CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

April 1, 2009 - March 31, 2010

VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL

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SUMMARY

Lettuce became a host of *V. dahliae* in the mid-1990s. The mechanisms involved in the expansion of the host range to encompass lettuce have remained elusive. Three hypotheses were deemed plausible, namely: 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. Using microsatellite, 2603 AFLP and IGS rDNA markers, we established that populations from lettuce and imported spinach seed are very similar. Strains isolated from spinach seed had the same virulence and aggressiveness attributes as those from lettuce, including the presence of race 2 strains. Furthermore, populations of *V. dahliae* isolated from lettuce seed grown in the Santa Clara Valley were significantly differentiated from those affecting lettuce plants in the Salinas and Pajaro Valleys. No migrants were identified in the population from lettuce plants that may be traced to lettuce seed, whereas numerous ones could be directly linked to one of four spinach seed populations tested (Chile, Denmark, the Netherlands and Washington State). This further highlights the significance of massive influx of *V. dahliae* inoculum in germplasm on the receiving population. Therefore, the efforts to identify chemical, physical or biological seed treatments and sources of resistance to both races of *V. dahliae* in spinach and to race 2 in lettuce, becomes even more critical in the absence of clean seed sources and the inability to use fumigants in many spinach seed production regions.

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

PROCEDURES AND RESULTS:

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

Methods. We applied four phenotypic and genotypic approaches to further the understanding of the genetic variability in sub-populations of *V. dahliae* isolated from spinach seed and several vegetable crops from coastal California. The virulence phenotype (*aka* race) of individual strains was assessed on two cultivars of lettuce that distinguish the two races of *V. dahliae*. We also applied three DNA-based molecular methods: sequencing of intergenic spacer region of the ribosomal DNA (IGS rDNA), fingerprinting using AFLP markers, and microsatellites or simple sequence repeat markers (SSR).

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.

We sequenced the IGS rDNA of a collection of *V. dahliae* strains isolated from several crops from coastal California (pepper, strawberry, artichoke, lettuce and weeds), in addition to strains isolated from spinach seed produced in the Pacific Northwest and northern Europe. Strains from other regions and hosts were also included. The ensuing information was used to build phylograms to explain the relationship among strains, and for migration analysis. Additionally, this information was used to attempt to re-trace the evolutionary history of these *V. dahliae* populations, which was performed using coalescent analyses.

An array of 15 primer combinations of amplified fragment length polymorphisms (AFLP) was used to study the genetic variability among 200 strains of *V. dahliae*. These data were then used to study the structure of the population of *V. dahliae* and to compare the various sampling groups.

Because of their relatively high speed of mutation, and subsequently their ability to differentiate among recently diverged populations, we used 22 SSR markers on 243 strains of *V. dahliae*. These strains were isolated from hosts in coastal California, or from imported spinach seed.

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 strains from spinach seed or those from lettuce.

The three genetic methods converged to indicate the high genetic variability of the *V. dahliae* strains included in these studies. Close to 90% of the variability was found at within the host or geographic sampling group. This information is exhibited in **Fig. 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 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 introduced on infested spinach seed produced from various countries and Washington State. This information is exhibited in **Fig. 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: **Assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach.**

Methods. DNA analysis using microsatellite markers and subsequent bioinformatics investigation were completed on strains isolated from lettuce grown in coastal California, infected spinach seed imported from 4 different geographic sources, and from infected lettuce seed grown in California.

A total of 65 *V. dahliae* strains isolated from plants affected with Verticillium wilt in the Salinas and Pajaro valleys were included in this analysis. These plants were collected between 1995 and 2007, from a number of different fields. Strains of *V. dahliae* isolated by S. Koike and L. du Toit from spinach seed were also included. These strains include 45 entries from Chile, 38 from Denmark, 32 from The Netherlands, and 54 from Washington State. Additionally, 56 strains isolated from lettuce seed that was submitted for the survey of *V. dahliae* on lettuce seed were also included. All of the latter entries were from seed produced in the Santa Clara Valley of California. The identity of all of these strains was confirmed morphologically and by amplification and sequencing of the ITS rDNA. This genomic region is generally highly conserved among strains of the same species, and has been previously used successfully to recognize several fungal species, including *V. dahliae*.

