

CALIFORNIA LETTUCE RESEARCH PROGRAM

April 1, 2009 - March 31, 2010

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE

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SUMMARY

There were six 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 if airborne phase of the pathogen could be a threat in seed production fields; d) simulation modeling of the impact of seedborne inoculum; e) continued characterization of the spinach and lettuce pathogen populations; and f) continued breeding program to identify and develop resistance in crisphead, leaf, and other lettuce types including screening of germplasm for resistance against race 2. Since the mid-1990s, repeated outbreaks of *Verticillium* wilt have caused complete losses in many lettuce fields. Since the initial discovery of the disease on lettuce, 2-3 new fields on average had developed *Verticillium* wilt on lettuce. In 2008, the number of fields in which the disease occurred jumped to 13 and was equally distributed among the Salinas and Pajaro Valleys. The problem fields discovered until 2008 clustered around three hot spots with two of these located in the Salinas Valley and one in the Pajaro Valley. In 2009, however, the disease was discovered in 43 fields accounting for greater than 50% increase over all the confirmed fields between 1995 and 2008. The three clusters around which the previous fields were located are progressively getting merged. We continued the assessment of commercial lettuce seed lots for *V. dahliae* infestation. This year, we specifically focused on commercial lettuce cultivars currently marketed for salad mixes. Of the 12 companies approached this year, 6 submitted 71 seed lots for evaluation but the soil samples were available from only two companies. The 8 soil samples assayed contained between 0 and 10 microsclerotia/g dry soil, well below the inoculum threshold at which disease develops on lettuce. Of the 71 seed lots evaluated on the *V. dahliae* semi-selective medium, two were positive for *V. dahliae* with one seed out of the 200 plated yielding the fungus for a seed infestation of 0.5%. The submitted seed was produced in the US, Chile, China, and The Netherlands and the two infested seed lots this year were produced in the US. 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. Experiments to evaluate this were indeed successful. In addition, an even more complicated picture of pathogen transmission was discovered this year. The pollen from one spinach germplasm line yielded *V. dahliae*. These results are preliminary and studies would be continued to confirm the role played by pollen in pathogen transmission into seed. Another major progress so far this year has been the development of a simulation model to determine the minimum number of infested seeds required to infest a field for *Verticillium* wilt to develop on lettuce. To date, we have screened 478 accessions using race 2 *V. dahliae* isolate VdLs17; we have confirmed susceptibility in 397 of these accessions. The remaining accessions are candidate sources of resistance that require further testing.

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

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OBJECTIVES:

1. Monitor 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. Simulation modeling of the impact of seedborne inoculum.
5. Continue the characterization of the spinach and lettuce pathogen populations.
6. 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. Monitor Verticillium wilt and soil inoculum density in coastal California.

Methods. Since there was a reasonably big spurt in the number of fields that developed Verticillium wilt in 2008, we suspended the annual monthly surveys that were undertaken until then to identify new fields with Verticillium wilt on lettuce and reappearance of the disease in previously fumigated fields in coastal California. 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. 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. Since the mid-1990s, repeated outbreaks of Verticillium wilt have caused complete losses in many lettuce fields. Since the initial discovery of the disease on lettuce, 2-3 new fields on average had developed Verticillium wilt on lettuce. In 2008, the number of fields in which the disease occurred jumped to 13 and was equally distributed among the Salinas and Pajaro Valleys. The problem fields discovered until 2008 clustered around three hot spots with two of these located in the Salinas Valley and one in the Pajaro Valley. In 2009, however, the disease was discovered in 43 fields accounting for greater than 50% increase over all the confirmed fields between 1995 and 2008. The three clusters around which the previous fields were located are progressively getting merged. There were several unique features among the new fields discovered this year. There were fields that had no history of spinach cultivation but also fields that have previously produced spinach. There were also several fields that had low incidence (<5%) of Verticillium wilt but the infected plants were randomly distributed within the field suggesting inoculum source other than field equipment and personnel. Yet more fields had disease concentrated in a corner of the field that we have previously proven to have been initiated by contaminated equipment or personnel. Furthermore, there was at least one field which had only been fumigated with methyl bromide + chloropicrin and planted to strawberries last year. The disease albeit at low levels appeared on the first lettuce crop planted. On further enquiry, it was apparent that the field had been planted to a grass cover crop between strawberries and lettuce. There is a body of literature that has clearly established that although *V. dahliae* is a pathogen of dicots, it is able to colonize and reproduce on several grass species, onion and tulips that are monocots. These monocots can serve as symptomless carriers while also dramatically increasing the soil inoculum levels. The new fields were located in Watsonville and the entire length of the Salinas Valley. Of the 43 fields, at least five fields were abandoned because the loss was near-total. Incidence of wilt in other fields varied between 5-60%.

