CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

April 1, 2012 - March 31, 2013

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE

Krishna V. Subbarao
Department of Plant Pathology
University of California, Davis

SUMMARY

There were eight proposed objectives and the report includes the progress on five of them. Three objectives could not be completed because resistance to race 2 in the germplasm has not been confirmed. The five objectives being reported on are: a) to determine the pathogenicity of *V. klebahnii* and *V. isaacii* relative to *V. dahliae* on lettuce and other hosts; b) to develop a rapid identification technique for various *Verticillium* species; c) to determine the potential of host-directed evolution of *V. dahliae* genotypes of differential virulence from a single genotype; d) to create a race 2-infested plot at the USDA Station; and e) to continue the breeding program to identify and develop race 1 resistance in crisphead, leaf, and other lettuce types. Isolates of *V. isaacii, V. kelbahnii,* and *V. dahliae* were evaluated for pathogenicity on lettuce and other hosts. While isolates of *V. dahliae* evaluated were pathogenic on most hosts, a majority of isolates of *V. isaacii* and *V. klebahnii* was non-pathogenic. However, some isolates each of *V. isaacii* and *V. klebahnii* caused extensive vascular discoloration in both lettuce and artichoke. Thus some isolates of species besides *V. dahliae* introduced into coastal California fields via spinach seed have the potential to cause Verticillium wilt on lettuce and artichoke. We also developed species-specific primers to identify all 10 species of *Verticillium* either singly or in groups of 4-5 species. These primers have been extensively tested and validated. Analysis of isolates of race 1 collected over the past 4 years to determine potential host-directed evolution with the microsatellite markers has begun. The creation of a one-acre area infested with race 2 did not lead to high enough levels of Verticillium wilt in a screening trial in 2012 fall. We have therefore planted another crop of inoculated lettuce to increase the soil inoculum density. Additional breeding lines were screened for resistance to race 1 in the field and against race 2 in the greenhouse.

CALIFORNIA LETTUCE RESEARCH PROGRAM

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PROJECT TITLE: **BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE**

PRINCIPAL INVESTIGATOR: **Krishna V. Subbarao**
Department of Plant Pathology
University of California, Davis

COOPERATING PERSONNEL: **Karun Maruthachalam, Patrik Inderbitzin, Suraj Gurung, and Dylan Short**
Department of Plant Pathology,
University of California, Davis

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

Ryan Hayes
USDA-ARS, Salinas

OBJECTIVES:

A. Determine the pathogenicity of *V. klebahnii* and *V. isaacii* relative to *V. dahliae* on lettuce and other hosts.
B. Develop a rapid identification technique for various *Verticillium* species.
C. Evaluate the effectiveness of *V. dahliae* race 1 resistance identified in lettuce against *V. klebahnii* and *V. isaacii*.
D. Screen germplasm (*L. serriola, L. georgica, L. virosa* in the order of priority) collected from Armenia and Georgia against a range of race 2 strains of *Verticillium dahliae*.
E. Determine the nature of putative resistance in *Lactuca serriola* accessions using GFP-tagged race 1 and 2 strains of *Verticillium dahliae.*
F. Determine the potential of host-directed evolution of *V. dahliae* genotypes of differential virulence from a single genotype.
G. Create a race 2-infested plot at the USDA Station.
H. Continue the breeding program to develop race 1 resistance in crisphead, leaf, and other lettuce types.

PROCEDURES AND RESULTS:

Objective A. Determine the pathogenicity of *V. klebahnii* and *V. isaacii* relative to *V. dahliae* on lettuce and other hosts.

Methods. The pathogenicity of *V. kelbahnii* and *V. isaacii* along with *V. dahliae* was tested on 4- to 5-wk-old seedlings of lettuce (Salinas and La Brillante), artichoke, cauliflower, eggplant, pepper, and tomato at the UCD greenhouse in Salinas and on spinach and strawberries at the UC Cooperative Extension greenhouse also in Salinas. For each species, four isolates were inoculated on each host and compared them to disease symptoms induced by two isolates of *V. dahliae.* All inoculated seedlings were transplanted into individual pots at both locations. Noninoculated plants were maintained as controls. Ten seedlings per replication in three replications were inoculated and the pots were arranged in a complete randomized design on greenhouse benches. All plants were incubated for six weeks under a 13/11 light/dark regime in the greenhouse maintained at 20 $\pm$ 5 C except for spinach plants that were supplemented with 5 additional hours of light. The plants were then uprooted, washed free of soil, root and stem split longitudinally to record the extent of vascular discoloration (disease severity) based on a scale that we have extensively used over the years. Mean disease severity was computed and the relative pathogenicity of each species determined for each host. The results are as follows:

Results. *V. dahliae* isolates Ls16 and Ls17 caused root discoloration on artichoke and the plants were significantly stunted and leaves dried relative to non-inoculated plants. Similarly, *V. isaacii* (Cello-A-Tri-P, PD 341, PD 661 and PD 752) and *V. klebahnii* (PD 347, PD 407, PD 458 and PD 659) also caused root discoloration. Disease severity caused by *V. isaacii* isolate Cello-A-Tri-P was significantly higher than that caused by *V. dahliae* isolate 'Ls16'. Overall, mean rankings of disease severity caused by isolates of *V. dahliae* were significantly higher ($P < 0.0001$) than those caused by *V. isaacii* and *V. klebahnii*.

