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RESEARCH ARTICLE

Biocontrol potential of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* on cucurbit fly, *Dacus ciliatus* (Diptera: Tephritidae)

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Entomopathogenic nematodes (EPNs) from the families Steinernematidae and Hererorhabditidae are considered excellent biological control agents against many insects that damage the roots of crops. In a regional survey, native EPNs were isolated, and laboratory and greenhouse experiments were conducted to determine the infectivity of EPNs against the cucurbit fly, *Dacus ciliatus* Loew (Diptera: Tephritidae). Preliminary experiments showed high virulence by a native strain of *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and a commercial strain of *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae). These two strains were employed for further analysis while another native species, *Steinernema feltiae*, was excluded due to low virulence. In laboratory experiments, larvae and adult flies were susceptible to nematode infection, but both nematode species induced low mortality on pupae. *S. carpocapsae* had a significantly lower LC_{50} value against larvae than *H. bacteriophora* in filter paper assays. Both species of EPNs were effective against adult flies but *S. carpocapsae* caused higher adult mortality. When EPN species were applied to naturally infested fruit (150 and 300 IJs/cm^2), the mortality rates of *D. ciliatus* larvae were 28% for *S. carpocapsae* and 12% for *H. bacteriophora*. Both EPN strains successfully reproduced and emerged from larvae of *D. ciliates.* In a greenhouse experiment, *H. bacteriophora* and *S. carpocapsae* had similar effects on fly larvae. Higher rates of larval mortality were observed in sandy loam and sand soils than in clay loam. The efficacy of *S. carpocapsae* and *H. bacteriophora* was higher at 25 and 30°C than at 19°C. The results indicated that *S. carpocapsae* had the best potential as a biocontrol agent of *D. ciliatus*, based on its higher virulence and better ability to locate the fly larvae within infected fruits.

Keywords: *Steinernema carpocapsae*; *Heterorhabditis bacteriophora*; cucurbit fruit fly; biocontrol; insect pathology

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1. Introduction

The cucurbit fruit fly, *Dacus ciliatus* Loew (Diptera: Tephritidae) is the most serious pest of cucumber (*Cucumis sativus*), melon (*Melon* sp.), water melon (*Citrullus lanatus*), musk melon (*Cucumis melo*) and related fruits in Iran, and is a major pest of a wide range of Cucurbitaceae in Africa, Asia and the Middle East (Arghand, 1979; EPPO/CABI, 2006). The European and Mediterranean Plant Protection Organization (EPPO) consider *D. ciliatus* to be an A1 quarantine pest, and recommend the development of novel control methods (EPPO/CABI, 2006). This fruit fly is multivoltine without any diapause in the southern part of the country, causing serious infestation of fruits from July to September (Arghand, 1979; Hancock, 1989). Adult flies lay their eggs beneath the fruit epidermis. The eggs hatch within 1–2 days and larvae feed for 5–6 days, destroying the pulp when making galleries. It has three larval instars (EPPO/CABI, 2006). The last instar larvae exit the fruit and reach the soil where they pupate and emerge as adults. Larval galleries in fruits also provide infection by other pathogens (Hancock, 1989).

Currently, the use of organophosphate insecticides is the main control method for *D. ciliatus.* Insecticides are combined with protein hydrolysate to form a bait spray (Roessler, 1989). In some extreme cases, farmers even dip infested fruits into insecticide solutions in containers. Therefore, it is critical to search for alternative control methods.

Fruit flies have numerous natural enemies from different groups of parasitoids, predators and entomopathogens. The stages of fruit flies that develop in the soil are protected from parasitoids, but are suitable for entomopathogens such as entomopathogenic nematodes (EPNs) and fungi. Different fruit fly species including *Rhagoletis indifferens* Curran, *Bacterocera oleae* Rossi, *Ceratitis rosa* Karsch, *Ceratitis capitata* Wiedemann, *Anastrepha serpentina* Wiedemann and *Anestrepha oblique* Macquart were found to be susceptible to various species/strains of both *Steinernema* and *Heterorhabditis* nematodes (Malan & Manrakhan, 2009; Sirjani, Lewis, & Kaya, 2009; Toledo et al., 2009; Yee & Lacey, 2003).

EPNs in the families Steinernematidae and Heterorhabditidae are available commercially in many parts of the world to control a number of different soil insect pests (Kaya & Gaugler, 1993, Kaya et al., 2006). They have a mutualistic relationship with bacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively (Kaya & Gaugler, 1993). The bacteria kill the host, provide nematodes nutrition and prevent secondary invaders from contaminating the host cadaver (Forst & Clarke, 2002). The infective juvenile stages (IJs) penetrate the host through spiracles, mouth or anus or directly through the cuticle (Peters & Ehlers, 1994). The nematodes release the bacteria that cause septicemia and the death of the insect. Once nutrients are consumed in the insect cadaver, nematodes develop into the IJs stage and with the bacteria stored in the oesophagus, exit the cadaver into the soil to search for a new host (Griffin, Boemare, & Lewis, 2005). The biology of EPNs, their safety to use and the ease with which they can be mass-produced and applied using standard spray equipment, makes them excellent candidates for controlling soil insect pests that spend part of their life cycle in the soil (Gazit, Rossler, & Glazer, 2000; Lindegren, Wong, & Mclnnis, 1990; Shapiro-Ilan, Fuxa, Lacey, Onstad, & Kaya, 2005; Wright, 1992).

The use of EPNs against fruit flies is a promising alternative to chemical products, especially for growers using organic production methods. Therefore, the aim of this study was to evaluate the susceptibility of several stages of the fruit fly *D. ciliatus* to strains of native and commercial EPNs under laboratory and greenhouse conditions. We also evaluated the effects of soil texture and temperature on their pathogenicity, reproduction rate in fruit fly larvae and adults, and their ability to penetrate fruit.

2. Materials and methods

2.1. Soil sampling, isolation and characterisation of EPNs

Fifty soil samples were collected from beneath trees in forests and orchards near the city of Bojnourd (North Khorasan Province, Iran, 27°32′N 57°12′E) during April–November 2011–2012. The composite samples were taken to a depth of 20 cm from beneath the tree canopies. EPN isolates were recovered from soil samples using the insect bait method of Bedding and Akhurst (1975). Subsamples from each sample were baited with seven *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae in 200 mL plastic containers. Larval mortality was checked daily and infected cadavers were rinsed in distilled water, transferred into White traps and incubated at 24°C (Kaya & Stock, 1997). After IJ emergence, nematodes were collected and used to confirm Koch's postulates for pathogenicity. The emerging IJs were harvested and kept for future study. All EPN strains were cultured in parallel on last instar wax moth, *G. mellonella*. The emerging, IJs were harvested from White traps and stored in tap water at 12°C (Kaya & Stock, 1997) for less than two weeks before using for experimental procedures.

