CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

Annual Report
April 1, 2009 to March 31, 2010

I. Abstract

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

Principle investigator:
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Cooperators:
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Summary:
At the request of the board, we have shifted our focus from research on Corky Root (CR) of lettuce to the development of tools to manage Bacterial Leaf Spot (BLS). We are continuing to provide phenotypic data for breeding lines essential to the development of BLS resistant germplasm. This year we evaluated baby leaf germplasm and identified red leaf and red romaine as two baby leaf types that could benefit from development of resistant lines. Other baby leaf types are either mostly resistant (green romaine and Latin) or are variable for resistance but include resistant individuals (lolla verde, red and green oak, green leaf and lolla rossa). Rankings of cultivars for resistance to BLS in Quebec differed from results achieved in our greenhouse assays although our assays also match those seen in the field. We will be evaluating strains used in the Quebec assays to determine if these differences are the result of differences in pathogens used. We developed tools to monitor populations of *Xanthomonas campestris* pv. *vitians* on plant leaf surfaces. We used these tools to evaluate the relationship between susceptibility of the host and the pathogen population dynamics. There was a significant correlation between populations of the pathogen causing BLS and disease development. The most susceptible and resistant cultivars had the highest and lowest bacterial populations, respectively, three weeks after inoculation. This experiment will be repeated and expanded to include additional detection and quantification methods. We previously demonstrated that a field soil from Watsonville harbored organisms that could overcome the resistance deployed in the majority of CR resistant cultivars including Green Lakes. The virulence was evaluated for 41 strains (members of the CR pathogen family) isolated from symptomatic Green Lakes roots grown in the Watsonville soil. No single organism evaluated reproduced the level of disease obtained on Green Lakes when it was grown in untreated Watsonville soil. This indicates that a mixture of organisms may be responsible for overcoming resistance. A previously designed primer pair and a PCR protocol were used to consistently detect *Sphingomonas suberifaciens* strain CA1 from diseased roots. Sequence comparisons indicate that DNA from all CR pathogens closely related to this strain should be amplified. Because CR is caused by a diverse group of bacteria including several different genera and species, we developed 2 primers pair-PCR protocol combinations that are promising for detection and quantification of all CR pathogens.

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Our project previously concentrated on Corky root (CR) of lettuce, caused by *Sphingomonas suberifaciens* (formerly *Rhizomonas suberifaciens*) and related organisms. Corky Root is an economically important disease in California and other major lettuce growing areas. Roots of infected plants develop yellow to brown lesions, which can become longitudinal corky ridges. In severely infested fields, yield losses can reach 30-70% due to reduction in head size. Resistant crisphead cultivars have reduced the impact of this disease. However, this disease still impacts yield and profit of romaine types because only a few tolerant romaine cultivars are available. We previously demonstrated that isolates of the pathogen from a Watsonville, Calif. field are overcoming resistance in crisphead cultivars. It is now even more critical to identify additional management strategies for this disease because resistance-breaking soils have been identified and there is an increase in planting of romaine types. Understanding pathogen biology and the effect of management strategies on pathogen populations is critical to development of alternative control methods.

This year we were asked to switch emphasis from work on Corky Root of lettuce to research on the detection *Xanthomonas campestris* pv. *vitians* (Xvc) and management of the disease it causes, Bacterial Leaf Spot (BLS) of lettuce. This transition will continue over the coming year as we finish and publish the results of our projects on Corky Root and develop the tools needed to answer the questions growers have about sources of inoculum for Bacterial Leaf Spot and disease development.

Bacterial Leaf Spot caused by *Xanthomonas campestris* pv. *vitians* is a sporadic but economically significant problem in head lettuce and was made a research funding priority by the CLGRP for lettuce grown for salad mix. BLS has been steadily increasing in incidence and severity over the past three seasons. The most significant outbreaks of BLS have always happened late in the year, with the most damaging cases occurring in August through October. This trend reached a peak in the fall of 2009, when numerous leaf, romaine, and iceberg lettuce fields in the Salinas and Santa Maria valleys were affected with BLS. BLS infections in 2009 were commonly followed by secondary decay activity due to soft rot bacteria and Botrytis gray mold; this secondary problem significantly reduced the quality and yields of affected fields.

