A QUANTITATIVE COMPARISON OF SOME METHODS FOR THE EXTRACTION OF NEMATODES FROM ROOTS[1]

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Accepted:
6.IV.1984
Aceptado:

ABSTRACT

McSorley, R., J.L. Parrado, and W.H. Dankers. 1984. A quantitative comparison of some methods for the extraction of nematodes from roots. Nematropica 14:72-84.

Methods for extracting various species of nematodes from root tissue were compared, including incubation in water, a mist chamber, plastic bags, or Mason jars; maceration of roots followed by centrifugation or suspension in Baermann funnels; or extraction in sodium hypochlorite solutions. Sodium hypochlorite solutions were superior to blender maceration and centrifugation for extracting eggs of *Meloidogyne incognita* or *Tylenchulus semipenetrans*. In most cases, blender maceration + centrifugation was superior for extracting migratory stages of *T. semipenetrans, Helicotylenchus multicinctus, Pratylenchus* spp., or *Hemicriconemoides mangiferae*. However, numbers extracted by this technique were not significantly different from those obtained in a mist chamber for *Pratylenchus* spp., nor from those obtained by incubation in plastic bags for *T. semipenetrans*. Results for extraction of larvae of *M. incognita* were inconsistent, possibly because of egg hatch during the incubation procedures. Maceration and centrifugation of young, *M. incognita*-infected squash roots without mature egg masses recovered more larvae than incubation in plastic bags.

Additional key words: techniques, Helicotylenchus multicinctus, Meloidogyne incognita, Hemicriconemoides mangiferae, Pratylenchus brachyurus, Pratylenchus coffeae, Tylenchulus semipenetrans.

RESUMEN

McSorley, R., J.L. Parrado, y W.H. Dankers. 1984. Una comparación cuantitativa de algunos métodos para la extracción de nematodos de las raices. Nematropica 14:72-84.

Se compararon los métodos para extraer nematodos de diferentes especies de las raices, incluyendo entre ellos: incubación en agua, cámara de neblina, bolsas plásticas o jarros Mason; maceración en la batidora seguido por centrifugación a suspensión en embudos Baermann; y solucionés de hipoclorito de sodio. El método de soluciones de hipcolorito de sodio fue superior a la maceración en la batidora y centrifugación para la extracción de huevos de *Meloidogyne incognita* y *Tylenchulus semipenetrans*. La maceración en la batidora seguido por centrifugación fué superior en la mayoría de los casos para la extracción de estadios migratorios de *T. semipenetrans, Helicotylenchus multicinctus, Pratylenchus* spp. y. *Hemicriconemoides mangiferae* de los tejidos de las raices. Sin embargo, para el *T. semipenetrans* el número extraido por dicho método no fué significativamente diferente a los obtenidos por los métodos de

incubación en agua o bolsas plásticas. Los resultados de la extracción de larvas de *M. incognita* de las raices fueron inconsistentes posiblemente debido a la emergencia de las larvas de los huevos durante el período de incubación. El método de maceración por batido y centrifugación recobró más larvas de *M. incognita* de raices (jovenes sin masas de huevecillos maduros) de calabacita que el método de incubación en bolsas plásticas.

Palabras claves adicionales: tecnicas, Helicotylenchus multicinctus, Meloidogyne incognita, Hemicriconemoides mangiferae, Pratylenchus brachyurus, Pratylenchus coffeae, Tylenchulus semipenetrans.

INTRODUCTION

Diagnosis of endoparasitic nematodes within root tissues is an important task which many nematology laboratories must perform on a day-to-day basis. Such a variety of methods are available for this procedure that it is often difficult to select a method which will be both accurate and practical for a given laboratory and nematode species. Ayoub (1) describes in detail the oldest methods for examining nematodes from plant tissue, i.e. dissection or staining of plant tissue. These methods are useful if qualitative information on the species present is desired, but may become tedious if routine quantitative data are needed.

