Estimation of entomopathogenic nematode population density in soil by correlation between bait insect mortality and nematode penetration

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Summary – We present a method for estimating entomopathogenic nematode densities in soil samples based on repeated sampling with wax moth, *Galleria mellonella* larvae as bait hosts. This method has a very high extraction efficiency and is less time consuming than other methods. Ten bait insects are added per sample and are replaced every 3 days with fresh insects until nematode infections cease. The estimation of nematode density is based on a strong linear relationship between the $\log_{10}$ of the number of nematode-infected bait insects and the $\log_{10}$ of the number of nematodes found in these bait insects. This relationship was tested for two nematode species in field samples of natural populations and for three nematode species and three soil types in the laboratory. The slope of the linear regression line varied only slightly among trial, species, and soil type.

Résumé – *Estimation de populations de nématodes entomopathogènes par corrélation entre densité des nématodes et mortalité des insectes appâts* – Il est décrit une méthode d'estimation des larves infestantes de nématodes entomopathogènes présentes dans des échantillons de sol, méthode basée sur des échantillonnages répétés des larves de *Galleria mellonella* jouant le rôle d'appât. L'efficacité d'extraction de cette méthode est très élevée et elle apparaît beaucoup plus rapide que les autres méthodes en usage. Dix larves sont placées dans chaque échantillon et sont régulièrement remplacées par de nouveaux insectes jusqu'à ce que l'infestation par les nématodes cesse. L'estimation de la densité des nématodes est basée sur une relation linéaire forte entre le $\log_{10}$ du nombre de larves infestées par les nématodes et le $\log_{10}$ du nombre de nématodes retrouvés dans ces larves. Cette relation a été calculée pour deux espèces de nématodes dans des échantillons provenant du champ (populations naturelles) et en laboratoire pour trois espèces de nématodes et trois types de sol. La pente de la courbe de régression linéaire ne présente qu'une faible variation entre essais, espèces et types de sol.

Keywords : baiting, *Galleria mellonella*, *Heterorhabditis*, nematode extraction, *Steinernema*.

Entompathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) occur naturally in soils throughout the world (Kaya, 1990) where they can play an important role in soil communities (Strong *et al.*, 1996). They are also used as biological control agents against many soil-dwelling insect pests (Kaya & Gaugler, 1993). Their only free-living stage is the infective juvenile (IJ) whose function is to find and infect a new insect host. Our understanding of entomopathogenic nematode ecology and the fate of nematodes released as biological control agents is limited, in large part, by the difficulties in assessing nematode population density in the soil. A technique is needed that is easy to perform, cost effective and yields quantitative estimates of nematode density.

Methods commonly used for the extraction of terrestrial nematodes from soil samples, such as Baermann funnel and centrifugal flotation, can provide good quantitative estimates of IJ density (Curran &

Heng, 1992). However, these techniques can be time consuming, expensive, and require a high level of expertise in nematode taxonomy because they are not specific for entomopathogenic nematodes. Bedding and Akhurst (1975) developed a simple technique for isolating entomopathogenic nematodes using larvae of the greater wax moth, *Galleria mellonella* L., as bait insects. This bait technique only enables qualitative estimates of entomopathogenic nematodes in soil and is therefore best suited for determining nematode presence or absence and for isolating new species and strains. Mrácek (1982) suggested that nematode numbers in soil samples could be estimated based on the percentage of nematode-infected bait insects, but one round of sampling, as pointed out by Fan and Hominick (1991), is frequently insufficient to estimate nematode numbers.

Quantitative data can be obtained if the bait insects are dissected and the number of nematodes pene-

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trated into the insects counted (Bednarek & Nowicki, 1991; Fan & Hominick, 1991). In addition, repeated baiting and dissecting of hosts increases the precision of recovering entomopathogenic nematodes from the soil (Fan & Hominick, 1991; Campbell *et al.*, 1995). The introduction of the pepsin digestion technique of nematode-infected insects (Mauléon *et al.*, 1993) has simplified the dissection and counting process. Although the dissection of cadavers is more specific for entomopathogenic nematodes and less time consuming than direct extraction methods (Curran & Heng, 1992), it is still very labor intensive for large scale studies.

