

# CALIFORNIA LETTUCE RESEARCH PROGRAM

April 1, 2010 - March 31, 2011

## BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE

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### SUMMARY

There were four objectives during the current funding cycle and included: a) monitoring *Verticillium* wilt and soil inoculum density in coastal California; b) continued assessment of seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation; c) determine the potential of host-directed evolution of *V. dahliae* genotypes of differential virulence from a single genotype; and d) 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 and to screen breeding material for race 1 resistance in infested plots at the USDA Station. Since the initial discovery of the disease on lettuce, 144 new fields had developed *Verticillium* wilt on lettuce through 2010. In 2010, the disease appeared in three fields as early as in May but was not seen again until late August and September. Fields with confirmed *Verticillium* wilt in 2010 exceeded 30. However, this number may not be an accurate as many growers that observed the disease on their ranches were unwilling to make it public or the disease had become so common that they stopped notifying us about the new fields. The new fields were located in Watsonville and the entire length of the Salinas Valley and losses ranged between 25 and 100%. We completed the assessment of commercial lettuce seed lots for *V. dahliae* infestation this year. During the course of this study, we screened a total of 265 seedlots, of which 48 were infested by *V. dahliae*. The infestation varied between 0.1 to 5%, a level considered too low to trigger *Verticillium* wilt epidemics in commercial fields by simulation modeling even after repeated lettuce crops. Lettuce seedlots assayed were drawn from seed produced in six countries and only seed produced in Australia and The Netherlands were free of *Verticillium* spp. Sixtyfour soil samples from US seed production fields were also assayed for *V. dahliae* microsclerotia and the highest inoculum density observed was 38 microsclerotia per gram soil. Again, this level of inoculum is too low to trigger *Verticillium* wilt development in the seed production fields. Field screening of breeding lines for race 1 resistance continued for the third season and significant progress is being made in incorporating resistance into horticulturally superior lettuce backgrounds. Since the breeding lines also included some with differential susceptibility to *Verticillium* wilt, it is conceivable that they exerted selection pressure on the resident race 1 population. We made the first collection of isolates over the spring season and are currently being curated. After two or three more seasons, we will begin our analysis to understand if the resident pathogen population has remained race 1 or if some isolates have developed into other pathogen genotypes. This may also predict the lifespan of a race 1-resistant cultivar that is deployed in commercial fields. To date, we have screened 601 accessions using race 2 *V. dahliae* isolate VdLs17 and confirmed susceptibility in 553 of these accessions. The remaining 48 accessions are being treated as candidate sources of resistance that require further testing. Among these are nine accessions (PIs 169511, 171674, 204707, 226641, 273582, 274366, 278074, 278080,

and 342450) that have demonstrated partial resistance in one to three independent replicated experiments.

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

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### **OBJECTIVES:**

1. Monitoring Verticillium wilt and soil inoculum density in coastal California.
2. Continued assessment of seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation.
3. Determine the potential of host-directed evolution of *V. dahliae* genotypes of differential virulence from a single genotype.
4. 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 and to screen breeding material for race 1 resistance in infested plots at the USDA Station.

### **PROCEDURES AND RESULTS:**

#### **Objective 1. Monitor Verticillium wilt and soil inoculum density in coastal California.**

**Methods.** We have been monitoring Verticillium wilt on lettuce in coastal California ever since the disease was first discovered in 1995 in Watsonville. During most of the 16 years since that initial discovery, we conducted monthly surveys of fields in coastal California to monitor the spread of the disease from this initial focus. However, with the sudden increase in the number of

