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Comparison of Three Methods for Estimating the Number of Entomopathogenic Nematodes Present in Soil Samples[1]

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Abstract: Numbers of *Steinernema* sp. (CB2B) and *S. carpocapsae* (Agriotos) exponentially declined after application into a clay loam soil. Over a 35-day sampling period, *Steinernema* sp. (CB2B) was more persistent than *S. carpocapsae* (Agriotos). The presence or absence of the second-stage cuticle on the third-stage juveniles (J3) at the time of application did not alter the rate of population decline of *Steinernema* sp. (CB2B). Nearly all J3 of *Steinernema* sp. (CB2B) and *S. carpocapsae* (Agriotos) lost their cuticle within 24 hours of being in soil. Centrifugal flotation recovered the greatest number of nematodes, with a lower variance than either the live bait or Baermann funnel techniques. A strong positive linear relationship was evident between numbers of nematodes present in the soil and the numbers that established in a bait insect. Approximately 40% of *Steinernema* sp. (CB2B) and 30% of the *S. carpocapsae* (Agriotos) present in the soil established in *Galleria mellonella* larvae. The extraction techniques had different efficiencies and gave different relative estimates of persistence for the two species. Persistence and infectivity was best measured using a combination of live bait and flotation techniques.

Key words: entomopathogenic nematode, extraction technique, *Galleria*, infectivity, nematode, persistence, *Steinernema carpocapsae, Steinernema* sp.

Reliable methods for estimating the number of nematodes present in soil samples are needed to monitor population changes of field-released entomopathogenic nematodes. Most frequently, population estimates are obtained using modifications of either Baermann funnel or centrifugal flotation techniques (7). In addition, a live bait technique using larvae of the greater wax moth, *Galleria mellonella,* has been developed for quantitative estimation of entomopathogenic nematodes in soil (2). The proportion of entomopathogenic nematodes extracted from soil by flotation, Baermann funnel, and live bait methods depends on different components of nematode biology. The mechanical separation of nematodes from soil by flotation relies on the physical properties of the infective-stage juvenile and does not assay behavioral attributes such as motility or infectivity. The Baermann funnel method is contingent on nematode motility, whereas the live bait method relies on infectivity.

The purpose of this study was to compare Baermann funnel, flotation, and live-bait extraction methods, and to determine if extraction efficiency was affected by nematode species or length of time the nematodes had been in soil. Moreover, as each technique measures different components of nematode biology, the relationships between the population estimates obtained were examined to see if insights could be gained into the behavior of entomopathogenic nematodes in soil. The possible role of the second-stage (J2) cuticle in conferring persistence of *Steinernema* sp. (CB2B) in unsterilized soil was also assessed because the J2 cuticle is alleged to afford protection from soil antagonists (6).

Materials and Methods

Steinernema carpocapsae (Agriotos) and an undescribed *Steinernema* sp. (CB2B) were cultured in vitro (1). All nematode inocula were equivalent in age (harvested 4 weeks after initiation of the culture).

The J2 cuticle of *Steinernema* sp. (CB2B) was removed by placing 50,000 infective-stage juveniles in 200 g washed fine sand

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(glass-making grade, pF 2, moisture content 8%) for 48 hours. The nematodes were collected by adding tap water to the sand, allowing the sand to sediment (30 seconds) and collecting the nematodes in the supernatant. None of the *Steinernema* sp. (CB2B) exposed to sand retained the second-stage cuticle, compared to 99.2% of the inoculum not exposed to sand.

Plastic specimen jars (5.5 cm deep, 4.2 cm diameter) were filled with 80 g clay loam soil (pF 1.8, moisture content 13%) and placed into a polystyrene box with constant 100% humidity at 23 C. After 5 days, 3 ml nematode suspension containing 400 infective-stage juvenile nematodes were inoculated into a centrally placed hole (15 mm deep, 7 mm diameter) in the soil surface. At 1, 5, 10, 15, 20, 25, and 35 days after inoculation, the number of entomopathogenic nematodes surviving in the soil was assayed by each of the three extraction methods. There were five replicates per sampling date for each method.

