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ORIGINAL PAPER

Evaluation of entomopathogenic nematodes and their combined application against *Curculio elephas* and *Polyphylla fullo* larvae

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Abstract The larvae of *Curculio elephas* (Coleoptera: Curculionidae) and *Polyphylla fullo* (Coleoptera: Scarabaeidae) are major agricultural pests of chestnut and roots of cultivated crops, respectively. Previous research showed that they are relatively resistant to nematode infection. Accordingly, we evaluated the efficacy of *Steinernema glaseri*, *S. weiseri* or *Heterorhabditis bacteriophora* alone or in combination against these two insect pests. A nematode concentration of 50 or 100 infective juveniles (IJs)/larva for *C. elephas* or 50 or 100 IJs/cm^2 for *P. fullo* at 25 °C was used. The highest (81 %) and the lowest (21 %) larval mortalities for *C. elephas* were obtained with *S. weiseri*+*H. bacteriophora* combined application and *S. glaseri* alone, respectively. The nature of the interactions (antagonism, additive, or synergy) for the larval mortality was evaluated. For *C. elephas*, *S. weiseri* combined with *S. glaseri* or *H. bacteriophora* was additive, whereas the combination of *S. glaseri*+*H. bacteriophora* was antagonistic. For *P. fullo*, the efficiency of nematodes used alone or combinations was very low, and there were no significant differences among the treatments at 50 or 100 IJs/cm^2. The interaction was additive with the combinations of *S. glaseri*+*H. bacteriophora* against *P. fullo* larvae. No synergistic effect was observed for any combination against *C. elephas* and *P. fullo* larvae. Our results show that the EPN species tested, either alone or in combination, are not economically feasible for use against *C. elephas* or *P. fullo*, but that further research with other combinations of EPN species or EPNs with other entomopathogens is warranted.

Keywords *Curculio elephas* · *Polyphylla fullo* · Biological control · Entomopathogenic nematodes · *Steinernema* · *Heterorhabditis*

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Introduction

Chestnut, *Castanea sativa* (Fagales, Fagaceae), is one of the most important agricultural crops in Turkey with annual production of more than 60,000 tons (Ertan and Seferoglu 2003). One of the most serious pests affecting chestnut production is the chestnut weevil, *Curculio elephas* (Coleoptera: Curculionidae), which has the potential to proliferate rapidly and is expanding its geographic range (Avtzis and Cognato 2013). The weevil adults emerge from the soil in August and the females lay their eggs on or in the chestnuts where the larvae feed on the kernel for about 2 months. In October, the majority of the last larval stage emerge from the infested kernels and enter the soil to pupate where they remain for at least 9 months (Desouhant 1998; Speranza 1999). Control of the larval stages of chestnut fruit pests is difficult because they occur within the chestnut fruit and then overwinter in the soil.

Polyphylla fullo (Coleoptera: Scarabaeidae) is a major polyphagous agricultural pest because the larvae feed on the roots of many important cultivated plants. It has a 2- to

3-year life cycle with three larval stages that feed on plant roots causing extensive damage, and, in severe infestations, can cause yield loss and plant death (Anonymous 2008). One of the main crops affected by *P. fullo* is strawberry. Because there is no registered pesticide against this white grub species in Turkey, strawberry producers resort to using chlorpyrifos-ethyl and parathion-methyl which are registered for insect control on fruit trees and vineyards, respectively (Anonymous 2008). These chemical pesticides are used individually or together at high concentrations, but the results have been unsatisfactory.

Research on developing alternative control methods for suppressing *C. elephas* and *P. fullo* is needed. One of the possible methods is using entomopathogenic nematodes (EPNs) against the larval stages that occur in the soil. For *C. elephas*, the approach is to reduce the overwintering population and to prevent or reduce damage to the next generation of chestnuts. For *P. fullo*, it is to reduce the larval population and protect the strawberry roots from damage.

