

An Assay for *Hirsutella rhossiliensis* Spores and the Importance of Phialides for Nematode Inoculation

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Abstract. A spore assay was developed to measure the relative density of spores of the nematophagous fungus *Hirsutella rhossiliensis* in soil. Orchard soil containing *H. rhossiliensis*-parasitized *Cricomonella xenoplax* was placed in vials and incubated for 0-120 days before the addition of probe nematodes, *Heterorhabditis heliothidis* juveniles. After 18 hours, *H. heliothidis* were extracted from the soil and examined for adhering spores of *H. rhossiliensis*. No spores were detected when *H. heliothidis* were added to freshly mixed soil, but the percentage of *H. heliothidis* with spores increased rapidly if soil was incubated undisturbed. Because mixing soil detaches spores from phialides, the results indicate that spores must be attached to phialides to adhere to nematodes. The spore assay was compared with a plate assay that measures the population density of *H. rhossiliensis*-parasitized *C. xenoplax*. Results from the two assays were highly correlated, suggesting that spores occur in three phases: reserves in nematodes that may be converted into spores; spores on phialides and therefore capable of adhering to nematodes; and spores detached from phialides and thus incapable of adhering to nematodes.

Key words: biocontrol, *Cricomonella xenoplax*, entomogenous, *Heterorhabditis heliothidis*, *Hirsutella rhossiliensis*, nematophagous, phialide, ring nematode.

Hirsutella rhossiliensis Minter & Brady is a fungus that parasitizes vermiform stages of many species of soil-borne nematodes but it has been most frequently associated with the ring nematode, *Cricomonella xenoplax* (Raski) Luc & Raski (3,4). Spores of *H. rhossiliensis* initiate infection by adhering to the cuticle of passing nematodes. The nematodes die shortly after infection, and hyphae emerge from the dead nematodes and sporulate. The adhesive spores are born singly on phialides (bottle-shaped cells about 30 µm long) spaced at regular intervals along the emergent hyphae (4).

An understanding of the interaction between a nematophagous fungus and soil-borne nematodes requires techniques to quantify parasitized and unparasitized nematodes and fungal inoculum. A plate assay (3) permits quantification of *H. rhossiliensis*-parasitized and unparasitized nematodes but does not directly measure fungal inoculum (spore density). The assay can provide an accurate estimate of spore density only if spore density is proportional

to the number of parasitized nematodes. The method could underestimate spore density, however, if spores detached from phialides by soil disturbance or other means (= detached spores) persist in soil and retain the ability to infect nematodes. Detached spores would not be detected by the plate assay because they would be washed through sieves used to collect nematodes or killed by treatment with sodium hypochlorite (6). In addition to their significance in quantification of fungal inoculum, detached spores may be important in utilizing *H. rhossiliensis* as a biocontrol agent. If detached spores are infective, the fungus can be added to soil as spores produced in vitro. An assay for spores of *H. rhossiliensis* capable of infecting nematodes in soil is needed.

Heterorhabditis heliothidis Khan, Brooks & Hirschmann is an entomogenous nematode that attacks its hosts in the soil. Third-stage juveniles (J3) are easily reared in the laboratory and have been reported to migrate up to 10 cm vertically in soil columns within a few days in the absence of hosts (2). Although spores of *H. rhossiliensis* adhere to the cuticle of the J3, the J3 retains the second-stage cuticle and resists infection (10). These characteristics suggest that *H. heliothidis* could serve as a probe to reveal *H. rhossiliensis* spores in soil. Similarly,

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Meloidogyne javanica was used to detect spores of *Pasteuria penetrans* in soil (9).

The objective of this research was to develop an assay for spores of *H. rhossiliensis* capable of adhering to nematodes in soil. We also tested the effect of soil disturbance on the number of spores detected by the spore assay and the correlation of the spore assay (direct method) and the plate assay (indirect method) for estimating numbers of spores in soil.

