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ORIGINAL PAPER

Quantification of *Fusarium solani* f. sp. *glycines* isolates in soybean roots by colony-forming unit assays and real-time quantitative PCR

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Abstract *Fusarium solani* f. sp. *glycines* (FSG; syn. *F. virguliforme* Akoi, O'Donnell, Homma & Lattanzi) is a soil-borne fungus that infects soybean roots and causes sudden death syndrome (SDS), a widespread and destructive soybean disease. The goal of this study was to develop and use a real-time quantitative polymerase chain reaction (QPCR) assay to compare the accumulation of genomic DNA among 30 FSG isolates in inoculated soybean roots. Isolates differed significantly ($P \leq 0.05$) in their DNA accumulation on a susceptible soybean cultivar when detected and quantified using a FSG-specific probe/primers set derived from the sequences of the nuclear-encoded, mitochondrial small subunit ribosomal RNA gene. QPCR results that were normalized as the fold change over the sample collection times after inoculation were significantly ($P \leq 0.001$) correlated with the $\log_{10}$ transformed colony-forming unit (CFU) values of FSG obtained from plating of inoculated ground roots on FSG semi-selective agar medium. Several isolates were identified that accumulated more FSG DNA and had higher CFU values than the reference isolate FSG1 (Mont-1). Compared to other isolates, FSG5 was the most aggressive root colonizer based on DNA accumulation and CFU values in infested roots. The described QPCR assay should provide more specificity, greater sensitivity, and less variability than alternatives to the culturing-dependent and time-consuming plating assays. Evaluation of isolate relative DNA differences on host plants using the QPCR approach provides useful information for evaluating isolates based on the extent and/or degree of colonization on soybean roots and for selecting isolates for breeding SDS-resistant soybean lines.

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Introduction

Fusarium solani (Mart.) Sacc. f. sp. *glycines* (syn. *F. virguliforme* Akoi, O'Donnell, Homma & Lattanzi) is the causal agent of sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Merr.; Roy 1997; Roy et al. 1989; Rupe 1989). SDS is a mid- to late-season disease and has become one of the most important diseases of soybean in the United States (Wrather et al. 2001). Yield losses from SDS range from slight to nearly 100% (Rupe and Hartman 1999). Although caused by a soil-borne fungal pathogen that infects only roots and lower stems, but not leaves (Roy 1997; Roy et al. 1989; 1997; Rupe 1989), foliar symptoms include chlorotic mottling, proceeding to interveinal chlorosis, necrosis and eventually defoliation with the petioles remaining attached to stems (Hartman et al. 1995; Rupe and Hartman 1999). Population densities of the fungus in soil were reported to be positively correlated with SDS foliar severity (Scherm et al. 1996).

Real-time quantitative polymerase chain reaction (QPCR) combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing

real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets (Higuchi et al. 1993, Schena et al. 2004; Valssek and Repa 2005). Since the early 1990s, numerous QPCR methods have been developed and used for detection and quantification of plant pathogens and for disease diagnostics (see reviews Schaad and Frederick 2002, 2003; Schena et al. 2004). For example, QPCR was used to develop a rapid and sensitive seed-health test for *Pyrenophora* species in barley seeds (Bates et al. 2001), and to quantify genomic DNA of two ecologically different fungi, the plant pathogen *F. solani* f. sp. *phaseoli* and the arbuscular mycorrhizal fungus *Glomus intraradices,* directly from soil (Filion et al. 2003a). It has also been used to quantify *Magnaporthe grisea* during infection of rice (Qi and Yang 2002); assay *Puccinia coronata* f. sp. *avenae* DNA in *Avena sativa* (Jackson et al. 2006); and detect and quantify *F. solani* f. sp. *glycines* in soybean roots (Gao et al. 2004; Li et al. 2004). However, QPCR methods have not been widely used to characterize fungal isolates, especially soil-borne pathogens that colonize and infect plant roots.

The objectives of this study were to develop a real-time QPCR assay to specifically and rapidly quantify the amount of *F. solani* f. sp. *glycines* genomic DNA in infected soybean roots and use this molecular approach to determine if isolates of *F. solani* f. sp. *glycines* differ in degree of root colonization in greenhouse grown soybean plants. Correlation between the culturing-independent QPCR assay and the culturing-dependent method as determined by the number of colony-forming units (CFU) was analyzed and compared. Preliminary experiments on the development of our QPCR assay for detection and quantification of *F. solani* f. sp. *glycines* in soybean roots and soil were reported (Li et al. 2004).

Materials and methods

Fungal isolates

Thirty *Fusarium solani* f. sp. *glycines* isolates (Table 1) with different degrees of aggressiveness were selected for use in this study based on the results of previous pathogenicity tests on over 123 isolates (Li et al. 2002) from the National Soybean Pathogen Collection Center (University of Illinois, Urbana, IL, USA). Fungal cultures were maintained on 2% water agar (w/v) at 4°C or stored in 15% (v/v) glycerol at −80°C. In our study, isolate FSG 1 (Mont-1) was used as a reference isolate because it has been widely used by the soybean community for basic and applied research (Hartman et al. 1997, 2004; Achenbach et al. 1996; Li and Hartman 2003; Iqbal et al. 2005; Lozovaya et al. 2006; Farias et al. 2006). This isolate was originally isolated from Monticello, Illinois in 1991 and has been re-isolated from infected soybean and maintained in the National Soybean Pathogen Collection Center at the University of Illinois and the USDA-ARS Crop Genetics and Production Research Unit in Mississippi.