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae are obligate pathogens in nature with the free-living, third-stage infective juveniles (IJs) searching for and infecting their insect host in the soil environment (Kaya and Gaugler 1993). They are associated with mutualistic bacteria in the genus *Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis*, and these bacteria are housed in the IJs in a specialized intestinal lumen in the case of steinernematids and in the intestine of heterorhabditids (Hazir et al. 2003). The IJ infects the insect host by entering through natural openings (mouth, spiracles, or anus) or thin areas of the host's cuticle (common in heterorhabditids) and penetrates into the host's hemocoel. The IJ then releases the bacterium which propagates and causes septicemia that kills the host in 48–72 h. The nematode resumes its development, feeding on the bacterial cells and host tissues that have been metabolized by the bacterium and goes through 1–3 generations, depending on host size. As the food resources in the host cadaver are depleted, a new cohort of IJs is produced and emerges from the host cadaver into the soil to search for new hosts (Kaya and Gaugler 1993). Most EPN species can infect a variety of insects, especially if ecological and behavioral barriers are removed. On other hand, some insect species are partially or completely resistant to EPN infection due to behavioral, morphological, or physiological defense mechanisms (Gaugler et al. 1994; Koppenhöfer et al. 2000).

In a laboratory study conducted in Turkey, Karagoz et al. (2009) demonstrated that the last instar larvae of *C. elephas* and *Cydia splendana* (Lepidoptera: Tortricidae), another pest of chestnut, responded differently to infection by three Turkish isolates of *Steinernema feltiae*, *S. weiseri*, and *Heterorhabditis bacteriophora*. *C. splendana* larvae were highly susceptible, whereas *C. elephas* larvae were relatively resistant to the tested EPN species. In another laboratory study, Kepenekci et al. (2004) showed that two Turkish isolates of *H. bacteriophora* caused 72.1 and 96.5 % mortality, respectively, of *C. elephas* larvae at 25 °C using 500 IJs/cup, whereas *S. feltiae* and *S. carpocapsae* were less effective. They also demonstrated that the two Turkish *H. bacteriophora* isolates required 266 and 494 IJs to kill 50 % of *C. elephas* larvae at 15 °C. Thus, *C. elephas* larvae are not easily killed by EPNs. Against *P. fullo* larvae, Karagoz et al. (2007) evaluated the efficiency of 35 new EPN isolates and reported that this white grub species was highly resistant to EPN infection. Similarly, Karimi et al. (2010) tested *H. bacteriophora* against the white grub *Polyphylla adspersa* in Iran and obtained only 42 % mortality. We focused our research on increasing the efficacy of EPNs on *C. elephas* and *P. fullo* because they are important pests in Turkey, are difficult to control with chemical insecticides, and have immature life cycle stages in the soil, and there is a need for alternate, safer control agents for these pests.

The standard approach of using EPNs against *C. elephas* and *P. fullo* is not feasible because they are resistant to EPN infection. A different application tactic has been used against insect pests that are naturally resistant to EPN infection, and this approach may be feasible against the two pests. For example, Choo et al. (1996) tested the efficiency of combinations of two EPN species against second-stage larvae of *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae) in the laboratory. They demonstrated that a combination of two nematode species was more efficacious than one species alone. In another study by Sankar et al. (2009), a combination of *H. indica* and *S. asiaticum* killed larvae of the rice leaf folder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) more quickly than each EPN species applied alone. Combined applications of three different nematode species, H. *bacteriophora*, *S. kushidai*, and *S. glaseri*, showed additive interactions against the third-instar masked chafer *Cyclocephala hirta* (Coleoptera: Scarabaeidae) and oriental beetle *Exomala* (=*Anomala*) *orientalis* (Coleoptera: Scarabaeidae) (Koppenhöfer et al. 2000). Accordingly, we hypothesized that using a combination of different EPN species against *C. elephas* and *P. fullo* larvae may be more efficacious than a single EPN species. Our objective was also to evaluate whether combined application of EPNs results in an antagonistic, additive, or synergistic effect on *C. elephas* or *P. fullo* larvae.

