

California Leafy Greens Research Program  
Spring Report  
April 1, 2011 to March 31, 2012

**Project Title:** Development of management strategies for Bacterial Leaf Spot of Lettuce.

**Principle investigator:** Carolee Bull, USDA/ARS, Salinas, CA. [Carolee.Bull@ars.usda.gov](mailto:Carolee.Bull@ars.usda.gov)

**Cooperators:** Ryan Hayes, USDA/ARS; Steven T. Koike, UCCE

### Summary

Bacterial leaf spot (BLS) of lettuce caused by the pathogen *Xanthomonas campestris* pv. *vitians* (*Xcv*) results in significant losses throughout California. Our long-term goal is to manage this disease through plant resistance and management of the pathogen inoculum. In addition to providing disease severity data for breeding material essential to the development of BLS resistant germplasm and the understanding of bacterial resistance we are investigating the role of the hypersensitive reaction in limiting bacterial growth in plant tissue. Among the cultivars evaluated the resistant cultivar was the only one on which *Xcv* elicited an HR within 24 hours. In plant tissue from these cultivars, bacterial populations levels did not increase at the same rate or to the same level as those on susceptible cultivars. We demonstrated differences in virulence among strain mixtures used to evaluate resistance here and abroad. A mixture of strains from Quebec, Canada was more virulent than the mixture of strains from California. Two of the three strains from Quebec belonged to a different genetic group than the third strain and the strains from California. According to analyses with three genes sequenced from 120 *Xcv* strains, there are three different genetic groups of *Xcv*. The Quebec strains belong to a group with few representatives and to which new pathogens from radicchio belong. Sequences from these three genes and an additional three genes are being used to design new specific primers for detection and quantification because the PCR primers and protocols currently available are not specific for *Xcv* strains.

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**Participating Personnel:** Ana Zacaroni -Visiting PhD student from the University of Lavras, Brazil; Samantha Gebben and Noemi Gomez - Hartnell College Intern; Mark Trent and Polly Goldman - USDA/ARS Salinas, CA

**OBJECTIVES:**

**Objective 1.** *Provide phenotypic data for breeding lines essential to the development of Bacterial Leaf Spot (BLS) resistant germplasm.*

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

**PROCEDURES:**

**Objective 1.** *Provide phenotypic data for breeding lines essential to the development of Bacterial Leaf Spot (BLS) resistant germplasm.*

The long-range objective for this project is to understand the genetics of resistance to *Xanthomonas campestris* pv. *vitians* (*Xcv*) and the mechanisms by which resistance functions. This year we have approached this goal by making progress on several different sub-objectives.

LETTUCE BREEDING AND GENETICS

We are working toward breeding and plant genetics sub-objectives in collaboration with Ryan Hayes of the USDA/ARS lettuce-breeding program. My laboratory is responsible for providing BLS phenotype data for the BLS is efforts to move resistance in Little Gem and other sources into additional germplasm and to study the genetics of resistance in these sources. We measure and evaluate relative disease severity using previously published methods (Bull et al., 2007). See the USDA/ARS Lettuce Breeding report for further information.

EFFECT OF CULTIVAR ON BACTERIAL POPULATIONS

One mechanism by which cultivars may impart resistance is by limiting the growth of the pathogen in or on lettuce tissue. We reported previously that bacterial populations increase to a greater extent and rate when applied to leaves of susceptible cultivars than when applied to leaves of resistant cultivars. Recently, we conducted experiments to evaluate changes in population levels of bacteria infiltrated into plant tissue thus, bypassing the processes required for internal colonization. This work was done additionally to evaluate the roll of the hypersensitive reaction (HR) on resistance in this pathosystem. The HR reaction involves rapid death of cells surrounding the bacterial infection that limits the spread of the pathogen.

