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Spatial Patterns of Entomopathogenic Nematodes in Microcosms: Implications for Laboratory Experiments[1]

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Abstract: Laboratory microcosms were used to: i) measure the effects of soil moisture on survival of *Steinernema riobravis* and ii) investigate the suitability of using microcosms to study motility and survival of these nematodes. Nematodes recovered from soil contained in petri dishes declined by more than 95% during 7 days, whereas nematodes recovered from the inner surfaces of dishes increased 35-fold. After 7 days in dishes, >20 times as many nematodes were recovered from dish surfaces than from soil. Nematodes exhibited a negative geotropism; greater numbers of nematodes were recovered from the lid surfaces than from the surfaces of dishes. Survivorship of nematodes in soil in plastic centrifuge tubes was somewhat greater than in petri dishes, and fewer nematodes ascended above the soil line in tubes than dishes. Downward migration of nematodes was inversely related to soil column diameter, possibly due to relatively unimpeded movement along container surfaces. An assay was developed by which nematodes were rinsed from the inner surfaces of centrifuge tubes into the soil. The resulting slurry was then processed on Baermann trays to recover motile nematodes. Nematode survival in soil in centrifuge tubes was higher at soil moistures between 2–4% than at lower (0.5–1.0%) and higher (4.0–12.0%) moisture levels. Survival of *S. riobravis* may be enhanced by quiescence induced by moisture deficits.

Key words: entomopathogenic nematode, spatial distribution, soil moisture, *Steinernema riobravis*, survival

Reports of the persistence of entomopathogenic nematodes added to soil vary widely depending on experimental conditions and methods. For example, *Steinernema carpocapsae* (Weiser) was shown to persist in vitro for 30 days at levels greater than 50% in sandy soil (13), whereas survival was reported to be <10% after 7 days in soils of similar texture in vitro (12) and in the field (5).

Water availability in soil is a major factor affecting the survival of entomopathogenic nematodes (3,11). Patterns of nematode vertical distribution in soil will affect nematode survival by exposing different portions of the population to different levels of soil moisture. Therefore, studies of the spatial patterns of these nematodes following application to soil as well as the impact of soil moisture on nematode survival are needed to understand the effects of soil moisture on nematode survival in the field.

Laboratory studies of the effects of soil variables on these nematodes require the containment of relatively small quantities of soil. Negative geotropism by some entomopathogenic nematode species (7,13) suggests that nematodes may distribute themselves in a manner that brings significant numbers of worms into contact with container surfaces. Depending on the extent of such contact, knowledge of the distribution of nematodes in microcosms may be important to design valid experiments. The following studies were conducted to validate a method to measure the effect of soil moisture on in vitro survivorship of *S. riobravis* (Cabanillas, Poinar & Raulston). We also evaluated the effect of container size on migration patterns of this nematode.

Materials and Methods

Candler fine sand soil (96% sand, 2% clay, 2% silt) was collected from the site of a field trial (6) that was conducted to determine the efficacy of *S. riobravis* against larvae of *Diaprepes abbreviatus*. Soil was air-dried to 1.5% moisture content (weight of water/dry weight of soil).

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(i) *Distribution of nematodes in petri dishes over time:* Plastic petri dishes (5.0-cm-diam. × 1 cm deep) were filled with 28.5 cm^3 soil that was previously adjusted to 5.45% moisture (w/w). Approximately 1,000 *Steinernema riobravis* third-stage infective juveniles (IJ) in 100 µl deionized water were pipetted into a conical depression in the soil of each dish and covered with additional soil. Final moisture level was 6.0%. Soil in the dishes was in contact with the lids. Dishes were sealed with parafilm and incubated in a humidified chamber in the dark at 28 °C for 0, 2, and 7 days. Following incubation, soil from 10 dishes was processed on Baermann trays lined with a single facial tissue. Nematodes were also rinsed from the inner surfaces of the dish lid and bottom and recorded separately from those recovered from soil.

A second experiment was conducted as described above, except that nematodes were recovered at 2 days only and nematodes recovered from soil, lids, and dish bottoms were enumerated separately. Nematodes were expressed per dish and per cm^3; those on dish surfaces were assumed to occupy a layer the thickness of the nematode body (30 µm).

