Turkish Journal of Agriculture and Forestry

Volume 39 | Number 2 Article 12

1-1-2015

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TÜBİTAK

**Turkish Journal of Agriculture and Forestry**

**http://journals.tubitak.gov.tr/agriculture/**

**Research Article**

Turk J Agric For
(2015) 39: 277-285

doi:10.3906/tar-1410-33

# Efficacy of the entomopathogenic nematode *Heterorhabditis bacteriophora* using different application methods in the presence or absence of a natural enemy

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**Received:** 09.10.2014 • **Accepted:** 22.11.2014 • **Published Online:** 06.04.2015 • **Printed:** 30.04.2015

**Abstract:** Various application methods for the entomopathogenic nematode *Heterorhabditis bacteriophora* were evaluated under laboratory and field conditions. Four different methods of applying the infective juveniles (IJs) of the nematode to soil were assessed including (1) insect cadavers (referred to as nematode-infected cadavers), (2) subsurface injection, (3) spraying, and (4) drip irrigation. In the laboratory experiment, except for the control with no nematodes, all treatments showed more than 95% insect mortality of the bait insect (*Galleria mellonella*) with no significant differences among treatments. The same experimental setup was conducted with the introduction of mites, *Sancassania polyphyllae* (Acari: Acaridae), which are natural enemies of entomopathogenic nematodes. The treatment groups with mites and the control group without mites showed more than 87% insect mortality and no significant differences were observed. The nematode-infected cadaver application method was further evaluated by using cadavers of different ages containing *H. bacteriophora* IJs in the presence of mites. Larval mortality of the bait insect was significantly lower when 3- or 6-day-old nematode-insect cadavers were used in the presence of mites. Different application methods were also tested in a corn field. No significant differences were observed among the application methods. Our results indicate that the different application methods had no significant effect on larval mortality, but the presence of mites had a negative effect on the cadaver application when the cadavers were 3 days old but had no significant effect when the cadavers were 6 and 9 days old.

**Key words:** Application methods, biological control, entomopathogenic nematode, *Heterorhabditis bacteriophora*

## 1. Introduction

Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) are mutualistically associated with insect-pathogenic bacteria and together they kill their insect hosts. The steinernematids are associated with the bacterial genus *Xenorhabdus*, whereas the heterorhabditids are associated with the genus *Photorhabdus*. These EPNs have adapted specific mechanisms to transmit the bacteria to their insect hosts (Dillman et al., 2012) and are considered good candidates for integrated pest management of soil insect pests (Lacey and Georgis, 2012). In fact, several nematode species are produced commercially and applied in a variety of cropping systems in many different countries (Alves, 1986; Garcia et al., 2008). These biological control agents must be delivered in a way that enables the infective juveniles (IJs) of the nematodes to survive and infect their hosts (Shapiro-Ilan et al., 2006; Brusselman et al., 2012). EPNs can be applied with nearly all agronomic or horticultural ground equipment including pressurized tank sprayers, mist blowers, electrostatic sprayers, drip irrigation systems, or even aerial sprayers (Georgis, 1990; Wright et al., 2005; Shapiro-Ilan et al., 2006, 2012).

Although EPNs can be applied and be effective against a number of soil insect pests, biotic and abiotic agents can influence the outcome of their applications. For example, antagonistic biotic factors such as nematode pathogens or predators including bacteria, protozoa, nematophagous fungi, mites, turbellarians, collembolans, and nematodes will feed on naturally occurring or applied EPN IJs (Kaya, 2002; Hazır et al., 2003); this predation may have a negative effect on their survival and persistence. Moreover, phages can reduce the efficiency of the nematode's mutualistic bacteria (Kaya, 2002), and abiotic factors such as ultraviolet radiation, soil moisture/relative humidity, and high temperatures can also reduce survival and efficacy of EPNs (Kaya, 1990; Shapiro-Ilan et al., 2006).

Effective and efficient delivery of EPNs can be achieved with careful consideration of available application

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technology coupled with an understanding of their assets and limitations (Shapiro-Ilan et al., 2006). However, because of issues with some biotic and abiotic factors affecting the application of IJs, an approach that has gained some attention is the delivery of nematode-infected hosts (also referred to as nematode-infected cadavers) (Jansson et al., 1993; Shapiro-Ilan and Glazer, 1996; Del Valle et al., 2008). Advantages of the cadaver application approach relative to standard IJ applications in aqueous suspension include increased nematode dispersal (Shapiro-Ilan and Glazer, 1996), infectivity (Shapiro-Ilan and Lewis, 1999), survival (Perez et al., 2003), and efficacy (Shapiro-Ilan et al., 2003), but other studies have not detected benefits in the nematode-infected cadaver approach (Bruck et al., 2005).

