Detection and Quantification of *Phialophora gregata* in Soybean and Soil Samples with a Quantitative, Real-Time PCR Assay

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ABSTRACT

Malvick, D. K., and Impullitti, A. E. 2007. Detection and quantification of *Phialophora gregata* in soybean and soil samples with a quantitative, real-time PCR assay. *Plant Dis.* 91:736-742.

Brown stem rot of soybean, caused by the soilborne fungus *Phialophora gregata*, is a common and widespread disease of soybean (*Glycine max*) in the midwestern United States. This pathogen is challenging to study due to a long latent period and slow growth. A TaqMan probe-based quantitative, real-time polymerase chain reaction (qPCR) assay was developed for sensitive and specific detection and quantification of genotypes A and B of *P. gregata* in plant and soil samples. It is sensitive with detection limits of 50 fg of pure genomic DNA, 100 copies of the target DNA sequence, and approximately 400 conidia. The qPCR assay is approximately 1,000 times more sensitive in detecting DNA and conidia of *P. gregata*, and is more rapid and less sensitive to PCR inhibitors from soybean stems than a standard PCR (sPCR) assay. Using this single-step qPCR assay, low levels of infection were detected in soybean stems at least 1 to 2 weeks prior to symptom development and before *P. gregata* was detected with sPCR. This assay also was used to detect the pathogen in field-grown plants and in naturally infested field soils. This new qPCR assay is a powerful tool for rapid, specific, and sensitive detection, diagnosis, and quantification of *P. gregata* in plants and soil, and for advancing studies of the ecology of *P. gregata* and its interactions with host plants.

Soybean (*Glycine max* L.) is produced on approximately 60 million acres in the north central region of the United States. Many diseases play an important role in productivity of soybeans in this region, and brown stem rot (BSR) is particularly common (7,10,16,38). The disease was detected in 73, 72, and 68% of soybean stems sampled from the three top soybean producing states of Illinois, Iowa, and Minnesota, respectively (37). Recent surveys indicate that BSR continues to be common in Minnesota and many other major soybean producing states (1,38).

Brown stem rot is a vascular disease of soybeans caused by the soilborne fungal pathogen *Phialophora gregata* f. sp. *sojae* (Allington & Chamberlain) Gams. BSR causes premature defoliation and yield loss (10). When BSR was first reported in central Illinois in the 1940s, it resulted in significant crop loss (2). Notable losses from BSR occurred frequently until genetic resistance was widely deployed in soybean varieties (2,10). Significant levels of disease continue to occur in scattered areas across the northern tier of the north central region; however, the overall impact of this disease is largely undefined due to differ-

ent types and symptoms of the disease and to varying levels of resistance and tolerance among varieties that are affected differentially by fungal populations and the environment.

Two different types of BSR, characterized by dissimilar symptoms, frequently occur (11). Type "LS" is characterized by interveinal leaf necrosis combined with internal stem browning in the pith, whereas type "S" is characterized by internal stem browning only with minimal or no leaf necrosis. Whether type LS or type S develops appears to be controlled primarily by different genotypes of *P. gregata* and by temperature and soil moisture (10,12). Two genotypes (subspecific populations) of *P. gregata* produce different symptoms in infected plants (11,15,20,22). Genotype A typically causes type LS symptoms, and genotype B typically causes only type S symptoms. The two genotypes can be distinguished based on sequence differences in the intergenic spacer (IGS) region of rDNA that can be determined with specific standard polymerase chain reaction (sPCR) assays (3). Infection can be latent for months with both genotypes, e.g., from infection in May or June until symptoms develop in August. The type LS stage of BSR continues to develop at low to high incidence levels in the northern states, but the type S stage may be equally or more common (15,23). The type S stage likely occurs much more than is recognized due to the effort required to split stems for field diagnosis. New diagnostic and detection meth-

ods based on the sPCR have markedly enhanced our abilities to readily distinguish BSR from other stem infections and to detect different genotypes of *P. gregata* (4,23).

P. gregata is a challenging fungus to study. It grows slowly, can be difficult to isolate from infected tissues, and due to long latent periods, infected plants typically do not develop symptoms until at least 5 weeks after inoculation in the greenhouse or until after growth stage R3 in the field. A semiselective medium was developed for isolating *P. gregata*, but is not sufficiently selective to prevent growth of other fungi found in soybean stems and soil (14,26,32). The sPCR test has improved diagnosis of BSR and is a great aid in distinguishing the two genotypes, but is not quantitative and is limited in sensitivity. Advanced methods are needed that are quantitative as well as highly sensitive for detection and rapid diagnosis, for studies of the infection process, and for elucidating endophytic and pathogenic interactions between *P. gregata* and soybean.

