Research Article

Efficacy and residual activity of commercially available entomopathogenic nematode strains for Mediterranean fruit fly control and their ability to infect infested fruits

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Abstract

BACKGROUND: Entomopathogenic nematodes (EPN) show potential in controlling larvae of the Mediterranean fruit fly (medfly) *Ceratitis capitate*, but previous studies mainly concern species and strains that are not commercially available. The use of EPN for control of Mediterranean fruit fly is further hampered by the cost of using nematodes. In this study, the efficacy and residual activity of commercial strains of three EPN species, *Steinernema carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora* medfly) *C. capitata*, in the soil substrate and inside fruits were evaluated.

RESULTS: Suspensions of these species were applied at a dose of 1.5 mi m^{-2} on a soil substrate wherein medfly larvae were added sequentially for a period of 4 weeks post application at 20 °C. *S. feltiae* provided the highest suppression up to 50% as assessed by adult medfly emergence because it had the highest immediate activity and long residual activity. Furthermore, *S. feltiae*, and to a lesser degree *S. carpocapsae*, were able to move and infect medfly larvae inside infested apples and oranges left in the surface of the substrate wherein EPN were applied, reducing significantly adult medfly emergence (60–78%).

CONCLUSION: These results support the efficacy and feasibility of applying a single, relatively low dose of *S. feltiae* in autumn, off-season, targeting overwintering medfly larvae with the scope of reducing the number of adult medflies emerging later in the new season.
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Keywords: *Steinernema carpocapsae*; *Steinernema feltiae*; *Heterorhabditis bacteriophora*; off-season control; overwintering; foraging behaviour

1 INTRODUCTION

Mediterranean fruit fly or medlfy, *Ceratitis capitata* (Wiedermann), is an extremely polyphagous species that attacks a wide variety of cultivated fruits.[1,2] Medfly not only causes significant economic losses to growers, sometimes reaching to 100%,[3,4] but also affects national and international fruit trading.[5,6] Control measures of medlfy include primarily insecticidal cover sprays and bait application, mass trapping of females and males using densely-spaced baited traps,[7] the sterile insect technique[8] and biological control using parasitoids.[9,10] The use of entomopathogens is an ever-increasing trend that is compatible with all these methods and further contributes to a sustainable environmentally friendly integrated pest management of medfly. Specifically, the use of entomopathogens such as entomopathogenic nematodes (EPN) and fungi against the soil-dwelling stage of tephritid flies offers an excellent opportunity for effective management, because, during its life cycle, medfly passes a significant amount of time in the soil because mature larvae drop and burrow in the soil to pupate.[11–15]

EPN are commercially produced and used against a wide range of insects.[16,17] The free-living stage of EPN infective juveniles enter their hosts and release symbiotic bacteria and toxins, which result in the death of their host within 24–48 h.[18,19] The use of EPN for controlling medfly has been experimentally explored.[11,20–22] Some species of EPN, such as *Steinernema riobravae*, *S. yirgalemense*, *Heterorhabditis baujardi* and *H. noenieputensis*, seem particularly adapted on medfly, showing increased efficacy in laboratory and field tests.[11,21,23] However, only a few EPN species are commercially produced and available, mainly

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S. carpocapsae, S. feltiae and H. bacteriophora,[24] and even for these species there are locally adapted strains that, although they show efficacy, are not commercially available.[15,20] Although there are numerous studies that investigate the efficacy of various EPN species in medlfy, their residual efficacy days after application has been rarely investigated; only one study showed that S. riobrave was active and controlled medfly larvae in the soil up to 10 days post application.[11] The ability of nematodes to infest medfly inside fruits is also relevant because in temperate climate regions such as the Mediterranean, medfly larvae overwinter inside fruits; in Spain they are found inside late-ripening varieties of orange,[25] whereas in Greece medfly larvae overwinter in apples which provide a suitable refuge, allowing slow growth and protection from natural enemies.[26] S. feltiae has been found to infect medfly larvae inside apricots,[15] whereas the ability of EPN to infect maggots of various fruit fly species inside fruits has been documented in other studies.[27–30] In this laboratory study we aimed to screen the efficacy and residual activity of commercially available species of EPN against mature medfly larvae burrowing in the soil and we assess their potential in infesting medfly larvae inside fruits, oranges and apples.