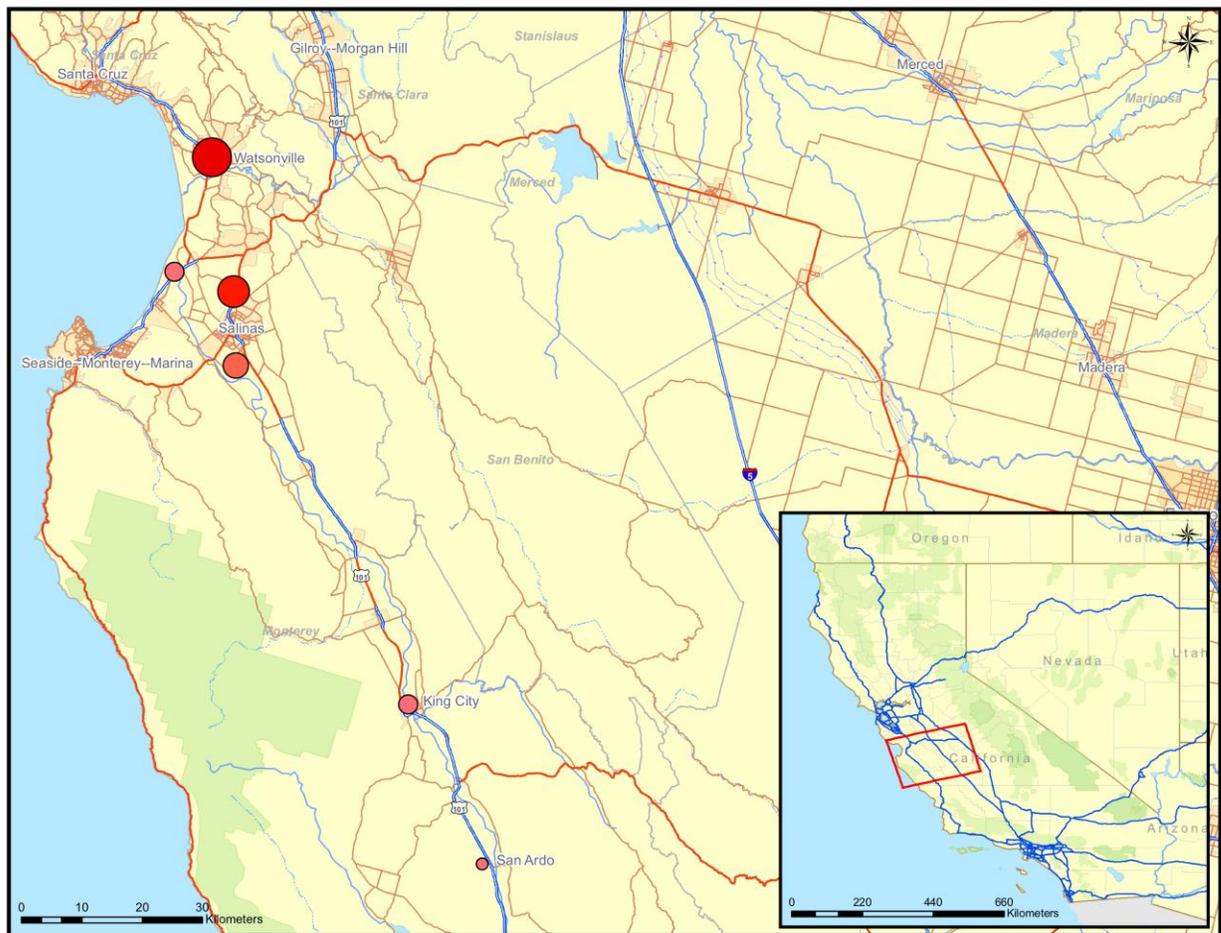
fields that developed *Verticillium* wilt in 2008, we could no longer keep track of all new fields that developed the disease between our monthly surveys and therefore suspended the monthly surveys. Instead, we relied on the well-trained cadre of PCAs to document both the new fields that developed *Verticillium* wilt on lettuce and also the fields in which the disease re-appeared following fumigation and strawberry cultivation. This changed strategy helped discover a more than significant jump in the number of new fields with *Verticillium* wilt in 2009. The PCAs were requested to document the type of lettuce, the acreage affected, and the incidence of the disease in each field when they were reasonably certain that it was *Verticillium* wilt. In 2010, however, even this became difficult with several growers expressing their inability to make the disease information on their ranches public and others thinking that it was less important to report new fields that developed *Verticillium* wilt since the disease was well established on their ranches. As the relationship between the soil inoculum levels and incidence of *Verticillium* wilt in commercial fields is well established, we discontinued collecting soil samples to assay for *V. dahliae* microsclerotia.

