CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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Development of a rapid, quantitative, DNA-based assay for *V. dahliae* in spinach seed

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SUMMARY

The fungal pathogen *Verticillium dahliae* can be seedborne in spinach, lettuce, and in some weeds. Spinach is not affected by Verticillium wilt in commercial production. However, planting *V. dahliae*-infested seed may contribute to Verticillium wilt epidemics on lettuce and other vegetable crops by increasing inoculum levels in the soil and by the introducing exotic strains of the fungus. To curtail the spread of the pathogen, a DNA-based assay (qPCR) that can be useful for the quick assessment of the amount of infection in spinach seeds was developed and validated. We have ascertained that the qPCR method detects *V. dahliae* in the background of spinach seed tissues, and employed the use of an analytical grinder to increase the number of seeds for analyses. We routinely ground 1000 seeds per sample for qPCR testing. The qPCR method reliably detected seed infection at the 2% level in an artificial seed infection gradient and at the 1.3% level in a commercial seed lot. A strong correlation was observed between the qPCR value (Cq) and the percent seed infected for the artificial seed infection gradient. In total, fifteen commercial spinach seed lots were analyzed for percentage of infected seed using NP10 plate assays and by qPCR. A weak correlation was observed between the mid range of Cq values and percent seed infected in the naturally infected commercial seed lots, likely due to variation in the amount of *V. dahliae* per seed. However, a Cq of $\geq$ 31 correlated with $\leq$ 1.3% infected seed and a Cq value of $\leq$ 29.6 was associated with a seed infection level of $\geq$ 5.8%. For this reason, it is advisable that the NP10 plate test be used to verify infection levels in the range of 29-31 (3 out of 15 seed lots tested in this study). qPCR analyses of fungicide-treated seeds from five different cultivars revealed decreased quantities of the pathogen DNA that was detectable as compared to the untreated controls, suggesting that the assay may also be useful for determining the efficacy of seed treatments. Additional validation of the assay revealed that the assay could potentially detect *Verticillium longisporum*. However, *V. longisporum* was not detected in spinach seed lots from Europe and the US, and therefore should not interfere with the assay. Additionally, similar Cq values were obtained on two different real-time PCR instruments, indicating that the assay can be applicable in other laboratories using a different instrument.

PROJECT TITLE: **Development of a rapid, quantitative, DNA-based assay for *V. dahliae* in spinach seed.**

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OBJECTIVE: To develop a rapid, quantitative, DNA-based assay for *V. dahliae* in spinach seed.

PROCEDURES:

Seeds from the USDA spinach accessions were obtained from the United States Department of Agriculture, North Central Regional Plant Introduction Station (NCRPIS) spinach germplasm collection. Twenty seeds were placed on NP10 plating medium for each accession to assess the number infected with *V. dahliae*. The seeds were scored for the presence or absence of *V. dahliae* after 14 days of incubation at 25^{o}C.

For qPCR sampling of the NCRPIS spinach germplasm collection, four seeds from each accession were ground with a micropestle in a microfuge tube. DNA was extracted from the samples using a commercially available DNA extraction kit and quantified.

For the seed health tests on the initial two commercial spinach seed lots (seed lots 6 and 14) on NP10 plates, 600 seeds from each seed lot were surface-sterilized for two min in % bleach, washed, dried overnight, and placed on NP10 medium, with 10 seeds per plate. Seeds were scored for the presence of *V. dahliae* after 10 days of incubation on the plates. There were three replicate experiments for the two commercial lots, each with 200 seeds. The seeds were scored for the presence or absence of *V. dahliae* after 10 days of incubation. The seeds from the remaining seed lots were handled similarly except that 400 seeds were plated for each seed lot (Table 2), in four replicate experiments with 100 seeds each.