Quantitative, real-time PCR (qPCR) is a powerful, specific, and sensitive tool for detection of fungi and for investigating quantitative interactions between fungi and plant hosts (8,9,18,28–30,34,36,39). This tool can determine the copy number of a specific target gene or DNA molecule from a fungal species, which can potentially be correlated to fungal biomass or population (8,9,31). Compared with sPCR, qPCR can have greater specificity and sensitivity, is faster, requires less labor, and can potentially detect multiple organisms simultaneously (29,30).

Thus, qPCR has much potential to advance work with detection, diagnosis, and infection of soybean by *P. gregata* and other pathogens. The primary objective of this work was to develop a quantitative, real-time PCR (qPCR) assay for specific and sensitive detection and quantification of *P. gregata* in plants and soil. The secondary objective was to compare qPCR and sPCR for their relative abilities to amplify and detect fungal DNA in plants and soil. This is the first complete report of a qPCR assay for specific and sensitive detection of *P. gregata* in plants and soil. This technique allows one to circumvent some of the challenges and problems with long infection periods and difficult isolation of *P. gregata*, while being quantitative and sensitive to low target DNA quantities.

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Accepted for publication 9 January 2007.

MATERIALS AND METHODS

Development of primers and probe for qPCR. Forward and reverse primers and a TaqMan minor groove binding (MGB) probe were developed for qPCR analysis of *P. gregata*. Primer and probe sequences were based on a previously reported 1,020-bp sequence from the *P. gregata* f. sp. *sojae* nontranscribed intergenic spacer (IGS) region of the nuclear rDNA (3). Sequences for the primers and probe were selected using default parameters with Primer Express software (Applied Biosystems [ABI], Foster City, CA). The code name and sequences for the forward primer, reverse primer, and TaqMan probe were BSRqPCRf1 (5'-CAAACCAGGGCCGAT CAG-3'), BSRqPCRr1 (5'-CGGATTCAG CGTAAAAATGG-3'), and BSRqPCRpb1 (5'-6FAM-CTCCCGTATGGTTTCT-MGB NFQ-3'), respectively. Using a nucleotide-nucleotide BLAST search from the National Center for Biotechnology Information (Bethesda, MD), all sequences in the database similar to the primer and probe were unique to *P. gregata*. The TaqMan probe was obtained from ABI and the primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Plant inoculations and sampling. Five seeds of a BSR-susceptible cultivar (AG2302) were sown in six 15-cm-diameter (6-in.) pots containing soil, sand, and perlite (40:40:20, vol/vol/vol). The soil was watered daily and pots were maintained at 22 to 24°C with a 14-h photoperiod in a greenhouse. Inoculum was prepared by growing the *P. gregata* isolates (genotype A, isolates 98F1-2 and 98G1-3) in soybean broth (18 g dry soybeans per liter of water) for 16 days at 23°C. Cultures were macerated by blending for 60 s, conidia and hyphal fragments were counted, and the inoculum suspension was adjusted to 5×10^6 CFU/ml. Treatments consisted of plants in three replicate pots inoculated by injection of 50 µl in the base of the stem at the soil line 3 weeks after sowing. The checks were injected with 50 µl of sterile soybean broth. Two samples from the apex of the inoculated stems in each pot were harvested 2 and 4 weeks after inoculation, and stems were split to assess symptom development. DNA was extracted from stem tissues as noted below.

DNA isolation from fungal, plant, and soil samples. Fungal samples grown as noted in the following section were homogenized in a FastPrep instrument (QBiogene, Irvine, CA), and DNA extractions were performed as outlined by the manufacturer of the FastPrep FastDNA kit. Before eluting the DNA from the glass beads in this system, the beads were vacuum-dried to standardize volumes. Extracted DNAs were diluted 1:10 for all reactions, which produced DNA concentrations that ranged from 10 to 1×10^{-6} ng/µl. Total DNA was routinely extracted from infected and noninfected soybean stems at

the R7/R8 growth stage by grinding dry stems with a Wiley Mini mill (Thomas Scientific, Swedesboro, NJ) and using a modified version of the FastDNA protocol (24). The mill was cleaned between samples with a stream of pressurized air. DNA was extracted from soil using the MoBio PowerSoil DNA isolation Kit (MoBio Laboratories, Carlsbad, CA). To determine if DNA extracts from the stem samples contained PCR-inhibitors, the β-actin gene from *Meloidogyne javonica* and specific primers and probes for this gene were used in inhibition tests (9) with DNA extracts (24) from ground stem samples from 13 different plants infected with BSR. In addition, a set of 18 soybean stem samples were extracted using the manufacturer's standard protocol for the FastDNA kit and with a modified protocol that removes additional PCR inhibitors and can increase the detection of fungal DNA from plants (9,24).

