Evaluation of Cultivar Resistance to Soybean Cyst Nematode with a Quantitative Polymerase Chain Reaction Assay

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Abstract

Lopez-Nicora, H. D., Craig, J. P., Gao, X., Lambert, K. N., and Niblack, T. L. 2012. Evaluation of cultivar resistance to soybean cyst nematode with a quantitative polymerase chain reaction assay. *Plant Dis.* 96:1556-1563.

Heterodera glycines, the soybean cyst nematode, is a major pathogen of soybean. Effective management of this pathogen is contingent on the use of resistant cultivars; thus, screening for resistant cultivars is essential. The purpose of this research was to develop a method to assess infection of soybean roots by *H. glycines* with real-time quantitative polymerase chain reaction (qPCR). This method will serve as a prelude to differentiation of resistance levels in soybean cultivars. A reproducible inoculation method was developed by means of a sand column to provide active second-stage juveniles (J2). Two-day-old soybean roots were infested with 0 or 1,000 J2/ml distilled water per seedling. Twenty-four hours after infestation, the roots were surface-sterilized and genomic DNA (gDNA) was extracted. For the qPCR assay, a primer pair for the single copy gene *HgSNO*, which codes for a

protein involved in the production of vitamin B6, was selected for *H. glycines* gDNA amplification within soybean roots. Compatible 'Lee 74', incompatible 'Peking', and cultivars with different levels of resistance to *H. glycines* were infested with 0 or 1,000 J2/ml distilled water per seedling. Twenty-four hours postinfestation, infected seedlings were transplanted into pasteurized soil. Subsequently, they were harvested at 1, 7, 10, 14, and 21 days postinfestation for gDNA extraction. With the qPCR assay, the time needed to differentiate highly resistant cultivars from the rest was reduced. Quantification of *H. glycines* infection by traditional means (numbers of females produced in 30 days) is a time-consuming practice. This qPCR assay has the potential to replace the traditional Female Index-based screening and improve precision in determining infection levels.

Heterodera glycines, the soybean cyst nematode (SCN), is a very important pathogen on soybean in the United States and worldwide (27,28). SCN infestations are responsible for significant losses incurred by soybean growers every year (33–35). The use of soybean varieties resistant to *H. glycines* remains the best-recommended method to reduce the impact of this pathogen on soybean production (27). Screening for soybean varieties with high levels of resistance to the nematode is therefore crucial. However, traditional screening methods rely on visually detecting and counting infective SCN propagules. These methods tend to be tedious and time-consuming. They are also not amenable to high throughput screening of soybean lines and varieties for resistance to SCN.

Resistance to *H. glycines* is a multigenic, quantitative trait (31). The breeding of soybean cultivars resistant to *H. glycines* and assessment of resistance is therefore problematic (31). Currently, the bioassay to determine soybean cultivar reaction to *H. glycines* takes 28 to 30 days after soil infestation with nematode eggs and/or juveniles, allowing development of females for enumeration. The plants' resistance response, however, takes place before 30 days. The use of a DNA-based molecular method could reduce the amount of time required to differentiate *H. glycines* genomic DNA (gDNA) levels among soybean cultivars differing in *H. glycines* resistance.

As an obligate parasite, *H. glycines* requires a living root to complete its life cycle. Root infection takes place regardless of soybean cultivar resistance (1,10). In a typical incompatible response to *H. glycines*, similar to the hypersensitive reaction, the syncytium degenerates and nematodes cannot develop because of the absence of a functional syncytium (1,26).

The cultivar Peking (Plant Introduction [PI] 548402) is resistant to *H. glycines* HG Type 0, a population for which the Female Index (FI) < 10 on all HG Type indicator plants (30); FI values are percentages derived from the results of 30-day bioassays, where $FI = [\text{mean number of } H. \text{ glycines females on a test soybean line}] / [\text{mean number of females on a standard susceptible line}] \times 100$. Five days after infection, the syncytia stop developing and the cells become necrotic in roots of Peking (17). In PI 437654, which is resistant to most *H. glycines* populations, the response to infection and eventual cell death is very similar to that of Peking (29). A second type of cellular response is expressed in PI 88788, which accumulates cisternae and rough endoplasmic reticulum, reducing syncytial function (29). Syncytial nuclei degenerate in roots of plants having both types of *H. glycines* resistance (17). This results in a partial or complete reduction of syncytia development, causing the delayed growth or death of the nematode. In resistant cultivars such as Peking, nematodes rarely reach the J3 stage, and no adults are observed (11).