The EPN strains were initially sorted as *Steinernema* spp. Travassos or *Heterorhabditis* spp. Poinar, based on the signs and symptoms (Kaya & Stock, 1997). Identifications of EPN strains were based on morphology and morphometrics (Stock & Hunt, 2005), cross-breeding trials (for *Steinernema* isolates) and finally, ITS sequences. For molecular characterisation, genomic DNA was extracted from an adult female using the Bioneer Genomic Extraction Kit (http://www.bioneer.com). The ITS region was amplified by Polymerase chain reaction (PCR) using the primer set of Vrain, Wakarchuk, Levesque, and Hamilton (1992) and conditions described by Nguyen (2007). The ITS sequences were edited manually using BioEdit software 7.0.5.3 (Hall, 1999), and compared with the DNA sequences in the GenBank database using Nblast (http://blast.ncbi.nlm.nih.gov). Valid and verified sequences were retrieved from GenBank and multiple alignments with other ITS rDNA sequences were obtained using CLUSTALW. The phylogenetic relationships of these sequences were conducted based on maximum parsimony (Thompson, Higgins, & Gibson, 1994) and neighbour-joining (NJ) methods of the ITS region using PAUP ver. 4.08b and MEGA 5 software (Saitou & Nei, 1987; Swofford, 2002; Tamura et al., 2011). Bootstrap analysis was carried out with 1000 datasets. Three different outgroups, *Pellioditis typica* Stefanski (AF036946), *Steinernema intermedium* Poinar (AY230172) and *Steinernema neocurtillae* Nguyen & Smart (AF122018) were used to produce an ITS-based phylogeny of Heterorhabditis and Steinernema species, respectively. Finally, we used a cross-breeding test to evaluate possible species (Nguyen, Malan, Antoinette, & Gozel, 2006). In this case, a strain of *Steinernema*

feltiae Filipjeve was used as a reference strain to check the cross and fertility results of the *Steinernema* isolates.

2.2. Insect rearing

The insect used for maintaining nematode isolates, *G. mellonella*, was reared in 1500 mL glass containers on an artificial medium. The fruit fly colony was started with naturally infested fruit collected through June–October 2010–2011 from cucumber fields that received no pesticide from North Khorasan Province, Iran. The fruit flies were reared under greenhouse conditions on cucumber plants (*Cucumis sativus* L.) to obtain colonies for assays. The rearing process was started with 50 adults (1:1, male: female). The colonies were kept in containers (25 cm $\times$15 cm $\times$15 cm) filled with pure sand under controlled conditions (25$\pm$2°C, 70$\pm$10% RH, and L12:D12 hours photoperiod). The sand substrate was passed through a 1-mm sieve, washed with deionised water and sterilised by autoclaving. Fly diet was prepared with a mixture of three parts sugar and one part enzymatic yeast hydrolysate.

For larval assay, the fruits were placed on wire screens at 15°C for 24 hours and third instar larvae were recovered while emerging. Pupae were recovered after the larvae dropped onto the soil by sieving the substrate sand. Pupae were used immediately. Adult flies were collected from pupae that were kept at 25°C for 8–10 days.

2.3. Laboratory bioassay

All native EPN strains were subjected to a preliminary assay on *D. ciliatus* to determine the larval and pupal lethal concentrations (LC_{50} and LC_{90}). The preliminary doses were selected based on previous works on related fruit flies species (Malan & Manrakhan, 2009; Sirjani et al., 2009; Toledo et al., 2009; Yee & Lacey, 2003). The experiment was repeated twice. The results of this initial assay showed low virulence of *S. feltiae* isolates (data not shown) and therefore, these isolates were excluded and HBoj strain of *Heterorhabditis bacteriophora* together with a commercial product of the Koppert Co. (Capsanem®) formulated with *Steinernema carpocapsae* were used in future experiments. Nematodes used in all the experiments were reared in the laboratory following the methods described above. Before each experiment, the IJs (less than two weeks old) were acclimatised at room temperature for one hour. The viability of EPNs was checked by confirming their motility under a stereo microscope.

2.3.1. Filter paper assay

The experiment was carried out in Petri dishes (5.5 cm diameter) containing a double layer of filter paper. The EPN concentrations (25, 50, 100, 200 and 400 IJs/cm^2) were prepared in a final volume of 0.5 mL and added to the filter paper surface. Unaugmented control treatments received only distilled water. Before the addition of the insects, the treated dishes were maintained at room condition (23–24°C) for one hour. Thereafter, ten third instar cucurbit fly larvae were placed in each Petri dish. A total of five replicates were performed per EPN species and concentration. The experiment was conducted twice. Experimental units were incubated under

controlled conditions (25 ± 1°C, RH 70 ± 10%). After three days, the numbers of dead and living insects were recorded according to body colour change of the cadaver. Dead insects were dissected under a stereo microscope to confirm nematode infection.

2.3.2. Soil assay

An assay with soil was performed in clear plastic containers (4 cm diameter, 4 cm high). Each unit was filled with 20 g of sieved potting soil (50.96% sand, 14.32% clay and 34.72% silt, pH 6.6), oven dried and then the moisture level was adjusted to 7% (w/w) by adding distilled water. EPNs were applied on the soil surface at a concentration of 0, 10, 25, 50, 100, 150 and 200 IJs/cm^2. Final soil moisture was 10% after the nematodes were added in a water suspension. The control treatment received only 2.5 mL distilled water. The containers were kept at room temperature (23–24°C) for one hour, then 10 last instar larvae per container were placed on the soil surface and immediately moved into the soil. The plates were covered with a lid and placed in a closed plastic container. Each treatment had 5 replicates that were maintained for 72 hours at 25°C in a growth chamber. The experiment was conducted twice. Three days after treatment, soil was sieved and dead insects were counted. Cadavers were dissected under a stereo microscope to confirm the presence of EPNs inside.

2.3.3. EPN reproductive potential in D. ciliatus

EPN reproduction potential in the fruit fly larvae was estimated at the concentration of 50 IJs/cm^2. Ten last instar larvae were placed in separate plastic containers (4 cm diameter, 4 cm high) filled with potting soil (10% moisture w/w). After application of the EPNs, the containers were kept for 72 hours at 25°C; afterwards, the cadavers were collected. Un-augmented control treatment received only distilled water. Cadavers were rinsed in distilled water to remove surface nematodes and placed individually on White traps at room temperature. There were five replicates for each strain with two repetitions of the whole experiment. The White traps were maintained for three weeks to provide enough time for EPN reproduction. Total number of emerged IJs were counted for each strain. The larvae from which no nematodes emerged were dissected to determine if EPNs were present inside cadavers.