**Results.** Until the mid-1990s, lettuce was considered resistant to *Verticillium* wilt, caused by *Verticillium dahliae*. However, in 1994, several fields on a farm in Watsonville (southern Santa Cruz County, Pajaro Valley) reported a loss of the entire lettuce crop to an unknown disease. *Verticillium* wilt was dismissed as the potential causal agent of the disease, although it was the only pathogen isolated from infected plants and *V. dahliae* microsclerotia were recovered from soil samples. The loss was blamed on herbicide damage, because lettuce was not a known host of *V. dahliae*. In 1995, *V. dahliae* was isolated from infected plants from the same fields and *V. dahliae* was proven to be the causal agent of wilt on lettuce, the first documented record of *Verticillium* wilt on lettuce in the literature.

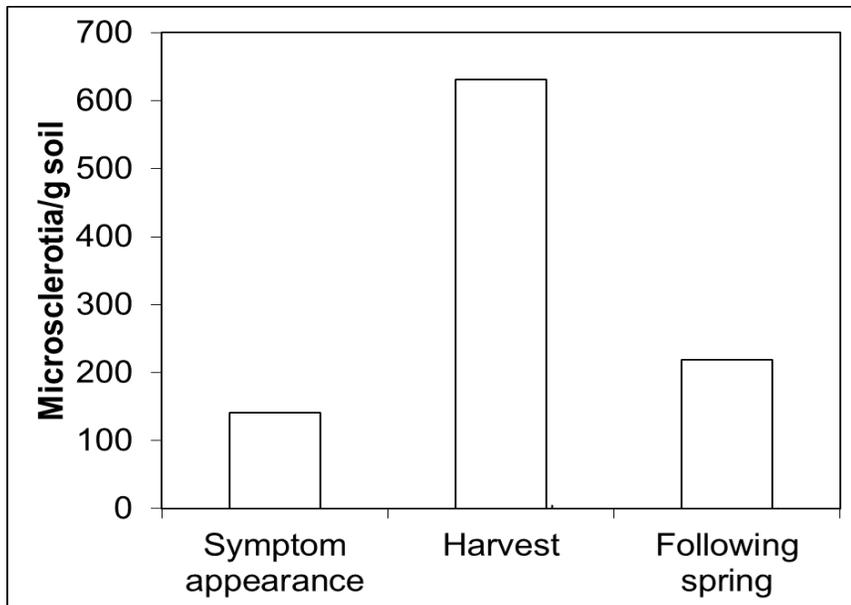
Since 1995, an increasing number of lettuce fields have shown varying levels of *Verticillium* wilt incidence. The total number of affected fields at the end of 2010 was over 150, amounting to ~1100 ha, with 73 new fields affected in 2009 and 2010, whereas only 77 fields had developed *Verticillium* wilt between 1995 and 2008 (Fig. 1). Following the initial appearance of *Verticillium* wilt in Watsonville, it wasn't until 1999 that it was first observed on lettuce in the neighboring Salinas Valley, where majority of lettuce production in the US occurs. *Verticillium* wilt was first observed just north of Salinas in 1999 and in 2003, the disease appeared on the south end of Salinas. By 2006, the disease was recorded at the southern end of the Salinas Valley, more than 100 Km south from Salinas in San Ardo, CA. While disease reports were confined to a small number of fields, by 2009 and 2010 most disease foci coalesced, and fields in a ~50 Km stretch of the prime lettuce production area had developed *Verticillium* wilt. The disease has not been identified in lettuce produced outside of the Salinas and Pajaro river valleys of coastal California, including the Santa Maria and Santa Clara valleys. Furthermore, significant areas of winter lettuce production located in parts of southern and central California and southwestern Arizona, have also not developed *Verticillium* wilt. The disease, however, has been reported from the Greek island of Crete and northern Italy.

