Detection and Quantification of *Fusarium solani* f. sp. *glycines* in Soybean Roots with Real-Time Quantitative Polymerase Chain Reaction

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ABSTRACT

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Fusarium solani f. sp. *glycines* is the causal organism of soybean sudden death syndrome (SDS). This organism is difficult to detect and quantify because it is a slow-growing fungus with variable phenotypic characteristics. Reliable and fast procedures are important for detection of this soybean pathogen. Protocols were optimized for extraction of DNA from pure fungal cultures and fresh or dry roots. A new procedure to test polymerase chain reaction (PCR) inhibitors in DNA extracts was developed. Novel real-time quantitative PCR (QPCR) assays were developed for both absolute and relative quantification of *F. solani* f. sp. *glycines*. The fungus was quantified based on detection of the mitochondrial small-subunit rRNA gene, and the host plant based on detection of the *cyclophilin* gene of the host plant. DNA of *F. solani* f. sp. *glycines* was detected in soybean plants both with and without SDS foliar symptoms to contents as low as 9.0×10^{-5} ng in the absolute QPCR assays. This is the first report of relative QPCR using the comparative threshold cycle (Ct) method to quantify the DNA of a plant pathogen relative to its host DNA. The relative QPCR assay is reliable if care is taken to avoid reaction inhibition and it may be used to further elucidate the fungus–host interaction in the development of SDS or screen for resistance to the fungus.

Additional key words: *β-actin*, *Glycine max*, QPCR inhibition, SYBR Green assay

Sudden death syndrome (SDS), caused by *Fusarium solani* (Mart.) Sacc. f. sp. *glycines* (= *Fusarium virguliforme* Akoi, O'Donnell, Homma & Lattanzi) (3) is one of the most economically important diseases (3) of soybean, *Glycine max* (L.) Merr. *F. solani* f. sp. *glycines* is a soilborne pathogen that infects soybean roots, causing root rot, crown necrosis, and vascular discoloration of the roots and stem. The mid- to late-season foliar symptoms, beginning with chlorotic mottling and proceeding to interveinal chlorosis, necrosis, and defoliation, are the most conspicuous (54). However, the foliar symptoms of SDS are unpredictable and are similar to those of other diseases, such as brown stem rot, making the disease difficult to diagnose based on symptoms alone. The fungus has been isolated from roots and lower stems, but not from leaves (53,55).

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Li and Hartman (36) used a polymerase chain reaction (PCR)-based method to detect *F. solani* f. sp. *glycines* because traditional methods to detect or isolate this fungus are limited by their lack of either sensitivity or specificity. Their protocol can be used for qualitative detection of *F. solani* f. sp. *glycines* in host roots and soil; however, it cannot be used easily to quantify *F. solani* f. sp. *glycines*. Semiselective media may be employed to enumerate the CFU of *F. solani* f. sp. *glycines* in roots (38,39), but this method is time consuming and labor intensive. To date, quantitative molecular methods have not been used to assay *F. solani* f. sp. *glycines* in plant roots or rhizosphere soil.

Real-time quantitative PCR (QPCR) is a relatively new molecular technology. The first documentation of QPCR was in 1993 (30), and yet this technology has only recently been used for gene quantification of different organisms (25). On the basis of detection of specific PCR products by utilizing the 5′→3′ exonuclease activity of *Thermus aquaticus* (*Taq*) DNA polymerase (31), Higuchi et al. (30) developed a QPCR assay that uses a video camera to monitor multiple PCRs simultaneously over the course of thermocycling. The video camera detects the accumulation of double-stranded DNA in each PCR using the increase of the fluorescence of ethidium bromide that results from its binding duplex DNA. They found that the kinetics of fluorescence accumulation during thermocycling is directly related to the starting number of DNA copies. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of DNA copies present. Though its basic principle remains the same, the QPCR assay has been modified and optimized for more than a decade (11,12,25,40,43,57). Currently, two common methods of analyzing data from QPCR experiments are employed: absolute quantification and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group, such as an untreated control or a sample treated at time zero in a time-course study (37). Absolute QPCR has been used to detect and quantify plant-pathogenic fungi (8–10,17–22,26,29,51), bacteria (7,42,47,48,56,58,61), and viruses (14,15,41,44,46), as well as biocontrol agents of plant pathogens (5,13,59). Avrova et al. (6) reported the application of real-time PCR to the relative quantification of gene expression of *Phytophthora infestans* during the early stages of infection. However, there are no reports on the use of relative QPCR to quantify plant pathogens relative to their host. The objectives of this study were to (i) test PCR inhibitors in sample DNA extracts with real-time PCR assays, (ii) develop real-time PCR protocols to quantify *F. solani* f. sp. *glycines* DNA in soybean roots and laboratory cultures, (iii) determine the absolute quantities of *F. solani* f. sp. *glycines* in soybean roots, and (iv) determine the relative quantities of *F. solani* f. sp. *glycines* in soybean roots using *F. solani* f. sp. *glycines* DNA as the target and host plant DNA as an endogenous control. Preliminary results of this project have been published (23).

MATERIALS AND METHODS

DNA extraction. The fungal species and isolates used in this study (Table 1) were maintained on 2% water agar (wt/vol) at 4°C. The method of Achenbach et al. (1) was adapted to obtain mycelium for DNA extraction. Fungal isolates were

grown on potato dextrose agar (PDA), and one plug (8 mm in diameter) of fungal mycelium was aseptically transferred to 50-ml aliquots of liquid potato dextrose broth (Difco Laboratories, Detroit) in 250-ml Erlenmeyer flasks. Cultures were grown at 24°C with shaking for 1 week. Mycelium was collected by vacuum filtration with a sterile Buchner funnel lined with sterile cheesecloth.

To produce *F. solani* f. sp. *glycines* inoculum, the fungus was grown on 250 cm^3 of sorghum (*Sorghum bicolor* (L.) Moench) seed that had been soaked overnight in distilled water and autoclaved twice in a 1-liter Erlenmeyer flask. Each flask was inoculated with 10 plugs (8 mm in diameter) of a 14-day-old fungal culture on PDA and incubated for 2 weeks. The field plots were infested with *F. solani* f. sp. *glycines* inoculum in a layer at the rate of 15 cm^3 of inoculum/plant before seeding.

To produce root tissue, seeds of the soybean cv. Williams 82 were disinfected by rinsing in 70% ethanol for 10 s and then in a 1% solution of sodium hypochlorite for 3 min. After three rinses in sterile water, the seeds were placed aseptically in petri dishes containing 2% water agar, sealed with paraffin film, and incubated at 24°C in the dark until the radicle was 2 to 3 cm long. The radicles were used for DNA extraction.

