A New TaqMan Real-Time Polymerase Chain Reaction Assay for Quantification of *Fusarium virguliforme* in Soil

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Abstract

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The quantification of the soilborne pathogen *Fusarium virguliforme* inoculum in soil is important for epidemiological studies of soybean sudden death syndrome (SDS). Classical dilution plating methods to determine inoculum density in soil have yielded inconsistent results due to slow growth, variable colony morphology of the pathogen, and the presence of other fungi with similar phenotype. A TaqMan real-time polymerase chain reaction assay was developed based on sequences of the *FvTox1* gene of *F. virguliforme*. The gene differed by four single-nucleotide proteins from the other SDS-causing species. Assay specificity was tested on 48 fungal isolates that varied in taxonomic relatedness. Assay sensitivity was appraised on 10-fold serial dilutions of genomic DNA, conidia suspensions, and soil spiked with conidia. Applicability of the assay was evaluated on field and greenhouse soil samples, and on roots of symptomatic plants. The assay detected only DNA sequences specific to *F. virguliforme*. The detection limit of the assay was 5 pg/µl, 1,000 conidia/ml, and 1,000 conidia/g soil for genomic DNA, conidial suspensions, and soil with conidia, respectively. The assay was specific to *F. virguliforme* and was used successfully to quantify inoculum density in soil and soybean roots. The assay can be used as a diagnostic tool for rapid screens of field and greenhouse soil, and for symptomatic and asymptomatic plants.

Fusarium virguliforme O'Donnell & T. Aoki is a soilborne fungus that infects soybean roots and causes root rot, crown rot, and vascular discoloration (20). When the fungus colonizes xylem tissues in roots, the toxins it produces are translocated to leaves, where they cause interveinal chlorosis, necrosis (or leaf scorch), premature defoliation, and flower and pod abortion (21,26). Collectively, these symptoms are referred to as sudden death syndrome (SDS) of soybean. This economically important disease is widely distributed throughout the soybean-producing regions of the United States (26), with estimated yield losses ranging from 11 to 75 million bushels per year between 1997 and 2007 (37). In recent years, SDS has caused particular concern in the Midwest, where outbreaks have been more severe (21) and several states have reported the disease for the first time (5,8,17,41). SDS also occurs in South America, where it is caused by *F. tucumaniae* T. Aoki, O'Donnell, Yosh. Homma & Lattanzi; *F. brasiliense* T. Aoki & O'Donnell; and an undescribed *Fusarium* sp. in addition to *F. virguliforme*, which is the only species known to cause SDS in the United States (1,25).

The mechanisms of long-distance spread of *F. virguliforme* are unclear but dispersal likely involves movement of soil, where the pathogen overwinters in the form of chlamydospores on plant debris and associated with the soybean cyst nematode (*Heterodera glycines* Ichinohe) (20,26). Chlamydospores, together with conidia and mycelia, may serve as inoculum for infection in subsequent growing seasons. Studies on pathogen distribution and inoculum density in soybean fields have been limited (28,29,32,35) due to the lack of practical methods to specifically detect and quantify *F. virguliforme* in soil. A tool to accurately and specifically quantify *F. virguliforme* in soil is needed to clarify the role of inoculum density in disease progress, facilitate SDS risk assessments in soybean fields, and provide insights about mechanisms of pathogen spread.

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SDS management is challenging and requires the use of multiple approaches, with the selection of resistant varieties being of primary importance (26). However, soybean varieties characterized as resistant to *F. virguliforme* exhibit partial resistance because resistance is multigenic (16,30), and resistance in some varieties can be overcome by high inoculum concentrations (2,14). Furthermore, screening for resistance to SDS in greenhouse assays does not consistently predict mature-plant responses in field conditions (26,34), possibly due, in part, to differences in inoculum levels in greenhouse versus field studies. Because SDS development in field conditions is highly dependent on environmental factors (26,31), accurate quantification of *F. virguliforme* in soil could help to improve reliability of resistance screening assays.

Soil dilution plating methods have been used to quantify *F. virguliforme* propagules in soil (28,29,32) but these methods are time consuming, laborious, and often underestimate pathogen density. Isolation is further compounded by fast-growing fungi that can overrun culture plates containing the very slow-growing *F. virguliforme* (26). Dilution-plate methods are also unsuitable for processing numerous samples that would be needed to assess SDS risk based on inoculum densities in soybean fields. In contrast, real-time polymerase chain reaction (PCR) can be used to quantify density of fungal propagules in soil (7,11,36) and may be applicable for studies of soilborne inoculum levels of *F. virguliforme*.

