Sporulation of the Nematophagous Fungus *Hirsutella rhossiliensis* from Hyphae Produced In Vitro and Added to Soil

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ABSTRACT

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After assimilating and killing host nematodes, *Hirsutella rhossiliensis* sporulates (i.e., produces external hyphae, phialides, and spores) from assimilative hyphae within the cadaver; the spores adhere to and infect nematodes. The ability of the fungus to sporulate from hyphae produced in vitro, rather than in host nematodes, was tested. Hyphae in the form of discrete vegetative colonies were grown in shake culture (potato-dextrose broth). In moist chambers, sporulation from vegetative colonies (rinsed free of broth) and from host nematodes was identical. To determine whether vegetative colonies sporulated in soil, vials were packed with nonheated, heated, or autoclaved loamy sand (17 cm^3) containing 0–50 rinsed vegetative colonies. After 14 days at 20 C, healthy juveniles (J2) of *Heterodera schachtii* were added and recovered 66 h later. The percentage of J2 with at least one attached spore of *H. rhossiliensis* increased nonlinearly with increased numbers of colonies added per vial. The percentage was not affected by soil treatment, and virtually all J2 with attached spores were infected by the fungus. Approximately four vegetative colonies per 17 cm^3 of soil were required to obtain 50% parasitism of J2 in loamy sand and in six other raw soils that varied in texture and other properties. Penetration of cabbage roots by J2 was suppressed in raw loamy sand containing rinsed vegetative colonies. Vegetative colonies did not affect growth of seven plant species in the absence of nematodes. Results suggest that hyphae of *H. rhossiliensis* produced in vitro and added to soil without organic substrate may be used for biological control of plant-parasitic nematodes.

Additional keywords: beet cyst nematode, formulation, hyperparasitism, inundative release.

The nematophagous fungus *Hirsutella rhossiliensis* Minter & Brady occurs naturally in agricultural fields (7,9,24) and parasitizes vermiform stages of many species of soilborne, plant-parasitic nematodes (8,24). Because natural infestations caused high levels of nematode mortality in the field (7) and in the laboratory (8), habitat manipulation (i.e., altering conditions to favor a natural enemy) (3) may be a means of utilizing the fungus for biological control. However, parasitism increases only slowly in response to high densities of hosts (11), and methods to enhance the activity of natural infestations are unknown.

Artificial infestations of *H. rhossiliensis*, achieved by adding fungal-colonized nematodes to soil, also caused high levels of nematode mortality in laboratory experiments (10). The fungus grows from colonized nematodes and produces phialides on external hyphae that radiate into the soil (12). Nonmotile spores are borne on the tips of the phialides (one per phialide) and adhere to passing, vermiform nematodes. A germ tube directly penetrates the cuticle, and assimilative hyphae ramify in the host, which is killed within several days. After all of the nematode except the cuticle is consumed by assimilative hyphae, the fungus sporulates from the cadaver, perhaps in response to depletion of the substrate. Sporulation involves utilization of reserves in the assimilative hyphae within the cadaver for synthesis of fungal structures external to the cadaver. The fungus is probably an obligate parasite in soil (13), although it grows on standard media in sterile culture.

Because *H. rhossiliensis* controlled nematodes when added to soil in the laboratory (10), we suspected that the fungus might also control nematodes when added to soil in the field. The addition of large amounts of an agent for immediate biological control is called *inundative release* (5). Inundative release may be especially useful for management of nematodes in annual crops for which short-term, early-season control is essential (6). In the case of *H. rhossiliensis*, colonized nematodes and spores are two obvious forms of inoculum that might be added to soil. The colonized nematode, however, is not practical for commercial production, and spores are not viable when added to soil, because they must be contacted by nematodes while attached to phialides to be infective (18).

Given the importance of assimilative hyphae in sporulation of *H. rhossiliensis* and the success obtained with colonized nematodes as inoculum, we hypothesized that nematodes could be parasitized by artificially infesting soil with hyphae. If hyphae similar to those in the colonized nematode could be grown in nutrient solution, these might sporulate when removed from the solution (i.e., when deprived of substrate) and added to soil.

The objective of this research was to determine whether hyphae of *H. rhossiliensis* sporulate when added to soil without added organic substrate. The effect of the hyphae on plant growth also was determined.

