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Virulence of entomopathogenic nematodes to larvae of the guava weevil, *Conotrachelus psidii* (Coleoptera: Curculionidae), in laboratory and greenhouse experiments

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Abstract

The guava weevil, *Conotrachelus psidii*, is a major pest of guava in Brazil and causes severe reduction in fruit quality. This weevil is difficult to control with insecticides because adults emerge over a long period, and larvae develop to the fourth-instar inside the fruit and move to the soil for pupation. We assessed the virulence of entomopathogenic nematodes to fourth-instar larvae in soil by comparing their susceptibility to nine species or strains: *Heterorhabditis bacteriophora* HP88, *H. baujardi* LPP7, and LPP1, *H. indica* Hom1, *Steinernema carpocapsae* All and Mexican, *S. feltiae* SN, *S. glaseri* NC, and *S. riobrave* 355. In petri dish assays with sterile sand at a concentration of 100 infective juveniles (IJs) of a given nematode species/strain, larval mortality ranged from 33.5 to 84.5%, with the heterorhabditids being the most virulent. In sand column assays with *H. baujardi* LPP7, *H. indica* Hom1, or *S. riobrave* 355 at concentrations of 100, 200, and 500 IJs, mortality was greater than the control only for *H. baujardi* (62.7%) and *H. indica* (68.3%) at the highest concentration. For *H. baujardi* LPP7 in a petri dish assay, the time required to kill 50 and 90% of the larvae (LT_{50} and LT_{90}) for 100 IJs was 6.3 and 9.9 days, whereas the lethal concentration required to kill 50 and 90% of the larvae (LC_{50} and LC_{90}) over 7 days was 52 and 122.2 IJs. In a greenhouse study with guava trees in 20-L pots, 10 weevil larvae per pot, and concentrations of 500, 1000 or 2000 IJs, *H. baujardi* LPP7 caused 30 and 58% mortality at the two highest concentrations. These results show that *H. baujardi* is virulent to fourth-instar larvae and has potential as a biological control agent in IPM programs.

Keywords: IPM; *Conotrachelus psidii*; *Steinernema*; *Heterorhabditis*; Guava; *Psidium guajava*; Entomopathogenic nematodes; Biological control

1. Introduction

The red guava, *Psidium guajava* L. (Myrtaceae), is native to America and widely cultivated in many tropical and subtropical countries (Menzel, 1985) with Brazil being the world's largest producer. In 2002, 400,000 tons of guavas were produced on 14,000 ha for the fresh market and juice processing (Piedade Neto, 2004). Insect pests such as fruit flies, *Ceratitis capitata* (Wiedeman) and *Anastrepha* spp., and the guava weevil, *Conotrachelus psidii* Marshall, are among the most important limitations for guava production (Barelli and Galli, 1998). In Brazil, the guava weevil is the main pest and can be found in virtually every guava orchard. The adult population peaks during summer (November–March) but adults can be found year round in some areas (Barbosa et al., 2001). Mated females lay eggs in green fruit (3–4 cm diameter) and larvae progress through four instars as the fruit develops. The presence of larvae accelerates fruit maturation and, when the fruit ripens and falls to the ground, the larvae crawl into the soil. After an unknown period of time in the soil, larvae develop into prepupae. The prepupal stage can last up to 6 months before pupation and development into

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the adult, which depends on environmental conditions such as soil moisture and temperature (Bailez et al., 2003; Boscán de Martinez and Cásares, 1982).

Control methods for the guava weevil involve weekly applications of insecticides such as organophosphates to suppress the adults, but no insecticide is currently registered for this pest in Brazil and most of those being used against this pest will be discontinued soon (Agência Nacional de Vigilância Sanitária, 2004; Souza et al., 2003). Without chemical control, the percentage of damaged fruit in heavily infested orchards can reach 100% (Boscán de Martinez and Cásares, 1980). The amount of fruit attacked has been increasing rapidly over the past 3 years perhaps because of the development of insecticide resistance, but poorly timed chemical applications and the tendency for adult weevils to hide in the litter around trees and avoid contact with the chemicals could also be involved (Denholm and Rolland, 1992). Integrated pest management (IPM) is a reasonable alternative to control various insect pests and could be effective against the guava weevil (Dolinski, 2003). One method of biological control that could be used is the application of entomopathogenic nematodes against life stages in the soil. Preliminary tests indicated that some entomopathogenic nematode species are effective against fourth-instar larvae (Dolinski and Samuels, 2002).