Two protocols were used for fungal and plant DNA extraction. DNA was extracted from pure fungal cultures (100 mg) and fresh soybean root tissues (200 mg) with the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) as described by the manufacturer (protocol 1). We used a second protocol (protocol 2) for dry root tissues (50, 100, or 200 mg) that was modified from the same manufacturer's recommendations by repetition of the DNA binding steps once to remove PCR inhibitors detected in early optimization experiments (D. Malvick, University of Illinois, *unpublished*). In both protocols, in order to enhance removal of PCR inhibitors, DNA extracts were centrifuged at 11,500 rpm for 5 min before the liquid was transferred to a new tube. DNA concentrations were determined with a Genesys 10 spectrometer (Thermo Spectronic, Rochester, NY).

Sequence data analysis and primer and probe designs. All the primers and probes were commercially synthesized. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and probes were synthesized by Applied Biosystems (Foster City, CA). One probe was dual labeled with the 6-carboxyfluorescein (6-FAM) fluorescent reporter dye and the minor groove binding nonfluorescent quencher (MGBNFQ), and the other probe used the VIC dye as fluorescence reporter and the 6-carboxyl-tetramethylrhodamine (TAMRA) dye as a fluorescence quencher.

Primer and probe sequences were designed with Primer Express software (Applied Biosystems). The GenBank database accession numbers used for the analysis of small-subunit mitochondrial rRNA genes of *F. solani* f. sp. *glycines* isolates and *F. solani* non-SDS-causing isolates were AF124995 and from AF125008 to AF125032 (35,36). Fsg-q-1 primers were: forward, 5′-GAT ACC CAA GTA GTC TTT GCA GTA AAT G-3′; reverse, 5′-TTA ATG CCT AGT CCC CTA TCA ACA T-3′; probe, 5′-6FAM-TGA ATG CCA TAG GTC AGA T-MGBNFQ-3′.

Primer sequences were designed for *Glycine max cyclophilin* (Cyp) (GenBank accession no. AF456323) and used as the endogenous control in the relative QPCR assay. *Cyclophilin* primers were: forward, 5′-CGC GTG ATC CCG AGT TTC-3′; reverse, 5′-CGT TTC CGG CGG TGA AG-3′.

For assays to determine whether the DNA extracts from fungal culture from fresh and dried soybean samples were PCR-inhibitor-free, the *β-actin* gene *(Mj-ba)* from *Meloidogyne javanica* (Treub) Chitwood was used in inhibition tests of fungal DNA extracts because the production and QPCR assay of *β-actin* already have been established successfully (49). *β-actin* primers were: forward, 5′-GCG ACA TTG ACA TCC GTA AAG AC-3′; reverse, 5′-CAA TGC CTG GAT ACA TGG TTG T-3′; probe, 5′-VIC-TTT ACG CCA ACA CTG TCC TTT CTG GAG G-TAMRA-3′ (49).

Real-time QPCR amplification. The QPCR assays were conducted in a 96-well plate format with the ABI PRISM 7900HT Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). The manufacturer's instructions were followed, except 25-µl reaction mixtures were used (24,28). Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, in addition to a 2-min pre-incubation at 50°C.

Absolute QPCR was employed to determine the quantities of *F. solani* f. sp. *glycines* DNA in fresh and dried soybean samples, and 10-fold dilutions were made for the DNA extracts from infected root samples. The samples and the negative control sample (molecular pure water) were run in triplicate. A subset of a 10-fold dilution series of the total DNA from the pure culture of *F. solani* f. sp. *glycines*, spanning from 1.8×10^{0} to 1.8×10^{-5} ng/µl, was incorporated in each absolute QPCR assay in triplicate to generate a standard curve.

Absolute QPCR assays were conducted to determine whether PCR inhibitors were present in the DNA extracts from fungal culture from fresh and dried soybean samples. Primers and probe for *Mj-ba* were used instead of primers and probe for *F. solani* f. sp. *glycines* in the procedure described in the previous paragraph. The purpose was to measure the amplification of *Mj-ba* when exposed to possible inhibitory factors in the sample DNA extracts. There were 7.0×10^{-3}, 7.0×10^{-4}, or 7.0×10^{-5} ng of *β-actin* in each reaction well (25-µl reaction mixture). The samples, the non-*F. solani* f. sp. *glycines* control (molecular pure water used to replace the same volume of fungal DNA extracts, with no DNA other than *β-actin* present in the reaction well), and the negative control with molecular pure water only were run in triplicate PCR reactions for each run.

Absolute QPCR was performed to test the specificity of the TaqMan probe of *F. solani* f. sp. *glycines* mitochondrial DNA. Eleven fungal isolates (Table 1) were used for the absolute QPCR assays. All the fungal DNA extracts were diluted to 0.1 ng/µl before the absolute QPCR assays were run. There were 0.5 ng of fungal DNA in each reaction well.

For relative QPCR, we used the comparative threshold cycle (Ct) method described in the manufacturer's instruction manual (PE Applied Biosystems). The threshold cycle is the cycle number when fluorescence of the sample exceeds the background fluorescence. In this method, Ct values obtained for the target (*F. solani* f. sp. *glycines* mitochondrial DNA), endogenous control (soybean genomic DNA), test sample, and a calibrator (con-

Table 1. Fungal isolates used for real-time quantitative polymerase chain reaction (QPCR) assays

Isolate	Species	Host	Geographic source
Mont-1[a]	*Fusarium solani* f. sp. *glycines*	*Glycine max*	Illinois
Carlock[a]	*F. solani* f. sp. *glycines*	*G. max*	Illinois
I502[a]	*F. solani* f. sp. *glycines*	*G. max*	Missouri
I504[a]	*F. solani* f. sp. *glycines*	*G. max*	Missouri
11-2-A[b]	*F. solani*	*G. max*	North Dakota
31-2-F[b]	*F. solani*	*G. max*	North Dakota
115-3-A[b]	*F. solani*	*G. max*	North Dakota
I1[a]	*F. solani*	*Solanum tuberosum*	Idaho
I16[a]	*F. solani*	*Medicago sativum*	New York
…[c]	*F. oxysporum*	*G. max*	Illinois
…[c]	*F. equiseti*	*G. max*	Illinois

[a] Isolates provided by the National Soybean Pathogen Collection Center.
[b] Isolates provided by Berlin Nelson, North Dakota State University.
[c] Isolates of *F. oxysporum* (Schlechtend.) emend. W. C. Snyder & H. N. Hans. and *F. equiseti* (Corda) Sacc. provided by Dean Malvick, University of Illinois.

trol sample, as defined in the next section) are used to calculate two parameters, ΔCt and ΔΔCt, as follows: $\Delta Ct = Ct_{target} - Ct_{endogenous\ control}$ and $\Delta\Delta Ct = \Delta Ct_{\ test\ sample} - \Delta Ct_{\ calibrator}$. The quantity of the target, normalized to the endogenous control and relative to the calibrator, is given by amount of target = $2^{-\Delta\Delta Ct}$. The derivation of the formula and more details are described in the manufacturer's instruction manual (PE Applied Biosystems). Note that, in relative QPCR, the sample quantity is divided by a calibrator quantity and, because both quantities have the same units, the relative quantity has none.