MATERIALS AND METHODS

Fungal inoculum and assay nematodes. *H. rhossiliensis* (IMI 265748) was maintained at 20 C on 50% potato-dextrose agar (Difco Laboratories, Detroit, MI). Two agar plugs, each with one colony 2 cm in diameter, were transferred to 50 ml of sterile 0.01% Triton X-100 surfactant (Sigma Chemical Co., St. Louis, MO) and vigorously agitated on a wrist-action shaker for 15 min to dislodge and suspend the spores. The spore suspension was aseptically filtered through two layers of cheesecloth, and 3×10^5 spores were added to a 1-L flask containing 500 ml of sterile potato-dextrose broth. The culture was incubated at 25 C on an orbital shaker (at 100 rpm) for 7 days and yielded $6–10 \times 10^4$ spherical, vegetative colonies. The colonies were collected on a sieve (351-μm pore diameter), rinsed thoroughly with 1 L of distilled water, and resuspended in 4.6 mM KCl for 1–2 h before

use. Mean colony diameter and dry weight were 1.7 mm and 76 μg, respectively, and were consistent among batches prepared for each experiment.

H. rhossiliensis in soil was assayed with second-stage juveniles (J2) of the beet cyst nematode *Heterodera schachtii* Schmidt (10). Cysts of *H. schachtii* were obtained from infected sugar beet (*Beta vulgaris* L. 'SSNB-2') plants in the greenhouse. Cysts were placed on Baermann funnels; J2 were collected every 2 h and stored with aeration at 10 C for less than 48 h before use.

Soils. Eight soils were collected from five counties in northern California (Table 1). All soils were mixed and passed through a sieve (2-mm pore diameter). The loamy sand (soil 3) used for most experiments was collected from a peach orchard after tree removal and 6 mo of fallow and was not used for at least 4 mo after collection. Although the peach orchard contained natural populations of *H. rhossiliensis* (8), the fungus was not detected in this soil in preliminary assays for spores. The loamy sand contained small numbers of nematodes (<100 nematodes per 100 cm^3 of soil). None of the soils contained *H. schachtii*.

General procedures for experiments in vials. Except as noted, 0–50 vegetative colonies in 0.5 ml of 4.6 mM KCl were manually mixed into loamy sand (dry weight 26.1 g). The soil (7.0% moisture) was packed to a volume of 17 cm^3 in vials (25 ml) with holes in the bottom (18). The tops of the vials were sealed, and the vials were kept in moist chambers for 2 wk at 20 C before assays were conducted.

At the initiation of each experiment, five vegetative colonies were placed in each of two moist chambers (plastic Petri dishes, 9 cm in diameter, with a moist filter paper in the lid). The dishes were sealed with laboratory film and incubated in the dark at 20 C. Colonies were examined periodically over 3 wk for growth and sporulation.

Infection of nematodes by *H. rhossiliensis* in soil. In the first experiment, parasitism of assay nematodes by *H. rhossiliensis* was compared in loamy sand (soil 3) that was nonheated or heated at 60 C for 2 h. The soil was air-dried, moistened, infested with 0–50 vegetative colonies, and packed into vials; four to eight replicate vials per combination of soil and fungal inoculum level were prepared, and the experiment was conducted twice (trials 1 and 2). After 14 days at 20 C, 300 assay nematodes in 0.5 ml of 4.6 mM KCl were added to the surface of the soil in each vial (10). After 66 h, the nematodes were extracted by wet sieving (25-μm pore diameter) and centrifugal flotation (14). The suspension was placed in a counting dish, and assay nematodes (at least 60 per replicate vial) with and without attached spores were counted at 70–140× magnification. The number of assay nematodes with attached spores that were colonized (infected and filled with hyphae of *H. rhossiliensis*) also was counted (8). To determine whether noncolonized nematodes with attached spores were infected (8), 30 from heated soil and 30 from nonheated soil (which had received two vegetative colonies per vial) were examined at 400× magnification in trial 1.

The second experiment was similar to the first, except the loamy sand was autoclaved rather than heated; 0, 2, 4, 6, or 10 vegetative colonies were added per vial; and each combination of soil (± autoclaved) and fungal inoculum level was replicated four times. Vials were assayed as in the first experiment. This experiment was not repeated, because results were generally consistent with the first experiment.

In the third experiment, parasitism of assay nematodes by *H. rhossiliensis* was compared in eight raw (not dried or heated) soils (Table 1). Soils were moistened as needed to a moderate water level (Table 1) with 4.6 mM KCl. Each vial contained 17 cm^3 soil and four vegetative colonies. Additional vials (two per soil) contained no vegetative colonies and served as controls. Vials were assayed as in the first experiment. Six replicate vials per soil were prepared, and the experiment was conducted twice (trials 1 and 2).