We propose herein a baiting technique that uses the number of nematode-infected bait insects after repeated baiting of soil samples as an estimate of the number of penetrating IJs. This technique is based on the sampling protocol used by Campbell *et al.* (1995, 1996) who used repeated baiting and dissection of all cadavers to determine the total number of infecting IJs; this was used as an estimate of nematode numbers in the soil. In summarizing these data, we found a correlation between total number of nematode-infected bait insects and total number of nematodes recovered from bait insects. In this study, we present that correlation, refine the sampling technique, assess its robustness to variation in nematode species and soil type, and test the correlation with the number of nematodes actually in the soil.

Material and methods

FIELD EXPERIMENT

Soil samples were collected as part of a study of endemic populations of *Heterorhabditis bacteriophora* Poinar and *Steinernema carpocapsae* (Weiser) in turfgrass (Campbell *et al.*, 1995, 1996). The field plots (15.3 × 15.3 m) were located at the Rutgers University Turfgrass Research Center in Adelphia, NJ, USA. *Heterorhabditis bacteriophora* samples were taken from a plot planted with tall fescue grass (*Festuca arundinacea* Schreb.) and *S. carpocapsae* samples were taken from a plot planted with Kentucky bluegrass (*Poa pratensis* L.). The soil was a sandy loam (56% sand, 28% silt, 16% clay). Samples were collected biweekly between 11 May 1992 and 7 July 1993.

A sample consisted of four soil cores (1.8 cm diameter and 15 cm deep, approximately 150 cm³) taken randomly within a 30 × 76 cm section that were mixed together in a 150 × 25 mm Petri dish. The soil moisture was adjusted, as needed, by adding water. Eight wax moth larvae were added as bait insects. The larvae were obtained from a commercial source (Northern Bait, Chetek, WI, USA), stored at 10°C, and used within 10 days of receipt. The dishes were held at room temperature (20-25°C) and every 3 days bait

insect mortality was recorded and dead insects were replaced with live wax moth larvae. Dead insects were individually digested in a pepsin solution (Mauléon *et al.*, 1993) and the number of nematodes recovered from the cadavers was determined. If no nematodes were found in a cadaver that showed signs of a nematode infection it was assumed that the insect had been infected by one nematode. Baiting was discontinued when no nematode-killed bait insects were recovered for at least two consecutive baiting rounds. The total number of positive samples was 28 for *H. bacteriophora* and 200 for *S. carpocapsae*. The total number of bait insects killed and nematodes recovered from these insects were recorded.

We considered the following as typical external signs for a wax moth larva having been killed by an entomopathogenic nematode. *i*) *Steinernema*-killed larvae are flaccid and creamy to tan in the case of *S. carpocapsae* and dark grey to brown in the case of *S. glaseri*, whereas *Heterorhabditis*-killed larvae are initially flaccid and orange and later become more rigid and dark red; and *ii*) the cadavers do not have a putrid odor. Upon dissection, the internal organs are partially dissolved but not liquified. In the case of *Heterorhabditis*-killed larvae, the contents of the cadavers have a ropey consistency.

LABORATORY EXPERIMENTS

To further refine the above baiting method, we increased the number of bait insects to ten and varied the following parameters: location of bait insects in the dish and baiting interval. To test the robustness of the sampling procedure, we varied nematode species and soil type. As in the field study, cadavers were dissected individually using pepsin digestion and the number of extracted nematodes recorded. The baiting procedure was continued until no nematodes were extracted from a baiting dish for two consecutive baiting rounds. If no nematodes were found in a cadaver that showed the typical symptoms of nematode infection, it was assumed that it had been infected by one nematode.

The nematodes used in the experiments, *S. carpocapsae* All strain, *S. glaseri* (Steiner) NC strain, and *H. bacteriophora* NC1 strain, were cultured in wax moth larvae (Woodring & Kaya, 1988). The IJs emerging from cohorts of simultaneously infected wax moth larvae were harvested from White traps over 5-7 days and stored in sterilized distilled water at 20°C. A mixture of all IJs that emerged from one cohort of wax moth larvae was used for experiments within 3 days of the final harvest. Wax moth larvae used for nematode rearing and as bait insects were obtained from a commercial source (Rainbow Mealworms, Compton, CA, USA), stored at 15°C, and used within 10 days of

receipt. All procedures and experiments were conducted at room temperature (22-25°C).

