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Comparison of Extraction and Shipping Methods for Cysts and Juveniles of *Heterodera glycines*[1]

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Abstract: Experiments to determine the effects of extraction techniques and the influence of shipping on extraction of *Heterodera glycines* life stages gave variable results. Shipping did not significantly affect numbers of nematodes extracted. More second-stage juveniles (J2) were extracted with Baermann funnels than with an elutriator, probably because incubation of encysted eggs on the Baermann funnel for 1 week allowed hatching to occur. Sieving was more efficient than elutriation for extracting cysts. Adding air agitation to the water pressure during elutriation increased extraction efficiency of cysts but not J2. Sample sizes of 250 cm^3 and 500 cm^3 did not influence extraction efficiency of cysts; however, sample size did influence extraction of J2.

Key words: Baermann funnel, centrifugal flotation, cyst, egg, elutriation, extraction, *Heterodera glycines*, methods, nematode, second-stage juvenile, shipping, soybean cyst nematode.

Soil-inhabiting nematodes are typically extracted from soil by a modification of the Baermann funnel technique (Christie and Perry, 1951; Cobb, 1918) or by manual or automated sieving plus centrifugation flotation (Jenkins, 1964). Each system has advantages and disadvantages. The Baermann funnel method is easy to use and inexpensive. This procedure is inefficiently for extraction of inactive nematodes such as *Criconemella* spp. (Barker et. al, 1969) but very effective for active nematodes such as *Rotylenchulus* spp. Hatching of eggs that occurs during the several days on the Baermann funnel increased the numbers recovered from the funnel of some species, such as *Heterodera glycines* (Riggs, unpubl.). On the other hand, centrifugal flotation extracts immobile and dead nematodes, which increases the number (Riggs, unpubl.).

Research on extraction of nematodes from soil has been discussed in a number of reviews (Barker, 1985; Kimpinski and Welch, 1971; McSorley, 1987). Different extraction methods have been compared in the same laboratory (Barker et. al, 1969; Dickerson, 1977; Weber and Williams, 1968). Barker et al. (1969) found that centrifugation-flotation usually was more efficient for most nematodes than the Baermann funnel (no sieving) method. Dickerson (1977) reported that sieving-centrifugation flotation was more efficient than sieving-Baermann funnel for extracting plant-parasitic nematodes but that greater total numbers of nematodes were extracted by sieving-Baermann funnel. Viglierchio and Schmitt (1983) found that extraction efficiency depended on soil type and nematode species. Cyst nematodes were not included in any of these studies.

Dunn (1969) described the extraction of cysts by sieving-centrifugation flotation. *Globodera tabacum solanacearum* was more efficiently extracted from soil by elutriation and flotation centrifugation than by sugar flotation and sieving (Reilly and Grant, 1985). Efficiency of extraction from field samples varied by month of sampling, perhaps because of changes in density of the nematode.

A number of factors are important for assessing the meaning of nematode numbers. Optimization of management recommendations depends on knowing the soil population densities. Comparability of extraction efficiency among laboratories is an important issue. A major aspect is to determine what impact soil shipment might have on

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nematode extraction. This latter concern is important because many samples are shipped to laboratories for nematode assay.

The purpose of this research was to compare the relative efficiencies of sieving and Baermann funnel and elutriation and centrifugation flotation for the extraction of *H. glycines* in laboratories in Arkansas and North Carolina, and to determine the effect of shipping soil samples on numbers of nematodes extracted.

Materials and Methods

Soil collection and preparation: Soil was collected from *H. glycines*-infested fields for most experiments. The selected Arkansas soils were silt loam (3% sand, 84% silt, 13% clay) from the Cotton Branch Experiment Station, Marianna, Arkansas, and sandy loam (69% sand, 26% silt, 5% clay) from a private farm near Kibler, Arkansas. Soils collected in North Carolina were a sandy soil from the Central Crops Research Station near Clayton, North Carolina (88% sand, 7% silt, 5% clay; 0.6% OM), and a loamy sand from a private farm near Weeksville, North Carolina (75% sand, 23% silt, 2% clay; 0.8% OM).

One extraction test involved infesting steamed soil with second-stage juveniles (J2) or cysts of *H. glycines*. In Arkansas, the numbers of J2 or cysts were estimated by counting a portion of the samples and calculating the total before mixing them with the soil. In North Carolina the numbers were more precisely determined by counting the total number or by picking them individually.

For each soil sample, half of the soil was retained at the originating state and half was sent to the other laboratory. Soils were shipped on the same date by the two laboratories. Soil was placed in styrofoam boxes with walls 1.25 cm thick and a 1-liter capacity, or in corrugated cardboard boxes 20 cm × 15 cm × 7.5 cm for shipment. After testing different types of containers and methods of shipment, container type was standardized for each set of samples. Soil retained at the origin was kept at room temperature (21–24 °C). When samples were received by both laboratories, all soil (shipped and nonshipped) was processed on the same day. The time required for shipment ranged from 4 to 10 days with an average of 7 days.

