Improved Diagnoses and Quantification of *Fusarium virguliforme*, Causal Agent of Soybean Sudden Death Syndrome

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ABSTRACT

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Fusarium virguliforme (syn. *F. solani* f. sp. *glycines*) is the primary causal pathogen responsible for soybean sudden death syndrome (SDS) in North America. Diagnosis of SDS is difficult because symptoms can be inconsistent or similar to several soybean diseases and disorders. Additionally, quantification and identification of *F. virguliforme* by traditional dilution plating of soil or ground plant tissue is problematic due to the slow growth rate and plastic morphology of *F. virguliforme*. Although several real-time quantitative polymerase chain reaction (qPCR)-based assays have been developed for *F. virguliforme*, the performance of those assays does not allow for accurate quantification of *F. virguliforme* due to the reclassification of the *F. solani* species complex. In this study, we developed a TaqMan qPCR assay based on the ribosomal DNA (rDNA)

intergenic spacer (IGS) region of *F. virguliforme*. Specificity of the assay was demonstrated by challenging it with genomic DNA of closely related *Fusarium* spp. and commonly encountered soilborne fungal pathogens. The detection limit of this assay was determined to be 100 fg of pure *F. virguliforme* genomic DNA or 100 macroconidia in 0.5 g of soil. An exogenous control was multiplexed with the assay to evaluate for PCR inhibition. Target locus copy number variation had minimal impact, with a range of rDNA copy number from 138 to 233 copies per haploid genome, resulting in a minor variation of up to 0.76 cycle threshold values between strains. The qPCR assay is transferable across platforms, as validated on the primary real-time PCR platform used in the North-central region of the National Plant Diagnostic Network. A conventional PCR assay for *F. virguliforme* detection was also developed and validated for use in situations where qPCR is not possible.

Additional keyword: diagnostic.

Within the United States, soybean (*Glycine max* (L.) Merr.) is the second most widely grown crop, with more than 3 billion bushels produced per annum (United States Department of Agriculture, National Agricultural Statistics Service, 2013). Numerous plant diseases threaten soybean production in the United States, including soybean sudden death syndrome (SDS) (43). In the past decade, SDS has ranked within the top five most yield-damaging soybean diseases in the United States, with estimated yield losses of 70 million bushels in 2010 (44). In North America, the predominant causal agent of soybean SDS is the soilborne fungal pathogen *Fusarium virguliforme* (3). *F. virguliforme* has been reported to colonize a wide range of host plant species (21), and can survive in soil or debris by producing conidia or chlamydospores (38). Diagnosis of SDS in the field can be difficult because several other diseases can produce similar symptoms, such as brown stem rot (BSR) caused by *Phialophora gregata* (= *Cadophora gregata*) or red crown rot caused by *Cylindrocladium parasiticum* (38).

Studies suggest that early infection of soybean plants by *F. virguliforme* is essential for foliar SDS symptom development (30), and infection by *F. virguliforme* was detected as early as the seedling stage (13). As a soilborne pathogen, *F. virguliforme* only colonizes the root and the lower stem of soybean plants (39); phytotoxins produced by the fungus are translocated through the xylem to the foliage, causing SDS foliar symptoms (7). Accurate

detection and quantification of *F. virguliforme* in root and soil samples are essential to study the epidemiology of *F. virguliforme*. Both culture-based and molecular polymerase chain reaction (PCR)-based methods have been developed for the detection of *F. virguliforme*. Culture-based methods utilize dilution plating or isolation from infected tissue on semiselective media (10,26,37,39). However, these culture-based methods are time consuming and can be difficult to implement given the slow growth rate and plastic morphology of *F. virguliforme* (3,10). These limitations of sensitivity and specificity led to the development of DNA-based molecular detection tools. Multiple conventional PCR assays have been developed for the detection of *F. virguliforme* but they are not specific due to the revised taxonomy of the *F. solani* species complex (1,23,32). Therefore, no specific conventional PCR assay is currently available for diagnosis of *F. virguliforme*.

Quantitative real-time PCR (qPCR) has been extensively applied in quantification and diagnosis of numerous plant pathogens. Compared with conventional PCR assay, qPCR assays can be more sensitive and specific and can detect multiple pathogens by multiplexing assays (35,40). Gao et al. (14) and Li et al. (24) reported two qPCR assays for quantification of *F. virguliforme* from plant samples using TaqMan probe assays designed against the mitochondrial small-subunit (SSU) ribosomal DNA (rDNA) sequence. Due to the reclassification of the *F. solani* species complex, the mitochondrial DNA (mtDNA) region used for design of these two assays was too conserved to differentiate *F. virguliforme* from the dry bean root rot pathogens *F. cuneirostrum* and *F. phaseoli* and other SDS causal agents such as *F. tucumaniae*, *F. crassistipitatum*, and *F. brasiliense*, which predominate in South America (5,33). Therefore, it is clear that qPCR assays for *F. virguliforme* need to be improved. O'Donnell et al. (33) and Aoki et al. (5) demonstrated that the intergenic spacer (IGS)

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region of the rDNA is one of the genetic loci that can resolve *F. virguliforme* from the other closely related *Fusarium* spp. in their multilocus genotyping studies of clade 2 *F. solani* species complex. The rDNA IGS region is a multicopy genetic locus in the eukaryotic genome (25); therefore, the sensitivity of the assay based on the IGS rDNA region is greater than a single-copy gene assay, and has been used for detection and quantification of numerous plant pathogens (6,9,15). Although the rDNA IGS region seems an ideal region for qPCR assay design, large rDNA copy number variations were reported in many fungal organisms (6,18), and accuracy of the quantification may be affected in plants infected by fungal strains with varied rDNA copy numbers. In addition, PCR inhibitors in DNA samples can cause false-negative results or low PCR amplification efficiencies. Strategies to deal with PCR inhibitors include dilution of DNA samples (27), additional DNA purification steps, or the use of PCR additives such as polyvinylpyrrolidone (19,28) and bovine serum albumin (BSA) (16). Despite attempts to reduce or remove PCR inhibitors, an internal or exogenous control is needed to monitor each reaction for PCR inhibition.

