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

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ABSTRACT
In this reporting period we finished testing selected endophyte isolates for antagonism with our *in vitro* assay. Isolates of *Bacillus pumilus, B. subtilis, Pantoea sp.,* and *Pseudomonas sp.* were the most effective inhibitors. In this year's movement assays we identified one more isolate, *Kocuria* sp, that could move into grape petioles. We subsequently used this isolate as one treatment in the mechanical inoculation part of the 2007 biological control experiment. While Pseudomonas species were good inhibitors, none were capable of moving past the point of inoculation.

Results were obtained from two biological control experiments completed in December 2006. We found that our vines in the field, originally part of the 2003 biological control experiment did not retain endophyte protection. All vines developed severe Pierce's Disease (PD) symptoms when challenged with X*ylella fastidiosa* (*Xf*). In the 2006 biological control experiment, we tested the protective capabilities of 3 *Bacillus* sp. strains, 3 *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomona*s sp. After 15 weeks post challenge with *Xf*, all vines across all treatments developed PD symptoms and tested positive for *Xf* infection. Based on this experiment and previous research in our lab, results suggest that mechanical, rather than insect, inoculation may overwhelm endophyte protection.

Our biological control experiment established in 2007 evaluates five endophtytes for their protective abilities against *Xf* in the vine: *Kocuria* sp., *Bacillus subtilis, B. cereus, Curtobacterium flaccumfaciens*, and *Pantoea agglomerans*. Endophyte treatments were split into two groups, those challenged with *Xf* by mechanical inoculation and those challenged by infective blue green sharpshooters (BGSS). We mechanically inoculated with 10^4 *Xf* cell suspensions so as not to overwhelm endophyte treatments. Symptoms have been rated on a weekly basis to compare disease progression and petioles have been cultured to examine if any endophyte treatment is capable of reducing *Xf* concentrations in the vine. Initial results indicate that both mechanical and insect inoculations were efficient with no fewer than 11 out of 15 plants infected. Concentration of *Xf* in petioles is similar among endophyte treatments and controls.

INTRODUCTION
The environment inside grape vine xylem vessels is a distinct ecological niche that supports a sparse microbial community. *Xf*, the causative agent of PD, is one 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). Some of these bacterial endophytes are antagonistic against *Xf in vitro* and may be niche competitors within grapevine xylem. Our library includes endophytes, such as *Pantoea agglomerans* and several *Pseudomonas* sp., already tested as biological control agents in other crop systems (Stockwell et al., 2002, Barka et al., 2002). We have also isolated strains of *Curtobacterium flaccumfaciens*, which is potentially associated with the control of citrus-variegated chlorosis, caused by a genetically different strain of *Xf* (Lacava, et al, 2004). While several endophyte isolates were capable of inhibiting *Xf* growth in vitro, those able to systemically colonize the vine were rarer. Furthermore, our results suggest that *Xf* inoculated at high concentrations may out-compete endophytes and overwhelm any biological control they might provide. In this year's biological control experiment we sought to achieve lower concentrations of *Xf* in endophtyte treated vines by using infective BGSS to inoculate some groups of plants and use lower *Xf* concentrations for mechanically inoculated vines. We also hoped to achieve better endophyte colonization of vines by using multiple inoculations. 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.

a. prevention of infection.
b. suppression of Pierce's disease symptoms in greenhouse and field studies.
c. 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

Conclusion of Antagonism assays The optimized screening protocol developed in the last reporting period was more efficient and during this reporting period we concluded antagonism assays on our endophyte library. This library included bacteria isolated from vines used for the first biological control experiment in 2003, new isolates collected spring 2006 from escape vines in Napa, and isolates collected in 2000-2002. In total 124 new isolates were tested in this reporting period. Fifty eight of these showed some ability to clear or reduce *Xf* growth and 34 of these isolates were identified as *Bacillus subtilis* by 16S rDNA sequencing or morphology. Isolates of *Bacillus subtilis*, various other *Bacillus* species, *Pseudomonas* sp, and *Pantoea* sp. showed the largest zones of inhibition in the antagonism assays (Table 1).

Table 1. Summary of representative bacterial isolates screened in 2005-2007 showing some degree of *Xf* inhibition.

