Suppression of the Nematode *Heterodera schachtii* by the Fungus *Hirsutella rhossiliensis* as Affected by Fungus Population Density and Nematode Movement

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ABSTRACT

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We quantified penetration of roots by *Heterodera schachtii* as affected by two factors: the population density of *Hirsutella rhossiliensis,* and the distance that nematodes moved through soil. The fungus was added to soil in the form of pelletized hyphae, and the nematode was added to soil as second-stage juveniles (J2). Polyvinyl chloride tubes were filled with 27 cm^3 of loamy sand containing 0.0, 0.25, 0.5, 1.0, or 2.0 pellets per cm^3 of soil. In vitro, hyphae grew from each pellet and produced approximately 104,000 spores after 14 days at 20°C. A cabbage seedling was planted in a hole 2 cm from one end of each tube, and J2 were added to holes either 2, 4, 6, or 8 cm from the seedling. Tubes were positioned vertically to prevent roots from growing toward nematodes and to confine roots to the bottom 2 cm of the tube. Penetration of roots by J2 was inversely related to pellet density and distance. Fifty percent suppression of root penetration required approximately 0.9, 0.3, and 0.2 pellets per cm^3 of soil at distances of 2, 4, and 8 cm, respectively.

Additional keywords: biological control, endoparasite, nematode motility, sugar beet cyst nematode.

The nematophagous fungus *Hirsutella rhossiliensis* Minter & Brady produces nonmotile spores on phialides. Transmission occurs when a motile, vermiform nematode encounters a spore, which adheres to the nematode's cuticle and detaches from the phialide. The host nematode is infected and killed within 3 days at 20°C (3), new spores are produced, and additional transmission may occur.

For *H. rhossiliensis* and similar parasites of nematodes, the probability of transmission depends greatly on spore density and on the distance that nematodes travel through soil (2,14,17). Transmission also may be affected by nematode species (5,15, 17), soil moisture and porosity (5,15), and other factors affecting nematode movement, fungal sporulation, or spore mortality.

To quantify transmission of *H. rhossiliensis* to the sugar beet cyst nematode *Heterodera schachtii* Schmidt, researchers (5,8,15) simplified experimental conditions as follows. First, a small volume of soil was used to minimize spatial heterogeneity, enable control of temperature and water, and obtain adequate replication. Second, a host plant was absent and time was eliminated as a variable: transmission was measured 66 h after host nematodes were added to soil. These experimental simplifications were useful for studying host-parasite population dynamics (6), the effects of soil moisture and texture on transmission (15), and formulation of the fungus for inundative application (8). However, the presence of a plant would likely affect the distance that nematodes move through soil and thus affect transmission. Because nematodes are attracted to host plants, a plant might stimulate nematode movement through soil and increase transmission. In contrast, the probability that an endoparasitic nematode, like *H. schachtii*, will contact a spore becomes zero once the nematode has penetrated the root.

If transmission results in infection and immobilization of nematodes in soil, their numbers in roots will be suppressed. Therefore, we quantified penetration of roots by *H. schachtii* as affected by the distance traveled by the nematodes and by *H. rhossiliensis* population density.

MATERIALS AND METHODS

Nematode, fungus, and soil. Cysts of *H. schachtii* were obtained from sugar beet (*Beta vulgaris* L. 'SSNB-2') pot cultures. Cysts were incubated on Baermann funnels, and healthy second-stage juveniles (J2) were collected hourly and stored at 10°C for less than 24 h before use.

Discrete vegetative colonies of *H. rhossiliensis* (isolate IMI 265748) were grown in potato-dextrose broth shake culture for 7 days at 25°C (8). Isolate IMI 265748 has been used in most of our laboratory experiments and is morphologically and pathogenically similar to many other isolates (16). Vegetative hyphae were pelletized in calcium alginate, and pellets were coated with sand and air dried for 1 day before addition to soil (9,10). *Hirsutella rhossiliensis* sporulates when growing from the pellets, which are about 2 mm in diameter and weigh 1.6 mg, when added to moist soil. Pellets without hyphae were used as controls.

A loamy sand (83% sand, 13% silt, 4% clay; pH 4.9; 0.2% organic matter) was collected from Merced County, California. The soil was heated for 2 h at 60°C to kill nematodes and soil fungi, screened through a sieve with 2-mm-diameter openings, and air-dried. For each experiment, the soil was moistened with dilute KCl (4.5 mM) to obtain a soil moisture of 9% (–18 J kg^{-1}) (15), which is moist enough for plant growth but not enough to greatly inhibit transmission of *H. rhossiliensis* (15).