Quantitative data are more easily obtained by first using some method of extracting the nematodes from the root tissue, the simplest of which is placing the material on a Baermann funnel (1). Young (14) attempted to improve on this technique by incubating roots in a closed Mason jar rather than in a funnel. This method was in turn improved upon by Tarjan (10), who increased the recovery of *Radopholus similis* (Cobb) Thorne from roots by incubating in plastic bags rather than in jars. He later refined the plastic bag technique by examining time and temperature of incubation as well as the effects of the addition of various concentrations of hydrogen peroxide. Thus he was able to propose optimal conditions, using the technique, for obtaining either *R. similis* (11) or *Tylenchulus semipenetrans* Cobb (12) from citrus roots. Chapman (3) also attempted to improve upon the original Baermann-funnel method of incubation, and examined the relationship between amount of root material incubated, funnel size, and incubation time. He concluded that Young's method (14) was an improvement over the Baermann funnel and permitted the use of larger samples. He went on to examine incubation of roots in water in Erlenmeyer flasks or jars, and attempted to optimize the recovery by adding aeration through shaking or bubbling air. Another incubation method yielding satisfactory recovery of migratory endoparasites is the mist chamber method described by Oostenbrink (9) and by Ayoub (1).

The above methods are all dependent on nematode motility to some extent, and were developed primarily as a means for extracting migratory endoparasitic genera such as *Pratylenchus* and *Radopholus*. Maceration of root tissues by a blender to facilitate nematode removal was introduced by Taylor and Loegering (13). The method was refined by Gowen and Edmunds (6) to include incubation in a Baermann funnel in the presence of hydrogen peroxide following the maceration step. Still, the method was intended primarily for extraction of *R. similis* and *Helicotylenchus multicinctus* (Cobb) Golden, both migratory endoparasites. Only when Coolen and D'Herde (4) combined maceration with sugar centrifugation was it possible to obtain quantities of sedentary endoparasites or eggs along with the mobile stages. In their tests (4), this method proved to be superior even for several of the migratory endoparasites. An alternative method for extracting eggs of *Meloidogyne* species has been developed by Hussey and Barker (8).

The present study was designed to make quantitative comparisons of the various extraction methods for a variety of nematode genera, to determine if one method was superior in all cases or if a different method should be used in specific instances. Several extraction methods were not considered in this study. Enzymatic maceration of plant tissue (5) has the advantage of providing clean inoculum for research studies, but may be difficult to use for large numbers of samples on a routine basis. Incubation in solutions of various types of chemical agents (2) is another practical extraction method, however this technique may stimulate egg hatching during the incubation period. This is useful if total population counts are required, but it may not provide a very meaningful comparison if only the migratory stages are to be assayed. This may be a problem with most incubation methods to some extent, but it probably would be maximized in this case.

MATERIALS AND METHODS

For each experiment comparing several different root extraction procedures, a large quantity (ca 500g) of small roots ($\leq$0.25 cm diameter) was removed from the host plant species examined. The roots were washed, cut into sections of $\leq$0.5 cm in length, mixed well, and divided into 5.0-g portions (fresh weight), which was the amount of material used for each extraction method. For most experiments, 7 to 9 different extraction methods were compared, and these served as the treatments, each replicated 8 times. Unless specified otherwise, extractions were carried out in the laboratory at a temperature of ca. 25 C. Extraction methods used were as follows:

Blender (short) + centrifuge. This method was similar to the root

extraction technique for swollen stages developed by Coolen and D'Herde (4), with the following modifications. The root sample was placed in 200 ml water and macerated for 15 sec at the lowest speed in a Waring® Futura II blender. The mixture was then washed through a 850-μm sieve and the residues caught on a 25-μm mesh sieve. Residues were centrifuged in water in 50 ml centrifuge tubes without kaolin in a Damon® IEC Clinical Centrifuge for 5 min at a relative centrifugal force (R.C.F.) of 1610 G. Residues were resuspended in a sucrose solution (sp. gr. = 1.13) and centrifuged under similar conditions for one min. The supernatant was then poured onto a 25-μm mesh sieve and the nematodes retained were counted.