Endophyte Isolate #	Identification	Zone of clearing[a]	Endophyte Isolate #	Identification	Zone of clearing[a]
Average 38 isolates	*Bacillus subtilis*	2mm- complete inhibition	200	*Pseudomonas sp.*	complete
197	*Pseudomonas viridiflava*	20mm-complete	11	*Pantoea agglomerans*	rg 3-8mm
393	*Pseudomonas viridiflava*	rg over entire plate	37	*Pantoea agglomerans*	rg 1-2mm
403	*Pseudomonas syringae*	complete	4	*Pantoea sp.*	rg over entire plate
N37[c]	*Pseudomonas syringae*	complete	W157[b]	*Bacillus pumilus*	rg 6-10mm
205	*Pseudomonas sp.*	complete	843	*B. pumilus*	8mm rg
329	*Pseudomonas sp.*	complete	139	*Bacillus sp.*	rg 10-15mm
168	*Paenibacillus sp.*	4mm rg	473	*Stenotrophomonas sp.*	4mm rg
177	*Paenibacillus sp.*	5mm			

[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.
[c] "N" indicates an isolate collected from escape vines in Napa spring 2006
rg = reduced growth in these areas, ie. *Xf* colonies aren't cleared but are much smaller compared to controls

Assessment of endophytes' ability to colonize and move systemically in grape xylem Isolates used in the 2007 movement assays included, *Bacillus megaterium,* three isolates of *Curtobacterium sp.,* two isolates *B. Subtilis*, two isolates of *Pantoea agglomerans*, *Stenotrophomonas* sp., *Bacillus pumilus, Pseudomonas syringae, Kocuria* sp., *Bacillus cereus*, and *Paenibacillus* sp (Table 2). Two Chardonnay vines per isolate were inoculated at two places on the stem near the third or fourth internode, reinoculated three days later, and then allowed to grow in the greenhouse. After seven weeks, movement past the point of inoculation (POI), was measured by culturing stem sections onto solid media. Four of the isolates were rifampicin resistant mutants: one isolate of *B. subtilis*, one isolate of *P. agglomerans*, one isolate of *Curtobacterium flaccumfaciens* and *Bacillus cereus*. All isolates maintained high concentrations at the POI and 8 out of 14 isolates moved up to 10cm past the POI. Only W218, *Bacillus subtilis* and *B. cereus* were able to colonize up to 30cm, and only D753, *Kocuria* sp., was capable of colonizing the petiole. We hypothesize that 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. However, endophyte colonization may not be consistent throughout a plant and may be affected by health and developmental stage (Rosenblueth and Martínez-Romero, 2006). Our Chardonnay vines used for this experiment were three years old and inoculated at the end of the dormant period. All of these factors could have restricted endophyte growth.

Table 2. Summary of isolates in movement assays completed in 2007.

Isolate	Identification	POI cfu/ml	Petiole[a] cfu/ml	10cm cfu/ml	30cm cfu/ml
N6	*Curtobacterium sp.*	8.74×10^5	0	9.15×10^2	0
W94-rif	*Curtobacterium flaccumfaciens*	9.00×10^4	0	3.30×10^3	0
D753	*Kocuria*	1.41×10^5	6.99×10^3	6.15×10^3	0
W121-rif	*B. cereus*	3.70×10^4	0	3.00×10^2	2.00×10^2
W218	*B. subtilis*	2.54×10^5	0	1.34×10^5	4.00×10^3
D843	*B. pumilus*	5.03×10^5	0	1.40×10^4	0
W127	*Pantoea agglomerans*	3.20×10^4	0	1.38×10^4	0
D37	*Pantoea agglomerans*	1.60×10^6	0	8.00×10^1	0

[a] First leaf petiole up from the POI.

Assessing Biological Control of Endophytes Against *Xf*.

Results of the biological control experiment for 2006 Seven groups of 15 Thompson seedless grapevines each were inoculated with different endophyte treatments to evaluate these isolates as potential biological control agents against *Xf* (Table 3). These isolates were chosen based on prior movement assays and antagonistic ability *in vitro*. We tested the protective capabilities of three *Bacillus* sp. strains, three *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomonas* sp. Endophytes tested are summarized in Table 3. Seven weeks post endophyte treatment, vines were inoculated with a 10^8 cfu/ml suspension of Stagg's leap strain (STL) of *Xf*. Pierce's disease symptoms rated at 15 weeks and 18 weeks post inoculation showed that across all treatments, vines developed similar symptom severity as compared to the non-endophyte control.

Although endophyte treatments in this experiment were not effective control against Pierce's disease, it is possible that a 10^8 cfu/ml cell suspension may overwhelm any protective effect that the endophytes might provide. While it is not known exactly how many *Xf* cells an infective insect introduces into the plant, populations as high as 10^5 cells have been cultured from a single BGSS head (Hill and Purcell, 1994). A cell suspension of 10^8 cfu/ml, or 10^6 cells in a 20µl drop, is far more cells than an insect would transmit to plants. Secondly, it is possible that endophyte protection was limited because isolates tested in the biocontrol experiment had not fully colonized the plant. Thompson seedless plants used for this experiment were, on average, one-two meters tall, and seven weeks was not enough time for these isolates to completely move up the vine. Plant defense response to pinprick injury may also slow endophyte colonization.