Modified Baermann funnel: The soil was placed on tissue paper supported by a nylon mesh screen suspended in a polystyrene container (6 cm deep, 9 cm diameter). Water (100 ml) was carefully added down the side of the container, so that the soil surface was evenly moistened. After 24 hours at 23 C, the nematode suspension was poured off into a counting tray.

Flotation: The soil was placed in a 1-liter beaker then suspended in 200–300 ml of tap water by vigorous stirring with a spatula. The suspension was allowed to settle for 30 seconds and the supernatant was poured off into a 1-liter polypropylene centrifuge bottle. This step was repeated twice more to give a final volume of approximately 600 ml. The suspension was centrifuged at 1,000 rpm (250*g*) for 4 minutes, and the supernatant was poured into a 1-liter beaker. Approximately 100 ml of a 20% w/v aqueous solution of NaCl was added to the soil in the centrifuge bottle, mixed well, and then centrifuged for 2 minutes at 250*g*, and the supernatant was combined with that from the previous centrifugation. The salt-flotation step was repeated once. The pooled supernatants were poured through a 63-μm-pore (240-mesh) wire sieve, and the collected nematodes were washed from the sieve into a counting tray.

Live bait method: Five late-instar larvae of *G. mellonella* were placed on the soil surface of each jar of soil. After 24 hours, the larvae were removed, washed in tap water, and replaced by five more larvae, which were exposed for 72 hours. This last step was repeated once more, using an additional five larvae. After exposure to the nematodes, the larvae were incubated at 23 C and 75% RH for 4 days in plastic specimen jars. They were then dissected in Ringer's solution, and the number of entomopathogenic nematodes was counted. The number of nematodes that established in *Galleria* over the three exposure periods was calculated for each soil sample.

In order to test cuticle retention after application, 2,000 *Steinernema* sp. (CB2B) or *S. carpocapsae* (Agriotos) were inoculated into jars containing 80 g soil. The nematodes were extracted from the soil by centrifugal flotation immediately and 24 hours after application. The percentage of nematodes without cuticles was determined before inoculation and after extraction from the soil. There were three samples of >70 individuals, with five replicates per nematode species.

The data for nematode counts were exponentially distributed and were transformed to $\log_e$ prior to regression analysis (model $y = a + bx$).

Results

There was a negative exponential decline in the number of nematodes recoverable from the soil over time (Table 1). The Baermann funnel method indicated that both species declined at a similar rate. However, as assayed by the flotation and live bait methods *S. carpocapsae* (Agriotos) declined at a faster rate than *Steinernema* sp. (CB2B).

TABLE 1. Regression constants ($\log_e$ mean nematode counts) for the persistence of *Steinernema* sp. (CB2B) and *Steinernema carpocapsae* (Agriotos) in soil derived by different extraction methods and measured over 35 days.

Nematode inoculum	Extraction method	*a*	*b*	R^2 (%)
Steinernema sp. (CB2B) with cuticle	Flotation	5.37	−0.048	89
	Live bait	4.99	−0.053	82
	Baermann	4.05	−0.111	95
Steinernema sp. (CB2B) without cuticle	Flotation	5.26	−0.050	97
	Live bait	4.83	−0.047	78
	Baermann	3.59	−0.100	85
S. carpocapsae (Agriotos)	Flotation	5.33	−0.091	95
	Live bait	5.07	−0.103	96
	Baermann	4.35	−0.084	88

The extraction efficiency of each extraction method was calculated as the number of nematodes recovered 1 day after application expressed as a percentage of nematode inoculum (Table 2). Centrifugal flotation recovered the greatest proportion of nematodes, with a lower variance than either the live-bait or Baermann funnel techniques. The lower variance associated with flotation, relative to the other two techniques, was evident throughout the experiment, as shown by the mean coefficient of variation over the eight sampling periods (Table 2).