Materials and methods

Insects

Last instar larvae of *C. elephas* emerged from the infested chestnuts were collected from a chestnut processing factory

in Aydin and used within 3 days. The 2nd and 3rd instar *P. fullo* larvae were collected from strawberry fields in the Umurlu district of Aydin, Turkey. The larvae were kept for a minimum of 1 week in 20-l plastic pots (8–10/pot) filled with sterilized soil collected from the strawberry fields (to detect and eliminate diseased and/or injured individuals) and fed sliced carrots before they were used in the experiments.

Late instar larvae of the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), reared on an artificial medium as described by Han and Ehlers (2000) were used to produce IJs.

Nematodes

Steinernema glaseri (Belgian isolate) isolated and identified by Ansari et al. (2005), and the Turkish isolates of *S. weiseri* (09-01) isolated and identified by Unlu et al. (2007) and *H. bacteriophora* (09-20) identified by Dr. Patricia Stock by morphological/morphometric and molecular techniques (unpublished) were reared in last instar *G. mellonella* larvae (Kaya and Stock 1997) and stored in distilled water at 10 °C in tetra pak boxes (Gulcu and Hazir 2012) for up to 2 weeks before use in experiments.

EPNs against *Curculio elephas* larvae

The experiments were conducted in 24-well tissue culture plates. Each well of the tissue culture plates was filled with 0.5 g autoclaved and air-dried sandy soil (87.4 % sand and 12.6 % silt) and 60 µl distilled water was added to each treatment. Soil moisture was 10 % (w/w). After 1 h at room temperature (23–24 °C) to allow for acclimatization of the IJs, one *C. elephas* larva was added to each well. Treatments were *S. glaseri*, *S. weiseri*, or *H. bacteriophora* alone at 50 and 100 IJs/larva, two-species combinations of each EPN species at 50 IJs per species (100 IJs total), and a water only control. There was one replicate (24 insects) for each treatment and the control, and the experiment was repeated three times on different dates.

The treated tissue culture plates were placed into plastic bags to minimize moisture loss and kept at room temperature. Each larva was checked daily for 10 days using a probe to determine whether it was alive or dead. All dead larvae were transferred individually to White traps to confirm EPN infection by the production of IJs emerging over a 2-week period.

EPNs against *Polyphylla fullo* larvae

The first experiment was conducted at room temperature in 350-ml plastic containers (30 cm^2 surface area) filled with 100 g autoclaved and air-dried loamy soil (48 % sand, 10 % clay, 42 % loam) prepared at 10 % (w/w) soil moisture. The soil had been collected from an infested strawberry field. One *P. fullo* larva was added to each container, and a slice of carrot was placed into the soil for food. After 1 h, treatments in 3 ml of water were applied to soil surface with a pipette. Treatments were *S. glaseri* alone and *H. bacteriophora* alone [both at 1,500 and 3,000 IJ per container (50 and 100 IJs/cm^2)], their combination (1,500 IJs of each species per container), and a water only control. There were 10 replications per treatment, and the experiment was conducted three times on different dates. The mortality of *P. fullo* larvae was recorded daily for 10 days. Dead larvae were transferred individually to White traps, incubated at room temperature, and checked for IJ emergence over a 3-week period.

A second experiment was conducted in plastic pots (1.3 l volume; 13 cm depth × 12 cm diameter; surface area = 113 cm^2) filled with 1 kg of the same sandy soil as used above. One larva along with a piece of carrot was placed into each pot. The treatments were (1) *S. glaseri* alone, (2) *H. bacteriophora* alone [both at 11,300 IJs per container (100 IJs/cm^2)], (3) their combination (11,300 IJs of each species per container), and (4) water only control.

There were 10 replications for each treatment and the experiment was repeated three times on different dates. The mortality of *P. fullo* larvae was recorded after 10 days, and dead larvae were transferred individually to White traps for monitoring nematode emergence for 3 weeks.

