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Optimization of the *Heterodera glycines* Race Test Procedure[1]

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Abstract: Effects of pot size, length of seedling radicle at the time of inoculation with *Heterodera glycines*, transplanting after inoculation, type and amount of inoculum, and temperature were tested to determine the optimum procedure for the *H. glycines* race test. Numbers of *H. glycines* females extracted from plants in 7.5-cm-d pots tended to be higher than numbers from 10-cm-d pots, but not significantly so. Radicle lengths from 2.5 cm to 7.5 cm had no effect. Transplanting after inoculation reduced the variation in the number of females extracted, but the numbers of females produced were very low. Plump females (40 per pot) or eggs (4,000 per pot) were the best inocula. A constant temperature of 28 C appeared to be optimum. More *H. glycines* females were extracted from plants 28 days after inoculation than at 35 days. Race tests in which all of these factors were included were still highly variable in the number of *H. glycines* females extracted from different replications of the same test host. Tests of several susceptible cultivars revealed differences in their capabilities as hosts of *H. glycines* races.

Key words: *Heterodera glycines*, race test, soybean cyst nematode.

Races of nematodes are based on differential reproduction on a prescribed set of differential host genotypes. Race tests should be conducted under a standard set of conditions but often are not. When races of *Heterodera glycines* Ichinohe were described, a set of differential host genotypes was prescribed, but no standard conditions under which a test should be conducted were set forth (2). There have been a number of reports of race tests with *H. glycines* (4,6–12), but none have reported the exact conditions under which they were conducted. In most *H. glycines* race tests, the variability in numbers of females obtained among replications has been great (9–12,14).

The objective of these studies was to determine whether the variability in numbers of females obtained among different replications of differential hosts in a race test could be reduced by using carefully controlled conditions.

Materials and Methods

All studies except two were conducted in environment control (Phytotron) growth chambers at the Southeastern Plant Environment Laboratories at North Carolina State University, Raleigh. The growth medium was a mixture of loamy soil and sand (81% sand, 14% silt, 5% clay). Soybean (*Glycine max* (L.) Merr.) seeds were germinated in vermiculite at 25 C, and seedlings were transplanted after about 3 days. Seedlings were selected for uniform radicle length as indicated in each study.

Nematode inoculum was obtained from stock cultures maintained in a greenhouse at 26 ± 2 C. *Heterodera glycines* races 1 and 3 were produced on the soybean cultivar Lee 68, and races 2, 4, and 5 were produced on Pickett. Inoculum was prepared as follows: Soil was soaked from the root ball, and females and cysts were dislodged from the roots with a high pressure water

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spray. Most of the debris was eliminated by decanting and sieving. Females and cysts were broken in a ground glass homogenizer, and eggs and second-staged juveniles (J2) that were released were washed through a 100-μm-pore sieve to eliminate coarse debris and unbroken females and cysts. Eggs and J2 per milliliter of suspension were counted with a microscope. Approximately 4,000 eggs and J2 were added to each pot except as otherwise indicated. *Heterodera glycines* race 1 was used in all tests except the tests in which races 1–5 were used.

Clay pots (7.5 cm d) were filled with the soil mix, and a 1.3-cm-d hole was made in the center of each pot to accommodate the length of radicle being transplanted. Inoculum was poured into the hole, a seedling was placed in the hole, and the soil was firmed around it. The pots then were placed in a Phytotron chamber maintained at 28 C for 16-hour light period and 24 C for an 8-hour dark period unless otherwise stated. Each treatment was replicated as indicated with the test, and each test was repeated; however, the data from the two tests were similar and were combined for analysis. After 28 days, the soil ball was removed from the pot, soil was gently washed from the roots, and roots were sprayed with a strong water jet to dislodge females. Water in which females were suspended was poured through nested 850-μm-pore and 250-μm-pore sieves to remove trash and collect females which were then washed into a counting dish and counted with a microscope.

