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Evaluation of entomopathogenic nematodes against the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae)

Farshid O. Sirjani, Edwin E. Lewis *, Harry K. Kaya

Departments of Entomology and Nematology, University of California, Davis, CA 95616, USA

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ABSTRACT

Infectivity of six entomopathogenic nematode (EPNs) species against *Bactrocera oleae* was compared. Similar infection levels were observed when third-instar larvae were exposed to infective juveniles (IJs) on a sand-potting soil substrate. When IJs were sprayed over naturally infested fallen olives, many larvae died within treated olives as well as in the soil; *Steinernema feltiae* caused the highest overall mortality of 67.9%. In addition, three laboratory experiments were conducted to optimize a time period for *S. feltiae* field application. (1) Abundance of fly larvae inside fallen olives was estimated over the 2006–2007 season with the highest number of susceptible larvae (3 mm and larger) per 100 olives being observed during December, 2006. (2) *S. feltiae* efficacy against fly larvae dropped to the soil post-IJ-application was determined. *B. oleae* added to the substrate before and after nematode application were infected at similar levels. (3) Effect of three temperature regimes (min–max: 10–27, 6–18, and 3–12 °C) corresponding to October through December in Davis, California on *S. feltiae* survival and infectivity was determined. After 8 weeks, the IJs at the 3–12 °C treatment showed the highest survival rate. However, the cold temperature significantly limited *S. feltiae* infectivity. Our results demonstrate that *B. oleae* mature larvae are susceptible to EPN infection both in the soil and within infested olives. Being the most effective species, *S. feltiae* may have the potential to suppress overwintering populations of *B. oleae*. We suggest that November is the optimal time for *S. feltiae* field application in Northern California.

1. Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi), is the most important insect pest of olive trees worldwide (Rice, 2000). It was first detected in California in October 1998 and has now spread to most areas where olives are grown within the state (Rice et al., 2003). In nature, this multivoltine pest is only known to reproduce in olive, *Olea* spp., fruit (Johnson et al., 2006). The first adults of each year emerge in spring and oviposit just under the skin of the olives that have remained on trees from the previous year. The larvae feed on fruit flesh and complete three instars before pupating. During warmer months of the year, pupation occurs inside the fruit (Rice, 2000). As the season progresses, an increasing proportion of the larvae leave the fruit as third instars, either after the fruit have dropped to the ground or when they are still on trees. They then pupate and over-winter in the soil (Kapatos and Fletcher, 1984) at a depth of 1–4 cm (Dimou et al., 2003). Infestation by *B. oleae* causes premature fruit drop and reduces fruit quality both in table olives and the olives processed for oil (Michelakis and Neuenschwander, 1983; Kapatos and Fletcher, 1983).

Currently, the main management tool for *B. oleae* consists of applications of an insecticidal material, based on spinosad (GF-120 NF Naturalyte Fruit Fly Bait, Dow AgroSciences LLC, Indianapolis, IN) (Johnson et al., 2006). Although spinosad is classified as a reduced-risk compound, developing sustainable and more environmentally safe control strategies is warranted. Such strategies may include the use of entomopathogenic nematodes (EPNs) against fly stages that are in contact with the soil during their over-wintering phase. Because of safety concerns associated with chemical applications and the small areas that need to be treated, the use of EPNs may be a safe and economically feasible control method to manage *B. oleae* in yards of home owners and in public grounds, both of which are important sources of infestation to commercial orchards annually.

EPNs are represented by the species in the genera *Steinernema* and *Heterorhabditis*. They have a mutualistic relationship with bacteria in the genera *Xenorhabdus* and *Photorhabdus* for steinernematids and heterorhabditids, respectively (Poinar, 1990). The bacteria kill the host by producing toxins, provide nematodes with nutrition, and prevent secondary invaders from contaminating the host cadaver (Forst and Clarke, 2002). Infective juveniles (IJs), which carry the bacteria in their intestine, are the only free-living stage in the EPN life cycle. IJs enter the host body mainly through natural openings and release their bacteria inside the hemocoel,

* Corresponding author. Fax: +530 752 3908.
E-mail address: eelewis@ucdavis.edu (E.E. Lewis).

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killing the host usually in 24–48 h. Nematodes feed on the bacteria and complete 1–3 generations, after which juveniles develop to the infective stage and exit the cadaver in search of new hosts (Boemare, 2002).

EPNs have been used effectively against a variety of insect pests including dipterans (Georgis et al., 2005; Jagadale et al., 2004; Jess et al., 2005; Grewal et al., 2005). No study has tested nematodes against *B. oleae*, but *Steinernema riobrave* Cabanillas, Poinar and Raulston, *S. carpocapsae* (Weiser), and *S. feltiae* (Filipjev) have shown promising results when tested against the tephritids *Ceratitis capitata* (Wiedemann) (Gazit et al., 2000; Lindegren et al., 1990) and *Rhagoletis indifferens* Curran (Yee and Lacey, 2003).

