Pierce's Disease Control Program

Research Progress Reports

Pierce's Disease and Other Designated Pests and Diseases of Winegrapes

RESEARCH PROGRESS BOARD

-December 2015-

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CALIFORNIA DEPARTMENT OF FOOD & AGRICULTURE

Research Progress Reports

Pierce's Disease and Other Designated Pests and Diseases of Winegrapes

- December 2015 -

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Note to Readers:
The reports contained in this document have not been subject to scientific peer review.

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Section 1:

Pierce's Disease and Its Vectors

ASSESSING PIERCE'S DISEASE SPREAD IN GRAPE LINES WITH NOVEL DEFENSIVE TRAITS

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Reporting Period: The results reported here are from work conducted July 2015 to September 2015.

ABSTRACT
This proposal expands on previous work funded by this program to develop Pierce's disease-resistant grapevine lines. Previous projects have successfully developed grapevine lines with promising traits conferring resistance against *Xylella fastidiosa*, including plants expressing the *rpfF* gene, the PdR1 major locus, and the HxfB protein (Meredith et al., 2001; Walker and Tenscher, 2014; Lindow et al., 2014). All these grapevine lines exhibit low symptom severity when mechanically inoculated with *X. fastidiosa*. We propose to expand upon previous work by testing the potential of Pierce's disease-defended grape lines to reduce the spread of *X. fastidiosa* using a multi-disciplinary combination of transmission experiments and mathematical modeling. Using this approach and HxfB-producing plants as a case study, we found that while HxfB plants are unlikely to eliminate Pierce's disease in the field, spread would nonetheless be significantly reduced. Further study will allow us to assess the impacts of these reductions on large-scale and long-term Pierce's disease spread in resistant grape lines.

LAYPERSON SUMMARY
The Pierce's disease research community has developed grapevine lines that exhibit novel and promising defenses against *Xylella fastidiosa* and have the potential to reduce crop damage from Pierce's disease. Yet it remains unknown if these novel defensive traits will increase or decrease large-scale spread of Pierce's disease within and among vineyards, which is a critical dimension of sustainable disease management. We propose to conduct transmission experiments with important insect vectors of *X. fastidiosa* and use data from these experiments to explore pathogen spread using mathematical models. We will assess the efficacy of defenses by comparing simulated spread in defended and susceptible vineyards and use these data to inform vineyard managers of how to minimize disease outbreaks across California.

OBJECTIVES
The overall goal of this project is to assess the potential for novel defensive traits in grapevine lines to reduce the transmission of *Xylella fastidiosa* by insect vectors and the prevalence of Pierce's disease within and among heterogeneous vineyards. We will assess Pierce's disease epidemiology in two defended lines: transgenic grape lines expressing the *rpfF* gene (Lindow et al., 2014) and conventionally bred grape lines with the *PdR1* dominant locus (Walker and Tenscher, 2014). The research consists of three specific objectives:

1. Estimate transmission of *X. fastidiosa* and vector feeding behavior on novel Pierce's disease-defended grape lines.
2. Assess large-scale and long-term Pierce's disease prevalence in defended grape vineyards.
3. Inform vineyard managers on the efficacy of novel Pierce's disease defenses.

We will address the above objectives using a multi-disciplinary approach involving transmission experiments, vector behavioral experiments, and mathematical modeling. The first objective will be accomplished through a series of greenhouse experiments to assess *X. fastidiosa* transmission by two important vectors in California -- the blue-green sharpshooter (BGSS; *Graphocephala atropunctata*) and the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) -- among novel Pierce's disease-defended grapevines and among susceptible near-isogenic vines. We will also experimentally investigate the host selection behavior of the vectors between defended and susceptible lines. These experiments will be designed to provide estimates of epidemiological parameters, which will be combined with mathematical models to accomplish the second objective -- to estimate long-term Pierce's disease prevalence in vineyards of defended and susceptible grapevine lines. The predictions will be compared to annual plant disease incidence and vector infection prevalence data collected from field trials of *PdR1* plants in Napa County. Finally, we will use the model results to develop and communicate recommendations to vineyard managers on the efficacy of deploying grape lines with novel defensive traits.

RESULTS AND DISCUSSION

During the first three months of the project, we have initiated acquisition and transmission trials with blue-green sharpshooter and transgenic *rpfF* plants. The experiments are ongoing and we have no results to report at this stage. Our timeline for the next year includes:

Table 1. Timeline.

	Summer 15	Winter 16	Spring/ Summer 16	Fall 16
Trials with BGSS + *rpfF* plants	X			
Epidemic model development		X		
Trials with BGSS + *PdR1* plants			X	
Trials with GWSS + *rpfF* plants			X	
Trials with GWSS + *PdR1* plants			X	
Reports, manuscripts, etc.				X

CONCLUSIONS

As the project was recently started, we have no conclusions at this stage.

REFERENCES CITED

Lindow, S., K. Newman, S. Chatterjee, et al. 2014. Production of *Xylella fastidiosa* diffusible signal factor in transgenic grape causes pathogen confusion and reduction in severity of Pierce's disease. *Mol. Plant-Microbe Interact*. 27: 244–254. doi: 10.1094/MPMI-07-13-0197-FI.

Meredith, C., A. Dandekar, B. Kirkpatrick, and J. Labavitch. 2001. Genetic transformation to improve the Pierce's disease resistance of existing grape varieties. Pp 76–77 in *Proceedings of the 2001 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA.

Walker, A., and A.C. Tenscher. 2014. Breeding Pierce's disease resistant winegrapes. Pp. 220-226 in *Proceedings of the 2014 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

EVALUATING POTENTIAL SHIFTS IN PIERCE'S DISEASE EPIDEMIOLOGY

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Reporting Period: The results reported here are from work conducted July 2015 to October 2015.

ABSTRACT
Pierce's disease of grapevine has reemerged in Napa and Sonoma counties, where disease incidence has been much higher than usual and the distribution of sick vines within vineyards often does not fall within expectations. These field observations, taken together with the very high number of vineyards affected in the region, indicate that a Pierce's disease epidemic is emerging. The goal of this project is to determine what factors are driving this epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different.

LAYPERSON SUMMARY
A Pierce's disease epidemic is emerging in Napa and Sonoma counties. Very high Pierce's disease prevalence is being reported throughout the region, with a large number of stakeholders reaching out to University of California Cooperative Extension Farm Advisors. More recently the UC Berkeley group working on Pierce's disease ecology had a series of joint meetings / field visits with the Farm Advisors during the last month. Two observations have been made that raised our concern about the problem. First, high prevalence of Pierce's disease in the North Coast is usually below 1-2% per vineyard; several vineyards visited had over 25% of vines symptomatic. Second, historically Pierce's disease is closely associated with riparian zones in the North Coast; we have visited several vineyards where Pierce's disease was not associated with riparian zones. We have observed these greater rates of disease incidence and dissociation with riparian areas throughout Napa and Sonoma counties -- they are not district specific. The goal of this proposal is to determine what factors are driving this epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different.

OBJECTIVES
The objectives of this project are necessarily intertwined, but are described here independently so that aims and expectations are more clearly described in the methods section.

1. Conduct vector, pathogen, and host community surveys to inform the development of a quantitative model to assess future Pierce's disease risk and develop integrated management strategies.
2. Investigate *Xylella fastidiosa* colonization of grapevines and the role of overwinter recovery in Pierce's disease epidemiology.
3. Determine the role of spittlebug insects as vectors of *Xylella fastidiosa*.
4. Data mine and disseminate existing information on vector ecology, vegetation management, and efficacy of pruning.

5. Develop a larger extension and outreach footprint with additional seminars, extended interviews made available on the web, and an update to the *Xylella fastidiosa* website, the main online resource for Pierce's disease information.

RESULTS AND DISCUSSION

Funds were just made available to the Principal Investigator in October, so there are no results to report.

CONCLUSIONS

There are no conclusions at this stage.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

EXPLOITING A CHITINASE TO SUPPRESS *XYLELLA FASTIDIOSA* COLONIZATION OF PLANTS AND INSECTS

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Reporting Period: The results reported here are from work conducted July 2015 to October 2015.

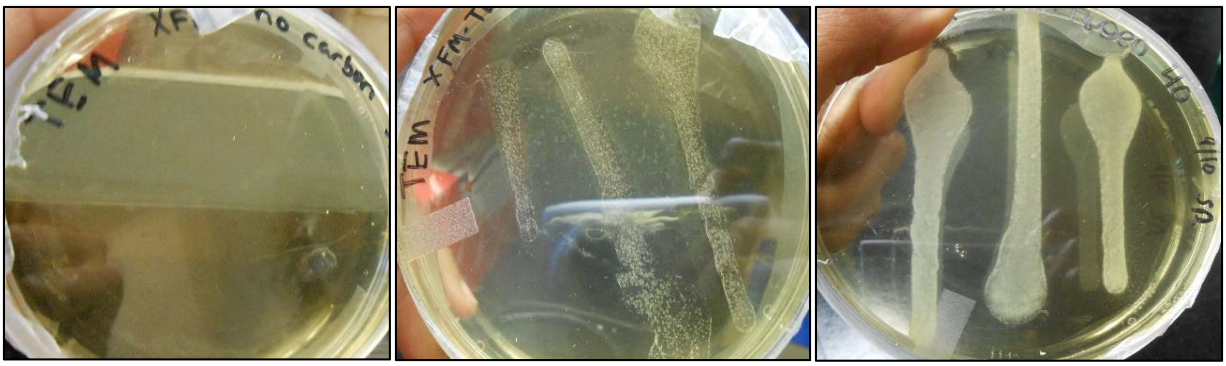
ABSTRACT
Previous research showed that *Xylella fastidiosa* has a chitinase (ChiA), which is required for sharpshooter vector colonization, transmission to plants, and plant colonization. The goals of this project are to understand the function(s) of ChiA so that it can be exploited as a tool for control of Pierce's disease by disrupting *X. fastidiosa* interactions with both plant and insect hosts. This report summarizes recent efforts aimed at experimentally determining carbon sources that can be used by *X. fastidiosa* in this context, as well as continuing our work to try to determine why the *chiA* knockout mutant is deficient in plant colonization.

LAYPERSON SUMMARY
The previously identified *Xylella fastidiosa* chitinase (ChiA) represents a unique opportunity to try to disrupt *X. fastidiosa* interactions with both insect and plant hosts, as well as sharpshooter transmission, because all of these processes are affected in the mutant strain that does not have this enzyme. The goal of this project is to better understand how ChiA impacts plant and insect colonization so that it can be exploited to limit Pierce's disease spread.

OBJECTIVES
Efforts during the report period focused on experimentally determining if *Xylella fastidiosa* chitinase (ChiA) is required for the degradation of one (or several) plant carbohydrates (movement and/ or carbon sources), and if ChiA is involved in evading the plant immune system.

RESULTS AND DISCUSSION
Although a substantial amount of information has been generated about the biology of *X. fastidiosa*, we still have very little experimental knowledge about the sources of carbon that *X. fastidiosa* can use. Because we hypothesize that the lack of *chiA* mutant strain colonization of host plants may be associated with reduced degradation of host polysaccharides, we screened for the ability of *X. fastidiosa* to utilize 192 different carbon sources. We used cells scraped from XFM plates and resuspended into a 25 µM glutamine solution to obtain a final 0D600 ranging from 0.1 to 0.2. One hundred µL of this solution as well as Dye G were added to each well of PM1 and PM2a MicroPlate Carbon Sources, each plate containing 96 different carbon sources (Biolog, USA). The plates were then incubated at 28°C for a week. The results were read at 0D590 on a VersaMax microplate reader (Molecular Devices). We further analyzed the carbon sources which were found to be positive on Biolog plates by comparing growth on XFM medium depleted for carbon sources initially present in the medium (**Δ**XFM) and supplemented or not with the carbon source of interest as previously performed (Killiny and Almeida 2009, 2010). This experiment was done with both *X. fastidiosa* wild-type and *chiA* mutant.

Among the 192 carbon sources tested, positive results were obtained with the following: Tween 20, Tween 40, Pyruvic acid, L-Malic acid, D,L-Malic acid, Fumaric acid, D-galactonic acid gamma lactone, L-ornithine, L-phenylalanine, L-pyroglutamic acid, L-arginine, Inulin, Mannan, and Pectin. We note that these represent our own clear positives; however, we cannot completely exclude other carbon sources for *X. fastidiosa*. So far we have been able to demonstrate with the confirmation assays on plates that both the wild-type and *chiA* mutant can use pectin and galacturonic acid as sole carbon sources. Interestingly, we were also able to confirm Tween 20 and Tween 40 as sole carbon sources. Additional experiments are being performed to follow up on these observations.



(a) (b) (c)

Figure 1. Wild-type Temecula cells grown on (a) **Δ**XFM, (b) **Δ**XFM-Tween 20, and (c) **Δ**XFM-Tween 40.

To determine if ChiA is involved in interactions with the immune system of plants, one-month *Nicotiana tabacum* cv. Xanthii 'Glurk' were needle-inoculated at the base of the fourth (or fifth) petiole with 20 µL of an inoculum containing log 9 CFU/ml of either wild-type, *chiA* mutant, or *chiA* mutant complemented with *chiA*. Mock inoculation with succinate-citrate phosphate buffer was performed on the same number of plants as a control (12-14 plants). Two days post inoculation, the inoculated leaves were harvested from half of the plants for each treatment. The other half was collected four days post-inoculation. The leaves were immediately frozen and kept at -80°C until further analysis. In parallel, 14 grapevines (var. Cabernet Sauvignon) per treatment were inoculated with five µL of an inoculum containing log 9 CFU/ml on the stem. Half of the leaves situated above the inoculation point were collected at two dpi whereas the other half were collected at four dpi. RNA is being extracted from these plants to determine if there are differences in key plant response genes among treatments.

CONCLUSIONS

Research has identified a series of new carbon sources that may be utilized by *X. fastidiosa*. Efforts are now focusing on experimentally confirming the results of a screen that targeted 192 different carbohydrates. In addition, experimental work to determine if ChiA is involved in interactions with the plant immune system have now moved from the greenhouse to the laboratory, where collected samples will be tested so that the expression of key plant genes in response to bacterial infections can be determined.

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Killiny, N. and Almeida, R.P.P. 2009. Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. *Proceedings of the National Academy of Sciences USA* 106: 22416-22420.

Killiny, N., Prado, S.S. and Almeida, R.P.P. 2010. Chitin utilization by the insect-transmitted bacterium *Xylella fastidiosa*. *Applied and Environmental Microbiology* 76: 6134-6140.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

IDENTIFICATION OF A NEW VIRULENCE FACTOR REQUIRED FOR PIERCE'S DISEASE AND ITS UTILITY IN DEVELOPMENT OF A BIOLOGICAL CONTROL

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Reporting Period: The results reported here are from work conducted July 2015 to October 2015.

ABSTRACT
Xylella fastidiosa is a serious pathogen that infects a number of important crops including citrus, almonds, and coffee. The *X. fastidiosa* Temecula strain infects grapevines and induces Pierce's disease. In efforts to understand infection better, we deleted the *X. fastidiosa* PD1311 gene encoding a putative acyl CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. We discovered that *X. fastidiosa* deleted of this gene is avirulent. Given the critical role of PD1311 in Pierce's disease development, we are determining its role in virulence. We have evidence that the ΔPD1311 strain may act as a biocontrol for management of Pierce's disease, as it significantly reduces the symptoms when inoculated prior to wild-type *X. fastidiosa*.

LAYPERSON SUMMARY
We discovered that deleting the *Xylella fastidiosa* Temecula gene, PD1311, results in a strain that does not induce Pierce's disease. We are conducting research to determine how PD1311 plays such a central role in symptom development. Given the agricultural importance of Pierce's disease, it is critical to understand how PD1311 exerts its effects. Additionally, we have evidence that the strain deleted for PD1311 may function as a biocontrol. When inoculated prior to wild-type *X. fastidiosa*, disease development becomes significantly reduced. Options for managing Pierce's disease are limited, which makes development of new biocontrols critically important. Together the results from these aims will expand our understanding of Pierce's disease and provide information in relation to preventing disease.

INTRODUCTION
Xylella fastidiosa is a Gram-negative, xylem-limited bacterium that causes Pierce's disease of grapevines (Chatterjee et al., 2008). *X. fastidiosa* is transmitted to plants by insect vectors and once in the xylem, *X. fastidiosa* is postulated to migrate, aggregate, and form biofilm that clogs the vessels, leading to Pierce's disease. We and others have studied *X. fastidiosa* proteins and regulators involved in these steps (Guilhabert and Kirkpatrick, 2005; Meng et al., 2005; Feil et al., 2007; Li et al., 2007; Shi et al., 2007; da Silva Neto et al., 2008; Cursino et al., 2009; Cursino et al., 2011; Cursino et al., 2015) with the goal of better understanding Pierce's disease and developing prevention strategies.

