Extraction of Root-associated *Meloidogyne incognita* and *Rotylenchulus reniformis*[1]

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Abstract: A technique based on physical maceration of root tissue was developed to extract vermiform and swollen stages of *Meloidogyne incognita* and *Rotylenchulus reniformis*. Experiments conducted on soybean and tomato evaluated the efficiency of method (stir, grind), NaOCl concentration (0%, 0.5%), and duration (1×, 2×) on extraction of nematodes and eggs from 60-day-old populations. Root-associated populations of *R. reniformis* were considerably lower than those of *M. incognita*, so development of the method focused on the latter. Grinding liberated more nematodes than stirring, but the reverse was true for egg extraction. Among grinding treatments, a duration of 10 seconds in 0.5% NaOCl provided the most efficient extraction of nematodes and eggs. Among stirring treatments, a duration of 10 minutes in 0.5% NaOCl provided the most efficient extraction of eggs. These techniques were compared on soybean roots 30 days older than those on which the procedures were first evaluated, with consistent results.

Key words: extraction, grinding, maceration, *Meloidogyne incognita*, method, nematode, reniform nematode, root-knot nematode, *Rotylenchulus reniformis*.

Studies involving phytoparasitic nematodes require accurate enumeration of multiple species from both the soil and root microenvironments. Changes in soil populations may reflect migrations out of or into root systems rather than actual changes in reproduction. The number of eggs extracted using the stirring procedure described by Hussey and Barker (1973) is often included in population assessments of phytoparasitic nematodes (Hirunsalee et al., 1995; Kirkpatrick et al., 1995; Montalvo and Esnard, 1994; Robbins et al., 1994; Sankaralingam and McGawley, 1994; Starr and Black, 1994; Walters and Barker, 1994; Weibelzahl-Fulton et al., 1996). Whereas this method provides an accurate population assessment for monospecific studies, it is not feasible in studies involving two or more species because of a lack of diagnostic features in egg morphology between species.

Many of the protocols for quantifying root-associated nematodes have limitations.

Baermann funnels can extract migratory nematodes from root tissue (MacGuidwin and Forge, 1991; Vrain, 1977) and soil (Kotcon et al., 1987; Robinson and Heald, 1989, 1991). This method does not work for sedentary endoparasites of plants. Clearing root tissue and staining endoparasitic nematodes (Byrd et al., 1983) also has been used routinely (Halbrendt et al., 1992; Hirunsalee et al., 1995; Zhang and Schmitt, 1995). However, staining may mask anatomical details required for identification of species or developmental stages. Additionally, it can be difficult to count individuals in galled tissue. Dislodging nematodes through enzymatic maceration of host tissue can be an effective method (Araya and Caswell-Chen, 1993; Dickson et al., 1970; Dropkin et al., 1960; Hussey, 1971; Kaplan and Davis, 1990). However, this technique can be time-consuming, as constant agitation of plant material in the enzyme solution for 5 to 36 hours is needed.

Physical maceration of host tissue in a blender used in conjunction with centrifugation was considered a reliable extraction method for eggs of *Meloidogyne incognita* (Kofoid & White) Chitwood (McClure et al., 1973), eggs and vermiform stages of *Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans Stekhoven (Dunn, 1973), and all developmental stages of *Tylenchulus semipenetrans* Cobb (Greco and D'Addabbo, 1990). The objective of this investigation was to es-

tablish a similar procedure to liberate vermiform and swollen stages (nematodes) of root-knot (*Meloidogyne incognita* race 2) and reniform nematodes (*Rotylenchulus reniformis* Linford & Oliviera) through mechanical disruption of the root tissue. Another objective was to compare the efficiency of the devised procedure with that of the standard egg extraction procedure (Hussey and Barker, 1973).

MATERIALS AND METHODS

General procedures: Tomato (*Lycopersicon esculentum* L. 'Rutgers') and soybean (*Glycine max* (L.) Merrill 'Davis') plants were grown in a greenhouse, where temperatures ranged from 22 °C to 35 °C. Supplemental incandescent and fluorescent lighting (ca. 260 $\mu E \cdot s^{-1} \cdot m^{-2}$) provided a minimum of 14 hours of light daily. These studies used 15-cm-diam. clay pots with approximately 1.6 kg of a soil mixture composed of three parts fumigated (67% methyl bromide, 33% chloropicrin) Convent silt loam soil (Aeric Fluvaquent, coarse-silty, mixed, non-acid, thermic) to two parts autoclaved sand.

