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SHORT COMMUNICATION

Infection of *Anastrepha ludens* (Diptera: Tephritidae) larvae by *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) under laboratory and field conditions

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Abstract

Laboratory and field experiments were performed to determine the efficiency of the entomopathogenic nematode *Heterorhabditis bacteriophora* against third instar larvae of the tephritid fruit fly *Anastrepha ludens*. Infection was affected by low (6%) and high (12–24%) soil moisture; the highest prevalence of infection was observed at 9% moisture. LC_{50} values were estimated under laboratory conditions at densities of 0.16, 0.26 and 0.64 larvae/cm^3 of sand in containers of different depths (2, 5 and 8 cm) at 10% moisture, and larval ages (third instar, early versus late stadium). Third instar *A. ludens* were significantly more susceptible to infection early in the stadium than late in the stadium, irrespective of host density (LC_{50} ~15 infective juvenile nematodes/cm^2 soil surface). Infection of late stadium third instars was significantly reduced at low density. Application of 115 and 345 infective juvenile nematodes/cm^2 (representing one and three times the laboratory LC_{50} at the lowest host density, respectively), in experimental plots in a commercial mango orchard, resulted in 46.7% (range of SE: 45.2–48.1) and 76.1% (SE: 74.8–77.3) infection, respectively. We conclude that *H. bacteriophora* merits further study as a natural enemy of *Anastrepha* spp. in tropical regions of the Americas.

Keywords: Heterorhabditis bacteriophora, *fruit fly,* Anastrepha ludens, *nematode, infection, host density, moisture, mango, insect stage*

The Mexican fruit fly *Anastrepha ludens* (Loew) (Diptera: Tephritidae) represents the most serious pest of fruit production in Mexico and several other Neotropical countries (Aluja 1994). This insect is particularly important in mango (*Mangifera*

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indica), peach (*Prunus persica*) and citrus (*Citrus* spp.) (Norrbom & Kim 1988; Hernández-Ortiz & Aluja 1993). The presence of *A. ludens* results in strict quarantine requirements by certain fruit importing countries including the United States, Australia and Japan (APHIS 1970). Integrated control practices targeted at this pest involve the use of insecticidal baits for adult control (Aluja 1994), and soil applications of the organophosphate diazinon for the control of prepupal and pupal stages in the soil (Mohamad et al. 1979; Saul et al. 1983; Penrose 1993). In southern Mexico, *A. ludens* is multivoltine with $\sim$10 overlapping generations annually. Peak infestation of fruits occurs from April to June and up to 80 larvae may emerge from a single mango to pupate in the soil.

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae have shown promise for the biocontrol of fruit flies (Beavers & Calkins 1984; Lindegren & Vail 1986; Lindegren et al. 1990; Lezama-Gutiérrez et al. 1996; Gazit et al. 2000; Toledo et al. 2001; Yee & Lacey 2003). Third instar *A. ludens* are susceptible to infection by nematodes during the period when they exit the host fruit and burrow into soil for pupation. The present study aimed to assess the efficacy of a soil dwelling nematode isolated in Central America, *Heterorhabditis bacteriophora* (Poinar), for the control of third instar *A. ludens* under laboratory and field conditions.

Larvae of *A. ludens* were obtained from the Moscafrut mass-rearing facility at Metapa, Chiapas, Mexico, where this species is reared on a semi-synthetic diet (Domínguez et al. 2000). A laboratory culture of *H. bacteriophora*, originating from material collected in Costa Rica (Castillo & Marbán-Mendoza 1996), was maintained in the laboratories of ECOSUR, Tapachula, Chiapas, Mexico, using a laboratory colony of wax moth larvae (*Galleria mellonella* L.) as the host. Infective stages were collected using White traps (Woodring & Kaya 1988). These stages were quantified by microscopic counting of ten different samples, each of 1 mL, then adjusted to a concentration of 800 nematodes/mL in sterile distilled water, and refrigerated at $10 \pm 2^{\circ}$C for up to 3 weeks, as required. Mortality during the storage period was minimal ($\sim$1%).