Soybean SDS is becoming one of the most devastating diseases threatening soybean production across most of the soybean production zones in the United States, and its range continues to expand. Despite this, an accurate, robust, and sensitive diagnostic

and quantitative assay is not available. Therefore, development of such an assay will facilitate the diagnosis of SDS, and can be used to study the epidemiology of *F. virguliforme*, which will improve our understanding and management of this pathogen. The objectives of this study were to (i) develop a qPCR assay with the inclusion of an internal control for the sensitive and specific detection and quantification of *F. virguliforme*, (ii) determine the transferability of the qPCR assay between platforms, (iii) develop a complimentary conventional PCR assay for use in situations where qPCR is not possible, and (iv) thoroughly validate the qPCR and PCR assays with plant and soil samples.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Thirty-six isolates of *Fusarium* spp. and other genera commonly found associated with soybean were used in this study (Table 1). Cultures of *Fusarium* spp. were grown on potato dextrose agar (PDA) media (Acumedia, Burton, MI) at room temperature for 14 days, and agar plugs with conidia were collected and stored at -80°C in 20% glycerol. To obtain mycelia for DNA extraction, seven to eight 4-mm³ pieces of colonized PDA media were transferred to 50 ml of potato dextrose broth (PDB) (Acumedia) in 250-ml Erlenmeyer flasks and shaken at room temperature on an orbital rotary shaker at

TABLE 1. Specificity test panel for quantitative polymerase chain reaction (qPCR) assay validation^a

Species ^b	Ct ^c	NRRL ^d	Host ^e	Geographic origin
<i>Fusarium acuminatum</i> ^f	35.75	—	<i>Solanum tuberosum</i>	Michigan
<i>F. avenaceum</i> ^f	35.07	—	<i>S. tuberosum</i>	Michigan
<i>F. brasiliense</i>	32.06	22678	<i>Glycine max</i>	California
<i>F. brasiliense</i>	36.21	22743	<i>G. max</i>	Brasilia, Distrito Federal, Brazil
<i>F. cerealis</i> ^f	34.11	—	<i>S. tuberosum</i>	Michigan
<i>F. crassiseptatum</i>	36.04	31949	<i>G. max</i>	Cristalina, Goias, Brazil
<i>F. cuneirostrum</i>	33.05	31157	<i>Phaseolus vulgaris</i>	Presque Isle, Michigan
<i>F. equiseti</i> ^f	34.29	—	<i>S. tuberosum</i>	Michigan
<i>Fusarium</i> sp.*	32.6	22574	<i>Coffea arabica</i>	Guatemala
<i>Fusarium</i> sp.*	35.54	22412	Bark	French Guiana
<i>F. graminearum</i> ^f	34.91	—	<i>S. tuberosum</i>	Michigan
<i>Fusarium</i> sp.*	31.35	22387	Bark	French Guian
<i>F. oxysporum</i> ^f	36.07	—	<i>S. tuberosum</i>	Michigan
<i>F. phaseoli</i>	37.18	22276	<i>P. vulgaris</i>	United States
<i>F. phaseoli</i>	36.47	31156	<i>P. vulgaris</i>	Michigan
<i>F. sambucinum</i> ^f	37.36	—	<i>S. tuberosum</i>	Michigan
<i>F. solani</i>	37.19	22395	Bark	Venezuela
<i>F. solani</i> ^f	35.21	—	<i>S. tuberosum</i>	Michigan
<i>F. torulosum</i> ^f	34.58	—	<i>S. tuberosum</i>	Michigan
<i>F. tricinctum</i> ^f	35.31	—	<i>S. tuberosum</i>	Michigan
<i>F. tucumaniae</i>	38.57	31096	<i>G. max</i>	San Agustin, Tucuman, Argentina
<i>F. tucumaniae</i>	37.15	31773	<i>G. max</i>	Brazil, PR, Ponta Grossa
<i>F. virguliforme</i>	21.78	22823	<i>G. max</i>	Indiana
<i>F. virguliforme</i>	20.88	31041	<i>G. max</i>	Illinois
<i>F. virguliforme</i>	21.08	34551	<i>G. max</i>	San Pedrom, Buenos Aures, Argentina
<i>F. virguliforme</i>	20.53	22292	<i>G. max</i>	Illinois
<i>F. virguliforme</i>	19.36	—	<i>G. max</i>	Counties across Michigan
<i>Phialophora gregata</i> (B)	34.84	—	<i>G. max</i>	—
<i>P. gregata</i> (A)	33.82	—	<i>G. max</i>	—
<i>Phytophthora sansomeana</i>	34.9	—	<i>G. max</i>	—
<i>P. sojae</i>	35.98	—	<i>G. max</i>	—
<i>Pythium sylvaticum</i>	34.21	—	<i>G. max</i>	—
<i>P. ultimum</i>	34.93	—	<i>G. max</i>	—
<i>Rhizoctonia solani</i> AG-2-IIIB	34.93	—	—	—
<i>R. solani</i> AG-4	34.99	—	—	—
<i>Sclerotinia sclerotiorum</i>	35.36	—	<i>G. max</i>	—

^a This table includes the *Fusarium* spp. that are closely related to the *Fusarium* spp. that cause sudden death syndrome and other commonly encountered soil fungal species. Cycle threshold (Ct) values listed in the table indicate the specificity performance of the *Fusarium virguliforme* quantitative polymerase chain reaction assay when 100 pg of genomic DNA were added to the reaction.

^b Asterisk (*) indicates unnamed *Fusarium* spp. nested within clade two of the *F. solani* species complex (34).

^c Ct values were determined by setting the threshold line at 0.1.

^d The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, United States Department of Agriculture—Agricultural Research Service.

^e Host plants for the microorganisms.

^f Species isolates obtained from the study published by Gachango et al. (12).

120 rpm for 3 to 4 days. Mycelia were collected by vacuum filtration on Miracloth (Calbiochem, Darmstadt, Germany) with a Buchner funnel, frozen, and lyophilized in sterile 2-ml microcentrifuge tubes overnight. Lyophilized mycelia (20 mg) were disrupted in 2-ml screw-cap tubes with one 6-mm ceramic bead and five 2-mm glass beads in a FastPrep FP120 Bio101 Savant machine (Qbiogene, Carlsbad, CA) at the speed setting 6 for 40 s. Mycelia were used for DNA extraction using the DNeasy Plant Mini kit (Qiagen, Germantown, MD). DNA concentration was determined with a Quant-iT double-stranded DNA (dsDNA) high-sensitivity assay kit (Invitrogen, Carlsbad, CA) on a 96-well SAFIRE microplate reader (TECAN, Männedorf, Switzerland).