Soybean seeds were treated with a commercial preparation of *Bradyrhizobium japonicum* (Kirchner) Jordan (The Nitragin Co., Milwaukee, WI) and sown in flats. Seedlings of uniform size were selected when plants were at growth stage V_1 (Fehr et al., 1971) and transplanted to each test pot. Tomato seeds were sown in flats, and 3- to 4-week-old seedlings were transplanted. Plants were fertilized 3 days after transplanting with 120 ml of a 23-19-17 N-P-K fertilizer solution (RapidGro; Chevron Chemical Co., San Ramon, CA). Plants received approximately 26 ppm N, 20 ppm P, and 33 ppm K.

Root-knot and reniform nematode isolates were derived from single egg masses and maintained on tomato cv. Rutgers in a greenhouse. Inoculum consisted of 1,000 vermiform nematodes extracted from the soil of 60- to 90-day-old cultures by hand-sieving and centrifugal flotation (Jenkins, 1964). Soil in each pot was infested by pipetting nematodes suspended in tap water into two depressions made in the soil. Each depression was 1 cm in diameter and 4 cm deep. After infestation, the depressions were filled with additional soil mix.

At harvest, root systems were freed from soil by gentle washing in tap water. Root systems were cut into 2.5-cm segments, and subsamples were selected at random for nematode extraction by stirring (Hussey and Barker, 1973) or grinding in 60 ml of water or 0.5% NaOCl. Root tissue was ground at maximum speed in a Waring commercial blender (model 31BL42) fitted with a 500-ml pulverizing container. The contents of the beaker (stirred treatments) or pulverizing container (ground treatments) were poured onto nested 75- and 25-μm-pore sieves. Nematodes and eggs collected on the 25-μm-pore sieve were counted.

Experiment 1: The objectives of this experiment were to optimize a grinding method for extracting root-knot and reniform nematodes from tomato and soybean roots and to compare the efficiency of stirring and grinding methods for extracting eggs. The experiment was conducted twice. Root-knot and reniform nematodes were introduced into the soil in pots containing three seedlings of uniform size. Monospecific populations were allowed to develop for 59 days (Test 1) or 64 days (Test 2). At harvest, the root tissue from all pots sharing the same nematode and host was combined and subsampled (1.5 g). Within each nematode species and host, treatments were combined in a factorial arrangement and assigned in a completely randomized design. Treatments were: method (stir, grind), carrier (water, 0.5% NaOCl), and duration (1×, 2×). Durations were 5 seconds and 5 minutes for the 1× level in ground and stirred treatments, respectively. Treatments were replicated four times. Data were tested with analysis of variance (ANOVA) and contrasts ("Fit Model" module of SAS JMP version 3.0) (SAS Institute, Cary, NC). Interactions that were significant on two or more occasions are presented as figures; those that occurred only once are described in the text.

Experiment 2: Results from Experiment 1 indicated that root-associated populations of reniform nematode were consistently small.

Therefore, Experiment 2 employed only root-knot nematode. The objective was to compare the optimized grinding procedure with the standard stirring extraction method for liberating nematodes and eggs from soybean roots of older plants. The experiment was conducted twice. One soybean seedling was transplanted into each of 10 test pots. One thousand second-stage juvenile (J2) root-knot nematodes were introduced into the soil in each pot, and populations were allowed to develop for 91 days (Test 1) or 93 days (Test 2). At harvest, two subsamples (2 g) were taken from the root system in each pot and either stirred in 0.5% NaOCl for 10 minutes or ground in 0.5% NaOCl for 10 seconds. Data were subjected to analysis of variance (ANOVA) ("Fit Model" module of SAS JMP version 3.0) (SAS Institute, Cary, NC).

RESULTS

Experiment 1: Root-associated populations of reniform nematode were considerably smaller than populations of root-knot nematode on both tomato and soybean (Tables 1 and 2). Egg and nematode counts for root-knot nematode were higher for tomato than for soybean, whereas the two hosts yielded similar numbers of reniform nematodes and eggs (Tables 1 and 2). Numerous test-by-treatment interactions were detected in the initial analyses, so each test was analyzed independently.