Postinoculation management: This study consisted of three treatments: 1) Plants left in the same pot undisturbed for 28 days. 2) Plants washed free of soil and transplanted to fresh, uninfested soil 48 hours after inoculation. 3) Plants washed free of soil and transplanted to fresh, uninfested soil 120 hours after inoculation. Each treatment was replicated four times.

Radicle length and pot size: Germinated seedlings were separated into four radicle length groups 2.0–3.0, 3.5–4.5, 5.0–6.0, and 6.5–7.5 cm. Seedlings in each radicle length class were planted in 7.5-cm-d or 10-cm-d pots. Each treatment was replicated six times, and the test was repeated twice.

Inoculum type and level: Seedlings with radicles 3.5–4.5 cm long were transplanted into 7.5-cm-d pots. They remained in the infested soil for the duration of the test. Treatments in this test were 1) 20 and 2) 40 plump cysts per pot; 3) 1,000, 4) 2,000, 5) 4,000, and 6) 6,000 eggs per pot; and 7) 500, 8) 1,000, and 9) 2,000 J2 per pot. Each treatment was replicated nine times. Juveniles were obtained by placing eggs in a hatching chamber with aerated water for 24 hours. Eggs and J2 were applied in 5 ml water with a repeating dispenser. Cysts were picked individually at 15× magnification, placed in small watch glasses, and carefully washed into the soil depression where the seedling was to be placed.

Temperature: Soybean seedlings with radicles 3.5–4.5 cm long were transplanted into the soil mix infested with 4,000 eggs and J2 of *H. glycines* in 7.5-cm-d pots. All treatments except one were in Phytotron growth chambers. Temperature regimes were 1) constant 24 C; 2) constant 28 C; 3) constant 32 C; 4) 28 C light period (16 hours), 24 C dark period (8 hours); 5) 32 C light, 24 C dark; 6) 36 C light, 24 C dark; and 7) 26 ± 1 C constant in a greenhouse. A second temperature study was set up 3 days after the initial test. Different inocula were used in the two tests, and pots from both were placed in the same Phytotron growth chambers set at the desired temperatures.

Methods check: Race tests were run using stock cultures of SCN races 1–5 maintained at North Carolina State University. The procedure was the same as that used in the temperature studies except the tests were run at constant temperature of 28 C. Eight replications of each differential were used. Two chambers set at 28 C were used and half of the plants of each differential for each race were placed in each chamber. A second race test was established 3 days after the first. Because of certain anomalies in the results of the first two tests, a third

TABLE 1. Average numbers of mature females of *Heterodera glycines* race 1 on the roots of Lee soybean inoculated with different types of inoculum at different levels.

Inoculum type	Level	Females/pot
Cysts	20	185 ± 98 b
	40	255 ± 93 a
Eggs	1,000	51 ± 20 de
	2,000	95 ± 37 cd
	4,000	256 ± 74 a†
	6,000	256 ± 108 a‡
Juveniles	500	25 ± 14 e
	1,000	56 ± 34 de
	2,000	129 ± 49 c‡

Numbers are means ± standard deviation of 17 replications except as otherwise noted. Numbers in columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.
† Fifteen replications.
‡ Sixteen replications.

TABLE 2. Average numbers of mature females of *Heterodera glycines* race 1 on roots of Lee soybean at different temperature regimes.

Temperature regime (C)	Females/pot
Constant 24	150 ± 98 ab
Constant 28	204 ± 127 a
Constant 32	139 ± 88 ab
28 day, 24 night	135 ± 90 b
32 day, 24 night	107 ± 86 b
36 day, 24 night	31 ± 39 c
Greenhouse 26	104 ± 78 b

Numbers are means ± standard deviations of 16 replications. Numbers followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

test was run at North Carolina State using Lee 74 as a susceptible check instead of Lee. The test also was repeated in growth chambers at the University of Arkansas using races 1, 2, 3, and 14 and including both Lee and Lee 74 as susceptible checks. The prescribed differential soybean genotypes Pickett, Peking, PI 88788, and PI 90763 were used in all race tests (2).

