CALIFORNIA LETTUCE RESEARCH PROGRAM

April 1, 2008 - March 31, 2009

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE

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

SUMMARY

There were five objectives during the current funding cycle and included: a) continued monitoring of Verticillium wilt and soil inoculum density in coastal California; b) continue assessing seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation; c) determine if airborne phase of the pathogen could be a threat in seed production fields; d) determine pathogenicity of spinach and French marigold isolates of *Verticillium dahliae* on lettuce and characterize them to the type of race on lettuce; and e) 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. Verticillium wilt of lettuce was observed on 13 new fields this year. Crop loss was near-total in two but varied between 20-60% in others. The number of microsclerotia in soil mimicked the association between high numbers and wilt documented previously. We continued the assessment of commercial lettuce seed lots for *V. dahliae* infestation. A total of 171 seed lots from 11 companies were evaluated for *V. dahliae* but the soil samples were available from fewer companies since many produced seed abroad. The number of microsclerotia in the 6 soil samples ranged from 0-20 per gram of soil. Forty-four seed lots were positive for *V. dahliae* and infestation ranged 1-9% with most seed lots carrying <4%. Since the soil infestation levels were too low to trigger extensive Verticillium wilt in the seed crop, airborne spread may have played a role. This is currently being investigated in the greenhouse. Pathogenicity of French marigold and spinach isolates was evaluated on lettuce and vice versa. Many French marigold and spinach isolates were pathogenic to lettuce and in turn the lettuce isolates were pathogenic to both crops. About 20% of the isolates of *V. dahliae* from spinach seed belonged to race 2. We also investigated potential sources of *V. dahliae* for lettuce using 22 polymorphic simple sequence repeats (SSR) by analyzing the genome of *V. dahliae* isolate Ls17 from lettuce. Differences between *V. dahliae* populations from lettuce and US and European spinach seed were minimal. In contrast, significant differences were observed with a tomato population from the San Joaquin Valley, where some lettuce seed is produced. Elevated symmetrical migration of isolates was measured between lettuce and spinach, but significantly smaller migrations were identified between these two crops and tomato. The breeding for resistance to Verticillium wilt has taken several approaches. Many sources of resistance to race 1 have been identified and several of these sources were released to commercial seed companies. Identification of resistance to race 2 is ongoing and putative sources of resistance have been identified. Evaluation of 98 isolates on lettuce differentials, Salinas and La Brillante was completed twice. Of these, 62 isolates belonged to race 1, 19 to race 2, and the remaining provided inconsistent results between the two virulence evaluations. Furthermore, we have developed a PCR technique that selectively identifies the race 1 strain from *V. dahliae* isolates from a variety of hosts.

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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: **Zahi Atallah and Karun Maruthachalam**
Department of Plant Pathology,
University of California, Davis

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

Ryan Hayes and Steve Klosterman
USDA-ARS, Salinas

OBJECTIVES:

1. Continued monitoring of Verticillium wilt and soil inoculum density in coastal California.
2. Continue assessing seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation.
3. Determine if airborne phase of the pathogen could be a threat in seed production fields.
4. Determine pathogenicity of spinach and French marigold isolates of *Verticillium dahliae* on lettuce and characterize them to the type of race on lettuce.
5. 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.

PROCEDURES AND RESULTS:

Objective 1. Continued monitoring of Verticillium wilt and soil inoculum density in coastal California.

Methods. Monthly surveys were undertaken to identify new fields with Verticillium wilt on lettuce and reappearance of the disease in previously fumigated fields in coastal California. The surveyed fields included all types of lettuce. During each survey, approximately 20 fields were evaluated. In each field, an average of 100 plants were examined for symptoms typical of Verticillium wilt, and any plants showing symptoms were further examined for the characteristic

vascular discoloration. A random sample of plants showing symptoms was brought to the laboratory for pathogen isolation from affected tissues on modified NP-10 medium semi-selective for *V. dahliae*. In fields, where the disease was found, incidence data was collected by sampling plants from an average 20 sites by walking the fields in an X-pattern. At each site, the total number of plants in a 1-m x 1-m area and the number of symptomatic plants were counted. Data from the 20 sites were averaged to give the mean disease incidence for each field. Soil samples were also collected in fields with Verticillium wilt to assay for *V. dahliae* microsclerotia.