Real-time PCR primer and probe design for *F. virguliforme*. The multicopy-number IGS region of the rDNA was chosen to design primers and probes. IGS rDNA sequences of *Fusarium* spp. (Table 1) were obtained from the National Center for Biotechnology Information GenBank database (FJ919498, FJ919499, FJ919507, FJ919510, FJ919511, FJ919512, FJ919515, and FJ919521). Eight IGS rDNA sequences from the soybean SDS and bean root rot (BRR) clade of *Fusarium* spp. (i.e., *F. virguliforme*, *F. tucumaniae*, *F. cuneirostrum*, *F. crassispitatum*, *F. brasiliense*, and *F. phaseoli*) were chosen for multiple sequence alignment to find the unique polymorphic regions of *F. virguliforme* using the multiple sequence comparison by log-expectation (MUSCLE) method (11). In addition, the sequence self-repeat structure was viewed using the 'dottup' package in EMBOSS (36) to avoid cross amplification within the IGS rDNA region. Primers were designed to amplify PCR product sizes of 50 to 150 bp. Polymorphic nucleotides unique to *F. virguliforme* were designed at the 3' end of the primers and the middle of probes. Melting temperature (T_m) for the primer oligos was predicted using the primer probe test tool in Primer Express 3.0 (Applied Biosystems, Carlsbad, CA). The secondary structure of the target amplicon was predicted with the 'mfold' software package (45) at the temperature of PCR annealing step and with the final [Mg²⁺] = 2 mM and [Na⁺] = 50 mM. The free energy (ΔG) was used to evaluate the stability of the amplicon secondary structure, and the PCR product with a higher ΔG was selected as test candidate for further evaluation. For the probe design, the following rules were applied: probe T_m 10°C higher than primer T_m; 15 to 30 nucleotides in length; avoid G on the 5' end; and place the mismatching nucleotides in the middle of the probe instead of both ends. The 5' end of the TaqMan probe (Life Technologies) for *F. virguliforme* was labeled with 6-fluorescein (6-FAM), and the 3' end was modified with a minor groove binder and nonfluorescent quencher. Other PrimeTime dual-labeled probes (IDT, Coralville, Iowa) were labeled with 5' 6-FAM, internal ZEN quencher, and 3' Iowa Black FQ quencher. The exogenous control HHIC assay primers and probe were ordered from IDT as describe by Haudenshield and Hartman (16).

qPCR amplification parameters. Real-time qPCR amplifications were performed on the ABI StepOnePlus thermocycler v2.3 (Applied Biosystems). Real-time qPCR was performed in a 20-μl total volume with at least two technical repeats. The qPCR mix consisted of 10 μl of TaqMan Universal real-time PCR master mix (2×) (Applied Biosystems), 2 μl of DNA template, 0.5 μl of FvPrb-3 (10 μM), 0.5 μl of F6-3 and R6 primers (20 μM), 0.6 μl of HHIC-F (20 μM) primer, 0.2 μl of HHIC-R (20 μM), 0.4 μl of HHIC-prb (10 μM), 0.5 μl of linearized HHIC DNA plasmid (10 fg/μl) (16), 0.4 μl of BSA at 10 mg/ml, and 4.4 μl of distilled water (Gibco, Grand Island, NY). qPCR conditions were initialized with 1 cycle of incubation at 50°C for 2 min to activate uracil-N-glycosylase, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min, with fluorescent data collection in the annealing and extension step.

Real-time PCR specificity and sensitivity tests. For the specificity tests, 100 pg of genomic DNA template of target and non-target species (Table 1) were tested against the designed assays.

Only the assays with specificity to *F. virguliforme* were evaluated in the validation step. To determine the PCR efficiency and sensitivity, a 10-fold *F. virguliforme* genomic DNA serial dilution from 10 ng to 1 fg was tested with the specific qPCR assay. PCR efficiency was calculated with the formula: efficiency = 10^(-1/slope) - 1 (slope was calculated from the linear regression between DNA log₁₀ [template DNA concentrations] and cycle threshold [Ct] values). *F. virguliforme* genomic DNA serial dilutions were diluted with salmon sperm DNA (Invitrogen) at 1 ng/μl to increase the stability of the low concentration DNA and protect against degradation or affinity to the plastic tubes.

Conventional PCR amplification parameters. Multiple primer sets were designed for the conventional PCR assay. To ensure ease of PCR amplification and product visualization in an agarose gel, the amplicon size was designed to be between 300 and 400 bp. Conventional PCR assays were performed with an ABI 2720 Thermal Cycler (Applied Biosystems). Conventional PCR was performed in a total volume of 25 μl, which included 2.5 μl of 10× DreamTaq DNA buffer (Thermo Fisher Scientific, Waltham, MA), 0.2 μl of 25 μM dNTPs (Promega Corp., Madison, WI), 1 μl of 10 μM primers (Sigma-Aldrich, St. Louis), 0.2 μl (5 U/μl) of DreamTaq DNA polymerase (Thermo Fisher Scientific), 0.5 μl of BSA at 10 mg/ml, 2 μl of DNA template, and distilled water to a total volume of 25 μl. PCR cycling conditions were set at 1 cycle at 94°C for 3 min; followed by 35 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C; and a final extension for 5 min at 72°C. PCR products were separated and visualized on a 1.5% agarose gel at 85 V for 45 min and stained with ethidium bromide at 1 μg/ml. Genomic DNA of *F. virguliforme* was serially diluted 1:10 from 10 ng to 1 fg to test the sensitivity of the conventional PCR assay. Also, 100 pg of genomic DNA from the specificity check panel (Table 1) was tested against the conventional PCR assay.

DNA extraction from soil. To determine the applicability of the qPCR assay to soil samples, DNA was extracted from artificially inoculated and naturally infested soil samples. In artificially inoculated soil samples, 0.5 g of soil was inoculated with 100 μl of 1:10 serially diluted *F. virguliforme* spore suspensions in sterile deionized water, with concentrations ranging from 10⁸ to 100 spores/ml. A half-gram of moist Riddles-Hillsdale sandy loam soil was placed into a 2-ml lysing matrix E tube with the FastDNA SPIN kit for soil (MP Bio, Solon, OH). After adding 978 μl of sodium phosphate buffer and 122 μl of MT buffer, the tubes were placed in a FastPrep FP120 instrument (MP Bio) and homogenized twice at speed 6 for 40 s. Subsequent steps were performed according to the manufacturer's recommendations; DNA was eluted with a final volume of 100 μl of DNase/pyrogen-free water (DES) (MP Biomedicals).

