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Parasitic and Saprophytic Abilities of the Nematode-Attacking Fungus *Hirsutella rhossiliensis*[1]

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Abstract: The ability of *Hirsutella rhossiliensis* to colonize various substrates in sterile and nonsterile soil was measured. *Hirsutella rhossiliensis* was recovered from 67% and 77% of living, inoculated *Criconemella xenoplax* incubated in sterile and nonsterile soil, respectively. In contrast, the fungus was recovered from 100% and 18% of heat-killed, inoculated nematodes incubated on sterile and nonsterile soil, respectively. *Hirsutella rhossiliensis* was readily recovered from inoculated, autoclaved wheat seeds incubated in sterile soil but not from seeds incubated in nonsterile soil. Autoclaved peach roots were a poor substrate for the fungus. Germination of *H. rhossiliensis* spores incubated on agar disks above soil was about 90% regardless of soil treatment. However, germ tube length was greatly suppressed by nonsterile soil. Our results suggest that *H. rhossiliensis* is a better parasite than saprophyte and that the fungus may be specialized for attacking nematodes.

Key words: biological control, *Criconemella xenoplax, Arthrobotrys oligospora.*

The fungus *Hirsutella rhossiliensis* Minter and Brady has been isolated from the plant parasitic nematode *Criconemella xenoplax* (Raski) Luc & Raski in the United States and from *Heterodera avenae* Woll. in Australia (5,15). Another species, *Hirsutella heteroderae,* probably synonymous with *H. rhossiliensis,* was isolated from *Heterodera humuli* Filipjev in Germany (5,17). *Hirsutella rhossiliensis* produces spores that adhere to passing nematodes. The fungus penetrates the nematode's cuticle, assimilates its body contents, and then emerges and sporulates (5–7). Although *H. rhossiliensis* is clearly capable of parasitic activity, it also can grow saprophytically on many laboratory media and on dead nematodes incubated in water or soil solution. The purpose of the present study was to determine the relative parasitic and saprophytic abilities of *H. rhossiliensis* in sterile and nonsterile soil.

Materials and Methods

General: Soil was collected from two South Carolina peach orchards (designated soils A and B) either 40 weeks before use (A-40) or 3 weeks before use (A-3 and B-3). *Hirsutella rhossiliensis* had been consistently isolated from *C. xenoplax* extracted from both soils before the samples were collected. Whereas soils A-3 and B-3 contained living, healthy *C. xenoplax* and *C. xenoplax* at various stages of infection and colonization, A-40 contained only dead *C. xenoplax* some of which were colonized (filled with hyphae). The colonized nematodes from A-40 were distorted and brown and appeared to have supported sporulation because the fungal hyphae within the cuticle were contracted (5,7). The absence of living *C. xenoplax* and the distinctive appearance of the colonized *C. xenoplax* from soil A-40 allowed us to separate nematodes experimentally introduced into this soil from nematodes already present. After 5 days of incubation in A-40, introduced nematodes were either alive and noninfected, alive and newly infected, or dead and newly colonized. Newly colonized nematodes were neither brown nor distorted and the hyphae within the cuticle were not contracted.

Hirsutella rhossiliensis (ATCC 46487, originally isolated from an adult *C. xenoplax* extracted from soil A) was maintained on cornmeal agar at 25 C; cultures were transferred monthly. *Criconemella xenoplax* was maintained on peach seedlings grown in autoclaved soil in a greenhouse. Nematodes were extracted from soil by elutriation followed by centrifugation. The incubation temperature for all experiments was 25 C.

Isolation of H. rhossiliensis from inoculated nematodes incubated in soil: Spores of *H. rhossiliensis* were placed on the anterior or posterior third of heat-killed (30 minutes at 60 C in 1 ml water) or alive *C. xenoplax* (5). Each nematode received five spores.

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Spores were obtained from *H. rhossiliensis* grown on cornmeal agar for 2–4 weeks. Inoculated nematodes were placed on the surface of 1 cm^3 moist (−0.5 bars), sterile (autoclaved 1 hour) or nonsterile soil (A-40) in BPI watch glasses (20 mm × 8 mm inside dimensions) and incubated in moist chambers for 5 days. The heat-killed nematodes were examined for fungal sporulation and then recovered from the surface of the soil with the aid of reflected light and a dissecting microscope. Soil to which living nematodes were added was washed into a 25-ml vial containing 15 ml distilled water (one vial per watch glass of soil). The vial was shaken vigorously for 30 seconds, and the supernatant was divided among six plastic 10-cm-d petri plates. This process was repeated three times for each vial. The nematodes were removed with a pick from the petri plates, examined at 140× for infection, surface disinfested, and placed on water agar (5). Each soil sample contained 10 live or heat-killed nematodes. Samples were replicated three times, and the experiment was performed three times.

