California Leafy Greens Research Program
Spring Report
April 1, 2010 to March 31, 2011

I. Abstract.

Project Title: Development of Management Strategies for Bacterial Leaf Spot of Lettuce.

Investigator: Carolee Bull, USDA/ARS, Salinas, CA. Carolee.Bull@ars.usda.gov

Summary:

Bacterial leaf spot (BLS) of lettuce caused by the pathogen *Xanthomonas campestris* pv. *vitians* can cause significant losses throughout California. Use of resistant cultivars represent an important part of the management strategy for this disease. We provided disease severity data for breeding material essential to the development of BLS resistant germplasm and the understanding of bacterial resistance. Using cultivars that we previously identified as susceptible or resistant, we began to evaluate the relative roles of environmental and pathogen diversity on disease development and performance of resistant cultivars. We collected strains of the BLS pathogen from California and around the world and have begun to evaluate their impact on disease development and performance of resistant cultivars. In separate experiments, we investigated the influence of host plant diversity on growth and survival of the pathogen. Evaluation of changes in population levels of *Xcv* on cultivars that were previously characterized as either susceptible or resistant indicated that resistant cultivars support lower numbers of the bacterial pathogen than susceptible cultivars support. Population dynamics are directly related to disease development and cultivars supporting higher populations of the pathogen had higher levels of disease in these experiments. These data indicate that resistance to BLS is in part due to differences in the ability of the pathogen to grow or survive on or in the lettuce leaves. Additional experiments will further investigate the location and timing of development of the differences in populations dynamics. In addition to understanding how cultivars influence the growth and survival of the pathogen, we began experiments to compare methods for detection and quantification of the pathogen from environmental samples. MMG was confirmed to be the best semi-selective medium for isolation and quantification of the pathogen. However, DNA-based detection methods were also improved as part of this research. We will continue to optimize these methods in the coming months with the goal of developing a rapid and sensitive method for detecting and quantifying the pathogen from plant and environmental samples.

II. Main Body of Report.

Project Title: Development of Management Strategies for Bacterial Leaf Spot of Lettuce.

Investigator: Carolee Bull, USDA/ARS, Salinas, CA. Carolee.Bull@ars.usda.gov

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

Objectives:
Objective 1. Provide phenotypic data for breeding material essential to the development of Bacterial leaf spot (BLS) resistant germplasm and the understanding of BLS resistance in lettuce.
Objective 2. Determine if resistant, susceptible and important parental cultivars differ in their influence on *Xanthomonas campestris* pv. *vitians* (*Xcv*) populations.
Objective 3. Develop a reliable and sensitive scheme for detecting and quantifying bacteria from potential inoculum sources.
Objective 4. Evaluate the effect of pathogen diversity on severity of disease on susceptible and resistant cultivars.

Objective 1. Provide phenotypic data for breeding material essential to the development of Bacterial leaf spot (BLS) resistant germplasm and the understanding of BLS resistance in lettuce.

The current individual objectives of the BLS breeding program can be found in the USDA/ARS lettuce-breeding report. We continue to analyze the BLS phenotype for families and lines developed to move resistance from Little Gem into iceberg types and to study the genetics of resistance conferred by Little Gem and have begun to evaluate resistance in baby leaf lettuce types. Bacterial leaf spot was prevalent in baby leaf lettuce fields this fall. We are screening germplasm to be released for resistance to other diseases for resistance to BLS. Additionally, we are evaluating the effect of pathogen diversity on disease development on a range of susceptible and resistant cultivars.

Objective 2. Determine if resistant, susceptible and important parental cultivars differ in their influence on *Xanthomonas campestris* pv. *vitians* (*Xcv*) population levels.

We selected rifampicin resistant variants of *Xanthomonas campestris* pv. *vitians* (*Xcv*) and compared them to the wildtype strain for virulence on the susceptible cultivar Vista Verde. Bacteria adjusted to approximately 1 x 10^8 CFU/ml were applied by spraying until run-off to leaves of lettuce plants with three fully expanded true leaves. After inoculation, plants were placed in a humidity chamber and disease was evaluated 7, 14, 21 and 28 days after inoculation using a previously reported disease rating scale (Bull et al., 2007). The area under the disease progress curve was calculated and was not significantly different for plants inoculated with the wildtype strain or the rifE variant (BS2885) in two independent experiments (data not shown). The rifampicin resistant strain BS2885 was therefore used in all experiments evaluating *Xcv* population levels on lettuce leaves.