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Sporulation from pellets in vitro. A direct assay for spores of *H. rhossiliensis* in soil is lacking, and spore numbers were estimated based on sporulation from pellets in 1.5-ml microfuge tubes. One pellet with hyphae was placed in a 5-µl drop of sterile, distilled water on the inside wall of each of 40 microfuge tubes. The tubes were capped, and five tubes were randomly selected after 7, 14, 21, 27, 36, 43, 49, or 62 days at 20°C. To dislodge and suspend spores, 1 ml of 0.26% NaOCl was added to each tube, and tubes were shaken for 1 min at maximum speed on a Vortex Genie 2 (Fisher Scientific, Pittsburgh, Pa.). Spores were counted in a hemacytometer. Two subsamples were counted from each tube, and the mean was recorded. The experiment was conducted three times using different batches of pellets. Data from all three trials were pooled for presentation.

Fungal growth from pellets in soil. Four dried pellets with hyphae were placed on the bottom of each of three 5-cm-diameter plastic petri dishes. The bottoms were completely filled with loamy sand (9% soil moisture), the tops were replaced, and sealed with tape. After incubation at 20°C, the bottom surface was examined using a dissecting microscope (100 to 140× magnification) with top lighting. Hyphae grew from the pellets into the soil and along the interface of soil and plastic. After 2, 4, and 5 weeks, the perimeter of the colony observed at the interface was demarcated by etching the plastic with a needle. The area of each colony, as delimited by the etchings, was recorded with a video camera attached to a personal computer and was analyzed using Jandel software (version 1.2) (Jandel Scientific, Corte Madera, Calif.). The experiment was conducted once.

Soil tubes. To quantify the effects of spore density and distance between nematodes and plant roots on nematode root invasion, soil tubes were used. Tubes were constructed from polyvinyl chloride pipe (1.8 cm inner diameter and 13.0 cm long). A 3.5-mm-diameter planting hole was drilled 2 cm from one end of each tube, and four 2.5-mm-diameter inoculation holes were drilled 4, 6, 8, and 10 cm from the same end (Fig. 1). A rubber stopper was inserted into the end of the tube nearest the planting hole. The soil (42 g dry weight) was moistened with dilute KCl to 9% soil moisture, and packed into tubes to a volume of 27 cm^3 (bulk density 1.56 g per cm^3). A rubber stopper inserted into the top of each soil tube was covered with a reflective metal cap to minimize moisture loss and heat absorption.

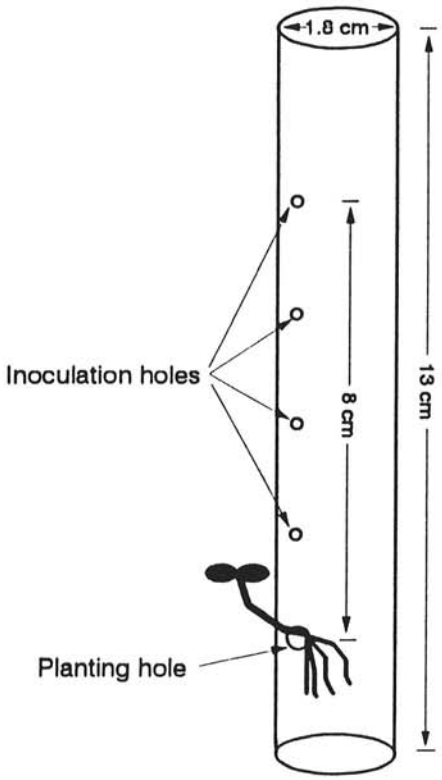

Fig. 1. Diagram of a soil tube. The planting hole is 2 cm from the lower end, and the spacing between holes is 2 cm.

Effects of pellet density and distance traveled by nematodes on root penetration. We conducted four independent but closely related experiments, because we lacked the labor and space to conduct one large factorial experiment. In experiment 1, tubes received 0, 7, 14, 27, or 54 pellets (equivalent to 0, 0.25, 0.5, 1.0, or 2.0 pellets per cm^3 of soil), and nematodes were added 2 cm from the plant. Experiment 2 was identical, but nematodes were added 8 cm from the plant. In experiment 3, tubes received seven pellets (0.25 pellets per cm^3) and nematodes were added 2, 4, 6, or 8 cm from the plant. Experiment 4 was identical to experiment 3, but 27 pellets (1.0 pellets per cm^3) were added to each tube. All four experiments were conducted twice (trials 1 and 2), with eight replicate tubes (experiments 1 and 2) or 10 replicate tubes (experiments 3 and 4) per combination of pellet type (± hyphae), pellet density, and distance in each trial.