Shipping method: The Arkansas silt loam and North Carolina loamy sand soils were collected from the root zone of soybean plants in August, mixed thoroughly, and divided into aliquants for processing. Two sets of samples were placed in styrofoam containers and one set in cardboard containers for shipping. One set of samples in styrofoam containers was shipped by U.S. Postal Service (USPS); the other two sets were shipped by United Parcel Services (UPS). A set of samples was retained at each origin to evaluate the effects of shipping. North Carolina used a semiautomatic elutriator and centrifugation flotation method (Byrd et. al., 1976) for nematode extraction, whereas Arkansas used a sieving and Baermann funnel extraction method (Christie and Perry, 1951). In the sieving-Baermann funnel procedure, 250 or 500 cm^3 (consistent for each test) soil was suspended in 8 liters of water, poured through nested 850-µm-pore (20-mesh) and 250-µm-pore (60-mesh) sieves into a plastic container, and then poured through a 37-µm-pore (400-mesh) sieve. *Heterodera glycines* cysts and females caught on the 250-µm-pore sieve were counted and then placed on folded tissue paper supported by a metal wire mesh in a Baermann funnel. The material caught on the 37-µm-pore sieve was placed either in the same funnel or in another funnel. The nematodes were withdrawn from each funnel 2 and 7 days later.

In the elutriation-centrifugation-flotation procedure, cysts were caught on a 250-µm-pore sieve and J2 on a 37-µm-pore sieve. Final extraction was with centrifugation-flotation using sucrose (commercial sugar) solutions of 680 g/liter of solution for cysts and 454 g/liter of solution for J2.

Each aliquant of soil was replicated five times. Nematode counts were compared with analysis of variance, and mean separation was performed with a protected LSD procedure (SAS-STATR User's Guide. 1989.

Version 6, 4th ed., vol. 2. SAS Institute, Cary, NC).

Sample size: All four soils (Arkansas sandy and silt loam and North Carolina sandy and loamy sand) were collected from infested soybean fields in October. Each soil was mixed and divided as described in the soil collection section and were shipped in styrofoam containers by USPS. To determine the effect of sample size on extraction efficiency, two sample sizes (250 cm^3 and 500 cm^3) were shipped. Nematodes were extracted by both sieving-Baermann funnel and elutriation-centrifugation flotation in Arkansas and North Carolina.

Elutriation with and without aeration: In December, North Carolina sandy soil samples were collected and mixed as described for the first exchange, and half were shipped to Arkansas. This soil was used to compare the effects of aeration during elutriation on the extraction of cysts and J2.

Comparison of extraction methods: The Arkansas silt loam and North Carolina loamy sand soils were collected, mixed, and divided as for the first exchange and shipped by USPS. Samples were processed by both sieving-Baermann funnel and elutriation-centrifugation flotation at both locations for the extraction of J2.

Artificially infested soil: Steam-disinfested sandy loam soil from Arkansas and sandy soil from North Carolina were divided into aliquants. Equal volumes of a suspension of cysts (394–463 in Arkansas and 149–151 in North Carolina) or J2 (501–593 in Arkansas and 245 in North Carolina) were added to each of five aliquants at each location, respectively. These samples were shipped by USPS in styrofoam containers. Samples were extracted by sieving-Baermann and elutriation-centrifugation flotation at both locations. Extraction efficiency was determined by the percentage of cysts or J2 recovered. Analyses were performed both on the original counts and percentage recovery. Data were transformed with $\log_{10}(x + 1)$ before analysis, where appropriate.

Statistical analyses: The significance of various factors was tested by analysis of variance for a completely randomized design with various factorial arrangements for each experiment. Treatment effects were separated into main effects and interactions. Planned contrasts were used to compare levels of factors and their interactions. In the absence of interactions among factors, main effect means were separated with Fisher's protected LSD. All statistical significances are reported at the 5% level unless otherwise stated.

Results and Discussion

Shipping method: Numbers of J2 extracted from samples sent by USPS and UPS and processed by sieving-Baermann funnel were not significantly different although those shipped by USPS yielded somewhat higher numbers (Table 1). Numbers of J2 extracted from shipped Arkansas soil appeared to be lower than those not shipped, but one set was extracted by elutriation-centrifugation flotation and the others by sieving-Baermann funnel. Because shipping was confounded by extraction method, comparisons could not be made. Comparisons of the means, which averaged shipping and extraction methods, revealed no significant difference (Table 1).

