California Leafy Greens Research Program
Final Report for 2012-2013

Project Title: Development of management strategies for Bacterial Leaf Spot

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Abstract
Xanthomonas campestris pv. *vitians* strains causing bacterial leaf spot of lettuce differ genetically and pathogenically on lettuce. This pathogen is a member of the species *X. hortorum* and the close genetic relationship among pathovars within *X. hortorum* make finding sequences for specific detection and quantification protocols difficult, but distinctions in pathogenic traits make it necessary to distinguish *X. campestris* pv. *vitians* from these strains. A major source of resistance to this pathogen relies on the hypersensitive response to reduce disease. However, strains have been identified that overcome this mechanism. Genetic analysis of 100 strains of *X. campestris* pv. *vitians* has allowed for the identification of one genotype responsible for overcoming resistance in lettuce cultivar Little Gem.

Objectives

Our long-range objective is to understand the genetics of resistance to *X. campestris* pv. *vitians* and the mechanisms by which resistance is conferred. It is important to this goal to understand how plant genotype influences *X. campestris* pv. *vitians* populations and how diversity within *X. campestris* pv. *vitians* influences the disease outcome. This will result in the wider deployment and longer durability of resistant germplasm. This research should lead improved resistance and/or allow for additional management strategies working in synergy with resistance mechanisms.

Immediate Objectives

Objective 1. Provide phenotypic data for breeding lines essential to the development of BLS resistant germplasm and the understanding of the genetics of BLS resistance.

Objective 2. Develop a reliable and sensitive scheme for detecting and quantifying bacteria from potential inoculum sources.

Objective 3. Evaluate seed for the presence of the pathogen.

Objective 4. Determine if pathogens from other members of the Asteraceae are closely related to *X. campestris* pv. *vitians* and therefore need to be considered in management strategies.

Procedures:

Objective 1. Provide phenotypic data for breeding lines essential to the development of BLS resistant germplasm and the understanding of the genetics of BLS resistance.

A mixture of *Xanthomonas campestris* pv. *vitians* strains Xav 98-12, BS339 and BS347 from the Salinas Valley (Barak and Gilbertson 2003; Bull et al., 2005) were used in most experiments comparing parents to progeny for resistance or susceptibility to bacterial leaf spot in lettuce. In some experiments plants were inoculated with strain BS347 which induces the HR reaction in cultivar Little Gem.

For all experiments inoculum was prepared by propagating bacteria as a lawn on NA for 48 hours at 27°C. Bacteria were recovered by flooding plates with 0.01 M sterile phosphate-buffer (pH 7.2) and the resulting suspension was adjusted to 0.6 OD at 600_{nm} (approximately 5 x 10^8 CFU/ml) using Shimadzu spectrophotometer model number UV-1601. The inoculum was sprayed onto the leaves of three-week-old plants until run-off, with each plant receiving approximately 1 ml, using a hand held spray bottle. Plants were incubated in the greenhouse on a misting table for a total of 21 days. Plant were evaluated for disease severity 7, 14 and 21 days after the initial inoculation. In some experiments plants were inoculated again at 7 and 14 days. A rating of 0 was given for plants with no disease; 1, for plants with few lesions of < 3mm; 2, for plants with individual lesions > 3mm or many lesions < 3mm in size; 3, for plants with a few coalesced lesions; 4, for plants with many larger coalesced lesions; 5, for plants with 30% or more of individual leaves diseased; 6, for plants with dead leaves. Experiments were designed and analyzed as randomized complete blocks with four to six replications per treatment. Area under the disease progress curve (AUDPC) of disease severity ratings were analyzed nonparametrically.