DNA extraction from soybean roots. Soybean roots with SDS-like foliar or root rot symptoms were collected from commercial fields and from samples submitted to the Michigan State University Diagnostic Services Laboratory. Roots were washed with tap water, rinsed with deionized water, and patted dry with a paper towel. Lateral or tap root tissues with obvious discoloration or lesions were preferred for DNA extraction; two technical replicates were made for each processed sample. Root tissue (100 mg) was added to a 2-ml lysing tube with five 1-mm-diameter glass beads and one 2-mm-diameter glass bead (except for samples processed in the Diagnostic Service Laboratory, where Lysing matrix A with two ceramic beads instead of the glass beads was used). AP1 buffer (400 μl) from the Qiagen Plant DNeasy mini kit (Qiagen) was added to each of the 2-ml lysing tubes and the mixture was homogenized in a FastPrep FP120 instrument (MP Bio) twice at speed setting 6 for 40 s. After homogenization, AP2 buffer (130 μl) was added to each tube and tubes were cooled on ice for 5 min, then spun at 16,000 × g for 6 min. Subsequent DNA purification steps were performed as per the manufacturer's instruction. DNA concentration was measured using the Quant-iT

dsDNA high-sensitivity assay kit (Invitrogen) on a 96-well SAFIRE microplate reader (TECAN).

Copy number of rDNA in *F. virguliforme*. Three single-copy genes— β -tubulin (HM453328.1), *G3PD* (glyceraldehyde 3-phosphate dehydrogenase), and *FvTox1* (JF440964.1)—were selected as single-copy reference genes to determine the rDNA copy numbers among *F. virguliforme* isolates collected from multiple soybean fields. These genes were confirmed as single copy by BLASTn search against the *F. virguliforme* genome sequence (41). Primers specific to each of these three genes were designed using Primer Express 3.0 (Applied Biosystems) (Table 2). The PCR efficiency of these three primer sets was evaluated by running a fivefold serial dilution of *F. virguliforme* genomic DNA ranging from 10 ng to 1 pg. Quantification of the rDNA and the three single-copy genes was performed using SYBR green based qPCR, which consisted of 10 μ l of 2 $\times$ SYBR Green PCR Master Mix (Life Technologies), 0.5 μ l of each primer (20 μ M), 0.2 μ l of BSA (20 mg/ml), and distilled water (Gibco) to a total-volume of 20 μ l in duplicate for each template DNA. Several genotypes were identified in a multilocus genotyping study of *F. virguliforme* (J. Wang and M. I. Chilvers, unpublished data). Therefore, to maximize the genetic diversity among the tested *F. virguliforme* isolates, multiple isolates from different genotype groups were included in the rDNA copy number variation test. PCR cycling conditions were set at 1 cycle at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C (fluorescent signal was collected at this stage). After the qPCR cycling stage, the melt curve stage was performed to determine the specificity of each primer set. The melt curve stage parameter was set as one step with 15 s at 95°C for dissociation, 60 s at 60°C for reassociation, and increase the temperature from 60 to 95°C with 0.3°C increments using the step and hold method.

Real-time PCR assay cross-laboratory and platform validation. Specificity and sensitivity of the qPCR assay was evaluated on a different platform, in collaboration with Michigan State University's Diagnostic Services Laboratory. The qPCR assay was performed on the Cepheid SmartCycler real-time PCR system (Cepheid, Sunnyvale, CA), in a 20- μ l total volume with two technical repeats. The qPCR master mix and cycling conditions

were the same as previously described and sensitivity was evaluated with serially diluted *F. virguliforme* genomic DNA from 10 ng to 10 fg with a 1:10 dilution factor. Specificity of this assay was tested against closely related *Fusarium* spp. (*F. tucumaniae*, *F. brasiliense*, *F. phaseoli*, *F. crassispitatum*, and *F. cuneirostrum*). Additionally, soybean samples from commercial fields submitted for disease diagnosis were tested with this assay. DNA extraction from soybean samples was performed as described above.

Data analyses. Real-time PCR data were collected and analyzed using StepOne Software v2.3. qPCR standard curve plots and DNA concentration correlations were plotted using the R ggplot2 package (42). The limit of detection was determined by 100% amplification of the lowest amount of target genomic DNA (8). Statistical analysis was performed with the R stats package.

RESULTS

Assay design and in silico screening. Based on the multiple sequence alignment conducted with MUSCLE, two polymorphic regions were identified as candidates for design of *F. virguliforme* specific assays. One of the polymorphic regions (from 481 to 777 bp) has a self-repeat (from 1,736 to 2,034 bp) in the IGS rDNA region (Supplemental Figure 1). Primers designed on the self-repeat region were determined to be less specific to *F. virguliforme* because of the ambiguous nucleotide mismatches with the self-repeat region. Instead, the other polymorphic region (between 1.2 and 1.4 kb) of the IGS rDNA with moderate polymorphism did not have any self-repeats and was chosen as the target to design primers and probes for the final assay. Multiple primers ($n = 35$) and dual-labeled probes ($n = 5$) were designed on the polymorphic sites. Before testing primer sets, the secondary structure of the designed PCR assay amplicons were predicted using the mfold (45) web server under PCR annealing conditions. The best amplicon candidates had higher free energy and fewer stem-loop structures, as demonstrated in the assay (Supplemental Figure 2).

Specificity and sensitivity of real-time PCR. In total, 30 primers and six probes were tested during assay development.

TABLE 2. Primers for determining ribosomal DNA intergenic spacer copy number variation

Names ^a	Sequences (5'–3')	Length (nt) ^b	Tm (°C) ^c	PCR efficiency (%)	Amplicon size (bp)
gpd_F	CTTGATGGCCTGCTTGATCTC	21	58.5	94.72	74
gpd_R	ACGTCTCCGTTGTCGACCTTA	21	58.3	...	...
b-tub_F	CTGCAGCAGCTTCATCATGAG	21	58.4	99.36	67
b-tub_R	CCGGTCTGGAGGTGAACCT	19	58.5	...	...
FvTox1_F	CAACAACACCCATCGCTAACG	21	60.0	95.12	68
FvTox1_R	GGGAGGACCCAGTTTTC	19	59.2	...	...

^a Abbreviations: gpd = designed based on the *Fusarium virguliforme* glyceraldehyde 3-phosphate dehydrogenase gene, b-tub = designed based on the β -tubulin gene, FvTox1 = designed based on the *FvTox1* gene, and *FvTox1* = phytoxin gene.