For our study, we had three objectives. The first objective was to evaluate the susceptibility of *B. oleae* to six commercially available EPN species in laboratory tests. Third-instar (prepupal) stage of the fly served as the target for EPN infection. The second was to determine the optimal time of year to apply selected EPN species to the field based on the abundance of susceptible stages of *B. oleae* in the field. And the third was to determine optimal EPN application times based on average field temperatures during fall and early winter.

2. Materials and methods

2.1. Nematodes and insects

Steinernema feltiae (SN strain), *S. carpocapsae* (All strain), *S. riobrave*, *S. glaseri* (Steiner) (NC strain), *Heterorhabditis bacteriophora* Poinar, and *H. marelatus* Liu and Berry were cultured in last instar *Galleria mellonella* (L.) at room temperature. Infective juveniles were harvested in White traps according to the procedure described by Kaya and Stock (1997). More information on nematodes is provided for specific experiments. Commercially produced *S. feltiae* (Nemasys®, Becker Underwood, Ames, IA) were used only in the efficacy experiment.

Third-instar *B. oleae* larvae used in experiments were obtained from naturally infested fruit collected from unmanaged edible olive, *O. europea* L., trees on the University of California-Davis (UCD) campus in November and December. Harvested olives were placed on wire screens over plastic tubs kept at 5–15 °C to collect the larvae as they exited the fruit for a 24-h period. In addition for other experiments, fallen olives were collected from beneath unmanaged infested trees in the city of Davis from November to January and used.

2.2. Susceptibility to nematodes

Two laboratory experiments were conducted to determine the susceptibility of *B. oleae* to each EPN species. For both experiments, harvested IJs were maintained in tap water at 20 °C and used within 3 weeks of emergence. The nematode application rate was 25 IJs/cm^2, total moisture content of the substrate was 10% (w/w), and five replicates were conducted per EPN species.

2.2.1. Experiment 1

Susceptibility of *B. oleae* larvae to infection was determined by exposing third instars that had emerged naturally from the olives to the IJs in 37-ml clear plastic cups filled to a depth of 3 cm with a substrate consisting of a 1:1 mixture of 30-mesh sieved sand and potting soil (Ace Organic Topsoil™) at 10% moisture (w/w). The sand was oven dried before starting the experiment and the initial moisture content of the potting soil was assumed to be 0%. Nematodes were applied to the sand/soil substrate in water using a pipette at the above rate. Within 1 h of IJ application, five larvae were added to each cup and allowed to burrow into the substrate. The cups were capped and incubated at 25 °C for 24 h, after which the insects were collected and dissected to determine infection status. Since the running time of the experiment was only for 24 h and infection status, not mortality, was to be determined, nematode-free control treatments were not included in this study.

2.2.2. Experiment 2

To confirm the results from Experiment 1 using infested olives, a second laboratory experiment was conducted. Plastic tubs (40 cm × 30 cm × 18 cm; 1200 cm^2 at soil surface) were filled with a 3:1 mixture of 30-mesh sieved sand and potting soil (10% moisture) to a depth of 4 cm, followed by placing 120 olives on the surface of the substrate. The olives were collected beneath olive trees and their infestation status was unknown; however, we assumed that each tub had approximately the same proportion of olives with larvae since the olives were mixed immediately before the experiment was initiated. Nematodes were sprayed over the olives using a hand-held water sprayer with the screen removed. Plain water was sprayed as a control. The tubs were covered with clear plastic wrap and stored at room temperature. The temperature and humidity above the olives were 24 °C and 18%, respectively. After 72 h, the olives were dissected and the soil (the sand-potting soil mixture) was sifted to collect larvae and pupae, which were dissected to determine infection status. Pupae inside dissected olives were not included in the analysis because we assumed that puparia had formed before nematode application when the fruit were still on trees and that *B. oleae* pupae with fully formed puparia were not susceptible, based on the results from studies on other dipterans (Belton et al., 1987; Ishibashi and Kondo, 1990; Lindegren and Vail, 1986; Yee and Lacey, 2003).

2.3. Optimal time period to apply a selected EPN species in the field

Only the most efficacious species from Experiments 1 and 2, *S. feltiae*, was tested further. For the IJ survival and infectivity experiments, IJs were harvested every other day. Those that were harvested on the 6th day after the beginning of emergence were maintained in tap water at 20 °C and used within 1 week.

