On the Methodology of Nematode Extraction from Field Samples: Comparison of Methods for Soil Extraction

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Abstract: The commonly used nematode extraction methods were compared using three soil types and four nematode species. The comparison was repeated in three trials by the same operator to estimate operator reproducibility. Extraction efficiency was dependent upon method, soil type, and nematode species, and reproducibility was not particularly satisfactory for routine analyses. Extraction by any method tested was less than 50% efficient. Quantitative nematode extraction methodology needs serious attention. *Key words:* sucrose flotation, sieving, misting, soil type, variability, efficiency.

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Comparisons of nematode extraction techniques have been biased to meet the needs of individual investigators using unknown substrate material (1,2,3,4,5,6,7). Method selection usually has been based upon the greatest number of nematodes extractable from a common soil sample containing an unknown number of nematodes. If nematode diagnostics are to develop a quantitative capability, it is mandatory to understand the parameters and to establish the limitations of extraction methodology. Ideally, for wide-scale routine diagnostic purposes, a method should be rapid, quantitative, independent of nematode species or soil type, free of operator error or variability, and faithfully reflective of the numbers of nematodes present in the soil. It was the aim of these experiments to compare the potential of a number of the more commonly used extraction methods for routine diagnostic implementation.

MATERIALS AND METHODS

The nematode extraction experiments employed three soil types. A sandy loam soil was steam sterilized and stored for a year in a closed container to allow nematodes to decompose. After the storage treatment no nematodes could be extracted. According to mechanical analysis, the soil consisted of 52.8% sand, 15.2% clay, 32.0% silt, and 0.2% organic matter. A clay loam soil type was prepared from this soil by the addition of clay. Mechanical analysis indicated 43.8% sand, 29.2% clay, and 27.0% silt. A loamy sand soil type was prepared from the sandy loam by the addition of sand. Mechanical analysis indicated 78.8% sand, 7.2% clay, and 14.0% silt. The three soil types were stored dry (moisture content less than 1%) until used.

Four different nematodes were used for extraction: *Xiphinema index,* Thorne & Allen, 1950; *Criconemella xenoplax,* (Raski, 1952) Luc & Raski, 1981; *Pratylenchus vulnus,* Allen & Jensen, 1951; and *Meloidogyne incognita,* (Kofoid & White, 1919) Chitwood, 1949. All nematodes were obtained daily from stock greenhouse cultures; each species was used separately and not mixed with other species.

Each of the following methods used to extract nematodes from the three soil types were replicated six times with each nematode. The experiment consisted of three trials, each one using different stock populations of nematodes. To reduce operational error, all trials were conducted by one individual who attempted to reproduce each step exactly.

Density flotation of soil using sucrose solutions: Twenty grams of soil was placed in each of six plastic centrifuge tubes (25 × 105 mm) and water was added to within 1 cm of the lip. The soil and water were mixed with a metal spatula; then an aliquant of nematodes was added and the suspension was re-mixed before centrifugation at 700 × g for one minute. The supernatant was decanted and sugar solution (sp gr 1.135) added and mixed as before. The sugar suspension was centrifuged at 700 × g for 1 min. after which the supernatant was decanted onto a 38-μM sieve. The nematodes were collected by backwashing from the sieve and counted.

Sieving then density flotation: Dry soil (100 ml) was mixed with a sevenfold volume of water after which an aliquant of nematodes was added. Each sample for each

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nematode was wet-sieved through a 850-μM sieve; *X. index* and *C. xenoplay* were collected on a 75-μM sieve, whereas *P. vulnus* and *M. incognita* were collected on a 38-μM sieve. The nematodes were obtained by backwashing the sieves; the sievings were subjected to density flotation as described above by substituting sievings for soil.

Sieve, funnel: Nematodes were sieved from soil as described above and the sievings placed upon Baermann funnels utilizing micro-wipe tissue for 24 hours, after which nematodes were collected and counted.

Sieving, mister: Nematodes were sieved from soil as described above and the sievings added to micro-wipe tissue on open stemmed funnels placed in a mist chamber for 7 days. The nematodes found in the collection tubes were counted.

Soil to mister: Dry soil (50 ml) was mixed with sufficient water to make a thick slurry. An aliquant of nematodes was added to the slurry which was again mixed and added to micro-wipe tissue on funnels which were placed in a mist chamber. The nematodes found in the collection tubes after 7 days misting were counted.

Sieving: Aliquants of nematodes were mixed with 250 ml of water and sieved through the appropriate screen for each nematode species. The nematode sievings were backwashed from the sieve and counted.

