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Inoculation Method for Studying Early Responses of *Glycine max* to *Heterodera glycines*[1]

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Abstract: An inoculation technique was developed for studying molecular responses of soybean to the soybean cyst nematode (*Heterodera glycines*). Effect of inoculum age (0–7 days after eggs were released from cysts) and inoculation site (meristem, elongation, or differentiation zone) on infection were tested on four soybean genotypes. Two genotypes (PI 437654 and cv. Peking) were resistant and two (cv. Essex and cv. Hutcheson) were susceptible to race 3 of *H. glycines*. Inoculum consisting of second-stage juveniles (J2) was prepared by gently agitating nematode eggs at 75 revolutions per minute at 28 °C for various intervals. Infection rates were monitored cytologically. The most consistent infection rate was obtained with 48-hour-old inoculum containing more than 80% J2. More than 100 juveniles/root were observed after inoculation with the 48-hour-old inoculum placed at the root elongation zone, in both resistant and susceptible soybeans. Horizontal orientation of roots during inoculation, the use of concentrated J2 inoculum (500 J2 in 125 µl/root), and restriction of inoculum to the root elongation zone facilitated synchronous root infection.

Key words: Glycine max, hatching, *Heterodera glycines,* infection, inoculation, method, nematode, root elongation zone, soybean, soybean cyst nematode.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is a major problem in soybean production. In the United States alone, soybean losses caused by SCN were estimated to be 215 million bushels in 1996 (Marking, 1997). Differential display reverse transcription PCR (Liang and Pardee, 1992) is a promising methodology for the isolation of genes for SCN resistance. This technique allows multiple comparisons of root tissue from different infection time periods and genotypes. Synchronous and uniform infection is prerequisite.

An early experiment on controlled infection concentrated nematode inoculum at a specific site on the root using the 'agar-cone' method (Endo, 1964). Later, Halbrendt et al. (1992) limited nematode access to roots to 8 hours and pruned infected seedlings to restrict infection to the taproot. A detailed procedure to procure cotton root segments synchronously infected by root-knot nematodes was reported by McClure and Robertson (1973). We adopted this procedure to obtain synchronously infected soybean roots to study ultrastructural responses to SCN infection (Mahalingam and Skorupska, 1996). In this paper, we describe a simple modification of McClure and Robertson's (1973) procedure that provides synchronous and uniform infection of root segments with the J2 of *H. glycines* and facilitates molecular investigations.

MATERIALS AND METHODS

Seed germination: Seeds of PI 437654, 'Peking', 'Hutcheson', and 'Essex' soybean were obtained from the USDA Soybean Germplasm Collection, Urbana-Champaign, IL. Seeds were washed in running tap water for 30 minutes, and then surface-sterilized in 10% household bleach (0.5% sodium hypochlorite) for 10 minutes. The residual bleach was removed by washing the seeds under running tap water for 30 minutes.

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Seeds were arranged in a single row between two sheets (38 × 12 cm) of moistened germination paper (Anchor Paper, Hudson, WI) so that each seed's hilum faced downward when the paper was rolled up and placed on end in a beaker containing 100 ml of tap water. The beaker was covered on the top with plastic wrap and placed in a dark incubator maintained at 29 °C for 48 hours.

Inoculum: A population of SCN race 3 was obtained from R. D. Riggs, University of Arkansas. This nematode population was inbred for more than 25 generations on Hutcheson soybean in a growth chamber. Race identity tests (Riggs and Schmitt, 1988) confirmed the population to be race 3. Thirty days after adding inoculum to tubs, plants were cut at the stem base and the roots were carefully extracted from the soil and rinsed gently in water. Mature yellow gravid females were washed off the roots and collected on a 150-µm-pore sieve, then gently crushed with a rubber stopper to release the eggs on a 25-µm-pore sieve. These eggs, suspended in water (ca. 4,000 eggs/ml), formed the inoculum. The flask containing the inoculum was placed in a shaker incubator at 28 °C with constant gentle agitation of 75 rpm for 48 hours. Five replicate 25-µl aliquots were drawn out every 12 hours to determine percentage of eggs hatched.

Inoculation: Two layers of germination paper (38 × 12 cm) were placed on a plastic tray covered with a thick blotter paper (Artistic Office Products, Port Morris, NY) soaked in tap water. Seedlings were arranged 1 cm apart on the germination paper. Each seedling was inoculated by pipetting 125 µl (ca. 500 J2) of inoculum onto the root above the root tip. A third piece of germination paper of the same size was soaked in water and laid on top of the inoculated seedlings. The entire tray was covered with plastic wrap and incubated at 20–24 °C for 24 hours under dim light. During the 24-hour period of infection, the horizontally oriented roots were in constant contact with the inoculum. After 24 hours the seedlings were washed gently and transferred to aerated water tanks maintained at 20–24 °C.