2.3.4. EPN efficacy against D. ciliatus *pupae*

EPN efficacy against *D. ciliatus* pupae was measured in soil experiments (4 cm diameter, 4 cm high). The containers were filled with 20 g sterilised potting soil and moisture was adjusted to 10% (v/w). Preliminary tests showed that *D. ciliatus* pupae had less susceptibility to EPNs than third instar larvae (data not shown), so a higher IJ concentration was selected. Ten pupae (one day old) were placed below the soil in each container. The EPN suspensions were adjusted to the LC_{90} calculated previously for larvae. Each treatment had five replicates. Un-augmented control treatments were exposed to distilled water. The containers were closed, placed into plastic bags and kept at 25–27°C and photoperiod L16:D8. Pupae were dissected and mortality was determined 14 days after treatment. The assay was conducted twice.

2.3.5. Efficacy of EPNs against adult D. ciliatus

The experiment was conducted in small plates (6 cm diameter, 4 cm height) filled with 35 g sterilised potting soil (10% w/w). The EPN concentrations were 0, 10, 50 and 150 IJs/cm^2. Before each test, pupae were maintained at 26 ± 1°C and 80% R.H. In each experimental unit, ten 9-day old pupae were buried 1–2 cm beneath the soil surface. There were five replicates for each strain. The experiment was conducted twice. An un-augmented control treatment received distilled water. After treatment, the plates were sealed with parafilm and kept in a controlled chamber at 25 ± 2°C, RH 70 ± 10% and 16 hours photoperiod. Four days after treatment, dead flies were removed from the plates and transferred onto White traps to verify IJ emergence.

2.3.6. Efficacy of EPNs against D. ciliatus *larvae within fruit*

Infectivity of EPN strains was evaluated against fruit fly larvae within infested fruits. Applied concentrations were 0, 150 and 300 IJs/cm^2. Experimental units were fruits obtained from the fruit fly colony. Plastic containers (12 cm × 6 cm × 6 cm height, 72 cm^2 soil surface) were partly filled with moist potting soil and four larvae-infested cucumbers were placed in each container. EPN suspensions prepared in 10 mL of distilled water were applied onto the surface of fruits using a manual sprayer. Control treatments were sprayed only with 10 mL water. Each treatment had seven replicates and the test was conducted two times. The containers were covered with clear plastic and kept in a temperature-controlled growth chamber. After four days, mortality was recorded by dissecting the fruit, sieving the soil and dissecting the cadavers.

In a separate experiment using the same procedures, except that plastic pots (15 cm diameter) were used as containers and EPNs were applied on the soil surface, the pots were kept at the greenhouse for an hour, and then three or four infested fruits were placed on the soil of each pot. The pots were covered and maintained in the controlled greenhouse at 25 ± 2°C, RH 70 ± 10% and 16 hours photoperiod. There were six replicates for each treatment combination. The experiment was conducted two times. Four days after EPN application, the infected fruits were dissected, soil was sieved and mortality rate was recorded after dissecting the cadavers.

2.4. Effect of soil type on pathogenicity of EPNs against D. ciliatus *larvae*

EPN efficacy against *D. ciliatus* larvae was assessed in three types of soil (Table 1). The IJs were adjusted to 40, 100 and 150 IJs/cm^2. An un-augmented control treatment received the same volume of deionised water, and no nematode suspension. The soils were sieved, sterilised and dried in an oven. Plates (4 cm diameter, 4 cm height) were filled with 20 g from the three types of soil texture and humidity was

Table 1. General characteristics of the soil used in texture assay.

Soil type	Locality	Sand %	Silt %	Clay %
Sand	Ferdowsi University paradise	96.2	0.7	3.1
Sandy loam	FUM greenhouse	62	34.72	14.32
Clay loam	FUM research farm	20.96	48.72	30.32

Note: FUM, Ferdowsi University of Mashhad.

adjusted to 10% (w/w) capacity. One hour after inoculation of IJs 10 third instar larvae of *D. ciliatus* were placed on each plate. The containers were closed with lids and kept at 25°C and 70% R.H. Three days after inoculation, EPN infectivity was determined by sieving the soil. Cadavers were dissected under a stereo microscope to confirm EPN infection. There were five replications per treatment. The experiment was conducted two times.

2.5. *Effect of temperature on EPNs efficacy*

The effect of EPNs against *D. ciliatus* larvae was evaluated at three different temperatures: 19, 25 and 30°C. Plastic containers (4 cm diameter, 4 cm high) were filled with sandy loam soil, and nematode suspensions at concentrations of 50 and 100 IJs/cm^2 were uniformly distributed on the soil surface. Un-augmented controls were prepared with 2.5 mL distilled water. Ten third-instar larvae were applied per container. The experimental units were examined after three days and dead insects counted. Fruit fly infection by nematodes was confirmed by dissection of cadavers. Each treatment was tested with five replicates. The experiment was conducted two times.

2.6. *Statistical analysis*

Insect mortality was corrected according to the control treatment values using Abbott's formula (Abbott, 1925), and the data were square-root transformed when necessary to meet assumptions of normality and homogeneity of variances. In the laboratory bioassays (filter paper and soil trials), Probit analysis on the larval mortality data was carried out to assess the LC_{50} and LC_{90} for each nematode strain.

Student's t-test was employed to detect significant differences between EPN efficacy against pupae ($P = 0.05$). To assess the effects of soil type on the pathogenicity of nematode strains, the data of larval mortality was subjected to three-way analysis of variance (ANOVA) (nematode strain × nematode concentration × soil type) and were followed by LSD test to compare larva mortality caused by interactive effects. Likewise, to study the effects of temperature on EPN efficacy, the data for larval mortality was analysed by three-way ANOVA (temperature × nematode strain × nematode concentration). The mean (± SEM) number of penetrating nematodes from the two nematode species and their reproduction potential were compared by *t* test ($P < 0.05$).

The mortality data for EPNs against fly larvae both within fruit and greenhouse assays were analysed by two-way analyses of covariance (ANCOVA) in which EPN species and concentrations were considered main factors and total number of fly larva was entered as a covariate. Where covariate was significant ($P < 0.05$), differences among means were determined by comparisons of LSD test. SAS software, version 9.1 was used for all statistical analyses (SAS Institute, 2002–2003).

3. Results

3.1. *Isolation and identification of EPNs*

Five of the 50 soil samples (10%) were positive for occurrence of EPNs. Analysis of the ITS rDNA region, based on NJ method, placed the isolates Boj1, Boj7, Boj8 and

Boj9 near other isolates of *S. feltiae*, with 99–100% similarities with isolates of *S. feltiae* from Iran (FUM1: JF920964; Arak7: JF920962) or Armenia (AY171256) and 96–100% query coverage. The ITS sequence of the HBoj isolate was 99% similar to BGKB-1/2007 (JX993984), Giza (JQ178380) and N-Arq (HQ225906) isolates of *H. bacteriophora*.