In the first three experiments, a loamy sand was used (87% sand, 7% silt, 6% clay; 0.4% organic matter; pH 6.9; 13% soil moisture [-6 kPa soil water potential]). In the fourth experiment, three soil types were used, the loamy sand, a sandy loam (74% sand, 19% silt, 7% clay; 0.7% organic matter; pH 7.2; 17 % soil moisture [-8 kPa water potential]), and a loam (54% sand, 37% silt, 9% clay; 4.6% organic matter; pH 6.9; 35 % soil moisture [-10 kPa water potential]). The soils were pasteurized (2 h at 62°C) and stored for at least 7 days before use. Their moisture release curves were determined using the filter paper method for determination of soil matric potential (Hamblin, 1981). The baiting dish was a Petri dish (100 × 25mm) filled with 100 cm^3 of soil (*ca.* 20 mm high).

In the first experiment, we determined whether inverting the baiting dishes (*i.e.*, covering the bait insects with soil) would affect nematode recovery. We hypothesized that *S. carpocapsae* and *S. glaseri* might be affected differently by this procedure because of their different foraging behavior. *S. carpocapsae* is a sit-and-wait or ambusher strategist adapted for finding mobile insects at the soil surface whereas *S. glaseri* is an actively searching or cruise forager adapted for finding less mobile insects below the soil surface (Campbell & Gaugler, 1993). To each baiting dish, 300 IJs of *S. carpocapsae* or *S. glaseri* were added in 0.5 ml of sterilized distilled water and ten wax moth larvae were placed on the soil surface. Half of the dishes of each nematode species was inverted, the other was left upright. Ten replicates of each treatment were conducted in two blocks with five replicates per block. The baiting dishes were examined every 2 days and nematode-killed bait insects were replaced with new bait insects.

In the second experiment, the length of time each bait insect was exposed to nematodes in the soil was standardized. Bait insects not infected by nematodes may remain in the soil for several baiting periods thus increasing the chance of death by other causes. This, potentially, increases the difficulty of assessing nematode-infected *versus* non-nematode infected cadavers and reduces the number of hosts available for infection. Ultimately, this may interfere with estimating nematode populations in the soil. Ten replicates, in two blocks of five replicates each, were performed for each combination of nematode species and baiting procedure. Each dish was inoculated with 300 IJs of *S. glaseri* or *S. carpocapsae*. Half of the dishes of each nematode treatment was baited as described in the first experiment for the upright treatment. In the other half, the bait insects were left in the dishes for 3 days and then all insects, dead or alive, were

removed. Nematode-infected insects were then evaluated as described above. Insects that were alive when recovered were incubated for 2 days and those that succumbed to nematode infection were evaluated as described previously.

In the third experiment, we varied the number of nematodes added to the dish and compared three entomopathogenic nematode species: *S. carpocapsae*, *S. glaseri*, and *H. bacteriophora*. Each species was examined at a different time. There were 32 dishes in block 1 and 40 dishes in block 2 for *S. carpocapsae*, 24 dishes in block 1 and 24 dishes in block 2 for *H. bacteriophora*, and 32 dishes in block 1, 32 dishes in block 2, and 18 dishes in block 3 for *S. glaseri*. Nematode density ranged from 5 to 4000 IJs/dish. All bait insects were replaced every 3 days.

In the fourth experiment, we tested the effect of soil type on the relationship between number of *S. glaseri* extracted and number of *S. glaseri*-killed bait insects. The soils were the loamy sand, the sandy loam, and the loam described previously. We added between 5 and 3000 IJ/dish for each soil type. The experiment was conducted in two blocks with nine dishes per block and soil type. All bait insects were replaced every 3 days.

STATISTICS

The number of nematode-infected cadavers and nematodes extracted from bait insects was summed over all the sampling rounds to determine total number of cadavers and nematodes. In the experiments testing the effect of inverting the baiting dishes and the effect of baiting procedure and interval, the number of nematodes extracted and the number of nematode-infected cadavers were analyzed by t tests (Anon., 1988). In the field study and in the laboratory experiments testing the effect of nematode species and soil type, the number of nematode-infected bait insects and the number of nematodes found within them were correlated on a log to log scale (Sigmaplot, Jandel Scientific, San Rafael, CA, USA). The slopes of the linear regression equations for each species or soil type were compared pairwise between blocks and, after combining data from blocks, between treatments, *i.e.*, nematode species or soil type, using a t-statistic (Zar, 1984). In the laboratory experiment testing the effect of nematode species, we also correlated the number of nematode-infected cadavers and the number of nematodes inoculated per dish on a log to log scale and the number of nematodes inoculated per dish and the number of nematodes penetrated in bait insects on a linear scale.