Table 2. Absolute real-time quantitative polymerase chain reaction (QPCR) assays to test for PCR inhibitors in DNA extracts from pure cultures of *Fusarium solani* f. sp. *glycines* or from fresh soybean root samples collected from field plots in Urbana, IL, in 2003[a]

Aliquot, sample code	Infestation[b]	Fungal DNA concentration (ng/µl)[c]	*β-actin* (Ct)[d]	ΔCt[e]
1	…	1.8×10^{0}	29.3	0.3
2	…	1.8×10^{-1}	29.5	0.5
3	…	1.8×10^{-2}	29.1	0.1
4	…	1.8×10^{-3}	29.6	0.6
5	…	1.8×10^{-4}	29.3	0.3
6	…	1.8×10^{-5}	29.1	0.1
7	…	1.8×10^{-6}	29.6	0.6
FR1	Artificial	…	29.4	0.4
FR2	Artificial	…	29.7	0.7
FR3	Natural	…	29.5	0.5
FR4	Natural	…	29.7	0.7
FR5	Natural	…	29.3	0.3
FR6	Natural	…	29.2	0.2
Control[f]	…	…	29.0	…

[a] Only *β-actin* primers were used to detect *β-actin* added to the master mix containing *F. solani* f. sp. *glycines* DNA extracts. DNA was extracted from 100 mg of fresh fungal culture or 200 mg of fresh roots with protocol 1 according to the manufacturer's instructions for the FastDNA kit (Qbiogene, Inc., Carlsbad, CA), with one additional centrifugation.

[b] Roots were obtained from plants grown in soil either naturally or artificially infested with *F. solani* f. sp. *glycines.*

[c] Concentration of *F. solani* f. sp. *glycines* total DNA. No further dilutions were made before adding to the reaction wells.

[d] Threshold cycle (Ct) number when fluorescence of the sample exceeded background fluorescence. The absolute QPCR assay was run with 7.0×10^{-4} ng of *β-actin* in each reaction well.

[e] ΔCt = (Ct of *β-actin* in sample tested) – (Ct of *β-actin* in non-*F. solani* f. sp. *glycines* control). Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.

[f] Non-*F. solani* f. sp. *glycines* control. Molecular pure water was used to replace the same volume of fungal DNA extracts, with no DNA other than *β-actin* present in the reaction wells.

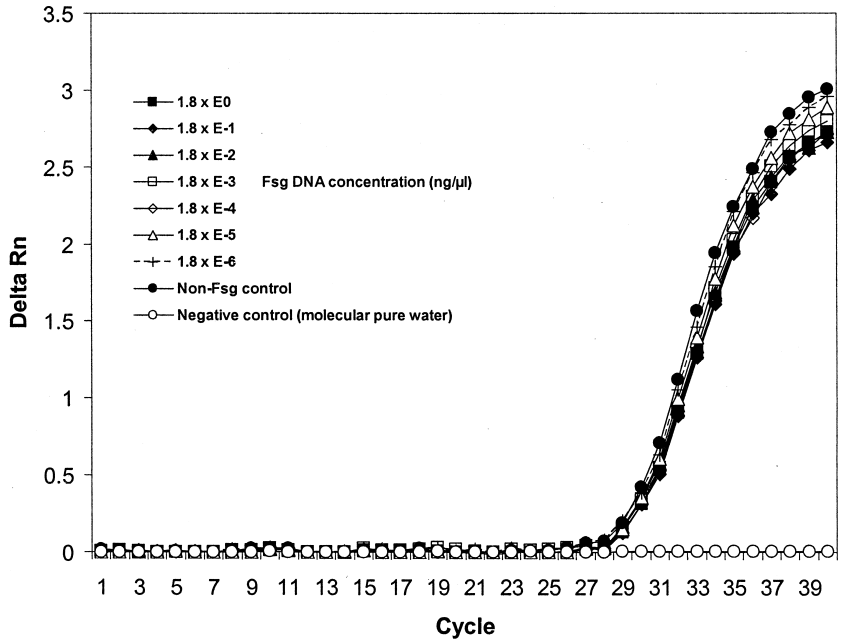

Fig. 1. Inhibition assays of DNA of *Fusarium solani* f. sp. *glycines* (Fsg) from the fungal mycelium on *β-actin* amplification using absolute quantitative polymerase chain reaction. DNA was extracted from 100 mg of fresh fungal culture with protocol 1 according to the manufacturer's instructions for the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) with one additional centrifugation. There were 7.0×10^{-4} ng of *β-actin* in each reaction well. Amplification plot shows cycle number versus normalized fluorescence (delta Rn). The fluorescent signal threshold was 0.2.

To use the ΔΔCt method, the first step is to validate the ΔΔCt calculation by showing that the efficiency of the target amplification and the efficiency of the endogenous control amplification are approximately equal. In a positive validation experiment, the absolute value of the slope of ΔCt versus log input should be <0.1 (37). To accomplish this, we used a subset of 10-fold dilution series of the total DNA from pure culture of *F. solani* f. sp. *glycines*, spanning from 1.0×10^{-1} to 1.0×10^{-4} ng/µl, assayed in triplicate. Similarly, a subset of 10-fold dilution series of the total DNA from the sterile soybean roots, spanning from 10^{-1} to 10^{-4} ng/µl, was assayed in triplicate. ΔCt ([Ct of fungal DNA] – [Ct of soybean DNA]) was calculated automatically for each dilution. The slope of the regression line of log total DNA on ΔCt was 0.0929. Therefore, the amplification efficiencies of the target and reference genes were similar, and relative QPCR may be applied.

Relative QPCR was used to determine the relative quantities (RQ) of *F. solani* f. sp. *glycines* mitochondrial DNA and soybean DNA relative to a control sample known as a calibrator, following the manufacturer's recommendation (PE Applied Biosystems). In order to get positive values for RQ of test samples, the calibrator was designed to use the lowest detectable dilution that contained *F. solani* f. sp. *glycines* total DNA solution at a concentration of 1.8×10^{-5} ng/µl, relative to the soybean total DNA at a concentration of 2.3 ng/µl that was diluted 100-fold from DNA extracted from sterile soybean roots. TaqMan universal PCR master mix (PE Applied Biosystems) was used for the target DNA assays and SYBR Green PCR Master mix (PE Applied Biosystems) for the endogenous control *cyclophilin* amplification. Both 10- and 100-fold dilutions were made from each sample DNA extract, the former for target amplification and the latter for endogenous control. Both target and endogenous control were run in triplicate. To make sure no formation of primer-dimer or other unspecific PCR products in *cyclophilin* amplification of DNA extracts from fresh or dried root tissues occurred, additional SYBR Green assays were run as absolute QPCR, including the dissociation curve analysis step recommended by the manufacturer (PE Applied Biosystems). The dissociation curve analysis of *cyclophilin* amplification products only generated one peak that had a mean temperature of 82.5°C, ranging from 79 to 84.5°C (*data not shown*), indicating that only the specific PCR product was generated in detectable amounts.

