# Effect of Nematode Inoculum on Suppression of Root-Knot and Cyst Nematodes by the Nematophagous Fungus *Hirsutella rhossiliensis*

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## ABSTRACT

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The effect of pelletized hyphae of *Hirsutella rhossiliensis* on penetration of tomato roots by *Meloidogyne javanica* and cabbage roots by *Heterodera schachtii* was measured in cups containing 100 $cm^3$ of field soil. The soil (±50 pellets) was infested with egg masses or cysts when placed into cups on day 0 or with juveniles on day 14. Seedlings were planted on day 17, and roots were measured and stained on day 22. The fungus always was more effective in soil infested with juveniles than in soil infested with egg masses or cysts, but substantial variability among trials within experiments limited our quantitative inferences. Suppression of nematodes per centimeter of root averaged 42 or 98% in soil infested with egg masses or juveniles of *M. javanica* and 83 or 98% in soil infested with cysts or juveniles of *H. schachtii*. *Hirsutella rhossiliensis* did not suppress *M. javanica* in egg mass-infested loamy sand when nematode density, as determined by bioassay, exceeded 1,200/100 $cm^3$ of soil but suppressed *H. schachtii* in cyst-infested loamy sand and loam regardless of nematode density.

*Additional keywords*: biocontrol, biological control.

In a recently described formulation of the nematophagous fungus *Hirsutella rhossiliensis* Minter & Brady, macerated hyphae were embedded in alginate pellets (4). When dried pellets were added to soil, the fungus utilized stored reserves in the macerated hyphae to grow and sporulate. The nonmotile spores adhered to and infected second-stage juveniles (J2) of the cyst nematode *Heterodera schachtii* Schmidt, and, thus, the addition of pellets increased the growth of seedlings in nematode-infested soil.

The formulation of *Hirsutella rhossiliensis* was evaluated in field soil infested with hatched J2 of *H. schachtii* (4). Efficacy, however, may be less in soil infested with eggs rather than with J2 if the eggs do not hatch until roots are near. Therefore, in the present study, we compared efficacy of pelletized *Hirsutella rhossiliensis* in soil infested with J2 or eggs of *H. schachtii* or *Meloidogyne javanica* (Treub) Chitwood.

## MATERIALS AND METHODS

**Fungal inoculum.** Hyphae of *Hirsutella rhossiliensis* (IMI 265748) were prepared and formulated into alginate pellets as described previously (3,4). Fresh pellets were coated with sand (No. 60 white silica sand, Corona Industrial Sand Co., Corona, CA), spread on wax paper, and dried for 24 h at room temperature and humidity. The sand coating did not affect pellet efficacy (B. A. Lackey, B. A. Jaffee, and A. E. Muldoon, *unpublished data*) but facilitated separation of the pellets for drying. Dried pellets weighed 1.6 ± 0.1 mg ($\bar{x} \pm$ SE) and were 2.0 ± 0.1 mm in diameter. Pellets prepared in this manner but without the sand coating contained 0.14 mg dry weight of hyphae, weighed 0.58 mg, and were 1.7 mm in diameter (4). Although Schuster and Sikora (7) reported that alginate pellets inhibited root growth, we did not use pellets without hyphae in our controls because we previously showed that rates of alginate two times greater than that used in the present study did not affect growth of cabbage or penetration of cabbage roots by *H. schachtii* in loamy sand (4). Moreover, we added much less (<5%) alginate per unit of soil than did Schuster and Sikora (7). In the present study, pellet density was 0 (control) or 0.5 pellets per cubic centimeter of soil, and pellets were added to soil within 24 h of drying.

**Soils and nematodes.** The loamy sand and loam used in this study were described previously (9). The loamy sand was collected from a sweet potato field in Merced County, CA, and the loam

was collected from a fallow field in Yolo County, CA. Neither soil contained detectable root-knot nematodes or cyst nematodes based on bioassay with cabbage (*Brassica oleraceae* L. 'Chieftain Savoy') and tomato (*Lycopersicon esculentum* Mill. 'UC82') seedlings (10) nor detectable *Hirsutella rhossiliensis* based on bioassay with *H. schachtii* (2). Soil was passed through a sieve (2-mm pore diameter) before use. Soil moisture at the initiation of each experiment was 7.0–7.9% (approximately $-25$ J kg$^{-1}$) for the loamy sand and 12.7–13.8% (approximately $-300$ J kg$^{-1}$) for the loam; these moisture levels were near optimum for transmission of spores of *Hirsutella rhossiliensis* (9).