(ii) *Distribution and survival of nematodes in centrifuge tubes and petri dishes:* Assays were performed in plastic petri dishes as in the previous experiment. Plastic culture tubes (9.5 × 1.5-cm-diam.) with snap-on plastic caps were filled with 10.6 cm^3 soil, and treatments were established as for the petri dishes. However, due to the smaller soil volume in tubes than in dishes, tubes were inoculated with 330 nematodes. Ten dishes and 10 tubes were incubated for 48 hours under conditions described in the previous experiment. Following incubation, soil from the dishes and tubes was placed in individual beakers and mixed with 25 ml deionized water. Soil slurries were also made with nematodes rinsed from the inner surfaces of petri dishes and tubes using 28.5 or 10.6 cm^3 soil (6% moisture) stored in plastic until needed for nematode recovery. All slurries were rinsed into Baermann trays lined with two coffee filters and incubated for 48 hours at 25 °C. Nematodes remaining in soil were extracted by centrifugal flotation (8). Data were analyzed using a factorial design to consider container type (dish vs. tube), substrate (soil vs. surface), and extraction method (centrifugation vs. Baermann tray). To compare results from tubes and dishes, numbers of nematodes recovered using different extraction methods were expressed as proportions of the inoculum density. Proportions were transformed (arcsin-square root) prior to hypothesis testing (Student's *t*-test, $P = 0.05$).

(iii) *Effect of tube diameter on nematode migration:* Four 5-cm-long pieces of polyvinyl chloride (PVC) tubing were secured end to end with tape to form 20-cm-long tubes. Wire mesh was fitted and taped to the bottom end of each tube. PVC tubing of two diameters (1.5 cm and 10.0 cm) were used. Eight 20-cm-long tubes of each diameter were filled with steam-sterilized Candler fine sand (3% moisture) and gently tapped to form soil columns that were supported vertically. Approximately 1,000 *S. riobravis* IJ in 100 µl water were pipetted onto the center of the sand surface of the 1.5-cm diameter tubes. Ten-centimeter-diameter tubes were treated in the same way, except that approximately 40,000 nematodes were used to adjust for increased soil volume in the larger tubes. Tubes were immediately irrigated in a greenhouse for 10 minutes using microjet emitters (1.5 cm water/hour) before being placed on a laboratory bench (25 °C). At 1 hour and 24 hours post-irrigation, four tubes of each diameter were disassembled. Soil in each tube section was mixed, and nematodes in 10-cm^3 subsamples were extracted using centrifugal flotation. Nematodes were rinsed from tube walls and enumerated. Average nematode counts in soil and on tube walls at depths of 0–5, 5–10, 10–15, and 15–20 cm were determined for tubes of each diameter.

(iv) *Effect of soil moisture on nematode persistence:* Treatments in plastic centrifuge tubes were established as in the second experiment except that, prior to inoculation,

six tubes each were adjusted to one of 10 different moisture levels. Final moisture levels following inoculation were: 2, 3, 4, 6, 7, 10, and 12% moisture. After incubating (28 °C) for 7 days, soil was placed in beakers with nematodes rinsed from the tube surface. Soil slurries were processed in Baermann trays as in the second experiment. A single treatment at 6% soil moisture was processed on day 0 to serve as a baseline to estimate persistence.

The experiment was repeated with modifications. The range of soil moisture levels (0.6, 1.4, 2.1, 3.4, 5.6, 7.3, 9.2, and 11.2%) was expanded to include moisture levels below 2.0%. Nematodes were inoculated in sand with a 10-cm cannula that permitted nematodes to be pipetted along the length of the sand column in the tube. Soil in tubes was then stirred with a wire to better distribute water in the inoculum throughout the sand column.

Nematode data in both experiments were normalized by dividing all data by the highest average recovery of nematodes for a treatment in the appropriate experiment.

Student's *t*-tests ($P = 0.05$) were used to evaluate differences between nematode counts. Differences reported in the results are significant unless stated otherwise.

Results

(i) *Distribution of nematodes in petri dishes over time:* Average numbers of *S. riobravis* recovered from soil with Baermann trays declined continually, from 685 at 0 days to 26.5 at 7 days (Fig. 1). Nematodes adhering to lids and bottoms of dishes increased from 15.5 to 531 during the same period. Total numbers of recovered nematodes declined 20% from 700 on day 0 to 557 on day 7.

By 2 days post-inoculation, the majority of nematodes adhering to the dish surfaces were on the lid (Table 1). On a volumetric basis, the distribution of nematodes was approximately 60-fold and 500-fold greater on the dish bottoms and lids, respectively, than in the soil.

