CALIFORNIA LEAFY GREENS RESEARCH BOARD

Annual Report
April 1, 2008 to March 31, 2009

I. Abstract

Project Title: Development and use of tools to aide in the development of management strategies for corky root of lettuce.

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Cooperators:
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Summary:
Corky root of lettuce, caused by *Sphingomonas suberifaciens* (formerly *Rhizomonas suberifaciens*) and related organisms, 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, pathogens in the soil from one field in Watsonville, Calif. are able to overcome resistance deployed in crisphead cultivars. Seventy-two bacteria were isolated from corked lesions on diseased roots of resistant cultivars. 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 have characterized the genetic diversity and pathogenicity of the organisms in this family. Only four of the 41 members of this family were identified as *Sphingomonas suberifaciens.* The remaining strains belong to related but different species and genera in the family, indicating that the pathogens responsible for overcoming resistance may be different than the well-studied *Sphingomonas suberifaciens*. Pathogenicity and virulence has been tested for 1/3 of the strains on resistant cultivars in multiple experiments. None of the organisms tested to-date is solely responsible for corky root symptoms seen on resistant cultivars. The causal agent may be found among the remaining organisms or the disease may be caused by a complex of organisms. We have developed DNA-based tools to detect and quantify various segments of the pathogen population. These tools can be used to determine what cropping practices increase various segments of the pathogen population and to determine what segments of the population are present in the Watsonville soil. Additionally, we have made progress in generating strains of *Sphingomonas suberifaciens* CA1 that will help us to monitor the infection process in resistant and susceptible cultivars. Differences in the infection process for resistant and susceptible cultivars may indicate targets for disease management.

II. Main Body of Report

Project Title: Development and use of tools to aide in the development of management strategies for corky root of lettuce.

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Objectives:
1. Molecular characterization of *cor* breaking members of the Sphingomonadaceae isolated from *c*BS soil.
2. Investigate the resistance identified in PI491239 and PI273597c to corky root strains including
3. Develop molecular detection and quantification methods for various genotypes.
4. Initiate research using confocal microscopy to follow the infection of susceptible and resistant cultivars of lettuce by *S. suberifaciens* CA1.

Corky root of lettuce is caused by *Sphingomonas suberifaciens* (formerly *Rhizomonas suberifaciens*) and related organisms in the family Sphingomonadaceae. 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. We previously demonstrated that pathogens in the soil from a field in Watsonville, Calif. were overcoming resistance in crisphead cultivars. Resistant cultivars became diseased when grown in this *cor*-breaking soil (*c*BS), but did not become diseased when grown in autoclaved soil or in autoclaved soil amended with *Sphingomonas suberifaciens* CA1. PI lines identified in our previous work (Mou and Bull, 2004) were relatively resistant when planted in *c*BS in greenhouse and field trials. Although the problem appears to be contained to one field, it is important to characterize the pathogens that are breaking the deployed corky root resistance.

Objective 1. *Molecular characterization of cor breaking members of the Sphingomonadaceae isolated from cBS soil.*

We previously demonstrated that cultivars resistant to corky root became diseased when grown in the putative *cor*-breaking soil (*c*BS) located in Watsonville, Calif., but did not become diseased when grown in autoclaved soil or in autoclaved soil amended with *Sphingomonas suberifaciens* strain CA1 (previously known as *Rhizomonas suberifaciens*). We isolated strains from diseased roots of resistant plants grown in this soil and have made significant progress characterizing the genetic diversity and pathogenicity of the isolates. These studies should identify and fingerprint the key isolates responsible for overcoming corky root resistance.

Proceedures:

S-agar amended with streptomycin was used isolated bacteria from the roots of corky root resistant lettuce (Glacier, Green Lake, Montello, or Telluride; double recessive for *cor*) grown in the *c*BS soil. For each strain the 16S rDNA gene was amplified from purified genomic DNA using universal primers fD1 and rP3 1492R using published reaction conditions (Weisburg *et al.*, 1991). After visually checking for amplification, amplicons were purified and sequenced directly by an outside vendor (McLab, South San Francisco, CA). Sequences were compared by using the multiple alignment function of Clustal W (Chenna, *et al.*, 2003; Pearson, 1990). Identifications were based on 97-99% sequence similarity for most strains. Identity for seven strains was based on sequence similarities between 91-97%.

