Infection of *Anastrepha ludens* following soil applications of *Heterorhabditis bacteriophora* in a mango orchard

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Abstract

To determine the efficacy of *Heterorhabditis bacteriophora* Poinar (Nematoda: Heterorhabditidae) for control of *Anastrepha ludens* (Loew) (Diptera: Tephritidae), field experiments were performed in a mango orchard with soil temperatures of 24–29 °C. The density of third-instar *A. ludens* (50–500 larvae per plot) released into 0.25 m^2 wood-framed experimental plots containing soil (16% wt/wt moisture) previously treated with 125 infective juveniles per square centimetre soil surface did not significantly influence the prevalence of infection by *H. bacteriophora*. In subsequent experiments, the percentages of infection of fly pupae were positively correlated with the concentration of infective stages applied to soil plots. The highest average percentage of infection (74% at 250 infective juveniles per square centimetre) was observed when fly larvae were released simultaneously onto soil, compared to larvae that emerged from laboratory-infested mangoes over a period of 8 days (52% infection at 500 infective juveniles per square centimetre). Double applications of infective juveniles at an interval of 4 days did not greatly improve the prevalence of infection (~10% higher) compared to single applications. Between 9 and 15% of larvae that remained within infested mangoes became infected by nematodes, irrespective of the concentration of nematodes applied to each experimental plot. We conclude that effective control of *A. ludens* requires very high densities of *H. bacteriophora*. The successful use of this nematode for biocontrol of *A. ludens* will depend on identifying ways of overcoming the fly's ability to avoid infection.

Introduction

Insect pests that develop inside plant structures are usually very difficult to control with chemical insecticide sprays or require costly applications of systemic insecticides. The Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), is widely distributed from the southern USA through central America. Larvae develop inside many species of fruits, typically causing direct losses of 10–25% in the production of mango (*Mangifera indica* L.) and citrus (*Citrus* spp.) (Enkerlin et al., 1989; Aluja, 1994; Aluja et al., 1996). Fruit grown in this region are subject to strict quarantine restrictions by the USA and other importing countries (APHIS, 1970).

Control of *A. ludens* usually involves the use of foliar sprays of synthetic insecticides formulated in baits for control of adult flies and soil application of compounds such as diazinon. This treatment is targeted at the control of final instar larvae emerging from the fruit to pupate, and adult flies emerging from pupae in the soil (Mohamad et al., 1979; Saul et al., 1983; Penrose, 1993). Large quantities of broad-spectrum insecticides applied to foliage or soil adversely affect non-target invertebrate fauna, particularly insect natural enemies (Troetschler, 1983). Given the interest in organic production, and the concern over levels of pesticide residues in food, the need for effective alternative

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strategies with reduced environmental impact for control of this pest is increasingly evident (Michaud, 2003).

Entomopathogenic nematodes have proved to be effective biological control agents of numerous insect pests, including soil-dwelling species (Gaugler, 1988). Infective juveniles penetrate the host via the spiracles, mouth and anus and release symbiotic bacteria that multiply and kill the infected insect by producing potent insecticidal toxins (Bowen et al., 1998).

The soil-dwelling nematode *Heterorhabditis bacteriophora* Poinar (Nematoda: Heterorhabditidae) has an established potential for control of dipteran pests (Mullens et al., 1987; Wozniak et al., 1993), including tephritid fruit flies (Beavers & Calkins, 1984; Lindegren & Vail, 1986; Lezama-Gutiérrez et al., 1996; Patterson-Stark & Lacey, 1999; Gazit et al., 2000; Toledo et al., 2001; Yee & Lacey, 2003). However, most of these studies have been performed in the laboratory and field evaluations are scarce (Lindegren et al., 1990; Toledo et al., 2005). The aim of the present study was to evaluate the efficacy of *H. bacteriophora* in the control of final instar *A. ludens* in a commercial mango orchard in southern Mexico. Specifically, we examined how infection varied with rate of application, pest density, and the efficacy of sequential treatments applied during the period when larvae were emerging from fruits to pupate in the soil.