Soils were air-dried in the laboratory (23 ºC ± 2 ºC), mixed thoroughly, and pulverized using mortar and pestle. From each sample, 10 g of soil were placed in snap cap vials and mixed with 2.5 ml of a dl-methionine solution (7.5 mg ml^{-1}). Vials were capped and incubated in the dark at 30 ºC for 1 week. The vials were then opened and allowed to air-dry for 1 week at 22 to 24 ºC. Samples were re-pulverized and dispensed onto Petri dishes containing modified NP-10 selective medium using the modified Anderson sampler. With the Anderson sampler, 0.5 g of pulverized soil from each sample was distributed over two replicates of six Petri dishes. Plates were incubated in the dark at 22 to 24 ºC for 3 weeks. After incubation, the surfaces of the agar media were gently washed under running tap water to dislodge and remove soil particles. Washed Petri dishes were examined for *V. dahliae* microsclerotia clusters using a dissecting microscope with transmitted light. Counts from the two replications were combined for mean values and expressed as microsclerotia g^{-1} dry soil.

Results. Verticillium wilt of lettuce was very active this year. Since the initial discovery of the disease nearly 14 years ago, there was never a year in which so many new lettuce fields succumbed to the disease. In 2008, there were 13 new fields in which Verticillium wilt on lettuce occurred. The new fields were located in Watsonville, Salinas, and Chualar areas but majority of the fields were in Salinas. At least two fields in Salinas were abandoned as the yield loss was near-total and were fumigated and planted to strawberries. At the last visit for this survey, plans for the other fields were not available. Incidence of wilt in others varied between 20-60%. The number of microsclerotia in soil again followed the strong association between high numbers and incidence of Verticillium wilt documented previously.

Objective 2: Continue assessing seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation.

As stated in the 2007 report, Verticillium wilt that occurs on many dicotyledonous plants is predominantly caused by *Verticillium dahliae* that colonizes xylem tissues and causes disease on a broad array of plants. In some instances these vascular pathogens may even invade the inflorescence, and subsequently the developing fruits and seeds. The seed-borne nature of *V. dahliae* has been documented in cotton, eggplant, tomato, and spinach, and in the cultivated composites, safflower and sunflower. We reported the seed transmission of *V. dahliae* in 2005 on *L. sativa*, which was previously reported to be a new host of *V. dahliae*. The recovery of *V. dahliae* following the disinfestation of seed surface suggests that the fungus resides within the achene, similar to findings in safflower and sunflower. In preliminary studies, the pericarps shed from germinating lettuce seeds were colonized by *V. dahliae*. Subsequent studies employing a green fluorescent protein-transformed race 1 strain of *V. dahliae* from lettuce suggested that the

fungus resides in the endosperm but never compromises the embryo. Thus, even if the pathogen resides in the seed, it may not reduce seed germination. The susceptibility of several weed species to *V. dahliae* and the infestation of seed, and subsequent infection of seedlings from these seed also has the potential to spread the pathogen in coastal California. The susceptibility of *Lactuca* species to isolates of *V. dahliae* from lettuce, in addition to the susceptibility of lettuce to several isolates of *V. dahliae* that were collected from weed species raises concerns about the potential of weed species to act as a reservoir of *V. dahliae* in California vegetable production areas.

The critical realization over the past year that a potentially significant role is played by *V. dahliae*-infested spinach seed that is planted in coastal California in Verticillium wilt of lettuce has become a highly emotive and controversial topic. Potentially, infested spinach and lettuce seed can augment soil inoculum levels and cause Verticillium wilt in lettuce. Other researchers, have isolated *Verticillium*-like fungi from infested spinach seed in addition to *V. dahliae* and have attempted to emphasize the importance of these fungi more to the exclusion of *V. dahliae*. The potential role played by these *Verticillium*-like fungi needs to be explored further. Even though they may add to the complexity of the issue, they will not lessen the importance of *V. dahliae*. Furthermore, the recent taxonomic revision of the genus *Verticillium* has reclassified *V. nigrescens* to *Gibbellulopsis nigrescens* and *V. nubilum* to *Musicillium nubilum*, the two *Verticillium*-like fungi frequently recovered from infested spinach seed. There are no reports of these two fungi causing significant disease on any crops and are no longer *Verticillium* species either (Table 1).

