A Real-Time PCR Assay for Detection and Quantification of *Verticillium dahliae* in Spinach Seed

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ABSTRACT

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Verticillium dahliae is a soilborne fungus that causes Verticillium wilt on multiple crops in central coastal California. Although spinach crops grown in this region for fresh and processing commercial production do not display Verticillium wilt symptoms, spinach seeds produced in the United States or Europe are commonly infected with *V. dahliae*. Planting of the infected seed increases the soil inoculum density and may introduce exotic strains that contribute to Verticillium wilt epidemics on lettuce and other crops grown in rotation with spinach. A sensitive, rapid, and reliable method for quantification of *V. dahliae* in spinach seed may help identify highly infected lots, curtail their planting, and minimize the

spread of exotic strains via spinach seed. In this study, a quantitative real-time polymerase chain reaction (qPCR) assay was optimized and employed for detection and quantification of *V. dahliae* in spinach germplasm and 15 commercial spinach seed lots. The assay used a previously reported *V. dahliae*-specific primer pair (VertBt-F and VertBt-R) and an analytical mill for grinding tough spinach seed for DNA extraction. The assay enabled reliable quantification of *V. dahliae* in spinach seed, with a sensitivity limit of ≈ 1 infected seed per 100 (1.3% infection in a seed lot). The quantification was highly reproducible between replicate samples of a seed lot and in different real-time PCR instruments. When tested on commercial seed lots, a pathogen DNA content corresponding to a quantification cycle value of ≥ 31 corresponded with a percent seed infection of $\leq 1.3\%$. The assay is useful in qualitatively assessing seed lots for *V. dahliae* infection levels, and the results of the assay can be helpful to guide decisions on whether to apply seed treatments.

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae*, is an economically important disease on a diverse range of crops worldwide (4,36,40). The fungus has a broad host range (4) and survives for years in the soil as microsclerotia. The microsclerotia can be dispersed via farm equipment, soil, and water (25). These features make the control of Verticillium wilt exceedingly difficult once established in a cropping system and, often, expensive soil fumigation is the only control measure available. As effective fumigants, such as methyl bromide, become unavailable for use, disease pressure from this pathogen will increase further. Of additional concern is the dispersal of the pathogen over long distances to new cropping systems, especially because *V. dahliae* can also be seedborne in weeds and crops (11,45).

Spinach seed production only occurs in areas with long-day-length summers to induce flowering, as in northern Europe, the

U.S. Pacific Northwest, and New Zealand (11). Spinach seeds produced in Europe and the United States are almost invariably infected with *V. dahliae* (11). *V. dahliae* is clearly localized inside the spinach seed, as revealed by laser-scanning confocal microscopy analyses of spinach seed colonized by a green fluorescent protein-tagged strain of *V. dahliae* (K. Maruthachalam, S. J. Klosterman, A. Anchieta, B. Mou, and K. Subbarao; *unpublished data*). Interestingly, Verticillium wilt symptoms on spinach are only apparent after bolting (seed stem elongation) (11). Therefore, due the short duration between planting and harvest (as few as 4 weeks), fresh and processing spinach crops do not show wilt symptoms (11). The crop residue that is returned to soil nevertheless harbors the pathogen inoculum that potentially remains in soils that are later planted to lettuce or other crops grown in rotation with spinach. Infected seeds may also introduce exotic strains of *V. dahliae* to new areas of crop production that could be consequential if the introduced genotypes are more aggressive and add to the genetic variability of the pathogen in the recipient areas.

Verticillium wilt was first reported on lettuce in the central coastal region of California in 1995 (43), although the disease was problematic on strawberry and other crops in the region decades prior to that discovery. Since the mid-1990s, Verticillium wilt on lettuce has spread throughout the central coastal California production region. Yield losses in lettuce crops due to *V. dahliae* of up to 100% have been reported (43,45). The increase in the incidence of the disease on lettuce coincided chronologically with changes in spinach cropping practices, including a trend toward year-around spinach production, an increase in acreage devoted to spinach, and a higher plant density of spinach

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per unit area (2). Because the majority of the U.S. lettuce and fresh spinach is produced in the central coastal region of California, the industry is potentially under long-term threat of *V. dahliae* imported to the region via spinach seeds produced in Europe and the U.S. Pacific Northwest.

Now that the threat posed by seedborne *V. dahliae* is well established, avoidance of planting seed lots with infection levels above a designated threshold is likely to slow the spread of *V. dahliae* via spinach seed. Currently, Mexico requires that imported seed have <10% (threshold) infection with *V. dahliae* (21). For avoidance to be effective, timely and accurate knowledge of seed infection levels is required. The conventional NP-10 plating assay, in addition to a widely used blotter assay (11), have been useful to assess the level of seed infected with *V. dahliae* (41). Although these assays can provide a clear view of numbers of infected seed per lot, there are also limitations associated with the administration of these assays. First, the NP-10 plate assay or blotter assay take $\approx$ 2 weeks to complete, which makes the plate assays unwieldy for testing a large number of seed lots in a reasonable time frame. Second, these assays do not easily distinguish between *V. dahliae* and closely related species, such as *V. tricornutus*, which can occur on a seed sample. The ideal assay for quantifying *V. dahliae* in commercial seed lots would be accurate, timely, and specific. A quantitative real-time polymerase chain reaction (qPCR)-based quantification of pathogens is a rapid, reliable, and ultrasensitive method and hence, has become the preferred technological platform for detection and quantification of plant pathogens in seed and environmental samples (7,13, 22,27).