We amplified the DNA of the above-mentioned *V. dahliae* strains using 16 SSR (or microsatellite) markers and separated these fragments to identify potential differences among DNA samples (polymorphisms). Individuals representing the various fragment sizes were also sequenced, to ascertain that the identified polymorphisms were solely caused by changes at the level of microsatellite motifs. Subsequently, the structure of these sub-populations was analyzed using two different methods. An AMOVA (analysis of molecular variance) analysis was conducted to identify the level at which the genetic variability becomes significant. For this the data was divided into three clusters: i) lettuce plants from coastal California, ii) spinach seed from 4 different sources, and iii) lettuce seed from the Santa Clara Valley. Migration or recent common ancestry would prevent sub-populations from differentiating. A second test of the structure of the tested population was performed by identifying the membership of individual strains to genetic clusters. In this analysis each strain is assigned a certain proportion of membership to a specific genetic population. This model was tested assuming 1-10 possible genetic clusters, over 1 million iterations. The data from this analysis is displayed graphically in a column diagram. The possibility of gene flow among the studied sub-populations was also tested through three different methods, each relying on a unique set of assumptions. The first of these tests was implemented in Migrate, which relies on the coalescent (retrospective re-tracing of common ancestry) approach and is biased toward recent samples, and does not require that populations be in equilibrium or of equal sizes. The second approach, implemented in BayesAss+, assumes that the loci are in gametic equilibrium (recombination is occurring and helps reshuffle the genome), however the Hardy-Weinberg equilibrium (allele frequencies are stable from one generation to the next generation) is not required, and it is assumed to be robust to deviations from the gametic equilibrium model and overlapping generations (parent and progeny populations exist at the same time in the same location). And the third method was implemented in GeneClass2 and employed a test that excludes populations that are not likely to be the sources of the individual genotypes tested. Using the exclusion approach allowed testing

the source of migrant genotypes, while acknowledging that not all possible source populations were included in the analysis.

Results. The analysis of the population structure using the AMOVA highlighted a lack of differentiation among the sub-populations from lettuce plants, spinach seed and lettuce seed. This indicates that these sampling groups have either recently separated, or that migration prevents them from differentiating. Furthermore, 3 distinct genetic clusters (populations) were identified across all six sampling groups, and were distributed pan-globally, which suggests gene flow among the various geographic regions and/or hosts of *V. dahliae* (**Fig. 3**). The analysis of the membership of individual strains to specific genetic clusters suggested that the lettuce sampling group shared significant similarity to spinach groups from Denmark, Chile and Washington state. And haplotypes from lettuce seed were affiliated to the same clusters as spinach haplotypes from Chile, Netherlands and Washington.

All three methods of migration analysis converged to suggest that the *V. dahliae* sub-population from lettuce seed, did not exchange "recent" (i.e. not since the sub-populations split from their most recent common ancestor, no chronological dates are currently available) migrants with the coastal valleys of the Salinas and Pajaro rivers. In contrast, spinach seed sources were clearly implicated, in as much as ~17 % of the haplotypes recovered from lettuce were determined to be first-generation immigrants from spinach populations (**Fig. 4**).