For qPCR sampling of the commercial spinach seed lots, mixtures of *V. dahliae*-infested and clean seed (0.5% infection) from seed lots 6 and 14 were prepared in different proportions (0.5, 2, 5, 10, 25, 50 and 64%) based on the results of the NP10 plating assay, creating an artificial seed infection gradient. One thousand seeds were ground with an IKA A-10 mill. The mill cleaning protocol between the grinding of samples involved thoroughly washing the grinding chamber with water, followed by a rinse with 70% ethanol and wiping the chamber. Three

samples of 200 mg each were taken from each respective proportion. DNA was extracted from each sample using a commercially available DNA extraction kit and quantified. DNA concentration was standardized to 1 ng/µl for qPCR. For the remaining seed lots, three samples of 1000 seeds were ground for each and the DNA was extracted from 200 mg of each sample.

The qPCR assays were set up using iQ™ SYBR® Green Supermix (Bio-Rad), *β-tubulin* primer mix, and 5 µl of DNA template in a 20 µl final volume. Primer sequences VertBt-F and VertBt-R for the *β-tubulin* primer mix were previously characterized [1]. Standard curves and reaction efficiency were performed using serial dilutions of the cloned *β-tubulin* sequence from *V. dahliae*. The reactions were run in a LightCycler 480 (Roche) and results analyzed with accompanying software (Roche). For inter-instrument comparisons, the results from both the Lightcycler and ICycler IQ (Bio-Rad) were compared. PCR products were sequenced at MCLAB (San Francisco, CA).

Verticillium longisporum-specific DNA primers [8] were used in conventional PCR on DNA extracted from each of the 15 commercial seed lots.

For additional details on the methods and results, refer to Durressa et al. [2].

RESULTS:

Tests were performed to validate the use of the *β-tubulin* primer set for qPCR. First, PCR using the primer set confirmed that only a single DNA product of the size expected (115 bp) was amplified from isolates of *V. dahliae* and also from *V. longisporum* but not the related species, *V. albo-atrum* (Figure 1).

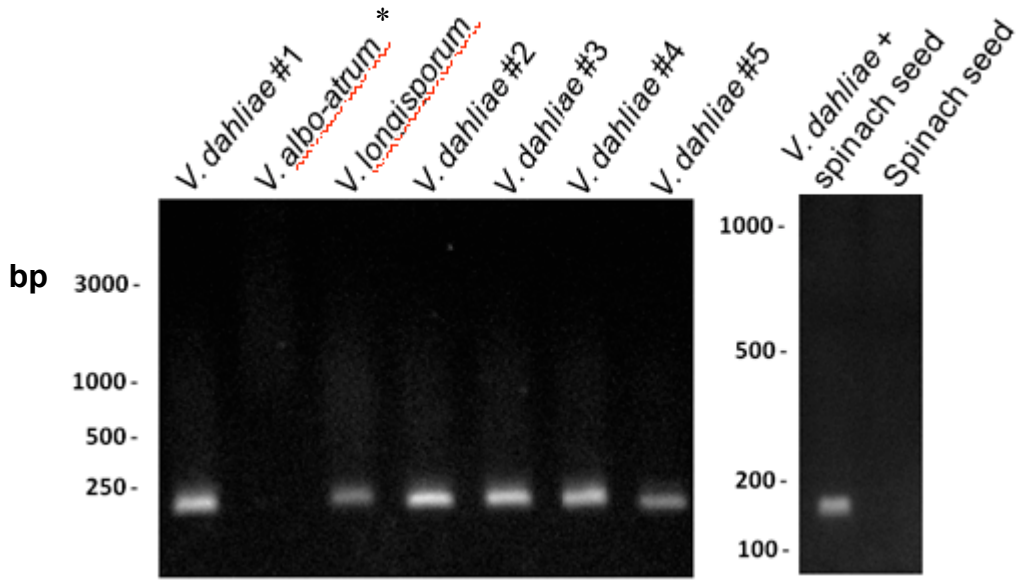

Figure 1. Amplification of a 115 base pair DNA product of the *β-tubulin* gene from *Verticillium dahliae* and *V. longisporum* using the primer pair VertBtF and VertBtR. The separate isolates of *V. dahliae* include those from tomato (#1), spinach (#2), spinach (#3), lettuce (#4), and lettuce (#5). *This type of *V. albo-atrum* strain was recently reclassified as the new species, *Verticillium alfalfae* [5]. The positions of the DNA size markers in base pairs (bp) are shown.

