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Impact of a Nematode-parasitic Fungus on the Effectiveness of Entomopathogenic Nematodes

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Abstract: The impact of the nematode-parasitic fungus *Hirsutella rhossiliensis* on the effectiveness of *Steinernema carpocapsae, S. glaseri,* and *Heterorhabditis bacteriophora* against *Galleria mellonella* larvae was assessed in the laboratory. The presence of *Hirsutella* conidia on the third-stage (J3) cuticle of *S. carpocapsae* and *H. bacteriophora* interfered with infection of insect larvae. Conidia on the J3 cuticle of *S. glaseri* and on the ensheathing second-stage cuticle of *H. bacteriophora* did not reduce the nematodes' ability to infect larvae. The LD_{50} values for *S. carpocapsae, S. glaseri,* and *H. bacteriophora* in sand containing *H. rhossiliensis* were not different from those in sterilized sand when *Galleria* larvae were added at the same time as the nematodes. However, when *Galleria* larvae were added 3 days after the nematodes, the LD_{50} of *S. glaseri* was higher in *Hirsutella*-infested sand than in sterilized sand, whereas the LD_{50} of *H. bacteriophora* was the same in infested and sterilized sand. Although the LD_{50} of *S. carpocapsae* was much higher in *Hirsutella*-infested sand than in sterilized sand, the data were too variable to detect a significant difference. These data suggest that *H. bacteriophora* may be more effective than *Steinernema* species at reducing insect pests in habitats with abundant nematode-parasitic fungi.

Key words: biological control, entomopathogenic nematode, fungus, *Heterorhabditis, Hirsutella rhossiliensis,* nematode, nematophagous fungus, *Steinernema.*

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are important biological control agents of certain soil-dwelling insect pests. All life stages of these nematodes occur in the insect host, except the infective third-stage juvenile (dauer). The function of the dauer is to survive in the environment, sense the presence of a host and orient toward it (host location), and penetrate into the insect's hemocoel and release its mutualistic bacterium (infection). The bacterial cells multiply within the hemocoel and kill the insect in 24–48 hours.

Numerous field evaluations of steinernematid and heterorhabditid nematodes for control of various insect pests have produced inconsistent results (6,14). The effectiveness of these nematodes is influenced by abiotic factors such as soil moisture, texture, and temperature, and biotic factors such as host defense mechanisms and host specificity of nematode species and strains (13). Although many natural enemies of nematodes kill entomopathogenic nematodes (3,8,17,18,20,22), their role in reducing the effectiveness of entomopathogenic nematodes has not been examined.

Parasites and predators of nematodes may diminish the effectiveness of entomopathogenic nematodes by reducing survival in soil, or by reducing host location or infection by dauers. In soil naturally infested with the nematode-parasitic fungus *Hirsutella rhossiliensis* Minter and Brady, entomopathogenic nematodes were infected by the fungus and their survival was reduced compared with those in sterilized soil (21). In the present study, we evaluated the impact of *H. rhossiliensis* on the efficacy of the entomopathogenic nematodes *Steinernema carpocapsae* (Weiser), *S. glaseri* (Steiner), and *Heterorhabditis bacteriophora* Poinar against larvae of the greater

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wax moth, *Galleria mellonella* (L.). Specifically, we determined whether the physical presence of *H. rhossiliensis* conidia on the nematode cuticle interferes with infection of the insect host and compared the effectiveness of the nematodes in sand with and without *H. rhossiliensis*.

Materials and Methods

Steinernema carpocapsae (All strain), *S. glaseri,* and *H. bacteriophora* (NC strain) were reared in *G. mellonella* (2,23) and stored in distilled water at 10 C for no more than 2 weeks before an experiment. *Hirsutella rhossiliensis* (ATCC 46487), isolated from the nematode *Criconemella xenoplax* (Raski) Luc and Raski, was cultured on 25% corn meal agar (1.5% agar) and used within 3–4 weeks of transfer. To avoid loss of virulence, the fungus was reisolated from infected *S. glaseri* after four consecutive transfers to corn meal agar.

