which pests reached 80% mortality within 10 days after treatment. The biology of the pest includes the female behavior of approaching the base of the plant (close to the ground) to oviposit, in which case nematodes present in soil may be control of the pest. Among the advantages of EPNs is their ability to search for hosts, and their symbioses with bacteria, which effectively kills insects. Moreover, in addition to killing the host quickly these agents are not harmful plants or vertebrates, are selective for insects, do not pollute soil or groundwater, are safe for the producers, and can readily establish the ground to maintain long-lasting, small populations to fight pests (FERRAZ, 1998; VOSS et al., 2009).

A successful biological control program depends on the existence of efficient control agents both under laboratory and in the field conditions, and in the case of *H. betulinus*, such products are rare, highlighting the need to search for new alternatives. This study aimed to evaluate and select isolates of EPNs to control *H. betulinus*, specifically those showing virulence and high productivity, as well as efficiency in semi-field conditions after soil and leaf application.

MATERIALS AND METHODS

Collection of nematodes and insects:

H. betulinus adults were obtained from a commercial erva mate plantation located in Ivaí, Paraná, Brazil. The insects were collected and kept in cages (60 cm × 40 cm wide × 40 cm long) containing erva mate branches and under controlled conditions (26 ± 1°C, 70% relative humidity, 14h photophase) until experiments began, and not exceeding seven days (ALVES, 2009a). Prior to beginning experiments intact insects were selected with movement and normal responses to stimuli (SOARES, 1998). We evaluated 18 isolates of *Steinernema* and *Heterorhabditis* nematodes from different research institutions in Brazil, reared in *G. mellonella* larvae as follows.

Selection of nematode isolates:

We uses 40 plastic 500 ml containers divided into four replications, each containing erva mate branches for food and closed with perforated plastic covers and lined with 150g of moist sand (Alves et al., 2009a).

Nematode suspensions were applied to dry sand (100 Infective Juveniles (IJs)/cm^2) (surface area). After application of the suspension, sterile distilled water it was added to obtain 20% substrate moisture. The adults insects were then released onto the branches (one insect/container). For controls, the sand was treated with distilled water (20% moisture).

The containers were closed and maintained at 26 ± 1°C with 14h photoperiod. Measurements were made daily for 15 days, and dead insects were transferred into a dry chamber composed of Petri dishes lined with dry filter paper where they remained for five days. After this time, 2 ml of distilled water was applied to the paper surface, and after 24h the cadavers were dissected and observed under stereomicroscope to search for nematodes. Isolates causing at least 80% mortality were selected for the next step.

Estimation of CL99 of isolate CB40 and *in vivo* production in *Galleria mellonella* larvae:

Nematode suspensions were inoculated into Petri dishes (9 cm dia.) lined with two sheets of filter paper at concentrations of 0, 500, 1000, 1500, 2000 and 2500 IJs/plate; 10 *G. mellonella* larvae were placed on each dish. Dished were incubated at 26 ± 1ºC, with 70 ± 10% RH and a 14 h photophase. Control dishes were treated only with distilled water. After 72 hours post-treatment, dead larvae were transferred into a dry chamber and maintained for five days to confirm nematode infection as described above. Each plate was considered one replicate, with a total of five replicates carried out per nematode concentration. The results were analyzed by linear regression using Excel 2007. After that, *in vivo* production it was evaluated. Therefore, 2 ml of a nematode suspension were inoculated onto Petri dishes lined by filter paper (630 IJs/plate). Ten previously weighed (lab

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reared) *G. mellonella* larvae were placed on the dish. Dishes were covered and sealed with plastic film, and incubated at 24 ± 1°C in the dark for 72 hours. After confirmation of death, cadavers were transferred into a dry chamber and maintained for five days at 24 ± 1°C after which nematode infection was confirmed (WOODRING; KAYA, 1988). After five days, the larvae were transferred to White traps for emergence of the IJs, which were collected and quantified daily until production ended. The most productive strain was selected for the next step.