MATERIALS AND METHODS

Plate assay: Soil (78% sand, 13% silt, 9% clay; < 1.0% organic matter; pH = 4.4 in 0.01 M CaCl₂; inflection point of moisture characteristic $\cong -4.0$ kPa) infested with *H. rhossiliensis* and *C. xenoplax* was collected from the root zone of peach trees in a California peach orchard (orchard M in 3) and stored at 10 C. Total *C. xenoplax* and *H. rhossiliensis*-parasitized *C. xenoplax* were determined by the plate assay of Jaffee et al. (3). Nematodes extracted by wet sieving and centrifugation (7) were surface disinfested with 0.5% sodium hypochlorite, suspended in water, and spread on water agar containing 200 mg streptomycin/liter. After 5 days, *C. xenoplax* were counted by stage, and those with emergent hyphae, phialides, and spores of *H. rhossiliensis* were scored as parasitized. Nematode species other than *C. xenoplax* were not identified to species or stage but were scored for parasitism. Nematode counts were corrected for experimentally determined extraction efficiencies of 0.50, 0.53, 0.58, and 0.78 for J2, J3, J4, and adult *C. xenoplax*, respectively. Low numbers of other species of nematodes were detected and assigned an extraction efficiency of 0.5.

Heterorhabditis heliothidis were reared on wax moth larvae (1). Harvested J3 were stored in distilled water at 10 C. Nematodes were never stored more than 1 month and were examined for vigor before each test.

Spore assay: Assays were performed on orchard soil samples in 25-cm³ plastic prescription vials with snap-on caps. Drain holes were made in the vial bottoms with

a heated nail, and a circle of polyester fabric was placed in the bottom to retain the soil. Soil was mixed and moistened to a uniform level by either a centrifugation method or a spray method. In the centrifugation method, 25 g (dry weight equivalent) of soil was placed in the sample vial and lightly compacted by tapping the vial on a counter top. Vials with soil were then placed on a sand bed and 7 ml distilled water was pipetted gently over the soil surface to saturate the soil. After initial drainage was complete (within 15 minutes), the vials were placed in 50-ml centrifuge tube shields using inverted vials as supports and spun at 250 rpm (12 g) for 15 minutes. This produced a consistent soil moisture content of (mean \pm SE) $18.5 \pm 0.7\%$ ($\cong -5.8$ kPa) and a soil bulk density of 1.5 g/cm³. In the spray method, soil was moistened in a plastic bag by alternately spraying with distilled water and mixing until the soil would just begin to hold together when squeezed. The soil moisture at this point was $11.6 \pm 2.1\%$ ($\cong -8.7$ kPa). Twenty-five grams (wet weight) of the moistened soil was placed in the vials and compacted to 17 cm³ (bulk density = 1.3–1.5 g/cm³) by tapping and tamping with a rubber stopper. Estimates of water potential were obtained from the moisture release curve for this soil.

For the assay, 2,000 active *H. heliothidis* in 0.5 ml distilled water were pipetted onto the soil surface of each vial and the vials were capped. Following incubation in a moist chamber (18 hours, 22 ± 2 C), *H. heliothidis* were recovered by wet sieving and centrifugation (7), and resuspended in 10 ml distilled water. One-milliliter samples were placed on Hawksley counting slide under a dissecting microscope (60 \times) and the first 50 moving *H. heliothidis* observed were scored for the presence or absence of adhering *H. rhossiliensis* spores.

To confirm the movement of *H. heliothidis* through the soil, some vials in early trials were tested qualitatively for depth of nematode penetration. The soil in these vials was pushed out intact and cut into top, middle, and bottom thirds with a razor

blade. The soil from each segment was mixed vigorously with water, allowed to settle for 5 seconds, and decanted. The decanted water was examined with a dissecting microscope for the presence of active *H. heliothidis*.