Testing specificity of primer and probe set. To determine whether the primers and probe were specific for amplification and detection of *P. gregata*, DNA was extracted as noted above from standardized quantities of mycelium from 30 isolates of fungi and oomycetes, consisting of 14 genera and 24 species (Table 1), which were grown in nutrient broth media for 7 to 16 days at 22°C. The DNA samples

were diluted 1:10 and tested (~10 ng per reaction) for amplification with qPCR. In addition, DNA was extracted from two soybean stems from plants with symptoms of charcoal rot, anthracnose, and root rot grown in a field near Urbana, IL with no history of BSR in order to confirm that the primers and probe would not amplify DNA from field-grown plants containing common soybean-infecting fungi. In addition, in order to confirm that *P. gregata* was present in plants tested as positive with the *P. gregata*-specific qPCR assay, *P. gregata* was isolated from three randomly selected plants by plating surface-disinfested stem tissue (1 min in 0.25% sodium hypochlorite) on green bean agar (3).

qPCR analysis. Analysis of samples with qPCR was conducted with an ABI 7000 sequence detection system. Reactions were conducted in a volume of 25 µl that contained 5 µl of the DNA sample, 12.5 µl TaqMan universal PCR master mix (ABI), 200 nM probe, 450 nM each of the forward and reverse primers, and 2.75 µl of molecular grade water. Thermal cycling parameters consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Ramp rates were 0.8°C/s up and 1.6°C/s down. Cycle threshold (Ct) values, which are inversely proportional to detected DNA content, were calculated using ABI soft-

Table 1. Fungal genera and species that were used to test specificity of a TaqMan primer and probe designed to specifically detect *Phialophora gregata*^a

Species and isolate	Origin	Detection ^b
<i>Colletotrichum</i> sp.	Illinois	ND
<i>Colletotrichum acutatum</i>	Illinois	ND
<i>Chaetomium cochliodes</i>	Illinois	ND
<i>Fusarium solani</i> (91-31-2)	North Dakota	ND
<i>F. oxysporum</i>	Illinois	ND
<i>F. equiseti</i>	Illinois	ND
<i>F. solani</i> f. sp. <i>glycines</i> M100	Illinois	ND
<i>F. solani</i> f. sp. <i>pisi</i> F1	Illinois	ND
<i>F. solani</i> f. sp. <i>pisi</i> F46	Illinois	ND
<i>Gaeumannomyces graminis</i> f. sp. <i>graminis</i>	Illinois	ND
<i>G. graminis</i> f. sp. <i>tritici</i>	Illinois	ND
<i>Leptosphaeria maculans</i>	Illinois	ND
<i>Macrophomina phaseolina</i> Dnt103s	Illinois	ND
<i>M. phaseolina</i> Unt107	Illinois	ND
<i>Periconia macrospinos</i>	Illinois	ND
<i>Phytophthora capsici</i> 34-3	Illinois	ND
<i>P. capsici</i> 38-12	Illinois	ND
<i>P. sojae</i> Hn-2	Illinois	ND
<i>Phytophthora</i> sp. Ds-1 from soybean	Illinois	ND
<i>Pythium</i> sp. (smooth oospores)	Illinois	ND
<i>Pythium</i> sp. (spiny oospores)	Illinois	ND
<i>Rhizoctonia solani</i>	Illinois	ND
<i>Stenocarpella maydis</i> M9181	Illinois	ND
<i>S. maydis</i> M9181	Illinois	ND
<i>Trichoderma</i> sp.	Illinois	ND
<i>Phialophora gregata</i> – genotype A	Illinois	Detected (Ct = 15.5)
<i>P. gregata</i> – genotype B	Minnesota	Detected (Ct = 15.8)
<i>Cadophora malorum</i>	Antarctica	ND
<i>C. fastigiata</i>	Antarctica	ND
<i>C. luteo-olivacea</i>	Antarctica	ND

^a Total DNA was extracted from each of these organisms, and detection was assessed as cycle threshold (Ct) values generated with quantitative polymerase chain reaction (qPCR) analysis using approximately 10 ng/µl of DNA per reaction.

