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Failure of a Mycelial Formulation of the Nematophagous Fungus *Hirsutella rhossiliensis* to Suppress the Nematode *Heterodera schachtii*

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Research was conducted to determine whether pelletized hyphae of *Hirsutella rhossiliensis* suppressed invasion of roots by the sugarbeet cyst nematode *Heterodera schachtii* in field microplots. The loamy sand in the microplots was infested with *H. schachtii* but not with *H. rhossiliensis.* Alginate pellets, with or without hyphae of *H. rhossiliensis,* were mixed into soil removed from the microplots (1 pellet/cm³ of soil). The soil was placed in cylinders positioned vertically in microplots; cylinders (6/microplot) were 10.1 cm wide and 15.3 cm deep and contained 1200 cm³ of soil. Pellets and soil also were placed in soil observation chambers, which were buried in the cylinders or kept at 20°C in moisture chambers in the laboratory. After 12 days, cabbage seeds were planted in each cylinder, and after 10 days of growth, the seedlings were removed from the soil and *H. schachtii* in the roots were counted. The number of *H. schachtii* in roots was large and was unaffected by addition of *H. rhossiliensis.* In soil observation chambers, *H. rhossiliensis* grew vigorously from the pellets in heat-treated soil but not in nonheated soil, and enchytraeids and collembolans were observed near damaged pellets. We suspect that organisms, possibly including enchytraeids and collembolans, fed upon or otherwise inhibited *H. rhossiliensis.* © 1996 Academic Press, Inc.

KEY WORDS: alginate; biological control; Collembola; Enchytraeidae; fungistasis; inundative release; mycelial or hyphal formulation; nematophagous; sugarbeet cyst nematode; *Hirsutella rhossiliensis.*

INTRODUCTION

The fungus *Hirsutella rhossiliensis* Minter & Brady produces conidia that adhere to motile nematodes in soil. Once attached to a nematode, the conidium germinates. The germ tube directly penetrates the host cuticle, and assimilative hyphae grow through and consume the nematode. After killing and colonizing the host, the fungus grows from the cadaver and produces a new cohort of conidia (Sturhan and Schneider, 1980; Jaffee, 1992).

One host of *H. rhossiliensis* is the sugarbeet cyst nematode, *Heterodera schachtii* Schmidt. Juveniles (J2) of this nematode hatch from eggs, move through soil pores, and penetrate host roots, where they develop into adults.

To achieve biological control of *H. schachtii* and other nematodes via inundative addition of *H. rhossiliensis* to soil, we have considered three forms of *H. rhossiliensis* inoculum: conidia, colonized hosts, and assimilative hyphae. Conidia of *H. rhossiliensis* are not useful for infestation of soil because they do not adhere to nematodes unless produced *in situ* (McInnis and Jaffee, 1989). In contrast, the colonized nematode is an effective form of inoculum. When *H. rhossiliensis*-colonized nematodes were added to soil microcosms, substantial proportions of *H. schachtii* were parasitized (Jaffee *et al.,* 1992). Moreover, addition of colonized nematodes initially caused high levels of parasitism of *H. schachtii* in field microplots (Tedford *et al.,* 1993). Although colonized nematodes can be produced in the laboratory, the procedure is labor intensive and probably unsuitable for commercialization or even for large-scale field experimentation.

For inundative addition of *H. rhossiliensis* to soil, assimilative hyphae have been considered as a substitute for the colonized nematode. Assimilative hyphae, which are produced within the host or in shake culture, supported sporulation when added to a variety of soils (Lackey *et al.,* 1992). The hyphae have been pelletized in alginate, dried, and stored for over 1 year at 5°C; in laboratory experiments, *H. rhossiliensis* grew from such pellets, formed conidia, and substantially suppressed *H. schachtii* (Lackey *et al.,* 1993).

The purpose of the present study was to determine whether pelletized hyphae of *H. rhossiliensis* suppressed root invasion by *H. schachtii* in field microplots. A second objective was to monitor the fate of the fungal inoculum after it was added to soil.

