

## QUANTIFICATION OF PARASITISM OF THE SOIL-BORNE NEMATODE *CRICONEMELLA XENOPLAX* BY THE NEMATOPHAGOUS FUNGUS *HIRSUTELLA RHOSSILIENSIS*

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(Accepted 9 March 1988)

**Summary**—A “dilution plate assay” was developed to quantify parasitism of *C. xenoplax* by *H. rhossiliensis*. Aliquots of *C. xenoplax* in suspension (treated with 0.5% NaOCl after extraction from soil by wet sieving and centrifugation) were spread onto 1.5% water agar plates supplemented with 200 parts  $10^{-6}$  streptomycin sulfate. After 5 days at  $22 \pm 2^\circ\text{C}$ , the numbers of *C. xenoplax* with or without sporulating *H. rhossiliensis* were determined using a dissecting microscope. The assay was used to measure the rate of decay (disappearance) of parasitized *C. xenoplax* in field samples incubated in the laboratory. Time required for 50% reduction in number of parasitized nematodes (averaged across all life stages) incubated at  $-33$  kPa soil water potential and at 10, 15 or  $20^\circ\text{C}$  was 61, 26 or 16 days, respectively. Rate of decay was much faster for parasitized second- and third-stage juveniles than for parasitized fourth-stage juveniles and adults. Rate of infection can be estimated if the number of parasitized nematodes and rate of decay of such nematodes are known.

### INTRODUCTION

*Criconebella xenoplax* Raski (Luc and Raski) is a soil-borne pathogen of stone fruit trees and is attacked by the endoparasitic fungus *Hirsutella rhossiliensis* Minter and Brady (Jaffee and Zehr, 1982). There are four juvenile stages (designated J1, J2, J3 and J4), the adult female and eggs. Except for the egg and J1 (which is in the egg), all stages are motile and live in the soil. *H. rhossiliensis* grows from nematodes that it has parasitized and produces non-motile spores on bottle-shaped phialides. Spores adhere to passing nematodes. After the nematode is infected and killed, the fungus converts the material within the body cavity into hyphae. Four to five days after infection of adult *C. xenoplax* at  $25^\circ\text{C}$ , the hyphae grow out of the nematode and produce distinctive phialides and spores. Sporulation continues, and the hyphae within the cuticle contract until the reserves within the cuticle are depleted (Jaffee and Zehr, 1982, 1983). *Hirsutella rhossiliensis* is a poor competitive saprophyte and appears to be specialized for parasitism (Jaffee and Zehr, 1985).

In our studies of California peach orchard soils, we have frequently observed high numbers and proportions of *H. rhossiliensis*-parasitized *C. xenoplax*; more than 8000 parasitized nematodes  $100\text{ cm}^{-3}$  of soil (representing about 80% of the total *C. xenoplax* present) have been observed (unpublished). Such observations and *in vitro* tests of pathogenicity (Jaffee and Zehr, 1982) suggest that *H. rhossiliensis* contributes to the regulation of *C. xenoplax* populations.

To understand the regulation of *C. xenoplax* populations by *H. rhossiliensis*, certain variables including the number of parasitized nematodes and the rate of infection must be quantified. Current methods, such as those described by Gray (1984) or Dackman

*et al.* (1987) permit qualitative or quantitative assessment of fungal inoculum but not quantification of parasitized nematodes.

We describe a quantitative assay for parasitized nematodes and a method to estimate the rate of infection. This method is based on the following relationship:  $P_{t+1} = P_t + NP - DP$  where  $P$  refers to the number of parasitized nematodes,  $NP$  refers to the addition of newly-parasitized nematodes during the period (from time  $t$  to  $t + 1$ ), and  $DP$  refers to the loss of parasitized nematodes due to degradation during the same period. In other words, the “pool size” of parasitized nematodes is a function of input into the pool (rate of infection) and output from the pool (rate of decay). Thus, rate of infection during the designated period can be estimated if the number of parasitized nematodes (at time  $t$  and  $t + 1$ ) and the rate of decay are known ( $NP = P_{t+1} - P_t + DP$ ). We describe rates of decay as influenced by nematode stage and soil temperature.