Assays of *F. solani* f. sp. *glycines* in root samples. Fresh soybean root samples from plants at growth stages V1 and R5

(16) were collected from field plots either naturally or artificially infested with *F. solani* f. sp. *glycines* in Urbana, IL in 2003. Roots were surface-disinfested with a 0.6% solution of sodium hypochlorite for 1 min and stored immediately in a sterile tube in a freezer at –20°C. Percent moisture of fresh root samples was averaged over three plants. Both absolute and relative QPCR were used to detect and quantify *F. solani* f. sp. *glycines* in fresh root samples.

In order to compare the efficiency of QPCR with a traditional method used to quantify fungal propagules in plant roots, we selected 14 dry root samples from previously conducted field studies (32–34) that represented a range of *F. solani* f. sp. *glycines* CFU/g. Soybean lines resistant and susceptible to SDS were planted in two Missouri and two Illinois fields infested with *F. solani* f. sp. *glycines* in 2000–02. Plants were evaluated for SDS foliar symptoms and taproots were collected at harvest maturity. Roots were surface disinfested with a 0.6% solution of sodium hypochlorite for 1 min and air dried at room temperature before being ground in a cyclone mill (Model 3010; UDY Corp., Fort Collins, CO). Roots were stored at 5°C following grinding. Serial dilutions of ground roots were made in sterile distilled water and plated on a modified Nash and Snyder semiselective medium (27,45). The plates were incubated at room temperature ranging from 24 to 26°C under constant fluorescent lighting and the *F. solani* f. sp. *glycines* colonies were counted after 10 days. Calculations for CFU/g for each sample were based on the mean number of colonies at a minimum of two dilution rates with five plates of each dilution.

Calculation. To generate the standard curve for absolute assays, the Ct data were graphed versus the total DNA quantities for the standard dilution sets. Based on the standard curve, the absolute quantity of fungal total DNA in each reaction well was calculated as ([fungal total DNA quantity] × [total dilution])/sample weight (mg). The fungal absolute total DNA was expressed as femtogram per milligram of fresh root sample, or nanogram per microgram of dry root samples. For the relative QPCR assays, the software SDS 2.1 (PE Applied Biosystems) was used to determine the RQ of the fungal mitochondrial DNA to the plant DNA in the calibrator sample.

RESULTS

Specificity and sensitivity. With the QPCR protocol using Fsg-q-1 primers, we detected all four isolates of *F. solani* f. sp. *glycines*. However, there was no amplification of DNA from sterile water, uninfected soybean, two isolates of *F. solani, F. oxysporum,* or *F. equiseti*. The standard curves (*data not shown*) indicated that we reproducibly detected and quantified total DNA of *F. solani* f. sp. *glycines* as low as 9.0×10^{-5} ng in an individual reaction well.

Inhibition effects of DNA extracts on *β-actin* amplification. Extracts of DNA from fungal cultures of *F. solani* f. sp. *glycines* and fresh and dried roots were tested to determine, using a *β-actin* assay, whether they contained PCR inhibitors. DNA extracts from both fungal cultures and fresh roots using protocol 1 did not show marked inhibition to *β-actin* amplification (Table 2; Fig. 1). The *β-actin* Ct of the non-*F. solani* f. sp. *glycines* control was 29.0, and *β-actin* Ct values of the seven dilution standard aliquots of *F. solani* f. sp. *glycines* were 29.1 to 29.6, with the *β-actin* ΔCt ranging from 0.1 to 0.6 (Table 2), indicating that *β-actin* was amplified at a similar speed in different dilution aliquots, and the fungal DNA extract carried no inhibitors to PCR. Similarly, the *β-actin* ΔCt of the DNA extracts from fresh roots ranged from 0.2 to 0.7 (Table 2), indicating that the DNA extracts from fresh root samples showed no PCR inhibition. However, most DNA extracts of *F. solani* f. sp. *glycines* from dry soybean roots with protocol 1 without the last centrifugation inhibited *β-actin* amplification to different degrees (Table 3; Fig. 2). The *β-actin* Ct value of the non-*F. solani* f. sp. *glycines* control was 24.9. The *β-actin* ΔCt of DNA extracts from dry roots ranged from 0.3 to 15.1. Amplification of *β-actin* was completely suppressed by DNA extracts of two samples, DR1 and DR4 (Table 3; Fig. 2). DNA extracts of *F. solani* f. sp. *glycines* from dry soybean roots with protocol 2 without the last centrifugation inhibited *β-actin* amplification to a lower extent (Table 3; Fig. 3). The *β-actin* Ct value of the non-*F. solani* f. sp. *glycines* control was 24.9. The *β-actin* ΔCt of the DNA extracts was 0.1 to 3.0. All the *F. solani* f. sp. *glycines* DNA extracts from dry soybean roots with protocol 2 showed

Table 3. Absolute real-time quantitative polymerase chain reaction (QPCR) assay to test for PCR inhibitors in DNA extracted from dry soybean root samples collected from field plots infested with *Fusarium solani* f. sp. *glycines* in Missouri and Illinois[a]

				Protocol 1[b]		Protocol 2[c]	
Sample code	Year	Source	Infestation[d]	*β-actin* Ct[e]	ΔCt[f]	*β-actin* (Ct)	ΔCt
DR1	2000	Ashland, MO	Natural	40.0	15.1	25.1	0.2
DR2	2000	Ashland, MO	Natural	32.4	7.5	25.0	0.1
DR3	2000	Ashland, MO	Natural	24.9	0.0	27.9	3.0
DR4	2000	Ashland, MO	Natural	40.0	15.1	25.3	0.4
DR5	2000	Ashland, MO	Natural	30.6	5.7	25.1	0.2
DR6	2001	Ashland, MO	Natural	32.1	7.2	25.7	0.8
DR7	2001	Ashland, MO	Natural	39.2	14.3	25.7	0.8
DR8	2001	Ashland, MO	Natural	38.2	13.3	26.2	1.3
DR9	2001	Ashland, MO	Natural	25.2	0.3	26.2	1.3
DR10	2002	Carmi, IL	Natural	25.4	0.5	26.2	1.3
DR11	2002	Carmi, IL	Natural	30.2	5.3	25.2	0.3
DR12	2002	Urbana, IL	Artificial	32.5	7.6	27.9	3.0
DR13	2002	Urbana, IL	Artificial	26.4	1.5	25.1	0.2
DR14	2002	Urbana, IL	Artificial	…	…	…	…
Control[g]	…	…	…	24.9	…	24.9	…

[a] Only *β-actin* primers were used to detect *β-actin* added to the master mix containing *F. solani* f. sp. *glycines* DNA extracts.
[b] Protocol 1 was according to the manufacturer's instructions for the FastDNA kit (Qbiogene, Inc., Carlsbad, CA), without one additional centrifugation; 200 mg of dry roots was used.
[c] Protocol 2 was modified from the same manufacturer's recommendations by repetition of the DNA binding steps once and without one additional centrifugation; 100 mg of dry roots was used.
[d] Roots were obtained from plants grown in soil either naturally or artificially infested with *F. solani* f. sp. *glycines.*
[e] Threshold cycle (Ct) number when fluorescence of the sample exceeded background fluorescence. The absolute QPCR assay was run with 7.0×10^{-3} ng of *β-actin* in each reaction well.
[f] ΔCt = (Ct of *β-actin* in sample tested) – (Ct of *β-actin* in non-*F. solani* f. sp. *glycines* control), and expressed as absolute value. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.
[g] Non-*F. solani* f. sp. *glycines* control. Molecular pure water was used to replace the same volume of fungal DNA extracts, with no DNA other than *β-actin* present in the reaction wells.

no PCR inhibition (Table 4). The *β-actin* ΔCt of the DNA extracts from 100-mg samples ranged from 0.1 to 0.8, similar to those from 50-mg samples.