One of the challenges with quantification of *F. virguliforme* in field soil is the presence of closely related *Fusarium* spp. that are ubiquitous in soil (22). Published real-time PCR assays (12,18) for *F. virguliforme* were based on the mitochondrial small subunit (mtSSU) rDNA region. However, primers developed from this region also amplify DNA from other SDS-causing *Fusarium* spp., as well as DNA from some *F. solani* (Mart.) Sacc. strains that do not cause SDS but have been isolated from soybean and corn roots in Iowa (*unpublished data*). The published primers were developed prior to recent findings that the mtSSU locus is highly conserved and unable to resolve species boundaries within the SDS-bean root rot (BRR) clade of the *F. solani* species complex (1,25), which groups the four SDS-causing species with a bean root rot *F. phaseoli* species.

Genetic loci that delimit species boundaries within the SDS-BRR clade would be useful for the design of species-specific prim-

ers and probes. *FvTox1* is a single-copy gene that has been recently shown to play a major role in causing the leaf scorch component of SDS caused by *F. virguliforme* (6). Because the ability to cause SDS leaf scorch is unique to the SDS-causing species, we hypothesized that the *FvTox1* gene would be useful for the development of a species-specific assay for *F. virguliforme*. The objective of this study was to develop a real-time PCR assay that distinguishes *F. virguliforme* from the other species within the SDS-BRR clade based on the *FvTox1* gene.

Materials and Methods

Fungal isolates and growth conditions. Modified Nash and Snyder medium (MNSM) (28) was used to isolate *F. virguliforme* from roots of diseased soybean plants collected in 2006 and 2007 from Iowa fields. Isolates were subcultured onto potato dextrose agar (PDA; Becton and Dickinson Co., Sparks, MD), and identified based on morphological characteristics (1). Single-spore isolates were obtained and species identification was confirmed using sequence analysis (1). Isolates were stored in sterilized soil (4) in 10-ml glass tubes at 4°C until further use. Isolates of the other three SDS-causing species (*F. tucumaniae*, *F. brasiliense*, and *F. cuneirostrum*) were obtained from the Agriculture Research Service Culture Collection (NRRL, National Center for Agricultural Utilization Research, Peoria, IL), and single-spore isolates of *F. solani* from corn and soybean were obtained from Dr. Gary Munkvold (Department of Plant Pathology and Microbiology, Iowa State University). Also included in this study were isolates of other species of fungi from the Iowa State University Mycology Teaching Laboratory Collection. Fungal cultures for all isolates (Table 1) were first grown on PDA for 4 days at 25 ± 2°C and then used to inoculate malt yeast extract broth (23). Mycelia were harvested by vacuum infiltration, freeze-dried overnight, and stored at 4°C for subsequent DNA isolation.

Design of primers and probe. The sequence of the *FvTox1* gene was obtained by PCR amplification using primer pair 1983 (5′-CAC CGG ATC CAC GAT GGC GAA GTC CAC ATT CAC

Table 1. Specificity of the TaqMan assay designed from the single-copy *FvTox*1 gene compared to two published assays designed from the mitochondrial small subunit (mtSSU) gene sequences