MATERIALS AND METHODS

Pellet preparation and viability. Preparation of alginate pellets, with and without (control) hyphae of *H. rhossiliensis* (IMI 265748), has been described (Lackey

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et al., 1993). Moist pellets were coated with quartz sand (Lackey *et al.,* 1994) and dried for 24 h. The viability of the dried pellets was measured using a standard soil assay as follows. Nonheated loamy sand (pH 4.9, −28kPa) (Tedford *et al.,* 1992) from a peach orchard and pellets with and without hyphae were packed into 25-ml vials (17 cm^3 of soil and four pellets per vial). After 14 days at 20°C, 100 J2 of *H. schachtii* were added to each vial. Vials were planted with cabbage (*Brassica oleraceae* L. "Chieftain Savoy") on Day 17, and roots were removed from soil and stained (Byrd *et al.,* 1983) on Day 22. The number of *H. schachtii* in roots was determined. Based on many identical tests in that soil (Lackey *et al.,* 1993), we expected pellets with *H. rhossiliensis* to suppress root invasion by at least 50%.

Microplots and soil preparation. Microplots were located on the campus of the University of California at Davis and consisted of plastic barrels 53 cm wide and 89 cm deep. The barrels were buried in soil so that the top 8 cm was above the soil surface. Barrels had holes in the bottom for drainage and contained, from bottom to top, 8 cm gravel, 25 cm sand, and 46 cm loamy sand (76, 16, and 8% sand, silt, and clay; 0.4% organic matter; pH 7.5 in 0.1 m*M* $CaCl_2$). The loamy sand was infested with *H. schachtii,* had been planted with sugarbeet (*Beta vulgaris* L. "SSNB-2") for 4 years, and did not contain *H. rhossiliensis.*

On April 11, 1994, sugarbeets and 9 liters of soil were removed from each of three microplots. The 27 liters of soil was mixed in a cement mixer for 6 min. Nine liters of the mixed soil was heated to 60°C for 2 h to kill nematodes and suppress other organisms, and 18 liters was not heated. The number of *H. schachtii* eggs in one 250-cm^3 sample of nonheated soil was determined by extracting cysts from soil and eggs from cysts (Caswell *et al.,* 1985). In a second determination of *H. schachtii* population density, four 100-cm^3 samples of nonheated and heat-treated soils were placed in cups, each of which was planted with six germinated cabbage seeds. The cups were placed under fluorescent lights at 20°C for 5 days. Roots were removed from soil and stained, and cyst nematodes in roots were counted.

Field experiments. On April 13, 1994 (spring experiment), the collected soil was divided into 1200-cm^3 lots (12 lots nonheated and 6 lots heat-treated) and placed in plastic bags. Alginate pellets with hyphae and controls without hyphae (stored at 5°C for 24 h) were mixed into the nonheated soil (1 pellet/cm^3 of soil); the heat-treated soil received no pellets. Thus, there were three treatments: nonheated soil containing pellets with hyphae, nonheated soil containing control pellets, and heat-treated soil with no pellets. The primary purpose of the two treatments in nonheated soil was to provide information on the effect of pellets on invasion of roots by *H. schachtii.* The third treatment, in which the soil was heat-treated, enabled observation of *H. rhossiliensis* growth from pellets in the absence of a full community of other organisms (see next section).

On April 13, the top 15 cm of soil was removed from the three microplots. Six cylinders were spaced evenly and vertically in each microplot. The cylinders were cut from plastic, corrugated drainage pipe (15.3 cm long and 10.1 cm wide); cylinder walls had 24 small holes (15 × 2 mm) arranged in six vertical rows. The cylinders were surrounded with the soil that had just been removed. Each cylinder then was filled with 1200 cm^3 of soil (treated as described in the preceding paragraph) and contained one soil observation chamber (Fig. 1, see next section). Using cylinders rather than microplots as the replicated unit reduced the volume of soil per replicate—this meant that the soil could be readily mixed to reduce variation in the initial distribution of nematodes and pellets. Moreover, we could not produce sufficient pellets at one time to infest all the soil in the microplots, and we did not have enough microplots to establish six replicates of three treatments with the microplot as the replicated unit. There were two replicate cylinders per treatment per microplot. The cylinders and microplots were watered after addition of soil and as needed thereafter. Soil temperature at 15 cm depth was measured daily at noon.

After 12 days, 12 cabbage seedlings were planted in each cylinder. The seeds were covered with 2 mm of steamed sand to improve emergence, and the microplots were covered with coarse screen to keep out birds. Ten days after addition of seeds, cylinders were removed from the microplots, and seedlings (and soil observation chambers) were removed from the cylinders. Seedlings were counted, lengths of tap roots were measured, roots were weighed and stained, and nematodes within roots were counted.