Root sample collection and preparation

To produce *F. solani* f. sp. *glycines* inoculum, sorghum grain (80 cm^3) was soaked in tap water in 250-ml Erlenmeyer flasks overnight. Floating sorghum seed and debris were removed. After soaking, the grain was washed with tap water three to five times. Excess water was drained, and the grain was autoclaved on two consecutive days for 40 min at 121°C. Each flask was infested with an individual isolate by transferring five 4-mm-diameter plugs from the edge of a 2-week-old *F. solani* f. sp. *glycines* culture on water agar. Cultures in flasks were incubated at 23°C in dark and shaken by hand every other day to promote uniform fungal growth. After 14 days, infested sorghum seeds were used to inoculate soybean.

Soybean seeds of a susceptible cultivar, Great Lakes 3202, were sown in Ray Leach Cone-Tainers (Stuewe & Sons, Inc., Corvallis, OR) and inoculated with 3 cm^3 of infested sorghum grains placed 2–3 cm below a soybean seed in each Cone-Tainer. Non-infested sorghum grain was used for control treatments. Cone-Tainers were placed in racks on a greenhouse bench under a 14-h photoperiod with a light intensity of 434 $\mu Em^{-2}\ s^{-1}$ at 25 ± 2°C and watered daily. Soil:sand mix (1:1 v/v) at pH 7.0 was pasteurized and autoclaved before filling the Cone-Tainer.

The experiment consisted of two greenhouse trials, which started in June 2004 and ended in December 2004. Within each trial, there were two randomized complete blocks based on the sample collection time. Roots in the first block were harvested 14 days after inoculation (DAI). Roots in the second block were harvested 21 DAI. Five replications of the 30 isolates and a non-inoculated control were completely randomized in both blocks. Two random fresh root samples for each isolate in each trial were used for the root colony forming unit (CFU) assay and two random root samples were washed with tap water and lyophilized immediately after removal from soil for DNA extraction. Each block contained 155 plants. A total of 310 root samples were collected for each trial.

DNA extraction, primer and probe designs

DNA was extracted from 20 mg of lyophilized roots of each sample (from two pooled plants) with DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA) as described by the manufacturer. Primer and probe sequences were

Table 1 Isolates used for specificity test of the real-time quantitative PCR assays using a minor groove-binding *Fusarium solani* f. sp. *glycines* probe and flanking primers

Fungus/oomycete[a]	Host/substrate	Number of isolates
Fusarium solani f. sp. *glycines*[b]	*Glycine max*	30
F. solani	*G. max*	10
	Phaseolus vulgaris	2
	Pisum sativum	2
	Medicago sativum	2
	Lycopersicum esculentum	2
	Cucurbita pepo	1
	Cucurbita sp.	2
	Solanum tuberosum	2
	Lupinus sp.	1
	Field soil	1
Alternaria sp.	*G. max*	1
Cercospora kikuchii	*G. max*	1
Colletotrichum truncatum	*G. max*	1
Diaporthe phaseolorum var. *caulivora*	*G. max*	2
D. phaseolorum var. *meridionalis*	*G. max*	3
D. phaseolorum var. *sojae*	*G. max*	2
F. graminearum	*G. max*	2
F. oxysporum f. sp. *glycines*[c]	*G. max*	1
Fusarium spp.	*G. max*	2
Macrophomina phaseolina	*G. max*	2
Neocosmospora vasinfecta[c]	*G. max*	1
Phomopsis longicolla	*G. max*	2
Phomopsis sp.	*G. max*	1
Phytophthora sojae	*G. max*	2
Phialophora gregata	*G. max*	2
Rhizoctonia solani	*G. max*	2
Septoria glycines	*G. max*	1
Sclerotinia sclerotiorum	*G. max*	2
Stachybotrys chartarum	*G. max*	1
Total isolates		86

[a] All cultures are maintained in the National Soybean Pathogen Collection Center in University of Illinois

[b] Cultures were obtained or isolated from nine States (Arkansas, Illinois, Indiana, Iowa, Kansas, Missouri, Mississippi, Ohio, and Wisconsin) and Argentina, Brazil, and Canada (Li et al. 2000)

[c] All fungi were from Illinois except *F. oxysporum* f. sp. *glycines* (NRRL 22598) and *N. vasinfecta* (NRRL 22166) that were obtained from the Northern Regional Research Laboratory (NRRL), Peoria, IL, USA

designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). 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 (Li et al. 2000; Li and Hartman 2003). A minor groove-binding (MGB) *F. solani* f. sp. *glycines*-probe (FSG-MGB probe) was designed based on the sequence of the Fsg1 primer (Li and Hartman 2003) and contained 6-carboxyfluorescein (6-FAM) fluorescent reporter dye at its 5′ terminus and the minor groove binding non-fluorescent quencher (MGB-NFQ) at its 3′ terminus (5′-6FAM-TCTTCTAGGATGGG CTGGT-MGBNFQ-3′). The specificity of the probe and flanking primers FSGq1 (5′-GGCTGAACTGGCAACTT GGA-3′) and FSGq2 (5′-CAAAGCTTCATTCAATCCT AATACAATC-3′) in QPCR was tested with DNA from pure cultures of 86 isolates that included 30 *F. solani* f. sp. *glycines*, 25 *F. solani* isolates that do not cause SDS, 31 isolates of 19 other soybean fungal pathogens, the Oomycete *Phytophthora sojae* (Table 1), and soybean DNA.