Our research program developed methods for field and greenhouse screening of cultivars for resistance to *Xanthomonas campestris* pv. *vitians* which causes bacterial leaf spot of lettuce (BLS). Importantly the methods allowed us to identify significant levels of resistance in the cultivars ‘Little Gem’ and ‘Batavia Reine de Glaces’ (Bull *et al*., 2007). We also used these

methods to develop breeding lines with moderate resistance to bacterial leaf spot and improved horticultural characteristics (Hayes *et al*., 2008). Our current research continues the phenotypic analysis of germplasm and identification of the nature of BLS resistance. Additionally we have developed tools that we are now using to understand pathogen biology and epidemiology to identify potential targets for disease management.

Bacterial Leaf Spot Objectives:

Objective 1. *Continue to provide phenotypic data for breeding lines essential to the development of BLS resistant germplasm.*

This year we conducted two replicated experiments to evaluate 36 baby leaf lettuce types for resistance to bacterial leaf spot using our greenhouse assay. Our goal was to identify baby leaf lettuce types that did not have resistant representatives in order to target breeding toward those with the greatest susceptibility and need.

The average disease severities for 'Little Gem' (resistant) and 'Vista Verde' (susceptible) were 0.64 and 1.96, respectively (Table 1). The most resistant types (green romaine and Latin) had average disease ratings of 0.76 for all cultivars tested. Resistance within these types appears to be sufficient for use in breeding programs.

The greatest susceptibility was found in red leaf and red romaine types, which had disease ratings of 1.45 and 1.52, respectively. The least susceptible cultivars from these types had disease severity ratings of 1.22 and 1.19, which are too high to have what we consider to be useable levels of resistance. Furthermore, the cultivars Cardinale (red leaf) and Rouge d'Hiver (red romaine) had higher disease severity levels than our susceptible control, Vista Verde. Therefore, a BLS resistance breeding program directed at red leaf and red romaine may be appropriate. The remaining types (lolla verde, red oak, green oak, green leaf, and lolla rossa) were more variable, and included resistant and susceptible individuals. Cultivar selection within these types will be more crucial when growing baby leaf lettuce in bacterial leaf spot prone environments.

Table 1. Variation for bacterial leaf spot resistance in 37 cultivars of nine types of baby leaf lettuce and Vista Verde.

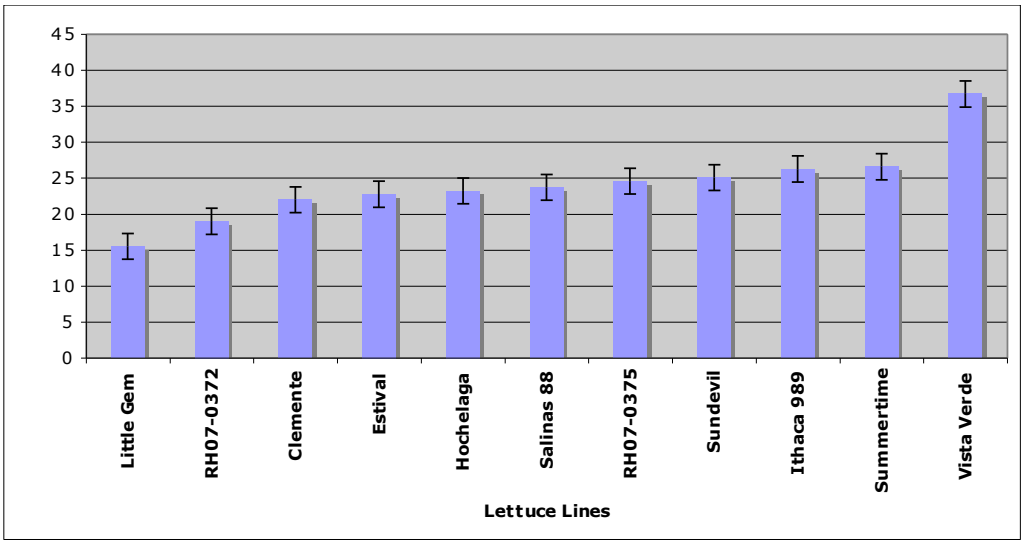
Lettuce Type	Number of cvs.	Mean Severity	Most resistant cv. (mean severity)	Most Susceptible cv. (mean severity)
Green Romaine	5	0.76	Mutiny (0.47)	Green Acres (1.03)
Latin	2	0.76	Little Gem^{R} (0.64)	Brigade (0.89)
Lolla Verde	1	1.02	Lolla Verde (1.02)	-
Red Oak	5	1.06	Crimson (0.82)	Red Tamale (1.37)
Green Oak	3	1.15	General (0.58)	Beijing Green (1.53)
Green Leaf	4	1.23	Sergeant (1.01)	Revenge (1.65)
Lolla Rossa	4	1.32	Lolla Rossa (0.82)	Rampage (1.55)
Red Leaf	7	1.45	Cavalry (1.22)	Cardinale (2.14)
Red Romaine	5	1.52	Flagship (1.19)	Rouge d'Hiver (1.83)
Vista Verde^{S}	-	1.96	-	-