Sample size: Results of extractions from samples of different size were variable (Table 2). No significant differences be-

Table 1. Mean numbers of *Heterodera glycines* second-stage juveniles (J2) extracted[a] from 500 cm^3 soil from samples collected in Arkansas or North Carolina and either not shipped (NS) or shipped, (USPS, UPSC or UPSS) to the other location for extraction.

Extraction method	Shipping method			
	NS	USPS	UPSC	UPSS
SB	1,750	2,076	1,551	1,269
EC	604	590	240	587
Mean	1,177	1,333	928	896ns[c]

Numbers are Least Squares Means based on five (SB-NS, SB-USM, SB-UPSC, SB-UPSS) or three (EC-USM, EC-UPSC, and EC-UPSS) replications.

[a] In Arkansas extractions were by sieving and Baermann funnel (SB); in North Carolina extractions were by elutriation and centrifugation-flotation (EC).

[b] USPS = U.S. Postalservice; UPSC = United Parcel Service in cardboard carton; UPSS = United Parcel Service in styrofoam carton.

[c] Differences among shipping-method means were not significant ($P = 0.05$).

TABLE 2. Mean numbers of second-stage juveniles (J2) and cysts of *Heterodera glycines* extracted from silt and sandy loam soil samples collected in Arkansas or a loamy sand or sandy soil, in North Carolina and shipped to the other location for extraction.

Extraction method[a]	No. J2 extracted			No. cysts extracted		
	500 cm³[b]	250 cm³	*P*	500 cm³	250 cm³	*P*
			Soils from Arkansas			
			Silt loam soil			
SB	3,991	3,610	ns	204	235	ns
EC	2,264	1,260	**	24	13	ns
			Sandy loam soil			
SB	1,908	1,613	ns	155	182	ns
EC	1,300	436	*	119	67	ns
			Soils from North Carolina			
			Loamy sand soil			
SB	215	430	ns	818	790	ns
EC	290	772	ns	73	78	ns
			Sandy soil			
SB	1,259	1,018	ns	136	144	ns
EC	1,590	3,392	**	15	11	ns

Numbers are means of five replications. Samples were shipped by the U.S. Postal Service in cardboard cartons. Soil texture analyses are presented in Materials and Methods section.

[a] SB = Second-stage juveniles were extracted by sieving and Baermann funnel, cysts by sieving and direct count; EC = Second-stage juveniles and cysts were extracted by elutriation and centrifugation flotation.

[b] Soil sample sizes were 500 and 250 cm³, and the numbers of nematodes extracted from the smaller sample size were multiplied by two to make the numbers comparable.

tween sample sizes were observed for cysts regardless of extraction method or soil type. Differences were observed with J2 extracted by elutriation-centrifugation flotation in three of the four soil types (Table 2). Higher numbers were of J2 were extracted from the 500-cm³ Arkansas silt loam and sandy loam samples than from the 250-cm³ samples, whereas higher numbers were extracted from the 250-cm³ North Carolina sandy samples than from the 500-cm³ samples. No differences between sample sizes were observed in extraction by sieving-Baermann funnel. Variation was so great that even relatively large differences were not significant (Table 2).

Elutriation with and without aeration: When aeration was added to the water stream during elutriation, the results varied between processing locations (Table 3). Numbers of J2 extracted were higher without aeration at one location but not at the other. However, the overall means showed no significant differences in the extraction of J2 with or without aeration added to the elutriation stream. In contrast, numbers of cysts extracted were

TABLE 3. Mean numbers of second-stage juveniles (J2) and cysts of *Heterodera glycines* extracted by elutriation and centrifugation flotation from naturally infested soil collected in North Carolina with half shipped to Arkansas for extraction.

Extraction location	No. J2/500 cm³ soil			No. cysts/500 cm³ soil		
	Aeration			Aeration		
	With[a]	Without	Mean	With	Without	Mean
A	432 aB	280 aA	356 A	78	50	64 a
B	90 bA	120 bA	105 B	37	17	27 b
Mean	261 A	200 A		57 A	33 B	

Numbers are means of four replications. Means followed by the same lowercase letter indicate no difference for comparing rows within a nematode stage; means followed by the same upper-case letter within a column indicate no difference in comparing the effects of aeration ($P = 0.05$). Samples were shipped in cardboard cartons by U.S. Postal Service.

[a] *With aeration* = elutriation and centrifugal-flotation with air added to the elutriation; Without aeration = elutriation and centrifugal-flotation without air added to the elutriation.

significantly higher with aeration plus water flow than without it (Table 3).

Comparison of extraction method: Overall, larger numbers of J2 were extracted by sieving and Baermann funnel from Arkansas soil infested with *H. glycines* than by elutriation flotation (Table 4). In addition, higher numbers were extracted at one location than at the other. However, numbers extracted from the NC soil were significantly greater by elutriation-centrifugation flotation at one location and not different at the other. Significantly higher numbers of J2 were extracted by elutriation-centrifugation flotation than by SB at one location but not at the other.