Identification of *H. glycines* with the polymerase chain reaction (PCR) opened new possibilities in diagnosis when Subbotin et al. (32) detected *H. glycines* J2 either alone or in mixtures with different soil inhabiting nematodes or *Pratylenchus* spp. Madani et al. (21) demonstrated a high correlation between nematode gDNA quantity with real-time quantitative polymerase chain reaction (qPCR) and the counted number of nematodes. Briefly, gDNA was extracted from 1, 5, 30, or 500 second-stage juveniles of *Heterodera schachtii* and *Globodera pallida* and the cycle threshold (Ct) values from the real-time qPCR increased as number of nematodes increased in the sample. Mekete et al. (25) developed a one-step multiplex PCR reaction using species-specific primers to simultaneously differentiate three different *Pratylenchus* spp. Agudelo et al. (3) used qPCR to accurately identify *Meloidogyne arenaria* from other *Meloidogyne* spp. in soil samples containing a mixture of different species. Their qPCR assay provided an accurate and sensitive approach to identify single juveniles from soil samples.

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Accepted for publication 15 May 2012.

We hypothesized that *H. glycines* gDNA content in resistant cultivars would be lower than in susceptible cultivars, and that a molecular method might be used to differentiate *H. glycines* gDNA levels among soybean cultivars differing in *H. glycines* resistance. The objective of this study therefore was to use real-time qPCR to reduce the time needed to identify resistance levels of soybean cultivars by detecting and quantifying *H. glycines* gDNA within soybean roots.

Materials and Methods

Two experiments were conducted. In the first one, soybean cultivars Lee 74 and Peking (PI 548402) were selected as compatible and incompatible hosts for *H. glycines*, respectively. The seed for these cultivars was obtained from the United States Department of Agriculture – Agricultural Research Service (USDA, ARS) Soybean Germplasm Collection (Urbana, IL). In the second experiment, soybean cultivars with three different levels of resistance to *H. glycines* HG Type 0 were selected from among entries in the 2009 and 2010 Illinois Soybean Variety Tests (Table 1). Resistance levels were based on previously determined FI values and were defined as highly resistant (FI < 10), moderately resistant (25 ≤ FI < 40), and susceptible (FI ≥ 60) (A. L. Colgrove, *personal communication*). The source of *H. glycines* resistance for each selected cultivar was PI 88788. Three different soybean cultivars were used to represent each level of resistance.

Seed germination. In order to generate uniform seedlings for infestation with *H. glycines* second-stage juveniles (J2), seeds were germinated as described by Mahalingam et al. (22). Briefly, with their hila facing downward, the seeds were rolled in moist, sterilized germination paper. The seeds were then placed into 1-liter beakers containing 100 ml of tap water to facilitate imbibition and germination. The beakers were covered with plastic wrap with air-holes and incubated for 48 h at 27°C.

Nematode inoculum and inoculation. *H. glycines* isolate UIUC0, HG Type 0, was grown on the susceptible soybean cultivar Lee 74. The plants were maintained in the greenhouse in 5.7-liter polypropylene pots partially submerged in a 27°C water bath under a 16-h photoperiod. Thirty days postinfestation (dpi), mature females were dislodged from the roots with a high pressure water spray. Eggs were released from the females by gentle crushing with a rubber stopper against a 150-μm-aperture sieve, and collected onto a 25-μm-aperture sieve (8). Eggs were concentrated by sucrose centrifugation (16), and the solution containing eggs was placed on a nylon sieve (41-μm-aperture) above a water reservoir for hatching (1). J2 were collected every day from hatching sieves

until the target inoculum level was reached. Twelve hours before use, J2 were placed on a sterile sand column above a water reservoir, and the active J2 were collected at the bottom of the column in sterile distilled water (9,18). The active J2 were suspended in sterile distilled water at a concentration of 1,000 J2/ml.