As a preliminary experiment for objectives proposed in 2010-2011, we compared cultivars from Quebec to Little Gem, Vista Verde and other cultivars or breeding lines of interest

to our program using our BLS screening methods. Previously, five cultivars (cv. Sundevil, Summertime, Ithaca 989, Estival, Hochelaga) were shown to be more resistant than Little Gem in greenhouse trials in Quebec using different strains of Xcv and assay conditions. The goal of this experiment was to evaluate the resistance of these cultivars using our methods and strains.

The results of this experiments supported previous conclusions from greenhouse and field experiments (Bull *et al*., 2007). Little Gem was the most resistant cultivar tested while Vista Verde was the most susceptible. Disease development was significantly less on Little Gem than on Vista Verde, RH07-0375, Summertime and Sundevil according to Tukey's HSD, although Sundevil was shown to be the most resistant cultivar in the trials in Quebec. In the coming year we will use the Xcv strains used in the Quebec experiments to determine if the differences seen in cultivar rankings are due to differences in pathogens used in the greenhouse assays.

Figure 1. Development of disease on resistant and susceptible cultivars and breeding lines.



Objective 2. *Develop Xanthomonas campestris pv. vitians strains to be used in population studies and future studies investigating the interaction between Xanthomonas campestris pv. vitians and resistant or susceptible hosts.*

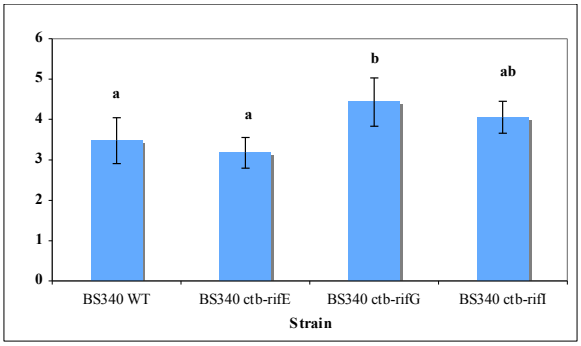
There are two methods currently available for monitoring populations of Xcv but both are too expensive for monitoring large numbers of samples. Therefore antibiotic resistant (resistant to rifampicin) variants of Xcv were generated. This will allow selective reisolation of the pathogen from plant tissue on media containing rifampicin to the media. These strains will allow us to ask several important questions about the interactions between the host and the pathogen.