Spinach seed tested for the amplification of DNA in the presence or absence of *V. dahliae* revealed that the expected product of 115 base pairs was present in seeds that were spiked with *V. dahliae*, but not in spinach seed only (spinach seed; Figure 1). Sequence analysis of the DNA product also confirmed that the *β-tubulin* sequence was amplified from *V. dahliae* (data not shown), and not from other sources.

Initial screening of spinach seeds using the qPCR assay was focused on seeds derived from spinach accessions from the NCRPIS spinach germplasm collection. The results of the qPCR assay were compared with the results of a plate assay for the detection of *V. dahliae* on NP10 medium. As shown in Table 1, an increased copy number was associated with a lower Cq value. In other words, the lower the Cq value, the more DNA from *V. dahliae* is present.

Table 1. Comparison of qPCR and NP10 plate assay to detect *V. dahliae* in the NCRPIS spinach germplasm collection.

Accession Name	Copy number[a]	Pathogen Mean Cq	qPCR assay[c]	Plate assay[d]
PI 174388	15.4	34.2	+	-
PI 200882	28.9	32.88	+	-
PI 179507	45.7	32.31	+	-
PI 171859	10.6	34.08	+	-
PI 358259	6.11	35.22	+	-
NSL28218	138	30.64	+	+
PI 254565	21.8	33.31	+	-
PI 368825	3250	29.03	+	+
PI 339546	101	30.97	+	-
PI 175930	228	30.05	+	+
PI 370602	144	30.9	+	-
PI 176771	151	31.18	+	-
NSL28216	983	28.01	+	+
NSL6082	96.9	31.02	+	-
PI 604791	3780	28.41	+	-
PI 361127	1.68	36.59	+	+
PI 206473	232	29.88	+	-
PI 370602	634	28.58	+	-
PI 220121	6750	25.39	+	+
NSL 184380	126000	21.49	+	-
PI 445784	0	N/A[b]	-	-

a = Copy number was estimated based on a standard curve using the *β tubulin* DNA sequence and primers VertBt-F and VertBt-R

b = N/A refers to no amplification or Cq values of $\geq$ 40 in the qPCR assay.

c = Four seeds were ground for each accession.

d = Twenty seeds were scored for the presence or absence of *V. dahliae* on NP10 plating medium.

Detection of *V. dahliae* in 20 out of 21 spinach accessions was possible using the qPCR assay (with Cq cutoff of $\geq$ 40; Table 1). In the seeds of 14 of 20 spinach accessions, *V. dahliae* was not detectable using the NP10 plating assay, although *V. dahliae* was detectable by the qPCR assay in all accessions except PI 445784. In the accession PI 445784, *V. dahliae* was not

detectable using either assay (Table 1). However, it is important to note that these results were obtained from only small seed samples (4 per accession for the qPCR assay, and 20 per accession in the NP10 plating assay).

To increase the number of commercial seeds that can be analyzed in one qPCR assay, we used a grinding mill to efficiently grind spinach seeds. We initially tested the qPCR assay by mixing clean seed (0.7% infected; seed lot 14 in Table 2) with infected seed (64% infected seed; seed lot 6 in Table 2) at different proportions to create an artificial seed infection gradient (0.7%, 2%, 5%, 10%, 25%, 50%, and 64%). Three samples for each of these grindings were tested by qPCR (Figure 2). The results of the qPCR assay indicated a strong correlation between copy number and percent seeds infected in the 0.7% to 64% range (Figure 2). On a 0 to 1 scale (with 1 representing a perfect correlation) we obtained a correlation value of 0.99 (Figure 2).

