

## CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

April 1, 2010 - March 31, 2011

### 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) continuation of the studies on genetic relationships among isolates of *V. dahliae* from spinach seed and other vegetables grown in coastal California; 2) assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach; 3) pre-screen seed of spinach germplasm lines *V. dahliae* infestation and only screen lines that are pathogen-free for Verticillium wilt resistance in the greenhouse; and 4) determine the role of pollen in *V. dahliae* transmission into seed. Lettuce became a host of *V. dahliae* in the mid-1990s. The mechanisms involved in the expansion of the host range to include lettuce have remained elusive. Three possible hypotheses examined were: a shift or adaptation in the local *V. dahliae* populations toward lettuce; or a sudden increase in population numbers in the region; or recurrent introductions of the pathogen into the area. These scenarios were primarily derived from the knowledge of the significant increase in the area planted with salad spinach in coastal California, and the published information on the high incidence of *V. dahliae* in spinach seed lots produced in multiple regions of the world. The disease is confined to this region, although *V. dahliae* and lettuce are present in other agricultural environs of California. Gene flow with heavily infested spinach seed was previously described, but the geographic sources of this immigration, as well as the impact of the imported sparsely-infested lettuce seed on the population causing disease on lettuce in coastal California were examined this year. Population analyses of *V. dahliae* were completed using 16 microsatellite markers on strains from diseased lettuce plants in coastal California, infected 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). Three globally distributed genetic populations were identified, indicating that sustained gene flow occurs among these distinct geographic regions. The population structure of *V. dahliae* from coastal California lettuce plants was heavily influenced by gene flow from spinach seed imported from Denmark and Washington. Conversely, the sparsely-infested lettuce seed showed no evidence of contribution to the Verticillium wilt epidemic. Studies on objective 2 could not be begun this year because of the unavailability of infected spinach seed. They are being initiated in 2011. Of the seed from 394 spinach germplasm lines pre-screened for *V. dahliae*, 173 were positive for *Verticillium* spp. The remaining 221 lines are being screened in the greenhouse for Verticillium wilt resistance. We confirmed the role of pollen in the transmission of *V. dahliae* in spinach again this year. Additional studies with a GFP-tagged strain are underway.

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**PROJECT TITLE:** **VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL**

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### **OBJECTIVES:**

1. Continue the genetic relationships among isolates of *V. dahliae* from spinach seed and other vegetables grown in coastal California.
2. Assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach.
3. Pre-screen seed of spinach germplasm lines *V. dahliae* infestation and only screen lines that are pathogen-free for Verticillium wilt resistance in the greenhouse.
4. Determine the role of airborne conidia and pollen in *V. dahliae* transmission into seed.

### **PROCEDURES AND RESULTS:**

**Objective 1. Continue the genetic relationships among isolates of *V. dahliae* from spinach seed and other vegetables grown in coastal California.**

#### **Methods.**

Two hundred and ninety five strains of *V. dahliae* were included in this study (Table 1). Sixty-five strains were from infected lettuce plants from Salinas and Pajaro valleys. Collections and isolations were carried out between 1995 and 2007 in commercial fields where Verticillium wilt had occurred. Analyses using the microsatellite markers, as well as IGS rDNA sequences, AFLPs and virulence, suggested a lack of differentiation among the various sampling dates. Therefore, all *V. dahliae* strains from coastal California lettuce were considered as one sampling group.

One hundred and sixty-nine strains were from spinach seed produced in Washington state (n= 54), Denmark (n= 38), The Netherlands (n= 32), and Chile (n= 45). These seed lots were destined for planting in coastal California and other spinach producing regions. Strains isolated from three seed lots were used from each spinach seed source. Additionally, 56 strains included in this study were from three lettuce seed lots produced in the Santa Clara Valley.

DNA was extracted from mycelia harvested from potato dextrose broth cultures using DNeasy Plant Mini Kit. Extracted DNA was quantified using a NanoDrop 1000 spectrophotometer and concentrations were standardized to 2 ng/ $\mu$ l by dilution in autoclaved purified water. DNA samples were kept at 4°C until used.