^b Samples with Ct values greater than 36 were considered to have nondetectable (ND) DNA with the TaqMan primer/probe set.

ware. Reactions were run in duplicate or triplicate on individual plates and analyzed based on absolute quantification using ABI software. Analysis of samples was repeated twice in different plates. Electronic pipettes (Rainin Instrument, LLC, Woburn, MA) were used to minimize variability in volume delivery to reaction wells. To check for cross contamination, DNase/RNase treated water controls were included in all experiments during the setup of qPCR reactions, and they were occasionally included during DNA extractions. Equipment was decontaminated with the product "DNA Away" (Molecular BioProducts, San Diego, CA), and aerosol barrier tips were used for all pipetting to minimize cross contamination.

Sensitivity testing and generation of three standard curves. To determine sensitivity of the primer/probe set and to generate standard curves for quantification of *P. gregata* with qPCR, three approaches were used. All qPCR assays were replicated at least three times. The goal was to develop calibration curves that would relate Ct values to known amounts of target DNA or pathogen conidia. (i) For the first approach, mycelium of isolates MNR (genotype A) and MNCL (genotype B) of *P. gregata* was grown for 20 days in soybean broth at 22°C in still cultures, and DNA was extracted from 100 mg of fresh mycelium as noted above. DNA was quantified with a spectrophotometer (Smartspec 3000, Bio-Rad, Hercules, CA), and serial dilutions were made to obtain concentrations from 1.0×10^5 to 1.0×10^{-4} pg/μl. (ii) For the second approach, *P. gregata* isolate MNR was grown in soybean broth for 14 days at 25°C, and conidia were harvested and counted with a hemacytometer. Conidia were diluted to 10^5 to 10^1 conidia per

ml and DNA was extracted (DNeasy Plant Kit, Qiagen, Valencia, CA) for use in qPCR reactions. (iii) For the third approach, the qPCR target sequence was cloned and standard curves based on this clone were developed for estimation of the target DNA sequence copy number in unknown samples. A 1,030-bp segment from the IGS region of ribosomal DNA from *P. gregata* isolate 98F1-2 (genotype A) was amplified with sPCR (3). The resulting amplicon, which contained one copy of the qPCR target sequence, was cloned using a TOPO II plasmid and *Escherichia coli* strain DH5 α (Invitrogen, Carlsbad, CA) to develop a target DNA standard. The plasmid was purified from *E. coli* (Quantum prep miniplasmid prep kit, Bio-Rad, Hercules, CA), and the cloned DNA fragment was sequenced to confirm that it was identical to the previously reported DNA sequence (3). The DNA quantity in the plasmid preparation was determined by UV spectrophotometry, and copies of target DNA/μl were calculated. A 10-fold dilution series was analyzed to develop amplification plots (Fig. 1) and standard curves based on 10^1 to 10^8 copies of target DNA per reaction.

Comparison of qPCR to sPCR and detection of *P. gregata* in plants. A series of comparisons were set up to compare qPCR to sPCR with regard to sensitivity to DNA quantity, susceptibility to PCR inhibition, and detection of *P. gregata* in field-grown soybeans. Sensitivity was tested with genomic DNA, different quantities of conidia, and cloned target DNA as noted above. Relative susceptibility to PCR inhibition and ability to detect *P. gregata* in soil samples (as noted below) and in soybean stem samples subjected to the standard and modified DNA extraction proce-

dures was evaluated. Soybean stems ($n = 34$) from one field each in Minnesota and North Dakota and three fields in central Illinois, as well as plants inoculated under greenhouse conditions, were tested for the presence of *P. gregata*. Soybean stems were collected at the R7 stage from the fields, and DNA was extracted as noted above and amplified with sPCR (3,23) or qPCR.

Detection of *P. gregata* in soil. Soil samples were collected from soybean fields in southern Minnesota with a history of BSR as well as from a southern Minnesota forest soil with no cropping history. In addition, soil samples with known numbers of conidia of *P. gregata* were prepared by autoclaving one soybean field soil sample from Minnesota and infesting it with a 10-fold dilution series of conidia of genotypes A and B of *P. gregata* produced as described above. DNA was isolated from soil using the MoBioPowerSoil DNA isolation kit, and DNA samples were diluted 1:10 prior to replicated qPCR analysis.

RESULTS

Specificity of primer and probe set.