The appropriate number of pellets were mixed into moistened soil using spatula, and soil was then packed into tubes. Tubes were incubated for 10 days at 20°C to allow the fungus to sporulate (10). On day 7, cabbage seeds were added to the surface of moist filter paper in a petri plate, and incubated at 20°C. On day 10, one germinated cabbage seed was partially embedded in the soil at the planting hole. To prevent roots from growing up the tube toward the inoculation holes, soil tubes were positioned vertically with the planting hole near the lower end (Fig. 1). Tubes were placed in a clear plastic box containing moistened paper towels and incubated at 20°C under fluorescent lights. After 4 days, approximately 200 (experiment 1) or 400 (experiments 2 through 4) newly hatched *H. schachtii* J2 in 0.1 ml of dilute KCl were added to the appropriate inoculation hole in each tube. Tubes were positioned horizontally while nematodes were added to prevent the suspension from moving down the inside wall of the tube. After nematodes were added, tubes were returned to a vertical position.

Because nematodes locate and penetrate roots faster when they are added close to plants than when they are added distant from plants, plants were harvested and the roots stained (1) at different times to achieve approximately equivalent root penetration regardless of distance. Staining times were determined based on a preliminary experiment in which nematodes were added to loamy sand (with no fungus added) either 2, 4, 6, or 8 cm from the planting hole and replicate root samples were stained daily. Based on data from this preliminary experiment, roots were removed from soil and stained 3, 5, 6, or 7 days after adding nematodes 2,

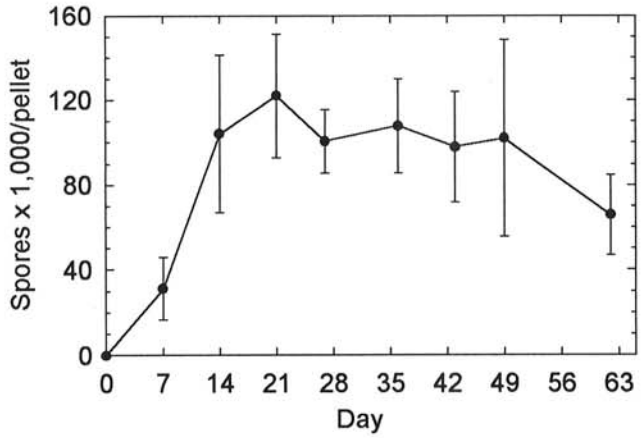

Fig. 2. Sporulation of *Hirsutella rhossiliensis* from alginate pellets as affected by time. Each value is the mean ± SE number of spores per pellet from 15 replicate pellets (pooled data from trials 1-3).

4, 6, or 8 cm from the planting hole, respectively.

Stained roots were observed at 100 to 140× magnification, and nematodes within the roots were counted. Suppression of root penetration was calculated using the equation: $S = (1 - x/y) \times 100$ where S = the percent suppression of root penetration, x = the number of nematodes in the roots when pellets with hyphae were added, and y = the number of nematodes in the roots when pellets without hyphae were added.

Nematodes are not immobilized or killed immediately following transmission, and nematodes with spores may penetrate roots (4). To determine the number of *H. schachtii* within roots having at least one adhering spore of *H. rhossiliensis*, roots were teased apart with forceps, and a minimum of 10 nematodes from each of five replicates were placed in a drop of water on a glass slide, covered with a coverslip, and observed at 400× magnification (unless stated otherwise).

A fresh batch of pellets was used for each trial, and variability among the eight batches was measured using a standard transmission assay (5). Briefly, 0, 4, 10, or 18 dried pellets with hyphae were added to each of five replicate samples of loamy sand (26.1 g dry weight). The soil was moistened with dilute KCl to 9% soil moisture and packed into 25-ml plastic vials to a volume of 17.4 cm^3 (bulk density 1.5 g per cm^3). After 14 days at 20°C to allow the fungus to sporulate, approximately 400 healthy *H. schachtii* J2 were added to the soil surface of each vial. Nematodes were recovered by wet sieving (25-µm pore diameter) and centrifugal flotation (7) 66 h later. At least 60 nematodes per replicate vial were examined at 100 to 140× magnification. Transmission was evaluated as the percentage of recovered nematodes that acquired one or more spores of *H. rhossiliensis*. Data on transmission versus pellet density were subjected to nonlinear regression (6).