Harnessing the power of qPCR technology for the purpose of pathogen quantification requires developing pathogen-specific DNA primers and a reliable assay protocol (9,10,14,32,44). Primers for qualitative conventional PCR analysis of *V. dahliae* in plant and soil samples have been previously reported (23,26,29). Atallah et al. (1) characterized a qPCR primer pair (VertBt-F and VertBt-R) derived from the single copy β -tubulin gene that could readily be adapted for quantification of *V. dahliae* in spinach seed. The specificity of this primer set for the amplification of a 115-bp amplicon of *V. dahliae* was confirmed against a range of other fungi and *Verticillium* spp. (1). However, the recent finding that *V. longisporum* is actually a hybrid fungus, containing a copy of *V. dahliae* DNA sequences in addition to a separate genomic complement (19), could potentially complicate the use of the VertBt primers for species-specific detection. Nevertheless, *V. longisporum* is a pathogen specific to cruciferous plants (12,18, 42) and, thus, an association of *V. longisporum* with spinach seed is not expected.

The objectives of this study were to (i) optimize a qPCR assay for the quantification of *V. dahliae* in spinach seed, (ii) determine whether a threshold real-time PCR quantification cycle (Cq) value can be correlated with the percentage of seed infected in commercial seed lots for use as a guide for whether to apply seed treatments or in regulatory decisions, and (iii) determine whether *V. longisporum* is detectable in commercial spinach seed lots.

MATERIALS AND METHODS

Seed sample collection and NP-10 plate assays. Fifteen seed lots were obtained from U.S. and European commercial spinach seed producers (Table 1) between December 2009 and May 2011. All seed were stored at -20°C until use for qPCR sampling. Upon receipt, subsamples of these seed lots were evaluated for percentage of seed infected with *V. dahliae* on NP-10 plates (41). Briefly, seed were surface sterilized with 1.2% commercial bleach for 1 min, triple-rinsed with sterile water, and subsequently air dried under a laminar flow hood on sterile paper towels. At least 400 seeds from each seed lot were plated, in replicates of 100 or 200 seeds for each lot. Plates were incubated at 25°C in the dark for 10 days and the percentage of infected seed was recorded (Table 1).

Seed samples from the United States Department of Agriculture, North Central Regional Plant Introduction Station (NCRPIS) spinach germplasm collection (Table 2) were surface sterilized as above and plated on NP-10 medium. However, due to limitations in the numbers of seed available for each accession, only 20 seeds were plated per accession. In addition, seed of this collection were evaluated after 14 days of incubation for the presence of *V. dahliae* and *V. tricornutus*, based upon colony morphology on the NP-10 plates. Unlike *V. dahliae*, which produces concentric rings of microsclerotia on NP-10 plates, *V. tricornutus* produces a diffuse arrangement of microsclerotia (picture not shown).

Seed lot subsampling and DNA extraction. The qPCR assay was conducted on three replicate seed samples, with each sample containing 1,000 seeds taken from the individual seed lots. The large numbers of seed per lot were sampled to provide an adequate sample to minimize the effect of seed-to-seed variation in pathogen load per seed. The seed samples were ground for $\approx$ 45 s to a fine powder using an IKA A10 analytical grinding mill (IKA Laboratory, Germany). Between samples, the stainless steel grinding chamber of the mill was washed with deionized water and rinsed with 70% ethanol.

DNA extractions were conducted from 200-mg fractions of 1,000 ground seeds ($\approx$ 10 g) using a FastDNA kit following the manufacturer's protocol (MP Biomedicals, Solon, OH), except

TABLE 1. Quantification of *Verticillium dahliae* using NP-10 plate and quantitative real-time polymerase chain reaction (qPCR) assays in 15 commercial spinach seed lots

Seed lot	NP10 plate assay		qPCR assay ^a	
	Infected seed/seed tested	Seed infected with <i>V. dahliae</i> (%)	Pathogen copy number	Cq value
1	341/400	85	544.68 $\pm$ 94.97	26.20 $\pm$ 0.26
2	307/400	77	2141.19 $\pm$ 728.40	25.38 $\pm$ 0.20
3	301/400	75	292.55 $\pm$ 135.17	27.01 $\pm$ 0.05
4	289/400	72	638.01 $\pm$ 186.33	26.32 $\pm$ 0.53
5	272/400	68	301.17 $\pm$ 120.47	27.78 $\pm$ 0.68
6	385/600	64	666.32 $\pm$ 185.07	25.94 $\pm$ 0.48
7	96/400	24	514.10 $\pm$ 88.69	26.45 $\pm$ 0.05
8	75/400	19	146.64 $\pm$ 10.10	28.77 $\pm$ 0.11
9	66/400	17	96.87 $\pm$ 6.99	29.02 $\pm$ 0.15
10	63/400	16	844.28 $\pm$ 165.10	26.09 $\pm$ 0.31
11	63/400	16	255.98 $\pm$ 47.16	27.20 $\pm$ 0.28
12	23/400	5.8	56.94 $\pm$ 8.02	29.60 $\pm$ 0.23
13	5/400	1.3	29.72 $\pm$ 4.87	31.11 $\pm$ 0.50
14	4/600	0.7	1.46 $\pm$ 0.45	35.81 $\pm$ 0.81
15	1/400	0.3	4.72 $\pm$ 1.32	34.44 $\pm$ 0.54

