CALIFORNIA LETTUCE RESEARCH BOARD

2007-2008 Final Report
May 16, 2008

I. Abstract.

Project Title: Development and use of tools to assist breeding for resistance to and management of bacterial leaf spot and corky root of lettuce

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Cooperators:
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Summary:

In collaboration with the USDA/ARS lettuce breeding program we continued progress toward developing lines resistant to bacterial leaf spot caused by *Xanthomonas campestris* pv. *vitians*. In a separate project we previously demonstrated that when cultivars for which resistance to corky root is conferred by a double recessive *cor* allele were planted in soil from a field in Watsonville, they became diseased. This year we demonstrated that PI lines that were resistant to the pathogen in the soil in growth chamber experiments were also the most resistant of the lines tested in the field. This indicates that our growth chamber experiments reflect field results. Of the 72 strains isolated, 25 strains from this soil are related to *Sphingomonas suberifaciens.* An additional 29 strains remain to be identified from our isolations. It is clear that several genetically distinct groups are involved in disease. We are looking for the specific groups that are involved in the resistance breaking in the Watsonville field soil. We are currently conducting experiments to evaluate the pathogenicity of strains isolated from this soil. Initially we worked on developing a method for evaluating individual strains that would reduce contamination issues. In these experiments we also tested several individual stains from the Watsonville soil. Although there have been some problems with the experiments, disease ratings on plants inoculated with several individual strains are higher or as high as those for plants treated with the Watsonville soil. We will finish evaluating pathogenicity on these strains this year. Additionally, we have developed Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR) protocols to detect and measure the populations of the pathogen from soil and roots. We are refining the quantitative PCR but have demonstrated detection and quantification from roots. We are in the process of transforming *S. suberifaciens* CA1 with green fluorescent protein. This will allow us to investigate the infection process in resistant and susceptible cultivars.

Main Body of Report.

Project Title: Development and use of tools to assist breeding for resistance to and management of bacterial leaf spot and corky root of lettuce

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Objectives:
Bacterial Leaf Spot of Lettuce
1) Evaluate and select progeny and breeding lines for resistance to BLS in greenhouse screenings.
2) Evaluate and select progeny and breeding lines for resistance to BLS in field evaluations.
3) Screen F2 and F3 families to determine the inheritance of BLS resistance in Little Gem and Batavia Reine des Glaces

Corky Root of Lettuce
1) Isolate and characterize *Sphingomonas* spp. from roots of several resistant cultivars grown in the putative *cor*-breaking soil (*c*BS).
2) Complete Koch's postulates on strains related to *Sphingomonas* spp. isolated from the roots of resistant plants grown in *c*BS.
3) Continue the development molecular detection and quantification system for the corky root pathogen(s).

PROCEDURES AND RESULTS

BACTERIAL LEAF SPOT

We are continuing to work with the USDA breeding program to evaluate and develop germplasm for resistance to bacterial leaf spot and to determine the mechanisms of resistance. Please see the breeding program report details. We are in the process of evaluating how *Xanthomonas campestris* pv. *vitians* populations respond to resistant and susceptible cultivars of lettuce. This work is not funded by the CLRB but we will inform you of the results.

CORKY ROOT

Objective 1. Isolate and characterize *Sphingomonas* spp. from roots of resistant cultivars grown in the putative *cor*-breaking soil (*c*BS).

We isolated 72 strains from corked roots of resistant cultivars (double recessive for *cor*) grown in the *c*BS. Strains were isolated using the semi-selective medium S-agar + 22 mg/L streptomycin sulfate. We began characterization of an initial 44 of the strains by sequencing and comparing 16S rDNA sequences to previously identified organisms. We are currently obtaining

16S rDNA sequences for the remaining 29 strains. Of the strains already sequenced, seven strains were very similar (> 98%) to *Sphingomonas spp*., nine strains were very similar (>98%) to the closely related genus *Sphingopyxis*, and seven additional strains matched these two genera, but at lower percent similarities. So far we have 25 isolates that appear to be members of the Sphingomonadaceae according to their 16S rDNA sequences. Because of the taxonomic ambiguity of this pathogen, we will test all of these isolates for their ability to cause corky root on resistant lettuce cultivars.