None of the *V. dahliae V. isaacii* and *V. klebahnii* isolates caused vascular discoloration in cauliflower. Plants looked healthy and plant height was not significantly different from control plants.

V. dahliae isolates Ls17 caused severe root discoloration in most inoculated plants of eggplant and the infected plants were stunted relative to the uninoculated plants. The four *V. isaacii* and *V. klebahnii* isolates each caused little or no root discoloration. Overall, mean rankings of disease severity caused by *V. dahliae* isolates were significantly higher ($P < 0.0001$) than those caused by *V. isaacii* and *V. klebahnii*, with the highest median disease severity of 4.2 for *V. dahliae* Ls17.

Both *V. dahliae* isolates Ls16 and Ls17 caused disease on lettuce cv. Salinas. Similarly, the four *V. isaacii* and *V. klebahnii* isolates also caused root discoloration of varying degrees. The *V. isaacii* isolate 'Cello-A-Tri-P' was as severe as Ls16 on Salinas. Overall, however, median disease rankings of *V. dahliae* were significantly greater ($P < 0.0001$) than those caused by *V. isaacii* and *V. klebahnii*. *V. dahliae* isolate Ls17 produced extensive root discoloration and wilting symptoms on La Brilliante. However, root discoloration caused by *V. dahliae* Ls16, *V. isaacii* and *V. klebahnii* isolates was negligible. These results suggest variability in the pathogenicity and virulence of *V. isaacii* strains on lettuce. In fall 2012, multiple *Verticillium* strains from commercial lettuce fields were collected in Salinas that were subsequently identified

as *V. isaacii*. So, *V. isaacii* introduced by spinach seed could also add to the problems caused by *V. dahliae* strains introduced on spinach seed. So far, however, all *V. isaacii* strains examined appear to belong to race 1.

None of the *V. isaacii and V. klebahnii* isolates caused vascular discoloration on peppers, nor was plant height reduced in inoculated plants. Except *V. dahliae* isolate Ls17, none of the other isolates including the race 1 strain from lettuce caused disease on tomato carrying the *Ve* gene. Again, this suggests that the *V. isaacii* and *V. klebahnii* strains evaluated belong to race 1.

Both *V. dahliae* isolates caused Verticillium wilt on spinach but none of the *V. isaacii* and *V. klebahnii* isolates evaluated in this study were pathogenic on spinach. All isolates of *V. isaacii* and *V. kelbahnii* were originally collected from commercial spinach fields, however. Thus, spinach appears to be a 'silent carrier' of these species, some of which may cause disease on crops that follow spinach such as lettuce and artichoke.

V. dahliae isolates, *V. isaacii* and *V. klebahnii* caused root discoloration on strawberries. One *V. klebahnii* isolate, PD659, had lower disease severity compared to other isolates. Overall, median rankings of the disease severity were significantly higher for *V. dahliae* isolates ($P < 0.0001$) than for *V. isaacii* and *V. klebahnii* isolates. We are currently awaiting the results from the repeat experiment involving strawberry.

All of these isolates have been inoculated on cotton seedlings and the results will be available in June 2013.

Table 1. Median and maximum disease severity and relative marginal effects along with 95% confidence intervals (CI) for Verticillium wilt severity ratings in relation to different hosts and isolate of *Verticillium dahliae*, *Verticillium isaacii* and *Verticillium klebahnii*

Hosts	Isolates[a]	Disease severity[b]		Relative marginal effect	
		Median	Maximum	Estimate	95% Confidence interval
Artichoke	LS16	1.43	2.50	0.69	(0.57, 0.79)
	LS17	2.75	3.50	0.92	(0.83, 0.94)
	Cello	2.00	2.43	0.71	(0.57, 0.81)
	PD341	0.92	1.29	0.46	(0.32, 0.60)
	PD661	0.47	1.14	0.33	(0.20, 0.49)
	PD752	0.31	1.40	0.36	(0.24, 0.52)
	PD347	0.69	2.17	0.45	(0.24, 0.68)
	PD407	0.32	1.67	0.37	(0.17, 0.65)
	PD458	0.21	1.86	0.34	(0.19, 0.55)
	PD659	0.34	2.29	0.37	(0.18, 0.63)
Cauliflower	LS16	0.37	0.70	0.59	(0.39, 0.75)
	LS17	0.57	1.00	0.62	(0.36, 0.82)
	Cello	0.05	0.60	0.42	(0.24, 0.62)
	PD341	0.58	1.30	0.65	(0.42, 0.81)