The primer and probe set was specific for *P. gregata*. No detectable amplification occurred for 30 isolates of fungi and oomycetes, which included 14 genera and 24 species (Table 1). In addition, DNA was not amplified from extraction controls (plants known to be free of BSR), indicating that amplification of DNA from soybean stems did not occur due to cross contamination. *P. gregata* was readily detected in plants inoculated with both genotypes A and B in the greenhouse (Ct values 17 to 23), but was not detected in noninoculated plants that were colonized by many different fungi based on plating assays (Table 2). DNA was not amplified or detected in any of the nontemplate controls that contained water in place of DNA.

Sensitivity of qPCR assay and standard curves. Genomic DNAs were extracted from genotypes A and B of *P. gregata*, and serial dilutions were analyzed using the absolute quantification method. Ct values for genotypes A and B ranged from 15 for 5.0×10^4 pg of DNA to 35 for 5.0×10^{-3} pg DNA (Fig. 2). The relationship between copy number of the target DNA sequence and Ct value appear virtually identical for both genotypes ($R^2 = 0.94$ to 0.95). Approximately 5 to 50 fg of pure genomic DNA from *P. gregata* was readily detectable with Ct levels below 34 in the qPCR assay (Fig. 2, Table 2). In the qPCR analyses, the standard deviation of Ct values was lowest when DNA concentration was high and increased as copy number decreased. For example, the average standard deviation was 0.05 with 1,000 pg of *P. gregata* DNA and was 0.5 for 0.01 pg.

Sensitivity of the primer and probe was also evaluated by constructing a dilution series with total DNA extracted from 10^1 to

Fig. 1. Amplification plots for real-time, quantitative polymerase chain reaction (qPCR). Cycle number is displayed for serial dilutions of *Phialophora gregata* cloned target DNA standards beginning with 5.0×10^7 target copies and continuing to 5.0×10^3 copies on the right side. Each set of lines represents two replicated amplifications of the same sample.

10⁵ conidia (Fig. 3, Table 2). Approximately 400 conidia were consistently detectable. For each doubling of conidia numbers, the Ct value decreased by approximately 1 unit. For example, the Ct values for 500, 1,000, and 2,000 conidia were approximately 32, 31, and 30, respectively.

Cloned standards of the qPCR target DNA sequence from *P. gregata* were also developed and analyzed for accurate copy number determinations. Known numbers of the qPCR target sequence, which is a portion of the cloned 1,030-bp segment of the IGS region, were diluted to obtain 10² to 10⁸ copies of the target DNA. These target DNA standards were amplified with qPCR to enable estimation of copy numbers of the target DNA in unknown samples (Figs. 1 and 4). Ct values ranged from 34 to 16 for 10¹ to 10⁸ copies, respectively, of the target DNA sequence. Approximately 100 copies were required for detection. The number of conidia was plotted against the estimated copy number detected (data not shown), indicating that an average of less than one copy of the IGS target sequence was detected for each conidium used for DNA extractions and the efficiency of detection was below 100%.

Detection of *P. gregata* in inoculated plants and field-grown plants. Inoculated soybean plants in a greenhouse were sampled at biweekly intervals after inoculation, and *P. gregata* was detected in the stem apex with qPCR 4 weeks after inoculation. This was prior to symptom development and prior to the time *P. gregata* was detected with sPCR. *P. gregata* was not detected in noninoculated plants. Soybean stems from fields in Minnesota, North Dakota, and Illinois were tested for the presence of *P. gregata* DNA using an established sPCR method (3,23) as well as the qPCR method reported here. All samples with symptoms of BSR tested positive for *P. gregata* with sPCR and with qPCR (Ct values 17 to 23). In addition, *P. gregata* was confirmed by isolation to be present in plants from the field tested as positive with the *P. gregata*-specific qPCR assay.

Comparison of qPCR to sPCR and sensitivity of assay to PCR inhibitors. Approximately 1,000 more copies of the IGS target sequence and 1,000 more conidia were required for detection of *P. gregata* with the sPCR assay compared with the qPCR assay (Table 2). Relative susceptibility of qPCR and sPCR to inhibitors was also tested with two different DNA extraction protocols and with β -actin qPCR inhibition tests. The Ct values from qPCR assays were the same for the DNA extracted with a standard and modified protocol that removes additional PCR inhibitors, whereas DNA was not amplified with sPCR from samples extracted with the standard DNA extraction protocol but was amplified from samples extracted with the modified protocol (data not shown).

Likewise with the β -actin inhibition test, Ct values (data not shown) were the same for the β -actin-spiked pairs of 13 samples extracted with the two DNA isolation methods. This test demonstrated, with a common and known starting concentration of target DNA, that inhibitors of qPCR reactions were not present in DNA extracts prepared from stems with either protocol.