The different extraction techniques gave different relative estimates of persistence for the two species. For example, data from the live bait method 10 days after application indicated that both nematodes species were equally persistent (means of 60.4, 47.2 and 69 nematodes for *S. carpocapsae* (Agriotos) and *Steinernema* sp. (CB2B) with and without cuticle, respectively). However, data from the flotation method indicated that approximately twice as many *Steinernema* sp. (CB2B) (means = 108.2 with cuticle, 101.4 without cuticle) as *S. carpocapsae* (Agriotos) (mean = 57) survived. By contrast, data from the Baermann funnel method suggested that *S. carpocapsae* (Agriotos) was approximately three times as persistent as *Steinernema* sp. (CB2B) (means of 29.8 cf. 12.4, 7.3, with and without cuticle, respectively). All three techniques showed no significant difference between numbers of *Steinernema* sp. (CB2B) with and without J2 cuticles. The different relative estimates of persistence for the two species using the three methods were not constant over

TABLE 2. Comparison of the efficiency of three extraction procedures for the recovery of infective-stage juveniles of *Steinernema* sp. (CB2B) and *Steinernema carpocapsae* (Agriotos) from soil.

Nematode inoculum	Extraction method	Extraction efficiency (%)†	CV‡ (%)
Steinernema sp. (CB2B) with cuticle	Flotation	60.8 ± 2.7	13.4 ± 7.5
	Live bait	41.7 ± 17.1	46.9 ± 15.4
	Baermann	16.9 ± 5.5	44.3 ± 28.8
Steinernema sp. (CB2B) without cuticle	Flotation	45.2 ± 1.8	7.4 ± 3.4
	Live bait	30.8 ± 11.4	31.2 ± 12.4
	Baermann	13.7 ± 3.4	38.9 ± 15.8
S. carpocapsae (Agriotos)	Flotation	46.4 ± 2.1	16.3 ± 13.2
	Live bait	33.4 ± 8.5	41.3 ± 15.9
	Baermann	17.9 ± 5.6	30.0 ± 7.5

† Mean ± standard deviation, calculated on data from 1 day after application as percentage of doseage applied.
‡ Mean coefficient of variation was calculated as the sum of coefficients of variation for each of the eight sampling periods/ the number of sampling periods.

time. The divergence between estimates of the persistence of *Steinernema* sp. (CB2B) based on the Baermann compared to live-bait and flotation methods increased with length of time the nematodes were present in the soil (Fig. 1). However, the Baermann funnel method recovered about half the numbers of *S. carpocapsae* (Agriotos) recovered by the other two methods, and this was independent of time (Fig. 1).

The data from the live-bait technique had comparable accuracy to that of Fan and Hominick (2). Using the sum of the three exposures as total recovery and averaging over the eight sampling dates, then the first exposure yielded 42.7% (CV 50%), 32.7% (CV 61%) and 73% (CV 23%) of the total nematodes recovered, and the cumulative mean recoveries after two exposures for 24 and 72 hours were 94% (CV 6%), 92% (CV 10%), and 95% (CV 8%) for *Steinernema* sp. (CB2B) with cuticle, for *Steinernema* sp. (CB2B) without cuticle, and for *S. carpocapsae* (Agriotos), respectively.

During the course of the experiment, the speed of infection steadily declined. Thus on day 1, 53% of *Steinernema* sp. (CB2B) with cuticle, 58% of *Steinernema* sp. (CB2B) without cuticle, and 78% of *S. carpocapsae* (Agriotos) that established did so in the first 24 hours, whereas by day 30 the corresponding percentages were 20%, 20%, and 58%.

Regression of numbers recovered by the live bait technique on the numbers present in the soil as estimated by flotation gave a