Suppression of nematode invasion of roots by *H. rhossiliensis*. Vials containing raw loamy sand (soil 3) with 0, 1, 3, 6, or 25 vegetative colonies were incubated at 20 C; eight replicate vials were prepared per level of fungus for each of two trials. After 14 days, each vial was inoculated with 114 ± 12 or 110 ± 6 (mean ± SE for trials 1 and 2, respectively) healthy J2 of *H. schachtii* in 0.5 ml of 4.6 mM KCl. After 3 days at 20 C, one germinated cabbage (*Brassica oleracea* L. 'Chieftain Savoy') seed was planted with minimal mechanical disturbance of the soil (18) 8 mm deep in each vial. Vials were placed in a clear plastic moist chamber under fluorescent lights with a 12-h photoperiod. After 5 days (temperature range 21–26 C), seedlings were removed from vials; root and shoot lengths were measured, and roots were stained (2). The number of J2 in each root was determined.

Direct effect of *H. rhossiliensis* on plants. Vials containing raw or autoclaved loamy sand (soil 3) with 0, 6, 12, 25, or 50 vegetative colonies were incubated at 20 C (four replicate vials per combination of soil and fungal inoculum level for each of two trials). After 14 days, one germinated cabbage seed was planted in each vial. Seedlings were removed from vials after 5 days, and root and shoot lengths were determined. Root systems were examined for general and localized necrosis and then were placed at room temperature in Petri dishes containing 1.5% water agar amended with streptomycin sulfate (200 mg/L). Dishes were examined at 70–140× magnification daily for 2 wk; the presence or absence of *H. rhossiliensis* on roots or in the agar was recorded.

A similar experiment was conducted in the greenhouse (temperature range 19–36 C). Steamed greenhouse sand (1.7 L) was infested with 0 or 500 vegetative colonies in 50 ml of distilled water. The sand was mixed and placed into a pot. Pots were

TABLE 1. Transmission of *Hirsutella rhossiliensis* to *Heterodera schachtii* in eight soils infested with vegetative colonies of the fungus[a]

Soil	Source[b]		Storage		Texture	pH	Organic matter (%)	Water (% of dry wt)	Transmission[c] (%)	Colonization[d] (%)
	Crop	County	Time (mo.)	Temp. before use (C)						
1	Tomato	Stanislaus	60	10	Silty loam	7.1	1.6	11	66 ± 3	63 ± 3
2	Fallow	Yolo	12	22	Sandy loam	7.0	1.2	11	53 ± 4	67 ± 3
3	Peach	Merced	4–18	22	Loamy sand	4.4	0.2	7	55 ± 4	64 ± 4
4	Tomato	San Joaquin	60	10	Sandy clay loam	6.8	1.6	9	54 ± 5	61 ± 3
5	Tomato	Stanislaus	60	10	Sandy clay loam	7.4	1.5	8	48 ± 6	57 ± 6
6	Greenhouse	...	4–12	22	Sand	8.3	<0.1	3	64 ± 4	68 ± 3
7	Tomato	Yolo	60	10	Clay loam	5.8	1.8	10	57 ± 5	65 ± 3
8	Potato	Siskiyou	3	10	Loam	7.0	16.0	28	30 ± 3	55 ± 5

[a] Vegetative colonies of *H. rhossiliensis* were added to raw soil, which was packed into vials (17 cm^3 of soil and four colonies per vial). After 2 wk at 20 C, assay nematodes (*H. schachtii*) were added to each vial; 66 h later, assay nematodes were recovered and examined. Control vials contained no vegetative colonies.

[b] Crop and county in California in which soil was collected. Soil 6 was a greenhouse sand and was not collected from the field.

[c] Percentage of assay nematodes with at least one spore of *H. rhossiliensis* attached to the cuticle. Transmission in control vials was 0% in all soils. Data from two trials were similar and were combined. Values are mean ± SE of 12 replicate vials.