Isolate	Species	Host[b]	Detection assays[a]		
			FvTox1	$mtSSU_1$	$mtSSU_2$
LL0028	*Fusarium virguliforme*	*Glycine max*	+	+	+
LL0009	*F. virguliforme*	*G. max*	+	+	+
NRRL31950	*F. tucumaniae*	*G. max*	–	+	+
NRRL31157	*F. cuneirostrum*	*G. max*	–	+	+
NRRL34938	*F. brasiliense*	*G. max*	–	+	+
42-T8	*F. solani*[c]	*G. max*	–	+	+
295-T9	*F. solani*	*G. max*	–	+	+
209-T4	*F. solani*	*G. max*	–	+	+
9T3	*F. solani*	*G. max*	–	+	+
5L5	*F. solani*	*G. max*	–	+	+
42-T8A	*F. solani*	*G. max*	–	+	+
76-L2	*F. solani*	*G. max*	–	–	–
9T3	*F. solani*	*G. max*	–	+	+
253-L4	*F. solani*	*G. max*	–	+	+
295-T6	*F. solani*	*G. max*	–	+	+
42-L10	*F. solani*	*G. max*	–	+	+
76-L6	*F. solani*	*G. max*	–	+	+
458-L10	*F. solani*	*G. max*	–	+	+
228-T2	*F. solani*	*G. max*	–	+	+
391-L4	*F. solani*	*G. max*	–	+	+
35-T7	*F. solani*	*G. max*	–	+	+
98-L3	*F. solani*	*G. max*	–	+	+
76-T10	*F. solani*	*G. max*	–	+	+
11-T3	*F. solani*	*G. max*	–	+	+
42-T1	*F. solani*	*G. max*	–	–	–
209-L6	*F. solani*	*G. max*	–	+	+
295-L5	*F. solani*	*G. max*	–	+	+
FSP185	*F. solani*	*G. max*	–	–	–
95-164	*F. solani*	*Zea mays*	–	+	+
FSP325PS	*F. solani*	*Z. mays*	–	–	–
FS94-1A	*F. solani*	*Z. mays*	–	–	–
F-1	*F. oxysporum*	*Lactuca sativa*	–	–	–
F-2	*F. culmorum*	*L. sativa*	–	–	–
F-5	*F. culmorum*	*L. sativa*	–	–	–
F-9	*F. concolor*	*L. sativa*	–	–	–
SR-8	*F. subglutinans*	*L. sativa*	–	–	–
17-JC	*F. oxysporum*	*L. sativa*	–	–	–
CY-6	*F. graminearum*	*L. sativa*	–	–	–
CY-10	*F. acuminatum*	*L. sativa*	–	–	–
1022	*F. proliferatum*	*L. sativa*	–	–	–
2192-001	*Alternaria alternata*	*Daucus carota*	–	–	–
269	*A. longissima*	*D. carota*	–	–	–
70-15	*Magnaporthe grisea*	*Oryza sativa*	–	–	–
ATCC 22959	*Rhizopus oligosporus*	ND	–	–	–
ATCC 11539	*Gloeophyllum trabeum*	ND	–	–	–
ATCC 24859	*Saccharomyces cerevisiae*	ND	–	–	–
ATCC 24725	*Phanerochaete chrysosporium*	ND	–	–	–
ATCC 1003	*Aspergillus oryzae*	ND	–	–	–

[a] Symbols: – not detected, + detected, and ND = host not known.
[b] Host plant from which fungus was isolated.
[c] *F. solani* isolates that did not amplify with mtSSU-based assays were not included.

CCT TG-3′) and 1986 (5′-CCG CGA ATT CTT ACT GTG GGT TGC GCA CAC AGT TG-3′) (M. K. Bhattacharyya, *unpublished*). These primers amplified a 657-bp fragment from representative isolates of the four *Fusarium* spp. that cause SDS, and the amplicons were sequenced. The sequences were aligned and parts of the nucleotide sequence that showed distinction among species were used to design the forward primer (FV-F, 5′-GCA GGC CAT GTT GGT TCT GTA-3′), reverse primer (FV-R, 5′-GCA CGT AAA GTG AGT CGT CTC ATC-3′), and probe (FV-MGB probe 5′-6-FAM ACT CAG CGC CCA GGA MGB N FQ-3′) specific to *F. virguliforme* (Fig. 1). Primers and probe were designed with Primer Express software and synthesized by Applied Biosystems (Foster City, CA).

DNA extraction from mycelia. DNA was isolated from lyophilized mycelia following the method of DeScenzo and Harrington (10) and concentrations were determined by absorbance at 260 or 280 nm with the Nanodrop system (Thermo Fisher Scientific, Wilmington, DE). Ten-fold serial dilutions of *F. virguliforme* genomic DNA were prepared in triplicate and used in real-time PCR reactions.

DNA extraction from *F. virguliforme* conidia. To determine whether conidia could be used for quantification of fungal inoculum density, 4-day-old plates of *F. virguliforme* (LL0028) were flooded with Tris EDTA (TE) buffer, and conidial concentrations were determined using a hemocytometer. Ten-fold serial dilutions (10^8 to 10^0 conidia/ml) of the conidia were prepared in 20% nutrient broth (40). For each conidial concentration, 1 ml of sample was transferred to 2-ml microcentrifuge tubes containing 1 ml of 0.5-mm-diameter silica beads (Biospec Products, Inc., Bartlesville, OK), and the conidia were homogenized at a speed of 6 for 40 s in a homogenizer (FastPrep-24; MP Biomedicals, Solon, OH). Three microcentrifuge tubes were prepared per dilution. The tubes were heated on a hot plate for 5 min at 100°C, cooled on ice for 5 min, and centrifuged for 1 min until the conidial debris formed a pellet. Supernatant containing the DNA (5 µl) was used directly for real-time quantitative PCR.