3.2. Laboratory bioassay

3.2.1. Filter paper and soil experiments: lethal concentration establishment and reproduction

The two substrates (filter paper and potting soil) in these trials influenced the virulence of the nematode against third instar larvae (Table 2). In the filter paper assay, the LC_{50} was 28 and 326 IJs/cm^2 for *S. carpocapsae* and *H. bacteriophora,* respectively. Both species exhibited good and similar activity against third instar larvae in potting soil. However, while the LC_{50} for *S. carpocapsae* in soil was equal to that on filter paper, *H. bacteriophora* performed much less effectively on paper than in soil (Table 2). For both nematode species, the mortality was always associated with nematodes in the dissected cadavers.

Both EPN strains successfully reproduced and emerged from larvae of *D. ciliatus* within 19–21 days. All nematode stages (female, male and IJs) were observed within host cadavers. But emergence rate of IJs from cadavers was very low. There was no significant difference between the two EPN species ($F_{1,\ 8}=1$, $P>0.05$). The larvae used in the experiments were near to pupation as *D. ciliatus* larvae pupate within a few hours of leaving the cucumbers.

3.2.2. EPN efficacy on pupae

There were no significant differences between efficacy of EPN species for control of pupae ($t_{1,\ 46}=1.23$, $P=0.22$). Both species killed few pupae with mortality percentages ranging from $8.9\pm1.8\%$ (*H. bacteriophora*) to $12.5\pm2.31\%$ (*S. carpocapsae*).

3.2.3. Efficacy of EPNs against adult flies

Both species of EPN were effective against adult flies and their effectiveness significantly differed ($F_{1,\ 54}=8.98$, $P=0.01$). *S. carpocapsae* caused higher adult mortality ($55.6\pm5.8\%$) than did *H. bacteriophora* ($44.6\pm4.5\%$). The mortality of

Table 2. LC_{50} values (IJs/cm^2) estimated for *S. carpocapsae* and *H. bacteriophora* on *D. ciliatus* on filter paper and in soil experiments.

P value[c]	χ^{2}[b]	Slope $\pm$ SE[a]	LC_{50} (95% fiducial limits)	Species	Experiment unit
0.03	8.354	0.947 ± 0.34	27.77 (9.39–55.18)	*S. carpocapsae*	Filter paper
0.45	2.63	1.058 ± 0.21	325.68 (211.83–720.95)	*H. bacteriophora*	
0.68	2.27	1.215 ± 0.16	27.76 (14.55–48.77)	*S. carpocapsae*	Soil
0.47	3.53	1.475 ± 0.167	45.89 (17.46–72.64)	*H. bacteriophora*	

[a]SE, standard error.
[b]Pearson χ^2 of the slope.
[c]*P*-values represent the probability of the slope.

adults increased with higher concentrations (10, 50 and 150 IJs/cm^2) of both nematodes ($F_{2, 54} = 112.7$, $P < 0.01$), ranging from $12 \pm 1.9\%$ to $75 \pm 3.7\%$, for the treatments with 10 and 150 IJs/cm^2, respectively. There were no significant interactions between nematode species and concentrations ($F_{1, 54} = 2.85$, $P = 0.09$).

3.2.4. Efficacy of EPNs against D. ciliatus *larvae within fruit*

In the first experiment, where EPNs were applied directly to fruits, larval mortality was significantly influenced by the total numbers of fly larvae which were analysed as covariates ($F_{1, 17} = 33.4$; $P < 0.01$). There was also a significant effect of the EPN species on larval mortality (Table 3). The percentage of larval mortality caused with *S. carpocapsae* (27.7 ± 3.1) was significantly greater than that produced with *H. bacteriophora* (12.8 ± 1.8). However, the percentage of larval mortality at a concentration of 150 IJs/cm^2 (20.3 ± 3.6) did not differ from that of 300 IJs/cm^2 (23.4 ± 3.1). There was no significant interaction of the two independent variables (Table 3). In the second experiment, where EPNs were applied to the soil surface, total larval numbers affected larval mortality in soil trials ($F_{1, 17} = 5.45$; $P < 0.05$). Therefore, after adjusting for differences in total larval number, the percentage of larval mortality induced by *S. carpocapsae* ($40.7 \pm 4.5\%$) was the same as that produced by *H. bacteriophora* ($39.9 \pm 3.3\%$; Table 3). Also, the percentage of larval mortality from 150 IJs/cm^2 ($38 \pm 4.1\%$) did not differ from that of 300 IJs/cm^2 ($42.1 \pm 4.5\%$). The interactive effect of EPN strain and concentration was not significant.

3.3. Effect of soil type on pathogenicity of EPNs against D. ciliatus *larvae*

The soil type ($F_{2, 162} = 35.6$, $P < 0.01$) and IJ concentration ($F_{2, 162} = 45.4$, $P < 0.01$) significantly affected larval mortality, and the two factors interacted ($F_{4, 162} = 2.45$, $P < 0.05$). The higher concentrations of EPNs (150 and 100 IJs) induced greater larval mortality of *D. ciliatus* both in sandy loam and sand soil types, whereas all concentrations of EPN species had lower virulence in clay loam (Figure 1). The differences between EPN species efficacy were not significant ($F_{1, 162} = 0.44$, $P = 0.53$). The interactive effects of nematode species * soil type ($F_{2, 162} = 1.64$, $P = 0.19$), nematode species * IJ concentration ($F_{2, 162} = 0.33$, $P = 0.71$) and nematode species * IJ concentration * soil type ($F_{4, 162} = 1.85$, $P = 0.11$) were not significant.

3.4. Effect of temperature on EPN efficacy

The temperature ($F_{2, 72} = 63.73$, $P < 0.01$) and IJ concentrations ($F_{1, -72} = 71.46$, $P < 0.01$) had a strong influence on infection of *D. ciliatus* larvae. However, there was no significant difference between EPN species on larval infection ($F_{1, 72} = 0.37$, $P = 0.55$). The interactive effect of EPN species and temperature on larval infection was significant ($F_{2, 72} = 5.98$, $P < 0.01$). The larval mortality caused by both nematodes at 25 and 30°C was higher than that at 19°C (Figure 2A). Moreover, larval mortality was affected by the interaction of IJ concentration * temperature ($F_{2, 72} = 12.37$, $P < 0.01$). The concentration of 100 IJs caused significantly higher larval mortality than did 40 IJs at 25 and 30°C, whereas the effects of the two IJ concentrations did not differ at 19°C (Figure 2B). The interaction between EPN

Table 3. Two-way analysis of covariance (ANCOVA) of the effects of two EPN species (*S. carpocapsae* and *H. bacteriophora*) and concentrations (150 and 300 IJs/ cm^2) on larval mortality of *D. ciliatus* within fruit and soil in greenhouse experiments.