Overall, these results indicate that qPCR detected lower levels of *P. gregata* than sPCR and was less sensitive to PCR inhibitors.

Detection of *P. gregata* in soil. Conidia of *P. gregata* were detected in soil using qPCR after extraction of DNA using the MoBio Power soil DNA extraction kit (Table 2). This pathogen was detected with

Table 2. Relative sensitivity and specificity for detection of *Phialophora gregata*, the causal agent of brown stem rot (BSR), in pure samples and in soybean stems and soil with quantitative polymerase chain reaction (qPCR) and standard PCR (sPCR) in replicated samples^a

Sample from <i>P. gregata</i> or field	qPCR Ct value ^b	sPCR detection ^c
10 ⁵ copies of target DNA sequence	21	+
10 ⁴ copies of target DNA sequence	24	–
10 ³ copies of target DNA sequence	28	–
10 ² copies of target DNA sequence	31	–
10 copies of target DNA sequence	34	–
10 ³ pg genomic DNA	17	+
10 ² pg genomic DNA	20	+
10 pg genomic DNA	23	+
1 pg genomic DNA	27	+
10 ² fg genomic DNA	31	+
10 fg genomic DNA	35	–
10 ⁵ conidia	24	+
10 ⁴ conidia	28	–
10 ³ conidia	32	–
10 ² conidia	34	–
10 conidia	35	–
10 ⁶ conidia per g soil	25	–
10 ⁵ conidia per g soil	29	–
10 ⁴ conidia per g soil	32	–
10 ³ conidia per g soil	34	–
Field soil with history of BSR	33	–
Forest soil with no BSR history	>36	–
Field plants with no infection by <i>P. gregata</i>	>36	–
Field plants with BSR symptoms	18–23	+
Greenhouse plants with BSR symptoms	17–23	+
Greenhouse plants, noninoculated	>36	–

^a Cycle threshold (Ct) values determined with qPCR are inversely proportional to the detected DNA quantity.

^b Samples with Ct values less than 36 were considered to have detectable levels of DNA with the *P. gregata*-specific TaqMan qPCR assay. Each value represents the mean of three replicate samples, and the range of Ct values was typically within one unit of the mean.

^c The + and – indicate whether a DNA amplicon was generated and detected with an sPCR assay that is specific for *P. gregata*.

Fig. 2. Relationship between log₁₀ concentrations of genomic DNA from *Phialophora gregata* and cycle threshold (Ct) values used as a measure of detection of a DNA target with quantitative polymerase chain reaction (qPCR). Each data point represents the mean of three replicated samples, and error bars (where visible) represent one standard deviation.

qPCR in field soils with a history of BSR, and was not detected from nonagricultural forest soil. From 1.0×10^3 to 1.0×10^5 conidia of *P. gregata* were detected in infested soil samples using qPCR, but conidia were not detected in infested soil samples using sPCR (Table 2). The results suggest that qPCR is more sensitive than sPCR for detection of conidia in soil.

DISCUSSION

This is the first complete report of a real-time, quantitative PCR (qPCR) assay for specific and sensitive detection of the widespread soybean pathogen *P. gregata* in plants and soil. A portion of this work was published previously in an abstract (25). Use of this technique reduces the challenges with difficult isolation of this pathogen using culture media, and extends the use of standard PCR (sPCR) by adding quantification, increased sensitivity to low

DNA quantities, and reduced sensitivity to PCR inhibition. The qPCR assay detected *P. gregata* in plants sooner after infection and at lower levels of infection than was possible with sPCR. This valuable new qPCR technique is a tool with great value for rapid, specific, and sensitive detection and diagnosis of *P. gregata* in plants and soil, and for advancing our understanding of the biology of *P. gregata*, its interactions with hosts and other pests of soybean, and its survival in soil. Thus, this qPCR technique has applications to field-based, applied studies as well as controlled studies of the infection process for BSR.

Specificity for a target organism or gene is a critical element in the design and use of any qPCR assay. The qPCR primer and probe set that is reported here is specific for *P. gregata*. It was tested against 14 genera and 24 species of fungi and oomycetes, of which most are pathogens of soy-

bean or are common in soils and plants in corn-soybean rotations in the midwestern United States. In addition, because *Cadophora* is the new proposed genus for *P. gregata* (13), we tested the qPCR assay against three species of *Cadophora*. There was no amplified DNA detected from any of these species, suggesting that the assay is also specific to the species level.

