EVALUATION OF GRAPEVINE ENDOPHYTIC BACTERIA FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT

In this reporting period we optimized our antagonism assay using our previously known antagonists. We found that a *Xylella fastidiosa* (*Xf*) lawn with a concentration of 10^5- 10^6 cfu/ml is optimal for visualizing zones of inhibition. Ten new isolates were shown to have antagonistic capability, including *Bacillus subtilis*, *Pseudomonas* sp., and *Pantoea agglomerans*.

Fourteen isolates chosen for their antagonistic or competitive ability *in vitro* were assayed *in planta* for systemic movement. Of these isolates, six were able to move upwards 30cm from the point of inoculation.

We continued our evaluation of Dr. Darjean-Jones' initial biological control experiment started in 2003. During fall of 2005, xylem sap extracted from these vines with a pressure bomb yielded approximately 220 new isolates. Of these, 169 are from vines inoculated with isolate #169 *Bacillus subtilis* and isolate #161*Bacillus* sp. Eighty-five of our new isolates were sequenced and 44 of these are *Bacillus* sp. Nine isolates had the same sequence as the *Bacillus* species that was originally inoculated into the vines. In February, bud-wood from Dr. Darjean-Jones' biological control experiment was collected from each treatment and propagated in the greenhouse. Ten vines per original treatment along with young Cabernet Sauvignon vines purchased from a nursery were inoculated with Stagg's Leap strain of *Xf* in mid July, 2006. At 14 weeks post inoculation these vines will be assessed for *Xf* infection using Immunocapture (IC)-PCR and rated for Pierce's disease symptom severity.

A new biological control experiment was started in the greenhouse this spring. This experiment is comprised of seven endophyte treatments in 15 Thompson seedless vines per treatment. Vines were mechanically inoculated with the endophytes and 6 weeks later they were inoculated with Stagg's Leap *Xf.* *Xf* infection and symptoms will be assessed after 14 weeks. We are testing the protective capabilities of three *Bacillus* sp. strains, three *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomonas* sp.

INTRODUCTION

The environment inside grape vine xylem vessels is a distinct ecological niche that supports a sparse microbial community. *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD), is one possible inhabitant. But our research, as well as work done in Nova Scotia reveals a diversity of other bacterial species capable of surviving in grape xylem (Bell et al., 1994). Endophytes are microbial organisms that do not visibly harm the host plant but can be extracted from surface sterilized tissue (Hallman et al., 1997). Some bacterial endophytes have been proven beneficial to plant health and are used to promote growth or as biological control treatments for fungal and bacterial pathogens. Previous researchers in our lab isolated an extensive library of endophytes collected from healthy grapevines, PD-infected vines, and asymptomatic vines in areas of high PD incidence (escape vines). We hypothesize that some of these bacterial endophytes may be antagonistic or compete with *Xf* for nutrients. Our library includes endophytes, such as *Pantoea agglomerans* and *Pseudomonas* sp., already tested as biological control agents in other crop systems (Stockwell et al., 2002, Barka et al., 2002). At this time our assays have yielded three additional endophytes that are antagonistic to *Xf in vitro* and are also capable of moving systemically within the grapevine. In this reporting period we have confirmed previous results and streamlined our *in vitro* antagonism procedure. Current PD management practices primarily involve keeping vector numbers low and removing infected vines. Biological control utilizing a systemic bacterial endophyte would be an implementable and environmentally desirable solution to this problem.

OBJECTIVES

1. Finish screening our existing library and recently acquired grape endophytic bacteria to identify potential antagonists of *Xf*.
2. Determine if *Xf*-antagonistic endophytes can systemically move in grapevines.
3. Evaluate the biocontrol abilities of endophytes against *Xf* including
   i) prevention of infection
   ii) suppression of Pierce's disease symptoms in greenhouse and field studies
   iii) long term health and survival of infected vines in the field
4. Isolate additional endophytes from escape vines and characterize these for antagonistic traits.

RESULTS AND CONCLUSIONS

Optimization of Antagonism assay

During this reporting period we optimized our procedure for the *in vitro* antagonism assay. Briefly, seven day old liquid cultures of *Xf* Temecula or our *Xf* hemagglutinin mutant (HxfA) which forms a confluent "lawn" of cells on solid medium are centrifuged and cells are re-suspended in PD3 media to a spectrophotometer OD 600 reading of 0.1-0.13. The resulting suspension contains 10^5– 10^6 cfu/ml. One hundred micro liters of this suspension are spread onto PD3 solid medium and incubated for 4 days. Each endophyte isolate, stored in glycerol solution at -80C, is streaked onto the media in which it was first isolated. The next day, one loop-ful of each overnight endophyte culture is suspended in sterile water. Three, 5µl droplets of each suspension are placed onto the 4 day old *Xf* lawns and allowed to dry. Endophyte inoculated plates are incubated for 5-7 days and then evaluated for zones of inhibition surrounding the endophyte colony.