Inoculation experiments: Infected roots were examined 48, 72, and 120 hours after inoculation. This experiment was done twice. Additional experiments examined the effects of inoculum age and placement on infection rates. For each time interval, seven roots per genotype were stained to determine the number and position of the juveniles as described by Dropkin et al. (1969). Briefly, roots were washed in 10% household bleach for 3 minutes and then rinsed in tap water for 20 minutes. One ml of acid fuschin stain (0.35 gram of acid fuschin in 25% glacial acetic acid) was added to a beaker containing 50 ml boiling water. Roots were boiled in the solution for 1 minute, then allowed to cool to room temperature and transferred to a beaker containing warm glycerol acidified with 10–12 drops of 5N HCl. After the solution cooled to ambient temperature, roots were placed on a paper towel to absorb the excess glycerol. The stained roots were squashed between two glass slides, and juveniles were counted with a stereomicroscope.

Results and Discussion

Seed germination rate was nearly 100% for all genotypes. After 48 hours the radicles were white, straight and 3–4 cm long. The proportions of eggs that had hatched after 0, 12, 24, 36, and 48 hours of incubation at 28 °C were 4, 20, 30, 60, and 85%, respectively.

When fresh suspensions of eggs, obtained immediately after the cysts were crushed, were used for inoculations, infection rates were poor. Forty-eight hours after inoculation, 10–12 juveniles were observed in some roots and none in others. Large variations in the egg hatching rates may be responsible for the inconsistent infections.

In the inoculum containing freshly hatched J2 obtained with gentle agitation at 28 °C for 48 hours, juveniles were highly active. When this inoculum was used, numbers of juveniles inside roots 48 hours after inoculation ranged from 60–145 per root, and average was 100 juveniles in both the SCN-resistant and susceptible genotypes.

Verdejo et al. (1988) reported that root-knot nematode J2, hatched less than 48 hours, also gave higher levels of infection than eggs. Seventy-two hours and 120 hours after inoculation the numbers of juveniles were similar to those at 48 hours, but the nematodes were more dispersed and deeper in root tissues. We observed that as the J2 aged they became sluggish and the intestines became translucent, indicating starvation, as observed in the 7-day-old inoculum. A few roots had up to 13 juveniles, but most had none when this inoculum was used.

More than 100 juveniles/root were recorded when the inoculum was placed on the root elongation zone (1 cm from the root tip). This high infection rate occurred repeatedly in tested soybean cultivars. Forty-eight hours after inoculation, juveniles within tissue were oriented parallel to the root axis. Juveniles were not found in the root meristem region. When inoculum was placed on root tips, an average of 20 juveniles/root in both the resistant and susceptible soybeans was observed. The juveniles were just above the darkly staining root apical meristem, but none were observed at the root tips 48 hours after inoculation. Observation of juveniles in the root elongation zone, even when the inoculum was placed at the root tips, corroborates earlier findings (Hale et al., 1978; Krusberg and Nielsen, 1958; Rovira, 1973; Wyss, 1981). When inoculum was placed in the root differentiation zone (about 4 cm from the root tip of the 3-day-old seedling), only one or two juveniles were seen in the primary root. The nematodes preferentially infected the junctions of newly emerging lateral roots in the differentiation zone. We speculate that, in the root elongation zone and sites of lateral root emergence, modifications of cell walls occur that might create a favorable ambience for nematode entry. The placement of inoculum at the root elongation zone or in the differentiation zone promoted penetration of the nematodes at these root zones and increased the overall rate of infection.

The experimental protocol described here is a modification of the method applied by McClure and Robertson (1973) to study root-knot nematode infection of cotton seedlings. They used boxes filled with vermiculite to perform the inoculations, and a 2-cm-wide strip of cellulose acetate fabric (Miracloth, Small Parts, Miami, FL) to trap the J2 at the root elongation zone. In our earlier experiments, Miracloth was used to retain the J2 in the root elongation zone and also to secure contact of roots with the inoculum, even when the germination paper was re-rolled (Mahalingam and Skorupska, 1996). A horizontal plane, as in the tray, probably contributes to energy-efficient juvenile orientation and direct movement toward roots, thereby aiding infection. The use of highly concentrated inoculum (500 juveniles/125 µl per root) and placement at the elongation zone obviate the use of Miracloth. Our method of inoculation also can allow efficient use of the inoculum, which is especially important in the winter months when nematode availability becomes a limiting factor.

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