Further verification of nematode-caused mortality

The color of the cadaver and the length of the new generation of IJs were used to determine which nematode species caused the larval mortality and reproduced in the cadaver. This verification was done for the nematode combination treatments for both *C. elephas* and *P. fullo* cadavers. Red cadavers indicated *H. bacteriophora* infection, whereas light-brown cadavers indicated *S. glaseri* or *S. weiseri* infections. A subsample was taken from each cadaver and the IJs were heat-killed by hot water (60 °C for 2 min). Twenty IJs were randomly selected and measured with a Leica IM50 microscope equipped with automatic measurement system.

Statistics

The treatment mortality was adjusted for control mortality (<10 %) using Abbott's formula (1925). Percentage data obtained from 3 replications were combined in the same analyses and arcsine-transformed before statistical analysis. One-way ANOVA was used to compare larval mortality among treatments and the means separated using Tukey's test ($P = 0.05$) (SPSS 2004).

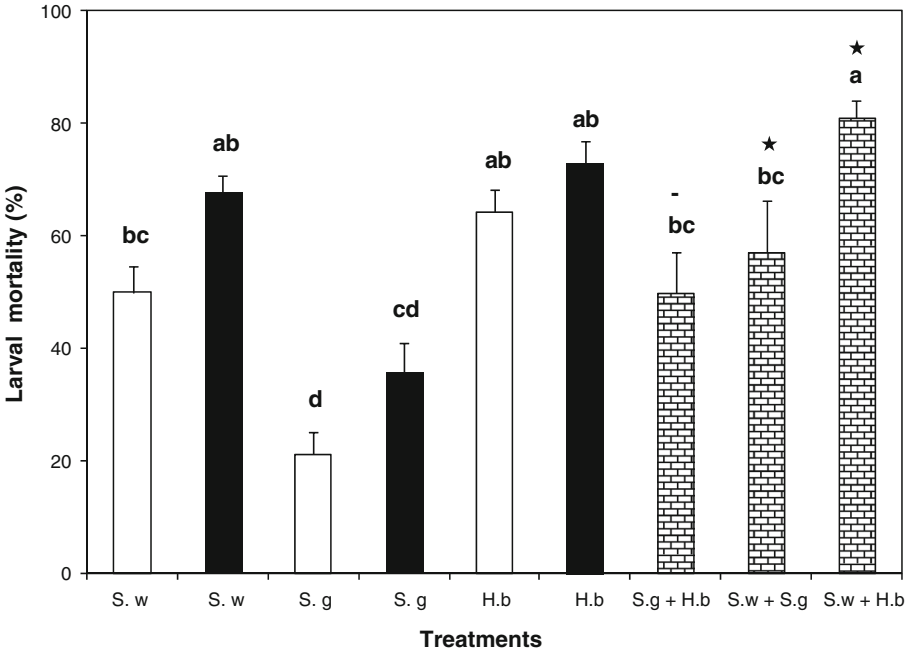

Fig. 1 Mean mortality (%) of *Curculio elephas* larvae by entomopathogenic nematode species alone or in combination. Different *lower case letters* above the *bars* indicate significant differences at $P < 0.05$. *S. w Steinernema weiseri*, *S. g S. glaseri*, *H. b Heterorhabditis bacteriophora*. *White bar* 50 IJ concentration of one nematode species; *black bar* 100 IJ concentration of one nematode species; *hatched bar* 100 IJ concentration with 50 IJs of one species and 50 IJs of another species. *Symbol* above *shaded bars* indicates (*hyphen*) antagonistic or (*filled star*) additive interaction