The microplot experiment and laboratory assays were repeated in fall 1994. Pellets were added to plots on September 21, seeds were planted on October 3, and the experiment was terminated on October 13.

Soil observation chambers. Understanding success or failure of biological control, even inundative biological control, requires information on what happens to the biological agent after it is released. Unfortunately, we lack a direct assay for *H. rhossiliensis* conidia, and although a bioassay for conidia has been developed, it is not effective in soil that has been disturbed by sampling (McInnis and Jaffee, 1989). We therefore placed pellets in soil observation chambers to obtain direct evidence of *H. rhossiliensis* growth, sporulation, and persistence in soil. One soil chamber was placed in each cylinder (8 cm depth) at the start of the spring and fall experiments, and each chamber contained the same soil (heat-treated or nonheated) as its host cylinder. Chambers with nonheated soil contained the same pellet type (with hyphae or control) as the host cylinder. Chambers with heat-treated soil contained pellets with hyphae.

We used heat-treated and nonheated soil to determine whether other organisms affected growth of *H. rhossiliensis* from the pellets. For every chamber in a cylinder, a second identical chamber was assembled, sealed in a plastic bag, and placed in a plastic box with moist paper towels; the boxes were kept in an incubator at 20°C.

Soil observation chambers were made with 6-cm-diameter × 1.5-cm-deep plastic petri dishes (Fig. 1). Two sections of plastic were cut from the bottom of each dish; the two openings were separated by a rectangular strip of plastic 1.5 cm wide. Three pellets were placed on the inner surface of the rectangular strip. Nylon mesh (0.5-mm openings), placed on the inner surface of the bottom, covered the holes but not the rectangular strip. The pellets and mesh were covered with sufficient soil (35 cm^3) to fill the bottom. Three additional pellets were placed on the soil surface. The petri dish top, which had two holes, a rectangular strip, and mesh identical to the bottom, was placed over the soil so that the rectangular strip of the top rested on the three pellets on the soil surface. The top of each chamber was sealed to the bottom with duct tape. The mesh-covered holes retained the soil in the chamber but allowed movement of gas, water, and organisms smaller than 0.5 mm. Six pellets could be observed through the two rectangular strips of each chamber. Chambers were oriented vertically in the field and horizontally in the laboratory.

Soil observation chambers that were incubated in the laboratory were examined weekly with a dissecting microscope and reflected light. The diameter of the colony that formed around pellets was determined at 10× magnification. The identity of the fungus forming the colony was determined at 140× magnification. Soil observation chambers recovered from the microplots were examined in a similar manner within 1 day of recovery.

Statistical analyses. Data from the cylinders (seedling emergence, root length, fresh root weight, and numbers of *H. schachtii* in roots) were analyzed by the analysis of variance (SAS general linear models procedure), and means were separated according to the Duncan's multiple range test (SAS, 1985). Each microplot was considered a block, with two replicate cylinders per treatment in each of three blocks. In one analysis, we compared two treatments (pellets with hyphae vs control pellets in nonheated soil), and in a second analysis, we compared all three treatments. Significance was determined at $P = 0.05$. We used means and variances to make inferences on the data obtained from soil observation chambers.

RESULTS

Spring experiment. In the determination of pellet viability, we counted 29 ± 5 and 60 ± 5 (mean ± SE) J2 of *H. schachtii* per replicate root system in vials containing pellets with and without hyphae, respectively. Thus, suppression of root invasion was 52%, and pellet viability was considered normal.

The nonheated soil in the cylinders contained 1600 *H. schachtii* eggs/100 cm^3 of soil at the start of the experiment. In the bioassay of nematode population density, 356 ± 57 and 0 ± 0 J2 penetrated roots of seedlings in cups containing 100 cm^3 of nonheated and heat-treated soil, respectively.