### MATERIALS AND METHODS

#### *Orchard soils and nematode extraction*

Three peach orchard soils (M, G and C) in Merced County, Calif., were selected. The orchards were mature (8–10 yr old), were planted on “Nemaguard” rootstock and contained both *C. xenoplax* and *H. rhossiliensis*. Soil texture was determined by the hydrometer method (Day, 1965); pH was measured on soil wetted to saturation with 10 mM  $\text{CaCl}_2$  (Smiley and Cook, 1972); electrical conductivity was measured on saturation extracts (Richards, 1954); percent organic matter and cation exchange capacity were determined by the Merkle Laboratory of the Pennsylvania State University (Table 1). Water release curves

Table 1. Characteristics of soils G, C and M

Characteristics	G	Soil C	M
Texture (%)			
Sand	88.0	86.2	78.4
Silt	6.2	6.0	12.8
Clay	5.8	7.8	8.8
Organic matter (%)	<1.0	<1.0	<1.0
pH	6.3	5.6	4.4
EC <sup>a</sup>	249	148	211
CEC <sup>b</sup>	4.7	4.6	5.1
Inflection point <sup>c</sup>	2.5	3.0	4.0

<sup>a</sup>Electrical conductivity ( $\mu\text{mhos cm}^{-1}$ ) of saturation extract.

<sup>b</sup>Cation exchange capacity (m equiv 100 g<sup>-1</sup>).

<sup>c</sup>Inflection point (-kPa) of moisture release curve.

were developed with tension funnels and a pressure plate (Vomocil, 1965).

Nematodes were extracted by mixing 100 cm<sup>3</sup> of soil in 900 ml of water (the suspension was poured back and forth between two 1-l. beakers 15 times), allowing the suspension to settle for 20 s, pouring the supernatant through a 38  $\mu\text{m}$  screen and centrifuging the material on the screen in water. The pellet was resuspended in sucrose and centrifuged (Jenkins, 1964). Nematodes in the supernatant were collected on a 38  $\mu\text{m}$  screen, rinsed and placed into a 30 ml vial. The suspended nematodes and debris in each vial are referred to as "nematode extract". Nematode counts were not corrected for extraction efficiency.

#### Sporulation on different media

Nematode extracts from orchards M, G and C were untreated or treated with bleach (0.5% NaOCl) for 25–30 s and then rinsed with distilled water. Adults showing symptoms or signs of *H. rhossiliensis* infection (Jaffee and Zehr, 1982) were transferred individually (with a nematode transfer needle) to 10-cm dia plastic Petri plates containing one of the following: 1.5% water agar (WA), acidified (pH = 4.4) water agar (AWA), water agar amended with 200 parts 10<sup>-6</sup> streptomycin sulfate (WA-S), 1% Gelrite<sup>®</sup> (GR) (Kelco, San Diego, CA 92123) or water (PL). In the PL treatment, nematodes were placed in 2  $\mu\text{l}$  drops of distilled water (one nematode per drop) on the bottom of a plastic Petri plate that had a moist filter paper in its lid. Sporulation of *H. rhossiliensis* from *C. xenoplax* and contamination by other fungi and bacteria were assessed after 2, 3, 5, 6 or 12 days at 22  $\pm$  2 °C. Ten nematodes were tested per plate for each combination of medium, bleach treatment and soil.

#### Soil sampling for determination of decay rates

Soil 33–66 cm from the soil surface was collected on 20 October 1986 from the root zone of 15 trees in each of the 3 orchards. Subsamples (100 cm<sup>3</sup> from each tree) were pooled to form one 1500 cm<sup>3</sup> composite sample per orchard. Four 100 cm<sup>3</sup> samples per composite were extracted within 24 h of collection (time 0) and after 2, 4 and 8 weeks of storage at 22  $\pm$  2 °C. Extracted nematodes were assayed by dilution plating as described below. Percent water in the soil was determined and was converted to water potential using the water release curves. The water potential of each soil was constant through the

experiment and ranged from -33 to -37 kPa (1 kPa = 0.01 bars) for the three soils.

Soil was also collected from orchard M on 12 December 1986. Subsamples (three 100 cm<sup>3</sup> samples per temperature) were processed as above after 0, 2, 4, 8 and 16 weeks of incubation at 10, 15 or 20 °C. The soil water potential was -33 kPa and was constant through the experiment.