We evaluated the effect of plant genotype on *Xcv* populations. We hypothesized that the changes in population levels of *Xcv* over time would be different on susceptible and resistant cultivars. The effect of cultivars on population levels of *Xcv* was evaluated on susceptible (Vista

Verde, Sniper) and resistant (Little Gem, Batavia Reine de Glace) cultivars, and on cultivars important to the BLS breeding program (Clemente and Salinas 88). For initial studies, relatively low population levels (approximately Log 4.0 CFU/cm^2) of *Xcv* were applied by spraying on plants with three fully expanded leaves. Populations on leaves were monitored by spreading dilutions of macerated tissue (0.8 cm^2) on nutrient agar amended with rifamipicin eight times over a four-week period. The average number of colonies that grew after incubating the inoculated media at 27°C for 4 to 7 days was reported as the Log (CFU/cm^2) and populations were compared statistically.

As previously reported, population levels on leaves of susceptible cultivars were higher than on leaves of resistant cultivars 7 to 14 days after inoculation with low levels of inoculum (Fall, 2010 report; Fig. 1). In the first experiment there was a positive correlation between disease severity and populations on leaf surfaces after 21 days (Fig. 2) and the resistant cultivar Little Gem had significantly lower populations on leaves 21 days after inoculation compared to the susceptible cultivar Vista Verde (data not shown). The differences were not as pronounced in the second experiment. Although there were significant differences among treatments on particular sampling dates, the data were inconclusive on other dates.

Figure 1. Changes in *Xanthomonas campestris* pv. *vitians* populations on the leaves of resistant and susceptible lettuce cultivars.