Primers for the soybean actin 1 gene (*SAc1*) were designed from the *SAc1* genomic sequence (Shah et al. 1983) and used as an endogenous control in the QPCR assay. The forward *SAc1* primer sequence was 5′-CCTG ATGGGCAGGTTATCACTAT-3′; the reverse primer sequence was 5′-GGTACAAGACCCCCGGACAT-3′ (Lim et al. 2005). Primers and the MGB probe were synthesized by Applied Biosystems.

Real-time QPCR amplification

The QPCR assays were conducted using a SmartCycler (Cepheid, Sunnyvale, CA, USA). All DNA samples from

two replicated greenhouse trials were analyzed by QPCR assays using the specific FSG-MGB probe and primers, and the soybean *SAc1* primers using SYBR Green I (Molecular Probes Co. Carlsbad, CA) as an endogenous control for data normalization. One microliter of DNA from each DNA sample was used in a 25-µl QPCR reaction using the OmniMix HS reagent with a lyophilized universal PCR reagent bead (Cepheid). Each DNA sample was tested three times by QPCR. Thermal cycling conditions were as follows: 95°C for 120 s, then 45 cycles of 95°C for 120 s and 60°C for 30 s. The specificity of the QPCR assay was tested with DNA from pure cultures of 30 *F. solani* f. sp. *glycines*, 25 *F. solani* non-SDS isolates, 31 isolates of 19 other soybean pathogens, and soybean (Table 1).

To compare *F. solani* f. sp. *glycines* DNA quantities of the different isolates inoculated on soybean roots, a relative QPCR method (Orlando et al. 1998) was used with threshold cycle (C_t) values representing relative target DNA among isolates. C_t is the PCR cycle number at which a statistically significant increase in the fluorescence above background was detected. Lower C_t values indicate fewer PCR cycles necessary for detection and greater quantities of DNA in the sample. In this study, default threshold setting by manufacturer (Cepheid, Sunnyvale, CA, USA) was 30. Any fluorescence signal below 30 fluorescence unit was considered background.

To estimate the absolute DNA quantity that corresponded to given C_t value, a standard curve of *F. solani* f. sp. *glycines* DNA was constructed using the FSG-MGB probe in QPCR. A 10 ng/µl solution of purified DNA from a pure culture of the reference isolate FSG1 was diluted tenfold serially to 1.0×10^{-6} ng/µl with 100 pg/µl of soybean root DNA. One microliter DNA from each dilution was added to the Smart-Cycler-PCR master mix and each dilution was tested three times. The DNA concentrations of *F. solani* f. sp. *glycines* and resulting C_t values were used to construct a standard curve.

To correct for differences in the amount of soybean root DNA in each sample, the C_t values from the *SAc1* reactions were subtracted from the corresponding FSG–MGB reactions as follows: $\Delta C_t = C_{t\ \mathrm{FSG\ DNA}} - C_{t\ \mathrm{soybean}\ SAc1}$. The fold change in the amount of fungal DNA from time 1 (14 DAI) and time 2 (21 DAI) were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) using the formula $2^{-\Delta\Delta C_t(T_1-T_2)}$, in which $T_1 = \Delta C_t$ (14 DAI) and $T_2 = \Delta C_t$ (21 DAI). To estimate the fold change in colonization normalized to an endogenous reference gene and relative to the non-inoculated control and to the reference isolate FSG1, $\Delta\Delta C_t C_K$ and $\Delta\Delta C_t$FSG1 were calculated as ΔC_t (each sample) $-\Delta C_t$ (non-inoculated control or the reference isolate FSG1).

Root colony forming unit assays

The CFU of infested roots for 30 selected isolates on 14 and 21 DAI and the non-inoculated roots from two replicated greenhouse trials were determined as previously described (Li et al. 2008). Briefly, roots were removed from soil, washed thoroughly with tap water, surface disinfected for 3 min in 0.5% (v/v) NaOCl solution, and rinsed twice with sterile deionized water. Roots were blotted dry with sterile filter paper and weighed. Roots from two plants (total eight roots of each isolate from two collection times in two trials) were randomly grouped and ground in 10 ml of sterile deionized water using a blender (Waring Inc., Santa Monica, CA, USA) at high speed for 1 minute. The homogenized root suspension was then diluted 10 and 100 fold with sterile deionized water and made up to 100 and 1,000-fold final dilutions. For each dilution, 100 µl samples were placed and evenly spread with a sterile glass rod on *F. solani* f. sp. *glycines* semi-selective medium (Huang and Hartman 1996) and three plates for each dilution. All plates were incubated at 25°C in the dark. Colonies of *F. solani* f. sp. *glycines* were identified and counted on each plate to determine the CFUs per gram of root for each isolate.