2.3.1. An estimation of B. oleae larval population inside fallen olives

To estimate the number of *B. oleae* larvae that are susceptible to *S. feltiae* inside fallen olives during the season, olives were collected from beneath 3 olive trees (20–30 cm trunk diameter at breast height; 5–10 m apart) on the UCD campus weekly from November, 2006 to March, 2007. The trees were selected based on their relatively high level of infestation observed the previous year. On each sampling date, collected olives were mixed together and 500 were taken randomly and used. *B. oleae* larvae inside the olives exited the fruit when incubated in sealed 1 gallon (3.75 l) plastic bags (Ziplock®, Johnson & Son Inc., Racine, WI) for 48 h at 25 °C. The larvae were counted and categorized into two groups: 3 mm and larger; smaller than 3 mm.

2.3.2. Efficacy against B. oleae dropped to the soil after IJ application

The efficacy of *S. feltiae* applied to the soil before placing infested olives on the surface was evaluated using commercially produced nematodes (Nemasys®). A small portion of the batch was added to 300 ml of tap water, stirred slowly for approximately 15 min, and applied within 2 h. Assay arenas and the experimental protocol were the same as those of Experiment 2. IJs were sprayed (25 IJs/cm^2) over infested olives resting on the substrate in the first treatment. In a second treatment, IJs were applied to the substrate 1 h prior to placing the infested olives on the soil. Subsequently, 10 ml of tap water was sprayed over the olives to provide moisture on the surface of the fruit. Tap water without nematodes was

sprayed over the olives as a control. Total moisture content of the substrate after the spray was 10% (w/w) in all treatments.

2.3.3. Effect of temperature on a selected EPN species survival

To determine the optimal period of time for nematode application, the effects of seasonal temperature on the survival of the most efficacious species from Experiments 1 and 2, *S. feltiae*, were examined. The experiment was conducted at three fluctuating temperature regimes based on average minimum and maximum daily air temperatures during the months of October through December, which is a period of time when a large proportion of *B. oleae* larvae exit the fruit to pupate in the soil (Kapatos and Fletcher, 1984). The temperature data recorded over the past 25 years were provided by a weather station (38°32′N/121°47′W) in Davis. Growth chambers were programmed with one of three thermal regimes with min–max temperature ranges of 10–27, 6–18, and 3–12 °C (the average temperature range for the months of October, November, and December, respectively). In a 24-h period, the temperature in the 10–27 °C regime fluctuated in the following pattern: 8 h at 27 °C, 4 h at 17 °C, 8 h at 10 °C, and the last 4 h at 17 °C. In the 6–18 and the 3–12 °C regimes, the number of hours at the maximum and at the minimum temperatures was the same as those in the 10–27 °C, but the 4-h temperatures were 12 and 7 °C, respectively.

One thousand *S. feltiae* IJs were added in distilled water to 25 g of autoclaved sand (Premium Play Sand, No. 1113, Quikrete®, Atlanta, GA) inside 50-ml plastic centrifuge tubes with conical bottoms. The total moisture content of the sand in all tubes was 10% (w/w). For each temperature regime, 90 arenas were prepared. As paired control tubes, 10 tubes were incubated at 25 °C for 24 h, after which the IJs were extracted from the sand (see below). The remaining 80 tubes were weighed individually, sealed with parafilm and incubated at the designated temperature regime. Every 4 days, the tubes were weighed, sufficient distilled water was added to them to maintain the sand moisture content at 10%, and the parafilm was replaced. The IJs were extracted from the sand in 10 randomly chosen tubes weekly and were counted under a dissecting microscope. The number of IJs obtained in the extraction process was taken as the representative of the number of survived IJs.

To extract the IJs from the sand, 40 ml of distilled water was added to each test tube. The openings were covered with 1-ply tissue paper (Kimwipes® EX-L, Kimberly-Clark® Corp., Roswell, GA) and secured by modified plastic caps. The tubes were inverted inside 100 mm^2 gridded petri dishes, to which 60 ml of distilled water was added. The inverted tubes were incubated at 25 °C for 48 h, after which they were removed and the IJs in the dishes were counted.

2.3.4. Effect of temperature on a selected EPN species infectivity

Infectivity of *S. feltiae* was evaluated in 24-well plates at the three temperature regimes described above. Twenty IJs were added in 50 µl distilled water to each well. Wells contained 3 g of autoclaved sand (Premium Play Sand, No. 1113, Quikrete®) with 10% (w/w) moisture content. Fifty wells with IJs and 50 treated with water, as a control, were tested per temperature regime. The lids were sealed with parafilm and the plates were incubated at the designated regime for acclimation of the IJs for 24 h. A surrogate insect, *G. mellonella*, was used in this experiment due to a lack of the olive fruit fly larvae. One *G. mellonella* larva (0.2–0.3 g) was added to each well after the acclimation period and the plates were covered and returned to their respective temperature treatments. Larvae were checked every 12 h for a week or until death, whichever occurred first. To determine larval death, each was gently probed after being held at room temperature for approximately 10 min; if it did not respond, it was considered dead. Dead larvae were rinsed with tap water to wash off any attached IJs, and incubated in clean petri dishes lined with moist filter paper at 25 °C for 5 days, after which they were dissected to determine infection status. Larvae that were found alive after a week were rinsed with tap water and incubated individually in clean 24-well plates at 25 °C for another 7 days, after which dead insects were dissected to determine infection status.

