Rates of Spore Transmission, Mortality, and Production for the Nematophagous Fungus *Hirsutella rhossiliensis*

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**ABSTRACT**


Transmission of spores of *Hirsutella rhossiliensis* to juveniles of *Heteroderida schachtii* (J2) was quantified in soil microcosms (17 cm³) containing loamy sand (−60 mbar matric potential) or coarse sand (−20 mbar) at 20 C. Transmission, measured as the probability (P) of a J2 acquiring at least one spore (as a function of numbers of spores × 10⁷ per microcosm [S]), was greater in loamy sand (P = 1.055/[S + 1]) than in coarse sand (P = −0.906 + 0.00165S). The value of P was constrained between 1 and 0. Differences in transmission were attributed to the effects of soil pore diameter on nematode motility and the probability of a nematode passing but not contacting a spore. The relative rate of spore mortality in microcosms (temperature and matric potentials as above) ranged from 0.072 to 0.103 per week. Some spores died shortly after sporulation and others were viable and virulent for at least 200 days. In moisture chambers at 20 C, *H. rhossiliensis* produced a mean of 112 spores per infected J2 after 12 days. Spore production per infected J2 was highly variable (range of 52 to 227).

*Additional keywords:* biological control, plant-parasitic nematode, soil porosity, sugar beet cyst nematode.

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A variety of soil fungi and bacteria parasitize and cause disease in populations of plant-parasitic nematodes (11,17). Quantitative information on the survival, reproduction, and transmission of most of these parasites is lacking but is needed for assessment of their biological control potential. Epidemiological information is fundamental to disease management, whether the intention is to suppress the disease in a population of a valued host or to enhance disease in a pest population.

Quantification of disease within populations of soilborne nematode pests is difficult because the environment is complex and opaque and the parasites and their target hosts are microscopic. However, the small size and low motility of nematodes and their antagonists allow the use of microcosms. Soil temperature, water potential, and porosity can be controlled in microcosms. Furthermore, microcosms can be replicated many times and incubated without disturbance until the entire replicate unit is destructively sampled. Replicates may be sacrificed periodically, thus providing data on how the undisturbed system changes through time. Finally, small volumes are appropriate for studying the dynamics of microscopic, slow-moving organisms (19).

The nematophagous fungus *Hirsutella rhossiliensis* Minter & Brady kills large numbers of plant-parasitic nematodes in some grower fields and in laboratory experiments (5,7). This fungus is especially suitable for epidemiological studies because methods for quantifying its activities are known (6,13) and its life history is simple. Hyphae of *H. rhossiliensis* extend from parasitized nematodes and bear nonmotile, adhesive spores on the tips of bottle-shaped phialides. Spores adhere to passing nematodes, detach from the phialide, germinate, and penetrate the nematode cuticle. The fungus grows through the nematode's body cavity, kills, and colonizes the nematode, and once again sporulates. Although the fungus grows slowly on laboratory media in the absence of competition, it has no saprophytic ability in nonsterile soil (9).

We are developing epidemiological models to understand how epidemics of fungal and bacterial parasites occur, or fail to occur, in populations of nematode hosts (15). These models require information on contact rates between parasites and hosts and how such rates are converted into probability of infection (1,14). Critical parameters include the probability that a nematode will encounter a parasite as a function of parasite density, the rate of parasite mortality, and the rate of parasite reproduction per infected host. The objective of this study was to estimate these parameters for the nematophagous fungus *H. rhossiliensis* and the plant-parasitic nematode *Heteroderida schachtii* Schmidt.