DNA extraction from soil infested with *F. virguliforme*. Soil samples were artificially infested with conidia to evaluate the ability to extract and subsequently amplify *F. virguliforme* DNA from soil. The effect of soil substrate and non-target soil microflora on efficiency of detection using PCR amplification was determined by comparing the yield of fungal genomic DNA amplified from sterilized and nonsterilized soil substrate spiked with conidia. Soil was collected from a soybean field with no history of SDS from Curtiss Farm of Iowa State University, Ames. The absence of *F. virguliforme* propagules in the soil was confirmed by extracting total soil DNA from three 250-mg subsamples and running PCR reactions with designed primers. One part of the soil sample was autoclaved for 60 min at 121°C for two consecutive days (sterilized soil sample), and the nonautoclaved part constituted the nonsterilized soil. *F. virguliforme* conidial suspensions (2 ml each of 10^7, 10^6, 10^5, 10^4, 10^3, 10^7, 10, and 1 conidia/ml) were added to 2 g of the sterilized and nonsterilized soil in 15-ml conical tubes and mixed. Three 250-mg samples of each of these mixtures were weighed and freeze dried. Controls consisted of 250-mg soil samples mixed with 2 ml of distilled water (11). Total soil DNA was isolated from each soil sample using the MoBio UltraClean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA) with the following modifications. After adding 60 µl of solution 1 from the MoBio kit, the soil-buffer mixture was homogenized at a speed level of 6 for 4 s in the Fast Prep homogenizer. The subsequent DNA extraction steps were performed as described in the instruction manual.

Sensitivity and specificity of the PCR assay. PCR reactions were carried out in a total reaction volume of 25 µl, containing 5 µl of DNA template, 0.3 µl of each primer (25 µM each), and 0.5 µl of the TaqMan probe (10 µM) in 12.5 µl of PerfeCTa qPCR SuperMix

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F. tucumaniae    ............................................................ 60
F. brasiliense   ............................................................ 60
F. cuneirostrum  ............................................................ 60
F. virguliforme  GGTCTACTTTTGCCGTGAGGAAAACTGGGGTCCTCCCTGCTTCGTCTACTACCCCGAACT 60

F. tucumaniae    ............................................................C..... 120
F. brasiliense   ............................................................C..... 120
F. cuneirostrum  ............................................................C..... 120
F. virguliforme  CGAGTACACATGCTCCGAGCTTGGGCCGGAGCTTGCAGGCCATGTTGGTTCTGTATTTGT 120
                                                                    FV-F

F. tucumaniae    ............................................................ 180
F. brasiliense   ............................................................ 180
F. cuneirostrum  ............................................................ 180
F. virguliforme  CGAGGCTGGTGCCATCTGCCGGATGGCAACgtacgtgaccctctcatacatgtcgaatac 180

F. tucumaniae    ............................T............................... 240
F. brasiliense   ............................T............................... 240
F. cuneirostrum  ............................T............................... 240
F. virguliforme  cgattactgacaaactgcagACTCAGCGCCCAGGACCGATGCGCCCCGATCGAGTTCTTT 240
                                          FV-MGB probe

F. tucumaniae    ............................................................ 300
F. brasiliense   ............................................................ 300
F. cuneirostrum  ............................................................ 300
F. virguliforme  GCCTGGCCCGAGACTGCGGCTGGCTGGCCCGACCTCTTCCAAAGGGACGGCCCCGACGGC 300

F. tucumaniae    ..............T............................G.............. 360
F. brasiliense   ..............T............................G.............. 360
F. cuneirostrum  ..............T............................G.............. 360
F. virguliforme  AAGGGCAAGCTTGGCGATGAGACGACTCACTTTACGTGCGCCGAATGTACCAACTGTGTG 360
                                          FV-R

F. tucumaniae    ........................... 386
F. brasiliense   ........................... 386
F. cuneirostrum  ........................... 386
F. virguliforme  CGCAACCCACAGTAAGAAATTCGCGG 386
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Fig. 1. Multiple sequence alignment of part of the *FvTox1* gene sequence and polymorphic sites used to design *Fusarium virguliforme*-specific primers and TaqMan MGB probe. Lowercase nucleotides represent the second intron of the gene. Dots represent matching nucleotides to the *F. virguliforme FvTox1* gene sequence.

with Rox (Quanta BioSciences, Inc., Gaithersburg, MD). The thermal profile was 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 45 s at 60°C (Bio-Rad IQ5 thermal cycler; Bio-Rad Laboratories, Hercules, CA). The threshold value of the real-time PCR machine was set manually at 100, and any signal <100 was considered background fluorescence.