2 MATERIALS AND METHODS

2.1 Mediterranean fruit fly and nematode rearing

A laboratory culture of C. capitata was established with flies recovered from field-infested bitter oranges (Citrus aurantium L.) collected during the summer of 2019 from the area of Attica-Greece. Bitter oranges were brought to the laboratory and placed in plastic containers. Mature larvae and pupae were collected daily. On emergence, adults were placed into wire-screened wooden holding cages (35 × 35 × 50 cm) and provided with water and a standard adult diet consisting of a mixture of yeast hydrolysate, sugar and water in a 4:1:5 ratio, ad libitum. Approximately 400 adults were placed into each cage, males and females in a 1:1 ratio. Females were allowed to oviposit into 5-cm prepunctured (30 holes of 0.5 mm in diameter), plastic, red, hollow domes that were fitted onto the lid of a Petri dish.[31] To stimulate oviposition, water and C. aurantium L. juice was placed under each dome. In these conditions, females typically lay at least 500 eggs in their lifetime after a pre-oviposition period of ca. 20 days. Eggs were collected daily, placed on wet filter paper and maintained in plastic Petri dishes until use. Larvae were reared on three round cotton pads, deposited in glass Petri dishes (9.5 cm in diameter, 1.5 cm in depth) and saturated with 48 g of an artificial diet (density of 200–300 eggs per food amount). Larval artificial diet was prepared by mixing 200 g of sugar, 200 g of brewer's yeast, 100 g of soybean flour, 4 g of salt mixture, 16 g of ascorbic acid, 16 g of citric acid, 3 g of sodium propionate and 1 L of water.[32] Cultures of flies were kept under laboratory conditions at 25 ± 1 °C, 55–65% relative humidity and 16-h light/8-h dark photoperiod.

The nematodes used in the experiments, H. bacteriophora Poinar, S. carpocapsae Weiser, and S. feltiae Filipjev, were obtained from E-nema GmbH (Schwentinental, Germany). All nematodes were cultured at room temperature 23–24 °C in Galleria mellonella (L.) larvae following methods by Kaya and Stock (1997). Harvested infective juveniles (IJs) in tap water were stored in Nunc™ Cell Culture Treated Easyflasks™ 175 cm² (Thermo Scientific™) at 9–11 °C until use. The nematodes used in experiments were at most 3 weeks old.

2.2 Efficacy and residual activity of nematodes in soil substrate

Each experimental unit consisted of a laboratory tray (W × D × H: 25 × 25 × 8 cm) with soil substrate (7:3 sand:potting soil) that was placed in a wooden holding cage (30 × 30 × 30 cm). The sand and potting soil were oven dried before starting the experiment and the moisture content of the substrate was adjusted to 10%. Nematode suspension (60000 IJs in 180 mL of water) was applied evenly in the substrate (estimated surface of 400 cm²), corresponding to a dose of 150 IJs cm⁻² (or 1.5 mi IJs m⁻²) and then late instar larvae of medfly, at the time of leaving the artificial diet, were placed on the top of the soil substrate (5–6 cm) to borrow and pupate. One hundred larvae were added at day 1, 100 larvae after 2 weeks and 100 larvae after 4 weeks. Control units received only water. The humidity of the soil substrate was adjusted to 10–15% by sprinkling it weekly with water. The wooden cages were kept at 20 °C, about 50% relative humidity and permanent light. We compared the efficacy of H. bacteriophora, S. carpocapsae and S. feltiae at 20 °C (20 replicates). Furthermore, we assessed if sequential addition of medfly larvae contributes to nematode recycling and consequently longer residual activity of applied S. carpocapsae and S. feltiae, the species that had the longer residual activity (see results). For this purpose, in separate units, 100 medfly larvae were added to the soil substrate only 4 weeks after the nematode application.

At our experimental conditions, adult medflies emerged within 20–24 days after the addition of the larvae at the substrate, and therefore they emerged in three different cohorts. They were left to starve to death (2–3 days) and then collected from the floor of the wooden cage and the surface of the substrate with forceps and counted. We were therefore able to assess EPN residual efficacy at different time intervals. In preliminary observations there were no dead flies infected with nematodes on dissection and therefore no adult flies were screened for nematode mortality in our experiments.