In the first experiment, we tested a method to consistently infect soybean seedlings with J2 and developed a real-time qPCR assay to detect *H. glycines* gDNA in infected soybean roots. Fifteen-cm-long polyvinyl chloride (PVC) tubes were cut in half lengthwise. These tubes were placed horizontally in trays and filled with a steam-pasteurized sandy loam soil, 77% sand, 11% silt, 12% clay, pH 7.8, and 0.9% organic matter. Uniform 3- to 5-cm-long soybean seedlings were placed horizontally at one end of each half-tube for infestation. To assess the consistency of our inoculation technique, 10 seedlings each of Lee 74 and Peking were infested with 0 or 1,000 J2/ml of distilled water per seedling, and treatments were distributed randomly in the inoculation trays. All seedlings were covered with moistened, pasteurized sandy loam soil and incubated horizontally at 27°C. After 24 h, the seedlings were removed, rinsed, and surface sterilized with 10% bleach for 30 s. Half the seedlings were frozen at –20°C for gDNA extraction, and the remaining seedlings were stained as described in Byrd et al. (6) for enumeration of J2 under a stereoscope. This experiment was conducted twice for the real-time qPCR assay and three times for the nematode counts.

For the qPCR assay, 100 seedlings each of Lee 74 and Peking were germinated, selected, and infested as described in the preceding paragraph. At 24-h postinfestation (hpi), all seedlings were washed under running tap water to remove free J2 that had not infected the seedlings (11). Infected seedlings were transplanted to 5.7-liter polypropylene pots. Each pot contained 22 PVC tubes (3 cm diameter × 15 cm long) filled with 100 cm³ of pasteurized sandy loam soil each. Seedlings were transplanted into each tube (7,8). The plants were maintained in the greenhouse with each pot three-fourths submerged in a 27°C water bath under a 16-h photoperiod until harvested. After 1, 7, 10, 14, and 21 dpi, five each of infested and mock-infested (sterile distilled water) Lee 74 and Peking soybean seedlings were surface sterilized and frozen at –20°C for gDNA extraction and real-time qPCR assay.

For the second experiment, soybean cultivars with different levels of resistance to *H. glycines* were used. Uniform seedlings of nine cultivars with different levels of resistance (Table 1) and Lee 74 (internal susceptible control) were used. Three seedlings of each cultivar were infested with 1,000 J2/ml of distilled water per seedling as described for the first experiment. Three seedlings (Lee 74) were mock-inoculated with sterile distilled water. At 24 hpi, all seedlings were washed and transplanted to inoculum-free pots as described for the first experiment. The infested soybean seedlings were surface-sterilized and frozen at –20°C for gDNA extraction at 7, 10, 14, and 21 dpi.

DNA extraction from nematodes and soybean roots. As nematodes develop, the amount of gDNA should increase. Because resistance derived from PI 88788 reduces male development much less than resistance derived from Peking, the quantity of gDNA in males had to be accounted for. To compare gDNA content in adult males and J2, nematodes were individually digested as described

Table 1. Selected soybean cultivar responses to infection with *Heterodera glycines* HG Type 0^a

Seed company	Cultivar	FI ^b	Rating ^c	Source of resistance ^d
Wilken	W 2672 NSTS	0	HR	PI 88788
Emerge Genetics	XC 3810	1	HR	PI 88788
FS Hisoy	HS 4366	1	HR	PI 88788
Merschman	NASHVILLE 749RR	27	MR	PI 88788
G2 (Nutech)	7438	27	MR	PI 88788
Great Lakes	GL 3259 R2	27	MR	PI 88788
Nutech	3255 L	150	S	PI 88788
Horizon	30N11 L	166	S	PI 88788
Mavrick	9298 LL	107	S	PI 88788

^a Female indices and resistance ratings were determined during the 2009 and 2010 Illinois Soybean Variety Tests (A. L. Colgrove, *personal communication*).

^b Female Index (FI) = (average number of females developed on the test soybean cultivar/average number of females developed on a standard susceptible) × 100 in a bioassay conducted for 30 days in a greenhouse. The *H. glycines* test population was UIUC0, an HG Type 0.

^c Resistance level for each cultivar based on the FI: HR = highly resistant, FI < 10; MR = moderately resistant, FI > 24 < 40; and S = susceptible, FI > 59.

^d Source of resistance to *Heterodera glycines* as reported by the seed company.

