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Enzymatic Digestion of Roots for Recovery of Root-knot Nematode Developmental Stages[1]

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Abstract: Developmental stages of *Meloidogyne javanica* were successfully released from roots by treatment with commercially available cellulase and pectinase. The average percentage recovery of nematode developmental stages from *Dolichos lablab, Elymus glaucus,* and *Lycopersicon esculentum* were as follows: eggs = 526%, J2 = 272%, J3 = 783%, J4 = 549%, adult females = 285%, and total = 425%, expressed as percentages of the counts obtained from stained roots spread on glass plates. Root digestion was more accurate and sensitive in detecting low numbers of nematodes in roots than was the glass plate method. No simple linear, quadratic, or cubic relationship was found between the two methods that would allow a conversion factor to be developed.

Key words: cellulase, demography, developmental stage, digestion, enzyme, extraction, *Meloidogyne javanica,* nematode, pectinase, root maceration.

Nematode management decisions are based on information about nematode population responses to control tactics, including changes in population density, growth rate, structure, and response time. Nematode population dynamics are the aggregate result of transition probabilities between stages of the nematode life cycle and fecundity. The characterization of population response (the number of eggs, juveniles, and adults over time) is especially important for research on nematode population dynamics or demography. The design of integrated management practices demands understanding of how individual control tactics affect the stages of the nematode life cycle.

Stage-specific responses of plant-parasitic nematodes to specific management practices are only beginning to be investigated and need detailed characterization. For example, different sources of resistance in soybean affect different developmental stages of *Heterodera glycines* (7). To measure nematode population-level responses, accurate enumeration of all nematode stages in a sample is necessary.

Current protocols for assessing the numbers of each life stage of *Meloidogyne* spp. within roots are inadequate. A method for nematode extraction from roots or soil is of quantitative value only when it provides an accurate estimation of the true nematode number present in a sample with minimum error (2). Commonly utilized methods achieve a recovery from roots or soil of less than half of the original nematode inoculum, and the variation obtained is generally too large for the use of correction factors (1,2).

Typically, the number of nematodes within roots is determined by observing cleared and stained roots sandwiched between glass plates for counting (3,9). The glass plate method is time consuming and does not yield accurate results when nematode numbers are high; in addition, certain developmental stages (J3, J4) are difficult to identify in roots.

Although there have been few advances in methodology for extracting nematodes from roots, adult female root-knot nematodes are readily released from roots (4–6). However, the methods (4–6) have not been used for quantitative extraction of all nematode developmental stages. Our objective was to compare the standard root staining and glass plate method for counting root-knot nematodes with a method that uses staining followed by enzymatic

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digestion to release root-knot nematodes from roots.

Materials and Methods

General procedures: All experiments involved the direct comparison of nematode counts obtained from stained roots held between glass plates with counts obtained by digesting those same roots with enzymes by one of four methods evaluated in numeric order. The methods were sequentially developed and assessed as problems in the recovery of root-knot developmental stages were detected.

Dolichos lablab cv. Highworth seeds were germinated on moist Whatman No. 4 filter paper in a 9.0-cm-d petri dish for 4 days at 30 C. Two germinated seeds were transplanted into each of five 53-liter black plastic pots filled with sterilized, washed mortar sand. *Elymus glaucus* cv. Berkeley seeds (50 per pot) were germinated in five 53-liter black plastic pots. The pots were placed in a lath house, irrigated with filtered water as necessary, and fertilized twice a week with Miracle-Gro fertilizer (Stern's Miracle-Gro Products, Port Washington, NY). When plants were 45 days old, they were inoculated with *M. javanica* J2 obtained from hydroponic culture (11). Using three inoculations at 2-day intervals, a total of ca. 106,000 J2 were added to the soil surface of each pot for an initial population density (Pi) of ca. 2.0 J2/cm^3 of sand. Approximately 90 days after the last inoculation, plants were gently uprooted, roots were rinsed free of sand, and 0.25 g of roots were randomly selected from each pot and were cleared and stained (3,9) in 20-ml glass scintillation vials.