Entomopathogenic nematodes (EPNs) in the genera *Heterorhabditis* and *Steinernema* (Rhabditida) are obligate parasites of insects (Poinar, 1990). These nematodes have a symbiotic relationship with bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Forst and Clarke, 2002). Infective juveniles (IJs), the only stage of the nematodes found in the soil, enter hosts through natural openings such as the mouth, anus or spiracles, but IJs of some species can also enter through the cuticle. After penetrating into the host's hemocoel, the nematodes release their symbiotic bacteria, which usually kill the host within 24 to 48 h. The bacteria are also responsible for antibiotic production and for providing nutrition for the nematodes (Dowds and Peters, 2002). The nematodes feed, develop, mate, and often complete 2–3 generations within the host cadaver. When resources within the cadaver are depleted, a new generation of IJs is produced and leaves the cadaver to search for new hosts (Kaya and Gaugler, 1993).

Entomopathogenic nematodes effectively control a variety of economically important weevil pests including *Diaprepes abbreviatus* (L.), *Conotrachelus nenuphar* (Herbst), *Otiorhynchus sulcatus* (Fabricius) and *Curculio caryae* (Horn) (Olthof and Hagley, 1993; Shapiro and McCoy, 2000; Shapiro-Ilan, 2001a,b; Shapiro-Ilan et al., 2002; Simons, 1981). The objective of our study was to evaluate various species and strains of entomopathogenic nematodes against fourth-instar larvae of *Co. psidii* in laboratory and greenhouse tests as part of an effort to develop entomopathogenic nematodes for an IPM program against the guava weevil.

2. Materials and methods

2.1. Nematodes, larvae, and experimental design

The nematode species and strains used in this study (Table 1) were reared in *Galleria mellonella* (L.) (Pyralidae: Lepidoptera) larvae at 25 °C, according to procedures in Woodring and Kaya (1988). Harvested IJs were kept at 16 °C for less than 1 week before the tests. *G. mellonella* larvae were reared in the laboratory in plastic pots, with a cereal, sugar cane and honey diet (I. Glazer, personal communication). Fourth-instar larvae of *Co. psidii* were derived from guava fruit obtained from orchards in São Francisco de Itabapoana, RJ, Brazil. The fruit was placed in plastic trays (70 × 40 × 10 cm) filled to 4 cm with autoclaved sand and maintained at 25–28 °C. After 3 to 4 days, all larvae left the fruit and were collected and placed in a Gerbox^{TM} (10 × 10 × 5 cm) filled with moist autoclaved sand. For the petri dish, sand column and greenhouse assays, the larvae were used within a few days of leaving the fruit. For the other tests, the larvae were kept at 25 °C for up to 1 month, with only those showing movement being used for testing.

2.2. Petri dish assay

For each replicate, 100 IJs were suspended in 0.5 ml of distilled water and distributed evenly onto a 6 cm-diameter plastic petri dish half filled with autoclaved sand at 10% moisture by weight. One fourth-instar larva of *Co. psidii* was placed in each dish. There were 20 replicates for each nematode strain tested (Table 1) and for the untreated control, which received 0.5 ml of distilled water without nematodes. The petri dishes were placed in plastic bags and incubated in the dark at room temperature (25 ± 2 °C). Larval mortality was recorded after 12 days. All dead insects were transferred to individual modified

Table 1
Species and strains of entomopathogenic nematodes used in this study

Species	Strain	Source/location
Heterorhabditis bacteriophora Poinar	HP88	R. Stuart, University of Florida, Lake Alfred
H. indica Poinar, Karanukar & David	Hom1	R. Stuart, University of Florida, Lake Alfred
H. baujardi Phan, Subbotin, Nguyen & Moens	LPP7[a]	C. Dolinski, Rondônia, Brazil
H. baujardi	LPP1[a]	C. Dolinski, Rondônia, Brazil
Steinernema glaseri (Steiner)	NA	R. Stuart, University of Florida, Lake Alfred
S. feltiae (Filipjev)	SN	R. Stuart, University of Florida, Lake Alfred
S. carpocapsae (Weiser)	All	R. Stuart, University of Florida, Lake Alfred
S. carpocapsae	Mex	R. Stuart, University of Florida, Lake Alfred
S. riobrave Cabanillas, Poinar & Rauston	355	R. Stuart, University of Florida, Lake Alfred

[a] LPP stands for Laboratório de Proteção de Plantas.