Effect of adjuvants with CB40 isolates:

We evaluated the effect of foliar fertilizer TEK-F® and Aureo® vegetable oil emulsifiable on the CB40 isolate for future application under field conditions. We used the IOBC protocol adapted by Negrisoli et al. (2008), in which a solution of the product is prepared with distilled water at double the dose recommended by the manufacturers. One mL of solution is then transferred to a glass tube, followed by 1 mL of the CB40 nematode suspension (2000 IJs/mL), yielding the product concentration indicated by the manufacturer. For controls, 1 mL of nematode suspension was added to 1 mL of distilled water. Five replicate tubes were prepared for each treatment. The tubes were kept at 22 ± 1 °C with 14 h photoperiod for 48h, after which the viability and infectivity of the isolate was evaluated. Data were subjected to ANOVA (the experiment having a completely randomized design) and means were compared by Tukey test ($p < 0.05$) using the statistical program Sisvar (FERREIRA, 2011).

Greenhouse experiment with erva-mate seedlings:

The CB40 isolate was applied to the aerial parts of the plants (foliar application) and to the soil, as follows.

Foliar application:

CB40 isolate was multiplied *in vivo* (see *in vivo* methods above) and stored in aqueous suspension, maintained with aeration to prevent sedimentation and death of IJs. Erva-mate seedlings (~50 cm height) were kept in pots and individually placed in wooden cages (55 × 40 × 40 cm) lined with nylon mesh. Ten adult insects were released in each cage, and the nematode suspension was applied throughout the shoot with manual spray to the point of run-off (approximately 14 mL of suspension/plant containing 5.0×10^{-3} IJs/mL). Treatments consisted of CB40 nematode suspension in distilled water, CB40 + Aureo®, nematode suspension, and Aureo® solution (both treatments with Aureo® following manufacturer recommendations). Control seedlings were treated with distilled water. Treatments were replicated four times. Five days after application, dead insects were counted and transferred to a dry chamber for confirmation of mortality, as described above. Data were subjected to analysis of variance (F test) and means compared by Tukey test ($p < 0.05$) according to a completely randomized experimental design using the statistical program Sisvar (FERREIRA, 2011).

Soil application:

Application to soil followed the same experimental procedures and statistical analysis described above, except that nematodes were applied directly to soil at a concentration of 100 IJs/cm^2 using a 10 mL pipette, and five *H. betulinus* adults were released onto the shoot. After five days, dead insects were counted and placed in a dry chamber for 5 days, then dissected to confirm mortality.

RESULTS AND DISCUSSION

Selection of nematode isolates:

There was strong variation in results, with only two out of 18 isolates causing mortality above 90% (CB40 and PI, both belonging to the genus *Heterorhabditis*). The CH3 isolated, SA, NEPET 11 and CB02 caused between 55 and 75% mortality, while the others caused less than 50% mortality (Table 1).

It's known both among and within-species differences in insect susceptibility to nematode genus (VAN TOL et al., 2004; VAN TOL; RAUPP, 2006; ALVES et al, 2009b). For example the CB40 and PI isolates both belonging to the genus *Heterorhabditis*, yet it is also impossible to affirm that this genus best suited to *H. betulinus* since other isolates in the genus showed very little activity.

Variation in cerambycid susceptibility to different nematode species and isolates has been previously reported. Machado et al. (2003) by selected EPNs for control of *Migdolus fryanus* (Westwood, 1863) (Coleoptera: Vesperidae) larvae and observed high susceptibility to genus *Heterorhabditis*, with up to 100% mortality. On the other hand, for *Plectrodera scalator* (Fabr., 1792) (Coleoptera: Cerambycidae) larvae with only *S. carpocapsae* All and *S. feltiae* SN isolates it was obtained mortality higher than 50%, and high concentrations were required to cause significant insect mortality (FALLON et al., 2006). Harvey et al. (2012)

evaluated the potential of *S. carpocapsae* and *H. downesi* for control of *Rhagium bifasciatum* Fabr., 1775 (Coleoptera: Cerambycidae) and found that both nematodes were capable of infecting larvae, pupae and adults both in laboratory and field conditions.