Herein, we report on the effects of different application methods including cadavers, subsurface injection, sprayers, and drip irrigation. As a model we used the EPN *Heterorhabditis bacteriophora* against a bait insect in the soil with corn plants. In previous studies, we investigated the impact of the nematode predator *Sancassania polyphyllae* on EPN IJs released into the soil and on insect cadavers containing EPNs or IJs emerging from the cadavers (Karagoz et al., 2007; Ekmen et al., 2010a; Cakmak et al., 2013) under laboratory conditions. Two female *S. polyphyllae* consumed more than 80% of *S. feltiae* IJs on an agar medium in 24 h and mites also found and consumed nematode-infected cadavers before IJ emergence was initiated in the soil profile. Therefore, in the present study, we evaluated the effects of different application methods in the presence and absence of *S. polyphyllae*.

## 2. Materials and methods

### 2.1. *Galleria mellonella*, *Sancassania polyphyllae*, and nematode culture

The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), was reared in the laboratory using an artificial medium containing 11% honey, 11% glycerol, 22% ground wheat, 22% ground maize, 11% milk powder, 5.5% yeast extract, and 17.5% bee wax in a glass jar at 25 ± 4 °C (Han and Ehlers, 2000). The last instar larvae of the waxworm were used for nematode rearing and evaluation of the application methods.

Living *Polyphylla fullo* (Coleoptera: Scarabaeidae) larvae that were confirmed to have the deutonymphal (hypopus) stage of *S. polyphyllae* mites were cut in half with a sterile scalpel, and the two halves of an individual larva were placed in a petri dish (9 cm diameter) containing a piece of moist filter paper. As the mites developed and fed on the insect host tissues, new dissected host tissues from frozen *P. fullo* (after thawing) were added every second or third day. Adult female mites that were 2 to 3 days old were collected as needed for the experiments.

The Turkish isolate *H. bacteriophora* (09–43) was reared in the *G. mellonella* larvae according to the methods described by Kaya and Stock (1997). Nematode-killed larvae were transferred to a White trap to collect the emerging IJs from cadavers. Freshly emerged IJs were harvested and rinsed three times in distilled water and stored at 15 °C in Tetra Pak juice boxes (Gülcü and Hazır, 2012). The IJs were used within a week after emergence for the experiments. To obtain nematode-infected cadavers for the experiments, five last instar *G. mellonella* were placed in a 9-cm plastic petri dish containing filter paper and exposed to 500 IJs for 2 days, and then transferred to a holding petri dish for an additional 8 days. Sufficient *G. mellonella* were infected to obtain 3-, 6-, 9-, or 10-day-old cadavers to be used in subsequent experiments.

### 2.2. Preparation of pots with corn plants

For the laboratory study, plastic pots (1.3 L) with a 100 $cm^2$ surface area were used in the experiments. Each pot was filled with 1 kg of sterilized and air-dried loamy soil (48% sand, 10% clay, 42% loam) and 100 mL of water was added. Then, a single corn seed was planted in each pot. An additional 100 mL of water was added to the bottom plate of the pot. The plants were watered as needed. The prepared pots were kept at 28 °C and 3-week-old plants (approximately 20 cm high) were used for the experiments.

### 2.3. Evaluation of different nematode application methods in the laboratory

Four living (i.e. "healthy") *G. mellonella* larvae were kept individually in a wire mesh cage and buried 5 cm deep in each pot. Five different application methods (i.e. treatments) were evaluated, and nematodes were applied at the rate of 25 IJs/$cm^2$. Each treatment had 10 pots and the experiments were conducted at room temperature and evaluated 7 days later. Experiments were repeated three times on different dates.

The following application methods (treatments) were evaluated:

Treatment-A (Nematode-infected cadaver) – A 10-day-old infected cadaver containing IJs was buried at 2 cm soil depth. New generation IJs of *H. bacteriophora* 09-43 isolate emerged from the cadavers 12–13 days after nematode infection.