We deleted the *X. fastidiosa* PD1311 gene (ΔPD1311), a putative acyl-CoA synthetase (ACS), as we were interested in potential genes involved in secondary metabolite production. ACSs catalyze long-chain fatty acyl-CoAs (Black et al., 1992) and are involved in numerous processes, including pathogenicity (Barber et al., 1997). We found that PD1311 is a functional enzyme (data not shown), and that the ΔPD1311 strain grows in PD2 and *Vitis vinifera* sap (**Figure 1**).

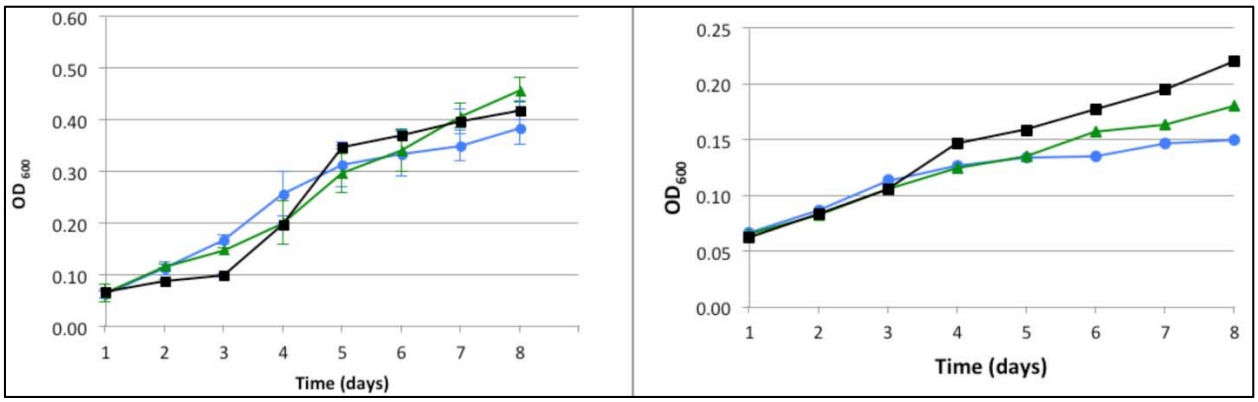

Figure 1. ΔPD1311 strain growth curve. Wild-type *X. fastidiosa* (black square), ΔPD1311 mutant (blue circle), and complemented mutant (green triangle) strains were grown for eight days in PD2 (left) or 100% *Vitis vinifera* cv. Chardonnay xylem sap (right) and growth was determined by OD_{600} readings.

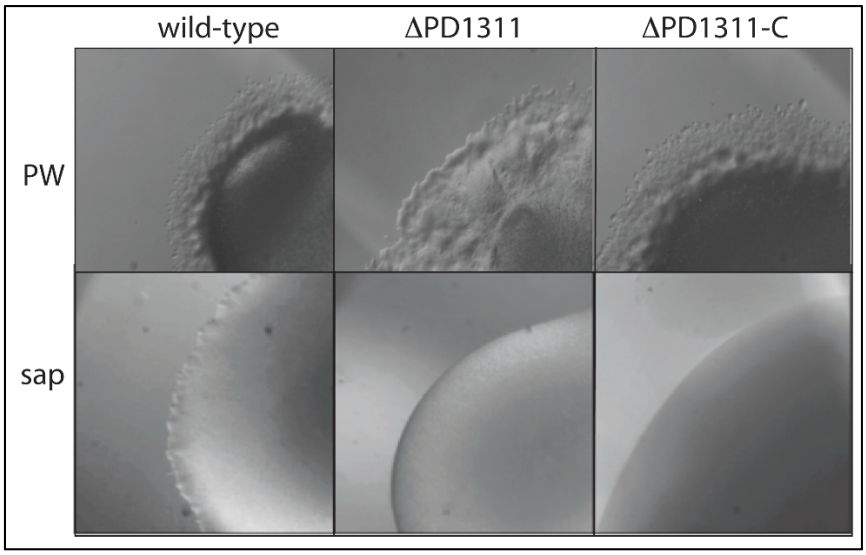

Figure 2. Motility of ΔPD1311 mutant strain. Colony fringes of wild-type, ΔPD1311 mutant, or ΔPD1311 complement (ΔPD1311-C) strains were assayed on PW agar or 80% *V. vinifera* sap agar. Colonies were assessed after five days of growth (Meng et al., 2005, Li et al., 2007). Colonies photographed at 90X magnification. Experiment was repeated three times.

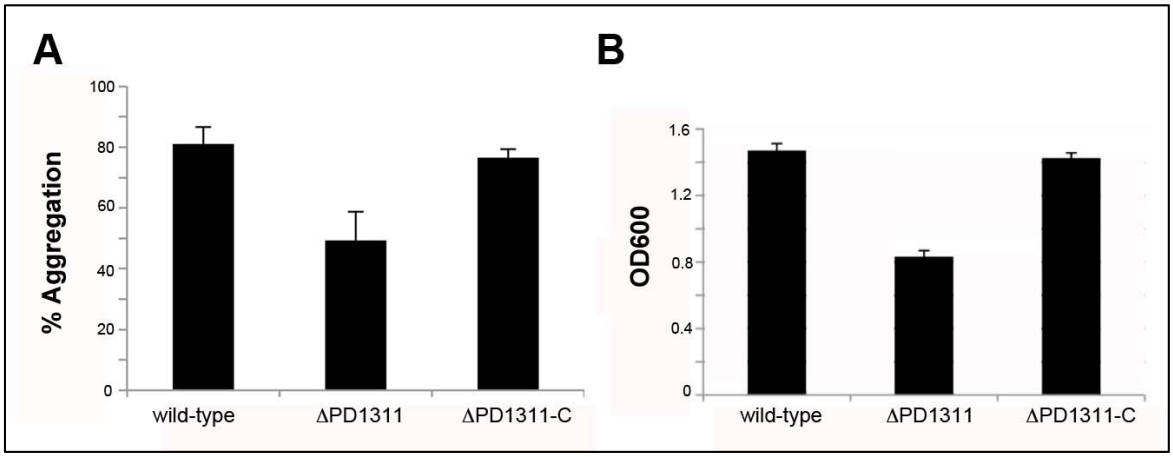

Figure 3. Aggregation and biofilm formation by ΔPD1311 strain. A) Aggregation of wild-type, ΔPD1311 mutant, or ΔPD1311 complement (ΔPD1311-C) strains grown in test tubes for five days in three ml of PD2 (Burdman et al., 2000, Davis et al., 1980, Shi et al., 2007). The experiment was repeated three times. B) Quantification of biofilm formation in 96 well plates (Zaini et al., 2009). Experiment was repeated three times with 24 replicates each.

Motility, aggregation, and biofilm production are key behaviors of *X. fastidiosa* that are associated with Pierce's disease (Chatterjee et al., 2008). The ΔPD1311 strain is reduced in type IV pili-mediated motility on PW-BSA plates and is non-motile on sap agar (**Figure 2**). In comparison to wild-type cells, the ΔPD1311 strain is reduced in aggregation and biofilm production (**Figure 3**). We therefore hypothesized that the ΔPD1311 strain might be less virulent in plants, as mutants with similar phenotypes have been shown to have reduced, but not eliminated, Pierce's disease (Cursino et al., 2009; Cursino et al., 2011). Unlike the other mutant strains, we found that the ΔPD1311 strain was avirulent and showed no Pierce's disease, even at twenty weeks post-inoculation (**Figure 4**).

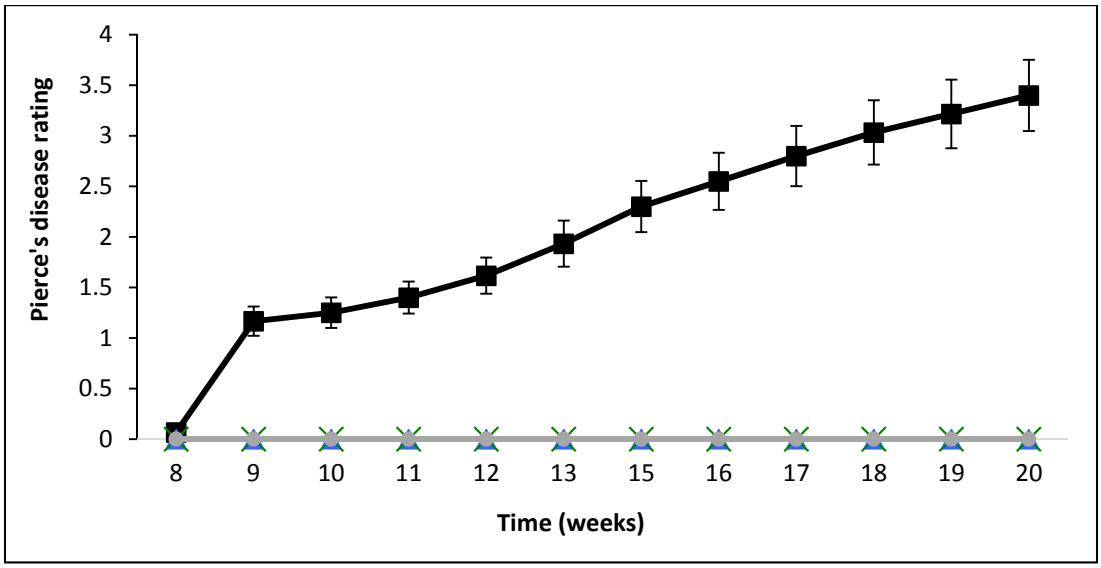

Figure 4. Development of Pierce's disease. Grapevines were inoculated with wild-type *X. fastidiosa* (black square), ΔPD1311 strain (blue triangle), ΔPD1311 complement (green x), and buffer (gray circle). Symptoms were monitored and rated on a scale of 0-5 (Guilhabert and Kirkpatrick, 2005, Cursino et al., 2009). Data represents averages of three trials.

Besides being avirulent, the ΔPD1311 reduced wild-type *X. fastidiosa* biofilm formation *in vitro* (**Figure 5**). We therefore wished to test if the ΔPD1311 strain reduced Pierce's disease by wild-type cells. Given our findings, we proposed that ΔPD1311 has potential as a biocontrol for Pierce's disease. The weakly virulent *X. fastidiosa* elderberry strain EB92-1 has been studied as a potential Pierce's disease biological control (Hopkins, 2005; Hopkins, 2012). Other approaches towards controlling Pierce's disease include resistant rootstocks (Cousins and Goolsby, 2011) and transgenic vines (Dandekar, 2014; Gilchrist et al., 2014; Gilchrist and Lincoln, 2014; Kirkpatrick, 2014; Lindow, 2014; Powell and Labavitch, 2014). Continued research of Pierce's disease controls is warranted. Given the avirulent phenotype of ΔPD1311 and its ability to limit wild-type induced Pierce's disease, this strain provides new potential for a commercialized biological control.

OBJECTIVES

The overall goal is to optimize the ΔPD1311 strain as a biological control for Pierce's disease and to understand the mechanisms of disease inhibition that will facilitate commercialization.

Objective 1. Examine aspects of the ΔPD1311 Temecula strain as a biological control of Pierce's disease.

a. Optimize application timing and conditions for the ΔPD1311 strain.
b. Determine if over-wintered ΔPD1311 inoculated plants maintain Pierce's disease resistance.
c. Explore leafhopper transmission of the ΔPD1311 strain.
d. Develop a clean deletion strain of ΔPD1311 that would be suitable for commercialization.

Objective 2. Determine the function of the PD1311 protein and the mechanism by which ΔPD1311 acts as a biological control.

a. Elucidate the role of the PD1311 protein.
b. Examine the impact of the ΔPD1311 strain on wild-type *X. fastidiosa in vitro* and *in planta*.

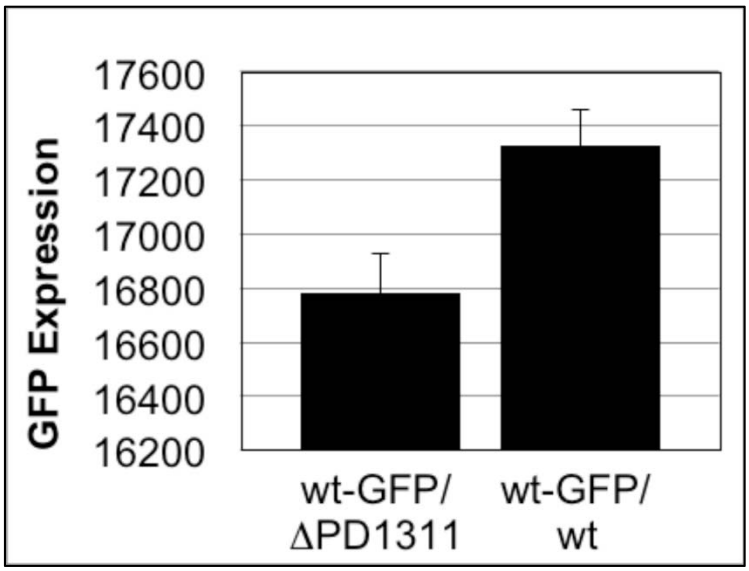

Figure 5. Mutant strain impact on wild-type strain biofilm formation. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type (wt) *X. fastidiosa* constitutively expressing green fluorescent protein (wt-GFP) and either wild-type *X. fastidiosa* (wt) or the ΔPD1311 strain (ΔPD1311). Experiment was performed once with 24 replicates. Fluorescence in artificial units.

RESULTS AND DISCUSSION

Objective 1. Examine aspects of the ΔPD1311 Temecula strain as a biological control of Pierce's disease.

Objective 1a. Optimize application timing and conditions for the ΔPD1311 strain.

To examine if the *X. fastidiosa* ΔPD1311 Temecula strain could act as a potential biocontrol, we inoculated *V. vinifera* cv. Cabernet Sauvignon vines per standard procedures (Cursino et al., 2011) and recorded disease development of Pierce's disease using the five-scale assessment (Guilhabert and Kirkpatrick, 2005). We created two different inoculation conditions: i) wild-type *X. fastidiosa* after a two week pre-treatment with the ΔPD1311 strain [following procedures used in *X. fastidiosa* elderberry EB92.1 strain biocontrol studies (Hopkins, 2005)] and ii) wild-type and ΔPD1311 strain co-inoculated. We previously found that inoculating the ΔPD1311 strain after a two week pre-treatment with the wild-type strain did not limit Pierce's disease (data not shown). Our controls included vines inoculated with wild-type Temecula, the ΔPD1311 strain, or buffer (Hopkins, 1984). We found that pre-treatment with the ΔPD1311 strain inhibits Pierce's disease, while co-inoculation does not alter disease development (**Figure 6**).

Objective 1b. Determine if over-wintered ΔPD1311 inoculated plants maintain Pierce's disease resistance.

In 2014 we had *V. vinifera* plants infected with wild-type *X. fastidiosa* or the ΔPD1311 strain. These vines were cut back and placed in nursery storage for the 2015 winter. The plants were then regrown in the greenhouse in Spring 2015. Vines were divided in half, and we followed one half for Pierce's disease development without any new treatments. The second half received fresh wild-type *X. fastidiosa* inoculations. Plants appeared to develop Pierce's disease independent of the previous year treatment (data not shown), suggesting that protective effects from the ΔPD1311 strain may not persist through the dormant period in cold storage. We are currently confirming these results by testing for *X. fastidiosa* infection in plants.

Objective 1c. Explore leafhopper transmission of the ΔPD1311 strain.

Xylem-sap feeding leafhopper vectors transmit *X. fastidiosa* from plant to plant (Chatterjee et al., 2008). The bacterium utilizes adhesins, such as FimA, HxfA, and HxfB, to attach and form biofilms on insect foreguts, which then becomes a source of inoculum for further disease spread (Killiny and Almeida, 2009; Killiny et al., 2010). Thus, interaction with insects is a known key step for *X. fastidiosa* to accomplish its life cycle. For development of the ΔPD1311 strain as a commercially viable biological control agent and for future field studies, it will be necessary to understand its insect transmissibility. Because ΔPD1311 has reduced aggregation and biofilm (**Figure 3**), we hypothesize that ΔPD1311 is altered in its ability to insect vectors. As an initial assay, we want to

examine the adhesion of the mutant strain to the hindwing of the leafhopper vector, as this assay has been found to mimic adhesion to the foregut region owning to similar chitinous nature of the cuticles (Killiny et al., 2010). We hope to begin these studies shortly.