### *SSR amplifications*

Primers amplifying the 15 SSR loci VD1, VD2, VD3, VD7, VD9, VD11, VD12, VD26, VD27, VD65, VD69, VD73, VD85, VD96, VD97, and VD98 were employed in polymerase chain reaction (PCR) assays. Subsequently, PCR fragments labeled with the various fluorophores were multiplexed for fragment analysis. A total of 1  $\mu$ l of each labeled PCR fragment was mixed with fragments labeled with the three remaining fluorophores. Then 1  $\mu$ l of this multiplex mixture was combined with Hi-Di formamide and 0.3  $\mu$ l of LIZ-500 size standard and separated on an ABI 3100 sequencer. Fragment analysis was completed using the GeneMarker software. The Ewens-Watterson neutrality test was completed and Nei's gene diversity ( $H_{obs}$ ) was calculated.

### *Population structure and identification of genetic clusters*

The affiliation of individual strains of *V. dahliae* to specific genetic clusters and the possibility of admixture were tested. A lack of significant differentiation between sampling groups may be explained by repeated gene flow. For each genetic cluster K (K = 1 through 6), 10 runs were performed with a burn-in period of 100000 generations and 1000000 Markov Chain Monte Carlo (MCMC) simulations. The admixture model was chosen, and the loci were considered independent, in light of previous analyses that indicated a lack of linkage among loci. The analysis was repeated five times to ensure convergence of results. The ad-hoc statistic  $\Delta K$  was computed to estimate the most likely number of genetic clusters K.

The potential for structuring within each obtained genetic cluster was investigated using the principal coordinates analysis (PCA). The structure was estimated first within each cluster, and included the sampling groups based on the affiliation of their individual strains to these clusters. Strains were assigned to individual genetic clusters if the value of their calculated  $Q$  was >70%. Furthermore, sampling groups were only retained if a minimum of 7 strains were identified; otherwise the sampling group was removed from further analyses within the particular genetic cluster.

### *Migration analysis*

Owing to the persistent nature of the exchange of *V. dahliae* among various geographic regions, it is reasonable to assume that equilibrium between the populations is not achieved. Therefore, the migration rates were studied using methods based on different evolutionary models. The first relied on the coalescent approach and the second employed a test that excludes populations that are not likely to be the sources of the individual genotypes tested. Two simulation methods were compared for this exclusion. 1) A migrant assignment method relying on a Bayesian approach to place genotypes into possible source populations based on allele frequencies. 2) A Monte-Carlo-based resampling method that generates hypothetical gametes rather than alleles, thereby reducing the Type I error rate, while modeling population processes more faithfully than other methods. The probability of finding first-generation immigrants was also explored by applying the  $L_h/L_{max}$  likelihood ratio, while applying  $\alpha = 0.01$  as critical threshold level. One known weakness of this method lies in its assumption that all possible source populations were sampled. The genetic clusters identified in the Structure analysis, and the sampling groups within each genetic cluster, were used in all of the migration tests.

Mutation rates at all 15 SSR loci were assumed to be constant for estimating migration rates. Five independent runs (a different random seed was chosen each time) were performed using the Brownian motion model, and the results of the run that generated the smallest Ln(Likelihood) value are discussed.

### *Divergence time*

The estimation of divergence time between the lettuce plant population from coastal California and the lettuce seed population from the Santa Clara valley was performed. This isolation by migration algorithm is aimed at populations that have recently diverged from a common ancestor. And through a Bayesian analysis, allows the evaluation of demographic models with varying levels of population size, migration rate, and divergence time. Based on the number of lettuce crops produced in coastal California, it was assumed that 3 generations of *V. dahliae* occur per year ( $u = 3$ ).