	PD661	0.00	1.22	0.37	(0.19, 0.62)
	PD752	0.05	0.33	0.36	(0.23, 0.51)
	PD347	0.50	1.25	0.64	(0.40, 0.82)
	PD407	0.00	1.33	0.41	(0.20, 0.67)
	PD458	0.27	0.44	0.47	(0.31, 0.64)
	PD659	0.15	1.22	0.48	(0.26, 0.70)
Eggplant	LS16	1.17	2.14	0.78	(0.64, 0.86)
	LS17	4.15	5.00	0.95	(0.63, 0.99)
	Cello	0.05	0.80	0.32	(0.17, 0.54)
	PD341	0.39	1.29	0.54	(0.36, 0.70)
	PD661	0.10	0.60	0.37	(0.20, 0.59)
	PD752	0.26	0.56	0.43	(0.26, 0.62)
	PD347	0.16	0.30	0.34	(0.22, 0.49)
	PD407	0.15	0.56	0.37	(0.21, 0.58)
	PD458	0.31	0.89	0.5	(0.33, 0.68)
	PD659	0.11	0.70	0.41	(0.23, 0.62)
Lettuce (Salinas)	LS16	3.00	3.50	0.81	(0.67, 0.89)
	LS17	4.50	4.67	0.85	(0.74, 0.91)
	Cello	3.26	3.96	0.69	(0.54, 0.80)
	PD341	1.00	3.00	0.41	(0.23, 0.62)
	PD661	1.75	3.13	0.53	(0.32, 0.73)
	PD752	0.75	2.13	0.29	(0.14, 0.52)
	PD347	1.04	1.78	0.31	(0.17, 0.51)
	PD407	1.05	2.33	0.36	(0.21, 0.55)
	PD458	1.25	2.17	0.46	(0.28, 0.66)
	PD659	0.95	1.50	0.3	(0.20, 0.43)
Lettuce (La Brilliante)	LS16	1.29	2.00	0.72	(0.52, 0.85)
	LS17	2.93	4.78	0.87	(0.75, 0.92)
	Cello	1.19	1.38	0.63	(0.41, 0.79)
	PD341	1.13	1.30	0.59	(0.45, 0.72)
	PD661	1.00	1.50	0.32	(0.19, 0.48)
	PD752	1.04	1.60	0.32	(0.29, 0.49)
	PD347	1.36	1.50	0.42	(0.23, 0.65)
	PD407	1.25	1.86	0.47	(0.24, 0.72)
	PD458	0.66	1.13	0.18	(0.12, 0.30)
	PD659	1.42	1.50	0.47	(0.30, 0.65)
Pepper	LS16	0.13	0.20	0.65	(0.46, 0.80)
	LS17	0.00	0.00	0.35	(0.30, 0.40)
	Cello	0.00	0.40	0.53	(0.33, 0.71)
	PD341	0.00	0.00	0.35	(0.30, 0.40)
	PD661	0.00	0.10	0.47	(0.34, 0.61)
	PD752	0.00	0.00	0.35	(0.30, 0.40)