Data analysis

Analysis of variance (ANOVA) using the mixed procedure (PROC MIXED) of SAS (version 9.1, SAS Institute, Cary, NC, USA) was performed on data combined from both trials as treatment by trial interaction was not significant and the error variances were homogeneous. The sampling time "DAI" was the main unit with "trial" being a source of replication for DAI. "Isolates" was a subunit with trials and "replications" within trial, both a source of replication. Fixed effects for isolates, DAI, and isolates X DAI were tested. The random effects included trial, trial X DAI, replications within trial X DAI, trial X isolates within DAI, and the residual error which was the replication by isolates within trial and DAI. Means were compared by least significant difference (LSD) at $P \leq 0.05$. The PROC CORR procedure of SAS was used to compute Pearson's correlation coefficients between the mean of variables obtained each DAI from QPCR assays and root CFU values (used both original and transformed to $\log_{10}$ values).

Results

Quantification of *F. solani* f. sp. *glycines* DNA in soybean roots

Specificity of the QPCR assay with the FSG-MGB probe and flanking primers was evaluated. All assays were highly

Table 2 Relative DNA quantities of *F. solani* f. sp. *glycines* isolates obtained from the real-time quantitative PCR assays on the roots of a soybean cv. Great Lakes 3202 that were sampled 14 and 21 days after inoculation in two replicated trials

Isolate[a]	Geographic origin	Year isolated/acquired	$C_{tFsg\ 14\ DAI}$[b]	$C^{c}_{tSAc1 14\ DAI}$	$\Delta C^{d}_{t14\ DAI}$	$C^{b}_{tFsg\ 21\ DAI}$	$C^{c}_{tSAc1 21\ DAI}$	$\Delta C^{d}_{t21\ DAI}$
FSG1	Illinois	1991	24.0	24.4	−0.4hijk	23.5	22.9	0.6bcdef
FSG2	Canada	2002	24.2	23.2	1.0bcdefghijk	23.7	23.1	0.6bcdef
FSG3	Brazil	2002	22.1	22.9	−0.8jk	21.8	22.9	−1.1f
FSG4	Illinois	2002	25.2	23.6	1.6bcdefg	24.5	22.9	1.6bcd
FSG5	Illinois	2003	24.0	25.0	−1.0k	23.1	22.4	0.7bcdef
FSG6	Argentina	2000	25.9	23.8	2.1bc	24.6	23.1	1.5bcd
FSG7	Missouri	2002	24.9	22.7	2.2bc	25.1	23.4	1.7bcd
FSG8	Missouri	2002	22.2	22.3	−0.1efghijk	21.7	22.4	−0.7ef
FSG9	Missouri	2002	25.3	22.9	2.4b	24.2	22.6	1.6bcd
FSG10	Missouri	2002	24.4	22.2	2.2bc	24.0	22.4	1.6bcd
FSG11	Arkansas	1996	25.0	23.2	1.8bcde	24.4	23.1	1.4bcd
FSG12	Arkansas	1996	24.6	23.5	1.1bcdefghij	24.6	22.6	2.1bc
FSG13	Illinois	1994	24.2	22.9	1.3bcdefghi	23.7	23.1	0.6bcdef
FSG14	Iowa	1996	25.0	23.2	1.8bcdefg	24.5	22.9	1.6bcd
FSG15	Illinois	1996	25.0	23.2	1.8bcde	24.1	23.0	1.1bcde
FSG16	Illinois	1999	23.5	23.8	−0.3ghijk	24.5	23.4	1.1bcde
FSG17	Illinois	1999	22.6	23.3	−0.7ijk	22.9	23.0	−0.1def
FSG18	Illinois	1999	24.3	23.4	0.9bcdefghijk	23.9	23.1	0.8bcdef
FSG19	Illinois	1999	22.0	22.0	0.0defghijk	22.6	23.2	−0.6ef
FSG20	Illinois	1999	22.9	23.3	−0.4hijk	23.1	23.4	−0.3def
FSG21	Illinois	2001	24.8	24.0	0.8bcdefghijk	25.0	24.1	0.9bcde
FSG22	Minnesota	2002	23.7	23.5	0.2cdefghijk	23.8	23.8	0.0def
FSG23	Illinois	2002	23.5	23.2	0.3bcdefghijk	23.6	23.1	0.5bcdef
FSG24	Indiana	2003	24.9	23.8	1.1bcdefghij	24.2	23.1	1.1bcde
FSG25	Arkansas	1996	22.6	23.1	−0.5ijk	23.9	23.6	0.3cdef
FSG26	Illinois	1998	24.3	23.6	0.7bcdefghijk	23.8	23.1	0.7bcdef
FSG27	Illinois	1998	25.7	23.8	1.9bcd	24.9	23.7	1.2bcde
FSG28	Wisconsin	1998	25.6	23.8	1.8bcdef	24.7	23.3	1.4bcd
FSG29	Wisconsin	1998	24.4	24.6	−0.2fghijk	23.8	23.7	0.1def
FSG30	Illinois	1993	24.8	23.2	1.6bcdefgh	25.2	22.9	2.3b
CK[e]			39.2	23.8	15.4a	37.1	22.1	15a
Mean			24.7	23.4	1.3	24.3	23.1	1.3

[a] Cultures located at the National Soybean Pathogen Collection Center, University of Illinois

[b] Threshold cycle (C_{tFsg}) of root sample DNA using FAM-labled *Fusarium salani* f. sp. *glycine* (Fsg)-specific MGBNFQ probe in the real-time quantitative PCR reaction. The C_t value was the mean of three QPCR runs for each DNA sample from two replicated greenhouse trials. The DAI stands for days after inoculation