The mean soil temperature in the cylinders was 19°C; the range was 17–24°C. Most cabbage seedlings emerged (Table 1). Tap root length and root fresh weight were similar in nonheated soil, whether *H. rhossiliensis* was added or not, but were greater in heat-treated soil than in nonheated soil (Table 1). Numbers of nematodes within roots grown in nonheated soil were similar whether *H. rhossiliensis* had been added or not. Although the heat-treated soil

TABLE 1

Effect of Pelletized Hyphae of *Hirsutella rhossiliensis* on Emergence, Root Length, and Root Weight of Cabbage Seedings and on Numbers of *Heterodera schachtii* in Seedling Roots[a]

Soil	Pellets	Seedlings emerged per cylinder	Root length (cm/cylinder)	Root weight (g/cylinder)	*H. schachtii* in roots per cylinder
Spring experiment					
Nonheated	Without hyphae	10.3 (0.4) b	72 (6) b	219 (9) b	1010 (88) a
Nonheated	With hyphae	10.3 (0.6) b	75 (6) b	239 (17) b	908 (105) a
Heat-treated	None	11.7 (0.3) a	152 (12) a	331 (34) a	451 (52) b
Fall experiment					
Nonheated	Without hyphae	10.0 (0.6) a	39 (6) b	166 (15) b	1159 (93) a
Nonheated	With hyphae	10.0 (0.9) a	40 (6) b	161 (13) b	1043 (116) a
Heat-treated	None	10.0 (0.4) a	90 (7) a	215 (9) a	516 (80) b

[a] Values are the means (standard error) of six replicates. Means within an experiment and column followed by the same letter are not significantly different ($P = 0.05$).

initially contained no *H. schachtii,* many were found in roots grown in heat-treated soil in cylinders (Table 1).

All control pellets were observed in nonheated soil in soil chambers recovered from the field (Table 2); the diameter of these pellets was the same at the start and end of the experiment, and overall the pellets appeared unchanged. In contrast, few pellets with hyphae were evident in chambers incubated in nonheated or heat-treated soil in the field (Table 2). Remnants of the pellet were always observed and consisted of the quartz sand (which had been used as a coating) and a slimy material on the plastic surface. When pellets with hyphae were present, colonies of *H. rhossiliensis* (consisting of hyphae, phialides, and conidia) were observed, and these colonies were larger in heat-treated than in nonheated soil (Table 2). Some pellets with *H. rhossiliensis* colonies contained tunnels and appeared to have been grazed upon or otherwise physically disturbed. Enchytraeids and collembolans were observed moving around and through many pellets with hyphae (and associated *H. rhossiliensis* colonies) in nonheated and in heat-treated soil (Table 2).

When soil chambers were incubated in the laboratory, all pellets were observed throughout the observation period (1 month). The diameter of control pellets incubated in nonheated soil remained constant at 2 mm (data not shown). Colonies of *H. rhossiliensis* formed around all pellets with hyphae; the colonies were larger and less variable in heat-treated than in nonheated soil (Figs. 1 and 2). In nonheated soil, small colonies of unidentified fungi grew on many pellets. Only one enchytraeid and no collembolans were observed in chambers incubated in the laboratory (data not shown).

Fall experiment. Based on the vial assay, the pellets formulated in the fall were more effective than those formulated in the spring: we counted 15 ± 2 and 53 ± 3 J2 per replicate root system in vials containing pellets with and without hyphae, respectively. Therefore, suppression of root invasion was 72%.

At the start of the fall experiment, the nonheated soil in the cylinders contained 480 eggs/100 cm^3 of soil. In the bioassay, 259 ± 39 and 0 ± 0 J2 penetrated roots of seedlings in cups containing 100 cm^3 of nonheated and heat-treated soil, respectively.

Soil temperatures were higher in fall than in spring. The mean soil temperature at 12 noon was 25°C; the range was 23–27°C.

Overall, the fall data were similar to the spring data (Tables 1 and 2). Most seedlings emerged; root length and weight were unaffected by addition of *H. rhossiliensis* but were greater in heat-treated than in nonheated soil; addition of *H. rhossiliensis* did not suppress nematode invasion of roots; and substantial numbers of *H. schachtii* were found in roots; growing in heat-treated soil, even though the bioassay had shown that the heat-treated soil contained no viable *H. schachtii.*

Enchytraeids but no collembolans were observed in many soil chambers recovered from the fall microplots, regardless of treatment (Table 2); as in spring, control pellets were unchanged, but all pellets with hyphae were gone, and only quartz sand and a slimy material remained. No enchytraeids or collembolans were observed in soil chambers incubated in the laboratory; as in the spring, all pellets were present and those with hyphae formed *H. rhossiliensis* colonies, which were larger and less variable in heat-treated soil than in nonheated soil (Fig. 2).