Tests of susceptible cultivars: In one test, seedlings of the *H. glycines*-susceptible cultivars Lee, Lee 74, Deltapine 105, and Bragg were transplanted into soil mix infested with *H. glycines* race 1 as described in the general methodology. The pots were placed in a growth chamber at 28 C light period and 24 C dark period. Each cultivar was replicated 10 times.

In a second test, seedlings of the cultivars Bragg, Deltapine 105, Essex, Lee 68, Lee 74, Lee from E. E. Hartwig, Delta Branch Experiment Station, Stoneville, Mississippi, and Lee from D. P. Schmitt, North Carolina State University, were transplanted into soil mix infested with *H. glycines* race 1, 2, 3, or 5. Twenty pots per race were planted with the respective cultivars; 10 pots were harvested after 28 days and 10 after 35 days.

Results of the two runs of each test were similar and data were combined for analysis. All data were subjected to analysis of variance, and Duncan's new multiple-range test using the SAS program (13).

RESULTS

Postinoculation management: The greatest number of females (380 ± 97/plant) ($P = 0.05$) were extracted from plants that were not transplanted after inoculation. Plants transplanted after 120 hours had 280 ± 47 females/plant and plants transplanted after 48 hours had 16 ± 2 females/plant. The coefficient of variation (CF) for the test was 77.

Radicle length and pot size: Radicle length of transplants had no effect on the number of females that developed. Treatment means ranged from 213 to 274 females/pot and the CV for the test was 54. Plants in 7.5-cm-d pots had a few more females (234) than those in 10-cm-d pots (194), but the difference was not significant.

Inoculum type and level: Plants in soil mix infested with 40 plump cysts, 4,000 eggs, or 6,000 eggs had the same number of females, but for these three treatments, variation in the number of females was lowest from plants inoculated with 4,000 eggs (Table 1). Fewer ($P = 0.05$) females were produced on plants in soil mix infested with all other inoculum types and levels.

Temperature: The numbers of females extracted were different ($P = 0.05$) among the temperature regimes (Table 2). Numerically, the highest number of females developed on plants at constant 28 C but not significantly more than at constant 24

TABLE 3. Average numbers of *Heterodera glycines* mature females on Lee and female indices on the differential host lines.

Race	Females on Lee (index = 100)	Indices†			
		Pickett	Peking	PI 88788	PI90763
1	219	3 ± 3 b	1 ± 2 b	58 ± 50 a	0.2 ± 0.5 b
2	268	112 ± 69 a	79 ± 54 a	87 ± 54 a	6 ± 7 b
3	257	0.7 ± 0.3 b	1 ± 1 b	35 ± 24 a	1 ± 2 b
3 (ARK)	287	0.3 ± 0.4 a	0.04 ± 0.1 a	1 ± 0.4 a	0.1 ± 0.2 a
4	247	102 ± 60 a	20 ± 15 b	93 ± 65 a	10 ± 23 b
5	177	122 ± 58 a	45 ± 40 b	121 ± 78 a	3 ± 9 c
14 (ARK)	116	150 ± 60 a	57 ± 34 b	4 ± 3 c	52 ± 23 b

Numbers are means of 24 replications except the Arkansas races 3 and 14 which had eight replications. Means in rows followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

† Indices were calculated as follows:

$$\frac{\text{No. females on differential}}{\text{No. females on Lee}} \times 100.$$

C or 32 C. Fewest females developed on plants placed at the 36 C day–24 C night regime.

Race tests: Mean numbers of females extracted from the susceptible cultivar Lee varied among the races, even though Lee was considered to be susceptible to all races. The test of races 1, 2, and 4 in North Carolina and races 1, 2, 3, and 14 in Arkansas gave the expected results but with extensive variation between plants within a test (Table 3).