Objective 2: Continue assessing 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.

Methods. We continued the assessment of commercial lettuce seed lots for *V. dahliae* infestation. This year, we specifically focused on commercial lettuce cultivars currently marketed for salad mixes because these cultivars are also planted in high seeding rates. 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 last year, we requested seed companies for information on lettuce types and the locations where the seed was produced. Of the 12 companies approached this year, 6 submitted 71 seed lots for evaluation but the soil samples were available from only two companies. 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°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°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 μl 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 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 8 soil samples assayed contained between 0 and 10 microsclerotia/g dry soil, well below the inoculum density at which disease develops on lettuce. Of the 71 seed lots evaluated on the *V. dahliae* semi-selective medium, two were positive for *V. dahliae* with one seed out of the 200 plated translating to a seed infestation rate of 0.5% (Table 1). The submitted seed was produced in the US, Chile, China, and The Netherlands and the two infested seed lots this year were produced in the US. Fumigation of the infested fields that had yielded infested lettuce seed lots last year, significantly reduced the seed infestation rates this year in the US. 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. 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.

A phylogenetic tree showing ITS rDNA phylogeny was constructed based on sequence alignments of the 560 bp ITS rDNA from 110 isolates of *V. dahliae*. The phylogenetic analysis revealed that nearly 95% of the isolates recovered from lettuce and spinach seed were *V. dahliae*. A few isolates formed clades that were distinct outgroups. These included *Gibbellulopsis nigrescens* and *V. tricorpus*. Thus, the majority of isolates recovered from infested lettuce and spinach seed were *V. dahliae*.

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.

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)

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 during 2007 and 2008

Year	Company	Seed lots	Number infested	Range (%)	Mode (%)
2007	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	-	-
Total		55	25	1-3	
2008	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	-	-
Total		171	44	1-5	
2009	1	9	2	0-0.5	0
	2	27	0	-	-
	3	6	0	-	-
	4	5	0	-	-
	5	14	0	-	-
	6	10	0	-	-
Total		71	2	0-0.5	

Methods and Results. Susceptible seedlings of cultivar Salinas were produced in 100-well seedling trays filled with pasteurized sand/potting soil mix (3:1 vol/vol). Three weeks after emergence, the seedlings were inoculated with 5-ml of VdLs17 conidial suspension adjusted to 1.0×10^7 conidia/ml. One week after inoculation, the seedlings were transplanted to 16-oz styroform cups filled with sand/potting soil mix. The inoculated plant was placed in the center of a green house bench. Pots containing un-inoculated plants were arranged alternatively in concentric circles 20-cm apart to a maximum distance of 1-m from the center (Fig 1). Inoculum was also applied in as aerosolized spray twice during the experiment from six feet distance in the prevalent wind direction to increase the probability of conidia landing on developing florets. After 14 post inoculation weeks, seeds were collected from inoculated and un-inoculated plants

and plated on the semi-selective NP10 medium to determine the presence/absence of *V. dahlia* in the seeds.

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 and 2009

Lettuce type	Number of seed lots evaluated	Number infested	Range (%)
Baby Leaf	28	3	0-1.5
Crisphead	72	10	0-2
Romaine	35	7	0-2
Red Romaine	7	1	1.5
Green Leaf	30	2	0-2
Red Leaf	21	2	0.5
Butterhead	5	0	-
Other/Unknown	44	22	0.5-5

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 and 2009

Country	Seed lots	Number infested	Range (%)
Australia	7	0	-
Chile	29	5	0.5-2.0
China	22	8	0.5-2.5
The Netherlands	5	0	-
U.S.A	179	33	0.5-5

Majority of the seeds collected from four un-inoculated healthy plants that were located in the prevalent wide direction when the inoculum was applied were positive to *V. dahliae* out of 40 plants that were arranged in concentric circles. Seeds from the lone inoculated plant that was placed in the center (Fig. 1) were also positive for *V. dahliae*.

In addition, an even more complicated picture of pathogen transmission was discovered this year. The pollen from one spinach germplasm line yielded *V. dahliae*. These results are preliminary and studies would be continued to confirm the role played by pollen in pathogen transmission into seed. 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*.