	EPN		Concentration		Total number of fly larva covariate		EPN × concentration	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Mortality by EPN applied on fruits	13.77	0.01	0.10	0.90	33.43	0.01	1.15	0.30
Mortality by EPN applied on soil	1.16	0.30	1.06	0.32	5.42	0.04	0.87	0.37

Note: Total number of fly larva was a covariate in the pathogenicity analysis.

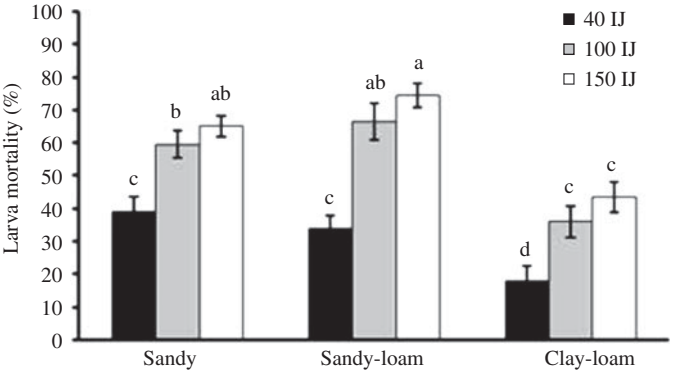

Figure 1. Mortality (%) of third instar *D. ciliatus* larvae in different soil types: sandy, sandy loam and clay loam. Two entomopathogenic nematodes species, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, were applied at three concentrations (40, 100 and 150 IJs/cm^2). Different letters indicate significant differences between interactive treatments (two-ways ANOVA and Fisher's protected LSD, $P < 0.05$). Data are presented as mean ($\pm$ SEM).

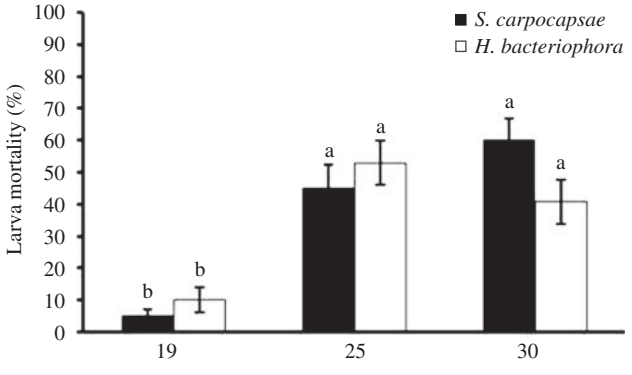

Figure 2. Mortality (%) of third instar *D. ciliatus* larvae. (a) Effect of EPN species, *S. carpocapsae* and *H. bacteriophora*, and temperatures, 19, 25 and 30°C. (b) Effect of concentration, 40 and 100 IJs of EPNs, and the same temperatures. Different letters indicate significant differences between interactive treatments (two-ways ANOVA and Fisher's protected LSD, $P < 0.05$). Data are presented as mean ($\pm$ SEM).

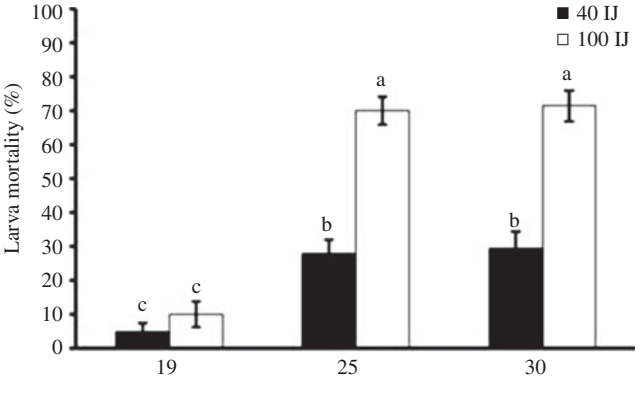

Figure 2. *(Continued)*

species and IJ concentration ($F_{1,\ 72} = 0.11$, $P = 0.94$) or EPN species * temperature * IJ concentration ($F_{2,\ 72} = 0.13$, $P = 0.91$) on larval mortality was not significant.

4. Discussion

Our results indicated that EPNs may be suitable for application against *D. ciliatus*. Toledo et al. (2005) reported that the lethal dose of *H. bacteriophora* to third instar larvae of *Anastrepha obliqua* was 49 IJs/cm^2, close to our LC_{50} estimation. In another report, the lethal concentration of *H. bacteriophora* against *A. serpentine* was determined to be 36 IJs/cm^2 (Toledo, Rojas, & Ibarra, 2006), a lower level compared to that on *D. ciliatus* larvae. This may be due to differences in methodology (Barbosa-Negrisoli et al., 2009). In contrast to our results, while *H. bacteriophora* was ineffective against the fruit fly, *Anastrepha fraterculus* (LC50 = 229 IJs/cm^2), it performed better than *Steinernema riobrave* Cabanillas (LC50 = 347 IJs/cm^2; Barbosa-Negrisoli et al., 2009).

The strong potential of *S. carpocapsae* against fruit fly larvae agree with Yee and Lacey (2003) who showed that *R. indifferens* larvae were highly susceptible to infection by *S. carpocapsae* and *S. feltiae*. Our results also agree with other reports of EPN efficacy against fruit flies such as *Anastrepha ludens* (Lezama-Gutierrez et al., 2006), *R. indifferens* (Yee & Lacey, 2003) and *C. capitata* (Karagoz, Gulcu, Hazir, Kaya, & Hazir, 2009). By contrast, Sirjani et al. (2009) showed higher susceptibility of the olive fruit fly larvae, *B. oleae* to *S. feltiae* than to *S. carpocapsae, S. riobrave*, *Steinernema glaseri* Steiner, *H. bacteriophora* and *Heterorhabditis marelatus* Liu & Berry. Differences in the efficacy of EPN species could be related to the foraging strategy of IJs and the behaviour of third instar larvae of the fruit fly. *S. carpocapsae* is an ambusher species, reported to be more efficient at finding mobile insects compared to cruiser species like *H. bacteriophora* (Campbell & Gaugler, 1993). Jumping rate varies considerably among species in *Steinernema*, and jumping can be an important mechanism in facilitating contact with mobile insects (Campbell & Kaya, 1999a, 1999b, 2000). Indeed, the inconsistency of efficacy by *H. bacteriophora* in soil and on filter paper in the present study may have resulted from behavioural differences of the insects in the two media. The fly larvae exhibited a jumping behaviour in the unnatural filter paper medium. This behaviour could reduce the chance of *H. bacteriophora* IJs penetrating host larvae because the natural behaviour of this species is to actively search for and infect sessile arthropods. By contrast, the similar LC50 values of *S. carpocapsae* in both media may be because IJs of this species are adept at jumping to attach themselves to motile prey.