Wet soil (dry soil suspended in water, centrifuged, and the supernatant discarded) and dry soil in quantities used previously for density flotation were suspended in sugar solutions of initial specific gravity 1.135. The density of the supernatant over wet and dry soil was measured with the aid of a hydrometer.

In an attempt to assess the fate of the nematodes subjected to the density flotation of loamy sand mix, 20 g of dry soil was mixed with water to make a slurry. *M. incognita* larvae were added in aliquants; the slurry was re-mixed then subjected to centrifugation (715 $\times$ *g*) for three minutes. The aqueous supernatant was collected and the nematodes counted. The soil pellet was resuspended in a sugar solution of specific gravity 1.135 then again centrifuged for 3 min. The sugar supernatant was poured through a 38-μM sieve. The nematodes retained by the sieve and those remaining in the sugar solutions were collected and counted. The sieves were examined with the aid of a microscope to detect any nematodes remaining in the mesh. The centrifuge tube walls were carefully rinsed to the pellet and the nematodes collected and counted. The entire procedure with water and sugar solution was repeated twice with the soil pellet before discarding. The experiment was replicated six times.

RESULTS AND DISCUSSION

The extraction efficiencies of selected methods for four nematodes and three soil types are indicated in Table 1 as a composite of three trials—each one representing the processing of more than 400 samples—done in sequence to obtain a gross notion of reproducibility by one operator. It is evident that in some systems (nematode-soil type-method) there is consistency in trial results, but in others there is enoromous variability. It appears that some methods are more effective for certain nematodes than are others and that extraction efficiency can vary substantially with soil type. The sieving without soil indicates an upper level efficiency of the process without the negative confounding influence of soil. The averages are not particularly meaningful other than to indicate that the recovery obtainable by these methods was usually less than 50%.

The density of the bouyant solution over wet or dry soil type is indicated in Table 2 for comparative purposes.

The fate of nematodes in a soil inoculum extracted three times in sequence by the density flotation method using sugar is indicated in Table 3. It was striking to note that less than half of the *M. incognita* juveniles added to the sample were recovered.

The findings reported in Table 1 confirm the observations of years of practical experience of experimental researchers. Nematode extraction as represented by the five selected methods was, within certain limits, poorer for a heavier soil than for a lighter one. It is evident that the efficiency of any method for the extraction of different nematodes was dependent upon the characteristics of that nematode. From these

Table 1. Mean percent efficiency of extraction methods applied to three soil types for four nematode species (inoculum in parentheses).

Soil type method*	*Xiphinema index* (125 ± 5)	*Criconomella xenoplax* (100 ± 4)	*Pratylenchus vulnus* (210 ± 7)	*Meloidogyne incognita* (200 ± 10)
Clay loam				
Su	2.91 ± 2.94	22.4 ± 9.2	22.7 ± 10.4	21.1 ± 10.2
Si-Su	2.48 ± 2.64	26.1 ± 12.4	17.1 ± 9.4	21.8 ± 12.3
Si-Fu	30.2 ± 21.7	2.55 ± 2.24	42.2 ± 16.7	29.6 ± 8.8
Si-Fu-M	26.9 ± 31.0	7.17 ± 3.75	21.7 ± 13.4	21.6 ± 6.4
M	15.5 ± 20.2	10.5 ± 8.4	35.4 ± 9.2	29.7 ± 22.4
LSD (P = .01)	12.6	7.13	8.1	12.6
(P = .05)	9.5	5.38	6.1	9.5
Sandy loam				
Su	3.41 ± 3.53	48.1 ± 8.2	36.3 ± 19.6	20.7 ± 4.3
Si-Su	6.19 ± 4.30	48.3 ± 7.3	24.4 ± 13.3	22.2 ± 8.0
Si-Fu	25.6 ± 22.5	5.67 ± 3.65	45.4 ± 10.7	40.2 ± 7.1
Si-Fu-M	32.5 ± 33.3	7.38 ± 3.40	36.0 ± 21.0	13.3 ± 6.9
M	18.9 ± 22.1	7.51 ± 5.97	40.1 ± 17.4	36.9 ± 9.7
LSD (P = .01)	13.4	15.4	8.1	6.4
(P = .05)	10.1	11.6	6.1	4.9
Loamy sand				
Su	5.21 ± 3.71	34.9 ± 23.8	37.3 ± 19.9	17.3 ± 5.7
Si-Su	8.68 ± 5.63	38.9 ± 25.8	29.1 ± 18.4	17.2 ± 7.2
Si-Fu	30.7 ± 23.7	15.1 ± 12.9	35.5 ± 11.7	44.3 ± 10.0
Si-Fu-M	34.6 ± 29.6	14.6 ± 9.2	35.8 ± 16.2	31.3 ± 11.8
M	21.8 ± 27.3	2.99 ± 3.84	32.3 ± 27.3	18.6 ± 13.5
LSD (P = .01)	13.4	5.4	9.9	9.5
(P = .05)	10.1	4.1	7.5	7.2
Si	88 ± 7	86 ± 9	61 ± 14	51 ± 2

