DETERMINATION OF SOYBEAN CULTIVAR RESISTANCE TO SOYBEAN CYST NEMATODE WITH QUANTITATIVE POLYMERASE CHAIN REACTION

BY

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THESIS

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ABSTRACT

Heterodera glycines, the soybean cyst nematode, is the major pathogen of *Glycine max* (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), a prelude to differentiation of resistance levels in soybean cultivars. Two experiments were conducted. In the first one, a consistent inoculation method was developed using to provide active second-stage juveniles (J2). Two-day-old soybean roots were infested with 0 and 1000 J2/mL. Twenty-four hours after infestation, the roots were surface sterilized and DNA was extracted with the DNA FastKit (MP Biomedicals, Santa Ana, CA)). For the qPCR assay, primer pair for single copy gene *Hg*SNO, which codes for a protein involved in the production of vitamin B6, was selected for *H. glycines* DNA amplification within soybean roots. In the second experiment, compatible Lee 74, incompatible Peking and cultivars with different levels of resistance to *H. glycines* were inoculated with 0 and 1,000 J2/seedlings. Twenty-four hours post inoculation they were transplanted into pasteurized soil. Subsequently they were harvested at 1, 7, 10, 14 and 21 days post inoculation for DNA 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; the qPCR method can replace the traditional one and improve precision in determining infection levels.

To my family

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TABLE OF CONTENTS

CHAPTER 1

HETERODERA GLYCINES – GLYCINE MAX INTERACTION

LITERATURE REVIEW

Soybean, *Glycine max* (L.) Merr., is one of the oldest cultivated crops (Hymowitz, 1970) with its origin tracing back to the northern and central regions of China (Gibson and Benson, 2002). Soybean was successfully established in the United States as a crop due to an important demand for soybean oil and meal, and the possibility of integrating it in a crop rotation system with corn and other crops (Riggs, 2004). After World War II, soybean production migrated from the southern part of the US to the "Corn Belt" where it is now a major cultivated crop, with 30,267,653.24 hectares planted in 2008 (United States Department of Agriculture, National Agricultural Statistics Service, 2008).

Soybean cyst nematode, *Heterodera glycines* Ichinohe, the most economically important pathogen of soybean in the north-central area of the United States (Workneh et al., 1999), was first detected in Illinois in 1959 (Noel, 1992). In the north-central region, *H. glycines* does not necessarily cause the "typical" aboveground symptoms described as chlorosis and stunting (Agrios, 1997); it has the ability to cause 15 to 30% yield loss with no visible symptoms (Niblack, 1993; Niblack, 2005; Noel, 1992; Wang et al., 2003; Young, 1996). The disease may have been mistaken for those caused by other environmental factors or simply not identified. The ability to survive in cysts and undergo a period of dormancy allows *H. glycines* to be disseminated and infest diverse

environments (Schmitt, 2004). This, and the fact that *H. glycines* can cause severe yield loss to soybean, makes this pathogen-crop association one of the most important and interesting in agriculture (Riggs, 2004). *Heterodera glycines* affected US soybean yield more from 1997 to 2007 than any other disease (Wrather and Koenning, 2009) and it will be a problem associated with soybean production for the foreseeable future.

Heterodera glycines - Soybean Cyst Nematode

Farmers in China gave the name of "fire-burned seedling" to a disease in soybean, but it was not until 1899 that a report from northeastern China indicated that this disease was caused by *H. glycines* (Liu et al., 1997). If soybean has its origins in China, where farmers long ago identified a disease as "fire-burned seedling," and the disease was proven afterwards to be caused by *H. glycines*, it seems likely that China is the origin of this pathogen and that both pathogen and crop evolved together (Riggs, 2004); however, there is controversy over the ancestral origins of *H. glycines* populations in the US (Noel, 1992). *Heterodera glycines* was not described as a species until 1952 (Ichinohe, 1952).

Heterodera glycines in North America

In North Carolina during the 1930-1940's, soybean reportedly expressed symptoms that resembled those caused by nutritional deficiency, but it was not until 1954 that *H. glycines* was determined to be the cause of the disease (Winstead, 1955). After this discovery the pathogen was reported in several other states in the US (Noel, 1992).

One of the most reasonable theories of how *H. glycines* arrived to the US is with the importation (from China) of infested soil that was used as a source of *Bradyrhizobium* inoculant (Riggs, 2004). The nematode can survive in storage for more than 22 months

(Epps, 1968), and thus could easily have been viable once it arrived in the US. The possibility of the nematode being indigenous to the US and simply adapting to the exotic legume (soybean) and the selection pressure imposed by agricultural practices is another strong theory (Riggs, 2004). Moreover, there is evidence of the presence of the sugar beet cyst in both Asia and the US before *H. glycines* was discovered in 1954. These two species are very similar and very closely related; in fact, they are able to interbreed (Colgrove et al., 2006; Miller, 1982). *Heterodera glycines* could have resulted from a selection of the sugar beet cyst (Riggs, 2004).

Whatever the means of arrival, once *H. glycines* became established in the US, different means of dissemination resulted in its distribution throughout soybean production areas. Birds, with their migratory paths and the ability of the eggs to survive in cysts through the birds' digestive tracts help to disseminate the pathogen (Epps, 1971; Riggs, 2004; Smith et al., 1992). Wind, flood water, and field runoff were other means of dispersion of the nematode (Riggs, 2004)

The known area of *H. glycines* infestation in the US increased steadily; surveys from 1961 confirmed the presence of the pathogen in 39 counties in 8 states (Spears 1964 cited in Riggs 2004). Now every state in the US where large hectarage of soybean is grown is infested with *H. glycines*. In addition, *H. glycines* is present in most countries of the world where soybeans are produced (Riggs, 2004).

***Heterodera glycines* Biology**

Heterodera glycines is an obligate parasite, which means that it needs a living root to complete its life cycle. The infective unit of the *H. glycines* is not the cyst nor the

eggs, but the second-stage juveniles (Niblack et al. 1986; Wrather and Anand, 1988). However, the protective cyst and the ability of the eggs to become dormant enable the nematode to be passively dispersed to diverse environments (Koenning, 2004).

The life cycle begins with the egg which undergoes embryonic development following fertilization, leading to development of a first-stage juvenile (J1), with fixed cell numbers except for the cells associated with reproduction (Hirschmann, 2004). The nematode grows and undergoes its first molt within the egg, becoming a second-stage juvenile (J2) (Koenning, 2004). Hatching is the next step in the nematode's life cycle. This process is regulated by the temperature of the soil, the presence of the host, and time (Yen et al., 1995). After hatching, the J2 (infective units) will locate a root, attracted by CO_2 gradients, thermal gradients, or host leachates (Dusenbery, 1987; von Mende et al., 1998) and penetrate it with the help of cellulases and other enzymes that allow the nematode to migrate intracellularly through the cortical cells to the vascular tissue (Davis et al., 2004; Niblack, 2005).

The J2 initiates the formation of a syncytium (Ross, 1958), a metabolic sink for the now sedentary nematode that will feed in the same location for the rest of its life (Niblack, 2005). This process takes place in compatible hosts (susceptible plants), but with incompatible hosts (resistant plants), the plant fails to form the syncytium and the nematode cannot develop in the root (Acedo et al., 1984; Kim et al., 1987; Koenning, 2004).

A J2 that successfully initiates a syncytium loses motility, begins feeding, and continues growing. Initial swelling transforms the J2 into a "sausage stage," and

development continues with the formation of the next two juvenile stages (J3 and J4) (Lauritis et al., 1983; Niblack, 2005). It is during the J3 stage that sexual differentiation is visible (Raski, 1950). This stage lasts 48 hours or less and the fourth stage likewise, lasting generally 24 hours for the males and 48 hours for the females (Lauritis et al., 1983). The J4 males and females are strongly dimorphic. The females continue swelling and remain sedentary while the males regain a vermiform morphology inside the fourth stage cuticle (Koenning, 2004). The last molt will lead into adulthood, which takes 8 to 9 days for males and 9 to 10 days for females (Lauritis et al., 1983; Niblack, 2005). Males have not been observed to feed after the J3 stage; they exit the root to locate females, whose posteriors protrude from the root at maturity. Sexual reproduction is obligatory for this species (Niblack, 2005).

The ratio of male to female is approximately 1:1 but this can be skewed by several conditions such as host resistance or environmental conditions (Colgrove and Niblack, 2005). After the female is inseminated, it will begin to lay fertile eggs in an external gelatinous matrix, and later to retain the eggs inside the body (Ichinohe, 1952). The range of egg production per female ranges from 40 to 600 or more (Sipes et al., 1992). A general average is 200 eggs per female (Niblack, 2005).

Under optimum conditions (25 °C) the *H. glycines* life cycle is 21 days long (Lauritis et al., 1983). However, under field conditions the range is from 14 to 28 days depending on the soil temperature (Alston and Schmitt, 1988).

Interaction with soybean plants

After hatching, the J2 will infect the roots. Primarily, infection of the roots results in a physical or mechanical damage (Ross, 1958) as the nematode enters the epidermis and cortical cells and destroys them during movement toward the vascular tissue (Noel, 2004). Secondly, initiation of a syncytium results in physiological damage during the interaction between enzymes produced by the nematode and host responses (Noel, 2004).

What is very interesting and important here is that these physiological and cellular responses are different in resistant (incompatible host) and susceptible (compatible) soybeans. Because penetration of roots by J2 takes place in both susceptible and resistant soybeans (Endo, 1970) and only the development (or not) of syncytia is associated with defense response, understanding of the development of these feeding sites is important.

After the J2 has chosen its feeding site, a "feeding plug" forms around the head of the nematode where it releases different enzymes (from amphids and stylet) (Endo, 1978, 1991, 1998). The initial syncytial cells surrounding this feeding plug undergo cytoplasmic changes (Endo, 1998), resulting in the dissolution of the cell walls and fusion of protoplasts to create multinucleate cells (Noel, 2004). Both hyperplasia and hypertrophy take place, the former very near the initial syncytial cells and the latter in cells distal to this place. This will contribute to the restriction of secondary phloem and xylem development (Noel, 2004).