	PD347	0.00	0.56	0.55	(0.33, 0.75)
	PD407	0.33	0.44	0.71	(0.46, 0.86)
	PD458	0.11	0.60	0.69	(0.45, 0.85)
	PD659	0.00	0.00	0.35	(0.30, 0.40)
Spinach	LS16	4.10	5.00	0.93	(0.87, 0.94)
	LS17	3.70	4.20	0.86	(0.81, 0.89)
	Cello	0.00	0.20	0.22	(0.15, 0.31)
	PD341	1.50	3.00	0.65	(0.48, 0.79)
	PD661	0.00	0.80	0.32	(0.36, 0.48)
	PD752	0.00	0.10	0.30	(0.32, 0.46)
	PD347	0.40	1.40	0.46	(0.47, 0.63)
	PD407	0.50	0.60	0.42	(0.31, 0.56)
	PD458	0.50	1.20	0.49	(0.32, 0.62)
	PD659	0.20	1.40	0.37	(0.42, 0.54)
Strawberry	LS16	2.20	2.60	0.39	(0.19, 0.64)
	LS17	3.60	5.00	0.93	(0.79, 0.94)
	Cello	2.20	2.40	0.40	(0.25, 0.57)
	PD341	2.20	2.40	0.30	(0.12, 0.59)
	PD661	2.40	3.20	0.51	(0.22, 0.78)
	PD752	2.40	3.60	0.48	(0.13, 0.85)
	PD347	2.60	2.80	0.67	(0.51, 0.79)
	PD407	2.60	2.80	0.53	(0.24, 0.78)
	PD458	2.20	2.80	0.43	(0.18, 0.72)
	PD659	1.80	3.50	0.36	(0.09, 0.80)
Tomato (Beef steak)	LS16	1.46	2.80	0.81	(0.58, 0.90)
	LS17	2.81	3.50	0.92	(0.87, 0.94)
	Cello	0.00	0.43	0.45	(0.33, 0.58)
	PD341	0.00	0.17	0.44	(0.33, 0.56)
	PD661	0.00	0.00	0.38	(0.34, 0.41)
	PD752	0.00	0.22	0.44	(0.33, 0.56)
	PD347	0.00	0.00	0.38	(0.34, 0.41)
	PD407	0.00	0.00	0.38	(0.34, 0.41)
	PD458	0.00	0.00	0.38	(0.34, 0.41)
	PD659	0.00	0.25	0.44	(0.33, 0.57)

[a] Isolates LS16 and LS17 were *V. dahliae* collected from lettuce, isolates Cello, PD341 PD661 and PD752 were *V. isaacii* and isolates PD347, PD407, PD458 and PD659 were *V. klebahnii*. Isolates from the last two species were collected from commercial spinach crops.

[b] Severity of Verticillium wilt was assessed visually on an ordinal 0 to 5 scale, where 0 = no vascular discoloration, 1 = 1 to 25% of the vascular tissue showing discoloration; 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% discoloration in the absence of foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms typical of Verticillium wilt (Hayes et al., 2007).

[c] The relative marginal effects and 95% confidence intervals were calculated from the analysis of rank values of disease severity.

Objective B. **Develop a rapid identification technique for various *Verticillium* species.**

Verticillium is a small group of agriculturally important, plant-associated fungus that causes Verticillium wilt resulting in significant losses in lettuce and other crops. Among the ten species currently recognized in *Verticillium*, *V. dahliae* is most widespread and most economically important, but *V. albo-atrum*, *V. alfalfae*, *V. longisporum*, *V. nonalfalfae*, *V. tricorpus* and *V. zaregamsianum* also cause significant losses, *V. nubilum* causes disease in pathogenicity tests, and both *V. isaacii* and *V. klebahnii* have been recovered from lettuce and artichoke, respectively. One of the characteristic features of *Verticillium* species is the formation of resting structures. The resting structures of *V. dahliae* consist of clusters of thick-walled cells that are referred to as microsclerotia, and remain viable in the soil for at least fourteen years. As few as two microsclerotia per gram of soil can result in plant infection and yield losses in strawberries, and knowledge about the abundance of microsclerotia and other resting structures in the soil is an important factor to consider for disease management. *Verticillium* species also differ in host range and pathogenicity, and thus, expedient detection, quantification and identification of *Verticillium* species is critical for disease management decisions. We used the DNA sequence data generated in conjunction with phylogenetic and taxonomic studies of *Verticillium*, and designed PCR assays for the identification of *Verticillium* species and *V. longisporum* lineages. The assays will be useful for diagnostics labs and research applications.

Methods

A total of 104 DNA sequences from ten *Verticillium* species and *Gibellulopsis nigrescens* were retrieved from GenBank or the Broad Institute website. Ninety-five of the *Verticillium* sequences were from 34 isolates that represented the genetic diversity at five loci in the ten *Verticillium* species. The loci evaluated were the ribosomal internal transcribed spacer (ITS) region, *actin* (*ACT*), *elongation factor 1-alpha* (*EF*), *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*), and *tryptophan synthase* (*TS*).

DNA sequences were aligned separately for each locus and eighteen primers were designed manually. Primer specificity was achieved by maximizing the number of mismatches between a primer's 3'-end and homologous sites in non-target lineages. Primer annealing temperatures were between 53°C and 58°C. Primer names were chosen to reflect primer specificity, for instance, forward primer 'If', named after *V. isaacii,* was used only for amplification of *V. isaacii*, whereas reverse primer 'IKr' that was named after *V. isaacii* and *V. klebahnii,* was part of both *V. isaacii* and *V. klebahnii*-specific primer pairs.