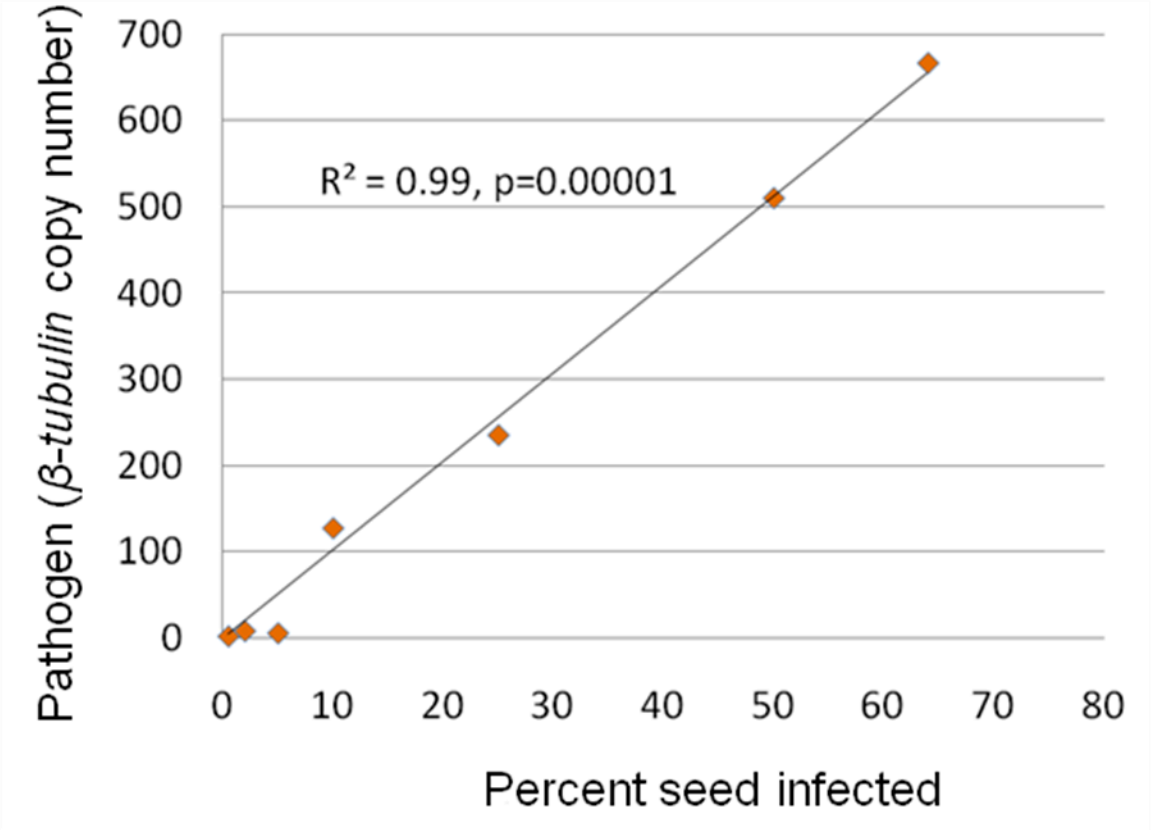

Figure 2. Linear regression of the *V. dahliae β-tubulin* copy number obtained by qPCR and percent seeds infected. Pathogen copy number values were obtained from three separate samplings of 1000 seeds and a standard curve using known quantities of *β-tubulin* DNA from *V. dahliae*. The percentage of seeds infected with *V. dahliae* was determined by analysis of seeds on NP10 plates.

