

A Technique for Evaluating *Heterodera glycines* Development in Susceptible and Resistant Soybean¹

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Abstract: A technique was developed to evaluate *Heterodera glycines* development in susceptible and resistant soybean. Roots of 3-day-old soybean were exposed to infective juveniles of *H. glycines* in sand for 8 hours followed by washing and transfer to hydroponic culture. The cotyledons and apical meristem were removed and plants were maintained under constant light, which resulted in a dwarfed plant system. After 15 or 20 days at 27 C, nematodes were rated for development. Emerged males were sieved from the culture water and females were counted directly from the roots. Nematodes remaining in the roots were rated for development after staining and clearing the tissues. The proportion of nematodes at each stage of development and the frequency of completed molts for each stage were calculated from these data. This technique showed that resistance to *H. glycines* was stage related and did not affect males and females equally in all resistant hosts. The resistance of plant introduction PI 209332 primarily affected development of third and fourth-stage juveniles; 'Pickett' mainly affected second and third-stage juveniles, whereas PI 89772 affected all stages. Male development was markedly affected in PI 89772 and 'Pickett' but not in PI 209332.

Key words: cyst nematode, *Glycine max*, *Heterodera glycines*, hydroponics, nematode, resistance, soybean.

Genetic resistance to the soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe) is assessed by bioassay. Soybeans (*Glycine max* (L.) Merr.) are inoculated with equal numbers of eggs or infective juveniles, and resistance is based on the relative number of females that develop compared to the number produced on a known susceptible cultivar (5). Generally, no data are collected on male emergence or juvenile development.

While investigating the host range of *H. glycines*, Ichinohe recorded the development of nematodes in various plants (8). In 12 plant species he found no juvenile penetration. In seven plant species, juveniles penetrated the roots but did not develop further, and in eight other plant species, nematodes were partially developed but no reproduction occurred. In Spanish runner bean (*Phaseolus coccineus*), females were almost mature but development was

arrested at the late fourth or early adult stage. These females failed to produce eggs, but many adult males were recovered from the same plant. In kidney bean (*Phaseolus vulgaris*), the females were poorly developed, produced few eggs, and rarely ruptured the root epidermis. Similar results were obtained by Riggs (11). Events necessary for successful reproduction of *H. glycines* include host finding, penetration, feeding, growth, development, sexual maturity, and egg production. Failure of the nematode to complete any stage denotes resistance in soybean, but standard bioassays do not characterize nematode development. Research on genetic resistance to *H. glycines* would benefit from phenotypic markers that would denote different resistance mechanisms. One of the most readily observable characteristics of host resistance is its effect on nematode development. Our technique evaluated *H. glycines* development in susceptible and resistant soybeans and identified stage-related resistance mechanisms.

Nematode development and concomitant histological responses of roots have been studied in detail (2-4,9,12). Second-stage *H. glycines* penetrate roots of susceptible and resistant soybean with equal frequency and may be found in the cortex within a few hours after inoculation. Syn-

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cytial development is evident within 2–3 days, and initial cell response is similar in susceptible and resistant plants (4,9). In susceptible plants, females feed until egg production ceases but males cease feeding after two molts (8). In resistant soybean, the level of nematode mortality varies depending on the genetic makeup of the SCN population and the source of resistance (2). Genetic heterogeneity usually ensures that some nematodes develop to maturity and reproduce (10,15).

Observations on *H. glycines* development have been made primarily by examination of thin, sectioned histological preparations of soybean roots. This technique allows detailed cytological analysis of both nematode and soybean but is labor intensive and time consuming. Generally, observations are made on relatively few nematodes and quantitative data are lacking. Previously, Halbrecht and Dropkin developed a hydroponic culture system utilizing pruned soybeans for evaluating *H. glycines* resistance (6). We used a modified version of this system to establish a development profile of seven selected *H. glycines* populations in susceptible and resistant soybean. By limiting nematode access to the roots to 8 hours, initial nematode development was synchronous in all plants. The developmental stage of every nematode was determined, and the proportion of nematodes at each stage was calculated. In resistant soybean, deterrents or blocks to development became evident by calculating the frequency of completed molts to successive stages relative to the checks.