All *D. ciliatus* larvae pupate in a short time after leaving the cucumbers. A short interval between leaving the fruit and pupation is also reported for other tephritids like *B. oleae* (Sirjani et al., 2009). Our observation that death of infected larvae occurred as pupae, agrees with other reports that most EPN-infected larvae of *C. capitata* and other tephritids died after forming puparia (Lindegren & Vail, 1986; Sirjani et al., 2009; Karagoz et al., 2009).

The high susceptibility to infection of larvae compared to pupae and adults might be related to several factors including their development duration, activity in soil, output of cues related to host finding by EPNs and larger natural openings. Both EPN species were found adhering to the pupae from natural openings, but we did not find any evidence of EPN entry via these openings. This is similar to the

results of Yee and Lacey (2003) with *R. indifferens* Beavers and Calkins (1984), *A. suspensa* Loew, Lindegren and Vail (1986) and Campos-Herrera and Gutiérrez (2009) with *C. capitata* and also in other dipterans like pupae of *Liriomyza trifolii* Burgess (LeBeck, Gaugler, Kaya, Hara, & Johnson, 1993). The main reason for this phenomenon might be the hard integument of the last instar larva which prevents EPN penetration through the puparium. Nevertheless, the pupal stage of *Anastrepha fraterculus* was susceptible to some EPN species, with highest susceptibility to *H. bacteriophora* and *S. riobrave* (Barbosa-Negrisoli et al., 2009). Also, Hernández (2003) observed 30% and 21% mortality of *Anastrepha ludens* pupae caused by *H. bacteriophora* and *S. riobrave*, respectively. Patterson Stark and Lacey (1999) found even higher mortality rates with *H. bacteriophora* (62.5%) and *S. riobrave* (40%) on *R. indifferens* pupae perhaps because EPNs entered through the inter-segmental membranes before final sclerotisation of the integument.

D. ciliatus adults were relatively susceptible to nematodes, particularly *S. carpocapsae*. Most IJs of *S. carpocapsae* probably attacked adult flies as they migrated up through the soil. Other studies showed that *S. carpocapsae* can infect adult fruit flies (Beavers & Calkins, 1984; Lindegren & Vail, 1986; Ghally, 1988) although in the case of *C. capitata*, application of *S. carpocapsae* to adult flies produced only 0.2% infection (Lindegren & Vail, 1986). When adult flies of *C. capitata* were exposed to *S. carpocapsae* in Petri dishes for longer periods, the EPNs caused high mortality (45–100%; Ghally, 1988). This rate was 70–92%, after exposure of 2 to 5 days old adult of *Anastrepha suspense* to *S. carpocapsae* (Beavers & Calkins, 1984).

Although not as effective as in soil, EPNs provided some control of *D. ciliatus* in fruit. Infection of *B. oleae* by EPNs in fruits, leaves or stems is also reported by Sirjani et al. (2009). LeBeck et al. (1993) and Hassani-Kakhki, Karimi, and Hosseini (2013) also found that EPN species entered the feeding tunnels of *L. trifolii* inside leaves and potato tuber moth mines inside tubers.

The greenhouse experiments provided the closest approximation to field conditions and thus, provide the best estimation of the potential of these EPNs for managing *D. ciliatus*. Here, both EPNs species induced moderate mortality at 150 and 300 IJs/cm^2. Lindegren et al. (1990) showed that *S. carpocapsae* at a concentration of 5000 IJs/cm^2 had higher infectivity compared to 150–500 IJs/cm^2 against last instar larvae of *C. capitata* in the field. While *S. carpocapsae* and *S. feltiae* at a concentration of 50–100 IJs /cm^2 were more effective than *S. intermedium* on third instar larvae of *R. indifferens* under field conditions, their efficacy was lower than that in laboratory trials (Yee & Lacey, 2003).

The effects of soil texture on virulence of the EPNs here were similar to reports by Toledo et al. (2009), Lezama-Gutierrez et al. (2006), Campos-Herrera and Gutiérrez (2009), Georgis and Poinar (1983), Gaugler (1988) and Choo and Kaya (1991). Clay content had a negative effect on infection rate of EPNs. Decreased virulence of EPNs in fine textured soil could be related to reduction of pore space or increased water content between soil particles (Barbercheck & Kaya, 1991; Barbercheck, 1992). Soils can also differ in their suitability for the survival of IJs and insects, thereby affecting EPN abundance and diversity (Kaya & Gaugler, 1993).

Temperature plays a critical role in the rate at which EPNs infect their hosts (Hazir, Stock, Kaya, Koppenhofer, & Keskin, 2001). It is advantageous that *S. carpocapsae* killed last instar larvae of *D. ciliatus* more effectively at 25 and 30°C

than at 19°C, because this approximates the average temperature in the fruit fly's habitat (North Eastern and Southern Iran as well as Africa, Asia and the Middle East) Similarly, *S. feltiae* caused the highest pathogenicity on larvae of *R. indifferens* at 25–27°C and higher temperature had a positive effect on mortality of *A. obliqua* larvae (Toledo et al., 2009). Nevertheless, excessive temperatures are deleterious to infection, reproduction and nematodes survival (Gray & Johnson, 1983).

All third instar larvae that were exposed to IJs, died as pre-pupae and pupae. *S. carpocapsae* reproduced in *D. ciliatus* pre-pupae and IJs emerged after three weeks. In addition, both species reproduced in *D. ciliatus* adults within a week. Similarly, Malan and Manrakhan (2009) demonstrated reproduction of *H. bacteriophora* and *Heterorhabditis zealandica* Poinar in larvae of *C. rosa* within 19–21 days. However, Sirjani et al. (2009) reported that last instar *B. oleae* larvae infected by six EPN species, subsequently pupated without IJ emergence after three weeks.

This study demonstrated the feasibility of using EPNs in *D. ciliatus* integrated pest management (IPM) programmes. Future work should emphasise collecting and screening more species/strains of EPNs to select the most virulent strains for field trials. Additionally, post-application biology of virulent EPNs should be characterised as well as their performance in combination with other biological control agents and agrochemicals.

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References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*, 265–276. doi:10.3109/13880209.2012.674950

Arghand, B. (1979). Introduction on Dacus sp and preliminary examination in the hormozgan province. *Applied Entomology and Phytopathology*, *51*, 3–9.

