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Suppression of Cyst Nematode by Natural Infestation of a Nematophagous Fungus

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Abstract: Penetration of cabbage roots by *Heterodera schachtii* was suppressed 50–77% in loamy sand naturally infested with the nematophagous fungus *Hirsutella rhossiliensis.* When *Heterodera schachtii* was incubated in the suppressive soil without plants for 2 days, 40–63% of the juveniles had *Hirsutella rhossiliensis* spores adhering to their cuticles. Of those with spores, 82–92% were infected. Infected nematodes were killed and filled with hyphae within 2–3 days. Addition of KCl to soil did not increase infection of *Heterodera schachtii* by *Hirsutella rhossiliensis.* The percentage of infection was lower when nematodes were touched to two spores and incubated in KCl solution than when nematodes naturally acquired two spores in soil.

Key words: biological control, *Brassica oleracea,* cabbage, *Criconemella xenoplax, Heterodera schachtii, Hirsutella rhossiliensis,* nematophagous fungus, peach, sugar beet cyst nematode, suppressive soil.

The nematophagous fungus *Hirsutella rhossiliensis* Minter & Brady (= *Hirsutella heteroderae* Sturhan & Schneider) parasitizes and kills nematodes in vitro (2,7,12,13), suppresses nematode numbers in greenhouse tests (3,11), and parasitizes high numbers of nematodes in grower fields (6,11). However, native populations of *Hirsutella rhossiliensis* do not suppress numbers of *Criconemella xenoplax* (Raski) Luc & Raski to an acceptable level in California peach orchards (6). High proportions of *C. xenoplax* are parasitized only if high numbers of *C. xenoplax* are present (6), and a high level of parasitism is not associated with a decline in nematode numbers (Jaffee and McInnis, unpubl.). In contrast, Timper and Kaya (pers. comm.) found that *Hirsutella rhossiliensis* decreased the persistence of certain entomogenous nematodes in orchard soil.

In the present study, juveniles of *Heterodera schachtii* Schmidt were added to orchard soil naturally infested with *Hirsutella rhossiliensis.* Our objective was to quantify the potential of this infestation to suppress penetration of roots by *Heterodera schachtii. Heterodera schachtii* was selected because cyst nematodes were associated with *Hirsutella rhossiliensis* in Germany (11,12), cyst nematodes are important pests of many crops, and inoculum of *Heterodera schachtii* is more uniform and more readily available than that of *C. xenoplax.*

Materials and Methods

Soil: Rhizosphere soil was collected 33–66 cm deep and 70 cm from the trunk of each of 10 peach trees on Nemaguard rootstock in an orchard containing high numbers of *Hirsutella rhossiliensis*-parasitized *C. xenoplax* (5,6). Soil containing very low numbers of *H. rhossiliensis*-parasitized *C. xenoplax* was collected 300 cm from each trunk of the same trees and was designated nonrhizosphere soil, although some peach roots were present. The soil contained 78.4% sand, 12.8% silt, 8.8% clay, and less than 1% organic matter. The soil pH (0.01 M $CaCl_2$) ranged from 4.5 to 5.3, and the inflection point of the moisture release curve was about −4 kPa (18% soil moisture). Rhizosphere and nonrhizosphere soils were stored 30 days at 10 C before use. In one experiment, autoclaved sand also was used.

Numbers of *H. rhossiliensis*-parasitized *C. xenoplax* in the soils were determined by a plate assay (5). Fungal inoculum in soil was a function of number and size of parasitized nematodes and was expressed as spore equivalents to account for the different number of spores produced when the fungus sporulates from nematodes of different sizes (10). For example, *H. rhossiliensis* produced about 700 spores when growing on an adult female *C. xenoplax* (8) and about 110 spores when growing on a second-stage

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juvenile of *Heterodera schachtii* (Jaffee, unpubl.).

The peach orchard soil contained low numbers of *Meloidogyne* and *Pratylenchus* spp. In preliminary experiments, cabbage seedlings (*Brassica oleracea* var. *capitata* cv. Late Flat Dutch) grown in the soil for 5 days contained less than one of any endoparasitic nematode per seedling.

Vials: Soil or sand was spray moistened and added to 25-cm^3 vials as described elsewhere (10) except the vials had one 0.5-mm-d hole in the lid and did not have holes in the bottom. Each vial contained about 17 cm^3 soil at a bulk density of 1.4–1.5 and 11–13% moisture. Vials were incubated in the dark at 22 ± 2 C for 10 days in a moist chamber before nematodes were added. Incubation without disturbance is required to allow infective spores of *Hirsutella rhossiliensis* to develop and persist (10).