Effect of soil disturbance on numbers of spores detected by the spore assay: Orchard soil was spray moistened and packed in 24 vials. Six vials were immediately assayed for spores by the spore assay (week 0). The remaining vials were incubated in a moist chamber at 22 ± 2 C. At week 1, six vials were assayed for spores; the 12 remaining vials were unpacked, and the soil from each was mixed and repacked. Six of these disturbed vials were assayed at once; the remaining six were returned to the moist chamber. At week 2, these last six vials were assayed without further disturbance. This experiment was performed once with soil stored 2 days and again with soil stored 30 days at 10 C before the experiment.

Density of spores as measured by plate and spore assays: To compare the efficacy of the two assays in measuring levels of fungal inoculum, two experiments were performed. In the first experiment, a homogeneous soil sample was assayed over time. Orchard soil was mixed, placed in 56 vials, and moistened by the centrifuge method (week 0). The capped vials were incubated in moist chambers at 22 ± 2 C. At week 0 and periodically thereafter to week 16, nine vials were removed. Three of these were used for gravimetric determination of soil moisture, and six were subjected to combined spore and plate assays. *H. heliothidis* were added to the six vials as described earlier, but following extraction the extract was divided into two 5-ml portions. One portion was examined for *H. heliothidis* with or without *H. rhossiliensis* spores as described in the spore assay, and the other was diluted to 10 ml and processed as described in the plate assay. The experiment was performed once with soil stored 2 days and again with soil stored 30 days at 10 C before the experiment. Numbers of parasitized *C. xenoplax* as determined from the plate assay were converted to spore equiv-

alents (potential number of spores produced per nematode) by multiplying the number of nematodes of each stage by 700, the mean total number of spores produced by adults (5), and by the relative body volume of the stage—0.05, 0.18, 0.34, and 1.00 for J2, J3, J4, and adult, respectively (H. Ferris, pers. comm.). Parasitized nematodes other than *C. xenoplax* were present in low numbers ($\cong 8$ /vial) and were assigned 200 spore equivalents.

In a second experiment, soil samples containing a wide range of *H. rhossiliensis*-parasitized *C. xenoplax* were assayed. A soil core (2 × 55 cm) was taken from the root zone of each of 15 randomly selected peach trees on 7 December 1987 and 22 additional trees on 10 February 1988 with a Veihmeyer soil tube. A 100-cm³ subsample from each core sample was extracted and subjected to the plate assay within 24 hours. Additional 25-cm³ subsamples were assayed by the spore assay (soil was moistened by the spray method). Spore assay vials were incubated 1 week before addition of *H. heliothidis*. For linear regression analysis (8), the number of parasitized nematodes detected in the plate assay was converted to spore equivalents as described.

Inoculum potential of spores added to soil: Lawn cultures of *H. rhossiliensis* (isolate HR 61 from orchard M) were established by evenly spreading 1 ml of a suspension of hyphal fragments of a blended culture over potato dextrose agar and incubating 14 days at 25 C. To obtain spores, 40 12-mm-d discs were cut from the cultures with a sterile cork borer, placed in 100 ml of a suspension medium (2.2 g KCl/liter of 0.01% Tergitol) in 250-ml Erlenmeyer flasks, and shaken vigorously for 15 minutes on a wrist-action shaker. The suspension was filtered through a double layer of sterile cotton gauze to remove the agar discs and large hyphal fragments. Spore viability was determined by spreading 1 ml of the suspension on water agar and after 24 hours at 25 C, 100 randomly selected spores in each of three fields of view were observed at 100× magnification for germination. Soil collected outside the root zone of peach

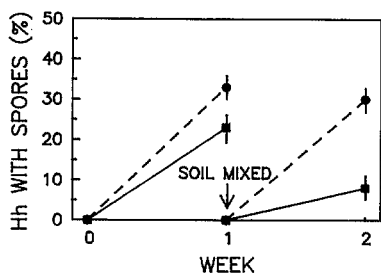


FIG. 1. Effect of soil incubation and disturbance on percentage of *Heterorhabditis heliothidis* (Hh) with spores of *Hirsutiella rhossiliensis* in spore assays. Soil was stored 2 days (●—●) or 30 days (■—■) at 10 C before the experiment. Soil was assayed immediately after packing in vials (week 0), following 1 week incubation (week 1, ± mixing), and following 1 week incubation of mixed soil (week 2). Each point is the mean of six replicates. Vertical bars indicate ± SE.