The hypersensitive response is a rapid plant cell collapse observed in response to the injection of virulent plant pathogenic bacteria into non-host plants. The hypersensitive response is a plant resistance mechanism that involves rapid (1-3 days) death of cells surrounding the bacterial infection that limits the spread of the pathogen. *X. campestris* pv. *campestris* elicits a hypersensitive response in resistant cultivar Little Gem. Susceptible cultivars Vista Verde and Salinas 88 do not express the HR, population levels of the pathogen increase to a higher level in these cultivars than in the resistant cultivars and disease develops within 7-14 days. In order to evaluate the ability of different *X. campestris* pv. *vitians* strains to induce the hypersensitive response, lettuce was injected with inoculum prepared as described above from individual pathovars of *X. hortorum* and *X. campestris* pv. *vitians* strains. *X. campestris* pv. *campestris* and sterile phosphate buffer were used as positive and negative controls, respectively. Plants were scored for severity of the reaction at 30 and 48 hours after inoculation. Intensity was recorded as 0 for inoculation sites with no reaction; 1 indicates water soaking occurred; 2 indicates some cell collapse on abaxial surface; 3 indicates some cell collapse on adaxial surface; 4 indicates complete tissue collapse at the site of injection.

For the titration experiment populations ranging from $1x10^3$ to 1 x 10^9 CFU/ml were used to inoculate susceptible cultivars (Vista Verde, Salinas 88, and Clemente), intermediately resistant cultivars (Iceberg and Batavia Reine de Glaces) and a resistant cultivar (Little Gem). Disease severity was monitored and recorded approximately 7, 14 and 21 days after the initial inoculation. Area under the disease progress curve (AUDPC) of disease severity ratings were analyzed nonparametrically.

We are working toward additional breeding and plant genetics sub-objectives in collaboration with Ryan Hayes of the USDA/ARS lettuce-breeding program and the current individual can be found in the USDA/ARS lettuce-breeding report.

Objective 2 -3. Develop a reliable and sensitive scheme for detecting and quantifying bacteria from potential inoculum sources and evaluate seed for the presence of the pathogen.

To develop PCR protocols that are specific to *X. campestris* pv. *vitians* we searched for sequences that are specific to this pathogen but are not present in other pathovars of *X. hortorum* (including *X. hortorum* pv. *hederae, X. hortorum* pv. *taraxaci, X. campestris* pv. *pelargonii* and *X. hortorum* pv. *carotae*). Multiple sequence alignments for 16S rDNA, 16S-23S intergenic region, *rpoD, dnaK, fyuA, gyrB* and *hrpB* sequences of *X. hortorum* strains were generated from sequence data. Regions of variability were searched for sequences that could serve as specific primer. Requests for infested seed did not yield any samples. We were unable to tested currently available methods on naturally infested seed. We will test artificially infested seed this fall, but still hope to identify sources of infested seed to verify methods.

Objective 4. Determine if pathogens from other members of the Asteraceae are closely related to *X. campestris* pv. *vitians* and therefore need to be considered in management strategies.

Vauterin et al., (1995) demonstrated that pathogenic strains of *X. campestris* pv. *vitians* belonged to the species *X. hortorum*. Although the pathogen is not yet called *X. hortorum*, most pathogenic *X. campestris* pv. *vitians* strains must be regarded as a member of this species. Thus, methods for identification and quantification of this pathogen should be specific and not include other members of *X. hortorum*. Multilocus sequence analysis (MLSA) was used to evaluate the genetic relatedness between *X. campestris* pv. *vitians* belonging to *X. hortorum* and the pathotype strains of other *X. hortorum* pathovars including *X. hortorum* pv. *hederae, X. hortorum* pv. *taraxaci, X. campestris* pv. *pelargonii, X. hortorum* pv. *carotae* and a potentially new pathovar from radicchio (Zacarroni et al., 2012). Additionally, MLSA was used to characterize over 100 *X. campestris* pv. *vitians* and data was used in Objective 1.

In order determine if *X. hortorum* pathovars were differed for pathogenicity from *X. campestris* pv. *vitians,* hypersensitive responses were evaluated on 3-week-old lettuce and radicchio plants using methods as described above.

Results and Discussion:

Objective 1. Provide phenotypic data for breeding lines essential to the development of BLS resistant germplasm and the understanding of the genetics of BLS resistance.