[c] Threshold cycle (C_{tSAc1}) of root sample DNA using soybean actin (*SAc1*) primers and SYBR Green I in the real-time quantitative PCR reaction. The C_t value was the mean of three QPCR runs for each DNA sample from two replicated greenhouse trials

[d] Delta threshold cycle = $C_{tFsg} - C_{tActin}$. Means followed by the same letter are not significant different by the least significant difference test ($P = 0.05$)

[e] Non-inoculated control

specific for detection of *F. solani* f. sp. *glycines* with C_t around 20; no fluorescent signals ($C_t > 38$) were obtained for DNA of other tested pathogens and soybean. QPCR assay detected *F. solani* f. sp. *glycines* DNA in all inoculated root samples collected but not in non-inoculated controls. Based on an *F* test of fixed effect from analyses of variance of DNA quantity, there were significant differences ($P \leq 0.001$) among isolates. Additionally, the *F* test for isolates within each DAI indicated strong isolate difference occurred in 14 DAI. The C_t values with the FSG-MGB probe and flanking primers ranged from 22.0 to 25.9 with a mean value of 24.7 at 14 DAI and 24.3 at 21 DAI in

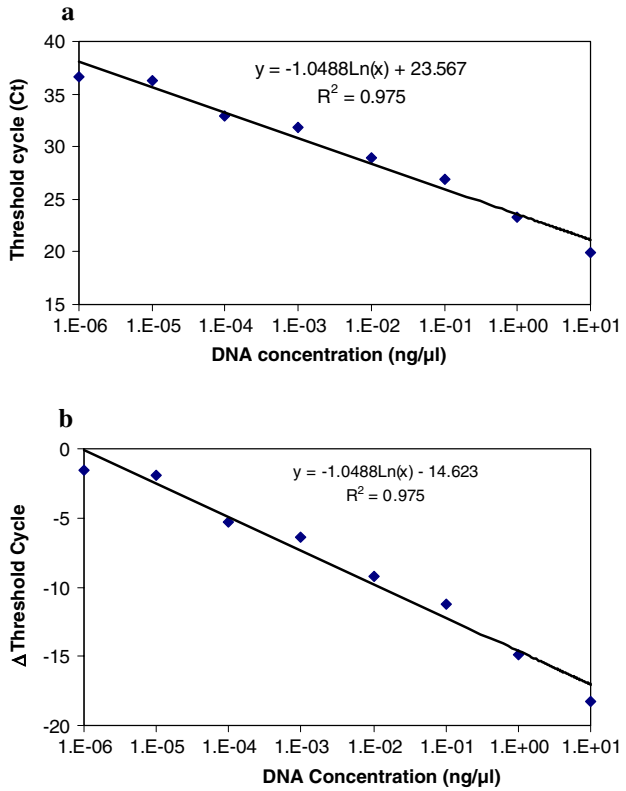

Fig. 1 Standard curve of *Fusarium salani* f. sp. *glycine* (FSG) DNA constructed using FAM-labeled FSG-specific MGBNFQ probe in the real-time quantitative PCR (QPCR) reaction. A 1 ng/µl solution of purified DNA from pure culture of the reference isolate FSG1 was diluted tenfold serially to 1.0 fg/µl with 100 pg/µl of soybean (Great Lakes 3202) root DNA. One microliter from each dilution was added to the Smart-Cycler-polymerase chain reaction master mix. Any signal greater than ten standard deviations of the background primary fluorescence was considered positive, and the cycle at which the signal exceeded the background fluorescence is the cycle threshold reported. **a** Threshold cycle (C_t) values were plotted against DNA concentrations and **b** Delta C_t (C_t of FSG $-C_t$ of non-FSG-control, in which molecular grade water was used to replace the same volume of fungal DNA extract in the QPCR reaction) values were plotted against DNA concentrations

the inoculated root samples, while the C_t values of non-inoculated roots ranged from 36.4 to 41.7 with means of 39.2 and 37.1 for 14 and 21-DAI samples, respectively (Table 2). The C_t values with the soybean *SAc1* primers in either inoculated or non-inoculated roots ranged from 22.0 to 25.2 with a mean value of 23.4 and 23.1 at 14 and 21 DAI samples, respectively (Table 2).

Based on the standard curves constructed with known concentrations of DNA from pure cultures of isolate FSG1 (Fig. 1), *F. solani* f. sp. *glycines* DNA quantities that were normalized by the non-inoculated control ranged from 0.093 to 2.242 pg, and 0.096 to 2.514 pg in 20 mg root samples collected 14 and 21 DAI, respectively (Table 3).

Colonization of soybean roots by *F. solani* f. sp. *glycines*

All isolates colonized soybean roots and caused SDS foliar symptoms (data not shown) in the susceptible cultivar, Great Lakes 3202, as previously tested (Li et al. 2002). No *F. solani* f. sp. *glycines* colonies were recovered from the non-inoculated root samples. Isolate by trial interactions were not significant ($P > 0.05$), therefore data from both trials were pooled and analyzed together. Log CFU values ranged from 2.92 to 3.90 with a mean 3.45 (LSD = 0.21 at $P = 0.05$) per gram of 14-DAI-root samples. In 21-DAI root samples, log CFU values ranged from 1.18 to 3.87 with a mean of 3.21 (LSD = 0.9 at $P = 0.05$; Table 4). Isolate FSG5 had the highest CFU value followed by FSG1 in 14-DAI root samples while isolates FSG16 and FSG5 had the highest CFU values at 21 DAI. Other fungi were also found in the CFU plates of 21-DAI root samples.

