

Parasitism of *Heterodera schachtii* and *Meloidogyne javanica* by *Hirsutella rhossiliensis* in Microplots over Two Growing Seasons¹

E. C. TEDFORD, B. A. JAFFEE, A. E. MULDOON, C. E. ANDERSON, AND
B. B. WESTERDAHL²

Abstract: Numbers of cyst and root-knot nematodes and percentage parasitism by the nematophagous fungus *Hirsutella rhossiliensis* were quantified in microplots over 2 years. The microplots contained either sugarbeets in loam infested with *Heterodera schachtii* or tomatoes in sand infested with *Meloidogyne javanica*. The fungus was added to half of the microplots for each crop. Although *H. rhossiliensis* established in both microplot soils, the percentage of nematodes parasitized did not increase with nematode density and nematode numbers were not affected by the fungus. The results indicate that long-term interactions between populations of the fungus and cyst or root-knot nematodes will not result in biological control.

Key words: *Beta vulgaris*, biological control, *Heterodera schachtii*, *Hirsutella rhossiliensis*, *Lycopersicon esculentum*, *Meloidogyne javanica*, microplot, nematode, nematophagous fungus, root-knot nematode, sugarbeet cyst nematode, tomato.

The population density of the endoparasitic fungus *Hirsutella rhossiliensis* Minter & Brady appears to change with that of its host. When many samples were collected at one time in mature peach orchards, the proportion of *Criconebella xenoplax* parasitized by *H. rhossiliensis* was positively correlated with the number of *C. xenoplax* in each sample (7). When samples were collected over time, the number and proportion of *C. xenoplax* parasitized by *H. rhossiliensis* were initially low in a newly planted orchard and then increased slowly as *C. xenoplax* populations increased over the next 6 years (Jaffee, unpubl.). In soil microcosms, the density of *H. rhossiliensis*, as inferred from the proportion of assay nematodes parasitized, increased when host density was high and decreased when host density was low (11). A close relationship between the fungus and nematodes is expected because the fungus is an obligate parasite (5).

Although fungal density may increase with host density, the effect of increased fungal density on suppression of nematodes is unclear. We assume that nematode densities would be higher in the absence of *H. rhossiliensis*, but the fungus may simply "track" nematode density without changing it. Tracking without suppression may occur if adult fecundity compensates for juvenile mortality (17) or if parasitism replaces but does not add to other mortality factors (14).

In addition to *C. xenoplax*, the host range of *H. rhossiliensis* includes species of *Heterodera* (23) and *Meloidogyne* (24), and preliminary results indicate that the fungus may suppress cyst nematodes in some sugarbeet fields in Germany (18,19). In the present study, we examined the interaction of *H. rhossiliensis* with *Heterodera schachtii* Schmidt or *Meloidogyne javanica* (Treub) Chitwood in microplots planted with sugarbeets or tomatoes over two growing seasons. We expected that fungal parasitism of nematodes would increase with increasing nematode density and eventually suppress nematode density.

MATERIALS AND METHODS

Microplots with Heterodera schachtii and sugarbeets: Microplots were located in Davis, California. Each of 12 microplots

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² Department of Nematology, University of California, Davis, CA 95616-8668.

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consisted of a plastic barrel, 53 cm wide and 89 cm deep, buried in the soil so that the top 8 cm extended above the soil surface. The bottom of each barrel had holes for drainage, and the top was open. To improve drainage, 10 cm of gravel was placed below the barrel. Each barrel contained, from bottom to top, 8 cm gravel, 25 cm sand, and 46 cm loam (48% sand, 36% silt, 16% clay; pH 7.0; 1.3% organic matter). The loam was obtained from a field that was fallow for 6 months following a barley crop. Preliminary analysis indicated that *H. rhossiliensis*, *H. schachtii*, and *Meloidogyne* spp. were not present in the loam.

Hirsutella rhossiliensis isolate IMI 265748 was introduced into the microplots as colonized *H. schachtii* juveniles (J2) obtained from deep-dish cultures of the fungus (10). Each deep dish contained 225 cm³ sand infested with 195,000 healthy *H. schachtii* J2 less than 3 days old. After 65 hours at 20 C, the sand from each of 12 cultures was mixed and a 100-cm³ sample was removed; the remaining sand was divided into six 433-cm³ lots. To determine the viability of fungus in the colonized nematodes, nematodes were extracted from the 100-cm³ sample by wet sieving (25- μ m-d pore) and centrifugal flotation (13), and a suspension was spread on water agar amended with 200 mg streptomycin sulfate per liter (6). All nematodes had spores attached to their cuticles (>40 spores per nematode) and were dead. After 2 days at 24 \pm 3 C, *H. rhossiliensis* had sporulated from 95% of the nematodes; the remaining 5% appeared to be parasitized but did not support sporulation for unknown reasons. To obtain sand without parasitized nematodes for control plots, 12 deep-dish cultures were treated in the same manner but were not inoculated with fungus or nematodes.