As mentioned above, resistant soybean (incompatible hosts) differs from susceptible soybeans (compatible hosts) in cellular responses. In soybean and snap beans, a very typical incompatible response to *H. glycines* is similar to what is known as the

hypersensitive reaction – HR (Acedo et al., 1984; Melton et al., 1984). The result is a small syncytium that will later degenerate, allowing new parenchyma cells to develop. Nematodes cannot develop without a functional syncytium (Acedo et al., 1986).

Three important sources of resistance used in Illinois are "Peking" (Plant Introduction [PI] 548402), PI 437654, and PI 88788. The responses expressed by each one of them are very similar. Five days after infection, the syncytia stop developing and the cells become necrotic (Kim and Riggs, 1996). In PI 437654, which expresses resistance to most *H. glycines* populations, the response is very similar to that of Peking (Noel, 2004). In contrast, syncytial cells in PI 88788 accumulate cisternae and rough endoplasmic reticulum, reducing their function (Endo, 1998). In all resistant responses the nuclei degenerate (Kim and Riggs, 1992). Consequently, responses from incompatible hosts result in a partial or complete reduction of the development of the syncytia resulting in delayed growth or death of the nematode.

Screening Soybean for Resistance to *Heterodera glycines*

In plant pathology, the preferred means to reduce yield losses caused by pathogens is the use of resistant cultivars; *H. glycines* is no exception (Shannon, 2004). Soon after *H. glycines* was first identified in North America, an intense search to find resistance sources to this pest began (Shannon, 2004). And because *H. glycines* was and continues to be the most important pathogen of soybean – one of the major agricultural crops -- breeding for resistant cultivars will continue to be needed.

One of the most important objectives in soybean breeding programs is to obtain resistant cultivars to *H. glycines* (Shannon, 2004). Screening resistant cultivars in highly

infested areas showed that resistant cultivars yielded 56% more than susceptible cultivars (Wheeler et al 1997; Young and Hartwig, 1988).

Breeding for resistance to *H. glycines* will be a perpetual process. Alice was told by the Red Queen in *Alice in Wonderland*: "Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!" (Carroll, 1872). Applied to plant pathology, this idea is known as the *Red Queen Hypothesis*, expressing the idea that even though resistance to pathogens exists, efforts to develop resistant plants must continue as the pathogens adapt. Adaptation of the pathogen to a host is evident in *H. glycines* populations, which exhibit diversity for virulence (Niblack et al., 2002) both among (Niblack et al., 1993; Rao-Arelli et al., 1991; Sikora and Noel, 1991) and within populations (Colgrove et at., 2002; Zhang et al., 1998). Fortunately, there are at least 118 plant introductions with resistance to *H. glycines* in the USDA soybean germplasm collection (Arelli et al., 2000) from which resistance to *H. glycines* can be obtained.

Assessing resistance is based on a bioassay (Niblack et al., 2002). To classify the resistance of a cultivar, a greenhouse test using the Female Index (FI) is conducted. Female Index is defined as a percentage relating the number of females that develop on a test cultivar and those that develop on 'Lee' (the susceptible standard). Four categories of resistance are commonly accepted for assessing breeding lines (Schmitt and Shannon, 1992): FI < 10, resistant (R); FI 10 to 29, moderately resistant (MR); FI 30 to 59, moderately susceptible (MS); and FI 60 or more, susceptible (S). An alternative scale has been used in screening released cultivars: FI < 10 highly resistant (HR); FI = 10 to 24,

resistant (R); FI = 25 to 39, moderately resistant (MR); FI = 40 to 59, low resistance (LR); and FI ≥ 60, non-effective resistance (N or NR) (Niblack, 2005).

Methodology

Initially, screening for resistance was very subjective, basing the preliminary data on symptoms during the reproductive phase of the soybean, either in the field or greenhouse (Young, 1998). Screening in greenhouses will always be better due to the reduction in variability caused by external factors and because nematode population density can be uniform. However, screening for resistance in the field is crucial for yield evaluation (Shannon, 2004). Currently a uniform protocol proposed by Niblack et al. (2002) is being used as a standard for screening for resistance to *H. glycines*.

A standard procedure is:

1. Small tubes filled with sterilized soil are inoculated with eggs of a determined HG Type (Anand et al., 1985; Niblack et al., 2002).
2. Seed of each line to be screened are sown into each pot and replicated according to the experimental design chosen (Anand and Gallo, 1984; Hartwig, 1985).
3. Environmental conditions are regulated: soil temperature at 27 to 28°C with 16 hr daylength.
4. At 25-32 days after seeding, the soil is washed carefully and the roots are examined for females.
5. FI is the preferred system for evaluating resistance (Niblack et al., 2002).

Assessing root infection with real-time PCR

Identification of *H. glycines* using the polymerase chain reaction (PCR) opened new possibilities in diagnosis (Subbotin et al., 2001). With the utilization of the restriction enzyme AvaI in combination with ribosomal DNA (rDNA)-RFLPs, *H. glycines* can be distinguished from other species of the *schachtii* group, rendering PCR a very sensitive and specific tool (Subbotin, 2000). This sensitivity was observed by Subbotin (2001) when detecting a single *H. glycines* J2 either alone or in mixture with different soil inhabiting nematodes or *Pratylenchus* spp.

Real-time quantitative PCR (qPCR) is a relatively new technology, first documented in 1993 (Higuchi et al., 1993), but the first qPCR assay for plant parasites was reported in 1996 (Okubara et al., 2002; Schaad and Frederick, 2002; Schoen et al., 1996). Advantages of qPCR over standard PCR have been demonstrated (Bustin, 2002; Mackay et al., 2002; McCartney et al., 2003; Okubara et al., 2002; Schaad et al., 2003), the main one being the possibility for the researcher to obtain quantitative data, which makes this technology a potential tool for substituting some traditional ones (Quader et al., 2008). Another advantage is its speed; it requires only two hours for the machine to accomplish a run in which 96 to 384 reactions can be accommodated in regular plates (Gao et al., 2004).

In order to replace the traditional time-consuming tools for reliable quantification of pathogens, it is necessary to relate the DNA quantities and the actual numbers of pathogens. Nematodes are ideal for this purpose, because they can be counted under magnification with dissecting scopes. Madani et al. (2005) demonstrated the high

correlation between DNA quantities of nematodes determined with qPCR and number of nematodes.

The current protocol for screening for resistance is very accurate. However, efficiency could be increased by reducing the time required to obtain results. Resistance is expressed much earlier than the formation of the females. The presence of a nematode in the root would not distinguish compatible and incompatible hosts, but quantitative differences in the levels of infection as the females begin to mature should allow us to predict the resistance level of the plant.

The objective of this study was to develop a new and faster protocol to replace the traditional and time-consuming one for screening for *H. glycines* resistance in soybean.

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CHAPTER 2

A CONSISTENT METHOD TO ASSESS INFECTION OF *GLYCINE MAX* ROOTS BY *HETERODERA GLYCINES* WITH QUANTITATIVE POLYMERASE CHAIN REACTION

Heterodera glycines is the most economically important pathogen of soybean in United States (Wrather and Koenning, 2009). Among the potential management options, the use of resistant cultivars is the most effective. Development of resistant cultivars requires screening for resistance through a combination of standardized and controlled techniques. One such technique, perhaps the most important, is the inoculation method. Previous works have reported variability and inconsistency in penetration of second-stage juveniles (J2) as the result of different inoculation techniques (Acedo et al., 1984; Colgrove and Niblack, 2005; Halbrendt and Dropkin, 1986; Mahalingam et al., 1998; Melton et al., 1986).

To address this issue, I conducted the experiments described below with two main objectives: 1) to develop a consistent method of inoculation which could reduce the variability and inconsistency in J2 penetration of soybean seedlings; and 2) to develop a sensitive and efficient real-time qPCR assay to detect, amplify, and quantify *H. glycines* gDNA inside infected soybean seedlings.

MATERIALS AND METHODS

Seed germination

Seeds of soybean cultivars Lee 74, a compatible host of *Heterodera glycines* and Peking (PI 548402), an incompatible host of *H. glycines*, were obtained from the United States Department of Agriculture, Agricultural Research Service (USDA, ARS) Soybean Germplasm Collection, Urbana, IL. Seeds were germinated as described previously (Mahalingam et al., 1998). Briefly, with their hila facing downward, seeds were rolled in moist sterilized germination paper. Rolls of germination paper with seeds were placed in 1,000 mL beakers containing 100 mL tap water to facilitate imbibition and germination. The beakers were covered with plastic wrap and incubated for 48 hours at 27 °C. Uniform 3 to 5 cm long seedlings were selected for inoculation.

Nematode inoculum

The *H. glycines* isolate used in this experiment was increased on susceptible soybean Lee 74 and maintained in the greenhouse in a water bath at 27 °C with 16-hr days. The isolate (UIUC0) was HG Type 0 (Niblack et al., 2002), with all Female Indices less than 10. For use as inoculum, the isolate was increased for 30 days, at which time females were dislodged from roots with high pressure water spray. Eggs were released by maceration of the females with a rubber stopper on a 150-µm-aperture sieve, and washed onto a 25-µm-aperture sieve (Colgrove and Niblack, 2008). Eggs were concentrated by sugar centrifugation (Hooper, 1986) and the solution containing eggs was placed on a nylon sieve with 41-µm-apertures for hatching (Acedo and Dropkin, 1982). J2 were collected every day from hatching sieves until the target inoculum level was reached. 12 hours before use, J2 were placed on a sterile sand column, and the active

J2 were collected in sterile distilled water (Lambert, 1999). Inoculum was prepared by suspending the active J2 in sterile distilled water at a concentration of 1,000/mL.