Correlation analysis between QPCR and log CFU showed that QPCR results normalized as the fold change ($2^{-\Delta\Delta C_t(T_1-T_2)}$) were significantly ($P \leq 0.001$) correlated with the log CFU values 14 and 21 DAI with correlation coefficients of 0.599 and 0.590, respectively (Table 5). In addition, normalization of the absolute DNA results from samples collected 14 DAI was also significantly ($P \leq 0.05$) correlated with CFU values (Table 6). Overall, QPCR results normalized as the fold change ($2^{-\Delta\Delta C_t(T_1-T_2)}$) gave the highest absolute correlation coefficients with log CFU when compared with other QPCR methods, such as ΔC_t, $2^{-\Delta C_t}$, $\Delta\Delta C_t C_K$, $2^{-\Delta\Delta C_t C_K}$, $\Delta\Delta C_t$FSG1, and $2^{-\Delta\Delta C_t\text{FSG1}}$ in each sample collection time (data not shown).

Discussion

In this study, a real-time QPCR assay was developed and used to study colonization of soybean by *F. solani* f. sp. *glycine* isolates based on the amount of *F. solani* f. sp. *glycine* genomic DNA in infested soybean roots. Even though genomic DNA was extracted from weighed 20 mg of lyophilized root from each sample, the amount of soybean DNA recovered varied as determined using the *SAc*1 gene primers. Since it would not be accurate to quantify *F. solani* f. sp. *glycine* DNA from different amounts of the host tissues, *SAc1* was used in both absolute and relative QPCR quantifications (Lim et al. 2005). Because amounts of the *SAc1* DNA in non-inoculated root samples on 14 and 21 DAI were not significantly different from inoculated roots, *SAc*1 appeared to be a reliable endogenous control for our experiments.

Absolute quantification requires a sample of known quantity of the gene of interest that can be diluted to generate a standard curve (Kuhne and Oschmann 2002).

Table 3 Estimation of absolute DNA quantities of *Fusarium solani* f. sp. *glycines* isolates based on the standard curve of isolate FSG1 from the real-time quantitative PCR assays

Isolate[a]	DNA (14 DAI)[b]	ΔDNA (14 DAI)[c]	DNA (21 DAI)[b]	Δ DNA (21 DAI)[c]
FSG1	0.655	1.302 cd	1.036	0.492defgh
FSG2	0.547	0.343ijkl	0.898	0.483defgh
FSG3	4.253	1.870b	5.296	2.514a
FSG4	0.205	0.179jkl	0.427	0.209ghi
FSG5	0.643	2.242a	1.503	0.423defghi
FSG6	0.106	0.112 l	0.363	0.205ghi
FSG7	0.283	0.114 l	0.243	0.181hi
FSG8	3.721	1.006ef	5.771	1.684b
FSG9	0.191	0.093 l	0.531	0.188hi
FSG10	0.460	0.109jkl	0.637	0.177hi
FSG11	0.248	0.151jkl	0.435	0.236ghi
FSG12	0.373	0.300hij	0.388	0.118hi
FSG13	0.557	0.250ijkl	0.889	0.502efghi
FSG14	0.267	0.169kl	0.427	0.205ghi
FSG15	0.248	0.148kl	0.607	0.321ghi
FSG16	1.087	1.161de	0.427	0.321ghi
FSG17	2.445	1.606b	1.945	1.006de
FSG18	0.492	0.381ijkl	0.756	0.415ghi
FSG19	4.590	0.898de	2.639	1.575bc
FSG20	1.926	1.302de	1.532	1.107 cd
FSG21	0.317	0.419ijk	0.245	0.356ghi
FSG22	0.864	0.687gh	0.840	0.933def
FSG23	1.026	0.607 fg	1.007	0.563defghi
FSG24	0.286	0.309ijkl	0.531	0.289ghi
FSG25	2.469	1.432bc	0.701	0.662defghi
FSG26	0.478	0.435hi	0.771	0.423fghi
FSG27	0.126	0.143jkl	0.280	0.286ghi
FSG28	0.146	0.155jkl	0.333	0.227ghi
FSG29	0.447	1.066e	0.832	0.801defg
FSG30	0.305	0.193jkl	0.203	0.096i
Mean	0.992	0.639	1.083	0.566

[a] Cultures located at the National Soybean Pathogen Collection Center, University of Illinois

[b] DNA concentration (ng) calculated based on the formula "$y = -1.0488\ \mathrm{Ln}(x) + 23.567$" using the threshold cycle (C_t) value of *F. solani* f. sp. *glycines* (See Table 2; Fig. 1a). The DNA value was the mean of three QPCR runs for each DNA sample from two replicated greenhouse trials. The "DAI" stands for days after inoculation

[c] Delta DNA (ΔDNA) concentration (pg) calculated based on the formula "$y = -1.0488\ \mathrm{Ln}(x) - 14.623$" using the delta threshold cycle value (ΔC_t) of *F. solani* f. sp. *glycines* (See Table. 2; Fig. 1b). The Delta DNA value was the mean of three QPCR runs for each DNA sample from two replicated greenhouse trials. Means followed by the same letter are not significant different by the least significant difference test ($P = 0.05$)