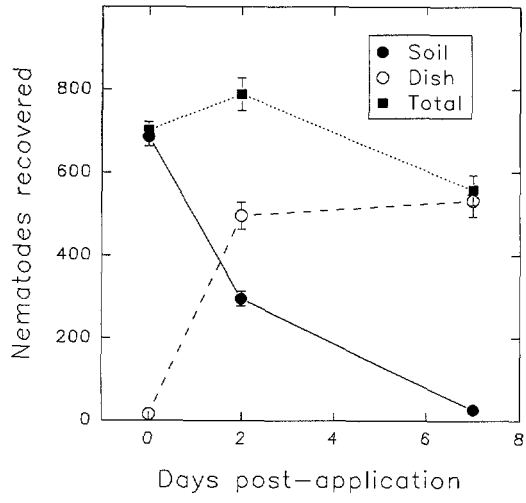

FIG. 1. Recovery of *Steinernema riobravis* from soil and from dish surfaces during 7 days in assay units.

(ii) *Distribution and survival of nematodes in centrifuge tubes and petri dishes:* The proportion of recovered nematodes that were extracted by centrifugal flotation was not different for soil (0.58) or container surfaces (0.42) using centrifuge tubes (Table 2). Proportionately more nematodes were recovered with centrifugal flotation from soil (0.75) than from surfaces (0.51) of petri dishes. Using both extraction methods, recovery of the inoculum was greater from tubes (49.7%) than from petri dishes (34.5%). The percentage of nematodes recovered was greater from container surfaces than from soil for both tubes (71.2%) and dishes (79.4%).

Direct observation of nematodes on the container surfaces showed that nematodes did not migrate more than a centimeter or two above the soil surface in the tubes. Moreover, nematodes on the tube walls be-

TABLE 1. Recovery of *Steinema riobravis* from soil, dish surfaces, and lid surfaces after 48 hours in petri dish assay units.

	Nematodes	Nematodes/cm^{3b}
Soil	259 (25)[a]	13 (1)
Dish	93 (15)	812 (154)
Lid	481 (35)	6,765 (559)

[a] Mean ($n = 10$) and standard error in parentheses.
[b] Nematodes on container surfaces assumed to occupy a volume 30 μm deep.

TABLE 2. Recovery of *Steinernema riobravis* from petri dish and centrifuge assay units after 48 hours using Baermann trays followed by centrifugal flotation.

	Petri dish		Centrifuge tube	
	BF[a]	CF	BT	CF
Container surface	0.135 (0.01)[b]	0.141 (0.01)	0.206 (0.03)	0.148 (0.02)
Soil	0.017 (0.01)	0.052 (0.01)	0.060 (0.01)	0.083 (0.02)

[a] BT = Baermann tray, CF = centrifugal flotation.
[b] Mean recovery as a proportion of inoculum (n = 10). Standard error in parentheses.

low the soil surface were much nearer to soil particles than were nematodes on petri dish lid surfaces. Despite efforts to secure the lids in tight contact with soil, large areas of the lid surface did not make contact.

(iii) *Effect of tube diameter on nematode migration:* One hour following inoculation and irrigation, fewer than 0.25% of nematodes recovered from soil in either diameter tube were found below 5 cm depth (Fig. 2). However, by 24 hours post-irrigation in the small-diameter tubes, nematode density in soil was highest 10–15 cm deep, followed by 5–10, 0–5, and 15–20 cm deep. By contrast, nematode density in soil in the large-diameter tubes was higher at 0–5 cm depth than at any deeper section in the tube. One hour following irrigation in the small diameter tubes, nematodes recovered from tube walls 0–5 cm deep were 4.4% as numerous as those recovered from soil (data not shown). The