White traps (White, 1927) and held for an additional 10 days for IJ emergence. Only insects showing obvious signs of nematode infection (appropriate color and odor) and producing IJs were considered killed by nematodes in this and all other tests (Woodring and Kaya, 1988). The experiment was repeated under the same conditions shortly after the first (January–February, 2003).

2.3. Sand column assay

Sand columns filled with autoclaved sand were used to evaluate the capability of IJs to find and kill *Co. psidii* larvae (Stuart et al., 1997). Three nematode strains originating in warm climates were selected for testing in this assay: *H. baujardi* LPP7 Phan, Subbotin, Nguyen and Moens, from Rondônia, Brazil; *H. indica* Hom1 Poinar, Karanukar and David, from Coimbatore, India; and *S. riobrave* 355 Cabanillas, Poinar and Raulston, from Texas, USA.

For each replicate, one fourth-instar larva of *Co. psidii* was placed in the bottom of a polyvinyl chloride tube (100 × 50 mm), which was then filled with autoclaved sand at 10% moisture by weight. Infective juveniles at one of four concentrations (0, 100, 200 or 500 IJs) were suspended in 1 ml of distilled water and added to the top of each column. There were 10 replicates per concentration for each nematode species. The sand columns were incubated at 25 °C in a germination chamber. Mortality was assessed 21 days after inoculation, and the entire experiment was repeated under the same conditions 6 months later (first experiment in March and second in September, 2003).

2.4. Lethal time and concentration

To determine the time needed for EPNs to kill 50 and 90% of the guava weevil larvae (LT_{50} and LT_{90}), 100 IJs were suspended in 1 ml of distilled water and homogeneously distributed onto a 6 cm-plastic petri dish half filled with autoclaved sand at 10% moisture by weight. This assay used the same three strains as the previous experiment, *H. baujardi* LPP7, *H. indica* Hom1 and *S. riobrave* 355. For each species, 20 petri dishes were set up with one larva in each, and 10 additional dishes served as an untreated control, which received only water. Each petri dish was sealed with plastic film and placed in a germination chamber at 25 °C. Mortality was recorded every 2 days for 21 days. The entire experiment was repeated 1 month later.

Only *H. baujardi* LPP7 was used for the lethal concentration test (LC_{50} and LC_{90}), and the concentrations tested were 0, 20, 50, and 100 IJs/larva. For each treatment (concentration), twenty-four 6-cm plastic petri dishes with filter paper on the bottom were set up with one larva in each. The petri dishes were sealed and kept in a germination chamber at 25 °C. Mortality was recorded after 7 days.

2.5. Greenhouse assay

In the greenhouse, 6-month-old guava trees (about 60 cm high) were planted in 20-L pots filled with a mixture of 76% sand; 6% silt; 18% clay; 16.6 g/cm^3 of organic matter, pH 6.4. Initially, soil moisture was set at 10% by weight, and subsequently 50 ml of distilled water was added as needed to a dish under each pot to maintain soil moisture. Ten fourth-instar larvae were placed in each pot and, 24 h later, a concentration of 0, 500, 1000, or 2000 IJs was added. The nematode used in this test was *H. baujardi* LPP7 because it had performed well in the previous assays and, as a native Brazilian strain, was most likely to be used in an IPM program. Each treatment had 10 pots, and the entire experiment was repeated a second time immediately following the first. Larval mortality was recorded 21 days post-treatment for both trials. The soil temperature was monitored weekly using a soil thermometer (Watchdog Data Logger, Model 450, Spectrum Tecnologies). No other treatments were applied to the plants during the test.

2.6. Statistical analyses

The percent mortality data were compared using ANOVA and Tukey's multiple range tests (PROC ANOVA, SAS, 1990; SAEG, 1990). Arcsine transformations were used where appropriate. For lethal time (LT_{50} and LT_{90}) and lethal concentration (LC_{50} and LC_{90}), the data were analyzed through PROBIT (Finney, 1964).