Results

FIELD EXPERIMENT

The pattern of nematode recovery from the field soil samples was highly variable. On average, nematodes were recovered over longer periods than in the laboratory experiments. For *S. carpocapsae* and *H. bacteriophora*, rarely more than 25% of the total number of nematodes recovered over all baiting rounds was recovered in a given baiting round. In approximately 80% of the samples, all nematodes were recovered within six baiting rounds. In some samples, nematode-infected hosts were recovered for more than twenty baiting rounds. In other samples, few or none of the bait insects were infected in the first sampling round, but infected hosts increased in subsequent sampling rounds. Sixty-eight percent of *H. bacteriophora* samples and 89% of *S. carpocapsae* samples had less than twenty nematode-infected cadavers. The number of nematodes per cadaver was variable but between 50 and 60% of the cadavers of both species contained fewer than five IJs.

Although the pattern of recovery was variable for both *H. bacteriophora* and *S. carpocapsae*, there was a significant linear correlation between the total number of nematode-killed bait insects and the total number of nematodes recovered when plotted on a log to log scale (Fig. 1). The slopes for *S. carpocapsae* and *H. bacteriophora* did not differ significantly ($P = 0.7$).

LABORATORY EXPERIMENTS

Most of the extracted nematodes (usually > 80%) were recovered in the first baiting round. In subsequent rounds, the number of nematode-infected insects and the number of nematodes per cadaver declined. The relationship between the number of nematodes recovered and the number of bait insects infected appears robust. Thus, inverting the baiting dishes did not affect the number of insects infected (*S. carpocapsae*: $P = 0.53$; *S. glaseri*: $P = 0.06$) or the number of nematodes extracted (*S. carpocapsae*: $P = 0.71$; *S. glaseri*: $P = 0.25$) for either nematode species (Table 1). Baiting procedure and interval also did not affect the number of nematode-infected bait insects (*S. carpocapsae*: $P = 0.35$; *S. glaseri*: $P = 0.06$) or the number of nematodes extracted (*S. carpocapsae*: $P = 0.23$; *S. glaseri*: $P = 0.82$) for either nematode species (Table 1).

The relationship between bait insect mortality and nematode recovery was relatively consistent among blocks, soil types, and nematode species. In the experiment testing the effect of nematode species, we found a linear relationship between the log of the number of nematode-infected bait insects and the log of the number of nematodes present inside these bait insects for *S. carpocapsae*, *S. glaseri*, and *H. bacterio-*
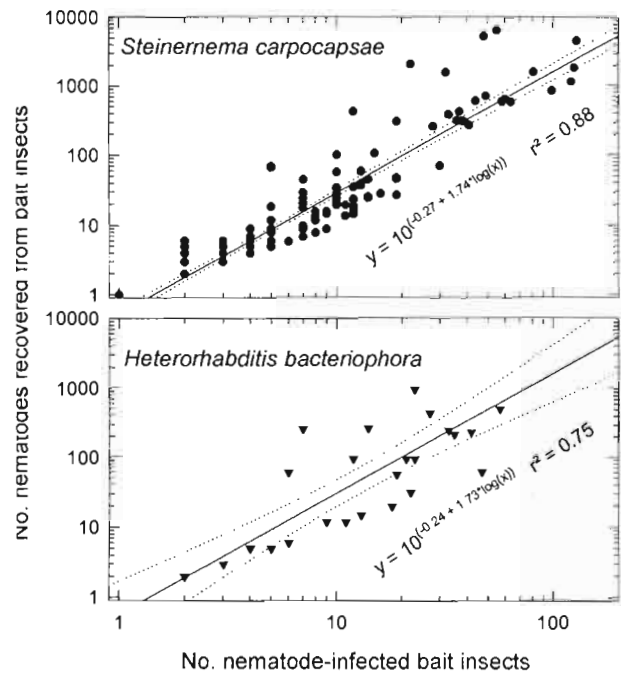

Fig. 1. *Relationship between number of nematode-infected bait insects and number of nematodes penetrated into bait insects in field soil samples. Dotted lines show 95% confidence interval.*

phora (Fig. 2). The slopes varied slightly between blocks within species but only for *S. glaseri* were the diffe-rences significant ($0.2 > P > 0.01$). Between species, the slopes differed significantly only between *S. glaseri* and *S. carpocapsae* ($P < 0.05$). Soil type did not signi-ficantly affect the equation (Fig. 3). The equations describing the relationship for *S. glaseri* were similar in the experiments testing the effect of nematode species and soil type. Because of the minor effect of nematode species and soil type on the equation, we pooled the data from the third and fourth laboratory experiment and also found a strong linear relationship (Fig. 4).