PCRs were performed using GoTaq Colorless Master Mix in GeneMate 0.2 ml 8-strip PCR tubes. Each PCR reaction comprised 10 µl template dilution containing 1, 10, or 100 ng DNA, 2.5 µl primer mixture (0.5 µM for each primer, except primers D3f and D3r that were 0.25 µM each when multiplexed) and 12.5 µl master mix, for a total volume of 25 µl. The PCR program consisted of a 2 min initial denaturation step at 94°C, 32 or 35 cycles of 10 sec at 94°C, 20 sec at the PCR assay-dependent annealing temperature, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. PCR reactions were set up at room temperature under sterile conditions in a laminar flow hood and using plugged pipet tips to minimize contamination. The reactions were run immediately, or were stored in a freezer. Agarose gel electrophoresis was performed in a RAGE RGX-60 gel box

with 20-sample comb or a larger Bio-Rad Wide Mini Sub Cell gel box with a 30-sample box. Gels were run between 30 to 70 minutes at 70-90 V, using various agarose concentrations.

Each PCR primer pair was initially validated in a simplex PCR assay that included one representative of the target species as a positive control, and negative controls that consisted of one representative of each species that differed from the target species by four or fewer substitutions at the more variable primer site. Further validation was performed with additional target and non-target isolates. Each PCR primer pair was tested in at least three different PCR runs, except for the Species A1, Species D1 and *V. dahliae* primer pairs. Multiplex PCR assays were validated with 24 *Verticillium* isolates representing the allelic diversity at *ACT*, *EF*, *GPD*, ITS and *TS* with the exception of *V. dahliae* where only three strains were used to represent *V. dahliae* lineages D2, D3 and the main group of *V. dahliae* and only one *V. alfalfae* isolate was included. The *V. dahliae* – *V. isaacii* – *V. klebahnii* – *V. tricorpus* multiplex PCR assay was also validated with eleven genetically uncharacterized isolates from lettuce in California. The PCR results were confirmed by DNA sequencing with the respective species-specific primers.

Results.

We designed eighteen PCR primers that were combined into eleven single-target (simplex) and four multi-target (multiplex) PCR assays for identification of all ten *Verticillium* species and *V. longisporum* lineages. PCR primer design was based on DNA sequence data of 257 *Verticillium* isolates at five loci, which were previously identified to species using type material. The reliability of the primer pairs was confirmed in PCR assays and various combinations of primer pairs were evaluated for simultaneous amplification of more than one target species or *V. longisporum* lineage in multiplex PCR assays. Four multiplex PCR assays were able to reliably amplify separate templates of *Verticillium albo-atrum, V. alfalfae* and *V. nonalfalfae*; *V. dahliae* including *V. longisporum* lineage A1/D3*, V. isaacii, V. klebahnii* and *V. tricorpus; V. dahliae* and *V. longisporum* lineages A1/D1, A1/D2 and A1/D3*; V. isaacii, V. klebahnii* and *V. tricorpus*. All multiplex PCR assays were validated with a set of 26 representative isolates. The PCR banding patterns for the multiplex assays are shown in Figure 1.

Verticillium dahliae and *V. longisporum* are the most difficult *Verticillium* species to identify by PCR assay. This is because *V. dahliae* is the parent of two of the three *V. longisporum* lineages, *V. longisporum* lineage A1/D2 and *V. longisporum* lineage A1/D3. Due to the high genetic similarity between *V. longisporum* and *V. dahliae*, PCR primers specific to *V. dahliae* protein-coding genes will in most cases amplify the orthologs in *V. longisporum* lineages A1/D2 and A1/D3. Our multiplex PCR assay differentiates *V. dahliae* from *V. longisporum* by targeting the Species A1 *EF* allele that is unique to *V. longisporum*. *Verticillium longisporum* lineage A1/D1 is differentiated from the other lineages by an amplicon of the Species D1 *GPD* allele, and *V. longisporum* lineage A1/D3 is the only lineage that has an ITS region derived from *V. dahliae*, the other two lineages' ITS regions are from Species A1. Due to concerted evolution, all of the *V. longisporum* lineages appear to have just one type of ITS region.

Thus, the *V. longisporum* lineage A1/D1 PCR banding pattern consists of the 310-bp Species A1 *EF* and the 1020-bp Species D1 *GPD* amplicons, the *V. longisporum* lineage A1/D2 banding pattern consists of the 310-bp Species A1 *EF* amplicon, and the *V. longisporum* lineage A1/D3 pattern

consists of the 310-bp Species A1 *EF* and the 490-bp ITS *V. dahliae* amplicons. The *V. longisporum* lineage A1/D2 banding pattern is identical to the pattern expected for Species A1. However, Species A1 has never been found and is only known as one of the parents of *V. longisporum*.