Nematode inoculation

Ten seedlings each of Lee 74 and Peking were inoculated with 0 or 1,000 J2/seedling. Seedlings germinated as described in the seed germination section were placed horizontally in 15-cm-long polyvinyl chloride (PVC) tubes cut in half longitudinally. Half-tubes were placed horizontally in trays and filled with steam-pasteurized soil mix: two parts sand to one part silty clay loam, giving a final mix of 77% sand, 11% silt, and 12% clay, with pH 7.8 and 0.9% organic matter. Seedlings were placed horizontally at one end of each half-tube for inoculation.

Nematodes were kept in a homogenous suspension with either agitation or aeration. Either 1 mL of J2 suspension or 1 mL sterile distilled water was pipetted onto each seedling. Treatments were randomly distributed in the inoculation trays. All seedlings were covered with moistened, pasteurized sandy loam soil and incubated horizontally at 27 °C for 24 hours. Seedlings were removed and washed 24 hours post inoculation (hpi) and surface sterilized with 10% bleach for 30 seconds.

Half of the seedlings were frozen for DNA extraction and the remaining seedlings were stained as described in Byrd et al. (1983) for enumeration under a stereoscope. This experiment was conducted twice for the DNA extraction and real-time qPCR assay and three times for the nematode counts.

DNA extraction

DNA was extracted from infected and noninfected roots with the FastDNA® SPIN Kit and the FastPrep® instrument (MP Biomedicals, Santa Ana, CA). DNA was extracted in all experiments with a slight modification of the manufacturer's protocol as described by Malvick and Grunden (2005).

Roots were placed in 2.0 mL lysing matrix in tubes containing garnet particles and ¼ ceramic sphere, 1000 µl Cell Lysing Solution (CLS-TC) extraction buffer, 80 µl polyvinylpyrrolidone solution (PVP in 1 mg/10 µl stock solution). A second ¼ ceramic bead was added and homogenized twice in the FastPrep® instrument for 30 seconds 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 µl 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 DNA from the J2 suspension, 1 mL suspended J2 was delivered to a lysing tube and the same procedure as used for the infected roots was performed. DNA concentrations were measured with a Nano Drop (ND-1000, Thermoscientfic, DE, USA) and stored at -20 °C.

Selection of primers, test for inhibitors and efficiency of the primers

To specifically detect *H. glycines* gDNA, a primer pair for amplification of the single copy gene *HgSNO* was selected. This nematode gene codes for a protein involved in vitamin B_6 metabolism (Craig et al., 2008). The qPCR primers were designed with ABI's Primer Express 2.0. DNA extraction was verified with primers for the soybean

lectin gene, as described in Berdal and Holst-Jensen (2001). All oligos were purchased from Invitrogen, Carlsband, CA (Table 2.1).

The efficiency of the *HgSNO* primer pair was tested as described in Livak and Schmittgen (2001). Briefly, qPCR was performed on a ten-fold dilution series starting at 89.6 ng/µl *H. glycines* gDNA and the ΔCt were calculated between each dilution. The efficiency of the primers was evaluated by observation of ΔCt for each ten-fold dilution.

To test for PCR inhibitors in the DNA samples, five 10-fold dilutions of soybean gDNA was prepared starting at 71 ng/µl. Each dilution was mixed in a 1:1 ratio with 7.8 ng/µl of *H. glycines* gDNA. QPCR assay was performed to amplify *HgSNO* from *H. glycines* gDNA mixed with 71, 7.1, 0.71, 0.071, 0.0071, and 0.00071 ng/µl of soybean 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 Plates where 25 µl reaction mixture was used in each well. Amplification reactions contained 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 0.25 µl of 20 pmoles/µl of each primer pair. In both the inoculation and primer efficacy tests, 5 µl template DNA and 7.25 µl molecular grade water were used. For the inhibition test, 5µl *H. glycines* and soybean gDNA was used in a 1:1 mixture, adjusting the water volume to 2.25 µl to maintain the overall 25 µl reaction mixture per well. A negative control with no DNA template and a positive control with pure *H. glycines* gDNA were included in each experiment. The

reaction cycles were: pre-incubation at 50°C for 2 min; template denaturation and activation of Taq polymerase at 95°C for 10 min; 40 cycles of 95°C for 15 s each; and finally, annealing and extension at 60°C for 1 min.

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 (Madani et al., 2005).

Statistical analysis

The experiment was completely randomized with each tube in the inoculation tray being the experimental unit and the treatments, the four cultivar × inoculation combination. Analysis of variance (ANOVA) was conducted with the MIXED procedure in SAS (SAS 9.2, SAS Institute, Cary, NC). The UNIVARIATE procedure was used to verify the normality of the residuals. Homoscedasticity was checked with the GLM (general linear model) 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

In a preliminary study to test the inoculation method, inconsistent results were obtained when J2 were taken directly from the hatching sieve and collected until the target inoculum level was reached because the inoculum is composed of both active and

inactive nematodes (Figure A.1). However, following movement through the sand column, only active J2 were obtained, which gave more consistent infection (Figure 2.1). Stained nematodes inside infected seedlings were counted 24 hpi. Infection of Lee 74 (compatible) and Peking (incompatible) did not differ ($P \leq 0.05$) within each experiment (Table 2.2). Consistency of the inoculation method showed no differences ($P \leq 0.05$) among three experiments (Table 2.2). The percentage of infection in both compatible and incompatible hosts within 24 hpi ranged from 30 to 41%. The mean number of J2 penetrating the seedlings ranged from 333 to 401/seedling (Table 2.2).

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 and sterile distilled water in qPCR plates. 89.6×10^{-5} ng/µl was the lowest *H. glycines* gDNA concentration detected and quantified (average Ct value = 32.72) on a ten-fold dilution series (Figure A.2).

Serial ten-fold dilutions were amplified with real-time qPCR and the efficiency of the primers was determined based on the ΔCt between each dilution. The highest concentration was 89.6 ng/µl *H. glycines* gDNA. The ΔCt between dilutions were similar, ranging from 3.49 to 3.66 (Figure 2.2).

The inhibition test showed that a known and constant concentration of *H. glycines* gDNA (7.8 ng/µl) in 1:1 ratio mixture with different concentrations of soybean gDNA (71, 7.1, 0.71, 0.071, 0.0071 and 0.00071 ng/µl) was amplified and quantified at similar

Ct values, ranging from 19.31 to 19.83. Amplification and quantification of mixed reactions did not differ at $\alpha = 0.05$ ($P = 0.6374$) (Figure 2.3).

Real-time qPCR assay

The results obtained with the SYBR Green real-time qPCR assay coincided with the results obtained in number of nematodes counted in stained infected roots. The content of *H. glycines* gDNA in Lee 74 and Peking roots did not differ ($P \leq 0.05$) within or between experiments (Table 2.3). Amplification plots for real-time qPCR showed a tight band of amplified reactions (Figure 2.4). The means of qPCR threshold cycle (Ct) values were compared for Lee and Peking within each experiment and a delta Ct was calculated (Table 2.3). 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 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.

DISCUSSION

In order to evaluate the efficiency of inoculation methods and to provide data on the penetration of soybean seedlings by *H. glycines*, I conducted a set of experiments *in vivo*. I optimized a method to infect soybean seedlings consistently with active J2 and subsequently detect, amplify, and quantify *H. glycines* gDNA in infected soybean seedlings. The molecular method chosen for this purpose was real-time quantitative polymerase chain reaction. Highly specific primers for *HgSNO* were designed and

employed to detect and quantify *H. glycines* gDNA inside infected soybean. The consistent inoculation method and the high specificity of the qPCR assay allowed comparisons to be made reliably.

Newly hatched J2 are the infective stage. However, if eggs are used for inoculum, variability in the hatching rate makes it difficult to collect enough J2 to reach a desired level in one day. Collecting J2 takes time, and results in inoculum of different ages and viability. Mahalingam et al. (1998) noticed that 7-day-old inoculum was composed of dead or starving nematodes, resulting in poor infection. Lambert et al. (1999) 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. An active J2 will crawl through the sand column in about 12 hours. Less active, older J2 are retained in this sand bio-filter.

A sand column was used in all the experiments to obtain uniform, surface-sterilized, active J2 which consistently infect soybean seedlings. Incorporating the sand column in the inoculation technique allowed reduced variability of J2 penetration into seedlings. This variation in penetration rate has been a problem in many studies of this sort (Acedo et al., 1984; Colgrove and Niblack, 2005; Halbrendt and Dropkin, 1986; Mahalingam et al., 1998). In my studies, about 30 to 40% of the J2 penetrated seedlings within 24 hours – an improvement, compared with the 10% infection rate reported by Acedo et al. (1984). The consistency of the method was demonstrated in three different experiments, performed with three different batches of hatched J2, in which no differences were observed within or among experiments. The sand column proved to be crucial to reducing the variability that previously was present even under standardized

and controlled inoculation conditions (Colgrove and Niblack, 2005; Halbrendt and Dropkin, 1986).

Even though adding a sand column reduced the variation in infection, the number of nematodes counted inside infected soybeans may be variable, especially at high infection rates. A molecular tool may help to reduce this source of variation. Previous studies demonstrated the use of qPCR to detect and quantify a specific target for subsequent comparison. Gao et al. (2004) developed a real-time qPCR protocol to quantify *Fusarium solani* f. sp. *glycines* in infected soybean root. Similarly, Malvik and Impullitti (2007) used qPCR to detect and quantify *Phialophora gregata* in soybean and soil samples. Madani et al. (2005) amplified DNA from *Heterodera schachtii* and *Globodera pallida* using qPCR in order to identify the species, and Motiul et al. (2007) identified different *Globodera* spp. using the same tool. Madani et al. (2005) reported a good relationship between the number of *H. schachtii* J2 and quantification of gDNA, but to my knowledge there are no reports on detection and quantification of *H. glycines* gDNA in infected roots.