In the experiment testing the effect of nematode species, the relationship between the logarithms of the number of nematode-infected cadavers and the number of nematodes inoculated per dish (*S. carpocapsae*: $y = 10^{(-0.44 + 2.41*log(x))}$, $r^2 = 0.77$; *S. glaseri*: $y = 10^{(-0.29 + 2.00*log(x))}$, $r^2 = 0.74$; *H. bacteriophora*: $y = 10^{(-0.35 + 2.31*log(x))}$, $r^2 = 0.82$; all species combined: $y = 10^{(-0.25 + 2.08*log(x))}$, $r^2 = 0.75$) was similar to the relationship between number of nematode-killed cadavers and number of nematodes penetrated. The proportion of inoculated nematodes that penetrated into bait insects, *i.e.*, the slope of the equation, was not affected by the inoculum number (*S. carpo-*

Table 1. *Effect of inverting baiting dishes and baiting intervals on number of nematode-infected bait insects and number of nematodes inside these bait insects.* [1]

Experiment	Treatment	*Steinernema carpocapsae*		*Steinernema glaseri*	
		No. killed bait insects	No. penetrated nematodes	No. killed bait insects	No. penetrated nematodes
1[2]	inverted	23.7 ± 0.9a[4]	275.7 ± 17.9a	24.2 ± 0.7a	240.4 ± 11.0a
	not inverted	22.9 ± 0.9a	284.7 ± 15.1a	27.0 ± 1.0a	223.2 ± 9.6a
2	2-day[2]	23.4 ± 1.0a	230.1 ± 25.8a	26.3 ± 0.8a	200.5 ± 12.9a
	3-day[3]	22.3 ± 0.4a	273.0 ± 22.9a	23.8 ± 0.9a	203.9 ± 6.1a

[1] To each baiting dish 300 IJs and 10 bait insects were added.

[2] Nematode-infected bait insects were recovered at 2-day intervals and replaced by new insects.

[3] All bait insects were recovered and replaced by new insects at 3-day intervals.

[4] Means ± SE followed by same letter in columns within experiments are not significantly different (t-test, $P \leq 0.05$).
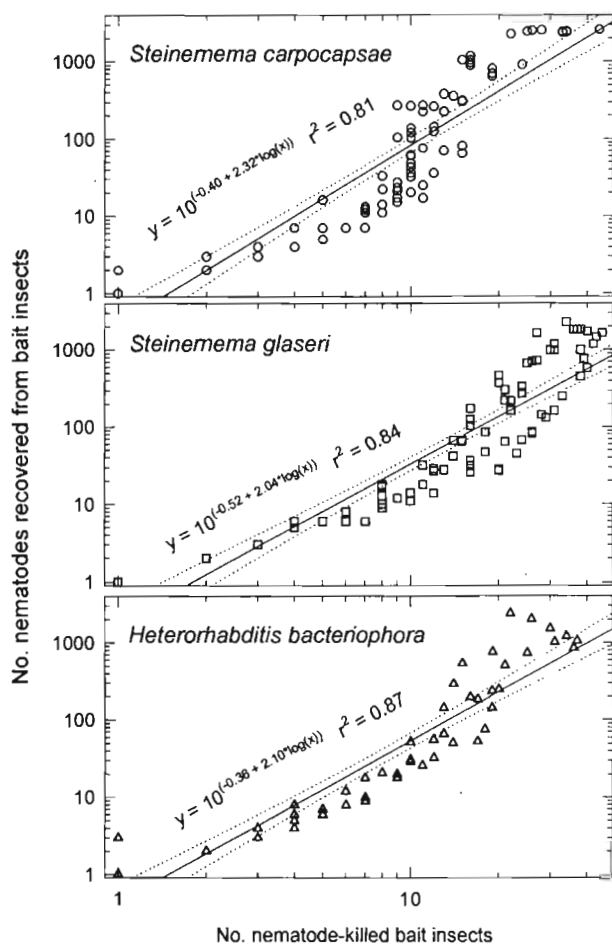