[d] Percentage of assay nematodes with attached spores that were filled with hyphae; note that percentage of colonization is less than percentage of infection. Values are mean ± SE of 12 replicate vials.

planted immediately with five seeds of either radish (*Raphanus sativus* L. 'Early Scarlet Globe'), spinach (*Spinacia oleracea* L. 'Bloomsdale Longstanding'), tomato (*Lycopersicon esculentum* Mill. 'UC82B'), cucumber (*Cucumis sativa* L. 'Poinsett 76'), cotton (*Gossypium hirsutum* L. 'Acala SJ-2'), or soybean (*Glycine max* (L.) Merr. 'Prize'). Each combination of inoculum level and plant species was replicated five times, and the experiment was performed twice. Fertilizer pellets (6 g, 17-6-10 [NPK]) were added to the soil surface of each pot at planting. Plants were thinned to one per pot within 10–14 days and were watered daily and alternately with deionized water and mineral nutrient solution. Insect pests were removed by hand, and plants were harvested after 30 days. Roots were removed from the sand, washed, and examined for macroscopic symptoms of disease. Shoot lengths and shoot and root dry weights were determined.

Statistical analysis. The design for all experiments was completely randomized, and general linear model and nonlinear regression procedures from Statistical Analysis Systems (23) were used. Except as indicated, data from trials 1 and 2 of each experiment were similar ($P > 0.05$) on the basis of analysis of variance or an approximate F test (19) for the linear or nonlinear model, respectively. An approximate F test also was used to compare transmission in heated vs. nonheated and autoclaved vs. nonheated soil. Data for each experiment were combined for presentation if variances were homogeneous across trials, if trial-by-treatment interactions were not significant, and if trials were similar.

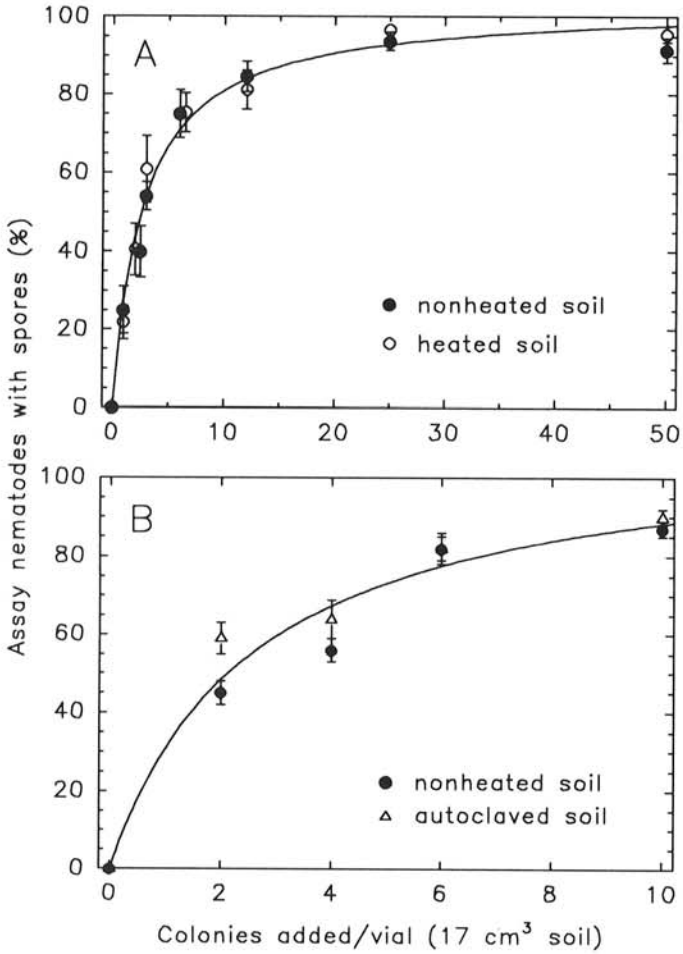

Fig. 1. Transmission of *Hirsutella rhossiliensis* (percentage of assay nematodes with spores) in vials containing loamy sand (nonheated, heated, or autoclaved) and vegetative colonies of the fungus. Soil in vials was incubated for 2 wk at 20 C before assay nematodes (*Heterodera schachtii*) were added. Assay nematodes were recovered after 66 h and were examined for spores of *H. rhossiliensis*. **A,** transmission in nonheated and heated (60 C for 2 h) soil containing 0, 1, 2, 3, 6, 12, 25, or 50 vegetative colonies of *H. rhossiliensis* per vial. Data from two trials were similar and were combined, and each value is the mean ± SE of four to eight replicate vials. For combined analysis of data from nonheated and heated soil, $Y = (105.4x)/(2.8 + x)$, $r^2 = 0.72$. **B,** Transmission in nonheated and autoclaved soil containing 0, 2, 4, 6, or 10 vegetative colonies of *H. rhossiliensis* per vial. Each value is the mean ± SE of four replicate vials. For combined analysis of data from nonheated and autoclaved soil, $Y = (111.3x)/(2.6 + x)$, $r^2 = 0.73$.