Hatched J2 of *M. javanica* were obtained from hydroponic cultures (5), and those of *H. schachtii* were obtained from cysts and recovered on Baermann funnels (2). Suspensions of J2 were stored at 10 C with aeration for less than 48 h before addition to soil.

**Infestation of soil with nematodes.** To infest soil with hatched J2, suspensions of J2 were added to the soil surface. To infest soil with eggs of *M. javanica* or *H. schachtii*, we either grew nematode-infected plants in the soil or, for *H. schachtii* in loamy sand, we mixed cysts into the soil. When infestation with eggs was achieved by growth of nematode-infected plants, the soil undoubtedly contained hatched J2, but we assumed that eggs predominated.

To obtain loamy sand infested with egg masses of *M. javanica*, field soil was placed into six 1.5-L pots, planted with 3-wk-old tomato plants, and infested with 8,000 J2 of *M. javanica* per pot. The J2 were obtained from the same hydroponic cultures described previously, and we assumed that the *M. javanica* in soil infested with hatched J2 and egg masses were genetically similar. Plants were kept in a greenhouse (20–29 C) and inoculated twice more at 2-wk intervals to achieve asynchronous reproductive cycles. Within 8–12 wk, the plants were removed, and soil from the pots was combined. The *M. javanica*-infested soil was stored in plastic bags at 10 C for 1–8 wk before use.

To obtain loamy sand infested with cysts of *H. schachtii*, cysts were mixed into the field soil. Cysts were added to the loamy sand because the soil puddled in the greenhouse and did not support good growth of host plants of *H. schachtii*. The cysts were from 12-mo-old sugar beet plants (*Beta vulgaris* L. 'SSNB-2') growing in pots of sand infested with 5,000 J2 of *H. schachtii* when plants were 1 mo old. We assumed that these greenhouse cultures provided cysts of various ages. Because the cysts and J2 were from the same greenhouse cultures, we also assumed that the *H. schachtii* in soil infested with hatched J2 and cysts were genetically similar. The cysts were extracted by repeated sieving (833- and 246-$\mu$m pore diameter) and decanting. Approximately 55,000 cysts, each with about 70 eggs, were suspended in 90 ml of tap water and added to 3 L of loamy sand; the soil was mixed and stored in a plastic bag at 10 C for 1–8 wk before use.

Loam infested with cysts of *H. schachtii* was obtained from experimental field plots infested with *H. schachtii* and planted with sugar beets for 2.5 yr (10). Approximately 5 L of the loam was mixed and stored in a plastic bag at 10 C for 1–16 wk before use. We did not investigate *M. javanica* in loam because crop damage by this nematode usually occurs in sandy soils.

Loamy sand and loam not infested with *M. javanica* or *H. schachtii* were stored in the laboratory. These noninfested soils were used for controls or to dilute infested soil.

**Suppression of *M. javanica* in soil infested with hatched J2 or egg masses (experiments 1 and 2).** In experiment 1, zero or 50 pellets of *Hirsutella rhossiliensis* were added to 100 cm$^3$ of loamy sand infested with egg masses or not infested with *M. javanica*. On day 0, the soil was placed in 190-ml polystyrene cups covered with aluminum foil to reduce water loss. Cups were incubated in a clear plastic box with moistened paper towels (moisture chamber) without light at 20 C. On day 14, 450 healthy J2 of *M. javanica* in 3 ml of 4.6 mM KCl were added to each of the cups that contained soil without *M. javanica*; the remaining cups, which contained egg mass-infested soil and zero or 50 pellets, received 3 ml of KCl solution without nematodes. On day 17, the foil was removed, six tomato seedlings (radicle <2 mm long) were planted per cup, 5 ml of tap water was added per cup, and the cups were returned to the moisture chamber and placed under fluorescent lights (12-h photoperiod) at 22 ± 2 C. On day 22, the seedlings were removed, shoot and root lengths were measured, and the roots were stained (1). The number of *M. javanica* in roots was determined. Each of the four treatments (infestation with egg masses or juveniles and zero or 50 pellets of *Hirsutella rhossiliensis* per cup) was replicated five times.