Materials and methods

Biological material, experimental units, and study area

Larvae of *A. ludens* were obtained from a laboratory colony maintained on semisynthetic diet at the Moscafrut rearing plant (SAGARPA-IICA), Metapa de Domínguez, Chiapas, Mexico, as described previously (Domínguez et al., 2000). Larvae were used at 9 days posthatching and were due to initiate the soil burrowing and pupation process.

For experiments involving infested fruits, 3200 pupae from the laboratory colony were placed in a cubic wooden cage (70 cm on all sides) sealed on all sides with fine nylon mesh. Velcro seals on the front provided access during the infestation process. Approximately 1500 adult pairs emerged in each cage and were fed with hydrolysed yeast (ICN Biomedical Inc., Costa Mesa, CA, USA), sucrose (in a 1:3 ratio), and water placed in 100 ml plastic pots with cotton wicks, and maintained at 26 ± 1 °C, 70 ± 5% r.h., and a L12:D12 photoperiod.

At 9 days postemergence, 60 mangos cv. Ataulfo, at 3/4 physiological maturity and with an average (± SE) weight of 380 ± 12 g, were placed on a pair of metal bars located on the base of the cage. Mangoes were exposed to oviposition for 12–24 h, which previous studies indicated would result in infestations of approximately 30–40 larvae per fruit. Infested fruits were removed from cages and incubated at 26 ± 1 °C for 15–18 days. When larvae reached the third instar, determined by dissection of five fruits from each batch, mangoes were taken for use in field experiments.

A laboratory colony of *H. bacteriophora* was continuously maintained using material originating from Costa Rica (Castillo & Marbán-Mendoza, 1996). Infective stages of *H. bacteriophora* were produced by inoculating larvae of the wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), and were collected using white traps (Woodring & Kaya, 1988). Infective stages were quantified by counting 10 samples per mililitre of suspension, each sample consisting of a 1-ml volume, divided into four drops of 250 µl on a plastic Petri dish, and counted under a binocular microscope. The concentration was adjusted to 800 nematodes per mililitre in sterile water and stored at 10 ± 2 °C for up to 3–4 weeks until required. Mortality during storage did not exceed 2% on any occasion and previous studies indicated no significant loss of infectivity during this period, provided that the water was changed at weekly intervals (J Toledo, unpubl.).

Field studies were performed during the months of January to April in a 0.6-ha area of a 15-ha mango cv. Ataulfo orchard, located 6 km west of the town of Tapachula, Chiapas, Mexico (14°55′N, 92°21′W), at an altitude of approximately 180 m. The climate in this tropical region is warm (annual mean 26 °C) with an annual precipitation of 2200 mm, almost all of which falls in the rainy season between May and November.

The area beneath 20 (experiment 1) or 24 (experiments 2, 3, and 4) randomly selected mango trees was manually cleared of fallen leaves and twigs. A square area of soil 50 × 50 × 10 cm depth was excavated at a randomly selected point beneath the canopy of each tree. To avoid interference by predators, particularly ants, each excavated plot was treated with 2 g of a powder formulation containing 2% (wt/wt) methyl parathion. A plastic sheet was then placed over the treated soil, and then a wooden frame, 50 × 50 cm and 25 cm tall with fine nylon gauze stapled onto the base, was placed in the excavated space. The gauze base prevented the escape of larvae and allowed aeration and drainage. Each wooden frame was filled to a depth of 8 cm with 10 kg of sieved soil (18 mesh) consisting of 34.5% sand, 33.4% silt, 32.1% clay, pH 5.4, and 2.5% organic matter. The external walls of the frame were treated with sticky trap glue to minimize entry of predators (Tangletrap, Gempler's, Madison, WI, USA). Soil moisture had been previously adjusted to 16% by weight, taking into account the volume of the suspension of nematodes to be applied. A clear plastic sheet was stapled over the wooden frame to avoid major changes in soil moisture due to rainfall. In all experiments, infective juvenile *H. bacteriophora* were applied to the soil surface in a volume of 200 ml of water using a hand held sprayer whereas control plots were treated with water alone.