**MATERIALS AND METHODS**

Preparation of colonized *H. schachtii* as fungal inoculum. Unless formed in situ, spores of *H. rhossiliensis* do not adhere to nematodes in soil (13). Therefore, inoculum of *H. rhossiliensis* was introduced to microcosms in the form of colonized *H. schachtii* (from which the fungus would sporulate). Colonized *H. schachtii* were obtained as follows: 300 g of sand (5% gravel, 3% silt and clay, and 7, 34, 39, 11, and 1% very coarse, coarse, medium, fine, and very fine sand, respectively) was moistened with 10 ml of 6 mM KCl and autoclaved in a deep petri dish (9 cm diameter and 7.5 cm height) for 65 min. KCl was added because ions are required for infection of nematodes by *H. rhossiliensis* (8). A colony of *H. rhossiliensis* (isolate 1M1 265748, 1.5 cm diameter, grown on potato-dextrose agar for 3 wk) was maturated in 4 ml of sterile distilled water and mixed with 50 ml of sterile, cleared V-8 juice broth (177 ml of V-8 juice centrifuged at 8,500 rpm for 20 min, with the supernatant diluted to 1 L with distilled water amended with 3 g of CaCO₃). The suspension of hyphae and spores in cleared broth was poured aseptically onto the surface of the sand in the deep petri dish. The lid was sealed with Parafilm, and the culture was incubated for 3-4 wk. The incubation temperature was 20 C for all preparations and experiments in this study. Reported values are means ± SE unless indicated otherwise.

Cysts of *H. schachtii* from pot cultures on sugar beet *Beta vulgaris* L. ‘SSVI1’ were incubated on Baermann funnels. The hatched second-stage juveniles (J2) were collected every 2 hr and were aerated and stored at 10 C for less than 48 hr. J2 (100,000
in 2 ml of 6 mM KCl were pipetted onto the surface of the H. rhossiliensis-infested sand in deep petri dishes. After 3 days, the nematodes were extracted by wet screening and centrifugal flotation (2,10). H. rhossiliensis had adhered to and infected more than 99% of the nematodes; more than 75% of the nematodes were colonized (i.e., the body cavity was filled with hyphae). Colonized nematodes usually were used in experiments within 6 hr after extraction from the deep petri dishes. In several instances, colonized nematodes were stored at 5°C for 24 hr before use.

**Spore production from colonized nematodes in moisture chambers.** Moisture chambers consisted of plastic petri dishes (9 cm diameter) containing water agar plus 200 mg of streptomycin sulfate per liter. Ten agar disks (1 cm diameter) were removed from each plate with a cork borer. One colonized H. schachtii was placed in a small drop of distilled water (each drop was just large enough to surround the nematode's body) on the surface of plastic in each of the 10 circular areas where agar was removed. The dishes were sealed with Parafilm, and each nematode was examined periodically at X140 magnification for spore production. The experiment was replicated six times with 10-20 nematodes per replicate. Replicates were performed at different times with different batches of colonized nematodes.

**Spore mortality in microcosms.** Rate of spore mortality was measured in coarse sand and in loamy sand. Coarse sand (19% very coarse, 57% coarse, 23% medium, and 1% fine) was obtained by repeatedly (15X) suspending 200 cm² of sand in 1 L of water and discarding the supernatant. The loamy sand (78.4% sand, 12.8% silt, 8.8% clay, <1% organic matter, pH 5.0 in 0.1 M CaCl₂) was collected from a peach orchard. Both sands were heated to 60°C for 2 hr and air-dried to kill any H. rhossiliensis and nematodes present. Colonized H. schachtii (2,274 ± 84 in 3.5 ml of 6 mM KCl) were mixed into 25.0 g of dried coarse sand, giving a soil moisture content of 14.0% (16.0% after addition of assay nematodes, see next paragraph). Colonized H. schachtii (1,010 ± 37 in 3.1 ml of 6 mM KCl) were also mixed into 26.1 g of dried loamy sand, giving a soil moisture content of 11.9% (13.8% after addition of assay nematodes). To establish microcosms, the sand was added to 25-cm³ plastic vials with lids (13) and packed to a volume of 17 cm³; thus, the bulk density was 1.47 for coarse sand and 1.54 for loamy sand. The rationale for selecting the indicated levels of soil water is discussed in the section concerned with spore transmission.