MATERIALS AND METHODS

Nematode populations were selected and maintained on various soybean lines. Populations N-20 and N-89 were the same populations used by Halbrecht and Dropkin (6). These populations initially were developed over nine generations of single female transfer to soybean introductions PI 209332 and PI 89772, respectively. Other selected populations were initiated by inoculating soybean with two juveniles

and increasing the progeny of successful matings. Populations N-ESA1 and N-ESA2 were selected from the offspring of a single female on 'Essex' soybean, populations N-20B1 and N-20F1 were selected from PI 209332, and population N-PiB1 was selected from Pickett. In each experiment, *H. glycines* development was evaluated on a resistant soybean, a susceptible check ('Williams' or Essex), and the selecting host.

All populations were increased on their selecting host in the greenhouse (7). After 33–35 days, nematodes were washed from the roots, females macerated, and eggs recovered on a sucrose gradient (1). Eggs were mixed in fine sand at 1,000 eggs/cm³ with sufficient water to allow the sand particles to loosely adhere. Approximately 200–250 cm³ of inoculum filled a 1,000-ml beaker to a depth of 3 cm. Soybeans with roots 3–5 cm long were placed 2–2.5 cm deep in the sand. The beaker was tapped gently on the bench to firm the sand around the roots and covered with plastic wrap to maintain humidity. In each experiment, all seedlings were inoculated simultaneously in one beaker with a wire divider separating the different soybean genotypes. The beaker was placed in a 27 C water bath for 8 hours.

After inoculation, roots were washed in a stream of water and the cotyledons were excised about 2 mm from the hypocotyl. Plants were transferred to test tubes and maintained in hydroponics according to the method of Halbrecht and Dropkin, except that fluorescent light (cool white) was applied continuously (6). Five to 7 days after inoculation, the hypocotyl was cut off above the cotyledonary node. After 15 or 20 days at 27 C, nematodes were counted and rated for development. Males were sieved from the water with a 20- μ m mesh nylon screen and counted under 8 \times magnification. Mature females were counted directly from the roots.

The segment of tap root containing undeveloped nematodes was about 2 cm in length and identified by the presence of lesions and mature females. Nematodes

were not found in lateral roots. Lateral roots and mature females were removed before cutting the tap root approximately 1 cm either side of the infected area. Infected root segments were stained in acid fuchsin and cleared in lactophenol/glycerin (2). Stained segments were flattened between two microscope slides, examined under low power of the microscope, and the developmental stage of each nematode determined. Each experiment was repeated and data from both trials were combined for analysis. A development profile was determined by calculating the proportion of nematodes at each stage. The values P_1 , P_2 , and P_3 , (where P_1 = the frequency of completed molts from second (J2) to third-stage (J3), P_2 = the frequency of completed molts from J3 to fourth-stage (J4), and P_3 = the frequency of completed molts from J4 to adult-stage), were calculated as follows: $P_1 = (J3 + J4 + \text{adults}) \div (\text{total nematodes})$, $P_2 = (J4 + \text{adults}) \div (J3 + J4 + \text{adults})$, and $P_3 = (\text{adults}) \div (J4 + \text{adults})$. Relative P values were calculated by dividing P values of test plants by the corresponding P value from the checks, which took into account the normal mortality rate for each stage (13). The number of nematodes at each stage and the sex ratios (male:female) obtained from resistant soybeans were compared to checks and analyzed by chi-square contingency table analysis.

RESULTS

The number of juveniles entering roots varied in each experiment, from a mean of 1.8 to 43.8 nematodes per plant. The proportion of adults also varied, ranging from 49 to 77% of the total in susceptible checks, but there was no correlation between infection level and adult development. All stages of development were present in each soybean genotype, although not in every plant. The proportion of nematodes that did not complete development in susceptible checks established a "normal mortality" rate for each experiment. The mortality rate in checks ranged from 23 to 51%

with a mean of 40%, but mortality rate was not uniform across all developmental stages. Mortality was higher for J4 in Williams and for J3 in Essex. Few J2 were found in any check (Table 1).