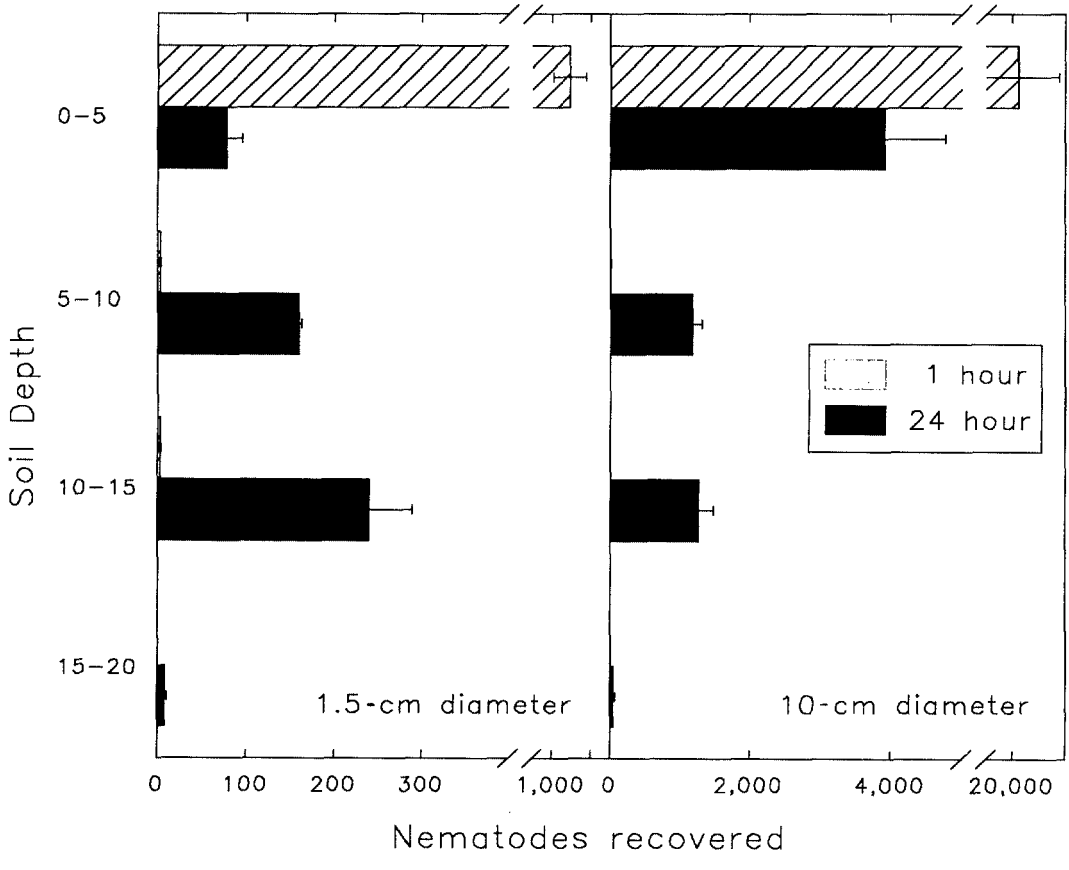

FIG. 2. Numbers of *Steinernema riobravis* in soil at different depths in PVC tubes of different diameters, 1 and 24 hours after inoculation.

corresponding percentage (1.3%) was lower for the large-diameter tubes. Nematodes were not detected on tube walls below 5 cm deep, one hour after irrigation. By 24 hours after irrigation, proportionately more of the total nematodes recovered were on walls in the small-diameter tubes (22.4%) than in the large-diameter tubes (6.9%) (data not shown).

(iv) *Effect of soil moisture on nematode persistence:* Recovery of nematodes was lower in the second than the first experiment, probably due to stirring of the soil following inoculation (data not shown). However, normalized data were consistent between the two experiments. In the range of 2–12% soil moisture, the recovery of nematodes was inversely proportional to moisture in both experiments (Fig. 3). At low moisture (0.5%, 1.0%) in the second experiment, nematode recovery was lower than at slightly higher (2%, 3%) moisture levels. Survival at 6.0% soil moisture was 49% and 54% in the first and second experiments, respectively. Direct observation of tubes in the second experiment revealed large numbers of nematodes on tube surfaces in all treatments but the lowest (0.5–1.0%) moisture levels.

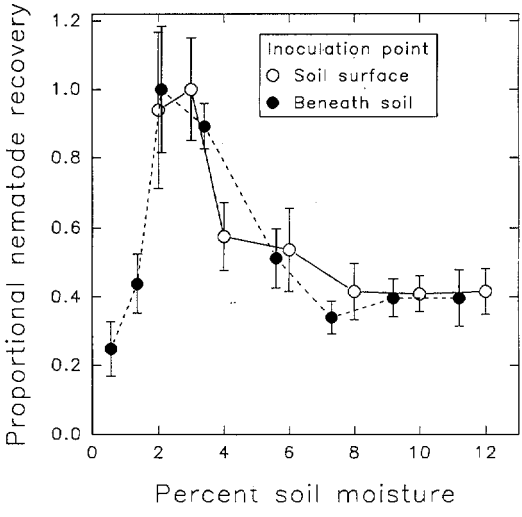

FIG. 3. Recovery of *Steinernema riobravis* following 7 days incubation at various soil moisture levels. Data from each trial were normalized by dividing by the highest average recovery for a treatment in the appropriate experiment.

