CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

August 1, 2008 - March 31, 2009

VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL

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SUMMARY

This is a new project that was begun in August of 2008 with two objectives: 1) Determine the genetic relationships among isolates of *V. dahliae* from spinach seed and other vegetables grown in coastal California; and 2) Estimation of the potential for gene flow of *V. dahliae* genotypes into coastal California, and the role immigrants on lettuce and other crops. A variety of traditional and molecular techniques were employed to address these objectives. A more detailed report on the pathogenicity of spinach isolates from seed on lettuce and vice versa along with their virulence phenotypes are in the lettuce report. Briefly, the spinach isolates of *V. dahliae* were pathogenic on lettuce and the lettuce isolates were pathogenic on spinach. All molecular methods employed did not differentiate spinach isolates from isolates that were endemic in coastal California. In contrast, both the endemic and spinach *V. dahliae* isolates were clearly differentiated from tomato isolates from San Joaquin Valley. High rates of migration were observed between spinach and crops in coastal California. The migration between lettuce, strawberry, non-lettuce Asteraceae and pepper, were also elevated suggesting that once introduced, genotypes become established and are distributed among all crops grown in rotation with lettuce.

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

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OBJECTIVES:

1. Determine the genetic relationships among isolates of *V. dahliae* from spinach seed and other vegetables grown in coastal California.
2. Estimation of the potential for gene flow of *V. dahliae* genotypes into coastal California, and the role immigrants on lettuce and other crops.

PROCEDURES AND RESULTS:

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

Methods. Four different approaches were employed to determine the relationship between strains of *V. dahliae* isolated from spinach seed and others collected from hosts grown in coastal California. These four methods are: identification of the isolate race, DNA sequencing, DNA fingerprinting, microsatellites.

1. Lettuce plants of two differential cultivars were inoculated with conidia (2 x 10^6 conidia/ml) from 29 isolates of *V. dahliae* from spinach to determine the race composition of isolates from spinach seed. Four-wk-old seedlings of cv. Salinas (iceberg type, race 1- and race 2-susceptible) and cv. La Brillante (Batavia type, race 1-resistant and race 2-suscpetible) were inoculated and the plants were maintained for eight to ten weeks in a greenhouse with a 16-h photoperiod and at ~24°C (±5°C) during the day and 12°C (±5°C) at night. Experiments were conducted on twelve replications per cultivar and isolate, and the whole experiment was repeated once. About 8-10 weeks after inoculation, the severity of Verticillium wilt on plants was assessed visually. Disease severity was rated on a scale of 0 to 5, with 0 = no disease and 5 = 100% of discoloration of vascular and foliar symptoms.

2. Sequences of the intergenic spacer region of the ribosomal DNA (IGS rDNA) from 142 strains of *V. dahliae* from several crops in coastal California, spinach seed produced in the Pacific Northwest and northern Europe, ornamentals shrubs and trees from Wisconsin, and

tomato from San Joaquin Valley. The IGS rDNA is a non-coding region that has been extensively used in studies of diversity of plants, animals and fungi, including *V. dahliae*. Our program has successfully used it for the comparison of isolates of *V. dahliae* from various crops. The genetic diversity present in this DNA sequence was visualized using the Bayesian phylogeny approach, and two phylogenetic network approaches. The Bayesian phylogeny is highly effective at the interspecific level, but may not necessarily provide adequate information when used at the intraspecific level. In contrast, the network methods, statistical parsimony and the neighbor-net, effectively identify the ancestral genotypes.

3. Fingerprinting of random DNA regions in the genome of *V. dahliae* was achieved by employing 15 different primer combinations of amplified fragment length polymorphisms (AFLP). AFLP data collection was accomplished on 200 strains of *V. dahliae* isolated from hosts in coastal California, spinach seed produced in the Pacific Northwest and northern Europe, and tomato grown in the San Joaquin Valley. The AFLP method allowed us to generate diversity information on 2603 random targets (loci) in the genome of *V. dahliae*. The AFLP results were visualized using two phylogeny methods, the maximum parsimony and neighbor-net approaches.

4. A total of 22 microsatellite markers were identified in the genomic sequence of *V. dahliae* isolate Ls17 (race 2 strain isolated from lettuce in 1995). Microsatellites are short sequences (2-10 nucleotides long) that are repeated in a string, and are commonly referred to as simple sequence repeats (SSR). Genetic differences among individuals would be observed as a change in the number of repeats in the chain (either decrease or increase). A total of 243 strains of *V. dahliae*, including 65 from lettuce, 15 from non-lettuce Asteraceae, 25 from pepper, 18 from strawberry, all collected in coastal California between 1995 and 2007. Additionally, we included the following groups: 43 from spinach seed produced in the Pacific Northwest and northern Europe, 17 from ornamental shrubs and trees from Wisconsin, and 60 from tomato collected in the San Joaquin Valley. Calculations showed that as few as 7 microsatellite markers explained 99% of the genetic variability in the studied *V. dahliae* strains. The genetic structure of the entire set of strains, and the membership of individual strains to genetic populations, were also determined.