We generated rifampicin resistant variants of the three strains of *X. campestris pv. vitians* used in our BLS bioassay. The genotypes of the rifampicin resistant variants were compared to the genotype of each wildtype strain for growth rate in culture, and strains that did not differ from the wildtype were tested further. The Xcv strain BS340 variants were compared to BS340 for virulence on the susceptible cultivar 'Vista Verde' using our standard BLS disease assay (Figure 2). We conducted two independent experiments comparing disease on plants inoculated with the wildtype BS340 strain and rifampicin variants. In both experiments the variant BS340 rifE (now BS2885) produced disease levels statistically equivalent to those produced with the

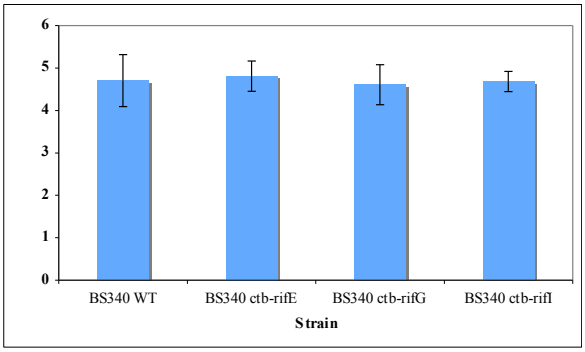
wildtype BS340 strain. Virulence of additional rifampicin variants generated from the other two Xcv strains used in our BLS bioassays will be compared to their respective wildtype strains.

Figure 2. Disease progress for *Xanthomonas campestris* pv. *vitians* strain BS340 and rifampicin resistant variants.

Experiment 1



Experiment 2



Treatments represented by bars with the same letter were not significantly different at 0.05 according to Tukey's HSD. No statistical differences were seen among treatments in the second experiment.

Objective 3. *Determine if resistant, susceptible and important parental cultivars differ in their influence on Xanthomonas campestris pv. vitians populations.*

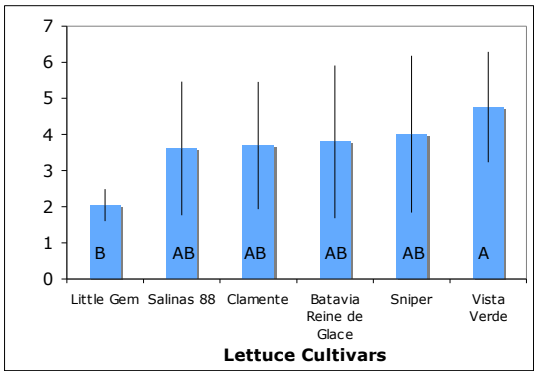
We have completed the first evaluation of the effect of lettuce cultivars on pathogen populations. Resistant cultivars Little Gem and Batavia Reine de Glaces, susceptible cultivars Vista Verde and Sniper and as well as Clemente and Salinas 88 were evaluated. The rifampicin resistant strain adjusted to 4.0 Log (CFU/ml) in 0.01M phosphate buffer (pH 7.0) was used to inoculate three-week-old lettuce seedlings by spraying until run-off. Populations were sampled from 0.5 cm^2 circular leaf tissue removed from the tip of inoculated leaves. Subsamples of a suspension of tissue crushed in 300 µl of phosphate buffer or dilutions were spread on Nutrient agar containing rifampicin (100 µg/ml) and cycloheximide (80 µg/ml). Populations were sampled on the day of inoculation, for the first three days after inoculation and then weekly for four weeks. On the second, third and fourth weeks after inoculation the disease severity was evaluated.

Bacterial population levels were approximately 4.0 Log (CFU/cm^2) on all cultivars directly after inoculation. By three weeks after inoculation the Xcv populations on cv. Little Gem (the most resistant cultivar tested) had dropped to an average of 178 CFU per cm^2 of leaf tissue (Figure 2A). In contrast the average population levels on Vista Verde increased slightly. There was a significant relationship between the disease ratings and the population of Xcv on the cultivars tested with Vista Verde and Little Gem having the highest and lowest average disease ratings and populations of Xcv, respectively, after three weeks.