Infection: To determine if dauers laden with *H. rhossiliensis* conidia could infect a host, a one-on-one assay was used (16). The nematodes, tested in separate experiments, were ensheathed *H. bacteriophora* and exsheathed *H. bacteriophora, S. carpocapsae,* and *S. glaseri.* The sheath (second-stage cuticle) was removed from dauers by treatment with 0.05% sodium hypochlorite for 15 minutes (20). Dauers were transferred with a wire pick either to a 3-week-old culture of *H. rhossiliensis* (*Hirsutella*-treated) or to sterile plates of ¼-strength cornmeal agar (untreated). Dauers were removed from the plates after 1–5 conidia were acquired (ca. 1–3 minutes). The one-on-one assay was performed by placing a dauer in 0.125 ml of 0.03 M KCl at the bottom of a tissue culture well (24-well plate), then adding 1.5 g dry sand and one last instar *G. mellonella* larva. Potassium chloride was added to moisten the sand and enhance nematode infection by *H. rhossiliensis* (9). The close proximity of the dauer to the insect host minimized host location. *Hirsutella*-treated and untreated nematodes were tested in separate plates to avoid cross contamination. The plates were placed in plastic bags and stored at 22 C. Daily, for 5 days, the larvae were examined for nematode infection. *Galleria* larvae infected with *S. carpocapsae* and *S. glaseri* were limp and appeared grey to light tan; those infected with *H. bacteriophora* were firm and appeared brick-red.

Mortality of dauers used for the one-on-one assay was determined by first transferring 20–40 *Hirsutella*-treated and untreated dauers with a pick to 0.03 M KCl and examining them 3 days later. Survival of nematodes within the insects was determined by dissecting infected *Galleria* larvae 3, 5, and 6 days after being infected by *S. glaseri, S. carpocapsae,* and *H. bacteriophora,* respectively.

There were 3–6 replicate plates (18 dauers/plate) per treatment and three experimental trials for each nematode species, except ensheathed *H. bacteriophora,* for which there were two trials. A two-way analysis of variance (ANOVA) on arcsine-transformed data was used to assess differences in percentage infection between *Hirsutella*-treated and untreated dauers, and among trials. Differences in percentage survival (arcsine-transformed) of nematodes within the insect cadaver were determined with a *t*-test.

Host location and infection: To determine if *H. rhossiliensis* affects the nematodes' ability to locate and infect a host, a dose–response assay was performed. Coarse sand (87.8% sand, 2.6% silt, 9.6% clay; <1% organic matter, pH 6.4 in 0.01 M $CaCl_2$) was moistened, heated at 60 C for 6 hours to eliminate most organisms, and air dried before the experiments. The sand was artificially infested with *H. rhossiliensis* (Hr sand) by adding dauers of *S. glaseri* infected with the fungus (12,21). Infected *S. glaseri* (4,311 ± 570, $\bar{x}$ ± SD) in 60 ml sterilized distilled water were mixed into 1,000 g dry sand. Because each infected *S. glaseri* produces 893 ± 396 conidia (Timper, unpubl. data), the sand contained an equivalent of ca. 4,000 conidia/cm^3. This conidial density is within the range of densities recorded from naturally infested soil (15). Uninfested sand (No Hr

sand) was prepared in the same manner as the Hr sand, except that no infected *S. glaseri* were added with the water.

Assay arenas were plastic containers (7 cm high × 17 cm d) with 11 holes (5 mm d) in the bottom. The holes were covered with masking tape. Containers were filled with 1,060 g Hr sand or No Hr sand, and the sand tamped down to a uniform level (908 cm^3). Because soil disturbances dislodge conidia from their conidiophores and inactivate *H. rhossiliensis* (15), space for the *Galleria* larvae (which were added 9 days later) was made in the sand before conidia formed. To make space for the larvae, the tape covering the holes in the bottom of the container was peeled back, and a 12-mm depression was made in the sand at each hole. The tape was sealed over the holes, and the containers with lids were placed into individual plastic bags along with a moist paper towel. The sand in the containers was incubated at 22 C for 9 days to allow *H. rhossiliensis* to sporulate from the infected *S. glaseri*. On day 3 of the incubation, 10 last-instar *Galleria* larvae were added to the sand surface to act as bait to remove *S. glaseri* that had not succumbed to *H. rhossiliensis* infection. Ten larvae were also added to containers containing No Hr sand. The bait larvae were removed 3 days later and discarded.