The IGS rDNA sequences and microsatellite and AFLP data were used to measure the contribution of genetic diversity at multiple hierarchical levels, using the analysis of molecular variance (AMOVA) approach. The AMOVA was applied on four clusters made up of the IGS rDNA sequences and microsatellite haplotypes: 1) all strains collected in the central coast of California, 2) strains from tomato, 3) strains from Wisconsin, and 4) strains from spinach seed. For the AFLP analyses, only three clusters were used, because of the lack of data on the tomato isolates. Also, pairwise comparisons among populations were also conducted to identify the level of differentiation among the various host or geographic groups that were studied. This pairwise analysis generates a Φ_{ST} value that varies between 0 and 1. The closer Φ_{ST} is to zero the lower the differentiation between populations. Conversely, the closer it is to 1, the higher the differentiation. It is also critical to remember that populations that are separated with no migration become differentiated over time, and populations that undergo repetitive migration are not differentiated.

Results. Isolates of *V. dahliae* that were recovered from spinach seed, and inoculated onto the lettuce cvs. Salinas and La Brillante, exhibited race 1 and race 2 virulence traits. No difference

was observed between the type and extent of symptoms induced by those strains from spinach seed compared with strains from lettuce.

The three genetic methods converged to indicate the high genetic variability of the *V. dahliae* strains in the Subbarao collection. Close to 90% of the variability was found at within the host or geographic sampling group. This information is exhibited in Table 1, which represents the results of microsatellite markers. Similar information was obtained from the IGS rDNA and AFLP analyses.

Upon comparing the host and geographic region from which isolates were derived in a pairwise manner, it became evident that the tomato isolates were genetically greatly differentiated from all other isolates. However, little genetic differentiation could be measured between populations collected from coastal California hosts such as lettuce or strawberry. Considering the broad host range of *V. dahliae*, it is not surprising that multiple hosts may harbor undifferentiated populations of the fungus. However, none of the three methods differentiated these populations from those collected from spinach seed, which was not planted in California. This indicates that a significant migration occurs that eliminates differences between coastal California populations of *V. dahliae* and those present in infested spinach seed. This information is exhibited in Figure 2, which displays the membership of individual strains to genetic populations. The population of *V. dahliae* isolated from tomato is evidently significantly differentiated from all the other groups. The IGS rDNA results are in agreement with the results of the microsatellite markers.

The phylogenetic analysis of the IGS rDNA was used to infer the evolutionary history of the strains isolated from multiple hosts in coastal California and those isolated from hosts from other geographic regions along with strains from spinach seed. The evolutionary relationships among the various haplotypes inferred from the IGS rDNA data is shown in Fig. 2. Using haplotypes allowed retracing of the ancestral form and eliminating the current differences that may be observed within the IGS rDNA sequences. *V. dahliae* strains isolated from spinach may be observed in the three major groups, which are at the center of the network. These groups may be considered as ancestral, and include representatives of each group. Interestingly, spinach strains are associated with populations from different hosts in coastal California.

Objective 2: **Estimation of the potential for gene flow of *V. dahliae* genotypes into coastal California, and the role immigrants on lettuce and other crops**.

Methods. Using the microsatellite data described above, we were able to calculate the extent of gene flow between the various hosts and geographic origins of the *V. dahliae* isolates. The migration analysis that was employed was implemented in the program Migrate. The algorithm calculates the "estimated" size of each population and the mutation rate in each population to compute the number of immigrants from each population and emigrants into each population (both incoming and outgoing individuals), and compares it to millions of distributions that are generated from re-sampling the entire dataset. Hence, this algorithm generates two values for each population, one describing the number of individuals going from population A into population B, and the second number describes the number of individuals coming into population A from population B.

The lack of differentiation between the various populations described in the previous objective is a clear indication that a high rate of migration takes place commonly among these hosts or geographic groups. A movement of individuals between populations leads to uniformity

Table 1. Analysis of molecular variance (AMOVA) of sampling groups of *Verticillium dahliae* arranged into four clusters (coastal California, ornamentals from Wisconsin, spinach seed, and tomato plants from the San Joaquin Valley of California)

Source of variation	df	Sum of Squares	Variance components	Percentage	Fixation index	P-value
Among clusters	3	8.62	0.043	8.4	0.006	0.027
Among sampling groups within clusters	3	1.74	0.005	0.9	0.006	0.229
Within sampling groups	236	108.12	0.458	90.6	0.012	<0.0001
Total	242	118.49	0.505			

Figure 1. Affiliation of individual genotypes of *Verticillium dahliae* as assessed using 22 polymorphic microsatellite loci. 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. The height of each colored column within an individual is the measure of proportional membership.