#### Dilution plate assay

We use the term "parasitized nematodes" to include both infected nematodes (i.e. living nematodes containing hyphae) and nematodes that died from infection. To estimate the numbers of parasitized and non-parasitized nematodes within each 100 cm<sup>3</sup> sample, a dilution plate assay was used. Each nematode extract was adjusted to 10 ml, treated with bleach for 25–30 s, rinsed on a 38  $\mu\text{m}$  screen and resuspended in 10 ml water. A 333  $\mu\text{l}$  aliquot was spread on the surface of each of three WA-S plates (three plates per extract) and held at 22  $\pm$  2 °C for 5 days. Plates were examined at 20–60 $\times$  to determine the number of *H. rhossiliensis*-parasitized or non-parasitized *C. xenoplax* per plate. Nematodes with sporulating *H. rhossiliensis* were designated "parasitized". *H. rhossiliensis* was identified by its distinctive phialide, spore and mode of sporulation. The life stage of each nematode was determined in the measurement of decay rates in soils G, C and M at 22  $\pm$  2 °C but not in the measurement of decay rates in soil M at 10, 15 or 20 °C. Instances when *H. rhossiliensis* occurred independently of *C. xenoplax* were also recorded. Totals from three plates were multiplied by 10 to estimate the numbers of nematodes 100 cm<sup>-3</sup> of soil.

#### Data analysis

Data were subjected to analysis of variance (SAS Institute, 1985) where appropriate. In the experiments concerned with the rate of decay, an appropriate model was selected for each curve and best fit parameter estimates were developed by linear transformation. Data were converted to percentages (value at time 0 = 100%) as indicated. In the analyses of decay rates of different nematode life stages, the three orchard soils were treated as blocks.

## RESULTS

#### Conditions for detection of *H. rhossiliensis*

After 5 days of incubation, *H. rhossiliensis* from bleach-treated, hand-selected, hand-transferred

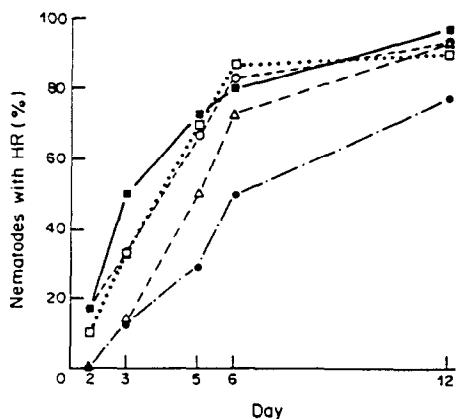


Fig. 1. Recovery of *Hirsutella rhossiliensis* (HR) from surface disinfested *Criconebella xenoplax* after incubation ( $22 \pm 2$  C) on 5 media. Values are percentages of all nematodes plated, are cumulative and are the means of 3 replications. 10 nematodes per replication. ■, WA-S; ○, WA; □, GR; △, PL; ●, AWA.

nematodes sporulated with greater frequency ( $P = 0.01$ ) on WA-S, WA and GR than on AWA or PL media (Fig. 1). Bleach initially delayed the emergence of *H. rhossiliensis* from *C. xenoplax*, but by day 5 the effect of bleach was not significant ( $P > 0.05$ , data not shown). Bleach suppressed contaminating organisms (Table 2) and therefore made it easier to detect *H. rhossiliensis*. Contaminating organisms did not appear to interfere with the growth and sporulation of *H. rhossiliensis* from *C. xenoplax* but made detection more difficult.

#### Decay of parasitized and non-parasitized nematodes in soils

The numbers of *H. rhossiliensis*-parasitized *C. xenoplax* had halved by day 17, 15 or 9 for soil C, M or G, respectively, when held at  $22 \pm 2^\circ\text{C}$  (Table 3, Fig. 2). Analysis by stage revealed that a significant proportion of the initial decline was due to reduction in J2 and J3 (Table 3, Fig. 3). The mean percentage

Table 2. Contamination of *Criconebella xenoplax* by bacteria and fungi as influenced by bleach treatment and medium

Treatment	Contaminated nematodes (%)	
	Bacteria	Fungi
Bleach <sup>a</sup>		
+	7a	13a
-	34b	36b
Medium <sup>b</sup>		
WA	40x	35x
AWA	3y	15x
WA-S	12y	25x
GR	27xy	22x

<sup>a</sup>Averaged across media.

<sup>b</sup>Averaged across bleach treatments. See text for description of media.