Table 1. Current taxonomic status of the genus *Verticillium*

Verticillium species in 2007	*Verticillium* species in 2008
V. albo-atrum	*V. albo-atrum*
V. dahliae	*V. dahliae*
V. tricorpus	*V. tricorpus*
V. theobromae	*V. theobromae*
V. nigrescens	*Gibbellulopsis nigrescens*
V. nubilum	*Musicillium nubilum*

Methods. We continued the assessment of commercial lettuce seed lots for *V. dahliae* infestation. Since very few commercial lettuce cultivars were evaluated last year, we specifically focused on commercial lettuce cultivars currently marketed for planting this year. In addition, we expanded the request to include seed of various lettuce types and the locations from which seed is produced. Of the 18 companies approached this year, nine submitted 171 seed lots for evaluation but the soil samples were available for fewer companies since many submitted seed produced abroad. From each seed lot, 200 seeds were plated onto *V. dahliae* semi-selective NP-10 medium and incubated at room temperature (22 $\pm$ 1ºC) for 10 days and fungal colonies that emerged from individual seeds were examined under a stereomicroscope. Number of seeds yielding *V. dahliae* colonies were counted and expressed as the percentage of seeds that yielded *V. dahliae*. These

colonies were transferred to fresh plates of NP-10 medium to confirm identity and also for isolate collection. After purification, single spore colonies were obtained and stored for future studies evaluating the populations. Soil samples were processed as described in objective 1 and the number of microsclerotia from each soil sample was expressed as the number per gram dry soil.

Results. The number of microsclerotia in the 6 soil samples ranged from 0-20 per gram of soil. Of the 171 seed lots evaluated in 2008 on the *V. dahliae* semi-selective medium, 44 were positive for *V. dahliae* and the level of infestation varied between 1-9% (Table 2). Nearly all infested seed lots came from two companies among the 19 that contributed samples for evaluation. Most seed lots positive for *V. dahliae* had <4% infestation (Table 3). All lettuce types except butterhead had some level of infestation but the majority of seed lots from undeclared or unknown lettuce types were infested followed by crisphead types (Table 4). Similarly, seed produced in all countries except The Netherlands were infested but the majority of those produced in the United States were infested (Table 5). One company that had many infested seed lots has fumigated their seed production fields with methyl bromide and chloropicrin. Since the soil infestation levels were too low to trigger extensive Verticillium wilt development in the seed crop, the seed infestation could possibly have come from the airborne spread of the pathogen. This aspect is currently being evaluated in the greenhouse. In addition to *V. dahliae*, other fungi that produce conidiophores similar to *Verticillium* are occasionally observed on the seed. However, many of these do not belong to the genus *Verticillium.* Whereas the role these fungi play in the overall seed pathogen complex is unclear at this time, they do not in any way lessen the impact of *V. dahliae*.

Table 2. Summary of seed lots and soil samples from seed production fields evaluated during 2007 -2008

Year	Seed companies approached	Number responded	Seed lots with *V. dahliae*/total assayed (Range %)	Soil samples with *V. dahliae*/total assayed (Range of microsclerotia)
2007	16	8	26/55 (0-6%)	33/37 (0-38 ms/g)
2008	19	9	44/171 (0-9%)	5/6 (0-20 ms/g)

Objective 3. Determine if airborne phase of the pathogen could be a threat in seed production fields.

Judging from historical trends, the level of microsclerotia in the soil samples from lettuce seed production fields was not high enough to cause Verticillium wilt and yet, the seed infestation levels suggest some level of disease incidence. Our recent studies employing a GFP-tagged race 1 isolate of *V. dahliae* from lettuce have shown a potential airborne phase for the pathogen during seed production. Large numbers of conidia were observed in the developing inflorescence and flower heads of early maturing lettuce lines and in susceptible head type lettuce cultivars that managed to produce an inflorescence. Massive quantities of conidia and

conidiophores were present on external and internal tissues of the pappus, which are easily disrupted mechanically, and could accelerate the movement of *V. dahliae* by airborne dispersal in lettuce seed production areas. Perhaps this plays a greater role spreading the pathogen on seed crops than we have so far realized.