^b Length in nucleotides.

^c Melting temperature (Tm) values were calculated using ABI PrimerExpress 3.0 (ABI)—Primer Probe Test Tool.

TABLE 3. Primers and probes used in the quantitative polymerase chain reaction (qPCR) quantification assays

Names	Sequences (5'–3')	Length (nt) ^a	Tm (°C) ^b
F6-3 ^{c,e,f}	GTAAGTGAGATTTAGTCTAGGGTAGGTGAC	30	57.8
R6 ^{c,f}	GGGACCACCTACCCTACACCTACT	24	59.6
FvPrb-3 ^{c,f}	6FAM-TTTGGTCTAGGGTAGGCCG-MGBNFQ	19	70.0
R9 ^{c,e}	CCATCCGCTCTGGGAATTTTAACTA	24	59.3
HHIC-F ^d	CTAGGACGAGAACTCCACAT	21	54.6
HHIC-R ^d	CAATCAGCGGGTGTTCAT	18	55.0
HHIC-prb ^d	5HEX-TCGGTGTGATGTTTGCCATGGT-3IABkFQ	23	65.2

^a Length in nucleotides.

^b Melting temperature (Tm) values were calculated using ABI PrimerExpress 3.0—Primer Probe Test Tool.

^c Primers and probes developed in this study.

^d Primers and probes developed by Haudenschild and Hartman (16).

^e Primers used for conventional PCR assay.

^f Primers and probes used for qPCR assay.

One primer set (F6-3 and R6) (Table 3) amplifying a 76-bp product from *F. virguliforme* and one TaqMan probe (FvPrb-3) (Table 3) were determined to be the most specific and sensitive primer and probe combination. The qPCR assay detected all tested target *F. virguliforme* strains at 100 pg of DNA with a Ct value range between 20.53 and 21.78, while the Ct value for the other nontarget species were all above the preset Ct values of the lower limit of detection (LOD – Ct = 30) (Table 1). There was a linear relationship ($y = -3.3x + 36$, $R^2 = 0.996$) between *F. virguliforme* genomic DNA serial dilutions (log transformed) and Ct values, and the corresponding PCR efficiency was 99% (Fig. 1). The genomic DNA serial dilution standard curve indicated that the qPCR assay consistently detected 10 fg of *F. virguliforme*

Fig. 1. Standard curve for absolute quantification of *Fusarium virguliforme* genomic DNA (fg). Genomic DNA samples were prepared from pure cultures grown in broth. The detection limit for pure culture genomic DNA was 100 fg. Two technical repeats for each *F. virguliforme* genomic DNA dilution level. PCR = polymerase chain reaction.

Fig. 2. Quantitative polymerase chain reaction standard curve plotted with serially diluted *Fusarium virguliforme* genomic DNA (log transformed) in femtograms against cycle threshold values with solid circles, open circles indicate nontarget species as indicated in Table 1. Sensitivity of the assay was determined to be 100 fg of *F. virguliforme* genomic DNA.

genomic DNA (Ct = 33.2). The cut-off Ct value threshold for positive amplification was set at 100 fg of *F. virguliforme* genomic DNA to remove nonspecific background amplifications (Fig. 2). In the artificially inoculated soil samples, our qPCR assay consistently detected 100 conidia artificially inoculated in 0.5-g soil samples (Fig. 3).

Conventional PCR assay. The conventional PCR assay developed is specific to *F. virguliforme*, amplifying a 375-bp DNA fragment. The conventional PCR was specific to *F. virguliforme* except for weak amplification of two nontarget *Fusarium* spp., *F. crassistipitatum* and *F. brasiliense* (product size = 375 bp). To overcome the nonspecific amplifications, the annealing temperature was raised from 60 to 65°C, which successfully eliminated amplification of these nontarget species (Supplemental Figure 3). The detection limit of the conventional PCR was determined to be 1 pg of *F. virguliforme* genomic DNA (Supplemental Figure 4).

Validation of real-time PCR assay and conventional PCR assay. Both conventional PCR and real-time PCR were used to diagnose soybean plants collected from commercial soybean fields in Michigan from 2011 to 2013. Based on the diagnostic results (Table 4), SDS-like symptomatic soybean plants were not always associated with the positive detection of *F. virguliforme* DNA from soybean root tissues. For example, samples collected from Midland County, MI (Midland-12-02) in 2012 were detected to be SDS negative but tested positive for *P. gregata*, the causal agent of BSR, with the BSR diagnostic qPCR assay (27). However, three plant samples with nontypical SDS foliar interveinal symptoms (MISO2-3, GR-12-02, and SA-11-2a) were determined to have positive *F. virguliforme* infection (Ct values < 30). Using the conventional PCR assay, most plant samples (27 of 38) were diagnosed with definitive results, as indicated with intense bands. However, the sensitivity of the conventional PCR assay was limited, potentially causing false-negative results for 11 of 38 samples. In contrast, the qPCR assay gave unambiguous diagnostic results by simultaneously utilizing quantitative Ct values and a reliable exogenous control assay. In diagnostic samples with abundant *F. virguliforme* DNA, the exogenous control may perform abnormally (e.g., no exponential amplification or early amplifications). The exogenous control assay began to be affected when the *F. virguliforme* genomic DNA reached 1 pg or more in the diagnostic samples (Supplemental Figure 5).

rDNA IGS copy number variation between isolates. Amplification efficiencies of the three single-copy genes were all very similar at 95, 99, and 95% for the G3PD, β -tubulin, and FvTox1

Fig. 3. Quantitative polymerase chain reaction (qPCR) quantification of DNA samples isolated from artificially inoculated soil samples with serially diluted *Fusarium virguliforme* macroconidia suspension. Detection limit was 100 macroconidia per 0.5 g of soil. Total soil DNA were isolated from six soil sample replicates, and qPCR was run twice for each soil DNA sample.

assays, respectively. Less than 5% difference in efficiency exists between the qPCR assay and the single-copy gene assays, indicating a valid rDNA copy number analysis. Melt curve analysis of the SYBR green PCR product detected a single peak for each primer set, demonstrating amplification of a single product. The PCR efficiency of the qPCR assay was calculated using the formula $IGS\ copy = (1 + 0.994)^{\Delta Ct}$, (e.g., ΔCt is the difference between Ct values of two amplified genes), given the PCR efficiency of 99.36%. The IGS rDNA copy numbers of *F. virguliforme* ranged from 138 to 233, with an average of 208 per haploid genome (Table 5).

qPCR assay cross-laboratory and platform validation. The qPCR assay performed similarly on both platforms tested. Assay sensitivity was determined to be 100 fg of *F. virguliforme* DNA and the assay was determined to be specific to this species. PCR efficiency of the *F. virguliforme* qPCR assay on the SmartCycler real-time PCR system and the StepOnePlus real-time PCR system was 99.8 and 99.0%, respectively. Diagnostic samples (16 soybean and 1 dry bean) were submitted from 14 counties in Michigan; 8 samples were diagnosed as positive for *F. virguliforme* with the qPCR assay (Table 6). Of the 17 diagnostic samples tested on the SmartCycler system, 4 were also tested on the StepOnePlus system, producing comparable results. Ct values of

DNA samples extracted from taproot versus lateral root were not significantly different ($P > 0.05$).