Stirring and grinding treatments liberated equivalent numbers of reniform nematodes (Table 1). Carrier and duration of treatment did not the influence number of nematodes extracted. A carrier-by-duration interaction was detected on tomato in Test 1 ($P \leq 0.01$). When treatments were processed in water, more nematodes were liberated from treatments of longer duration ($P \leq 0.05$). Treatments processed in 0.5% NaOCl liberated equivalent numbers of nematodes from the 1× and 2× durations ($P \leq 0.05$).

Stirring liberated significantly more intact reniform eggs than grinding from tomato roots in Test 1 ($P \leq 0.05$) (Table 1). On all other occasions, the methods liberated equivalent numbers of intact eggs. Carrier and duration of treatment did not influence the number of eggs extracted. Examination of a method-by-carrier interaction on tomato in Test 1 showed that 0.5% NaOCl increased extraction of eggs only for stirred treatments ($P \leq 0.01$).

Grinding liberated more root-knot nematodes than stirring for soybean and tomato

TABLE 1. Effect of treatment method, carrier, and duration on extraction of vermiform or swollen individuals (nematodes) or eggs of *Rotylenchulus reniformis* from tomato or soybean roots in two tests.

Treatment	Level	Nematodes per g fresh root tissue				Eggs per g fresh root tissue			
		Soybean		Tomato		Soybean		Tomato	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Method (M)	Stir	8	3	15	1	40	13	38	3
	Grind	4	6	12	4	14	11	17	3
Carrier (C)	Water	6	4	16	1	19	7	24	3
	0.5% NaOCl	5	4	12	4	36	17	31	3
Duration (D)	1×[a]	6	3	11	4	38	17	34	6
	2×	6	6	17	1	17	7	21	0
Source									
M		NS	NS	NS	NS	NS	NS	*	NS
C		NS	NS	NS	NS	NS	' NS	NS	NS
D		NS	NS	NS	NS	NS	NS	NS	NS
M × C		NS	NS	NS	NS	NS	NS	**	NS
M × D		NS	NS	NS	NS	NS	NS	NS	NS
C × D		NS	NS	**	NS	NS	NS	NS	NS
M × C × D		NS	NS	NS	NS	NS	NS	NS	NS

Data are means of four replicates.
[a] 1× = 5 seconds or 5 minutes in grinding or stirring treatments, respectively.

TABLE 2.   Effect of treatment method, carrier, and duration on extraction of vermiform or swollen individuals (nematodes) or eggs of *Meloidogyne incognita* race 2 from tomato or soybean roots in two tests.

Treatment	Level	Nematodes per g fresh root tissue				Eggs per g fresh root tissue			
		Soybean		Tomato		Soybean		Tomato	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Method (M)	Stir	40	59	98	158	2,613	966	5,442	1,232
	Grind	78	226	124	420	738	427	1,897	686
Carrier (C)	Water	45	114	74	294	182	92	448	195
	0.5% NaOCl	72	171	148	284	3,169	1,301	6,892	1,723
Duration (D)	1×[a]	54	112	121	192	2,023	620	2,557	856
	2×	64	174	100	386	1,328	773	4,783	1,062
Source of variation									
M		NS	***	NS	***	***	*	***	NS
C		NS	NS	*	NS	***	***	***	***
D		NS	NS	NS	**	NS	NS	**	NS
M × C		NS	NS	NS	NS	***	*	***	*
M × D		NS	NS	NS	NS	*	NS	**	NS
C × D		NS	NS	NS	NS	*	NS	**	NS
M × C × D		NS	NS	NS	NS	*	NS	**	NS

Data are means of four replicates.
[a] 1× = 5 seconds or 5 minutes in grinding or stirring treatments, respectively.

in Test 2 ($P \le 0.001$) (Table 2). More nematodes were extracted from treatments processed in 0.5% NaOCl for tomato roots in Test 1 ($P \le 0.05$) than from those processed in water. Doubling the duration of treatment resulted in more nematodes extracted from tomato roots in Test 2 ($P \le 0.01$).