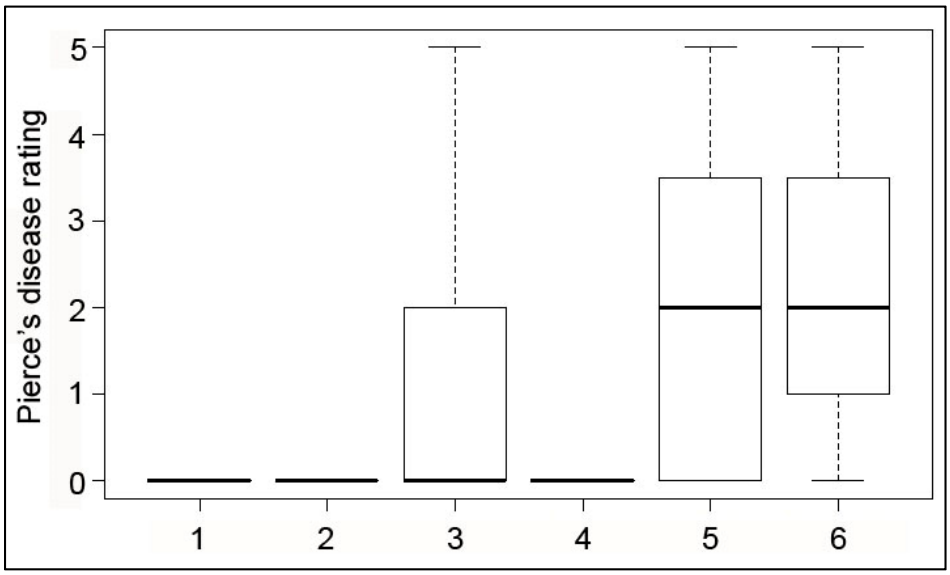

Figure 6. ΔPD1311 strain reduces Pierce's disease. Grapevines were inoculated with 1) buffer, 2) ΔPD1311 complement strain, 3) pre-treat with ΔPD1311 two weeks before wild-type, 4) ΔPD1311 only, 5) wild-type cells only, and 6) ΔPD1311 and wild-type *X. fastidiosa* co-inoculated. Bold lines represent the median values and boxes representing one standard deviation. Symptoms have been monitored on 30 plants for each treatment for 20 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick, 2005).

Objective 1d. Develop a clean deletion strain of ΔPD1311 that would be suitable for commercialization.
The ΔPD1311 strain was created via site-specific recombination of a kanamycin cassette into the *X. fastidiosa* chromosome (Matsumoto et al., 2009, Shi et al., 2009). For commercial viability, the antibiotic marker needs to be removed from the strain. Unlabeled *Agrobacterium tumefaciens* mutants have been created (Merritt et al., 2007), which will be the first approach we will attempt. This work will begin soon.

Objective 2. Determine the function of the PD1311 protein and the mechanism by which ΔPD1311 acts as a biological control.
The *X. fastidiosa* PD1311 gene has motifs suggesting it encodes an ACS protein (acyl- and aryl-CoA synthetase) (Chang et al., 1997; Gulick, 2009). ACS metabolite intermediates are involved in beta-oxidation and phospholipid biosynthesis. ACS proteins have also been implicated in cell signaling (Korchak et al., 1994), protein transportation (Glick and Rothman, 1987), protein acylation (Gordon et al., 1991), and enzyme activation (Lai et al., 1993). Importantly, ACSs are involved in pathogenicity (Banchio and Gramajo, 2002; Barber et al., 1997; Soto et al., 2002). We plan to characterize the role of the PD1311 protein in order to understand how the deletion strain is avirulent and functions as a biological control. Additionally, we plan to explore the general mechanism by which the deletion strain suppresses wild-type *X. fastidiosa* induced Pierce's disease. Basic understanding of its function will facilitate development and acceptance as a viable biological control.

Objective 2a. Elucidate the role of the PD1311 protein.
ACS proteins metabolize fatty acids through a two-step process to form a fatty acyl-CoA precursor utilized in any downstream metabolic pathways (Roche et al., 2013; Watkins, 1997; Weimar et al., 2002). To confirm enzymatic activity, we expressed and purified a PD1311-His tag protein, and we tested it for ligase activity using acetate as the substrate. Acetate is the simplest substrate for fatty acid synthetase reaction, as a two-carbon (C2) chain length molecule. We used a standard colorimetric assay that measures acyl-CoA production (Kuang et al., 2007). The PD1311 protein exhibited a functional ATP/AMP binding domain that performed the following reaction: ATP + acetate + CoA ⇄ AMP + pyrophosphate + acetyl-CoA. While we confirmed activity, we are determining if PD1311 is an acyl-CoA synthetase (metabolizes acids greater than C2), as oppose to acetyl CoA synthetase

(metabolizes C2 acetate). Therefore we propose to determine the substrate affinity of the PD1311 protein. This work is currently being performed.

The deletion of the PD1311 gene is non-lethal, suggesting that it has a role in non-essential fatty acid metabolism. One possibility is that PD1311 plays a role in diffusible signal factor production, however, our preliminary results do not support that role (data not shown). An alternative potential role for the PD1311 protein is in precursor production of lipopolysaccharide (LPS). LPS is found on the outer membrane of gram-negative bacteria and is composed of a lipid A innermost component, a core saccharide, and an outer most O-antigen. Upstream of PD1311 are three genes annotated as LPS-associated enzymes: lipid A biosynthesis N-terminal domain protein (PD1312), dolichol-phosphate mannosyltransferase (Dpm1) (PD1313), and WbnF nucleotide sugar epimerase (PD1314) (Simpson et al., 2000). Dolichol-phosphate mannosyltransferase proteins are involved in N-linked oligosaccharides in the LPS core (Kapitonov and Yu, 1999), while nucleotide sugar epimerases are involved in O-antigen synthesis (Lam et al., 2011). LPS is a known major virulence factor of *X. fastidiosa*, and changes in LPS integrity renders bacteria more susceptible to environmental stress and defective in virulence (Clifford et al., 2013).

Considering the avirulent phenotype of ΔPD1311 on grapevines, PD1311 may be involved in lipid A biosynthesis or membrane production. Therefore, the ΔPD1311 cells may be more sensitive to oxidative stress. When wild-type and ΔPD1311 cells were exposed to hydrogen peroxide on agar plates in a Kirby-Bauer type assay, the zone of inhibition was greater for the mutant strain than wild-type cells (**Figure 7**). We are exploring how this sensitivity may be associated with the biocontrol response.

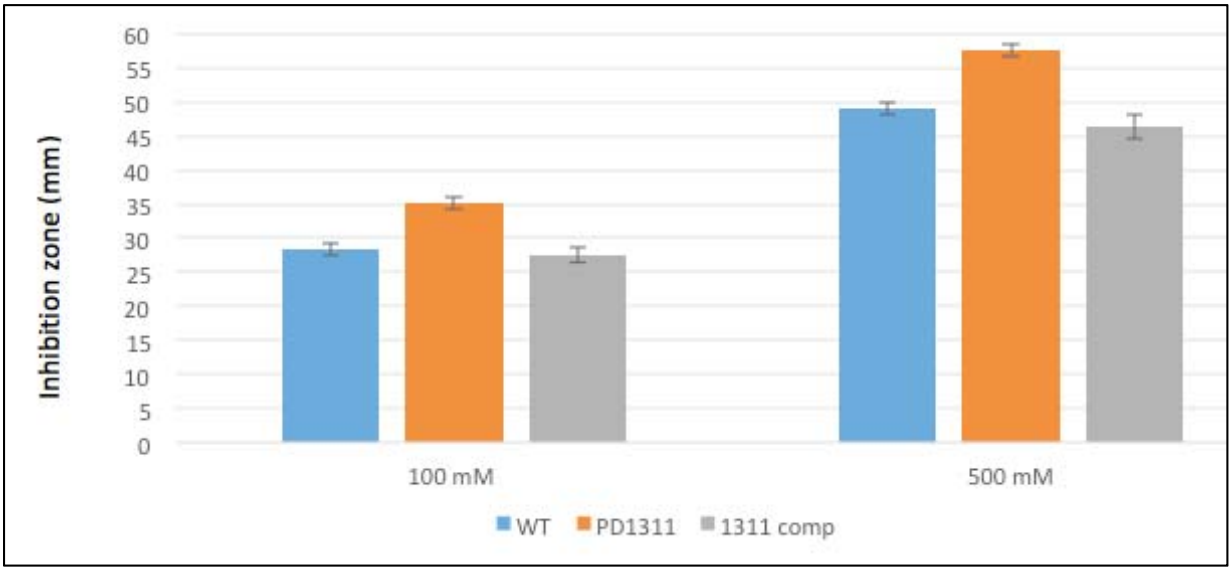

Figure 7. The ΔPD1311 strain has increased sensitivity to oxidative stress. Each strain was grown individually in PD2 broth for seven days, then 400 uL of bacterial suspension (~OD_{600} 0.25) was mixed with 3.6 mL of 0.8% of warm PD2 agar respectively and poured onto a PD2 plate. A paper disk containing 10 uL of 100 or 500 mM of H_2O_2 was placed in the middle of each plate. The diameters of inhibition zones were quantified seven days after incubation at 28°C. Each treatment included three replicates and the experiment was repeated twice.

Objective 2b. Examine the impact of the ΔPD1311 strain on wild-type *X. fastidiosa in vitro* and *in planta*.
To have better grounding on why the ΔPD1311 strain acts as a biological control, we need to explore the mechanism by which the mutant strain impacts wild-type cells. We have preliminary results showing that the ΔPD1311 strain impacts wild-type cells *in vitro* (**Figure 5**) and limits wild-type induced disease (**Figure 6**). Therefore we would like to know how the two strains spread through the plant when both are inoculated. The ΔPD1311 strain does not secrete a toxin that affects wild-type cells; we grew wild-type cells in supernatant from ΔPD1311 cells and found no growth changes (data not shown). Understanding how the mutant cells impact wild-type *X. fastidiosa* is important for understanding not only how the biological control is achieved but also how the treatment would be most effectively applied in the field.

CONCLUSIONS

X. fastidiosa motility, aggregation, and biofilm formation are key steps in Pierce's disease development (Chatterjee et al., 2008). Concerning Objective 1, we confirmed that the ΔPD1311 strain is avirulent, and we found that it can significantly reduce Pierce's disease development by wild-type *X. fastidiosa* by pre-inoculating plants with the mutant strain. For Objective 2, we are determining how the mutant strain impacts the wild type strain resulting in Pierce's disease suppression. Overall, this work is furthering our understanding of Pierce's disease and its prevention.

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CHIMERIC ANTIMICROBIAL PROTEIN AND POLYGALACTURONASE-INHIBITING PROTEIN TRANSGENIC GRAPEVINE FIELD TRIAL

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Reporting Period: The results reported here are from work conducted March 2010 to September 2015.

ABSTRACT

We successfully established a field trial to validate two greenhouse-tested strategies to control the movement and clearance of *Xylella fastidiosa*. *X. fastidiosa* is a xylem-limited, Gram-negative bacterium and is the causative agent of Pierce's disease. Key to *X. fastidiosa* virulence is its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements. This action enhances long-distance movement and vector transmission. Our first strategy enhanced clearance of bacteria from *X. fastidiosa*-infected xylem tissues using a chimeric antimicrobial protein, neutrophil elastase-cecropin B (NE-CB). Our second strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) derived from pear to counteract *X. fastidiosa* virulence associated with polygalacturonase activity. Expressing these proteins is expected to prevent *X. fastidiosa* movement and reduce its inoculum size, curbing the spread of Pierce's disease in California vineyards.

Ninety-six (96) transgenic Thompson Seedless (TS; *Vitis vinifera*) grapevines expressing either NE-CB or PGIP, used as rootstocks and grafted with untransformed TS scions, were planted together with 12 grafted, untransformed controls in Solano County in 2011. The grafted transgenic vines were evaluated phenotypically using the first 12 descriptors from the "Primary Descriptor Priority List" proposed by the International Organization of Vine and Wine (OIV). No phenotypic or horticultural differences were observed between grafted transgenic and grafted untransformed TS vines. NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County were genotyped, confirming the presence of the inserted transgene in all lines.

Half of the Solano County grafted transgenic and control plants were inoculated with *X. fastidiosa* in 2012 and 2013. Leaf scorching, a characteristic symptom of Pierce's disease, was observed in inoculated grafted transgenic and grafted control lines in 2013. *X. fastidiosa* presence in stem extracts from grafted lines was confirmed by enzyme-linked immunosorbent assay (ELISA) in 2013. In previous years, Pierce's disease symptoms were assessed using a standardized score based on percentage leaf area scorching. Cane survival and grapevine vigor were also assessed. On May 27, 2014 and on May 27, 2015, following the recommendation of the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Product Development Committee (PDC), at least four new canes per year of all grafted transgenic and control plants at the Solano site were mechanically inoculated with *X. fastidiosa*. Grafted transgenic and control grapevines inoculated in 2012 and 2013 and the individual grafted transgenic canes inoculated in 2014 were rated for the absence or severity of Pierce's disease using a zero to five Pierce's disease symptom severity scale in summer 2014 and in fall 2015. The Pierce's disease symptom severity

score was lower in most grafted inoculated transgenic lines from each strategy than in grafted untransformed controls.

On July 22, 2014, one 2014-inoculated cane of each grafted transgenic plant was harvested to quantify *X. fastidiosa* by quantitative polymerase chain reaction (qPCR). *X. fastidiosa* was detected in grafted transgenic vines, but at lower density than in grafted control grapevines. Another set of canes from the 2014-inoculated grafted transgenic and control individual canes was harvested in fall 2015 and *X. fastidiosa* quantification is in progress. Bud break success of grafted individual canes inoculated in 2014 was assessed on March 26, 2015. Bud break success was greater in most grafted, inoculated transgenic lines from each strategy than in grafted untransformed controls, and Pierce's disease symptom severity scores were lower.

The current USDA Animal and Plant Health Inspection Service (APHIS) field permit for Solano and Riverside County was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The permit was extended by USDA APHIS, with a new end date of April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trial. The issues that require regulatory oversight are listed in the permit. Timely reporting and inspections are regularly conducted to maintain compliance with USDA APHIS.

LAYPERSON SUMMARY

Ninety-six (96) grafted, transgenic Thompson Seedless (TS; *Vitis vinifera*) grapevines expressing either a chimeric antimicrobial protein (neutrophil elastase-cecropin B; NE-CB) or polygalacturonase-inhibiting protein (PGIP), and 12 grafted, untransformed TS control vines were planted in Solano County. Grafted transgenic grapevines are being evaluated as rootstocks grafted with untransformed scions to demonstrate the field efficacy of two strategies to control Pierce's disease in California grapevines. The first strategy tests whether transgenic rootstocks expressing NE-CB can clear *Xylella fastidiosa* infections in xylem tissue. The second tests whether transgenic rootstocks expressing PGIP can limit the movement of the bacterium *X. fastidiosa* in water-conducting xylem.

At the Solano County site, ~ 50% of the grafted plants were mechanically inoculated in 2012 and the rest in 2013. The presence of *X. fastidiosa* in stem extracts was confirmed using enzyme-linked immunosorbent assay (ELISA). In addition, we evaluated Pierce's disease symptoms, cane survival, and grapevine vigor, and found some grafted transgenic lines from each strategy that consistently scored better than the control and others that did not. Resistant transgenic lines can transmit their resistance from the rootstock to the untransformed scion. On May 27, 2014 and May 27, 2015, following the recommendation of the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Product Development Committee (PDC), at least four new canes of each grafted transgenic and control vine at the Solano County site were mechanically inoculated with *X. fastidiosa*. Severity or absence of Pierce's disease symptoms and grapevine survival were evaluated in summer 2014 using a Pierce's disease symptom severity rating system of zero to five, where 0 = healthy vine, all leaves green with no scorching; 1 = first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the cane shows scorching; 4 = the whole cane is sick and in decline; and 5 = the cane is dead. The Pierce's disease symptom severity score was lower in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. In summer 2014, one cane of each grafted plant was harvested to quantify *X. fastidiosa* by quantitative polymerase chain reaction (qPCR). Grape stem and *X. fastidiosa* DNAs were extracted using a modified hexadecyltrimethyl-ammonium-bromide (CTAB) method that yields DNA of a quantity and quality suitable for qPCR. An *X. fastidiosa* 16s primer pair was used to quantify *X. fastidiosa*. qPCR standard curves were obtained using concentrations of *X. fastidiosa* from 102 to 106 cells. *X. fastidiosa* was detected in grafted transgenic vines, but at lower concentrations than in control grapevines.

Bud break success of grafted individual canes inoculated in 2014 was assessed on March 26, 2015. Bud break success was greater in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. Survival of grafted transgenic grapevines inoculated in 2012/2014 and in 2013/2014 was assessed on April 28, 2015 using a one to five score. Grapevine survival was greater in some grafted inoculated transgenic lines using either strategy than in grafted untransformed controls.