The specificity assays were conducted with pure cultures. This assay will be used with plants grown in the greenhouse and fields that are colonized by many different fungi and other organisms, and must be specific for *P. gregata* from plants in those situations. DNA was not amplified from field-grown soybean stems that were collected from field locations in Illinois that did not have a history of BSR. This confirmed that the qPCR assay would not detect many different pathogenic and likely endophytic fungi (such as *Phomopsis*, *Plectosphaerella*, and *Acremonium*) that commonly infect or colonize soybean (14,32). The assay also did not detect fungi from greenhouse-grown plants, which contained several different fungal genera when stems were cultured on potato dextrose agar. Further, although the qPCR assay did not detect other fungi in pure culture or in greenhouse- or field-grown plants free of BSR, it readily detected *P. gregata* with Ct values of 17 to 23 in greenhouse- and field-grown plants that were infected with different levels of *P. gregata* and had symptoms of BSR. Therefore, all evidence suggests this new qPCR assay is specific for *P. gregata*.

High sensitivity, or the ability to detect small quantities of the target pathogen DNA, is another critical element in the development of a qPCR assay. While some studies have demonstrated high sensitivity by performing nested PCR (21,39), i.e., PCR done with two different sets of reactions and primer sets, this work focused on single-stage reactions with a goal of developing a relatively rapid, easily conducted, and less expensive assay. Considerable effort was put into sensitivity testing and development of standard curves that relate target DNA quantity with Ct. Based on head-to-head comparisons between qPCR and sPCR, qPCR was about 1,000 times more sensitive than sPCR for detection of *P. gregata*. It detected about 1/1,000 as many copies of the IGS target sequence and about 1/1,000 as many conidia as the sPCR assay. The sensitivity of qPCR was also demonstrated by detection of *P. gregata* in infected plants 4 weeks after inoculation, which is 1 to 2 weeks before BSR symptoms developed and before *P. gregata* was detectable with sPCR. These results clearly demonstrate that qPCR is a great deal more sensitive and can detect much lower levels of *P. gregata* than can the sPCR that has been used for *P. gregata* (3,22).

Three different types of standard curves were developed for sensitivity testing and

Fig. 3. Relationship between $\log_{10}$ number of conidia of *Phialophora gregata* and cycle threshold (Ct) values used as a measure of quantitative detection with quantitative polymerase chain reaction (qPCR). Data points represent the mean from three replications, and error bars represent one standard deviation.

Fig. 4. Relationship between $\log_{10}$ copy number of *Phialophora gregata* target DNA from the intergenic spacer (IGS) region and cycle threshold (Ct) values used as a measure of quantitative detection with quantitative polymerase chain reaction (qPCR). Each data point represents the mean from three different experiments, and vertical lines represent one standard deviation.

relating pathogen levels to Ct levels measured with the qPCR assay. The first was simply testing the ability of the qPCR test to detect serial dilutions of genomic DNA from the target organism. If we use 34 as an example Ct, we detected as little as 5 to 50 fg of genomic DNA from both genotypes A and B of *P. gregata*. The Ct value that represents realistic detection of a pathogen must be determined based on the instrument, the pathogen, the type of sample used, and the results obtained with nontemplate controls. We used 34 here as an example to illustrate detection at a particular limit. The upper threshold limit for a positive reaction in this assay is 34 to 36. This range represents a conservative yet realistic detection limit based on results from analysis of different types of samples and nontemplate controls. Hughes et al. (19) set the cutoff at 36 cycles for *Phytophthora ramorum* (10% below the 40 cycles used for thermal cycling), with only samples having a Ct level below 36 recorded as positive. In other studies, for example, detectable amounts of pure pathogen DNA in nonnested qPCR SYBR green assays ranged from 1 fg for *Fusarium oxysporum* (39), to 10 fg for *Pythium* spp. (31), and 2 pg for *Colletotrichum coccodes* (6). In addition to determining quantity of pure genomic DNA that can be detected, we also related detection limits to more biologically meaningful measures of conidia and copies of the target DNA. Using the threshold Ct value of 34, we could reliably detect approximately 400 conidia of *P. gregata*. Experiments are in progress to relate the Ct values to different levels of infection in soybean.