Validation of the qPCR assay was achieved by checking the efficiency of the primer pairs used in the experiment and by testing for PCR inhibition. The protocol used for DNA extraction provided soybean gDNA extracts with no PCR inhibition, allowing amplification of known and constant *H. glycines* gDNA in mixtures with different concentrations of soybean gDNA. Bessetti (2007) reported that soil and plant material may contain PCR inhibitors and Malvick and Impullitti (2007) expressed how critical it is to test for PCR inhibitors and account for them in qPCR assays. If PCR inhibitors are detected in DNA samples they must be addressed by further purification of the samples.

Livak and Schimittgen (2001) reported that the sensitivity and efficiency of the primers can be assessed by observing how Ct varies with each dilution. The primers for *HgSNO* used in all the experiments were found to be highly sensitive and efficient.

The robustness of the techniques described herein allows comparisons between compatible and incompatible hosts. Detection and quantification of *H. glycines* gDNA with qPCR from infected soybean seedlings did not differ between Lee and Peking 24 hpi. Therefore, J2 penetrated seedlings regardless of the level of soybean resistance. The same results were observed when infected seedlings were stained and nematodes counted. J2 penetration of compatible as well as incompatible hosts was observed in early studies (Acedo et al., 1984; Endo, 1965). Furthermore, when soybean lines with different sources of resistance were inoculated, J2 penetration 5 days after inoculation was equivalent (Colgrove and Niblack, 2005).

Two achievements were described in this chapter. First, an inoculation method to reduce the variability in J2 penetration of soybean seedlings was generated and optimized. Variability in J2 penetration and inconsistent inoculation results observed in early works were major issues to be addressed, especially to prevent misleading results in breeding programs. With the inoculation method described herein, variation and inconsistency of J2 penetration were reduced. Second, a sensitive and efficient assay with SYBR Green real-time qPCR was developed to detect, amplify, and quantify *H. glycines* gDNA inside infected soybean roots. The combination of a consistent inoculation method and qPCR assay showed a tight band of amplified reactions demonstrating a consistent detection of *H. glycines* gDNA inside radicles and allowing comparisons to be made. The next phase of this research is to quantify differences in *H. glycines* gDNA content in

soybean cultivars with different resistance genotypes when observed at different time points after inoculation.

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TABLES

Table 2.1. Primers used for real-time quantitative polymerase chain reaction (qPCR) assays targeting *Heterodera glycines* and *Glycine max.*

Amplicon	Accession no.	Primers (5' → 3') [a]	Size (bp)
HgSNO	EU747298	F: AGGCAACGTGCAGCAACAT R: CTGATCGCCAGTCTTCACTATGA	76
Lectin	K00821	F: CTTTCTCGCACCAATTGACA R: TCAAACTCAACAGCGACGAC	102

[a] F and R indicates the forward and reverse primers

Table 2.2. Penetration of compatible soybean host 'Lee 74' and incompatible 'Peking' (PI 548402) by *Heterodera glycines* second-stage juveniles (J2) 24hrs after inoculation with 1,000 J2/seedling.

		Juveniles [b]				
Experiment [a]	Host	Range	Mean	Infection (%)	*P*	Pooled [c]
1	Lee 74	235 – 553	366	37	0.6765	
	Peking	221 – 589	401	40		
2	Lee 74	300 – 580	413	41	0.2131	0.272
	Peking	244 – 415	334	33		
3	Lee 74	255 – 350	306	31	0.3447	
	Peking	280 – 398	333	33		

[a] In each experiment, 5 Lee 74 and 5 Peking (PI 548402) were inoculated with active J2, collected after being allowed to crawl through a sand column.

[b] Infected seedlings were stained with acid fuchsin (Byrd et al., 1983) and nematodes were counted at × 64 magnification with a stereoscope. Range, mean, and infection rate were calculated from the nematodes inside the 5 replications for each host.

[c] The data were pooled for the three experiments and did not differ at $\alpha = 0.05$.

Table 2.3. Comparison of *Heterodera glycines* DNA content in compatible soybean 'Lee74' and incompatible 'Peking' (PI 548402) 24 hours after inoculation with 1,000 *H. glycines* second-stage juveniles/seedling according to a SYBR Green real-time quantitative polymerase chain reaction (qPCR) assay with a primer pair designed to amplify the *HgSNO* gene in *H. glycines*.

	Average qPCR Ct value [b]						
Experiment [a]	Lee 74	Peking (PI 548402)	SD[c]	Δ Ct	$2^{-\Delta Ct}$ [d]	*P*	Pooled [e]
1	23.33	22.84	0.5848	- 0.49	1.40	0.4264	0.0653
2	24.16	23.53	0.5010	- 0.63	1.55	0.2427	

[a] In each experiment, 5 Lee 74 and 5 Peking (PI 548402) were inoculated.

[b] DNA extracted from soybean roots was amplified and quantified using primers for *HgSNO*. The cycle threshold (Ct) values were averaged. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.

[c] SD: Standard deviation.

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

[e] The consistency of the inoculation method and the efficiency of the qPCR assay was corroborated when experiments were pooled and did not differ at $\alpha = 0.05$.

FIGURES

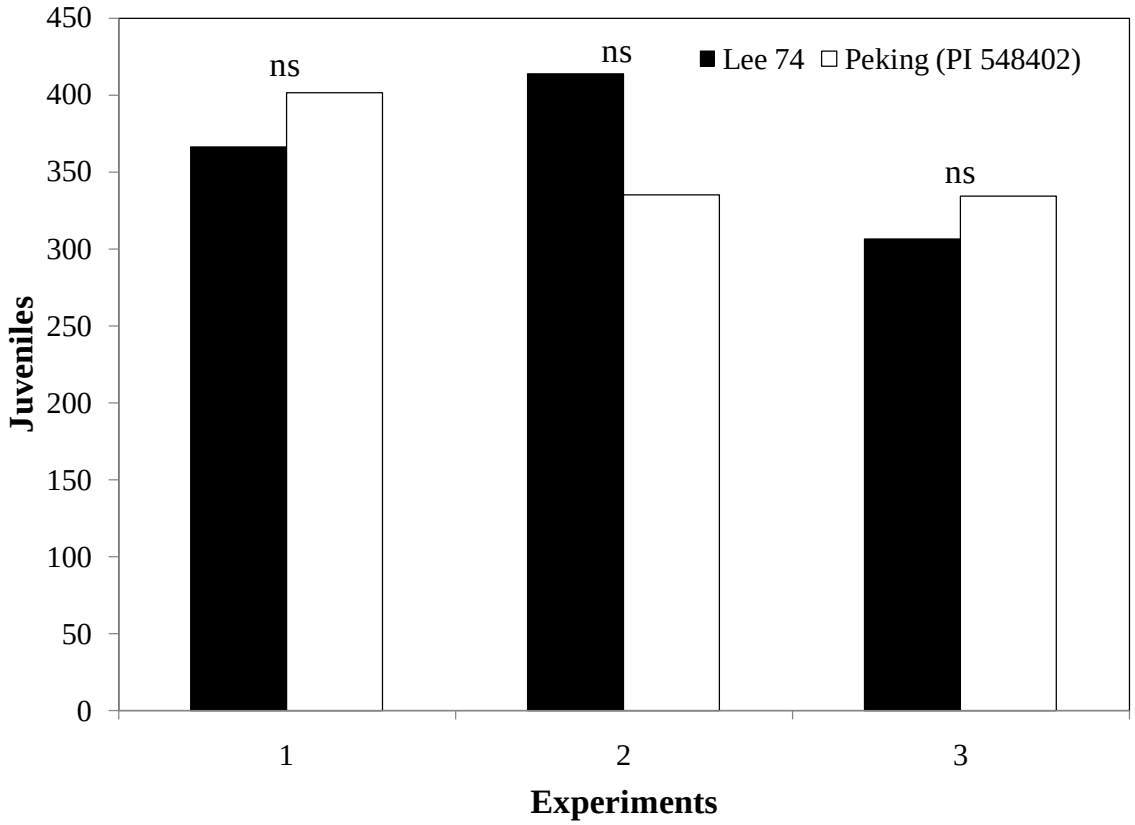

Figure 2.1. Numbers of *Heterodera glycines* second-stage juveniles (J2) within roots of compatible soybean host 'Lee 74' and incompatible 'Peking' 24 hrs after inoculation with 1,000 J2/seedling when a sand column was used to obtain active inoculum. Pairs of means superscribed by 'ns' are not significant at $P \leq 0.05$.