RESULTS

Colony characteristics and sporulation in moist chambers. Vegetative colonies were examined at 400× before placement in moist chambers. Colonies were spongy, consisting of tightly packed hyphae with cells that were cylindrical and filled with cytoplasm. Thick-walled hyphae grew radially from the vegetative colonies along the surface of the chamber and into the atmosphere surrounding each colony within 1.5 days. Within 2 days, the first phialides and spores were observed. Radial growth and sporulation continued for at least 3 wk, when the mean diameter of the sporulating colonies was 1.3 cm. As colonies sporulated in the moist chamber, the volume of the original vegetative colonies decreased, the original hyphae darkened and appeared empty, and the original hyphal cells became increasingly spheroidal. In general, the growth habit and sporulation were identical to that from parasitized nematodes. The number of spores produced per colony was not determined, because spores were numerous and occurred on many planes.

Infection of nematodes in soil. In the first experiment, assay nematodes recovered from soil infested with vegetative colonies had spores of *H. rhossiliensis* adhering to the cuticles (Fig. 1A). Transmission (percentage of assay nematodes with at least one attached spore) increased nonlinearly with numbers of colonies added per vial (Fig. 1A) and was similar ($P > 0.05$) in nonheated and heated soil. The data were described by a saturation function, transmission $= 100(b \times x)/(c + x)$, where x equals the number of colonies added per vial, and b and c are transmission parameters. In vials infested with two colonies, 30 of 30 noncolonized nematodes with attached spores were infected in both nonheated and heated soil.

Transmission was described by the saturation function in nonheated and autoclaved loamy sand in the second experiment as well (Fig. 1B). Transmission was not affected ($P > 0.05$) by soil treatment.

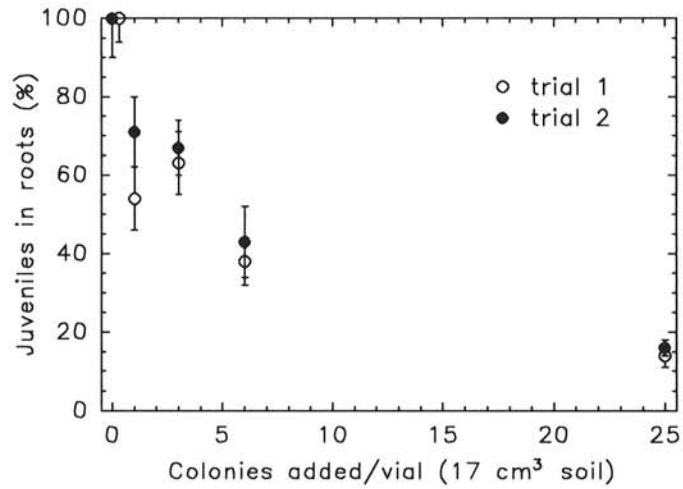

Fig. 2. Penetration of cabbage roots by juveniles (J2) of *Heterodera schachtii* as affected by number of vegetative colonies of *Hirsutella rhossiliensis* added per vial. Penetration is expressed as a percentage of the number of J2 that penetrated roots in control vials that contained no *H. rhossiliensis*. Raw loamy sand in vials was incubated for 14 days at 20 C before 114 (trial 1) or 110 (trial 2) J2 were added per vial. One cabbage seed was planted in each vial on day 17; roots were removed from the soil and stained on day 22. Each root contained 48 ± 8 (trial 1) and 63 ± 11 (trial 2) J2 when no vegetative colonies were added. Each value is the mean ± SE of eight replicate vials.

In experiment 3, transmission occurred in all soils but was greater ($P < 0.05$) in soil 1 than in soil 8 (Table 1) on the basis of Tukey's Studentized range test. The percentage of assay nematodes with attached spores that were colonized by *H. rhossiliensis* was similar ($P > 0.05$) for all soils (Table 1). Because previous observations indicated that virtually all nematodes with one attached spore became infected, and because infection was advanced in most nematodes with attached spores, infection of noncolonized nematodes with attached spores was not confirmed. The fungus was not detected in noninfested soils.