The analysis of gene flow using the coalescent approach implemented in Migrate, yielded evidence that the lettuce plant sampling group exchanged significantly more migrants with the spinach sampling groups from The Netherlands and Washington. The Bayesian migration analysis implemented in BayesAss+ suggested that between 0 and 9 % of haplotypes recovered from each sampling group is a likely immigrant from another sampling group. This analysis also indicated that 4 and 6 % of the haplotypes from lettuce were migrants from Chile, Denmark and Washington spinach, while 2 % may be migrants from The Netherlands. In contrast, The Netherlands, Chile and Washington exchanged 6 to 8 % of migrants. And finally, the haplotype assignment tests by exclusion assigned more than 80% of the haplotypes to source populations. Between 3 and 18 % of haplotypes from lettuce could be linked to a specific spinach source, whereas none could be associated with lettuce seed. Actually, no haplotypes from lettuce seed could be assigned to any other population tested, and similarly no haplotypes in other sampling groups may be assigned to lettuce seed.

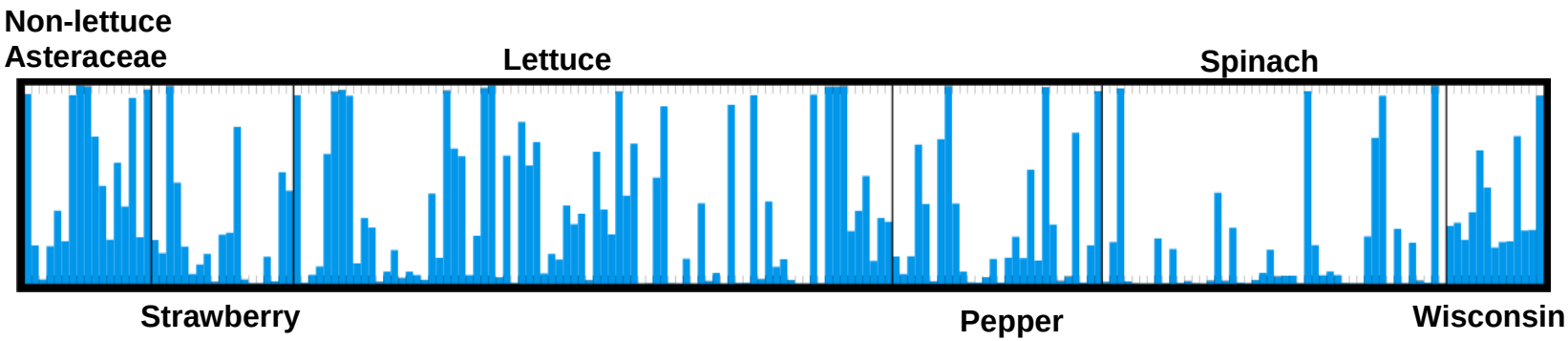

Figure 1. Affiliation of individual genotypes of *Verticillium dahliae* as assessed using 2603 AFLP loci. Individual haplotypes are separated into discrete vertical bars organized by sampling groups. The presence of two colors (white and blue) within a vertical bar indicates a bi-population affiliation of an individual genotype (admixture). The height of each color within an individual bar is the measure of proportional affiliation. K= 2 was determined to be the most likely number of genetic clusters for the six *Verticillium dahliae* sampling groups from lettuce plants, strawberry, non-lettuce Asteraceae and pepper grown in coastal California, spinach seed from northern Europe and the US Pacific Northwest, and ornamentals from Wisconsin.