The current USDA Animal and Plant Health Inspection Service (APHIS) field trial permit was changed from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The USDA APHIS permit end date is

April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trial. Timely reporting and inspections are conducted to maintain compliance with USDA APHIS permit reporting requirements.

INTRODUCTION

Thompson Seedless (TS; *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from neutrophil elastase (NE) and the lytic domain cecropin B (CB). The NE domain specifically binds to the outer-membrane protein MopB of *Xylella fastidiosa*, the causative agent of Pierce's disease, while the CB domain clears *X. fastidiosa* (Dandekar et al., 2012). We also transformed TS grapevines with a gene encoding polygalacturonase-inhibiting protein (PGIP). PGIP expression in transgenic plants inhibits the action of polygalacturonase, a virulence factor expressed by *X. fastidiosa*. Inhibiting polygalacturonase interferes with long-distance movement of *X. fastidiosa*, providing resistance to Pierce's disease (Agüero et al., 2005). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions. Several lines that showed resistance to Pierce's disease were identified by mechanically inoculating plants with *X. fastidiosa* (Dandekar et al., 2012). Selected transgenic grapevines expressing either NE-CB or PGIP, grafted with non-transgenic TS, were planted in 2011 in Solano County to validate their Pierce's disease resistance and horticultural characteristics under field conditions.

OBJECTIVES

The goals of this project are to finish field testing of four NE-CB and four PGIP transgenic grapevine clones by evaluating their horticultural characteristics and resistance to Pierce's disease. Transgenic rootstocks grafted with untransformed scions, grafted transgenic vines, and untransformed control grapevines were tested in field locations with no Pierce's disease pressure by mechanical inoculation with *X. fastidiosa*.

- Objective 1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *X. fastidiosa* infection in xylem tissue and to pass through the graft union under field conditions.
  - Activity 1. Propagation, field planting, and grafting of NE-CB and PGIP transgenic grapevines.
  - Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines used as rootstocks.
  - Activity 3. Evaluate Pierce's disease resistance of grafted NE-CB and PGIP transgenic grapevines after inoculation with *X. fastidiosa*.
- Objective 2. Assume permit holder status for existing USDA APHIS field permit and maintain regulatory oversight and compliance with permit reporting requirements.
  - Activity 4. Participate with the Public Intellectual Property Resource for Agriculture (PIPRA) during transition and assume permit holder status.
  - Activity 5. Maintain regulatory oversight of both field locations and compliance with reporting requirements.
  - Activity 6. Maintain active regulatory compliance inspections.

RESULTS AND DISCUSSION

Objective 1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *X. fastidiosa* infection in xylem tissue and to pass through the graft union under field conditions.

Activity 1. Propagation, field planting, and grafting of NE-CB and PGIP transgenic grapevines.

Four independent transgenic events expressing NE-CB (40-41G, 40-89G, 40-92G, and 41-151G), four expressing different PGIP constructs (31-25G, 45-77G, 52-08G, and TS50G), and an untransformed control (TS-G), all grafted with untransformed TS scions (**Table 1**), were planted at a site in Solano County on June 27, 2011. Genotyping of NE-CB- and PGIP-expressing grafted transgenic grapevine lines in Solano County has confirmed the presence of the inserted transgene in all lines.

Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines used as rootstocks.

To verify that horticultural and varietal characteristics of the parental genotype were unchanged, NE-CB- and PGIP-expressing grafted transgenic lines were evaluated phenotypically in Solano County in 2012 and 2013. This examination was accomplished using the first 12 descriptors from the "Primary Descriptor Priority List" proposed by the International Organization of Vine and Wine (OIV 1983). The descriptors used were (1) aperture of young shoot tip/opening of young shoot tip, (2) density of prostrate hairs between main veins on 4th leaf lower side of blade, (3) number of consecutive shoot tendrils, (4) color of upper side of blade on 4th young leaf, (5) shape of

mature leaf blades, (6) number of lobes on mature leaf, (7) area of anthocyanin coloration on main veins on upper side of mature leaf blades, (8) shape of teeth on mature leaves, (9) degree of opening of mature leaves/overlapping of petiole sinuses, (10) mature leaf petiole sinus bases limited by veins, (11) density of prostrate hairs between main veins on lower side of mature leaf blades, and (12) density of erect hairs on main veins on lower sides of mature leaf blades. No differences between grafted transgenic and parental TS grapevines were observed.

Table 1. Grafted transgenic and control grapevines planted in Solano County.

Event ID	Event ID (Vector)	# Planted
NE-CB lines		
40-41G	pDU04.6105	12
40-89G	pDU04.6105	12
40-98G	pDU04.6105	12
41-151G	pDU04.6105	12
PGIP lines		
31-25G	pDU05.1002	12
45-77G	pDU06-0201	12
52-08G	pDU05.1910	12
TS50G	pDU94.0928	12
Control line		
TS-G	N/A	12

Activity 3. Evaluate Pierce's disease resistance of grafted NE-CB and PGIP transgenic grapevines after inoculation with *X. fastidiosa*.

At the Solano County site (**Figure 1**), half of the grafted transgenic lines were mechanically inoculated as described by Almeida et al. (2003) on May 29, 2012 and half on June 17, 2013. On May 27, 2014 and on May 27, 2015, following the recommendation of the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Product Development Committee (PDC), at least four new canes from all grafted transgenic and control plants were mechanically inoculated with *X. fastidiosa*. Inoculation dates from 2012 to 2015 are shown in a color-coded map (**Table 2**).

Figure 1. Solano County grafted transgenic grapevines inoculated in spring 2014 and spring 2015. Summer 2015 (left); fall 2015 (right).

Table 2. Solano County grape field map, color-coded by *X. fastidiosa* inoculation date, from 2012 to 2015.

Vine	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Row 9	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	TS-G	
Row 8	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G
Row 7	31-25-G	40-92-G	40-92-G	40-92-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	40-92-G	40-92-G	40-92-G	41-151-G	41-151-G	41-151-G	45-77-G	45-77-G	45-77-G
Row 6	TS-50-G	TS-50-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	41-151-G	41-151-G	41-151-G	52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G
Row 5													52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G	31-25-G	45-77-G	45-77-G	45-77-G	TS-50-G

North

Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.
Grapevine inoculatation with Xf (Temecula) 250,000 per 20ul on 6/17/2013.
Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.
Grapevine inoculatation with Xf (Temecula) at 500,000 per 20ul on 5/27/2015.

On July 22, 2014, one 2014-inoculated cane per grafted transgenic plant was harvested to quantify *X. fastidiosa* by qPCR using an Applied Biosystems SYBR green fluorescence detection system. Grape stem and *X. fastidiosa* DNA was extracted using a modified hexadecyltrimethyl-ammonium-bromide (CTAB) method that yielded DNA of a quantity and quality suitable for qPCR. The *X. fastidiosa* 16s primer pair (Forward 5'-AATAAATCATAAA AAAATCGCCAACATAAACCCA-3' and (Reverse 5'- AATAAATCATAACCAGGCGTCCTCACAAGTTAC-3') was used to quantify *X. fastidiosa*. qPCR standard curves were obtained using concentrations of *X. fastidiosa* from 102 to 106 cells per 0.1 gm tissue. *X. fastidiosa* was detected in grafted transgenic vines, but at lower concentrations than in grafted control grapevines (**Figure 2**). Another set of canes from the 2014-inoculated grafted transgenic and control individual canes was harvested in fall 2015 and *X. fastidiosa* quantification is in progress.

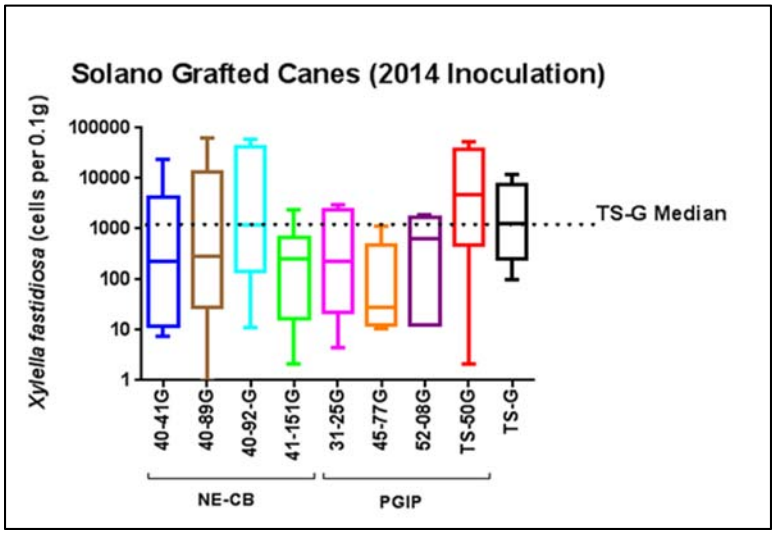

Figure 2. *X. fastidiosa* quantification by qPCR of Solano County grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014.

Bud break success of grafted individual transgenic canes inoculated in 2014 was assessed on March 26. 2015 (**Figure 3**). Bud break success was greater in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. Survival of grafted transgenic grapevines inoculated in 2012/2014 and in 2013/2014 was assessed on April 28, 2015 using a 1 to 5 score, where 1 = very healthy and vigorous grapevine, 2 = healthy grapevine with slightly reduced vigor, 3 = slightly reduced spring growth, 4 = much reduced spring growth, and 5 = dead (**Figure 4**). Grapevine survival was greater in some grafted inoculated transgenic lines with both constructs than in grafted untransformed controls.

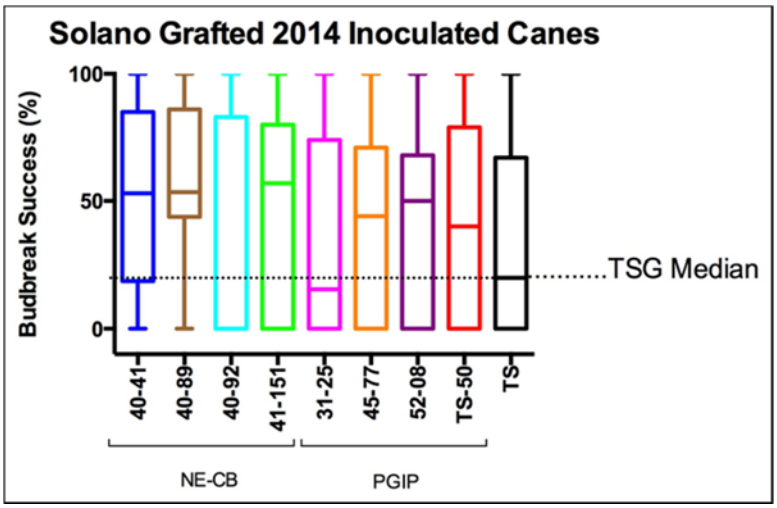

Figure 3. Bud break success for Solano County grafted inoculated grapevines scored on March 26, 2015.

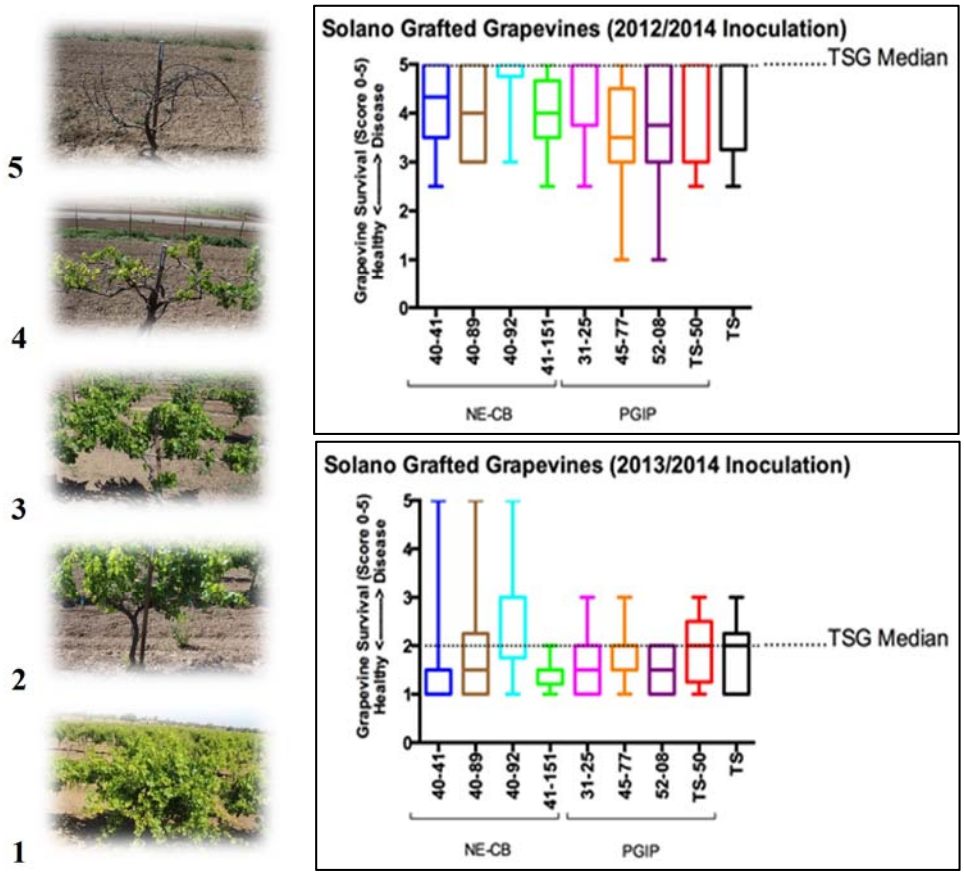

Figure 4. Survival of Solano County grafted transgenic grapevines inoculated in 2012/2014 (upper right) and 2013/2014 (lower right), scored on April 28, 2015, using a scale of 1 to 5 (left).

Severity or absence of Pierce's disease symptoms for all Solano County grafted transgenic grapevines inoculated from 2012 to 2015 was assessed in fall 2015 using the Pierce's disease symptom severity rating system 0 to 5, where 0 = healthy vine, all leaves green with no scorching; 1 = first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the cane shows scorching; 4 = the whole cane is sick and is declining; and 5 = the cane is dead (**Figure 5**). Pierce's disease symptom severity scores were lower in most grafted inoculated transgenic lines from either strategy than in grafted untransformed controls.

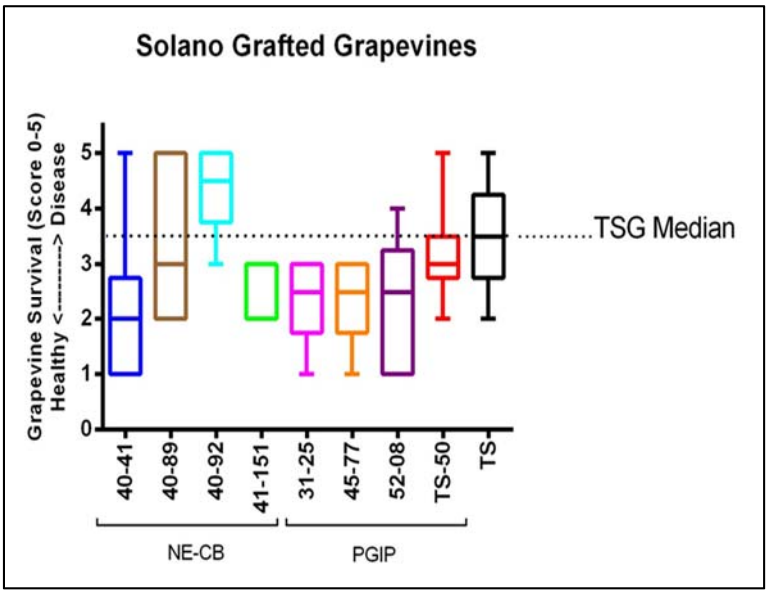

Figure 5. Severity or absence of Pierce's disease symptoms for all Solano County grafted inoculated grapevines scored on September 29, 2015.

Objective 2. Assume permit holder status for existing USDA APHIS field permit and maintain regulatory oversight and compliance with permit reporting requirements.

Activity 4. Participate with PIPRA during transition and assume permit holder status.

The current USDA APHIS field permit was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The permit was extended by USDA APHIS, with a new end date of April 1, 2016.

Activity 5. Maintain regulatory oversight of field location and compliance with reporting requirements.

During the transition period beginning October 1, 2013, personnel from the Dandekar laboratory worked with PIPRA personnel to obtain all documentation and records necessary to maintain regulatory oversight of the field trial. This process was completed in January 2014, with the transfer of full responsibility to the new permit holder. We have worked closely with the Environmental Health and Safety Unit, University of California, Davis to modify our existing Biological Use Authorization (BUA) to include this permit, a process that integrated the institutional biosafety committee into the chain of custody for regulatory oversight compliance management. Although the responsibility for regulatory compliance rests with the new permit holder, UC Davis was included during the transition to maintain their oversight of campus BUAs. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trial. The issues requiring regulatory oversight are listed in the permit.