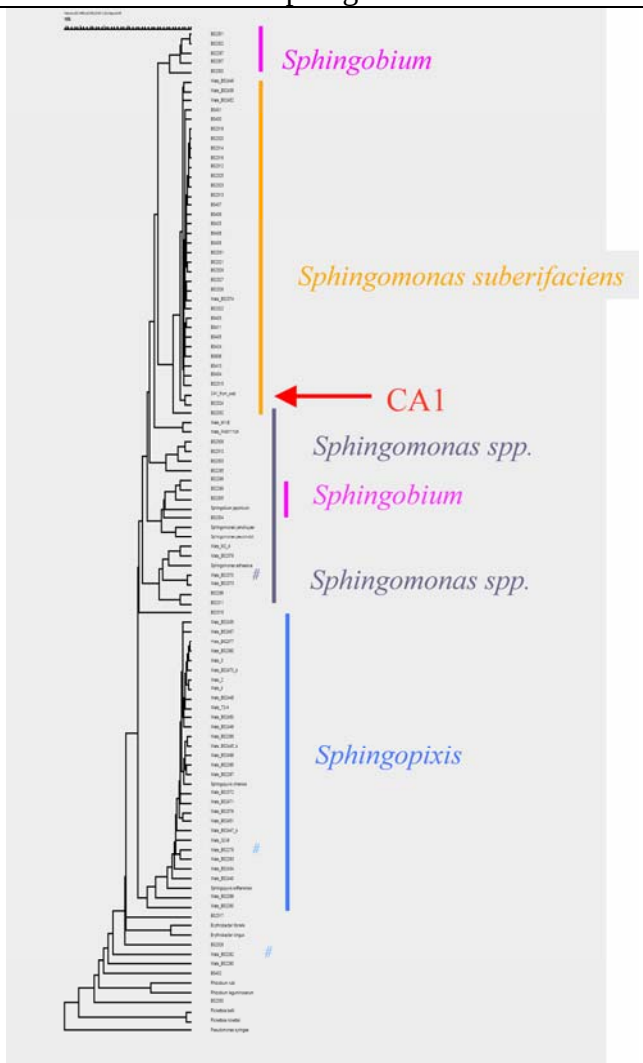
The BOXA1R primer, designed amplify DNA that is repeated throughout the bacterial genome, was used in the polymerase chain reaction on purified genomic DNA (rep-PCR). Amplified DNA fragments were examined by agarose gel electrophoresis and fingerprints generated from different strains were compared visually. Patterns were analyzed for their degree of similarity and dendrograms were generated using Applied Maths BioNumerics software. Multi Locus Sequence Analysis (MLSA) is being used for the final aspect of genome analysis for this project. We have selected the genes we will sequence for the analysis and are developing protocols using published primers for amplification of *rpoD* and *gyrB* genes. MLSA will be completed once all sequences are available. MLSA will help to provide a more robust understanding of the relationships between the *c*BS strains and those previously identified. Additionally these sequences serve as targets for primers that may allow for amplification and quantification of specific groups of pathogens.

Pathogenicity of individual isolates has been examined in growth chamber bioassays conducted either in an open system using Plantel trays or a closed system using Magenta boxes. For assays in Plantel trays, seeds are 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 are evaluated and rated based on the scale developed by Brown and Mitchemore (1988). For assays conducted in Magenta boxes, eight seedlings were established in sterile vermiculite or sterile and untreated *c*BS. Individual bacterial strains or a suspension of *c*BS were used to inoculate sterile vermiculite two to three weeks after planting. The roots were evaluated three weeks after inoculation.