At the end of the incubation, nematodes were pipetted in 2.75 ml distilled water evenly over the sand surface of separate containers of Hr or No Hr sand. The nematodes, tested in separate experiments, were *H. bacteriophora, S. carpocapsae,* and *S. glaseri*. There were four dosages of nematodes plus a water control. The dosages ranged from 17–346, 10–64, and 6–56 dauers/container for *H. bacteriophora, S. carpocapsae,* and *S. glaseri,* respectively. The level of fungus in the Hr sand was bioassayed at this time by adding 1,267 ± 222 *H. bacteriophora* dauers to 76-cm^3 cylinders containing Hr sand (set up 9 days earlier), extracting the dauers 24 hours later, and counting the number of conidia adhering to their J2 cuticles (15,21). On the same day the nematodes were added, the tape was peeled back from the holes in the bottom of the container, one last-instar *Galleria* larva was introduced into each hole, and the tape was resealed over the holes. Four days later, the larvae were removed and examined for nematode infection. Additionally, *S. carpocapsae*-infected larvae were dissected and the number of invading nematodes was recorded.

There were three replicate containers for each sand treatment–nematode dosage combination. The experiment was performed twice for *S. carpocapsae* and *S. glaseri* and once for *H. bacteriophora*. The relationship between nematode dosage (log_{10}) and larval mortality was determined with probit analysis using the PROC PROBIT procedure of SAS (19). Differences between No Hr and Hr treatments were based on nonoverlap of the 95% fiducial limits of the LD_{50}. A two-way ANOVA on arcsine-transformed data was used to compare the percentage of *S. carpocapsae* dauers invading larvae in Hr sand vs No Hr sand.

Survival, host location, and infection: This experiment was similar to the previously described experiment, except that last-instar *Galleria* larvae were introduced into the holes 3 days after the nematodes were pipetted onto the sand surface. Thus, the nematodes had to survive for 3 days before hosts could be located. The nematode dosages ranged from 14–64, 11–65, and 8–66 dauers/container for *H. bacteriophora, S. carpocapsae,* and *S. glaseri,* respectively. Infected larvae were dissected and the number of invading nematodes was recorded. There were three replicate containers for each sand treatment–nematode dosage combination and two experimental trials for each nematode species. Statistical analyses were the same as for the previous experiment.

Results and Discussion

Infection: Fewer *Galleria* larvae were infected by *S. carpocapsae* ($P = 0.003$) and *H. bacteriophora* ($P = 0.003$) when *H. rhossiliensis* conidia were present on the J3 cuti-

cle than when conidia were absent (Fig. 1A). The presence of *H. rhossiliensis* conidia did not affect infection of larvae by exsheathed *S. glaseri* or ensheathed *H. bacteriophora*. The number of conidia adhering to the cuticle was 2.9 ± 0.3 ($\bar{x}$ ± SD), 4.6 ± 1.3, 2.6 ± 0.5, and 4.5 ± 0.1 for exsheathed *S. carpocapsae, S. glaseri, H. bacteriophora,* and ensheathed *H. bacteriophora,* respectively. Mortality of the *Hirsutella*-treated dauers placed in 0.03 M KCl was 88 ± 8, 93 ± 7, 93 ± 7, and 0% for exsheathed *S. carpocapsae, S. glaseri, H. bacteriophora,* and ensheathed *H. bacteriophora,* respectively. Mortality of untreated dauers was ≤2%.

The physical presence of *H. rhossiliensis* conidia on the nematode cuticle may have hindered the penetration of exsheathed *S. carpocapsae* and *H. bacteriophora* dauers into the insect hemocoel. However, penetration by exsheathed *S. glaseri* was not hindered by the conidia. Another explanation is that some *S. carpocapsae* and *H. bacteriophora* dauers were killed by *H. rhossiliensis* before they could infect the insect. *Hirsutella rhossiliensis* kills *S. carpocapsae* and *H. bacteriophora* dauers within 2 days and *S. glaseri* dauers within 3 days of conidial attachment (20); consequently, *S. glaseri* dauers have more time than *S. carpocapsae* and *H. bacteriophora* dauers to infect larvae. Very few exsheathed *H. bacteriophora* dauers infected larvae compared with ensheathed dauers (Fig. 1A). Sodium hypochlorite reduces the ability of *H. bacteriophora* either to locate or infect a host (1).