Vials were assayed for spores by adding 500 healthy J2 of H. schachtii (assay nematodes) in 0.5 ml of 6 mM KCl. Each vial was assayed only once (10, 20, 30, 60, 90, 120, or 150 days after seeding with colonized nematodes for coarse sand and after 20, 40, 60, 90, 150, or 210 days for loamy sand). Five vials (replicates) per date were assayed for coarse sand and four vials per date for loamy sand. The decline in the number of spores detected on assay nematodes was considered a measure of spore mortality. The assay nematodes were extracted from coarse sand after 3 days by washing the contents of the vial into a bottle containing 200 ml of water. The bottle was inverted six times, and the supernatant poured onto a screen (25 μm pore diameter). Material collected on the screen was washed into a glass vial. Material and solution passing through the screen were collected and returned to the bottle. This procedure was repeated twice per sample. Assay nematodes were recovered from loamy sand after 3 days by wet screening and centrifugal flotation. The suspension of recovered nematodes was placed in a Hawkesley slide, and nematodes with and without spores were counted at X100-140 magnification. At least 100 nematodes per vial were counted. The assay nematodes were distinguished easily from the colonized nematodes added to the vials 10 or more days before. After 10 days at 20°C, colonized nematodes have supported extensive sporulation and are mostly degraded. After 3 days at 20°C, assay nematodes that acquired spores showed little degradation (insufficient time had passed for sporulation) and those that had not acquired spores appeared healthy. In several experiments, cultures confirmed that vials receiving colonized nematodes at day 0, but no assay nematodes, contained no nematodes with spores at day 10 that could be confused with assay nematodes with spores (data not shown).

In addition to determining the percentage of assay nematodes with spores, 15 assay nematodes with spores per replicate were examined at X1000 magnification with differential-interference contrast optics to determine the number of spores adhering per nematode and the percentage of nematodes infected as evidenced by hyphae of H. rhossiliensis in the body cavity. The number of spores detected per vial was calculated.

The experiment was repeated three times in coarse sand and two times in loamy sand, but only the percentage of assay nematodes with spores was measured. Nonlinear regression (an iteratively reweighted least squares procedure) was used to determine the rate of spore mortality. SAS (16) programs were used for all analyses in this study.

**Transmission of spores in microcosms.** Transmission was measured as the probability of a nematode acquiring at least one spore (or the percentage of assay nematodes with spores). Transmission was compared in coarse sand at 16.0% water and loamy sand at 13.8% water (percentages include water added with assay nematodes). These moisture levels approximated the inflection points of the moisture release curves (Fig. 1), which were determined with tension funnels (3) and tensiometers (12). Wallace (18) showed that nematodes usually move fastest at matrix potentials near the inflection point. The estimates of matrix potential in the vials were only approximations because the moisture characteristics were from drying soils, whereas the soils in vials were re-wetted. The percentage of air-filled porosity was 35% in coarse sand and 58% in loamy sand. Data from the moisture release curves were used to calculate pore size distributions (4). Most (87%) pores in the coarse sand were greater than 80 μm in diameter, and 4% were between 38 and 80 μm. Pore diameters

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**Fig. 1.** Moisture release curves for loamy sand and coarse sand. Matric potential is measured in centimeters of water (1 cm of water = 1 mbar = 0.1 kPa).

**Fig. 2.** Rate of spore production from individual second-stage juveniles of Heterodera schachtii parasitized by Hirsutella rhossiliensis. Each point is the mean ± SD of six replicates, 10-20 parasitized nematodes per replicate.
in the loamy sand were more variable than in coarse sand; 44% were greater than 80 \( \mu m \) and 20% were between 38 and 80 \( \mu m \).