Lycopersicon esculentum cv. UC 204 C (Sunseeds Genetics, Hollister, CA) seeds were germinated identically to the *D. lablab* seeds. Three germinated seeds were transplanted into each of five 3.7-liter black plastic pots filled with sterilized washed mortar sand. Pots were placed in a greenhouse maintained at ca. 24 C, and the plants were watered as necessary and fertilized twice weekly with Hoagland's solution. After the plants were 4 weeks old, they were inoculated three times at 15-day intervals with a suspension of at least 15,000 *M. javanica* J2 collected from hydroponic culture (11). The inoculations (45,000 J2 total) assured the presence of different growth stages in the roots at sampling. Sixty days after the first inoculation, the plants were gently uprooted and roots were rinsed free of sand. Randomly selected 0.25-g root samples were cleared and stained (3,9) in 20 ml glass scintillation vials. After the roots were stained, the four different methods were compared by determining the numbers of each nematode developmental stage counted from roots.

Method 1—Basic enzymic digestion: This basic protocol was varied in methods 2–4. *Elymus glaucus* root segments (ca. 1 mm) were spread on a glass plate (7.5 × 5.0 cm), and a second glass plate was placed on top of the first with moderate pressure so that the roots were sandwiched between the glass plates. The nematode stages counted were eggs, J2, J3 + J4, and adult females (13). After the nematodes were counted on the glass plates with an inverted compound microscope at 4–40×, the roots together with the 5 ml of stain solution from the vials were transferred into 50-ml polycarbonate centrifuge tubes and treated with 3 ml of the following stock solutions in milli-Q-water (Millipore, Bedford, MA): cellulase from *Penicillium funiculosum* 0.83 units/ml (Sigma Chemical Co., St. Louis, MO) and 0.06 v/v antibiotic-antimycotic solution (10,000 units penicillin/ml, 10 mg streptomycin sulfate/ml, 25 μg amphotericin B/ml from Sigma). After adding the cellulase stock solution to each tube, pH was adjusted to 5 with 1 N NaOH, and tubes were incubated at 37 C for 36 hours on a rotary shaker at 22 rpm (Model RD 4512 with the sample platform set at an angle of 30–35 degrees from the horizontal, Glas-Col, Terre Haute, IN). Next, 1.0 ml of pectinase solution (from *Aspergillus niger,* 68 units/ml, with 1 unit liberating 1.0 mMole galacturonic acid from polygalacturonic acid per minute at pH 4.0 at 25 C, Sigma) was added to each tube and vortexed. The pH was than adjusted to 4 with

1 N HCl. Tubes were incubated at 28 C for 36 hours on a rotary shaker at 22 rpm and then stored at 5 C. Immediately before counting nematodes, the sample vials were vortexed vigorously for 15 seconds at full speed. The contents of each tube were poured through nested 250- over 20-μm-pore sieves and rinsed into counting dishes; the nematode developmental stages were then enumerated using a dissecting microscope (10–70× magnification). Fifteen replicate pairs of plate–digestion samples of *E. glaucus* roots were counted in the single experiment.

Method 2—Vortexing omitted: The protocol was the same as method 1, except that samples were not vortexed before counting, and J3 and J4 were counted separately. Fifteen replicate samples of *L. esculentum* roots were counted in each of the two repetitions of the experiment. When the experiment was repeated, only J4 and adult females were counted, because these stages were recovered poorly in the first experiment.

Method 3—Vortexing and plate squeezing omitted: The procedure was the same as method 1 with several modifications. *Lycopersicon esculentum* roots were sandwiched between glass plates but without pressure to avoid crushing swollen stages; the post-digestion samples were not vortexed before counting; and J3 and J4 were counted separately. Fifteen replicate samples were assessed in each of two experiments. When the experiment was repeated, only J4 and adult females were counted, because of poor recovery during the first experiment.

Method 4—No plate squeezing, slower rotation: Method 1 was modified as follows. Roots of *D. lablab, E. glaucus,* and *L. esculentum* were sandwiched between glass plates, but without pressure to avoid rupturing or damaging J4 and females. To avoid further damage, the 50-ml centrifuge tubes were replaced by 20-ml glass scintillation vials, and rotor speed was changed to 5 rpm from 22 rpm. Fifteen replicate samples were counted in single experiments performed with each plant species.

For each method, nematode developmental-stage data were expressed as numbers per gram of root for the plate count and digestion methods. For method 4, the numbers of each stage recovered by plate counting and digestion were compared using paired *t*-tests ($P \leq 0.05$) to define increases in recovery. Data for each stage and from all plant species in method 4 were pooled and subjected to regression analysis using PC-SAS (12).