Experiment 2 was similar to experiment 1, except that we used two levels of nematodes and attempted to achieve similar or higher nematode inoculum potential in J2-infested soil than in egg mass-infested soil. Based on the nematode inoculum potential in egg mass-infested soil (determined by bioassay; data not shown), the egg mass-infested soil was undiluted or diluted 1:8 (v/v) with noninfested loamy sand, and the J2-infested soil received 1,370 or 840 J2 per cup. Other procedures and data collection were as in experiment 1. There were four replicate cups per treatment.

**Suppression of *H. schachtii* in soil infested with J2 or cysts (experiment 3).** Experiment 3 was similar to experiment 2, except that soils infested with J2 and cysts of *H. schachtii* were compared. To achieve high and low levels of cysts, loamy sand infested with cysts of *H. schachtii* was diluted 1:8 or 1:32 with noninfested soil. Two levels of J2 infestation were achieved by adding 1,590 or 850 J2 per cup. Other procedures were as in experiment 2, except that the host plant was cabbage. There were four replicate cups per treatment.

**Effect of level of *M. javanica* on suppression in egg mass-infested loamy sand (experiment 4).** Loamy sand infested with egg masses of *M. javanica* was undiluted or diluted with noninfested loamy sand to yield 100, 50, 25, and 13% infested soil (v/v) in trial 1 and 25, 13, and 6% infested soil in trial 2. Pellets were added to soil (zero or 50 per 100 cm$^3$), and other procedures and data collection were as in experiment 1, except dilute KCl was not added on day 14. Each level, with and without pellets, was replicated four times.

**Effect of level of *H. schachtii* on suppression in cyst-infested loamy sand and loam (experiment 5).** Experiment 5 was identical to experiment 4, except that loamy sand and loam were used and the soils were infested with cysts of *H. schachtii* and planted with cabbage. In addition, the levels of nematode-infested soil were 100, 50, 25, 13, 6, and 3%.

**Nematode inoculum potential.** We measured inoculum potential, which reflects both nematode density in soil and its infection efficiency, rather than nematode density in soil because nematode viability could vary greatly between infestation methods and among different trials using the same infestation method. Inoculum potential was inferred from the number of nematodes in roots in soil without added fungus. The limitations of this determination are that nematode density in soil is unknown, and inoculum potential could be underestimated if root mass is inadequate or if some nematodes do not have sufficient time to penetrate roots.

**Statistical analysis.** Experiments were conducted two times (trials 1 and 2), except for experiment 1 and part of experiment 5, which were conducted three times. The design was completely randomized. We determined the means and standard errors for the number of nematodes in roots per cup, number of nematodes per centimeter of root, and root length per cup. For statistical analysis, we focused on the number of nematodes per centimeter of root because this parameter appeared to be the most sensitive indicator of nematode suppression by the fungus. We calculated percent suppression as $(1 - w/z)\ 100$, in which $w$ is the number of nematodes per centimeter of root in cups with pellets and $z$ is the mean number of nematodes per centimeter of root in cups without pellets. The percents were transformed (arcsine) for analysis by the SAS general linear models procedure (6).

In experiments 1–3, analyses of variance (ANOVA) were used to determine whether suppression of nematodes differed ($P < 0.05$) in J2-infested soil versus egg-infested soil. In the model statement, the dependent variable was the percent suppression in the number of nematodes per centimeter of root, and the

independent variables were trial, infestation method, and the interaction between trial and infestation method. For experiments 2 and 3, this analysis was conducted independently for high and low nematode densities. When a significant interaction was detected, we repeated the analysis for each trial to elucidate the nature of the interaction. In experiments 4 and 5, we used ANOVA and linear regression to determine whether percent suppression decreased as nematode density increased.

## RESULTS

**Suppression of *M. javanica* in soil infested with hatched J2 or egg masses (experiments 1 and 2).** Suppression in the number of nematodes per centimeter of root was 47, 7, and 19% in egg mass-infested soil and 94, 99, and 99% in J2-infested soil in trials 1–3 of experiment 1, respectively. Although suppression was significantly greater ($P < 0.05$) in J2-infested soil than in egg mass-infested soil in each trial and over all trials, an interaction between trial and infestation method occurred because suppression was inconsistent in egg mass-infested soil. We combined the data from the trials for presentation, because the interaction was quantitative and not qualitative.