Activity 6. Maintain active regulatory compliance inspections.

Timely reporting and inspections are conducted to maintain compliance with USDA APHIS. Regulatory compliance is enforced by working closely with the participant investigators and the two field coordinators and their crews. Pierce's disease field trial activities information is updated quarterly using the principal investigators' activity monitoring logs. Two individuals from the Dandekar lab are entrusted with the tasks of documentation, training, and inspection to ensure regulatory compliance.

CONCLUSIONS

We successfully established two field trials to validate two greenhouse-tested strategies to control movement and clearance of *X. fastidiosa*, a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease. A key virulence feature of *X. fastidiosa* resides in its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements, enhancing long-distance movement and vector transmission. The first strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *X. fastidiosa* polygalacturonase activity. Our second strategy enhances clearance of bacteria from *X. fastidiosa*-infected xylem tissues using a chimeric antimicrobial protein, NE-CB. The expectation is that expressing these proteins will prevent *X. fastidiosa* movement and reduce inoculum size, curbing the spread of Pierce's disease in California vineyards.

Ninety-six (96) grafted transgenic grapevine plants expressing either NE-CB or PGIP and 12 grafted untransformed controls were successfully planted in Solano County in 2011. These grafted transgenic grapevines were evaluated as rootstocks grafted with untransformed TS scions. NE-CB- and PGIP-expressing grafted transgenic lines in Solano County were evaluated phenotypically using the first 12 descriptors from the "Primary Descriptor Priority List" proposed by the International Organization of Vine and Wine (OIV). No phenotypic/horticultural differences were observed between grafted transgenic and untransformed TS vines. Grafted grapevines were also genotyped, confirming the presence of the inserted transgene in all lines. At the Solano County site, grafted vines were mechanically inoculated with *X. fastidiosa* in 2012 and 2013 to validate resistance to Pierce's disease under field conditions. Leaf scorching, the characteristic symptom of Pierce's disease, was observed in Solano Country grafted transgenic and control lines in 2013 and *X. fastidiosa* presence was confirmed by ELISA in stem extracts from samples collected in the same season. On May 27, 2014 and May 27, 2015, following the recommendation of the CDFA Pierce's Disease and Glassy-winged Sharpshooter's Product Development Committee (PDC), at least four new canes per year from all grafted transgenic and control plants were mechanically inoculated with *X. fastidiosa*.

The severity or absence of Pierce's disease symptoms on all inoculated canes was assessed in summer 2014 and fall 2015 using the Pierce's disease symptom severity rating system with a 0 to 5 scale, where 0 = healthy vine, all leaves green with no scorching; 1 = first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and in decline; and 5 = the cane is dead. Pierce's disease symptom severity scores were lower in at least two NE-CB and two PGIP grafted inoculated transgenic lines than in grafted untransformed control. Bud break success of grafted individual canes inoculated in 2014 was assessed on March 26, 2015, using a 0 to 5 score. The data showed that bud break success is greater in most grafted inoculated transgenic lines with either construct than in grafted untransformed controls. Survival of grafted transgenic grapevines inoculated in 2012/2014 and 2013/2014 was assessed on April 28, 2015, using a 1 to 5 score. Grapevine survival was higher in some grafted inoculated transgenic lines from each strategy than in grafted untransformed control grapevines.

In summer 2014, one cane per grafted plant was harvested to quantify *X. fastidiosa* by qPCR. *X. fastidiosa* DNA from grape stem was extracted using a modified CTAB method that yields DNA of a quantity and quality suitable for qPCR. An *X. fastidiosa* 16s primer pair was used for *X. fastidiosa* quantification. qPCR standard curves were obtained using concentrations of *X. fastidiosa* from 102 to 106 cells. *X. fastidiosa* was detected in grafted transgenic vines, but at lower concentrations than in grafted untransformed control grapevines. Another set of canes from the 2014-inoculated grafted individual canes was harvested in fall 2015 and *X. fastidiosa* quantification is in progress.

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DEFINING THE ROLE OF SECRETED VIRULENCE PROTEINS LESA AND PRTA IN THE PATHOBIOLOGY OF XYLELLA AND IN THE DEVELOPMENT OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2014 to September 2015.

ABSTRACT

Our goal is to understand the virulence mechanisms of *Xylella fastidiosa* that lead to the leaf scorching symptoms observed in Pierce's disease and to exploit this information to develop new strategies to control Pierce's disease in grapevines. The analysis of *X. fastidiosa* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA, that appear to be causal to the leaf-scorching phenotype observed in Pierce's disease. We generated mutant *X. fastidiosa* that are defective for each of these two genes and they show alterations in disease phenotype -- lesA1 is less virulent, while prtA1 is more virulent. LesA protein displays lipase/esterase activities and is the most abundant but is very similar to two additional less abundant proteins, LesB and LesC, also secreted by *X. fastidiosa*. Expression of LesA, B, and C individually in *Escherichia coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and *X. fastidiosa*-prtA1 mutants are highly virulent. We are analyzing the microbiome of plants infected with the wild-type Tem 1 and comparing it with uninfected and with plants infected with our various mutant strains, lesA1, lesA3B1, and prtA1. A fairly large dataset composed of different tissue types and stages of infection has been generated and is currently being analyzed. We will look at differences in microbiome composition in different tissues and at different stages of infection. Additionally, we have built a vector to test expression of PrtA in transgenic tobacco. The three transgenic plants tested so far do not display any anti-virulence activity; 10 more transgenic lines will be tested. An understanding of how these two secreted proteins function and their associated pathobiology will provide new insights into this disease and deliver new avenues of therapy.

LAYPERSON SUMMARY

Pierce's disease of grapevines is caused by the bacterium *Xylella fastidiosa*, a xylem-limited bacterium that is responsible for several economically important diseases in many plants. A characteristic symptom of Pierce's disease is leaf scorching, with marginal regions of leaves developing chlorosis progressing to necrosis. Blockage of xylem elements and interference with water transport by *X. fastidiosa* and its associated biofilm have been posited to be the main cause of Pierce's disease symptom development. An analysis of *X. fastidiosa* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA, that may play a role in the development of Pierce's disease symptoms. We generated mutant *X. fastidiosa* that are defective for each of these two proteins and show alterations in disease phenotype. Mutant bacteria defective in making LesA are less virulent and display a biofilm behavior in culture, while the bacteria defective in PrtA appear highly virulent and correspondingly display planktonic growth in culture. Our experiments show that these two proteins play a role in disease progression. We are also examining the role of these secreted proteins with respect to the grapevine microbiome with respect to the normal flora present in different tissues of a healthy grapevine plant. We have extracted the microbiome and are analyzing the effects that these two proteins may have on the composition and relative distribution of the natural flora or microbiome as this could affect vine health and disease outcome. An understanding of how these two proteins work will provide new insights into this disease and deliver new avenues of therapy.

INTRODUCTION

Xylella fastidiosa is a fastidious, xylem-limited gamma-proteobacterium that causes several economically important diseases in many crops including grapevine, citrus, periwinkle, almond, oleander, and coffee (Davis et al., 1978; Chatterjee et al., 2008). In the field, *X. fastidiosa* is vector-transmitted by various xylem sap-feeding sharpshooter insects (Purcell and Hopkins, 1996; Redak et al., 2004). The *X. fastidiosa* subspecies *fastidiosa* (*Xff*), as exemplified by the California strain Temecula 1, causes Pierce's disease in grapevine. The *X. fastidiosa* life cycle and virulence mechanism are not entirely understood (Chatterjee et al., 2008). This research seeks to understand the pathobiology of *X. fastidiosa* that leads to disease; specifically, the underlying mechanism that leads to leaf scorching symptoms. Understanding the underlying mechanism could help develop new strategies to control Pierce's disease in grapevines in California as well as diseases in many other economically important crops.

The secretion of virulence factors by pathogens is an important mechanism by which many plant diseases are triggered. Unlike closely-related pathogens from the genus *Xanthomonas*, *Xff* does not possess the type III secretion system (T3SS) (Van Sluys et al., 2002). However, *Xanthomonas* and *X. fastidiosa* have in common a similar type II secretion system (T2SS) for a battery of important extracellular enzymes that are responsible for virulence (Ray et al., 2000). In *Xff*, genes have been identified that code for plant cell-wall degrading enzymes (CWDEs) such as polygalacturonase, cellulase, lipase/esterase, and several proteases (Simpson et al., 2000). These enzymes may aid *Xff* migration inside xylem vessels by degrading the pit membrane and also by helping to release the carbohydrates necessary for bacterial survival. Cell wall degradation by CWDEs releases oligosaccharides as products, which can induce potent innate immune responses from plants. The plant defense responses include production of phytoalexins, fortification of cell walls through deposition of callose, oxidative burst, and induction of programmed cell death (Darvill and Albersheim, 1984; Ryan and Farmer, 1991; Braun and Rodrigues, 1993). One T2SS secreted protein, a polygalacturonase virulence factor encoded by pglA, lost pathogenicity when it was mutated and resulted in *X. fastidiosa* that was unable to colonize grapevine (Roper et al., 2007). This confirmed an earlier finding that expression of a polygalacturonase inhibitory protein blocked the action of pglA, providing resistance to Pierce's disease (Aguero et al., 2005).

OBJECTIVES

The goal of this project is to define the role that *Xylella* secreted proteins LesA and PrtA play in the Pierce's disease phenotype of grapevine.

- Objective 1. Define the mechanism of action of LesA and PrtA gene products.
  - Activity 1. Express LesA, B, C, and PrtA individually and examine their role in the virulence response of *Xylella* cultures.
  - Activity 2. Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C, and PrtA.
  - Activity 3. Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence.

RESULTS AND DISCUSSION

Objective 1. Define the mechanism of action of LesA and PrtA gene products.

Our previous analysis revealed 24 secreted proteins in cultures of *X. fastidiosa* Temecula 1. Of these, we have characterized two proteins, PrtA and LesA. A proteomic analysis of infected leaf tissues revealed five of the 24 secreted *X. fastidiosa* proteins, the most abundant of which is LesA. To further characterize the role of these proteins, we used insertional mutagenesis of *X. fastidiosa* cultures and expressed the respective proteins in *Escherichia coli* to identify their function in Pierce's disease.

Activity 1. Express LesA, B, C, and PrtA individually and examine their role in the virulence response of *Xylella* cultures.

The secreted protein PrtA was previously annotated as an uncharacterized protein. We analyzed the structure of PrtA, comparing it with proteins in the Protein Data Bank (PDB) database, and found a close structural match to an extracellular alkaline serine protease. Based on this structural prediction, we were able to align the amino acid residues in the active site showing a perfect alignment of these active site residues. Also, prtA is highly conserved among various *Xylella* strains, but interestingly not among *Xanthomonas* strains. The protease activity of prtA was confirmed by expressing the encoded protein, PrtA in *E. coli* and we were able to demonstrate a lack/lesser activity in a mutant (prtA2) where one of the active site residues (S280 mutated to A280) was mutated as compared to the wild-type PrtA enzyme using fluorescent-labelled casein as the substrate. To investigate the

function of this protein we created a functional knockout strain via homologous recombination where the genomic region encoding PrtA was disrupted via the insertion of a gene encoding resistance to the antibiotic gentamycin. This mutant strain was called prtA1. We confirmed via polymerase chain reaction (PCR) analysis that the coding region of prtA was disrupted in the prtA1 mutant. Expectedly, the mutant strain displayed less protease activity and PrtA was not detectable in secreted proteins from prtA1 cultures, while PrtA was detectable in wild-type cultures. Growth characteristics of prtA1 cultures revealed that it was markedly more planktonic than the wild-type *X. fastidiosa* Tem1 strain. Growth on plates showed less aggregation, and when grown in flasks, a clear biofilm ring was formed by wild-type but not prtA1 cultures. We used scanning electron microscopy (EM) to confirm that wild-type cultures showed marked aggregation whereas prtA1 appeared to be exclusively planktonic. Since planktonic forms have reportedly displayed more virulence (Newman et al., 2004; Chatterjee et al., 2008), we infected grapevine plants as described earlier (Dandekar et al., 2012) in the main stem, 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible with prtA1 infections starting at six to eight weeks, much earlier than that observed with the wild-type. A comparison of prtA1 and wild-type colonies shows twitching motility at the margins of prtA1 colonies, confirming enhanced movement consistent with the observed enhanced virulence.

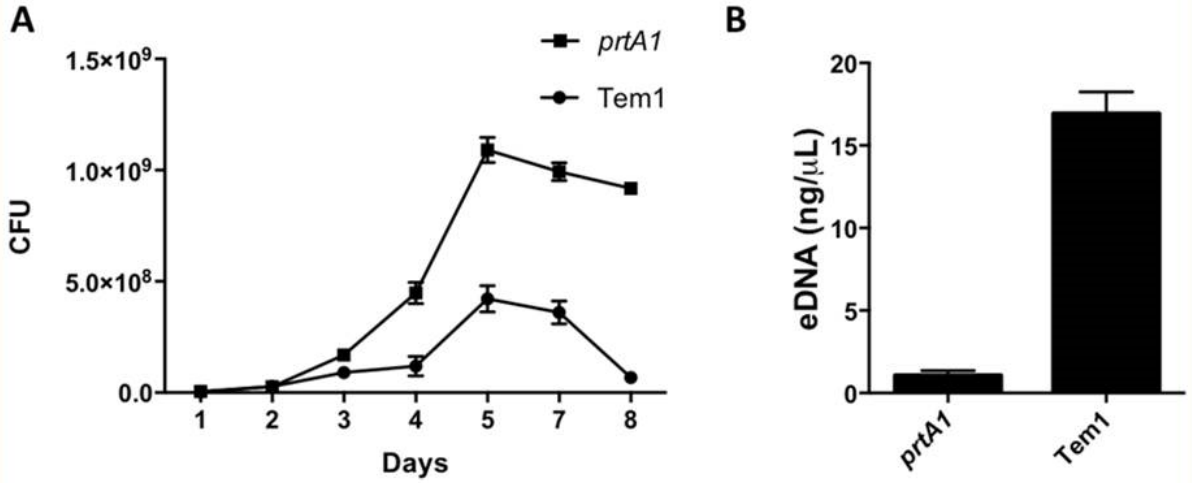

Fig 1: Cell lysis and eDNA quantification. (A) *X. fastidiosa prtA1* mutant and Tem1 wild type growth curves by plating on agar plate and counting number of colony-forming units (CFU) for 8 days. (B) eDNA quantification for *prtA1* mutant and Tem1 strains grown in liquid media for one week.

The secreted protein LesA was also previously annotated as an uncharacterized protein. It has a 35 amino acid secretion peptide consistent with it being secreted. Immunogold localization of LesA in fixed cells using antibodies against LesA revealed that most of the protein was embedded within the secreted matrix surrounding *X. fastidiosa* cells, confirming that LesA is a secreted protein. We compared the structure of lesA to proteins in the PDB database and found a close structural similarity to a *Xanthomonas oryzae* pv. oryzae (*Xoo*) LipA that has lipase and esterase activity (Aparna et al., 2009). Lipase activity was confirmed by growing *X. fastidiosa* cultures on plates containing tributryn, a triacylglyceride of butyrate; zones of clearance were clearly visible surrounding the colonies, indicating lipase activity. Based on this structural prediction, we aligned the active site residues S200, D360, and H402 of LesA with LipA from *Xoo*. We then threaded LesA with the known structure of the *Xoo* LipA and there was an excellent alignment of active site residues. Additionally, LesA was found to be highly conserved among both *Xylella* and *Xanthomonas* strains. To determine whether LesA had both lipase and esterase activities, we expressed LesA in *E. coli* and made a mutant version, LesA2, in which the S200 serine in the protein was substituted with an alanine residue. We then analyzed the activity by growing/harvesting *E. coli* strains that displayed both lipase activity evaluated on agar plates containing tributryn as well as esterase activity by assaying the *E. coli* extracts using the substrate 4-methyl umbelliferone butyrate and measuring the formation of 4-methyl umbelliferone (4MU), the product of the reaction. Esterase activity was clearly seen in strains expressing LesA and to a lesser extent in those expressing LesA2 or the empty vector (EV). *E.coli* expressing

LesA showed zones of clearance but not those expressing LesA2. In addition, LesA protein was detected on western blots from cultures expressing both LesA and LesA2. To investigate the function of the LesA protein we created a functional knockout strain via homologous recombination where the genomic region encoding LesA was disrupted via the insertion of a gene encoding resistance to the antibiotic kanamycin. This mutant strain was called lesA1. Among the 24 proteins secreted by *X. fastidiosa* cultures, LesA was the most abundant, but we also identified LesB and LesC, proteins with strong homology to LesA. An alignment of the protein sequences revealed a conservation of the active site residues of Les A in LesB and C. Les B is located adjacent to the lesA on the *X. fastidiosa* genome, but LesC is located at some distance away. Since lesA and B were located together, we created a double knock-out using kanamycin, this particular strain is designated lesA3B1. We expressed both LesB and LesC in *E.coli*. We confirmed by PCR comparison of wild-type *X. fastidiosa* with lesA1 and LesA3B1 that we had knocked out one and both genes. LesA1 and LesA3B1 show less lipase and esterase activities. Additionally, there are some differences among the activities of LesA, B, and C. Les A has both lipase and esterase activities, LesB has neither lipase nor esterase activity, and LesC has lipase but no esterase activities directed at butyrate substrates. LesA1 and lesA3B1 cultures displayed increased aggregation, in contrast to wild-type *X. fastidiosa* Tem1 strains. When grown in flasks, a clear biofilm ring was visible for the wild-type, but a much larger and more profound ring of biofilm was visible for lesA1. We further confirmed this observation using scanning EM, where lesA1 showed marked aggregation of cells. To investigate the role of lesA in the virulence response and Pierce's disease, we infected grapevine plants as described earlier (Dandekar et al., 2012), inserting ~10 million bacteria at the bottom of grapevines about 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible starting at 10 weeks for the wild-type Tem1 strains, but neither the lesA1 nor the lesA3B1 strains showed symptoms. Infiltrating the LesA protein into grapevine leaves led to scorching, but infiltrating the LesA2 mutant protein that lacks both lipase and esterase activities did not. These results clearly show that the secreted LesA protein is related to leaf scorching and that the activity of the lipase/esterase is necessary for full virulence.