Barbosa-Negrisoli, C. R. C., Garcia, M. S., Dolinski, C., Negrisoli, Jr, A. S., Bernardi, D., & Nava D. E. (2009). Efficacy of indigenous entomopathogenic nematodes (Rhabditidae: Heterorhabditidae, Steinernematidae), from Rio Grande do Sul Brazil, against *Anastrepha fraterculus* Wied (Diptera: Tephritidae) in peach orchards. *Journal of Invertebrate Pathology*, *102*, 6–13. doi:10.1016/j.jip.2009.05.005

Barbercheck, M. E. (1992). Effect of soil physical factors on biological control agents of soil insect pests. *Florida Entomologist*, *75*, 539–548. doi:10.2307/3496136

Barbercheck, M. E., & Kaya, H. K. (1991). Effect of host condition and soil texture on host finding by the Entomopathogenic nematodes *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) and *Steinernema carpocapsae* (Rhabditida: Steinernematidae). *Environmental Entomology*, *20*, 582–589.

Beavers, J., & Calkins, C. O. (1984). Susceptibility of *Anastrepha suspensa* (Diptera: Tephritidae) to Steinernematid and Heterorhabditid nematodes in laboratory studies. *Environmental Entomology*, *13*, 137–139.

Bedding, R. A., & Akhurst, R. J. (1975). A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica*, *21*, 109–110. doi:10.1163/187529275X00419

Campbell, J. F., & Gaugler, R. (1993). Nictation behavior and its implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour*, *126*, 155–169. doi:10.1163/156853993X00092

Campbell, J. F., & Kaya, H. K. (1999a). How and why a parasitic nematode jumps. *Nature*, *397*, 485–486. doi:10.1038/17254

Campbell, J. F., & Kaya, H. K. (1999b). Mechanism, kinematic performance, and fitness consequences of entomopathogenic nematode (*Steinernema* sp) jumping behavior. *Canadian Journal of Zoology*, *77*, 1947–1955. doi:doi/abs/10.1139/z99-178

Campbell, J. F., & Kaya, H. K. (2000). Influence of insect-associated cues on the jumping behavior of entomopathogenic nematodes (Steinernema spp). *Behaviour*, *137*, 591–609. doi:10.1163/156853900502231

Campos-Herrera, R., & Gutiérrez, C. (2009). Screening Spanish isolates of steinernematid nematodes for use as biological control agents through laboratory and greenhouse microcosm studies. *Journal of Invertebrate Pathology*, *100*, 100–105. doi:10.1016/j.jip.2008.11.009

Choo, H. Y., & Kaya, H. K. (1991). Influence of soil texture and presence of roots on host finding by *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology*, *58*, 279–280. doi:10.1016/0022-2011(91)90074-Z

EPPO/CABI. (2006). *Dacus* species. In I. M. Smith, D. G. McNamara, P. R. Scott, & M. Holderness (Eds.), *Quarantine Pests for Europe* (2nd ed., pp. 1–4). Wallingford: CAB International.

Forst, S., & Clarke, D. (2002). Bacteria-nematode symbiosis. In R. Gaugler (Ed.), *Entomopathogenic Nematology* (pp. 54–77). Wallingford: CAB International.

Gaugler, R. (1988). Ecological considerations in the biological control of soil-inhabiting insects with entomopathogenic nematodes. *Agriculture, Ecosystems and Environment*, *24*, 351–360. doi:10.1016/0167-8809(88)90078-3

Gazit, Y., Rossler, Y., & Glazer, I. (2000). Evaluation of entomopathogenic nematodes for the control of Mediterranean fruit fly (Diptera: Tephritidae). *Biocontrol Science and Technology*, *10*, 157–164. doi:10.1080/09583150029297

Georgis, R., & Poinar, G. O. (1983). Effect of soil texture on the distribution and infectivity of *Neoaplectana glaseri* (Nematoda: Steinernematidae). *Journal of Nematology*, *15*, 308–311.

Ghally, S. E. (1988). Pathogenicity of the nematode *Steinernema feltiae* Filipjev in relation to different insect hosts. *Journal of the Egyptian Society of Parasitology*, *18*, 297–304.

Gray, P. A., & Johnson, D. T. (1983). Survival of the nematode *Steinernema feltiae* in relation to soil temperature, moisture and time. *Journal of the Georgia Entomological Society*, *18*, 454–460.

Griffin, C. T., Boemare, N. E., & Lewis, E. E. (2005). Biology and behavior. In P. S. Grewal, R. U. Ehler, & D. I. Shapiro-Ilan (Eds.), *Nematodes as Biocontrol Agents* (pp. 47–64). Wallingford: CAB International.

Hall, T. A. (1999). Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hancock, D. L. (1989). Pest status, southern Africa. In A. S. Robinson & G. Hooper (Eds.), *World crop pests. Fruit flies; their biology, natural enemies and control* (pp. 51–58). Amsterdam: Elsevier.

Hassani-Kakhki, M., Karimi, J., & Hosseini, M. (2013). Efficacy of entomopathogenic nematodes against potato tuber moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae) under laboratory conditions. *Biocontrol Science and Technology*, *23*, 146–159. doi:10.1080/09583157.2012.745481

Hazir, S., Stock, S. P., Kaya, H. K., Koppenhöfer, A. M., & Keskin, N. (2001). Developmental temperature effects on five geographic isolates of the entomopathogenic nematode

Steinernema feltiae (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology*, *77*, 243–250. doi:10.1006/jipa.2001.5029

Hernández, M. A. R. (2003). *Patogenicidad de nematodos entomopatogenos (Nemata: Steinernematidae, Heterorhabditidae) em larvas y pupae de mosca de la fruta Anastrepha ludens Loew (Diptera: Tephritidae)* [Pathogenecity of entomopathogenic nematodes (Nematoda : Steinernematidae, Heterorhabditidae) against larvae and pupae of fruit fly Anasterpha luden Loew (Diptera : Tephritidae) M.Sc thesis, University of Colima]. Dissertacao (Mestrado em Ciencias/Biotecnologia), Universidada de Colima, Mexico.

Karagoz, M., Gulcu, B., Hazir, C., Kaya, H. K., & Hazir, S. (2009). Biological control potential of Turkish entomopathogenic nematodes against the Mediterranean fruit fly *Ceratitis capitata*. *Phytoparasitica*, *37*, 153–159. doi:10.1007/s12600-008-0020-5

Kaya, H. K., Aguillera, M. M., Alumai, A., Choo, H.Y., de la Torre, M., Fodor, A., … Ehlers, R. U. (2006). Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biological Control*, *38*, 134–155. doi:10.1016/j.biocontrol.2005.11.004

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology*, *38*, 181–206. doi:10.1146/annurev.en.38.010193.001145

Kaya, H. K., & Stock, S. P. (1997). Techniques in insect nematology. In L. A. Lacey (Ed.), *Manual of techniques in insect pathology* (pp. 281–324). Great Britain: Academic Press.