Values indicate the mean percentage of nematodes contaminated with at least one bacterial or fungal colony (other than *Hirsutella rhossiliensis*) after 5 days incubation. Means in column for bleach or medium followed by the same letter are not significantly different ( $P = 0.05$ ) according to Duncan's multiple range test.

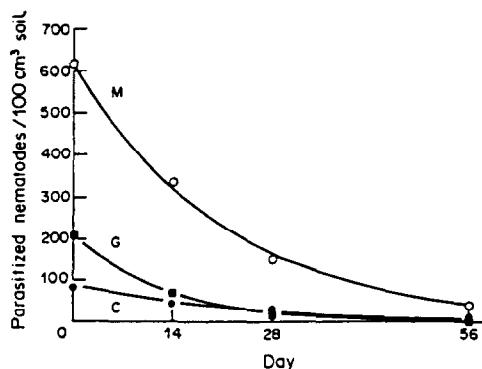


Fig. 2. Recovery of *Hirsutella rhossiliensis*-parasitized *Criconebella xenoplax* in three peach orchard soils incubated at  $22 \pm 2^\circ\text{C}$ . Values are the means of 4 replications. Equations are shown in Table 3. At time 0, SEM = 49, 25 and 26 parasitized *C. xenoplax*  $100\text{ cm}^{-3}$  soil for soil M, G and C, respectively.

of J2, J3, J4 and adult females at time 0 were 7, 23, 36 and 33%, respectively, and these percentages are fairly typical for the entire year and for data not corrected for stage-specific extraction efficiency.

Decline of parasitized nematodes was slower in colder soils (Table 3, Fig. 4). The mean percentage of J2, J3, J4 and adult females at time 0 were 12, 25, 32 and 31%, respectively.

The decline of non-parasitized nematodes was also rapid (Table 3). In fact, comparison of decline by stage indicated that non-parasitized J4 and adults were less persistent than parasitized J4 and adults (Table 3).

*H. rhossiliensis* was observed parasitizing nematodes other than *C. xenoplax* in many samples; however, the numbers of these parasitized nematodes were always very low. The fungus was associated with substrates other than nematodes only once when it was observed growing from unidentified organic debris. In other experiments, examination of such debris under high magnification usually revealed the presence of a nematode (unpublished).

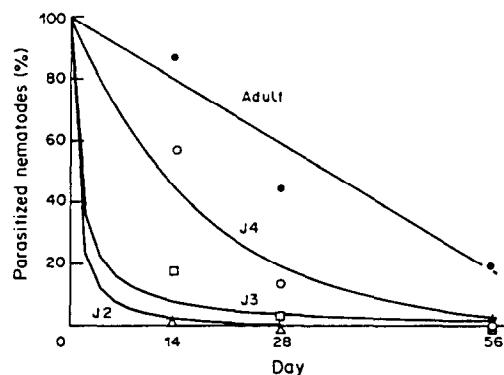


Fig. 3. Recovery of *Hirsutella rhossiliensis* from adult female (●), J4 (○), J3 (□) and J2 (△) stages of *Criconebella xenoplax* incubated at  $22 \pm 2^\circ\text{C}$ . Densities at time 0 were considered 100%. Values are the means of 3 replications. Equations are shown in Table 3.

Table 3. Influence of soil, nematode stage and soil temperature on rate of decay of *Hirsutiella*-parasitized and non-parasitized *Criconeimella xenoplax* (Cx)

Soil	$D_{50}$ for parasitized Cx	Regression of parasitized Cx on days	$r^2$	$D_{50}$ for non-parasitized Cx	Regression of non-parasitized Cx on days	$r^2$
G	9	$y = 208e^{-0.07x}$	0.97	1	$y = 582(x+1)^{0.777}$	0.88
C	17	$y = 90e^{-0.04x}$	0.96	2	$y = 788(x+1)^{0.679}$	0.99
M	15	$y = 615e^{-0.04x}$	0.99	4	$y = 785(x+1)^{0.460}$	0.98
Stage						
J2	1	$y = 100(x+1)^{1.271}$	0.98	1	$y = 100(x+1)^{1.028}$	0.92
J3	1	$y = 100(x+1)^{0.912}$	0.79	1	$y = 100(x+1)^{0.799}$	0.99
J4	12	$y = 100e^{-0.038x}$	0.76	2	$y = 100(x+1)^{0.626}$	0.98
Adult	34	$y = 100 - 1.480x$	0.91	4	$y = 100(x+1)^{0.415}$	0.94
Temperature						
10	61	$y = 303(x+1)^{0.418}$ for $x > 11$	0.84	45	$y = 220(x+1)^{0.386}$ for $x > 6$	0.93
15	26	$y = 600(x+1)^{0.757}$ for $x > 9$	0.86	16	$y = 300(x+1)^{0.612}$ for $x > 4$	0.94
20	16	$y = 1500(x+1)^{1.306}$ for $x > 8$	0.88	7	$y = 180(x+1)^{0.625}$ for $x > 1$	0.90