Quantification of *F. solani* f. sp. *glycines* from roots. Absolute QPCR assays were run to determine the quantities of *F. solani* f. sp. *glycines* DNA in soybean roots. The standard curves (there was one in each run; *data not shown*) indicated that the correlations between Ct and DNA quantities were high. The average of R^2 of three parallel runs was 0.9963. Based on the results of assays of *F. solani* f. sp. *glycines* in fresh root samples from fields, both seedlings (FR1 to FR4) and adult plant (FR5 and FR6) roots were infected (Fig. 4). The absolute total DNA quantities in fresh root samples ranged from 4.4 to 4.4×10^4 fg/mg. The total DNA quantities of *F. solani* f. sp. *glycines* in the two root samples from infested plots, FR1 and FR2, were 8.2×10^3 and 4.4×10^4 fg/mg, respectively, more than 50-fold higher than those in the roots from noninfested plots, which ranged from 4.4 to 1.6×10^2 fg/mg. The seedlings in infested plots developed typical SDS foliar symptoms at growth stage V1 in the field, but seedlings in noninfested plots did not. Fourteen dry field samples from Missouri and Illinois were assayed and the absolute quantities of total DNA in the field samples ranged from 1.1 to 2.2×10^2 (ng/μg) (Table 5). *F. solani* f. sp. *glycines* DNA was detected with absolute QPCR in all of the six dry root samples from plants with typical foliar symptoms (Table 5), whereas CFU was determined only in one of them by the semiselective medium. Moreover, DNA was detected with absolute QPCR in nine samples in which *F. solani* f. sp. *glycines* was not detected with the semiselective medium (Table 5).

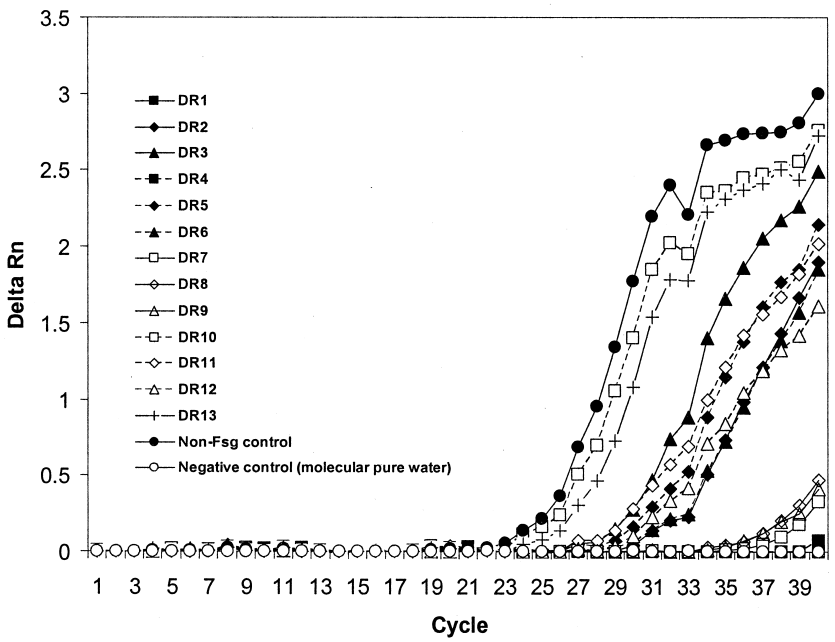

Fig. 2. Inhibition assays of DNA extracts of *Fusarium solani* f. sp. *glycines* (Fsg) from dry soybean roots on *β-actin* amplification using absolute quantitative polymerase chain reaction. DNA were extracted with protocol 1 according to the manufacturer's instructions for the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) without one additional centrifugation; 200 mg of dry roots was used. Root samples DR1–13 were obtained from plants grown in soil infested with *F. solani* f. sp. *glycines.* There were 7.0×10^{-3} ng of *β-actin* in each reaction well. Amplification plot shows cycle number versus normalized fluorescence (delta Rn). The fluorescent signal threshold was 0.2.

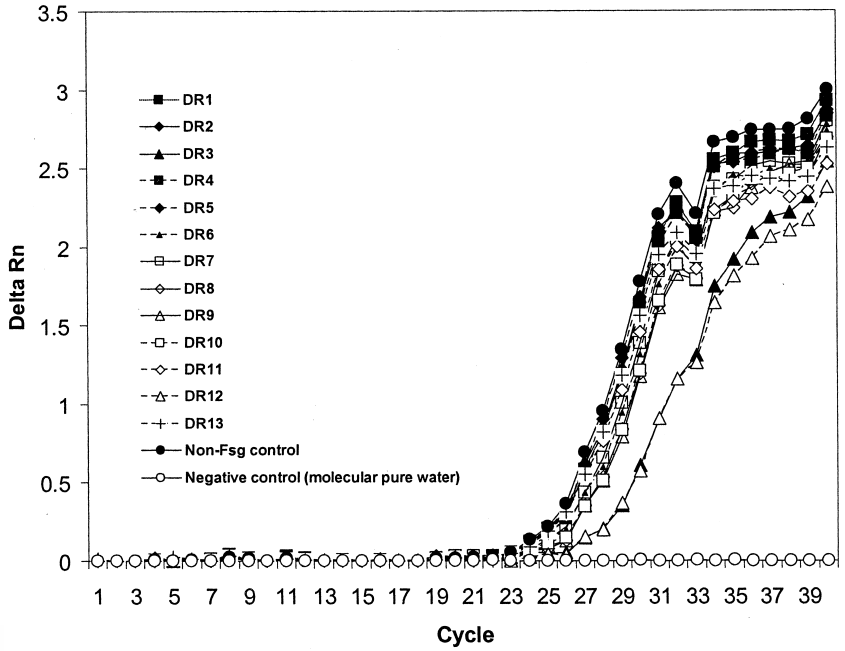

Fig. 3. Inhibition assays of DNA extracts of *Fusarium solani* f. sp. *glycines* (Fsg) from dry soybean roots on *β-actin* amplification using absolute quantitative polymerase chain reaction. DNA was extracted with protocol 2 that was modified from the recommendations of the manufacturer of the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) by repetition of the DNA binding steps once and without one additional centrifugation. Root samples DR1–13 were obtained from plants grown in soil infested with *F. solani* f. sp. *glycines.* There were 7.0×10^{-3} ng of *β-actin* in each reaction well. Amplification plot shows cycle number versus normalized fluorescence (delta Rn). The fluorescent signal threshold was 0.2.