trees (containing no *H. rhossiliensis*-parasitized *C. xenoplax*) was amended with harvested *H. rhossiliensis* spores by adding 2 ml of the spore suspension to 200 cm³ soil in a plastic bag and mixing. Additional water was added by spraying to increase the moisture level to 14%. This yielded a density of 365,000 harvested spores/100 cm³ of soil, about 10 times the mean number of spore equivalents found in soil heavily infested with *H. rhossiliensis*. Six vials were packed with the spore-amended soil and six with unamended soil. The spore assay was immediately performed on all vials.

RESULTS

Effect of soil disturbance on spore density: No spores were observed on *H. heliothidis* added to freshly mixed soil (week 0) even though *H. heliothidis* penetrated to the bottom of the vials (Fig. 1). Spores were observed on *H. heliothidis* added to soil incubated for 1 week. If week 1 soil was remixed before addition of *H. heliothidis*, spores were not detected. Following incubation of this redisturbed soil, however, spores were once again observed on *H. heliothidis* (week 2). Spore density was lower in 30-day-old soil than in fresh soil, but the pattern of spore recovery was the same in both soils.

Comparison of the two assays: The spore and plate assays initially gave different es-

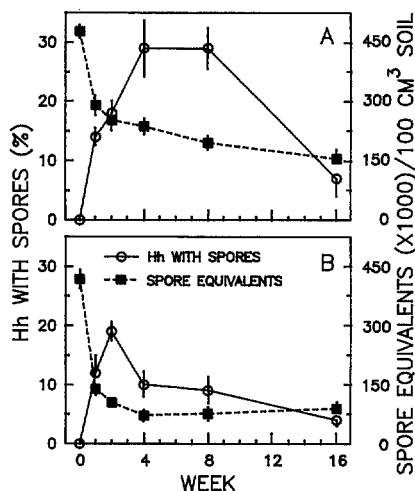


FIG. 2. Relative population density of spores of *Hirsutiella rhossiliensis* (Hh) as determined by plate (■—■) and spore (○—○) assays. Soil was stored 2 days (A) or 30 days (B) at 10 C before placement in vials. Each point is the mean of six replicates. Vertical bars indicate ± SE.

timates of fungal inoculum when homogeneous soil was incubated in vials at constant water content and periodically sampled (Fig. 2). Soil incubation increased the percentage of *H. heliothidis* with spores for 2–4 weeks (Fig. 2). Spore numbers were lower, and peaked and declined faster, in stored soil (Fig. 2B) than in fresh soil (Fig. 2A). Spores were never detected at week 0 (freshly mixed samples) using the spore assay. In the plate assay, however, the maximum numbers of parasitized *C. xenoplax* (and consequently the largest number of spore equivalents) were observed at week 0 and the numbers declined thereafter.

Analysis of soil samples from 37 trees showed a strong correlation ($P = 0.0001$) between numbers of *H. heliothidis* with spores from the spore assay and numbers of spore equivalents from the plate assay (Fig. 3). The regression analysis also included date of soil collection and date of soil collection × spore equivalents as independent variables; neither of these variables was significant ($P > 0.20$).

Inoculum potential of spores added to soil: Spores (viability = 87%) were not observed on *H. heliothidis* added to either spore-amended soil or to unamended soil.