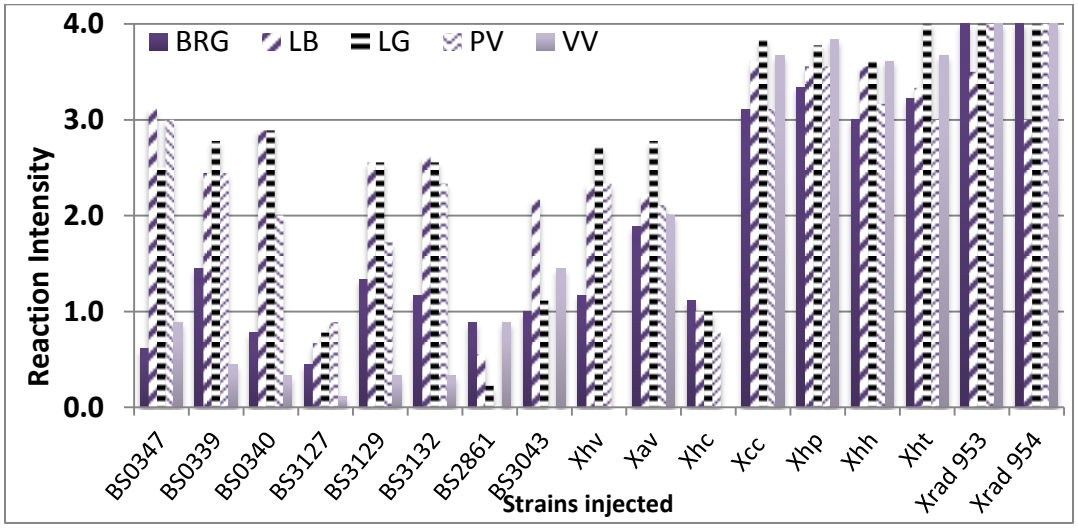
Twenty five lettuce lines bred for resistance to pests other than *X. campestris* pv. *vitians* were screened for resistance and susceptibility to this pathogen. The majority of strains evaluated were not significantly different from the parents used to generate the lines for severity of BLS in these experiments. A few of the lines were significantly more susceptible than their parents to *X. campestris* pv. *vitians.* Information on the status of BLS resistance and susceptibility will be

included in germplam release statements for these lines.

Previously we demonstrated plants spray inoculated with a mixture of Xcv strains from Quebec were more virulent than the mixture of Xcv strains from California used in breeding and phenotyping experiments. Experiments conducted in fall 2012 demonstrated that this difference is largely due to virulence of individual strains. In replicate experiments, strains from the original Quebec mix (BS3127 and BS3132) and BS347 from the California mix were significantly more virulent than the other three strains in the mixtures on both susceptible and resistant cultivars. The resulting mixture from Quebec was more virulent because it contained 1/3 more of the highly virulent cells than did the California mixed inoculum.

One of the Quebec strains also overcame the hypersensitive reaction. Resistant cultivars, Little Gem, Pavane and La Brilliante, expressed the HR when injected with a high concentration ($1x10^8$ CFU/ml) of Xcv (BS347 from California and other strains; Figure 1). However, injection of leaf tissue with Xcv strain BS3127 from Quebec did not result in the HR and severe BLS symptoms develop on La Brillante and Little Gem. Because this strain overcomes resistance mediated by the HR in these two cultivars, our next step will be to identify germplasm which expresses the HR when injected with strain BS3127. Once identified the genes for resistance to these two different pathogen types can be combined. Likewise, additional cultivars and strains need to be evaluated in order to develop a differential panel of cultivars to screen for strains that overcome resistance.

Figure 1. Non-host and hypersensitive reactions on susceptible and resistant cultivars of lettuce 48 hours after injection of bacteria into leaf tissue.