Blender (long) + centrifuge. This was identical to the previous method, except that roots were macerated in the blender for 120 sec, the length of time recommended by Coolen and D'Herde (4) for extraction of eggs and mobile stages.

Blender + funnel (short). The procedure used here was similar to that which Gowen and Edmunds (6) used to extract nematodes from banana roots. Root samples in 100 ml of water were macerated for 10 sec in the blender and the resulting mixture was suspended in a Baermann funnel constructed from Kimwipes® Disposable Wipers in glass funnels. Ten ml of 3% H_2O_2 were added to each funnel, which was then drained after 2 days.

Blender + funnel (long). This was identical to the previous method, except that funnels were drained after 5 instead of 2 days.

Mist chamber. A mist extraction system was constructed according to the specifications outlined by Ayoub (1). Root samples were placed on Kimwipes Disposable Wipers in the funnels and a 1.5-min-on/8.5-min-off mist cycle was maintained for 5 days. The temperature of the water used in the mist system was 26.5 C.

Incubation in water. The incubation of root material in water was similar to that described by Chapman (3). Root samples were incubated in 200 ml of aerated water in 250 ml beakers for a period of 7 days. Aeration was accomplished by bubbling a continuous stream of air through the beakers with aquarium pumps. After the incubation period, the mixture of water, roots, and nematodes was poured through a piece of cheesecloth to remove the roots, which were rinsed well. The filtrate was concentrated into glass vials for counting.

Plastic bag. Root samples incubated in sealed plastic bags (12) containing 10 ml of 3% H_2O_2 were maintained for 5 days. After this time, the bags were washed out with water and the mixture washed through a 850-μm mesh sieve to remove the roots. The filtrate was then passed

through a 25-μm mesh sieve, and the residue containing the nematodes transferred to vials for counting.

Mason jar. Root samples were placed in Mason jars (14) for an incubation period of 5 days. To retain consistency with the plastic bag method, 10 ml of 3% H_2O_2 was added to each jar. After 5 days, jars were rinsed with water and the contents filtered and prepared for counting as in the plastic bag method.

Sodium hypochlorite. This method, intended primarily for extracting nematode eggs, was identical to that developed by Hussey and Barker (8), using a 1.05% NaOCl solution.

Extraction experiments were planned so that data could be obtained for the following endoparasitic and semi-endoparasitic nematodes: *Helicotylenchus multicinctus, Hemicriconemoides mangiferae* Siddiqi, *Meloidogyne incognita* (Kofoid & White) Chitwood, *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuurmans Stekhoven, *Pratylenchus coffeae* (Zimmermann) Filipjev & Schuurmans Stekhoven, and *Tylenchulus semipenetrans.* The host plants used and the sampling dates varied (Table 1). With the exception of squash, all hosts were established plants at least 8 months old, and for each of these hosts, 7 to 9 different extraction methods were examined on each sampling date.

The experiment with squash plants differed somewhat from the other experiments, since the test involved plants grown in plastic pots in a shadehouse. The plants were grown from seed planted on 16 Dec., 1983, in a soil mix infested with *M. incognita.* On 6 Jan., 1984, the plants were removed, the root systems washed, cut into 0.5-cm-long pieces, mixed, and divided into 14 portions of 0.5 g each. Extraction of 7 of these portions was by the plastic bag method and extraction of the other 7 portions was by the blender (short) + centrifuge method. The objective of this small experiment was to insure that any *M. incognita* larvae extracted originated as endoparasites within the root tissue and not from eggs that may have hatched during the incubation process from egg masses attached to the roots. When roots of this experiment were harvested, only a few very slight swellings on the roots were observed, and no mature egg masses were noted.

For each experiment, nematodes obtained for each extraction sample were maintained in glass vials, killed by gentle heat, and 1/25 of each sample counted shortly thereafter. Results were analyzed by an analysis of variance followed by mean separation by the Waller-Duncan K-ratio t-test (7).

RESULTS AND DISCUSSION

The relative efficiency of several methods in extracting *Pratylenchus*

Table 1. Host plants and nematode species examined by various extraction techniques.

