Supplement to Journal of Nematology 25(4S):778–784. 1993.

Comparison of Two Inoculum Preparation Methods for *Rotylenchulus reniformis*[1]

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Abstract: Three greenhouse experiments were conducted to determine whether NaOCl-extracted eggs would provide an acceptable inoculum source for *Rotylenchulus reniformis.* Two tests (one each on loamy sand and sandy clay) were designed to compare eggs extracted from roots with sodium hypochlorite (NaOCl) with mechanically extracted vermiform males, females, and juveniles from soil as inoculum sources. Infection rates for both inoculum types were low (<1–3%) on roots of 'Ransom' soybean 14 days (loamy sand soil) or 30 days (sandy clay soil) after inoculation. A larger number of infective females from the mechanically extracted than from NaOCl-extracted inoculum penetrated the roots in the loamy sand; however, in the heavier soil (sandy clay), NaOCl-extracted eggs were the better inoculum source. Significant reproduction occurred on infected plants, regardless of inoculum preparation method or soil type. Extraction of eggs by the NaOCl method is much easier and quicker than mechanical extraction of vermiform nematodes from soil. A third test was conducted to determine the infectivity of *R. reniformis* from eggs extracted at different NaOCl concentrations. Five initial inoculum levels (0, 500, 2,500, 5,000, and 10,000) and four NaOCl concentrations (0.25, 0.50, 0.75, and 1.0%) were compared on 'Rutgers' tomato harvested on two dates, 17 and 23 days after inoculation. Again, infection rates of roots were low (≤0–3%). By 23 days after inoculation, the largest number of females penetrating the roots were from the highest inoculum level extracted with 0.25% NaOCl. The lowest infection rates in both harvests occurred when inoculum was prepared with 1.0% NaOCl.

Key words: *Glycine max,* inoculum, *Lycopersicon esculentum,* nematode, reniform nematode, *Rotylenchulus reniformis,* soybean, tomato.

The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) is an important plant parasite throughout tropical and subtropical areas of the world, including the southeastern United States (5). This pathogen has been found in more than 38 countries and infects a wide range of crops (6). Currently, much research is being conducted with this parasite on several crops (8).

The vermiform adult female is the infective stage of *R. reniformis.* Following infection, adult females swell and become sedentary and reniform in shape. They produce an external gelatinous matrix into which most of the eggs are deposited (10). Because the root-knot nematodes, *Meloidogyne* spp., also produce eggs in an external gelatinous matrix that can be extracted using a 0.5–1.0% sodium hypochlorite solution (7), we evaluated this procedure for the extraction of *R. reniformis* eggs for use as inoculum. Previously, Clark and Thomas (4) used a 0.53% NaOCl solution when extracting reniform nematode eggs. They found that a 4-minute extraction did not reduce egg hatch compared with a 2-minute extraction, but the 4-minute procedure extracted higher numbers of eggs; however, they did not evaluate infectivity.

The objective for two of three tests was to compare the infectivity of *R. reniformis* females from inocula of NaOCl-extracted eggs and mechanically extracted vermiform life stages on two different soil types (loamy sand and sandy clay). A third test was conducted to determine the effects of NaOCl and initial inoculum levels on the number of successful infections following inoculation with eggs extracted at various NaOCl concentrations.

Materials and Methods

Comparison of two inoculum preparation methods (loamy sand soil): A greenhouse ex-

Received for publication 10 April 1992.

[1] The research reported in this publication was funded in part by the North Carolina Research Service, North Carolina State University, Raleigh, and USAID/USDA/OICD Grant 58-319R-1-008.

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The authors gratefully acknowledge the technical assistance of Mr. J. A. Phillips and the editing assistance of Dr. S. R. Koenning. The authors thank Dr. J. P. Noe for supplying us with the population of *Rotylenchulus reniformis* used in this study.

periment focused on the infectivity of females produced from NaOCl-extracted eggs compared with mechanically extracted inoculum (vermiform males, females, and juveniles) on a loamy sand soil. An isolate of *R. reniformis* used in this experiment was maintained on Puerto Rico sweetpotato (*Ipomoea batatas* (L.) Lam.) and was initially obtained from a cotton field in Oconee County, Georgia. Sweetpotato plants were inoculated with 600 vermiform *R. reniformis* 20-cm-d pot and allowed to grow for 3–4 months. Eggs were then extracted from the roots by stirring them for 4 minutes in a 1.0% NaOCl solution (7). Vermiform nematodes were extracted from the soil by a modified decanting and sieving method (11) with a 400-μm-pore sieve nested over a 40-μm-pore sieve. Nematodes obtained from soil were not centrifuged.