Sand also was used to add nematode inoculum (healthy *H. schachtii*) to the microplots. Sand infested with healthy *H. schachtii* was obtained from 4- to 6-month-old pot cultures of sugarbeet (*Beta vulgaris* L. 'SSNB-2'). The sand contained approximately 67,000 eggs and J2 per liter (as de-

termined by Baermann funnel extraction for 4 days) and was mixed in a cement mixer. Each microplot received 3 liters of sand infested with healthy *H. schachtii*. Half of the microplots received an additional 433 cm³ sand containing fungal-colonized *H. schachtii*, and the other half received sand without the fungus. These treatments were added by removing the top 5 cm of loam from each microplot, mixing the sand (nematode-infested plus fungus-infested or control) and 100 g of slow-release fertilizer (17-6-10) into the soil to a depth of 15 cm, and returning the surface loam.

Microplots with Meloidogyne javanica and tomatoes: Twelve microplots were established adjacent to the sugarbeet microplots. The procedures for installing the microplots were the same as described for the sugarbeet microplots with the following modifications. The barrels contained 8 cm gravel below 71 cm sand (98% sand with 50, 37, and 13% coarse, medium, and fine sand, respectively; pH 8.1; <0.1% organic matter). The sand was untreated. Preliminary analysis indicated that the sand did not contain plant-parasitic nematodes or *H. rhossiliensis*.

Hirsutella rhossiliensis was introduced into the tomato microplots in the form of colonized *Steinernema glaseri* Steiner. *Steinernema glaseri* was used instead of *H. schachtii* because more spores were produced from *H. rhossiliensis*-colonized *S. glaseri* (25) than from colonized *H. schachtii* (10); thus, fewer nematodes were needed to introduce the fungus. In addition, large numbers of *S. glaseri* were readily available (26), and we did not want to risk introducing viable *H. schachtii* into these microplots. *Hirsutella rhossiliensis*-colonized *S. glaseri* were obtained from deep-dish cultures (25). Sand in each deep dish was infested with 35,200 healthy *S. glaseri* third-stage (dauer) juveniles. After 66 hours at 20 C, the sand from 12 deep dishes was mixed, and nematodes were extracted from a 100-cm³ sample, as described in the sugarbeet microplot section. The remaining soil was divided into six 433-cm³ lots. Each mi-

croplot received either 433 cm³ sand containing *H. rhossiliensis*-colonized *S. glaseri* or 433 cm³ sand without colonized nematodes (control) as described in the sugarbeet microplot section. To test the viability of the fungal inoculum, nematodes were extracted from the 100-cm³ sample by wet sieving and centrifugal flotation (13), and a suspension was spread on water agar amended with 200 mg streptomycin sulfate per liter (6). After 2 days at 25 ± 3 C, *H. rhossiliensis* had sporulated from 100% of the *S. glaseri*.

Meloidogyne javanica was introduced into each microplot by planting six 8-week-old tomato plants (*Lycopersicon esculentum* Mill. 'UC82') previously inoculated with 2,500 and 1,000 *M. javanica* J2 when plants were 6 and 7 weeks old, respectively. After the second inoculation, the plants were kept in a lath house for 1 week before transplanting.

Planting and maintenance of microplots: Three healthy sugarbeet plants, grown for 6 weeks in pots containing 400 cm³ loam, were transplanted equidistant (23 cm apart) into each cyst-nematode-infested microplot on 7 May 1990. Microplots were drip irrigated one to three times a week from May to November, but otherwise received only rainfall. Soil temperature at 15 cm was measured hourly in two microplots. The sugarbeets were removed on 5 April 1991, and three new sugarbeet plants (6 weeks old) per microplot were planted in their place. Following planting, 100 g slow-release fertilizer (17-6-10) was added to the surface of the loam in each microplot.

On 17 July 1990, six tomato plants infected with *M. javanica* were planted 17 cm apart, with five outer plants and one plant in the center, into each of the remaining 12 microplots. Microplots were shaded by a double layer of cheesecloth for 4 days to reduce transplant shock. The microplots were drip irrigated daily from July to November, but otherwise they received only rainfall. The tomato plants died by November 1990, at which time shoots were cut and roots were left in the soil. At the

start of the second growing season (25 March 1991), six uninoculated tomato plants (8 weeks old) were planted in each microplot. One week after planting, 100 g of slow-release fertilizer (17-6-10) was added to the surface of the sand in each microplot. All of the tomato plants died by August.