2.4. Nematode reproduction within B. oleae larvae

Reproductive ability of *S. feltiae* inside *B. oleae* was evaluated. Fifty third-instars were exposed to 50 IJs/cm^2 inside ten 30-ml plastic cups, 5 larvae in each cup, at 25 °C. The substrate consisted of a 1:1 mixture of 30-mesh sieved sand and potting soil with 10% (w/w) moisture content. After 2 days, the insects were collected and placed individually on White traps at room temperature. Within 3 weeks, the pupae that developed to adult flies were discarded; the remaining ones were considered infected and were maintained on the traps for 3 weeks to collect possible emerging IJs. The pupae from which no IJs emerged were dissected to determine the EPN presence inside cadavers.

2.5. Statistical analysis

Susceptibility, survival and efficacy data were analyzed with ANOVA (SAS Institute, version 9.1, 2002–2003), followed by the Student–Newman–Keuls means separation test when $P < 0.05$. Data collected from 3 mm and larger larvae were included in analyses; smaller larvae were never infected during our tests. *t*-tests (SAS Institute, version 9.1, 2002–2003) compared infection rates inside olives and in the soil for each EPN treatment. *G. mellonella* mortality and *S. feltiae* survival at different temperatures were corrected for the control mortality and survival, respectively (Abbott, 1925). The χ^2 test was used to compare *S. feltiae* infectivity at different temperature treatments. Data recorded as % infection or % survival were subject to arcsine transformation before analysis. Untransformed means (±SEM) are presented in all figures.

3. Results

3.1. Susceptibility to nematodes

3.1.1. Experiment 1

All six species of EPNs infected *B. oleae* larvae at similar levels ($F = 2.3$; $df = 5, 23$; $P > 0.05$) (Fig. 1). *S. feltiae* infected 100% of exposed larvae. *S. riobrave* and *S. glaseri* caused the lowest levels of 76%. Many larvae exposed to nematodes died as pupae except those infected by *S. feltiae*, all of which died before pupating.

3.1.2. Experiment 2

All six species of EPN caused greater mortality than the control treatment, in which no infection was observed ($F = 14.21$; $df = 6, 28$; $P < 0.0001$); *S. feltiae* was the most efficacious species, inducing 67.9% infection (Table 1). Infection levels inside the olives versus in the soil ($F = 5.51$; $df = 5, 24$; $P = 0.0016$) are also presented in Table 1. All infected larvae were 3 mm and larger. Infected pupae were found only in the soil. Due to variable availability of *B. oleae* larvae despite mixing the collected olives, the total number of dissected larvae and pupae differed among treatments.

3.2. Optimal time period to apply a selected EPN species in the field

3.2.1. An estimation of B. oleae larval population inside fallen olives

The highest total number of larvae estimated per 100 olives was 23 (6 larvae at 3 mm and larger and 17 larvae smaller than 3 mm)

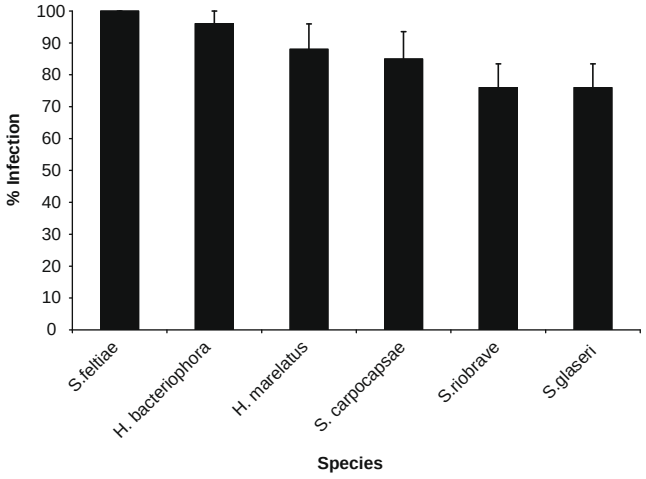

Fig. 1. Infection levels (±SEM) of third-instar larvae of *Bactrocera oleae* by *Steinernema feltiae* (SN strain), *S. carpocapsae* (All strain), *S. riobrave*, *S. glaseri* (NC strain), *Heterorhabditis bacteriophora*, and *H. marelatus* at 25 infective juveniles/cm^2 after 24-h exposure on a sand-potting soil substrate inside 37-ml plastic cups at 25 °C. The means are not significantly different among treatments ($P > 0.05$).