DISCUSSION

Soybean SDS is a major threat to soybean production in the United States. Specific and sensitive molecular diagnostic assays are lacking, which hampers diagnosis and limits epidemiological studies of the disease. To address this need, we developed a specific and sensitive qPCR assay to quantify *F. virguliforme* in soybean tissue, soil, and environmental samples. The assay was specific to *F. virguliforme* when tested against closely related *Fusarium* spp. and other commonly encountered fungi in soybean fields. The assay is very sensitive and can detect as little as 100 fg of *F. virguliforme* genomic DNA or 100 macroconidia per half-gram of soil. An exogenous control was multiplexed with the *F. virguliforme* assay to detect false negatives and monitor for the presence of PCR inhibitors. In addition, the assay was validated with soybean samples collected from commercial soybean fields. The qPCR assay is transferable and successfully performed on a Cepheid SmarterCycler real-time PCR platform at the North-Central region's hub laboratory of the National Plant Diagnostic Network.

TABLE 4. Diagnostic results for commercial soybean samples on StepOnePlus real-time polymerase chain reaction (PCR) system^a

Sample ID	County ^b	Isol ^c	CPCR	qPCR	Fv Ct1 ^d	Fv Ct2	HHIC Ct1 ^e	HHIC Ct2	Description of symptoms ^f
Coleman	Midland	NEG	POS	POS	15.64	14.90	UD	UD	Foliar SDS symptoms, leaf chlorosis and necrosis
Razjer	Van Buren	NEG	NEG	NEG	UD	UD	UD	UD	Root rot
Avery	Van Buren	NEG	?	POS	29.48	31.36	29.48	28.64	SDS-like foliar symptoms
EA-13-1	Eaton	POS	POS	POS	31.42	29.55	28.26	28.21	SDS-like foliar symptoms discoloration of taproot
MtC-13-1	Montcalm	POS	POS	POS	29.59	27.76	28.23	28.15	SDS-like foliar symptoms
IN-13-1	Ingham	NEG	NEG	NEG	32.78	31.31	28.10	28.09	White mold symptoms and chlorosis on leaves
MISO2-7	Midland	NEG	NEG	NEG	31.38	31.27	29.42	29.47	Foliar symptom on the lower part plant, possible herbicide
CL-12-01	Clinton	NEG	NEG	NEG	34.97	33.44	29.04	29.31	Foliar SDS-like symptom, but not typical
IO-12-01	Ionia	NEG	NEG	NEG	34.62	33.55	29.25	29.23	Foliar chlorosis symptom
EA-12-01	Eaton	NEG	?	NEG	32.33	32.28	29.08	29.21	no SDS foliar symptoms
TU-12-01	Tuscola	NEG	?	NEG	32.93	30.88	29.27	29.18	Manganese deficiency symptoms
GR-12-01	Gratiot	POS	POS	POS	31.34	24.15	29.34	28.93	Typical SDS-like symptoms, wilting
IS-12-01	Isabella	NEG	NEG	NEG	34.67	34.52	29.16	28.99	no SDS foliar symptoms
MISO2-3	Allegan	NEG	POS	POS	29.68	26.78	29.22	29.06	Both oomycete and fungal wilting symptom
IN-12-01	Ingham	NEG	NEG	NEG	33.18	35.22	29.20	29.39	Interveinal chlorosis, lack of hairy roots, may be BSR pith dark brown, but BSR assay negative
GR-12-02	Gratiot	NEG	POS	POS	33.09	27.96	29.22	28.95	Wilting and leaves chlorosis
Midland-12-02	Midland	NEG	?	NEG	35.52	34.88	29.29	29.13	SDS-like foliar symptoms, brown pith, BSR assay positive
DL201203875	Monroe	POS	POS	POS	18.59	—	UD	—	Brown stem, leaves discoloration
EA-12-02	Eaton	NEG	?	NEG	32.71	31.7	29.13	29.236	Phytoplasma symptoms, but no SDS symptoms
SA-11-2a	Saginaw	POS	POS	POS	20.55	—	*23.24	—	Plant wilting, defoliation, and Dead
SA-11-2b	Saginaw	NEG	POS	POS	21.16	—	UD	—	Foliar SDS-like symptom
HU-11-1b	Huron	POS	POS	POS	21.50	—	UD	—	Foliar SDS-like symptom
HU-11-1 (DL-6)	Huron	POS	POS	POS	21.89	—	*23.90	—	Foliar SDS-like symptom
DL-5	Lenawee	POS	POS	POS	20.21	20.83	UD	UD	Foliar SDS-like symptom
SA-11-1	Saginaw	POS	POS	POS	24.32	25.84	28.18	27.766	Foliar SDS-like symptom
CL-11-1	Clinton	POS	POS	POS	24.00	—	*26.97	—	N/A
GR-11-2b	Gratiot	POS	POS	POS	28.22	—	28.2	—	Foliar SDS-like symptom
CL-11-2	Clinton	NEG	POS	POS	28.23	—	28.09	—	N/A
GR-11-2a	Gratiot	NEG	POS	POS	28.03	—	28.31	—	Plant Defoliation and Dead
IN-11-1	Ingham	NEG	POS	POS	29.75	—	29.29	—	Foliar SDS-like symptom
TUS-11-2a	Tuscola	NEG	?	NEG	33.58	—	28.43	—	Leaf burn/ herbicide/desiccant damage
TUS-11-2b	Tuscola	NEG	?	NEG	33.38	—	28.09	—	Foliar SDS-like symptom
TUS-11-1b	Tuscola	NEG	?	NEG	33.85	—	28.08	—	Stunted beans/yellowing/root rot
HU-11-2	Huron	NEG	?	NEG	32.55	—	28.34	—	SDS-like symptoms
TUS-11-1a	Tuscola	NEG	?	NEG	33.79	—	28.18	—	N/A
DL-4	Kalamazoo	NEG	?	NEG	34.71	33.94	28.32	28.476	Foliar SDS-like symptom
TUS-11-3	Tuscola	NEG	NEG	NEG	34.35	—	28.14	—	Foliar SDS-like symptom

^a Results include isolation on semiselective media, conventional PCR (CPCR), plant symptoms, and quantitative PCR (qPCR) cycle threshold (Ct) values. NEG = negative results, POS = positive results, UD = undetected, ? = unclear results (i.e., weak band), and — = not applicable.