Host plant	Sampling date	Nematodes extracted	Mean temperature (°C)[x]	
			Sampling date	Week preceeding[y]
Pummelo, *Citrus grandis* (L.) Osbeck	21 Sept. 1983	*Tylenchulus semipenetrans* *Pratylenchus coffeae*	26	28
Banana, *Musa* x 'Dwarf Cavendish' (AAA Group)	5 Oct. 1983	*Helicotylenchus multicinctus* *Meloidogyne incognita*	27	27
Longan, *Euphoria longana* Lam.	9 Nov. 1983	*Hemicriconemoides mangiferae*	21	22
Banana, *Musa* x 'Burro' (ABB Group)	30 Nov. 1983	*Helicotylenchus multicinctus* *Meloidogyne incognita*	23	23
Costa Rican Guava, *Psidium friedrichsthalianum* (Beng.) Nied.	7 Dec. 1983	*Pratylenchus brachyurus*	24	24
Cassava, *Manihot esculenta* Crantz, cv. Senorita	14 Dec. 1983	*Meloidogyne incognita*	17	21
Banana, *Musa* x 'Burro' (ABB Group)	28 Dec. 1983	*Helicotylenchus multicinctus*	16	17
Grapefruit, *Citrus paradisi* Macf.	4 Jan. 1984	*Tylenchulus semipenetrans*	16	17
Squash, *Cucurbita pepo* L., cv. Early Yellow Summer Crookneck	6 Jan. 1984	*Meloidogyne incognita*	13	16
Cassava, *M. esculenta*, cv. Senorita	11 Jan. 1984	*Meloidogyne incognita*	17	16

[x]Air temperatures obtained from a weather station located at the Tropical Research and Education Center, Homestead, Florida.

[y]Mean of 7 days preceeding the sampling date.

spp. from roots of two hosts is shown (Table 2). The mist chamber was among the best methods for both hosts, but the short blender maceration + centrifuge was comparable for pummelo, while the long blender maceration + centrifuge was best when Costa Rican guava was the host. In either case, modifications of Coolen and D'Herde's (4) blender + centrifuge technique were comparable to mist extraction as the best methods. The discrepancy between the long (120 sec) blender maceration and the short (15 sec) maceration is not surprising, since the roots of Costa Rican guava are relatively hard and more difficult to macerate than the relatively soft small citrus feeder roots. Blender times should be adjusted in relation to the hardness or softness of roots since this combination of factors can affect recovery (4). Other methods were not as satisfactory for recovery of *Pratylenchus* spp. as the mist chamber or blender + centrifuge.

The blender (long) + centrifugation was generally superior to other methods for recovery of *Helicotylenchus multicinctus* from banana roots (Table 3). In one instance, incubation in water gave similar results, but in general, the relative performance of the other methods was intermediate or inconsistent. A long blender time was preferable to a short time for

Table 2. Comparison of various methods for extraction of *Pratylenchus* spp. from plant roots.

Method	*Pratylenchus* spp.[x] per gram of fresh root by host[y]	
	Pummelo	Costa Rican Guava
Mist chamber	78 a	72 ab
Blender (long) + centrifuge	45 b	88 a
Blender (short) + centrifuge	85 a	54 bc
Incubation in water	27 bc	53 c
Plastic bag	27 bc	28 d
Mason jar	0 d	30 d
Blender + funnel (long)	17 cd	40 cd
Blender + funnel (short)	10 cd	—
Sodium hypochlorite	0 d	—

[x]*Pratylenchus coffeae* from pummelo; *P. brachyurus* from Costa Rican guava.

[y]Means followed by the same letter are not significantly (P = 0.05) different, according to Waller-Duncan test. A dash (—) indicates method not evaluated.

Table 3. Comparison of various methods for extraction of *Helicotylenchus multicinctus* from banana roots.