3. Results

3.1. Petri dish assays

Significant differences in mortality were detected among the species/strains tested (ANOVA; $F = 268.83$; df = 9, 10; $P < 0.0001$), and all species/strains showed higher mortality compared to the control (Fig. 1). The four *Heterorhabditis* strains were the most virulent in this test and caused mortality ranging from 75.5 to 84.5%. The *Steinernema* species caused mortality ranging from 33.5 to 66.0%.

3.2. Sand column assay

Significant differences in mortality were found among species and concentrations (ANOVA, $F = 3.00$, df = 11, 12; $P < 0.05$). At concentrations of 100 and 200 IJs, mortality for all three species was not significantly different from the control (Fig. 2). At 500 IJs, only *H. indica* Hom1 and *H. baujardi* LPP7 showed significantly greater mortality than the controls (68.3 and 62.7% versus 12.8 and 13.0%, respectively), but there was no significant difference in mortality between these two nematode species (Fig. 2).

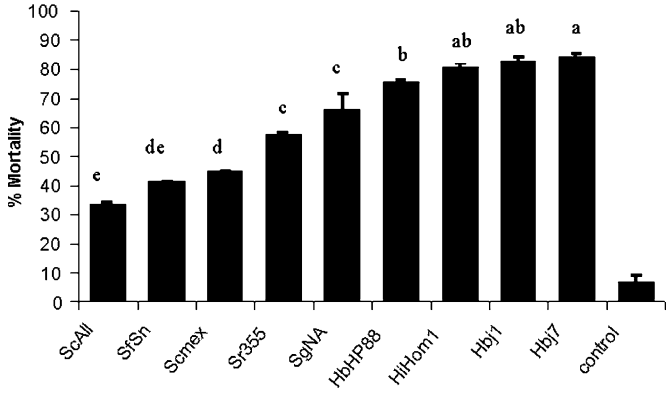

Fig. 1. Percent mortality (mean + standard error) of guava weevil larvae in a petri dish assay after exposure to various species/strains of entomopathogenic nematodes. Different letters above bars indicate significant differences ($P < 0.05$). See Table 1 for the complete names of the nematode species/strains.

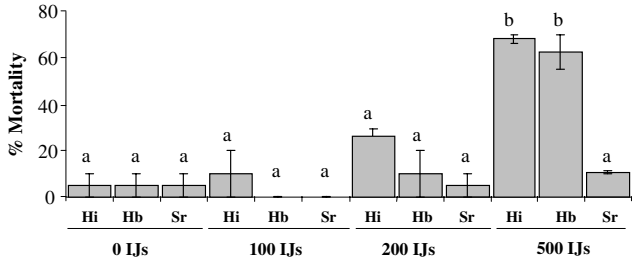

Fig. 2. Percent mortality (mean + standard error) of guava weevil larvae in a sand column assay after exposure to various concentrations of infective juveniles (IJ) of various species/strains of entomopathogenic nematodes. Different letters above bars indicate significant differences ($P < 0.05$). See Table 1 for the complete names of the nematode species/strains.

3.3. Lethal time and concentration

The time needed for *H. baujardi* LPP7, *H. indica* Hom1 and *S. riobrave* 355 to cause 50 and 90% mortality in guava weevil larvae was 6.5 and 9.9 days, 6.5 and 10.6 days, and 27.4 and 43.8 days, respectively. The concentration for *H. baujardi* LPP7 to cause 50% mortality in *Co. psidii* larvae (LC_{50}) was 52 IJs (lower fiducial limit = 36.7; upper fiducial limit = 70.5) whereas the concentration to cause 90% (LC_{90}) mortality was 122.2 IJs (lower fiducial limit = 95.8; upper limit = 183.3).

3.4. Greenhouse assay

Significant differences in mortality were detected among the different concentrations tested (ANOVA; $F = 18.61$; df = 3, 36; $P < 0.0001$). The two highest concentrations, 1000 and 2000 IJs, produced significantly higher levels of larval mortality than the control, and the highest mortality (60%) was achieved with the highest concentration (Fig. 3). The average soil temperature during the testing period (January–February, 2005) was 26.8 ± 0.22 °C.