^b County in Michigan from which samples were collected.

^c Positive isolation (Isol) was identified by colony morphology.

^d Two DNA extractions were made for qPCR detection.

^e Internal control to assess the potential PCR inhibitors; * indicates abnormal Ct values affected by *F. virguliforme* assay competition.

^f SDS = sudden death syndrome and BSR = brown stem rot.

The choice of genetic locus and in silico sequence analysis plays a pivotal role in development and performance of a qPCR assay. Revision of the taxonomy of *Fusarium* spp. that cause SDS or BRR has resulted in the obsolescence of previously developed assays. However, the taxonomic revision and loci used for phylogeny construction also provided an opportunity for the development of more specific diagnostic assays. The phylogenetic tree constructed with IGS rDNA sequences explicitly separated the *Fusarium* spp. in the SDS-BRR clade with high branch support (33). Therefore, the IGS rDNA was determined to be a suitable target locus to design an assay for the specific detection of *F. virguliforme*. The multicopy nature of the IGS rDNA target was also desirable from the standpoint of assay specificity.

An optimized multiple sequence alignment is essential for identifying suitable polymorphic regions within a locus for species-specific primer and probe design. Different sequence alignment methods (MUSCLE, MAFFT, ClustalW, and T-Coffee) were used to align the IGS rDNA sequence of SDS-BRR clade *Fusarium* spp. The multiple sequence alignments of the IGS rDNA loci required the insertion of a considerable number of gaps, which increased the complexity of the alignment. Alignments with ClustalW (22) and T-Coffee (31) resulted in alignments that were not optimal for locating polymorphic loci. However, the use of methods such as MUSCLE (11) and MAFFT (20) resulted in the identification and validation of polymorphisms suitable for specific species discrimination.

Sensitivity is also a significant factor that determines the applicability of a qPCR assay. For the previous qPCR assays designed for *F. virguliforme*, two targeted the small subunit of the mtDNA and the other was designed on the *FvTox1* toxin-coding gene (14,24,29). Although the limit of detection for the mtDNA assays was 90 fg of genomic DNA, these two assays are not specific to *F. virguliforme* based on the discovery of four SDS and three BRR *Fusarium* pathogens (4,5). The assay designed to the *FvTox1* is specific; however, the assay is not as sensitive, because it requires 25 pg of genomic DNA. We designed our qPCR assay to the IGS rDNA region, and the limit of detection was determined to be 100 fg of genomic DNA (approximately equivalent to the DNA quantity of four haploid genomes of *F. virguliforme*), which is 100 times more sensitive than the single-copy *FvTox1* gene assay.

rDNA copy number variation and IGS rDNA sequence variation among isolates have been reported in several fungal species (2,6,18). Variation in rDNA copy number within species may cause variability in qPCR assay sensitivity. IGS rDNA sequence variation within species may affect the specificity of the qPCR

assay as well. In this study, we tested our qPCR assay with numerous isolates of *F. virguliforme* obtained from isolates representing the primary genetic groups (J. Wang and M. I. Chilvers, unpublished data) from geographically distant locations within the United States. Although the rDNA copy number ranged from 138 to 233, with an average number of 208 copies per haploid genome, the largest difference in Ct value was 0.76 cycles, which is within the same order of magnitude. Therefore, the differences in rDNA IGS copy numbers did not cause significant differences in qPCR quantification or diagnostic detection.

Although improved sensitivity and specificity are ideal, consistent performance in dealing with real-world diagnostic soybean samples was of primary concern. The molecular diagnostic assay results are strong evidence in determining a diagnostic conclusion, especially when the submitted plant samples lack other specific symptoms or signs. Because of PCR inhibitors in plant DNA or plant tissue sampling bias, the molecular diagnostic assay detection results can be inconsistent (Table 5). To avoid the potential inconsistent detection of *F. virguliforme* during diagnostics, we implemented PCR additives (e.g., BSA), which alleviated the PCR inhibition effects. Multiple ($n \geq 2$) biological and technical repeats are recommended to improve consistency in the diagnostic assay. Normally, without competition or inhibition, the HHIC exogenous control assay will produce a Ct value of 29. However, inhibitors in the DNA samples can prevent PCR amplification for the *F. virguliforme* assay and exogenous control assay. To overcome the PCR inhibition issues, magnetic bead-based DNA sample purification can be used to reduce PCR inhibitors in DNA samples. Furthermore, PCR inhibitions are not the only cause for lack of amplification in the exogenous control assay. Too much *F. virguliforme* genomic DNA in the sample can also cause no amplification or malfunction of the HHIC exogenous control assay. When the *F. virguliforme* genomic DNA reaches 1 pg in the reaction, the HHIC exogenous control assay is affected. Therefore, interpretations of the HHIC exogenous control results help evaluate the performance of the *F. virguliforme* assay.