The RQ of DNA in fresh root samples ranged from 0.2 to 3.7×10^3 (Fig. 4). However, the RQ of *F. solani* f. sp. *glycines* DNA in the two root samples in infested plots were 1.5×10^3 and 3.7×10^3, respectively, more than 1,000-fold higher than in the noninfested roots (Fig. 4). Field samples from Missouri and Illinois were assayed and the RQ of *F. solani* f. sp. *glycines* DNA in the noninfested field samples ranged from 4.8×10^2 to 4.1×10^6. All dried root samples were infested with *F. solani* f. sp. *glycines* (Table 5).

Impact of sample processing and storage conditions on soybean DNA degradation. The Ct and ΔCt values of relative QPCR assays of *cyclophilin*, the endogenous control, in 14 dry and 6 fresh soybean root samples are shown in Table 6. DNA was extracted from 50 and 200 mg of dry and fresh root samples, respectively. Moisture contents of samples FR1 to FR4 and FR5 to FR6 averaged 85.5 and 72.5%, respectively. Therefore, 200 mg of fresh root tissue was equivalent to 29.0 and 55.5 mg of dry root material, respectively. The Ct values ranged from 26.7 to 28.8 in fresh root samples and 29.8 to 36.1 in dry root samples. The ΔCt values were 0.2 to 1.2 in fresh root samples (FR1 to FR4), much smaller than 3.0 to 9.3 in dry root samples, although the dry root weight of the former was less than that of the latter (29 versus 50 mg), indicating that more total soybean

DNA was found in fresh soybean root samples than in the dry root samples. The ΔCt values of the dry root samples from Ashland, MO in 2000 were 6.9 to 9.3, much higher than those from the same locality in 2001, which were 3.0 to 4.4, indicating that less soybean DNA was detected in the dry root samples stored at 5°C for a longer period of time.

DISCUSSION

Achenbach et al. (2) reported that *F. solani* f. sp. *glycines* isolates from throughout the United States compose an almost clonal population with an extremely low level of genetic variation among individuals. Therefore, there is a solid basis for developing molecular protocols to detect and quantify *F. solani* f. sp. *glycines* from soybean roots. Our protocol only amplified mitochondrial DNA of *F. solani* f. sp. *glycines* and did not detect *F. solani*, *F. oxysporum*, or *F. equiseti* from soybean and other plants when all the fungal DNA extracts were diluted to the same concentration. This protocol is sensitive, having detected *F. solani* f. sp. *glycines* total DNA as low as 9.0×10^{-5} ng. Our QPCR protocol to detect *Fusarium solani* f. sp. *glycines* is among the most sensitive ones to date for detection of plant pathogens. This level of detection has been reported for *Phytophthora* spp. (10) and *Xylella fastidiosa* (47,58).

QPCR is especially useful to detect and quantify nonculturable or slow-growing organisms. For *F. solani* f. sp. *glycines*, there have been no quantitative protocols available other than the use of semiselective media. We developed a QPCR protocol to quantify *F. solani* f. sp. *glycines* in various samples, including pure cultures and fresh and dry roots from fields. Our method allowed detection of *F. solani* f. sp. *glycines* in all of the field samples, although CFU was detected in only 5 of the 14 samples using the semiselective medium. Our QPCR protocol clearly is more sensitive than the semiselective medium. Another advantage of QPCR is its speed, because it requires only approximately 2 h for the machine to accomplish a run in which at least 96 reactions can be accommodated in regular plates.

Our protocol is currently being used in the investigation of interactions between *F. solani* f. sp. *glycines* and *Heterodera glycines* to quantify the fungus in plant tissues infested with different nematode inoculum levels. In addition, it is difficult to evaluate soybean cultivars for resistance to *F. solani* f. sp. *glycines* because of the unpredictable and inconsistent nature of foliar symptoms in the field. Our QPCR protocol may be useful for the evaluation of SDS resistance by quantifying *F. solani* f. sp. *glycines* in roots of soybean varieties with different levels of resistance to the pathogen. Moreover, this protocol will be useful for quantifying *F. solani* f. sp. *glycines* in asymptomatic plants for the early diagnosis of SDS and determining the distribution of the pathogen. *F. solani* f. sp. *glycines* has been reported in major soybean production areas in the United States, Argentina, and Canada (4,50,54). Awareness of phytosanitation and biosecurity recently has been heightened (57). This QPCR protocol can be employed as a biosecurity tool to detect and guard against the threat posed by this pathogen in soybean-growing regions where *F. solani* f. sp. *glycines* is not known to occur.

It should be emphasized that pure DNA extract containing no inhibitors to PCR is critical for precise comparison of fungal DNA quantities in root samples. However, there is little published information available on this aspect in real-time PCR assays in clinical, food, and environmental samples. Rossen et al. (52) identified inhibitory factors of regular PCR in foods, bacterial culture media, and various chemical compounds. Wilson (62) reviewed the inhibition and facilitation of nucleic acid amplification in regular PCR reactions and stated that reaction inhibition may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity. This phenomenon also occurred in our real-time PCR assays of fungal mitochondrial DNA from roots, especially in the insufficiently purified DNA extracts from dry roots when an inappropriate DNA extraction protocol was used. At the beginning of the development of this protocol, we encountered inhibition of PCR amplification of DNA extracts from dried root samples with protocol 1. However, the inhibition to PCR from DNA extracts from dried roots was eliminated with protocol 2. To ensure the best performance in DNA extraction with protocol 2, we recommend that 50- or 100-mg dry root samples be used. Otherwise, too much moisture will be removed from the extraction buffer by the dry root tissues.

Too often, the sensitivity and other advantages of QPCR have been emphasized. However, the purity of DNA extracts for QPCR assays have been overlooked. Most authors have paid great attention to the specificity, sensitivity, precision, and accuracy of the QPCR protocols themselves,

Table 4. Absolute real-time quantitative polymerase chain reaction (QPCR) assay to test for PCR inhibitors in DNA extracted from dry soybean root samples collected from field plots infested with *F. solani* f. sp. *glycines* in Missouri and Illinois[a]