Table 2. Primers used for real-time quantitative polymerase chain reaction (qPCR) assays targeting *Heterodera glycines* and *Glycine max*

Amplicon	Accession no. ^a	Primers (5′ → 3′) ^b	Size (bp) ^c
<i>HgSNO</i>	EU747298	F: AGGCAACGTGCAGCAACAT R: CTGATCGCCAGTCTTCACTATGA	76
<i>Lectin</i>	K00821	F: CTTTCTCGCACCAATTGACA R: TCAAACCTCAACAGCGACGAC	102

^a Primer sequences available through the National Center for Biotechnology Information (NCBI).

^b F and R indicate forward and reverse primers.

^c bp = base pairs.

by Craig et al. (9). Briefly, 10 males and 10 J2 were handpicked and individually digested in 5 μ l buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.5) containing 4 mg/ml of protease K (Invitrogen, Carlsbad, CA). The protease K was inactivated after 24 h by incubation at 80°C for 30 min. Genomic DNA was further used in qPCR.

DNA was extracted from infected and noninfected roots with the FastDNA SPIN Kit and the FastPrep instrument (MP Biomedicals, Santa Ana, CA) following the manufacturer's manual with modifications as described by Malvick and Grunden (23). J2 infection was observed to occur in the 3 to 5 cm of root that was originally infested; therefore, roots from which DNA was collected were standardized to 7 cm length at harvest. Surface-sterilized roots were placed in 2.0 ml of lysing matrix in tubes containing garnet particles and a 6.35-mm ceramic sphere, 1,000 μ l of Cell Lysing Solution (CLS-TC) extraction buffer, and 80 μ l of polyvinylpyrrolidone solution (PVP in 1 mg/10 μ l stock solution). A second 6.35-mm ceramic bead was added and homogenized twice in the FastPrep instrument for 30 s at a speed setting of 4.5 in intervals of 5 min. Samples were centrifuged for 7 min at 11,500 rpm. The supernatant was transferred to a new 1.5-ml tube and the same procedure repeated; 600 μ l of the supernatant was transferred to 1.5-ml tubes containing an equal volume of the binding matrix. From this point onward, steps were followed as described in the manufacturer's protocol. For extraction of gDNA from the J2 suspension, 1 ml of suspended J2 was pipetted into a lysing tube and the same procedure used for the roots was performed. *H. glycines* gDNA concentrations were measured with a Nano Drop (ND-1000, ThermoScientific, DE) and stored at -20°C.

Fig. 1. Number of *Heterodera glycines* second-stage juveniles (J2) within roots of compatible soybean host cultivar Lee 74 and incompatible cultivar Peking (PI 548402) 24 h postinfestation with 1,000 J2/ml of distilled water per seedlings. Nematodes were passed through a sand column to obtain active inoculum. Within each experiment, cultivars followed by 'ns' did not differ at $\alpha = 0.05$.

Selection of primers, test for inhibitors and efficiency of the primers. To specifically detect *H. glycines* gDNA, a primer pair amplifying the single-copy nematode gene *HgSNO* was selected (9). To detect soybean genomic DNA and verify successful DNA extraction from the infected and noninfected roots, a primer pair amplifying a soybean lectin gene was selected (4) (Table 2).

The efficiency of the *HgSNO* primer pair was tested as described in Livak and Schmittgen (20). Briefly, qPCR was performed on a 10-fold dilution series starting with 89.6 ng of *H. glycines* gDNA, and the Δ Ct was calculated between each dilution. The efficiency of the primers was evaluated by observation of Δ Ct for each 10-fold dilution.

To test for PCR inhibitors in the DNA extracted from soybean roots, five 10-fold dilutions of soybean gDNA was prepared starting at 71 ng/ μ l. Each dilution was mixed in a 1:1 ratio with 7.8 ng/ μ l of *H. glycines* gDNA. QPCR was performed to amplify *HgSNO* from the dilution series spiked with *H. glycines* gDNA.

Real-time qPCR assay. The SYBR Green real-time qPCR assay was conducted on an ABI PRISM 7000 sequence detection system instrument (PE Applied Biosystems, Foster City, CA). The amplification reactions were performed on a 96-well Optical Reaction Plate with a 25- μ l reaction volume. Reaction mixtures contained 12.5 μ l of SYBR Green PCR Master Mix (Applied Biosystems), 0.25 μ l of each 5 pmoles of *HgSNO* and *Lectin* primers, 5 μ l of template DNA, and 7.25 μ l of sterile distilled water. The reaction cycles were: preincubation at 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s each, and 60°C for 1 min, dissociation stage.