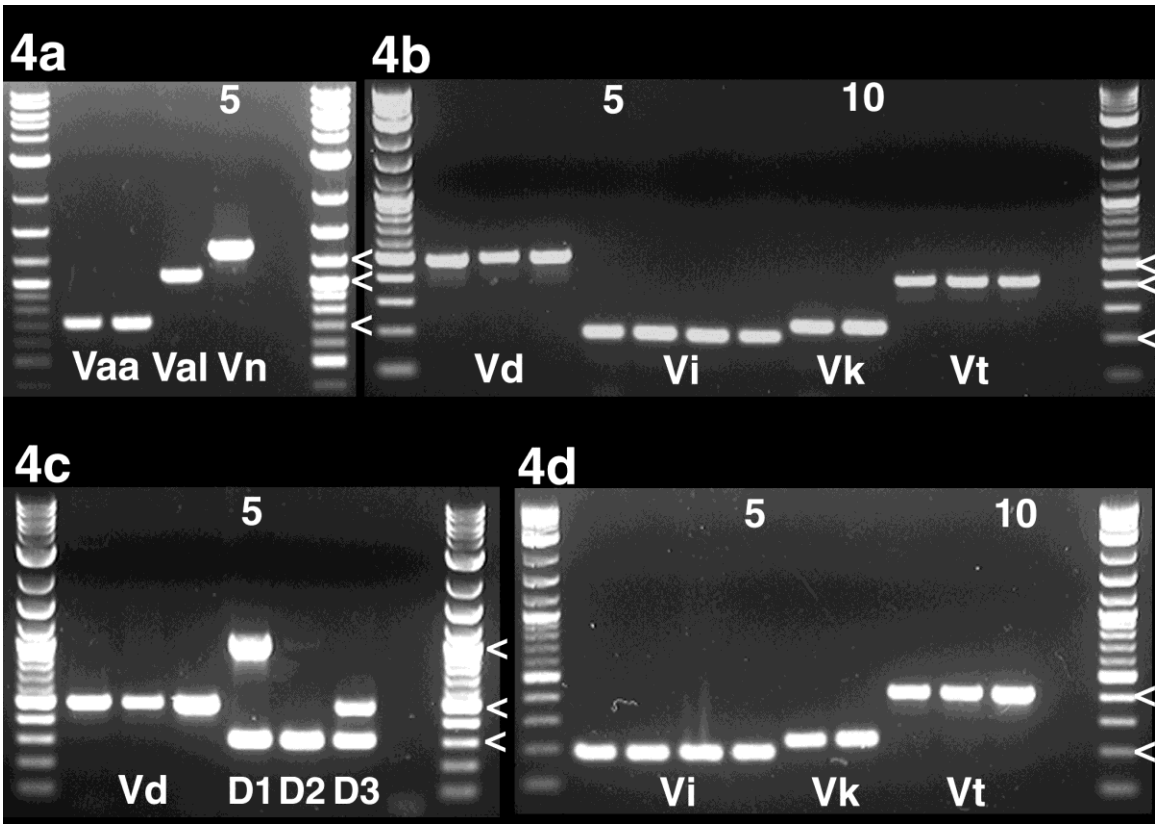

Figure 1. Multiplex PCR assays identify genetically diverse target isolates. Each agarose gel displays the results of one of the four multiplex PCR assays, controls with none-target isolates. Gels are delimited by 2-log ladders, penultimate wells are negative controls, and relevant size markers are indicated by '<'. Lanes are numbered from left to right, numbers are given for every fifth lane. Abbreviations below bands indicate species and *V. longisporum* lineages. All lanes contain 100 ng template DNA. 4a. *Verticillium albo-atrum – V. alfalfae – V. nonalfalfae* multiplex PCR assay. Lanes 2, 3: *V. albo-atrum* strains PD670, PD693. Lane 4: *V. alfalfae* strain PD338. Lane 5: *V. nonalfalfae* strain PD592. Size markers = 700 bp, 1000 bp, 1200 bp. 4b. *Verticillium dahliae – V. isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2-4: *V. dahliae* strains PD322, PD327, PD502. Lanes 5-8: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 9, 10: *V. klebahnii* strains PD347, PD407. Lanes 11-13: *V. tricorpus* strains PD593, PD685, PD703. Size markers = 200 bp, 400 bp, 500 bp. 4c. *Verticillium dahliae – V. longisporum* PCR multiplex PCR assay. Lanes 2-4: *V. dahliae* strains PD322, PD327, PD502. Lane 5: *V. longisporum* lineage A1/D1 strain PD348. Lane 6: *V. longisporum* lineage A1/D2 strain PD356. Lane 7: *V. longisporum* lineage A1/D3 strain PD589. Size marker = 300 bp, 500 bp, 1000 bp. 4d. *Verticillium isaacii – V. klebahnii – V. tricorpus* multiplex PCR assay. Lanes 2-5: *V. isaacii* strains PD341, PD343, PD618, PD752. Lanes 6, 7: *V. klebahnii* strains PD347, PD407. Lanes 8-10: *V. tricorpus* strains PD593, PD685, PD703. Size markers = 200 bp, 400 bp.