Published research from van Bruggen and coworkers demonstrated that corky root of lettuce was caused by three genetically distinct groups of pathogens related to *Sphingomonas suberifaciens* (formerly known as *Rhizomonas suberifaciens*). The first group is represented by *S. suberifaciens* strain CA1, the second by *Sphingomonas sp.* represented by WI4, and a third group of strains were not similar to either WI4 or CA1. Some of the strains from Australia including AU40, AU45 and AU46 were in this third group. All are suspected to either be genus *Sphingomonas* or at least in the family Sphingomonadaceae. We confirmed these results by demonstrating that strains previously identified as *Sphingomonas spp*. did not group with strains previously demonstrated to be *S. suberifaciens* using phylogenetic analysis of 16S rDNA sequences. The 25 members of the Sphingomonadaceae from *c*BS analyzed to-date fall into groups other than that represented by CA1. Several of the strains were in a group different than the three previously identified genetic groups of corky root causing strains. We used rep-PCR as an additional measure of genetic diversity. Again, *S. suberifaciens* grouped separately from the *c*BS strains. These data coupled with pathogenicity data will allow us to determine if specific genotypes are responsible for the resistance breaking character in the *c*BS.

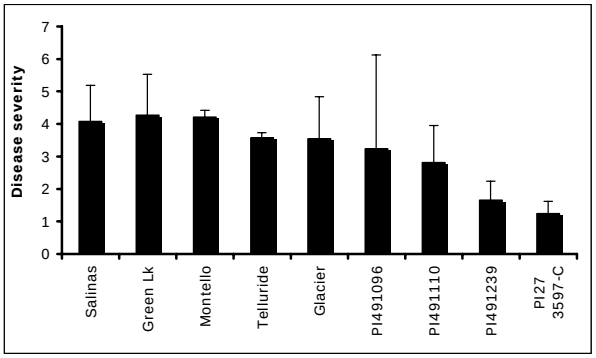
Objective 2. **Complete Koch's postulates on strains related to Sphingomonads isolated from the roots of resistant plants grown in *cor* breaking soil (*c*BS).**

We previously demonstrated and reported (spring 2007) that plants resistant to *S. suberifaciens* CA1 grown in soils collected from one field in the Watsonville area developed corky root (Figure 1A). During this funding cycle we completed a field trial using the same cultivars and compared our results from growth chamber assays to results obtained in the field. We direct seeded five cultivars and four PI lines into the field shown to have *c*BS. In addition, for two of the cultivars, Green Lake and Montello, transplants provided by Headstart Nursery were planted four weeks after seeds were sown. PI lines that were relatively resistant to corky root when planted in *c*BS in greenhouse trials were also resistant in field trials (Figure 1B). The commercial cultivars tested were significantly more diseased than the PI lines in both the field and greenhouse trials. For Montello or Green Lake, transplants did not reduce disease severity in the field. This indicates that transplanting may not be a useful strategy for reducing disease in fields infested with the corky root resistance breaking strains.