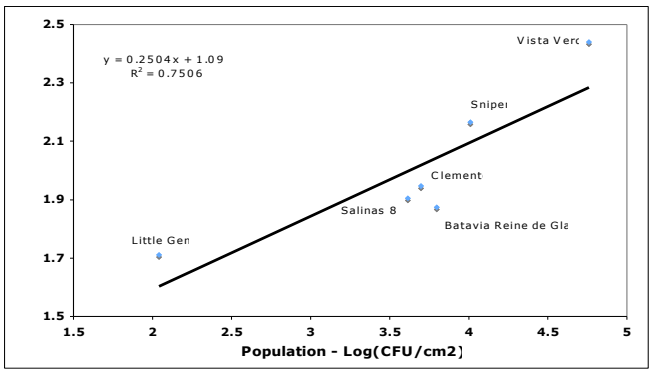
A second experiment has been planted to verify these results. In addition to evaluating the effect of cultivars on populations applied at a relatively low inoculum concentration (4.0 Log (CFU/ml)) we will also evaluate the fate of bacteria applied at relatively high inoculum levels (8.0 Log (CFU/ml)) to leaves susceptible and resistant cultivars. These experiments will be completed this summer.

Figure 3. Bacterial populations and disease severity on susceptible and resistant cultivars 21 days after inoculation with *Xanthomonas campestris* pv. *vitians* at 4.0 Log (CFU/ml).

A. Bacterial populations



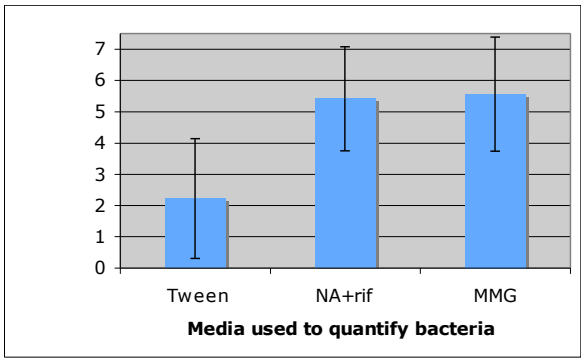
B. Relationship between disease severity and Xcv populations



Treatments represented by bars with the same letter were not significantly different at 0.05 according to Tukey's HSD.

For the 2010-2011 funding year, we proposed to evaluate the ability of previously published media (MMG, Toussaint et al., 2001; tween media, McGuire et al., 1986 and CKTM, Sijam et al., 1991) for quantification of populations of *X. campestris* pv. *vitians*. As a preliminary evaluation of the media, we determined bacterial populations from six replications of Vista Verde sampled at 21 days after inoculation by spreading aliquots of the samples or dilutions on MMG, Nutrient Agar amended rifampicin (NA + rif) and MMG (Figure 4). Fewer colonies developed on Tween media than on either MMG or NA amended with rifampicin. We will repeat this experiment, evaluating additional media and using populations from plant tissue, purified cultures and soil to which bacteria have been added.

Figure 4. Evaluation of media for quantification of *Xanthomonas campestris* pv. *vitians.*



Corky Root Objectives:

Objective 4. *Completion of virulence evaluations for isolates from corky root resistance breaking soils.* We previously demonstrated that soils from a Watsonville lettuce planting harbored organisms that could overcome the resistance deployed in the majority of CR resistant

cultivars including Green Lakes. This soil was designated *cor* Breaking Soil (*c*BS). Seventy-two bacteria were isolated from corked lesions on diseased roots of resistant cultivars grown in *c*BS from Watsonville. Among these were 41 strains that were identified as members of the bacterial family *Sphingomonadaceae*, of which the most well studied corky root pathogen, *Sphingomonas suberifaciens* CA1, is a member. We characterized the genetic diversity of the organisms in this family (see previous reports). Many of these strains belonged the family *Sphingomonadaceae* but to species and genera other than *S. suberifaciens*.