Nematode inoculum: Heterodera schachtii was obtained from greenhouse pot cultures on sugar beets (*Beta vulgaris,* cv. SSY1). Cysts were collected by wet screening and placed on Baermann funnels. Second stage juveniles (J2) were collected every 2 hours and were aerated at 10 C for less than 48 hours before being added to vials. Nematode inoculum was added to vials as J2 in 0.5 ml of 0.003 M KCl unless indicated otherwise.

Infection of roots: In trial 1, vials were packed with rhizosphere soil (1,428 ± 46 × 10^3 spore equivalents of *Hirsutella rhossiliensis* per 100 cm^3 soil), nonrhizosphere soil (12 ± 8 × 10^3 spore equivalents per 100 cm^3 soil), or sand (no *Hirsutella rhossiliensis*), and 204 ± 23 ($\bar{x}$ ± SD) *Heterodera schachtii* per vial were added after 10 days. One cabbage seedling (radical < 2 mm long) was placed in each vial 3 days after addition of nematodes. In trial 2, vials were packed with rhizosphere soil (1,319 ± 162 × 10^3 spore equivalents per 100 cm^3 soil) or nonrhizosphere soil (0 ± 0 spore equivalents per 100 cm^3 soil) and 48 ± 13 or 375 ± 27 *Heterodera schachtii* per vial were added after 10 days; cabbage seedlings were planted 3 days later. Vials with seedlings were placed in clear plastic moist chambers under fluorescent lights. Roots were removed, measured, and stained (1) 5 days after the seedlings were planted. All nematodes in each root system were counted. Treatments were replicated six times, and the trials were performed once.

Infection in soil: Heterodera schachtii (537 ± 48) J2 were added to rhizosphere soil (1,359 ± 104 × 10^3 spore equivalents per 100 cm^3 soil) in vials. The J2 were subsequently extracted by wet screening and sucrose centrifugation (9) after 2, 4, 6, or 9 days at 22 ± 2 C. The percentage of J2 with *Hirsutella rhossiliensis* spores adhering to their cuticles or with spores and hyphae-filled body cavities was determined by examining the first 50 nematodes observed per replicate in a Hawksley slide at 70–140× magnification. At day 2, 25 live nematodes with spores per replicate were examined at 400× magnification with differential interference contrast microscopy; *Heterodera schachtii* containing infection bulbs (as in Fig. 1A) were scored as infected. There were four replicate vials per extraction day, and the experiment was performed once.

The effect of KCl on infection of *Heterodera schachtii* by *Hirsutella rhossiliensis* in soil was examined because KCl and certain other ions enhanced parasitism of *C. xenoplax* in vitro (7). *Heterodera schachtii* (473 ± 38) in 0.5 ml distilled water, 0.003 M KCl, or 0.03 M KCl were added to vials containing rhizosphere soil with 1,320 ± 335 × 10^3 spore equivalents per 100 cm^3. Two days later, nematodes were extracted and 25 nematodes per vial were examined for spores. Twenty nematodes with spores per vial were examined for signs of infection. There were three replicate vials per treatment, and the experiment was performed once.

In vitro infection: Individual *Heterodera schachtii* J2 on a nematode pick were touched to two spores of *Hirsutella rhossiliensis* produced by a 7 to 53-day-old culture on quarter-strength corn meal agar and placed in a petri dish containing 3 ml of 0.03 M KCl. After 3 days at 25 C, the number of *H. rhossiliensis* spores adhering