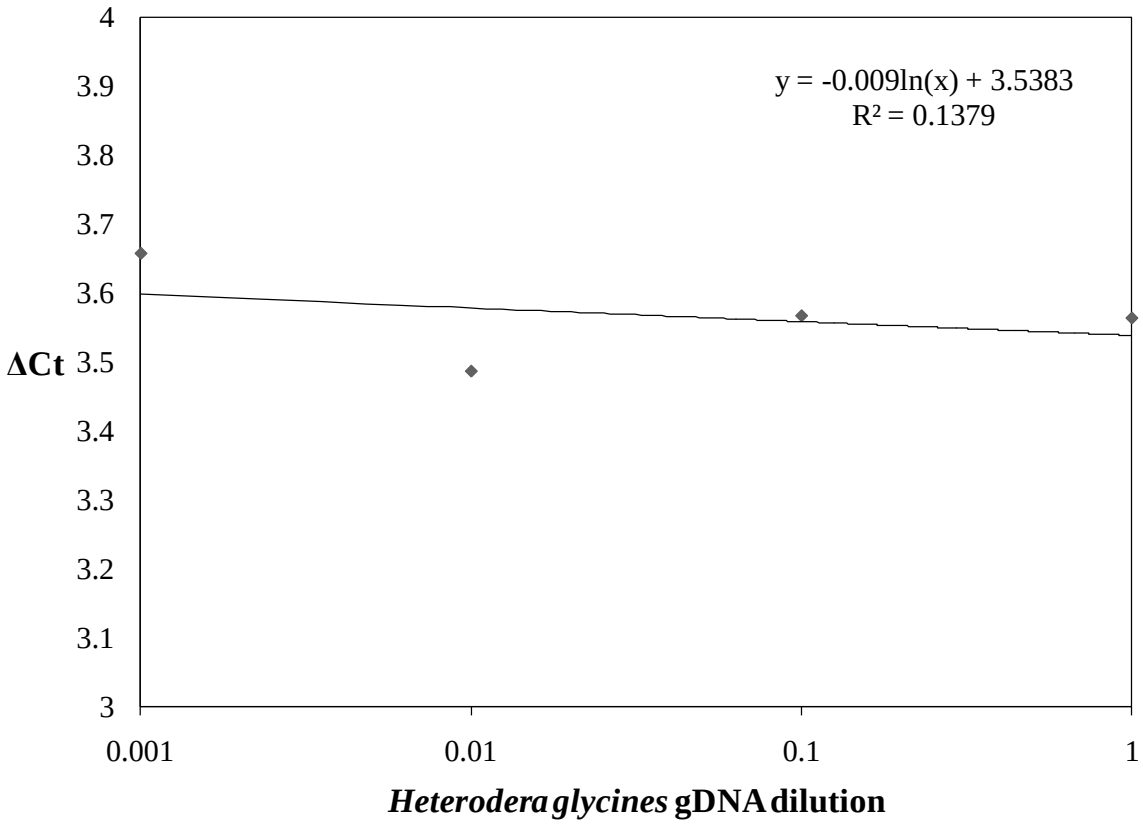

Figure 2.2. ΔCt values (differences in cycle threshold values) in a quantitative PCR analysis of the efficiency of a primer pair for the *Heterodera glycines* gene *HgSNO* tested with a ten-fold serial dilution of genomic *H. glycines* DNA (starting at 89.6 ng/µl).

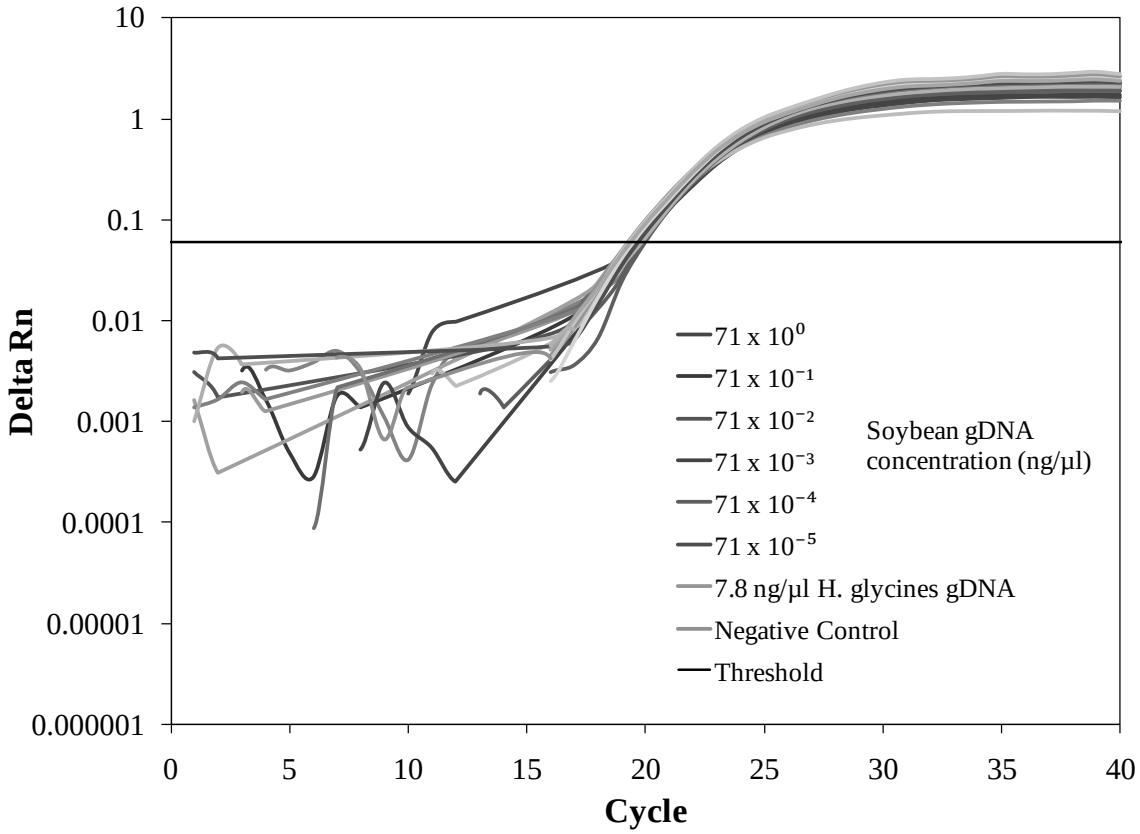

Figure 2.3. Amplification plot of *Heterodera glycines* genomic DNA with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and a primer pair designed to amplify the nematode *HgSNO* gene in serially diluted soybean genomic DNA combined in a 1:1 mixture with 7.8 ng/µl *H. glycines* genomic DNA. Distilled water was used instead of DNA for the negative control.

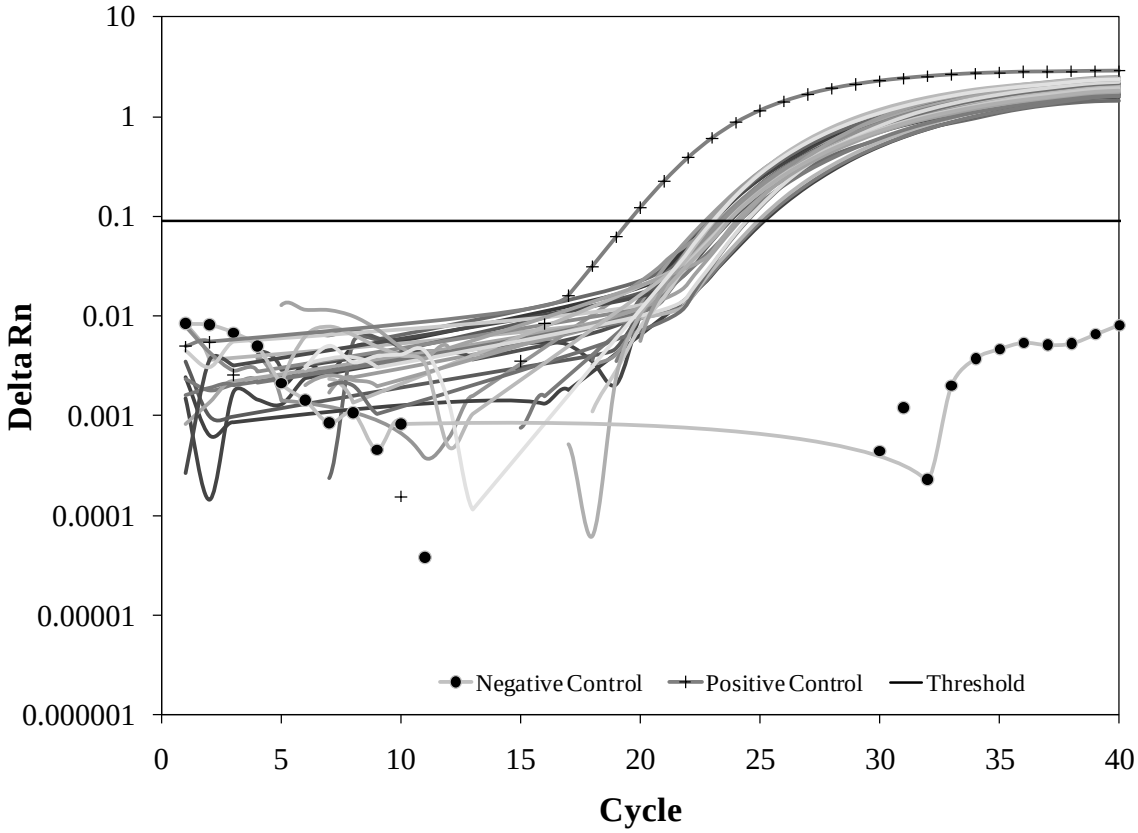

Figure 2.4. Amplification plot of *Heterodera glycines* genomic DNA with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and a primer pair designed to amplify the nematode *HgSNO* gene from infected Lee 74 and Peking roots 24 hrs after inoculation with 1,000 *H. glycines* second-stage juveniles/seedling. Distilled water was used instead of DNA for the negative control, and 7.8 ng/µl *H. glycines* gDNA was the positive control.

CHAPTER 3

DIFFERENTIATION OF SOYBEAN CULTIVAR RESISTANCE TO *HETERODERA GLYCINES* WITH QUANTITATIVE POLYMERASE CHAIN REACTION

Endo (1965) reported the histological responses of resistant and susceptible soybean cultivars (Peking and Lee, respectively) to entry and development by *Heterodera glycines*. These soybean cultivars represent the extreme responses to *H. glycines* in the continuum from fully compatible to incompatible hosts. In this chapter, I describe a set of experiments intended to verify whether the methods developed in Chapter 2 could be used to detect differences in the responses of the *H. glycines*-compatible cultivar Lee 74 (derived from Lee) and the incompatible Peking (PI 548402) without having to stain and count nematodes in infected roots or wait 30 days until adult females can be extracted and counted, as is currently done to evaluate resistance.