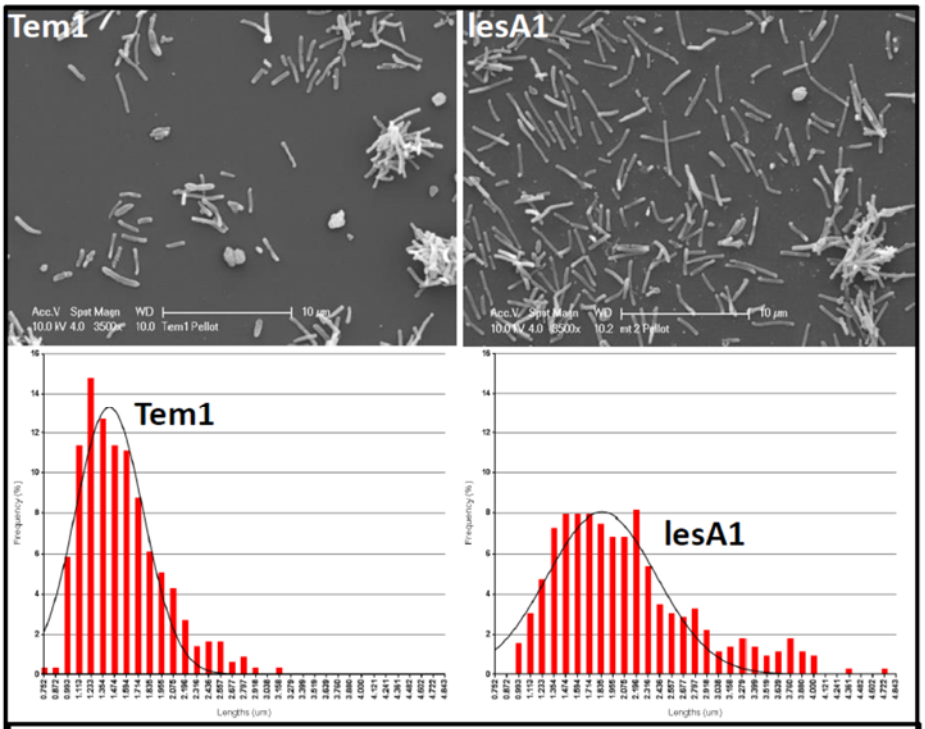

Fig 2: SEM comparison of wild type Tem1 and lesA1 bacteria and their length distribution.

Growth of the lesA1 in culture showed that a predominant proportion of the bacteria were in an aggregated state and when grown in a flask the culture displayed a strong ring of biofilm (**Figure 3**). Further, the optical density (OD) of the culture was low compared to the wild-type Tem1 strain. Shown in **Figure 2** are scanning electron

microscope (SEM) image panels on the top showing wild-type and leaA1 cells grown in culture and on the bottom are the measurements of bacterial length of this population.

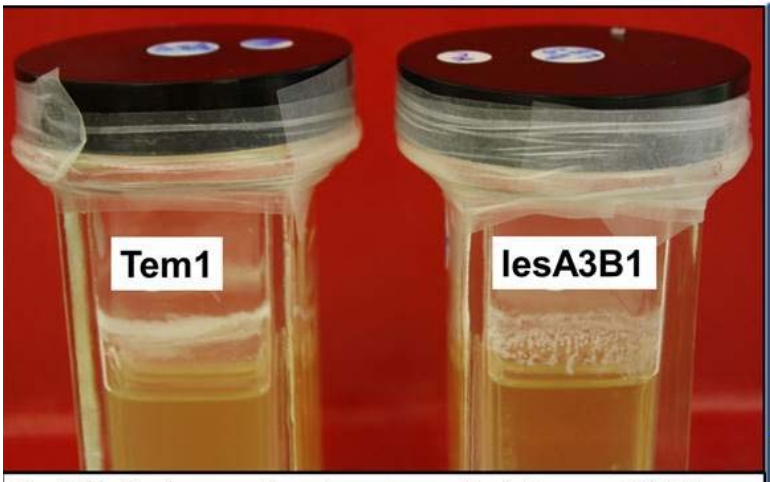

Fig. 3: Biofilm/aggregation phenotypes of wild type and *Xylella* mutated for lesAB

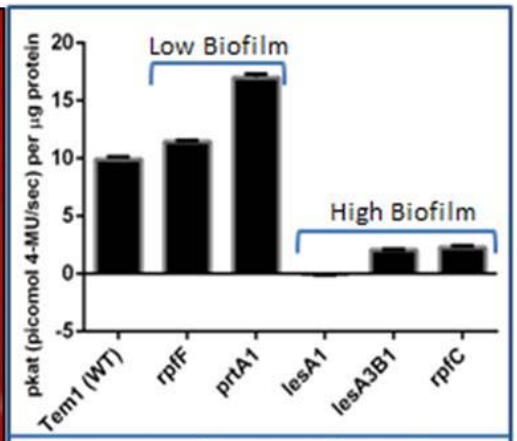

Fig 4: Activity of LesA in cultures of different mutants compared to wild type Tem1 cells

The lesA1 strain that displayed a greater proportion of aggregated cells had a greater proportion of longer cells (**Figure 2**). Expectedly, lesA1 was less pathogenic as compared to wild-type *Xylella*. The biofilm and planktonic states have been shown to be regulated by quorum sensing behavior, regulated by diffusible signal factor (DSF) as C12 fatty acid molecule (Chatterjee et al., 2008). DSF synthesized by rpfF triggers biofilm formation and down regulates pathogenesis. Mutants in rpfF are more virulent than wild-type *Xylella*. The DSF is sensed by a receptor rpfC that is part of a two-component regulatory system that senses DSF on the outside and triggers rpfG to mediate the response (Buttner and Bonas, 2009). LesA is required for pathogenesis and so lesA1 that does not make LesA appears to be a nonpathogenic biofilm in culture (**Figure 3**). In **Figure 4** we measured the levels of LesA and there appears to be a good correlation between the presence of lesA and pathogenesis. Wild-type Tem1, prtA1, and rpfF that do not make biofilm and that are very pathogenic make a lot of LesA, whereas mutants that appear to make more biofilm like lesA1, lesA3B1, and rpfC express lower levels of LesA (**Figure 4**).

Activity 2. Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C, and PrtA. The secreted proteins could influence the grapevine microbiota and that interaction could influence the disease outcome. Previously, we conducted a preliminary alpha diversity survey in which we took Thompson Seedless (TS) samples infected with *X. fastidiosa* unable to make PrtA (prtA1), wild-type *Xf* (Tem1), and uninfected tissue. Samples were kept frozen and then ground into powder using Qiagen's grinding jar set and associated TissueLyser. DNA was extracted using the MoBio PowerPlant Pro DNA isolation kit. PCR and sequencing of the V4 region of the 16S rRNA gene using region-specific primers and PCR and sequencing were performed using standard protocols as agreed upon in the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/) using Illumina MiSeq (Caporaso et al., 2012).

The immediate problem which emerged from the preliminary sequencing data was the high proportion of host chloroplast sequences that came from the samples. Preliminary data demonstrated the low number of sequences after chloroplast removal. To compensate for low sequencing depth due to the abundance of chloroplast sequences, we obtained PCR blockers that selectively inhibited amplification of chloroplast sequences (Orum, 2000). Using PCR blockers, a rarefaction plot verified that the number of observed species was plateauing, indicating that we sampled a majority of the 16S community (**Figure 5**).

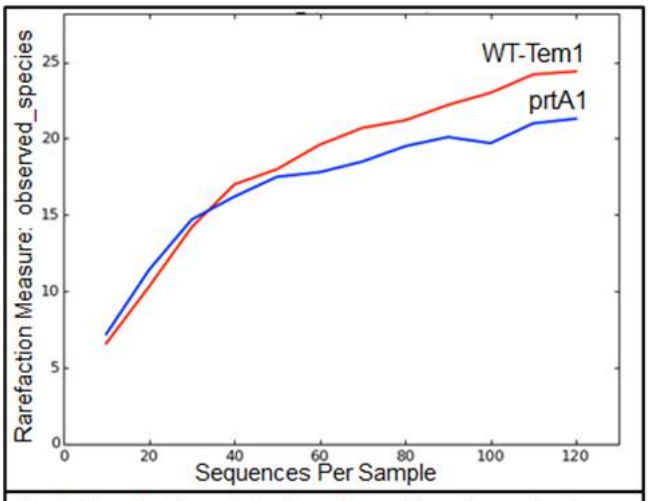

Fig 5: Rarefaction plots show how diversity varies in a sample with the number of sequences sampled.

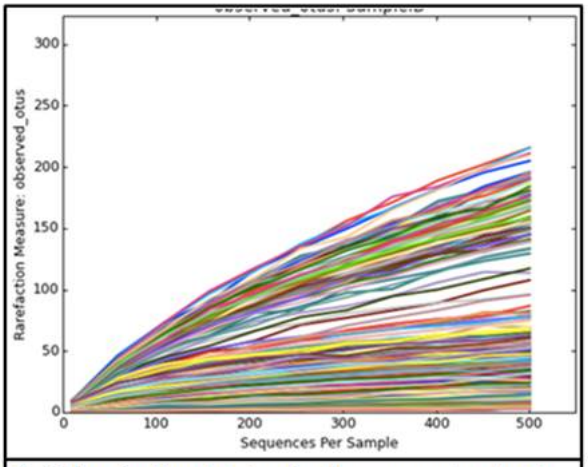

Fig 6: Rarefaction plot showing the sequencing depth of samples as it pertains to new microbial OTU discovery in the x.f. infection experiment.

We have since used the data and experience we obtained from our preliminary work to begin work on an infection study to determine differences in grapevine microbiome structure upon infection with *X. fastidiosa*. A rarefaction plot of this new data shows a wide range in microbial diversity per sample (**Figure 6**). For this infection study, we harvested non-leaf samples at a much closer distance to the inoculation point, as this is where symptoms are first observed and where probable changes in the microbiota will first be evident. TS grapevines were grown 18 weeks and harvested at six different times. Six plants were sampled from each of five treatments: uninfected or infected with *X. fastidiosa* Tem1 (Wild-Type), *X. fastidiosa* lesA1, *X. fastidiosa* lesA2B3, or *X. fastidiosa* prtA1) at each time. From each plant we obtained root tissue, stem tissue from two to three nodes above the infection point, and stem tissue between nodes 10 and 11. Samples were placed on ice, brought to the lab, washed with 0.04% Tween 20 and rinsed with ddH20 to minimize background surface microbiota. Samples were then frozen in liquid nitrogen and maintained at -80C until analysis. Samples were ground into powder using Qiagen's grinding jar set and associated TissueLyser. Unfortunately, the previously used MoBio PowerPlant Pro DNA isolation kit and several other kits and protocols were insufficient to obtain high-quality DNA from these high-phenolic, woody samples. As a kit was preferred to decrease possible microbiota background variability arising from user contamination during a phenol-chloroform DNA extraction, high-quality DNA was finally obtained using Qiagen's DNAeasy Plant Mini Kit with an additional 5% sodium metabisulfite in the lysis buffer. We made a library of these DNA extractions and sequenced using Illumina MiSeq. Data was demultiplexed and mitochondrial, singleton, and remaining chloroplast sequences were removed. Unique operational taxonomic units (OTUs) were defined as having no more than 97% similarity. We have begun our initial analysis of the sequencing data (**Figure 7**), and have begun comparing diversity changes between tissues (**Figure 8**). This will be followed by a diversity comparison between time points followed by a comparison of treatments at a single time point and then across time points. Shown in **Figure 7** is our analysis of 16S DNA sequences extracted from two locations (upper and lower) of the same stem segment of the grapevine at 0 time point of infection. Pre-infection, the top classes which dominate the microbiome sampled from 2-3 nodes (lower) and from 10-11 nodes (upper) above the pre-infection point show the top classes in these tissues are Gammaproteobacteria, Bacilli, Alphaproteobacteria, and Betaproteobacteria (**Figure 7**).

Activity 3. Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence.

To test the anti-virulence phenotype of PrtA, we cloned the prtA coding region into a binary vector under the CaMV35S promoter after codon optimization for expression in tobacco (**Figure 9**). The binary vector construct was introduced into a disarmed strain of *Agrobacterium* (EHA105) via electroporation to create a functional system for plant transformation. Thirteen transgenic SR1 tobacco lines have been generated at the UC Davis Parson Transformation Facility. We have screened these plants and they are positive for the presence of the kan genes and express PrtA as detected using an an-FLAG antibody. We did not detect the protein using an anti-PrtA antibody. The resulting plants are currently being propagated to collect F1 seed that germinate on medium containing kanamycin. Kan-resistant transplants have been moved as they mature to the greenhouse and grown up

to six to eight leaves. We have infected the fully expanded leaves with a virulent strain of *X. fastidiosa* for three of the lines to evaluate whether PrtA has anti-virulence activity. Thus far the three lines tested showed no protection from the disease development. We have 10 more lines remaining to be evaluated.

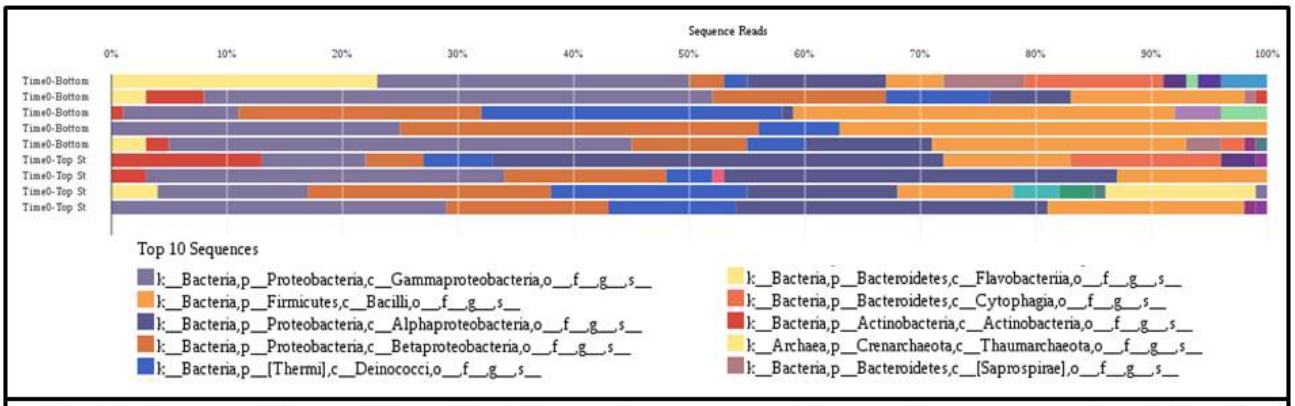

Fig. 7: Composition of the microbiome in upper and lower stem segments at the 0 time point of the infection.