Methods and Results. Susceptible Salinas and PI 251246 and resistant LaBrillante seedlings were produced in flats. Three weeks after emergence, the seedlings were transplanted to larger Styrofoam cups filled with autoclaved sand:potting soil mixture. Prior to the transplanting, the seedlings of PI 251246 was dipped in a suspension of 10^6 conidia^{-1} ml of a race 1 isolate (VdLs.16). Four pots containing the inoculated PI 251246 were placed in the center of a greenhouse bench. Pots containing Salinas and La Brillante seedlings were arranged alternatively in concentric circles 20-cm apart (both cultivars were next to each other in each circle) to a maximum distance of 1-m from the focus (PI 251246). Before the floral tissues and seed from Salinas and LaBrillante plants could be sampled, many plants died from *Botrytis* infection. The experiments are therefore being repeated currently.

Table 3. Number of seed lots evaluated from individual seed companies that contributed samples for evaluation, the number of lots that were infested with *Verticillium dahliae*, range and mode for the infested seed lots during 2007 and 2008

Year	Company	Seed lots	Number infested	Range (%)	Mode (%)
2007	1	5	0	-	-
	2	7	1	3	3
	3	20	14	1-3	<2
	4	15	8	1-2	1
	5	6	2	2	2
	6	1	0	-	-
	7	1	0	-	-
Total		**55**	**25**	**1-3**	
2008	1	28	22	1-9	<4
	2	25	0	-	-
	3	4	0	-	-
	4	30	0	-	-
	5	2	0	-	-
	6	2	0	-	-
	7	60	22	1-3	<2
	8	16	0	-	-
	9	4	0	-	-
Total		**171**	**44**	**1-9**	

Table 4. Number of seed lots of different lettuce types evaluated, the number of seed lots that were infested with *Verticillium dahliae* and the percentage range of infestation during 2008

Lettuce type	Number of seed lots evaluated	Number infested	Range (%)
Baby Leaf	15	3	0-3
Crisphead	59	10	0-3
Romaine	26	7	0-4
Red Romaine	3	1	3
Green Leaf	20	2	0-3
Red Leaf	11	2	1
Butterhead	4	0	-
Other/Unknown	33	19	0-9

Table 5. Lettuce seed lots produced in five countries, the number of seed lots infested by *Verticillium dahliae* and the range expressed in percentage during 2008

Country	Seed lots	Number infested	Range (%)
Australia	7	0	-
Chile	19	5	1-3
China	12	8	1-5
The Netherlands	4	0	-
U.S.A	129	31	1-9

Objective 4. Determine pathogenicity of spinach and French marigold isolates of *Verticillium dahliae* on lettuce and characterize them to the type of race on lettuce.

Methods: The determination of the pathogenicity of French marigold and spinach isolates on lettuce and other ornamental plants and the virulence of these isolates on lettuce differentials was carried out in a number of experiments at two locations. The first experiment involved the studies of French marigold and lettuce isolates and the second of spinach and lettuce isolates. Six isolates of *V. dahliae* from French marigold and one isolate each from lettuce representing races 1 and 2 were inoculated on lettuce differentials Salinas and La Brillante as also several French marigold cultivars, 3 *Alyssum* species, two *Lobelia* species, and one each of *Dahlia* and *Zinnia* spp. Seedlings of each cultivar or species were inoculated with a 10^6 conidial suspension of each isolate three times one week apart. All inoculated seedlings were transplanted into larger Styrofoam cups individually and arranged on greenhouse benches in a completely randomized design. For each isolate, 5 plants of each cultivar/plant species were maintained and the experiment was repeated two times. After 10 wk incubation on greenhouse benches, the plants were washed free of soil, the crown and root split longitudinally to examine the vascular discoloration. The number of plants infected plants was expressed as a percentage of the total inoculated. Similarly, 18 isolates from spinach, 7 from lettuce representing both races 1 and 2,

two from strawberries and one each from *Sonchus* spp. and pepper were inoculated onto spinach seedlings in flats two times and the seedlings were transplanted into larger Styrofoam cups one week later. For each isolate, 10 plants were inoculated, and the entire experiment was repeated once. The pots with inoculated plants were arranged in a completely randomized design and incubated on greenhouse benches with supplemental lighting during the night to trigger flowering and symptom development. After 12-15 wk incubation, the plants were examined for typical Verticillium wilt symptoms and random samples showing symptoms were plated on NP-10 medium to confirm that the symptoms were caused by *V. dahliae.*