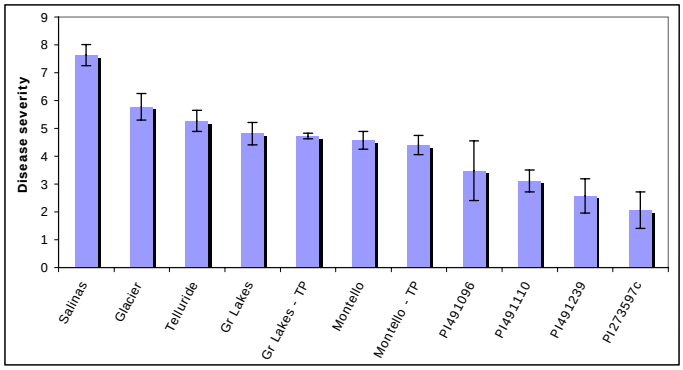
In the previously described greenhouse experiments (reported last year and Figure 1A), seed were sown directly into soil collected from the Watsonville field site. We evaluated the possibility of using less soil by growing seedlings in sterile vermiculite and then drenching them with a slurry made from Watsonville *c*BS ("Wats drench" treatment). In addition to reducing soil volumes, this method has the added advantage of not exposing the seedlings to the pathogen in their first weeks of growth; when planted directly into *c*BS, seeds frequently did not germinate, and some plants died as seedlings. This problem was reduced when using the soil drench

Figure 1. Resistance of cultivars and PI lines to corky root strains in *c*BS.

A. Green house evaluation



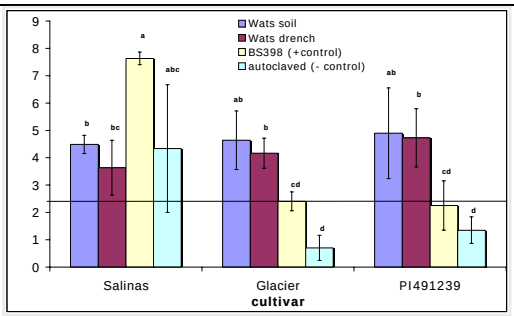
B. Field evaluation



'TP' = transplants. Disease severity in greenhouse and field experiments was rated using Brown and Michelmore (1988) and O'Brien and van Bruggen (1992) rating scales, respectively.

method. The soil drench treatment results were not significantly different than planting directly into soil (Figure 2). As demonstrated previously, there were significantly higher levels of disease on resistant cultivars in the soil or soil drench treatments as compared to an autoclaved soil control or those inoculated with strain *S. suberifaciens* CA1 (BS398). The soil drench method successfully reduced by 85% the amount of soil needed for each experiment, which reduced the amount of *c*BS we needed to collect and store.

Figure 2. Evaluation of soil drench of vermiculite to replace planting and autoclaving soil.

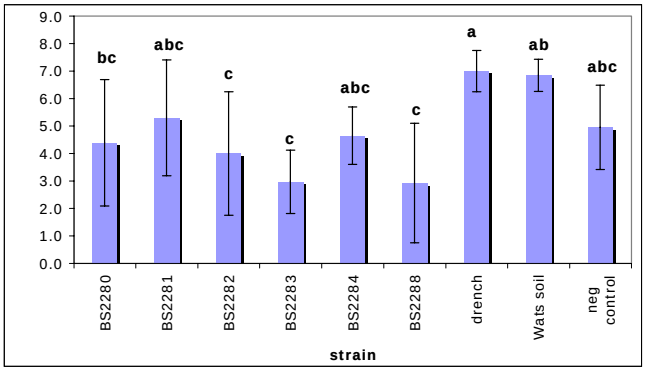


Wats soil = Lettuce seeded directly into *c*BS. Rest of treatments were seeded into autoclaved vermiculite. Wats drench = Inoculated with *c*BS slurry 2 weeks after planting; BS398 = inoculated with *S. suberifaciens* (CA1) 2 weeks after planting; autoclaved = inoculated with an autoclaved *c*BS slurry 2 weeks after planting. Disease severity rated 3 weeks after inoculation using Brown and Michelmore (1988) scale.