In experiment 1, pelletized *Hirsutella rhossiliensis* suppressed the number of *M. javanica* in roots in J2-infested loamy sand but not in egg mass-infested loamy sand (Fig. 1A). Nematode inoculum potential as inferred from the number of nematodes in control roots, however, was greater in the egg mass-infested soil than in the J2-infested soil (Fig. 1A). The percent suppression in number of nematodes per centimeter of root was greater ($P < 0.05$) in J2-infested soil than in egg mass-infested soil (Fig. 1B). When pellets were added, root length increased substantially in J2-infested soil but only marginally in egg mass-infested soil (Fig. 1C).

In experiment 2, infestation method and the interaction between infestation method and trial were significant sources of variance with the high nematode level, but only infestation method was significant with the low nematode level. As in the first experiment, data from trials were combined for analysis and presentation because qualitative inferences based on individual or combined trials were identical. *Hirsutella rhossiliensis* suppressed the number of *M. javanica* in roots with both infestation methods and nematode levels (Fig. 1D). In contrast to experiment 1, nematode inoculum potential, as inferred from the number of nematodes in control roots, was greater in J2-infested soil than in

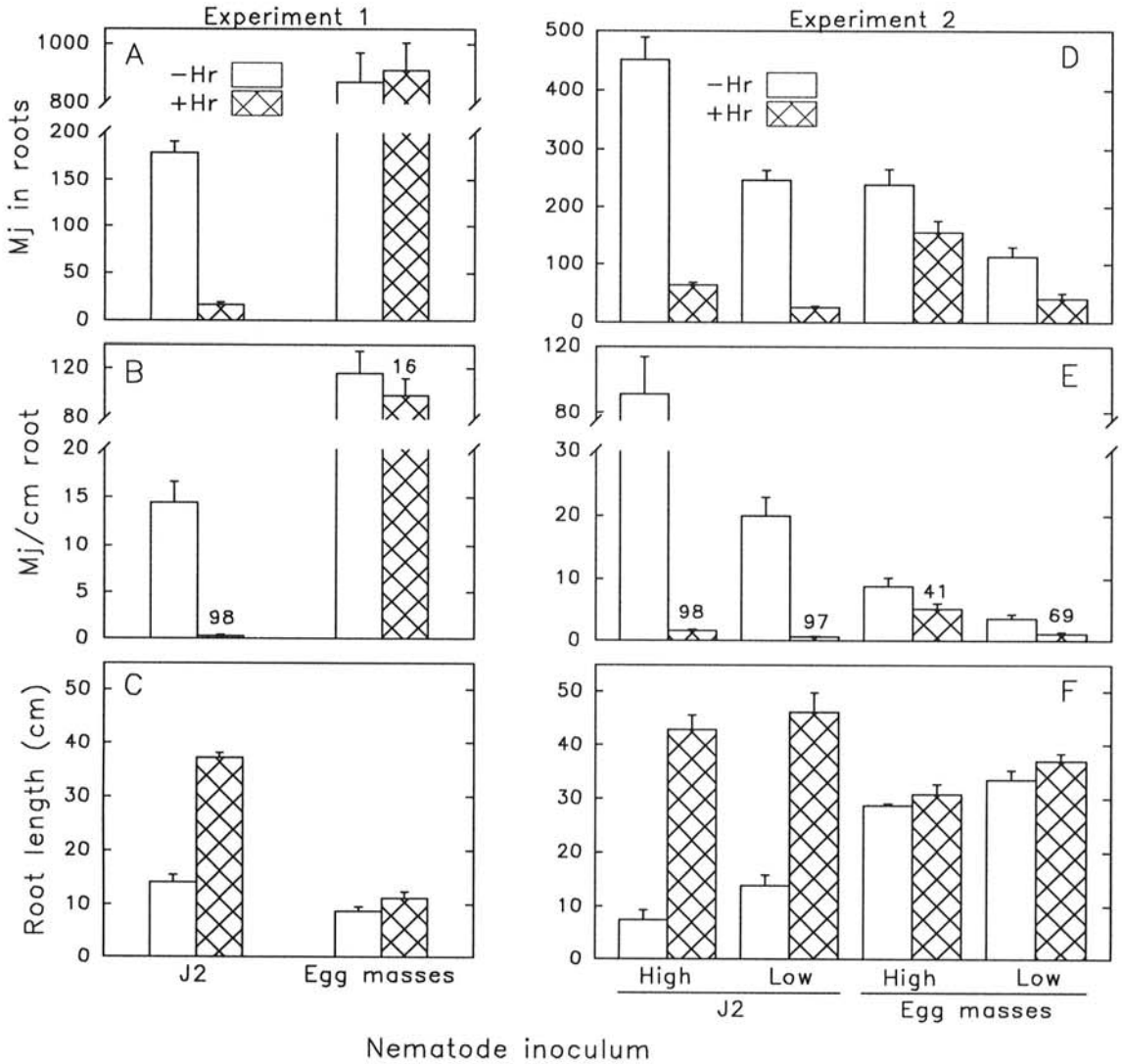


**Fig. 1.** Efficacy of pelletized *Hirsutella rhossiliensis* (Hr) in loamy sand infested with hatched juveniles (J2) or egg masses of *Meloidogyne javanica* (experiments 1 and 2). Zero or 50 pellets of *Hirsutella rhossiliensis* and 100 cm$^3$ of soil (infested or not infested with egg masses of *M. javanica*) were added to cups. After 14 days, cups without *M. javanica* were infested by adding a suspension of hatched J2 to the soil surface. There was one nematode level per infestation