Next, we examined all 15 naturally infected commercial spinach seed lots by seed health tests on NP10 plates and by qPCR. Although the correlation between copy number and percent seeds infected was much lower for the 15 commercial spinach seed lots, these results revealed that all seed lots with a Cq value of $\leq$ 29.6 were infected at a level of 6 % or higher (Table 2). A

lower Cq value correlates with a higher amount of DNA amplified from *V. dahliae*. As shown in Table 2, seed lots 13, 14 and 15 had the highest Cq values (31.1, 35.8 and 34.4, respectively, ± SD) and the lowest numbers of infected seeds (1.3, 0.7 and 0.3 %, respectively). In contrast, all Cq values below 29.6 were correlated with seed infection levels of ≥ 16%. The levels of the external control (spinach actin) amplification were similar for all seed lots tested (data not shown).

Table 2. Comparison of qPCR and NP10 plate assays to detect *V. dahliae* in commercial spinach seed lots.

Seed lot[a]	Infected seed/ non-infected seed[c]	Percent seed infected	*β-tubulin* Cq ± SD
1	341/400	85	26.21 ± 0.52
2	307/400	77	25.38 ± 0.20
3	301/400	75	27.01 ± 0.05
4	289/400	72	26.32 ± 0.53
5	272/400	68	27. 78 ± 0.68
6	385/600	64	25.94 ± 0.48
7	96/400	24	26.45 ± 0.05
8	75/400	19	28.77 ± 0.11
9	66/400	17	29.02 ± 0.15
10	63/400	16	26.09 ± 0.31
11	63/400	16	27.20 ± 0.28
12	23/400	5.8	29.60 ± 0.23
13	5/400	1.3	31.11 ± 0.50
14	4/600	0.7	35.81 ± 81
15[b]	1/400	0.3	34.44 ± 0.54

a = Among the 15 seed lots shown, seed lots 1, 2 and 5 are different seed lots of the same spinach cultivar. Seed lots 6 and 14 are also of the same cultivar. The remaining seed lots (3, 4, 7, 8, 9, 10, 11, and 13) each represent a different spinach cultivar.
b = For this seed lot, 1000 seeds were ground. For all other seed lots, 3 or more replicates of 1000 seeds were tested for qPCR.
c = The numbers of infected seeds in each lot were determined in seed health tests on NP10 plates.

The next aim was to use the qPCR assay to examine the level of pathogen DNA in spinach seeds that were either treated or untreated with a fungicide. The seed lots used in the comparison included 3, 4, 5, 7, and 8 (Table 2). Seed health tests conducted by NP10 plating using intact seeds revealed that each of the treated seed lots was free of the pathogen (0/400 seeds infected for each lot, not shown). However, when the seeds were broken open and plated, growth of the pathogen was detectable on the NP10 medium (see report of Dr. Krishna Subbarao). The levels of detectable pathogen per seed for each of these untreated seed lots are shown in Table 2. Quantitative PCR analyses revealed a significant decrease in the amount of detectable DNA from the fungus in each fungicide-treated seed lot, as compared to the untreated controls (Figure 3).

