

An Innovative Method for Counting Females of Soybean Cyst Nematode with Fluorescence Imaging Technology

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Abstract: Use of resistant cultivars is one of the major tactics for combating soybean cyst nematode, *Heterodera glycines* Ichinohe, which is the most destructive pathogen affecting soybean seed production. However, developing new *H. glycines*-resistant soybean cultivars is a very labor-intensive process, partially due to the lack of a quick method for counting the *H. glycines* females that develop on soybean roots. We have developed a fluorescence image-based system for counting females on excised seedling roots cultured on nutrient media in petri dishes. In this system, the females fluoresced when exposed to a wavelength of 570 nm. The fluorescent images were captured with a digital camera, transferred to a computer, and displayed on a monitor. The image of an entire sample was viewed at once, and the fluorescing females were counted manually. This system significantly improved the efficiency and accuracy of counting females developed on cultured seedling roots compared to a microscope counting method. The potential for applications in the screening of nematode-resistant crops is discussed.

Key words: female counting, fluorescence image, *Heterodera glycines*, imaging technology, soybean, soybean cyst nematode.

Soybean cyst nematode (*Heterodera glycines* Ichinohe) is one of the most devastating pathogens affecting worldwide soybean (*Glycine max* (L.) Merrill) production (Williamson and Hussey, 1996; Wrather et al., 1992). This sedentary endoparasite (Ichinohe, 1961) targets soybean root tissues and causes severe yield losses in the *H. glycines*-infested field (Wang et al., 2003; Wrather et al., 2001).

Several practices for managing *H. glycines* have been developed, including resistant cultivars and crop rotation (Wrather et al., 1992), nematicides (Rodríguez-Kábana, 1992), and biological control (Liu and Chen, 2000). Recently, transgenic approaches have been initiated for engineering nematode resistance in plants (Atkinson et al., 2003) because use of soybean cultivars resistant to *H. glycines* is the most effective tactic for managing *H. glycines* and many other nematodes. Plant resistance to nematodes is defined as the ability of host plants to restrict nematode development and reproduction (Jung and Wyss, 1999). Bioassays are used to screen plants during the development of new resistant cultivars. Counting the number of females per plant in these bioassays is an accepted parameter for indicating plant resistance or susceptibility to *H. glycines* (Starr et al., 2001).

A few terms are used to express *H. glycines* resistance in genetic research, including the index of parasitism (IP) (Rao-Arelli and Anand, 1988) and mature female index (MFI) (Davis et al., 1996; Hancock et al., 1987). However, both terms are based on empirically determined numbers of females formed on soybean roots. Therefore, counting female numbers is an important task in developing *H. glycines*-resistant cultivars.

Female counting is normally done manually, with a

magnifying glass such as the optiVISOR or a microscope, because the *H. glycines* females are only 0.6 to 0.8 mm in length and 0.3 to 0.5 mm in diameter. Manual counting is very labor intensive and tedious because females are lemon-shaped and white to yellow, and cysts are the same shape but brown in color. They both exhibit low color contrast with the root surface color of most soybean varieties. We have developed an imaging system to make the process of counting females on infected soybean seedling roots more efficient. This method significantly increased the effectiveness of female counting and has great potential in screening *H. glycines* resistance.

MATERIALS AND METHODS

Plant materials and growth conditions: Seeds of *Glycine max* cv. Pioneer YB17E were surface-sterilized for 3 minutes in 50% (v/v) bleach (Ultra Clorox) and germinated on 1% agar plates in a growth chamber (26 °C, 16 hr/8 hr of light/dark respectively). Root explants (~5 cm long) were dissected from the seedling roots 1 week after germination and transferred onto Gamborg's B5 (Sigma, St. Louis, MO) medium in petri dishes (9 cm × 2 cm) (2 root segments/dish) for inoculation.

Nematode sterilization, hatching, and inoculation: *Heterodera glycines* (race 3) cysts were collected from infected soybean roots in the greenhouse. Second-stage juveniles (J2) were prepared following the procedures essentially as described by Hermsmeier et al. (1998). Briefly, the cysts were crushed to release eggs, which were collected on a 25-µm-pore sieve. Eggs were separated from silt and plant debris by centrifuging in a 35% sucrose solution at 350g for 5 minutes. The cleaned eggs were sterilized in 10% (v/v) bleach (Ultra Clorox) solution for 3 minutes and rinsed with sterile double-distilled water. The eggs were placed on a 20-µm-pore nylon screen in a hatching chamber composed of a plastic dish containing 3.14 mM ZnSO₄ solution. The nylon screen supported the eggs to allow direct contact with the air and the solution. After 7 days of incubation, the J2 were collected from the hatching