To determine specificity, the primer pairs were tested on total genomic DNA (50 ng) from 49 fungal isolates representing *Fusarium* spp. diversity and species commonly found in association with soybean roots (Table 1). The same samples were tested using the previously published real-time PCR assays (12,18). To determine the sensitivity of the assay, the ability of the primers to amplify the target fragment from decreasing concentrations of *F. virguliforme* using the DNA obtained from 10-fold serial dilutions of *F. virguliforme* total genomic DNA, conidial suspensions, and spiked soil was tested. The quantification cycle (C_q; fractional PCR cycle at which the target is quantified in a given sample) values obtained for each dilution series were used to generate standard curves for absolute quantification of the target DNA in soil and root samples.

Assay validation. To assess reliability of the assay in detecting and quantifying *F. virguliforme* in native soil, total DNA was extracted from four soil samples obtained from infested potted soil used in greenhouse pathogenicity tests and eight samples obtained from soybean fields in Iowa with a history of SDS. DNA was extracted in triplicate from each soil sample as previously described and the *FvTox1* gene was amplified using the TaqMan assay. DNA was also extracted from roots of diseased soybean plants collected from a soybean field infested with SDS and the target gene was quantified using the TaqMan assay. Because quantification cycle values vary between experiments, DNA obtained from 10-fold serial dilutions of the conidia in nonsterilized soil was included in each run of the test soil samples for calibration purposes. For all runs, the 18S rDNA primers and probe were included as the endogenous amplification control (IAC) (33). Foliar and root disease severity were assessed on the greenhouse plants in pots from which soil samples were collected. SDS foliar disease severity, defined as the percentage of leaf area showing chlorosis or necrosis typical of SDS, was rated at 10, 14, 18, 22, 26, and 30 days after inoculation. Root rot severity was rated 30 days after inoculation as the percentage of root area showing brown discoloration.

Data analysis. A linear-regression equation was obtained from each standard curve. The concentrations of target DNA in test root and soil samples were calculated by comparing the C_q values obtained for these samples with the crossing point values of the linear-regression line of the standard curve of both the mycelia and the spiked-soil standards. The amount of the target DNA in soil was expressed as number of conidia of *F. virguliforme* per gram of soil. Amplification efficiency (E) was calculated according to the equation $E = [10^{(-1/slope)}] - 1$. Pearson correlation analysis was conducted to determine the association between conidial density in soil, foliar area under disease progress curve (AUDPC), and root rot severity.

Results

Specificity of *F. virguliforme* primers and probe. The *FvTox1* gene showed sufficient interspecies divergence to produce amplification products specific to *F. virguliforme*. The sequence of the *FvTox1* gene in *F. virguliforme* differed by four single nucleotide polymorphisms (SNPs) from each of the three SDS-causing species from South America (Fig. 1). The TaqMan probe and primers amplified a 200-bp single amplicon from pure cultures of *F. virguliforme* and not from any of the other fungal isolates tested (Table 1). The *FvTox1* assay was more specific than the published *F. virguliforme* mtSSU-based assays (12,18) because it detected only *F. virguliforme* (Tables 1 and 2).

Sensitivity of the primers and probe. There was a linear relationship (R^2 = 0.999; E = 1.0) between *F. virguliforme* genomic DNA concentration and real-time quantification cycles using the *FvTox1* primers and probe (Fig. 2). The pathogen was detected at template concentrations of 5 pg/µl (Fig. 2). A similar linear pattern of amplification was obtained from DNA derived from dilution series of conidia (R^2 = 0.979, E = 2.0). The detection limit of the target amplicon was 1,000 conidia/ml of suspension (Fig. 3A). When conidia were added to both sterilized and nonsterilized soil, a consistent and proportionate amount of DNA was extracted and quantified across the range of inoculum concentrations (R^2 = 0.960; E = 1.1) (Fig. 3B). At 10^3 conidia/g soil, *F. virguliforme* was detected in all three replicates whereas, at 10^2 conidia/g of soil, the DNA target was detected in only two of the three replicates (G. C. Y. Mbofung, *unpublished data*). Thus, the limit of detection was defined as 10^3 conidia/g soil. Dilution series from artificially infested soils resulted in amplicons of the same size as from pure cultures. Quantification cycle values for DNA obtained from both sterilized and nonsterilized soil were similar (for sterilized soil, *data not shown*). The *FvTox1* assay detected its target five quantification cycles later than the mtSSU assay (Table 2).