In regression equations,  $y = Cx$  100 cm<sup>3</sup> soil in analyses by soil and  $y = \%Cx$  (time 0 = 100%) in analyses by stage or temperature.  $D_{50}$  refers to the days required for 50% reduction in nematode numbers.  $y = 100\%$  if  $x$  is < the indicated number of days.

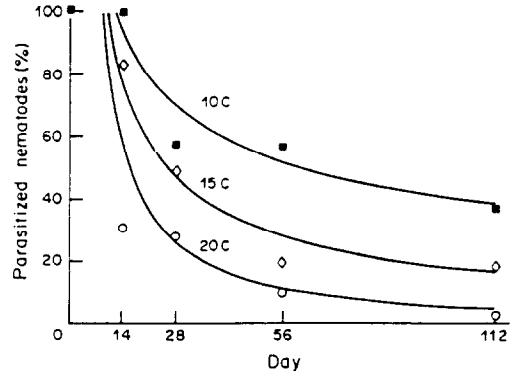


Fig. 4. Recovery of *Hirsutiella rhossiliensis* from *Criconeimella xenoplax* incubated at 10 (■), 15 (◇) or 20°C (○). Densities at time 0 were considered 100%. Values are means of 3 replications. Equations are shown in Table 3.

DISCUSSION

WA-S was the most suitable medium for the dilution plate assay because its use resulted in a high level of detection of *H. rhossiliensis* (Fig. 1) but a relatively low level of bacterial contamination (Table 2). Bleach treatment improved the assessment of samples as bleach suppressed growth of contaminating organisms. Also, bleach treatment killed *H. rhossiliensis* spores on the surface of nematodes (Jaffee and Zehr, 1983) and thus eliminated the possibility that the *H. rhossiliensis* detected grew from nematodes that were superficially infested but not parasitized. We selected 5 days as the incubation period because assessment was difficult after longer incubation due to contamination. Figure 1 indicates that about 75% of the *H. rhossiliensis*-parasitized *C. xenoplax* were detected by day 5.

The dilution plate assay described in this paper combines two standard techniques: extraction of nematodes (live and dead) from soil by screening-centrifuging and enumeration of soil microorganisms by spreading aliquots of soil suspensions onto agar media. This assay differs from others (e.g. the soil sprinkle and the Baermann funnel assays, see Gray, 1984) for endoparasites of vermiform nematodes because it directly quantifies parasitized nematodes, whether living or dead. In the soil sprinkle and related assays (Dackman *et al.*, 1987), soil slurries or suspensions are spread onto agar plates to which high numbers of a bait nematode are added. As the bait nematodes move across the plate, they contact spores, become infected, and support sporulation that permits identification of the nematophagous fungi. Level of parasitism among the bait nematodes is only indirectly related to level of parasitism among the nematodes naturally present in the soil. The Baermann funnel assay does not depend on bait nematodes but only detects living nematodes. Both the soil sprinkle and Baermann funnel assays have been used successfully to determine distribution and other ecological characteristics of nematophagous fungi. However, collection of quantitative data on parasitism of specific nematodes by specific fungi requires an assay such as the dilution assay described here. An additional advantage of the dilution plate assay is that parasitism of soil arthropods

can be detected. Mites and collembola that were parasitized by three species of *Hirsutiella* were observed in a related study (unpublished).