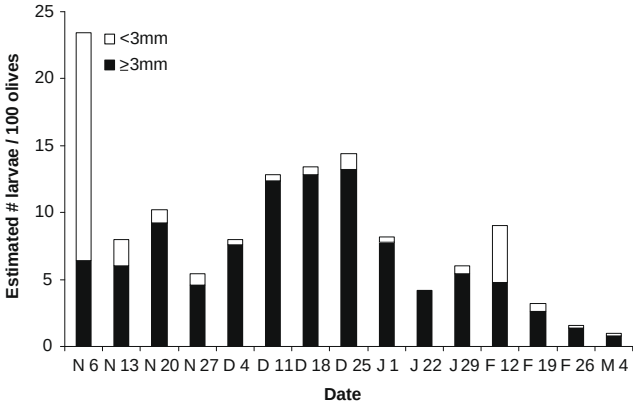

Fig. 2. Estimated number of *Bactrocera oleae* larvae per 100 fallen olives collected on a weekly basis during 2006–07 season. **N:** November; **D:** December; **J:** January; **F:** February; **M:** March. Legend indicates the size of collected larvae.

and was obtained from the olives collected on the first sampling date, November 6, 2006 (Fig. 2). The highest numbers of 3 mm and larger larvae were obtained from the olives collected on December 11, 18, and 25, 2006 (average of 12.8 larvae/100 olives). Collection was disrupted on 2 sampling dates in January and 1 in February of 2007 because of heavy rain or mowing of the grass beneath the trees. The experiment was terminated on March 4, 2007 due to insufficient availability of fallen olives at the site.

3.2.2. Efficacy against B. oleae dropped to the soil after IJ application

Infection induced by *S. feltiae* that were sprayed over infested olives was similar to that caused by IJs applied to the soil prior to adding the olives, and both were better than the control ($F = 46.4$; $df = 2, 12$; $P < 0.0001$). Many nematode-treated *B. oleae* died as pupae in the soil only. When sprayed over the infested olives, *S. feltiae* induced 50% overall infection with 16.4% of *B. oleae* dead inside the olives and 33.6% dead in the soil. The IJs that were applied to the substrate prior to adding the olives induced an overall infection level of 47.2%. In the latter treatment, 10.2% of *B. oleae* died inside the olive and 37% died in the soil. No infection was observed in the control.

3.2.3. Effect of temperature on a selected EPN species survival

Survival of *S. feltiae* was affected by both time ($F = 50.91$; $df = 7, 216$; $P < 0.0001$) and temperature ($F = 106.47$; $df = 2, 216$; $P < 0.0001$) (Fig. 3). For the first 2 weeks of incubation, *S. feltiae* survival was similar for all three temperature treatments. After 4 weeks, the IJs in the coldest (3–12 °C) and the warmest (10–27 °C) temperature regimes had lower survival than those in the medium (6–18 °C) treatment. On week 8, however, *S. feltiae* incubated at the coldest and at the warmest regimes showed the highest (87.6%) and the lowest (26.3%) survival rate, respectively. Percent survivals ($F = 37.86$; $df = 23, 216$; $P < 0.0001$) of *S. feltiae* at the three temperature regimes are presented in Table 2.

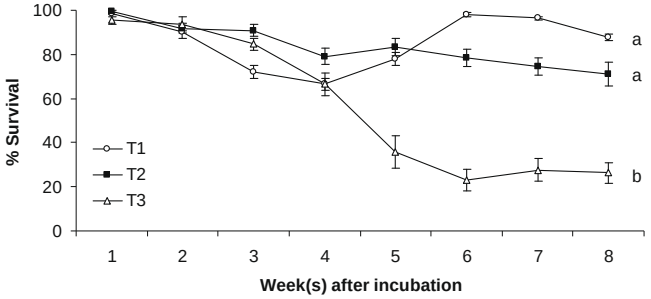

Fig. 3. Mean percent survival (±SEM) of *Steinernema feltiae* IJs incubated on a sand substrate (10% moisture w/w) at three different temperature treatments. Legend indicates the three temperature regimes: **T1:** 3–12 °C (min–max); **T2:** 6–18 °C; **T3:** 10–27 °C. In a 24 h period, the temperature fluctuated in the following pattern: 8 h at the maximum and 8 h at the minimum temperature with two 4-h intervals at 7, 12, and 17 °C, respectively. Data were transformed ($\sqrt{}$arcsin) and analyzed with ANOVA ($P < 0.05$), following by SNK test ($\alpha = 0.05$). Lines with the same letter are not significantly different ($P > 0.05$).