Quantification of *H. glycines* infection by traditional means (numbers of females produced in 30 days) is a time-consuming practice. I hypothesized that the real-time qPCR assay reported in Chapter 2 can replace the traditional bioassay and improve precision in determining infection levels. The objective of this experiment was to quantify differences in *H. glycines* gDNA content in roots of infected soybean cultivars with different resistance genotypes over time.

MATERIALS AND METHODS

Soybean seeds

Soybean seeds were obtained from the USDA, ARS Soybean Germplasm Collection, Urbana, IL. Cultivars Lee 74, a compatible host, and Peking (PI 548402), an incompatible host to *H. glycines*, were used in the first experiment. For the second experiment, soybean cultivars with three different levels of resistance to *H. glycines* HG Type 0 (highly resistant [HR], FI < 10; moderately resistant [MR], FI ≥ 25 and < 40; and susceptible [S], FI ≥ 60) were selected from among entries in the 2008 and 2009 Soybean Variety Testing program (Table 3.1) (http://vt.cropsci.illinois.edu). Three different soybean cultivars were used to represent each level of resistance. Resistance levels were based on previously determined and reported female indices (FI) (http://vipsoybeans.org). The source of *H. glycines* resistance for each selected cultivar is PI 88788. For seedling inoculation, uniform 3 to 5 cm long, straight seedlings were produced as described in the previous chapter.

Nematode inoculum

For both experiments, 1,000 J2/seedling was the inoculum level. The *H. glycines* isolate was HG Type 0, which was increased, harvested, and collected as described in the previous chapter. All nematode J2 used were passed through a sand column 12 hours before seedlings were inoculated as described in the previous chapter.

Nematode inoculation

In the first experiment, uniform 3 to 5 cm long, straight seedlings of Lee 74 and Peking (PI 548402) were inoculated with 1,000 infective J2 pipetted onto each seedling.

Control seedlings received an equal amount of sterile distilled water. Twenty-four hours post inoculation (hpi), all seedlings were lifted from the inoculation environment and washed under running tap water to remove free J2 that had not infected (Endo, 1965). Five each of Lee 74 and Peking, and 3 mock-inoculated soybean seedlings, were surface-sterilized and frozen for DNA extraction at 1, 7, 10, 14, and 21 days post inoculation (dpi).

Soybean cultivars with different levels of resistance to *H. glycines* were used in the second experiment. Uniform seedlings of nine cultivars with different levels of resistance, and control (mock-inoculated) soybean seedlings were used. Three replications of each cultivar were inoculated with 1,000 J2/seedling as described in chapter 2. Control seedlings were mock-inoculated with sterile distilled water. At 24 hpi, all seedlings were washed as describe above. Three replications of each cultivar and three mock-inoculated soybean seedlings were surface-sterilized and frozen for DNA extraction at 7, 10, 14, and 21 dpi.

DNA extraction

Single male and juvenile DNA extraction: Nematodes were individually digested for qPCR assay as described by Craig et al. (2008). Briefly, 10 males and 10 J2 were handpicked and individually digested in 5µl buffer (50 mM Tris-HCL pH 7.5, 50 mM NaCl) containing 4 mg/mL of fungal protease K (Invitrogen, Carlsbad, CA). The protease K was inactivated after 24 hours at 80°C for 30 min.

Infected and noninfected soybean DNA extraction: DNA was extracted from surface-sterilized, frozen soybean roots at each harvest point with a FastDNA® SPIN kit

and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The protocol used for DNA isolation in these experiments was described in Chapter 2. Each root was standardized by the length at the moment of inoculation +2 cm (about 7 cm).

Real-time qPCR assay

On an ABI PRISM 7000 sequence detection system instrument (PE Applied Biosystems, Foster City, CA), SYBR Green real-time qPCR assay was conducted. A 25 µl reaction mixture containing 5 µl target soybean DNA or 5 µl digested single nematode DNA was used in each well of a 96-well optical reaction plate for the amplification reactions. A duplicate of each reaction was run on each plate. Analysis and cycle threshold (Ct) values were calculated as described in Chapter 2. Similarly, validation of the qPCR assay was the same as described in the previous chapter. Specificity and efficiency of the primers for *HgSNO* were verified as well as the absence of PCR inhibitors.

Statistical analysis

Both experiments were completely randomized with each tube in the inoculation tray being the experimental unit and the treatments, the four cultivar × inoculation combination. Analysis of variance (ANOVA) for each harvest point was done with the MIXED procedure in SAS (SAS 9.2, SAS Institute, Cary, NC). The UNIVARIATE procedure was used to verify whether the assumptions of ANOVA were met. 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

Single male vs. second-stage juvenile

Males have four times more gDNA than J2. The results obtained with the SYBR Green real-time qPCR assay showed that the content of gDNA in fully developed males differed at $\alpha = 0.05$ ($P = 0.0001$) from gDNA content of J2 (Figure 3.1). Mean average Ct values for 10 digested males was 25.3 compared with a mean of 23.3 for the same number of J2. The ΔCt value between an adult male *H. glycines* and a J2 was 2.

Lee 74 vs. Peking (PI 548402)

Heterodera glycines gDNA content in compatible host Lee 74 and incompatible Peking was compared at different days post inoculation with SYBR Green real-time qPCR (Figure 3.2). At 10 and 21 dpi, Lee 74 and Peking differed at $\alpha = 0.05$. At 21 dpi, females were visible and countable; however, at 10 dpi Lee 74 and Peking roots showed no visible differences (data not shown). Lee 74 and Peking did not differ at $\alpha = 0.05$ at 1, 7, and 14 dpi. The same results were obtained when this experiment was repeated (Table 3.2).

Soybean cultivars with different level of resistance to *Heterodera glycines*

The content of *H. glycines* gDNA in nine soybean cultivars with different levels of resistance (3 highly resistant cultivars, 3 moderately resistant, and 3 susceptible) was compared at 7, 10, 14, and 21 dpi with SYBR Green real-time qPCR. Even though the cultivars had different level of resistance, the source of resistance for all of them was PI

88788. Cultivars did not differ at $\alpha = 0.05$ at 7 and 10 dpi; however, they differed at 14 and 21 dpi (Table 3.3).

Cultivars with the same level of resistance (highly resistant, moderately resistant and susceptible) were pooled; the means were calculated and single-degree-of-freedom contrasts were performed for each harvest point. Highly resistant cultivars differed from those both moderately resistant and susceptible at 14 and 21 dpi, at $\alpha = 0.05$; however, cultivars with moderate resistance and susceptible did not differ (Table 3.4). The same result was obtained when the experiment was repeated.

DISCUSSION

Differences in DNA content of *H. glycines* were detected between Lee 74 and Peking at different days post inoculation with SYBR Green real-time qPCR. The difference between Lee and Peking was detected as early as 10 dpi. Likewise, cultivars with different levels of resistance derived from PI 88788 differed at 14 dpi. For both experiments, there was no difference between cultivars at day 7 dpi.

J2 of *H. glycines* generally penetrate roots of soybean irrespective of the level of resistance. In my study, *H. glycines* gDNA content did not differ 1 dpi in Lee 74 and Peking. Previous studies showed that in incompatible hosts such as Peking, nematodes rarely reach the J3 stage, and no adults are observed (Endo, 1965). However, at day 7 after penetration, the DNA of dying nematodes is still amplified and quantified, resulting in an inability to detect differences between cultivars at this harvest point. This was observed in both experiments. However, males were observed in cultivars with resistance derived from PI 88788. Resistance effective against females in highly resistant soybean

cultivars (FI < 10) is not effective against male development (Colgrove and Niblack, 2005). Development of males may account for differences observed in these experiments.

Endo (1964) and Lauritis et al. (1983) reported development of *H. glycines* in susceptible soybean plants and showed the presence of fully developed males inside the roots at 10 dpi. During 11-15 dpi, males started to gradually leave the root and were found in the proximities of adult females. By 10 days after root penetration, real-time qPCR assay revealed that compatible host Lee 74 differed from Peking suggesting that the former contained fully developed males as opposed to the latter.

Colgrove and Niblack (2005) demonstrated that the female-male ratio in Lee 74 does not differ from 1. In contrast, nematodes do not complete their life cycle in incompatible host Peking; few reach the J3 stage (Endo, 1965). Results from SYBR Green real-time qPCR assay with single digested nematodes revealed that males contain 4 times more gDNA than juveniles.

By 10 days after penetration, Lee 74 contained more *H. glycines* gDNA amplified and quantified from adult nematodes, and differed from Peking in which *H. glycines* gDNA from dead J2 (and maybe a few J3) was still amplified and quantified. However, by 14 days after infection, *H. glycines* gDNA content in Lee 74 and Peking surprisingly did not differ anymore, even though big developed females were observed in stained roots. Since the female-male ratio in Lee 74 is 1:1, between 11 and 15 days, half of the nematodes (males containing four times more gDNA than juveniles) emerged from the roots to complete their reproductive life cycle. On the other hand, gDNA from dead

juveniles in Peking were still detected, amplified, and quantified. At 14 days after infection the number of (dead) juveniles in Peking is twice the number of female juveniles in Lee 74, thus the gDNA content did not differ between the compatible and incompatible host. At 21 days after inoculation, the nematode gDNA content in Lee 74 and Peking differed significantly, perhaps due to the content of fertilized eggs (each one containing *H. glycines* gDNA) inside females on the compatible host (Endo, 1965; Lauritis et al., 1983).

Shannon et al. (2004) described resistance to *H. glycines* as a complex and multigenic process, requiring major genes and some minor genes to confer resistance to the nematode. Resistance in *H. glycines* is therefore quantitative. Based on the female index; resistance ranges from highly resistant to susceptible, passing through different categories such as moderately resistant and moderately susceptible (Schmitt and Shannon, 1992).