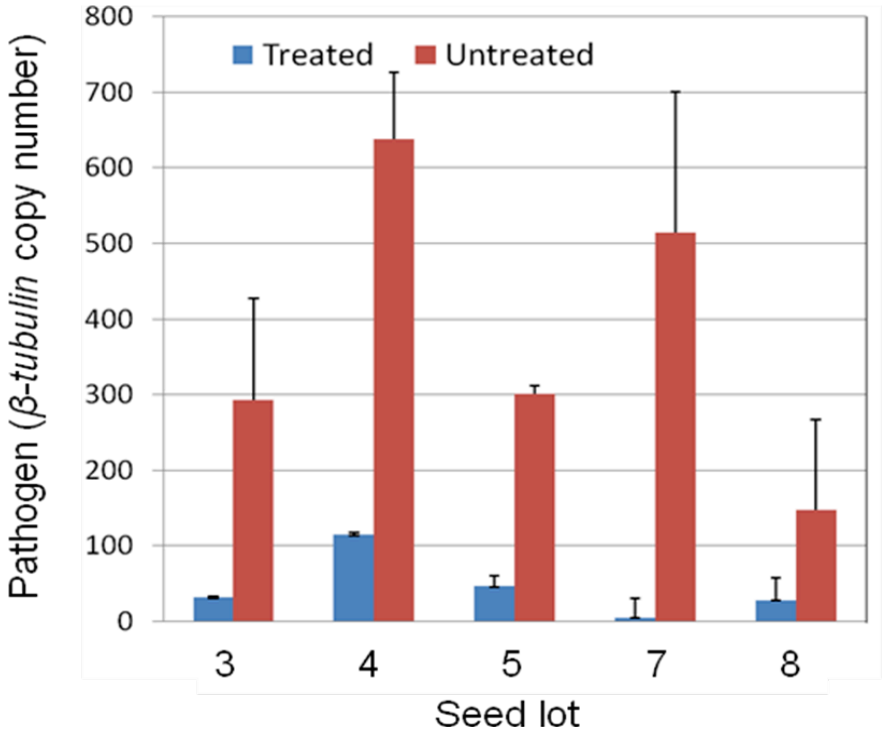

Figure 3. Bar chart of *β-tubulin* copy number from the pathogen, *V. dahliae*, in five seed lots that were treated (blue bars) with a fungicide or untreated (red bars). The *β-tubulin* copy number was determined using a standard curve using known quantities of *β-tubulin* DNA from *V. dahliae* versus quantification cycle (Cq) derived from qPCR on the seed lots. Error bars are ± SD.

Additional experiments were conducted for further validation of the technique. These experiments included tests i) to determine whether *Verticillium longisporum* could be detected in spinach seeds since we had obtained evidence that the *β-tubulin* primers could amplify DNA from both *V. dahliae* and *V. longisporum* and ii) to compare the Cq values obtained from two different real-time PCR instruments to assess the variability between instruments. Not all laboratories have the lightCycler instrument, and therefore minimal variation between instruments could indicate wider applicability of the assay in other laboratories.

A primer pair was selected for specific DNA amplification of *V. longisporum* to determine whether *Verticillium longisporum* could be detected in spinach seeds. There was amplification of *V. longisporum* DNA from DNA extracted from a pure *V. longisporum* culture (Figure 4, lane 1) and from *V. longisporum* DNA spiked into spinach seed (Figure 4, lane 2), but not from any of the seed lots (Figure 4, lanes 4-18). 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 ± 0.80 µm compared with a conidia length of 8.75 ± 1.55 µm for *V. longisporum* isolate Bob70 (data not shown), providing additional evidence that the long-spored *V. longisporum* was not present in these seed lots.

The variation in the quantification values between two real-time PCR instruments was minimal (Figure 5A and 5B).

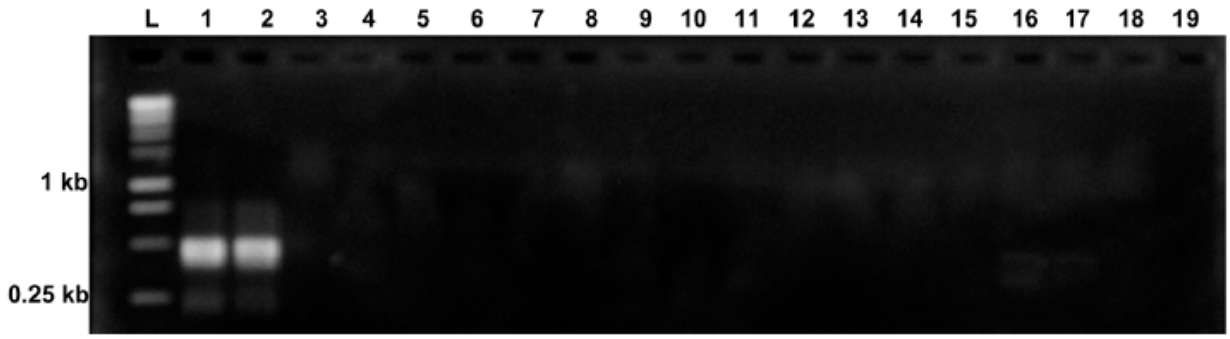

Figure 4. Polymerase chain reaction (PCR) analyses of spinach seed lots for the presence of *Verticillium longisporum* using a *V. longisporum*-specific primer pair. Lane 1, PCR amplification of DNA from DNA template extracted from a pure culture of *V. longisporum,* strain Bob70; lane 2, DNA template 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).

