CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM
April 1, 2013 - March 31, 2014

VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL

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SUMMARY

There were four objectives during the current funding cycle and included: 1) assessing the impact of immigrant populations of *V. dahliae* introduced from spinach seed on crops that follow spinach; 2) sampling spinach seedlings in organic and conventional production systems in Monterey, San Benito, Santa Barbara and Santa Cruz counties and assaying for *Verticillium*; 3) refining the race 2-specific PCR and analyzing the race structure of *V. dahliae* isolates from spinach seed from multiple seed production locations; and 4) continuing studies on the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables from coastal California. We initiated a controlled field experiment to assess the role of *V. dahliae* introduced via spinach seed into soil in causing *Verticillium* wilt on lettuce. Over the past three years, three spinach crops each in treatments with different proportions of *V. dahliae*-infested spinach seed were grown in replicated microplots during 2011 followed by two lettuce crops in all microplots during 2012. This was followed by two crops of spinach and a lettuce crop in 2013. Microsclerotia levels in the soil increased following spinach production using infested seeds. *Verticillium* wilt developed in both lettuce crops and the incidence during the spring season was proportional to the spinach seed infestation treatment established. *Verticillium dahliae* was isolated from each infected plant and identified to species using the species-specific primers. Direct evidence of the spread of *V. dahliae* from spinach seed to lettuce roots was obtained using a green fluorescence tagged strain. The second objective to examine why *Verticillium* wilt of lettuce is confined to the two valleys in CA even though cropping patterns are similar in other areas was continued. Twenty plants from 100 commercial spinach fields in Salinas, Pajaro, Santa Maria and San Juan valleys were sampled between 2011 and 2012, and the roots and petioles from each were plated on the NP-10 medium. More than 885 isolates recovered were characterized to species. The majority of these isolates were *V. isaacii* followed by *V. dahliae*. The frequency of recovery of *Verticillium* species did not explain the current distribution of the disease in lettuce but was correlated with the magnitude of spinach production in the four valleys. For the third objective, we successfully developed a PCR assay to rapidly identify race 2 *V. dahliae* isolates based on race-associated DNA sequences. We were able to characterize the races of *V. dahliae* isolates from multiple spinach seed lots from Chile, Denmark, the Netherlands, and Washington. Of the 340 isolates from spinach seed characterized, 4% were race 1 and 96% were race 2. Thus, both races are present in commercial spinach seed, but the vast majority of them are race 2. For the fourth objective, we used global collection of *V. dahliae* isolates was genotyped using microsatellite markers and analyzed to determine the genotypes of *V. dahliae* associated with different crop hosts. In addition to confirming the migration of isolates from spinach seed production regions into California, the study also identified genotypes associated with spinach seed and other crops including lettuce.
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OBJECTIVES:

A. Assess the impact of immigrant populations of V. dahliae introduced from spinach seed into soil on crops that follow spinach.

B. Characterize V. dahliae, V. isaacii, and V. klebahni collected from surveys of spinach production fields in four coastal counties.

C. Refine the race 2-specific PCR and analyze the race structure of V. dahliae isolates from spinach seed from multiple seed production locations.

D. Continue determining the genetic relationships among populations of V. dahliae from spinach seed and other vegetables grown in coastal California.

PROCEDURES AND RESULTS:

Objective A. Assess the impact of immigrant populations of V. dahliae introduced from spinach seed into soil on crops that follow spinach.

Verticillium dahliae can be easily disseminated or introduced into new fields or areas via infested seeds, planting materials, and soil. Up to 89% of the commercial seed lots from Denmark, New Zealand, The Netherlands, and the United States were infested with Verticillium species and individual seed lots with up to 90% infestation were not uncommon. On infested seed, V. dahliae colonizes the pericarp, seed coat, cotyledons, and radicle, and can form more than 250 microsclerotia per seed. Seedborne V. dahliae is transmitted systemically into new spinach seedlings and to the developing seeds in spinach plants grown for seed. In spinach production fields, the crop is harvested prior to symptom development, though the pathogen can become a part of the resident soil microbial community when the crop residue is incorporated into the soil.