Results and Discussion:

We have sequenced the 16S rDNA for all strains isolated from lesions on resistant plants grown in *c*BS. Of the 72 strains isolated, 41 were members of the Sphingomonadaceae and are being used in further experiments. Only four of the strains isolated were identified as *S. suberifaciens*, although the majority of corky root pathogens isolated previously have been identified as this species. Previously, strains causing corky root were also classified as other undefined *Sphingomonas* spp.. Sixteen of the *c*BS strains were identified as alternative *Sphingomonas* spp.. Additionally, 19 isolates were closest in identity to *Sphingopixis* spp. and two additional strains were identified as *Sphingobium* spp. (Figure 1).

Figure 1. Dendrogram of relationship of 16S rDNA sequences from members of the *c*BS isolates and other members of the Sphingomonadaceae.



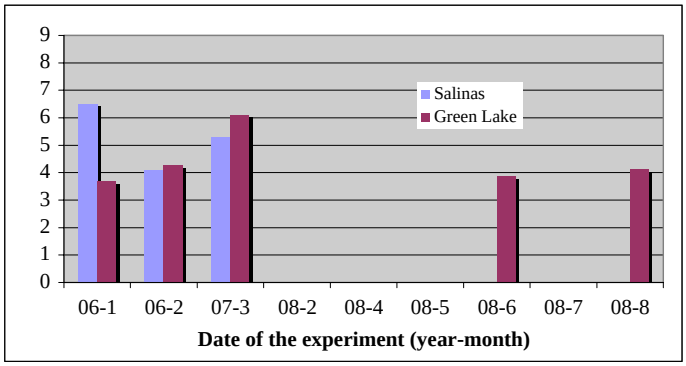
In addition to the *c*BS isolates this figure includes sequences from isolates from around the world (*S. suberifaciens* and *Sphingomonas* spp.) previously by van Bruggen et al.

Analysis of rep-PCR patterns from strains isolated from diseased roots grown in *c*BS, indicate that few strains from *c*BS are related to *S. suberifaciens*. Strain CA1 and other well-characterized *S. suberifaciens* strains grouped separately from most of the *c*BS strains. We are currently analyzing the relationship between the rep-PCR fingerprints and 16S rDNA sequences to determine if rep-PCR fingerprint patterns can predict taxonomic groupings. It would be useful if rep-PCR provided a rapid method for allocating isolated organism to various taxonomic groups.

We are in the process of optimizing amplification of genes for MLSA for the final aspect of genome analysis for this project. In preliminary experiments, we used published protocols and primers in an attempt to amplify *rpo*D and *gyr*B from *S. suberifaciens* CA1. These protocols did not work for *S. suberifaciens* CA1 and we will test other protocols and primers for amplification of this group. MLSA will be completed once all sequences are available.

We began evaluating the virulence of *c*BS strains on resistant cultivars. Initial experiments indicated that several strains were virulent on the resistant cultivars Green Lake or Glacier. We have conducted five experiments in which individual strains were evaluated for pathogenicity. Nineteen of 72 strains have been evaluated in more than one experiment. In at least two experiments, four strains have produced disease symptoms on resistant cultivars that are more severe than symptoms on the negative controls. In one experiment Green Lake inoculated with strain BS2370 had disease levels that were equal to the levels of disease seen on Green Lake grown *c*BS. However, no single strain has consistently resulted in levels of disease similar to those on resistant cultivars grown in *c*BS soil. We are conducting additional experiments to evaluate the remaining strains isolated from *c*BS.

Figure 2. Level of disease conferred by *cor*-breaking soil (*c*BS) on the resistant cultivar Green Lake over time.



Bioassays have been conducted with stored *c*BS for two years. The disease severity has not declined for resistant plants grown in *c*BS, during that time (Figure 2). This indicated that storage of *c*BS did not reduce the pathogens in the soil. Thus, we should be able to continue to use the stored soil for a considerable period of time.