Table 2. Specificity and sensitivity of the TaqMan assay designed from the single-copy *FvTox*1 gene compared to a TaqMan assay designed from the mitochondrial small subunit (mtSSU) gene in distinguishing *Fusarium virguliforme* from other sudden death syndrome (SDS) and non-SDS-causing species

		Detection[a]			
		FvTox1		$mtSSU_2$	
Isolate	Species	C_q value	Detection	C_q value	Detection
2T2	*Fusarium solani*	34.77	–	19.42	+
FS94-1A	*F. solani*	33.83	–	33.41	–
209L6	*F. solani*	33.99	–	19.23	+
FSP185	*F. solani*	34.61	–	33.33	–
FB15II	*F. virguliforme*	21.05	+	15.55	+
NRRL22292	*F. virguliforme*	21.03	+	14.16	+
Mont 1	*F. virguliforme*	20.29	+	14.33	+
LL0009	*F. virguliforme*	21.89	+	15.99	+
NRRL31950	*F. tucumaniae*	33.94	–	14.24	+
NRRL31793	*F. tucumaniae*	34.99	–	13.11	+
NRRL31085	*F. tucumaniae*	33.79	–	14.62	+
NRRL34938	*F. brasiliense*	35.27	–	15.22	+
NRRL22743	*F. brasiliense*	NA	–	14.67	+
NRRL22678	*F. brasiliense*	34.94	–	15.08	+
NRRL31104	*F. cuneirostrum*	34.02	–	15.13	+
NRRL31157	*F. cuneirostrum*	33.45	–	14.88	+
NRRL22275	*F. cuneirostrum*	NA	–	14.48	+
NRRL22158	*F. cuneirostrum*	34.74	–	19.21	+
Control	Water	33.34	–	33.36	–

[a] Symbols: – not detected and + detected. Quantification cycle (C_q) values ≥ the value obtained for a water control were considered not detected; NA = no amplification.

Validation of the real-time PCR assay. From the 12 samples used to test the applicability of the assay for detection in soil, the detectable number of conidia per gram of soil ranged from 0 to 10^6, with infested greenhouse samples having the highest conidial densities (Fig. 4). Soil PCR products exhibited 100% sequence homology to the pure culture (G. C. Y. Mbofung, *unpublished*). In soil from the greenhouse experiment, inoculum density of *F. virguliforme* was positively correlated (r = 0.853, P = 0.004) with foliar AUDPC but not correlated (P = 0.266) with root rot severity. The amount of target DNA estimated in the roots of symptomatic plants ranged between 2.5 and 3.4 ng/mg of fresh root tissue.

Discussion

The real-time PCR assay reported here is the first to directly quantify *F. virguliforme* in soil substrate. The assay was highly specific to *F. virguliforme* and enabled the quantification of genomic DNA of *F. virguliforme* in both artificially and naturally infested field soils. The assay developed was sensitive enough to detect genomic DNA at 25 pg/g of soil and *F. virguliforme* at 1,000 conidia/g of soil. Because the pathogen persists in soil after cultivation of soybean, the capability to quantify *F. virguliforme* in soil will facilitate epidemiological studies on the relationship between inoculum density and disease development, and permit identification of high-risk fields that can either be avoided or else managed to reduce yield losses due to SDS (32).

The primers and probe used in the assay were developed based on the *FvTox1* gene of *F. virguliforme* (6). This gene codes for a proteinaceous phytotoxin involved in the development of SDS leaf scorch. Two other phytotoxins, monorden (3) and a 17-kDa proteinaceous toxin (15), have been isolated from cell-free culture filtrates of *F. virguliforme* and shown to cause necrosis on soybean leaves. Because *F. virguliforme* has never been isolated from aboveground plant parts (27), the toxins are thought to be produced by the fungus in colonized roots and translocated to the leaves via the xylem (21).

Comparison of the four SDS-causing species showed that the sequences of the *FvTox1* gene contained four SNPs unique to *F. virguliforme*, three of which were used to develop the specific primers and probe. Because the *FvTox1* gene sequences were identical for *F. tucumaniae*, *F. brasiliense*, and *F. cuneirostrum*, this gene cannot be used to distinguish among the causal agents of SDS in South America (25). Preliminary work to amplify the *FvTox1* gene in *F. solani* isolates (*unpublished data*) showed very low similarity of the gene with the SDS-causing species, consistent with the inability of *F. solani* to cause SDS foliar symptoms.