More intact root-knot nematode eggs were extracted from stirred treatments than from ground treatments on three occasions ($P \le 0.05$) (Table 2). On all four occasions, more eggs were extracted from treatments processed in 0.5% NaOCl ($P \le 0.001$) than in water. The longer treatment duration yielded more eggs from tomato in Test 1 than the shorter duration ($P \le 0.01$). A three-way interaction involving method, carrier, and duration was detected on both hosts in Test 1 ($P \le 0.05$) (Fig. 1). For both hosts, duration of stirring influenced egg extraction only when treatments were processed in 0.5% NaOCl. On soybean, more eggs were extracted from treatments of shorter duration than longer duration (Fig. 1). On tomato, more eggs were extracted from treatments of longer duration than shorter duration (Fig. 1). In addition, 0.5% NaOCl improved extraction of eggs from ground tomato roots only for treatments of longer duration (Fig. 1). Examination of a

method-by-carrier interaction for both hosts in Test 2 showed that processing treatments in 0.5% NaOCl improved egg yield more from stirred treatments than from ground treatments ($P \le 0.05$) (Fig. 2).

Experiment 2: Findings from Experiment 1 indicated that extraction of root-knot nematodes by grinding occasionally was improved by using 0.5% NaOCl instead of water as a carrier and by increasing the duration of grinding to 10 seconds. This optimized grinding treatment yielded almost three times as many nematodes as stirring in 0.5% NaOCl for 10 minutes ($P \le 0.05$) (Table 3). Again, stirring liberated more intact eggs than did grinding ($P \le 0.05$) (Table 3).

DISCUSSION

Grinding root tissue in 0.5% NaOCl for 10 seconds gave optimum extraction of vermiform and swollen root-knot nematodes from tomato and soybean roots supporting populations for approximately 60 days. This technique was applied to soybean roots 30 days older than those on which the procedure was first evaluated with consistent results. When used to extract reniform nematodes from host roots supporting comparatively lower populations, the technique did not improve extraction efficiency.
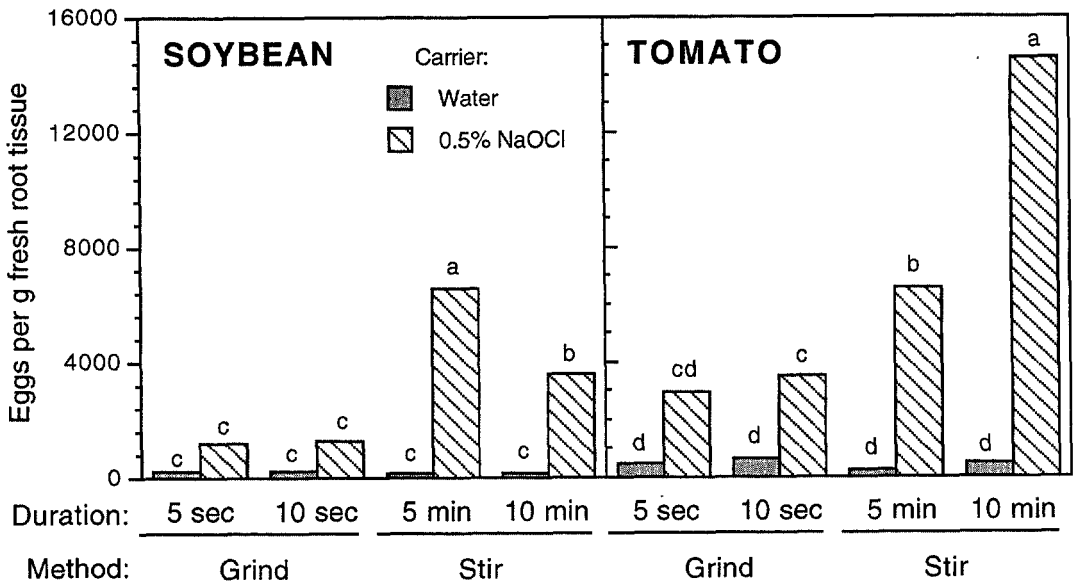
FIG. 1. Influence of treatment method, duration, and carrier on number of *Meloidogyne incognita* race 2 eggs extracted from soybean and tomato roots in Test 1. Data are means of four replicates. Within figures, means designated with the same letter do not differ ($P \le 0.05$).