The nature of interaction in all experiments between nematode species on larval mortality was determined through a comparison of expected and observed percentage larval mortality (Shapiro-Ilan et al. 2004). Expected mortality was calculated with the formula $P_E = P_0 + (1-P_0)(P_1) + (1-P_0)(1-P_1)(P_2)$, ($P_E$: expected mortality; P_0: control mortality; P_1: mortality from one nematode applied alone; P_2: mortality from the other nematode applied alone). A Chi square (χ^2) test was applied to the observed and expected results. $\chi^2 = (L_0-L_E)^2/L_E + (D_0-D_E)^2/D_E$ (Lo: the number of living larvae observed; L_E: the number of living larvae expected; Do: the number of dead larvae observed; D_E: the number of dead larvae expected). If the value of $\chi^2 < 3.84$ = additive, $\chi^2 > 3.84$ and Pc–P_E positive = synergistic, $\chi^2 > 3.84$ and P_C–P_E negative = antagonistic (P_C: observed mortality; P_E: expected mortality).

Results

EPNs against *Curculio elephas* larvae

Statistically, the highest larval mortalities were obtained with *S. weiseri*+*H. bacteriophora* combination application followed by *H. bacteriophora* and *S. weiseri* at 100 IJs alone, and *H. bacteriophora* at 50 IJs alone. These mortalities were significantly different from *S. glaseri* alone at 50 and 100 IJ concentrations, respectively ($F = 15,\ 414$; $df = 8,\ 17$; $P < 0.0001$) which had the lowest larval mortality (Fig. 1). However, there were no significant differences among *H. bacteriophora* or *S. weiseri* at 100 IJs alone or *H. bacteriophora* at 50 IJs alone or *S. weiseri* at 50 IJs alone, or *S. weiseri*+*S. glaseri* combination, or *S. glaseri*+*H. bacteriophora* combination. In addition, there were no significant differences among *S. weiseri* at 50 IJs alone, or *S. weiseri*+*S. glaseri* or *S. glaseri*+*H. bacteriophora* combination when compared with *S. glaseri* at 100 IJs alone ($P > 0.05$) (Fig. 1). *S. weiseri*+*S. glaseri* (57 % ± 9.3) and *S. weiseri*+*H. bacteriophora* (81 % ± 3.1) resulted in additive effects, whereas the combination of *S. glaseri*+*H. bacteriophora* (50 % ± 7.1) showed an antagonism interaction (Fig. 1; Table 1).

Verification of nematode-caused mortality in *Curculio elephas* cadavers

Combination of S. glaseri+*H. bacteriophora*

When *S. glaseri* and *H. bacteriophora* species were applied together, *H. bacteriophora* reproduced in 56 % (±11.0) and 53 % (±15.6) of the cadavers, whereas *S. glaseri* species reproduced in 44 % (±13.5) and 47 % (±15.7) of the nematode-killed insects at 50 and 100 IJs/larva, respectively. *S. glaseri* and *H. bacteriophora* did not produced progeny from the same cadavers.

Combination of S. weiseri+*H. bacteriophora*

At both concentrations, *S. weiseri* had more cadavers with progeny than *H. bacteriophora*. At 50 IJs/larva, *S. weiseri* and *H. bacteriophora* had 62 % (±22.0) and 38 % (±22.5)

Table 1 Interactions observed when combining the entomopathogenic nematode species, *Steinernema weiseri*, *S. glaseri*, or *Heterorhabditis bacteriophora* for suppression of *Curculio elephas or Polyphylla fullo larvae*

Nematodes	Host	Observed mortality	Expected mortality	χ^2	Interaction
S. weiseri+*S. glaseri*	*C. elephas*	57	60	0.20	Additive
S.weiseri+*H. bacteriophora*	*C. elephas*	81	82	0.01	Additive
S.glaseri+*H. bacteriophora*	*C. elephas*	50	71	6.49	Antagonism
S.glaseri+*H. bacteriophora*	*P. fullo*	9.9	8.5	0.21	Additive
S.glaseri+*H. bacteriophora*[a]	*P. fullo*	19	13	2.00	Additive

Interactions were based on a χ^2 ratio of expected:observed mortality

[a] Pot experiment

of the cadavers with progeny, respectively. At 100 IJs/larva, 60 % (±24.9) and 40 % (±24.9) reproduction were observed for *S. weiseri* and *H. bacteriophora*, respectively. No progeny of *S. weiseri* and *H. bacteriophora* together was observed in the same cadaver.