Effect of moisture on prevalence of infection

Experimental units were constructed of PVC tubes of 5 cm diameter and 8 cm tall. The base of each tube was sealed using the base of a plastic Petri dish. Tubes were filled with 120 g of sand that had previously been sieved, washed and sterilized in an autoclave. The moisture of soil samples was adjusted to 6, 9, 12, 15, 18, 21 and 24% (w/w) moisture by the addition of sterile distilled water. The surface area of sand exposed in each experimental unit was 19.6 cm^2. Each moisture treatment was replicated six times. Each experimental unit was treated with 125 infective juvenile nematodes/cm^2 (2450 nematodes per unit) applied uniformly to the soil surface in a volume of 1 mL water using a micropipette. Moisture calculations took into account the addition of the nematode suspension. Ten minutes later, 25 late stadium third instar *A. ludens* were placed on the sand surface and allowed to burrow into the sand. This represented a density of 0.16 larvae/cm^3 of sand. After 7 days, the sand from each unit was gently sieved (18 mesh) to separate fly pupae. Mortality due to infection was quantified by dissection under a binocular microscope. Results were analyzed in the GLIM program (Generalized Linear Interactive Modeling, Numerical Algorithms Group 1993) with binomial error structure. Moisture was considered as a factor with

seven levels. A slight degree of overdispersion was taken into account by scaling the error distribution (scale parameter 1.18). An alternative regression analysis considering moisture as a continuous parameter involved a fifth-order polynomial model and was deemed unrealistically complex. Soil moisture had a highly significant effect on the prevalence of infection ($F(6,34) = 16.1$, $P < 0.001$). A distinct peak in prevalence of infection was observed in soil with 9% moisture (Figure 1) compared to soil of lower (6%) or higher ($\geq 12\%$) moisture levels. For this reason, subsequent studies were performed at 10% soil moisture, which facilitated all calculations of soil water content.

Effect of larval development and soil depth on infection

Bioassays were performed using the PVC experimental units described above at one of three heights: 2, 5 and 8 cm. Tubes were filled with either 30, 70 or 120 g, of damp sand that had previously been sieved, washed and sterilized in an autoclave and adjusted to 10% (w/w) moisture.

Infective juvenile *H. bacteriophora* were uniformly applied to the surface of each unit in a volume of 1 mL using a micropipette: for early stadium third instars at 6, 13, 25, 51, 76, 102, and 127 nematodes/cm^2 of sand surface, whereas for late stadium third instars, an additional concentration of 204 nematodes/cm^2 was included (total 118–4000 nematodes/experimental unit). These concentrations were selected based on previously published work (Lezama-Gutierrez et al. 1996). Control units were treated identically but without the addition of nematodes. The moisture of each experimental unit took into account the 1 mL addition of water necessary for the application of nematode suspensions. After 10 min, groups of 25 insect larvae of two ages (early and late stadium third instars, aged 8 and 9 days post-hatching, respectively) were placed on each unit and allowed to burrow into the sand. The density of larvae was 0.64, 0.26 and 0.16 larvae/cm^3 sand for containers of 2, 5 and 8 cm tall, respectively. Each treatment was replicated three to five times. Experimental

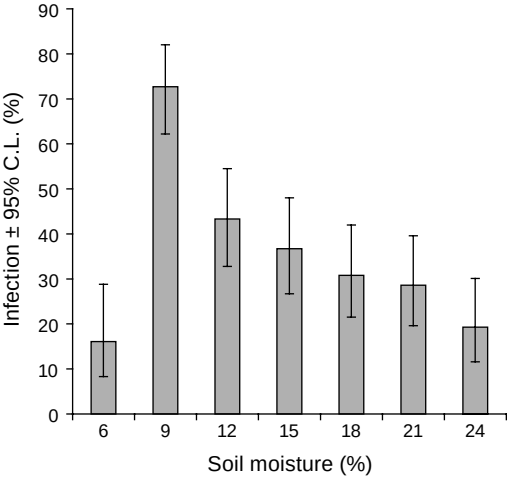

Figure 1. Effect of sand moisture content on prevalence of infection by *Heterorhabditis bacteriophora* on late stadium third instar larvae of *Anastrepha ludens* at a density of 0.16 larvae/cm^3 of sand in 8-cm deep containers.

units were incubated in a bioclimatic chamber at 26 ± 1°C, 70 ± 5% RH and 12:12 h L:D photoperiod. Seven days later, the sand of each unit was gently sifted to separate fly pupae. The prevalence of dead pupae attributable to infection was determined by dissection using a binocular microscope. Results were subjected to logit analysis in GLIM. Evidence of overdispersion was determined by dividing the error model deviance by the error degrees of freedom. Where this value was substantially >1, scaling of the error distribution was performed (Crawley 1993). LC_{50} values and 95% C.L.s were calculated using the procedure described by Collett (1991). To determine the significance of treatment and interaction effects, bioassay results were subjected to analysis of deviance in GLIM with a binomial error structure in which larval stage and sand volume were considered as factors with two and three levels, respectively, and $\log_e$ (concentration of nematodes/cm^2 of surface area) was considered as a continuous variable. Changes in model deviance by sequential removal of terms approximate to a χ^2 distribution. The error specification and structure of models were examined by plotting (i) scaled residuals against fitted values and (ii) ranked residuals against standard normal deviates, and found satisfactory (Crawley 1993).