Treatment-B (Subsurface injection) – A surgical syringe (60 mL) was used for nematode inoculation (2500 IJs) on the subsurface of the soil. Before injection, the syringes were shaken vigorously to prevent settling of IJs.

Treatment-C (Spraying method) – A 300-mL hand sprayer was used to dispense the nematodes. A nematode suspension with 2500 IJs was sprayed on the surface of the soil in 50 mL of water. After 5 min, an additional 10 mL of water was applied to allow the nematodes to get into the soil.

Treatment-D (Drip irrigation) – Glass serum bottles were used in this experiment. The bottles were filled with 60 mL of nematode suspension (2500 IJs) and the nozzles were opened to dispense approximately 15 drops/minute. The bottles were shaken to distribute the IJs in the bottle.

Treatment-E (Control) – The pots in controls received 60 mL of distilled water in the bottom plate of the pot without any nematodes.

All the experimental groups were examined for the mortality of the *G. mellonella* larvae by destructive sampling 1 week after treatment. Each corn plant was uprooted and the insect larva in the wire mesh cage was checked for mortality. Cadavers were transferred individually to White traps to verify that they had been killed by the *H. bacteriophora*.

**2.4. Effect of *S. polyphyllae* on nematode application methods in the laboratory**

The effect of *S. polyphyllae* on different nematode application methods was determined. The same experimental setup was arranged as described above and 100 female mites were added to the soil surface of each pot 1 day before the nematode treatments. Larval mortality was checked after a week. Only IJs were added in the control group (no mites). Each group had 10 replicates and the experiments were conducted on different dates and repeated three times. Cadavers were transferred individually to the White traps to verify that they had been killed by nematodes.

**2.5. Effect of *S. polyphyllae* on nematode-infected cadavers of different ages in the laboratory**

In this experiment, the effect of *S. polyphyllae* was evaluated against nematode-infected cadavers of different ages. The experiment was conducted in pots (as described above) containing 3-week-old corn plants. A hundred mites were added to each pot 1 day before treatment for acclimatization. Four *G. mellonella* larvae were caged in a wire mesh individually and buried 5 cm deep in each pot. Then, a single 3-, 6-, or 9-day-old nematode-infected cadaver was buried 2 cm below the soil surface. The control group received only a 3-, 6-, or 9-day-old nematode-infected cadaver without mites. Each group had 10 pots. Larval mortality was checked after a week. The experiments were conducted on different dates and repeated three times. Cadavers were transferred individually to the White traps to verify that they had been killed by *H. bacteriophora*.

**2.6.Evaluation of different nematode application methods in the field**

The efficacy of different application methods were tested in a corn field in Serçeköy village in Aydın, Turkey. Two experimental plots were selected separately for each group of nematode application methods. Plants that were approximately 40 cm tall were selected at random (with one buffer plant between each within the row), and four *G. mellonella* larvae held individually in a wire mesh cage were buried 5 cm deep around each treated plant. A 15-cm white string was tied to the cage and the string was exposed on the soil surface so that each wire cage could be easily located. The treatments were: 1) IJs sprayed around the soil surface of the plant, 2) one 10-day-old nematode-infected cadaver buried 2 cm deep near the plant, 3) IJs injected 2-3 cm deep into the soil with a syringe near the base of the plant, 4) IJs applied by drops on the soil surface from a serum bottle around the plant, and 5) only water was applied to the plant that served as the control. Except for the nematode-infected cadaver treatment, the number of IJs per plant was 2500 in 60 mL of water. As soil moisture was adequate, no additional water was added to the field soil after treatment. Applications were carried out between 2000 and 2100 hours to avoid UV radiation and high ambient temperatures. The soil temperature was 32 °C when the nematodes were applied and the soil water content was 17% (w/v). Each treatment was randomly assigned to each block. The treatments were evaluated 6 days later by retrieving the wire cages and checking each larva for mortality and nematode infection. Each application method had 2 replicates in the same field and 10 plants were used for each treatment in each plot. The experiment was conducted twice on different dates (four replicates total).

**2.7. Statistical analysis**

Treatment effects were analyzed using two-way analysis of variance (ANOVA). Means were compared at the $P = 0.05$ level, and Tukey's test was used to separate the means (SAS, 1989). The data obtained from the effect of *S. polyphyllae* on nematode-infected cadavers were analyzed by Student's t-test after Abbott (1925) and arcsine transformation.