In conclusion, a wide range of organisms have been isolated from lesions of resistant plants grown in the putative *cor*-breaking soil (*c*BS) from Watsonville including a wide range of members of the Sphingomonadaceae. *Sphingomonas suberifaciens* CA1 is the most well characterized corky root pathogen, but few of the isolates from *c*BS resemble this pathogen. The analysis of genetic diversity among the *c*BS isolates and corky root pathogens is facilitating primer design for rapid identification and quantification of these organisms. Ideally the genetic methods will distinguish and quantify the *c*BS strains. However, our biggest challenge has been to correlate these taxonomic groups to the pathogens responsible for breaking of disease resistance. Although we have screened nearly one third of the strains isolated no individual has caused the levels of disease expected on resistant plants. In addition to completing the evaluation of all the organisms isolated, we are considering additional strategies to determine if there is a complex of organisms that are causing disease on resistant plants grown in *c*BS.

Objective 2. *Investigate the resistance identified in PI491239 and PI273597c to corky root.*

As proposed, Objective 2 will be initiated as soon as we have identified the most virulent strains from *c*BS. We will continue to work on this objective in the coming year if individual strains are shown to be responsible for the disease seen in *c*BS.

Objective 3. *Develop molecular detection and quantification methods for various genotypes.*

The goal of this objective is to have rapid detection and quantification methods for various taxa of corky root pathogens. Ideally this will allow us to detect and quantify the pathogens present in *c*BS from new locations. The genetic characterization conducted for objective 1 has been essential in developing these tools.

Procedures:

Sequences of 16S rDNA from all known corky root pathogens and members of the Sphingomonadaceae isolated from lesions on resistant cultivars grown in *c*BS were compared by using the multiple alignment function of Clustal W (Chenna, *et al.*, 2003; Pearson, 1990).

Sequences potentially useful as primers were identified manually and using various primer design software. We designed primers to the different taxa within the Sphingomonadacae (*Sphingobium*, *Sphingomonas suberifaciens*, additional *Sphingomonas spp*. and *Sphingopixis spp*.). In addition to primers designed by our group, we evaluated two sets of previously published primers and amplification protochols that have the potential to amplify a wide range of Sphingomonads (Kim et al., 1989; Leys et al., 2004). In both manuscripts, *Sphingomonas suberifaciens* CA1 was amplified using these methods. For all potential primers, their usefulness was evaluated by *in silico* hybridization (using computer programs to compare primer sequences to the target and non-target genes) and empirically by developing PCR protocols and visualization after electrophoresis.

Results and Discussion:

Results from experiments using protocols and primers designed in our lab were reported (see 2008 report). These primers and protocols were used in preliminary experiments for detection and quantification of *Sphingomonas suberifaciens* in roots using qPCR. We hope to accomplish the same with the new primers being evaluated in the coming year.

We completed *in silico* hybridization studies with previously published primers for the detection and quantification of broad taxonomic groups within the Sphingomonadaceae. After removal of the final G from the forward primer, the first set of primers (Leys et al., 2004) hybridized *in silico* to all but 8 of the corky root isolates from our collection. An additional set of primers hybridized *in silico* to all *Sphingomonas suberifaciens* strains (Kim et al., 1989). We tested the primers for amplification empirically using a range of magnesium concentrations because the magnesium concentration of the reaction conditions was not given. For the Leys et al (2004) primers and protocol, there was poor correlation between the *in silico* hybridization results and the empirical results. The correct size fragment was amplified from four of 13 strains that *in silico* hybridization predicted would be amplified. Additionally, some strains that were predicted to not be amplified by *in silico* hybridization were amplified. The reaction conditions for this protocol need to be optimized and evaluated for the range of organisms that it amplifies. Although a range of magnesium and temperature conditions were tested, the Kim et al., (1989) primers have not amplified DNA from any of the corky root pathogens and further modifications will be made to this protocol.

Objective 4. *Initiate research using confocal microscopy to follow the infection of susceptible and resistant cultivars of lettuce by S. suberifaciens CA1*.

The first task for this objective is to label *S. suberfaciens* strain CA1 with green fluorescence protein (gfp) to allow CA1 to be monitored during plant infection. This should allow us to compare the process of infection by the pathogen in resistant and susceptible cultivars. Differences in the infection process for resistant and susceptible cultivars may indicate targets for disease management.