3.2.4. Effect of temperature on a selected EPN species infectivity

Cold temperature significantly limited *G. mellonella* mortality by *S. feltiae* ($\chi^2 = 29.8$; $df = 4$; $P < 0.0001$). No mortality was observed in the 3–12 °C treatment during the first 7 days of inoculation (Fig. 4). Within 4 days, *S. feltiae* induced 2% and 18.4% mortalities

Table 1
Overall infection levels of *Bactrocera oleae* and percentage that died in the soil compared to that inside infested olives resting on a sand-potting soil substrate and treated with *Steinernema* and *Heterorhabditis* species (25 infective juveniles/cm^2) in the laboratory.

Nematode species	Percent[a] overall infection ± SEM	Percent[b] of all *B. oleae* infected ± SEM		Total larvae and pupae
		Inside olives[a]	In the soil	
S. feltiae	67.9 ± 6.0a	42.1 ± 9.0a	25.8 ± 4.2a	78
S. carpocapsae	45.6 ± 4.9b	30.2 ± 6.2ab	15.4 ± 2.5a	73
S. riobrave	35.8 ± 4.6bc	5.5 ± 2.4b	30.3 ± 4.2a	70
S. glaseri	33.7 ± 6.4bc	28.5 ± 4.1ab	5.2 ± 2.8c	117
H. bacteriophora	37.3 ± 6.4bc	10.2 ± 6.1b	27.1 ± 5.9b	60
H. marelatus	19.1 ± 8.4c	10.3 ± 5.4b	8.8 ± 3.9b	63

[a] Means followed by the same letter are not significantly different (SNK test, $P < 0.05$).
[b] Two means followed by the same letter within each row are not significantly different (t-test, $\alpha = 0.05$).

Table 2
Mean percent[a] survival (±SEM) of *Steinernema feltiae* incubated on a sand substrate (10% moisture) at three different temperature (°C) treatments.

Treatment[b]	Week(s) after incubation							
	1	2	3	4	5	6	7	8
T1	98.7 ± 0.5a	90.1 ± 2.8b	72.1 ± 3.0cd	66.5 ± 2.6c	78.1 ± 2.9d	97.9 ± 1.0a	96.4 ± 0.9a	87.6 ± 1.4e
T2	99.5 ± 0.5a	91.9 ± 2.3b	90.9 ± 2.5b	79.1 ± 3.5cd	83.1 ± 3.9c	78.4 ± 3.8cd	74.7 ± 3.9cd	71 ± 5.4cd
T3	95.5 ± 1.7a	93.8 ± 3.1a	84.7 ± 2.8b	66.6 ± 5.1c	35.8 ± 7.5d	23.1 ± 5.0e	27.6 ± 5.0e	26.3 ± 4.5e

[a] Means followed by the same letters within rows are not significantly different (SNK test, $P < 0.05$).
[b] **T1**: 3–12 °C (min–max); **T2**: 6–18 °C; **T3**: 10–27 °C; In a 24 h period, the temperature fluctuated in the following pattern: 8 h at the maximum and 8 h at the minimum temperature with two 4 h intervals at 7, 12, and 17 °C, respectively.

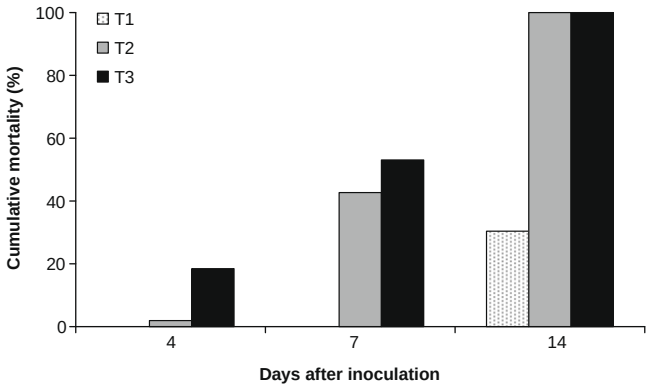

Fig. 4. Mortality of *Galleria mellonella* exposed to *Steinernema feltiae* (20 infective juveniles/larvae) on a sand substrate at three different temperature treatments. The larvae were incubated at the designated temperature regime for the first 7 days of inoculation. Afterwards, live insects were rinsed with tap water and transformed to 25 °C. Legend indicates the three temperature treatments: **T1**: 3–12 °C (min–max); **T2**: 6–18 °C; **T3**: 10–27 °C. In a 24 h period, the temperature fluctuated in the following pattern: 8 h at the maximum and 8 h at the minimum temperature with two 4-h intervals at 7, 12, and 17 °C, respectively.

in the 6–18 °C and the 10–27 °C treatments, respectively. Seven days after inoculation, observed mortalities were 42.8% in the 6–18 °C and 53% in the 10–27 °C temperature regimes. After incubating live larvae at 25 °C for another 7 days, all insects exposed to *S. feltiae* from both the 6–18 °C and the 10–27 °C treatments died, whereas 30.2% mortality was observed for the insects from the 3–12 °C temperature regime.