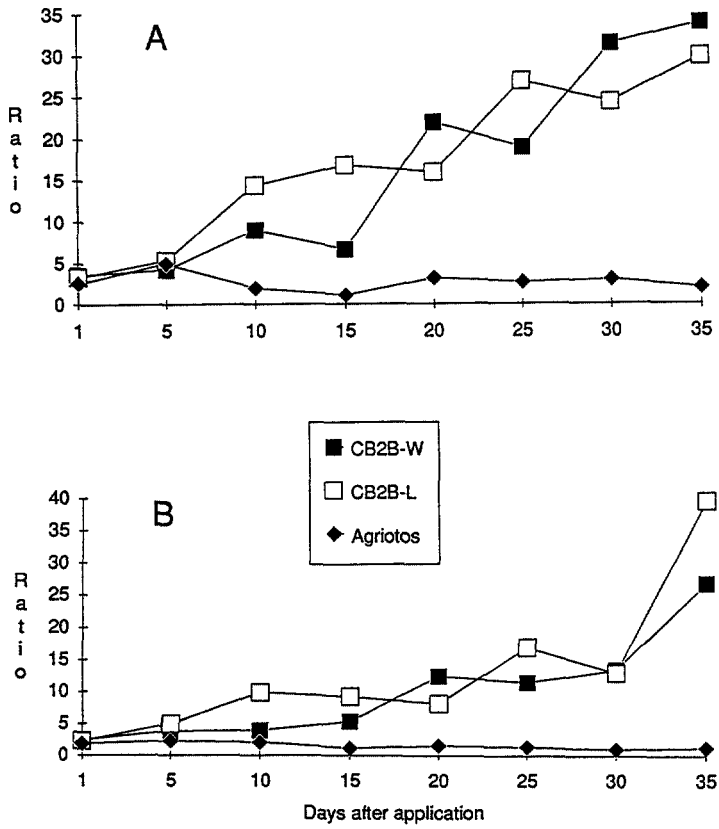

FIG. 1. Comparison of the number of entomopathogenic nematodes recovered from a clay loam soil by different extraction techniques over 35 days. A) Numbers recovered by centrifugal flotation divided by numbers recovered by Baermann funnel. B) Numbers recovered by live-bait method divided by numbers recovered by Baermann funnel. CB2B-W, *Steinernema* sp. (CB2B) with cuticle; CB2B-L *Steinernema* (CB2B) without cuticle; Agriotos, *Steinernema carpocapsae* (Agriotos).

linear relationship with the following coefficients for untransformed data: *Steinernema* sp. (CB2B) with cuticle $y = -12.32 + 0.791x$, $R^2 = 90\%$; *Steinernema* sp. (CB2B) without cuticle $y = -8.38 + 0.801x$, $R^2 = 87\%$; and *S. carpocapsae* (Agriotos) $y = 4.34 + 0.605x$, $R^2 = 85\%$.

Ensheathed infective-stage juveniles of *Steinernema* sp. (CB2B) and *S. carpocapsae* (Agriotos) rapidly lost their J2 cuticles in the soil. The centrifugal flotation method itself removed the cuticle from a significant proportion of nematodes (approximately 9% of the ensheathed inoculum of *Steinernema* sp. (CB2B) and 18% of *S. carpocapsae* (Agriotos)), but 24 hours after entry into the soil, 98% of *Steinernema* sp. (CB2B) and 89.7% of *S. carpocapsae* (Agriotos) had lost their J2 cuticle.

Discussion

Steinernema sp. (CB2B) was more persistent than *S. carpocapsae* (Agriotos) in the soil tested, but both species underwent a period of rapid decline immediately after application. The cause of this decline is unknown, but the soil used is known to contain nematode antagonists such as predatory mites, nematodes, and predatory and pathogenic fungi. Both nematode species survive longer in sterilized than in unsterilized samples of this soil (unpubl. data). A similar exponential decline has been reported for *S. carpocapsae* (DD-136) and *S. glaseri* in unsterilized sandy soil, with greater persistence of both species in sterilized soil (5). The presence of the second-stage cuticle on the J3 at the time of application did not alter the rate of population decline of *Steinernema sp.* (CB2B). It has been proposed that the J2 cuticle may protect entomopathogenic nematodes, particularly *Heterorhabditis,* from antagonists (6,8). However, because nearly all J3 of *Steinernema* sp. (CB2B) and *S. carpocapsae* (Agriotos) lost their cuticle within 24 hours of entering the soil, it is questionable whether the function of the J2 cuticle is to protect infective-stage juveniles of *Steinernema* from soil antagonists.

Estimates of the relative persistence of each species were significantly influenced by extraction method. Over the 35-day period, the data obtained using the Baermann funnel method indicated that *S. carpocapsae* (Agriotos) was more persistent than *Steinernema* sp. (CB2B), whereas the live-bait and flotation methods consistently showed the reverse, with *Steinernema* sp. (CB2B) being the most persistent. Thus, choice of technique can markedly alter estimates of relative persistence between species.