## 3. Results

**3.1. Evaluation of different nematode application methods in the laboratory**

The percentage mortality of the insect larvae was calculated for all application methods. All nematode treatments showed more than 95% insect mortality, with control mortality being less than 2%. However, there was a significant difference between the nematode treatments and the control ($F = 218.67$; $df = 4, 143$; $P < 0.0001$) (Figure 1). No significant differences were observed among the nematode treatments (Figure 1).

**3.2. Effects of *S. polyphyllae* on nematode application methods in the laboratory**

All nematode applications with mites and the nematode control without mites had more than 87% insect mortality and no significant differences were observed among the treatments ($F = 2.48$; $df = 4, 143$; $P > 0.05$).

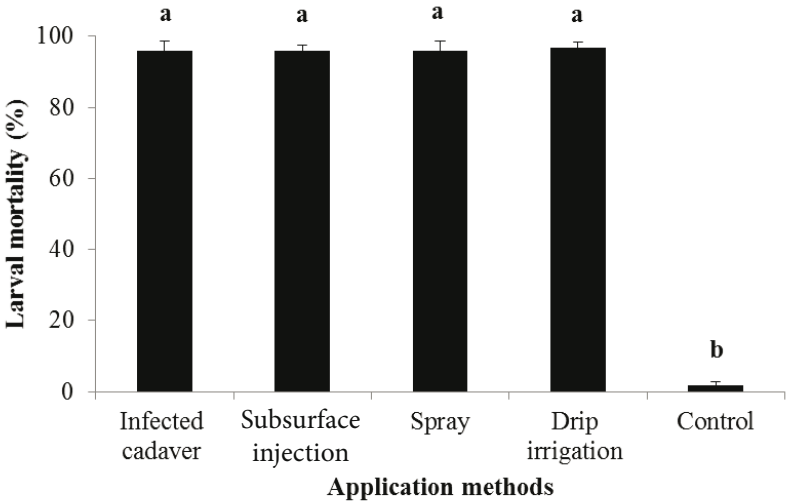


**Figure 1.** Percentage mortality (mean + SE) of *Galleria mellonella* larvae in pot experiments treated with different application methods. Different letters above bars indicate significant differences ($P < 0.05$).

### 3.3. Effects of *S. polyphyllae* on nematode-infected cadavers of different ages in the laboratory

The age of the nematode-infected cadaver applied had a significant negative effect on efficacy in the presence of *S. polyphyllae*. The 3-day-old nematode-infected cadaver treatments ($t = 1.96$; $df = 58$; $P = 0.027$) had significantly lower *G. mellonella* mortality when the mites were present compared to when mites were absent (Figure 2). However, if the nematode-infected cadavers were 6 days old ($t = 2.25$; $df = 58$; $P = 0.094$) and 9 days old ($t = 0.31$; $df = 58$; $P = 0.791$), there was no significant difference between treatments with mites and without mites (Figure 2). When we compared the effects of 3-, 6-, and 9-day-old cadaver treatments with each other, the 9-day-old cadaver produced higher *G. mellonella* mortality than the other treatments and the others ($F = 33, 77$; $df = 2, 87$; $P < 0.05$) (Figure 2).

### 3.4. Evaluation of different nematode application methods in the field

Larval mortalities were 68.12%, 61.25%, 61.25%, and 56.87% for spray, subsurface injection, drip irrigation, and infected-cadaver, respectively. No significant differences were observed among the treatments. There was a significant difference between the treatments and control ($F = 14.76$; $df = 4, 192$; $P = 0.0001$) (Figure 3).

## 4. Discussion

EPNs have been applied as biological control agents to suppress a variety of economically important insect pests (Kaya and Gaugler, 1993; Hazır et al., 2003; Grewal et al., 2005). In our study, we used the bait insect *G. mellonella* to establish whether different application methods affected efficacy in the laboratory and field. Our data showed that there were no significant differences among the tested application methods in the laboratory and field experiments using *G. mellonella*. In addition, we investigated whether a mite predator of nematodes can affect efficacy using the different application methods and whether the mite predator affects efficacy using the different ages of insect-infected cadavers in the laboratory. Our results show that *S. polyphyllae* female mites found and consumed 3-day-old nematode-infected cadavers in the soil because there were significant differences between larval mortality with and without mites.