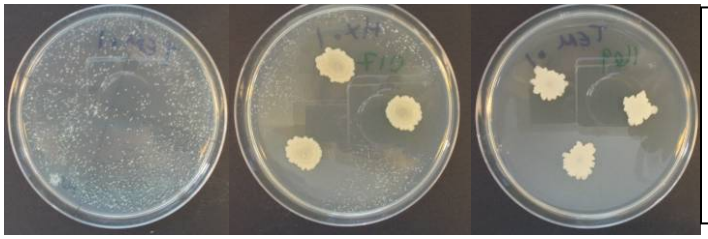
During this period we screened approximately 100 new isolates from our existing library and from our new additions collected during fall 2005. The optimized screening protocol is more efficient than our previous protocol and we expect to finish the library evaluation by December 2006. Table 1 summarizes our current results with 17 isolates that show some degree of *Xf* inhibition *in vitro*. Strains of *B. subtilis* and *Pseudomonas* have shown the greatest antagonism *in vitro*. Isolates #4, #11, and #37 have been included on the table even though their inhibition zones are smaller because *Pantoea agglomerans* strains have been successfully used in biocontrol research (Stockwell et al., 2002), and may be good candidates for further study in this project.

In addition to the antagonism assay we have started evaluating each isolate showing some degree of inhibition on crystal violet polypectate media (CVP). A positive result on this media indicates pectin degradation. It is possible that isolates capable of pectin degradation may also be able to degrade pit membranes connecting xylem vessels and be able to move systemically within the vine.

Table 1. Bacterial isolates screened in 2005-2006 showing some degree of antagonism toward *Xf*.

Endophyte	Identification	Zone of clearing (a)
169	*Bacillus subtilis*	16-20mm
69	*Bacillus subtilis*	complete
17	*Bacillus subtilis*	7-15mm
197	*Pseudomonas virdiflava*	20mm-complete
393	*Pseudomonas virdiflava*	rg over entire plate
403	*Pseudomonas syringae*	complete
205	*Pseudomonas sp.*	complete
329	*Pseudomonas sp.*	complete
100	*Bacillus subtilis*	15-20mm
147	*Bacillus subtilis*	rg over entire plate
11	*Pantoea agglomerans*	rg 3-8mm
37	*Pantoea agglomerans*	rg 1-2mm
4	*Pantoea sp. (Erwinea sp.)*	rg over entire plate
W157 (b)	*Bacillus pumilus*	rg 6-10mm
154	*Bacillus subtilis*	8-12mm
139	*Bacillus sp.*	rg 10-15mm
200	*Pseudomonas sp.*	complete

(a) zone attained on lawn plates with *Xf* concentration of 10^5-10^6 cfu/ml.
(b) "W" indicates an isolate collected October 2005 from our 2003 biocontrol experiment in the field.
rg = reduced growth in these areas, ie. *Xf* colonies aren't cleared but are much smaller compared to controls



A. Temecula *Xf* lawn control plate
B. Isolate 17 on a lawn of HxfA showing zones of inhibition
C. Isolate 169 on a Temecula plate showing almost complete inhibition

A B C

Figure 1. *In vitro Xf* Antagonism assay.

Assessment of endophytes' ability to colonize and move systemically in grape xylem