method in **A–C,** experiment 1 and two levels in **D–F,** experiment 2. On day 17, six tomato seedlings were planted in each cup, and roots were measured and stained on day 22. **A and D,** Number of *M. javanica* in roots per 100 cm$^3$ of soil. **B and E,** Number of *M. javanica* per centimeter of root; the numbers above the bars indicate the percent suppression relative to the control. **C and F,** Root length per 100 cm$^3$ of soil. Each value is the mean ± SE of 15 (**A–C**) or eight (**D–F**) replicate cups from two or three pooled trials.

egg mass-infested soil (Fig. 1D). Percent suppression in number of *M. javanica* per centimeter of root was greater ($P < 0.05$) in J2-infested soil than in egg mass-infested soil at both nematode levels (Fig. 1E). In egg mass-infested soil, roots were relatively long and were not greatly affected by *Hirsutella rhossiliensis*; in J2-infested soil, roots were much longer in *Hirsutella rhossiliensis*-amended soil than in the control (Fig. 1F).

**Suppression of *H. schachtii* in soil infested with J2 or cysts (experiment 3).** Infestation method and the interaction between infestation method and trial were significant sources of variance with high nematode level, but only infestation method was significant with low nematode level. Data from trials were combined for analysis and presentation because qualitative inferences based on individual or combined trials were identical. Fewer *H. schachtii* were found in cabbage roots growing in soil with *Hirsutella rhossiliensis* than in soil without the fungus, regardless of infestation method or nematode level (Fig. 2A). Suppression was greater ($P < 0.05$) in J2-infested soil than in cyst-infested soil at both nematode levels (Fig. 2B). Root length was longer in both soils and at both nematode levels in *Hirsutella rhossiliensis*-amended soil than in the control (Fig. 2C).