Populations produced similar numbers of females on both the selecting host and check plants but fewer on the nonselecting host. The number of males did not always correspond to the number of females. As a general rule, relatively fewer males developed on PI 89772 and 'Pickett' than on PI 209332 or the checks, regardless of selecting host for the population (Table 1).

The sex ratio (male:female) on the checks was approximately 1, except in Experiment 7, where the ratio was 0.4 (Table 2). Three populations selected on PI 209332 produced as many adults on PI 209332 as the check but significantly fewer on PI 89772 and Pickett. The sex ratio was different from the check only on PI 89772, which had fewer females than males (Table 2, Experiments 1, 3, and 4). Population N-89 produced fewer adults on both PI 89772 and PI 209332 than on Williams. The sex ratios were also different from the check, but females were more abundant than males on PI 89772 and males were more abundant than females on PI 209332 (Table 2, Experiment 2). Population N-PiB1 produced as many adults on Pickett as on Essex but fewer on PI 209332. The sex ratios differed from the check on both test plants, but females were more abundant than males on Pickett and males were more abundant than females on PI 209332 (Table 2, Experiment 5). Two populations selected on Essex produced fewer adults on PI 209332 and Pickett than on Essex. The sex ratio of population N-ESA1 was not different among treatments but N-ESA2 produced more males than females on PI 209332 (Table 2, Experiments 6 and 7).

The values of P_1 , P_2 , and P_3 indicated the frequency of completed molts from stages J2 to J3, J3 to J4, and J4 to adult, respectively, relative to the number of survivors at each developmental stage (data not shown). The P values in Table 3 were

TABLE 1. Development of selected SCN populations on susceptible and resistant soybean lines in seven experiments†.

Experiment no. and soybean‡	No. of plants	Total SCN	Stages				
			Juveniles			Adults	
			J2	J3	J4	M	F
Experiment 1			N-20 (selected on PI 209332)			Day 15	
Wms	30	564	0.05§	0.18	0.21	0.29	0.27
PI 89	30	660	0.36***¶	0.41***	0.10***	0.12***	0.01***
PI 20	30	582	0.14***	0.17 NS	0.14*	0.27 NS	0.28 NS
Experiment 2			N-89 (selected on PI 89772)			Day 15	
Wms	30	1313	0.06	0.06	0.11	0.35	0.42
PI 89	30	1239	0.06 NS	0.14***	0.16**	0.23***	0.41 NS
PI 20	16¶	688	0.09*	0.18***	0.18***	0.30 NS	0.25***
Experiment 3			N-20B1 (selected on PI 209332)			Day 20	
Esx	27	329	0.09	0.14	0.04	0.38	0.35
Pick	30	435	0.21***	0.16 NS	0.10**	0.28*	0.25*
PI 20	24	356	0.08 NS	0.10 NS	0.05 NS	0.36 NS	0.41 NS
Experiment 4			N-20F1 (selected on PI 209332)			Day 20	
Esx	13¶	90	0.03	0.31	0.12	0.28	0.26
Pick	33	211	0.25***	0.41 NS	0.10 NS	0.13*	0.11*
PI 20	33	217	0.07 NS	0.44 NS	0.05 NS	0.19 NS	0.25 NS
Experiment 5			N-PiB1 (selected on Pickett)			Day 20	
Esx	40	415	0.04	0.32	0.09	0.25	0.30
Pick	53	560	0.06 NS	0.38 NS	0.11 NS	0.13***	0.32 NS
PI 20	48	510	0.05 NS	0.42*	0.12 NS	0.28 NS	0.13***
Experiment 6			N-ESA1 (selected on Essex)			Day 20	
Esx	27	52	0.08	0.32	0.02	0.31	0.27
Pick	27	65	0.63***	0.29 NS	0.02 NS	0.06**	0.00***
PI 20	30	55	0.17 NS	0.42 NS	0.15 NS	0.11 NS	0.15 NS
Experiment 7			N-ESA2 (selected on Essex)			Day 20	
Esx	27	134	0.08	0.40	0.03	0.14	0.35
Pick	28	118	0.59***	0.29 NS	0.01 NS	0.05*	0.06***
PI 20	29	111	0.07 NS	0.68*	0.10*	0.11 NS	0.04***

† Data are the combined results of two similar trials.