				50-mg sample		100-mg sample	
Sample code	**Year**	**Source**	**Infestation[b]**	***β-actin* Ct[c]**	**ΔCt[d]**	***β-actin* Ct**	**ΔCt**
DR1	2000	Ashland, MO	Natural	33.3	0.2	33.3	0.2
DR2	2000	Ashland, MO	Natural	33.3	0.2	33.8	0.7
DR3	2000	Ashland, MO	Natural	33.6	0.5	33.4	0.3
DR4	2000	Ashland, MO	Natural	33.8	0.7	33.8	0.7
DR5	2000	Ashland, MO	Natural	33.5	0.4	33.3	0.2
DR6	2001	Ashland, MO	Natural	32.0	1.1	33.2	0.1
DR7	2001	Ashland, MO	Natural	33.9	0.8	32.8	0.3
DR8	2001	Ashland, MO	Natural	33.7	0.6	33.7	0.6
DR9	2001	Ashland, MO	Natural	32.6	0.5	33.1	0.0
DR10	2002	Carmi, IL	Natural	33.9	0.8	33.5	0.4
DR11	2002	Carmi, IL	Natural	33.1	0.0	33.9	0.8
DR12	2002	Urbana, IL	Artificial	33.3	0.2	32.9	0.2
DR13	2002	Urbana, IL	Artificial	32.8	0.3	33.5	0.4
DR14	2002	Urbana, IL	Artificial	33.7	0.6	33.2	0.1
Control[e]	…	…	…	33.1	…	33.1	…

[a] Only *β-actin* primers were used to detect *β-actin* added to the master mix containing *F. solani* f. sp. *glycines* DNA extracts. DNA was extracted with protocol 2 that was modified from the recommendations of the manufacturer of the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) by repetition of the DNA binding steps once, and with one additional centrifugation.

[b] Roots were obtained from plants grown in soil either naturally or artificially infested with *F. solani* f. sp. *glycines.*

[c] Threshold cycle (Ct) when fluorescence of the sample exceeded background fluorescence. The absolute QPCR assay was run with 7.0×10^{-5} ng of *β-actin* in each reaction well.

[d] ΔCt = (Ct of *β-actin* in sample tested) – (Ct of *β-actin* in non-*F. solani* f. sp. *glycines* control), and expressed as absolute value. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence.

[e] Non-*Fusarium solani* f. sp. *glycines* control. Molecular pure water was used to replace the same volume of fungal DNA extracts, with no DNA other than *β-actin* present in the reaction wells.

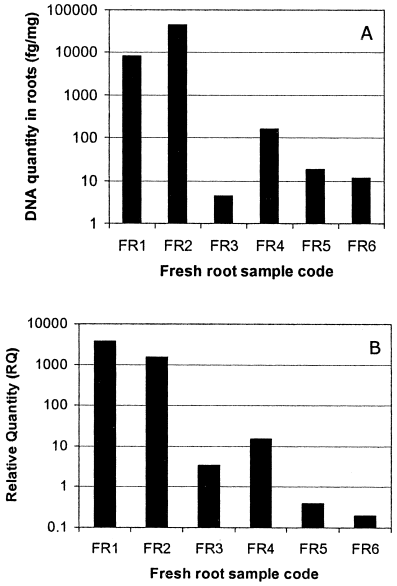

Fig. 4. Quantitative polymerase chain reaction (QPCR) assays of total DNA of *Fusarium solani* f. sp. *glycines* in fresh soybean roots sampled in fields at Urbana, IL. Y-axis is on the logarithmic scale. **A,** Absolute QPCR assay. Samples FR1–4 were 16-day-old seedlings, and FR5–6 were from adult plants at growth stage R5. Sample FR1 and 2 were from plots infested with *F. solani* f. sp. *glycines* at 15 cm^3 of infested sorghum seed per plant. All other samples were from noninfested field sites. The equation of the standard curve: $Y = -3.6323X + 45.9526$. R^2 = 0.9969. The standard deviations of absolute quantities of *F. solani* f. sp. *glycines* DNA in sample FR1 and FR2 were 0.4 and 3.1, respectively. The standard deviations of absolute quantities of *F. solani* f. sp. *glycines* DNA in all the other samples were less than 0.1. **B,** Relative QPCR assay of *F. solani* f. sp. *glycines* DNA in fresh soybean roots sampled in fields at Urbana, IL. Samples were the same as those in **A.** The standard deviations of relative quantities of *F. solani* f. sp. *glycines* DNA in all the samples were less than 0.1.

but little information is available on how to assay QPCR inhibitors in DNA extracts and how to overcome QPCR inhibition. More than 20 QPCR protocols have been developed to detect and quantify plant pathogens and their biocontrol agents since the use of fluorescence technology in plant pathology (10), but only a few researchers noticed there were inhibitors in their DNA extracts for QPCR assays (14,60). Van de Graaf et al. (60) reported that there was significant PCR inhibition in some of their DNA extracts of *Spongospora subterranea* from soil. In addition, usually there is no PCR inhibition information in the manufacturer's manuals of DNA-extracting kits. Therefore, we propose that DNA purity should be examined when a QPCR protocol is developed or employed to detect and quantify plant pathogens.

The sensitivity of most QPCR protocols has been determined with DNA extracts from fungal or bacterial cultures that probably contained no or fewer inhibitors than DNA extracts from plant tissues or soil. It should be noted that high sensitivity of a QPCR protocol does not necessarily mean it will generate reliable data with DNA extracts from plant roots or rhizosphere soil. If considerable PCR inhibitors exist in the DNA extract, DNA Products amplified with QPCR protocols could be undetectable, resulting in a false negative reaction. The QPCR protocol to quantify *β-actin* originally was employed to study the transcript expression profile of the gene *chorismate mutase* in the *Meloidogyne javanica* esophageal gland (49). It is sensitive and can reliably detect *β-actin* as low as 7.0×10^{-5} ng in each reaction well (25-µl reaction mixture). Based on this QPCR protocol, we developed a new procedure, called the ΔCt PCR inhibition test, to quantitatively and precisely determine the PCR inhibition level in individual DNA extracts. DNA extracts were considered to be PCR inhibitor-free if the *β-actin* ΔCt ([Ct of *β-actin* in sample tested] – [Ct of *β-actin* in non-*F. solani* f. sp. *glycines* control]) in an absolute QPCR assay with 7.0×10^{-5} ng of *β-actin* in each reaction well is less than 1.0. The less *β-actin* in a reaction mixture, the more sensitive to PCR inhibition it is. However, when the *β-actin* amount is too low in a reaction mixture, the amplification of *β-actin* is not stable (*data not shown*). We found that a *β-actin* content of 7.0×10^{-5} ng per reaction well is the most appropriate for the PCR inhibition test in our assays. Our PCR inhibition procedure will be helpful for the evaluation of the efficiency of different DNA extraction methods.