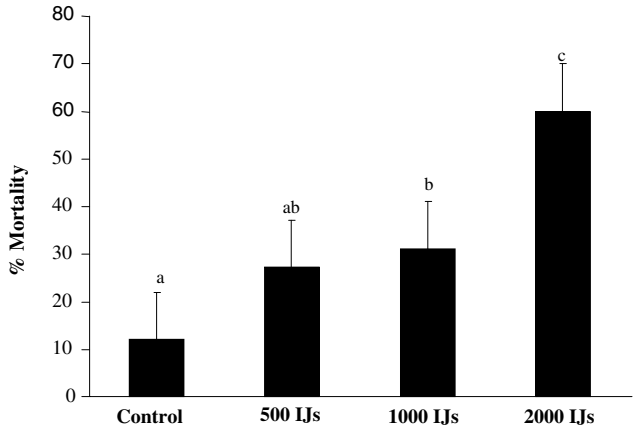

Fig. 3. Percent mortality (mean + standard error) of guava weevil larvae in a greenhouse assay after exposure to various concentrations of infective juveniles (IJ) of *Heterorhabdits baujardi* LPP7. Different letters above bars indicate significant differences ($P < 0.05$).

4. Discussion

Laboratory screening of entomopathogenic nematode species and strains in petri dish assays showed that the *Heterorhabditis* species tested were more virulent to guava weevil larvae than the *Steinernema* species. Sand column assays with a subset of these species supported this result and indicated that both *H. indica* Hom1 and *H. baujardi* LPP7 were capable of finding and killing the target insect whereas *S. riobrave* 355 performed poorly. In greenhouse tests with *H. baujardi* LPP7, larval mortality greater than the control was obtained only for nematode concentrations of 1000 and 2000 IJs/pot. These tests demonstrated the ability of this nematode to find and kill guava weevil larvae at temperatures characteristic of Brazilian guava orchards during summer.

Bedding et al. (1983) suggested testing EPNs at a concentration of 100 IJs/insect as a preliminary assessment of host susceptibility and to begin the process of selecting nematode species or strains as potential biological control agents. Our petri dish assays were conducted with this in mind, and all nine species and strains tested produced mortality levels greater than the control. However, all four heterorhabditids tested were significantly more virulent than the five steinernematids tested. In a previous study, Dolinski and Samuels (2002) tested certain EPN species against guava weevil larvae and found similar results with *S. feltiae* Israeli producing mortality of only 33% whereas *H. indica* Hom1 produced mortality of 85%. The two studies reinforce the view that heterorhabditids are more virulent than steinernematids against fourth-instar *Co. psidii* larvae.

The sand column assay provided a more rigorous test of the search and infection capability of different EPN species and produced results similar to those of the petri dish assay. *H. indica* Hom1 and *H. baujardi* LPP7 demonstrated greater capabilities than *S. riobrave* 355 for finding and

killing *Co. psidii* larvae at a concentration of 500 IJs. This concentration is close to 2.5 billion IJs/ha, which is generally recommended for field applications (Georgis, 1992).

The results of our assays with *Co. psidii* larvae differ in various ways from those conducted with other weevil species. Shapiro-Ilan (2001a) conducted tests using various EPN species against larvae of the pecan weevil, *Cu. caryae*, in plastic cups filled with sand and found that *H. indica* Hom1 and *S. riobrave* 355 at concentrations of 500 IJs caused 55 and 52% mortality, respectively. Shapiro-Ilan et al. (2002) tested various EPN species against another closely related weevil, the plum curculio, *Co. nenuphar*, and concluded that *S. riobrave* 355 and *S. feltiae* SN had the greatest potential for larval control. *S. riobrave* is highly virulent against *D. abbreviatus* larvae, and is considered the most efficient entomopathogenic nematode for inundative biological control of this citrus pest (Shapiro and McCoy, 2000; Stuart et al., 2004). In contrast, our results indicate that *S. riobrave* 355 and other steinernematids are less virulent to *Co. psidii* larvae than various heterorhabditids, including *H. indica* Hom1 and *H. baujardi* LPP7.

Previous studies have shown that adults of some weevil species are especially susceptible to EPNs and this includes the pecan weevil, *Cu. caryae*, and the plum curculio, *Co. nenuphar* (Shapiro-Ilan, 2001b; Shapiro-Ilan et al., 2002). In preliminary tests against guava weevil adults using *H. bacteriophora* HP88 and *S. glaseri* NA, mortality was extremely low and ranged from 0 to 10% (Dolinski, unpublished data). This is another example of how guava weevils differ from certain other curculionids in their susceptibility to EPNs.