## **Results**

No clones were detected among the haplotypes of *V. dahliae* from the coastal California lettuce plant sampling group, even when strains were isolated from the same field. However, varying levels of clonality were observed in the spinach and lettuce seed sampling groups (Table 1). The percentage of clones in these populations ranged from 15.5% to 31.2%. Clones were identified among strains from seeds collected from different seed lots, as well as among strains from seeds within a seed lot. All of the population analyses were performed after clone-corrections within each sampling group. No evidence of positive selection was concluded from the Ewens-Watterson neutrality test (Table 1).

*Three genetic clusters are spread in various geographic regions*

Three distinct genetic clusters ( $K=3$ ) were identified among the six sampling groups included in this study. The rate of change in the log probability between the successive  $K$  values was highest for three genetic clusters ( $\Delta K=3158.7$ ) compared with any other  $K$  value tested ( $\Delta K < 87$ ). All three clusters were recovered in varying levels from the four spinach sampling groups, the green cluster predominated in the lettuce plant sampling group, while the red population predominated in the lettuce seed sampling group. The red cluster (hereafter referred to as genetic cluster 1) was particularly prevalent in the Chile, Netherlands, Washington, and lettuce seed sampling groups. The yellow cluster (hereafter referred to as genetic cluster 2) was recovered from Denmark, lettuce plant and the Netherlands (6 individuals only) sampling groups. And finally the green cluster (hereafter referred to as genetic cluster 3) was identified in the lettuce plant, Chile and Washington sampling groups (Figure 1). These results suggest that significant amounts of admixture occur between the various sampling groups.

No significant differentiation was calculated between the Denmark spinach seed and lettuce plant sampling groups within genetic cluster 2 in the PCA analysis. In contrast, the lettuce seed sampling group was significantly differentiated from other sampling groups in both genetic clusters 1 and 3 (Figure 2). And the lettuce plant sampling group was not differentiated from the Chile and Washington spinach sampling groups in genetic cluster 3. No differentiation was observed among the spinach seed sampling groups Chile, Netherlands and Washington in genetic cluster 1. Similarly, no significant differentiation was measured between the spinach seed sampling groups Chile and Washington in genetic cluster 3.

### *Gene flow*

The analysis of gene flow using the coalescent approach indicated that the highest migration was between genetic clusters 1 and 3, which exceeded 1 migrant per generation in both directions (Figure 3). Conversely, the migration rates between genetic cluster 2 and the other two clusters were significantly smaller than one migrant per generation. Within genetic cluster 1, the lettuce seed sampling group consistently exchanged fewer than 0.7 migrants per generation with the Chile, Netherlands and Washington spinach seed sampling groups (Figure 3). In contrast, all three spinach seed sampling groups exchanged more than 1 migrant per generation, with the exception of evidence of a unidirectional migration from the Netherlands into Washington, rather than the opposite. In genetic cluster 3, only the Washington spinach seed sampling group contributed 1.5 migrants per generation to the lettuce plant sampling group, whereas the latter only contributed 0.5 migrants to the former (Figure 3). The remaining pairwise comparisons among the four sampling groups within cluster 3 yielded less than 0.7 migrants per generation.

Strikingly, in the lettuce seed sampling group that comprises strains affiliated with genetic clusters 1 and 3, all assigned genotypes were from lettuce seed, and did not include any strains from other sampling groups (Figure 4). In each of the three genetic clusters, the majority of genotypes were recruited from within a sampling group. However, there was one exception in genetic cluster 1, where more genotypes in the Netherlands sampling group were assigned to the Washington sampling group.

In genetic cluster 1, evidence of significant gene flow was found among all three spinach seed sampling groups, especially from Washington, a large spinach seed producer (Figure 4A). For instance, 53 and 87% of genotypes in Chile and Netherlands, respectively, were assigned to Washington, and 73% of Washington genotypes were self-recruited. The Netherlands sampling group was assigned 15 and 30% of genotypes from Chile and Washington, respectively, and only 62% were self-assigned. In contrast, Chile contributed 6 and 8% of the genotypes of the Netherlands and Washington sampling groups. In genetic cluster 2, 75% of the genotypes in the lettuce plant sampling group were assigned to Denmark (Figure 4B). And in genetic cluster 3, 14% of the genotypes in the lettuce plant sampling group were assigned to Washington (Figure 4C).