We are evaluating the pathogenicity of strains isolated from *c*BS. Resistant cultivar Glacier (double recessive for *cor*) was grown in vermiculite drenched with a slurry made from *c*BS or inoculated with individual strains from *c*BS. For the initial experiment we analyzed the effect six *c*BS isolates (Figure 3A). Disease ratings on roots were not significantly different

among plants treated with three *c*BS strains or plants drenched with or grown in *c*BS soil. However, this experiment needs to be repeated because the negative control (a drench made using autoclaved *c*BS) had high levels of disease. The high disease level in the control may be due to incomplete kill of the pathogen in the soil or contamination. To improve the experiment we now use *S. suberifaciens* CA1 as an additional control since resistant cultivars are resistant to CA1.

Figure 3. Evaluation of disease severity caused by individual strains from *c*BS.

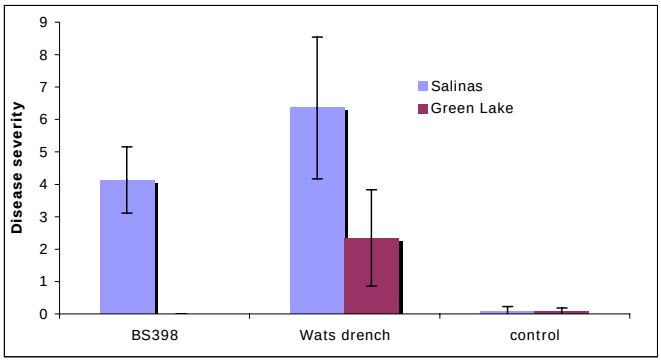


Disease severity rated using Brown and Michelmore (1988) scale.

In an additional attempt to improve the experiment, we compared two methods to reduce contamination. These methods involved different planting strategies including either 1) planting in Magenta boxes which allow for isolation of treatments from each other, or 2) a method developed by S. Klosterman of inoculating plants suspended in liquid in Falcon tubes. The Falcon tube method did not work for evaluating corky root but the method using Magenta boxes reduced contamination. In the Magenta box experiment there was no disease in the uninoculated controls, as expected (Figure 4). There was disease on Salinas but not Green Lakes inoculated with the CA1 control (BS398). There was significant disease on both Salinas and Green Lakes inoculated with the slurry of *c*BS (Wats drench). This indicated that the Magenta box method would provide a sterile environment and good method for evaluating individual strains.

We began large-scale testing of the strains isolated from *c*BS strains using the Magenta box method (Figure 5A). The first experiment was designed to evaluate 19 strains and 6 control treatments but the experiment failed due to poor germination. We repeated this experiment and inoculated two week-old seedlings and evaluated disease three weeks later. There were two inconsistencies with the experiment (Figure 5B). First the drench treatment, in which a slurry of *c*BS soil was used to inoculate the seedlings in vermiculite in the Magenta boxes, did not result in the high levels of disease seen in other experiments. Secondly the negative controls had disease. In spite of these problems with the controls, several strains from the *c*BS soil caused significant disease on the resistant cultivar Green Lakes. Three strains had corky root ratings

Figure 4. Evaluation of potential of Magenta box method for evaluation of corky root breaking.



Disease severity rated using Brown and Michelmore (1988) scale.

numerically greater than the ratings on Green Lakes inoculated with CA1. We have planted the next experiment using the same method with changes in the amount of liquid present in the system. The saturated water status in the Magenta box experiments to date may have predisposed the seedlings to non-target diseases and affected our results. If these changes do not produce the results in the controls that we anticipate, we will return to the Plantel cell method to complete the testing of these strains.

Figure 5A. Magenta boxes planted with inoculated seedlings.