Immigration of novel genotypes or races is a particularly serious concern as it interferes with the ability of breeders to select for durable resistance. More than 90% of the V. dahliae isolates
collected from spinach seed were identified as race 2 based on PCR assays. Although *V. dahliae* populations sampled from lettuce fields in the Salinas and Pajaro valleys between 1995-2013 were dominated by race 1, the unrestricted influx of novel *V. dahliae* genotypes or races via infested spinach seed will likely cause irreversible changes to the population and race structure of *V. dahliae* in these regions. Successful efforts over the past 15 years in developing *V. dahliae* race 1 resistance in lettuce cultivars will be compromised. Additionally, *V. dahliae* isolates from spinach seed have been shown to cause severe Verticillium wilt in several crops, including lettuce and tomato under greenhouse conditions. Despite these greenhouse experiments, direct evidence linking *V. dahliae* introduced into a field from spinach seeds and Verticillium wilt in subsequent lettuce crops is lacking. Furthermore, species of *Verticillium* other than *V. dahliae* have also been isolated from spinach seed. The frequency of these species introductions into spinach production fields and their eventual impact on other crops are also unknown. This objective investigates the potential role of *V. dahliae* inoculum introduced via infested spinach seed on Verticillium wilt in subsequent lettuce crops and assesses the reasons why the disease is currently restricted to the Salinas and Pajaro valleys in California.

**Methods.**

Six different *V. dahliae* infestation level treatments in spinach seed were established (64%, 33%, 15%, 10%, 0% infested spinach seed) with four replications each in 2011. Prior to planting spinach, soil samples were collected from each microplot and assayed for *V. dahliae* microsclerotia and determined that soil in these microplots did not contain *V. dahliae*. Thus, any *V. dahliae* microsclerotia recovered from soil in subsequent samplings is likely to have come from spinach seed planted in the microplots. Three spinach crops representing each treatment were grown in the microplots in 2011. Both soil and spinach root samples were collected twice each season and assayed for *Verticillium* spp. The fungus isolates from plants were identified to species using species-specific markers. After three successive crops of spinach in 2011, lettuce cultivar Salinas was planted in all microplots in 2012 during spring and fall, and evaluated for Verticillium wilt at crop maturity. During 2013, two crops of spinach were grown during spring and summer followed by a lettuce crop in the fall. Plants displaying symptoms typical of Verticillium wilt were collected, washed, surface disinfested, and the excised tissue plated on NP-10 medium. Two weeks after plating, colony and morphological traits of *V. dahliae* were examined, including zonate growth, microsclerotia, and verticillate conidiophores.

Finally, we conducted a follow up experiment using a green fluorescence protein-tagged isolate of *V. dahliae* (generated from an isolate originally collected from spinach seed), to track the movement of the fungus from spinach seed, into soil and to the site of infection on lettuce and spinach roots grown in the same soil. Seeds of spinach cv. Hector, infested with the GFP-expressing spinach strain VdSo925-316, were planted (10 seeds per 0.94 liter Styrofoam cup) in a pasteurized 3:1 sand:potting soil mix and grown in a greenhouse. Nearly 75% of the spinach seeds were infested (234/312) as determined by NP-10 plate analyses described previously. Approximately 6 weeks after planting, whole spinach plants were uprooted, chopped into 1 cm pieces, and allowed to dry on the surface of the sand soil mixture for five days. After the drying period, more of the SoVd316-GFP-infested spinach seeds were planted into the same cups and soil mix (10 seeds per cup) and grown for approximately six weeks. The plants were uprooted, chopped into pieces as before, and allowed to dry on the surface of the sand soil mixture for approximately one week. Following two spinach crop cycles, lettuce cv. Salinas was planted, also at 10 seeds per cup, in the same cups containing the dried spinach debris. Seeds of cv. Hector not infested with SoVd316-GFP were
planted at a rate of 10 seeds per cup for the final spinach planting. After five weeks, both lettuce and spinach plants were uprooted, the roots were rinsed vigorously with distilled water, and examined for the presence of the GFP-expressing *V. dahliae* strain.