The qPCR target DNA sequence was cloned and used to develop target copy number standards to allow accurate quantification of actual copy numbers of the target DNA of *P. gregata* in pure culture and plant samples. Using this approach, 100 copies were required for reliable detection, and it was estimated that we detected about one copy of the target IGS sequence in each conidium. It is probable that more than one copy of the target sequence was present in each conidium, but the lower value indicates that the detection efficiency was below 100%. When analyzing qPCR data and developing qPCR assays, the copy numbers of the target sequence can be of great significance in developing highly sensitive assays and for developing useful biological interpretations of the results. The different isolates of *P. gregata* tested had very similar quantities of the target sequence in conidia and in pure DNA samples. This assay was designed to detect both genotypes A and B of *P. gregata* with equal success, and this goal was achieved. For some experiments, differential and specific detection of genotypes A and B is desirable, and work is in progress to accomplish this.

Another goal of this study was to compare qPCR to sPCR for susceptibility to PCR inhibition. It is critical that PCR inhibition be considered, tested, and accounted for in qPCR assays. For detection and quantification of fungal pathogens in soybean plants, PCR inhibition can affect whether or not a pathogen is detected with sPCR and can affect the quantity of DNA detected as measured by Ct values with qPCR (9,24). Relative susceptibility of qPCR and sPCR to PCR inhibition was tested with two different DNA extraction protocols and with β -actin inhibition tests. For the tests to compare sensitivity of qPCR and sPCR for DNA extracts of different purity, the Ct values from qPCR assays were within 0.5 Ct units for the DNA extracted with both methods. However, the sPCR was sensitive to PCR inhibitors to the point that DNA was not amplified from the samples extracted with the standard, less-purified DNA extraction protocol. Similarly, with the β -actin inhibition test, Ct values were the same for the pairs of 13 samples extracted with the two DNA isolation methods, hence the qPCR assay for β -actin also appeared to be insensitive to PCR inhibitors present in the DNA sample extracted with the standard extraction method that does not remove all PCR inhibitors (24). The same β -actin inhibition assay and two DNA extraction protocols were used by Gao et al. (9) to study PCR inhibition in soybean roots infected with *Fusarium solani* f. sp. *glycines*. However, their results contrast with those from this study; the Ct values they reported were higher for the less-purified DNA extracted with the standard procedure. The reason for the contrasts in these results is unknown, but may be due to tissues sampled (roots versus stems), the pathogen, reagents, or other factors. It appears that some samples and qPCR assays are more prone to PCR inhibition than others, and inhibition must always be considered (6,24).

Although this work focused on detection and quantification of *P. gregata* in plant samples with qPCR, a method was also established for detection of this pathogen in soil samples with qPCR. *P. gregata* was detected in soil samples that were spiked in the laboratory with known numbers of conidia. This fungus was also detected in soil samples with a history of BSR, and conversely was not detected in soil that had no history of BSR or soybean production. This is the first report of successful detection of *P. gregata* from soil samples using a PCR technique. Thus, *P. gregata* can be detected in soil samples with a combination of this qPCR assay and an effective soil DNA extraction system such as the MoBio PowerSoil DNA isolation kit. This kit was found to be the most effective DNA extraction procedure for this application among the different techniques that were evaluated. Other kits and methods tested to extract "amplifiable" DNA of

P. gregata from soil included the MoBio UltraClean soil DNA, MoBio UltraClean Mega soil DNA (MoBio Laboratories, Carlsbad, CA), Epicentre Soil Master (Epicentre Biotechnologies, Madison, WI), FastPrep Soil Spin Kit (QBiogene, Irvine, CA), and five published protocols (5,17,27,33,35). More work is required to determine extraction efficiencies and to account for variable PCR inhibition in different soil types to reliably quantify this pathogen from "unknown" soil samples.

The goal of developing a specific and sensitive qPCR test for detection and quantification of *P. gregata* was achieved. The new qPCR assay described in this paper is a robust method that has been validated with plant and soil samples. The assay has much value for rapid diagnosis of BSR and detection of *P. gregata*, and for studies of BSR infection, its ecology in plants and soil, and its interaction with plants and soybean pests. Further, the qPCR approaches also have potential to be used for many other fungal pathogens and diseases.

ACKNOWLEDGMENTS

Support for this project is gratefully acknowledged from the North Central Soybean Research Program, Department of Plant Pathology and College of Food, Agricultural, and Natural Resources Sciences at the University of Minnesota, the Department of Crop Sciences at the University of Illinois, and the NSF Center for Integrated Pest Management. This work was partially conducted while the first author was assistant professor at the University of Illinois. The expert technical assistance of E. Grunden in obtaining fungal DNAs is much appreciated. We thank X. Gao, Z. Islam, M. Babadoost, H. Fouly, D. White, and R. Blanchette for fungal isolates.

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