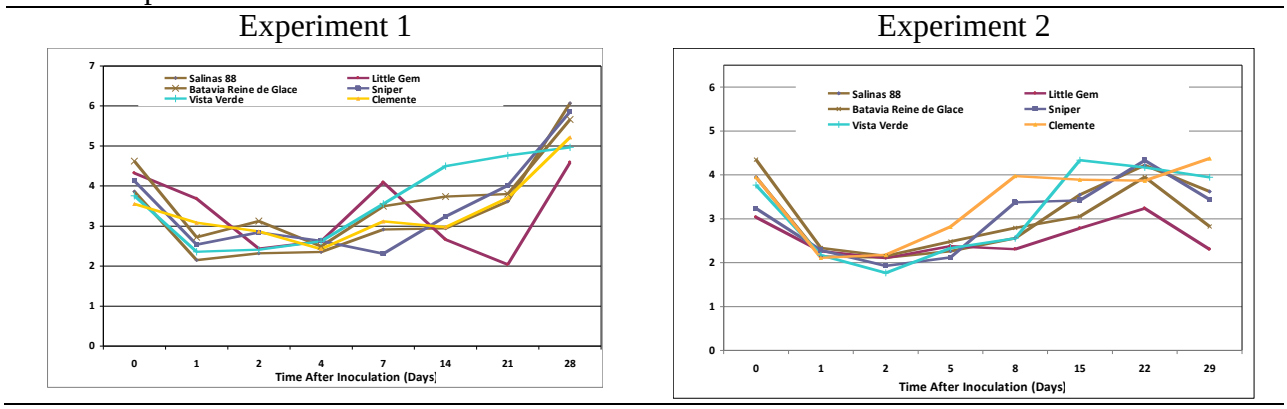
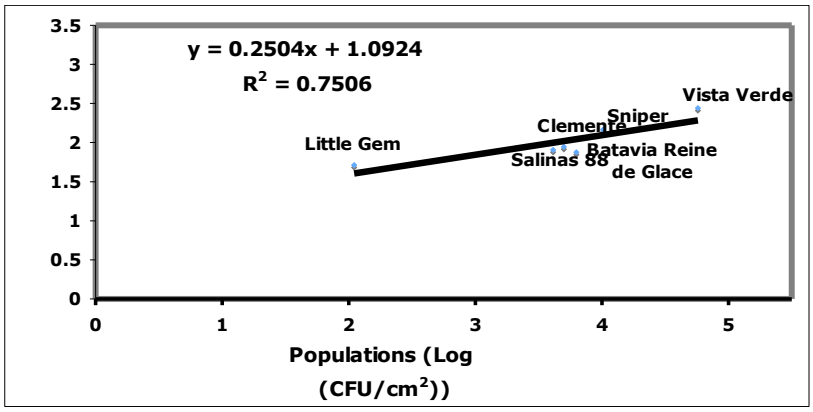
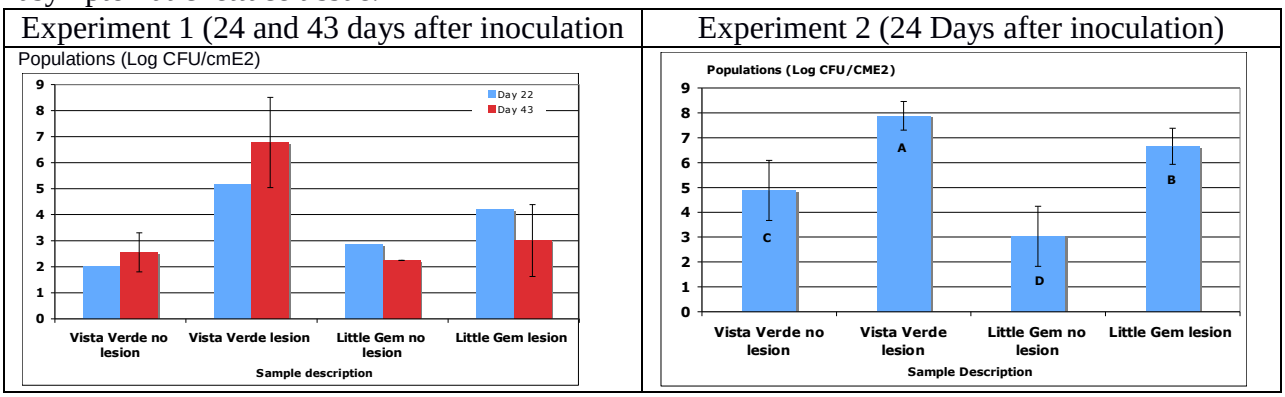


Figure 2. Relationship between disease severity and population levels of *Xanthomonas campestris* pv. *vitians* 21 days after inoculation (Experiment 1).



In the experiments reported in Figures 1 and 2, samples were taken from a standard location on the oldest leaf, regardless of the presence or absence of symptoms at the designated sampling location on the leaf. In order to determine if high within-treatment variability could be attributed to the variation in the presence or absence of lesions we estimated population levels for both the most severe lesions (once symptoms developed) and non-symptomatic tissue for each plant of Little Gem and Vista Verde on which disease had developed. Population levels on samples of asymptomatic tissue of Little Gem and Vista Verde were significantly lower on those samples for which there was a lesion present (Fig. 3). Thus, in subsequent experiments, samples for estimating population levels consisted of the most severe lesion on symptomatic leaves or asymptomatic tissue if no symptoms were present.

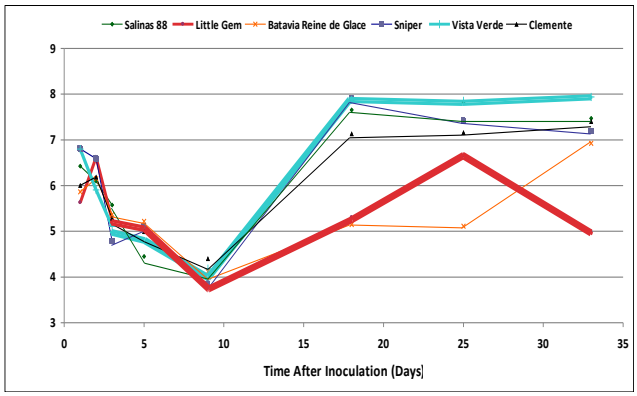
Figure 3. Populations of *Xanthomonas campestris* pv. *vitians* from symptomatic and asymptomatic lettuce tissue.