Whole root mounts were examined with an epifluorescence Olympus BX60 compound microscope or a Nikon compound microscope with filter blocks for GFP (450-490 nm excitation, 590 nm longpass emission), coupled to a Leica confocal laser scanning microscope (CLSM; Leica Microsystems Inc., Buffalo Grove, IL). Confocal images were captured as outlined by Maruthachalam et al. (22). Out of five lettuce root systems from five plants examined, a portion of the root system of a single plant was determined as positive for GFP expression by epifluorescence microscopy. Similarly, of the roots of five spinach plants examined, one was positive for GFP expression. The positive samples were examined further by CLSM analyses.

**Results.**

Generally, the number of microsclerotia per gram soil was correlated with level of seed infestation, and the quantities of microsclerotia were estimated at 0-120 per gram soil.

![Figure 1](image_url)  
**Figure 1.** Recovery of *Verticillium dahliae* from microplots at different times of soil sampling. Y1 = first year (2011), S1 = First planting of spinach crop, SM1= First spinach sampling, SM2= Second spinach sampling, S2 = Second planting of spinach crop, S3 = Third planting of spinach crop, Y2 = Second year (2012), LS = Lettuce crop planting, respectively. Vertical bar represents standard error.

*V. dahliae* was routinely isolated from the spinach seedlings (Figure 2) demonstrating that the fungus was viable within the seed and grew systemically within spinach seedlings as the plant developed.

Four microplots in the first year and three microplots in the second year contained lettuce plants infected with *V. dahliae*. These infections were verified as *V. dahliae* using PCR (Figure 3).

Interestingly, we observed *V. dahliae* infections of lettuce in soils which contained ≤6
microsclerotia/gram soil, which is lower than what has been postulated as a requirement for Verticillium wilt to occur in lettuce. Verticillium wilt epidemics of lettuce were previously thought to only occur when the microsclerotia thresholds reached significantly higher quantities, but the results from this experiment show that *V. dahliae* infections of lettuce are possible even with low microsclerotia densities in soils.

Analyses of the spinach-to-lettuce transmission was carried out by two plantings of spinach seeds infested with the *V. dahliae* green fluorescent protein (GFP)-tagged strain VdSo925-316 followed by a planting of lettuce, and subsequent observations five weeks later of lettuce roots using confocal laser scanning microscopy (CLSM). The GFP-tagged spinach strain VdSo925-316 colonized the lettuce root surface five weeks after planting lettuce in soil following two prior plantings of the infested spinach seeds and incorporation of the residue (Fig. 4A). In addition to the result in Figure 4A, another portion of the same lettuce root system was clearly positive for the GFP-expressing fungus (data not shown). The spinach-to-spinach transmission of strain VdSo925-316 was carried out similarly, and the strain also colonized the spinach root surface (Fig. 4C). The GFP-tagged spinach strain penetrated the root surface as observed by CLSM (Arrow in Fig. 4C).

**Figure 2.** Recovery of *Verticillium dahliae* from microplots at different times of soil sampling. Y1 = first year (2011), S1 = First planting of spinach crop, SM1= First spinach sampling, SM2= Second spinach sampling, S2 = Second planting of spinach crop, S3 = Third planting of spinach crop, Y2 = Second year (2012), LS = Lettuce crop planting, respectively. Vertical bar represents standard error.

**Objective 2.** Characterize *V. dahliae*, *V. isaacii*, and *V. klebahnii* collected from surveys of spinach production fields in four coastal counties.