‡ Soybean germplasm used in these experiments: Williams (Wms), PI 89772 (PI 89), PI 209332 (PI 20), Essex (Esx), and Pickett (Pick).

§ Number of nematodes at a stage given as a fraction of the total.

¶ Number of nematodes at each stage compared to the check (Williams or Essex); significance determined by chi-square contingency table analysis of raw data (with continuity correction), NS = not significant, * = ($P \leq .05$), ** = ($P \leq .01$), and *** = ($P \leq .001$).

¶ Plants of one trial developed a fungal infection and were discarded.

calculated relative to the checks to account for mortality rate in the checks. High *P* values indicated low mortality and low *P* values indicated high mortality of J2, J3, and J4 respectively.

Second-stage mortality was generally low and usually not significant on PI 209332, regardless of the selecting host for the population. In contrast, J2 mortality was significant on PI 89772 and Pickett, except when populations had been se-

lected on those soybeans (Table 3). Mortality of J3 varied. Of three populations selected on PI 209332, N-20B1 showed low J3 mortality on Pickett and PI 209332, N-20 showed low mortality on PI 209332 but high mortality on PI 89772, and N-20F1 showed significant J3 mortality on both Pickett and PI 209332; however, mortality on Pickett was greater (Table 3, Experiments 1, 3, and 4). Although populations N-89 and N-PiB1 both showed sig-

TABLE 2. Comparison of total adults and sex ratio of each SCN-soybean combination to the check (Williams or Essex).

Experiment no. and soybean	Total adults	Sex ratio (male:female)
Experiment 1		
		<u>N-20</u>
Williams	.56†	1.07
PI 89772	.13***‡	12.00***
PI 209332	.55 NS	0.96 NS
Experiment 2		
		<u>N-89</u>
Williams	.77	0.83
PI 89772	.64**	0.56***
PI 209332	.55***	1.20**
Experiment 3		
		<u>N-20B1</u>
Essex	.73	1.09
Pickett	.53**	1.12 NS
PI 209332	.77 NS	0.88 NS
Experiment 4		
		<u>N-20F1</u>
Essex	.54	1.08
Pickett	.24***	1.18 NS
PI 209332	.44 NS	0.76 NS
Experiment 5		
		<u>N-PiB1</u>
Essex	.55	0.83
Pickett	.45 NS	0.41***
PI 209332	.41*	2.15***
Experiment 6		
		<u>N-ESA1</u>
Essex	.58	1.15
Pickett	.06***	• NS
PI 209332	.26*	0.73 NS
Experiment 7		
		<u>N-ESA2</u>
Essex	.49	0.40
Pickett	.11***	0.83 NS
PI 209332	.15***	2.75**

† Number of adults given as a fraction of the total.

‡ Significance determined by chi-square contingency table analysis of raw data (with continuity correction), NS = not significant, * = ($P \leq .05$), ** = ($P \leq .01$), and *** = ($P \leq .001$).

nificant J3 mortality on resistant soybean, mortality rates were higher on PI 209332 compared to the selecting host (Table 3, Experiments 2 and 5). Two populations selected on Essex showed high J3 mortality on Pickett, but only N-ESA2 showed high J3 mortality on PI 209332 (Table 2, Experiments 6 and 7).

Fourth-stage mortality was not evident on PI 209332 when populations had been selected on PI 209332, but J4 mortality was significant when populations had been selected on PI 89772, Pickett, or Essex (Ta-

TABLE 3. Relative development success: Frequency of completion of J2 to J3 molt (P_1), J3 to J4 molt (P_2), and J4 to adult molt (P_3) of each SCN-soybean combination relative to the check (Williams or Essex).