Table 1. Mortality in *Hedypathes betulinus* adults caused by entomopathogenic nematodes under laboratory conditions.

Tabela 1. Mortalidade de adultos de *Hedypathes betulinus* causada por nematoides entomopatogênicos em condições de laboratório.

Isolate	Genus/Species	Location	% Mortality
NEPET 8	*Heterorhabditis* sp.	Origin unknown	0.0
CB36	*Steinernema* sp.	Naviraí – MG - Brazil	0.0
SORGO	*Heterorhabditis* sp.	Lavras – MG – Brazil	0.0
RSC02	*Heterorhabditis* sp.	Benjamin Constant – AM – Brazil	17.8
JPM3	*Heterorhabditis* sp.	Lavras – MG – Brazil	19.3
CB44	*Heterorhabditis* sp.	Santa Adélia – SP – Brazil	22.0
RSC01	*Heterorhabditis* sp.	Benjamin Constant – AM – Brazil	33.3
RSC05	*H. amazonensis*	Benjamin Constant – AM – Brazil	36.6
ALHO	*Heterorhabditis* sp.	Lavras – MG – Brazil	37.5
JPM4	*Heterorhabditis* sp.	Lavras – MG – Brazil	44.8
NEPET 36	*Heterorhabditis* sp.	Origin unknown	45.0
HP88	*H. bacteriophora*	New Jersey - USA	46.8
CH3	*Steinernema* sp.	Campinas – SP – Brazil	55.0
SA	*S. arenarium*	North Carolina – USA	68.7
NEPET 11	*Heterorhabditis* sp.	Palmeiras das Missões – RS – Brazil	75.0
CB02	*S. carpocapsae*	Florida – USA	75.0
CB40	*Heterorhabditis* sp.	Taboporã – SP – Brazil	92.5
PI	*Heterorhabditis* sp.	Terezina – PI – Brazil	92.5

These data reinforce the need to carefully select entomopathogenic nematode isolates, because biological features and adaptations to the environment can cause great variation among species, and even among isolates of the same species (GAUGLER et al., 1997). Our results demonstrate this variability, and the importance of conducting tests in order to choose the most appropriate isolate for insect control. So, CB40 and PI isolates were selected to continue the research.

Estimation of CL99 of isolate CB40 and *in vivo* production in *Galleria mellonella* larvae:

The PI isolate showed low productivity, requiring a large number of hosts to obtain a small number of individuals, and thus, it was discarded at this stage of the study. The CL_{99} of isolate CB40 in *G. mellonella* larvae was estimated at 9 IJs/cm^2, which is considered low and indicative of high virulence (Figure 1).

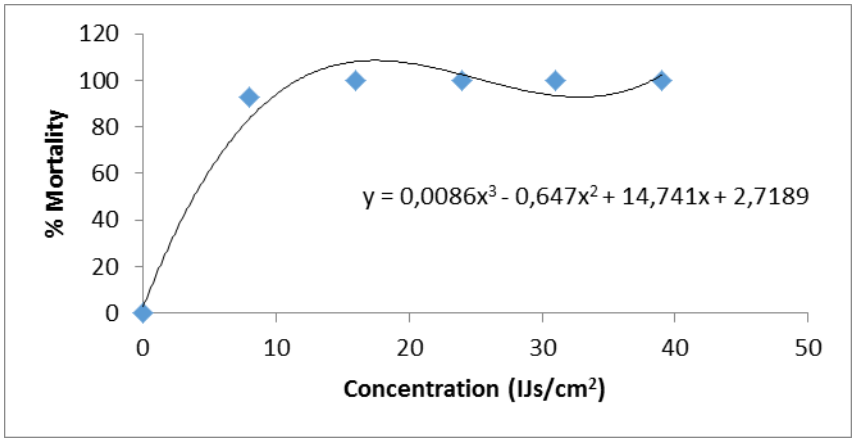

Figure 1. Mortality of Galleria mellonella 72 hours after exposure to CB40 isolates of different concentrations.