3.3. Nematode reproduction within *B. oleae* larvae

All insects that were exposed to the EPN pupated after 2 days. No IJ emergence was observed after 3 weeks, during which 19 out of 50 pupae developed to adult flies. When dissected, 35.5% of the remaining 31 puparia contained high numbers of live IJs, 9.7% contained high numbers of dead IJs, 26% contained dead female nematodes, and 9.7% contained different parasitic stages many of which were dead. No nematodes were observed in the remaining 19%.

4. Discussion

Our results indicate that *B. oleae* larvae that are 3 mm and larger are susceptible to EPN infection not only in the soil but also while still inside fallen olives. This may provide a reasonable window of opportunity for nematodes to be used against *B. oleae*, since many infested olives that fall to the ground late in the season contain mature larvae (Sirjani, personal observation). Our findings further demonstrate that *S. feltiae* can survive and remain infectious to *B. oleae* at temperatures typical of fall and early winter, when the larvae drop beneath olive trees in large numbers to overwinter in the soil in areas with similar climatic conditions to those of Davis, California. Thus, *S. feltiae* shows potential as a biocontrol agent to reduce *B. oleae* overwintering populations. We suggest that the month of November is the optimal period of time to apply *S. feltiae* against *B. oleae* in the field in Northern California due to temperature and moisture conditions in the field.

Third-instar larvae showed similar susceptibility to infection by *Steinernema* and *Heterorhabditis* species when exposed to nematodes on a sand-potting soil substrate (Experiment 1). Many of the larvae died as pupae, which is similar to results found with *R. indifferens* exposed to *S. feltiae* and *S. carpocapsae* (Patterson Stark and Lacey, 1999; Yee and Lacey, 2003). Also, larvae of *C. capitata, Dacus dorsalis* Hendel and *D. cucurbitae* Coquillett (all Tephritidae) exposed to *S. carpocapsae* (referred to as *S. feltiae* = *Neoaplectana carpocapsae* Weiser) (Lindegren and Vail, 1986) were infected as larvae but died after pupating. *S. feltiae* causing 100% mortality before pupation is surprising and impressive, since fly larvae pupate within a few hours of leaving the fruit at 25 °C (Sirjani, personal observation). This may be due to a relatively high pathogenicity ("the ability to produce disease") and/or virulence ("the degree of pathogenicity") (Shapiro-Ilan et al., 2005) of *S. feltiae* against *B. oleae* larvae specifically and dipterous larvae in general (Lewis et al., 2006). When tested against *C. capitata* larvae, however, *S. feltiae* was out-performed by *S. riobrave* (Gazit et al., 2000).

When applied over olives infested with *B. oleae* (Experiment 2), EPN species had different infection levels with *S. feltiae* being the most efficacious species. This experiment was designed to mimic natural conditions, in which nematodes' potential to infect the larvae that reside inside olives may improve their overall efficacy. A single dropped olive, with or without exit holes on its surface, may contain more than one larva of different stages (Sirjani, personal observation). When the olives were collected, we did not know which ones were infested and which ones were not, so we included 120 olives in each replicate, with the aim of having a sufficient number of infested ones to tease apart differences among the EPN species tested. The exclusive susceptibility of 3 mm and larger larvae was probably caused by their high CO_2 output, which attracts nematodes (Lewis et al., 1993), and their larger natural openings, which are the most common portals of entry for IJs (Griffin et al., 2005).

Many larvae died inside treated olives, which indicate that IJs were able to find and infect them before they exited the fruit. Nematodes most likely entered feeding canals in the olives via exit holes, oviposition punctures, or tears on the surfaces of the fruit. Similarly, *S. feltiae* and *S. carpocapsae* infected *Anaplophora glabipennis* (Motchulsky) (Coleoptera: Cerambycidae) larvae inside tunnels bored in an artificial diet (Fallon et al., 2004). In addition, LeBeck et al. (1993) demonstrated that *S. carpocapsae* entered the leaf mines of *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) larvae via oviposition holes or unnatural openings and subsequently infected them. EPN species have been categorized into "ambusher", those that have a sit-and-wait foraging strategy (e.g. *S. carpocapsae*), "cruiser", those that actively search for their hosts

(e.g. *S. glaseri*), or "intermediate" (e.g. *S. feltiae* and *S. riobrave*) classes (Campbell and Lewis, 2002). The cruiser, *S. glaseri* killed larvae while still inside olives but so did *S. feltiae* and *S. carpocapsae*. Thus, even ambushing EPN species move enough to get inside olives and parasitize larvae. Similar results are seen in field tests using *S. carpocapsae* against *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) that overwinters inside fallen pistachio nuts (Siegel et al., 2004).