RESULTS

Sporulation from pellets in vitro. Vegetative hyphae grew from the hydrated pellets in microfuge tubes, and normal phialides and spores developed. Variation in number of spores

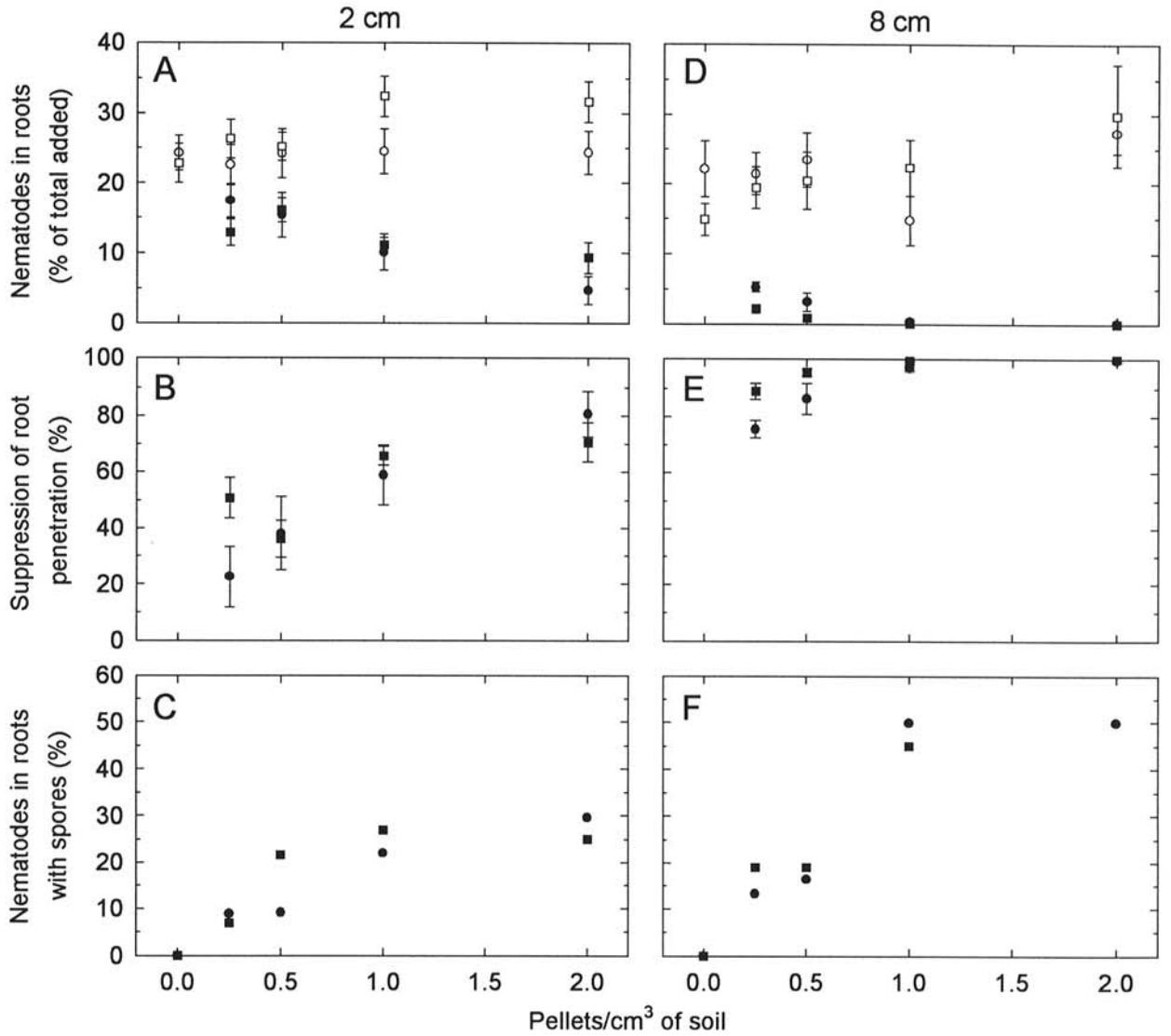

Fig. 3. Penetration of cabbage roots by juveniles (J2) of *Heterodera schachtii* as affected by distance between nematodes and plant and density of *Hirsutella rhossiliensis*. Data are from soil infested with zero pellets (open symbols), pellets without hyphae (open symbols), and pellets with hyphae (closed symbols). Circles and squares indicate data from two independent trials. **A** and **D,** Percentage of J2 in roots relative to total number added. **B** and **E,** Suppression in number of J2 in roots relative to that in tubes receiving pellets without hyphae. **C** and **F,** Percentage of nematodes in roots with spores of *H. rhossiliensis* after nematodes traveled through infested soil. For A, B, D, and E, values are the means ± SE of eight replicate tubes. For C and F, fewer than eight nematodes were observed per trial when the distance was 8 cm and pellet densities were 1.0 or 2.0 pellets per cm^3 of soil; in all other combinations, at least 50 nematodes were observed per trial. For F, only one value is shown when pellet density was 2.0 pellets per cm^3 of soil because no nematodes were observed in the roots of that treatment in the second trial.

produced per pellet was substantial (Fig. 2). Spore number increased for 2 to 3 weeks, remained steady for another 4 to 5 weeks, and then declined. Sporulation was maximum when pellets were incubated for 21 days. On day 14, the time allowed for the fungus to grow and sporulate before nematodes were added to the soil tubes, 104,000 spores were produced per pellet (Fig. 2).