California growers planted 206,200 acres of lettuce in 2011. A large portion of this acreage is located in the Monterey and Santa Cruz counties. In 1995, Verticillium wilt, caused by *V. dahliae*, appeared suddenly and unexpectedly on lettuce in Watsonville, Santa Cruz County. Despite the nearly 18-year history of Verticillium wilt on lettuce, the disease is largely confined to the Monterey and Santa Cruz counties. Since our discovery of *V. dahliae*-infected spinach seed as a pathway for the introduction of the pathogen into lettuce production fields, a frequent but very legitimate question that is asked is why the disease is currently confined to lettuce fields in Monterey and Santa Cruz counties despite the similarity in cropping patterns in San Benito and
Santa Barbara counties. In an attempt to answer this question, we initiated this objective in 2011 and continued in 2012.

**Figure 3.** PCR results from fungi cultivated from lettuce taproots with characteristic Verticillium wilt symptoms. The double banding pattern indicates the molecular signature of *V. dahliae*.

![PCR results from fungi cultivated from lettuce taproots with characteristic Verticillium wilt symptoms.](image)

**Figure 4.** Analyses of the transmission of *Verticillium dahliae* strain VdSo925-316 from spinach debris in soil to lettuce or spinach roots by confocal laser scanning microscopy. Strain VdSo925-316 expresses green fluorescent protein (GFP) and was visualized using the appropriate UV excitation and emission filters (see materials and methods). A, Analysis of lettuce root sample (cv. Salinas) five weeks after planting in soil infested with VdSo925-316-infected spinach debris. B, Same lettuce root sample shown in A, without the filter to detect fungal GFP fluorescence. C, Analysis of spinach root sample (cv. Hector) five weeks after planting in soil infested with VdSo925-316-infected spinach debris. D, Same spinach root sample shown in C, without the filter to detect fungal GFP fluorescence.

**Methods.**

A total of 69 and 31 major commercial spinach fields from Monterey, San Benito, Santa Barbara and Santa Cruz counties in California were extensively surveyed for Verticillium wilt diseases during 2011 and 2012, respectively. Each field regardless of the cultivar or crop age was walked in an X pattern and 20 plants were randomly sampled and returned to the laboratory. The roots and petioles of all spinach plants were washed free of soil, surface sterilized with 10% bleach, rinsed 2-3 times with sterile water, dissected and plated on NP-10 media. The numbers of plants yielding *Verticillium* colonies were examined 9 to 14 days after plating. *Verticillium* species were identified based on the colony morphology and later confirmed using the species-specific PCR tests.

**Results.**

Of the 1380 total spinach plants analyzed in 2011, 585 plants yielded at least one *Verticillium* species; of the 620 plants analyzed in 2012, *Verticillium* was recovered from 300 plants. Three
Verticillium species, *V. dahliae*, *V. isaacii*, and *V. klebahnii* were recovered from spinach plants in this commercial field survey (Table 1). *Verticillium isaacii* was the most frequently isolated, followed by *V. dahliae*. Based on an initial assessment of colony morphology and pigment production, 496 putative *V. isaacii* and 89 *V. dahliae* isolates were collected in 2011 while 218 putative *V. isaacii* and 82 *V. dahliae* isolates were collected in 2012. The recovery of *V. dahliae* and *V. isaacii* from spinach roots and petioles were similar in four counties during 2011 and 2012, except in Monterey County during 2012, where the recovery of *V. isaacii* from spinach roots were almost three-fold higher than those from petioles. On a per unit field basis, *V. isaacii* was the most predominant *Verticillium* species isolated in all counties. In 2011, the highest number of *V. isaacii* isolates was recovered from Monterey County, followed by Santa Barbara County. In 2012, the highest number of *V. isaacii* isolates was recovered in Monterey County, followed by San Benito County. The per unit field basis indicated that recovery of *V. dahliae* was highest from Santa Barbara County followed by Monterey County during 2011, and Santa Cruz County followed by Monterey County during 2012.