The experiment was set up as two parallel 2 × 7 factorials arranged in five randomized complete blocks. Two parallel 2 × 7 factorials were used because data were collected and analyzed on two different dates. Each treatment consisted of one inoculum preparation method and one inoculum level (Pi) in a 15-cm-d (1,750 cm^3 volume) clay pot containing moist, sterilized loamy sand soil (85% sand, 10% silt, 5% clay). A 3-day-old 'Ransom' soybean (*Glycine max*) seedling (cotyledon stage) was transplanted into each pot. A commercial preparation of *Bradyrhizobium japonicum* was added to each pot to ensure normal nodule development. After transplanting, each seedling was inoculated at one of seven Pi (0, 100, 500, 2,500, 5,000, 10,000, or 20,000 *R. reniformis*). Greenhouse temperatures averaged 30 C (days) and 22 C (nights).

Plants were harvested on one of two dates: 14 or 90 days after inoculation. Fourteen days after inoculation, fresh shoot and root weights were recorded, and whole root systems were stained (3) and examined to determine the number of females that penetrated the roots. Ninety days after inoculation, fresh shoot and root weights were measured and numbers of eggs on the roots were determined by NaOCl extraction (2). Numbers of eggs and juveniles in the soil were determined by elutriation and centrifugation (1).

Data from each harvest were analyzed separately as a 2 × 7 factorial and were subjected to analyses of variance (ANOVA) and correlation analysis. Regression models were used to determine the relationships between inoculum preparation method and inoculum level with root penetration (14-day test) and numbers of eggs on roots, eggs in soil, vermiform males and females in soil, and total number of nematodes (90-day test).

Comparison of two inoculum preparation methods (sandy clay soil): The experiment was similar to the first test in that it evaluated two inoculum preparation methods (NaOCl-extracted eggs and mechanically extracted vermiform *R. reniformis*); however, only Pi of 0, 2,500, 5,000 10,000 or 20,000 15-cm-d (1,750 cm^3) clay pot were included, and the soil was a sandy clay (54% sand, 10% silt, 36% clay). Soil was obtained from Central Crops Research Station near Clayton, NC, and steam pasteurized for 2 hours at 80–82 C. Greenhouse temperatures averaged 29 C (days) and 21 C (nights). Plants were harvested either 30 days or 90 days after inoculation. Data collected for each harvest and data analysis were identical to those of the first test.

NaOCl concentration experiment: A third test was conducted to determine the number of infective females obtained from NaOCl-extracted eggs at various concentrations and inoculum levels. This experiment was conducted because of the small percentage of reniform nematodes that penetrated soybean in the first two tests described above. 'Rutgers' tomato (*Lycopersicon esculentum* Mill.) was inoculated with eggs of the reniform nematode extracted with different concentrations of NaOCl. Tomato was used in this test because it has a more fibrous root system than soybeans, providing more infection sites for *R. reniformis*.

The experiment was a 2 × 4 × 5 facto-

rial with two harvest dates (17 and 23 days after inoculation), four NaOCl concentrations (0.25, 0.50, 0.75, and 1.0%) for 4 minute extractions (7), and five Pi (0, 500, 2,500, 5,000, and 10,000 eggs/plant) in a randomized complete block design with four replications. Tomato seedlings at the cotyledon stage (3 days old) were transplanted into 7.5-cm-d (190 cm^3) styrofoam cups containing a steam-sterilized loamy sand soil (85% sand, 10% silt, 5% clay) and inoculated 1 week after transplanting.

Seedlings were watered twice daily and fertilized weekly with Peter's® 20-20-20 (N-P-K) (W.R. Grace, Fogelsville, PA). Temperatures in the greenhouse averaged 33 C (days) and 23 C (nights). Tomato plants were harvested either 17 days or 23 days after inoculation. Fresh shoot and root weights, dry shoot weight, and the number of females that penetrated the roots were determined (3). Data for both harvests were analyzed together by ANOVA. Regression models were used to determine whether a relationship existed between number of females penetrating roots and NaOCl concentration.