We evaluated the virulence of these strains on the resistant cultivar Green Lakes. In these evaluations, seeds were planted directly into *c*BS (soil), autoclaved *c*BS, or autoclaved vermiculite. Plants were inoculated two weeks after planting with a suspension of *c*BS, individual bacterial strains or a negative control. After six weeks the plant roots were evaluated and rated based on the scale developed by Brown and Mitchemore (1988). We evaluated virulence of the 41 members of the *Sphingomonadaceae* from the *c*BS soil in 11 experiments. No single organism evaluated reproduced the level of disease obtained on Green Lakes when it was grown in untreated *c*BS. This indicates that perhaps more than one organism is responsible for the level of disease seen on Green Lakes grown in *c*BS. We are developing a new strategy to determine what organisms are responsible for severe CR on Green Lakes grown in *c*BS.

Objective 5. *In situ detection and quantification of various corky root populations.* We have generated and characterized rifampicin resistant CA1 variants using the methods described above for Xcv strains and our standard CR bioassay described in previous reports. We identified two strains that had similar growth rates in culture. Plants inoculated with the two rifampicin resistant variants (1B and 2C) did not differ from the wildtype for disease severity three weeks after inoculation. These strains can be used to monitor the basic biology and population dynamics of the pathogen in relation to disease management strategies.

In previous years we reported that we had identified primers (Rs665F/Rs773R) and a PCR protocol useful for detecting and quantifying *Sphingomonas suberifaciens* strains including the type strain CA1. We evaluated the ability of this method to detect the pathogen from plant tissue grown in the presence or absence of the pathogen. Seeds of the susceptible cultivar Salinas or resistant cultivar Green Lakes were grown in untreated or autoclaved *c*BS from Watsonville. Seeds were also planted in autoclaved soil inoculated with strain CA1 as a positive control. Disease ratings were recorded for all roots sampled according to the scale of Brown and Michelmore (1988). DNA was isolated from lyophilized roots of diseased and healthy lettuce plants using the Macherey-Nagel NucleoSpin Plant DNA Isolation kits.

The method consistently detected the pathogen in diseased cv. Salinas roots (those with a disease rating of greater than 5) grown in autoclaved soil and inoculated with CA1. In contrast, there was no DNA amplification from Salinas roots planted in untreated autoclaved Watsonville soil. Likewise there was no amplification from diseased roots of the resistant cultivar Green Lake grown in natural Watsonville soil indicating as our other data do, that the strains from the Watsonville soil causing disease on Green Lakes are different than CA1 and are not detected using this method.

It will be useful to understand how management practices influence different portions of the pathogen population especially because of the differences in pathogenicity seen on different cultivars. However, it will also be important to detect and quantify the entire population of organisms causing CR on lettuce. Therefore we developed two additional PCR protocols that amplify DNA from all known CR pathogens. We sequenced the 16S rDNA from all strains in

our collection including those isolated from the Watsonville *c*BS. The DNA sequences were aligned and DNA Workbench was used to help define potential primer sequences. In initial experiments using the two new protcols, DNA was amplified from the complete range of pathogens but not from negative controls. We will continue to test these methods for detection of pathogens in plant tissue and soil.

Objective 6. *Comparison of disease resistance in cultivars resistant due to cor and resistance identified in PI491239 and PI273597c.* This work has not been completed because we have not identified single isolates that are responsible for the over coming of resistance in the Watsonville fields. This experiment will be completed at a later date.

References

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- Bull, C. T., Goldman, P. H., Hayes, R., Madden, L. V., Koike, S. T., and Ryder, E. 2007. Genetic diversity of lettuce for resistance to bacterial leaf spot caused by *Xanthomonas campestris* pv. *vitians*. Online. Plant Health Progress doi:10.1094/PHP-2007-0917-02-RS.
- Hayes, R.J., Ryder, E.J., and Bull, C.T. 2008. Release of Iceberg Lettuce Germplasm with Resistance to Bacterial Leaf Spot Caused by *Xanthomonas campestris* pv. *vitians*. United States Department of Agriculture, Agricultural Research Service. Beltsville, MD.