^a Mean $\pm$ standard deviation.

that the IKA A10 mill was used for sample homogenization in place of the FastPrep Instrument. DNA extracts were further purified with AMPure magnetic beads and a SPRI 96-Ring Magnet Plate (Agencourt, Beverly, MA) to cleanse DNA of potential PCR inhibitors. DNA concentration was measured using a Qubit fluorometer and Quant-iT dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). All DNA samples were diluted to 1 ng/μl in nuclease-free water and 5 μl of the diluted DNA was used for qPCR. To determine the number of DNA extractions needed per seed lot to accurately determine *V. dahliae* concentration, preliminary qPCR data were generated from ≤18 extractions of three seed lots. Using the pooled standard-deviation from this data, the 99% margin of error was calculated as the standard error $\times t$ value using hypothetical numbers of DNA extractions of 2 to 31. Using the approach, six DNA extractions should result in a Cq margin of error of 0.82. Based on this result, six 200-mg fractions of the ground seed were taken from each of three samples (1,000 seeds/sample) for DNA extraction and qPCR for all subsequent experiments.

For the NCRPIS seed samples (Table 2), DNA was extracted from 4 seeds/accession using a MoBio PowerPlant Extraction kit (MO BIO Laboratories, Inc., San Diego) and a micropestle for grinding the seed in a microfuge tube. Seed were soaked in a 1.5-ml microcentrifuge tube with 550 μl of PowerPlant Bead Solution for 1 h and crushed with a micropestle, and the resulting homogenate was transferred to PowerPlant Bead Tubes for DNA purification following the procedures in the PowerPlant DNA Isolation Kit. DNA concentration was measured as described above. AMPure DNA extract (50 ng) was then used in qPCR analysis.

qPCR assay. Quantitative PCR was carried out in the LightCycler 480 model II (Roche Diagnostics, Indianapolis, IN). Reactions were prepared in 96-well plates (USA Scientific, Orlando, FL) in total reaction volumes of 20 μl containing 10 μl of 1× IQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.4 μl of forward (VertBt-F 5'-AACACAGTC CGATGGATAATTC-3') and reverse (VertBt-R 5'-GTACCGGGCTCGAGATCG-3') primers (1) at concentrations of 200 nM each, 5 μl of template DNA (1 or 5 ng total), and 4.6 μl of nuclease-free water. Each DNA sample was loaded in triplicate in a 96-well plate. The temperature profile for qPCR was as follows: 95°C for a 5-min denaturation step; followed by 40 cycles of amplification at 95°C for 10 s and 65°C for 35 s; and melt curve analysis of heating to 95°C, cooling to 65°C for 1 min, and heating to 97°C at a rate of 1°C/5 s. The spinach actin was used as a reference gene (GenBank accession JN987183) and was amplified in each sample using primer pair SO385-F, 5'-TCCGTGCAGGTATTGTG CTG-3' and SO489-R, 5'-AGCAAGGTGAGACGAAGG-3' at a concentration of 300 nM under the same qPCR cycling conditions as described above. Known standards for quantification of the *V. dahliae* β-tubulin gene were prepared. To prepare the standard, the β-tubulin gene fragment was PCR amplified from DNA extracted from naturally infected seed of the NCRPIS spinach accession 165560C using the VertBt primer pair and cloned into a PCR4.0TOPO vector (Invitrogen). The quantity of DNA from *V. dahliae* in the seed samples was then estimated using copy-number-based, five-point (500,000, 50,000, 5,000, 500, and 50 copies) internal standard curves constructed by serial dilution of linearized plasmid vector DNA carrying the β-tubulin gene fragment from *V. dahliae*. The absolute quantification module of the LightCycler software was used to determine Cq and pathogen copy number. The threshold Cq value was manually adjusted to 1.0 for all qPCR experiments to enable run-to-run comparisons. DNA melt peaks were analyzed at the end of each run to ensure specificity of amplification of the β-tubulin gene fragment.

For the NCRPIS seed samples, qPCR analysis was conducted on a Bio-Rad ICycler real-time PCR system (Bio-Rad) using the same VertBt and actin primers and identical PCR conditions, with

the exception that the annealing temperature was adjusted to 63°C and the adjustment to 50 ng of DNA template. DNA melt peaks were analyzed at the end of each run to ensure specificity of amplification of the β-tubulin gene fragment. Additionally, the 115-bp product of the β-tubulin gene was amplified from DNA template derived from accession NSL184380 (Table 2) and sequenced directly for verification of the correct product (matching with 100% sequence identity to the corresponding 115-bp sequence from *V. dahliae*, GenBank accession DQ840621).

Inter-instrumental reproducibility of the qPCR assay. Seed samples with artificial infection levels of 0.7, 2, 5, 10, 25, 50, and 64% were created by blending seed lot 6 (64% infection) and seed lot 14 (0.7% infection), hereafter referred to as seed samples with artificial infection gradient. The samples were then assayed independently on the Roche LightCycler 480 II and the Bio-Rad ICycler real-time PCR instruments under the same reaction and cycling conditions (annealing temperature of 65°C) to examine the inter-instrumental reproducibility of the qPCR assay.