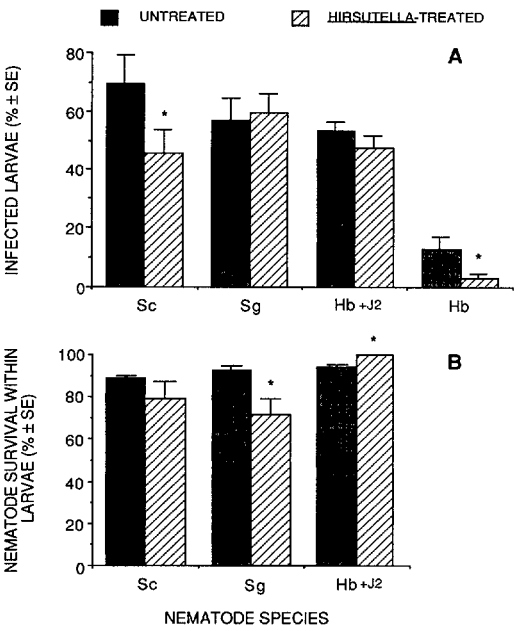

FIG. 1. Infection of *Galleria mellonella* larvae by exsheathed *Steinernema carpocapsae* (Sc), *S. glaseri* (Sg), *Heterorhabditis bacteriophora* (Hb), and ensheathed *H. bacteriophora* (Hb + J2) with (*Hirsutella*-treated) and without (untreated) *Hirsutella rhossiliensis* conidia adhering to their cuticles. A) Infection of *Galleria* larvae. B) Post-infection survival of nematodes within the larvae. Columns with asterisks are different from untreated columns ($P \leq 0.05$). There were insufficient insect larvae infected by exsheathed *H. bacteriophora* to determine percentage survival of nematodes.

The percentage survival of nematodes within the insect cadavers was similar ($P = 0.34$) for *Hirsutella*-treated and untreated *S. carpocapsae,* lower ($P = 0.04$) for *Hirsutella*-treated than for untreated *S. glaseri,* and higher ($P = 0.01$) for *Hirsutella*-treated than for untreated ensheathed *H. bacteriophora* (Fig. 1B). Too few exsheathed *H. bacteriophora* infected *Galleria* larvae to compare survival of *Hirsutella*-treated and untreated nematodes. The lower survival of *Hirsutella*-treated, compared with untreated, *S. glaseri* suggests that some dauers penetrated the insect hemocoel, released their bacteria, and then died from fungal infection. We are unable to explain the small, but significant ($P < 0.05$) difference in survival of *Hirsutella*-treated and untreated ensheathed *H. bacteriophora* within the insect cadaver, but doubt that *H. rhossiliensis* conidia on the J2 cuticle of *H. bacteriophora* would increase nematode survival.

Some conidial-laden *S. carpocapsae* and *S. glaseri* dauers appeared to escape *H. rhossiliensis* infection by infecting the larvae. For instance, 34 and 43% of *S. carpocapsae* and *S. glaseri* dauers infected insects and survived to maturity within the cadaver, whereas only 12 and 7% survived in KCl. We suggest two possible mechanisms of escape: either the conditions within the insect inhibit germination of conidia or fungal infection is interrupted when the dauer molts to the fourth stage. Plant-

parasitic nematodes may also escape fungal infection by penetrating roots. For example, J3 of *Heterodera schachtii* Schmidt were occasionally found in cabbage roots with *H. rhossiliensis* conidia attached to the molted second-stage cuticles (11).

Host location and infection: Hirsutella rhossiliensis did not significantly affect the LD_{50} values of the nematode species when nematodes were added to sand the same day as the hosts (Table 1); however, the LD_{50} values were consistently higher in Hr sand than in No Hr sand. In the one-on-one assay, fewer *S. carpocapsae* dauers infected larvae when they were laden with *H. rhossiliensis* conidia than when no conidia were present. Therefore, we expected to observe a difference in larval mortality by *S. carpocapsae* between Hr and No Hr sand. The absence of any observable difference between the two sand treatments may be due to the lack of sensitivity of the assay. Insect mortality is not an accurate measure of nematode infection because of the frequency of multiple infections (7).