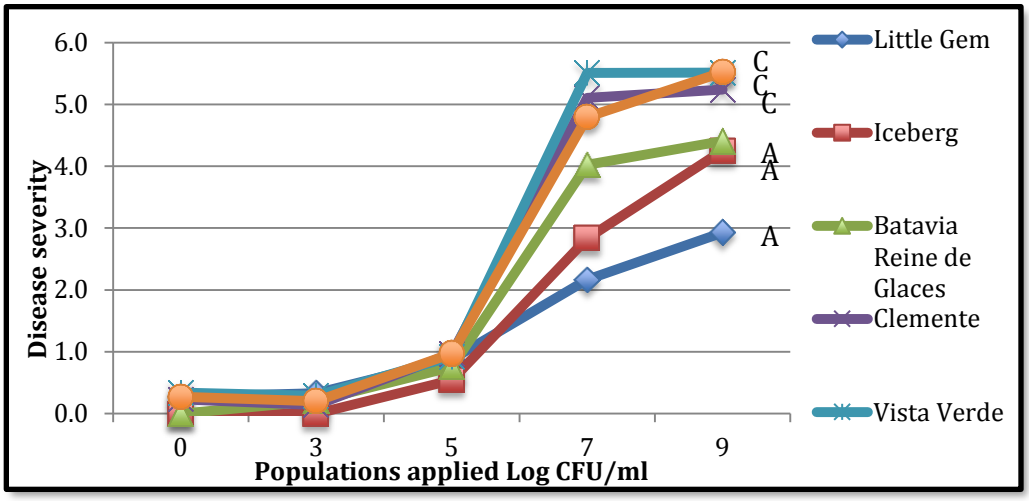
-Labels for strains: *X. campestris* pv. *campestris* (Xcc); *X. hortorum* pathovars (Xhp, Xhh, Xht, and an avirulent isolate of Xhc); strains from radicchio (Xrad 953, Xrad 954); Other strains are *X. campestris* pv. *vitians*.
-Intensity was recorded as 0 for inoculation sites with no reaction; 1 indicates water soaking occurred; 2 indicates some cell collapse on abaxial surface; 3 indicates some cell collapse on adaxial surface; 4 indicates complete tissue collapse at the site of injection.

Cultivar Batavia Reine des Glace is resistant to BLS but did not express the HR when inoculated with any of the Xcv strains from Quebec or California in the experiment described above. This indicated that resistance is mediated by a different mechanism. However, the HR

might be expressed in Batavia Reine des Glace if it were inoculated with a strain for which it had specific resistance genes. Thus, we will evaluate all strains collected to determine if any will elicit the HR in Batavia Reine des Glace.

The titration experiment confirmed the assignment of resistant cultivars and susceptible cultivars (Figure 2) and confirmed the differences seen in the types of disease resistance described. The pathogen induces a hypersensitive reaction in Little Gem (Figure 3) but not in any of the other cultivars tested. Little Gem had the lowest level of disease by all measurements. The hypersensitive response is not elicited by the inoculum in the other two moderately resistant cultivars (Iceberg and Batavia Reine de Glaces) tested. These cultivars had significantly more disease on some individual days at some inoculation levels, but disease progress as measured by AUDPC was not different than that for Little Gem. Three susceptible cultivars had the highest disease severity ratings by all measurements. Additionally, for all three rating periods the effect of inoculum concentration on disease severity was greater for susceptible cultivars than for resistant cultivars.

Figure 2. Influence of bacterial population levels applied on disease severity on resistant and susceptible cultivars.



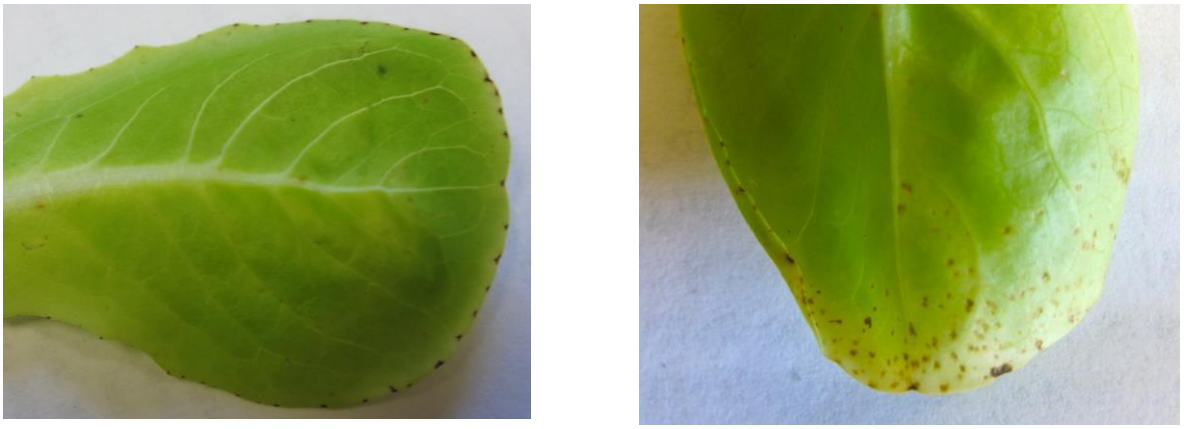
-Letters associated with each cultivar indicate significant differences in AUDPC as analyzed using nonparametric statistics.
-Data shown for the disease severity 21 days after inoculation.