**Table 1.** Numbers of *Verticillium* species isolates collected from different counties of California tested with *Verticillium* species-specific PCR assays.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample size</th>
<th><em>Verticillium isaacii</em></th>
<th><em>Verticillium dahliae</em></th>
<th><em>Verticillium klebahnii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Monterey</td>
<td>52</td>
<td>62</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td>Santa Barbara</td>
<td>80</td>
<td>4</td>
<td>70</td>
<td>4</td>
</tr>
<tr>
<td>San Benito</td>
<td>13</td>
<td>102</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>Santa Cruz</td>
<td>16</td>
<td>30</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>160</strong></td>
<td><strong>198</strong></td>
<td><strong>135</strong></td>
<td><strong>152</strong></td>
</tr>
</tbody>
</table>

*Verticillium dahliae*, *V. isaacii*, and *V. klebahnii* were recovered from all sampled commercial spinach fields during 2011 and 2012. Notably, *V. isaacii* was recovered in higher frequencies than *V. dahliae* and *V. klebahnii*. Since it is virtually impossible to recover *Verticillium* from petioles at the seedling stage of spinach growth if the pathogen did not originate from within the spinach seed, the recovery of *V. isaacii* or *V. dahliae* from petioles of young spinach plants in this study also suggests that these *Verticillium* species are seedborne and occur inside spinach seed.

**Objective C. Refine the race 2-specific PCR and analyze the race structure of *V. dahliae* isolates from spinach seed from multiple seed production locations.**

Polymerase chain reaction (PCR) techniques for the characterization of races in *V. dahliae* are valuable, as screening disease reactions on plant differential lines is time consuming and resource intensive. Molecular techniques that rapidly distinguish races are convenient for population biology, disease surveillance, and resistance breeding. Prior to the identification of the gene encoding the race-1 specific fungal effector (*Ave1*), the PCR primers Tr1-Tr2 were used to positively identify race 1 isolates. Subsequently, it has been shown that both the *Ave1* locus and the Tr1-Tr2 primer
binding sites reside within a unique 50-kb region specific to \textit{V. dahliae} race 1. However, currently there is no complementary assay to positively identify race 2 isolates of \textit{V. dahliae}.

\textbf{Methods.} We developed a PCR assay to rapidly identify race 2 isolates based on race-associated DNA sequences. The specificity of the race 2-specific primer pair VdR2F-VdR2R was achieved in a 256-bp region containing 12 nucleotide polymorphisms between race 1 strain Ls16 and race 2 strain Ls17 by designing the primers in such a way that the binding sites within each primer differed from homologous sites in the non-target race by at least two substitutions. Specifically, the forward primer VdR2F was designed to contain two substitutions, while the reverse primer VdR2R was designed to contain three substitutions. PCR optimization for the primers was completed (Figure 5). Validation of PCR results was performed in greenhouse experiments by testing the virulence of a large number of isolate on differential lettuce lines.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig5.png}
\caption{A. Results of the race 1-specific PCR assay and B. race 2-specific PCR assay. The two tests are concordant and amplify products in only one of the two races. Isolate Sb52 collected from Strawberry, CA, USA; isolate Le1087 collected from tomato, CA, USA; isolate Cs1616 collected from artichoke, CA, USA; isolate Sb50, collected from Strawberry, CA, USA; isolate 84 collected from spinach seed, WA, USA; isolate Ci1, collected from watermelon, CA, USA; isolate PP48 collected from cotton, Australia; isolate Ls775 collected from lettuce, CA, USA; isolate PD494 collected from strawberry, CA, USA; isolate G2D5 collected from spinach seed, WA, USA; Cb86.7.2 collected from potato, WA, USA; isolate VD57, collected from mint, WA, USA; isolate Ls16 collected from lettuce, CA, USA; isolate Ls17 collected from lettuce, CA, USA; isolate Sm1, collected from eggplant, CA, USA. Ls16 and Ls17 were used as positive controls for race 1 and race 2, respectively.}
\end{figure}

\textbf{Results.}

Using a combination of race 1 and race 2-specific primers, we were able to characterize the races of over three hundred \textit{V. dahliae} isolates originating from multiple spinach seed lots from Chile, Denmark, the Netherlands, and Washington, USA (Figure 6). Of the 340 isolates from spinach seed characterized, 4% were race 1 and 96% were race 2. Thus, both races are present in commercial spinach seed, but the vast majority of them are race 2. Race 1 isolates are generally more aggressive because they possess the virulence factor encoded by the Ave1 gene.