DISCUSSION

We found a high proportion of these entomopathogenic nematodes on the surface of experimental containers. The assays in these studies utilized sandy soil at moisture levels generally less than 10%. Consequently, almost no soil adhered to the surfaces of emptied dishes or tubes. Nevertheless, we found nematodes to distribute themselves in microcosms in a manner requiring careful recovery from container surfaces as well as from soil to assess survivorship in the experimental units. Thus, the potential for distribution-related artifact from laboratory survivorship studies is high and may have contributed to the variability between reported survivorship estimates (2,10–13).

The effect that nematode migration onto container surfaces may have on survival is unclear. Since dead or nonmotile nematodes must remain in soil, it could be expected that centrifugal flotation would recover proportionately more nematodes than Baermann funnels from soil rather than container surfaces if survival in the two substrates were equal. While this was observed in petri dishes, the trend was not significant in tubes. Normal pore-space relationships, which vary between soils, do not exist at the soil-container surface interface. Gas exchange in water drops on the surfaces of containers will be different than in more confined spaces between soil particles. Poor contact of surfaces with soil particles also results in nematodes being trapped within drops of water, disrupting normal migration behavior. The probability of contact with biotic antagonists may change. Presumably, diffusion of some biologically derived materials is impeded in water drops on container surfaces as is a nematode's ability to respond to detrimental concentrations of such chemicals.

Attempts to reduce the affinity of nematodes for container surfaces by using glass vs. plastic containers, and by incubating plates in the light, had little effect. The possibility exists of coating container surfaces with repellent compounds; however,

potentially confounding effects of such compounds would pose hazards as well. Of the two bioassays reported herein, use of centrifuge tubes appeared preferable because nematodes were maintained in somewhat more intimate contact with the soil substrate and survival was greater.

A simple method to maintain nematodes within soil is to increase the size of the experimental units. Larger units could increase the need for sampling rather than processing the entire contents of units, negating one of the advantages of microcosm compared to field research. For some studies, however, it may be necessary to increase the size of bioassay units. Relatively unimpaired movement of nematodes along container surfaces could result in erroneous conclusions regarding their ability to migrate in soil. The effects of tube diameter on migration patterns in the present study indicate clearly that, to study migration rate, it is necessary to ensure that container surfaces are farther from the point of inoculation than distances of migration to be measured.

The relative effects of moisture on the survival of nematodes was reasonably consistent in the two experiments. Highest survivorship occurred in relatively dry soils (2–3% moisture), possibly due to quiescence induced by low moisture availability (11). When nematodes were mixed in soils with <2% moisture, survivorship was reduced. Many of the nematodes added to soil in such a dry state may have desiccated too rapidly to complete biochemical changes necessary for anhydrobiotic survival (1,14). Declining nematode recovery at higher moisture levels (4–12%) likely resulted from depletion of energy reserves due to high activity levels (9,11). A similar inverse relationship between soil moisture content and survivorship was reported for *S. carpocapsae* in loamy sand (11).

These data demonstrate that recovery of *S. riobravis* from soil is affected by a range of moisture levels normally encountered in sandy soils in Florida citrus orchards, and this effect may explain the high mortality rates observed for these nematodes (6). Duncan et al. (6) estimated nematode survivorship to be less than 5% within 1 week after application in mature citrus orchards. In a related study (5), survivorship was shown to be inversely related to the vertical distribution of nematodes in the soil. While nematode density at depths below 1 cm was relatively stable over time, large numbers of nematodes at a depth of 0–1 cm declined to nearly nondetectable levels within 1 week following application. Rapid, profound changes in soil moisture near the soil surface could reduce nematode densities by direct moisture stress or by repeated induction of the anhydrobiotic state in the nematodes (4). A smaller proportion of nematodes residing deeper in soil would be less subject to rapid mortality due directly to moisture stress. Rather, densities of these nematodes should decline more slowly due to loss of energy reserves, combined with other factors such as biological antagonism.

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