Effect of DNA template clean-up and evaluation of PCR inhibitors. All of the qPCR assays described were conducted using AMPure purified DNA, because PCR inhibitors are commonly found in seed tissue samples (8,15). To evaluate the results in the absence of this template clean-up step, AMPure magnetic bead-purified DNA and unpurified DNA were assayed by qPCR to determine the necessity of incorporating a DNA template clean-up step in the qPCR assay protocol. This was done using the seed samples with artificial infection gradient created by blending seed lots 6 and 14. For PCR inhibition tests, sensitivity of detection of plasmid DNA containing 50 copies of *V. dahliae* β-tubulin gene in water (pure plasmid DNA) was compared with detection in the background of 5 ng of AMPure (or unpurified) seed DNA from seed lot 14 (0.7% infection). The assumption made was that any difference between Cq values of the spiked seed DNA samples and pure plasmid DNA would be due to the presence of PCR inhibitors in the seed DNA samples.

Survey of spinach seed lots for contamination with *V. longisporum*. To determine whether *V. longisporum* was present on the

TABLE 2. Comparison of quantitative real-time polymerase chain reaction (qPCR) and NP-10 plate assays for detection of *Verticillium dahliae* in spinach germplasm seed collections

Accession	β-Tubulin copy number (qPCR) ^a	Average Cq (qPCR)	NP-10 plate assay ^b
PI 171859	11	34.08	N
PI 254565	22	33.31	N
PI 174388	15	34.20	N
PI 339546	101	30.97	N
PI 604791	3,780	28.41	N
PI 175930	228	30.05	<i>V. dahliae</i>
PI 358259	6	35.22	<i>V. tricornus</i>
PI 4661	1,330	27.71	<i>V. tricornus</i>
PI 176771	151	31.18	N
PI 339259	154	30.42	Not determined
PI 6082	97	31.02	<i>V. tricornus</i>
PI 179507	46	32.31	N
PI 361127	2	36.59	<i>V. dahliae</i>
NSL 6093	358	29.66	<i>V. dahliae</i>
PI 200882	29	32.88	N
PI 368825	3,250	29.03	<i>V. dahliae</i>
NSL 28216	983	28.01	<i>V. dahliae</i>
PI 206473	232	29.88	N
PI 370602	144	30.90	<i>V. tricornus</i>
NSL 28218	138	30.64	<i>V. dahliae</i>
PI 220121	6,750	25.39	<i>V. dahliae</i>
PI 370602	634	28.58	<i>V. tricornus</i>
NSL 184380	126,000	21.49	<i>V. tricornus</i>
PI 445784	0	NA ^c	<i>V. tricornus</i>

^a In all, 4 seeds were used for DNA extraction.

^b In all, 20 seeds were plated and scored. N = *V. dahliae* not detected.

^c NA = not amplified.

spinach seed lots, 15 commercial seed lots were screened using the *V. longisporum*-specific primer pair 5'-TCTCCTCTC TACGAGAACGA-3' and 5'-CACTTTCTAAGTATCCTTCCTAT-3' (42) by conventional PCR. PCR cycling parameters included an initial denaturation at 94°C for 2 min; followed by 30 cycles of 94°C for 2 min, 48°C for 2 min, and 72°C for 3 min; and a final extension at 72°C for 10 min. PCR amplifications on the commercial seed lots were carried out in a total reaction volume of 50 µl, containing 1 µl of the FastDNA extracted seed DNA template (25 ng) from the commercial spinach seed lots, 15 pmol of each primer, and GoTaq PCR Mastermix (Promega Corp., Madison, WI). To obtain a positive control for PCR amplification of *V. longisporum* DNA in the background of spinach seed DNA, a 1-µl dilution containing 25 ng of DNA template from *V. longisporum*, strain Bob70 (38), was mixed into 1 µl of 25 ng of DNA extracted from seed lot 10 (Table 1), and 1 µl of this template mixture was used in PCR. In addition, 25 ng of DNA template from *V. dahliae*, strain VdLs.16 (38) was used as a negative control. DNA templates from strains VdLs.16 and Bob70 were prepared using a standard bead beat procedure for fungal DNA isolation (39).

Measurements of the lengths of conidia from isolates of commercial seed lots also were compared with those of *V. longisporum*, to examine whether *V. longisporum* was present in the seed lots. The lengths of conidia for *V. dahliae* and *V. longisporum* were expected to be ≈4.4 and 7.9 µm, respectively, based on a previous report of the lengths of conidia of short-conidia and long-conidia strains of *Verticillium* spp. (24). Fungal isolates from seed lots 1, 4, 6, 10, and 11 from diverse geographic locations (United States and Europe) were obtained by culturing 15 seeds/seed lot on NP-10 plates following the procedure for NP-10 plate assay described above. Subsequently, the lengths of 4,700 conidia (7 to 10 isolates/seed lot and 100 conidia/isolate) were measured using an Olympus BX60 microscope fitted with a DP70 digital camera and DP manager software (Olympus America, NY), and compared with the lengths of conidia of *V. longisporum* isolate Bob70.

Data analysis. Linear regression analysis was used to evaluate the relationship between percent seed infection and pathogen DNA quantity in spinach seed.