Enumeration of nematodes and fungal parasitism: Soil samples were collected every 1 to 4 months over two growing seasons. Five soil cores (2-cm-d by 34-cm-deep) were collected on each sampling date and combined in one 500-cm³ soil sample per microplot. The holes created by the sampling tool were refilled with noninfested loam or sand.

Soil samples were processed within 2 hours of collection. A 100-cm³ subsample from each sample was soaked in water for 10 minutes and suspended in 1 liter of water. After 20 seconds, the supernatant was poured through nested 833- and 38-µm-pore sieves. Material collected on the 38-µm-pore sieve was centrifuged in water. The pellet was suspended in sucrose (454 g/liter) and centrifuged, and the supernatant was poured through a 25-µm-pore sieve. The material collected on the sieve was washed into a vial, and the volume was adjusted to 5 ml. The suspension was mixed, and 0.5 ml was spread onto each of five 9-cm-d petri plates containing water agar amended with streptomycin (6). Plates were incubated at room temperature for 48 to 72 hours and then examined at 100× magnification; the number of nematodes supporting sporulation of *H. rhossiliensis* was determined. The total from five dishes was multiplied by two to calculate the number of parasitized nematodes per 100 cm³ soil. The remaining 2.5 ml of suspension in each vial was increased with water to 5 ml, and nematodes (J2 of *H. schachtii* and *M. javanica*, males of *H. schachtii*, and other nematodes) in 1 ml were counted. The number of nematodes in 1 ml was multiplied by 10 to calculate the number of vermiform nematodes per 100 cm³ soil.

A bioassay was used to estimate the

number of living nematodes in soil samples. A 100-cm³ subsample from each soil sample was placed into a styrofoam cup with holes in the bottom, and six germinated cabbage (*Brassica oleracea* L. 'Chieftain Savoy') or tomato ('UC 82') seeds (for microplots with sugarbeets or tomatoes) were planted in each cup. Cups were placed in a clear plastic box with moistened paper towels and were incubated under fluorescent lights (12-hour photoperiod) at 23 ± 3 C. After 6 days, roots were removed from the soil and stained (1), and the number of nematodes within the roots were counted at 100–140× magnification.

Heterodera schachtii eggs were quantified from sugarbeet microplot soil samples every 3–4 months. Soil samples (250 cm³) from each microplot were dried, and cysts and eggs within cysts were extracted and counted (2).

RESULTS

Microplots with Heterodera schachtii and sugarbeets: Average high and low soil temperatures for each month were calculated based on daily high and low soil temperatures (Fig. 1). Numbers of *H. schachtii* J2 in soil peaked in July 1990, 2 months after planting, and in February of 1991 and 1992 (Fig. 2A). Males were present in soil at much lower levels than were J2 throughout the study (Fig. 2B). Numbers of eggs increased from the initial date of planting until April of the second growing season, when their density was extremely high

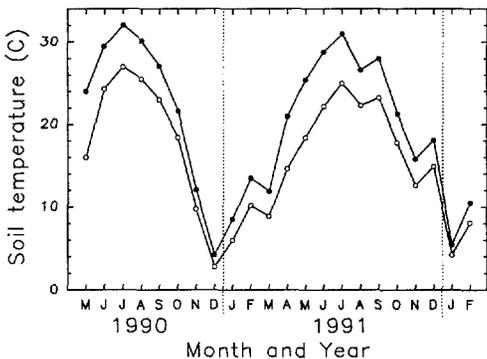


FIG. 1. Average monthly high and low soil temperatures at 15 cm depth in sugarbeet microplots.