Experiment no. and soybean	Relative P values		
	P_1	P_2	P_3
Experiment 1			
		<u>N-20</u>	
Williams	1.00	1.00	1.00
PI 89772	0.67***†	0.44***	0.77***
PI 209332	0.91***	0.99 NS	1.10*
Experiment 2			
		<u>N-89</u>	
Williams	1.00	1.00	1.00
PI 89772	1.00 NS	0.90***	0.91***
PI 209332	0.96*	0.85***	0.85***
Experiment 3			
		<u>N-20B1</u>	
Essex	1.00	1.00	1.00
Pickett	0.87***	0.94 NS	0.88***
PI 209332	1.01 NS	1.05 NS	0.99 NS
Experiment 4			
		<u>N-20F1</u>	
Essex	1.00	1.00	1.00
Pickett	0.77***	0.66***	0.86 NS
PI 209332	0.96 NS	0.76*	1.11 NS
Experiment 5			
		<u>N-PiB1</u>	
Essex	1.00	1.00	1.00
Pickett	0.98 NS	0.90*	0.93 NS
PI 209332	0.99 NS	0.84**	0.90*
Experiment 6			
		<u>N-ESA1</u>	
Essex	1.00	1.00	1.00
Pickett	0.40***	0.32**	0.82 NS
PI 209332	0.89 NS	0.75 NS	0.66**
Experiment 7			
		<u>N-ESA2</u>	
Essex	1.00	1.00	1.00
Pickett	0.45***	0.51**	0.99 NS
PI 209332	1.01 NS	0.47***	0.61***

† Significance determined by chi-square contingency table analysis of raw data; NS = not significant, * = ($P \leq .05$), ** = ($P \leq .01$), and *** = ($P \leq .001$).

ble 3). Population N-20B1 showed significant J4 mortality on Pickett but other populations did not, regardless of the selecting host (Table 3, Experiments 3-7). Populations N-20 and N-89 both showed significant J4 mortality on PI 89772, but the relative P_3 value was considerably greater for N-89, indicating lower mortality (Table 3, Experiments 1 and 2).

DISCUSSION

Previous work demonstrated that the number of female *H. glycines* developing

on pruned, resistant, and susceptible soybean plants provided a measure of resistance similar to that obtained from a standard bioassay (6,7). This has also been observed for *H. glycines* in adventitious roots of rooted leaf cuttings (unpubl.). These data suggest that genetic resistance to *H. glycines* affects nematode development similarly in pruned and intact plants. Therefore, development in pruned soybean can indicate stage-related resistance mechanisms by identifying blocked developmental stages.

Evidence for stage-related resistance is provided in part by Endo's study of *H. glycines* development in soybean 'Lee,' 'Peking,' and a Lee \times Peking cross (4). In Peking, development of J2 was arrested and no adult males or females were found. In Lee most nematodes completed development, and in the Lee \times Peking cross there was noticeable J2 mortality (but some males and females developed). Although quantitative data were not provided, these results appear similar to those of selected populations in our study. Development of N-ESA1 and N-ESA2 on Pickett (resistance derived from Peking) was similar to Endo's population on Peking (i.e., mortality was greatest at the J2 stage and virtually no adults developed). Development of N-20B1 and N-20F1 on Pickett was similar to Endo's population on the Lee \times Peking cross (i.e., there was considerable J2 mortality but some males and females developed). Development of all four populations on Essex was similar to Endo's population on Lee (Table 1, Experiments 3, 4, 6, and 7). The ability of our technique to process a relatively large sample size and to evaluate nematode development quantitatively suggests its usefulness for research on genetic resistance to *H. glycines*.

Variable levels of infection were possibly due to differences in the general level of maturity of each batch of eggs. Fluctuations of greenhouse temperatures and variation in development time may account for some of the observed differences. It was shown that infection levels could be increased by incubating the egg/

sand mixture a few days at 27 C before use; the inoculum remained viable for weeks provided it was not allowed to dry (unpubl.). Presumably high levels of infection could interfere with nematode development by creating competition for feeding sites and food. Our data did not suggest abnormal development at these infection levels, however, because the proportion of adults did not decline with increasing nematodes in the root. Heavily infected roots were more difficult to examine than lightly infected roots, and an infection level of 10 to 20 nematodes per plant was considered optimum. Additionally, uniform and moderate infection levels would simplify comparisons of results between experiments because sample size affects statistical significance. For example, a relative P_1 value of 0.96 was significant in Experiment 2, but a P_1 value of 0.89 was not significant in Experiment 6 (Table 3). The sample sizes were very different in these experiments.