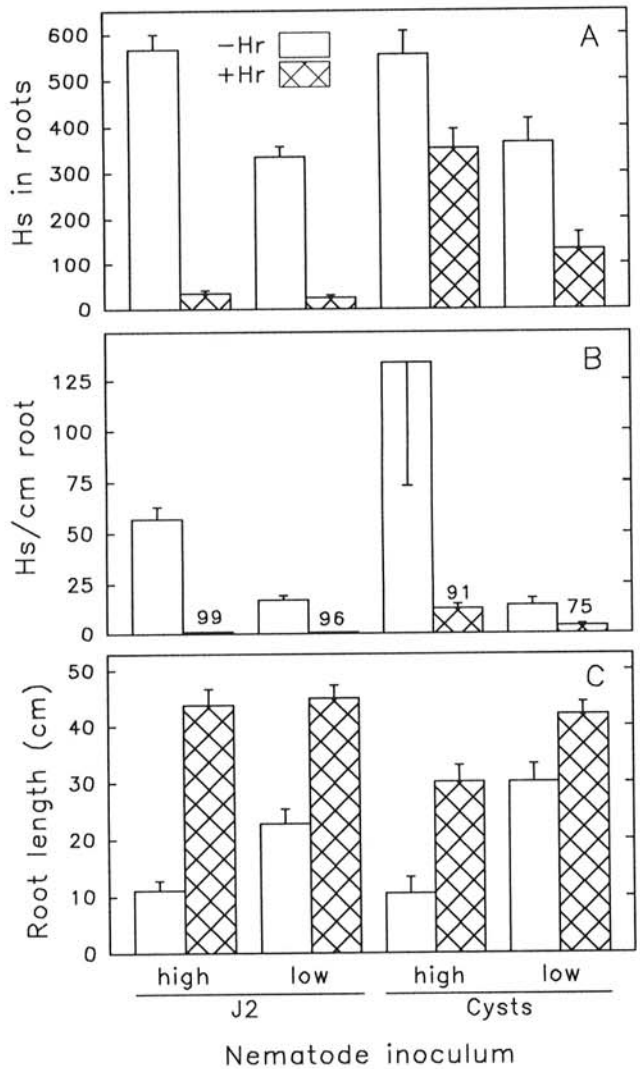


**Fig. 2.** Efficacy of pelletized *Hirsutella rhossiliensis* (Hr) in loamy sand infested with hatched juveniles (J2) or cysts of *Heterodera schachtii* (experiment 3). Zero or 50 pellets of *Hirsutella rhossiliensis* and 100 $cm^3$ of soil (infested or not infested with cysts of *H. schachtii*) were added to cups. After 14 days, cups without *H. schachtii* were infested by adding a suspension of hatched J2 to the soil surface. There were two nematode levels per infestation method. On day 17, six cabbage seedlings were planted in each cup, and roots were measured and stained on day 22. **A,** Number of *H. schachtii* in roots per 100 $cm^3$ of soil. **B,** Number of *H. schachtii* per centimeter of root; the numbers above the bars indicate the percent suppression relative to the control. **C,** Root length per 100 $cm^3$ of soil. Each value is the mean ± SE of eight replicate cups from two pooled trials.

**Effect of level of *M. javanica* on suppression in egg mass-infested loamy sand (experiment 4).** In loamy sand infested with egg masses of *M. javanica*, the fungus appeared unable to suppress the nematode when nematode level was high (Fig. 3). In trial 1, suppression in number of *M. javanica* per centimeter of root was −43, 26, 75, and 87% in 100, 50, 25, and 13% infested soil, respectively. In trial 2, suppression was 84, 85, and 77% in 25, 13, and 6% infested soil, respectively. An ANOVA with data from both trials indicated that suppression was greater ($P < 0.05$) in 25, 13, and 6% infested soil than in 50 and 100% infested soil. Root length was inversely related to the percentage of soil infested with *M. javanica* and was greater in the *Hirsutella rhossiliensis*-amended soil than in the control, except at the highest (Fig. 3C) and lowest (Fig. 3F) levels of nematode infestation.

**Effect of level of *H. schachtii* on suppression in cyst-infested loamy sand and loam (experiment 5).** Qualitative inferences based on trials 1 and 2 in cyst-infested loamy sand and on trials 1 and 3 in cyst-infested loam were identical, and data were combined for each soil type for presentation and analysis. The viability of fungal inoculum in trial 2 with loam was low, determined