The designed primers and probe amplified a single fragment only from *F. virguliforme* but not from pure isolates of other fungal species or other *Fusarium* spp. from nonsterilized soil, confirming their reliability for specifically detecting *F. virguliforme* and indicating no undesired cross-reactivity with non-target genomic DNA. Furthermore, the *FvTox1* gene region was shown to be more specific for *F. virguliforme* than previously used regions because no amplification occurred with the *F. solani* strains that cross-reacted with published assays based on the mtSSU region (12,18). This region was recently reported as unsuitable for distinguishing species within the SDS-BRR clade of the *F. solani* complex (25). The fact that most of the *F. solani* isolates tested in this study amplified with the published mtSSU real-time PCR assays while other *F. solani* isolates did not may be a consequence of the genetic variation found within the *F. solani* species complex (24). The specificity of the assay developed in this study will allow detection of *F. virguliforme* in field soil where other *Fusarium* spp., including *F. solani*, are present.

The similar quantification cycle values obtained for DNA extracted from both sterilized and nonsterilized soil indicated a high efficiency in DNA extraction and effectiveness of the assay to detect the target despite the presence of other soil organisms. Because the target fragment was detected consistently from dilutions of genomic DNA of 5 pg/µl, the limit of detection was defined as DNA at 5 pg/µl. The sensitivity of the assay was set to 1,000 conidia/g of nonsterilized soil due to consistent amplification at this inoculum density but the assay was sensitive enough to often detect 100 conidia/g of soil. Similar soil densities (10^2 to 10^3 CFU/g of

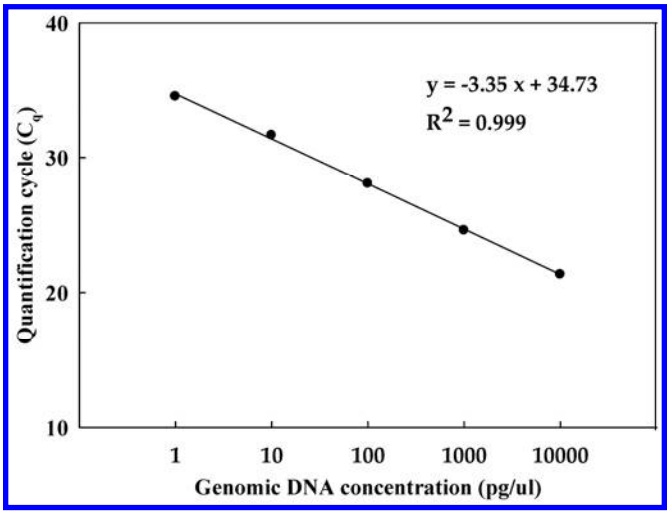

Fig. 2. Standard curve for absolute quantification of genomic DNA generated with 10-fold serial dilutions of genomic DNA isolated from pure cultures of *Fusarium virguliforme*. The detection limit for genomic DNA was 25 pg.