Results and Discussion

Comparison of two inoculum preparation methods (loamy sand soil): Infection rates were low (0.1–2.5%) for both inoculum preparation methods, NaOCl-extracted eggs and mechanically extracted vermiform inoculum, on roots of Ransom soybean by 14 days after inoculation (Table 1). Numbers of infective females that penetrated roots increased quadratically with Pi for both inoculum preparation methods (Table 1). Infection rates were greater for the mechanically extracted inoculum than for the NaOCl-extracted eggs (P = 0.0001). The highest infection rate of soy-

Table 1. Numbers of *Rotylenchulus reniformis* on 'Ransom' soybean grown in a loamy sand soil at 14 days and 90 days after inoculation following inoculum preparation by two methods.

Initial population/pot†	14-day test‡ (females penetrated)	Final populations (×1000) per pot (90-day test)§ Eggs (roots)	Eggs (soil)	Nematodes (soil)	Total
		Mechanically extracted inoculum			
0	0	0	0	0	0
100	2	37	21	23	81
500	9	75	78	135	288
2,500	63	81	139	143	363
5,000	110	216	381	292	889
10,000	163	321	184	602	1,107
20,000	428	306	203	684	1,193
		NaOCl-extracted inoculum			
0	0	0	0	0	0
100	1	4	12	6	22
500	1	16	16	13	45
2,500	4	18	45	61	124
5,000	3	73	112	149	334
10,000	5	152	292	268	712
20,000	31	175	333	384	892

Data are means of five replications of one plant each. Plants were inoculated during transplanting and evaluated either 14 days or 90 days later.

† Initial populations (Pi) were either eggs (NaOCl-extracted) or vermiform nematodes (mechanically extracted).

‡ ANOVA for number penetrated (14-day test): Pi (P = 0.0001), method (P = 0.0001), and Pi × method (P = 0.0001). ANOVA for total number of nematodes (90-day test): Pi (P = 0.0001), method (P = 0.0008), and Pi × method interaction (NS).

§ Quadratic regression models provided the best fit for all data analyzed. Coefficients of determination for soil-extracted inoculum were as follows: females penetrated (R^2 = 0.90, P = 0.0001), eggs (roots) (R^2 = 0.52, P = 0.0001), eggs (soil) (R^2 = 0.31, P = 0.0029), nematodes (soil) (R^2 = 0.43, P = 0.0001), and total nematodes (R^2 = 0.59, P = 0.0001). Coefficients of determination for NaOCl-extracted inoculum were as follows: females penetrated (R^2 = 0.90, P = 0.0001), eggs (roots) (R^2 = 0.49, P = 0.0001), eggs (soil) (R^2 = 0.49, P = 0.0001), nematodes (soil) (R^2 = 0.89, P = 0.0001), and total nematodes (R^2 = 0.83, P = 0.0001).

bean roots was detected when mechanically extracted inoculum was used, and this was directly related to the higher number of adult females found in roots. The largest numbers of adult females in roots were obtained when 20,000 vermiform nematodes were used as inoculum; however, the highest infection rate (2.5%) was observed when 2,500 vermiform nematodes were used.

Nematode reproduction occurred in all *R. reniformis* treatments, regardless of inoculum preparation method (Table 1). In a comparison of the two inoculum preparation methods for total numbers of nematodes, higher numbers were seen in all treatments in which mechanically extracted inoculum was used (Table 1). The low percentage of infection in all treatment combinations 14 days after inoculation, followed by the high reproduction rate, indicated that most females from the initial inoculum may have penetrated roots later than 14 days after inoculation.

The increases of total numbers of nematodes with increasing Pi were described by quadratic equations, $R^2 = 0.59$, $P = 0.0001$ (mechanically extracted inoculum) and $R^2 = 0.83$, $P = 0.0001$ (NaOCl-extracted eggs). Numbers of eggs in roots, eggs in soil, and juveniles in soil also increased quadratically with increasing Pi, regardless of the inoculum preparation method (Table 1).

Vermiform nematodes were a more efficient inoculum source than eggs extracted with NaOCl. For each Pi, more nematodes were obtained when vermiform nematodes were applied to plants (Table 1). Some differences in penetration at 14 days may be due to developmental differences and not from damage to eggs by NaOCl, because females from mechanically extracted inoculum are more developmentally advanced compared with NaOCl-extracted eggs. Differences in penetration between the two inoculation methods may be due to many eggs being killed by NaOCl. Some infectivity was lost with eggs, but eggs are much easier and quicker to obtain than mechanically extracted inoculum.