Objective 2 -3. Develop a reliable and sensitive scheme for detecting and quantifying bacteria from potential inoculum sources and evaluate seed for the presence of the pathogen.

Detection and identification of *X. campestris* pv. *vitians* is currently being accomplished by amplification of DNA using the B162 primers and protocols (Barak et al., 2001) and/or culturing on MMG (Toussaint et al., 2001) followed by additional sequence analysis. We are seeking infested seed lots to test the currently available strategies. Because the B162 protocol is not specific to *X. campestris* pv. *vitians* and because the PCR protocol can't be efficiently adapted for quantitative PCR because the size of the fragment amplified is too large, we are exploring the potential of developing additional PCR protocols. Polymorphisms in the16S

rDNA, 16S-23S intergenic region, *rpoD*, *dnaK*, *fyuA*, *gyrB* and *hrpB* sequences from strains of *X. campestris* pv. *vitians*, *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci* and *X. campestris* pv. *pelargonii* were not large enough to design primers that would result in specific amplification of the genes from *X. campestris* pv. *vitians.* We are taking three approaches to overcoming this problem. First we are sequencing one additional gene that has had promise in other systems (*fliC*), second we have isolated phage specific to *X. campestris* pv. *vitians* that might be developed into a light-based detection system (Schofield et al., 2012) and lastly we are in the process of having whole genomes of these strains sequenced in order to find *X. campestris* pv. *vitians* specific sequences.

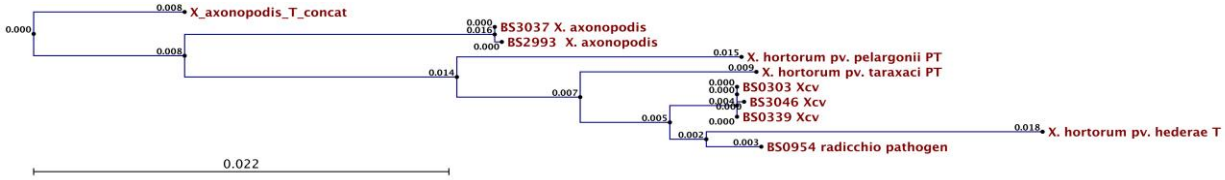
Figure 3. Hypersensitive reaction elicited on Little Gem to which 9.0 Log CFU/ml of *X. campestris* pv. *vitians* strain BS0347 was applied as a foliar spray.



Objective 4. Determine if pathogens from other members of the Asteraceae are closely related to *X. campestris* pv. *vitians* and therefore need to be considered in management strategies.

Pathovars withing *X. hortorum* are genetically similar and are clearly all located within one species. The genetic distances among pathovars within *X. hortorum* range from 0.01 to 0.03 for all four genes evaluated (*rpoD*, *dnaK*, *fyuA*, *gyrB*). The *X. campestris* pv. *vitians* strains pathogenic on lettuce and commonly found in California (BS0303, BS3046, BS0339) are most closely related to *X. hortorum* pv. *taraxaci* (98% identity) and a new pathovar from radicchio (99% identity; Zacaroni et al., 2012) (Figure 4). The genetic distances between these pathovars fall within the range described by Young et al., (2010) for distinct pathovars within *Xanthomonas* species.