Hyphal growth from pellets in soil. The fungus formed a circular colony consisting of hyphae bearing phialides and spores around each pellet. The area (and diameter) of the colony, measured along the interface of soil and plastic, was 24 ± 3 mm^2 (5.5 mm ± 0.01), 43 ± 6 mm^2 (7.4 mm ± 0.02), and 47 ± 9 mm^2 (7.7 mm ± 0.02) after 2, 4, and 5 weeks, respectively. Although spores were not quantified, their numbers were much greater at the center than at the edge of the colony.

Effects of pellet density and distance traveled by nematodes on root penetration. Pellets without hyphae had little or no effect on the percentage of nematodes that penetrated cabbage roots (Fig. 3A and D). In contrast, pellets with hyphae suppressed the percentage of nematodes in roots (Figs. 3A and D, 4A and D). Suppression was directly related to pellet density (Fig. 3B and E) and distance of nematodes from roots in *H. rhossiliensis*–infested soil (Fig. 4B and E). The combination of pellet density and distance giving 50% suppression was estimated (Fig. 5), using linear regression for data in Figures 3B and 4B and interpolation for data in Figures 3E and 4E. Interpolation rather than linear regression was used because the data in Figures 3E and 4E were not linear and were lacking for 0 pellets per cm^3 and 0 cm distance.

The percentage of nematodes both in the roots and with adherent spores was directly related to population density of pellets with hyphae in experiments 1 and 2 (Fig. 3C and F). This percentage, however, was unrelated to distance in experiments 3 and 4 (Fig. 4C and F).

Based on the transmission assay, the eight batches of *H. rhossiliensis* pellets were not identical (Fig. 6). Nevertheless, a saturation-type equation (see legend of Fig. 6) described the data in all cases. Values of r^2 ranged from 0.26 to 0.73, with a mean of 0.54. Values of P were < 0.01.

DISCUSSION

As expected, suppression of the cyst nematode *H. schachtii* by the fungus *H. rhossiliensis* increased with the population density of the fungus and the distance that nematodes traveled through soil. The value of our study lies not in the confirmation of this