There were no differences ($P \leq 0.05$) among fresh shoot weights within the 14-day test or 90-day test for either the method or inoculum level used. So, even after 90 days, damage due to this parasite was not measurable on Ransom soybean in the greenhouse.

Comparison of two inoculum preparation methods (sandy clay soil): After 30 days, only small infection rates (0.6–2.5%) of Ransom soybean roots were detected, regardless of the inoculum preparation method (Table 2). Numbers of adult females found in roots again increased quadratically with Pi. The method of inoculum preparation had no significant effect on the number of vermiform females that penetrated soybean roots; however, more nematodes were found in roots when eggs were used. The largest number of adult females were found in roots when 20,000 eggs were used, which was also the largest infection rate (2.5%). When the 30-day penetration test is compared with the 14-day penetration test, NaOCl extraction of eggs is the better form of inoculum and supports the idea that differences in penetration in the first experiment were due to developmental differences. More time was needed to allow females to develop before they could penetrate roots.

Again, no differences ($P \leq 0.05$) were observed between fresh shoot weights of plants from various treatments within the 30-day test or 90-day test for either the method or Pi used. Thus, the numbers of *R. reniformis* used herein were not damaging Ransom soybean after 90 days in a sandy clay soil in the greenhouse.

Significantly higher total numbers of nematodes ($P \leq 0.05$) were seen on all treatments when NaOCl-extracted eggs were used (Table 2). The increases of total numbers of nematodes with increasing initial inoculum levels were described by quadratic equations, $R^2 = 0.46$, $P = 0.0017$ (mechanically extracted inoculum) and $R^2 = 0.50$, $P = 0.0005$ (NaOCl-extracted

TABLE 2. Numbers of *Rotylenchulus reniformis* on 'Ransom' soybean grown in a sandy clay soil at 30 days and 90 days after inoculation following inoculum preparation by two methods.

Initial population/pot†	30-day test‡ (females penetrated)	Final populations (×1,000) per pot (90-day test)§ Eggs (roots)	Eggs (soil)	Nematodes (soil)	Total
		Mechanically extracted inoculum			
0	0	0	0	0	0
2,500	38	24	7	89	120
5,000	44	72	27	99	198
10,000	103	66	8	70	144
20,000	220	102	24	189	315
		NaOCl-extracted inoculum			
0	0	0	0	0	0
2,500	15	89	21	139	249
5,000	71	191	43	267	501
10,000	154	127	43	387	557
20,000	490	179	48	400	627

Data are means of five replications of one plant each. Plants were inoculated during transplanting and evaluated either 30 days or 90 days later.

† Initial populations (Pi) were either eggs (NaOCl-extracted) or vermiform nematodes (mechanically extracted).

‡ ANOVA for number penetrated (30-day test): Pi ($P = 0.0001$), method (NS), and Pi × method (NS). ANOVA for total number of nematodes (90-day test): Pi ($P = 0.0001$), method ($P = 0.0001$), and Pi × method (NS).

§ Quadratic regression models provided the best fit for all data analyzed. Coefficients of determination for soil-extracted inoculum were as follows: females penetrated ($R^2 = 0.78$, $P = 0.0001$), eggs (roots) ($R^2 = 0.37$, $P = 0.0084$), eggs (soil) ($R^2 = 0.15$, NS), nematodes (soil) ($R^2 = 0.30$, $P = 0.0252$), and total nematodes ($R^2 = 0.46$, $P = 0.0017$). Coefficients of determination for NaOCl-extracted eggs were as follows: females penetrated ($R^2 = 0.47$, $P = 0.0009$), eggs (roots) ($R^2 = 0.27$, $P = 0.0311$), eggs (soil) ($R^2 = 0.38$, $P = 0.0055$), nematodes (soil) ($R^2 = 0.54$, $P = 0.0002$), and total nematodes ($R^2 = 0.50$, $P = 0.0005$).

eggs). In this test, NaOCl-extracted eggs provided a more efficient inoculum source than did mechanically extracted inoculum. For each Pi, more nematodes were obtained when NaOCl-extracted eggs were applied to plants (Table 2).