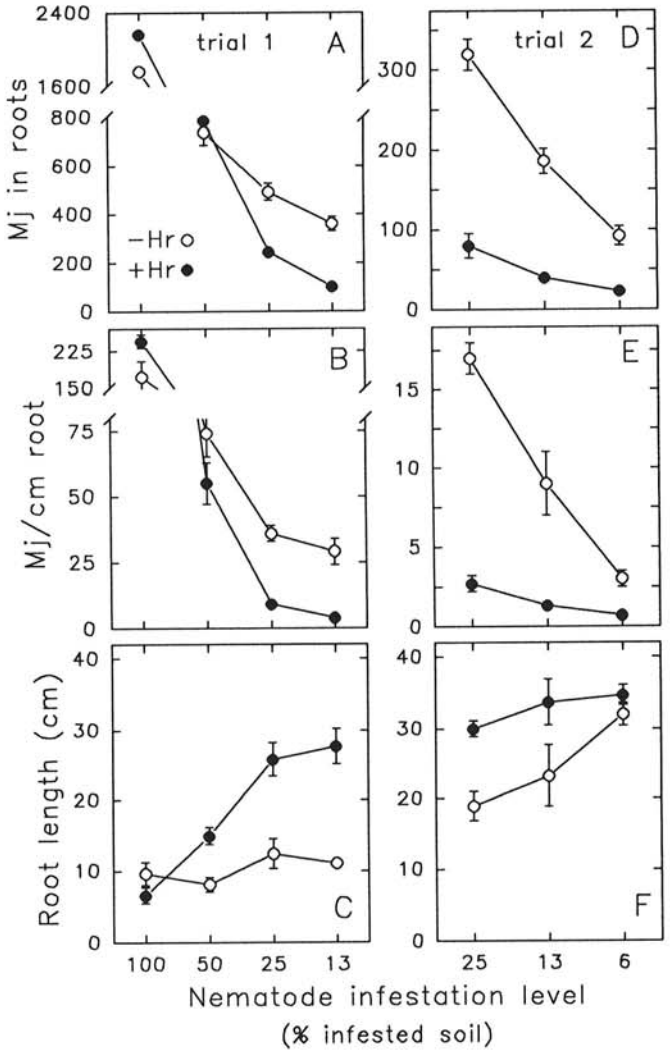


**Fig. 3.** Efficacy of pelletized *Hirsutella rhossiliensis* (Hr) as influenced by level of *Meloidogyne javanica* in egg mass-infested loamy sand (experiment 4). On day 0, pellets of *Hirsutella rhossiliensis* were added to cups containing egg mass-infested loamy sand that was or was not diluted with noninfested soil (zero or 50 pellets and 100 $cm^3$ of soil per cup). Six tomato seedlings were planted on day 17, and roots were measured and stained on day 22. Data are from trials 1 (**A–C**) and 2 (**D–F**). **A and D,** Number of *M. javanica* in roots per 100 $cm^3$ of soil. **B and E,** Number of *M. javanica* per centimeter of root. **C and F,** Root length per 100 $cm^3$ of soil. Each value is the mean ± SE of four replicate cups.

by an independent bioassay (data not shown); data from trial 2 were excluded (no suppression occurred). Because trials with loamy sand and loam were conducted at different times, they could not be compared directly.

Regardless of nematode level, *Hirsutella rhossiliensis* suppressed the number of *H. schachtii* in roots (Fig. 4A and D) and the number per centimeter of root (Fig. 4B and E). In loamy sand, percent suppression in number of *H. schachtii* per centimeter of root was 92, 92, 77, 84, 64, and 99% in 100, 50, 25, 13, 6, and 3% infested soil, respectively. In loam, percent suppression in number of *H. schachtii* per centimeter of root was 71, 74, 68, 69, 67, and 22% in 100, 50, 25, 13, 6, and 3% infested soil, respectively. Based on linear regression, suppression was not related ($P > 0.05$) to nematode level in either case. Root growth was inversely related to nematode level and was greater in the presence of *Hirsutella rhossiliensis* than in the control, except at the lowest nematode density (Fig. 4C and F).

## DISCUSSION

At the outset of this study, we were concerned that our standard bioassay, in which hatched J2 are allowed to move through soil for 66 h (4), overestimated the probability that J2 would encounter spores. The first experiments described here with *M. javanica* appeared to support this concern; suppression was substantial with hatched J2 but minimal with egg masses. One interpretation is that the J2 in egg masses remained in eggs until roots were near, traveled short distances through the soil, and, thus, minimized their chance of encountering spores (8,11). Data from subsequent experiments, however, did not support this explanation, because suppression was substantial when the level of egg masses was reduced. Moreover, the fungus suppressed cyst nematodes regardless of the quantity and form of nematode inoculum, although the suppression was somewhat greater with hatched J2 than with cysts.