Early stadium third instar larvae were significantly more susceptible to infection than late stadium third instars (Tables I and II). LC_{50} values of early stadium larvae were similar at all host densities (14.0–15.8 nematodes/cm^2), whereas LC_{50} values increased significantly with decreasing host density in late stadium insects (47.0–111.5 nematodes/cm^2), reflected in a significant stadium × density interaction (Table II). Natural mortality in control insects was less than 1.6% and no infection was observed.

Field trial

In March 2002, a field trial was performed in a mango orchard (cv. 'Ataulfo'), on the Pacific coast of Chiapas, Mexico (14°10′N, 92°10′W, altitude 180 m). During the experiment, soil temperature fluctuated between 25 and 29°C. Random areas of soil surface 50 × 50 cm were selected under the canopies of 18 full grown trees and excavated to a depth of 10 cm. A wooden frame, 50 × 50 cm and 25 cm high with fine nylon gauze stapled onto the base was placed in the excavated space. The gauze base prevented escape of larvae and allowed aeration and drainage, although no rainfall was recorded during the experiment. Each wooden frame was partially filled with 10 kg of sieved sandy soil (depth ~8 cm) previously adjusted to 10% moisture. The soil comprised approximately 96% sand, 3% clay, 1% silt, 0.18% organic matter, pH 6.6. One of three treatments was then applied to the soil in each frame: (i) 287 500 infective juvenile nematodes, equivalent to 115 nematodes/cm^2 (approximately the laboratory LC_{50} for mature larvae in 8-cm deep containers), (ii) 862 500 nematodes, equivalent to 345 nematodes/cm^2 (approximately three times the laboratory LC_{50} value), (iii) water control. Each treatment was applied to six frames in a fully randomized design. Nematodes were applied in 200 mL water using a hand-held atomizer. Ten minutes later, 200 late stadium third instar *A. ludens* were placed on the soil surface of each frame. The frame was sealed with a nylon gauze roof and sticky fly-trap glue was applied to the upper outer edges of the frame to prevent predation of experimental larvae. Soil moisture was monitored by daily sampling and maintained at 10% (±1%) by the addition of 100 mL of water to the soil surface at 24-h intervals using an atomizer. At 8 days post-application, each frame was removed from the soil

Table I. Logit regression of lethal infection of early and late stadium third instar *Anastrepha ludens* on concentration of infective juvenile *Heterorhabditis bacteriophora* at densities of 0.16, 0.26 and 0.64 larvae/cm^3 of sand (10% moisture).

Stadium and host density[1] (depth of container)	*N* (replicates)	Regression[2]	SE of intercept	LC_{50}^{3}	Range of 95% C.L.	Error deviance/ error df[4]
Early third instar						
0.16 (8 cm)	699 (5)	$y=0.7134x-1.992$	0.2909	15.8	12.2–20.6	0.393
0.26 (5 cm)	588 (4)	$y=0.8885x-2.346$	0.3240	14.0	10.7–17.4	0.891
0.64 (2 cm)	432 (3)	$y=0.8250x-2.213$	0.3671	14.6	10.4–19.1	0.326
Late third instar						
0.16 (8 cm)	981 (5)	$y=0.9005x-4.245$	0.5374	111.5	86.1–157.1	2.450[5]
0.26 (5 cm)	692 (4)	$y=1.1720x-5.075$	0.4853	76.0	65.9–88.1	0.580
0.64 (2 cm)	818 (5)	$y=0.6490x-2.499$	0.2874	47.0	37.6–59.7	1.178

[1]Density given as larvae/cm^3 of sand. [2]Regression presented as $\log_e$ odds ratio (p/q). [3]LC_{50} values expressed as nematodes/cm^2 of surface area. [4]Indicates degree of overdispersion of data from binomial distribution (1.0). [5]Model was scaled by 2.450 to account for moderate overdispersion.

and the contents were gently passed through a sieve (18 mesh) to separate *A. ludens* pupae. Pupae were taken to the laboratory, placed on moist filter paper in plastic containers and incubated in a bioclimatic chamber at $26\pm1^{\circ}$C, $70\pm5\%$ RH and 12:12 h L:D photoperiod. Pupae that did not emerge were dissected to determine the presence of nematodes.

