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. We are developing a DNA-based assay (qPCR) that can be useful for the detection of *V. dahliae* and for rapidly quantifying the amount of infection in spinach seeds. 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 analysis. We routinely ground 1000 seeds per sample for qPCR testing. The results indicated that the qPCR method can reliably detect seed infection at the 2% level. We obtained eleven additional commercial spinach seed lots, and analyzed them for percentage of infected seed using the NP10 plate assay. Quantitative PCR values were obtained from each of the eleven additional seed lots, revealing that a qPCR value of $\leq$ 29.6 correlated with > 6% infected seed. Additionally, 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. Because recent evidence indicates that the long-spored isolates of *V. dahliae* represent a distinct species, *Verticillium longisporum*, and the qPCR assay also detected *V. longisporum*, we are currently examining strains of *Verticillium* obtained from spinach seeds to ascertain if long-spored strains are present among the isolates. We are also conducting additional testing with an alternate DNA extraction procedure.

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 Ames, Iowa 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 C.

For qPCR sampling of the Ames, Iowa spinach germplasm accessions, 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 12) 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 12 were prepared in different proportions (0.5, 2, 5, 10, 25, 50 and 64%) based on the results of the NP10 plating assay. 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 (Atallah et al. 2007). 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). Quantitative PCR analysis was performed with the LightCycler 480 software release 1.5.0 SP3 (Roche). PCR products were sequenced at MCLAB (San Francisco, CA).

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).

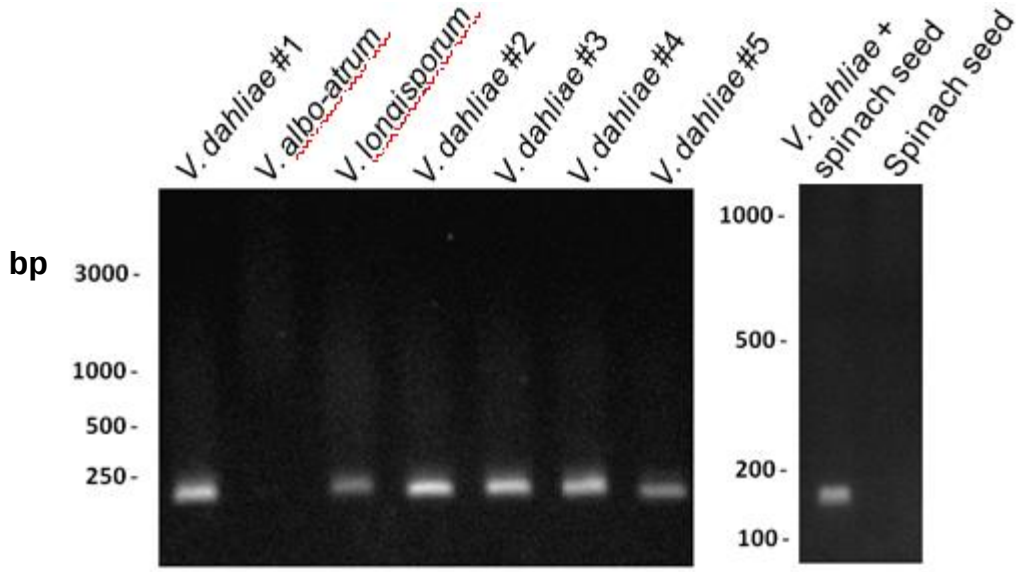

Figure 1. Amplification of a 115 base pair DNA product of the *β-tubulin* gene from *Verticillium dahliae* and *V. longisporum* using the primer pair VertBtF and VertBtR. The separate isolates of *V. dahliae* include those from tomato (#1), spinach (#2), spinach (#3), lettuce (#4), and lettuce (#5). 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 *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 Ames, Iowa 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 Ames, Iowa 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.

We were able to detect *V. dahliae* in 20 out of 21 spinach accessions using the qPCR assay (with Cp 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. 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 incorporated the use of a grinding mill to efficiently grind spinach seeds. We initially tested the qPCR assay by mixing clean seed (0.7% infected; seed lot 12 in Table 2) with infected seed (64% infected seed; seed lot 6 in Table 2) at different proportions. We were able to thoroughly grind 1000 seeds for each of the seed infection levels (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).