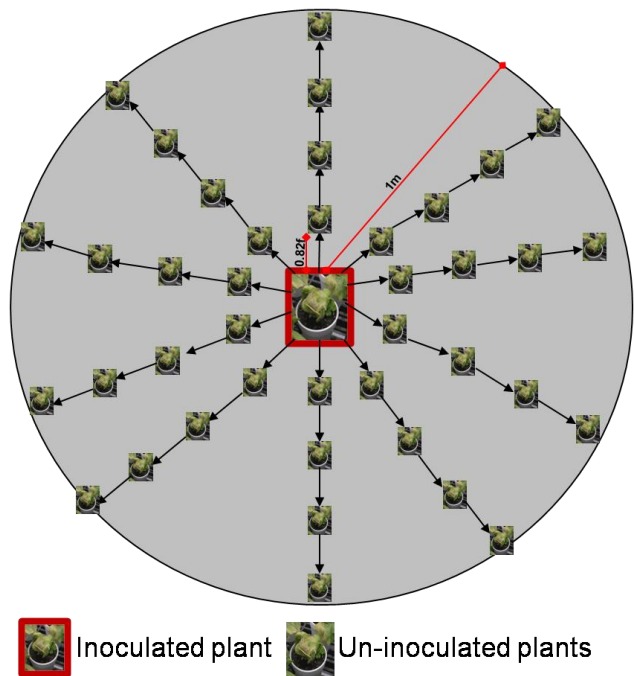


Figure 1. Arrangement of lettuce plants to determine the potential role of airborne inoculum in producing seed carrying *Verticillium dahliae*.

In a preliminary experiment, pollen were collected randomly from symptomatic spinach plants and plated on NP10 medium to detect the presence of *V. dahliae*. Uninoculated control plant pollen was also sampled to verify the absence of *V. dahliae*. Based on preliminary screening, pollen from the germplasm line NSL 184378 was positive for *V. dahliae* out of 16 germplasm lines tested (Table 6). Pollen from uninoculated control plants did not yield *V. dahliae*. Currently we are testing pollens from 30 additional germplasm lines for this year to determine the presence of *V. dahliae*. The implications of this discovery on the spread of *Verticillium* wilt in lettuce production may be minimal since the crop is self-pollinated. However, for a cross-pollinated crop such as spinach, infected pollen may provide a new mode of pathogen transmission in spinach seed production.

Objective 4. Simulation modeling of the impact of seedborne inoculum.

We have also developed a simulation model to determine the minimum level of *V. dahliae* infested seeds required for the establishment of *Verticillium* wilt in a lettuce field, and the duration over which *Verticillium* wilt epidemics develop and result in significant economic loss given different seed infestation levels. The model consists of four major components, survival of microsclerotia in the field, infection of lettuce via germination of viable sclerotia, reproduction of microsclerotia on diseased plants, and dispersal of microsclerotia following tillage in both the horizontal and vertical planes. During this study, we used empirical data for calibration of model parameters for each component. We ran the simulation model with parameters that varied over a wide range to determine the effects of parameters on the establishment of *V. dahliae* in soil and the epidemics of *Verticillium* wilt in lettuce crops. We also looked at how the multiple-year disease epidemics change as the input variables (including the initial infestation level of lettuce

seeds, crops per year) change. Based on this model, the important factors determining the establishment of *V. dahliae* in a new field, and progress of Verticillium wilt in lettuce crops include: i) level of resistance in the lettuce cultivar, reflecting on how many microsclerotia will be reproduced on each infected lettuce plant, and the quantum of infected plants resulting from a certain number of viable microsclerotia in the top soil profile; ii) The rotation arrangement, the longer the gap between two consecutive lettuce crops, the slower is the accumulation of inoculum in the soil, and the smaller is the risk of pathogen establishment in the soil; and iii) the tillage, greater uniformity of inoculum dispersal across the field is achieved through increased tillage, resulting in both the disease spread in space and dilution of inoculum density at the disease foci. This dilution of inoculum can result in lowering of the soil inoculum density below the threshold at which the disease no longer develops on the subsequent crops. Lettuce indeed has the highest inoculum threshold requirement of all the hosts for which data are available for *V. dahliae* to cause wilt. A minimum of 100 microsclerotia/g soil is required for wilt to develop on lettuce. This model still requires additional empirical data, which are unavailable for calibration and the simulations were run at a small scale. However, it seems likely that for successful epidemics of Verticillium wilt to develop on a susceptible lettuce cultivar, a minimum of 3.5% of the planted seed has to be infested by the pathogen, and has to be planted over six to seven consecutive lettuce crops (2-3 chronological years). The impact of infested spinach seed planted in fields where lettuce crops follow is currently not known. It is also desirable to determine how environmental conditions and resistance affect infection efficiency (ID-DI), and reproduction of microsclerotia in infected plants. Obtaining empirical data on all of these factors and varying these factors at the same time would tie the land up for many years and perhaps also is an experimental impracticality. Simulation modeling would bypass these obstacles and at the same time provide answers in the quickest possible time based on which decisions can be made on a variety of factors. Even though the Leafy Greens Board is not funding the continuance of this work, we will further develop this study using funding from alternate sources and make results widely available to the industry as well as other end users.