2.3 Ability of nematodes to infect medfly larvae inside fruits

Oranges (Valencia and Merlin) and apples (Starkin) were artificially infested each with 50 neonate medfly larvae by opening small incisions and transferring the larvae with fine brushes. The infested fruits were placed in soil substrate (7:3 sand:potting soil) within a plastic container (W × D × H: 12 × 12 × 13 cm) on which an EPN suspension of 21 600 IJs in 80 mL of water was applied. Therefore, the dose used was the same as in the first experiment. The oranges and apples were removed from the substrate after 20–24 days and 27–30 days, respectively (medfly typically develop at slower rate in apples; Papadopoulos et al., 1996) and were dissected and examined. The condition of larvae found was assessed and any larvae with signs of nematode infection were dissected to confirm nematode presence. The soil was also thoroughly examined and pupae found were examined and counted. The experiments were run at 20 °C, about 50% relative humidity and permanent light. Overall, there were 20 replicates for each treatment (H. bacteriophora, S. carpocapsae, S. feltiae and control).

2.4 Statistical analysis

One-way comparison of mean flies emerging in experimental units of various treatments (combination of treatments and timing of larvae addition post treatment) was performed with a Welch's ANOVA followed by a Games–Howell multiple

comparisons *post hoc* test, which is a more robust test when the variance among groups is unequal, as was the case in our data.[33,34] In the second experiment, we used a two-way ANOVA with treatment and fruit as factors for exploring their influence in the number of flies that successfully developed in the fruits followed by Tukey–Kramer *post hoc* tests ($P < 0.05$). All analyses were performed with SPPS vs21.

3 RESULTS

3.1 Efficacy and residual activity of nematodes in soil substrate

Variances among groups were unequal (Levene test = 2.2268, df$_{11,228}$, $P = 0.012$). Overall, there were significant differences among treatments (Welch ANOVA, $F_{11,89.126} = 101.664$, $P < 0.001$). All nematode treatments resulted in a smaller number of emerging adult flies compared to the control (Fig. 1); *S. feltiae* was the most effective at 0 days (addition of medfly larvae immediately after application) among the three EPN species. At 2 weeks post EPN application, *S. carpocapse* and *S. feltiae* had higher residual activity than *H. bacteriophora*, whereas at 4 weeks post application all three EPN species had similar efficacy. Overall EPN applications reduced the emergence rates of adult medflies from the soil substrate with application of *S. feltiae* leading to an almost 50% reduction of adult medflies (Table 1).

Sequential addition of medfly larvae did not have any effect on nematode recycling; the number of flies emerging from soil substrate wherein larvae were added only after 4 weeks from nematode application was not statistically different to those emerging
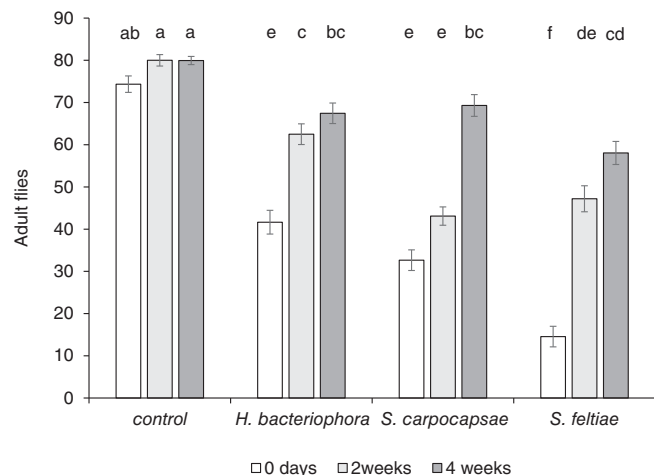
from soil substrate 4 weeks post nematode application in the experimental units wherein fly larvae were added every 2 weeks (*S. carpocapsae*: 4 weeks 65.15 ± 2.13, 4 weeks with sequential larvae addition 69.3 ± 2.57, two tailed *t*-test = 1.241, $P = 0.222$; *S. feltiae*: 4 weeks 61.85 ± 1.9, 4 weeks with sequential larvae addition 58 ± 2.73, two tailed *t*-test = 1.141, $P = 0.261$).