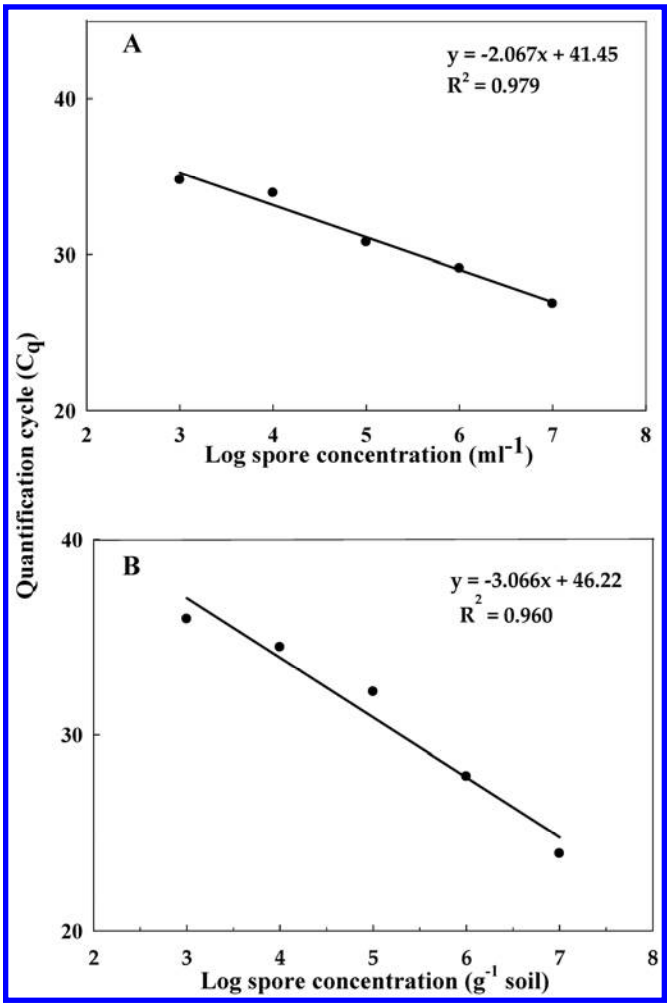

Fig. 3. A, Standard curve for absolute quantification of DNA from conidial suspensions. Detection limit for conidial suspension was 1,000 conidia/ml. **B,** Standard curve for absolute quantification of DNA from soil artificially infested with conidia. Detection limit for conidia in soil was 1,000 conidia/g of soil. All curves show a linear relationship between quantification cycle values and initial DNA concentrations.

soil) of SDS pathogen have been reported in symptomatic soybean fields based on soil dilution plating methods (28,29,32), suggesting that the PCR assay is adequate for detecting *F. virguliforme* at densities capable of causing disease. However, a major limitation with using PCR to detect targets from complex environmental samples, such as soil, is the presence of PCR inhibitors in the extracted DNA (12). This limitation was indicated by the lower R^2 values of the standard curves obtained for soil artificially infested with conidia compared with pure conidial suspensions. Although total soil DNA obtained using commercially available kits was of high quality and suitable for the assay, it is possible that continued optimization of DNA extraction and purification protocols could further improve the assay sensitivity.

Although *F. virguliforme* propagules in soil include mycelia, conidia, conidiophores, and chlamydospores (26), conidia were used as a calibrator for generating standard curves and as the unit for expression of inoculum density in this study. Conidia have been consistently used as a calibrator for quantification of inoculum densities of plant pathogens from soil because of ease of calculation (7,9,11,19,38), whereas calibration using mycelia or chlamydospores is possible but complicated. Nevertheless, it is theoretically possible to quantify target DNA from any source if standard curves based on known concentrations of genomic DNA are used. The assay could, for example, be used to quantify the pathogen in infected soybean plants, as shown by the amplification of the *FvTox1* gene from roots of symptomatic field plants.

The availability of species-specific primers to quantify *F. virguliforme* in soil will contribute significantly to epidemiological studies on SDS. A better understanding of the relationship between inoculum density in soil and disease development (12,13,32) would facilitate predictions of disease risk in individual fields and improve reliability of screening assays for disease resistance (22). The strong correlations obtained in this study between inoculum density and foliar severity shows that the real-time PCR assay will be useful for studying these relationships. Quantification of pathogen density in soil may also provide essential insights about the effectiveness of cultural practices for SDS management. Recent findings that *F. virguliforme* survives on corn residue (39), for example, stress the importance of monitoring pathogen dynamics in soil under different cropping systems (29). Studies on *F. virguliforme* distribution in soybean production areas and its interactions with SCN (20) may also clarify the mechanisms of pathogen spread. Finally, quantification of *F. virguliforme* in roots could be useful for evaluating SDS resistance in soybean varieties and for early diagnosis of the pathogen in asymptomatic plants.

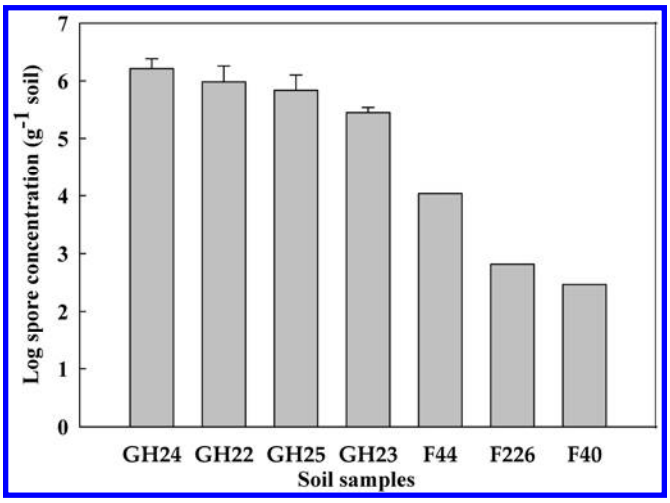

Fig. 4. *Fusarium virguliforme* conidial density in 7 of the 12 soil samples tested, 4 from greenhouse studies (GH22, GH23, GH24, and GH25) and 3 from soybean fields (F44, F226, and F40). Conidial densities detected in each soil sample are the means of three replications and were calculated using the regression equation $y = -3.066x + 46.22$. Error bars represent standard error of the mean.

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