Fig. 2. *Relationship between number of nematode-infected bait insects and number of nematodes penetrated into bait insects for three nematode species. Dotted lines show 95% confidence interval.*
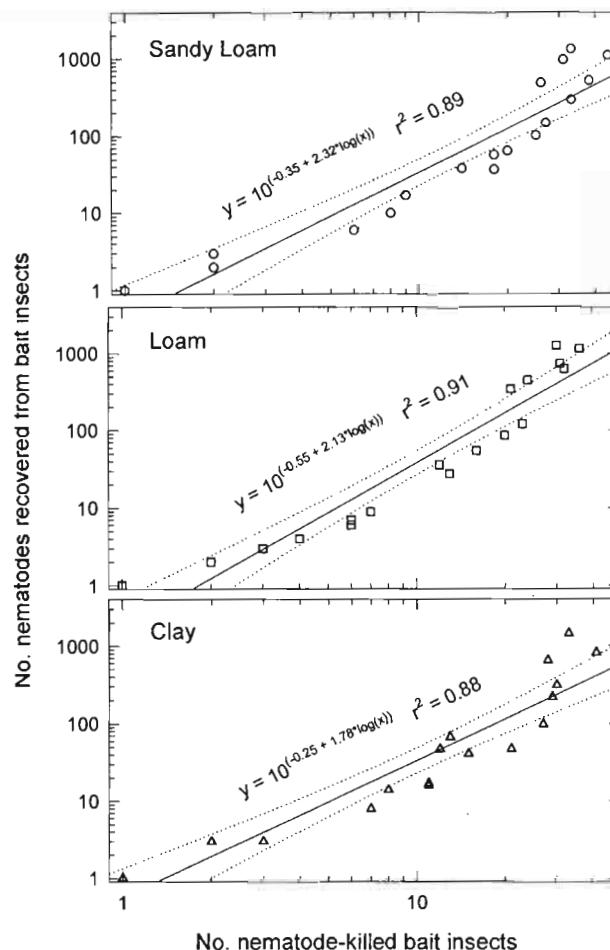
Fig. 3. *Relationship between number of nematode-infected bait insects and number of nematodes penetrated into bait insects for* Steinernema glaseri *in three soil types. Dotted lines show 95% confidence interval.*
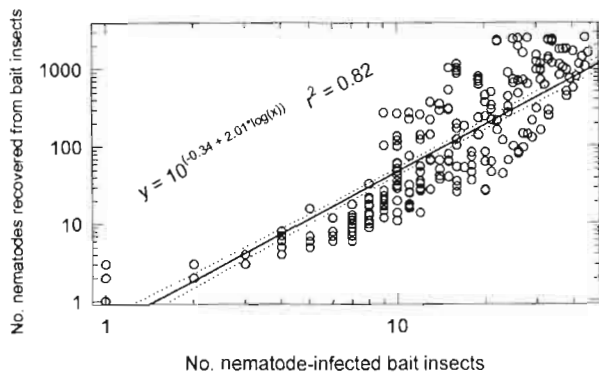
Fig. 4. *General relationship between number of nematode-killed bait insects and number of nematodes penetrated into bait insects. Dotted lines show 95% confidence interval. Graph combines data from the third and fourth laboratory experiment.*

capsae: $y = -0.96 + 0.81x$, $r^2 = 0.99$; *S. glaseri*: $y = -20.04 + 0.54x$, $r^2 = 0.93$; *H. bacteriophora*: $y = -21.61 + 0.51x$, $r^2 = 0.90$; all species combined: $y = 17.25 + 0.58x$, $r^2 = 0.91$).

Discussion

The linear relationship between bait insect mortality and number of nematodes penetrated into bait insects is consistent and can be used as a basis for estimating nematode densities in soil samples. Our experiments show that the method is quite robust and slight variations in the sampling protocol do not appear to significantly affect the relationship. Although the method with 3-day intervals and complete replacement of all bait insects after each baiting round requires more bait insects, it is the better procedure because it reduces the number of bait insect that die from causes other than nematode infection.