In all cases, soil moisture was carefully monitored during the experimental period and, if necessary, adjusted to 16% moisture by the application of water using a hand-held atomiser at 24-h intervals. At the end of each trial, soil moisture was determined in eight randomly selected plots based on 100 g (wet weight) samples. Soil temperatures were registered using a laboratory glass thermometer inserted to a depth of 6 cm in five randomly selected plots (four plots in experiment 3) at 2-h intervals from 08:00 to 18:00 hours every other day during each experiment.

Experiment 1: effect of host density

To determine the influence of varying host densities on the efficacy of the nematode, a concentration of 125 infective stages per square centimetre soil surface (total 312,500 nematodes per plot) was applied to the soil surface. At 15 min after application, one of the following densities of third-instar *A. ludens* was released onto the surface of each plot and allowed to burrow into the soil: 50, 150, 300, or 500 larvae per plot. Previous studies had indicated that the interval between soil inoculation of the insect larvae and the infective juveniles did not significantly alter the prevalence of infection for periods up to 4 days postapplication (Pérez, 2000). Control plots were each infested with 200 larvae (the median density used in nematode plots) but were not treated with nematodes. Each treatment was applied to four replicate plots distributed in a fully randomized design. At 8 days after the start of the experiment, soil was removed from plots and transported to the laboratory where it was sieved (18 mesh) and the mortality of pupae due to nematode infection was determined by dissection of pupae. Control pupae were placed in Petri dishes with damp filter paper, incubated at 26 ± 1 °C and adult emergence was determined daily. In all cases, dead pupae were dissected to determine nematode infection.

Experiment 2: effect of nematode density

The prevalence of mortality of a fixed density of *A. ludens* larvae released onto the surface of plots that had been inoculated with varying concentrations of *H. bacteriophora* infective stages was determined. Volumes of 200 ml of nematode suspension containing the equivalent of 0 (control), 25, 50, 75, 125, or 250 infective stages per square centimetre of soil surface (0–625,000 nematodes per plot) were applied to randomly selected experimental plots using a hand-held sprayer. At 15 min after application, 500 *A. ludens* third instars were gently scattered over the surface of the treated soil and allowed to burrow into it. There were four replicate plots of each treatment. At 8 days postinoculation, soil from each plot was transported to the laboratory, sieved, and the prevalence of nematode infection determined by dissection. The emergence of insects from control plots was determined as described previously.

Experiment 3: effect of nematode density on larvae emerging from fruits

This study was performed to determine the efficacy of *H. bacteriophora* in infecting larvae emerging naturally from infested mango fruits. Treatments consisted of 0, 25, 50, 75, 125, or 250 infective juveniles per square centimetre of soil surface. At 15 min after the application of nematodes, 15 infested fruits were uniformly distributed on the soil surface within each plot and remained there for the duration of the experiment. Each treatment was applied to four replicate plots in a fully randomized design. At 8 days postinoculation, the soil of each plot was transported to the laboratory and the prevalence of infection by nematodes and emergence of insects from control plots were determined as described previously.

Experiment 4: effect of two nematode applications on larvae emerging from fruits

In this experiment, we determined the mortality of fly larvae emerging from infested fruit following two applications of nematodes, as compared to a single application in the previous experiment. For this, nematodes were applied at one of the following concentrations: 0, 25, 50, 75, 125, or 250 infective juveniles per square centimetre in a volume of 200 ml water using a hand-held sprayer. At 15 min after the application, 15 mangoes infested with third-instar *A. ludens* were uniformly distributed on the surface of each plot. After 4 days, a second application was made using the same concentrations. Each treatment was applied to four replicate plots. Insects were recovered from each plot and infection by nematodes and emergence of insects from control plots were determined as described previously.