The greater capability of heterorhabditids compared to steinernematids in locating and killing guava weevil larvae as demonstrated in our study might be explained by various aspects of host finding, host recognition, and host defense. Heterorhabditid IJs have an anterior "tooth-like structure" that could enable enhanced penetration of larval cuticle (Bedding and Molyneux, 1982). This capability might be especially important for virulence against guava weevil larvae, since there is some evidence based on histopathological studies that IJs enter the larvae mainly through the cuticle and less often though the anus (Dolinski, unpublished data). Heterorhabditids also generally search for their hosts by moving through the soil matrix with what is referred to as a "cruiser" foraging strategy whereas some steinernematid species (e.g., *S. carpocapsae*) are much less mobile and use an "ambusher" strategy, and others have an intermediate strategy with both cruiser and ambusher capabilities (e.g., *S. riobrave* 355; Campbell and Gaugler, 1997; Grewal et al., 1994; Lewis, 2002). Guava weevil larvae are immobile and stay in the soil for long periods, and a cruiser foraging strategy is likely to be the most effective for finding this host. In our assays, *S. glaseri* NA, which exhibits a cruiser foraging strategy (Campbell and Gaugler, 1997; Grewal et al., 1994; Lewis, 2002), performed nearly as well as the heterorhabditids but *S. riobrave*, which has proven to be highly effective against other weevil larvae in similar habitats, performed poorly. Thus, a difference in foraging strategy is unlikely to explain all of our results. Insect defense strategies against EPNs can be morphological, behavioral or immunological and can be highly specific to particular EPN species (Wang et al., 1994). Our results suggest that *Co. psidii* larvae have defense strategies that are much more effective against *S. riobrave* 355 and other steinernematids than against the heterorhabditids that were tested. Further studies are necessary to determine the nature of these defenses.

Larval mortality from EPN infection is usually observed within 3 days (Del Valle, 2004) and, therefore, the time necessary for *H. baujardi* LPP7 to cause 50 and 90% mortality to *Co. psidii* larvae (6.3 and 9.9 days, respectively) was longer than expected. Our assay was conducted at the end of the season when the larvae had been in the laboratory for up to a month. As in the pecan weevil, older larvae might be more resistant to infection by EPNs than younger larvae (Shapiro-Ilan, 2001a). Moreover, in *Co. psidii*, the fourth-instar larva is the stage that leaves the fruit, burrows into the soil, becomes inactive, and develops into the prepupa. The prepupa is morphologically extremely similar to the fourth-instar larva but is somewhat smaller, and the larval cuticle appears to lose moisture and become harder during prepupal formation. These and other developmental changes might influence IJ penetration rates (Omar Bailez, personal communication). However, other studies have also shown unusually long intervals for weevil larvae to succumb to EPNs (e.g., *D. abbreviatus*, Stuart et al., 2004).

The LC_{50} and LC_{90} calculated in our study for IJs of *H. baujardi* LPP7 infecting *Co. psidii* fourth-instar larvae were higher than that calculated in similar studies of the plum curculio, *Co. nenuphar* (Shapiro-Ilan et al., 2002). Such differences would be expected for different nematode and host combinations. However, it should also be noted that our LC_{50} and LC_{90} were evaluated after only 7 days of exposure and, based on the results of the LT tests, it seems likely that a longer test period might have produced higher mortality at lower concentrations. Unfortunately, our LC tests were conducted before the results of the LT tests were available, and the unusually long intervals indicated in the LT tests were not expected.

Our greenhouse tests showed a clear dose response and provide evidence that *H. baujardi* LPP7 is capable of finding and killing *Co. psidii* larvae under conditions that more closely resemble Brazilian guava orchards. These experiments were conducted during the summer when the average soil temperature was almost 27 °C and similar to that often found in guava orchards. To date, surveys for EPNs in guava orchards in Rio de Janeiro State have failed to produce any positive samples (Dolinski et al., 2003). Nonetheless, EPNs are often difficult to isolate in agricultural areas (Hominick, 2002) and we expect that a native nematode such as *H. baujardi* LPP7 that is well adapted to local climate and soil conditions could be very effective for biological control of *Co. psidii* in this agroecosystem. Future research will test this nematode in the field.

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