In heavily infected lettuce plants, tissues are invariably lined with microsclerotia. We previously recovered 2500-100000 cfu/g of dry taproot tissue from severely affected plants. Hence several million microsclerotia are incorporated into the soil by tilling each infected lettuce plant. Depending on the incidence of the disease in a field and the severity of colonization of individual

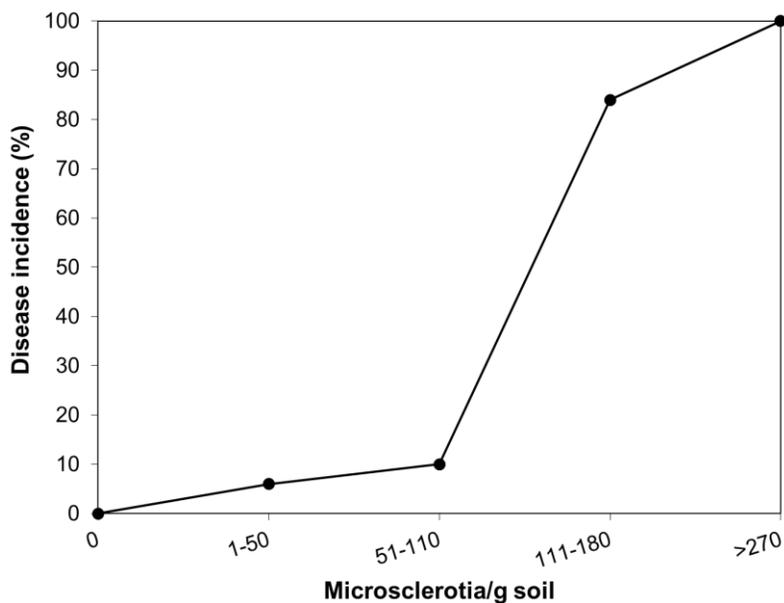
lettuce heads, the amount of microsclerotia returned to the soil may differ greatly. While disease incidence may be assessed visually by counting diseased plants, microsclerotial densities are assessed by quantifying pathogen amounts in representative soil samples. In the soil from an infected lettuce field (Fig. 2) the number of microsclerotia per gram of soil increased by about six-fold between the appearance of symptoms and the time of harvest and tillage. In the following spring, after a cover crop and repeated tillage, close to a two-fold higher number of microsclerotia was recorded prior to the following lettuce crop. Verticillium wilt development on lettuce was invariably associated with soil inoculum densities of >100-150 microsclerotia/g soil (Fig. 3). Such high inoculum densities are rare in other cropping systems affected by Verticillium wilt and most crops suffer near-total losses at densities of <50 microsclerotia/g soil.



**Figure 1.** Distribution of Verticillium wilt on lettuce in coastal California’s Salinas and Pajaro river valleys. The disease was reported in 114 lettuce fields between 1995 and 2009. The size and the intensity of the red color of circles are proportional to the number of affected fields. Many of the affected fields showed wilt incidence that exceeded 80% and the crops were abandoned.



**Figure 2.** Fluctuation in the number of microsclerotia of *Verticillium dahliae* per gram of soil associated with the infection of lettuce plants in coastal California fields. The number of microsclerotia generally increased after the incorporation of infected plant material following harvest. The level of inoculum remained high for the following crop unless the plots were fumigated.



**Figure 3.** Relationship between inoculum density of *Verticillium dahliae* (expressed as microsclerotia per gram of soil) and disease incidence compiled from surveys in 54 lettuce fields in coastal California.

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

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 such as 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 flow of novel pathogen genotypes is an extremely serious phenomenon that may disrupt the ability of breeders to select for a durable resistance. Hence, incoming lettuce seed has been screened over three years to document the extent of seed infestation with *V. dahliae* to identify potential sources of migration.

**Methods.** We continued the assessment of commercial lettuce seed lots for *V. dahliae* infestation. Again this year, we specifically focused on commercial lettuce cultivars currently marketed for salad mixes because these cultivars are also planted in high seeding rates (approximately 150,000 per acre). Should the cultivars carry *V. dahliae* in seed, they have the potential to introduce the pathogen to new areas or enhance the soil inoculum density in areas the pathogen is already present. Similar to the last three years, we requested seed companies for information on lettuce types and the locations where the seed was produced. Of the 12 companies approached this year, 3 submitted 23 seed lots for evaluation but the soil samples were available from only two companies and they submitted 10 samples. From each seed lot, 200 seeds were plated onto *V. dahliae* semi-selective NP-10 medium and incubated at room temperature ( $22 \pm 1^\circ\text{C}$ ) 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. Isolates were grown on potato dextrose broth in an orbital shaker at  $25^\circ\text{C}$  at 200 rpm for 4 to 5 days. Mycelia were harvested by filtration, freeze-dried, and ground with liquid nitrogen, and stored at  $4^\circ\text{C}$ . Total DNA of the individual isolates was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). To determine if species other than *V. dahliae* are also recovered from the seed, the complete ITS region of rDNA, encompassing ITS1, ITS2 and 5.8S rDNA was amplified by PCR using universal ITS-1 and ITS-4 primers. All PCR amplifications were performed in a Bio-Rad DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, CA). PCR was carried out in 30  $\mu\text{l}$  reactions containing 10 ng