Stable performance across labs is one of the most important criteria of a qPCR diagnostic assay. The performance of a qPCR assay can be vulnerable to changes in the PCR reagents or real-time PCR systems (17). It may require efforts to optimize the qPCR conditions to perform a robust qPCR assay with the desired performance. Use of a qPCR assay on a new platform, change in PCR master mix, or use in a new lab should always be implemented with the appropriate validation. To explore the applicability of our qPCR assay on another real-time PCR systems, we

TABLE 5. *Fusarium virguliforme* ribosomal DNA (rDNA) intergenic spacer (IGS) copy number estimation using three single copy reference genes

Isolates ^a	Mean Ct difference between IGS and single-copy gene ^b			Mean Ct difference ^c	Copy number ^d
	Btub-IGS	FvTox1-IGS	G3PD-IGS		
INMO_A6	7.57	7.64	7.66	7.62	190
INMO_C5	7.69	7.53	7.76	7.66	195
KSSH_E5	7.83	7.54	7.84	7.74	205
MIBer_A6	7.59	7.51	7.71	7.60	187
MIBer_B7	7.76	7.56	7.83	7.72	202
MIBer_E1	7.99	7.76	8.02	7.92	233
MIIN_B7	7.89	7.67	7.93	7.83	219
MISA_C4	7.30	7.26	7.50	7.36	158
MISTJ_E4a	7.80	7.71	8.06	7.86	223
MISTJ_G5	8.02	7.66	8.04	7.91	231
MITU_B4	7.90	7.79	8.03	7.91	231
MIVB_A5	7.21	7.02	7.25	7.16	138
22292-Mont1	7.54	7.56	7.87	7.66	194

^a *F. virguliforme* isolates collected from Indiana (INMO), Kansas (KSSH), and Michigan (MIBer, MIIN, MISA, MISTJ, MITU, and MIVB).

^b Cycle threshold (Ct) differences between IGS rDNA assay and single-copy gene assay. Btub = β tubulin gene, FvTox1 = phytotoxin gene produced by *Fusarium virguliforme*, and G3PD = glyceraldehyde 3-phosphate dehydrogenase gene.

^c Average Ct for all three single copy genes.

^d Copy number determined by mean Ct differences.

TABLE 6. Soybean and dry beans samples submitted to Michigan State University Diagnostic Services Laboratory for diagnosis assayed on a SmartCycler real-time polymerase chain reaction system

Sample ID	Root parts ^a	County ^b	Dilution ^c	Ct ^d	Results
2192	Lateral	Branch	1	19.32	Positive
	Lateral	Branch	1:10	22.74	...
	Tap	Branch	1	14.12	...
	Tap	Branch	1:10	18.48	...
2193	Lateral	Branch	1	29.22	Positive
	Lateral	Branch	1:10	33.25	...
	Tap	Branch	1	26.31	...
	Tap	Branch	1:10	29.73	...
2198	Lateral	Lenawee	1	26.42	Positive
	Lateral	Lenawee	1:10	30.19	...
	Tap	Lenawee	1	23.24	...
	Tap	Lenawee	1:10	27.11	...
2371	Lateral	Hillsdale	1	19.44	Positive
	Lateral	Hillsdale	1:10	23.17	...
	Tap	Hillsdale	1	22.32	...
	Tap	Hillsdale	1:10	26.09	...
2905 ^e	Lateral	Huron	1	37.99	Negative
	Lateral	Huron	1:10	UD	...
	Tap	Huron	1	UD	...
	Tap	Huron	1:10	UD	...
3006	Lateral	Allegan	1	24.41	Positive
	Lateral	Allegan	1:10	23.97	...
	Tap	Allegan	1	19.66	...
	Tap	Allegan	1:10	23.51	...
2603	Lateral	Shiawasee	1	UD	Negative
	Lateral	Shiawasee	1:10	UD	...
	Tap	Shiawasee	1	UD	...
	Tap	Shiawasee	1:10	UD	...
2591	Lateral	Clinton	1	UD	Negative
	Lateral	Clinton	1:10	UD	...
	Tap	Clinton	1	UD	...
	Tap	Clinton	1:10	UD	...
3059	Lateral	Ingham	1	18.97	Positive
	Lateral	Ingham	1:10	23.07	...
	Tap	Ingham	1	19.36	...
	Tap	Ingham	1:10	22.81	...
3062	Lateral	Van Buren	1	32.22	Negative
	Lateral	Van Buren	1:10	36.39	...
	Tap	Van Buren	1	37.13	...
	Tap	Van Buren	1:10	UD	...
3240	Lateral	Tuscola	1	36.41	Negative
	Lateral	Tuscola	1:10	UD	...
	Tap	Tuscola	1	34.37	...
	Tap	Tuscola	1:10	UD	...
3415	Lateral	Tuscola	1	35.25	Negative
	Lateral	Tuscola	1:10	38.65	...
	Tap	Tuscola	1	37.56	...
	Tap	Tuscola	1:10	UD	...
3505	Lateral	Van Buren	1	18.98	Positive
	Lateral	Van Buren	1:10	22.28	...
	Tap	Van Buren	1	17.48	...
	Tap	Van Buren	1:10	20.45	...
3525	Lateral	Saginaw	1	UD	Negative
	Lateral	Saginaw	1:10	UD	...
	Tap	Saginaw	1	37.97	...
	Tap	Saginaw	1:10	UD	...
3851	Lateral	Lapeer	1	31.08	Negative
	Lateral	Lapeer	1:10	35.28	...
	Tap	Lapeer	1	31.48	...
	Tap	Lapeer	1:10	34.21	...
3872	Lateral	Arenac	1	UD	Negative
	Lateral	Arenac	1:10	UD	...
	Tap	Arenac	1	39.22	...
	Tap	Arenac	1:10	UD	...
4532	Lateral	Lenawee	1	26.73	Positive
	Lateral	Lenawee	1:10	31.16	...
	Tap	Lenawee	1	36.86	...
	Tap	Lenawee	1:10	UD	...

^a Root portion used for DNA extraction.

^b Michigan county.

^c DNA dilution level from the original DNA extraction.

^d Ct = cycle threshold and UD = undetected.

^e Dry bean samples.

collaborated with the Michigan State University Diagnostic Services Laboratory. Assay performance was tested on a Cepheid SmartCycler real-time PCR system, which is the most popular system in the North-Central region of the National Plant Diagnostic Network. Without changing the reagents or cycling parameters, this assay successfully achieved the desired performance (specificity, sensitivity, and PCR efficiency), demonstrating high transferability of this assay between platforms. In addition, the Michigan State University Diagnostic Services Laboratory used this assay for the diagnostic evaluation of soybean and dry bean plants with SDS or root rot symptoms (Table 6). Although both taproot and lateral root tissues were used for DNA extractions, the detection results (Ct values) were not significantly different.

The *F. virguliforme*-specific qPCR assay presented here allows for the rapid and accurate quantification of *F. virguliforme*, which will facilitate epidemiological studies and diagnosis of this pathogen in plant, soil, and environmental samples. The assay may be used to screen for root rot resistance where visual estimation or culture-based methods are difficult or not practical. In addition, the assay may be used to potentially predict the preplant risk of SDS development if *F. virguliforme* inoculum thresholds can be established.

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