Figura 1. Mortalidade de Galleria mellonella após 72 horas de esposição ao isolado CB40 em diferentes concentrações.

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Barbosa (2005) show an estimated CL_{99} of 43 IJs/cm^2 for *H. bacteriophora* in *G. mellonella* larvae. *G. mellonella* has high susceptibility to most known entomopathogenic nematodes, and it can also be assumed that smaller nematodes can penetrate hosts with greater ease, which may have been a factor in the virulence of isolate CB40. Further, CB40 *Heterorhabditis* are able to penetrate not only natural openings of hosts, but also the cuticle (GIOMETTI, 2011); this is another feature that may be associated with virulence of this isolate.

About *in vivo* production**,** CB40 IJs emerged from *G. mellonella* larvae for up to 10 days, with peak emergence between the third and fourth days. The total accumulated production in the host was 6.5×10^4 IJs/larva or 4.2×10^4 IJs/g larva (Figure 2).

The results obtained are lower than previously reported in studies with different isolates in *G. mellonella*, including Barbosa (2005), which report 4.9×10^5 IJs/g larva for *H. bacteriophora.*

Saenz and Lopes (2011) also evaluated the production of *Heterorhabditis* sp. isolate SL0708 and obtained between 1.5 and 2.8×10^5 IJs/larva. According to the authors, the differences in the production of IJs in *G. mellonella* are due to various factors involved in the infection process, including the host penetration site (mouth, anus, spiracles or cuticle), inoculum concentration, the number of IJs infecting the host, and the local release of symbiotic bacteria in the hemolymph, all of which can interfere with J1 development within the host. The CL_{99} of our CB40 isolate is quite low by comparison, which may have been a factor affecting productivity. This indicates the need to evaluate other inoculum concentrations for improved productivity.

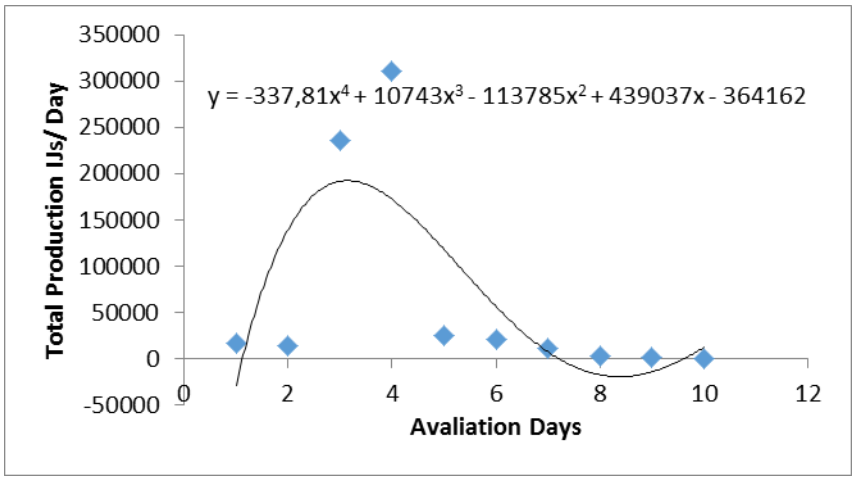

Figure 2. Production of CB40 infectious juveniles in *Galleria mellonella.*
Figura 2. Produção de juvenis infectantes do isolado CB40 em *Galleria mellonella.*

Effect of formulation adjuvants isolated on CB40:

The TEK-F® product significantly reduced the viability and infectivity (by 84 and 82%, respectively) and is classified as incompatible with CB40 according to the IOBC protocol. The Aureo® product vegetable oil base did not affect the viability or infectivity of nematodes and results did not differ from the controls, highlighting the possibility of joint use of this product and the nematode (Table 2).