Objective C. Evaluate the effectiveness of *V. dahliae* race 1 resistance identified in lettuce against *V. klebahnii* and *V. isacii*.

Because the host range analyses of *V. isaacii* and *V. klebahnii* is still ongoing, we could not begin the experiments we outlined for objective B during the current project year. From the host range

analysis described in objective A, it is clear that the lettuce cultivar La Brillante is resistant against all four isolates each of *V. isaacii* and *V. klebahnii* tested. We can thus tentatively conclude that all isolates of these two species tested belong to race 1. Thus, testing of the cultivars and breeding lines resistant to *V. dahliae* race 1 now is therefore even more meaningful, as is confirming the race identity of these isolates using race-specific primers. These studies will be completed during 2013-14.

Objective D. Screen germplasm (*L. serriola, L. georgica, L. virosa* in the order of priority) collected from Armenia and Georgia against a range of race 2 strains of *Verticillium dahliae*.

Germplasm lines from the *Lactuca* species collected from Armenia and Georgia screened in Salinas were susceptible to race 1 isolate of *V. dahliae*. These results are at variance with the results obtained by Michelmore's group in Davis. Since plants obtained from different aliquots of seed were screened at the two locations, we have obtained the selections from Davis that exhibited various degrees of resistance for screening in Salinas. These and the crosses generated from low-susceptible lines were planted in the race 2-infested field for screening. Unfortunately, the disease did not develop in the field. Thus, the crosses have been carried to advanced generations in the greenhouse and we plan to screen them in the field again this year.

Objective E. Determine the nature of putative resistance in *Lactuca serriola* accessions using GFP-tagged race 1 and 2 strains of *Verticillium dahliae.*

Because we could not confirm the resistance in the *Lactuca serriola* accessions as explained in Objective C, proposed studies with the GFP-tagged races 1 and 2 strains had to be postponed. We will await the results from Objective C during the current project year and then complete the studies to determine the nature of resistance in the different *Lactuca* accessions. We already have the tagged strains from both races ready and will just await the confirmation of resistance in the *Lactuca* accessions to complete this objective.

Objective F. Determine the potential of host-directed evolution of *V. dahliae* genotypes of differential virulence from a single genotype

The study of the evolution of individual genotypes of fungi over time has not been attempted. Additionally, no information is currently available on the impact of diverse genotypes of a plant host on soilborne pathogens. This information is critical to devising strategies for the long-term success of breeding efforts targeting diseases such as Verticillium wilt. The race 1-infected transplants of the cultivar Salinas were produced in a growth chamber, transplanted into the fumigated field at the USDA Station where they were grown until maturity, and incorporated by tillage. This field had no history of Verticillium wilt on lettuce, and race 1 infestation was performed over two growing seasons. This field is currently serving as a Verticillium wilt screening nursery. In screening trials performed over the past three seasons, all race 1-susceptible lettuce cultivars and breeding lines planted in this plot developed typical Verticillium wilt. It may be safe to claim that a single genotype of *V. dahliae* was introduced into the field. Since the breeding lines also included some with differential susceptibility to Verticillium wilt, it is quite likely that they exerted selection pressure on the resident pathogen population. Over the successive lettuce cropping cycles in this field plot, we have been collecting samples from each lettuce season in order

to genotype them using the microsatellite markers that we developed. This will allow us to study the evolution of this individual genotype (race 1) in the natural environment, but most importantly it will provide us with insights into the impact of lettuce on this evolution. Does the host apply selective pressure on *V. dahliae* leading to its genotypic diversification? In a situation where the host does apply a selection pressure, then it is likely that a monoculture of one host genotype will lead to a rapid diversification of the pathogen and an increase in its fitness. Subsequently, this may mean that using near-isogenic lines carrying the gene(s) coding for resistance may be preferable to provide for a long-term success. Alternatively, if the host applies little or no selection pressure, the expectation is that the genotype that was introduced in the field plot will remain unchanged over the successive growing seasons. This would mean that if the introduction of new genotypes is curbed, no new alleles would appear in the resident population, unless mutations occur. Subsequently, breeders may expect that a monoculture of resistant lettuce cultivars is likely to be sustainable over a number of years.