As shown in our study, we can achieve high mortality of the bait insect in the laboratory using different application methods. In fact, EPNs have provided high mortality against a great number of insect pests under laboratory conditions, but the same successful results do not always occur in the field (Klein, 1990; Shapiro-Ilan et al., 2002). To achieve successful applications in the soil environment, a variety of abiotic and biotic factors such as temperature, moisture, UV light, and natural enemies must be considered (Kaya, 1990). Application equipment and methods are also crucial factors that can affect efficacy in the field (Curran, 1992; Bullock et al., 1999; Hayes et al., 1999; Shields et al., 1999). Yet, a number of studies have reported significant differences between and among nematode application methods. For example, Curran (1992) reported that trickle irrigation was inferior to surface spray or multiple injections of nematodes, and Hayes et al. (1999) reported that sprinkler irrigation was inferior to a boom sprayer.

Each application method has advantages and disadvantages. In general, IJs are usually applied in aqueous

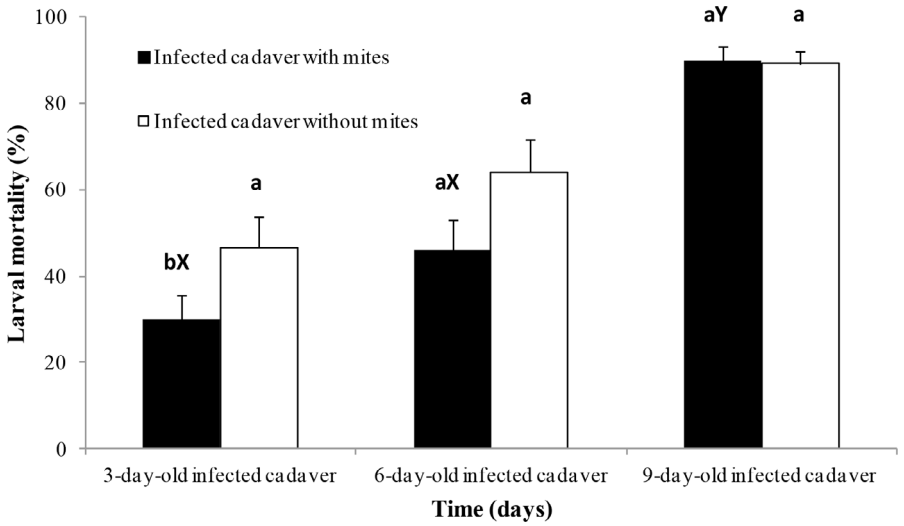


**Figure 2.** Percentage mortality (mean + SE) of caged *Galleria mellonella* larvae in treatments with *Heterorhabditis bacteriophora*-infected cadavers of different ages in the presence or absence of the natural enemy, *Sancassania polyphyllae*. *S. polyphyllae* feeds on insect cadavers and is also predacious on entomopathogenic nematodes. The same lowercase and uppercase letter indicates no significant difference (P > 0.05). Lowercase letters (a, b) above bars show the comparison of infected cadavers with and without mites whereas uppercase letters (X, Y) show the comparison of 3-, 6-, and 9-day old infected cadavers with mites.

suspensions using nearly all agronomic or horticultural ground equipment (Georgis, 1990; Wright et al., 2005; Shapiro-Ilan et al., 2006), but standard spray systems that are designed for chemical application do not perform very efficiently when applying particulate materials such as nematode IJs (Lello et al., 1996; Mason et al., 1998). Fife et al. (2003, 2004), for example, reported that EPNs can experience a variety of physical stresses when they pass through the spray system.

When compared to conventional spraying, delivering nematodes by irrigation was generally more successful (Cabanillas and Raulston, 1996), but we found that there was no difference among application methods including spraying, subsurface injection, drip irrigation, and infected cadavers. However, irrigation application has its advantages and disadvantages. One advantage of drip irrigation systems is that they are less labor-intensive, but the irrigation systems must be in good condition to allow for an increase in pressure and the use of carboxymethylcellulose (CMC) to prevent sedimentation of the nematode IJs in the tubing. On the other hand, there are several disadvantages; for example, the black irrigation hoses can heat up considerably unless buried and most nematodes will be negatively affected by temperatures > 35 °C for more than 30 min. Moreover, the solubility of oxygen decreases dramatically with increasing temperature and low oxygen concentrations will inactivate nematodes. Leakages in the drip irrigation hose can also result in substantial losses of nematodes and will decrease pressure and flow velocity in the remaining part of the hose. Reed et al. (1986) recovered only 37%–59% of the nematodes injected into a trickle irrigation system, and Conner et al. (1998) demonstrated that such losses were due to EPNs settling in tubing further away from the injection point.