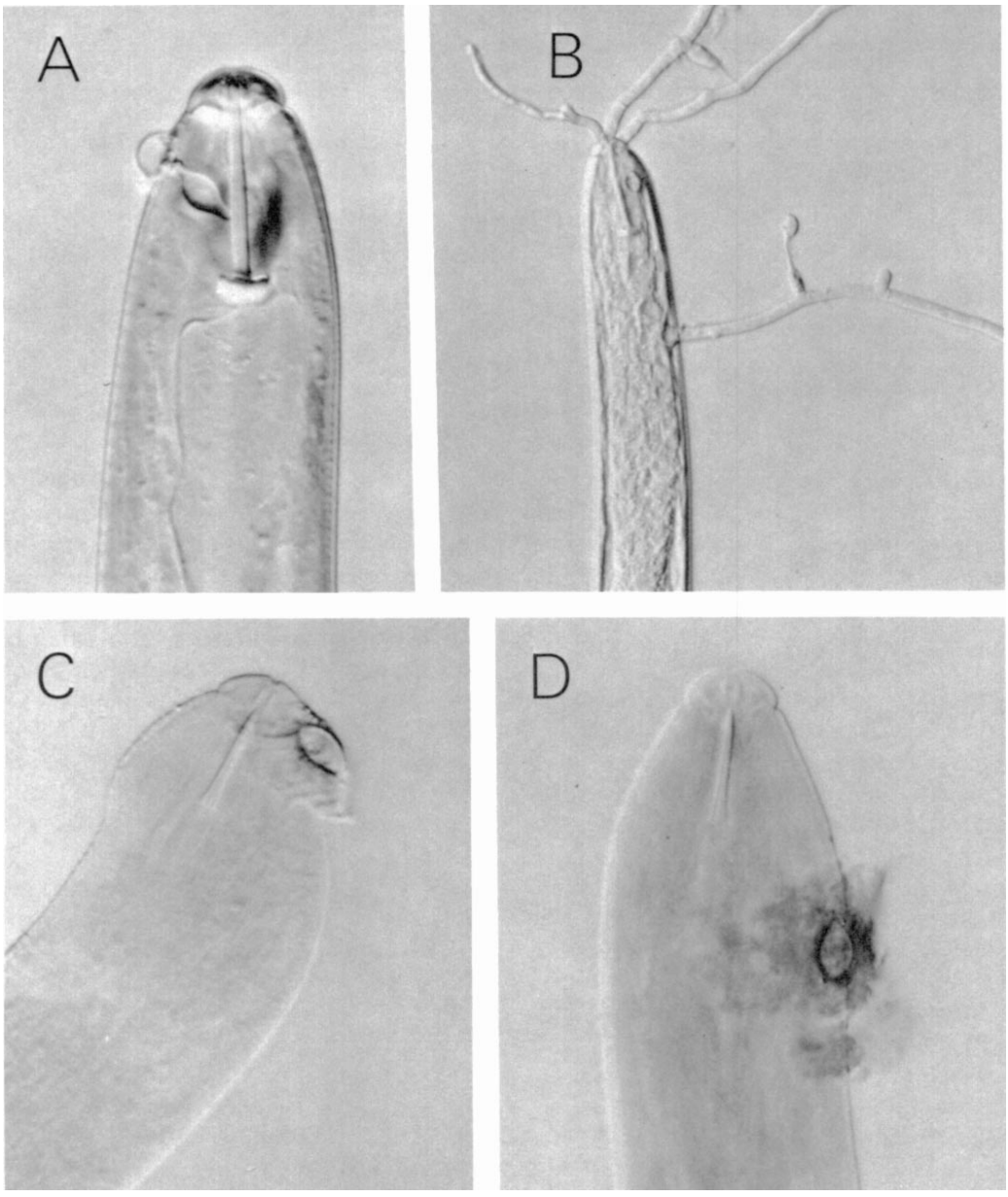

FIG. 1. *Heterodera schachtii* juveniles recovered from soil naturally infested with *Hirsutella rhossiliensis*. A) Spore that germinated through the cuticle and formed an infection bulb within the body cavity of a living juvenile. Juvenile had been added to soil 2 days earlier. B) Juvenile that was infected, killed, and filled with hyphae after incubation for 4 days. Spore that initiated the infection is to the right of the stylet. Fungus had begun to sporulate. C, D) Nematodes dissected from cabbage roots. Although each juvenile had a spore on its cuticle, it penetrated the root and began development without becoming infected.

to the cuticle of each nematode and the presence of infection bulbs or hyphae in the body cavity were recorded. There were 20 nematodes per replicate and six replicates. A different isolate of *H. rhossiliensis* was used with each replicate, including two isolates from parasitized *C. xenoplax* extracted from the rhizosphere soil (isolates Hr 61 and Hr 68), two isolates from parasitized mites extracted from the rhizosphere soil (isolates Hr 63 and Hr 65), one isolate from a parasitized *M. incognita* ex-

TABLE 1. Infection of cabbage roots by *Heterodera schachtii* (Hs) as affected by *Hirsutella rhossiliensis* (Hr).