Lettuce cultivars, susceptible (Vista Verde, Clemente and Salinas 88), moderately resistant (Batavia Riene de Glace) or resistant (Little Gem), were grown for approximately five-weeks in the greenhouse. Bacteria were removed from an agar plate and were suspended in phosphate buffer and adjusted to 0.6 OD<sub>600nm</sub>. Approximately 0.5 ml of the bacterial solution was infiltrated into leaf tissue. Plants were incubated after injection in a growth chamber with 16 hours light and 8 hours dark at 20°C. The HR response was documented at 24-hour intervals for 7 days. Bacterial populations were estimated directly after infiltration and on 4 of the 7 days. Samples were sterilely excised from the leaves using a 0.5 cm<sup>2</sup> cork borer (#3) and macerated in phosphate buffer. Dilutions were spread on nutrient agar amended with rifampicin. Bacterial colonies were counted after plates were incubated at 27°C for four days. The Log of the average number of colonies per cm<sup>2</sup> of leaf tissue was calculated and populations were compared.

#### GENETIC VARIATION AMONG XCV STRAINS AND THE IMPACT OF PATHOGEN DIVERSITY ON DISEASE RESISTANCE

It is important for researchers and growers to know if the previously described (Bull et al., 2007) and deployed (Hayes et al., 2006) resistance is effective against all populations of the pathogen or if it is effective against only a sub-set of pathogen strains. Thus, a sub-objective of our collaborative work is to evaluate the role of pathogen genotype of disease severity. We are approaching this question in two ways: 1) by evaluating differences that may occur between two specific sets of strains from Canada and California; and 2) by evaluating a large number of pathogens from a wide geographic range first for genetic diversity and to be followed with evaluation of diversity in virulence (also necessary for Objective 2).

Previously, we hypothesized that strains from Quebec, Canada might differ from California strains with regard to virulence. In preliminary experiments, cultivars and breeding lines were ranked differently for relative BLS levels depending on the location of the experiments, California or Quebec. This was unexpected because previous results indicated that environment did not influence ranking of resistant and susceptible cultivars (Bull et al., 2007). To determine if the differences in these experiments were due to differences in pathogens used in the experiments we compared disease severity on cultivars inoculated with either California or Quebec strains in experiments conducted in California.

In the first experiment, 4 replications of 13 cultivars or breeding lines (Clemente, Estival, Hochelaga, Ithica 989, Little Gem, Vista Verde, RH07-0372, RH07-0375, Salinas 88, Summertime, Sundevil, Chief, Eruption) were germinated in a growth chamber and then moved to the greenhouse. Three-week-old plants were inoculated using a mixture of *Xcv* strains Xav 98-12, BS339 and BS347 from the Salinas Valley (Barak and Gilbertson 2003; Bull et al., 2005) or a mixture of strains from Quebec (BS3127, BS3129 and BS3132). Plants held in a greenhouse maintained at 100% humidity and were inoculated and rated weekly using published methods (Bull et al., 2007). Differences in treatments for disease severity were analyzed using nonparametric statistics. A second experiment was conducted which was identical to the first experiment except that two additional cultivars were included (La Brillante and Batavia Reine de Glace).

A second approach to defining diversity among *Xcv* strains is defined below as part of Objective 2. Once the diversity is defined using a sequencing approach, virulence of strains will be evaluated.

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

We evaluated the methods for identification and quantification of *Xcv* from environmental sources and identified culturing on MMG (Toussaint et al., 2001) as the most reliable method currently available. However, this method is time and resource intensive and requires training to recognize *Xcv* among those growing on the medium. We began experiments using culturing of organisms on MMG to test environmental samples to begin identifying potential sources of inoculum. There are not results to report at this time. We will request infested seed from the industry in the coming weeks.

Detection and identification of *Xcv* was previously accomplished by amplification of DNA using the B162 primers and protocols (Barak et al., 2001). This PCR protocol can't be efficiently adapted for quantitative PCR because the size of the fragment amplified is too large. Thus, our immediate research goal was to sequence this fragment and identify sequences from within the fragment that could serve as primers for amplification of a smaller fragment for quantitative PCR. To this end we used the published protocol to amplify sequences from several *Xcv* strains and related pathogens. We identified a similar sequence using publically available databases and the BLAST algorithm. We compared sequences and tried to define unique fragments about the *Xcv* sequences.

