CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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

Steve Klosterman
USDA-ARS
Salinas, CA

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 specific detection of *V. dahliae* and for rapidly assessing the level of infection in spinach seeds. We have thus far ascertained that the qPCR method is specific for detection of *V. dahliae* in the background of spinach seed tissues. In addition, we obtained two commercial spinach seed lots of the same cultivar, and analyzed them both for percentage of infected seed using the NP10 plate assay and the qPCR method. The seeds from both lots were mixed at different proportions and were analyzed using the qPCR method. The results indicate that the qPCR method can reliably detect seed infection at the 5% level. We are currently conducting additional testing with increased numbers of seeds per sample and alternate DNA extraction procedures that may help to further differentiate seed samples with levels of seed infection in the 2% to 25% range.

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

INVESTIGATOR: **Steve Klosterman**
USDA-ARS
1636 E. Alisal St
Salinas, CA 93905

COOPERATING PERSONNEL: **Krishna Subbarao**
University of California Davis, Salinas, CA

Beiquan Mou
USDA-ARS, Salinas, CA

Steven T. Koike
U. C. Cooperative Extension, Salinas, CA

Ryan Hayes
USDA-ARS, Salinas, CA

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.

Seeds from two commercial spinach seed lots were assessed for percent infestation with *V. dahliae* by the NP10 plating method. Specifically, 600 seeds from each seed lot were placed on NP10 medium, with 10 seeds per plate. 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.

For qPCR sampling of the Ames, Iowa spinach germplasm accessions, four seeds from each accession were placed in PowerPlant® DNA Isolation Kit buffer (MO BIO Laboratories Inc.) for 1 hour and ground with a micropestle in a microfuge tube. DNA was extracted from the samples following the manufacturer's instructions (MO BIO Laboratories Inc.), purified, and quantified with a Qubit® fluorometer (Invitrogen) using a Quant-iT™ dsDNA BR Assay Kit (Invitrogen).

For qPCR sampling of the commercial spinach seed lots, mixtures of *V. dahliae*-infested and clean seed (0.5% infection) were prepared in different proportions (0.5, 2, 5, 10, 25, 50 and 64%) based on the results of the NP10 plating assay. Seeds (1000 seeds) were ground with an IKA A-10 mill (figure 1) for 5-8 sec. The mill cleaning protocol between samples involved thoroughly washing the grinding chamber with water, followed by a rinse with 70% ethanol. Three samples of 100 mg were taken from each respective proportion. DNA was extracted from each sample using a FastDNA kit (MPBio) and quantified with a Qubit® fluorometer (Invitrogen) using a

Quant-iT™ dsDNA BR Assay Kit (Invitrogen). DNA concentration was standardized to 1 ng/ul for qPCR.

The qPCR assays were set up using iQ™ SYBR® Green Supermix (Bio-Rad), *β-tubulin* primer mix, and 1 µl of DNA template in a 20ul 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 *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* from different hosts and not the closely related species, *V. albo-atrum* (Figure 1). Second, 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 (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.