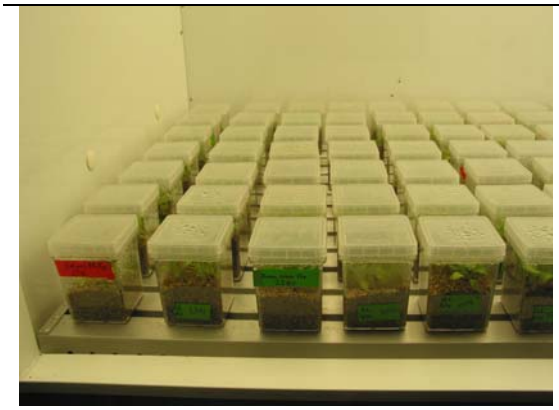
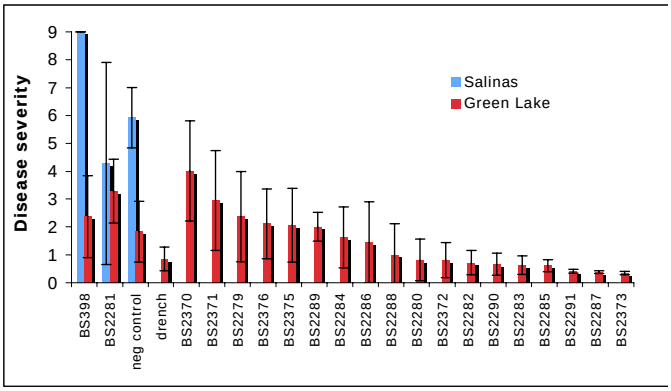


Figure 5B. Disease severity on plants inoculated with individual strains from *c*BS.



Disease severity rated using Brown and Michelmore (1988) scale.

Objective 3. Continue the development of molecular detection and quantification systems for the corky root pathogen(s).

We have made progress toward developing methods to study the ecology of *Sphingomonas suberifaciens* and the infection process in resistant and susceptible hosts. We are

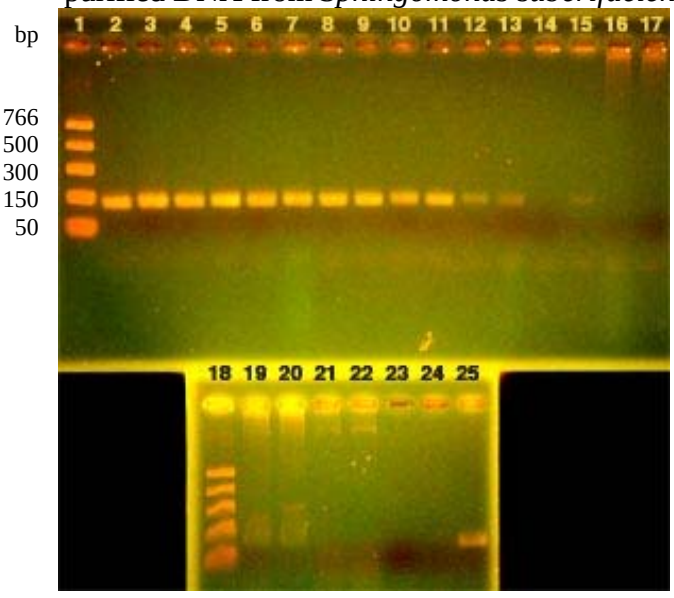
using two separate approaches. The first involves the development of PCR methods that will quantify the numbers of various *Sphingomonas suberifaciens* populations present in soils. The second approach will allow us to visualize the pathogen during infection and help us to understand the infection process in susceptible and resistant cultivars.

PCR and Q-PCR detection and quantification.

We optimized the PCR protocol for detection using primers, 665F/773R. The final protocol uses touchdown PCR, starting with 94° for 1 min and then the annealing temperature for 30 sec. The annealing temperature starts at 68°C and drops over 8 cycles to 62°C. This is followed by 18 cycles using the final 62° annealing temperature. After optimization we evaluated the protocol for detection of corky root strains. To-date, this PCR protocol amplified bands from strains originally identified as "*Sphingomonas suberifaciens.*" These primers did not amplify bands from strains designated as other species (originally "*Sphingomonas* spp."). Additionally, none of the members of the Sphingomonadaceae from *c*BS tested were amplified by this protocol and this is understandable considering they may be different species and potentially different genera. Thus, the protocol should be able to specifically monitor *S. suberifaciens* populations. Development of primers specific to each biologically relevant group will allow us to: monitor the spread of the more virulent populations, determine how current cropping practices influence various populations in the soil, and evaluate how chemicals applications influence various populations.