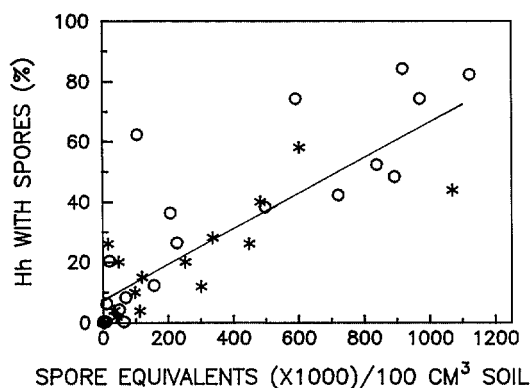


FIG. 3. Estimated spore density of *Hirsutella rhossiliensis* as determined by spore assay (% *Heterorhabditis heliothidis* [Hh] with spores) and plate assay (spore equivalents). Each of the 37 points was derived from a single soil sample (one sample/tree) collected on 7 December 1987 (asterisks) or 10 February 1988 (circles). $R^2 = 0.74$.

DISCUSSION

The spore assay described here was easy to perform and reproducible. Replicate samples had low variances and repeated experiments on soils of differing age had similar trends (Figs. 1, 2). Because soil water potential and bulk density affect fungal sporulation and nematode motility, they should be controlled and described. Sufficient water must be present to permit nematode movement, but excess water inhibits nematode movement and prevents fungal sporulation (4).

The spore assay might not be applicable to some soils. Adjustment of soil moisture and bulk density would be more difficult in soils that contained more silt or clay than the soil used in this study. The centrifuge method could leave such soils too wet or too compacted, whereas the spray method could leave the soil too sticky to pack evenly in the vials. The presence of other fungi, the spores of which adhere to *H. heliothidis*, or nematodes similar in appearance to *H. heliothidis* in the sample could confound interpretation. In this study, spores other than those of *H. rhossiliensis* were rarely observed and *Heterorhabditis*-like nematodes were not naturally present in the soil.

No spores were observed on *H. heliothidis* added to freshly mixed soil naturally in-

festated with *H. rhossiliensis*, nor to soil amended with harvested *H. rhossiliensis* spores. One interpretation of these results is that spores must be on phialides to adhere to nematodes and that mixing of soil disrupts hyphae and detaches spores from phialides. We base this on observations that spores readily detach from phialides when touched, and mature spores have not been observed on phialides when emergent hyphae are recovered from soil (unpubl.). Furthermore, emergent hyphae are usually broken off parasitized nematodes during nematode extraction (4,5). If our interpretation is correct, harvested spores cannot be used to establish or increase *H. rhossiliensis* in soil. In addition, soil cultivation is likely to reduce the inoculum potential of *H. rhossiliensis* in the field because some spores would become detached from phialides.

Detached spores may not adhere to nematodes in soil because they may adhere to soil particles (we have observed nonspecific adhesion to glass and plastic), they may not be properly positioned for contact with nematodes passing through soil pores, or they may germinate prematurely (6). Presumably, germinated spores would be non-infective and would perish because of low competitive saprophytic ability (6).

The spore and plate assays initially provided inverse estimates of spore density in incubated soil (Fig. 2). This inverse relationship can be explained if spores are considered to exist in three phases: potential spores, i.e., fungal reserves in parasitized nematodes (mainly *C. xenoplax* in this study) not yet converted into spores; spores on phialides (and thus capable of adhering to nematodes); and spores detached from phialides. The plate assay detects only potential spores (fungal reserves) in the sample, since existing spores are detached and lost during the assay. As the fungus sporulates, fungal reserves are depleted (3) and the estimation of spore equivalents declines through time (Fig. 2). In contrast, the spore assay detects spores on phialides rather than reserves, and the estimate of spores initially increases through time as

reserves are converted into spores. It follows that the number of spores detected by the spore assay after 1 week of incubation will be related to fungal reserves measured by the plate assay at week 0. Therefore, the two estimates of spore density are highly correlated when the spore assay follows the plate assay by 1 week of incubation (Fig. 3). This correlation indicates that both assays provide good estimates of spore density in soil. The lower numbers of spores and the more rapid decline of spores in older soil (Figs. 2, 3) can be explained by the depletion of fungal reserves during storage (3). Neither assay detects detached spores, but detached spores appear to be noninfective and therefore of no value in biocontrol of pest nematodes.

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