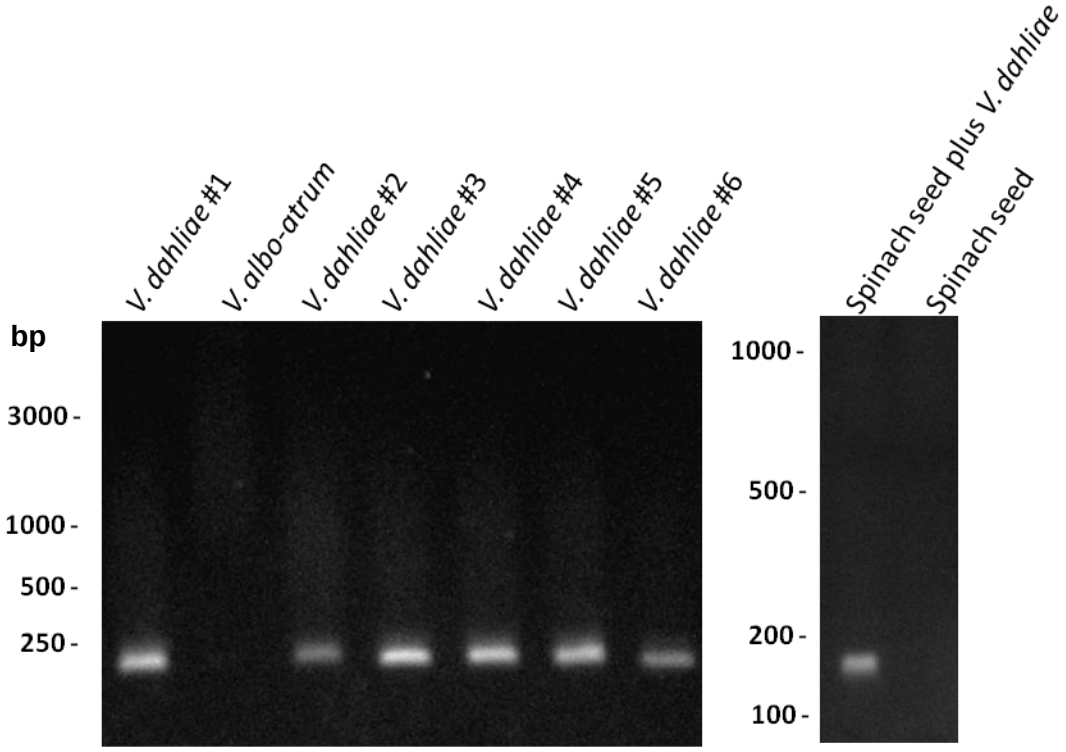

Figure 1. Specific amplification of a 115 base pair DNA product of the *β-tubulin* gene from *Verticillium dahliae* using the primer pair VertBtF and VertBtR. The separate isolates of *V. dahliae* included those from tomato (#1), cauliflower (#2), spinach (#3), spinach (#4), lettuce (#5), and lettuce (#6). The positions of the DNA size markers are shown in bp.

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 Cp value. In other words, the lower the Cp value, the more DNA from *V. dahliae* is present. We were able to detect *V. dahliae* in 18 out of 21 spinach accessions using the qPCR assay (with Cp cutoff of $\geq$ 35; Table 1). In the seeds of 12 of 20 spinach accessions, *V. dahliae* was not detectable using the NP10 plating assay, although *V. dahliae* was detectable by the qPCR assay in these same accessions. In the accessions PI 445784 and PI 358259, *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).

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 Cp**	**qPCR assay[b]**	**Plate assay[c]**
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	-	-

a = copy number was estimated based on a standard curve using the *β tubulin* DNA sequence and primers VertBt-F and VertBt-R
b = Cp values of $\geq$ 35 were counted as negative for detection of *V. dahliae* in the qPCR assay. Four seeds were ground for each accession.
c = Twenty seeds were scored for the presence or absence of *V. dahliae* on NP10 plating medium.

To increase the numbers of commercial seed that can be analyzed in one qPCR assay, we incorporated the use of a grinding mill to efficiently grind spinach seeds. We were able to thoroughly grind 1000 seeds for each of the seed infection levels (0.5%, 2%, 5%, 10%, 25%, 50%, and 64%). At least two samples for each of these grindings were tested by qPCR (Figure 2). The instrument used in qPCR analyses did not differentiate Cp values $\geq$ 35. Therefore, samples that were consistently recorded with Cp values $\geq$ 35 at the lower percent seeds infected (0.5% to 2%) were excluded from further analysis. The results of the qPCR assay indicated a strong correlation between Cp and percent seeds infected in 5% to 64% range (Figure 2). On a 0 to 1 scale (with 1 representing a perfect correlation) samples 1 and 2 had correlation values of 0.9184 and 0.9074, respectively.