3.2 Ability of EPN to infest medfly larvae inside fruits

There was a significant difference among the number of flies (pupae exiting in soil + alive larvae inside the fruit) developing in fruit among different treatments ($F_{3,152} = 104.186$, $P < 0.001$), whereas there was no difference between oranges and apples ($F_{1,152} = 0.133$, $P = 0.716$). The interaction between treatment and fruit was also not significant ($F_{3,152} = 31.823$, $P = 0.603$). *S. feltiae* was more effective than *H. bacteriophora* in reducing the number of medflies developing inside apples and oranges, but not more effective than *S. carpocapsae* (Fig. 2). *S. feltiae* resulted in 72% and 78% reduction of medflies developing inside apples and oranges, respectively.

4 DISCUSSION

EPN demonstrate a relative high efficacy against medfly larvae in the soil, at least in laboratory bioassays where soil or sand substrates have been used[10,15,20–23] and in a field trial.[21] The majority of these studies use either indigenous species of EPN or local strains of species that are widely commercially available, such as *S. feltiae*,[15,20] in a variety of experimental conditions such as temperature, soil/substrate composition and dose of nematodes. In our study we evaluated the efficacy of commercial strains of the most used nematodes *S. carpocapsae*, *S. feltiae* and *H. bacteriophora*; *S. feltiae* was the most effective nematode species as assessed by adult medfly emergence in substrates where
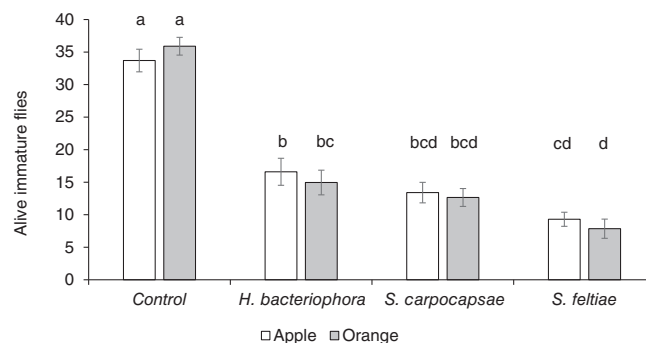

Figure 1. Emerging adult medflies from substrates to which different treatments were applied [*Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *Steinernema feltiae* suspensions (150 IJs cm^{-2}) and water (control)] and 100 third-instar larvae of medlfy were added immediately, 2 and 4 weeks after application (means ± SE, bars with the same letter do not differ significantly at $P \leq 0.05$).

Figure 2. Immature flies that developed inside apples and oranges (pupae in soil + alive larvae inside fruit) that were placed on substrate to which different treatments of *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *Steinernema feltiae* suspensions (150 IJs cm^{-2}) and water (control) were applied (means ± SE, bars with the same letter do not differ significantly at $P \leq 0.05$). Fruits were each artificially infested with 50 neonate medfly larvae.

Table 1. Percentage reduction (%) of medfly adults after application of entomopathogenic nematodes

Nematode species	0 days	2 weeks	4 weeks	Cumulative
H. bacteriophora	43.99	21.875	15.64	26.77
S. carpocapsae	56.09	46.125	13.33	38.1
S. feltiae	80.44	41	27.4	48.87

EPN suspension was applied. *Steinernema carpocapsae* was also effective, leading to a significant medfly suppression but to a lesser extent. The superior efficacy of various strains of *S. feltiae* against medfly has also been reported in other studies,[15,20] whereas the same species has also been found effective against other fruit fly species such as *Rhagoletis cerasi*,[35] olive fruit fly *Bactrocera oleae*,[28] *Anastrepha suspensa*,[36] *Bactrocera tryoni*[37] and *Bactrocera indifferens*.[38] In our study we applied a dose of 150 IJs cm^{-2}, which is similar to the dose of *S. feltiae* applied in other studies[11,20] and close to others[15] that used any kind of soil substrate in bioassays. However, in our study we had 0.25 larvae cm^{-2}, which is similar to the study of Mokrini et al.[15] (estimated 0.26–0.3 larvae cm^{-2}), lower than that of Karagoz et al.[20] (estimated 0.26–0.3 larvae cm^{-2}) and higher than that of Gazit et al.[11] (estimated 0.075 larvae cm^{-2}). Host density influences EPN efficacy because as host numbers increase, fewer nematodes are available to parasitize these hosts successfully; this is further exacerbated by the decline of EPN post application and by possible decreased susceptibility of the targeted hosts.[39–41]