We developed an absolute QPCR protocol and a relative QPCR protocol. The absolute QPCR can quantify the absolute quantity of the target DNA and requires only one probe and two primers. However, because it is sensitive to PCR inhibition, pure DNA extracts must be used. The relative QPCR allows the quantification of the RQ of the target DNA compared with a calibrator. An endogenous control gene is used to correct for the total amount of the sample. Therefore, for relative QPCR, one assay is used to amplify the target *F. solani* f. sp. *glycines* mitochondrial DNA and another assay is used to measure the endogenous plant DNA. In our experiments, we used SYBR Green to assay the endogenous control, but a sequence TaqMan probe also was used. Because two probes are used in the relative QPCR assays, it is not as sensitive to inhibition as the absolute QPCR method. Both assays should be of similar efficiency (37). In our study, the

Table 5. Quantities of *Fusarium solani* f. sp. *glycines* DNA determined by absolute real-time quantitative polymerase chain reaction (QPCR) and relative QPCR in dried soybean roots from field-grown plants

Sample code	Year	Source	Infestation[a]	SDS[b]	CFU/g[c]	Quantity	
						Absolute DNA (ng/µg of root)[d]	Relative DNA[e]
DR1	2000	Ashland, MO	Natural	+	1.4×10^3	122.6	4.1×10^6
DR2	2000	Ashland, MO	Natural	+	ND	18.3	9.1×10^5
DR3	2000	Ashland, MO	Natural	+	ND	1.1	7.5×10^4
DR4	2000	Ashland, MO	Natural	–	ND	4.5	7.7×10^5
DR5	2000	Ashland, MO	Natural	–	ND	10.1	1.2×10^6
DR6	2001	Ashland, MO	Natural	–	ND	68.7	3.4×10^5
DR7	2001	Ashland, MO	Natural	–	8.3×10^3	221.7	2.1×10^5
DR8	2001	Ashland, MO	Natural	–	1.4×10^2	78.7	3.3×10^4
DR9	2001	Ashland, MO	Natural	–	ND	2.3	4.8×10^2
DR10	2002	Carmi, IL	Natural	+	ND	26.1	1.9×10^5
DR11	2002	Carmi, IL	Natural	–	2.1×10^3	23.9	1.1×10^5
DR12	2002	Urbana, IL	Artificial	+	ND	10.4	9.9×10^3
DR13	2002	Urbana, IL	Artificial	+	ND	6.5	1.5×10^4
DR14	2002	Urbana, IL	Artificial	–	8.4×10^2	3.5	1.6×10^4

[a] Roots were obtained from plant samples that were grown in soil either naturally or artificially infested with *F. solani* f. sp. *glycines*.
[b] SDS = soybean sudden death syndrome. Plants were evaluated based on the presence (+) or absence (–) of SDS foliar symptoms.
[c] CFU of *F. solani* f. sp. *glycines* per gram of root tissue were determined by serial dilution plating on a modified Nash and Snyder semiselective medium (27,45). ND = not detectable.
[d] Quantities of DNA were determined with assays for absolute QPCR. The equation of the standard curve: $Y = -3.7276X + 46.6732$. $R^2 = 0.9981$. DNA was extracted from 50 mg of root samples with protocol 2 that was modified from the recommendations of the manufacturer of the FastDNA kit (Qbiogene, Inc., Carlsbad, CA) by repetition of the DNA binding steps once, and with one additional centrifugation.
[e] Quantities of DNA were determined with assays for relative QPCR. DNA was extracted from 50 mg of root samples with protocol 2.

relative quantification of *F. solani* f. sp. *glycines* in soybean roots was determined in both fresh and dry root samples. The relative QPCR better reflects the interactions between the pathogen and its host plant because the data related the fungal DNA quantities with host plant DNA quantities. In our assays of *F. solani* f. sp. *glycines* DNA in fresh root samples, the RQ of the fungal DNA in roots from the infested plots were more than 1,000-fold higher than in the noninfested plots, but the absolute quantities of the total DNA of *F. solani* f. sp. *glycines* in the same samples from infested plots were only about 50-fold higher than those from noninfested plots. In addition, the relative QPCR data were ready for statistical analysis because they were normalized when relative quantification was performed. Relative QPCR is more expensive than absolute QPCR, but more suitable for high-throughput screening. To our knowledge, this is the first report of relative QPCR using the Ct method to quantify the DNA of a plant pathogen relative to its host DNA.

Our study showed that the methods used to process or store samples have a marked impact on plant DNA. Less soybean DNA was detected in dry root samples stored at 5°C for longer periods. In addition, less soybean DNA was found in dry root samples than in fresh root samples with similar or less dry root tissues. It is suggested that samples be stored at –20 or –80°C immediately after processing. In addition, different sample types may require different protocols for target DNA extraction and real-time PCR assays. It is suggested that each laboratory make sure the target DNA extracts from their own samples are inhibitor-free for absolute QPCR.

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Table 6. Relative real-time quantitative polymerase chain reaction (QPCR) assay to test the effects of sample processing and storage conditions on soybean DNA degradation in root samples collected from field plots infested with *Fusarium solani* f. sp. *glycines* in Missouri and Illinois[a]

Sample code	Year	Source	Infestation[b]	Root sample type	Ct[c]	ΔCt[d]
DR1	2000	Ashland, MO	Natural	Dry	33.7	6.9
DR2	2000	Ashland, MO	Natural	Dry	34.2	7.4
DR3	2000	Ashland, MO	Natural	Dry	35.5	8.7
DR4	2000	Ashland, MO	Natural	Dry	36.1	9.3
DR5	2000	Ashland, MO	Natural	Dry	35.6	8.8
DR6	2001	Ashland, MO	Natural	Dry	30.4	3.6
DR7	2001	Ashland, MO	Natural	Dry	31.2	4.4
DR8	2001	Ashland, MO	Natural	Dry	30.7	3.9
DR9	2001	Ashland, MO	Natural	Dry	29.8	3.0
DR10	2002	Carmi, IL	Natural	Dry	34.6	7.8
DR11	2002	Carmi, IL	Natural	Dry	34.0	7.2
DR12	2002	Urbana, IL	Artificial	Dry	31.9	5.1
DR13	2002	Urbana, IL	Artificial	Dry	33.5	6.7
DR14	2002	Urbana, IL	Artificial	Dry	34.7	7.9
FR1	2003	Urbana, IL	Artificial	Fresh	28.1	0.5
FR2	2003	Urbana, IL	Artificial	Fresh	27.8	0.2
FR3	2003	Urbana, IL	Natural	Fresh	28.8	1.2
FR4	2003	Urbana, IL	Natural	Fresh	26.7	0.9
FR5	2003	Urbana, IL	Natural	Fresh	28.3	0.5
FR6	2003	Urbana, IL	Natural	Fresh	27.9	0.9

[a] Assays were run as a relative QPCR. DNA extracts were from 50- and 200-mg dry and fresh root samples, respectively. Fresh root weight was equivalent to 29.0- and 55.5-mg dry root weights in samples FR1 to FR4 and FR5 to FR6 at 85.5 and 72.5% moisture, respectively.

[b] Roots were obtained from plant samples that were grown in soil either naturally or artificially infested with *F. solani* f. sp. *glycines*.

[c] Threshold cycle (Ct) when fluorescence of the sample exceeded background fluorescence.

[d] ΔCt = Ct of sample DNA – Ct of the endogenous control of the calibrator, and expressed as absolute value. Ct = threshold cycle number when fluorescence of the sample exceeded background fluorescence. The Ct value of the endogenous control of the calibrator for the dry root samples (DR1 to DR14) was 26.8. The Ct value of the endogenous control of the calibrator for the fresh samples of seedling plants (FR1 to FR4) was 27.6. The Ct value of the endogenous control of the calibrator for the fresh samples of adult plants (FR5 to FR6) was 28.8.

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