The different techniques had different extraction efficiencies with flotation recovering a greater proportion of nematodes than either live bait or Baermann funnel methods. Differences in extraction efficiency have been reported (7) in a previous study of the extraction of nematodes from sand inoculated with *S. carpocapsae* (Agriotos), but with greater recovery with a Baermann funnel method (71.2%) than with a centrifugal flotation method (45.8%). Such disparity in results may be related to differences in experimental method (sucrose vs. salt flotation), nematode strain (*S. carpocapsae* (All) versus (Agriotos)), or substrate used (sand vs. soil).

The live-bait and Baermann funnel methods had larger coefficients of variation than the flotation method. In addition to general measurement error, estimates from all three techniques were subject to variability due to differences in nematode persistence in individual soil samples. However, the influence of the soil environment or infectivity and (or) motility would be an additional source of biological variability for both the live bait and Baermann funnel methods. The Baermann funnel method is the simplest method to use, but biased estimates (due to influence of species and time) reduce its general applicability. Centrifugal flotation consistently gave the highest recoveries with lowest variability; however, sample processing ca-

pacity is limited by the centrifugation steps. The live-bait method has appeal in studies of entomopathogenic nematodes because it measures infectivity of surviving nematodes (4). In our laboratory, each live-bait sample could be processed in approximately half the time required for centrifugal flotation. A subsequent study has demonstrated that the number of *Galleria* per sample can be reduced from five to three and confirmed that only two exposures are required (Curran and Hartley, unpubl.).

With the flotation data as the best estimate of the number of nematodes present in a soil sample, a strong positive linear relationship is evident between numbers of nematodes present in the soil and the numbers that establish in a bait insect. This agrees with published data relating the number of nematodes establishing per insect to exposure to different dosages of nematodes (2). Correcting for extraction efficiency, approximately 40% of *Steinernema* sp. (CB2B) and 30% of the *S. carpocapsae* (Agriotos) present in the soil are capable of establishing in *Galleria* at any one time. This is remarkably similar to the results of Fan and Hominick (2) and leaves unanswered their question of why the majority of nematodes fail to establish when conditions are optimal.

Although our study did not extract each soil sample to exhaustion, the results support the findings of Fan and Hominick (2) that two exposures recover a high percentage of the nematodes recoverable by this technique and considerably reduce the error of the estimate. This extends the use of this technique to two more species of entomopathogenic nematodes, *S. carpocapsae* (Agriotos) and *Steinernema* sp. (CB2B), and the close correspondence of results in both studies confirms the general applicability of the live-bait technique.

Each of the extraction techniques recorded the rapid decline of entomopathogenic nematodes after application, and a strong positive correlation existed between population estimates obtained by all methods. However, the efficiency of each technique is dependent on different components of nematode biology and each provides a different insight into nematode behavior in soil. Flotation estimates indicated that *Steinernema* sp. (CB2B) was more persistent than *S. carpocapsae* (Agriotos). The overrepresentation of *S. carpocapsae* (Agriotos) by the Baermann funnel method suggests that *S. carpocapsae* (Agriotos) is more motile than *Steinernema* sp. (CB2B) in wet soil. Flotation measures persistence and does not measure the infectivity of the nematodes recovered. However, as noted here and reported by Fan and Hominick (2), only 30–40% of the entomopathogenic nematodes present in a soil sample can infect *Galleria* at any time.

Estimates of the relative persistence of entomopathogenic nematodes in soil are significantly influenced by species differences, extraction method, and length of time the nematodes had been in soil. Flotation gave the best estimate of persistence. The flotation method may be superior to the live-bait method because flotation is less dependent on the behavioral attributes of the nematodes. As a consequence, however, reliance on flotation alone would mask important behavioral characteristics of entomopathogenic nematode populations, such as infectivity and motility. Thus, to better characterize nematode populations, we routinely use the live-bait method in combination with flotation to study the biology of entomopathogenic nematodes in soil.

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