### *Divergence time*

The time of divergence of the *V. dahliae* from lettuce plants in coastal California and lettuce seed from the Santa Clara Valley was in the range of 58000-68000 years ago. And the mean divergence time was *ca.* 64000 years ago.

### **Conclusions**

The population structure in *V. dahliae* in coastal California was heavily influenced by infected spinach seed planted in this region. Conversely, lettuce seed that is infected with *V. dahliae* and is produced 30 miles away, had a limited impact on the structure of the pathogen in commercial lettuce production. Previous studies using SSR markers and rDNA sequences and AFLP loci had highlighted the ongoing gene flow of *V. dahliae* from spinach into lettuce. However, the actual sources of this gene flow among the various spinach production regions had not been identified, and the impact of infested lettuce seed remained uncertain.

### **Objective 2. Assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach.**

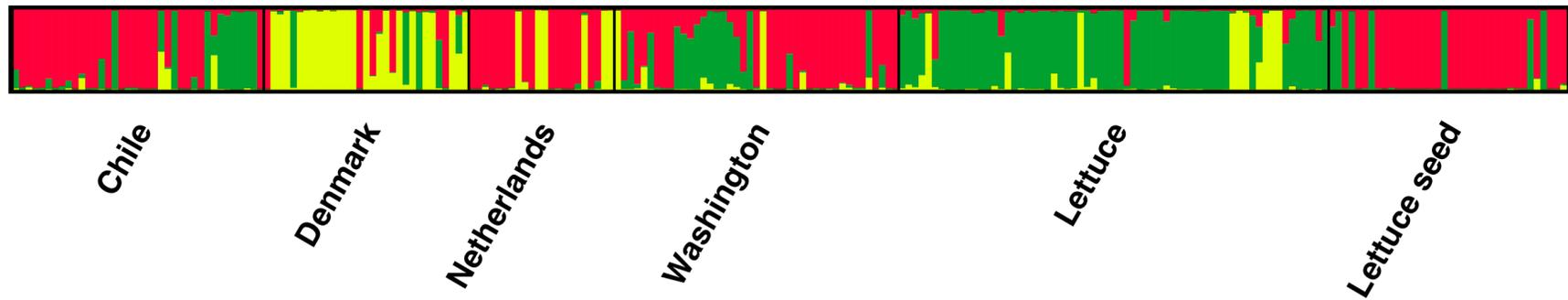
Many unanswered questions remain regarding the development of Verticillium wilt on lettuce based on the previous cropping history in the field. For example, how many microsclerotia are added to the soil after planting infested spinach seed in a field, how *V. dahliae* populations change over time depending on the crops that follow spinach, and the time it takes for microsclerotia to begin affecting lettuce crops in the field, etc. We decided to pursue studies to answer these questions in microplots where we can control all factors during this project year. However, unavailability of highly infested seed prevented the initiation of these studies last year and hence no results are available to report. We have now secured large quantities of infested seed from two different sources and are able to begin the studies in 2011. Microplots where these studies will be conducted have had the soil excavated to a depth of 3 ft and refilled with soil with no history of *V. dahliae*. The refilled soil also has been assayed to ensure that there were no *V. dahliae* microsclerotia. The results from these studies will be available in the 2012 report.