Artificially infested soil: When J2 were added to the soil and then extracted, the average percentage recovered ranged from 12% to 35% from the Arkansas soil and 17% to 46% from the North Carolina soil (Table 5). The lowest recovery was by elutriation-centrifugation flotation and the highest by sieving-Baermann funnel, whether from Arkansas or North Carolina soil. For the North Carolina soil, sieving-Baermann funnel was significantly more efficient than elutriation-centrifugation flotation for extracting J2. Overall, the two methods gave similar results with J2 regardless of location.

TABLE 4. Mean numbers of second-stage juveniles (J2) of *Heterodera glycines* extracted from field soil from Arkansas and North Carolina by sieving and Baermann funnel (SB) or elutriation and centrifugation flotation (EC) at both locations.

Extraction location	No. J2/500 cm^3 soil		
	EC	SB	Mean
	Soil from Arkansas		
A	81	319	200 B
B	387	637	512 A
Mean	234 b	478 a	
	Soil from North Carolina		
A	68 aB	108 aA	88
B	280 aA	55 bA	168
Mean	174	82	

Numbers are means of five replications. Means within a row followed by different lowercase letters indicate significant difference in method for a given row (source by extraction method combination). Means within a column followed by different uppercase letter indicate a significant difference between extraction laboratories ($P = 0.05$).

Samples were shipped in cardboard cartons by the U.S. Postal Service.

Recovery of cysts was more variable than recovery of J2, ranging from 23% to 44% for the Arkansas soil and from 9% to 60% for the North Carolina soil (Table 5). Sieving Arkansas soil at one location and elutriating it at the other location gave similar results. Recovery of cysts from North Carolina soil was low at one location regardless of extraction method but was significantly higher at the other location (Table 5).

General comments: The population densities of cysts and J2 of SCN extracted from Arkansas silt loam and sandy loam soil or North Carolina sandy or loamy sand soil were extremely variable. Numbers of nematodes extracted from shipped samples were not significantly different from unshipped samples. Earlier studies indicated that handling of soil reduced the numbers of nematodes extracted (R.E. Motsinger, pers. comm.). The 250-cm^3 sample size did not yield larger numbers of J2 even though earlier studies (Riggs, unpubl.) indicated that extraction from the smaller samples was more efficient. The recommended sample size for the extraction of cereal cyst nematodes is 200 g of soil (Fidler et al., 1959).

Suspending the soil plus cysts in a pail of water and sieving generally resulted in higher extraction of cysts than elutriation except when additional agitation was provided by forcing air through the funnels in the elutriation. The roiling process likely suspended more cysts than the moderately gentle water action in the elutriator.

Other researchers have compared extraction procedures (Chapman, 1958; Dickerson, 1977; Dunn, 1969; Malo, 1960; Rickard and Barker, 1982; Viglierchio and Schmitt, 1983; Weber and Williams, 1968); however, in no case was a cyst nematode involved. Chapman (1958) indicated that sieving and Baermann funnel extraction was too poor and inconsistent to be considered for quantitative studies. In a test of the centrifugal flotation technique, Weber and Williams (1968) indicated that 53.3% extraction was the highest efficiency attained with three different vermiform species. Viglierchio and Schmitt (1983) extracted four different nematode species from three soil types. Ef-

Table 5. Mean percentages of second-stage juveniles (J2) and cysts of *Heterodera glycines* extracted from soil artificially infested in Arkansas or North Carolina.

Extraction location	J2			Cysts		
	EC	SB[a]	Mean	EC	SB	Mean
			Soil from Arkansas			
A	12.0 bA	34.8 aA	23.4	22.8 B	43.8 A	33.3
B	20.8 aA	27.8 aA	24.3	42.6 A		
Mean	16.4	31.3		32.7		
			Soil from North Carolina			
A	16.8	46.2	31.5 A	10.2 B	9.2	9.7
B	20.0	37.2	28.6 A	59.6 A		
Mean	18.4 b	41.7 a		34.9		

Numbers are means of five replications. Means followed by the same letter are not different for soil within a source within a nematode stage. Blank spaces indicate no sample. Samples were shipped by U.S. Postal Service in cardboard cartons.

† SB = sieving and Baermann funnel; EC = elutriation and centrifugation flotation.

ficiency depended on nematode species and soil type. The efficiencies of both sieving-Baermann funnel and sieving-centrifugation-flotation were below 50% in their study.

From our study and from previous studies, we conclude that the available extraction procedures are not efficient for vermiform stages. Extraction results depend on soil type and sometimes change between extraction locations, shipment method, and sample size. Efforts should be made to improve extraction methods.

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