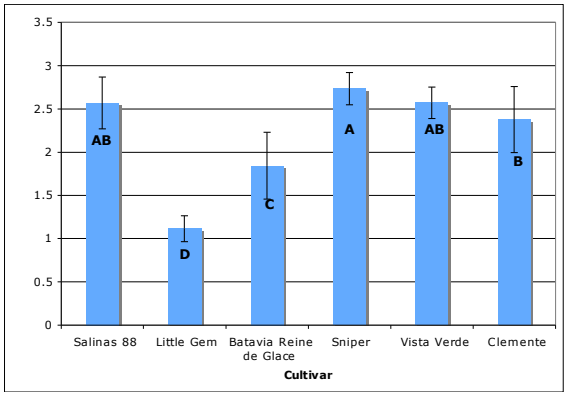
Experiments evaluating the effect of plant genotype on *Xcv* populations were repeated using the modified sampling technique described above and relatively high population levels (approximately 7.0 Log (CFU/cm^2)) of *Xcv* applied to leaves. Variability in populations within treatments was reduced using this new sampling method (data not shown). During the first 8 days of the experiment, population levels on all cultivars dropped from approximately 7.0 to 4.0 Log (CFU/cm^2), and were not significantly different (Fig. 4). Subsequently, population levels on susceptible cultivars increased to between 7.0 and 8.0 Log (CFU/cm^2). The subsequent increase in population levels on resistant cultivars was not as dramatic. At 32 days after inoculation the population levels on Little Gem were approximately 5.0 Log (CFU/cm^2). As expected, disease severity was lowest for Little Gem and among the highest for Vista Verde. The data indicated that resistant cultivars support lower levels of bacteria than are supported on susceptible cultivars and increases in population levels correspond with lesion development on all cultivars. These data indicate that further understanding the nature of population dynamics may provide an understanding of disease resistance mechanisms.

Figure 4. Disease severity and population levels of *Xanthomonas campestris* pv. *vitians* on the leaves of resistant and susceptible lettuce cultivars inoculated with high (8.0 Log (CFU/cm^2)) population levels.

Population levels



Disease severity 32 days after inoculation



Objective 3. **Develop a reliable and sensitive scheme for detecting and quantifying bacteria from potential inoculum sources.** Testing of seed and environmental samples for *Xcv* will be most useful when the detection and quantification of *Xcv* in these samples is rapid, accurate and sensitive. Currently, grow-out tests and evaluations including plant inoculations are standard and time consuming. In order to begin to develop improved methods we first wanted to evaluate the methods that are currently available for detection and quantification. There are currently two common methods used for detection and quantification of *Xcv* in or on lettuce leaves or seed; one relying on the growth of the pathogen on semi-selective media and the second relying on DNA-based detection methods.

The first method involves macerating surface-sterilized tissue, making serial dilutions of the liquid from this tissue, plating on semi-selective media, and then counting the colonies produced after approximately one week of incubation. We evaluated the semi-selective media available for detection and quantification of *Xcv.* Samples from the population dynamics studies described above were used for these experiments. The number of colonies of the rifampicin resistant strain of *Xcv* that developed on Nutrient Agar amended with rifampicin was compared the number of colonies estimated on MMG (Toussaint et al., 2001), tween media (McGuire et al., 1986), CKTM (Sijam et al., 1991), YDC. The population levels were evaluated at four separate sampling dates during three experiments (data not shown). In the first experiment, there were significant differences in the population levels estimated on NA amended with rifampicin and Tween media. In the second experiment, these differences were not detected. We tested MMG because, although it is expensive, *Xcv* colonies are distinct when grown on this media and are easy to recognize in comparison to the other media tested. Population levels estimated by MMG were not different than NA amended with rifampicin in any of the experiments. After optimization of PCR based techniques, we will compare MMG and PCR-based detection and quantification methods in the next experiments.