Table 1. Origin of *Verticillium dahliae* strains, type of the host tissue from which they were isolated, and number of strains used in population analyses, the number of different haplotypes (clones removed) obtained using 15 SSR markers, Nei's corrected gene diversity ( $H$ ), and Ewens-Watterson neutrality test

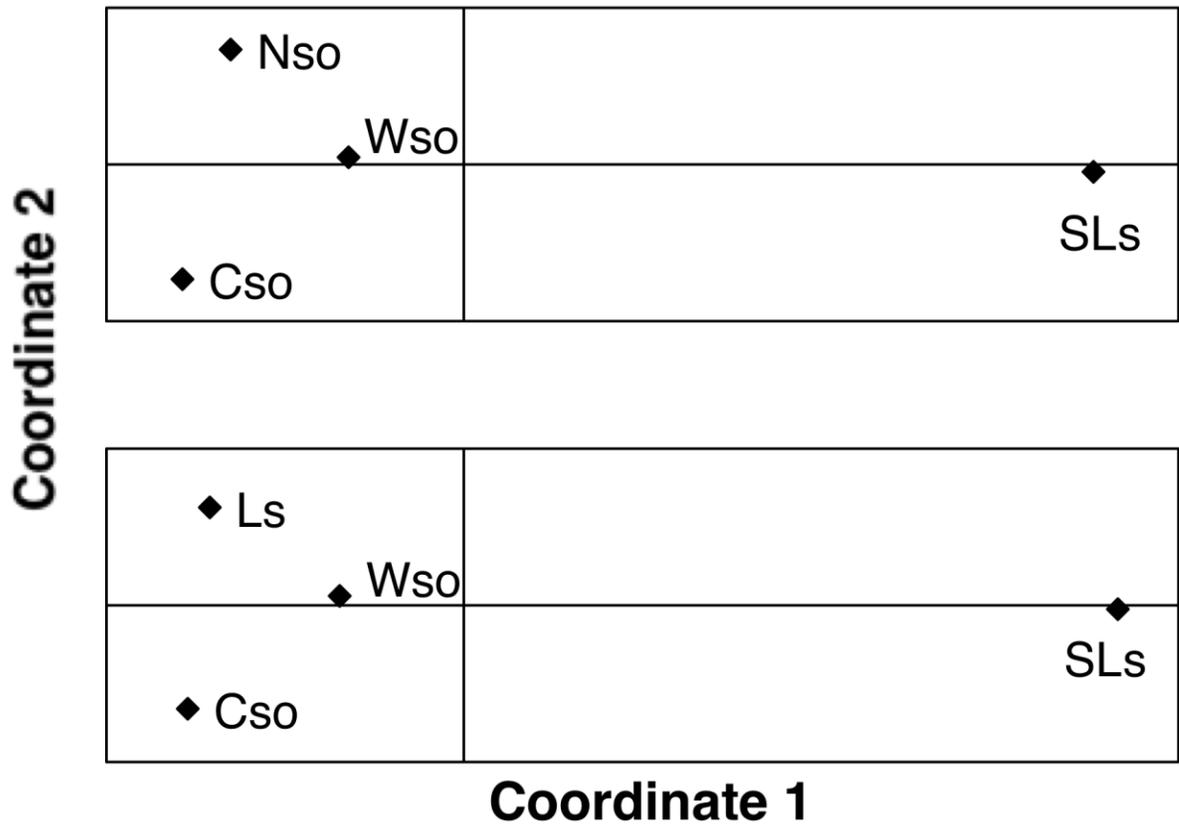
Host	Origin	Tissue type	# Individuals	# Haplotypes	$H_{obs}^a$	EW <sup>b</sup> (p-value)
Spinach	Chile	seed	45	38	0.306	0.969
	Denmark	seed	38	31	0.704	0.823
	Netherlands	seed	32	22	0.465	1.000
	Washington	seed	54	43	0.320	1.000
Lettuce	Coastal California, USA	symptomatic plants	65	65	0.409	1.000
	Santa Clara valley, California, USA	seed	56	36	0.204	0.845

<sup>a</sup> Nei's corrected gene diversity ( $H$ ) values were generated in Genodive.