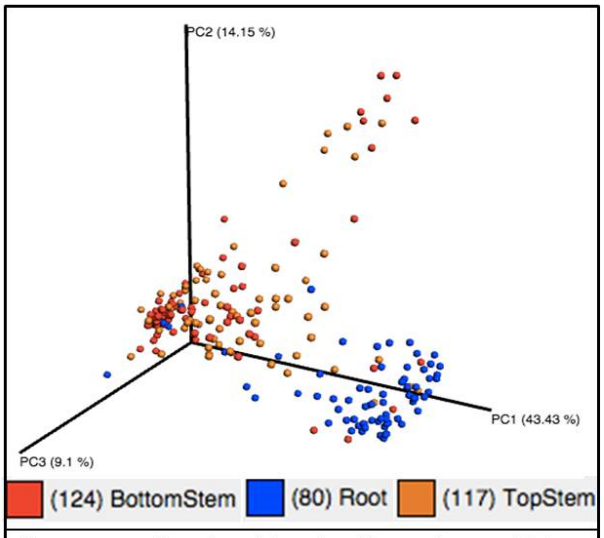

Fig. 8: PCoA plot of Weighted Unifrac Values. Initial data shows a clear difference between root tissue (blue) and stem(red and orange). Bottom Stem tissue is not highly differentiable from Top Stem tissue.

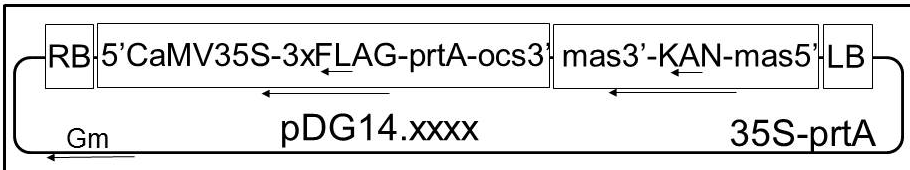

Fig. 9: Binary vector construct developed for *in planta* testing of anti-virulence activity of Xf prtA encoded protein in transgenic tobacco plants

CONCLUSIONS

The goal of this project is to understand the virulence mechanisms of *X. fastidiosa* that lead to the leaf scorching symptoms observed in Pierce's disease and to exploit this information to develop new strategies to control Pierce's disease in grapevines. The blockage of xylem elements and the interference with water transport by *X. fastidiosa* is regarded to be the main cause of Pierce's disease symptom development. The analysis of *X. fastidiosa* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA. We generated mutant *X. fastidiosa* that are defective for each of these two genes and they show alterations in disease phenotype -- lesA1 is less virulent while prtA1 is more virulent. LesA displays lipase/esterase activities and is the most abundant but is very similar to two additional less abundant proteins, LesB and LesC, also secreted by *X. fastidiosa*. Expression of LesA, B, and C individually in *E.coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and *X. fastidiosa* prtA1 mutants are highly virulent, suggesting that this protein may somehow block disease. We are also investigating what role these particular proteins have on the composition and distribution of the microbiome. A fairly large dataset has been generated and is being currently analyzed to evaluate the differences in the composition of the microbiome in different tissues and at different stages of infection. We have built vectors to test the anti-virulence activity of PrtA by expressing it in transgenic SR1 tobacco plants. The analysis of the first three transgenic tobacco plants has revealed no difference compared to controls. There are 10 more lines that need to be tested. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

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THE RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM IN THE TEMECULA VALLEY

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ABSTRACT
For approximately 15 years the Temecula Valley has been part of an area-wide control program for an invasive vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*). The goal of this program is to limit Pierce's disease spread by suppressing vector populations in commercial citrus, an important reproductive host for this insect, before they move out into vineyards. To achieve effective control of the glassy-winged sharpshooter, spring applications to citrus of the systemic insecticide imidacloprid have been made in years past. As part of this treatment program there is ongoing monitoring of glassy-winged sharpshooter populations to ensure that the treatments are effective. Notably, since 2013, reimbursements to citrus growers have not been made. As a result, over the past three seasons, apparently no Temecula Valley citrus acreage was treated specifically for the glassy-winged sharpshooter – the consequences of which are not well understood. Approximately 140 yellow sticky traps were inspected on a biweekly basis throughout 2015 to monitor glassy-winged sharpshooter in citrus. The results show a typical phenology for this pest in the region, with a total of less than 400 glassy-winged sharpshooters caught during the summer peak (July through September). Overall glassy-winged sharpshooter catch was intermediate this year - modest compared to high years in 2008 and 2009 but slightly higher than recent low years (e.g., 2010, 2011).

LAYPERSON SUMMARY
The glassy-winged sharpshooter (*Homalodisca vitripennis*) constitutes one of the primary threats to the wine, table grape, and raisin industries in California owing to its ability to spread a pathogen that causes Pierce's disease. In the Temecula Valley, an area-wide control program has been in place for more than 10 years, which relies on insecticide applications in citrus groves to control the glassy-winged sharpshooter before it moves into vineyards. This program is viewed as critical for reducing the disease spread in vineyards. As part of the control program, citrus groves are monitored regularly for glassy-winged sharpshooters. This year, like the past two, despite no insecticide applications being made to target the glassy-winged sharpshooter, the glassy-winged sharpshooter catch in Temecula was relatively modest; intermediate between very high years such as 2008 and 2009 and very low years such as 2010 and 2011.

INTRODUCTION
The wine grape industry and its associated tourism in the Temecula Valley generate $100 million in revenue for the economy of the area. Following the invasion of the glassy-winged sharpshooter into Southern California from the Southeastern USA, a Pierce's disease outbreak occurred. This outbreak resulted in a 30% loss in overall vineyard production over a few years, with some vineyards losing 100% of their vines during the initial years of the outbreak. An area-wide glassy-winged sharpshooter management program initiated in the spring of 2000 saved the industry from even more dramatic losses. Since the initiation of the Temecula glassy-winged sharpshooter area-wide management program several hundred new acres of grapes have been planted and multiple new wineries have been built.

The glassy-winged sharpshooter has the potential to develop high population densities in citrus. Fortunately, the glassy-winged sharpshooter is also highly susceptible to systemic insecticides such as imidacloprid. Insecticide treatments in citrus groves, preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments, have been used to manage this devastating insect vector and disease.

As part of the area-wide treatment program, monitoring of glassy-winged sharpshooter populations in citrus has been conducted since program inception. This monitoring data has been used to guide treatment decisions for

citrus, to evaluate the efficacy of the treatments, and to guide vineyard owners, pest control advisors, and vineyard managers on the need for supplementary vector control measures within vineyards.

In 2013, the decision was made by state and federal regulators not to reimburse citrus growers for insecticide applications intended to target the glassy-winged sharpshooter in the Temecula Valley. This change was motivated by the expectation that citrus growers would likely be treating already for the Asian citrus psyllid, *Diaphorina citri*, an invasive vector of the pathogen associated with huanglongbing or citrus greening disease. Sharpshooter and psyllid integrated pest management rely on largely the same insecticides. However the timing of applications differs slightly depending on the focal pest. Therefore, monitoring of sharpshooter populations continues to be important, to determine whether glassy-winged sharpshooter populations, which already show substantial interannual variability, appear to be rebounding.

OBJECTIVES

1. Regularly monitor glassy-winged sharpshooter populations in citrus groves throughout the Temecula Valley to evaluate the effectiveness of prior insecticide applications and to provide a metric of Pierce's disease risk for grape growers.
2. Disseminate a newsletter for stakeholders on sharpshooter seasonal abundance in citrus throughout the region.

Double-sided yellow-sticky cards **(**14 x 22 cm; Seabright Laboratories, Emeryville, CA**)** are being used to monitor adult sharpshooters in citrus. A total of 137 such sticky traps have been placed in citrus groves throughout the Temecula Valley. All traps are labeled, numbered, and bar-coded to identify the site within the management program. Each trap is then georeferenced with a handheld global positioning system monitor. Most traps are placed at the edge of the groves at the rate of approximately one per 10 acres. Traps are attached with large binder clips to wooden stakes around the perimeter of the grove. For large groves traps are also placed in the interior. The total number of traps depends on the size of the orchard block. Sharpshooters found on the traps are counted and then removed from the trap.

The yellow cards are inspected and replaced every two weeks during the summer and fall (May through October) and monthly the rest of the year. At each inspection the number of adult glassy-winged sharpshooters and smoketree sharpshooters (*Homalodisca liturata*) is recorded, along with the abundance of common generalist natural enemy taxa.

After collecting all data for a given sharpshooter census date, these data are collated into a newsletter that shows the number of sharpshooters caught, where they were caught, and the seasonal phenology of sharpshooter populations to date. This newsletter is disseminated to stakeholders via e-mail and on a blog hosted by UC Riverside's Center for Invasive Species Research (http://cisr.ucr.edu/temeculagwss/).

RESULTS AND DISCUSSION

The results for 2015 are shown in **Figure 1**. This includes monthly censuses of glassy-winged sharpshooters in citrus through April, then biweekly censuses from May through October. Census results show seasonal patterns of glassy-winged sharpshooter abundance and activity that are typical for this region. Glassy-winged sharpshooter catch is low for much of the year; it increases dramatically at the beginning of the summer and then drops off through August and September. As of early-October, glassy-winged sharpshooter populations appear to have declined substantially.

Figure 2 shows glassy-winged sharpshooter catch in 2015 relative to other years. 2015 shows qualitatively the same seasonal phenology as in other years, with a moderate overall catch compared to others (i.e., 2009).

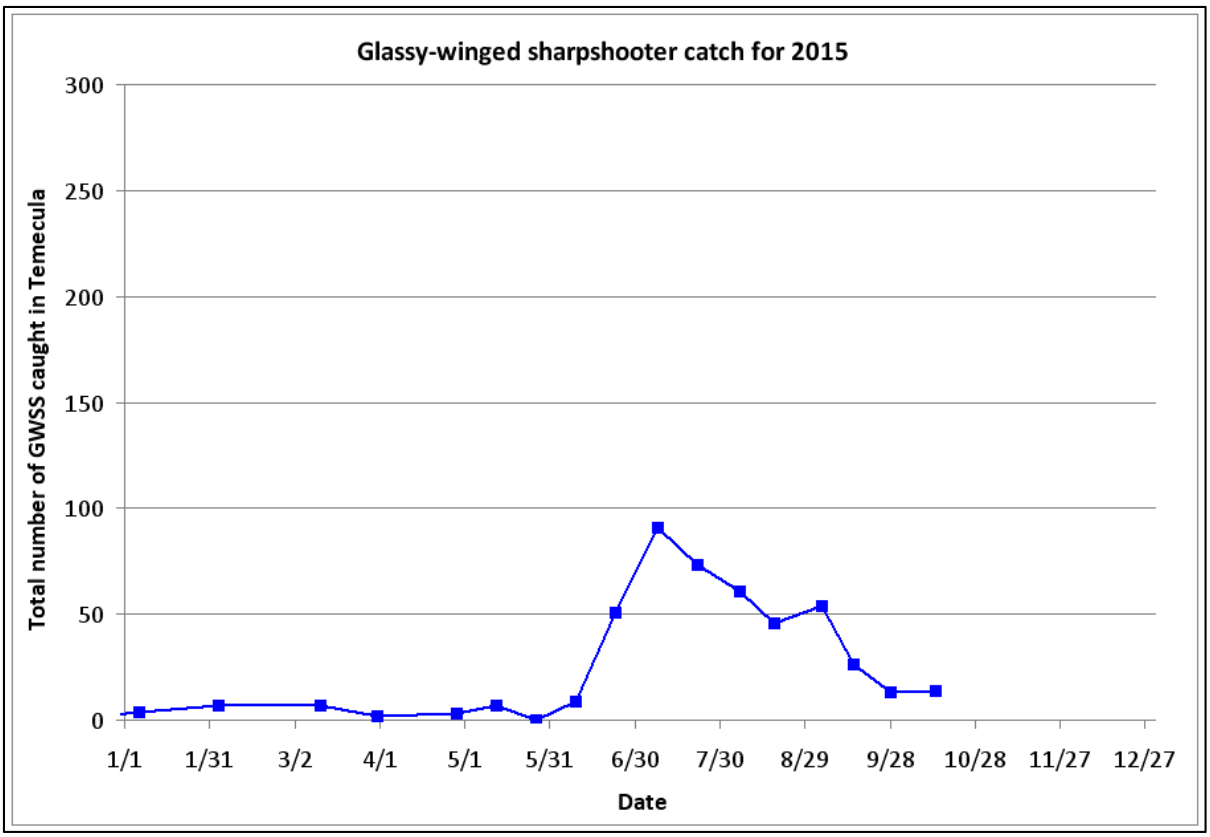

Figure 1. Seasonal total glassy-winged sharpshooter catch in 2015 for 137 traps throughout the Temecula Valley.

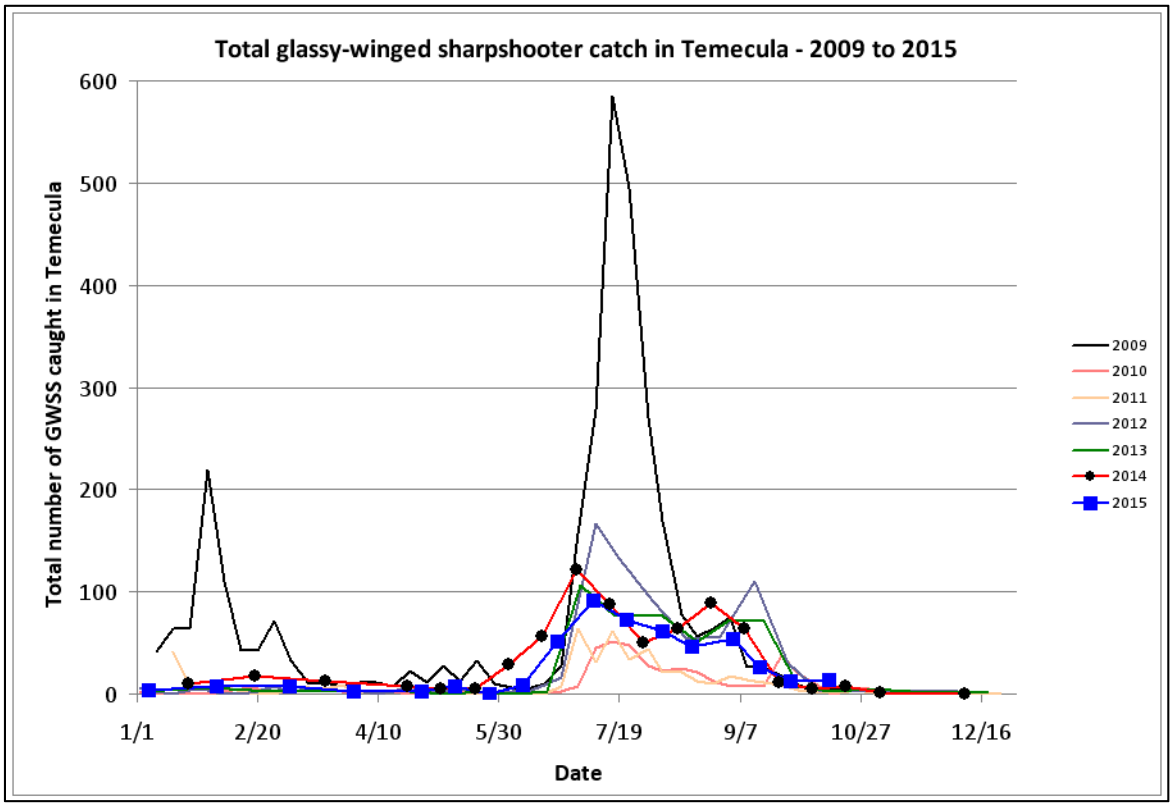

Figure 2. Seasonal total glassy-winged sharpshooter catch in the Temecula Valley from 2009-2015.

CONCLUSIONS

The results for 2015 continue to suggest that there is no clear evidence of a glassy-winged sharpshooter resurgence in the Temecula Valley region, as is occurring in portions of the southern Central Valley. At least some of the explanation may be because of the potential for treatments made for the Asian citrus psyllid, which is controlled primarily via the same classes of insecticides. Although the recommended treatment timings are slightly different for Asian citrus psyllid versus glassy-winged sharpshooter, applications made for its control may aid somewhat with glassy-winged sharpshooter control.

FUNDING AGENCIES

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ACKNOWLEDGEMENTS

We would like to thank Ben Drake and Nick Toscano for their help in initiating this project. Thanks also to the Temecula Valley citrus growers for their continued cooperation in making this work possible.

RNA INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted July 1, 2014 to October 31, 2015.

ABSTRACT
RNA interference (RNAi) is a natural biological activity for controlling gene expression and for anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNAi directed toward different types of insect plant pests is becoming more feasible and promising. RNAi is already used in commercial agriculture for plant virus control, and the many new publications demonstrating experimental successes with various plant-feeding insects suggest that RNAi could have a role in helping to manage Pierce's disease of grapevines. In our efforts, we were able to induce RNAi effects in the glassy-winged sharpshooter (*Homalodisca vitripennis*) and evaluate initial transgenic plants as a means to initiate RNAi to help control the glassy-winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. Furthermore, we are currently developing plant-infecting viruses to express interfering RNAs in plants as an additional means for glassy-winged sharpshooter control.