In the second experiment, nine cultivars were used, all with PI 88788 as the source of resistance. Based on the female indices, three were highly resistant, three moderately resistant, and three susceptible to *H. glycines*. Nematode penetration at 10 dpi in both highly resistant and susceptible cultivars contained approximately the same amount of *H. glycines* gDNA and did not differ, contrary to the results observed in the first experiment. Differences were observed only at 14 and 21 days after infection between cultivars. In both cases, the qPCR assay could only detect differences between highly resistant and moderately resistant and between highly resistant and susceptible cultivars; however, moderately resistant and susceptible cultivars did not differ. Our results support findings by Colgrove and Niblack (2005) who reported that effective

resistance from PI 88788 does not reduce male development. Halbrendt and Dropkin (1986) also reported that the number of males produced on soybean with resistance to *H. glycines* differed from those of females.

In conclusion, a method to detect, amplify, and quantify *H. glycines* gDNA in infected roots of soybean cultivars with different levels of resistance was developed. As nematodes develop inside infected soybean roots their DNA content increases. This observation was the basis of the two experiments described in this Chapter. I successfully detected differences in *H. glycines* gDNA content between compatible and incompatible hosts at 10 dpi, reducing by 20 days the time needed to distinguish these cultivars. With PI 88788 as a source of resistance, resistance could be distinguished by 14 dpi, but only highly resistant cultivars were different. Moderately resistant cultivars did not differ from susceptibles with the real-time qPCR assay.

These results were encouraging because in both experiments the time needed to differentiate highly resistant cultivars from moderately resistant and susceptible cultivars was reduced by more than half the time needed for traditional screening based on the Female Index. Moreover, results showed that male development is a key factor when differences are observed between cultivars.

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TABLES

Table 3.1. Soybean cultivars[a] distinguished by Female Indices (FI) and resistance rating selected for inclusion in a study of the use of quantitative PCR as a substitute for traditional means of screening for resistance to *Heterodera glycines*.

Seed company	Cultivars	FI [b]	Rating [c]	Source of resistance [d]
FS HISOY	HS 38C60	0	HR	PI 88788
KALTENBERG	KB 2609 RR	0	HR	PI 88788
WILKEN	W 2672 NSTS	0	HR	PI 88788
LG SEEDS	C 4488 NRR	38	MR	PI 88788
NK BRAND	S 23-N7	33	MR	PI 88788
STONE SEED GROUP	3A319 NRR	31	MR	PI 88788
MERSCHMAN	MOHAVE 1029LL	107	S	PI 88788
MERSCHMAN	OLYMPUS 1051LL	122	S	PI 88788
SOUTHERN STATES	RT 4808 N	107	S	PI 88788

[a] 2008 and 2009 Soybean Variety Testing program (http://vt.cropsci.illinois.edu)

[b] Female index = (average number of females developed on an indicator line / average number of females developed on a standard susceptible) x 100.

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

[d] PI 88788 source of resistance to *Heterodera glycines* HG Type 0.

Table 3.2. Mean comparisons of real-time quantitative polymerase chain reaction (qPCR) cycle threshold (Ct) values for *Heterodera glycines* genomic DNA content in Lee 74 and Peking (PI 548402) at 1, 7, 10, 14 and 21 days after inoculation.

		Average qPCR Ct value [b]					
DAI	Experiment [a]	Lee	Peking (PI 548402)	SD [c]	Δ Ct	$2^{-\Delta Ct}$ [d]	*P*
1	1	23.57	23.61	0.448	0.04	1.03	0.9345
	2	23.26	23.30	0.315	0.04	1.03	0.9068
7	1	21.47	22.12	0.484	0.65	1.57	0.2148
	2	21.80	22.99	0.433	1.19	2.28	0.0251
10	1	19.00	20.07	0.289	1.07	2.10	0.0059
	2	21.34	22.88	0.296	1.55	2.92	0.0008
14	1	23.74	26.31	1.829	2.57	5.93	0.1979
	2	19.97	20.40	0.211	0.43	1.35	0.0805
21	1	25.92	29.81	1.326	3.88	14.74	0.0191
	2	19.57	23.82	0.964	4.25	19.01	0.0031

[a] In each experiment, 5 Lee 74 and Peking (PI 548402) were inoculated with 1,000 *Heterodera glycines* second-stage juveniles/seedling.

[b] *Heterodera glycines* genomic DNA content in infected Lee 74 and Peking (PI 548402) was determined using 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 fluoresce of the sample exceeded background fluorescence.

[c] SD: Standard deviation.

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

Table 3.3. Probability values for the main effect of cultivars when *Heterodera glycines* genomic DNA content in soybean cultivars with different resistance levels was quantified with real-time quantitative polymerase chain reaction (qPCR) 7, 10, 14 and 21 days after inoculation. [a]

	Experiment	
Days after inoculation	1	2
7	0.3509	0.2254
10	0.2405	0.2151
14	0.0178	0.0015
21	0.0155	<.0001

[a] Nine cultivars with different levels of resistant to *Heterodera glycines* (three highly resistant, moderately resistant, and susceptible) were inoculated in three replications with 1,000 *H. glycines* second-stage juveniles/seedling and harvested at 7, 10, 14 and 21 days after inoculation. *Heterodera glycines* genomic DNA content in infected cultivars was determined using SYBR Green with a primer pair designed to amplify the *HgSNO* gene in real-time quantitative polymerase chain reaction (qPCR).

Table 3.4. Probability values and standard deviations of means for single-degree-of-freedom contrasts of *Heterodera glycines* DNA content in soybean cultivars with different levels of resistance at 7, 10, 14 and 21 days after inoculation with 1,000 second stage juveniles with real-time quantitative polymerase chain reaction (qPCR).

		Days after inoculation							
		7		10		14		21	
Experiment	Comparison [a]	SD [b]	*P*	SD [b]	*P*	SD [b]	*P*	SD [b]	*P*
1	HR vs. MR	0.4592	0.4112	0.8595	0.1930	0.6344	0.0037	1.0196	0.0018
	MR vs. S	0.4592	0.3306	0.8595	0.0915	0.6536	0.6151	0.9191	0.8020
	HR vs. S	0.4397	0.0809	0.8595	0.0057	0.6808	0.0022	1.0510	0.0014
2	HR vs. MR	0.4682	0.5342	1.6341	0.1630	0.3445	0.0021	0.6432	0.0001
	MR vs. S	0.4682	0.1708	1.6341	0.4752	0.3198	0.3293	0.6432	0.0507
	HR vs. S	0.4499	0.0463	1.6341	0.4777	0.3328	0.0002	0.6432	0.0001

[a] Single-degree-of-freedom contrast between Highly resistant (HR) and Moderately resistant (MR), Moderately resistant (MR) and Susceptible (S), and Highly resistant (HR) and Susceptible (S).

[b] SD: Standard deviation.

FIGURES

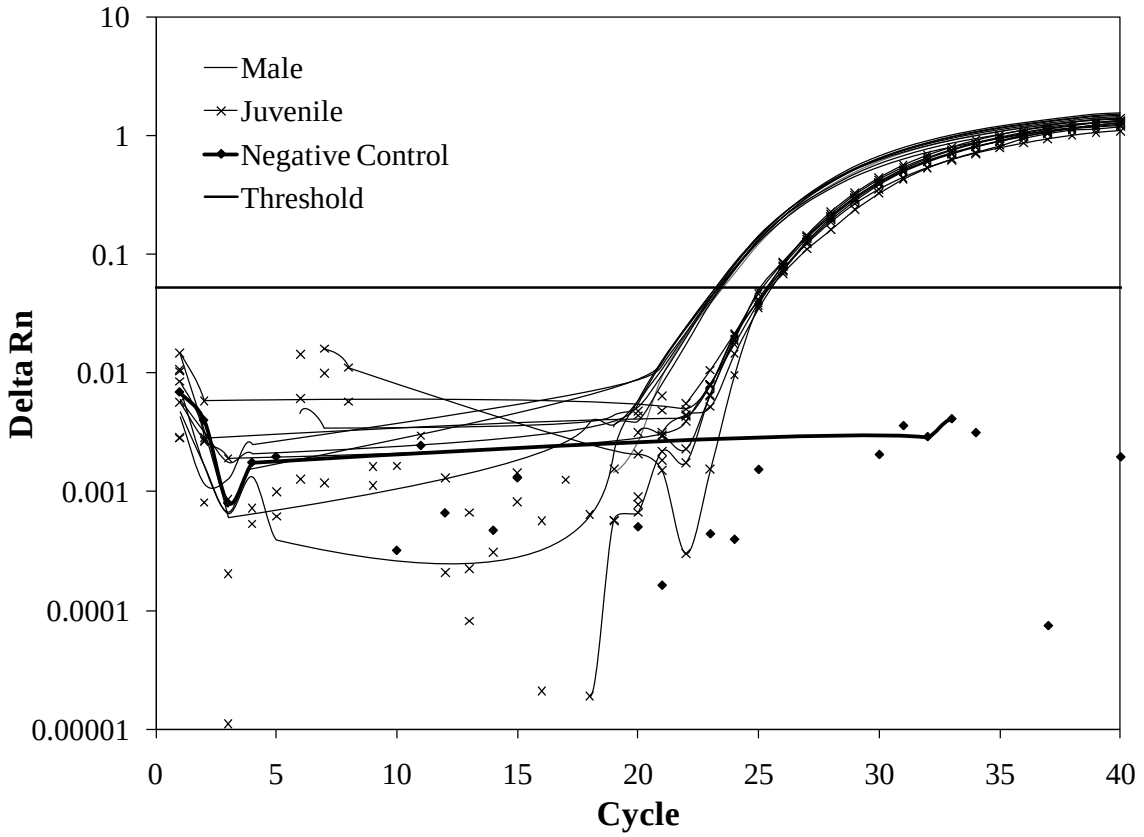

Figure 3.1. Real-time quantitative polymerase chain reaction (qPCR) amplification plots of genomic DNA of *Heterodera glycines* males and juveniles with SYBR Green and a primer pair designed to amplify the *HgSNO* gene. Distilled water was the negative control.