NaOCl concentration experiment: Considering all treatments, only 0–3% of eggs produced infective females that penetrated the roots of Rutgers tomato by 17 or 23 days after inoculation. This response agrees with the previous tests in which only a small number of females penetrated the roots of soybean. More penetration ($P = 0.0001$) occurred in all treatments when plants were harvested at 23 days than at 17 days (Table 3).

Increasing NaOCl concentration resulted in lower ($P = 0.0001$) root infection by infective females (Table 3). However, no significant linear or quadratic relationship existed between numbers of females found in roots and NaOCl concentration. The highest numbers of adult females in roots were found 23 days after inoculation when 10,000 eggs were extracted using 0.25% NaOCl ($P \leq 0.05$). This response was expected because the eggs were exposed to a lower NaOCl concentration as they were extracted. While extracting eggs, however, we observed that the number of eggs released from egg masses was dependent on concentration: more eggs were released as the concentration increased.

The lowest percentage of infection in both harvests occurred when 1.0% NaOCl was used. Although numbers of females penetrating roots were lowest when 1.0% NaOCl was used, this treatment could be used when extracting *R. reniformis* eggs, because of the greater release of viable eggs. Even though a large number of eggs are killed with 1.0% NaOCl, more eggs are extracted than at any other concentration. Extraction with 0.25% NaOCl results in a higher survival rate; however, all NaOCl concentrations tested apparently killed large numbers of eggs.

The containers used in this test may

TABLE 3. Numbers of vermiform adult females of *Rotylenchulus reniformis* that penetrated roots of 'Rutgers' tomato 17 days and 23 days after inoculation.

NaOCl concentration (%)	Pi†	Harvest 1 (17 days)	Harvest 2 (23 days)
Control		0	0
0.25	500	4	15
0.25	2,500	8	61
0.25	5,000	15	110
0.25	10,000	35	275
0.50	500	0	8
0.50	2,500	5	51
0.50	5,000	15	70
0.50	10,000	36	97
0.75	500	0	9
0.75	2,500	4	40
0.75	5,000	12	75
0.75	10,000	34	92
1.00	500	0	8
1.00	2,500	4	34
1.00	5,000	10	59
1.00	10,000	18	95

Data are means of four replications of one plant each. Plants were inoculated 1 week after transplanting and evaluated either 17 or 23 days later. All main effects and interactions were significant ($P = 0.0001$) in the analysis of variance.

† Pi = initial population (inoculum level) in eggs per plant.

have been unfavorable for nematode hatch or root infection due to the oxygen or moisture conditions found within the containers. In the first two tests, 15-cm-d clay pots were used. A low percentage of infection was obtained with both inoculum preparation methods, regardless of the soil type. The pots may have been too large for females to locate roots, or many of the females may have been washed out of the pot. In the third test, styrofoam cups were used instead of clay pots, and drainage holes were not used to prevent females from washing out of cups. However, styrofoam cups may have restricted air flow through the soil and allowed for waterlogging to occur, because plants were being watered twice daily. Wallace (12) showed that the hatching rate of *M. javanica* eggs decreased as oxygen levels were reduced. Thus, low soil aeration due to waterlogging of styrofoam cups could be detrimental to *R. reniformis* egg hatch. Freshly hatched juveniles of *M. javanica* that are kept at low oxygen levels for extended periods lose much of their normal activity and movement (12). Infection of roots by reniform nematode females may also be lower at reduced oxygen levels.

Several problems may be encountered in obtaining eggs of *R. reniformis*. First, females of this nematode only produce about 40 eggs per egg mass (9,10), whereas *Meloidogyne* females produce hundreds or thousands of eggs per egg mass depending on species and host. So, inoculum in the form of eggs is more difficult to obtain from *R. reniformis* compared with root-knot nematodes because fewer eggs are produced per egg mass. Second, when roots are washed with tap water to remove soil, egg masses tend to fall off easily. One way to avoid this is to shake as much soil off the roots as possible and then extract the eggs without washing the roots with tap water.

For studies on *R. reniformis* in field plots and microplots, we use infested soil and infected roots as inoculum (which are probably more viable). The low infectivity obtained through NaOCl extraction of eggs was unexpected and too low for field plot infestations. Eggs may be used for greenhouse tests where less inoculum is needed because NaOCl extraction of eggs is a much easier and quicker method of obtaining inoculum than mechanical extraction. Although much time is needed to obtain large numbers of eggs for use as inoculum, they provide a more easily quantified and precise source of inoculum than do infected roots and infested soil.

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