We developed a QPCR protocol using the regular touchdown PCR as a starting point. The final QPCR protocol starts with 8 cycles of 95° for 30 seconds followed by an annealing temperature of 64° for 30 seconds; after 8 cycles, the annealing temperature drops to 61° for 28 more cycles. Using the Biorad iQ SYBR Green Supermix (Biorad #170-8880), we have been able to clearly detect down to 100fg of purified BS398 (CA1) DNA, which represents between 17 and 29 cells/sample. Continued optimization may improve this detection limit.

Figure 6. Detection and quantification of purified DNA from *Sphingomonas suberifaciens*.



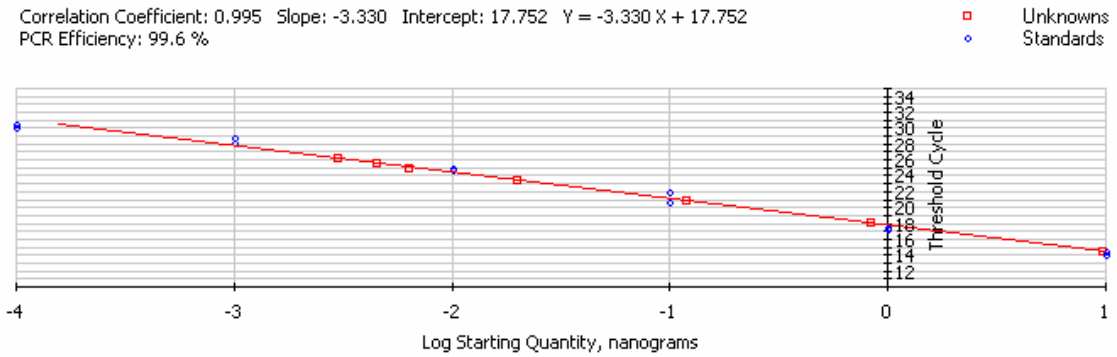
Agarose gel of final QPCR products. Expected product is 130 bp. Lanes 1 and 18, DNA ladders; lanes 1-17 purified BS398 (CA1) DNA: 10ng (lanes 2 and 3); 1ng (4, 5); 100pg (6, 7); 10pg (8, 9); 1pg (10, 11); 100fg (12, 13); 10fg

(14, 15); 1fg (16, 17). Lanes 19-24 negative controls: *P. syringae* pv. *alisalensis* (19, 20) ; *Xanthomonas campestris* pv. *vitians* (21, 22), and *Myxococcus xanthus* (23, 24). Lane 25 positive control (10ng CA1).

Next we needed to determine whether this protocol could detect *S. suberifaciens* in mixed samples of DNA isolated from biological materials. We conducted a preliminary experiment (with one replication per treatment) with two primary goals: 1) to determine if materials present in root DNA extractions would inhibit the QPCR, and 2) to determine whether we could use the QPCR on DNA extractions to successfully detect the pathogen in samples in which it was clearly present, while at the same time not detecting the pathogen in negative samples. For this experiment, 3-week old Salinas seedlings were inoculated with *S. suberifaciens* (CA1). After 3 additional weeks of incubation, the roots were cleaned, rated for disease, and ground in liquid nitrogen. DNA was isolated using DNeasy Plant Mini Kits (Qiagen #69104). Controls were uninoculated Salinas seedlings, Green Lake (a resistant cultivar) seedlings inoculated with CA1, both cultivars inoculated with a strain from *c*BS (BS2281) that is not expected to amplify, and both cultivars exposed to CA1 immediately prior to DNA isolation.