Method	*H. multicinctus* per gram of fresh root by sampling date[a]		
	5 Oct.	30 Nov.	28 Dec.
Blender (long) + centrifuge	816 a	604 a	1062 a
Blender (short) + centrifuge	602 b	258 bc	893 b
Incubation in water	311 cd	580 a	477 c
Mist chamber	378 c	366 b	461 c
Plastic bag	292 cd	199 c	362 c
Mason jar	246 cd	318 bc	362 c
Blender + funnel (long)	615 b	27 d	40 d
Blender + funnel (short)	185 d	—	—
Sodium hypochlorite	10 e	—	—

[a]Means followed by the same letter are not significantly (P = 0.05) different, according to Waller-Duncan test. A dash (—) indicates method not evaluated.

extracting this species. Even though the roots were relatively soft in consistency, a 15-sec blender time was insufficient for complete maceration of the roots.

Recovery of *Hemicriconemoides mangiferae* from roots of longan was low (results not shown). Average numbers of nematodes extracted by the blender (long) + centrifuge, blender (short) + centrifuge, and mist chamber methods were 0.6, 1.0, and 0.4 nematodes per gram fresh root weight, respectively. No nematodes were recovered by the other methods. Even though the blender + centrifuge methods were best, numbers were still too low for meaningful comparisons. Frequently these methods produced broken specimens, since the nematode is semi-endoparasitic in its association with the roots, and therefore exposed to damage by the blender.

The plastic bag method developed by Tarjan for extraction of *T. semipenetrans* from citrus roots (12) was among the best methods for extracting larvae and females from citrus roots (Table 4). A short blender maceration followed by centrifugation gave comparable results, the two methods not being significantly different. Incubation in water was comparable for recovery of larvae on one sampling date, but in general, other methods yielded significantly lower numbers of nematodes. The good recovery of females by the plastic bag method, essentially an

Table 4. Comparison of various methods for extraction of *Tylenchulus semipenetrans* from *Citrus* roots.

Method	*T. semipenetrans* per gram of fresh root[x]			
	Larvae		Females	
	21 Sept.	4 Jan.	21 Sept.	4 Jan.
Plastic bag	234 ab	1939 a	58 a	244 a
Blender (short) + centrifuge	216 abc	1710 ab	56 a	239 a
Blender (long) + centrifuge	154 bcd	1341 cd	27 b	130 c
Mason jar	43 e	1459 bc	6 c	185 b
Incubation in water	278 a	1121 de	0 c	51 d
Mist chamber	132 cd	934 e	0 c	16 de
Blender + funnel (long)	41 e	257 f	0 c	4 e
Blender + funnel (short)	36 e	—	0 c	—
Sodium hypochlorite	90 de	—	2 c	—

[x]Means followed by the same letter are not significantly (P = 0.05) different, according to Waller-Duncan test. A dash (—) indicates method not evaluated.

incubation technique, was unexpected and may have resulted from the washing of the roots following incubation in the plastic bags. Yet recovery of females by incubation in Mason jars or in water was poor, as expected.

Recovery of *Meloidogyne incognita* larvae from banana or cassava host tissue did not show consistent patterns with the methods used (Table 5). Contradictory results were obtained with cassava, depending on the sampling date. On the earlier sampling date, techniques which involved various methods of incubation were superior to the mechanical methods using the blender + centrifuge. On the later sampling date, however, following a period of cold weather, the blender + centrifuge methods were significantly better than the other methods. The blender + centrifuge method modified from Coolen and D'Herde (4) was always among the most effective techniques for extracting the other nematode species sampled here. The inconsistency obtained with *M. incognita* suggested that further investigation was needed. Since many eggs are present in the mature egg masses of this species, it is possible that some of these eggs may hatch if kept in water for a period of time, as would happen if incubation was the primary method used, whether it be in water, plastic bags, Mason jars, or a mist chamber. These recently hatched eggs could then be counted as larvae, possibly inflating the larval extraction

Table 5. Comparison of various methods for extraction of *Meloidogyne incognita* larvae from plant roots by host and sampling date.