Combination of S. weiseri+S. glaseri

At 50 IJs/larva, *S. weiseri* had 58 % (±11.5) of the cadavers with progeny followed by *S. glaseri* at 36 % (±8.0) and S. *weiseri* and *S. glaseri* together in the same cadaver at 6 % (±6.9). Only 2 from 34 cadavers produced co-progeny. At 100 IJs/larva, *S. glaseri* reproduced in 69 % (±14.4) of the cadavers, whereas *S. weiseri* (±14.4) reproduced in 31 %. At this concentration, no progeny of *S. weiseri* and *S. glaseri* occurred together in the same cadaver.

EPNs against *Polyphylla fullo* larvae

In the plastic container experiments conducted at 50 IJs/cm^2, the larval mortality was 2.9, 5.7, and 6.3 % for *S. glaseri*, *H. bacteriophora,* and the *S. glaseri*+*H. bacteriophora* combination, respectively. No statistical difference was observed among the treatments ($F = 0.601$; $df = 2, 6$; $P > 0.05$).

The combination of *S. glaseri*+*H. bacteriophora* resulted in an additive interactions of *P. fullo* mortality (Table 1). At 100 IJs/cm^2, *S. glaseri* resulted in 8.8 % larval mortality followed by *H. bacteriophora* at 12 % and *S. glaseri*+*H. bacteriophora* at 9.9 %. There was no significant difference among the groups ($F = 0.275$; $df = 2, 6$; $P > 0.05$).

In the pot experiments, although *S. glaseri*+*H. bacteriophora* combination showed numerically more mortality (24 % ± 20.4) than *S. glaseri* (7 % ± 4.9) and *H. bacteriophora* (11 % ± 6.0) alone applications, there was no statistical difference among the treatments ($F = 0.46$; $df = 2, 6$; $P > 0.05$). However, the combination treatment of *S. glaseri*+*H. bacteriophora* did cause an additive interaction of *P. fullo* mortality (Table 1).

Verification of nematode-caused mortality in *Polyphylla fullo* cadavers

In the plastic container experiments, when *S. glaseri* and *H. bacteriophora* were applied together, only *S. glaseri* reproduced into the cadavers. In the pot experiments, 71 % of the cadavers produced *S. glaseri* IJs, whereas 29 % of the cadavers produced *H. bacteriophora*. No mixed progeny were observed in the same cadaver.

Discussion

The combined application of entomopathogens can result in antagonistic, additive, or synergistic effects on the target pest. In our study, additive and antagonistic interactions were encountered in certain nematode combinations against C. *elephas* and *P. fullo* larvae. However, no synergistic effect was observed from any of the combinations.

Entomopathogenic nematodes are often applied to systems that are regularly treated with many other agents, including chemical or biorational pesticides, other biological control agents, soil amendments, and fertilizers (see review by Koppenhöfer and Choo 2005). With entomopathogens, synergism was observed with the combine application such as the nematode–fungal interactions (Barbercheck and Kaya 1990; Choo et al. 2002). In a field study conducted in Korea, *Beauveria brogniartii*, *H. bacteriophora*, and *S. carpocapsae* species alone and their combinations were tested against *Exomala orientalis* larvae. The nematode combination did not increase the efficacy compared to treatments with one nematode species alone. However, the EPN and fungal combination (*S. carpocapsae*+*B. brongniartii*) significantly enhanced grub mortality over the application of the fungus alone (Choo et al. 2002). In another study, the combinations of *Beauveria bassiana* with *S. carpocapsae* or *H. indica* showed an antagonistic interaction for suppression of *Curculio caryae* (Coleoptera: Curculionidae) larvae (Shapiro-Ilan et al. 2004). Antagonism was also observed between the fungus,

Paecilomyces fumosoroseus, combined with *H. indica* or *S. carpocapsae*.