Soil	Hr density†	Hs added/vial	Hs within each root	Tap root length (mm)
		Trial 1		
Rhizosphere	1,428 ± 46	204	36 ± 9	40 ± 12
Nonrhizosphere	12 ± 8	204	77 ± 9	29 ± 9
Sand	0 ± 0	204	100 ± 23	29 ± 7
		Trial 2		
Rhizosphere	1,319 ± 162	48	7 ± 3	50 ± 12
Nonrhizosphere	0 ± 0	48	30 ± 6	38 ± 7
Rhizosphere	1,319 ± 162	375	46 ± 9	45 ± 8
Nonrhizosphere	0 ± 0	375	92 ± 34	10 ± 2

Values are means ± SD of six replications.
† Spore equivalents: $\bar{x}$ ± SD × 10^3/100 cm^3 soil.

tracted from a California peach orchard (isolate Hr 57, isolated by Graham Stirling), and one isolate from a parasitized *C. xenoplax* extracted from a South Carolina peach orchard (isolate IMI 265748).

RESULTS

Infection of roots by Heterodera schachtii: In trials 1 and 2, penetration and damage of cabbage roots by *H. schachtii* were suppressed in soil containing high levels of *Hirsutella rhossiliensis* (Table 1). In trial 2, *Heterodera schachtii* were removed from the stained roots grown in rhizosphere soil inoculated with 375 juveniles per vial. The dissected nematodes were examined at 400× and 1,000× magnification for spores on the cuticle or hyphae within the body cavity. Of 120 nematodes (20 per replicate root system), 5.8% had spores on their cuticle and contained hyphae typical of *Hirsutella rhossiliensis.* An additional 4.2% had acquired spores but were not infected (Fig. 1C, D).

Infection in soil: Most *Heterodera schachtii* incubated in, and recovered from, rhizosphere soil (1,359 × 10^3 spore equivalents per 100 cm^3) acquired *Hirsutella rhossiliensis* spores by day 2 and were dead and colonized (i.e., filled with fungal hyphae) by day 6 (Fig. 2). After an initially rapid increase, spore acquisition and colonization leveled off. On day 2, 88 ± 9% of those *Heterodera schachtii* with spores were already infected (Fig. 1A). Infection was not determined after day 2. Of the *H. schachtii* without spores, 80 and 83% were healthy and active on days 4 and 6, respectively. The number of spores per *H. schachtii* fit a negative binomial distribution on day 2 (Fig. 3A). The percentage of nematodes with spores but not infected decreased with increasing numbers of spores per nematode (Fig. 3B). The extraction efficiency (J2 extracted ÷ J2 added per vial × 100) was 38 ± 6% and did not change significantly from day 2 to day 9.

Infection of *Heterodera schachtii* by *Hirsutella rhossiliensis* in rhizosphere soil (1,320 × 10^3 spore equivalents per 100 cm^3) was not affected by KCl. Of the *Heterodera schachtii* added in distilled water, 0.003, or 0.03 M KCl treatments, 40 ± 23, 46 ± 11, and 42 ± 7%, respectively, had spores on their cuticles on day 2. Of those with spores, 92 ± 3, 82 ± 10, and 88 ± 10% were infected in the distilled water, 0.003, or 0.03 M KCl treatments, respectively.

In vitro infection: After 3 days, 1.1 ± 0.4 spores per juvenile adhered to the cuticle of *Heterodera schachtii. Hirsutella rhossiliensis* infected 25 ± 20% of *Heterodera schachtii*; of the *H. schachtii* with one or two spores, 32 and 41%, respectively, were infected.

DISCUSSION

As reported by Muller (11), natural infestations of *Hirsutella rhossiliensis* can suppress *Heterodera schachtii.* The peach rhizosphere soil undoubtedly contained antagonists in addition to *Hirsutella rhossiliensis,* but the mortality induced by the

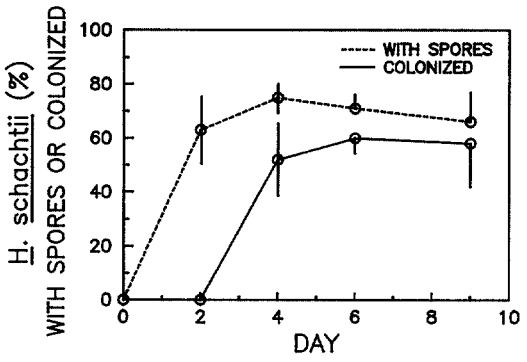

FIG. 2. Percentage of *Heterodera schachtii* juveniles that acquired spores (broken line) or that were colonized (solid line) in soil naturally infested with *Hirsutella rhossiliensis*. Colonized nematodes are dead nematodes with spores on their cuticles and with hyphae-filled body cavities. The percentage of nematodes colonized is a subset of the percentage that acquired spores. Each value is the mean ± SD of four replications.