Table 3. Summary of results for 2006 Biocontrol Experiment

Endophyte treatment	Identification (16S rDNA)	*Xf* Infected Vines	Average severity rating 15 weeks	Average severity rating 18 weeks
147	*Bacillus subtilis*	15/15	2.1	3.2
100	*Bacillus subtilis*	13/15	2.7	3.6
169	*Bacillus subtilis*	14/15	2.8	4.0
329	*Pseudomonas* sp.	15/15	2.0	3.4
197	*Pseudomonas viridiflava*	15/15	2.8	3.8
329/197	*Pseudomonas* sp./ *P. viridiflava*	15/15	2.4	3.7
Control	*No endophyte*	14/15	2.0	3.1

Continuing evaluation of biocontrol experiment initiated in 2003 During this reporting period we finished an experiment that continued evaluation of vines growing in the field that were originally part of a biological control experiment in 2003. In the 2003 experiment, Cabernet Sauvignon vines were inoculated with six endophyte treatments and challenged with infective BGSS. Vines with significantly lowered symptom severity were planted in the field. We wanted to determine if, after two years in the field, these vines were still protected against Pierce's disease. In spring of 2005, propagated bud wood cuttings from these vines were challenged via mechanical inoculation with Stagg's leap strain *Xf*. Symptom rating at 14 weeks showed that all vines inoculated with *Xf* had developed severe symptoms and these propagated cuttings showed no continued protection against Pierce's disease. Again, the concentrated *Xf* inoculum may have obscured possible endophyte effect. However, given the diversity of genera isolated from these same vines during fall 2005, we know the endophyte community has changed since these vines were originally used in the biocontrol experiment. Protection effected by the original endophyte treatment could have been diluted or inactivated.

Biological control experiment 2007 In this year's biological control experiment, we wanted to make sure that endophytes fully colonized test vines and that *Xf* was inoculated in a lower concentration such that potential endophyte protection would not be overwhelmed. We also wanted to compare potential endophyte protection between mechanically inoculated vines and vines inoculated with *Xf* infective insects. Five endophyte isolates were chosen for this year's biological control experiment: *Pantoea agglomerans* (D11), *Bacillus subtilis* (D147), *Curtobacterium flaccumfaciens* (W94), *Bacillus cereus* (W121), and *Kocuria* sp. (D753). Both isolates W121 and W94 were spontaneously generated rifampicin resistant mutants.

To achieve better colonization, endophytes were pin-prick inoculated into grapevines, first at the third internode and then at 15cm intervals up the stem. After seven weeks, one group of endophyte treated vines was mechanically inoculated with STL *Xf* and one group was inoculated with infective adult BGSS, *Graphocephala atropunctata* with the assistance of the Almeida lab at UC Berkeley. We tried to avoid overwhelming endophytes with high concentration of *Xf* in the mechanical inoculations by inoculating with 10^5cfu/ml instead of 10^8cfu/ml. Unfortunately culturing confirmed that we had inoculated with only a 10^4cfu/ml suspension which we felt was too low. Given these results we reinoculated vines three weeks later. Again the concentration was too low, 10^4 cfu/ml, and we inoculated a last time and achieved a cell concentration of 10^5 cfu/ml. In summary, vines were inoculated three times with STL *Xf* over a seven week period. Insect transmission was achieved with four infective BGSS per plant that were allowed to feed on endophyte treated or control vines for four days. First symptoms on all vines were rated eight weeks after the first inoculation.

Table 4 indicates endophyte treatments and preliminary results for the 2007 biological control experiment. Insect transmission was efficient although symptoms in these vines are developing more slowly that in mechanically inoculated vines. Unfortunately, mechanically inoculating three times, even with low concentrations, may have introduced too many *Xf* cells. Petioles cultured at 12 weeks post inoculation across all treatments contain similar concentrations of *Xf*. Symptom ratings will continue through November and *Xf* will be isolated and quantified from petioles a second time. Surviving vines will be cultured to determine, if possible, the concentration of endophytes in the vine.

Table 4. Endophytes tested and preliminary results for 2007 Biocontrol Experiment

Endophyte treatment	Identification	Vines infected BGSS inoculated	Vines infected mechanically inoculated	Average *Xf* concentration petiole BGSS inoculated	Average *Xf* concentration petiole mechanically inoculated	Average rating 12 weeks post BGSS inoculation	Average rating 12 weeks post first mech. inoculation
D147	*Bacillus subtilis*	11/15	15/15	$1.62x10^7$	$4.83x10^7$	1.1	2.2
D11	*Pantoea agglomerans*	13/15	15/15	$1.21x10^7$	$1.04x10^7$	1.2	2.4
W121-rif	*B. cereus*	14/15	14/15	$1.38x10^7$	$4.41x10^6$	1.4	2.1
W94-rif	*Curtobacterium flaccumfaciens*	14/15	15/15	$2.32x10^7$	$5.80x10^7$	1.3	2.4
D753	*Kocuria sp.*	---	14/15	---	$4.66x10^6$	---	2.1
Positive control	Water inoculated	13/15	13/13	$9.49x10^7$	$5.35x10^7$	1.5	2.8
Negative Control	Water inoculated endophyte, no *Xf*	---	8/8	---	0	---	0

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