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solution, sterilized in 0.001% HgCl₂ for 3 minutes, rinsed with sterile double-distilled water, and suspended in 1% low-gelling agarose (SeaPlaque Agarose [0.8%] + SeePrep Agarose [0.2%]) (Cambrex Corp., East Rutherford, NJ) at a concentration of 10 J2/μl. Each root was infected with 100 μl of this mixture. The plates were incubated in a growth chamber at 26 to 28 °C and in the dark for 7 weeks. *Heterodera glycines* (race 3) can complete its life cycle in the soybean roots within 3 to 4 weeks, and the eggs from the first generation can hatch, infect, and reproduce in the cultured roots on Gamborg's B5 medium (data not shown). The females and cysts were counted 7 weeks after inoculation to determine if the imaging system can detect the developing females at various stages. Three experiments with 15 replicates/experiment were conducted.

The imaging system: Pioneer Hi-Bred International Inc. developed the imaging system. It consists of an enclosed lighting system (KL 2500 LCD, Leica, Solms, Germany), a camera (Spot RT, Diagnostic Instruments Inc., Sterling Heights, MI), and a computer (Pentium 4, Dell, Austin, TX). The high-sensitivity gray-scale camera is equipped with a zoom lens. A bandpass filter was placed before the lens to allow optimal wavelengths through to produce a high-contrast image. Halogen light was used as the excitation light source. To allow required light to excite the samples, another bandpass filter was placed on the excitation light path. The power of the light was carefully adjusted to give an adequate excitation without photo bleaching of the sample. Guides were used to introduce the light to the sample. The imaging system is illustrated in Figure 1A.

Counting females using the imaging system and microscope: The root samples in the petri plates were examined under the microscope and imaging system from the bottom side only for direct comparison. The *H. glycines*-infected root sample was placed under the camera of the imaging system and uniformly illuminated with excitation light. After catching the clear and high-contrast image of the root sample and displaying it onto the computer screen, the females were counted manually from the images (Fig. 1B). Because a cyst is the dead body of a mature female, we use "females" to refer to both developing females and cysts. The imaging system can detect developing females. The females on the roots also were counted manually using a dissecting microscope. To compare the efficiency of the two counting methods, the time was recorded for finishing one experiment in a continuing time period.

RESULTS

Development of a fluorescence imaging system for counting *H. glycines* females: The natural colors of mature females of *H. glycines* and soybean roots are very similar to each other. The key to using imaging technology is to find a way to enhance the color contrast between *H. glycines*

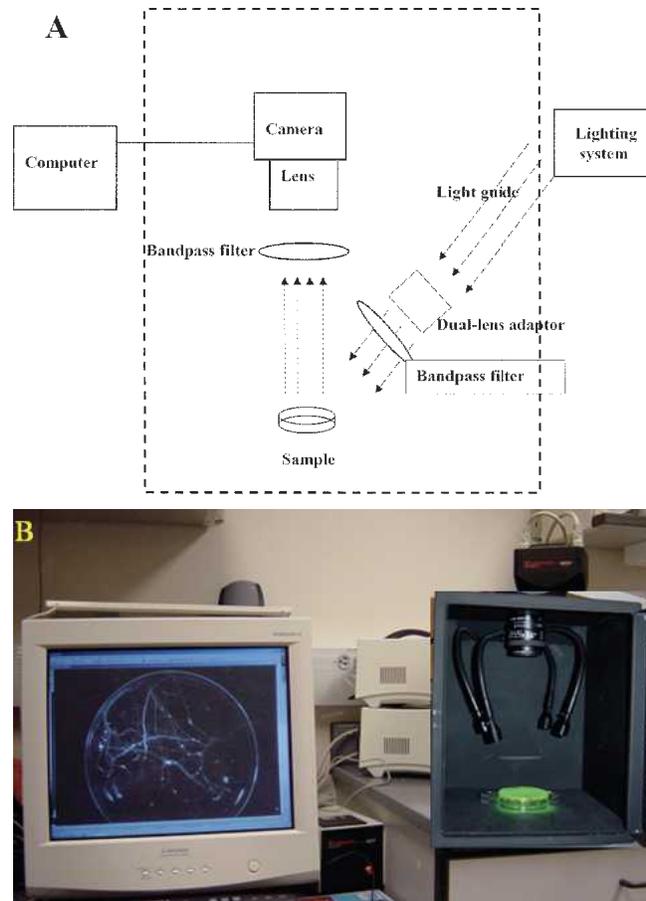


FIG. 1. Illustration (A) and photograph (B) of the fluorescence imaging system. A) Illustration of the designed imaging system. The dashed line represents a dark box. B) Photograph of the imaging system in operation. Camera captured the fluorescence from the infected soybean root samples (the green fluorescing plate in the box), transferred, and displayed the gray-scale images onto the computer screen.