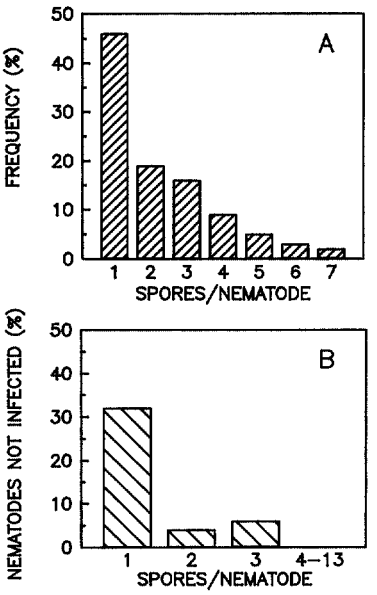

FIG. 3. Infection of *Heterodera schachtii* by *Hirsutella rhossiliensis* after incubation for 2 days in soil naturally infested with the fungus. A) Frequency distribution of numbers of *Hirsutella rhossiliensis* spores per *Heterodera schachtii* juvenile. Sixty-three juveniles that acquired spores were examined. B) Absence of infection of *Heterodera schachtii* as related to numbers of spores per nematode. Live nematodes with 1 (n = 25), 2 (n = 23), 3 (n = 17), or 4–13 (n = 18) spores per nematode were examined at 400× for infection bulbs or hyphae within the body cavity.

fungus (Fig. 2) appeared to explain most of the suppression in root penetration (Table 1).

Heterodera schachtii is not a pest in peach orchards, and cabbage and peach are not rotated. Thus, our results are not immediately useful to culture of either crop. However, we have provided quantitative evidence that natural populations of *Hirsutella rhossiliensis* have the potential to suppress plant-parasitic nematodes. These results and those of Muller (11) encourage us to quantify the spread, survival, and significance of *H. rhossiliensis* in populations of cyst nematodes. In addition to the short-term experiments described here, long-term studies are needed on the temporal feedback between fungus and nematode reproduction and mortality.

Many *Heterodera schachtii* infected the roots in the present study even when spore density was high (Table 1). The probability of a nematode contacting a spore is partially a function of spore numbers and distance nematodes travel through soil before contacting roots (4). Greater suppression may have occurred if the nematodes had been placed farther from the seedling or if planting had been delayed.

The number of *Heterodera schachtii* with *Hirsutella rhossiliensis* spores did not increase after day 2 (Fig. 2). Perhaps nematode motility, and thus the probability of contacting spores, declined due to reduced vigor of the nematodes, suboptimum soil water potential, or the absence of root diffusate. Another possible explanation is that more nematodes than spores were present within certain soil pores. Nematodes moving through soil pores may have acquired many spores before dying (Fig. 3), thus reducing the inoculum for subsequent nematodes moving through the same pores. Finally, some of the nematodes may have been resistant.

Certain salts including KCl stimulate infection of *C. xenoplax* by *Hirsutella rhossiliensis* in vitro, and sandy soils may have suboptimum concentrations of these salts (7). However, KCl amendments did not increase parasitism of *C. xenoplax* by *H. rhos-*

siliensis in greenhouse experiments (3) and did not affect fungal parasitism of *Heterodera schachtii* in the present study.

Nematodes that were touched to two spores and incubated in 0.03 M KCl had relatively few spores adhering to their cuticles after 3 days. Of those with two spores on day 3, fewer than 50% were infected. In contrast, over 90% of the nematodes that acquired two spores in soil were infected by day 2 (Fig. 3B). Possible explanations for reduced spore adherence and infection in vitro include low virulence of laboratory vs. field isolates of *Hirsutella rhossiliensis,* difference in spore–cuticle contact, or differences in the in vitro vs. the soil environment.

One spore of *Hirsutella rhossiliensis* per nematode was sufficient to infect *Ditylenchus dipsaci* on agar plates (2). Our results indicate that one spore per nematode may not be sufficient to infect *Heterodera schachtii* (Fig. 3B). However, nematodes with one spore may have been spore encumbered for less time than nematodes with two or more spores. Thus, the nematodes with one spore that were not infected at day 2 (Fig. 3) may have become infected if examined 1 day later.

High rates of nematode reproduction and low rates of spore transmission may partially explain the failure of *Hirsutella rhossiliensis* to reduce *C. xenoplax* numbers to low levels. Because the spores of *H. rhossiliensis* are nonmotile, moving nematodes must contact them for infection to occur. Timper and Kaya (13) previously suggested that motility of entomogenous nematodes could influence their interaction with nematophagous fungi. Similarly, *H. rhossiliensis* might have a greater effect on *Heterodera schachtii* than on *C. xenoplax* because of the greater motility of *H. schachtii.*

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