A more accurate measure of nematode infection is to count the invading nematodes per infected insect (4). Dissection of larvae infected with *S. carpocapsae* revealed a lower ($P = 0.002$) percentage of infection in Hr sand than in No Hr sand. Percentage of infection, combined across dosages, was 38.0 ± 4.1% ($\bar{x}$ ± SE) in the Hr sand and 51.6 ± 4.7% in the No Hr sand. There was also an effect of dosage on percentage of infection ($P = 0.004$); however, because nematode dosages could not be duplicated in the experimental trials, there were only three replicates per dosage. Random variation in such a low number of replicates may have resulted in the dosage effect. Although the number of dauers invading the hosts increased linearly with dosage ($r^2 = 0.79$, slope ± SE = 0.43 ± 0.09 in No Hr sand and $r^2 = 0.77$, 0.30 ± 0.07 in Hr sand), there was no linear relationship between nematode dosage and percentage of infection. These results confirm those reported previously (4).

Survival, host location, and infection: When nematodes were added 3 days before hosts, the LD_{50} values against *Galleria* larvae were higher in Hr sand than in No Hr sand for *S. glaseri* but not *H. bacteriophora* (Table 1). In both *S. carpocapsae* trials, mortality of *Galleria* larvae was highly variable

TABLE 1. Effect of *Hirsutella rhossiliensis* (Hr) on the LD_{50} response of *Galleria mellonella* larvae exposed to *Steinernema carpocapsae, S. glaseri,* and *Heterorhabditis bacteriophora* in sand artificially infested with and without the fungus (No Hr).

	Treatment	Total *Galleria*	Slope ± SE†	LD_{50}	95% fiducial limits of LD_{50}
		Nematodes added same day as hosts‡			
S. carpocapsae	No Hr	254	2.04 ± 0.32	1.4	1.1–1.6
	Hr	254	1.30 ± 0.29	1.8	1.3–2.4
S. glaseri	No Hr	254	1.29 ± 0.25	2.6	1.9–4.0
	Hr	257	0.94 ± 0.24	3.1	2.0–7.3
H. bacteriophora	No Hr	126	1.76 ± 0.30	3.3	2.0–4.7
	Hr	118	1.36 ± 0.28	3.8	2.0–5.7
		Nematodes added 3 days before hosts§			
S. carpocapsae	No Hr	202	1.22 ± 0.61	8.8	—‖
	Hr	187	1.52 ± 0.65	22.5	9.2–>2000
S. glaseri	No Hr	256	1.32 ± 0.28	4.0	3.0–6.7
	Hr	243	1.19 ± 0.33	14.0*	7.2–115.8
H. bacteriophora	No Hr	228	2.32 ± 0.44	4.2	3.5–5.6
	Hr	215	1.44 ± 0.44	7.2	4.8–29.4

LD_{50} values (nematodes/*Galleria* larva) followed by an asterisk are different from No Hr sand based on non-overlap of the 95% fiducial limits.

† Based on $\log_{10}$ of the nematode dosage, three replicates per dosage.

‡ Data from host location and infection experiment; 54 ± 15 ($\bar{x}$ ± SD) conidia/cm^3 detected by bioassay in the Hr sand.

§ Data from survival, host location, and infection experiment; 102 ± 23 conidia/cm^3 detected by bioassay in the Hr sand.

‖ Data did not fit the probit analysis model (20); therefore, no fiducial limits were calculated.

in the No Hr sand; consequently, the data would not fit the probit analysis model. This variability in larval mortality was not observed when the larvae were added at the same time as *S. carpocapsae*. During the 3 days before larvae were added, many of the *S. carpocapsae* dauers may have died or moved up the side of the containers, and thus they were unable to infect the larvae.