Objective 5. Continue the characterization of the spinach and lettuce pathogen populations.

The results from this objective are described in the report on the spinach Verticillium project.

Objective 6. 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 to the Salinas Valley. 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. 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 and 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*, and subsequent identification of resistance to race 1 isolates in diverse lettuce types. None of this germplasm is suitable for direct commercial production, and all of this germplasm was susceptible to race 2. Regardless, we have used race 1 resistant lines as parents to develop resistant iceberg, romaine and leaf types. Among these are three resistant iceberg breeding lines that were released to the lettuce industry. Seed of race 1 resistance materials were deposited into the WRPIS (Western Regional Plant Introduction Station) for inclusion in the collection.

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 spinach seeds, which is widely grown in rotation with lettuce. Therefore, identification and subsequent development of lettuce cultivars with resistance to race 2 is imperative for sustaining the lettuce industry.

Table 6. Pollen from spinach germplasm lines evaluated for potential *Verticillium dahliae* recovery

Spinach germplasm lines	<i>V. dahliae</i> (+/-)
PI 254565	-
PI 165504	-
PI 166044	-
PI 165560	-
PI 169026	-
PI 165994	-
PI 103063	-
AMES 20169	-
AMES 26246	-
AMES 26244	-
AMES 25467	-
AMES 25862	-
AMES 25788	-
NSL 184378	+
NSL 92009	-
NSL 4649	-

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 (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 478 accessions using race 2 *V. dahliae* isolate VdLs17; we have confirmed susceptibility in 397 of these accessions. The remaining accessions are candidate sources of resistance that require further testing. Among these are 62 PIs selected from an unreplicated experiment and 12 PIs selected from a single replicated experiment. In addition, seven advanced PIs have been evaluated three replicated greenhouse experiment. Based on all three experiments, all seven had significantly lower disease incidence than Salinas and La Brillante (**Figure 2**). 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*. All selected PIs will be re-tested in greenhouse, growth chamber, or micro-plot experiments.

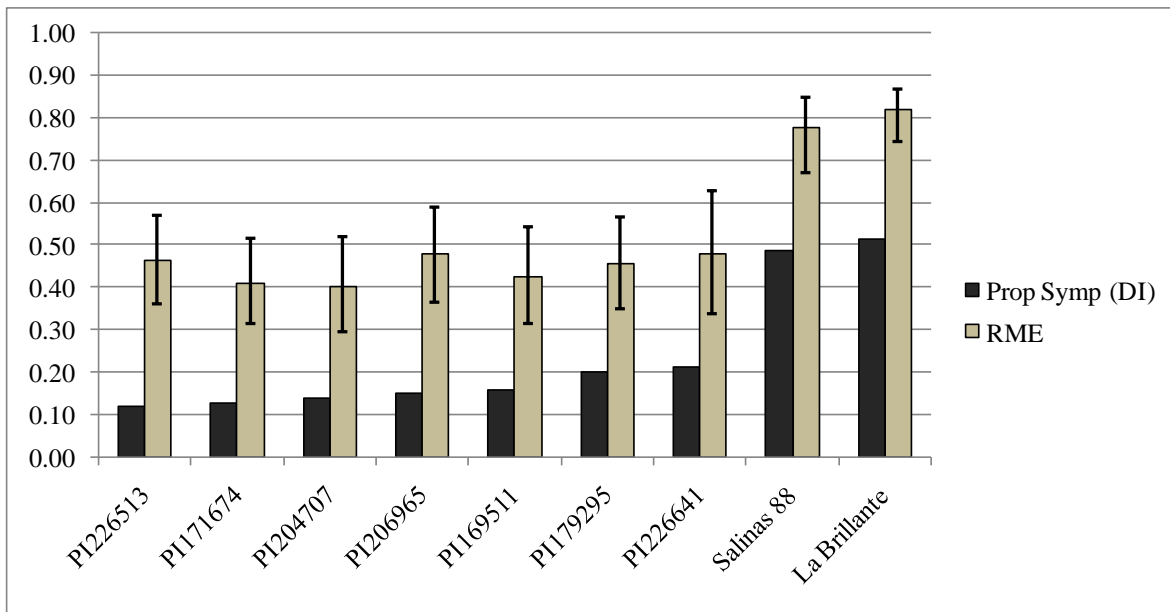


Figure 2. *Verticillium* wilt disease incidence from three independent replicated greenhouse experiments inoculated with a race 2 isolate of *Verticillium dahliae* (VdLs17). Prop Symp (DI) = disease incidence expressed as the proportion of symptomatic plants. RME = Relative marginal effect from nonparametric analysis of disease incidence data, error bars indicate 95% confidence intervals.