The slope of the equation varies slightly with nematode species and sampling conditions. A standard correlation averaged across the most common nematode species and conditions should provide estimates of nematode density adequate for many applications. Based on the combined data from our laboratory experiments, the following correlation was obtained: $y = 10^{(-0.34 + 2.01 \ast log(x))}$ ($r^2 = 0.82$) where x = total number of nematode-infected cadavers and y = total number of nematodes that penetrated (Fig. 4). This relationship is in turn related to the actual density of nematodes in the soil: $y = 10^{(-0.25 + 2.08 \ast log(x))}$ where x = total number of nematode-infected cadavers and y = number of nematodes inoculated. Therefore, reasonable estimates of nematode density can be obtained by measuring *G. mellonella* mortality.

Accordingly, we recommend the following protocol for the estimation of entomopathogenic nematode densities in soil samples:

i) Place soil sample (100 cm^3) in a Petri dish (100 × 25 mm). Break up soil clumps and adjust soil moisture as needed.

ii) Add ten wax moth larvae onto the soil surface. Do not invert the dish.

iii) Incubate samples for 3 days (at 22-25°C).

iv) Recover all wax moth larvae.

    a. Record number of nematode-infected larvae.

    b. Incubate alive larvae for 3 days, then record the number of nematode-infected larvae.

v) Repeat steps *ii-iv* until no more nematode-infected larvae are recorded for two consecutive baiting rounds.

vi) Calculate total number of nematode-infected larvae in all baiting rounds and read the corresponding number of nematodes from the correlation graph (Fig. 4).

For some applications it may be desirable to determine the correlation with greater precision, *e.g.*, when sampling a new entomopathogenic nematode species or using different temperatures. The relationship between cadaver number and number of nematodes recovered can be determined easily. Because of the good correlation, a low number of concentrations and replicates would be needed, although multiple blocks should be performed. For example, in the experiment testing the effect of soil type, two blocks of nine dishes each, with each dish having a different nematode inoculum, was sufficient to obtain a high r^2-value for the relationship.

The degree of correlation between number of cadavers and number of penetrating nematodes does vary with number of cadavers. This occurs because on a linear scale the relationship is sigmoid. When the number of nematode-infected cadavers is low, the estimates are less precise; with five or less cadavers there is little relationship to the number of nematodes. However, the total number of nematodes recovered only varies between one and ten so the inaccuracy of the estimate is unlikely to strongly influence estimates of nematode density. With more than twenty cadavers, the estimate of nematode number also becomes less reliable. In our samples of endemic populations, 68% of *H. bacteriophora* positive samples and 89% of *S. carpocapsae* positive samples had twenty or less infected cadavers. Therefore, for most samples the correlation will be good, but caution should be used in interpreting the relationship at high densities. It should be noted that high IJ densities also present problems for the accuracy of other baiting estimates of nematode density.

Our method has two major advantages over other methods using multiple baiting rounds to extract nematodes from inoculated soil samples (Fan & Hominick, 1991; Curran & Heng, 1992; Koppenhöfer *et al.*, 1996). First, the extraction efficiency, *i.e.*, the percentage of the inoculum extracted, of our method is higher (40 to 95%) than that of the other methods (20 to 60%). We suspect that the higher host density allowed for this higher extraction efficiency; our observations concur with those made by Epsky and Capinera (1993). Second, our method is less labor intensive because it does not require cadaver dissection, pepsin digestion, and nematode counting. In the case of field samples, the repeated baiting of the samples also appears to overcome some of the variability in the pattern of extraction and extraction efficiency of natural populations of IJs by baiting. Reliance on one or even a few rounds of baiting for assessing population densities of IJs in field-collected samples can be misleading.

Our lack of understanding of nematode ecology has impeded the successful implementation of entomopathogenic nematodes as biological control agents (Hominick & Reid 1990). Determining temporal and spatial patterns to entomopathogenic nematode distribution is fundamental to understanding their impact on host populations. This applies to both natural populations and to nematodes released as biological control agents. Understanding the persistence of released populations of entomopathogenic nematodes is especially important from the standpoint of determining the *i*) causes of biological control successes and failures, *ii*) fate of inoculative releases, *iii*) impact to non-target organisms, and *iv*) ability to recycle. An impediment to this understanding has been the difficulty of assessing nematode populations in the field. We propose that our sampling protocol can greatly reduce the labor involved in sampling entomopathogenic nematode populations while providing good quantitative estimates of density. The protocol will enable more researchers to monitor both native and introduced nematode populations and ultimately lead to a greater understanding of entomopathogenic nematode ecology.

Acknowledgments

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