Pathogenicity tests were conducted on lettuce and radicchio because our results indicated that strains causing radicchio leaf spot were similar to the *Xcv* strains from Quebec. In a series of three experiments, three to five radicchio (cv Leonard) and lettuce plants (cv. Vista Verde) were inoculated with *Xcv* and related pathovars of *X. hortorum* or *Xanthomonas* strains previously demonstrated to be pathogenic on radicchio. Inoculum for all strains was prepared from bacteria grown on agar medium, suspended in phosphate buffer and adjusted to 0.6 OD<sub>600nm</sub>. Bacteria were applied to plant leaves by spraying until run-off. Plants were incubated in the greenhouse at 100% humidity for the first 48 hours and then watered from the bottom for the remainder of the experiment. Symptoms were recorded within 14 days after inoculation and the pathogen was considered to be a pathogen if strain inoculated could be isolated from symptomatic tissue and identified by rep-PCR.

In addition, we are evaluating the genetic diversity of a larger set of *Xcv* strains and related pathogens. To this end we are sequencing six additional genes (16S rDNA, *gyrB*, *dnaK*, *fyuA*, *rpoD* and *hrpB*) from approximately 120 strains. The sequencing work was funded by a CDFFA grant but is reported here because these data are required for completion of this and other objectives. Primers used for amplification of genes were previously published (Young et al., 2008 and Obradovic et al., 2004). Sequencing was completed using a commercial laboratory. Gene fragments were cut and analyzed according to a new scheme developed by Zacaroni et al.,

(unpublished data) and will become publically available in the Plant Associated and Environmental Microbes Database (PAMDB). These data should also be useful in advancing Objective 2.

**Results and Discussion:**

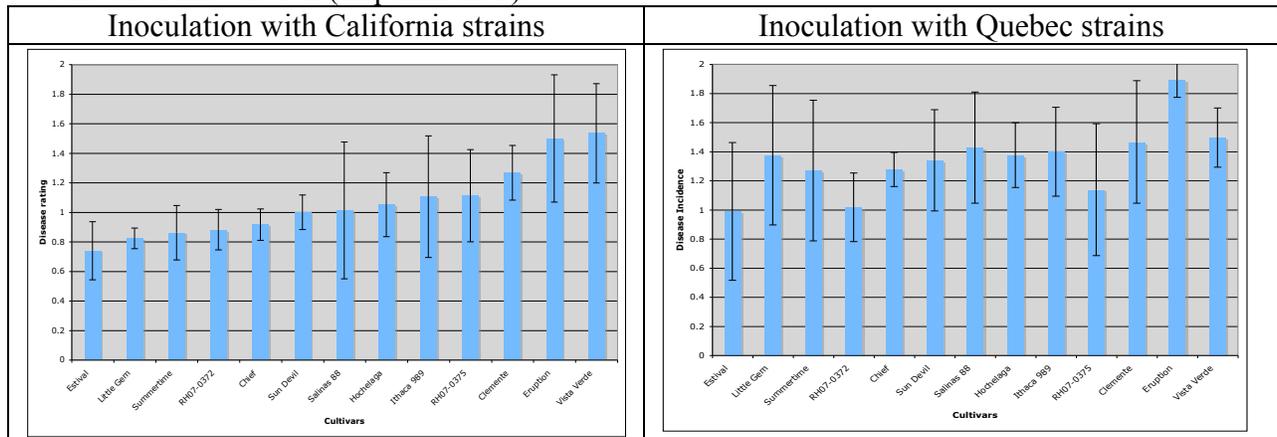
Because the results from the main objectives are related the results are presented together.

A MIXTURE OF QUEBEC *Xcv* STRAINS WAS MORE VIRULENT THAN A MIXTURE OF CALIFORNIA STRAINS CAUSING BLS OF LETTUCE.

In both experiments, the plants inoculated with the Quebec strains had significantly greater disease ratings and area under the disease progress curve (AUDPC) than those inoculated with the California strains (Fig. 1;  $p < 0.0001$ ). Within each inoculum source, there were significant differences in disease rating and AUDPC among cultivars for each treatment ( $p < 0.0001$ ). There were not significant strain-by-cultivar interactions in either experiment. However, the rankings were different for disease severity means for cultivars inoculated with Quebec versus California strains, thus confirming initial observations. Data from only the first experiment are shown below.