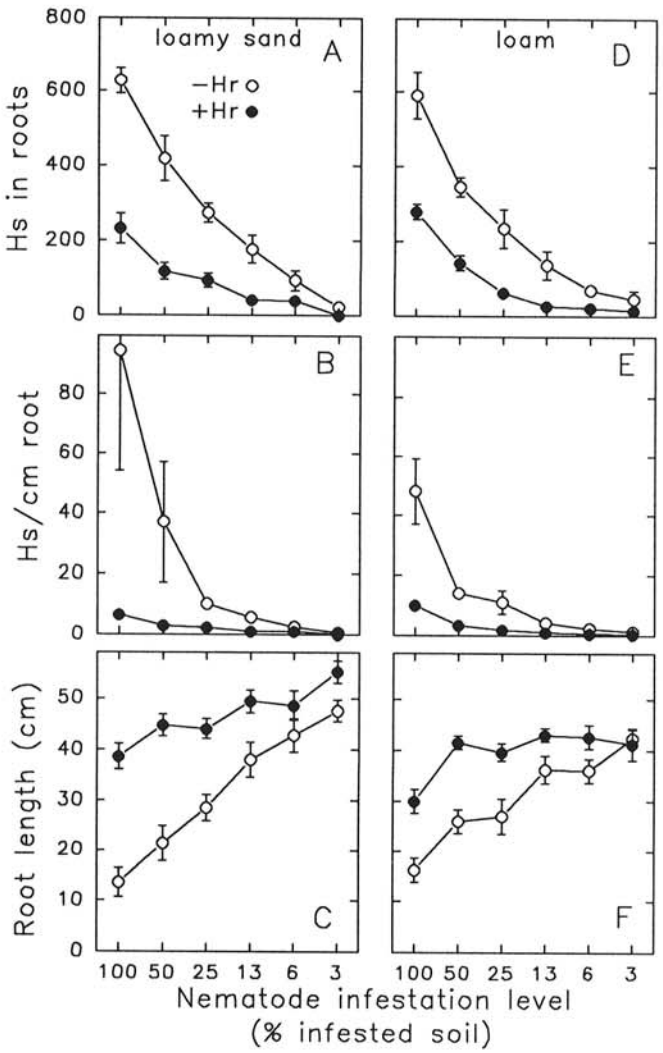


**Fig. 4.** Efficacy of pelletized *Hirsutella rhossiliensis* (Hr) as influenced by level of *Heterodera schachtii* in cyst-infested loamy sand and loam (experiment 5). Pellets of *Hirsutella rhossiliensis* were added to cups containing loamy sand or loam (zero or 50 pellets and 100 $cm^3$ of soil per cup) on day 0. The cyst-infested loamy sand (**A–C**) and loam (**D–F**) was or was not diluted with noninfested soil. Six cabbage seedlings were planted on day 17, and roots were measured and stained on day 22. **A and D,** Number of *H. schachtii* in roots per 100 $cm^3$ of soil. **B and E,** Number of *H. schachtii* per centimeter of root. **C and F,** Root length per 100 $cm^3$ of soil. Each value is the mean ± SE of eight replicate cups from two pooled trials.

The distance that nematodes move through soil remains a possible explanation for the observed effect of nematode inoculum on fungal suppression, but other possibilities cannot be ruled out. Perhaps the egg mass-infested soil contained an agent(s) that inhibited sporulation or increased spore mortality. In this respect, our handling of the soils was flawed. The same loamy sand was used for all the experiments with *M. javanica*, but the loamy sand used to dilute the egg mass-infested soil and to test hatched J2 inoculum was not planted with tomato and was incubated in the laboratory rather than in the greenhouse. Perhaps unknown factors introduced into greenhouse-incubated soil but not into laboratory-incubated soil inhibited the fungus.

The failure to include alginate pellets without hyphae in our controls is another potential problem, because high rates of alginate may suppress root invasion by nematodes (7). We previously showed, however, that low rates of alginate do not affect nematodes or seedlings (4). Moreover, the observed suppression of nematodes was entirely consistent with previous experiments in which controls contained alginate pellets and in which suppression was correlated with spore acquisition by nematodes (4). Our main problem is not explaining suppression but rather the lack of suppression in some experiments. Although we should have included alginate in the controls to eliminate possible side effects, we doubt that addition of alginate would have altered the results or helped us to understand why the pellets sometimes performed poorly.

Our formulation shows some potential, but we must continue to study the conditions that reduce or increase efficacy. In particular, we need more information on how far J2 move through soil to roots and on the relationship between distance moved, spore density, and the probability of encountering a spore. We also need direct measurements of spore density to determine whether variation in efficacy reflects sporulation, spore mortality, and/or spore acquisition (9).

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