genomic DNA, 2X GoTaq master mix (Promega, Madison, WI), and 5 pmol each of ITS-1 and ITS-4 primers.

Soil samples were processed using the Anderson Sampler technique described in previous reports and the number of microsclerotia from each sample was expressed as number per gram dry soil.

**Results.** The 10 soil samples assayed contained between 0 and 24 microsclerotia/g dry soil, well below the inoculum density at which disease develops on lettuce. Of the 23 seed lots evaluated on the *V. dahliae* semi-selective medium, four were positive for *V. dahliae* with 7 seed out of the 200 plated translating to a maximum seed infestation rate of 3.5% (Table 1). The submitted seed was produced in the Australia, Chile, China, The Netherlands, and the US, and the four infested seed lots this year were produced in China and the US. Fumigation of the infested fields in the US again significantly reduced the seed infestation rates. 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 or infested seed that was planted.

Most seed lots positive for *V. dahliae* had <4% infestation (Table 2). 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 3). Similarly, seed produced in all countries except The Netherlands were infested but the majority of those produced in the United States were infested (Table 4). One company that had many infested seed lots fumigated their seed production fields with methyl bromide and chloropicrin. In 2009, they were able to reduce seed infestation by nearly 95%. However, the two seed lots with less than 0.5% seed infestation could possibly have come from the airborne spread of the pathogen. This aspect is currently being evaluated in the greenhouse.

**Table 1.** Summary of seed lots and soil samples from seed production fields evaluated during 2007 -2009

Year	Seed companies approached	Number responded	Seed lots with <i>V. dahliae</i> /total assayed (Range %)	Soil samples with <i>V. dahliae</i> /total assayed (Range of microsclerotia)
2007	16	8	26/55 (0-6%)	33/37 (0-38 ms/g)
2008	19	9	44/171 (0-5%)	5/6 (0-20 ms/g)
2009	12	6	2/71 (0-0.5%)	2/8 (0-10 ms/g)
2010	12	3	4/23 (0-3.5%)	4/10 (0-24 ms/g)

Over the four years of this study, lose to 18% of the 250 seed lots tested positive for *V. dahliae*, but the maximum incidence of the seed infection by the fungus among seed lots was less than 5% (Tables 3 and 4). Higher levels of seed infestation (both the number of seed lots infested and the number of infested seed within a seed lot) have been documented in crops grown in rotation with lettuce, such as spinach. For instance, a large percentage of tested spinach seed lots were infested

with *V. dahliae*, and the incidence of the fungus was >75% in many seed lots. Preliminary simulation modeling results of *Verticillium* wilt epidemics in lettuce fields suggest that a minimum of 5% infested lettuce seed planted over 3-5 seasons is required for the disease to appear, and that at least five additional years are required for disease incidence in lettuce crops to reach an asymptote in coastal California