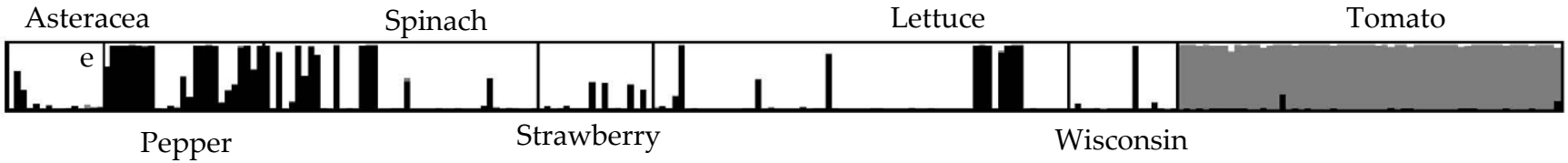
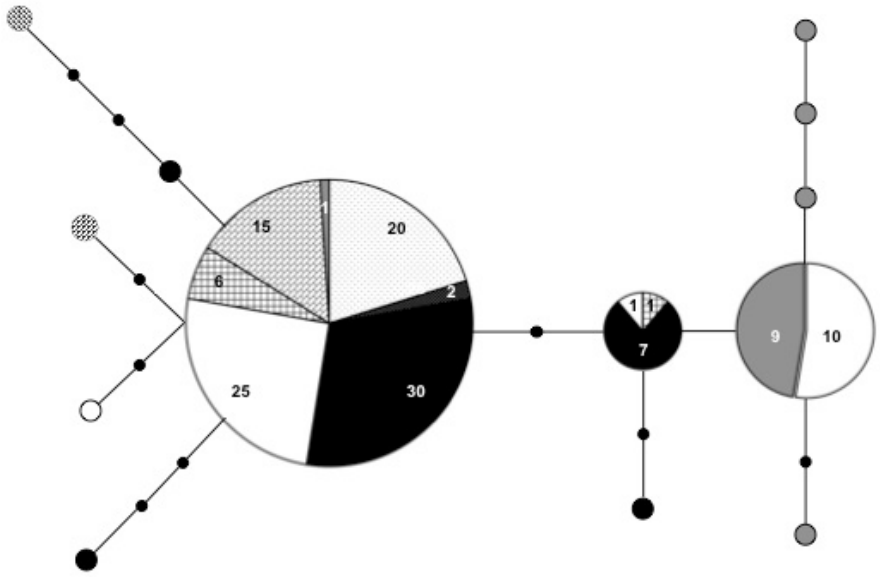


Figure 2. Statistical parsimony network of the IGS rDNA sequences from 141 *Verticillium dahliae* strains from non-lettuce Asteraceae (light brick pattern), lettuce (black), pepper (hatched squares), spinach (grey), strawberry (grayish black), tomato (white) and ornamentals for Wisconsin (clear grey). Haplotypes at interior nodes would be considered ancestral, from which haplotypes at the tips have evolved more recently. Small black circles are putative haplotypes not found in the studied samples. The size of colored circles is proportional to the number of individuals that form each haplotype. Numbers in circles indicate the number of individuals from each host.



of both populations. Conversely, a lack of exchange of individuals leads to a differentiation of populations over time.

Results. Significant migration was observed among all hosts and geographic origins with the exception of tomato (Table 2). Migration among the sampling groups in coastal California was high; additionally, high rates of migration were observed between spinach and hosts in coastal California (Table 2). The migration between lettuce, strawberry, non-lettuce Asteraceae and pepper, were also elevated suggesting that once introduced, genotypes become established and are distributed among all crops grown in rotation with lettuce. Agricultural production in coastal California targets the fresh market and hence is not used as a source of germplasm in other growing regions. This makes the possibility of emigration of *V. dahliae* from this region remote. Therefore, coastal California likely constitutes a net sink to the immigrant populations of *V. dahliae* carried on seed and other planting material produced outside this region, and used as planting material.

Table 2. Migration between pairs of populations of *Verticillium dahliae* with the source populations in columns and recipient populations in rows

Population	Asteraceae CA	Pepper	Strawberry	Lettuce	Spinach	Wisconsin	Tomato
Asteraceae[a]	-	1.168	0.609	1.021	0.835	0.655	0.806
Pepper	1.018	-	1.168	1.173	0.682	0.884	0.598
Strawberry	1.111	0.735	-	1.285	1.115	0.737	0.535
Lettuce	0.925	1.328	1.064	-	1.203	0.792	0.632
Spinach	1.006	0.975	1.364	1.036	-	0.743	0.649
Wisconsin	1.301	1.022	0.429	0.632	0.868	-	0.561
Tomato	0.414	0.477	0.392	0.508	0.417	0.330	-

[a] Non-lettuce isolates of Asteraceae