Statistical analysis

The numbers of insects or the percentage of insects recovered from experimental plots (where numbers of released insects varied between treatments) were subjected to one-way analysis of variance. The relationship between the prevalence of lethal infection by *H. bacteriophora* and host density, or the concentration of infective juveniles applied to experimental plots, was determined by linear regression. In all cases, assumptions of normality and homogeneity of variances were examined and, where necessary, arcsine transformation was applied to percentage data prior to analysis. All analyses were performed in SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). None of the insects recovered from control plots were found to be infected by nematodes, and these were therefore excluded from the analyses.

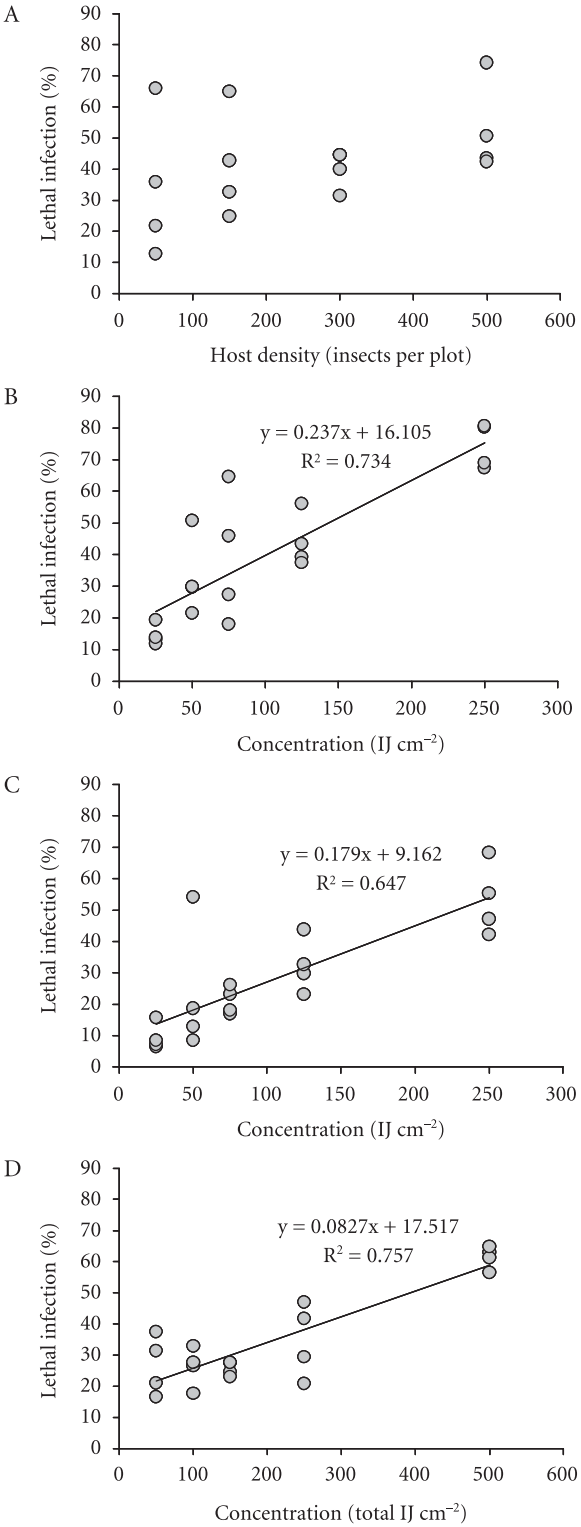

Figure 1 Infection of third-instar *Anastrepha ludens* larvae by *Heterorhabditis bacteriophora* under field conditions. Percentage lethal infection was determined at 8 days following the application of (A) 125 infective juveniles (IJ) per square centimetre soil surface at densities of 50–500 fly larvae per plot, (B) 25–250 IJ cm^{-2} of soil surface at a density of 500 larvae per plot, (C) 25–250 IJ cm^{-2} in plots subsequently inoculated with 15 mangoes infested by third-instar fly larvae, and (D) 25–250 IJ cm^{-2} on two occasions over an interval of 4 days (total 50–500 IJ cm^{-2}) in plots subsequently inoculated with 15 mangoes infested by third-instar fly larvae. Regression lines with corresponding equations and R^2 values are shown for significant linear regressions ($P<0.05$). Data points represent individual replicates. Nematode infections were not observed in any untreated control plots (data not shown).