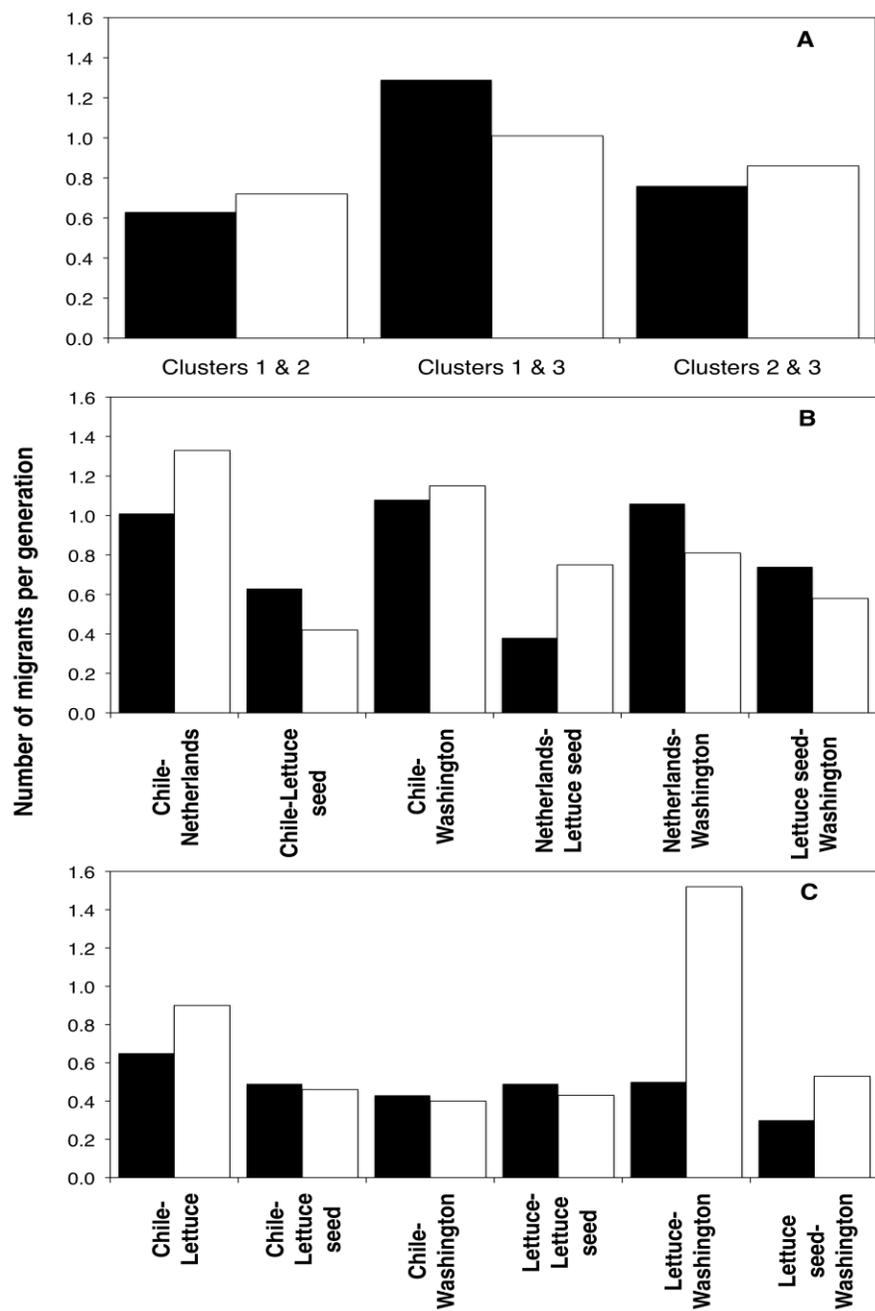
<sup>b</sup> Ewens-Watterson neutrality test calculated in Arlequin.