However, it was not feasible to construct standard curves for each isolate for comparison. Based on the assumption that all *F. solani* f. sp. *glycine* isolates contain a similar number of copies per genome of the sequences complementary to the probes and primers, absolute DNA of isolates were estimated and compared using the standard curve constructed with known concentrations of FSG1 DNA. The absolute *F. solani* f. sp. *glycine* DNA from root

Table 4 Mean transformed root colony-forming units obtained on semi-selective medium from infected roots of a soybean cv. Great Lakes 3202 that had been inoculated 14 or 21 days individually with 30 *Fusarium solani* f. sp. *glycines* isolates

Isolate[a]	Log CFU/g[b]	
	14 DAI[c]	21 DAI
FSG1[d]	3.88ab	3.46ab
FSG2	3.57efgh	3.11ab
FSG3	3.68bcdefg	3.49ab
FSG4	3.07 ml	3.13ab
FSG5	3.90a	3.62ab
FSG6	3.54gh	3.45ab
FSG7	3.29jk	3.37ab
FSG8	3.80abc	3.50ab
FSG9	3.05mn	2.89abc
FSG10	2.99mn	3.45ab
FSG11	3.49hi	3.07abc
FSG12	3.67cdefg	3.55ab
FSG13	3.24kl	3.42ab
FSG14	3.07 ml	2.86bc
FSG15	3.57efgh	3.09bc
FSG16	3.72bcdef	3.87a
FSG17	3.57efgh	3.28ab
FSG18	3.60defgh	3.05ab
FSG19	3.59defgh	2.80bc
FSG20	3.09klm	3.47ab
FSG21	3.69bcdefg	3.59ab
FSG22	2.92n	3.27ab
FSG23	3.61defgh	3.09ab
FSG24	3.78abcd	3.05ab
FSG25	3.76abcde	3.40ab
FSG26	3.59defgh	3.36ab
FSG27	2.86n	3.27ab
FSG28	3.51gh	1.18d
FSG29	3.03mn	3.05ab
FSG30	3.43hij	3.01ab
Mean	3.45	3.21

[a] Cultures located at the National Soybean Pathogen Collection Center, University of Illinois

[b] Colony forming units (CFU) per gram root sample. Data were the mean of six replications for each isolate from two replicated trials and were transformed to Log_{10}. Means followed by the same letter are not significant different by the least significant difference test ($P = 0.05$)

[c] Days after inoculation

[d] Isolated from Mont-1 isolate-inoculated soybean (Great Lakes 3202) roots in 2003

Table 5 Pearson's correlation coefficients and significance showing relationship between relative real-time quantitative PCR values normalized over the sample collection times and soybean root colony forming units by *Fusarium solani* f. sp. *glycines*

	$\Delta\Delta C_t$ (T1 − T2)[a]	$2^{-\Delta\Delta C_t}(T_1 - T_2)$[b]	CFU (14 DAI)[c]	CFU (21 DAI)[c]
$\Delta\Delta C_t$ (T1 − T2)	1.000	−0.968 (<0.0001)[d]	−0.574 (0.001)	−0.534 (0.002)
$2^{-\Delta\Delta C_t}(T_1 - T_2)$		1.000	0.599 (0.001)	0.590 (0.001)
CFU (14 DAI)			1.000	0.412 (0.024)
CFU (21 DAI)				1.000

[a] Threshold cycle (C_t) is the PCR cycle number at which a statistically significant increase in the fluorescence (greater than background) can be detected. Delta threshold cycle $\Delta C_t = C_{t\ Fsg} - C_{t\ Actin}$ in which threshold cycle of root sample DNA using FAM-labeled *Fusarium solani* f. sp. *glycine* (Fsg)-specific MGBNFQ probe was subtracted by the threshold cycle of root sample DNA with soybean actin (*SAc1*) primers using SYBR Green I in the real-time quantitative PCR reaction. $\Delta\Delta C_t (T_1 - T_2) = \Delta C_t$ (14 DAI) − ΔC_t (21 DAI), in which DAI stands for days after inoculation

[b] A calculated method developed by Livak and Schmittgen (2001)

[c] Colony-forming unit count per gram of infested roots collected 14 and 21 DAI, respectively

[d] Values in parentheses are probabilities

Table 6 Pearson's correlation coefficients and significance showing relationship between absolute real-time quantitative PCR values and soybean root colony forming units of *Fusarium solani* f. sp. *glycines* on semi-selective medium

	DNA (14 DAI)[a]	ΔDNA (14 DAI)[b]	DNA (21 DAI)[a]	ΔDNA (21 DAI)[b]	CFU (14 DAI)[c]	CFU (21 DAI)[c]
DNA (14 DAI)	1.000	0.614 (<0.0001)[d]	0.848 (<0.0001)	0.907 (<0.0001)	0.307 (0.099)	0.127 (0.504)
ΔDNA (14DAI)		1.000	0.556 (0.001)	0.628 (<0.0001)	0.543 (0.002)	0.437 (0.016)
DNA (21 DAI)			1.000	0.905 (<0.0001)	0.341 (0.065)	0.142 (0.455)
ΔDNA (21DAI)				1.000	0.200 (0.288)	0.084 (0.658)
CFU (14 DAI)					1.000	0.412 (0.024)
CFU (21 DAI)						1.000