Results

Experiment 1: effect of host density

The mean percentage of recovery of *A. ludens* released onto experimental plots at different densities (50–500 larvae per plot) was 97.8% (range 92.0–100%, total n = 4723) and was not significantly affected by insect density ($F_{3,12} = 0.53$, $P = 0.64$, following arcsine transformation, excluding control plots). Emergence of adults from pupae recovered from control plots was 87.2% and nematode infections were not observed in control insects. The prevalence of infection in *A. ludens* larvae exposed to a single concentration of 125 infective juveniles per square centimetre varied from 12 to 72% in different plots (Figure 1A), but was not significantly affected by insect density ($F_{1,14} = 2.56$, $P = 0.13$). Mean (± SD) soil temperatures ranged from 24.2 ± 0.1 °C at 08:00 hours to 27.8 ± 0.1 °C at 16:00 hours. Mean soil moisture at the end of the experiment was 16.1 ± 0.5%.

Experiment 2: effect of nematode density

Of the 500 larvae released into each plot, the mean number recovered as pupae from experimental plots was 477 ± 25.3 (95.4%, total n = 11,448) and did not differ between any of the nematode treatments and the control ($F_{5,18} = 2.45$, $P = 0.073$). Emergence of adults from pupae recovered from control plots was 96.6% and nematode infections were not observed in control pupae. A clear positive relationship was detected between the concentration of infective juveniles applied and the resulting prevalence of lethal infection ($F_{1,18} = 49.54$, $P<0.001$, excluding control plots). The mean prevalence of infection increased from 14.5% at 25 infective juveniles per square centimetre to 74.3% at 250 infective juveniles per square centimetre (Figure 1B). The coolest soil temperatures were registered at 08:00 hours (mean ± SD: 24.1 ± 0.1 °C) and the warmest temperatures at 16:00 hours (27.7 ± 0.2 °C) over the course of the experiment. Mean soil moisture at the end of the experiment was 16.5 ± 0.5%.

Experiment 3: effect of nematode density on larvae emerging from fruits

The mean (± SD) number of insects recovered from experimental plots inoculated with mangoes infested by third-instar *A. ludens* was 441 ± 129 pupae per plot (total n = 10,577) and did not differ between any treatment including the control ($F_{5,18} = 2.25$, $P = 0.094$). Emergence of adults from pupae recovered from control plots was 82.9% and no nematode infections were observed. The prevalence of lethal infection of pupae was positively correlated with the concentration of infective juveniles applied to the soil surface (Figure 1C), increasing from an average of 9.4% at 25 infective juveniles per square centimetre to 52.1% at 250 infective juveniles per square centimetre ($F_{1,18} = 32.9$, $P<0.001$, excluding control plots). Moreover, when mango fruits were manually dissected, an average (± SD) of 41.3 ± 37.0 larvae in each group of 15 fruits were found not to have left the fruits to pupate. Of these insects, an average of 15.0% (range 0–57%) were observed to be infected by *H. bacteriophora*. However, no significant relationship was detected between the concentration of nematodes applied to the soil and the percentage of infection of insects that remained in the mangoes ($F_{1,17} = 1.07$, $P = 0.32$, excluding control plots). One replicate plot of the treatment involving 50 infective juveniles per square centimetre was excluded due to predation by ants. Average (± SD) soil temperatures ranged from 25.8 ± 0.4 °C at 08:00 hours to 28.5 ± 0.1 °C at 14:00 hours. Mean soil moisture at the end of the experiment was 16.3 ± 0.6%.