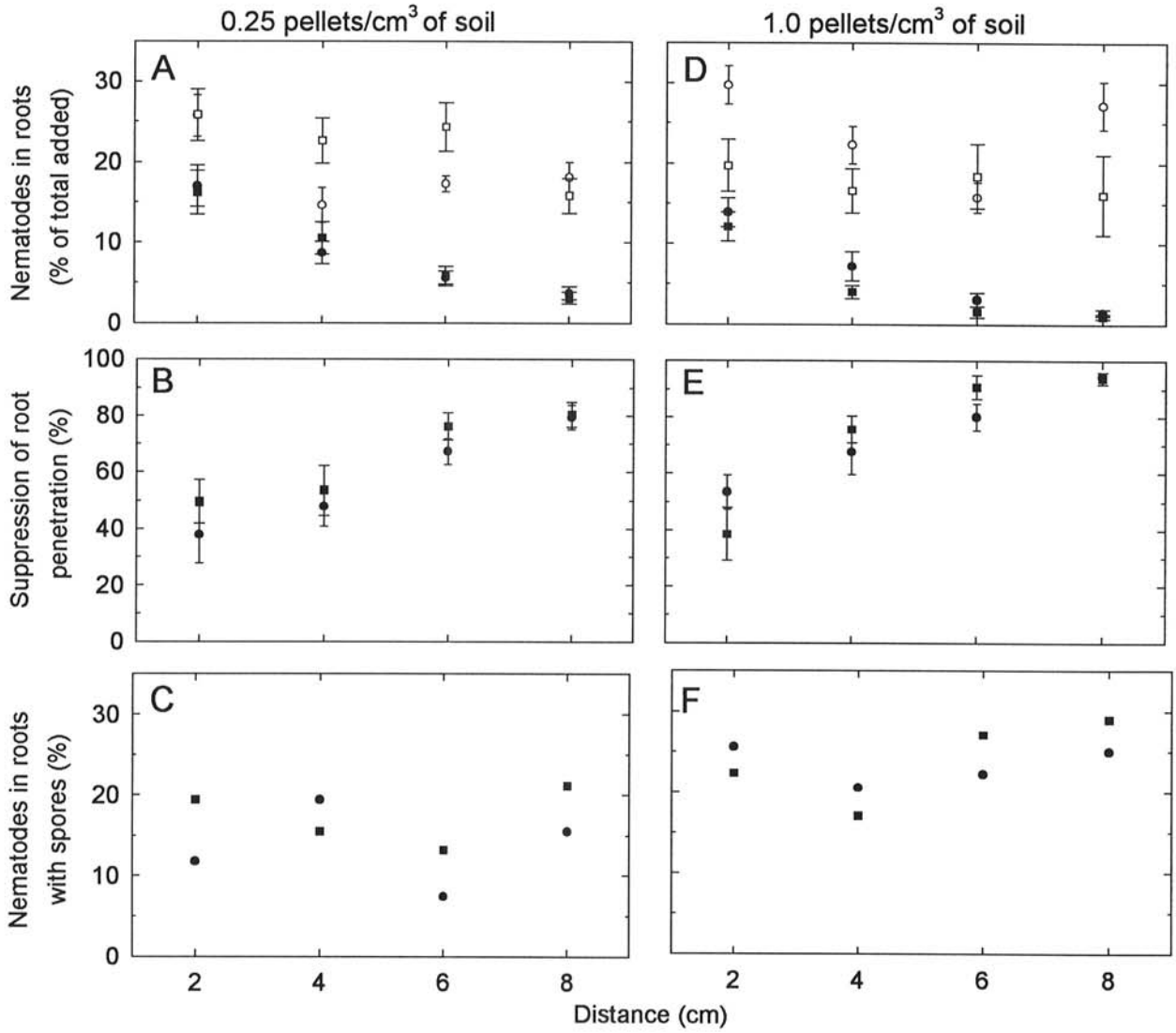

Fig. 4. Penetration of cabbage roots by juveniles (J2) of *Heterodera schachtii* as affected by initial distance between nematodes and plant and density of *Hirsutella rhossiliensis*. Data are from pellets without hyphae (open symbols) or with hyphae (closed symbols). Circles and squares indicate data from two independent trials. **A** and **D,** Percentage of J2 in roots relative to total number added. **B** and **E,** Suppression in number of J2 in roots relative to that in tubes receiving pellets without hyphae. **C** and **F,** Percentage of nematodes in roots with spores of *H. rhossiliensis*; data are from tubes receiving pellets with hyphae (except at the 0.0 level). For A, B, D, and E, values are the means ± SE of 10 replicate tubes. For C and F, each value is based on observation of at least 50 nematodes.