Some major constraints of nematode application to the soil surface are exposure to high soil temperatures, desiccation, and UV radiation. Therefore, it is best to apply nematodes to the soil in the evening or early morning hours, and pre- and postirrigation are also recommended (Wright et al., 2005). In our study, nematodes were applied in the field between 2000 and 2100. Given these constraints of spray technology for applying EPNs to the soil surface, other equipment and approaches have been developed, including subsurface (Cabanillas and Raulston, 1995) and nematode-infected cadaver applications (Shapiro-Ilan et al., 2003). For subsurface application, a seed-driller adapted to apply an aqueous nematode suspension has been found to improve the delivery of *S. glaseri* to turfgrass four-fold compared to an application with a boom sprayer (Smits, 1999). When using a subsurface applicator for EPN application on a golf course against the garden chafer (*Phyllopertha horticola*), excellent efficacy was achieved with one-third the rate used with a boom sprayer (Wright et al., 2005). Shetlar et al. (1993) recorded good control of billbug (*Sphenophorus parvulus*) larvae using a similar subsurface injector to apply 2.6 billion *S. feltiae*/ha at a

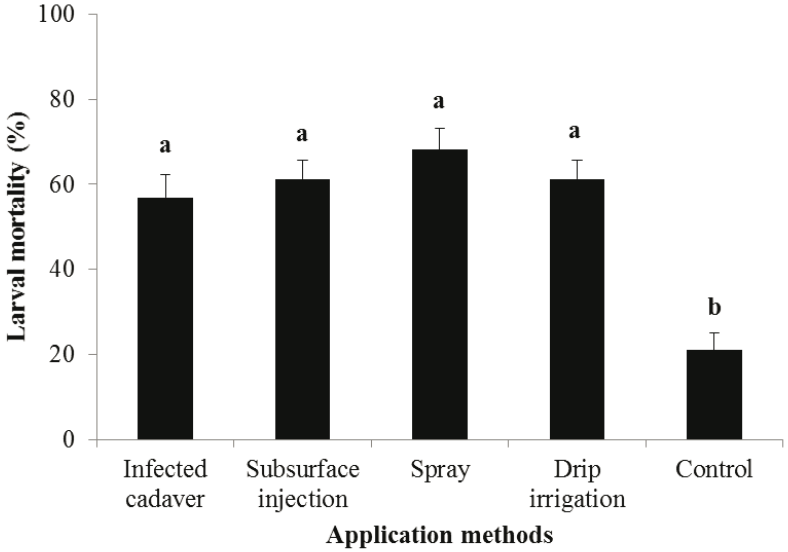


**Figure 3.** Percentage mortality (mean + SE) of *Galleria mellonella* larvae stemming from different application methods in the field. Different letters above bars indicate significant differences ($P < 0.05$).

depth of 2 cm at 1200 L/ha. Subsurface injection could improve the effectiveness of nematodes by placing them throughout the root system using a lance. This approach is particularly important for effective use in soil with high clay content and/or when less active nematode species (i.e. ambushers) are used (Lewis et al., 1992). Likewise, soil injectors have been used to treat strawberry plants under plastic mulch. Curran (1992) tested subsurface injection in field-grown strawberries naturally infested with black vine weevil larvae. *Heterorhabditis* sp. (isolate T390) applied 10 cm deep and six injections per plant was compared with a surface spray and surface application through drip emitters. In one test, the surface spray provided 86% control compared with 65% control with delivery through drip irrigation. In another test, multiple injections were more effective (79% weevil mortality) compared with application through drip irrigation or a single soil injection per plant (61% and 63% mortality, respectively). We also found that subsurface injection of IJs was a successful method, resulting in 96% and 80% larval mortality in the laboratory and field studies, respectively.