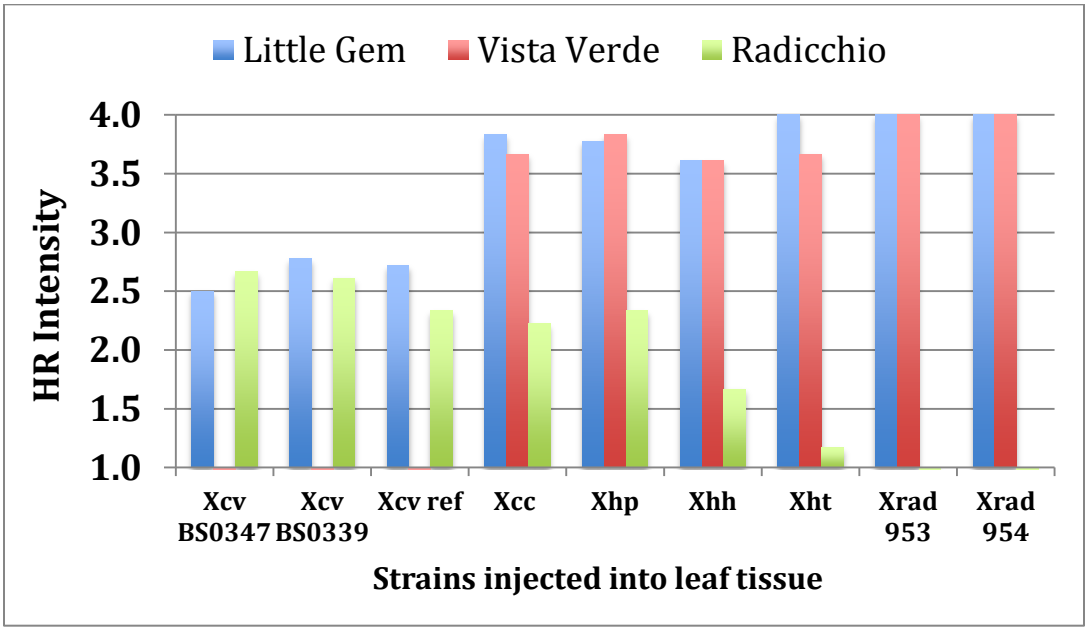
Figure 4. Neighbor Joining Tree for *X. hortorum* pathovars, radicchio strain and strains of Xcv that have been moved to *X. hortorum*.



Although they are closely related genetically, *X. campestris* pv. *vitians* is pathogenically

distinct from all other members of *X. hortorum* (Figure 4). The non-host control, *X. campestris* pv. *campestris* (Xcc), all *X. hortorum* pathovars (Xhp, Xhh, Xht) and the two strains from radicchio (Xrad 953, Xrad 954), resulted in rapid cell death in both lettuce cultivars tested. In contrast the pathogens of lettuce (Xcv labeled strains) induced a less intense HR reaction in Little Gem but not in Vista Verde (the resistant cultivar) as expected. This indicated that like *X. campestris* pv. *campestris* the *X. hortorum* pathovars and the pathogens from radicchio were pathogenically distinct from *X. campestris* pv. *vitians.* Likewise, the because *X. campestris* pv. *vitians* strains, the *X. hortorum* strains and *X. campestris* pv. *campestris* induced a rapid cell death in radicchio indicating that they are not pathogens of radicchio. The response induced by *Xanthomonas hortorum* pv. *taraxaci* on radicchio was less intense than the responses of the other pathogens. Evaluation of pathogenic distinctions between the radicchio strains and *Xanthomonas hortorum* pv. *taraxaci* will be conducted with funding from other sources. The pathogenic distinctions among the pathovars in *X. hortorum* makes it clear that detection and quantification methods must distinguish *X. campestris* pv. *vitians* from the other pathovars.

Figure 4. Non-host and hypersensitive response to injection by pathogens of *Xanthomonas hortorum* pathovars.



-Labels for strains: *X. campestris* pv. *campestris* (Xcc); *X. hortorum* pathovars (Xhp, Xhh, Xht); strains from radicchio (Xrad 953, Xrad 954); *X. campestris* pv. *vitians* (Xcv).
-HR intensity was measured at 30 and 48 hrs after inoculation. Data shown here were collected at 48 hrs. Results from 30 hrs after inoculation are similar. This is the second of two experiments showing similar results.
-Intensity was recorded as 0 for inoculation sites with no reaction; 1 indicates water soaking occurred; 2 indicates some cell collapse on abaxial surface; 3 indicates some cell collapse on adaxial surface; 4 indicates complete tissue collapse at the site of injection.

Acknowledgements
Much of this work was done in collaboration with Mark Trent, Polly Goldman and Isaiah Martinez.

References

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