Results

Method 1: The use of cellulase and pectinase improved the recovery of *M. javanica* growth stages to 167.3% for eggs, 200% for J2, and 138.7% for J3 + J4 extracted from *E. glaucus* roots relative to the standard plate count method (Table 1). Recovery of adults, however, was only 48.1% of that in the plate method.

Method 2: The elimination of vortexing before counting maintained the good recovery of eggs, J2, and J3 observed in method 1, but did not improve recovery of J4 and adults (Table 1). Recovery of adults by digesting roots with enzymes was 11.2 and 31.7% of that in the standard plate method in trials 1 and 2, respectively. The recovery of J4 was 46.8 and 48.3% in trials 1 and 2.

Method 3: Gently pressing roots without smashing them against the plate provided good recovery of eggs, J2, and J3 after digestion, but the recovery of J4 and adults remained poor (Table 1).

Method 4: Root maceration with cellulase and pectinase in scintillation vials rotator-shaken at 5 rpm improved ($P \leq 0.05$) the recovery of all developmental stages from three plant species (Table 2). Improvements in recovery from *L. esculentum* roots with method 4 varied from 169.5% for J2 to 563.8% recovery of J4 (Table 2). From *D. lablab* roots processed with method 4, the lowest and highest recoveries were 262.6 and 989.3% for adults and J4, respectively (Table 2). Although nematode numbers in *E. glaucus* roots were low, the enzyme treatment allowed detection and

TABLE 1. Numbers of *Meloidogyne javanica* per gram of root obtained by staining roots, pressing them between glass plates, and counting nematode growth stages within roots of *Elymus glaucus* or *Lycopersicon esculentum*. Roots were then sequentially digested with cellulase and pectinase to release nematode growth stages for counting.

	E. glaucus		*L. esculentum*			
Stage	Pressed plate method	Method 1†	Pressed plate method	Method 2‡	Pressed plate method	Method 3§
Eggs	1,712.9	2,865.7	5437.6	43,750.9	6,984.3	22,335.8
J2	408.0	816.0	132.0	493.3	168.0	1,418.6
J3			54.6	226.2	12.8	455.1
J4			54.1	25.3	17.6	10.7
Adult females	29.0	13.9	302.4	33.8	324.8	159.5
J3 + J4	86.0	119.3	108.8	192.9	30.4	772.8
Total juveniles	494.0	878.8	240.7	461.4	198.4	3,201.4
Total	2,235.9	3,285.2	5,980.7	18,396.6	7,507.5	23,956.4
			Experiment 2		Experiment 2	
J4			9.3	4.5	14.3	5.2
Adult females			49.7	15.8	48.2	16.7

Data are means of 15 replicates and were not subject to statistical analysis because of poor recovery of J4 and adults.
† Roots were removed from the plates and digested with cellulase and then pectinase.
‡ As method 1, but samples were not vortexed before counting.
§ As method 1, but plates were not pressed together.

enumeration of more ($P \leq 0.05$) nematodes of each developmental stage than did the glass plate method (Table 2).

The data from all three plant species were pooled for regression analysis, and the slope (m) of the regression of digestion counts on the plate counts was greater than one (Table 3) for all developmental stages, indicating greater recovery of all stages using enzyme digestion. Highly significant F values were observed for each nematode developmental stage (Table 3). Regression analysis revealed that there was not a general relationship between the plate counts and digestion counts that would serve as a quantitative factor for converting plate counts to equivalent digestion counts.

TABLE 2. Numbers of *Meloidogyne javanica* counted within roots of three plant species by staining and placing roots between glass plates. Roots were subsequently digested with cellulase and pectinase to release nematode growth stages.