More importantly, we have shown that all EPN species showed significant residual activity, although it reduced over a period of 4 weeks. Significantly long residual activity for 2 weeks was observed in *S. carpocapsae* and *S. feltiae*. Residual activity of EPN application against medfly has not been evaluated in previous studies, except for one study reporting that *S. riobravae* efficacy dropped within 10 days and was completely lost by 2 weeks post application,[11] which is significantly shorter than the residual activity reported in our study. The increased residual activity of *S. feltiae* and *S. carpocapsae* could be attributed to recycling of nematodes given the sequential addition of medfly larvae on the substrate, which simulates real conditions of larvae falling on the soil.[40] However, our complementary experiments suggest that long residual activity due to recycling is negligible. A possible explanation is that small medfly larval hosts cannot support the production of such a high number of IJs capable of sustaining a high nematode population in the substrate. In another study it was found that when *S. feltiae* infected *B. oleae* larvae, the larvae pupated and only 35.5% of these pupae contained live IJs.[28] It is likely that our experimental conditions, such as substrate, relatively low temperature and humidity, likely sustained a relatively high number of nematodes for a long period. *Steinernema feltiae* residual activity against cherry fruit fly *R. cerasi* spanned to 1 week at temperatures of 25 °C.[35,38] However, *S. feltiae* is a nematode that is relatively cold tolerant and better adapted to cooler temperatures.[42,43] Variability of different geographic strains to different temperatures[43] should be accounted for when interpreting results from various studies but nonetheless the broad consensus of *S. feltiae* superior efficacy in relatively cool temperatures is supported.

Our experiments also suggest that all three nematode species can infect medfly larvae inside apples and oranges, as assessed by adult fly emergence. Parasitized larvae were found inside 20% of the fruits; we hypothesize that smaller larvae infected with nematodes are completely decayed and not visible after weeks at the time of inspection and assessment. The foraging behaviour of different EPN species explains the difference in their efficacy. 'Cruiser' nematodes, such as *H. bacteriophora*, actively move in the substrate searching for hosts and therefore they can move to deeper soil depths and infect soil-dwelling pests. 'Ambushers', such as *S. carpocapsae*, employ a 'sit-and wait' strategy and infect insects in the soil surface, e.g. fly larvae digging into the soil, whereas species such as *S. feltiae* employ an intermediate strategy.[44–48] *S. feltiae* is thus ideally suited to infect medfly larvae both within soil and fruits and in the surface of the soil, but also *S. carpocapsae* was capable to penetrate inside fruits, despite its ambushing foraging behaviour strategy; *S. carpocapsae* was also successful in infecting *B. oleae* larvae inside olives.[28] The role of organic content in the soil also influences nematode movement; *S. feltiae* moved better than *S. carpocapsae* in a substrate of 75% sand and 25% peat soil.[49] This is relevant to our study because medfly larvae pupate within 5–10 cm of top soil,[50] which can be typically high in organic matter, as it is in the case of mulches used in citrus orchards.[51]

The cost of applying EPN against medfly limits their use because the medfly has many generations per year, develops high populations and consequently large numbers of larvae are in the soil for a long period. As a result, many applications of EPN per year in high doses are required to suppress their population. As an alternative strategy, a single application of EPN could be carried out off-season from the beginning to the end of autumn depending on the crop and/or early in the season, targeting the last cohort of larvae that fall on the soil to pupate and also the overwintering generation, thus preventing population growth later in the season. Our experimental conditions simulated a scenario wherein EPN are applied in the autumn (off-season) once at a relatively low dose (1.5 mi m^{-2}) to the soil beneath the tree canopy, making the application affordable with the scope of reducing the load of overwintering flies that will be emerging in the next spring. The dose we used in our experiments (1.5 mi m^{-2}) is not considered excessively high given that EPN applications are in many cases recommended to be at least 2.5 mi m^{-2}.[52] Relatively cooler autumn temperatures could improve the residual activity and therefore the efficacy of *S. feltiae* against medfly larvae in the soil and in fruits, complementing or even replacing the sanitation of the infested fruits that fall on the ground, which can be a laborious and costly task. These laboratory results should also be confirmed in field trials and furthermore similar interventions with EPN drenches could be tested in early spring, targeting first-generation larvae that fall to the ground to pupate or even the first adult flies from overwintering populations that emerge.[53]

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CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

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