Experiment 4: effect of two nematode applications on larvae emerging from fruit

The mean (± SD) recovery of pupae was 460 ± 124 insects per plot (total n = 10,585) and this did not differ between treatments including the control ($F_{5,17} = 1.47$, $P = 0.25$). Emergence of adults from pupae recovered from control plots was 85.5% and no nematode infections were observed, although one replicate control plot was excluded due to predation by ants. The relationship between total concentration of nematodes applied (the sum of both applications) and percentage of lethal infection of pupae ranged from a mean of 27% at 25 + 25 infective juveniles per square centimetre to 62% at 250 + 250 infective juveniles per square centimetre (Figure 1D) and was highly significant ($F_{1,18} = 56.0$, $P<0.001$, excluding control plots). Dissection of infested mangoes following the experiment revealed an average (± SD) of 39.1 ± 32.1 larvae that remained inside each group of 15 mangoes. The average percentage of infection of these insects by *H. bacteriophora* was 9.7% and this was not significantly affected by the total concentration of nematodes applied to each plot ($F_{1,18} = 4.22$, $P = 0.055$, excluding control plots). Mean daytime soil temperature ranged from 25.9 ± 0.4 °C at 08:00 hours to 28.4 ± 0.1 °C at 14:00 hours. Mean soil moisture at the end of the experiment was 16.2 ± 0.6%.

Discussion

The high recovery of insects from experimental plots in all experiments indicated that insecticidal treatment of the soil outside the experimental plot applied to reduce interference by ants did not adversely affect survival of *A. ludens*. No infections were observed in dissections of insects from control plots, indicating that entomopathogenic nematode populations were below the detection threshold at our experimental site. Host density had no significant effect on the percentage of infected *A. ludens* pupae, across a 10-fold range of densities. The densities employed in this study were similar to those that are likely to occur naturally in orchards when chemical control is not applied to the soil. For this reason, and also because large samples are desirable for statistical purposes, experimental plots were inoculated with over 400 insects in all the subsequent experiments.

Infection rates reached high levels (>70%) only at the two highest tested concentrations of 250 and 500 infective juveniles per square centimetre of soil surface, equivalent to 2.5 and 5×10^{10} infective juveniles per hectare, respectively. This is more than 10 times the usual rate for inundative soil applications of nematodes, typically ~2.5×10^9 infective juveniles per hectare (Grewal et al., 1994; Wilson et al., 2003), that can provide effective control of other soil dwelling pests (Zimmerman & Simons, 1986; Shanks & Agudelo-Silva, 1990; Berry et al., 1997; Koppenhöfer et al., 2000a). However, targeted applications restricted to the area of fruit fall beneath the canopy of mango trees may help to reduce the amount of nematodes required for the control of *A. ludens*. Direct release of larvae on treated soil (experiment 2) resulted in a high prevalence of infection (mean 74%) at the highest concentration tested (250 infective juveniles per square centimetre). In contrast, an average of 52% infection was observed at the same concentration when insects emerged from fruits (experiment 3). This was probably due to the extended period of emergence of insects from infested fruits and a greater degree of heterogeneity of developmental stages in insects from fruits. The prevalence of infection was not greatly increased by consecutive applications of infective juveniles (experiment 4). The mean percentage of infection at the highest concentration increased by approximately 10% compared to single treatments, despite twice the amount of nematodes being applied at an interval of 4 days.

Previous studies have also reported difficulties in achieving high levels of infection of fruit fly larvae, even

under seemingly optimal laboratory conditions. Beavers & Calkins (1984) observed a maximum of 79 and 87% infection of third-instar *Anastrepha suspensa* (Loew) following treatment with *Heterorhabditis heliothidis* Khan, Brook and Hirschmann and *H. bacteriophora*, respectively, in the laboratory. Similarly, *H. bacteriophora* infection of third-instar *A. ludens* did not exceed 63% in the laboratory (Lezama-Gutiérrez et al., 1996), whereas infection of *Ceratitis capitata* (Wiedemann) larvae was high (70–80%) shortly after emerging from a laboratory diet but fell off rapidly thereafter (Gazit et al., 2000). In a previous field trial performed at 10% soil moisture, application of 115 and 345 infective juveniles per square centimetre resulted in 47 and 76% infection of *A. ludens*, respectively (Toledo et al., 2005).