[a] Absolute *F. solani* f. sp. *glycines* DNA that calculated based on the formula "$y = -1.0488\ \mathrm{Ln}(x) + 23.567$" (see Fig. 1a)

[b] Delta absolute DNA that calculated from delta threshold cycle ($C_{t\ Fsg} - C_{t\ non\text{-}inoculated\ control}$) based on the formula "$y = -1.0488\ \mathrm{Ln}(x) - 14.623$" (see Fig. 1b)

[c] Colony forming unit count per gram of infested roots collected 14 and 21 days after inoculations (DAI), respectively

[d] Values in parentheses are probabilities

samples collected 14 DAI that were normalized with non-inoculated soybean was significantly correlated with the CFU data. The lack of correlation between absolute QPCR data at 21 DAI and log CFU values may have been the result of more fungal DNA degradation at 21 DAI than at 14 DAI.

To study the colonization of isolates, the relative DNA quantification approach provided a convenient way to compare isolates. Using this approach, C_t values were used to compare the difference among isolates. Since the C_t values are proportional to the DNA content, it is not necessary to compare the absolute DNA among isolates. In our study, several data normalization methods were used. These included normalizing the QPCR data with the non-inoculated control, use of a reference/standard isolate to make the comparison, and the fold change over the sample collection times (Livak and Schmittgen 2001). Results showed that normalization of the QPCR data as the fold change over sample collection times ($2^{-\Delta\Delta C_t(T_1-T_2)}$) gave the highest correlation coefficients with CFU when compared with other normalization methods. The fold change in DNA reflected the fungal growth and integrity of fungal DNA in plant tissues during infection period. The derivation, assumptions, and applications of the $2^{-\Delta\Delta C_t}$ method are well-documented, and this method has been shown to be useful in analysis of data from different gene expression experiments (Livak and Schmittgen 2001).

Development of specific probe and/or primers is an important step to quantify pathogen DNA in infected plant tissues using QPCR because different microbes may co-exit in the same host. The specificity of our PCR assay was validated by testing DNA from 86 isolates that included *F. solani* f. sp. *glycines*, *F. solani* non-SDS causing isolates, other soybean pathogens and soybean.

Determination of CFU is commonly used to quantify fungal pathogens in infected plant tissues (Njiti et al. 1997, 1998; Lightfoot et al. 2007) and has been used to analyze

soybean yield loss to SDS in relation to symptom expression and root colonization by *F. solani* f. sp. *glycines* (Luo et al. 2000; Kazi 2005). However, CFU measures viable cells by counting the number of colonies formed on agar plates. The number of CFU is estimated by visual identification, which may be less accurate if different contaminating microbes have similar colony morphology. In addition, at high plating densities, each colony may contain more than one cell. *F. solani* f. sp. *glycine* is a slow growing fungus and other microbes may inhibit its growth in the root, which may interfere with the CFU counts. QPCR, in contrast, is a specific and culturing-independent method. In a study on quantification of *F. solani* f. sp. *phaseoli* in mycorrhizal bean plants and surrounding mycorrhizosphere soil, CFU values were not correlated with real-time PCR data (Filion et al. 2003b). This situation could be attributed to the variation of mechanical strength applied to dislodge and break Fusarium propagules from soils for subsequent CFU enumeration (Filion et al. 2003b). In our study, QPCR results normalized as the fold change over the sample collection times after inoculation were significantly ($P = 0.001$) correlated with log CFU values of *F. solani* f. sp. *glycine* with correlation coefficients of 0.599 and 0.590 on 14 and 21 DAI, respectively.

QPCR has been used routinely to detect and identify pathogens in plant tissues (Bates et al. 2001; Schaad and Frederick 2002; Jackson et al. 2006). Since the DNA can be quantified, it can be used to evaluate relative pathogen growth in host plants and compare the colonization differences of isolates. Several isolates have been identified that produced higher quantities of DNA in soybean roots than other isolates. In view of QPCR and CFU data, isolates FSG1, 5, 12, 16, and 25 have greater *F. solani* f. sp. *glycine* DNA quantities (lower C_t values) and higher CFU counts than other isolates. Those isolates were from different geographic origins. Based on genomic DNA accumulation or CFU counts of the fungus from soybean roots, there did not appear to be any relation to the geographic origin of the isolates. Isolate FSG5 from Illinois was the most aggressive root colonizer based on the QPCR and CFU assays. Illinois isolate FSG 27 had the third highest C_t value of fold change from 14 to 21 DAI, the third lowest absolute *F. solani* f. sp. *glycine* DNA quantities, and the lowest CFU count from 14 DAI. Isolates FSG 2 from Canada and FSG18 from Illinois were moderate root colonizers. QPCR is a culturing-independent method, which is faster, and more specific and sensitive than the traditional plating methods. To our knowledge, this is the first reported study using QPCR to evaluate the relative differences in accumulation of DNA of *F. solani* f. sp. *glycine* isolates in soybean. Evaluation of isolate relative DNA differences on host plants using QPCR provides useful information for evaluating isolates based on their colonization on soybean roots and for selecting isolates for breeding SDS-resistant soybean lines.

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