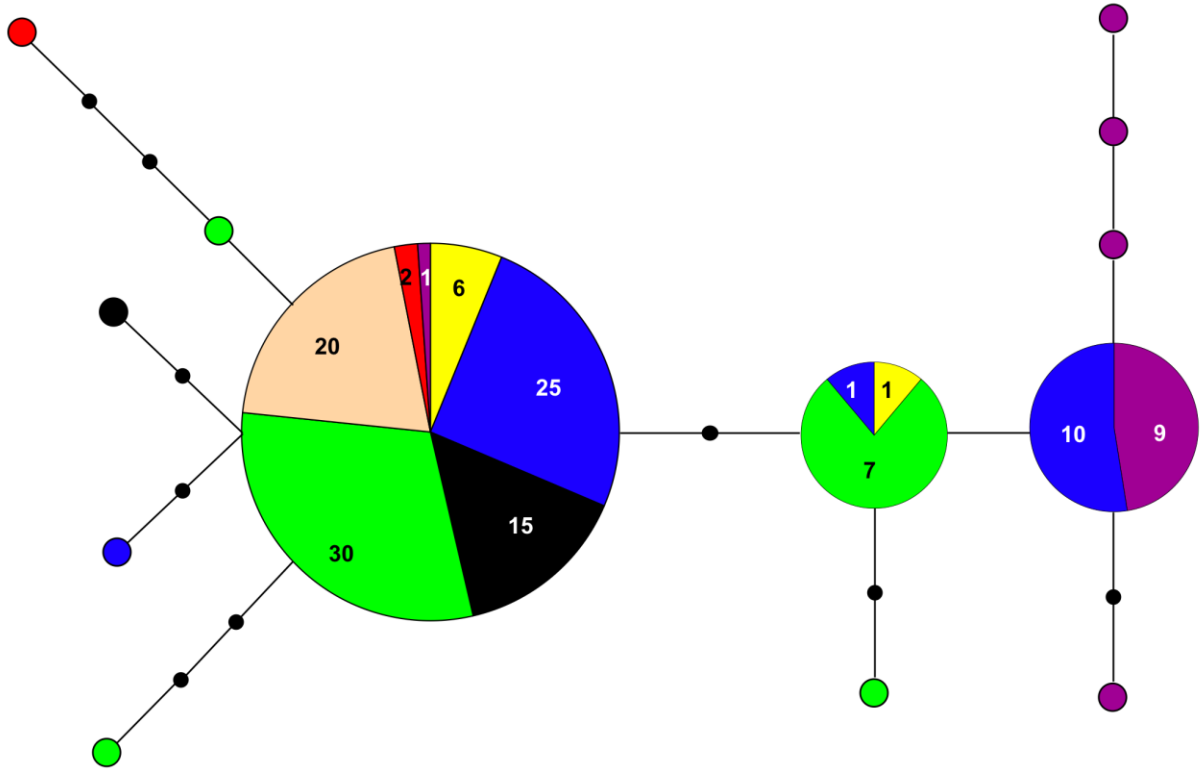

Figure 2. Statistical parsimony network [33] of the IGS rDNA sequences from 141 *Verticillium dahliae* strains from non-lettuce Asteraceae (yellow), lettuce (green), pepper (purple), spinach (blue), strawberry (red), tomato (salmon) and ornamentals for Wisconsin (black). Small black circles are putative 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 sampling group.

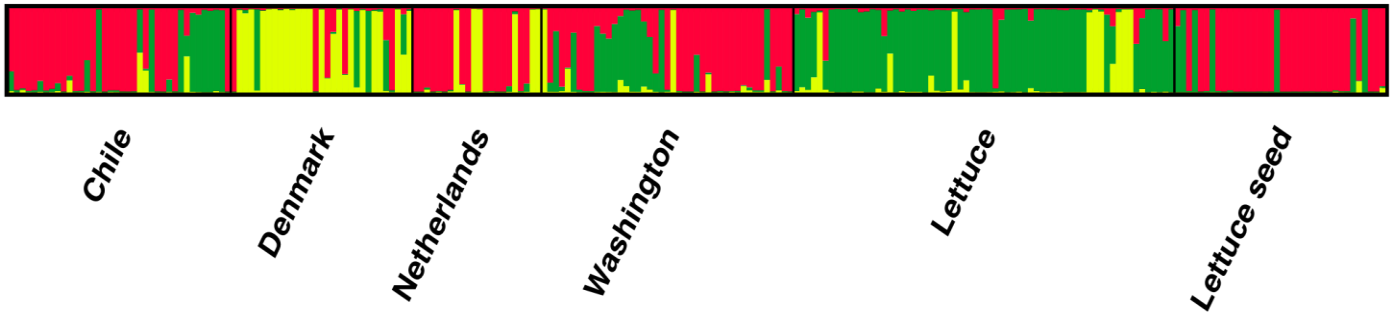

Figure 3. Affiliation of individual genotypes of *Verticillium dahliae* as assessed using 15 polymorphic SSR loci as measured in Structure ver. 2.2 (Pritchard et al. 2000). Individual haplotypes are separated into discrete vertical bars that are organized by sampling groups. Differences in color within 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.