Vials were packed with coarse or loamy sand that had been heated to 60 C for 2 hr before addition of colonized \textit{H. schachtii} (0 \( \pm \) 0, 91 \( \pm \) 6, 539 \( \pm \) 40, 1,124 \( \pm \) 41, or 2,247 \( \pm \) 82 per vial). To estimate the number of spores present in each vial at day 10, the number of colonized nematodes added per vial was multiplied by 112 (see Results, Fig. 2). After 11 days, 250 assay nematodes in 0.5 ml of 6 mM KCl were added to the surface of the sand in each vial. These nematodes were extracted by wet screening and centrifugation 3 days later. Between 50 and 100 nematodes per vial were examined for spores. There were two replicate vials per level of fungal inoculum, and the experiment was performed twice; levels of colonized nematodes were slightly different in the second trial.

In a similar experiment, spore acquisition was compared in loamy sand and a well-structured silty clay (14, 45, and 41% sand, silt and clay; 1.8% organic matter; 15.0% water but matric potential was unknown; bulk density 0.91). There were two replicate vials per level of fungal inoculum, and the experiment was performed once because data from vials with loamy sand were consistent with data from previous experiments. Data from loamy sand or silty clay were subjected to nonlinear regression; a ‘satisfaction function’ (given in the Results) described the data and was used as the model. Values of \( r^2 \) were calculated from the residual and adjusted total sums of squares. Data from coarse sand were subjected to linear regression.

To examine the effect of numbers of assay nematodes on spore acquisition, vials containing coarse sand infested with 756 \( \pm \) 56 colonized nematodes were inoculated with 47 \( \pm \) 4, 263 \( \pm \) 14, 478 \( \pm \) 43, or 994 \( \pm \) 24 assay nematodes. Numbers of nematodes with spores after 3 days were determined. All of the assay nematodes extracted from vials that received 47 assay nematodes were examined, and 50–100 assay nematodes per vial were examined for the other levels. A similar experiment was conducted with loamy sand. Each vial contained 107 \( \pm \) 14 colonized nematodes and was assayed with 40 \( \pm \) 4, 239 \( \pm \) 17, or 1,023 \( \pm \) 49 assay nematodes.

To determine rate of spore acquisition, sand infested with 856 \( \pm \) 20 or 262 \( \pm \) 11 colonized \textit{H. schachtii} (coarse or loamy sand, respectively) was added to vials. After 11 days, 250 assay nematodes were added per vial. Assay nematodes were extracted and examined for spores after 1, 2, 3, 4, or 6 days. The experiment was performed once because data were consistent with previous observations on rate of spore acquisition in naturally infested soil (7). There were three replicate vials per extraction time in coarse sand and four replicate vials per extraction time in loamy sand.

**RESULTS**

**Spore production.** Rate of spore production peaked after 3 or 4 days and then declined until day 13, when no spores were produced (Fig. 2). Nematodes became increasingly transparent as their body contents were assimilated by the fungus and converted into external hyphae, phialides, and spores. By day 13 the nematodes looked like shrunken, empty sacs, and it was assumed that the nematode substrate had been depleted. Total spores produced per nematode were 88 \( \pm \) 5, 123 \( \pm \) 5, 111 \( \pm \) 7, 91 \( \pm \) 4, 132 \( \pm \) 8, and 124 \( \pm \) 9 for each of the six replicates. The range in spore production per nematode was 52–227, and the mean of all trials was 112 \( \pm \) 7. This mean was used to calculate initial numbers of spores in spore transmission and mortality experiments; number of spores per vial at day 10 equaled number of colonized nematodes added at day \( 0 \times 112 \).

**Fig. 3.** Detection of spores of \textit{Hirsutella rhossiatisis} over time in coarse sand. Each point is the mean ± SD of five replicates; SD smaller than the symbols do not appear. A. Percentage of \textit{Heterodera schachtii} that acquired spores. B. Numbers of spores acquired per nematode with spores (left axis) and the percentage of those nematodes with spores that were infected by \textit{H. rhossiatisis} (right axis). C. Number of spores detected per vial. The dotted line indicates survival with a relative mortality rate of 0.103/wk.