Procedures:

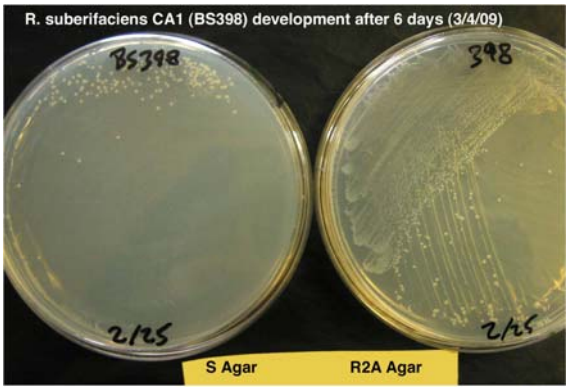
There are several potential methods by which *S. suberifaciens* CA1 may potentially be transformed with the gfp-gene. We initially tried to mobilize the broad host range plasmid pKT-kan-GFP into strain CA1 by electroporation using standard parameters. Concentrations of the plasmid and

recipient as well as electroporation conditions were altered in additional transformation experiments. Transformation was also attempted using triparental matings between *S. suberifaciens* CA1 and *Escherichia coli* strains harboring either pKT-kan-GFP or the helper plasmid pRK2013. *Pseudomonas syringae* pv. *alisalensis* BS91 was used as a control in some transformation experiments.

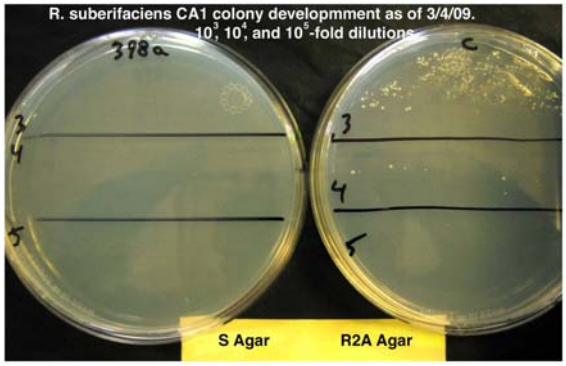
One problem with using *S. suberifaciens* in triparental matings is that it does not grow on most bacteriological media and grows slower than most other organisms on S-agar. We evaluated growth of *E. coli* (used to deliver the gfp-containing plasmid) and *S. suberifaciens* on ten media including some which are regularly used to grow other members of the Sphingomonadaceae (S-agar, TSA, M53, M54, M381, M830, M464, M988, M535, M220, M545; media numbers according to DSMZ). Replicate bacterial suspension of *S. suberifaciens* CA1 were used to streak for single colonies or make serial dilutions that were spread on each medium. Plates were incubated at 28 C and the date of colony appearance and the final populations were recorded.

Figure 3. Growth of *Sphingomonas suberifaciens* CA1 on S-Agar and R2A.

Equal amounts of bacteria streaked for single colonies



Serial dilutions of the same sample



transformation experiments.

In order to facilitate selection of *S. suberifaciens* CA1 transformed with gfp we have generated rifampicin resistant isolates of this strain. An overnight culture of CA1 was concentrated and spread onto media containing rifampicin. Single colonies that grew within two weeks were restreaked and the morphology of the colonies was compared to CA1.

Results and Discussion:

We have tried several protocols for transformation without success although the control organism was transformed using triparental matings. We plan to next use a fluorescently labeled dextran molecule as a control in electroporation experiments. This will allow us to monitor the conditions under which CA1 is transformed by plasmid sized molecules. *S. suberifaciens* CA1 grew significantly better on Reasoner's 2A (R2A) agar than on S-Agar, however growth in S-broth is better than in R2-broth. We will test R2A for selection and support of the triparental matings but continue to use S-broth until we study growth in broth further. In addition, we have generated rifampicin resistant isolates that are putatively *S. suberifaciens* CA1. These isolates will be compared to the wildtype *S. suberifaciens* CA1 for identity, growth and pathogenicity prior to using them in