Table 2. Viability and infectivity (mean ± SE) of CB40 isolate infective juveniles (*Heterorhabditis* sp.) after exposure to adjuvants, and their compatibility.
Tabela 2. Viabilidade e infectividade (média ± DP) de juvenis infectantes do isolado CB40 (*Heterorhabditis* sp.) após exposição aos adjuvantes, e sua compatibilidade.

Treatment	% Viability	Classification*	% Infectivity	Classification
CB40 (Control)	93.2±1.50A**	-	52.0±8.60AB	-
CB40+Aureo®	87.4±0.98A	Compatible	78.0±5.83A	Compatible
CB40+TEK-F®	16.4±6.38B	Incompatible	18.0±7.35B	Incompatible
CV.	10.88		39.34	

* Product classification according to IOBC protocol (VAINIO, 1992).
**Different letters in columns indicate significant differences between treatment groups (Tukey test, $P \leq 0.05$).

Greenhouse experiment – soil application:

The isolate showed promising results in the laboratory, causing over 90% mortality in the insects, and presented ease of in vivo production in *G. mellonella* larvae, however it did not show activity against *H.*

betulinus when applied to the soil. This may have been due to the surface available on the plant for the insect to remain upon, perhaps decreasing the chance of contact with the soil and consequently, with the nematode.

Greenhouse experiment – foliar application:

Foliar application caused 82.5% mortality of insects treated with nematodes in pure suspension, with no increased nematode action when combined with adjuvant. Furthermore, although the product does not have toxicity to IJs, it is toxic to insects (Table 3).

Bellini and Dolinski (2012) evaluated foliar application efficiency of *H. baujardi* (LPP7 isolate) and *S. carpocapsae* (Ncall isolate) for control of *Diatraea sacharalis* under greenhouse conditions, with and without adjuvants similar to those used in this study (Joint* Oil- Dow AgroSciences and Gotafix® - Milenia) and as in our study, found no differences between treatments.

Table 3. Mortality (mean ± SE) of *Hedipathes betulinus* after spraying CB40 isolate under greenhouse conditions.

Tabela 3. Mortalidade (média ± DP) de *Hedipathes betulinus* após aplicação do isolado CB40 em condições de casa-de-vegetação.

Treatment	% Mortality
Control	7.3 ± 7.14A*
CB40 nematodes + Aureo®	46.3 ± 6.00B
Aureo®	58.5 ± 6.98BC
CB40 nematodes	82.5 ± 11.09C
CV: 33.07	

**Different letters indicate significant differences between treatment groups (Tukey test, $P \leq 0.05$).

While foliar application of EPNs is somewhat unusual, the literature does support the action of some isolates capable of infecting cerambycid larvae while still inside the stem of infected plants (FINNEY; WALKER, 1977; FINNEY; WALKER, 1979; SOLTER et al., 2001), suggesting its potential as a strategy for controlling this pest. Further, Fallon et al. (2004) evaluated control methods for *Anoplophora glabripennis* and showed that nematode isolates may have capacity for to move, attracted by host presence or host excrement, which serve as a stimulus for localization of larvae or even adult insect hosts in the plant, enabling application on aerial parts of the plant or areas of trunk infested with larvae. In the case of the *H. betulinus*, the adult remains most of the time in the aerial region of the plant and females moving to inferior part of the plants only to oviposit within the branches, making control on leaf areas important; this study indicates foliar application as a viable method of control. Another alternative would be to use EPNs in conjunction with other control measures, such a insect sex pheromone traps if identified (FONSECA et al., 2010), which would enhance insect encounter with effective nematodes.

CONCLUSION

- We conclude that the CB40 isolate has potential for control of *H. betulinus*, however utilization strategies and the use of adjuvants should be further evaluated to enable field application.

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