To address our first goal, DNA extractions of uninoculated Salinas roots were added to a QPCR dilution series of purified DNA from CA1. The samples in this series amplified at the expect rate at each concentration, falling very close to the points on the standard curve generated with purified DNA alone (Figure 7) and thus demonstrating that extraneous materials from the extraction of DNA from lettuce plants did not inhibit the QPCR.

Figure 7. Standard curve with purified DNA alone and with added DNA from uninoculated cv Salinas roots.



The circles represent the standard curve consisting of amplicons from purified DNA in a dilution series. The squares represent the same dilution series added to DNA extracts from cv Salinas roots.

The second goal of this preliminary experiment was to determine if the DNA extraction procedure coupled with the QPCR reaction could detect the pathogen from root samples in which it was clearly present. The concentration of the pathogen in the samples in which it was clearly present (treatments 1, 9) were numerically higher by at least 100 fold than from samples that were negative controls (treatments 3, and 7; Table 1). The concentration of the pathogen was also higher (by at least 100 fold) in samples from cv Green Lakes in which the pathogen could be hypothesized to be present (treatment 2) compared to negative controls (treatments 4, and 8).

Development of GFP strains of S. suberifaciens

We are progressing toward establishing strains of *S. suberifaciens* CA1 that produce the green fluorescent protein (GFP). The presence of the GFP will allow us to visualize the infection

process. We received approval from the USDA/ARS Institutional Biosafety Committee on January 24, 2008 for transforming *S. suberifaciens* CA1 with plasmids that confer GFP to bacteria. We have made two attempts to transform *S. suberifaciens* with these plasmids. For the two attempts we used published cell preparation and electroporation methods for *E. coli*.

For both we used a BioRad Micropulser electroporator with a Gene Pulser Cuvette (0.1 cm path length). The proposed transformant would be streptomycin resistant as is CA1 and kanamycin resistant conferred by the plasmid. In the first experiment we electroporated with a single 1.8kV pulse for 5 msec. When this didn't result in any transformants we modified the protocol to encourage more rapid bacterial growth; increased the stringency of the preparation conditions to make the bacteria more susceptible to transformation; and increased the pulse to 2mV. No clones were derived that were streptomycin and kanamycin resistant.

One problem we have had is getting the cells to grow quickly enough to insure that the cultures are in log phase. We will be evaluating different media to see which culture conditions provide the most rapid growth rates. A second issue is the method of cell preparation and electroporation. We will test additional methods and use an *E. coli* as a control for transformation. Lastly because the plasmids are broad host range plasmids, they should be suitable for transformation of *S. suberifaciens*. However, there is a slight likelihood that *S. suberifaciens* is not a host for these plasmids. We will contact other researchers working with *Sphingomonas* spp. and attempt to get plasmids that have worked in the past with this genus.

Table 1. QPCR amplification of *Sphingomonas suberifaciens* from plant roots

Treatment	Hypothesis	Disease status (visual)	Threshold Cycle	Sample DNA concentration
1) cv Salinas inoculated with *S. suberifaciens* CA1 three weeks before sampling	Expected to amplify	Diseased	21.0	0.1 ng
2) cv Green Lakes inoculated with *S. suberifaciens* CA1 three weeks before sampling	Unknown	Not diseased	23.8	0.02 ng
3) cv Salinas inoculated with BS2281 three weeks before sampling	Not expected to amplify	Not diseased	27.5	0.002 ng
4) cv Green Lakes inoculated with BS2281 three weeks before sampling	Not expected to amplify	Not diseased	29.2	0.0004 ng
7) cv Salinas negative control (These data are the concentrations of the original sample back calculated from the results of a QPCR on a 1/10 dilution because the original was contaminated during PCR)	Not expected to amplify	Not diseased	28.2	0.007 ng
8) cv Green Lakes negative control	Not expected to amplify	Not diseased	29.9	0.0003 ng
9) cv Salinas positive control, *S. suberifaciens* CA1 added to extraction mixture	Expected to amplify	Not diseased	15.4	5.6 ng