All 53 isolates that yielded amplicons with VdR2F-VdR2R and failed to amplify with Tr1-Tr2 and Ave1F-Ave1R induced severe wilt symptoms in both differential lines of lettuce suggesting that they belonged to race 2. \textit{Verticillium dahliae} was recovered from symptomatic roots plated on the semi-selective NP-10 medium. As expected, reference race 1, Ls16, caused severe wilt symptoms on cv. Salinas but failed to cause symptoms on La Brillante. As expected, DNA from all 53 isolates evaluated for virulence in the greenhouse amplified a ~256 bp product with the primer pair VdR2F-VdR2R, but did not amplify with the race 1-specific PCRs.

From these results, we speculate that while race 1 isolates are much less common in spinach seed, because of the high planting density of spinach (~9 million seeds/ hectare), spinach seed should be considered a significant reservoir of both race 1 and race 2 \textit{V. dahliae}. 
Objective 4. Continue the research of the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables grown in coastal California.

Since 1995, lettuce in coastal California, where more than half of the crop in North America is grown, has consistently suffered from severe outbreaks of Verticillium wilt. The disease is confined to this region, although the pathogen (*Verticillium dahliae*) and the host are present in other crop production regions in California. Migration of the pathogen with infested spinach seed was previously documented, but the geographic sources of the pathogen, as well as the impact of lettuce seed sparsely infested with *V. dahliae* produced outside coastal California on the pathogen population in coastal California had remained unclear. Population analyses of *V. dahliae* were completed using 16 microsatellite markers on isolates from lettuce plants in coastal California, infested lettuce seed produced in the neighboring Santa Clara Valley of California, and spinach seed produced in four major spinach seed production regions: Chile, Denmark, the Netherlands, and the United States (Washington State). California produces 80% of spinach in the US and all seed planted (the majority infested by *V. dahliae*) comes from the above four sources. Three globally distributed genetic populations were identified, indicating sustained migration among these distinct geographic regions with multiple spinach crops produced each year and repeated every year in coastal California. The population structure of *V. dahliae* from coastal California lettuce plants was heavily influenced by migration from spinach seed imported from Denmark and Washington. Conversely, the sparsely infested lettuce seed had limited or no contribution to the Verticillium wilt epidemic in coastal California. The global trade in plant and seed material is likely contributing to sustained shifts in the population structure of *V. dahliae*, affecting the equilibrium of native populations, and likely affecting disease epidemiology. We have expanded this study to determine the global movement of *V. dahliae* and its contribution to Verticillium wilt on a variety of hosts.
Methods

To study the genetic relationships among populations of *V. dahliae* from spinach seed and other crops, we used 13 polymorphic microsatellite markers to genotype over 1000 isolates from our *Verticillium* culture collection. After genotyping, we performed several analyses on the data, including the minimum spanning network constructed using PhyloViz (Figure 7).

![Minimum spanning network diagram](image)

**Figure 7.** Minimum spanning network diagram depicting the relationships of multilocus microsatellite genotypes of *V. dahliae*. Over 1000 isolates from a variety of crops and geographic locations were genotyped using 13 microsatellite markers. Colors indicate the host of origin from which the isolates are derived. The different sizes of the points represent the number of individuals that shared an identical genotype.

Results

Figure 7 shows that genotypes of *V. dahliae* associated with spinach are highly diverse (black points) and, more interestingly, identical genotypes associated with spinach also occurred in lettuce, tomato, and artichoke. This means that there is considerable overlap between the genetic diversity of *V. dahliae* that occurs in spinach seed and other crops grown in coastal California. Other genetic groups were discovered in the spinach sample that were entirely genetically unrelated, as indicated by the unconnected clusters of black points, which represent unique genotypes seen only in spinach.