Percentage of invading dauers was lower in Hr sand than in No Hr sand for all nematode species (Table 2). Additionally, there was a significant effect of nematode dosage on percentage of invading *H. bacteriophora,* but not *S. carpocapsae* or *S. glaseri* (Table 2). As with *S. carpocapsae* in the previous experiment, random variation in the three replicates of a nematode dosage may have resulted in the dosage effect observed with *H. bacteriophora.*

These data indicate that a nematode-parasitic fungus can reduce the effectiveness (percentage mortality of hosts) of entomopathogenic nematodes and that some nematode species are more affected than others. The effectiveness of *H. bacteriophora* was not reduced by the presence of *H. rhossiliensis*. This species may be unaffected by the fungus due to retention of the protective J2 cuticle. When the J2 cuticle was removed, *H. rhossiliensis* infected *H. bacteriophora* and severely reduced their survival in soil after only 4 days (22). The effectiveness of *S. glaseri* was not reduced by the presence of *H. rhossiliensis* when hosts were available immediately, but it was reduced when there was a 3-day delay in host availability. During the 3 days before hosts were added, the dauers may have moved randomly through the sand, thus increasing their chances of encountering conidia.

The density of the fungus and the distance nematodes move before locating a host will affect survivorship of nematodes in the presence of a sedentary parasite such as *H. rhossiliensis* (5). In this study, the density of *H. rhossiliensis* conidia was relatively high. In many soils, the density of nematode-parasitic fungi will probably be lower. Nevertheless, obligate nematode-parasitic fungi can increase in response to nematode densities (10). High populations of entomopathogenic nematodes, resulting from recycling within the insect population or repeated introductions into the same habitat, may increase the density of nematode-parasitic fungi. In our study, the dauers did not have far to move to locate hosts (2–3 cm). In the field, most dauers applied to the soil surface will probably have to move more than 3 cm to control root-feeding insects. The further the nematode has to move to infect a host, the greater will be the probability of encountering a sedentary parasite.

Entomopathogenic nematodes have traditionally been applied inundatively to control a single generation of insect pest (short-term control). However, these nematodes are capable of recycling in the host population (13) and may control the

TABLE 2. Percentage of *Steinernema carpocapsae, S. glaseri,* and *Heterorhabditis bacteriophora* infecting *Galleria mellonella* larvae when nematodes were added 3 days before larvae to sand artificially infested with *Hirsutella rhossiliensis* (Hr) and to uninfested sand (No Hr).

	Percentage of nematodes infecting *Galleria*†		F-ratio (*P*-value)‡	
	Hr	No Hr	Hr treatment	Nematode dosage
S. carpocapsae	2.2 ± 0.4	8.5 ± 0.9	23.51 (0.0001)	0.27 (0.9623)
S. glaseri	6.9 ± 1.2	27.2 ± 3.1	47.77 (0.0001)	0.63 (0.7282)
H. bacteriophora	8.1 ± 1.2	14.8 ± 4.8	8.03 (0.0079)	3.07 (0.0136)

† Values are means ± SE of four nematode dosages, three replicates per dosage.
‡ Two-way ANOVA.

insect population over many generations (14). Under these conditions, nematode reproduction is important. For amphimictic species such as *S. carpocapsae* and *S. glaseri,* the number of invading dauers influences whether reproduction occurs within the host. Multiple nematode infections that result in a mating pair should be more common in soils with high populations of nematodes than in soils with low populations. The presence of *H. rhossiliensis* in sand reduces the percentage of invading *S. carpocapsae* when hosts are located immediately and reduces the percentage of invading *S. carpocapsae* and *S. glaseri* when hosts are located after 3 days. The percentage of invading *H. bacteriophora* also was affected by the fungus, but one nematode is sufficient for reproduction with this species. Thus, natural enemies may reduce reproduction of amphimictic nematodes without diminishing short-term efficacy.

Our results suggest that *Heterorhabditis* spp. rather than *Steinernema* spp. should be used to control insect pests in soils where nematode-parasitic fungi are abundant. The effectiveness of *H. bacteriophora* against *Galleria* larvae was not reduced in sand containing a high level of *H. rhossiliensis* and, because this nematode genus is hermaphroditic, moderate reduction in the percentage of invading dauers will not severely affect recycling in the pest population. However, if other ecological considerations justify the use of a *Steinernema* species, then higher rates of dauers should be applied to overcome the effects of parasites.

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