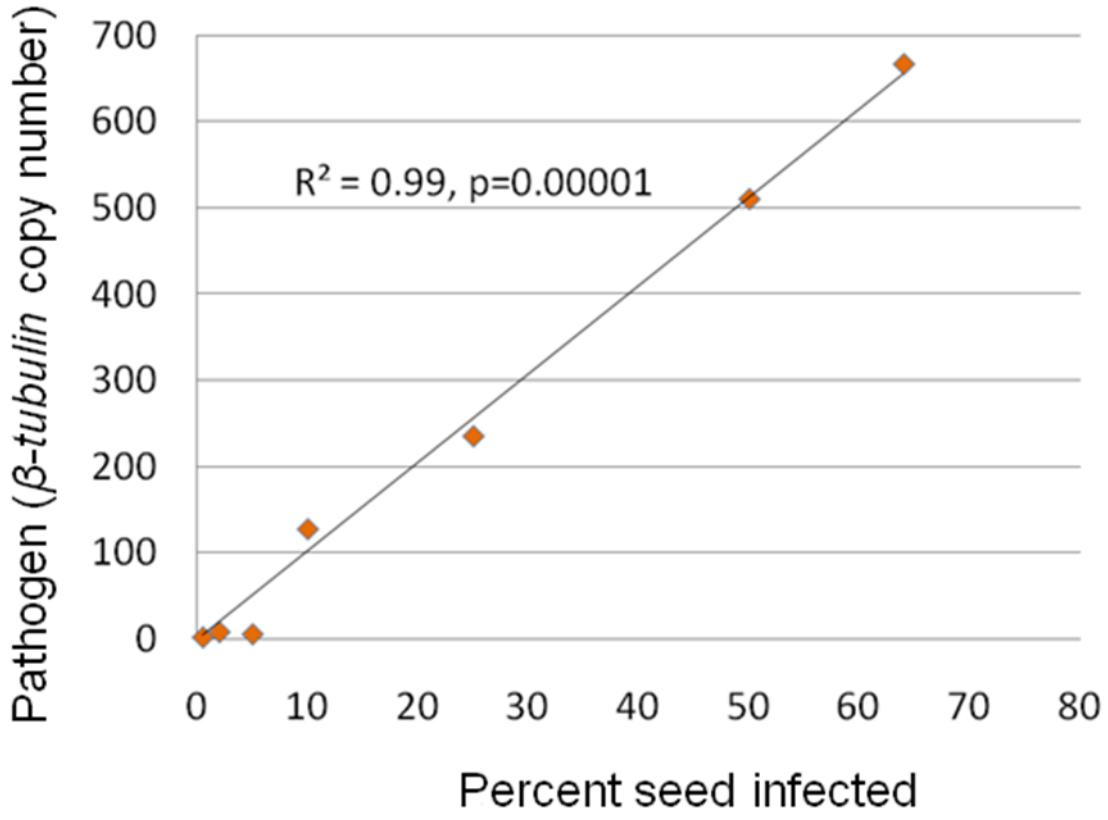

Figure 2. Linear regression of the *V. dahliae β-tubulin* copy number obtained by qPCR and percent seeds infected. Pathogen copy number values were obtained from three separate samplings of 1000 seeds and a standard curve using known quantities of *β-tubulin* DNA from *V. dahliae*. The percentage of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

Next, we examined an additional eleven commercial spinach seed lots by seed health tests on NP10 plates and by qPCR. Comparison of these results revealed that all seed lots with a Cq

value of ≤ 29.6 were infected at a level of 6 % or higher (Table 2). In qPCR, there is an inverse relationship between the Cq value and the amount of target DNA amplified. A lower quantification cycle value correlates with a higher amount of DNA amplified from *V. dahliae*. As shown in Table 2, seed lots 12 and 13 had the highest Cq values (35.8 and 34.4, respectively, ± SD) and the lowest numbers of infected seeds (0.7 and 0.3 %, respectively). In contrast, all Cq values between 25.38 and 28.77 were correlated with seed infection levels of ≥ 16%. The levels of the exogenous 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	63/400	16	26.09 ± 0.31
10	63/400	16	27.20 ± 0.28
11	23/400	5.8	29.60 ± 0.23
12	4/600	0.7	35.81 ± 81
13[b]	1/400	0.3	34.44 ± 0.54

a = Among the 13 seed lots shown, seed lots 1, 2 and 5 are different seed lots of the same spinach cultivar. Seed lots 6 and 12 are also of the same cultivar. The remaining seed lots (3, 4, 7, 8, 9, 10, 11, and 13) each represent of 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 revealed that each of the treated seed lots was free of the pathogen (0/400 seeds infected for each lot, not shown). 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).