In an estimate of *B. oleae* larval population inside fallen olives, the olives collected on the last three sampling dates in December, 2006 contained the highest number of 3 mm and larger larvae, which are susceptible to infection by *S. feltiae*. However, temperatures during this period are not ideal for application. As fall begins, a large proportion of the larvae exit the fruit that are still on the trees to pupate in the soil (Kapatos and Fletcher, 1984). This proportion was not determined in our study. Therefore, the data represent only a portion of larvae that are potential targets in EPN applications. Nevertheless, our results may be considered as a representative of the *B. oleae* larval population that is susceptible to infection by *S. feltiae*.

To determine the optimal time period during the season for field application of selected EPN species, it is essential to examine the effects of abiotic factors on EPN survival and infectivity during the potential time period. Temperature is one of the major factors that may limit EPNs activities (Griffin, 1993; Grewal et al., 1994) as it influences development (Kaya, 1977), foraging behavior (Byers and Poinar, 1982), infectivity (Molyneux, 1986; Saunders and Webster, 1999; Chen et al., 2003), and survival (Kung et al., 1991) of the nematodes. The average highs and lows during January, February, and March are within the ranges during December, November, and October, respectively, so we only included the latter three months' temperature regimes.

Our IJ survival experiment demonstrates that IJs of *S. feltiae* tolerate low temperatures and may survive the cold weather during the months of November and December in Northern California. When the survival of *S. feltiae* incubated at three temperature regimes with min–max temperatures of 3–12, 6–18, and 10–27 °C was evaluated over an 8-week period, the IJs at the coldest (3–12 °C) temperature treatment showed the highest survival at the end of the experiment. Although the rates for the medium (6–18 °C) treatment compared to that for the coldest regime was lower over the last 3 weeks, the IJs at the former regime showed a high survival rate on average (89%) for the first 5 weeks. *S. feltiae* has been reported to be cold tolerant and adapted to cooler temperatures (Grewal et al., 1994; Wright, 1992; Hazir et al., 2001). Even though low temperatures negatively affect *S. feltiae* mobility (Chen et al., 2003), the EPN actively searches for and induces significant infectivity to *B. oleae* within 72 h given the optimal temperature (Data presented in Table 1).

Temperature is also a significant factor in infectivity of EPNs (Griffin, 1993; Hazir et al., 2001). When *G. mellonella* larvae were exposed to *S. feltiae* at the temperature treatments described above, larval mortality was significantly lower at the coldest regime. Similar to our results, *S. feltiae* did not infect *G. mellonella* larvae at 8 °C for the initial 10 days of incubation (Saunders and Webster, 1999). In other studies, *S. feltiae* (100 IJs/larvae) infected *Delia radicum* (L.) (Diptera: Anthomyiidae) larvae after 4 days at 10 °C (Chen et al., 2003) and caused 50% mortality of *G. mellonella* (50 IJs/larvae) larvae after 20 days at 8 °C (Grewal et al., 1994). Similar mortalities induced by *S. feltiae* in the medium and the warmest (10–27 °C) treatments suggest that the lower temperature during the month of November, compared to that of October, does not affect *S. feltiae* infectivity.

Our results further indicate that the coldest temperature regime induced latent infection (i.e., prevention of host death at low temperature until optimal conditions resume) (Brown et al., 2002) of *S. feltiae* to *G. mellonella* larvae. EPNs may adopt this strategy to survive the cold and over-winter inside the host. However, the mortality caused by *S. feltiae* after transferring the larvae to 25 °C was low (30%). Thus, *S. feltiae* is not expected to cause significant host mortality in the temperature range that represented the month of December. Infection latency has been reported for other EPN species as well; Brown et al. (2002) showed that 100% *G. mellonella* larvae that survived exposure to *S. carpocapsae* at 5 °C died within 72 h of being transferred to 25 °C.

S. feltiae that was already applied to the soil sought and infected *B. oleae* inside the olives resting on the soil when the surface of the fruit was moistened. Our findings suggest that *S. feltiae* is as effective against *B. oleae* dropped to the soil after the EPN application as it is against *B. oleae* present in the soil prior to the application, when optimal conditions are present. This leads to more flexibility in *S. feltiae* application timing and the number of applications, thus providing a wider window of opportunity for, as well as a greater amount of savings in, *B. oleae* management plans using *S. feltiae*. Suggesting an optimal time period between the EPN application and *B. oleae* larvae falling to the soil needs more investigation.

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