RESULTS

Optimization of seed grinding procedure and template DNA for qPCR assay. The IKA grinder was effective for rapidly grinding 1,000 seeds/sample, enabling the timely processing of samples. Further, by increasing the template DNA amount used for qPCR from 1 to 5 ng, we were able to detect DNA from *V. dahliae* in the spinach seed background with sufficient sensitivity. Using 1 ng of template DNA extracted from three replicates of 1,000 seeds from an artificial seed infection of 2%, no amplification was recorded in 24 of 63 reactions (38%) (data not shown). In contrast, amplification was recorded in all reactions (100%) when the spinach DNA template of 5 ng was applied for qPCR on an artificial seed infection level of 2% (data not shown). Subsequently, the spinach DNA template concentration of 5 ng was used in the qPCR testing of all commercial seed lots.

Correlation between percent seed infection and pathogen DNA quantity. The relationship between pathogen DNA copy number and percent seed infection was linear and highly significant ($R^2 = 0.99$, $P = 0.0001$) when assessed on seed samples with an artificial infection gradient of 0.7 to 64% (Fig. 1A). However, when the relationship between pathogen copy number and percent seed infection was examined in 15 naturally infected seed lots, the correlation was numerically small, though statistically significant ($R^2 = 0.32$, $P = 0.027$). Cube root transformation of pathogen copy number and percent seed infection data improved the correlation ($R^2 = 0.66$, $P = 0.0002$) (Fig. 1B). Despite the reduced correlation in 15 naturally infected seed lots compared with seed samples with artificially infection gradient, the overall trend was that pathogen DNA quantity decreased as percent seed infection decreased. An important observation in this regard was that a pathogen DNA quantity corresponding to a Cq value of ≥31 correlated with a percent seed infection of ≤1.3% (Table 1) in the naturally infected seed lots.

Sensitivity and reproducibility of the qPCR assay in naturally infected seed lots. The qPCR assay was highly sensitive and reproducible between replicate samples of a seed lot. qPCR assays of the 15 naturally infected seed lots with infection levels determined by NP-10 plate assays of 0.3 to 85% showed detection of *V. dahliae* at the 1.3% infection level for seed lot 13

Fig. 1. Correlation between percentage of spinach seed infected by the NP-10 method and the quantitative real-time polymerase chain reaction values for DNA quantity from *Verticillium dahliae*. **A**, Correlation between pathogen DNA quantity, as determined by the copy number of β -tubulin from *V. dahliae*, and percent seed infected in an artificial infection gradient created by mixing seed lots 6 and 14. **B**, Correlation between pathogen DNA quantity, as determined by the copy number of β -tubulin from *V. dahliae*, and the percentage of seed infected in 15 commercial seed lots. Data were cube root transformed.

(Table 1), which translates to a sensitivity limit of ≈ 1 infected seed per 100. At infection levels below this, detection was possible but was not reproducible (Cq values were recorded for all replicate samples for seed lot 13 but not for seed lots 14 and 15). The assay was also highly reproducible between replicate samples of a seed lot using 5 ng of DNA template, as revealed by the low standard deviation associated with the Cq values (Table 1).

Reproducibility on two different qPCR machines. Comparative testing of seed samples with artificial infection gradients of 0.7 to 64% on the Roche LightCycler and the Bio-Rad ICycler real-time PCR instruments under similar PCR conditions produced nearly identical results (Fig. 2A), showing a high interinstrumental reproducibility of the test. Correlation between pathogen copy numbers quantified with the Roche LightCycler and the Bio-Rad ICycler was highly significant ($R^2 = 0.99$, $P = 6.84E-7$) (Fig. 2B).

Germplasm screening. Twenty-four germplasm accessions obtained from the NCRPIS spinach germplasm collection were tested for *V. dahliae* infection. Because low numbers of seed were available for each of the accessions, the IKA A10 grinding mill was not employed for sample homogenization in these experiments. In spite of the low number of seed assessed for the germplasm accessions, the qPCR assay enabled detection of *V. dahliae* in 23 of 24 of the spinach germplasm accessions, whereas the NP-10 plate detected *V. dahliae* in 7 of 23 of the accessions examined (Table 2). In 16 of the accessions, the NP-10 plate assay test revealed negative results for the detection of *V. dahliae* whereas, in 7 of the accessions, *V. tricornutus* was detected.

Effect of DNA purification on the sensitivity of the qPCR test. We evaluated the effect of DNA template clean-up intended to remove potential PCR inhibitors purified with DNA on the sensitivity of the qPCR. DNA purification slightly increased (decreased Cq by ≈ 0.5) the sensitivity of detection of *V. dahliae* in spinach seed in the 2 to 64% infection range in seed samples with artificial infection gradient (Table 3). However, the effect of PCR inhibitors was evident when testing purified β -tubulin template spiked in DNA from seed. Comparison of Cq values obtained

using purified plasmid DNA corresponding to 50 copies of the *V. dahliae* β -tubulin gene and Cq values of the same plasmid amplified in the background of 5 ng of AMPure (or unpurified) spinach seed DNA showed a decrease in sensitivity of the qPCR when seed DNA is present. Cq values were 30.20 ± 0.44 for pure plasmid DNA, 32.98 ± 0.93 for plasmid DNA amplified in the background of AMPure seed DNA, and 35.67 ± 1.05 for plasmid DNA amplified in the background of unpurified seed DNA.