Fourteen isolates exhibiting good antagonism or competition *in vitro* were assessed for their ability to move systemically in grape xylem. Two Chardonnay vines per isolate were pinprick inoculated with an endophyte suspension of approximately 10^8 cfu/ml. Mechanical pinprick inoculation with the endophyte suspension is similar to mechanical inoculation of *Xf*: a 20 µl drop of suspension is pipetted onto the stem and the drop is punctured with a needle 1-5 times until the drop is sucked into the stem. We inoculated plants on two places on the stem near the 3rd or 4th internode from the graft junction. Plants were inoculated once and then again three days later to ensure success. After seven weeks, four, 2 cm sections including the point of inoculation (POI), first petiole after the POI, 9-11 cm, and 28-30 cm from the POI, were cut from the vine and cultured. Each 2 cm section or petiole was surface sterilized in 10% bleach, 70% ethanol, and three washes in sterile water for one minute. The section was then put into a sterile grinding bag with 2 ml phosphate buffered saline (PBS) and pulverized. One hundred micro liter aliquots of this solution were plated onto solid media of the same type on which the endophyte was first isolated. Total colonies that were morphologically similar to the endophyte were counted and recorded. Representative colonies morphologically resembling the original endophyte at the POI and 28-30 cm were sequenced to confirm their identities. Out of 14 isolates tested for systemic movement, 5 were able to move past the point of inoculation and up to the 28-30cm section. Isolates capable of moving 30cm or into the petiole, are likely capable of degrading pectins in the pit membranes connecting xylem elements, and would thus be able to colonize the entire vine over time.

Table 2. Endophytes that possessed some ability to systemically colonize Chardonnay vines. Numbers reflect cfu/ml resembling original isolate in each stem section.

Isolate	POI	Petiole (a)	9-11cm	28-30 cm
W121	1.00×10^6	3.00×10^5	5.25×10^6	3.00×10^5
69	2.60×10^5	128	270	69
169	6.37×10^5	lawn	7.90×10^4	3.50×10^3
17	3.07×10^5	0	1.20×10^4	1.09×10^3
11	5.99×10^5	9.50×10^4	3.35×10^4	2.89×10^3
Agro556 (b)	6.07×10^5	1.66×10^5	2.55×10^5	10

(a) First leaf petiole up from the POI.
(b) *Agrobacterium vitis* 556 is an avirulent strain obtained from Thomas Burr.

Continuing evaluation of biocontrol experiment initiated in 2003

During September and October 2005, xylem sap from vines in Dr. Darjean-Jones' original biocontrol experiment, was extracted with the pressure bomb technique as described in previous reports. These vines had been in the field for two years. Approximately 220 bacterial isolates were streaked to purity and then frozen at -80C for further characterization. Of these, 176 isolates were isolated from vines originally inoculated with a putative *Cellulomonas* species (#169) or *Bacillus* species (#161). In the 2003 experiment these two isolates best protected vines from developing PD symptoms in the field. Last fall we re-sequenced #169 and found that the putative *Cellulomonas* isolate was in fact a *Bacillus subtilis* strain. Because three different researchers have worked independently on this project, we cannot be sure when the misidentification occurred. It is possible that Dr. Darjean-Jones' vines were inoculated with *Cellulomonas* and the isolate has been lost, or her vines were initially inoculated with *B. subtilis*. However, our current research shows that isolate #169, *B. subtilis*, has strong antagonistic ability *in vitro* and moves well within the vine, properties that were originally reported for the *Cellulomonas* strain #169.

We also wanted to determine, if after 2 years in the field, the original endophytes used for the experiment could be re-isolated. Out of 220 newly isolated samples, 122 of these were extracted from vines originally treated with strain #169. The 16S rDNA of 45 of these was sequenced. Twenty-five were identified as *Bacillus* species and three were specifically identified as *Bacillus subtilis*. While it is possible we were able to re-isolate some of the original #169 endophyte strain, we

also isolated *Bacillus subtilis* strains from field grown vines that were not originally inoculated with #169. Out of 54 isolates from vines originally treated with *Bacillus* sp (#161), 20 were sequenced, and six were identified also as species of *Bacillus*. We will do more sensitive genetic typing of these isolates using rep-PCR to determine if the *Bacillus* strains that were isolated from endophyte inoculated vines are the same as those that were originally inoculated in 2003. We expect to finish characterizing the other isolates this winter 2006-2007.

To determine if these vines were still protected against *Xf*, bud wood cuttings from these vines were propagated in the greenhouse this spring and challenged with *Xf*. Cuttings were taken from all vines in the field testing negative for *Xf* infection by IC- PCR. Each cutting had 2-4 buds and was rooted in a plastic callus chamber for 6 weeks. Cuttings with callus formation were grown in the greenhouse and trained to a single shoot for 3 months. Ten propagated vines representing each original endophyte treatment were then mechanically inoculated with Stagg's leap strain of *Xf*. Stagg's leap strain was chosen because it is one of the more virulent *Xf* strains. Nursery grown Cabernet Sauvignon plants were used as positive and negative controls. These plants will be evaluated for *Xf* infection with IC-PCR, rated for symptoms at 14 weeks post inoculation, and planted in the field in spring, 2007. Final results from this experiment should determine if our propagated cuttings or the original vines retain their resistance to PD.