Duplicates or triplicates of each reaction were run on each plate. Analysis was based on absolute quantification generating amplification curves for each reaction with ABI sequence detection software. Cycle threshold (Ct) values were calculated with the automatic baseline analysis option (21).

Statistical analysis. Each experiment consisted of completely randomized tubes in the inoculation tray. Treatments were the cultivar-inoculation combinations. Analysis of variance (ANOVA) was conducted with the MIXED procedure in SAS (Version 9.2, SAS Institute, Cary, NC). Effects were considered significant at $P \leq 0.05$. The UNIVARIATE procedure was used to verify the normality of the residuals. Homoscedasticity was checked with the GLM (general linear models) procedure with the MEANS statement on the residuals. Brown and Forsythe's HOVTEST option was used to compute statistics to test for the homogeneity of variance assumption.

Results

Root infection. Active J2 produced consistent infection (Fig. 1). Stained nematodes inside infected seedlings were counted at 24 hpi. Infection of Lee 74 (compatible) and Peking (incompatible) did not differ within each experiment (Table 3), and this result was consistent among all three experimental repetitions (Table 3). The percentage of infection in both compatible and incompatible hosts within 24 hpi ranged from 31 to 41%. The mean

Table 3. Penetration of compatible soybean cultivar Lee 74 and incompatible cultivar Peking (PI 548402) by *Heterodera glycines* second-stage juveniles (J2) 24 h postinfestation with 1,000 J2/ml of distilled water per seedling

		Juveniles ^a				
Experiment ^b	Host	Range	Mean (no.)	Infection (%)	<i>P</i>	Pooled <i>P</i> ^c
1	Lee 74	235 – 553	366	37	0.677	0.272
	Peking	221 – 589	401	40		
2	Lee 74	300 – 580	413	41	0.213	
	Peking	244 – 415	335	34		
3	Lee 74	255 – 350	306	31	0.345	
	Peking	280 – 398	334	33		

^a Infected seedlings were stained with acid fuchsin (7) and nematodes were counted with a stereoscope at $\times 64$ magnification. Range, mean, and infection rate represent five replications for each cultivar within an experiment.

^b In each experiment, five seedlings each of Lee 74 and Peking were infested with *H. glycines* J2, collected after being allowed to crawl through a column of sterilized sand.

^c P value for three experiments when pooled.

number of J2 penetrating the seedlings ranged from 306 to 401/seedling (Table 3).

Sensitivity and efficiency of primers for *HgSNO* and inhibitors tests. The primer pair for *HgSNO* detected, amplified, and quantified *H. glycines* gDNA from infected Lee 74 and Peking soybean seedlings, and from pure J2 DNA. However, there was no amplification of *H. glycines* gDNA from noninfected seedlings or sterile distilled water. The lowest *H. glycines* gDNA concentration detected and quantified on a 10-fold dilution series was 89.6×10^{-5} ng/ μ l with an average Ct value of 32.72 (data not shown). Primer efficiency for the real-time qPCR was determined by comparison of the Δ Ct of serial 10-fold dilutions of the gDNA. The highest *H.*

Fig. 2. Differences in cycle threshold (Δ Ct) values with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and analysis of the efficiency of a primer pair for the *Heterodera glycines* gene *HgSNO* tested with a 10-fold serial dilution of genomic *H. glycines* DNA starting at 89.6 ng/ μ l.

glycines gDNA concentration was 89.6 ng/ μ l. The Δ Ct between dilutions were similar, ranging from 3.49 to 3.66 (Fig. 2).

To test the qPCR assay for inhibition from soybean roots, 7.8 ng/ μ l of *H. glycines* gDNA was mixed in a 1:1 ratio with 71, 7.1, 0.71, 0.071, 0.0071, and 0.00071 ng/ μ l of soybean gDNA. The results demonstrated that the *H. glycines* gDNA amplification at each soybean gDNA concentration was not different ($P = 0.637$) (Fig. 3). This indicated that there was no inhibition in the presence of soybean gDNA.