Recovery of pupae from experimental plots was high (range of means: 186–196 insects/plot) and did not differ significantly between treatments, including the control ($F(2,15)=1.48$, $P=0.26$). Application of 115 nematodes/cm^2, approximately the laboratory LC_{50} concentration in late stadium third instar larvae in 8-cm deep containers, resulted in 46.7% (range of SE: 45.2–48.1) infection of pupae. Application of 345 nematodes/cm^2 (approximately three times the laboratory LC_{50} value), resulted in 76.1% (range of SE: 74.8–77.3) infection of pupae, which was significantly greater than observed in the single LC_{50} treatment ($t=6.31$, $df=10$, $P<0.001$). Mortality of 3.4% was observed in control pupae, none of which was due to infection by nematodes.

Laboratory and field experiments demonstrated that *H. bacteriophora* has the ability to infect larvae of *A. ludens*. Younger larvae were more susceptible to infection, possibly because the period of exposure, from inoculation to pupation, was longer in young larvae than in older larvae of the same instar. In contrast, pupae are totally refractory to penetration by nematodes, probably due to the toughness of the puparium, and the limited possibility to penetrate pupal spiracles (Beavers & Calkins 1984; Toledo et al. 2001).

In the laboratory, nematode efficacy did not change with host density for early stadium third instars, whereas late stadium larvae were clearly less likely to become infected at lower densities. This may reflect vigorous burrowing behavior in late stadium larvae that are about to pupate, which facilitates their escape from high concentrations of juvenile *H. bacteriophora* near the soil surface, whereas such behavior is not highly developed in early stadium third instars that would normally be on the point of leaving the host fruit to seek a pupation site.

Table II. GLIM analysis of effect of larval stadium (early versus late stadium third instar), density of host larvae, and natural logarithm of concentration of infective juvenile *Heterorhabditis bacteriophora* on mortality of *Anastrepha ludens* in laboratory bioassays.

Source	Contribution to model deviance (χ^2)	df	P
Log_e(nematode conc.)	404.20	1	<0.001
Stadium	339.30	1	<0.001
Density	43.60	2	<0.001
Density × conc.	5.60	2	0.061
Stadium × density	20.11	2	<0.001
Stadium × conc.	1.32	1	0.250
Stadium × density × conc.	7.29	2	0.026
Error	165.90	162	

Error deviance/error df for full model was 1.08. Log_e(nematode concentration, nematodes/cm^2) was taken as a continuous variable, larval stadium (early versus late instar) and density were taken as factors with 2 and 3 levels, respectively.

Despite this, infection by *H. bacteriophora* was more uniform in containers of different depths compared to infection by *Steinernema feltiae* which declined dramatically with increasing soil depth (Toledo et al. 2001). These two species of nematodes disperse differently: *S. feltiae* tends to move horizontally close to the soil surface, whereas *H. bacteriophora* can disperse vertically and infect hosts at greater depths (Campbell et al. 1996).

Many physico-chemical variables can influence the performance of soil nematodes as agents of biological control, including soil moisture, texture, temperature, the degree of soil compaction (Portillo-Aguilar et al. 1999; Yee & Lacey 2003), as well as biotic factors, such as the depth at which host insects pupate (Eskafi & Fernández 1990; Jackson et al. 1998; Alyokhin et al. 2001). *Heterorhabditis bacteriophora* was highly sensitive to very low soil moisture, displaying the highest prevalence of infection at 9% moisture. In contrast, other nematode species such as *S. riobrave* can function across a range of soil moistures (Gazit et al. 2000).

Cryptic pests such as fruit flies that inhabit fruits and soil during their development may be particular difficult to control due to the physical refuge from attack offered by the substrate they infest. The only natural enemy currently used for *Anastrepha* spp. control in southern Mexico in the braconid parasitoid *Diachasmimorpha longicaudata* (Ashmead) that typically causes ~40% parasitism of larvae in mango (Montoya et al. 2000). This underlines the need to seek additional natural enemies for fruit fly control in Mesoamerica. We conclude that *H. bacteriophora* may represent a potentially important agent for biological control of fruit flies of the genus *Anastrepha* in tropical regions of the Americas. Additional field studies are required to validate the efficacy and persistence of this nematode in commercial fruit orchards of the region.

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