To determine if the disinfestation method killed *H. rhossiliensis* spores on the surface of *C. xenoplax,* 20 living and 20 heat-killed nematodes were inoculated, immediately disinfested, and incubated on water agar as described earlier.

Isolation of H. rhossiliensis from inoculated wheat seeds or peach roots incubated in soil: Ten milliliters of wheat seeds (*Triticum aestivum* L. 'Blue boy') plus 10 ml distilled water in glass petri plates were autoclaved for 1 hour on 2 successive days. The seeds were placed on the surface of a 1-month-old *H. rhossiliensis* colony grown on Difco potato-dextrose agar. The number of spores adhering to each seed was not determined. Ten infested seeds were transferred immediately to sterile moist chambers. Additional infested seeds were buried in sterile and nonsterile soil (A-40) in petri plates (15 seeds/plate, replicated three times). Seeds in moist chambers were examined periodically for evidence of *H. rhossiliensis.* After 2 weeks, seeds in soil were removed, washed in running tap water for 20 minutes, disinfested in 1% NaOCl plus Tween 20 for 1 minute, rinsed three times in sterile distilled water, blotted dry, placed on water agar plates, and examined daily for 1 week and again after 30 days. Autoclaved Lovell peach roots (1 cm long and 2 mm in diameter) were infested and incubated in moist chambers and soil (15 roots per plate, replicated three times) as described for the seeds. The experiments were performed two times with seeds and once with roots. In a related experiment, 50 autoclaved wheat seeds were inoculated and incubated for 7 days in moist chambers. The seeds were then incubated for 2 weeks in sterile or nonsterile soil, and the fungi were isolated. This experiment was performed once.

Isolation of H. rhossiliensis from noninoculated, heat-killed nematodes incubated on soil: C. xenoplax adults were heat-killed, placed on the surface of moist soil (A-3 or B-3) in watch glasses, and incubated in moist chambers for 5 days as described earlier. Forty-five nematodes were placed on each soil (15 nematodes/watch glass). After incubation the nematodes were recovered, disinfested, and placed on water agar. The experiment was performed twice.

Isolation of H. rhossiliensis from peach root fragments: Approximately 2 cm^3 soil (A-3 or B-3) was placed in 25-ml vials (2 cm^3 soil/vial) containing 20 ml distilled water. Each vial was shaken vigorously, and the supernatant was poured into a petri plate. Ten peach root fragments (1–3 mm × 0.1–0.2 mm) were obtained from five 2-cm^3 soil samples of soil A-3 or B-3 (50 root fragments/orchard). The root fragments were disinfested in 0.5% NaOCl for 15 seconds, rinsed in sterile distilled water, blotted dry, and incubated on water agar. This experiment was performed once.

Sensitivity of H. rhossiliensis to fungistasis: The system used was similar to that described by Bailey et al. (1). Water agar (2%) was added to 10-cm-d petri plates, and a 20 × 4-mm cylinder of agar was removed from the center. Water-saturated sterile or nonsterile soil (A-40) was added to the cavity, and a 4.7-cm-d cellulose esters membrane with 0.4-μm pores was placed on the soil. A disk (10 × 1 mm) of 2% water agar adjusted to −0.3 bars with KCl was incubated on the membrane above the soil for 4 hours. Spores of *H. rhossiliensis* were then placed on the agar disk by gently touching a small portion of a colony (grown on Difco potato-dextrose agar for 10 days) to the disk. The spores detached from the phialides and adhered to the disk. Contact be-

TABLE 1. Recovery of *Hirsutella rhossiliensis* from *Criconemella xenoplax* incubated in sterile or nonsterile soil.*

Conditon of nematodes	Soil	Sporulation on soil surface (%)†	Nematodes colonized at harvest (%)‡	Fungi isolated (%)§	
				Hr	Other
Heat-killed	Sterile	100 ± 0		100 ± 0	0 ± 0
Heat-killed	Nonsterile	15 ± 4		18 ± 2	67 ± 12
Alive	Sterile		52 ± 31	67 ± 17	0 ± 0
Alive	Nonsterile		64 ± 29	77 ± 13	0 ± 0

* Adult *C. xenoplax* were heat-killed or alive, inoculated with five spores of *H. rhossiliensis* per nematode, and incubated in sterile or nonsterile soil for 5 days at 25 C. All values are means ± SD of three replications, 20–30 nematodes per replication.

† After 5 days of incubation on soil surface, heat-killed nematodes were examined in situ for *H. rhossiliensis* sporulation.

‡ After 5 days of incubation in soil but before disinfestation, nematodes (not heat-killed) were examined for fungal hyphae within the body cavity.