Results from the amplification of spinach actin gene as an external control in the different seed lots revealed similar Cq values across all seed lots (Table 4), suggesting amplification with similar efficiencies in the different seed lots and the absence of any significant variation in PCR inhibitors in spinach seed DNA template obtained with the FastDNA kit and AMPure purification.

Survey of *V. longisporum* contamination of the seed lots. The 115-bp β -tubulin amplicon was detectable by PCR using the VertBt primers from both *V. dahliae* isolates and the *V. longisporum* isolate, Bob70 (data not shown). Therefore, extracts from the 15 commercial seed lots used in this study were screened with

TABLE 3. Effect of additional DNA purification on the sensitivity of quantitative real-time polymerase chain reaction (qPCR) as reflected in quantification cycle (Cq) in seed samples with artificial infection gradients created by mixing seed lots 6 and 14

Seed infection (%)	Cq (purified DNA) ^a	SD ^b	Cq (unpurified DNA) ^c	SD
0.7	35.66	0.95	34.70	1.51
2	32.26	0.49	32.86	0.64
5	33.02	0.20	33.67	0.83
10	29.02	0.19	29.61	0.28
25	27.65	0.22	28.28	0.13
50	26.43	0.30	27.64	0.32
64	26.03	0.26	26.86	0.20

^a FastDNA kit extracted DNA purified with an AMPure kit; average of 18 replicate assays.

^b SD = standard deviation.

^c FastDNA kit extracted DNA; average of 18 replicate assays.

Fig. 2. Interinstrumental reproducibility of the quantitative real-time polymerase chain reaction (PCR) assay for quantification of *Verticillium dahliae* in spinach seed with artificial infection levels. **A**, Scatter plot of the average threshold real-time PCR quantification cycle (Cq) readings by the Roche LightCycler and Bio-Rad ICycler real-time PCR instruments on seed samples with varying artificial infection levels. **B**, Correlation between Cq values measured by LightCycler and ICycler real-time PCR instruments.

a *V. longisporum*-specific primer pair (42). The 340-bp amplicon unique to *V. longisporum* was amplified from DNA obtained from *V. longisporum* and in spinach seed DNA that was spiked with *V. longisporum* (Fig. 3). However, the amplicon of the expected size of 340 bp was not amplified from any of the spinach seed lots examined in this study (Fig. 3). Further, measurements of 4,700 conidia of the multiple individual fungal isolates obtained from five seed lots revealed an average conidia length of $3.91 \pm 0.80 \mu\text{m}$ compared with a conidia length of $8.75 \pm 1.55 \mu\text{m}$ for *V. longisporum* isolate Bob70 (Supplemental Figure 1).

DISCUSSION

The discovery of the widespread contamination of commercial spinach seed lots with *V. dahliae* (11) prompted a need to develop a rapid, sensitive, and specific seed health testing method that may help curb the spread of the fungus. The conventional NP-10 plate assay for *V. dahliae* is unwieldy for rapid testing of the large number of seed lots produced by commercial seed companies. Additionally, the conventional NP-10 plate assay may not always easily differentiate *V. dahliae* from other fungi in seed samples. In this study, an alternative qPCR assay for pathogen quantification was developed using primers derived from the single copy β -tubulin gene (VertBt primers) of *V. dahliae* (1) and evaluated using spinach seed lots from growing regions in Europe and the United States.

Spinach seed sampling for qPCR required an alternate sample homogenization procedure because dry spinach seed is exceed-

ingly difficult to grind with a mortar and pestle method commonly used for sample homogenization for DNA extraction. Even wet spinach seed are difficult to grind using a mortar and pestle, because of the thick, hard, outer fruit wall present in spinach seed. Further, the numbers of seed that could be partially homogenized with a mortar and pestle or micropestle technique are too small to adequately represent the voluminous lots from which the seed are drawn. The initial approach of sample preparation for DNA extraction and subsequent qPCR was based on homogenization of four seeds using a micropestle in a microcentrifuge tube. As such, the mortar and pestle grinding technique was not optimal for obtaining the necessary homogeneity and yield of the extracted DNA (5). Seed homogenization with a micropestle procedure was laborious for the operator and the sample size was deemed inadequate as a representative sample of the population for quantitative analyses. Therefore, we optimized the grinding protocol by adopting the use of IKA A10 grinding mill, which is capable of grinding large seed samples with remarkable speed (≈ 45 s), and upscaled the sample size used for DNA extraction to 1,000 seeds/sample.

Another requirement of DNA preparation for a qPCR assay is elimination or reduction of PCR inhibitors which are commonly found in DNA extracts from seed (8,15,28,32,37). PCR inhibitors in DNA extracts may increase false negative readings, and could detrimentally affect a qPCR test result by reducing amplification efficiency. A trace amount of inhibitor is sufficient to obscure pathogen detection at the lower end of the dynamic range of qPCR. DNA purification with commercial kits and dilution of the DNA extract have proven effective in minimizing the effect of inhibitors on PCR reactions (5,17,32). Treatment of DNA with polyvinylirrolidone has also been reported to greatly improve the sensitivity of PCR-based tests by reducing the effect of inhibitors (30,31,35,46). In developing the qPCR assay reported herein, DNA was extracted with a FastDNA kit and subsequently purified with AMPure magnetic beads to eliminate or reduce the amount of potential PCR inhibitors. Nonetheless, we found that addition of the AMPure purification step had only a slight effect on the sensitivity of the qPCR test (decreasing the Cq value by ≈ 0.5 in the 2 to 64% seed infection range). Further, a valid quantitative DNA analysis such as a qPCR assay requires that the DNA template be of homogeneous quality for the samples being compared (5,20). As an indirect measure for homogeneity and integrity of the DNA extracts from the different seed lots, amplification of a spinach actin gene was used as a control in all DNA amplifications. The actin amplification efficiencies, as deduced from Cq values, were similar among all seed lots, confirming that the DNA extracts were of similar quality and that variation in DNA purity should not affect the results obtained. Dilution of the template DNA may further reduce the impact of PCR inhibitors.