DISCUSSION

Pelletized hyphae of *H. rhossiliensis* failed to suppress *H. schachtii* in our small-scale field tests. We cannot attribute this failure to low pellet viability, because the pellets suppressed nematodes in laboratory bioassays. We also cannot attribute the failure to inadequate pellet dosage: the field rate was four times greater than that which provided at least 50% suppression in the laboratory. Because *H. rhossiliensis* efficacy

TABLE 2

Persistence of Pellets and Diameter of *H. rhossiliensis* Colonies Growing from Pellets in Soil Observation Chambers Incubated in Microplots

Soil	Pellets	Pellets observed per chamber[a]	Colony diameter (mm)[a]	Chambers with enchytraeids[b]	Chambers with collembolans[b]
Spring Experiment					
Nonheated	Without hyphae	6.0 (0.0)	2.0 (0.0)	0	0
Nonheated	With hyphae	1.3 (0.6)	2.3 (0.5)	5	2
Heat-treated	With hyphae	2.8 (1.3)	4.8 (0.6)	4	0
Fall Experiment					
Nonheated	Without hyphae	6.0 (0.0)	2.0 (0.0)	4	0
Nonheated	With hyphae	0.0	—	5	0
Heat-treated	With hyphae	0.0	—	4	0

[a] Values are the means (standard error) of six replicate chambers. Each chamber initially contained six pellets.
[b] Values are the number of chambers (of six total) with at least one enchytraeid or collembolan.

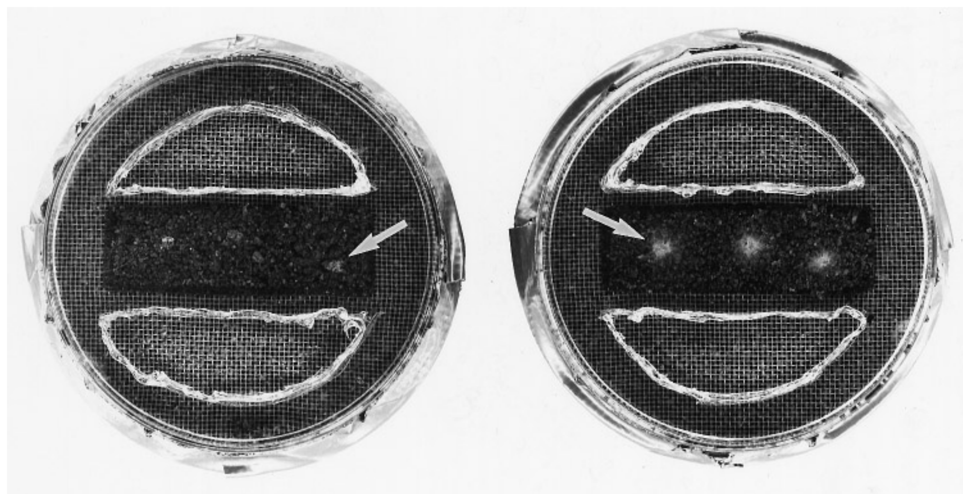

FIG. 1. Pellets and colonies of *Hirsutella rhossiliensis* in soil observation chambers (actual size) kept in the laboratory for 2 weeks at 20°C. The chambers contained nonheated soil (left) or heat-treated soil (right). Arrows point to one pellet and colony in each chamber. Substantial colonies of *H. rhossiliensis* developed in heat-treated but not in nonheated soil.

has been documented in a variety of soils differing in pH, texture, organic matter, etc., (Lackey *et al.,* 1992; 1993; Tedford *et al.,* 1992), we also do not believe that abiotic conditions were responsible for the lack of biological control.

The lack of control could relate to the distance that nematodes moved through soil: nematodes moving short distances may not contact conidia (Timper *et al.,* 1991; Tedford *et al.,* 1995) or may contact conidia but nevertheless penetrate roots (Tedford *et al.,* 1995). The large number of nematodes found in roots growing in cylinders that initially contained no nematodes, however, indicated that many nematodes moved substantial distances through the soil; we assume that these nematodes moved into the cylinders from the surrounding soil via the cylinder bottom or the small holes in the cylinder wall. We don't know the distance moved, but the rate of pellets used in this study (1/cm^3) reduced root penetration by >50% when *H. schachtii* juveniles were placed only 2–3 cm from roots (Tedford *et al.,* 1995).