Suppression of nematode invasion of roots. Vegetative colonies of *H. rhossiliensis* suppressed penetration of cabbage roots by J2 in raw soil (Fig. 2); penetration declined exponentially, and the variance decreased with number of colonies added per vial. Approximately four colonies per vial were required to suppress penetration by 50%. Only cyst nematodes were observed in roots.

Effects on plants. Symptoms of root disease were not observed on cabbage seedlings grown in raw or autoclaved soil containing zero to 50 vegetative colonies of *H. rhossiliensis* per vial. Shoot and root lengths were not affected ($P > 0.05$) by addition of vegetative colonies or by soil treatment (raw or autoclaved) in trials 1 or 2 but were greater in trial 1 than in trial 2. Mean shoot and root lengths ranged approximately from 3.0 to 4.0 cm in trial 1 and 4.5 to 5.0 cm in trial 2, regardless of the number of colonies added per vial. The fungus was not observed on roots removed from the soil and incubated on 1.5% water agar.

In the greenhouse experiment, dry weights of shoots and roots of six plant species grown in steamed sand without plant-parasitic nematodes were not affected ($P > 0.05$) by vegetative colonies of *H. rhossiliensis* (data not shown).

DISCUSSION

The growth of *H. rhossiliensis* from vegetative colonies in the present study was similar to that from colonized nematodes described in previous studies (10,12). In both cases, the fungus sporulated in soil and the spores adhered to, infected, and killed nematodes. In both cases, phialides and spores were borne on thick-walled hyphae that grew externally from the source. Thus, vegetative hyphae produced in liquid culture may be similar to the assimilative hyphae within colonized nematodes, but additional comparative data on structure and physiology are needed to support this hypothesis.

Whereas the organic substrate (potato-dextrose broth) was rinsed from the vegetative colonies of *H. rhossiliensis* in the present study, substrates are often included in formulations of other biological control fungi (20). *Trichoderma harzianum, T. viride, T. hamatum, Talaromyces flavus, Gliocladium virens, G. roseum, G. catenulatum*, and *Aspergillus ochraceus* are relatively good competitive saprophytes and thus can utilize the added substrate (16). In fact, *T. harzianum* did not proliferate in soil when added without substrate (16). We did not add substrate, because *H. rhossiliensis* is a weak competitive saprophyte (13); we also assumed that the rinsed vegetative colonies contained minimal residual (i.e., unassimilated) broth. Rather than improve the performance of the fungus, substrate could reduce efficacy by promoting antagonism by opportunistic indigenous organisms (4). Thus, the fungus was allowed to assimilate the substrate in aseptic broth culture before transfer to soil, a situation analogous to assimilative growth of the fungus in nematodes, in that assimilation occurs without interference from other microorganisms.

In contrast to assimilation, sporulation by *H. rhossiliensis* is probably insensitive to competition. Sporulation involves the conversion of captured resource into new fungal structures and does not require competition for additional resource. Thus, transmission of the fungus was similar in nonheated and heated soil. The vegetative colonies, however, are not protected by the nematode cuticle, as are the assimilative hyphae in colonized nematodes. Exposed colonies may be sensitive to predation, parasitism, or antibiosis in some soils.

Other biological control agents have been applied without external substrate and, in some cases, as hyphae. The nematophagous fungus *Verticillium chlamydosporium* was successfully added to soil as chlamydospores; chlamydospores in colonized sand-bran were less effective than chlamydospores alone (4).

Entomologists are developing mycelial preparations of *Beauveria bassiana, Metarhizium anisopliae*, and other entomopathogenic fungi. These preparations may be stored dry (21,22) and have suppressed insect pests when applied to soil (15) and to leaves (1,17).

Despite the potential exhibited in our laboratory experiments, *H. rhossiliensis* has some important limitations relative to biological control. The fungus is sensitive to high soil moisture (25) and to mechanical soil disturbance (18). The spatial distribution of spores around each colony may be quite limited, and near-complete control of nematodes may require high inoculum density of the fungus. A related limitation is the temporal delay associated with sporulation from vegetative colonies; substantial numbers of endoparasitic nematodes could infect roots after colonies were added to soil but before high densities of spores were present. Even when spores are present, endoparasitic nematodes that hatch and travel only short distances to host roots may escape parasitism. Nevertheless, additional research is warranted on the use of vegetative colonies of *H. rhossiliensis* for biological control of nematodes. The host range of this fungus includes many economically important plant-parasitic nematodes, and the vegetative colonies were simple to produce and effective in several different soils.

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