Data suggested that at least two of the strains in the Quebec inoculum mixture belong to a group of strains that are genetically distinct from the majority of *Xcv* strains to which the California strains belong (see below). It is not know if all members of the rare group of strains or if individual strains are more virulent then strains from the more common group. Additional experiments will be conducted comparing individual strains selected based on the results of the sequencing analysis.

Figure 1. Disease ratings on cultivars inoculated with strains from California or Quebec five weeks after inoculation (Experiment 1).

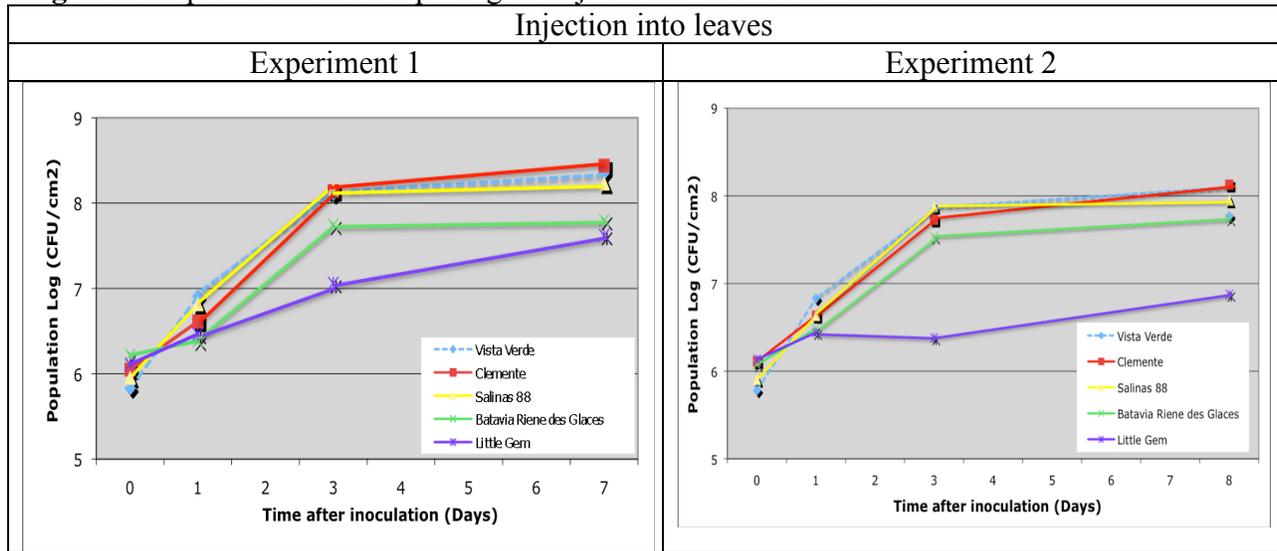


THE HYPERSENSITIVE REACTION CAUSED BY *Xcv* INJECTED INTO THE LEAVES OF RESISTANT CULTIVARS IS ASSOCIATED WITH REDUCED POPULATION GROWTH OF THE PATHOGEN.

In two experiments, population levels of *Xcv* began to increase within 24 hours of infiltration into lettuce leaves (Fig. 2). Little Gem expressed the hypersensitive response within 24 hours of infiltration (see the USDA/ARS Lettuce Breeding report for further information). In the first experiment, the rate of increase in population levels was significantly lower in the resistant cultivars (expressing HR) than in the susceptible cultivars that did not express HR ( $p > 0.0006$ ).