What limits the efficacy of *H. bacteriophora* as a biological control agent against *A. ludens*? First, the developmental stage of the insects appears to be highly influential; older larvae are less susceptible to infection and the temporal window of susceptibility is restricted as they tend to pupate quickly and are then almost completely resistant to infection (Toledo et al., 2005). Second, only a fraction of the infective juvenile population is infective at any moment in time (Bohan & Hominick, 1997), a phenomenon known as phased infectivity (Campbell et al., 1999). Infectivity is also dependent on physiological status (Greshman & Womersley, 1991; Womersley, 1993), ensheathment (Dempsey & Griffin, 2003), storage duration prior to application (Fitters & Griffin, 2004), and method of production (Ehlers, 2001). Third, the survival of *H. bacteriophora* infective stages in soil is affected by numerous biotic and abiotic factors that have been reviewed in detail elsewhere (Gaugler, 1988; Kaya, 1990; Kaya & Koppenhöfer, 1996). In general, the Heterorhabditidae do not survive as well as the Steinernematidae (Molyneux, 1985). Infective juveniles of *H. bacteriophora* remained infective for up to 18 days in laboratory soils with 15% moisture content, although the prevalence of infection observed in bioassays using *Anastrepha obliqua* (Macquart) fell significantly 4 days after the start of the experiment (Pérez, 2000). Now, if a single application resulted in recycling of the nematode and pest suppression over an extended period, the use of *H. bacteriophora* might be a viable option in integrated pest management programmes in tropical orchards. Nonetheless, the integrated use of chemical pesticides with entomopathogenic nematodes would require careful insecticide selection to avoid adverse effects on nematode survival and infectivity (Head et al., 2000; Cuthbertson et al., 2003), although synergism has also been reported (Koppenhöfer et al., 2000b).

Soil type and structure is also highly influential in nematode function (Shapiro et al., 2000). The soil used in the present study was clay loam, which appears to favour the survival and dispersal of infective juveniles (Choo & Kaya, 1991). This is due to the moisture-retaining properties of the soil and the presence of pores around sand particles that facilitate oxygenation of the soil and movement of the nematode (Wallace, 1958; Georgis & Poinar, 1983).

Temperature is an additional factor that may have affected *H. bacteriophora* performance in the present study. The optimal temperature for Heterorhabditidae is usually between 20 and 25 °C (Griffin, 1993). A laboratory study on *H. bacteriophora* infection of *A. obliqua* indicated that 24 °C was optimal for infection by this species (Pérez, 2000). Daily air temperatures fluctuated by over 15 °C (22–37 °C) during the experimental period, although the soil acted as an effective buffer with average maximum temperatures of 27–29 °C each day of the experiments. High soil temperatures are also likely to have reduced the interval between larvae entering the soil and pupation, resulting in lower infection rates.

Interestingly, nematodes were capable of infecting insects still in the fruit, albeit at a fairly low prevalence (10–15% on average). This observation underlines the superior host-seeking behaviour of *H. bacteriophora*, which includes horizontal and vertical displacement in the soil (Gaugler & Kaya, 1990; Cohen et al., 2002). The fact that the infection of insects in the fruits was not dependent on the concentration of infective juveniles applied indicates that a large proportion (85–90%) of the fruit-dwelling insects occupied a refuge from parasitism, whereas the remainder were susceptible to parasitism, possibly because of their developmental stage, the accessibility of their location within the fruit, or some aspects of their physiology or behaviour that rendered them vulnerable to attack by infective juveniles.

We conclude that, in all cases, the relationship between the density of infective juvenile *H. bacteriophora* and the percentage of infection of *A. ludens* in the soil of a tropical mango orchard was linear and positive over the range of densities tested. Very high densities of infective juveniles were required to achieve a high prevalence of infection (>70%). Such densities are unlikely to be commercially viable for the control of this pest. Fruit fly larvae appear capable of escaping infection by remaining in the fruit or by pupating rapidly once having entered the soil. The successful use of this nematode for the biological control of *A. ludens* will depend on identifying ways of overcoming or bypassing the insect's ability to avoid infection.

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