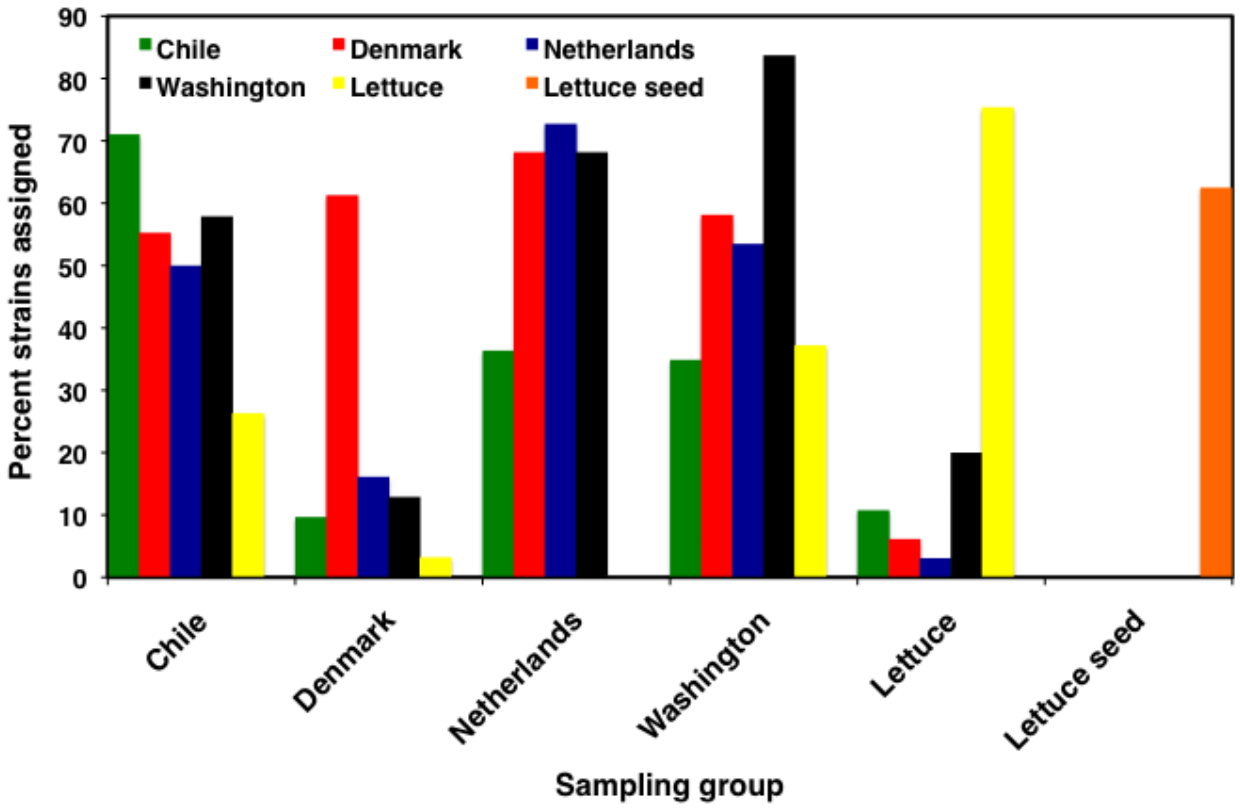

Figure 4. Percentage haplotype assignment among six *Verticillium dahliae* sampling groups from lettuce plants grown in coastal California, spinach seed from Europe and North and South America, and lettuce seed from the Santa Clara Valley of California.