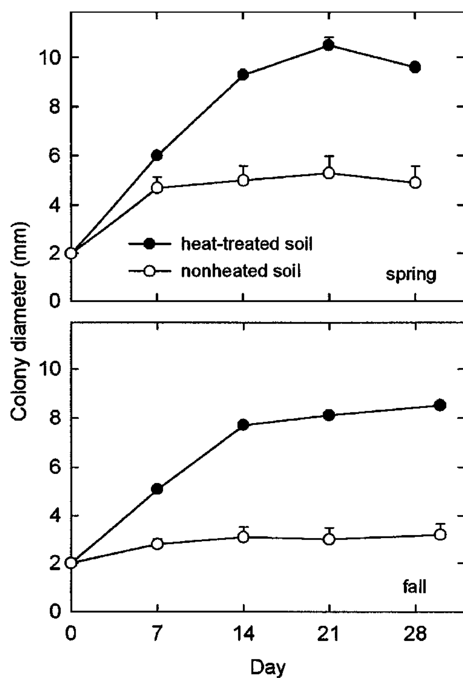

FIG. 2. Colony diameter of *H. rhossiliensis* growing from pellets in soil observation chambers kept in the laboratory at 20°C. The chambers contained nonheated or heat-treated soil collected in spring and in fall. Each value is the mean of six replicate chambers. Vertical bars equal one standard error; bars that do not appear are smaller than the symbol.

Although we cannot dismiss suboptimum abiotic conditions and limited nematode movement as explanations for the lack of biological control, we suspect that inhibition of *H. rhossiliensis* by other organisms was more important. In soil observation chambers incubated in the field, pellets and associated colonies appeared to have been fed upon. In many cases, only remnants of the pellet and colony were present, and enchytraeids and collembolans, which may eat fungi (e.g., Didden, 1993; Lartey *et al.,* 1994; Hedlund and Augustsson, 1995; van Vliet *et al.,* 1995), were observed nearby. Both organisms are motile in soil and sufficiently small to move into the soil chambers. We assume that heating soil to 60°C for 2 h killed enchytraeids and collembolans and that those observed in cylinders and chambers containing heat-treated soil in the field moved from the surrounding, nonheated soil.

In contrast to pellets incubated in the field, those kept in soil observation chambers in the laboratory seldom exhibited signs of feeding but formed smaller

colonies in nonheated than in heat-treated soil. Here we suspect that the inhibition was caused by bacteria, fungi, or other microscopic organisms.

Nonsterile soil is generally considered a hostile environment for introduced microorganisms, including biological control agents, because resident microorganisms often are superior competitors or are otherwise antagonistic (e.g., Mankau, 1962; Cooke and Satchuthananthavale, 1968; Guima and Cooke, 1974; Cook and Baker, 1983; Pereira *et al.,* 1993). We were aware of this concern but had not anticipated biotic inhibition in general, and microbial inhibition in particular, for several reasons. First, hyphae of *H. rhossiliensis* produced many conidia when added to eight nonsterile soils (Lackey *et al.,* 1992). Second, although sporulation from hyphae was less in nonheated than in heat-treated loamy sand from a peach orchard, sporulation was nevertheless substantial in both (Lackey *et al.,* 1993). The eight soils studied by Lackey *et al.* (1992) had been stored for 2–60 months, however, and antagonists of *H. rhossiliensis* may have declined with storage. Moreover, the peach orchard soil used by Lackey *et al.* (1993) contained naturally high densities of *H. rhossiliensis* and may have been unusually conducive to *H. rhossiliensis* establishment and survival.

We also failed to anticipate biotic inhibition because of two assumptions regarding colonized nematodes and hyphal pellets. Our first assumption was that, as a natural form of fungal inoculum that sometimes occurs in high densities, the colonized nematode is resistant to biotic inhibition. The second assumption was that the hyphal pellet is similar to the colonized nematode. The sensitivity of the colonized nematode to biotic inhibition has not been measured, however, and the hyphal pellet differs from the colonized nematode in that the pellet lacks a cuticle and contains macerated hyphae.

This study has generated questions requiring additional research. Which organisms inhibited *H. rhossiliensis* and are enchytraeids and collembolans important mortality factors for *H. rhossiliensis* and other fungal control agents? Is biotic inhibition common to many soils? How is inhibition affected by the abiotic environment? Does the cuticle of the parasitized nematode reduce inhibition of hyphae? If so, can a protective covering be made for hyphae in pellets? Are macerated hyphae, as presently used in our pellets, more leaky and thus more sensitive to biotic inhibition than are intact hyphae?

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