The second method uses PCR to specifically amplify DNA isolated from *Xcv* from environmental samples (Barak et al., 2001). It is less prone to false positives and can provide results more rapidly than the first method; however, because of inhibitors present in lettuce

tissue, this second method requires that DNA be isolated from the infested tissue prior to performing the PCR for detection. DNA isolation kits are available and although they result in rapid and consistent DNA isolation, DNA isolation adds expense both in time and money and an additional step that can negatively influence quantification. Using currently available methods, the detection limit of the PCR-based method is 2×10^3 CFU per PCR reaction. However, no amplification was achieved directly from samples containing lettuce tissue. We have therefore begun to optimize methods for direct amplification. Our first experiments with these methods have allowed us to omit traditional DNA isolation, thus also decreasing sample preparation expense, while achieving a detection limit of about 2×10^4 CFU per PCR reaction. This first attempt is only 10-fold less sensitive than DNA isolation techniques. We are continuing to improve these methods, and will focus on protocols that are transferable to real-time PCR, which will further decrease the total time from sampling to detection.

Objective 4. Evaluate the effect of pathogen diversity on severity of disease on susceptible and resistant cultivars.

Previously, we ranked disease development on a range of susceptible and resistant cultivars inoculated with strains from California (Figure 1 CLGRB report May 2010). The greenhouse trials supported data obtained in field trials (Bull et al., 2007), but the rankings of cultivars differed from results obtained for the same cultivars in Canada where strains isolated from diseased plants from Quebec were used for inoculation (Table 1). Although, Little Gem was the most resistant cultivar tested in California trials, six other cultivars ranked as more resistant than Little Gem in the Canadian trials. These differences may be due either to differences in the pathogens used to inoculate these experiments or environmental factors. Because our results demonstrated that rankings were similar in both the field and greenhouse studies, we assumed that the environment did not influence the ranking of cultivars for disease. Additionally there is evidence of diversity among strains of the pathogen. *Xanthomonas campestris* pv. *vitians* strains are heterogeneous and were placed in two different species in a taxonomic study conducted by Vauterin et al (1991) and three different rep-PCR groups by Rademaker et al. (2005). Thus, we initiated experiments designed to determine whether pathogen or environmental diversity account for differences in performance of resistant and susceptible cultivars. It is important to know if pathogen or environmental diversity influences disease resistance rankings so that BLS breeding programs can take this in to account. We recently received the strains used in the Canadian studies. We will evaluate disease on the same cultivars comparing those inoculated with the Canadian strains to the same cultivars inoculated with California strains. The same experiment will be conducted under Canadian conditions.

In addition, we received funding from the CDFA to further investigate the role of pathogen diversity on disease development. We collected 93 strains obtained either by purchasing them from culture collections or by donations from researchers. The strains included the type strain of *X. campestris* pv. *vitians* and the pathogenic reference strain assigned to *X. hortorum*. All strains are stored at the USDA/ARS and additional strains may be collected throughout the grant period as outbreaks are reported from regional BLS outbreaks in order to have representative isolates from current disease events in central coastal California. This fall, these strains will be compared to strains isolated in throughout the country and the world. We

will determine if genetic differences correlate to differences in disease severity on susceptible and resistant hosts.

Table 1. Ranking of lettuce cultivars for resistance or susceptibility to bacterial leaf caused by *Xanthomonas campestris* pv. *vitians*.

Ranking (1 = most resistant)	Salinas California USA	Quebec Canada[a]
1	Little Gem	Sundevil
2	RH07-0372	Summertime
3	Clemente	Ithaca 989
4	Estival	Estival
5	Hochelaga	Hochelaga
6	Salinas 88	RH07-0375
7	RH07-0375	Little Gem
8	Sundevil	RH07-0372
9	Ithica 989	Clemente
10	Summertime	Vista Verde
11	Vista Verde	

[a]Data provided by Drs. Sylvie Jennie and Vicky Toussaint of Agriculture Canada

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