**Fig. 4.** Detection of spores of \textit{Hirsutella rhossiatisis} over time in loamy sand. Each point is the mean ± SD of four replicates; SD smaller than the symbols do not appear. A. Percentage of \textit{Heterodera schachtii} that acquired spores. B. Numbers of spores acquired per nematode with spores (left axis) and the percentage of those nematodes with spores that were infected by \textit{H. rhossiatisis} (right axis). C. Number of spores detected per vial. The broken line or dotted line indicate survival with a relative mortality rate of 0.072 or 0.103/wk.
Spore mortality. Because most sporulation occurred by day 10 (Fig. 2), spore age was arbitrarily set to 0 on day 10. In coarse sand, the percentage of assay nematodes with adhering spores generally declined through time (Fig. 3A). The number of spores per nematode with spores also declined (Fig. 3B). The percentage of nematodes that had spores and that were also infected was high and did not decline (Fig. 3B). Decline in spore detection (survival) (Fig. 3C) was described by the following function: Fraction of spores detected = \((1 - M)^a\), where \(M = 0.103 \pm 0.005\) (parameter estimate \pm asymptotic standard error) and \(a\) is the age of spores in weeks. The results of three other experiments in coarse sand, in which only the percentage of assay nematodes with spores was determined, were similar to those in Fig. 3A (data not shown).

A higher percentage of nematodes acquired spores and there were initially more spores per nematode with spores in loamy sand than in coarse sand (Fig. 4A and B), even though the coarse sand was seeded with twice as many colonized nematodes. The percentage of nematodes that had spores and that were also infected was high and declined about 10% (Fig. 4B). Decline in spore detection (Fig. 4C) was described by the same function used for coarse sand but \(M = 0.072 \pm 0.003\) (broken line). Decline with \(M = 0.103\), as observed in coarse sand, is shown for comparison (Fig. 4C, dotted line). Saprophytic nematodes were observed in the last sampling of loamy sand but not in other samplings of loamy or coarse sand. The results of two other experiments in loamy sand, in which only the percentage of assay nematodes with spores was determined, were similar to those in Fig. 4A (data not shown).

**Discussion**

Differences in soil porosity probably explained the higher spore transmission in loamy vs. coarse sand. The coarse sand contained...
mostly large pores about 150 μm diameter, whereas the loamy sand contained a range of pores, many with diameters of 38-80 μm. Pores wider than 60 μm may limit motility of H. schachtii (18) and thus limit contact between nematodes and spores. Furthermore, pores wider than 53 μm (the length of the phialide plus spore plus the diameter of the nematode) allow nematodes to pass spores without contacting them (Fig. 8). The probability of contacting a spore will also be affected by the diameter of the nematode (hence, nematode species and stage), nematode behavior, and the orientation of the phialide and spore. In 150-μm-diameter pores (Fig. 8A), H. schachtii would not contact a spore unless the nematode lifted its head or tail off the pore wall or unless the distal end of the phialide was directed toward the pore wall rather than toward the center of the pore. Although transmission in a fine soil was examined in only one experiment, the results (Fig. 5B) and consideration of the relationship between pore diameter and spore acquisition suggest that transmission in fine soils may be at least as great as transmission in coarse soils.

Differences in water status also may have contributed to lower spore transmission in coarse sand vs. loamy sand. The matric potential in each soil was near the inflection point of the moisture release curve, but the coarse sand was less drained and had less air-filled porosity than the loamy sand. Transmission in coarse sand may have been greater than observed if matric potentials were reduced.

Coarse sand was used in initial experiments because it permitted rapid extraction and high extraction efficiency (efficiencies were 90% in coarse sand and 60% in loamy sand) and resulted in clean, easily read samples. Furthermore, coarse sand is physically and chemically simple and thus may be easier to describe and understand than a more complex soil. Spore transmission, however, was much lower in coarse sand than in loamy sand or silt clay, suggesting that coarse sand should not be used for assessing biological control potential of H. rhossiliensis, at least not without more information on the effects of soil water.