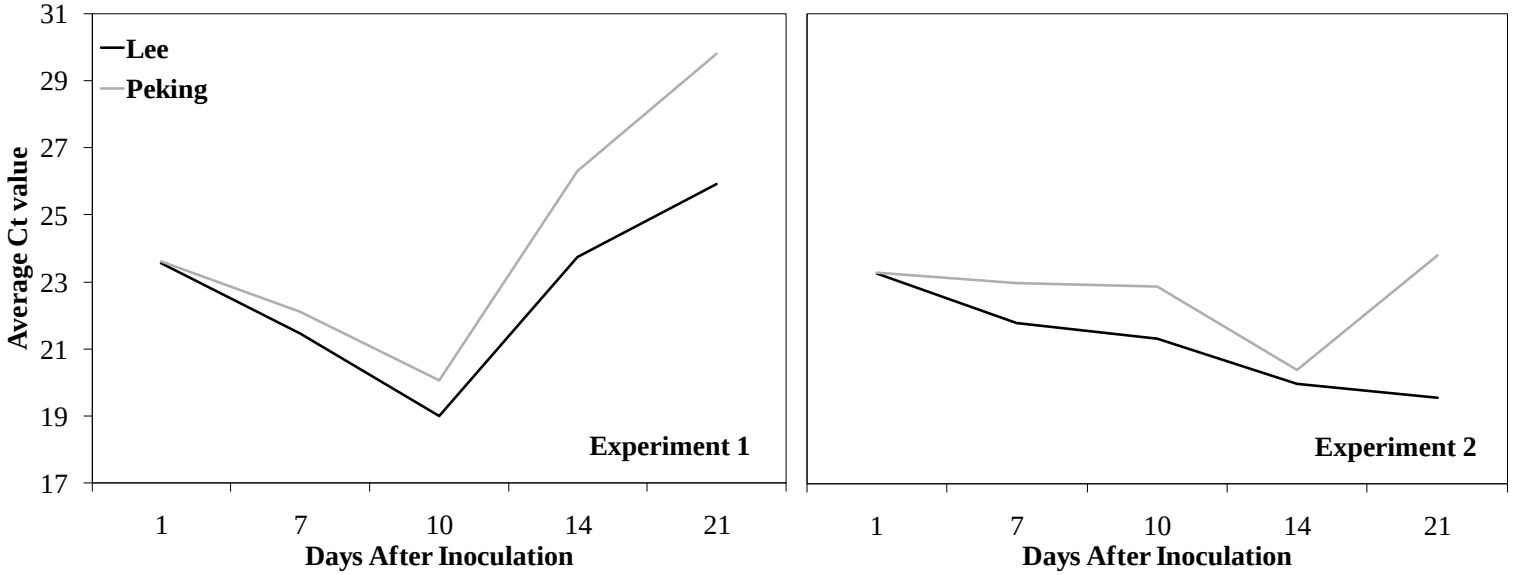

Figure 3.2. *Heterodera glycines* genomic DNA content in compatible host Lee 74 and incompatible host Peking (PI 548402) at different days after inoculation with SYBR Green real-time quantitative polymerase chain reaction (qPCR) and a primer pair designed to amplify the *HgSNO* gene. Soybean radicles were inoculated with 1,000 *H. glycines* J2 and transplanted to pasteurized soil for the designated number of days after inoculation. Plants were maintained in a water bath at a constant 27 C and 16 hour daylength.

APPENDIX

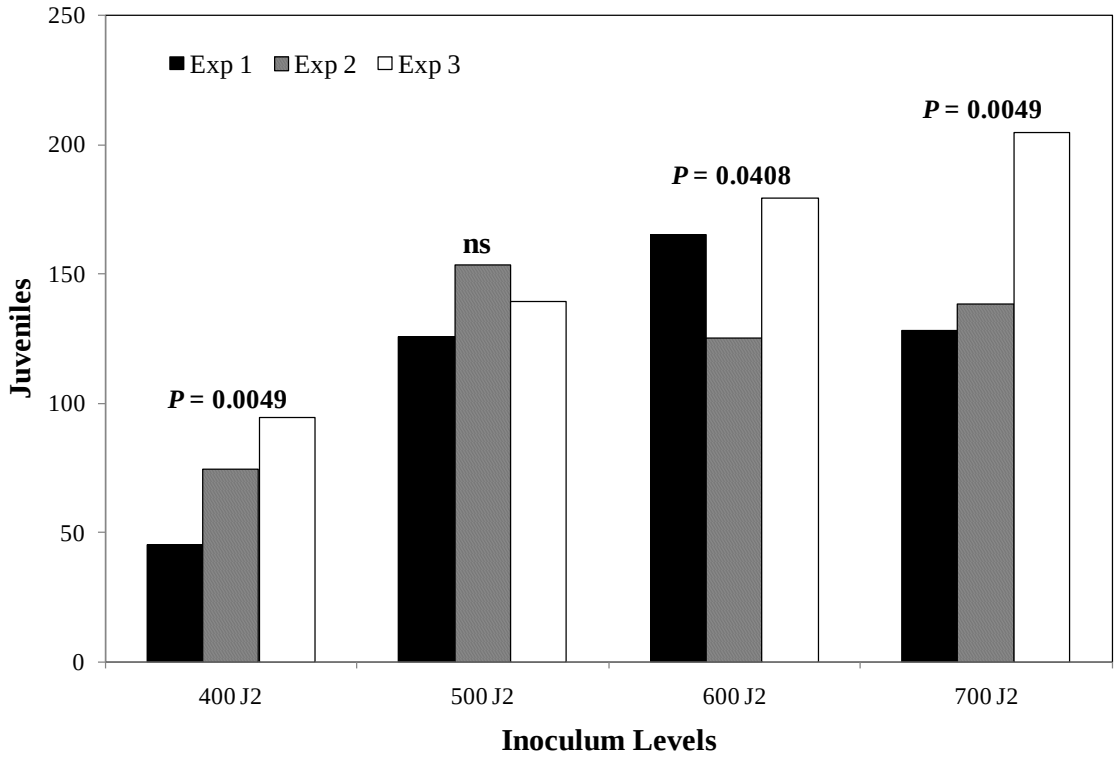

Figure A.1. Effect of different inoculum levels of *Heterodera glycines* second-stage juveniles (J2) on penetration of soybean roots 24hrs after inoculation. Nematodes were collected after hatching until the target inoculum level was reached and used directly to inoculate seedlings without further preparation. Bars labeled 'ns' are not different at $P \leq 0.05$.

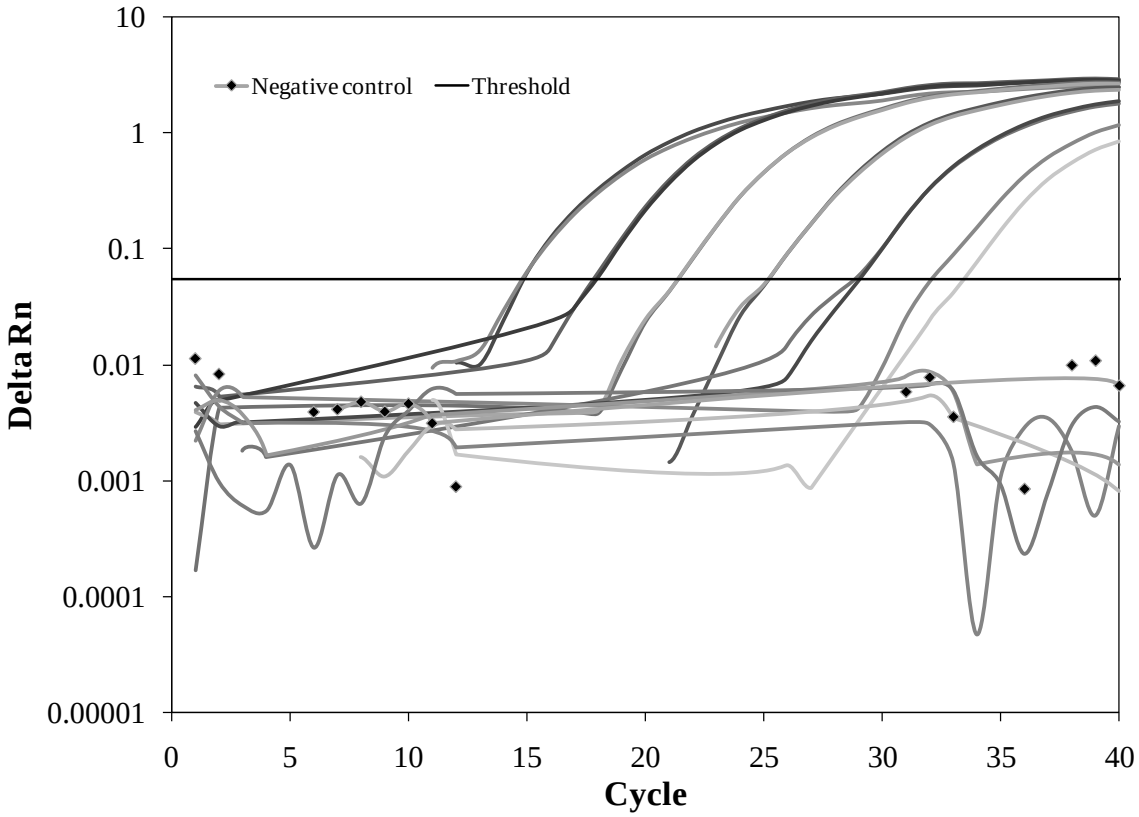

Figure A.2. Amplification plot for real-time quantitative polymerase chain reaction (qPCR) of a 10-fold dilution series of *Heterodera glycines* genomic DNA (starting at 89.6 ng/µl) with SYBR Green and a primer pair designed to amplify the *HgSNO* gene. Distilled water was the negative control.