	Lycopersicon esculentum		*Dolichos lablab*		*Elymus glaucus*	
Stage	Pressed plate method†	Enzymic digestion‡	Pressed plate method	Enzymatic digestion	Pressed plate method	Enzymic digestion
Eggs	3,386.8	11,115.0**	2,666.1	7,913.0**	1,322.2	12,605.9**
J2	101.8	172.6**	55.2	145.3**	70.0	141.7*
J3	46.9	178.8**	3.6	29.2**	0.5	5.8*
J4	12.0	67.7**	1.0	9.9**	0.0	0.01*
Adult females	89.6	285.6**	32.4	85.1**	16.8	45.9*
J3 + J4	58.9	218.3	4.6	44.4	0.5	7.2
Juveniles	160.7	381.2	59.8	298.7	70.4	181.8
Total	3,637.2	11,348.1**	2,758.4	8,129.0**	1,409.6	9,427.4**

Data are means of 15 replicates compared using paired t-tests (*$P \leq 0.05$; **$P \leq 0.01$).
† Developmental stage numbers were obtained by staining roots, placing them between glass plates without squeezing the plates together, and counting nematode growth stages within roots.
‡ Roots were taken from the glass plates after counting and sequentially exposed to cellulase and pectinase on a rotary shaker at 5 rpm in 20-ml scintillation vials to release nematodes from roots.

TABLE 3. Regression of numbers of *Meloidogyne javanica* obtained by enzymatic root digestion on counts obtained from the same stained roots pressed between glass plates. Data pooled from roots of *Dolichos lablab, Elymus glaucus,* and *Lycopersicon esculentum,* with $n = 15$ for each plant species.

Stage	Regression			
	m	R^2	F	P
Eggs	1.9	0.45	36.3	0.0001
J2	1.25	0.74	128.0	0.0001
J3	2.52	0.69	98.4	0.0001
J4	2.38	0.37	26.6	0.0001
Adult females	1.29	0.59	62.7	0.0001
J3 + J4	2.64	0.77	147.5	0.0001
Juveniles	1.53	0.69	97.8	0.0001
Total	1.95	0.48	39.3	0.0001

DISCUSSION

We have developed an enzymatic root digestion method that releases all growth stages of *Meloidogyne javanica* from roots and may be suitable for use in research on nematode population dynamics, demography, nematode biological control, and resistance assessment. Enzymatic digestion of infected roots provides an easy, inexpensive, effective, and fast method for extraction, identification, and counting of root-knot nematode growth stages. The method was more accurate and sensitive than the pressed glass plate method at different nematode densities, and allowed recovery of larger numbers of all nematode growth stages in three different plant hosts. The digestion method could possibly also be used to observe antagonists on or within nematodes recovered from roots. This technique does not involve sucrose flotation, a problem for studies on biological control agents because residual sucrose may promote growth of microbial contaminants (6).

Digestion released the nematodes from roots and thereby expedited determination of developmental stage. Enumerating these stages within roots is essential for demographic experiments, especially those involving resistance and host plant status. Using a slightly more difficult protocol, Halbrendt et al. (7) demonstrated that soybean resistance to *Heterodera glycines* is stage specific and that males and females are differently affected in different cultivars. The standard glass plate method for counting stages in roots is limited, because heavily infected roots may contain nematode aggregates that make it difficult to count individual nematodes or to identify nematode developmental stages. For example, Halbrendt et al. (7) observed that roots heavily infected with *Heterodera glycines* were difficult to examine with their glass plate method, and that an infection level of only 10 to 20 nematodes per sample was optimum for enumeration.

The experiments described here were initially conducted with protocols similar to those in related studies (5,6,8,10), but the protocols were adjusted to avoid crushing J4 and adult females. The root digestion protocol in method 4 was successful because of vial size, the low rpm incubation, and the lack of vortexing. The large tube size, high rpm incubation, and vortexing in methods 1–3 likely destroyed the fragile swollen nematode stages.

The incubation period selected was 36 hours with each enzyme; however, the optimal period may vary with the species of host plant and enzyme concentration (6,10). Adult females and fractions of egg masses were retained on the 250-μm sieve, while J2, J3, and J4, occasionally adult females, and small root segments were retained on the 20-μm sieve. Staining the nematodes facilitated counting. This procedure worked well with root samples as large as 1.0 g stored for one year (Araya and Caswell-Chen, unpubl.).

In summary, root digestion improved detection of *M. javanica* growth stages in roots of three different plant species, indicating that the technique should have wide applicability. The method is probably suitable for quantifying other endoparasitic nematodes within roots. This technique will allow nematologists to assess stage-specific attributes of host–parasite interactions, and will be useful in assessing host status, resistance, and nematode population dynamics and demography.

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