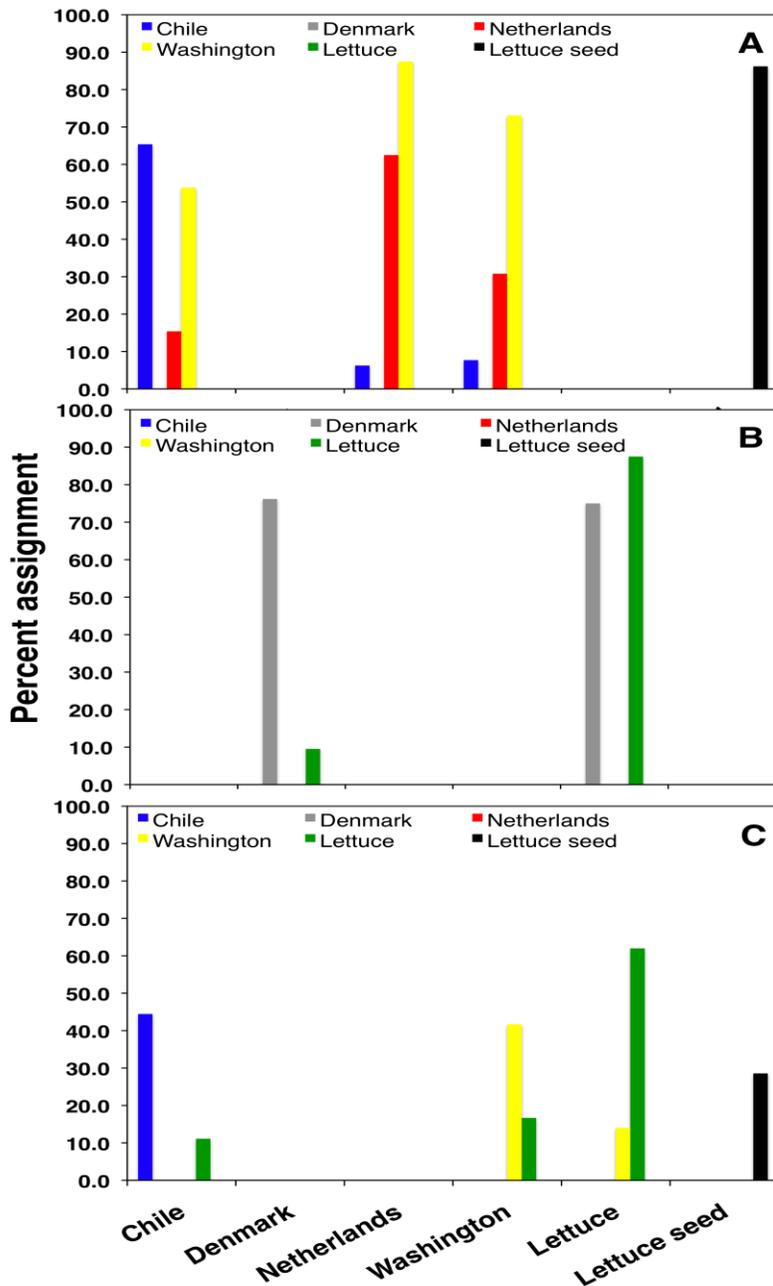
**Figure 1.** Affiliation of individual genotypes of *Verticillium dahliae* as assessed using 15 polymorphic SSR loci as measured in Structure ver. 2.2. Individual haplotypes are separated into discrete vertical bars that are organized by sampling groups. Differences in color within a vertical bar indicate a multi-population affiliation of an individual genotype (admixture). The height of each color within an individual is the measure of proportional affiliation.  $K=3$  was determined to be the most likely number of genetic clusters for six *Verticillium dahliae* sampling groups from lettuce plants grown in coastal California, spinach seed from Europe and North and South America, and lettuce seed from the Santa Clara Valley of California.



**Figure 2.** Principal coordinates analysis (PCA) of *Verticillium dahliae* from 6 sampling groups and sub-divided into 3 genetic clusters based on the affiliation of individual strains. The analysis was performed in GenAlEx v. 6.2 and only two principal components were detected. Only the analyses from genetic clusters 1 (A) and 3 (B) are shown, as there was no significant difference among sampling groups within genetic cluster 2.