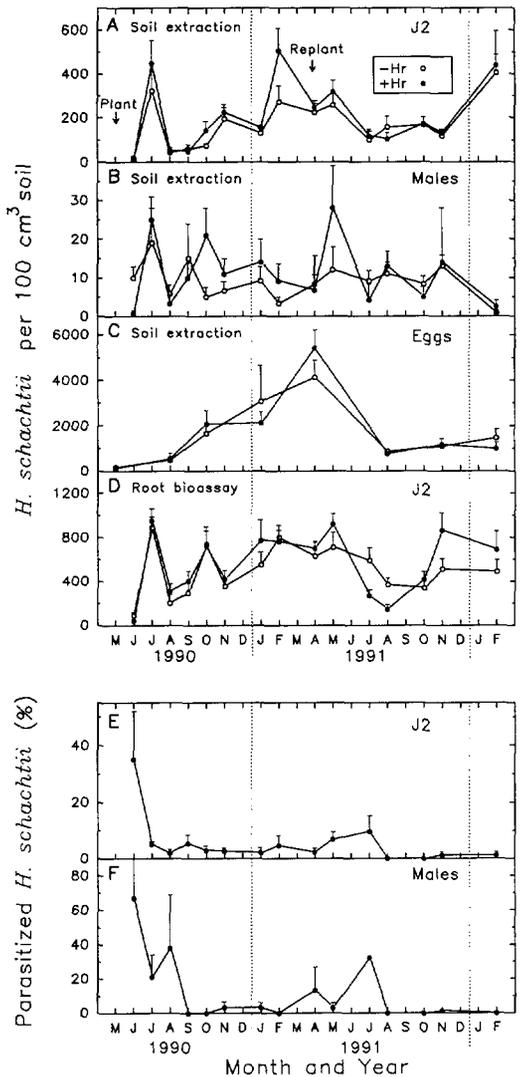


FIG. 2. Numbers of *Heterodera schachtii* and percentage parasitized by *Hirsutella rhossiliensis* in sugarbeet microplots. A) Juveniles (J2), B) males, or C) eggs extracted per 100 cm³ soil. D) Number of *H. schachtii* J2 in roots of six bioassay cabbage seedlings grown in 100 cm³ microplot soil. E, F) Percentage of J2 and males parasitized by *H. rhossiliensis*. For A–D, each value is the mean ± SE of six replicate microplots with (+ Hr) or without (– Hr) *H. rhossiliensis*; for E and F, each value is the mean ± SE of six replicate + Hr plots. No variance is shown for percentage of parasitized males in July 1991 because males were detected in only one + Hr plot. No parasitism was observed in the – Hr plots.

(Fig. 2C); egg numbers then declined to an intermediate level for the remainder of the second growing season. Numbers of nematodes detected in the root bioassay were

somewhat higher than those detected in soil (Fig. 2D).

The addition of *H. rhossiliensis* did not affect the numbers of *H. schachtii* detected in soil (Fig. 2A–C) or in roots (Fig. 2D).

One month after planting, nearly 40% of the J2 in soil were parasitized by *H. rhossiliensis* (Fig. 2E). These parasitized J2 had one to four spores adhering per nematode, in contrast to the 40 or more spores per parasitized J2 added at planting. The percentage of parasitized J2 dropped to a low level for the remainder of the experiment.

Parasitized males were present in summer of the first season and in spring and summer of the second season (Fig. 2F). As with the J2, the highest percentage of parasitized males was detected 1 month after planting, and parasitism appeared unrelated to change in nematode density.

Microplots with Meloidogyne javanica and tomatoes: Numbers of *M. javanica* J2 in soil increased to extremely high levels by the end of the first growing season (Fig. 3A) and then declined to lower levels for the remainder of the experiment. Nevertheless, numbers were greater than 500 J2 per 100 cm³ of soil, except in the spring and fall of the second growing season.

Fewer J2 were found in the root bioassay (Fig. 3B) than were extracted from soil (Fig. 3A). The bioassay plants, however, were heavily galled and stunted, and the roots contained high numbers of J2 at most sampling dates.

As with cyst nematodes in the sugarbeet microplots, *H. rhossiliensis* did not suppress root-knot nematodes in the tomato microplots (Fig. 3A,B). Parasitized J2 were detected in soil in the fall and winter of the first growing season and in the spring of the second growing season (Fig. 3C). The percentage of J2 parasitized never exceeded 0.2%.

DISCUSSION

Contrary to our expectations, fungal parasitism did not increase with nematode density, and *H. rhossiliensis* did not sup-