females and the roots. Both soybean roots and females produce auto-fluorescence. The fluorescence of collected root samples from several soybean varieties and *H. glycines* females at different developmental stages was scanned at different wavelengths in the visible light region. By comparing the profiles, a wavelength of 570 nm was determined as the excitation wavelength at

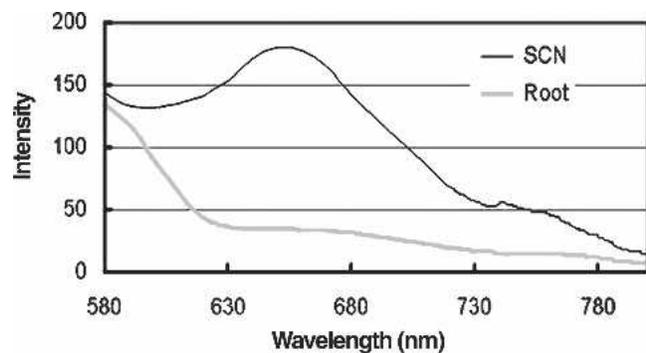


FIG. 2. Emission spectra of *H. glycines* females and soybean root under 570 nm of excitation. SCN-soybean cyst nematode.

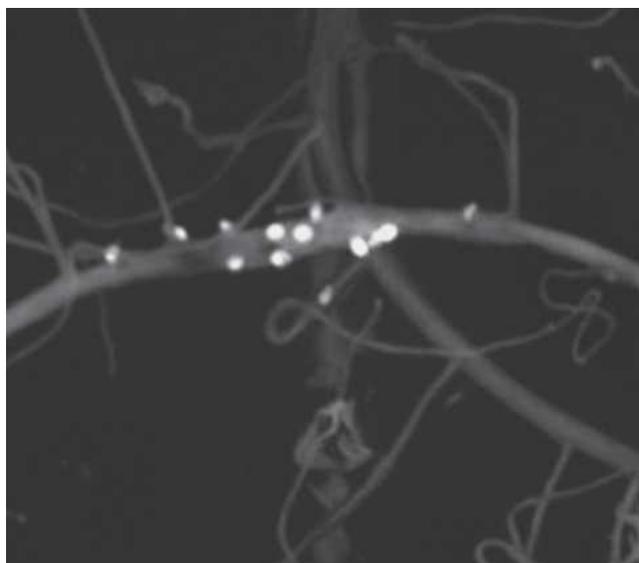


FIG. 3. A gray-scale image of a root sample 7 weeks after *H. glycines* infection under the imaging conditions. Highly contrasting lemon-shaped spots represent the *H. glycines* females on the roots.

which the females and root samples exhibit the best peak separation of fluorescence intensity (Fig. 2). The excitation and emission bandpass filters are 560DF55 and 645DF75 (Omega Optical, Inc, Brattleboro, VT), respectively. Under the defined conditions, the *H. glycines* females and soybean roots exhibit high contrast in the image as shown in Figure 3.

The fluorescence images of the infected root samples were captured by a camera and displayed on a computer screen. The image of the whole sample can be captured and displayed on the computer screen (Fig. 4A). On the captured image, the females appear as white spots against a darker background, thus enabling manual counting (Figs. 2; 4A). Any part of the image could be enlarged for finer resolution for cyst confirmation. *Heterodera glycines* females are too small for unaided manual counting (Fig. 4B). With the microscope counting method, only part of the sample could be viewed at one time (Fig. 4C). Therefore, the imaging

system is a more accurate method for counting *H. glycines* females, when compared with the microscope method.