Araya and Caswell-Chen (1993) reported extraction of 194 to 706 juveniles and females of *Meloidogyne javanica* (Trueb) Chitwood per gram of root tissue with enzymatic maceration, though numbers varied depending on the host plant species. The grinding procedures used in this study liberated 78 to 420 juveniles and adults of *M.*

incognita per gram of tissue from two host species. The number of individuals extracted by grinding appears to be within the range reported for enzymatic digestion, although caution must be used in drawing conclusions from such different experiments. However, the data suggest that the grinding technique is a quick, simple, and
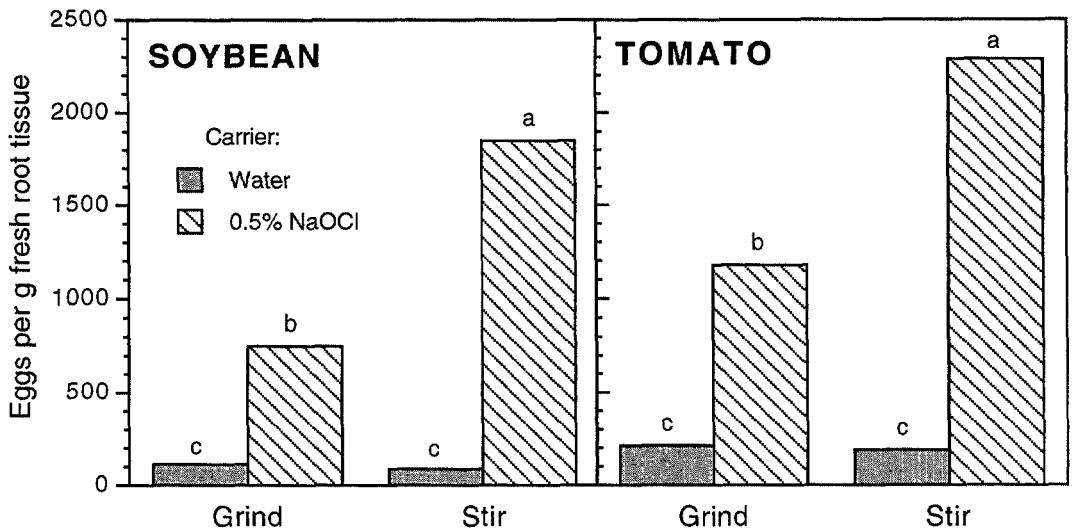

FIG. 2. Influence of treatment method and carrier on number of *Meloidogyne incognita* race 2 eggs extracted from soybean and tomato roots in Test 2. Data are means of four replicates. Within figures, means designated with the same letter do not differ ($P \le 0.05$).

TABLE 3. *Meloidogyne incognita* race 2 nematodes and eggs extracted from Davis soybean roots by stirring for 10 minutes in 0.5% NaOCl or by grinding for 10 seconds in 0.5% NaOCl.

Treatment	Level	Nematodes per g	Eggs per g
Test (T)	1	122	1,779
	2	21	201
Method (M)	Grind	107	573
	Stir	36	1,407
Source of variation			
T		**	***
M		*	*
T × M		NS	NS

Data are means of 20 replicates in two tests.

inexpensive alternative to the enzymatic maceration procedure for extracting juvenile and adult root-knot nematodes.

For extraction of eggs, the grinding procedure was not an improvement over the standard extraction procedure described by Hussey and Barker (1973) or the enzymatic digestion method described by Araya and Caswell-Chen (1993) in terms of the number of eggs recovered. The results of the current study confirmed the efficiency of stirring in 0.5% NaOCl, though the relative impact of the duration of this treatment was not consistent. Grinding takes about 20% of the time that stirring does but liberates only about 37% of the eggs. In situations where it is not necessary to extract "every" egg (e.g., inoculum preparation, qualitative comparisons), a substantial amount of time may be saved using the grinding method. Even though three grinding events are required to yield approximately as many eggs as one stirring event, the processing would be completed in 40% less time. Where circumstances call for extraction of as many developmental stages as possible, a single sample may be processed first by stirring and subsequently by grinding, providing a more complete assessment of root-associated nematode populations.

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