The results obtained with EPN and entomopathogenic bacterium combinations varied from synergism to antagonism. Some studies reported synergistic interaction (Thurston et al. 1993, 1994; Koppenhöfer and Kaya 1997; Koppenhöfer et al. 1999), whereas antagonism was also observed from EPN and entomopathogenic bacterium combinations (Shapiro-Ilan et al. 2004). Laboratory and field studies showed that synergistic or additive effect occurred when *Bacillus thuringiensis* subsp. *japonensis* was combined with *H. bacteriophora* or *S. glaseri* against second or early third instar *Cyclocephala hirta* and *C. pasadenae* and third instar *E. orientalis* (Koppenhöfer and Kaya 1997; Koppenhöfer et al. 1999).

In the combined application of two different EPN species against different insect hosts, synergism or additive interactions have been reported. Choo et al. (1996) tested the efficacy of four different nematode species or strains applying them alone or in combination with one of the other species against *D. undecimpunctata* larvae. In the laboratory study, there was no significant difference between expected and observed mortality and the interaction of the nematodes was additive ($\chi^2 = 0.076$), but in the greenhouse study, no advantage was obtained from the nematode combination. Sankar et al. (2009) applied *H. indica*+*S. asiaticum* against the rice leaf folder, *C. medinalis*, and observed faster and higher percent mortality in the combination treatment than with one EPN species alone. As the authors did not calculate the expected and observed mortality and χ^2 values, it is not known whether the interactions were synergistic or additive. In our study, combinations of *S. weiseri* showed additive interactions, whereas combinations of *S. glaseri* and *H. bacteriophora* resulted in antagonistic interaction against *C. elephas* larvae. *S. glaseri* alone was not as effective as other EPN species when it was used alone. On the other hand, when host species changed, S. *glaseri* and *H. bacteriophora* combination showed additive effects on *P. fullo* larvae. Several hypotheses have been proposed to explain these different results varying from synergism to antagonism. It was speculated that the differences might be related to the isolates or species of nematodes and combined pathogens (Koppenhöfer and Kaya 1997; Choo et al. 2002; Shapiro-Ilan et al. 2004, 2011), host species and stages (Koppenhöfer et al. 1999), timing of application (Koppenhöfer and Kaya 1997), and interaction between their mutualistic bacteria and the environmental conditions such as temperature and humidity (Sankar et al. 2009). The control potential of *H. bacteriophora*+*S. kushidai*, *H. bacteriophora*+*S. glaseri*, and *S. kushidai*+*S. glaseri* combinations were evaluated against the third instar *C. hirta*, and *E. orientalis* in the greenhouse. Mortality in the combinations was not significantly higher than single nematode treatments and the interactions were additive for all combinations (Koppenhöfer et al. 2000).

As discussed above, the studies with combining an EPN species with another EPN species or other entomopathogens have provided mixed laboratory, greenhouse, and field results. The results depended on which EPN species is combined with the other entomopathogens including other EPN species, the target pest, and environmental conditions. As far as we are aware, the only EPN combination field studies have been conducted by Choo et al. (2002) combining EPNs with entomopathogenic fungi and by Koppenhöfer et al. (1999) combining EPNs with an entomopathogenic bacterium. In our laboratory study, although we showed an additive effect with *S. weiseri* combined with *S. glaseri* or *H. bacteriophora* against *C. elephas* larvae, the increased mortality was not sufficient to justify field applications. Koppenhöfer and Grewal (2005) stated that "Combined aaplication of two agents is only useful if target mortality is synergistically increased." With EPNs and other entomopathogens, they further stated that only the *B. thuringiensis* ssp. *japonensis*+*H. bacteriophora* or *S. glaseri* combination has provided synergistic interactions and can be recommended as a control measure against certain white grub species. However, *B. thuringiensis* subsp. *japonensis* is currently not commercially available, and, even if it is, the economic feasibility of the combination with EPNs would depend on the cost of each agent alone versus combining the two agents.