New Biological control experiment for 2006

This year we started a new biological control experiment consisting of eight different endophyte treatments. Each treatment consists of 15 Thompson seedless vines inoculated with endophyte suspensions or H_2O controls in the same manner as movement assay vines. We are testing 3 *Bacillus* isolates, 3 *Pseudomonas* isolates and one co-inoculation of 2 *Pseudomonas* species. Six weeks after endophyte inoculation, the vines were inoculated with Stagg's leap strain of *Xf*. After 14 weeks or when PD symptoms appear these vines will be evaluated for *Xf* infection with IC-PCR and rated for symptoms. During spring of 2007, surviving vines will be planted out in the Armstrong field plots at UC Davis, California.

Isolate additional endophytes from escape vines in the field

This spring we sampled xylem sap from 10 escape vines in Napa vineyards. The first sampling took place in late April 2006 when vines were "bleeding". Sap was collected in a 40 ml Falcon tube and directly plated onto 523 and PD3 media. Resulting colonies were streaked to purity and then stored in glycerol at -80C. In June we sampled the same vines and extracted xylem sap with the pressure chamber. All isolates have been cataloged and will be tested for antagonism later this winter.

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IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA*- INFECTED GRAPEVINES

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ABSTRACT
Pierce's disease does not occur in colder regions of North America. Although the "cold curing" phenomenon has been well documented, little is known about the physiological mechanisms that mediate cold therapy. To better understand the cold therapy phenomenon, we planted control and *Xylella fastidiosa (Xf*) infected *Vitis vinifera* "Pinot Noir" (PN) and "Cabernet Sauvignon" (CS) grapevines across 4 locations in Northern California and exposed control and infected grapevines across 4 temperature conditions in cold rooms. After treatment periods, xylem sap was extracted using a pressure bomb and the composition of the sap was analyzed for pH, osmolarity, glucose, sucrose, fructose, Ca^{2+}, and Mg^{2+}. Differences were found across the different field locations and cold rooms. Similar results for CS inoculated vines were found for the cold room experiments. Results for the PN field plots revealed differences in pH for both inoculated and control vines across the field plot locations. Osmolarity values of PN control vines were different across the field plot locations and in the cold room treatments.

Effects of buffer and xylem sap on the survival of *Xf* and various cold temperatures were reported in the 2004 PD/GWSS Proceedings. Abscisic acid (ABA) levels are elevated in many cold-treated plants and ABA has been shown to induce the synthesis of certain pathogenesis related (PR) proteins that in some case possess anti-fungal properties. However, we are proceeding with experiments to determine if exogenous applications of ABA on non-chilled grapevines can elicit PR proteins.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes Pierce's disease (PD) in grapevines. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. Purcell (1977, 1980) demonstrated that relatively brief exposures to sub-freezing temperatures eliminated *Xf* in cold treated *Vitis vinifera* grapevines. Purcell also found that a higher percentage of grapevines that were moderately susceptible to PD such as 'Cabernet Sauvignon', were cured by cold therapy treatments compared to susceptible varieties such as 'Pinot Noir.' More recently, Purcell's group also showed that whole, *Xf* infected potted vines exposed to low temperatures had a higher rate of recovery than PD-affected detached bud sticks exposed to the same cold temperatures (Feil, 2002). This implies that some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Despite documentation of the cold curing phenomenon, little is known about the physiological/biochemical basis that mediates cold therapy. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate Xf. If such a factor(s) is/are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

OBJECTIVES
1. Develop an experimental, growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osomolarity, total organic acids, proteins and other constituents that occur in the xylem sap of cold-treated versus non- treated susceptible and less susceptible *Vitis vinifera* varieties.
3. Assess the viability of cultured *Xf* cells growing in media with varying pH and osomolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

RESULTS
Objective 1
Using the same varieties used in our 2004-2005 field and cold room studies (PD vs. temperature treatment results were reported in the 2005 Pierce's Disease Symposium Proceedings), Pinot Noir (PD-susceptible) and Cabernet Sauvignon (moderately resistant to PD) grapevines were prepared as described in the 2005 Pierce's Disease Symposium Proceedings. In October/November 2005, prepared grapevines were transported to 4 sites that were selected because of their varying winter temperatures. Sites included: UC Hopland Research Station (Mendocino County), McLaughlin Reserve (Lake County), Foresthill (Placer County), and UC Davis (Yolo County).