Female counting with imaging system is reliable: To understand the accuracy of the imaging counting system, we compared female numbers obtained using both the microscope and the imaging system. The root samples in the petri plates were examined under the microscope and imaging system. The three experiments consistently showed that imaging counts are higher than the counts using a microscope (Table 1). Three factors might contribute to microscope counting errors. First, obscure females were not detected during initial screening and thus not counted with a microscope. Some of the obscure females include young females that were still developing inside the roots and females that were partially blocked by roots. The imaging system, however, is able to detect certain obscure females. Figure 5 demonstrates instances where females are visible using imaging technology while they are invisible to the microscope reader from the same view. In all cases, the females detected with the imaging system were confirmed by viewing the spots in enlarged images or positively identified as females using the microscope. Second, because only a portion of a plate can be visualized under the microscope (Fig. 4C), it is possible that some areas might be under- (or over-) counted. Third, the similar colors of the mature females and root surfaces cause human eyes to miscount (or overlook) females.

The microscope counts represented in this article were counted from one side of the dish only for the purpose of direct comparison of accuracy and efficiency. During routine screening with the microscope before the imaging system, both sides of the plate were counted with great care to avoid over- or under-counting. Counting females using a magnifier (such as optiVISOR) has the same issues as microscope counting. Very few females might be missed by the imaging system due to the weak fluorescence or high-root fluorescence. When the fluorescence level of soybean root

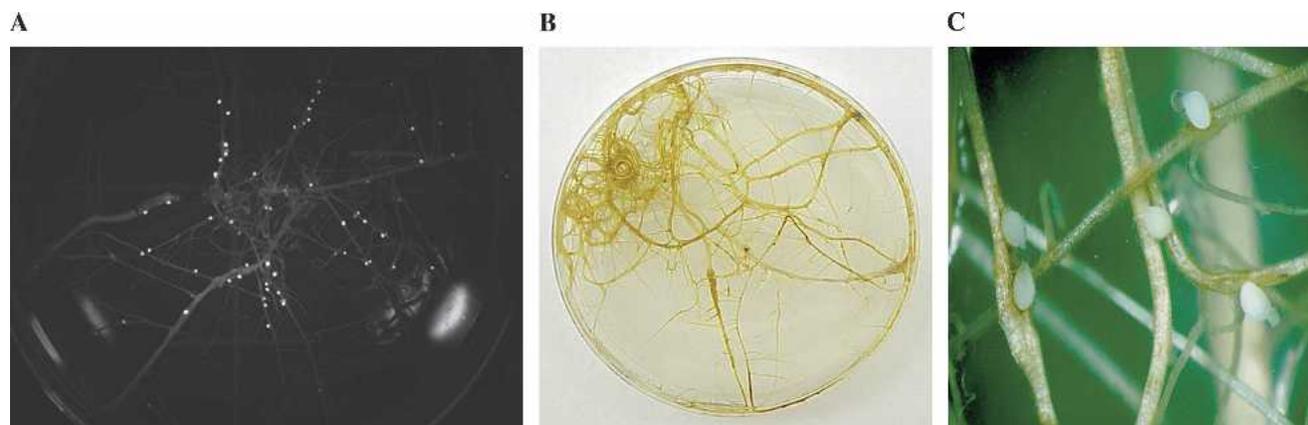


FIG. 4. Comparison of the views of an infected soybean root sample under the imager (A), direct observation (B), and microscope (C).

TABLE 1. Comparison of female counts using microscope and imager. Only one side of the petri dish was counted. Fifteen samples were counted per experiment.

	Experiment 1		Experiment 2		Experiment 3	
	scope	imager	scope	imager	scope	imager
Average females	48	56	57	65	69	72
Difference ^a		8		9		3
Percent Diff. (%) ^{b*}		14		14		4

^a Female number difference between imager counting and microscope counting.

^b Difference/imager count $\times 100$.

* The data were analyzed with the general mixed models procedure of SAS (SAS Institute, Gary, NC). Statistical analysis indicates that the difference between the two counting methods is significant ($P < 0.005$).

is similar to the female fluorescence level, imaging counting can be difficult. Female counting using the imaging system is repeatable and independent of the sample orientation in the system. These results indicate that the counts from the imaging system are reliable.

Imaging counting is more efficient than microscope counting: Counting efficiency is another important factor for screening a large number of samples. As indicated in Table 2, counting nematodes in a dish using a microscope took about 2.1 minutes, whereas counts using the imaging system required 1.3 minutes. The imaging system was more than 60% faster than the microscope counting. When considering eyestrain breaks during routine two-side counting for accurate results using the microscope, the imaging system is closer to two to three times faster for counting a large number of samples. Further, counting females using the imaging system is much more ergonomic than the microscopic method.

TABLE 2. Comparison of counting times using microscope and imager. Only one side of the petri dish was counted. Fifteen samples were counted for each experiment.