TABLE 4. Average quantification cycle (Cq) values obtained from quantitative real-time polymerase chain reaction (qPCR) analyses of a spinach actin gene used as a control in 15 seed lots

Seed lot	Cq actin	SD ^a	Number of tests
1	23.51	0.67	54
2	22.96	0.54	90
3	22.39	0.40	18
4	22.93	0.46	36
5	22.86	0.75	18
6	23.42	0.31	126
7	22.65	0.45	18
8	22.44	0.23	18
9	23.08	0.14	18
10	23.02	0.25	18
11	22.93	0.16	18
12	22.98	0.25	18
13	23.06	0.15	18
14	23.16	0.24	18
15	22.70	0.12	18

^a SD = standard deviation.

Fig. 3. Polymerase chain reaction (PCR) analyses of spinach seed lots for the presence of *Verticillium longisporum* using a *V. longisporum*-specific primer pair. Lane 1, PCR amplification of DNA from DNA template extracted from a pure culture of *V. longisporum*, strain Bob70; lane 2, DNA template of *V. longisporum*, strain Bob70, in spinach seed DNA background; lane 3, DNA template extracted from *V. dahliae*, strain VdLs.16; lanes 4 to 18, DNA template extracted from spinach seed lots; lane 19, water (no template) control; lane L, DNA molecular weight ladder (Promega Corp., Madison, WI).

In this study, the initial attempt to quantify *V. dahliae* by qPCR using 1 ng of template DNA was not successful because the results were not reproducible. Hence, we subsequently increased the template DNA to 5 ng and were able to detect and quantify *V. dahliae* with sufficient sensitivity.

For a diagnostic assay to be successfully employed, a high level of sensitivity and specificity is required (6,9,31). The VertBt primers detect as little as 148 fg (equivalent of five nuclei) of *V. dahliae* DNA in the background of potato tissue as indicated by Atallah et al. (1), based upon one *V. dahliae* genome in one nucleus ≈ 28 fg (42). In spinach seed extracts, we found that the VertBt primers reliably quantified *V. dahliae* at a sensitivity limit of 1 infected seed per 100 (≈ 29 nuclei or 812 fg of DNA), which is well below the current Mexican government importation standard for *V. dahliae*-infected spinach seed of $\leq 10\%$ (21). At limits of seed infection $< 1.3\%$, detection was possible but quantification was not reproducible because there were cases in which the test was negative in some replicates of $< 1.3\%$ infection levels. Compared with the standard NP-10 plate assay, the analytical and diagnostic sensitivity of the qPCR assay described herein is high. Diagnostic assays of 24 spinach germplasm accessions for seed infection with *V. dahliae* using the qPCR assay and NP-10 plate assay showed that the qPCR assay detected infected seed with 100% accuracy (23 of 23) whereas the NP-10 plate assay detection accuracy was only 29% (7 of 24). Similarly high diagnostic sensitivity of PCR over plate assays for detection of *V. dahliae* has been previously reported (1,23,26,29). These results clearly show the greater diagnostic sensitivity and specificity of the qPCR assay over the NP-10 plate assay.

The recent discovery that *V. longisporum* is a hybrid species sharing genomic sequence in common with *V. dahliae* (19) prompted examination of possible cross-amplification of *V. longisporum*. Even though *V. longisporum* is a crucifer-specific pathogen and not expected to be associated with spinach seed (12,18,42), *V. longisporum* may nevertheless be a surface contaminant on spinach seed, and empirical proof is desirable for the satisfaction of the end users of the assay. Therefore, we screened the commercial seed lots with a *V. longisporum*-specific primer pair (42). In addition, conidia length measurements (4,700 conidia) of fungal isolates from five seed lots did not indicate the presence of *V. longisporum*. The conidia length differences between *V. dahliae* and *V. longisporum* reported in this study (3.91 ± 0.80 μm *V. dahliae* versus 8.75 ± 1.55 μm *V. longisporum*) and those reported by Karapapa et al. (24) (4.4 ± 0.23 μm *V. dahliae* versus 7.9 ± 0.08 μm *V. longisporum*) were similar. In summary, both molecular evidence and that obtained from conidia size measurements indicated that *V. longisporum* was not present on the spinach seed lots examined and, thus, did not interfere with the accuracy of the qPCR assay.