Real-time qPCR assay. The results obtained with the SYBR Green real-time qPCR assay matched with the results of nematodes counted in stained, infected roots. The content of *H. glycines* gDNA in Lee 74 and Peking roots did not differ within or between experiments (Table 4). Amplification plots for real-time qPCR showed a tight band of amplified reactions (Fig. 4). The means of qPCR Ct values were compared for Lee 74 and Peking within each experiment, and a Δ Ct was calculated (Table 4). The range of Ct values was similar between the first experiment (Ct values 22.25 to 25.40) and the second (Ct values 22.38 to 24.88). An endogenous control for the soybean lectin gene was used to verify successful DNA extraction. Amplification and quantification of the lectin gene was observed in every reaction where amplification of *H. glycines* gDNA was recorded, with Ct values ranging from 17.67 to 35.80 (data not shown).

Genomic DNA content in male versus second-stage juvenile. The results obtained with the SYBR Green real-time qPCR assay showed that the content of gDNA in fully developed males differed ($P \leq 0.001$) from gDNA content of J2 (Fig. 5). Mean average Ct values for 10 digested males was 25.3 compared with a mean Ct value of 23.3 for the same number of J2. The Δ Ct value between an adult male *H. glycines* and a J2 was 2, which indicated a fourfold greater gDNA content in males than in J2.

Genomic DNA content in Lee 74 versus Peking (PI 548402). *H. glycines* gDNA content in compatible host Lee 74 and incompatible Peking was compared at different dpi with SYBR Green

Fig. 3. Amplification plot of *Heterodera glycines* genomic DNA in a SYBR Green real-time quantitative polymerase chain reaction (qPCR) with a primer pair designed to amplify the nematode *HgSNO* gene in serially diluted soybean genomic DNA combined in a 1:1 mixture with 7.8 ng/ μ l *H. glycines* genomic DNA. Distilled water was used in place of DNA for a negative control.

real-time qPCR (Table 5). At 10 and 21 dpi, Peking had less *H. glycines* gDNA than Lee 74, as demonstrated by the higher Ct values. *H. glycines* gDNA in Lee 74 and Peking did not differ at 1 and 14 dpi. The same results were obtained when this experiment was repeated (Table 5). At 7 dpi, however, results were inconsistent. In experiment one, *H. glycines* gDNA content in Lee 74 and Peking did not differ. In experiment two, on the other hand, gDNA content in Lee 74 differed from that of Peking (Table 5).

Soybean cultivars with different levels of resistance to *H. glycines*. *H. glycines* gDNA content was compared at 7, 10, 14, and 21 dpi with SYBR Green real-time qPCR in three highly resistant, three moderately resistant, and three susceptible soybean cultivars as well as cultivar Lee 74 (Table 6). The source of resistance for all nine cultivars was PI 88788, and Lee 74 was the susceptible check. Cultivars with the same FI values were pooled, and the mean Ct values were calculated. Single-degree-of-freedom contrasts were performed for each harvest point. Results from the two experimental repetitions were similar only at 10 and 21 dpi (Table 6). At 10 dpi, *H. glycines* gDNA content in highly resistant, moderately

resistant, susceptible, and cultivar Lee 74 differed from each other. However, at 21 dpi, *H. glycines* gDNA content in cultivars with high resistance and that in those with moderate resistance did not differ. Likewise, *H. glycines* gDNA content in Lee 74 did not differ from that in susceptible cultivars (Table 6).

Discussion

This study successfully developed a qPCR assay to detect differences in *H. glycines* gDNA content between compatible and incompatible hosts at 10 dpi. Likewise, differences between highly resistant, moderately resistant, and susceptible cultivars were observed 10 dpi, reducing by 20 days the time needed to distinguish these cultivars using traditional FI-based screening.

Incorporation of a sand column in the inoculation technique reduced the variation of J2 penetration into seedlings. Variable rates of penetration have been a problem in similar studies (2,7,15,22). Due to variability in the hatching rate, collecting J2 until the desired inoculum level is reached takes time, and results in inoculum of different ages and viability. Mahalingam et al. (22) noticed that

Table 4. Comparison of *Heterodera glycines* genomic DNA (gDNA) content in compatible soybean cultivar Lee 74 and incompatible cultivar Peking (PI 548402) 24 h postinfestation with 1,000 *H. glycines* second-stage juveniles (J2)/ml of distilled water per seedling based on a SYBR Green real-time quantitative polymerase chain reaction (qPCR) assay with a primer pair designed to amplify the *HgSNO* gene in *H. glycines*

Experiment ^b	Average qPCR Ct value ^a		SD ^c	Δ Ct	$2^{-\Delta Ct}$ ^d	<i>P</i>	Pooled <i>P</i> ^e
	Lee 74	Peking					
1	23.33	22.84	0.585	-0.49	1.40	0.426	0.065
2	24.16	23.53	0.501	-0.63	1.55	0.243	

^a Genomic DNA extracted from infected soybean roots was amplified and quantified with primers for *HgSNO*. The cycle threshold (Ct) values were averaged. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.