	Experiment 1		Experiment 2		Experiment 3	
	Scope	Imager	Scope	Imager	Scope	Imager
Time (Min)	33.2	18.7	29.9	18.9	33.3	20.8
Min/plate	2.2	1.2	2.0	1.3	2.2	1.4

DISCUSSION

The soybean cyst nematode can propagate and complete its life cycle in soybean root explants (Lauritis et al., 1982). Therefore, this imaging system may be adapted into a high-throughput screening method to support *H. glycines* resistance-breeding program using root explants. Soybean hairy roots can be generated by *Agrobacterium rhizogenes*-mediated transformation and support the complete life cycle of *H. glycines* (Cho et al., 2000; Narayanan et al., 1999). This cultured hairy root system is ideal for testing candidate *H. glycines* resistance genes toward engineering *H. glycines* resistance in soybean (Cho et al., 2000). The imaging system reported herein should be useful for the hairy root bioassay. Additional experiments are under way to evaluate this counting method for *H. glycines*-infected samples in soil from greenhouse and field trials. To successfully count females on soil-grown roots, the imaging system must distinguish the females from contaminating soil and sand particles. This technology may be used for other potential applications such as counting females of other nematodes and measuring root growth pat-

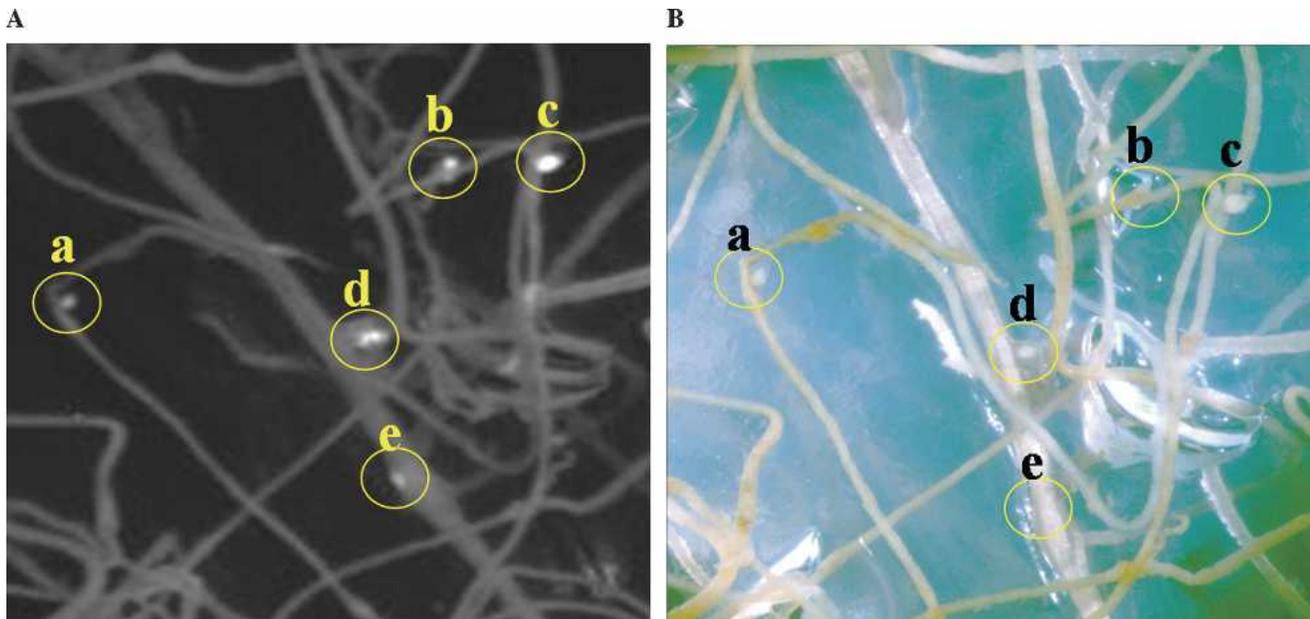


FIG. 5. Comparison of the imaging counts and microscope counts. A) Image examination detected five females (a, b, c, d, and e) in the rectangular area. B) Only three females (a, c, and d) were visible under microscope. Female "b" is hidden behind the root tissues and not visible from this view with the microscope, but it was captured by the imaging system. Female "e" was developing inside the root. When the sample was re-examined closely under the microscope, both "b" and "e" were confirmed as females.

terns, nodulation, and pathogen-induced lesions. A patent application has been filed for the technology described in this paper (US 20030142852).

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