Repeatability or reproducibility of test results is another key consideration in seed health test assay development (3,34). The qPCR assay developed here is reproducible between replicate samples of a seed lot and real-time PCR instruments. The Cq standard deviation for the experiments of the naturally infected seed lots was 0.05 to 0.81, which is within limits of minor random fluctuations in instrumental measurement. Assays of seed samples with artificial infection gradient of 0.7 to 64% on both the Bio-Rad ICycler and Roche Diagnostics LightCycler real-time PCR instruments yielded nearly identical measurements of pathogen DNA from spinach seed samples. These two real-time PCR instruments are in widespread use, enabling applicability of the test in many research laboratories.

A challenging aspect in developing a PCR-based pathogen assay is finding a significant correlation between seed pathogen DNA quantity as measured by qPCR and seed pathogen incidence as determined by plate assays. We and others have demonstrated that these measures are highly correlated in seed samples with artificial infection gradient created by mixing infected seed and

uninfected seed (or those with a very low level of infection), or in seed samples artificially inoculated with an incremental pathogen dose (8,17,33). However, in naturally infected seed lots, the correlation between seed pathogen DNA content and percentage of seed infected is often low or not evident (8,13,22), with few exceptions (3,13). Such incongruity is not due to flaws in DNA purification or qPCR parameters. Rather, the major causes for this incongruity may be (i) seed lot to seed lot variability in the amount of pathogen among individual seed in a seed lot or (ii) the ability of qPCR to quantify both live and dead fungi. If one seed lot contains only a few infected seed with a high pathogen load per seed, compared with another seed lot with a high number of infected seed at low pathogen load per individual infected seed, qPCR quantification may give similar results for the two seed lots but NP-10 plate would record a higher percentage infection in the latter seed lot. Similarly, more fungi would be detectable using the NP-10 plate assay in seed lots with more live fungi, regardless of the total fungal biomass in the seed lot. A qPCR assay based on a sampling scheme of one seed per DNA extraction could provide a robust assessment of the relationship between seed pathogen DNA content and percent seed infection (8,13), although the technologies to make this approach commercially feasible do not currently exist. Increasing the amount of seed per DNA extraction might also be a sound alternative to narrow the discrepancy between plate and qPCR assays. In developing the present assay, 1,000 seeds were used per DNA extraction. The large number of seed per DNA extraction was undoubtedly useful in decreasing inter-replicate variations. However, even the testing of this high number of seed per sample did not lead to a strong correlation between the percentage of seed infected determined by a plate assay and seed pathogen DNA quantity determined by qPCR in the 15 naturally infected commercial seed lots.

Even though a high correlation between the percentage of seed infected and pathogen DNA quantity is not the main focus of many of the qPCR assays currently available for seed pathogen detection and quantification, the assays provide important qPCR thresholds of practical value. For example, McNeil et al. (33) found that 44 pg of *Tilletia caries* DNA corresponds to < 1 conidia/seed of wheat, Bates et al. (3) found that 2.8 pg of *Pyrenophora* sp. DNA corresponds with 0.3% barley seed infection, Justesen et al. (22) found that 8.6 pg of *Pyrenophora graminea* DNA corresponds with 5% seed infection in a susceptible barley variety, and Guillemette et al. (16) found that 2% infection of cruciferous seed with *Alternaria brassicae* corresponds with pathogen DNA quantity equivalent of a 34 Cq value. These examples demonstrate that seed pathogen DNA quantity in conjunction with a regulatory threshold could be a useful guide in making seed treatment and regulatory decisions for plant pathogens. In the present study, even if Cq was not strongly correlated with percent seed infection from seed lot to seed lot, the overall trend is that as the Cq value increases the percentage of seed infected decreases. We observed that a Cq value of ≥ 31 corresponds with a percent seed infection of $\leq 1.3\%$ in the 15 naturally infected spinach seed lots. Based on this relationship, the qPCR assay may be applied for seed treatment decisions to limit the migration of *V. dahliae* in spinach seed. Although the correlation between percent seed infection and seed pathogen DNA content was not high for the naturally infected spinach seed lots, the assay also can provide a qualitative measure of infection to narrow down the numbers of seed lots to test for the verification of infection levels. Hence, for seed samples with Cq values of 29 to 31 (3 of 15 seed lots in this study) (Table 1), the NP-10 plate or blotter assay for the verification of percent seed infection would be advisable.

Bates et al. (3) stipulated that a seed health test should also be sensitive, specific, reliable, and rapid. The assay developed herein is sufficiently sensitive, with a detection limit of 1 infected seed per 100. In addition, the qPCR assay takes a day to complete

compared with ≈ 2 weeks required for a conventional NP-10 plate or blotter assay. The use of the IKA analytical grinding mill for sample homogenization represented a key feature of the assay, enabling analyses of an increased number of seed per sample and the enhanced robustness of the assay. The qPCR assay reliably quantified DNA of *V. dahliae* between replicate samples drawn from a seed lot, PCRs, and real-time PCR instruments.

Future improvement of the assay will depend on advances in related technologies such as an automated high-throughput seed grinding system to enable parallel processing of large numbers of samples, and kits specifically designed for purification of DNA from seed tissue. At present, however, it is difficult to conceive of a different seed lot sampling design that may improve the co-linearity between percent seed infection determined by the plate assay and pathogen DNA quantity. In the current format, the qPCR assay described herein can be useful to assess the infection levels of *V. dahliae* in spinach seed lots, representing a valuable tool in the management of Verticillium wilt. The qPCR assay also may be useful as a tool to assist in screening spinach germplasm collections for resistance to *V. dahliae*.

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