LeBeck, L. M., Gaugler, R., Kaya, H. K., Hara, A. H., & Johnson, M. W. (1993). Host stage suitability of the leaf miner *Liriomyza trifolii* (Diptera: Agromyzidae) to the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida: Steinernematidae). *Journal of Invertebrate Pathology*, *62*, 58–63. doi:10.1006/jipa.1993.1074

Lezama-Gutierrez, R., Molina, O. J., Pescador-Rubio, A., Galindo-Velasco, E., Angel-Sahagun, C. A., Michel-Aceves, A. C., & Gonzalez-Reyes, E. (2006). Efficacy of Steinernematid nematodes (Rhabditida: Steinernematidae) on the suppression of *Anastrepha ludens* (Diptera: Tephritidae) larvae in soil of differing textures: Laboratory and field trials. *Journal of Agriculture and Urban Entomology*, *23*, 41–49.

Lindegren, J. E., & Vail, P. V. (1986). Susceptibility of Mediterranean fruit fly, melon fly, and oriental fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema feltiae* in laboratory tests. *Environmental Entomology*, *19*, 383–386.

Lindegren, J. E., Wong, T. T., & Mclnnis, D. O. (1990). Response of Mediterranean fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema feltiae* in field tests in Hawaii. *Environmental Entomology*, *19*, 383–386.

Malan, A. P., & Manrakhan, A. (2009). Susceptibility of the Mediterranean fruit fly *Ceratitis capitata* and the natal fruit fly *Ceratitis rosae* to entomopathogenic nematodes. *Journal of Invertebrate Pathology*, *100*, 47–49. doi:10.1016/j.jip.2008.09.007

Nguyen, K. B. (2007). Methodology, morphology and identification. In K. B. Nguyen & D. J. Hunt (Eds.), *Entomopathogenic nematodes: Systematics, phylogeny and bacterial symbionts* (pp. 59–119). Leiden and Boston: Brill.

Nguyen, K. B., Malan, A. P., Antoinette, P., & Gozel, U. (2006). *Steinernema khoisanae* n. sp. (*Rhabditida: Steinernematidae*), a new entomopathogenic nematode from South Africa. *Journal of Nematology*, *8*, 157–175. doi:10.1163/156854106777998728

Patterson Stark, J. E., & Lacey, L. A. (1999). Susceptibility of western cherry fruit fly (Diptera: Tephritidae) to five species of entomopathogenic nematodes in laboratory studies. *Journal of Invertebrate Pathology*, *74*, 206–208. doi:10.1006/jipa.1999.4854

Peters, A., & Ehlers, R. U. (1994). Susceptibility of leatherjackets (*Tipula paludosa and T. oleracea; Tipulidae: Nematocera*) to the entomopathogenic nematode *Steinernema feltiae*. *Journal of Invertebrate Pathology*, *63*, 163–171. doi:10.1006/jipa.1994.1031

Roessler, Y. (1989). Control; insecticides; insecticidal bait and cover sprays. In A. S. Robinson & G. Hooper (Eds.), *World crop pests 3 (B). Fruit flies; their biology, natural enemies and control* (pp. 329–336). Amsterdam: Elsevier.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425.

SAS Institute. (2002–2003). *SAS 9.1 for windows*. Cary, NC: Author.

Shapiro-Ilan, D. I., Fuxa, J. R., Lacey, L. A., Onstad, D. W., & Kaya, H. K. (2005). Definitions of pathogenicity and virulence in invertebrate pathology. *Journal of Invertebrate Pathology*, *88*, 1–7. doi:10.1016/j.jip.2004.10.003

Sirjani, F. O., Lewis, E. E., & Kaya, H. K. (2009). Evaluation of entomopathogenic nematodes against the olive fruit fly, *Bacterocera oleae* (Diptera: Tephritidae). *Biological Control*, *48*, 274–280. doi:10.1016/j.biocontrol.2008.11.002

Stock, S. P., & Hunt, D. J. (2005). Nematode morphology and systematics. In P. S. Grewal, R. U. Ehlers, & D. I. Shapiro-Ilan (Eds.), *Nematodes as biological control agents* (pp. 3–43). Wallingford, UK: CAB International.

Swofford, D. L. (2002). *PAUP/Phylogenetic Analysis Using Parsimony (and other methods)*. Sunderland, MA: Sinauer.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731–2739. doi:10.1093/molbev/msr121

Thompson, J. D., Higgins, D. G., and Gibson, T. J (1994). CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680. doi:10.1093/nar/22.22.4673

Toledo, J., Ibarra, J. E., Liedo, P., Gómez, A., Rasgado, M. A., & Williams, T. (2005). Infection of *Anastrepha ludens* (Diptera: Tephritidae) larvae by *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) under laboratory and field conditions. *Biocontrol Science and Technology*, *15*, 627–634. doi:10.1080/09583150500089049

Toledo, J., Rasgado, M. A., Ibarra, J. E., Gomez, A., Liedo, P., & Williams, T. (2006). Infection of *Anastrepha ludens* following soil applications of *Heterorhabditis bacteriophora* in a mango orchard. *Entomologia Experimentalis et Applicata*, *119*, 155–162. doi:10.1111/j.1570-7458.2006.00412.x

Toledo, J., Rojas, R., & Ibarra, J. E. (2006). Efficiency of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) on *Anastrepha serpentina* (Diptera: Tephritidae) larvae under laboratory conditions. *Florida Entomologist*, *89*, 524–526. doi:10.1653/0015-4040(2006)89[524:EOHBNH]2.0.CO;2

Toledo, J., Williams, T., Perez, C., Liedo, P., Valle, J. F., & Ibarra, J. E. (2009). Abiotic factors affecting the infectivity of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) on larvae of *Anastrepha obliqua* (Diptera: Tephritidae). *Biocontrol Science and Technology*, *19*: 887–898.

Vrain, T. C., Wakarchuk, D. A., Levesque, A. C., & Hamilton, R. I. (1992). Intraspecific rDNA restriction fragment length polymorphisms in the *Xiphinema americanum* group. *Fundamental and Applied Nematology*, *15*, 563–574.

Wright, P. J. (1992). Cool temperature reproduction of Steinernematid and Heterorhabditid nematodes. *Journal of Invertebrate Pathology*, *60*, 148–151. doi:10.1016/0022-2011(92)90088-L

Yee, W. L., & Lacey, L. A. (2003). Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of Steinernema nematodes. *Biological Control*, *27*: 349–356. doi:10.1016/S1049-9644(03)00029-X