**Table 2.** 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 between 2007 through 2010

Year	Company	Seed lots	Number infested	Range (%)	Mode (%)
<b>2007</b>	1	5	0	-	-
	2	7	1	1.5	2
	3	20	14	0.5-1.5	<2
	4	15	8	0.5-1.0	1
	5	6	2	1	1
	6	1	0	-	-
	7	1	0	-	-
<b>Total</b>		<b>55</b>	<b>25</b>	<b>1-3</b>	
<b>2008</b>	1	28	22	1-5	<4
	2	25	0	-	-
	3	4	0	-	-
	4	30	0	-	-
	5	2	0	-	-
	6	2	0	-	-
	7	60	22	1-2	<2
	8	16	0	-	-
	9	4	0	-	-
<b>Total</b>		<b>171</b>	<b>44</b>	<b>1-5</b>	
<b>2009</b>	1	9	2	0-0.5	0
	2	27	0	-	-
	3	6	0	-	-
	4	5	0	-	-
	5	14	0	-	-
	6	10	0	-	-
<b>Total</b>		<b>71</b>	<b>2</b>	<b>0-0.5</b>	
<b>2010</b>	1	7	0	-	-
	2	10	4	0-3.5	0
	3	6	0	-	-
<b>Total</b>		<b>23</b>	<b>4</b>	<b>0-3.5</b>	

**Table 3.** 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 - 2010

Lettuce type	Number of seed lots evaluated	Number infested	Range (%)
Baby Leaf	34	0	-
Crisphead	84	13	0.1-4
Romaine	38	8	0.5-2
Red Romaine	7	1	3
Green Leaf	31	2	0.5-2
Red Leaf	22	2	0.5
Butterhead	5	0	-
Other/Unknown	44	22	0.5-5
<b>Total</b>	<b>265</b>	<b>48</b>	

**Table 4.** Lettuce seed lots produced in five countries, the number of seed lots infested by *Verticillium dahliae* and the range expressed in percentage during 2008 - 2010

Country	Seed lots	Number infested	Range (%)
Australia	7	0	-
Chile	33	5	0.5-2.0
China	22	8	0.5-2.5
The Netherlands	5	0	-
UK	3	2	0.0-4
U.S.A	195	33	0.5-5
<b>Total</b>	<b>265</b>	<b>48</b>	

Similar to the results from last year nearly 95% of the isolates recovered from lettuce seed based on the analysis of the ITS rDNA were *V. dahliae*. A few isolates formed clades that were distinct outgroups. These included *Gibbellulopsis nigrescens* and *V. tricorpus*.

**Objective 3.** 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 for the past two years. In screening trials performed over the past two 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 plan on collecting samples from each lettuce season and genotype them using the microsatellite markers that we have developed. This will allow us to study the evolution of this individual genotype in the natural environment, but most importantly it will provide us with insight into the impact of lettuce on this evolution. Does the host apply selective pressure on *V. dahliae* leading to its genotypic diversification? In the 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 it is induced by mutation. 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, were collected from symptomatic lettuce plants. The stem pieces were plated on NP-10 medium to isolate *V. dahliae*. A total of 76 and 40 isolates were collected, purified, and stored from spring and summer seasons, respectively. We have thus far analyzed 46 isolates from the spring season using the recently developed race 1-specific primers, which confirmed 43 isolates as race 1. The remaining three isolates from this season and all isolates from the summer seasons will be analyzed during 2011. Additional isolates will be collected from the screening nursery again this year for analyses.

**Objective 4. 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 California Leafy Greens Board 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. Race 1 resistant iceberg

breeding lines were subsequently developed. Seed of race 1 resistance 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.

We are currently screening the Western Regional Plant Introduction Station (WRPIS) *Lactuca* collection for resistance to race 2 isolates of *V. dahliae*. More specifically, our research is using a working collection of the WRPIS located in Salinas, CA. 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, and in some genotypes symptoms are not expressed until the plant reaches flowering. 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 601 accessions using race 2 *V. dahliae* isolate VdLs17. In unreplicated and replicated experiments, we confirmed susceptibility in 553 of these accessions. The remaining 48 accessions are being treated as candidate sources of resistance that require further testing. Among these are nine accessions (PIs 169511, 171674, 204707, 226641, 273582, 274366, 278074, 278080, and 342450) that have demonstrated partial resistance (disease incidence significantly lower than ‘Salinas’) in one to three independent replicated experiments. However, all of these PIs have had at least a few symptomatic plants, and all but PI171674 have had non-symptomatic plants that are nonetheless colonized by *V. dahliae*. A manuscript is in preparation to report the existence of partial resistance in lettuce to race 2 isolates of *V. dahliae*. Complete resistance to race 2 has not yet been found, and we will continue to screen the collection in hopes of finding this trait.