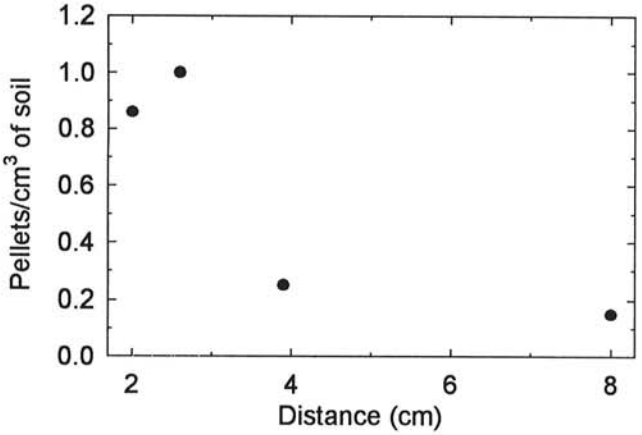

Fig. 5. Combinations of initial distances between plants and nematodes and densities of *Hirsutella rhossiliensis* pellets (pellets per cm^3 of soil) required for 50% suppression of root penetration by *Heterodera schachtii*. Values were estimated from data in Figures 2B and E, and 3B and E. We used linear regression of data in Figures 2B and 3B. For Figure 2E, we interpolated between 0.0 and 0.25 pellets per cm^3 of soil. For Figure 3E, we interpolated between 2 and 4 cm.

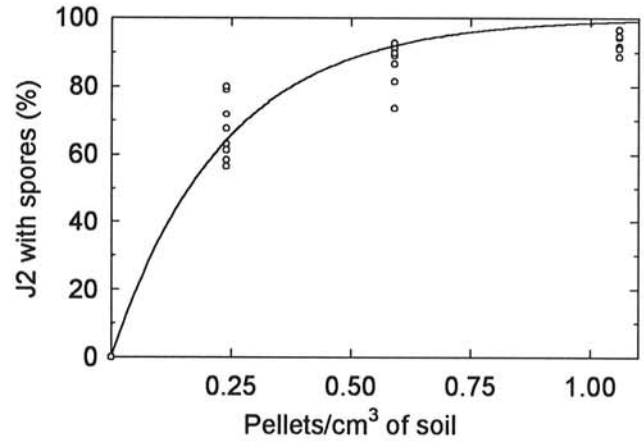

Fig. 6. Variation in transmission among eight batches of *Hirsutella rhossiliensis* pellets. Vials were packed with soil containing 0, 4, 10, or 18 pellets. After 14 days at 20°C, approximately 400 healthy second-stage juveniles (J2) of *H. schachtii* were added to each vial. After 66 h, J2 were recovered and examined for spores. Each value is the mean of five replicate vials. Data were analyzed by nonlinear regression with the equation $Y = 1 - e^{-b \cdot x}$. Based on pooled data from the eight batches, $b = 0.25$, $r^2 = 0.41$, and $P < 0.01$.

relationship, but in the estimation of fungal densities and distances required to suppress nematodes. Thus, 50% suppression of root penetration required 0.9 pellets (about 94,000 spores) per cm^3 of soil if *H. schachtii* were placed 2 cm from roots but only 0.3 pellets (about 31,000 spores) per cm^3 of soil if nematodes were placed 4 cm from roots. Although these estimates are limited to one soil and environmental regime, they nevertheless provide a baseline for future studies and modeling. A model, in particular, would allow us to explore how characteristics of the environment, the fungus, and the nematode affect biological control. This model should be mechanistic, i.e., it should consider fungal growth and sporulation from pellets and from parasitized nematodes, nematode movement, infection of nematodes by the fungus, and infection of roots by nematodes.

We conducted our experiments in pasteurized soil so as to eliminate uncontrolled effects of native fungi and nematodes. The efficacy of fungal pellets, however, is less in unheated soil than in heated soil (10), and thus we cannot interpret our data in terms of field efficacy. Two other limitations also should be mentioned. First, we measured net movement and not the actual distance that nematodes traveled through soil. Second, soil tubes were incubated for appropriate times so as to obtain equivalent root penetration in soil without fungus regardless of distance between nematodes and plants; we assume that other incubation times would not have changed our inferences.

In our laboratory experiments, *H. rhossiliensis* was substantially more effective if nematodes were placed 4 cm rather than 2 cm from roots. Do nematodes move 4 cm through soil in the field? Some certainly do. For example, juveniles of *Meloidogyne* spp. moved over 40 cm through microplot soil to infect susceptible tomato roots (12). Many juveniles, however, may move much shorter distances, especially when crops support multiple generations of cyst or root-knot nematodes per season (11,13). Eggs of second and subsequent generations may hatch adjacent to or even within roots, and thus juveniles may move only millimeters or not at all through soil. Although the question "How far do nematodes travel through soil?" is complex, the distance may be greatest when the overwintering stage attacks roots of newly planted crops. We therefore suggest that pelletized hyphae will be most effective early in the growing season.

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