§ Nematodes were removed from soil, surface disinfested, and incubated on water agar. The fungi growing from the nematodes were identified as *H. rhossiliensis* (Hr) or other fungi.

tween the potato-dextrose agar and the disk did not occur. Each treatment was replicated four times. After inoculation, the plates were incubated for 20 and 40 hours at 25 C. After incubation, 50 randomly selected spores from each disk were observed for germination and 10 for germ tube length. The experiment was performed twice, but in the second experiment, observations were made only after 40 hours.

RESULTS

Isolation of H. rhossiliensis from inoculated nematodes incubated in soil: H. rhossiliensis was frequently isolated from inoculated, living or heat-killed nematodes incubated in sterile soil and from living nematodes incubated in nonsterile soil (Table 1). However, the fungus was infrequently isolated from inoculated, heat-killed nematodes incubated in nonsterile soil. Fungi other than *H. rhossiliensis* were isolated only from nematodes that were heat-killed and incubated in nonsterile soil. All 180 heat-killed nematodes and 128 (71%) of the living nematodes added to soil were recovered. *Hirsutella rhossiliensis* did not grow from any of the 20 living or heat-killed nematodes that were inoculated, immediately disinfested, and incubated on water agar.

Isolation from inoculated wheat seeds or peach roots incubated in soil: After 7 days in moist chambers, *H. rhossiliensis* overgrew the entire surface of wheat seeds and produced abundant spores. In contrast, growth and sporulation on peach roots in moist chambers were very sparse. The fungus was isolated from all wheat seeds but from only 20% of peach roots incubated in sterile soil (Table 2). Whereas seeds and roots incubated in nonsterile soil never yielded *H. rhossiliensis,* they frequently yielded other fungi, especially *Arthrobotrys oligospora* Fres.

Inoculated, autoclaved wheat seeds also were incubated for 7 days in moist chambers to give *H. rhossiliensis* an advantage in colonizing the seeds. Then they were placed in sterile or nonsterile soil for 2 weeks, and the fungi were isolated. *Hirsutella rhossiliensis* was recovered from 25 of 25 seeds incubated in sterile soil but only 1 of 25 seeds incubated in nonsterile soil. *Arthrobotrys oligospora* was recovered from 22 of 25 seeds incubated in nonsterile soil.

Isolation from noninoculated, heat-killed nematodes incubated on soil and from peach roots in nonsterile soil: H. rhossiliensis was recovered from only 1 of the 180 noninoculated, heat-killed nematodes incubated on nonsterile soil. Other fungi were recovered from approximately 80% of the nematodes. Neither *H. rhossiliensis* nor *A. oligospora* was recovered from peach root fragments naturally present in soil A-3 or B-3.

Sensitivity to fungistasis: Observations for the two experiments were similar; data shown here are from the first experiment. Most spores germinated within 20 hours in both sterile and nonsterile soil treatments (Table 3). Germ tube lengths in both treatments were similar at 20 hours, but at 40 hours germ tubes incubated above sterile soil were about four times longer than those incubated above nonsterile soil. Germ tube diameters in sterile and nonsterile soil

TABLE 2. Isolation of *Hirsutella rhossiliensis* and *Arthrobotrys oligospora* from inoculated wheat seeds and peach roots incubated in soil.*

Substrate	Soil	Seeds or roots with fungi (%)	
		H. rhossiliensis	*A. oligospora*
Wheat seeds	Sterile	100†	0
Wheat seeds	Nonsterile	0	95
Peach roots	Sterile	20‡	0
Peach roots	Nonsterile	0	95

* Autoclaved wheat seeds or peach roots were inoculated with *H. rhossiliensis* spores, incubated in sterile or nonsterile soil, recovered after 2 weeks, surface disinfested, and incubated on water agar.

† Based on a total of 90 seeds examined after 7 days of incubation on water agar.

‡ Based on a total of 45 roots examined after 30 days of incubation on water agar.

TABLE 3. Germination and germ tube length of *Hirsutella rhossiliensis* spores incubated above sterile and nonsterile soil for 20 and 40 hours.*

Soil	20-hour incubation		40-hour incubation	
	Germination (%)	Germ tube length (μm)	Germination (%)	Germ tube length (μm)
Sterile	89 ± 8†	18 ± 2‡	93 ± 4	128 ± 13
Nonsterile	91 ± 6	16 ± 1	93 ± 8	33 ± 5

* Spores of *H. rhossiliensis* were placed on water agar disks. The agar disks were placed on membranes positioned on moist sterile or nonsterile soil.

† Values are the means ± SD of four replications with 50 spores examined per replication.