The dilution plate assay also has disadvantages. First, it is time consuming. Examination of a plate requires 4–7 min, or longer when there are increased amounts of silt or organic matter in the soil. Second, the assay does not directly assess fungal inoculum; however, fungal inoculum levels are correlated with numbers of parasitized nematodes (McInnis and Jaffee, unpublished). Third, identification of nematodes becomes more difficult as the fungus consumes and alters the nematodes. This is not a problem with *C. xenoplax* because its unique shape and annulations persist during degradation. Fourth, the dilution plate assay would be less useful when parasitized nematodes are present in very low numbers; the 10 × dilution could result in failure to detect parasitized nematodes. Fifth, the pool of parasitized nematodes might be too small to detect with this assay if the rate of decay is much faster than the rate of infection. Finally, the suitability of this technique for quantification of other nematophagous fungi is unknown.

When high numbers of *H. rhossiliensis*-parasitized *C. xenoplax* were initially detected in soil samples from California peach orchards, we suspected that this reflected accumulation (slow rates of decay) rather than high rates of infection. However, peach orchards in the Central Valley of California are irrigated and soil temperatures are 15°C or greater for about 8 months of the year. Our results suggest that, under these warm and moist conditions, *H. rhossiliensis*-parasitized *C. xenoplax* persist for relatively short periods. Persistence of parasitized nematodes is probably a function of sporulation of *H. rhossiliensis* from those nematodes. Parasitized nematodes are no longer recognized as parasitized after the body contents have been depleted and substrate is no longer available to support sporulation. Decay of individual *H. rhossiliensis*-parasitized nematodes in soil should therefore be fastest when conditions are optimum for sporulation (25°C and water potentials above –500 kPa) (Jaffee and Zehr, 1983). Time required to complete sporulation (i.e. to deplete the nematode substrate) may be related to nematode mass; younger stages of *C. xenoplax* decayed faster than older stages (Fig. 3) and J2 are about 20 × smaller than adult females. As expected, decay of parasitized and non-parasitized nematodes decreased as soil temperature decreased.

The rapid decay of non-parasitized *C. xenoplax* can be explained by the relatively rapid growth and activity of saprophytic fungi on dead but non-parasitized nematodes. *C. xenoplax* is an obligate parasite. In warm, moist soil lacking a plant host, *C. xenoplax* may die rapidly and may be rapidly degraded by saprophytes. In contrast, *H. rhossiliensis* grows slowly and presumably protects the parasitized nematodes from saprophytes by "possession" of the substrate (Bruehl, 1975) or by production of antibiotics. Consequently, the bodies of parasitized nematodes may persist longer than those of non-parasitized nematodes. Sporulation over an extended period would be advantageous for a fungus that does not produce resting spores and is dependent on

encounters between spores and nematodes for transmission and long-term persistence. Therefore, the results indicate that parasitized J4 and adult females are important for transmission and survival of *H. rhossiliensis*.

Rate of decay of parasitized nematodes can be estimated from the decrease in number of parasitized nematodes through time if rate of infection is zero ( $DP = P_t - P_{t-1} + NP$ ). In the present study, we assumed that no parasitism occurred during incubation because the soil was too dry to support significant nematode movement. As stated before, the soil water potentials (–33 to –37 kPa) were considerably lower (more negative) than the inflection points (–2.5 to –4.0 kPa) of the moisture release curves. Wallace (1958) and Blake (1962) showed that, for sandy soils with inflection points greater than –5 kPa, nematode movement was minimal if matric potential was less than –20 kPa. *H. rhossiliensis* spores are not motile, and nematode movement is required for inoculation.

The rate of decay of parasitized nematodes in a soil sample depends on how recently the nematodes were infected. Nematodes that were recently infected will persist longer than those previously infected. In the present study, samples processed at time 0 probably contained a mixture of recently and less recently infected nematodes. Thus, the measured decay rates were faster than would be the case if all the nematodes had been freshly infected. Definitive data on rates of decay could be obtained by introducing known numbers of dead, synchronously-infected nematodes into soil, maintaining environmental regimes, and assaying the soil as described here. Synchronous infection of large numbers of *C. xenoplax* is not possible at this time.

Rate of infection significantly affects the influence of a parasite on a host population. As stated earlier, rate of infection (input into the pool of parasitized nematodes) and rate of decay (output from pool) determine the pool size of parasitized nematodes at any time. The dilution plate assay and equations described here permit estimation of the pool size and rate of decay, and thus permit estimation of rate of infection. Rate of infection as influenced by nematode population density, soil temperature, soil water potential and other factors can now be determined.

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