Tests of susceptible cultivars: When Lee, Lee 74, Deltapine 105, and Bragg were grown in soil mix infested with *H. glycines* race 1, mean numbers of females extracted after 28 days were 79 ± 73, 126 ± 58, 101 ± 46 and 49 ± 21, respectively. Numbers of females on Lee 74 and Deltapine 105 were greater ($P = 0.05$) than on Lee or Bragg. In soil mix infested with one of four *H. glycines* races, six cultivars, including two sources of Lee, had 20–403 females per plant 28 days after inoculation (Table 4). Few females of races 1 and 3 matured on Lee from Hartwig, and Lee from Schmitt had about 20% resistant plants. The mean numbers of females produced by all four races on each cultivar were not significantly different, except the Lee from Hartwig had fewer ($P = 0.05$) than any of the others, particularly when inoculated with race 1 or race 3. When the number of females produced was averaged across cultivars, the number of race 3 females was highest followed by race 5, race 2, then race 1 ($P = 0.05$).

A second set of tests on susceptible cultivars screened after 35 days had differences among races and among cultivars similar to those at 28 days (Table 4). The number of females produced per plant was lower ($P = 0.05$) at 35 days than at 28 days even though the soil was screened to extract females and cysts that were dislodged from the roots when the soil was removed. At 35 days Bragg, Lee 68, and Lee 74 had similar numbers of females, whereas Deltapine 105, Essex, and Lee from both sources had fewer females ($P = 0.05$).

The CV for the test of susceptible cultivars in the greenhouse was 44. This compares with a CV of 50 for the test of only four susceptible cultivars inoculated with only one race in the growth chamber at 28 C and with uniform environmental conditions.

DISCUSSION

Attempts to develop standard procedures for *H. glycines* race tests had only limited effectiveness. Even though inoculum preparation and the number of propagules applied were the same for two comparable tests, the numbers of females produced varied greatly among replications and among tests. Nevertheless, some variables could be eliminated. Because the numbers

TABLE 4. Average numbers of mature females of four races of *Heterodera glycines* from the roots of seven susceptible soybean cultivars grown in a greenhouse for 28 or 35 days after inoculation.

Host	Race 1	Race 2	Race 3	Race 5	Mean
			28 days		
Bragg	166 ± 76.5	236 ± 114.4	403 ± 211.3	248 ± 109.1	259 ± 161.7 a
Deltapine 105	30 ± 58.6	242 ± 89.7	374 ± 162.5	243 ± 125.4	245 ± 147.8 a
Essex	127 ± 34.2	273 ± 111.8	398 ± 196.1	265 ± 125.7	249 ± 159.5 a
Lee 68	170 ± 74.9	278 ± 101.2	337 ± 161.7	319 ± 150.6	274 ± 140.5 a
Lee 74	88 ± 79.7	211 ± 101.4	324 ± 131.7	279 ± 97.1	246 ± 117.2 a
Lee (Hartwig)	20 ± 57.3	258 ± 84.6	22 ± 50.5	288 ± 119.0	147 ± 150.5 b
Lee (Schmitt)	165 ± 102.6	184 ± 92.0	346 ± 231.1	291 ± 108.8	242 ± 163.3 a
Mean	120 ± 85.4 D	241 ± 100.9 C	311 ± 95.1 A	276 ± 118.6 B	
			35 days		
Bragg	128 ± 49.6	221 ± 115.6	280 ± 113.7	228 ± 83.7	214 ± 107.0 a
Deltapine 105	111 ± 54.2	178 ± 51.8	220 ± 115.1	151 ± 97.9	165 ± 90.7 a
Essex	115 ± 48.2	167 ± 67.7	190 ± 104.9	222 ± 97.9	173 ± 88.5 cd
Lee 68	119 ± 43.5	175 ± 81.5	284 ± 134.6	201 ± 79.4	194 ± 106.3 abc
Lee 74	143 ± 34.6	235 ± 121.7	252 ± 111.7	207 ± 101.3	209 ± 104.1 ab
Lee (Hartwig)	6 ± 10.3	195 ± 90.6	28 ± 95.7	200 ± 77.5	107 ± 117.8 e
Lee (Schmitt)	131 ± 80.8	156 ± 58.5	253 ± 141.5	202 ± 92.9	185 ± 106.3 bcd
Mean	108 ± 64.5 C	190 ± 88.6 B	215 ± 141.0 A	201 ± 90.0 B	