*Methods: Su Sugar flotation of soil.
Si-Su Sieving of soil followed by sugar flotation of sievings.
Si-Fu Sieving of soil followed by Baermann funnel extraction of sievings (1d).
Si-Fu-M Sieving of soil followed by Baermann funnel extraction of sievings in mist chamber (7d).
M Soil placed directly on funnel in mist chamber (7d).
Si Sieving of aqueous nematode suspension.

trials the worst procedures could be quickly identified and discarded from future use; of those remaining, the selection of the best was open to question. Of the 60 combinations tested, three barely achieve a 50% recovery of the inoculum.

It is axiomatic in separation technology that an extraction process incurs a loss of target substrate; the loss is progressive with an increasing number of steps. Several of the methods tested use two such steps; sieving followed by a funnel extraction or by density flotation with sugar solution. The variable losses incurred by sieving (Table 1) depend upon the nematode used. These losses occur under good sieving conditions (i.e., careful operator handling and a suspension of nematodes in water); the addition of soil to the system would be expected to decrease the efficiency of the sieving process. Other studies (8) have shown that the paper tissue used in the Baermann funnel extraction could retain substantial numbers of nematodes. The combination of losses from sieving with those from paper retention ac-

Table 2. Density (sp.g) of resultant supernatant after wet and dry soils were suspended in sugar solution of initial specific gravity 1.135.

	Dry soil	Wet soil
Clay loam	1.125	1.116
Sandy loam	1.118	1.103
Loamy sand	1.117	1.111
	LSD (P = .01) = 0.007	

Table 3. Percent recovery of nematodes at various extraction stages, in the implementation of a density flotation separation technique: *Meloidogyne incognita* from loamy sand soil type (20 g), as mean percent of inoculum.

Extraction	Extraction Stage					Overall recovery
	Loss with water discard	Loss with sugar discard	Sieve collection from sugar solution	Loss on sieve	Adhered to tube wall	
First	7.82 ± 3.77	0.37 ± 0.28	29.22 ± 2.1	0.09 ± 0.22	2.06 ± 0.68	38.3 ± 4.44
Second	0.65 ± 0.90	0.09 ± 0.22	5.45 ± 1.87	0.37 ± 0.28	1.03 ± 0.42	7.61 ± 2.10
Third	0.0	0.0	1.88 ± 1.27	0.09 ± 0.22	0.0	1.97 ± 1.16
				Total recovery		47.9

count for the losses incurred by the two-step processes indicated in Table 1.

The efficiency of direct nematode extraction processes using density flotation with sugar was of the same order and showed a similar variability exhibited by the other processes. Since this was a one-step process, it was pertinent to examine parameters which could account for some of the losses. Literature reports indicate the density of the prepared sugar solution but not the resultant density of the solution floating the nematodes. It was noted that the density of the floating solutions over all soil types, whether dry or wet, was significantly less than that of the prepared sugar solution (Table 2). Furthermore, there were significant differences between all soil types when wet and certain soil types when dry, as well as between wet and dry of the same soil types. The reduction in the density of the solution over dry soil suggests a preferential binding of sugar to soil particles so as to render the bathing solution less dense. The additional reduction in density of the floating solution of wet soil may be explained in part by preferential binding of sugar and dilution by residual water.

The fate of nematodes in the density process using *M. incognita* and loamy sand soil indicated that a low percentage was lost with the water discard, very few nematodes were lost with the sugar discard through the screen, the bulk of the recovery was collected from the sugar solution sieving, no nematodes were lost on the sieve, and a small percentage was lost by adherence to the tube wall. Approximately 38% of the inoculum was accounted for in the first extraction, 7.5% in the second extraction, and approximately 2% in the third extraction. Overall, less than half of the inoculum was accounted for; apparently at least half of the nematodes were sufficiently bound to the soil as to be unavailable to this extraction process.

In summary, the results of these experiments imply that the methods tested were exceedingly poor quantitative diagnostic tools. A conversion factor in excess of two to arrive at an estimate of the initial population level in a sample is highly risky for a quantitative method and untrustworthy for disease threshold determinations. The statistical analysis should be viewed with caution to preclude the misinterpretation of observations as recommendations. It should be readily apparent that current extraction methodology is in urgent need of substantial improvement to achieve near quantitative status.

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