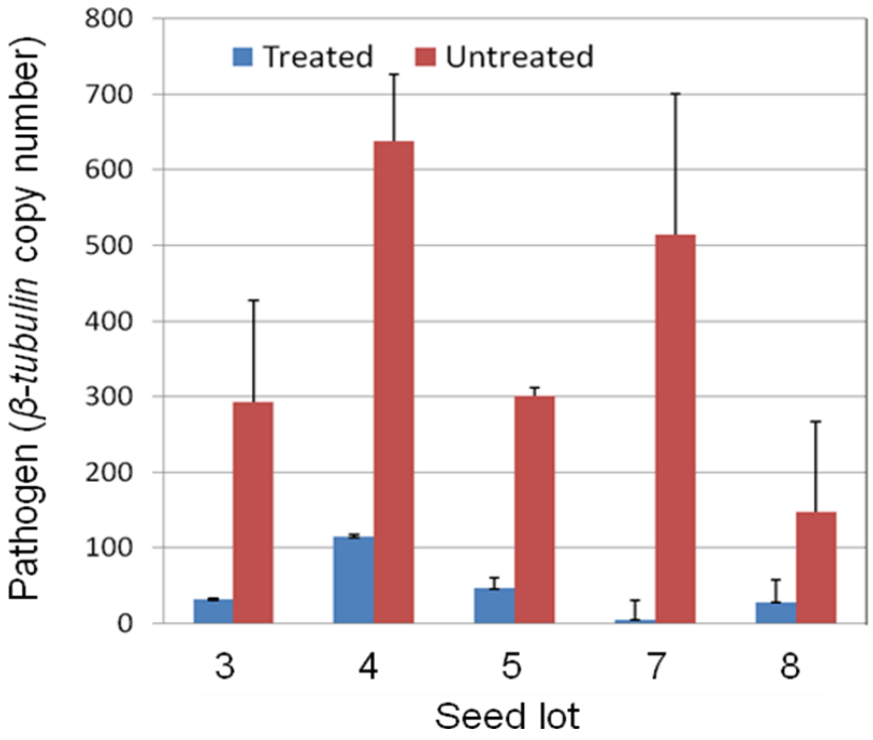

Figure 3. Bar chart of *β-tubulin* copy number from the pathogen, *V. dahliae*, in five seed lots that were treated (blue bars) with a fungicide or untreated (red bars). The *β-tubulin* copy number was determined using a standard curve using known quantities of *β-tubulin* DNA from *V. dahlia*e versus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

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* (du Toit, 2005), 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 has been some controversy as to whether long-spored isolates of *V. dahliae* should be considered a separate species (Klosterman et al. 2009). However, recent analyses of the long-spored isolates of *Verticillium* suggest that these isolates represent a distinct hybrid *Verticillium* species known as *Verticillium longisporum* (Inderbitzin et al. 2011). 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 another closely related species, *V. albo-atrum* (Fig. 1, and Atallah et al 2007). Based on the *β tubulin* DNA sequence comparisons between *Verticillium* isolates, it is clear why these primers do not amplify DNA from *V. albo-atrum* and *Verticillium tricorpus*. *V. tricorpus* is regarded as a weak pathogen on many hosts

(Klosterman et al. 2009), 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* accessions may sometimes be mistaken for *V. dahliae* in the NP10 plating assays. *V. tricorpus* was identified in seeds of the Ames, Iowa spinach germplasm collection.

The presence of *V. longisporum* in spinach seeds is not known at this time. Because *V. longisporum* is primarily a pathogen of cruciferous plants, such as cauliflower, we do not anticipate that these long-spored isolates are present in spinach seed. Nevertheless, we are currently assessing whether *V. longisporum* can be recovered from commercial spinach seed lots from Europe and the US Pacific Northwest.

The qPCR assay enabled detection of DNA from *V. dahliae* in the Ames, Iowa 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 Ames, Iowa spinach accessions 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. The grinding mill is durable and also easily cleaned since the grinding chamber is stainless steel. 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 at this level. However, the assay was consistent for detection of *V. dahliae* at the 2% level of infection (~2/100 seeds infected), where qPCR values were recorded in all samples tested. Analyses of all thirteen 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.

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.

We are currently in the process of collecting two additional commercial seed lots to further test the sensitivity of the assay for the quantification of *V. dahliae* in the 4 to 14% range of seed infection. The current DNA extraction protocol may be compared with others available, and we will assess the variability of the qPCR assay on two different real-time PCR machines from two different manufacturers. As mentioned above we are assessing whether long-spored isolates can be obtained from spinach seeds collected from the US and Europe.

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 2% infection threshold. Currently, the Mexican importation standard requires that seed have less than 10% infestation with *V. dahliae* (IPC, 2003). Information provided by the qPCR assay on the levels of seed infestation with *V. dahliae*, in addition to the NP10 plating method, can be useful to make informed decisions concerning acceptable infestation levels in spinach seeds.

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Citations:

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