Numbers for races are means of 10 replications. Means of varieties or races followed by the same letter are not significantly different ($P = 0.05$) according to the Waller-Duncan k-ratio *t*-test.

of females in the 7.5-cm-d pots were at least as high as the numbers from 10-cm-d pots, there is no advantage to using larger pots that take more space. The size of seedlings transplanted did not affect the number of females that developed, but the variability in female numbers was somewhat lower when plants with radicles 3.5–4.5 cm long were used. The number of females produced from 40 cysts was as great as the number produced from 4,000 eggs, but the variation was greater. When the variation was less than that with 4,000 eggs, the number of females was lower than that considered necessary for a good comparison of numbers.

Previous temperature studies indicated that 24–28 C was optimum for *H. glycines* in terms of length of its life cycle (1,3,5). No previous studies reported the effect of temperature on the number of females produced in a given period of time. Constant 24 C, constant 32 C, or 28 C in the day and 24 C at night resulted in adequate female numbers and lower variation than other temperature regimes. A temperature regime of 36 C in the day and 24 C at night allowed some female maturation, whereas a constant temperature of 34 C inhibits female maturation (3). These results indicated that a greenhouse in summer, when temperatures may be 36 C or above for part of the day, may result in lower female numbers. Actual results, however, indicate that tests run in a greenhouse in the summer have adequate female maturation (Riggs, unpubl.).

Problems with the Lee cultivar resulted in a need to run four race tests. There is no explanation of why Lee, previously reported to be susceptible to all races of *H. glycines*, suddenly became resistant to races 1 and 3 of *H. glycines*. Similar results were reported in Iowa with Lee (Ruff, pers. comm.), and in race tests run later in Arkansas, Lee was still resistant to race 3 (Riggs, unpubl.). Lee 74 is being used as the susceptible check until the situation with Lee is resolved. The variation among host replications in race tests conducted under standardized conditions was considerable but not as much as in greenhouse tests where only inoculum is standardized.

Possible sources of variation include 1) the plants, even though seed stocks were built from a single seed taken from a uniform source; 2) the number of propagules applied, even though the number of eggs

was calculated and a measured amount was placed in each pot; 3) the number of eggs that hatch; 4) the ability of juveniles to find the roots; 5) the ability of juveniles to penetrate the roots; and 6) the male : female ratio. The last could be influenced by the number of nematodes that penetrate.

The tests of susceptible cultivars in the growth chambers were conducted to evaluate the uniformity of cyst nematode development in different susceptible cultivars. Results indicated that the susceptible cultivar used could greatly affect the apparent race of a population of *H. glycines*. The greenhouse test was conducted to further investigate the problem with the Lee cultivar, to check other susceptible cultivars, and to determine whether 35 days rather than 28 days would make a difference in the number of females extracted. There is no explanation for the low number of females produced on Lee, whereas Lee 68 and Lee 74 had abundant female production. Also, the reason for the greater number of females extracted at 28 days than at 35 days is not known. If the roots alone had been processed at 35 days, the difference might have been caused by some females coming free in the soil when the roots were extracted; however, when the females were extracted from both roots and soil, fewer were obtained at 35 days than at 28 days. Females or cysts do not normally deteriorate that soon or that rapidly.

In conclusion, these studies show that uniformity in seedling size, inoculation, and temperature may help reduce the variability in number of females produced by *H. glycines*. The numbers of females on the differentials in the race test, however, were still quite variable.

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