The relationship between spore transmission and spore density (Fig. 5) was based on data collected after 3 days. The experiments were not run longer than 3 days because spore density would have increased on day 4 as the fungus sporulated from infected assay nematodes and because most acquisition occurred within 3 days (Fig. 7). Nematode exposure to spores in the field might be much less than 3 days if host roots are near; the nematode is an endoparasite and would not encounter spores once inside a root.

The percentage of assay nematodes with spores should decline with increased numbers of assay nematodes if the nematodes ‘competed’ for a limited number of spores. Although there was some evidence for such an inverse relationship, the number of assay nematodes added per vial affected the variance more than the mean of spore acquisition (Fig. 6). The decreased variance in transmission with increased numbers of assay nematodes can be explained by probabilistic arguments. For example, in experiments involving five trials and 10 or 100 coin tosses per trial, variation in the percentage of heads among trials would be greater with 10 tosses than with 100 tosses per trial. Variable results can be expected and increased replication may be required in experiments involving low numbers of assay nematodes.

Spore production from colonized nematodes was rather variable (the maximum was four times greater than the minimum) concerning that the nematodes were all second-stage juveniles of one population. Possible causes include variance in the size or in other characteristics of the nematodes, variance in the number of spores infecting (competition among thalli in a host may reduce sporulation from that host), and variance among the spores that infected.

Spore mortality was inferred from changes in spore acquisition by assay nematodes. We assumed that spores were dead if they were unable to adhere to hosts, an assumption supported by previous studies (9,13). To simplify our findings, we also assumed that spores were 0 days old on day 10 when some spores were 8 days old and others were yet to be produced (Fig. 2). Because the parameters quantified in this study will be used in simulations of disease development over 25 to 50 wk, this error in estimation of spore age is probably insignificant.

Spore transmission differed greatly in loamy and coarse sand (Fig. 5A), but spore mortality did not (Fig. 5C). This is reasonable because porosity directly affects the probability of spore contact but only indirectly affects spore mortality. Spores die naturally as they age; as the environment becomes too extreme; as predators, parasites, or other antagonists attack them; or as the spores are removed from the phialides by any agent other than a host (13). Nonhost agents that can remove spores from phialides include moving soil particles and nonhost animals. Both the coarse sand and loamy sand were heated and dried and contained no animals other than assay nematodes (except in the last sampling of loamy sand). The sand in the vials was not disturbed until sampling, and the moisture and temperature were moderate and relatively constant. Thus, natural senescence would appear to be the primary cause of mortality. However, the soil was not sterile, and bacteria, fungi, or protozoa could have affected spore survival.

Observations of high levels of parasitism in naturally infected (7) and in artificially infested soils (Fig. 5B) are exciting but should be supported with ecological and epidemiological data on how epidemics occur. Rates of spore production, mortality, and transmission greatly influence the occurrence and severity of epidemics (1). Although more research is needed on the effect of the environment, the parameters quantified in this study should permit detailed and realistic exploration of disease dynamics of H.

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**Fig. 7.** Percentage of assay nematodes (second-stage juveniles of Heteroderda schachtii) with spores of Hirsutella rhossiliensis as affected by time. Assay nematodes were added on day 0; 11 days before, vials had been seeded with 856 ± 20 or 262 ± 11 colonized H. schachtii for coarse or loamy sand, respectively. Each value is the mean ± SD of four replicates in loamy sand and three replicates in coarse sand.

**Fig. 8.** Cross-sectional diagrams of second-stage juvenile of Heteroderda schachtii (filled circle), female Cricogramma xenopelax (circle with stripes), and phialide and spore of Hirsutella rhossiliensis in A, a 150-μm-diameter pore or, B, a 40-μm-diameter pore.
rhossiliensis in populations of H. schachtii. In particular, the parameter values are being used in computer models to predict the host threshold density (1) and the effect of nematode population density on the proportion of nematodes parasitized (15).

LITERATURE CITED