^b Each experiment comprised five replications per soybean cultivar.

^c SD = standard deviation.

^d $2^{-\Delta Ct}$ represents the fold difference in gDNA content between cultivar Lee 74 and cultivar Peking (PI 548402).

^e *P* value for three experiments when pooled.

Fig. 4. Amplification plot of *Heterodera glycines* genomic DNA with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and a primer pair designed to amplify the nematode *HgSNO* gene from infected roots of cultivar Lee 74 and cultivar Peking (PI 548402) 24 h postinfestation with 1,000 *H. glycines* second-stage juveniles/ml of distilled water per seedling. Distilled water was used in place of DNA as a negative control and 7.8 ng/μl *H. glycines* gDNA was used as a positive control.

7-day-old inoculum was composed of dead or starving nematodes, causing poor infection. Lambert et al. (18) designed a sand column for the purpose of cleaning and surface-sterilizing nematodes by allowing them to crawl through the sand and be collected in sterile distilled water. Active J2 will crawl through the sand column in about 12 h. Less viable J2 are retained in this sand biofilter, and the remaining J2 are highly infective.

To detect, amplify, and quantify *H. glycines* gDNA in infected roots, we first validated the qPCR assay by checking the efficiency of the primer pairs used and by testing for PCR inhibition. Livak and Schmittgen (20) reported that the sensitivity and efficiency of

the primers can be assessed by observing how Ct values vary with each dilution. Bessetti (5) reported that soil and plant material may contain PCR inhibitors, and Malvick and Impullitti (24) expressed how critical it is to test for PCR inhibitors and account for them in qPCR assays. To our knowledge, this is the first report on detection and quantification of *H. glycines* gDNA in infected roots.

As nematodes develop inside infected soybean roots, their gDNA content increases. However, in incompatible hosts such as Peking, nematodes rarely reach the J3 stage, and no adults are observed (11). *H. glycines* J2 generally penetrate roots of soybean irrespective of the level of resistance (12,13). Syncytia are initiated

Fig. 5. Real-time quantitative polymerase chain reaction (qPCR) amplification plots of genomic DNA of *Heterodera glycines* males and juveniles amplified with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and a primer pair designed to amplify the *HgSNO* gene. Distilled water was used as a negative control.

Table 5. Mean comparisons of real-time quantitative polymerase chain reaction (qPCR) cycle threshold (Ct) values for *Heterodera glycines* genomic DNA content in cultivar Lee 74 and cultivar Peking (PI 548402) at 1, 7, 10, 14, and 21 days postinfestation (dpi)

Experiment ^b	dpi	Average qPCR Ct value ^a		SD ^c	Δ Ct	$2^{-\Delta Ct}$ ^d	P
		Lee 74	Peking				
1	1	23.57	23.61	0.448	0.04	1.03	0.935
	7	21.47	22.12	0.484	0.65	1.57	0.215
	10	19.00	20.07	0.289	1.07	2.10	0.006
	14	23.74	26.31	1.829	2.57	5.94	0.198
	21	24.66	29.81	0.468	5.15	35.51	<0.001
2	1	23.26	23.30	0.315	0.04	1.03	0.907
	7	21.80	22.99	0.433	1.19	2.28	0.025
	10	21.34	22.88	0.296	1.54	2.91	0.001
	14	19.97	20.40	0.211	0.43	1.35	0.081
	21	20.25	23.82	0.888	3.57	11.88	0.007

^a *H. glycines* genomic DNA (gDNA) content in infected Lee 74 and Peking (PI 548402) was determined with SYBR Green with a primer pair designed to amplify the *HgSNO* gene in real-time quantitative polymerase chain reaction (qPCR). Cycle threshold (Ct) values were averaged. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.