The normal mortality rate varied and was thought to be influenced by the general condition of the nematodes. Some nematodes may fail to develop fully, even under optimum conditions. Failure to find a suitable feeding site and general health of the nematodes are some factors that may influence success. The mortality level in a susceptible check provides a benchmark for evaluating resistant interactions. This is particularly useful when the mortality rate in the resistant host is relatively low.

Populations N-ESA1 and N-ESA2 produced few adults on Pickett and PI 209332, indicating that resistance to these populations was expressed by both soybeans. The number of adults on Pickett or PI 209332 was not different from Essex, however, if the population had been selected on Pickett or PI 209332 (Table 2, Experiments 3-7). Similarly, population N-20 produced comparable numbers of adults on both PI 209332 (the selecting host) and Williams (Table 2, Experiment 1). These data illustrate the advantage of using selected populations to demonstrate

genetic resistance. All populations showed varying levels of compatibility with the nonselecting hosts, however, indicating that they were still heterogeneous for virulence. Experiments on genetic resistance should be improved if populations of *H. glycines* were inbred and selected for specific virulence traits.

Developmental blocks were most prominent in combinations showing high mortality. The two populations selected on Essex provided the best examples of stage-related resistance. In Pickett virtually all mortality occurred at the J2 and J3 while in PI 209332, the J3 and J4 were most affected (Table 3, Experiments 6 and 7). This same basic pattern was evident in other combinations, although it was not as distinct and not always at significant levels because mortality was not as great. This can be attributed to the genetic heterogeneity of these populations. The genetic makeup of the *H. glycines* population is an important factor in expression of resistance. A resistant soybean could conceivably have two or more different resistant mechanisms, but this could go undetected unless challenged with appropriate SCN populations of known genetic background.

Significant differences in sex ratios resulted from differential mortality of the sexes. Except for populations N-89 and N-PiB1, male mortality was generally associated with resistance that affected J2 development and was common in PI 89772 and Pickett (Table 1). This may be linked to differences in feeding habit of males and females, because males only feed during early development. Once males have stopped feeding, perhaps they continue to develop while being effectively isolated from host resistance. The mechanism of resistance is not known, but if expressed through feeding, both males and females would be exposed to an "early type" of resistance factor, whereas only females would be exposed to factors influencing later development. Supporting evidence comes from females of N-89 and N-PiB1 on PI 209332 which were fewer and smaller (pers. obs.) compared to those on

the selecting host and check. Male development was apparently unaffected (Table 1, Experiments 2 and 5). Developmental blocks in PI 209332 affected J3 and J4 stages, suggesting a resistance mechanism that affects late stages of development, thus having a greater impact on females (Table 3, Experiments 2 and 5). This observation is supported by other reports of differential resistance where poorly developed females were associated with abundant and active males (6,8).

Although stage-related resistance mechanisms may partially explain differential mortality of the sexes, they do not provide a satisfactory explanation for all of the observed results. Populations N-89 and N-PiB1 produced fewer males than females on their own selecting host, even though early mortality was relatively low (Table 1, Experiments 2 and 5). In these examples, it appears that resistance was specific against males, whereas females were relatively unaffected. In these combinations, the possibility of sex-linked genes for virulence cannot be ruled out and could possibly account for this pattern of development (14).

The standard bioassay for *H. glycines* resistance is based exclusively on the relative number of females that develop to maturity. The method is relatively quick and convenient but does not distinguish different types of resistant mechanisms. Most research has not focused on details of the SCN-soybean interaction because it has been difficult to study the progress of nematode development in whole plants. Our technique overcame several problems by using a timed inoculation to establish a localized and synchronous SCN infection. One effect of pruning was to reduce the volume of cortex tissue, which facilitated identification of nematode stages. The closed hydroponic system also permitted recovery of all egressed males. The technique is useful for obtaining quantitative data on *H. glycines* stages and has shown that different resistant soybean lines arrest nematode development at different stages. When compared to a susceptible check,

nematode mortality in resistant hosts could be attributed to arrested development at specific stages. This technique may be a useful tool for future research on the genetics and mechanism of *H. glycines* resistance in soybean.

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