Method	*M. incognita* larvae per gram of fresh root[a]			
	Banana		Cassava	
	5 Oct.	30 Nov.	14 Dec.	11 Jan.
Blender (long) + centrifuge	284 b	32 a	56 b	44 a
Plastic bag	169 c	19 ab	91 a	20 b
Blender (short) + centrifuge	312 ab	8 ab	28 c	46 a
Mason jar	281 b	11 ab	91 a	22 b
Incubation in water	112 c	19 ab	74 ab	17 bc
Mist chamber	126 c	18 ab	66 ab	9 cd
Blender + funnel (long)	398 a	2 b	4 c	1 d
Blender + funnel (short)	178 c	—	—	—
Sodium hypochlorite	5 d	—	—	—

[a]Means followed by the same letter are not significantly (P = 0.05) different, according to Waller-Duncan test. A dash (—) indicates method not evaluated.

totals of less efficient methods. Thus, a short experiment with the squash plants was performed to compare extraction of larvae in plants without mature egg masses. An average of 93 larvae/gm of fresh root was recovered by the short blender maceration followed by centrifugation, while only 14 larvae/gm were recovered following incubation in plastic bags (one of the best incubation methods), significantly (P = 0.001) less according to a t-test. Thus it may be that the blender + centrifuge method is actually better than incubation methods in recovering *M. incognita* larvae from tissues, particularly the less mobile stages, and that counts obtained by the latter method can be inflated by hatched eggs.

The blender + centrifuge method is also quite useful for extracting nematode eggs from root tissues (4), but it was not as effective as the sodium hypochlorite method of Hussey and Barker (8) when the techniques were compared here (Table 6). The blender + centrifuge method was the most consistent method in extracting a wider variety of life stages, although in these experiments, females of *M. incognita* were usually broken and no counts were made. Besides efficiency, the method has the additional advantage in that life stages can be assayed as they occur in the root. With incubation methods, development, egg hatch, and reproduction can occur during the extraction process, so that the resulting nematode stages that are counted may be different from those initially present.

Table 6. Comparison of several methods for extraction of nematode eggs from root tissue for several hosts and sampling dates.

Method	Nematode eggs per gram of fresh root[a]			
	Tylenchulus semipenetrans Pummelo 21 Sept.	*Meloidogyne incognita* Banana 5 Oct.	*Meloidogyne incognita* Banana 30 Nov.	*Meloidogyne incognita* Cassava 14 Dec.
Sodium hypochlorite	2810 ab	345 a	369 a	2775 a
Blender (long) + centrifuge	2630 b	198 b	138 b	1222 b
Blender (short) + centrifuge	2975 a	112 c	34 b	738 c

[a]Means followed by the same letter are not significantly (P = 0.05) different, according to Waller-Duncan test.

There are several disadvantages to the Coolen and D'Herde method and its modifications. One is that recovery will vary with the length of the maceration time, the extent of this depending on the nature of the roots used. If this method is to be used routinely for a given nematode-crop combination, then the relationship between numbers recovered and blender time must be optimized, as Coolen and D'Herde did for a number of examples (4). The method includes many steps and is therefore more difficult and time consuming to carry out than most of the other methods tested, some of which require very little labor. In addition, use of a blender + centrifugation produces much debris in the samples to be counted. This is a problem with many of the other methods as well, but samples collected in the mist chamber were by far the cleanest. Thus, if specimens were needed for taxonomic studies, that method may be superior. For quantitative work, however, methods involving incubation will usually not be as efficient as those based on maceration and centrifugation, and the performance of the various incubation techniques may vary more with the nematode and crop combination. For *T. semipenetrans*, incubation in plastic bags worked as well as maceration and centrifugation, but for *Pratylenchus* spp., incubation in a mist chamber gave results comparable to maceration and centrifugation.

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ACKNOWLEDGEMENTS

The authors are grateful to Dr. V.H. Quimi for his help and suggestions with the preliminary investigations which preceded this work. We are also grateful to Mrs. Callie Sullivan for typing the manuscript and to Mr. Mike Roessner and Mr. Charles Brunson for the construction of the mist chamber.

Received for publication:
14.III.1984
Recibido para publicar:

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[1]Florida Agricultural Experiment Stations Journal Series No. 5466.