**Figure 3.** Number of *Verticillium dahliae* migrants per generation calculated in Migrate for pairs of genetic clusters (A), and pairs of sampling groups within genetic cluster 1 (B) and 3 (C). Because Migrate produces bi-directional migrant estimates, two columns per sampling group, or genetic cluster, pair are displayed. The black column exhibits the migration from population 2 into population 1, whereas the white column shows the opposite migration.



**Figure 4.** Percentage haplotype assignment among *Verticillium dahliae* strains from 6 sampling groups sub-divided into 3 genetic clusters based on the affiliation of individual strains. Exclusion analyses were implemented in GeneClass2. A: genetic cluster 1, B: genetic cluster 2, and C: genetic cluster 3. The abscissa indicates receiving sampling groups, whereas colored columns indicated the proportion of genotypes associated with donor sampling groups.

**Objective 3. Pre-screen seed of spinach germplasm lines *V. dahliae* infestation and only screen lines that are pathogen-free for Verticillium wilt resistance in the greenhouse.**

### **Methods**

Because of the seedborne nature of *V. dahliae* in spinach and other crops, it is important to pre-screen germplasm lines for seedborne inoculum. Sources of germplasm that are already infested are unlikely to be sources of *V. dahliae* resistance and this pre-screening will prevent the more expensive greenhouse evaluation of these susceptible lines. A total of 394 accessions in the USDA spinach germplasm collection were pre-screened for *V. dahliae* infestation. For each accession, 20 seeds (since seed availability is low) were surface-sterilized by soaking in 1.2% NaOCl for 60 seconds and triple-rinsed in sterile deionized water. After drying on sterile blotter paper, the seeds were plated onto the NP-10 medium in two 10-cm Petri dishes. The plates were sealed with Para film, incubated in the dark at 24<sup>0</sup>C for 12 days. The seeds were observed under a microscope 14 days after plating for microsclerotial colonies and/or conidiophores and conidial characteristics of *V. dahliae*.

### **Results**

Of the lines pre-screened for the presence of *V. dahliae*, 173 were positive for the pathogen. The remaining 221 lines that did not yield *V. dahliae* are being evaluated in the greenhouse for Verticillium wilt resistance.

**Objective 4. Determine the role of airborne conidia and pollen in *V. dahliae* transmission into seed.**

The first part of the objective has already been addressed in the 2010 report and the studies detailed in the report confirm the ability of airborne conidia to land on receptive flowers and initiate flower infection. This is also another avenue for seed contamination without the plant being infected. Since airborne pollen is the primary avenue of pollination in many cross pollinated crops, we attempted to explore the role of pollen from Verticillium wilt-infected plants in transmitting the pathogen into the developing seed.

### **Methods**

Twenty-seven spinach germplasm lines were inoculated with a conidial suspension from spinach isolate So923 and incubated in the greenhouse. Uninoculated plants were maintained as controls for each line. When the plants began shedding pollen, pollen was collected from inoculated and uninoculated plants of each germplasm line and gently tapped individually onto two separate plates of NP-10 medium and incubated in the laboratory. The plates were periodically examined under a stereoscope to identify

colonies typical of *V. dahliae*. The number of germplasm lines from which *V. dahliae* was isolated from pollen was recorded.

## Results

Of the 27 lines evaluated, *V. dahliae* was isolated repeatedly from only one germplasm line (Table 2). These studies are being repeated with the GFP tagged strain of *V. dahliae* with funding from the USDA-SCRI grant.

Table 2. Isolation of *Verticillium dahliae* from the pollen of 27 spinach germplasm lines

Germplasm line	<i>V. dahliae</i> (+/-)
NSL 184380	-
PI 179042	-
PI 174389	-
PI 169676	-
PI 169679	-
PI 206474	-
PI 174388	-
PI 171858	-
PI 205235	-
AMES 262483	-
PI 177557	-
PI 175925	-
PI 173129	-
PI 173131	-
PI 181809	-
NSL 4655	+
PI 175931	-
NSL 4661	-
PI 204733	-
PI 175929	-
PI 129588	-
PI 171863	-