Methods. During the course of the evaluation of the breeding material in the spring and summer seasons of 2010, 140 and 120 stems, respectively, during the summer season of 2011, 91 stems and during the summer of 2012, 50 stems were collected from symptomatic lettuce plants. The stem pieces were plated on NP-10 medium to isolate *V. dahliae*. Currently, we have 102 isolates from 2010, which came from two separate harvests, 14 isolates from 2011 and 28 isolates from 2012. Isolations and subculturing of *Verticillium* is ongoing and we expect to increase the number of isolates from 2011 and 2012. A final collection of isolates from infected lettuce is planned for Fall 2013. DNA is available for 68 isolates from the 2010 collection. We will begin molecular characterization of these isolates using both race-specific PCRs and a set of microsatellite markers during the 2013-14 project year.

Objective G. Create a race 2-infested plot at the USDA Station.

To have a field site infested with only the race 2 strains of *V. dahliae* available, a 1-acre site at the USDA Station in Salinas was identified. The field site was fumigated with methyl bromide and chloropicrin in the spring of 2011. During the summer of 2011, 12,000 seedlings of lettuce cultivar 'Salinas' were produced in greenhouse trays and inoculated by soil drench method three times before being transplanted at the fumigated field site. The crop developed very high levels of Verticillium wilt incidence. At maturity, the crop was incorporated into the soil. A second crop was transplanted in the spring of 2012 and was incorporated into the soil in July. Assuming the inoculum densities will be similar to the race 1 screening site after two inoculated crops, we planted the germplasm and breeding material for evaluation during the fall of 2012. No disease developed even in highly susceptible lines and the soil inoculum density evaluations revealed low numbers of microsclerotia. This was surprising but quite possibly due to the fact that the race 2 strain used may have a lower reproductive potential in infected plants compared with race 1. We therefore transplanted 24,000 seedlings of lettuce cv. Salinas on March 27 that had been grown in plug trays in the UC Davis greenhouse at the USDA-ARS research station in Salinas. These transplants had also been inoculated three times at weekly intervals beginning with three weeks after emergence with 3 ml of a *V. dahliae* strain Ls17 spore suspension at a concentration of 1×10^{-8} conidia per ml. The last inoculation occurred on March 20. We will evaluate the disease incidence in mid-June and incorporate the residue in late June. With this infected crop incorporated, we expect the inoculum density in soil to reach high levels required for Verticillium wilt development in lettuce.

Objective H. Continue the breeding program to identify and develop resistance in crisphead, leaf, and other lettuce types including screening of germplasm for resistance against race 2.

Methods and Results. Since the initial discovery of Verticillium wilt in lettuce in 1995, the disease has spread within the Salinas Valley. In affected fields, losses have ranged between 30 to 90%. Therefore, Verticillium wilt of lettuce caused by *Verticillium dahliae* has become a major concern to the California lettuce industry. The soilborne pathogen produces long-term resting structures called microsclerotia that remain dormant in the soil for 10 to 15 years. Fumigation is not economically feasible for lettuce, and crop rotation is ineffective due to the broad host range of *V. dahliae*. Therefore, the development of lettuce cultivars resistant to Verticillium wilt is important to the survival of the lettuce industry in California.

Funding from the National Plant Germplasm System has allowed us to screen lettuce germplasm for resistance to Verticillium wilt. Through this we identified two distinct pathogenic races of *V. dahliae* as well as resistance to race 1 isolates in diverse lettuce types. A single gene named Verticillium resistance 1 was identified in the Batavia cultivar La Brillante, and race 1 resistant iceberg breeding lines have been developed and released developed. Seed of race 1 resistant materials were deposited into the WRPIS (Western Regional Plant Introduction Station). All of this germplasm is susceptible to race 2 isolates.

The existence of race 2 isolates in California lettuce production fields is certain. Moreover, it is highly probable that widespread use of race 1 resistant germplasm will select for and increase the economic damage caused by race 2 isolates. Even more concerning is the finding that race 2 isolates can be introduced on infested seed of spinach, a crop widely grown in rotation with lettuce in the Salinas Valley. Therefore, identification and subsequent development of lettuce cultivars with resistance to race 2 is imperative for sustaining the lettuce industry.

To date, we have screened over 850 *L. sativa* and *L. serriola* accessions using race 2 *V. dahliae* isolate VdLs17. We have confirmed partial resistance (disease incidence significantly lower than 'Salinas') in four accessions (PIs 169511, 171674, 204707, 226641) (Hayes et al. 2011c). However, all of these PIs have had at least a few symptomatic plants, and all but PI 171674 have had non-symptomatic plants that are nonetheless colonized by *V. dahliae*. It does not appear that these PIs have a sufficient level of resistance to control the disease, and we are pursuing research to determine if intercrossing these four accessions will result in progeny with greater levels of resistance. Complete resistance to race 2 needs to be found, and we will continue to screen the collection in hopes of finding this trait.