‡ Values are the means ± SD of four replications with 10 spores measured per replication.

treatments were about 2 μm and 1 μm, respectively. Germ tubes in the sterile soil treatment were of uniform diameter, but those in the nonsterile soil treatment were constricted and swollen.

DISCUSSION

Nematode-attacking fungi differ in their saprophytic and parasitic abilities. Some, such as *Nematophthora gynophila,* appear to be obligate parasites whereas others, such as *A. oligospora,* compete saprophytically for certain substrates (2,8,11). In our study, *H. rhossiliensis* was a poor competitive saprophyte. The fungus colonized dead nematodes and autoclaved wheat seeds in the absence, but not in the presence, of other soilborne fungi. The fungus failed to colonize wheat seeds incubated in nonsterile soil even when given a time advantage over other organisms.

Parasitic and saprophytic abilities of nematode-attacking fungi are often inversely related; i.e., efficient parasites are inefficient saprophytes and vice versa (2,8,9). The behavior of *H. rhossiliensis* appears to be consistent with this statement. Its competitive saprophytic ability is limited, whereas its parasitic ability is substantial. Approximately 70% of inoculated living nematodes were parasitized, and the level of parasitism did not appear to be affected by soil microflora.

It is not certain why *H. rhossiliensis* competes well for living nematodes but poorly for heat-killed nematodes. Perhaps this fungus is relatively specialized for parasitism and depends on the intact cuticle of its host to avoid antagonism. During germination, penetration, infection, and colonization, the cuticle of the host nematode remains intact, and the hyphae of *H. rhossiliensis* are thus physically separated from potential antagonists (5,7,17). The fungus does not emerge and sporulate until the contents of the nematode are assimilated and thus unavailable to competitors. In contrast, the cuticle of heat-killed nematodes may permit penetration by less specialized fungi that can then compete successfully with *H. rhossiliensis* for nematode substrate.

The different sensitivities of spore germination and germ tube elongation to fungistasis also suggest that *H. rhossiliensis* is specialized for parasitism. There is no obvious advantage for a detached *H. rhossiliensis* spore to remain dormant (i.e., sensitive to fungistasis) when adhering to the cuticle of the host nematode. Apparently, the spore does not distinguish between the surface of a nematode, agar disk, or wheat seed and germinates well on all of these. However, the agar disk and wheat seed do not protect the germ tube from antagonism, and the growth of the fungus on agar disks or wheat seeds on or in nonsterile soil is greatly suppressed.

Unlike *H. rhossiliensis,* nematode-trapping fungi usually germinate poorly in nonsterile soil (3,12). Like *H. rhossiliensis,* the germ tubes of three nematode-trapping fungi were stunted and appeared weak in nonsterile soil. Based partly on this ob-

servation, Mankau (12) concluded that the three nematode-trapping fungi he studied were poor competitive saprophytes.

Wheat seeds are a good substrate for *H. rhossiliensis* under axenic conditions, and their size facilitates their recovery from the soil. Wheat seeds colonized with *H. rhossiliensis* also might be used to introduce the fungus into soil or increase its inoculum potential. Seeds of wheat, rye, and rice have been used to introduce other nematophagous fungi into soil (10). However, our results indicate that *H. rhossiliensis* cannot be readily introduced into nonsterile soil on wheat seeds. In contrast, *A. oligospora* was frequently recovered from autoclaved wheat seeds or peach roots incubated in nonsterile soil. Whereas *H. rhossiliensis* grows slowly on laboratory media and is an endozoic fungus, *A. oligospora* grows rapidly and is a nematode-trapping fungus (2). The organic matter and saprozoic nematodes associated with biodegrading seeds may have been responsible for the frequent recovery of this fungus, but *A. oligospora* is also a mycopathogen and may have been parasitizing *H. rhossiliensis* (18).

Stirling and Mankau (16) demonstrated that *Dactylella oviparasitica* parasitized eggs of root-knot nematodes in peach orchard soil. This fungus was also isolated from dead peach roots (14). The ability of *D. oviparasitica* to grow saprophytically on dead peach roots was considered a desirable characteristic that might permit the fungus to persist when few nematode hosts were present (13). Unlike *D. oviparasitica, H. rhossiliensis* was a poor colonizer of dead peach roots.

Although our results indicate that *H. rhossiliensis* is a better parasite than competitive saprophyte, we do not conclude that *H. rhossiliensis* has no saprophytic potential in soil. Competitive saprophytic ability is greatly influenced by substrate and environment (4). For example, dead wheat seeds were good substrates for *A. oligospora* but dead *C. xenoplax* were not. Given different substrates and environmental conditions, *H. rhossiliensis* might be more competitive than was demonstrated in our experiments.

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