The nematode-infected cadaver application method can serve as slow-release systems for EPNs (Shapiro-Ilan et al., 2003). Advantages to the cadaver application approach relative to standard application in aqueous suspension have been reported, such as increased infectivity (Shapiro and Lewis, 1999), survival (Perez et al., 2003), and efficacy (Shapiro-Ilan et al., 2003). Shapiro and Glazer (1996) reported that the dispersal ability of *S. carpocapsae* and *H. bacteriophora* was significantly greater when nematodes were applied in cadavers compared to when they were applied in aqueous suspension. They speculated that the enhanced dispersal may have been caused by physiological or behavioral differences between nematodes exiting hosts and those kept in aqueous suspension. Recently, Kaplan et al. (2012) showed that nematode-infected cadavers emanate an ascaroside (ascr#9) compound that signals IJs to disperse; this finding may explain the differences in dispersal observed by Shapiro and Glazer (1996). On the other hand, when the efficacy of nematode-infected cadavers and aqueous applications for the black vine weevil (*Otiorhynchus sulcatus*), larval control was compared in outdoor trials and the aqueous application showed complete control after 14 days, whereas the cadaver applications provided complete control after 28 days. The authors reasoned that cool soil temperatures delayed or potentially reduced IJ emergence from cadavers, resulting in delayed control (Bruck et al., 2005). Our data did not show better efficacy with the nematode-infected cadaver application compared to the other treatments. Interestingly, although no statistically significant differences were observed among the application methods, we noted that the larvae of a scavenger fly (an unidentified phorid species) infested approximately 20% of the nematode-infected cadavers, which probably negatively affected our results.

A number of biotic agents in soil can also have detrimental effects on EPN applications. The nematodes are subject to infection or predation by certain bacteria, protozoans, nematophagous fungi, predacious mites, nematodes, etc. (Kaya, 2002). Previous research showed that the mite *Sancassania polyphyllae* (Acari: Acaridae) could potentially interfere with biological pest control by feeding on purposely released EPN IJs, cadavers containing EPNs, or IJs emerging from the cadavers

(Karagoz et al., 2007; Cakmak et al., 2010, 2011; Ekmen et al., 2010a, 2010b). In a laboratory study, 10 adult females of *S. polyphyllae* consumed 86% of 500 *H. bacteriophora* IJs on an agar substrate within 48 h (Karagoz et al., 2007). Ekmen et al. (2010b) reported that significantly more *S. polyphyllae* gathered near or on nematode-killed larvae of the medfly (*Ceratitis capitata* (Diptera: Tephritidae)), compared to freeze-killed larvae or bamboo pieces used to mimic medfly larvae, and consumed 96% of the IJs that emerged from a cadaver. Ekmen et al. (2010b) hypothesized that a chemical or an odor from the nematode-killed larvae attracted the mites. Thus, in soil containing a nematode-killed insect, the average number of *S. feltiae* IJs recovered was < 30 when mites were present, whereas the average number of IJs recovered was > 375 when mites were absent. When the IJs alone were placed at different depths in relation to mites in the soil column for 4 and 10 days, *S. polyphyllae* was not as efficient at finding the IJs when they were separated from each other in the soil. Finally, Ekmen et al. (2010a) demonstrated that when offered different food choices *S. polyphyllae* preferred tissues of its phoretic host, *Polyphylla fullo* (Coleoptera: Scarabaeidae), over wax moth *G. mellonella* tissues or living *S. feltiae* or *H. bacteriophora* IJs. It was established that *S. polyphyllae* may recognize the volatiles that emanated from either its dissected phoretic host larvae or insect larvae infected with EPNs over EPN IJs (Cakmak et al., 2013). Our results show that *S. polyphyllae* female mites found and consumed 3-day-old nematode-infected cadavers in the soil because there were significant differences between larval mortality with and without mites. If 6- and 9-day-old and older cadavers were used, there was no significant effect of mites on larval mortality. Shapiro-Ilan et al. (2003, 2010) have demonstrated that applying nematode-killed insects into the soil and allowing the IJs to emerge is an effective biological control tactic of insect pests. However, natural scavengers such as mites or flies in an area to be treated should be taken into consideration before applying nematode-infected cadavers. On the other hand, many insect scavengers do not feed on insects killed by EPNs, especially *Heterorhabditis* species, because of the presence of a scavenger deterrent factor (Gulcu et al., 2012).

**Acknowledgments**

We thank Dr Harry K Kaya, University of California, Davis and David Shapiro-Ilan from the USDA for editing the manuscript. We also thank TÜBİTAK for supporting Dr Ramalingam Karthik Raja with a fellowship. This study was funded by Adnan Menderes University (Project no: FEF – 12002).

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