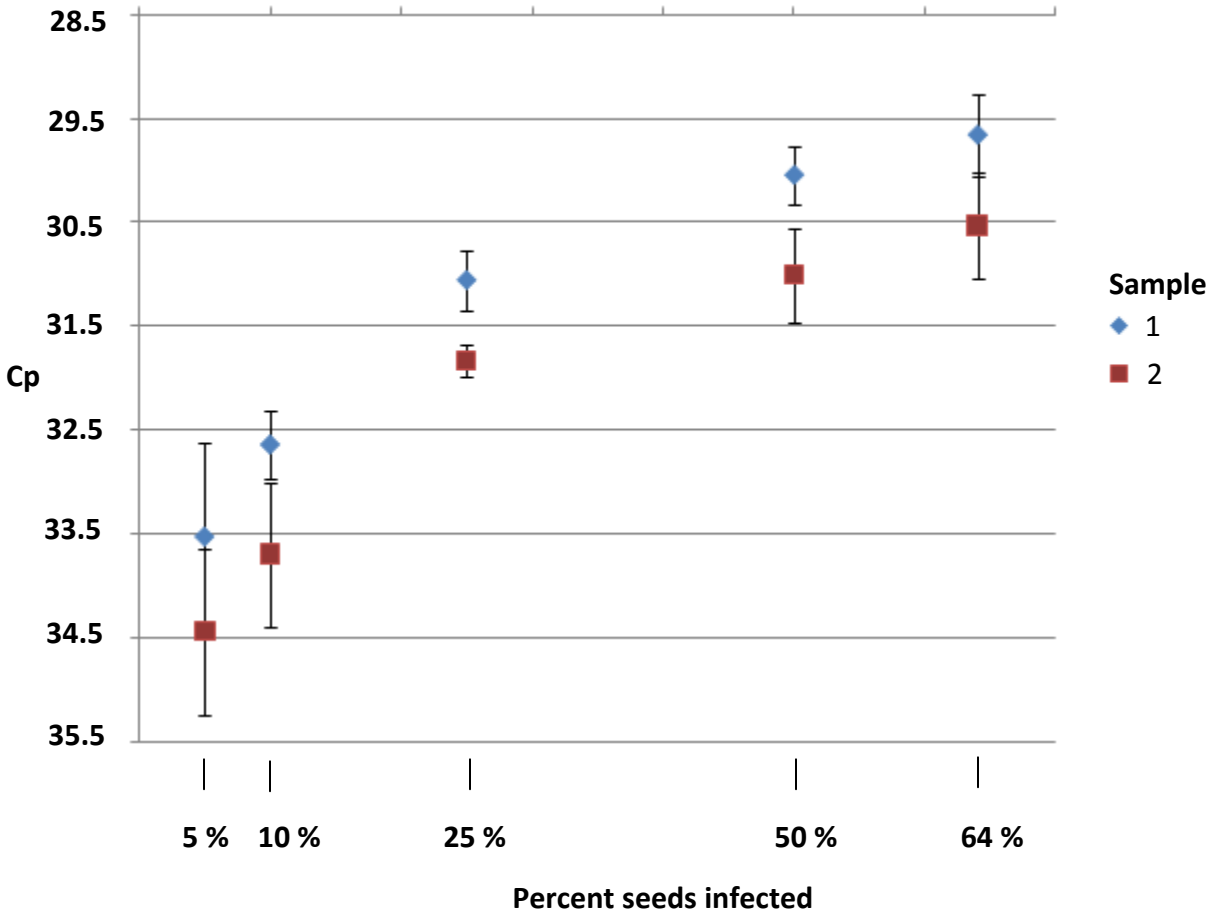

Figure 2. Graph of crossing point (Cp) values obtained by qPCR vs. percent seeds infected. Cp values were obtained from two separate samplings of the same grinding of 1000 seeds. The percent seeds infected with *V. dahliae* was determined by analysis on NP10 plates. Error bars indicate standard deviation. A lower Cp value represents more DNA of *V. dahliae* that is present.

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 or lettuce seed tissues. The DNA primers are also specific for the detection of *V. dahliae* and not other fungi, including the closely related species, *V. albo-atrum*. Based on sequence comparisons with *Verticillium tricorpus* isolates, it is also clear why these primers do not amplify DNA from *V. tricorpus*. *V. tricorpus* accessions may sometimes be mistaken for *V. dahliae* in the NP10 plating assay, which does not differentiate between these *Verticillium* species.

The qPCR assay enabled detection of DNA from *V. dahliae* from the Ames, Iowa spinach germplasm collection, even from 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. Nevertheless, results from both assays confirmed that seeds from 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 large numbers of seeds ($\geq$ 1000) is required. The results indicate that the grinding mill that we employed for seed grinding will allow large quantities of seed to be analyzed.

The results indicate that we cannot reliably detect *V. dahliae* at less than 5% seed infection at this time. We are investigating several modifications to further increase the sensitivity of the assay for the quantification of *V. dahliae* in the 5 to 25% range of detection. We will narrow the focus of the qPCR assay testing to the lower percent range (2% to 25%) of infected seed. The current DNA extraction protocol may be compared with others available. Also, we will test more commercial spinach seed lots and examine lettuce seeds.

The qPCR assay under development may be a useful tool to limit the spread of the pathogen. The qPCR assay is rapid, with detection of *V. dahliae* possible within one day. The qPCR assay allowed for detection to a 5% 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 or lettuce seeds.

Citations:

Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., Stevenson, W. R. 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology. 97:865-872.

du Toit, L.J., Derie, M.L., Hernandez-Perez, P. 2005. Verticillium wilt in spinach seed production. Plant Disease. 89:4-11.

IPC, 2003. International Phytosanitary Certificate No. 4051. Phytosanitary Federal Law of the Mexican United States.