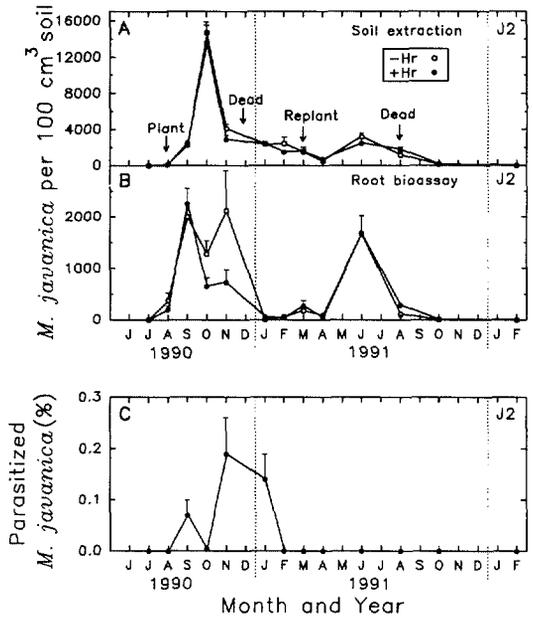


FIG. 3. Numbers of *Meloidogyne javanica* and percentage parasitized by *Hirsutella rhossiliensis* in tomato microplots. A) Juveniles (J2) extracted per 100 cm³ soil. B) J2 in roots of six tomato seedlings grown in 100 cm³ of tomato microplot soil. C) Percentage of J2 parasitized by *H. rhossiliensis*. For A and B, each value is the mean \pm SE of six replicate microplots with (+ Hr) and without (- Hr) *H. rhossiliensis*; for C, each value is the mean \pm SE of six replicate + Hr plots. No parasitism was observed in the - Hr plots.

press numbers of cyst and root-knot nematodes. These results might reflect inadequate methods for adding an alien fungus to soil (3,20), but our data suggest that *H. rhossiliensis* did establish, albeit at low levels. The recovery of parasitized nematodes over one (Fig. 3) or two (Fig. 2) growing seasons indicates that sporulation and transmission occurred repeatedly, because parasitized nematodes rapidly disappear from soil due to degradation by the fungus (6,9).

Several other lines of evidence besides the rapidity with which parasitized nematodes disappear indicate that the parasitized cyst nematodes recovered after introduction were not those added to the soil. Each parasitized nematode added to the soil had more than 40 spores, whereas those recovered on day 30 and thereafter had fewer than 5 spores. Moreover, parasitized males were recovered but had not

been added. In the tomato microplots, *H. rhossiliensis* was introduced as parasitized *S. glaseri*, and the presence of parasitized *M. javanica* therefore must reflect sporulation and transmission from the original source of inoculum. We conclude that the introduction of *H. rhossiliensis* to the microplots was successful but that the fungus subsequently did not respond to the high nematode densities for unknown reasons.

Abiotic or biotic factors may explain why parasitism did not increase with increasing host density, but no one factor stands out. Soil texture affects transmission (the probability of a nematode contacting a spore), but transmission of *H. rhossiliensis* to *H. schachtii* and *M. javanica* occurred in both microplot soils in microcosms (24). Transmission in microcosms was greater in the loam than in the sand (24), and this may account for the higher levels of parasitism in the sugarbeet microplots. Soil matric potential also affects transmission (24) but was not measured in this study. Soil temperature in this study was frequently above or below the optimum (25 C) for sporulation (12), and the effect of temperature on other aspects of *H. rhossiliensis* biology is unknown. The effect of antagonists on *H. rhossiliensis* also is unknown.

If isolates of the fungus were host-specific, the low levels of parasitism could reflect the use of an inappropriate isolate. However, the isolate used in this study (IMI 265748), although originally obtained from *C. xenoplax*, was as virulent to *H. schachtii* and *M. javanica* as were isolates from *H. schachtii* or other nematodes (Tedford and Jaffee, unpubl.).

Failure of *H. rhossiliensis* to suppress cyst and root-knot nematodes may be due to limited exposure of nematodes to spores. Both nematode species are in roots for much of their life cycle, and only juveniles and males move through soil. The probability of contacting a spore depends, in part, on the volume of soil traversed (4,22,25). This volume may be quite small if juveniles reinfect the same roots infected by their parents (21). In addition, transmission does not always prevent nematodes

from penetrating roots, and nematodes may escape infection if they molt before being penetrated by the fungus (8).

The decline in nematode numbers over time was most likely due to nematode-induced damage to the plants. Our experiments did not include nematode-free controls, but nematode densities were above damaging levels: The sugarbeet plants did not look healthy but survived, and the tomato plants were extensively galled and died midseason.

Spore density is a critical parameter in this host-parasite system but was not measured because suitable methods are lacking. Spore density was highly correlated with parasitism in microcosm studies (11), where known numbers of assay nematodes were added to soil for known lengths of time and with close control of temperature and water. Conditions in our field microplots were not controlled, however, and it is possible that spore density and parasitism were not tightly coupled. Quantification of spore density would have enhanced our understanding of the results, and a direct assay for spores of *H. rhossiliensis* is needed.

Our experiments focused on long-term biological control, which may develop as the fungus cycles within the host population over several growing seasons. The results were not encouraging, at least for root-knot and cyst nematodes under our experimental conditions. Nevertheless, the fungus may be useful for short-term control of these nematodes if high spore densities can be obtained by addition of fungal inoculum to soil at or before planting (15,16).

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