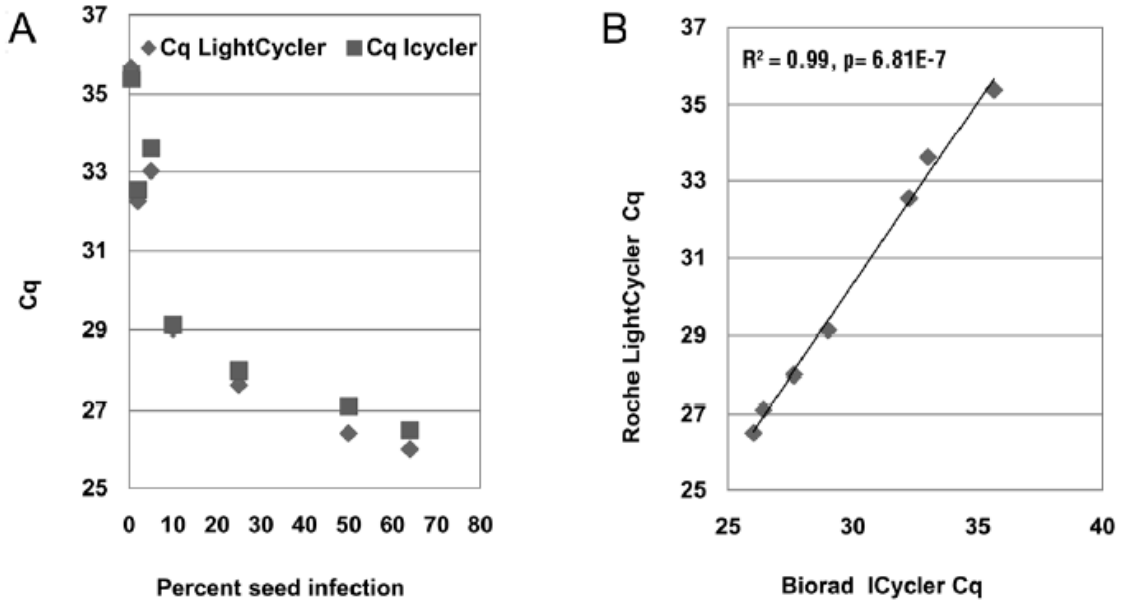

Figure 5. Interinstrumental reproducibility of the quantitative real-time polymerase chain reaction (PCR) assay for quantification of *Verticillium dahliae* in spinach seed with artificial infection levels. **A,** Scatter plot of the average threshold real-time PCR quantification cycle (Cq) readings 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.

DISCUSSION:

Because all spinach seed in California is imported, and the seed imported from Washington State or abroad are known to be infested with *V. dahliae* [3], an assay that enables accurate identification and quantification of the pathogen in seed has implications in limiting the spread of the pathogen.

The results indicate that the qPCR primers are specific for the detection of *V. dahliae* in the background of spinach seed tissues. Analyses of DNA amplification from other fungi indicate that the primers also detect DNA from the long-spored isolates of *Vertcillium*. Until recently, there had been controversy as to whether long-spored isolates of *V. dahliae* should be considered a separate species [7]. Recent analyses of the long-spored isolates of *Verticillium* suggest that these isolates represent a distinct hybrid *Verticillium* species known as *Verticillium longisporum* [4]. Since the hybrid species shares a copy of the DNA sequence of *V. dahliae*, the DNA primers used in this study detect *V. longisporum* in PCR, but not other fungi, including other related species [1]. *V. tricorpus* is regarded as a weak pathogen on many hosts [7], and the presence of this additional species in spinach seed could cause confusion in identification on plate assays. Without adequate training to discriminate fungal colony morphologies, *V. tricorpus* may sometimes be mistaken for *V. dahliae* in the NP10 plating assays. *V. tricorpus* was identified in seeds of the NCRPIS spinach germplasm collection.