By three days after inoculation bacterial population levels in resistant cultivars (Little Gem and Batavia Reine des Glaces) were significantly lower than the populations in susceptible cultivars (Vista Verde, Clemente and Salinas 88) and remained significantly lower throughout the experiment lasting 7 days ( $P>0.0001$ ). Results for Little Gem were similar in a second experiment, however, population levels and the rate of population level increase in Batavia Reine des Glaces, although numerically lower, did not differ significantly than the more susceptible cultivars. The pathogen population data was less variable for experiments in which the pathogen was infiltrated into rather than sprayed onto leaves apparently because the processes needed for internal colonization of the leaf were bypassed. However, the general response of *Xcv* population levels was similar for resistant and susceptible cultivars for both inoculation methods.

**Figure 1.** Population levels of pathogens injected into lettuce leaves.



THE B162 PCR PROTOCOL IS NOT SPECIFIC FOR *Xcv* BECAUSE THE AMPLIFIED GENE HAS IDENTICAL SEQUENCES IN RELATED PATHOGENS.

In 1995, Vauterin et al. reclassified pathovars in the species *Xanthomonas campestris*. These authors transferred the pathotype strain of *Xcv* to *X. axonopodis* but the remaining strains were not related to the pathotype and were transferred to a different species *X. hortorum*. For most strains it is not know if they are more similar to the *X. axonopodis* or the *X. hortorum* strains. The published B162 primers and protocols amplified fragments from *Xcv* strains hypothesized to be members of *X. hortorum*. This included the three strains from Canada and the three strains from California used in mixtures for experiments reported here.

A BLAST search using the sequence of the B162 fragment of *Xcv* showed that the fragments amplified were nearly identical to a glycosyl hydrolase gene from a variety of plant pathogens including *X. gardneri*. Because the B162 primers were identical to sequences in this gene from *X. gardneri* we hypothesized that the B162 based detection protocol probably amplifies DNA from other related organisms. The B162 primers did amplify pathogens related to *Xcv* including *X. hortorum* pv. *taraxaci* and pathogens we are in the process of describing from radicchio. The protocol did not amplify DNA from *X. hortorum* pv. *pelargonii*, *X. hortorum* pv. *hederae* or the pathotype strain of *Xcv* which was previously shown to unrelated to the *X. hortorum* strains. We

did not have *X. gardeneri* in our collection to test but the sequence identity at the primer sites would indicate that the fragment would be amplified from this species. Comparisons of the amplified sequences demonstrated that *Xcv* strains from Canada and elsewhere were closely related to *X. hortorum* pv. *taraxaci* and *X. hortorum* strains from carrot and radicchio and were distinct from the sequences of the gene fragment for all California strains tested to date. Moreover, radicchio strains were pathogenic on lettuce and reciprocally *Xcv* strains were pathogenic on radicchio. Though the taxonomy of the radicchio pathogen is not yet clear, these results have implications of management of BLS in locations rotating lettuce crops with radicchio and dandelion.

Because the B162 primers and protocols are not adequate for specific detection and quantification of *Xcv*, alternative PCR protocols need to be developed. Sequence analysis indicated that there are no primers suitable within the B162 gene fragment. Therefore, additional genes were investigated for the development of new primers for specific detection and quantification of *Xcv*.

#### THERE ARE AT LEAST THREE GENETICALLY DISTINCT GROUPS OF *XCV* STRAINS

MultiLocus Sequence Types (MLST) were defined for *Xcv* strains and related pathovars based on three of the genes sequenced (*gyrB*, *dnaK*, *fyuA*). Most *Xcv* strains were in the same MLST and had identical sequences for all genes, however, there were three distinct MLST among the strains evaluated. The remaining genes will be added to the MLST this summer and the number of MLST may increase with these additional sequences. These analyses confirmed the data from analysis of the B162 gene fragment because two of the strains from the Quebec inoculum mixture belonged to one of the less common MLSTs. Significantly, two strains from Florida belonged to a completely different species *X. axonopodis*. This summer we will test the hypothesis that strains from these less common MLSTs are more virulent than strains from the *X. hortorum* *Xcv* strains. This work has significant implications for breeding efforts. Additionally sequence differences were detected between *Xcv* strains and *X. hortorum* pathovars including *X. hortorum* pv. *taraxaci*. These sequences may be useful in designing new primers specifically to the pathogens causing bacterial leafspot on lettuce.

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