^b In each experiment, five seedlings of cultivars Lee 74 and Peking (PI 548402) were infested with 1,000 *H. glycines* second-stage juveniles/ml of distilled water per seedling.

^c SD = standard deviation.

^d $2^{-\Delta Ct}$, represents the fold difference in *H. glycines* gDNA content between cultivars Lee 74 and Peking (PI 548402).

Table 6. Probability and *F* values for single degree of freedom mean comparisons of different soybean cultivars to penetration and development of *Heterodera glycines* 7, 10, 14, and 21 days postinfestation (dpi) with SYBR Green real-time qPCR

dpi	Single df contrast	Experiment 1		Experiment 2	
		<i>F</i> value	<i>P</i>	<i>F</i> value	<i>P</i>
7	Lee 74 vs. HR	10.54	0.004	5.390	0.031
	Lee 74 vs. MR	0.38	0.543	0.650	0.430
	Lee 74 vs. S	3.02	0.098	0.020	0.892
	HR vs. MR	13.81	0.001	19.57	<0.001
	MR vs. S	11.11	0.003	0.890	0.356
	HR vs. S	49.68	<0.001	12.10	0.002
10	Lee 74 vs. HR	28.94	<0.001	56.45	<.001
	Lee 74 vs. MR	13.97	0.001	33.27	<.001
	Lee 74 vs. S	4.44	0.048	9.18	0.007
	HR vs. MR	5.39	0.031	6.09	0.023
	MR vs. S	5.32	0.032	14.99	0.001
	HR vs. S	21.42	<0.001	40.19	<.001
14	Lee 74 vs. HR	23.11	<0.001	2.39	0.138
	Lee 74 vs. MR	15.05	0.001	0.04	0.835
	Lee 74 vs. S	0.03	0.876	0.01	0.934
	HR vs. MR	1.72	0.205	3.57	0.073
	MR vs. S	32.62	<0.001	0.03	0.859
	HR vs. S	49.31	<0.001	4.28	0.052
21	Lee 74 vs. HR	40.61	<0.001	26.38	<0.001
	Lee 74 vs. MR	33.85	<0.001	24.54	<0.001
	Lee 74 vs. S	2.99	0.099	0.010	0.913
	HR vs. MR	0.61	0.442	0.070	0.798
	MR vs. S	33.45	<0.001	46.90	<0.001
	HR vs. S	43.13	<0.001	50.51	<0.001

and formed in both compatible and incompatible hosts within 2 dpi (11,30). At this point, there is no difference between syncytia formed in resistant cultivar Peking and the ones observed in Lee (14). Riggs et al. (30) showed that at 4 dpi, syncytia in Peking start deteriorating, but only at 7 dpi was a remarkable degeneration of the feeding site observed. Therefore, consistent differences in nematode gDNA content between incompatible and compatible hosts could not be detected at 7 dpi.

Endo (10) and Lauritis et al. (19) reported development of *H. glycines* in susceptible soybean plants and showed the presence of fully developed males inside the roots by 10 dpi. At this time, compatible (susceptible) soybeans contained more *H. glycines* gDNA than any soybean cultivar with some degree of resistance to SCN, and consistent differences in gDNA content between incompatible and compatible hosts could be detected due to the presence of males.

At 14 dpi, difference in gDNA content between incompatible and compatible hosts was inconsistent. Colgrove and Niblack (7) demonstrated that the female-male ratio in compatible host Lee 74 is 1:1. Therefore, between 11 and 15 dpi, half of the population of nematodes (males containing four times more gDNA than J2) emerged from the roots to complete their reproductive life cycle. However, gDNA of dead nematodes (males and females) within the incompatible host was still detected and quantified.

Our study indicates that out of five different harvest points (1, 7, 10, 14, and 21 dpi), it is by 10 dpi that we can detect lower *H. glycines* gDNA content in a resistant relative to a susceptible cultivar. This is consistent with previous histological and ultrastructural studies (7,10-13,19,30). With the proposed qPCR assay, we were able to reduce the time to detect difference between soybean cultivars by at least 20 days compared with the traditional FI-based screening.

Acknowledgments

We thank Alison Colgrove and Ursula Reuter-Carlson for helping with DNA extraction and qPCR assay. This research was supported by the Illinois Soybean Association (ISA).

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On 23 October 2012, in the Literature Cited section, number 25 was corrected by deleting an incorrect citation and inserting the correct one.