The qPCR assay enabled detection of DNA from *V. dahliae* in the NCRPIS spinach germplasm collection, including seed samples that were not inoculated with *V. dahliae*. Comparing the results of the NP10 plating method and the qPCR assay indicated that a much lower number of seeds from these accessions were positive for *V. dahliae* using the NP10 plating assay. This finding indicated the high sensitivity of the qPCR assay. However, the seed samples tested for *V. dahliae* infection in Table 1 were pulverized by grinding seeds in a microcentrifuge tube using a micropestle followed by a lengthy DNA extraction protocol. For large scale screening of seed lots for *V. dahliae* infection, the micropestle approach and a lengthy DNA extraction protocol would be impractical. Results from both assays confirmed that seeds from most of the NCRPIS spinach germplasm collection were naturally infested with *V. dahliae*.

To rapidly quantify the percentage of seeds infected with *V. dahliae* from commercial seed lots, the grinding of sufficient numbers of seeds is required. The results indicate that the IKA grinding mill that we employed for seed grinding is suitable to grind large quantities of seed (1000 seeds) for qPCR sampling. We tested commercial spinach seed lots using the grinding mill and subsequent qPCR. The pathogen was detectable at the 0.7% level of seed infection (4/600 seeds infected) but detection was not consistent (not detected in all samples) at this level. However, the assay was consistent for detection of *V. dahliae* at the 1.3% level of infection (~1/100 seeds infected), where qPCR values were recorded in all samples tested. Analyses of all fifteen commercial seed lots revealed that seed lots with a qPCR value of $\leq$ 29.6 were infected at a level of about 6 % (5.8%) or higher. However, the correlation between percent seed infection and pathogen DNA content for all fifteen commercial seed lots was low. Nevertheless, the assay 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 in the range of 29 to 31 (3 of 15 seed lots in this study), the NP-10 plate assay for the verification of percent seed infection would be advisable.

Quantitative PCR analyses revealed a significant decrease in the amount of detectable DNA from the fungus in each fungicide-treated seed lot, as compared to the untreated controls. Since the fungal DNA within seeds was anticipated to remain largely intact with only limited

degradation after the death of the fungus, this result was unexpected. However, measurements of *β-tubulin* copy number clearly indicated a reduction in the amount DNA detectable in the qPCR assay following fungicide treatment. These results suggest that following seed treatment and death of the fungus, there is concomitant degradation of the fungal DNA that can be detected. This result suggests that the qPCR technique could be useful to assess the efficacy of seed treatments as well.

Additional validation and testing of the qPCR assay revealed that there is minimal variation between two different real-time PCR instruments, and therefore the assay may be more widely applied in laboratories with different real-time PCR instruments. Also, based on spore measurements and PCR analysis, *V. longisporum* was not detectable in spinach seeds collected from the US and Europe, and therefore will not interfere with detection of *V. dahliae*.

The qPCR assay for *V. dahliae* may be a useful tool to limit the spread of the pathogen. The qPCR assay is rapid, with quantification of *V. dahliae* possible within one day. The qPCR assay was consistent at detection of the pathogen at a 1.3% infection threshold in a commercial seed lot. Currently, the Mexican importation standard requires that seed have less than 10% infestation with *V. dahliae* [6]. As noted above, it is advisable to verify the amount of seed infection within a given range of Cq values (29-31). Thus, the qPCR can be useful to substantially narrow the number of seed lots to test with the NP10 plating method. Initial analyses also indicate that the assay can have further utility for assessing resistance of spinach germplasm to *V. dahliae*.

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