What is the future of combining EPN species or EPNs with other entomopathogens? There are so many EPN species and other entomopathogens that are being discovered that certain combinations may provide synergistic interactions and prove useful against a given notably resistant soil pest (Koppenhöfer and Kaya 1997; Koppenhöfer et al. 1999).

Interspecific competition of the nematodes within the same cadaver showed that nematode species, host species, inoculum size, and physical factors such as experimental arena were crucial factors that determined which nematode species reproduced (Akhurst 1983; Dunphy et al. 1985; Alatorre-Rosas and Kaya 1990; Koppenhöfer et al., 1995). With the *H. bacteriophora*+*Steinernema* spp. combinations in *C. elephas* larvae, *H. bacteriophora* species produced progeny in some cadavers in the combined application with *S. weiseri* or *S. glaseri*, but only one nematode species successfully reproduced in the cadaver. In the *H. bacteriophora*+*S. glaseri* combination, more cadavers produced *H. bacteriophora* progeny than *S. glaseri*. However, when the host was *P. fullo* larva, *H. bacteriophora* did not produce any progeny in the pathogenicity tests, whereas *S. glaseri* did. Koppenhöfer et al. (1995) indicated that some host species are more

susceptible and better hosts for some nematode species. On the other hand, *H. bacteriophora* or *S. glaseri* produced progeny from *P. fullo* cadavers in the pot experiments. This result may be related to the physical conditions of the experimental arena. Pathogenicity tests were conducted in a small arena (350 ml volume and 100 g soil), whereas the pot experiments were conducted in larger arena (1,300 ml volume and 1 kg soil). *H. bacteriophora* is a cruiser characterized by high motility and is distributed throughout the soil profile (Campbell et al. 2003). Therefore, this species could have used its foraging strategy more effectively in the larger arena and compete with the cruiser, *S. glaseri*, for a host.

In our study, coexistence and progeny production were observed between *Steinernema* spp. but not between steinernematids and heterorhabditids. It is known that the association between nematode and bacterial symbiont is not completely specific. That is, several *Steinernema* spp. have been cultured with the *Xenorhabdus* symbionts isolated from other *Steinernema* spp. (Akhurst 1983). Accordingly, the occurrence of more than one *Steinernema* spp. in an insect host has been reported in several studies (Kaya 1984; Dunphy et al. 1985; Choo et al. 1987; Kondo 1989; Koppenhöfer et al. 1995). We confirmed that *S. weiseri* and *S. glaseri* can reproduce in the same *C. elephas* cadaver. In contrast, Akhurst (1983) found that *Steinernema* spp. could not be cultured on the *Photorhabdus* symbionts of *Heterorhabditis* spp. and Alatorre-Rosas and Kaya (1990) reported that both *Steinernema* and *Heterorhabditis* could infect the same insect, yet neither nematode species survived to produce progeny. In our study, we did not check for nematode development within the cadavers, but we did observe the emergence of new generation IJs. As only one nematode species emerged when challenged with both a *Steinernema* spp. and *H. bacteriophora*, we conclude that only one species successfully infected a *C. elephas* and *P. fullo* host.

In conclusion, although the combination of *S. weiseri*+*H. bacteriophora* did produce higher larval mortality of *C. elephas* than the other treatments, it was not significant compared with some of the one nematode species alone treatment. Therefore, the use of combining nematode species to control *C. elephas* cannot be justified until a more effective nematode or other pathogen combination is found. We did observe an additive effect on the combined application of *S. glaseri*+*H. bacteriophora* against *P. fullo* larvae, but the result was far from satisfactory. If a combination of biological control agents is to be used for *P. fullo* larvae or even *C. elephas*, different control tactics with different combinations of pathogens such as entomopathogenic fungus, virus, or bacterium may be needed. Possibly, a more effective pathogen combination that controls these pests can be found.

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