The same isolates were inoculated onto lettuce differentials Salinas and La Brillante as described above. After 10-wk incubation on greenhouse benches, the plants were washed free of soil, the crown and root split longitudinally to examine the vascular discoloration. The number of plants infected plants were expressed as a percentage of the total inoculated.

Results. All *V. dahliae* isolates from French marigold and lettuce were pathogenic on each French marigold cultivar tested. However, none of these isolates were pathogenic on the 3 *Alyssum* species, two *Lobelia* species, and one each of *Dahlia* and *Zinnia* spp. evaluated (Table 6). The French marigold isolates were all pathogenic on lettuce cultivar Salinas, however, only 3 of the 6 isolates caused wilt on La Brillante. Thus, the French marigold isolates were of both race 1 and 2 types. A molecular marker that has been developed specifically for race 1 also confirmed these results.

Fifteen of 18 isolates from spinach, 5 of 7 from lettuce, both strawberry isolates and the lone isolate from *Sonchus* evaluated on spinach were pathogenic. The pepper isolate was non-pathogenic to spinach. In contrast, all of these isolates were pathogenic on lettuce but their virulence phenotypes differed. Six of 18 isolates from spinach, 4 of 6 from lettuce, one of two from strawberry, the isolates from *Sonchus* and pepper were virulent to both Salinas and La Brillante and thus belonged to race 2. All other isolates were of race 1 type. Once again, the race 1 isolates were confirmed by the molecular marker specific to race 1. Thus, all spinach isolates from seed that was previously planted in the Salinas Valley were not only pathogenic to lettuce but nearly 30% of these isolates belonged to race 2.

The genetic relationship between isolates from lettuce, tomato, spinach, artichoke, strawberries, French marigold among others were studied using a variety of molecular techniques. The details of this work are in the report on the spinach project.

Objective 5. 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. We are currently screening *Lactuca* collection for resistance to race 2 of *V. dahliae* from lettuce. Our strategy uses greenhouse testing to screen up to eight plants of 160 accessions per year in unreplicated plots to identify candidate sources of resistance. This is followed by replicated greenhouse and field-micro-plot experiments to confirm resistance. Verticillium wilt disease development in lettuce is dependent on plant development (Hayes et al. 2007), and in some genotypes symptoms are not expressed until the plant reaches flowering

(Hayes, Vallad, Subbarao, personal communication). We maintain the plants in the greenhouse until flowering begins, at which time disease evaluations are conducted. This substantially lengthens the duration of each experiment, but is necessary to reduce the number of false positives. At all stages of testing, crown sections of asymptomatic plants are plated on NP10 media to determine the presence / absence of *V. dahliae* stem infection. To date, we have screened 323 accessions in unreplicated greenhouse experiments using a race 2 isolate of *V. dahliae*; we have confirmed susceptibility in 247 of these accessions. The remaining 76 accessions are candidate sources of resistance. Sixteen have been characterized in two replicated greenhouse experiments; five of these had significantly lower disease incidence than Salinas and La Brillante. Detailed characterization of the remaining 60 accessions will be completed in during the coming year. Details of the lines identified as resistance will be in the report by Ryan Hayes.

Table 6. Ornamental crop species evaluated against *Verticillium dahliae* isolates from French marigold. None of the isolates were pathogenic on these crops species.

Genus	Cultivar
Zinnia state	Fair rose shade
Dahlia figaro	Red shades
Lobelia spp.	Crystal palace
Lobelia spp.	Regatta sapphire
Alyssum spp.	Snow crystals
Alyssum spp.	Deep rose
Alyssum spp.	EB violet