LAYPERSON SUMMARY
This work presents fundamental efforts towards understanding the feasibility of applying RNA interference (RNAi) to help combat Pierce's disease of grapevines. Pierce's disease is a significant threat to grape production in California and other parts of the USA, and the causal agent, *Xylella fastidiosa*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *X. fastidiosa*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (*Homalodisca vitripennis*), and other sharpshooter vectors of *X. fastidiosa*.

We focused our recent efforts on evaluating transgenic potato plants to evaluate their potential for inducing RNAi effects in the glassy-winged sharpshooter, and for identifying optimal RNAi inducer delivery systems. Potatoes are easier and faster to transform and regenerate than grapes, and the glassy-winged sharpshooter feeds readily on these plants. We also generated large-scale genomic data along with small RNA datasets, which will help us for future genetic/genomic efforts against the glassy-winged sharpshooter.

INTRODUCTION
Our primary objectives are to evaluate and demonstrate RNA interference (RNAi) activity against the glassy-winged sharpshooter (*Homalodisca vitripennis*). We have previously demonstrated induction of RNAi effects in the glassy-winged sharpshooter and evaluated different strategies to induce RNAi effects in the glassy-winged sharpshooter. We envision that RNAi approaches can be part of long-term strategies to help control the glassy-winged sharpshooter and other sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevines, but only if we understand how they work and optimize the delivery of RNAi inducers. We are focused now on answering the latter questions. We have built upon our previous progress and initiated some new directions in our glassy-winged sharpshooter RNAi efforts. We also continue to work closely with Dr. Shizuo George Kamita.

We continue to use potato plants as surrogates for transgenic RNAi-based approaches for the glassy-winged sharpshooter. We have generated stable transgenic potato plants using the constitutive, non-tissue-specific 35S promoter, and a *Eucalyptus gunii* minimal xylem-specific promoter (EgCAD) to control the spatial expression of

candidate interfering RNAs. Glassy-winged sharpshooters feed on potatoes for our experiments, but we are still optimizing how to evaluate RNAi effects from transgenic potatoes. It is also now our intent to directly use grapevines, but instead of using transgenic grapevine plants, we are now attempting to develop grapevine-infecting viruses to express interfering RNAs in grapevines.

OBJECTIVES

1. Generate transgenic plants for novel effective targets of the glassy-winged sharpshooter and other sharpshooters.
2. Generate and use microRNAs from different developmental stages of glassy-winged sharpshooter insects.
3. Assess the potential of using plant viruses for delivery of small RNA effectors.

RESULTS AND DISCUSSION

Objective 1. Generate transgenic plants for novel effective targets of the glassy-winged sharpshooter and other sharpshooters.

We compared transgenic potato plants engineered to express interfering RNAs to target the glassy-winged sharpshooter. We used two different promoters for these experiments, the 35S constitutive promoter and the EgCAD promoter from *Eucalyptus gunii* which has shown the ability to target developing xylem tissues. A table showing our plants and their analysis by small RNA hybridization analysis was presented in our December 2014 report published in the *Proceedings of the 2014 Pierce's Disease Research Symposium* (pp. 16-22); that data is not included here. These assays showed that all transgenic plants produced specific small interfering RNAs; we were also able to induce RNAi effects in the glassy-winged sharpshooter as determined by RT-qPCR analysis of target mRNAs, but we failed to generate a detectable phenotype. We now believe that this may be due at least in part because of how we performed our assays, and we are repeating feeding experiments in a different format as described below.

Our first experiments used potato cuttings with caged fourth and fifth instar glassy-winged sharpshooter nymphs. The cuttings were placed in dilute nutrient solution and the glassy-winged sharpshooters remained on cuttings for approximately seven days. It is possible rooted plants may produce better RNAi effects in the glassy-winged sharpshooter than the nutrient solution cutting experiments. For that reason, our most recent experiment used small rooted cuttings in soil, as opposed to the dilute nutrient solution, with caged fourth and fifth instar glassy-winged sharpshooter nymphs. The glassy-winged sharpshooter nymphs were allowed to feed on the plants for five days at which point the insects were harvested and RNA was extracted to test for target gene knockdown using RT-qPCR. Unfortunately, these feeding trials did not induce a detectable phenotype or result in reduced target gene expression when compared to the wild-type and green fluorescent protein (GFP) negative control plants (**Figure 2**). Our ongoing efforts with phloem-feeding hemipterans have shown similar results, but we have been able to see negative phenotypes only when we allow target insects to develop on test plants -- they must go through nymphal instar stages and molt. For the glassy-winged sharpshooter this is a little problematic as they like to move among plants and feed on different species. In fact, in order to have sufficient reproduction, we rear them in cages containing basil, cotton, and cowpea plants. Our current, ongoing studies are now using small rooted cuttings in soil. We are starting experiments using second and third instar nymphs and maintaining the test times as long as possible, or until nymphs molt into adults. After optimizing the RNAi assays we will move on to assessing additional targets and even using artificial microRNAs (Objective 2).

Objective 2. Generate and use microRNAs from different developmental stages of glassy-winged sharpshooter insects.

We have begun evaluating three approaches for expressing artificial microRNAs (amiRNAs) in plants, which will be described later. Our intent here is two-fold: one is to use specific amiRNAs to target glassy-winged sharpshooter mRNAs and reduce the possibilities for potential RNAi off-target effects which are more possible with longer, dsRNA RNAi inducers (Nunes, 2013); and second, we have identified several glassy-winged sharpshooter novel miRNAs by Illumina-based sequencing and bioinformatics analysis (see **Figure 1**). We have so far only identified miRNAs in adult glassy-winged sharpshooters, but our goals are to identify potential miRNAs that may be glassy-winged sharpshooter instar-stage specific and evaluate their potential for use in RNAi towards the glassy-winged sharpshooter.

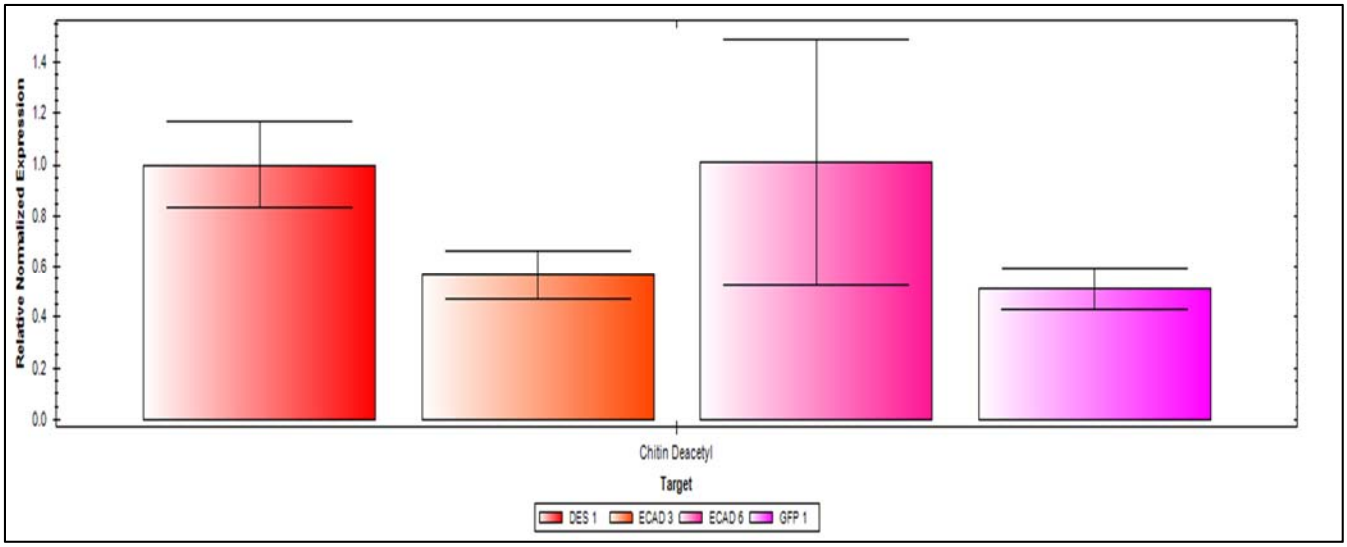

Figure 1. Relative normalized expression of the glassy-winged chitin deacetylase gene after glassy-winged sharpshooter feeding on wild-type and transgenic plants expressing dsRNA, showing no difference in target gene expression between wild-type and transgenic plant lines. DES 1 is the wild-type potato control. ECAD 3 and ECAD 6 are separate transgenic lines expressing dsRNA for glassy-winged sharpshooter chitin deacetylase under control of the EgCAD promoter. GFP 1 is a control transgenic line expressing dsRNA for GFP. The glassy-winged sharpshooter ubiquitin gene was used as an internal control for the RT- qPCR. Error bars represent the standard error of the data.

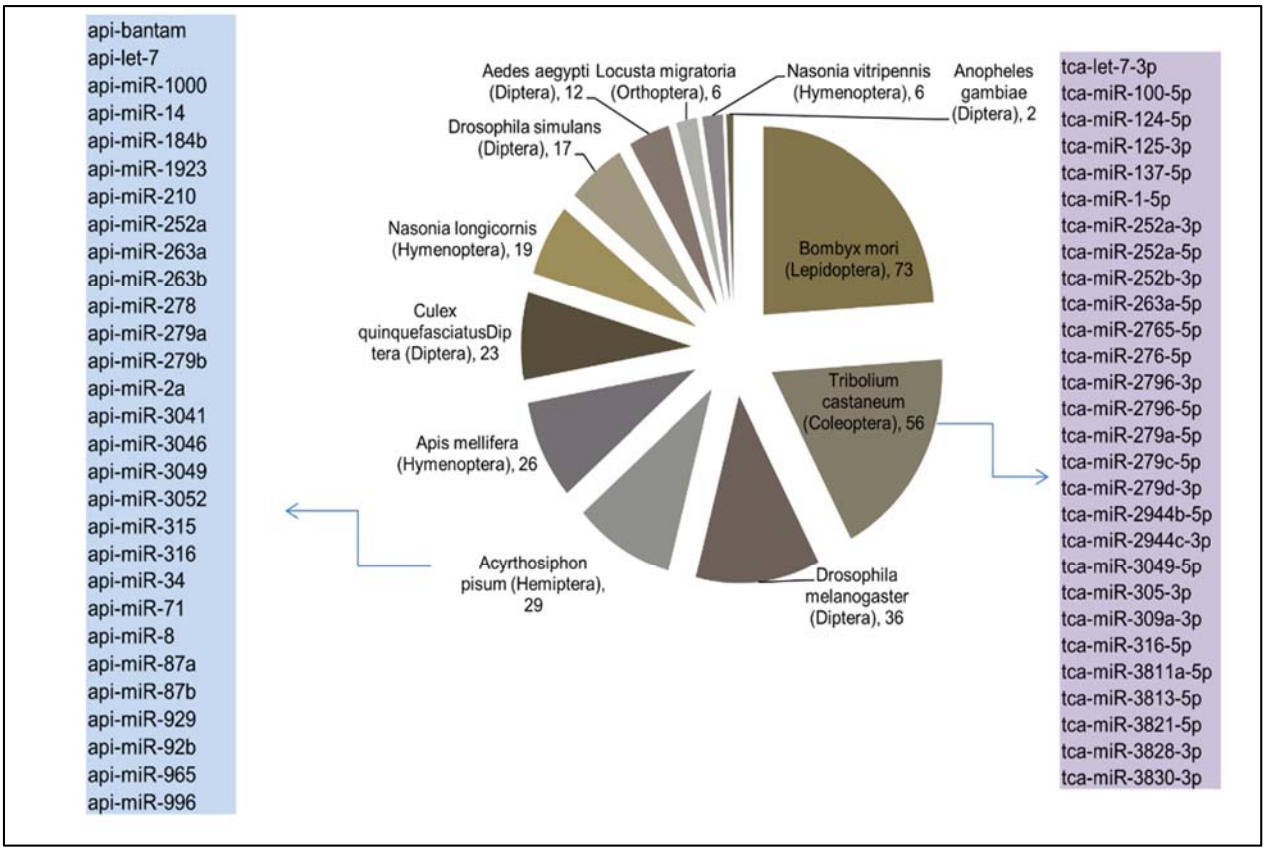

Figure 2. The microRNA profile analysis of glassy-winged sharpshooter adult insects revealed the presence of microRNAs that are conserved between different insects. Glassy-winged sharpshooter adults also share some microRNA conservation with plants.

We have used agroinfiltration of *Nicotiana benthamiana* plants, followed by small RNA hybridization and Illumina sequencing to assess production of amiRNAs. These experiments showed that we can produce specific amiRNAs in plants by two methods: one by using a binary plasmid vector to produce the specific amiRNA; and the second by using a modified begomovirus A component to replicate and express higher levels of amiRNAs in plants. The latter suggests that it is worth investigating using grapevine red blotch associated virus (GRBaV) (Krenz et al., 2014) as a means for generating specific amiRNAs in grapevines. We are considering this at least as an experimental approach (see Objective 3).

Objective 3. Assess the potential of using plant viruses for delivery of small RNA effectors.
Our efforts here are based on our previous successes using plant-infecting viruses to express interfering RNAs in plants, which produced negative phenotypes in specific phloem-feeding target insects. We are attempting to engineer grapevine leafroll-associated virus-*7* (GLRaV-7), a phloem-restricted virus from the complex family Closteroviridae, as our primary virus for these studies. This is based on successes by others using viruses from the same family such as Citrus tristeza virus in citrus (Dawson and Folimonova, 2013; Folimonov et al., 2007; Hajeri et al., 2014), and GLRaV-2 in grapevines (Dolja and Koonin, 2013). In both cases the plant virus-based vectors were capable of regulating the expression of endogenous genes via virus-induced gene silencing in their respective host plant and also were capable of expressing foreign genes for long periods of time showing a significant stability and durability.

We have a GLRaV-7 isolate from California in culture and we have access to the complete genome sequence of the same isolate (Genbank accession number: JN383343; Al Rawhnih et al., 2012), which allows us to design specific primers to successfully amplify the entire genomic RNA of GLRaV-7. Currently we have been able to amplify the complete GLRaV-7 genomic RNA and to clone it as a cDNA in an intermediate plasmid vector in which we can confirm the accuracy of the viral cDNA sequence by DNA sequencing. As a future step and to enable more efficient delivery of the viral construct into plant tissue, we will subclone the entire cDNA of GLRaV-7 into a binary vector, $pCAMBIA_{1380.}$ The new recombinant plasmid will be used then to inoculate plants through agro-inoculation using vacuum infiltration of grapevine plants as it was successfully reported for GLRaV-2 in grapevines (Kurth et al, 2012).

Based on our success with expressing amiRNAs in plants (see Objective 2 above) and comments from the grant review panel last year, we are now considering using GRBaV also as at least an experimental tool to express amiRNAs in grapevines. We have established cultures of GRBaV and have cloned the genomic ssDNA. We are attempting to generate an infectious cDNA version for use in grapevines.

CONCLUSIONS

RNAi is a natural biological activity for controlling gene expression and for anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNAi directed toward different types of insect plant pests is becoming more feasible and promising. In our efforts, we were able to induce RNAi effects in the glassy-winged sharpshooter and evaluated initial transgenic plants as a means to initiate RNAi to help control the glassy-winged sharpshooter and other leafhopper vectors of *X. fastidiosa*.

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HIGH-THROUGHPUT LIVE CELL SCREEN FOR SMALL MOLECULES TARGETING THE TOLC EFFLUX PUMP OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted July 1, 2015 to October 30, 2015.

ABSTRACT
The goal of this new project is to exploit the known vulnerability of the single TolC gene of the *Xylella fastidiosa* Type I secretion system using a high-throughput screen for *X. fastidiosa* sensitivity to a mild surfactant following small molecule treatments that may disable Type I secretion directly or indirectly. Two combinatorial small molecule libraries (1,280 chemicals in the Prestwick Chemical, and 320 chemicals in the Prestwick Phytochemical) and *X. fastidiosa* resistant grapevines will be screened as treatments. For this purpose, both the wild-type *X. fastidiosa* strain Temecula and a *tolC*$^{-}$ mutant of Temecula, M1, were electroporated with a stable plasmid (pBBR1MCS-5) expressing an enhanced green fluorescent protein (GFP) fluorescence marker. To establish growth characteristics in a 96-well plate format, initial inoculation densities (OD_{600}) ranging from 0.01 to 0.05 in culture volumes ranging from 100 to 300 µl were used to establish an experimental protocol for evaluating growth in the presence and absence of 20 ppm Silwet L-77. Preliminary results indicate that optimal growth without contamination in the 96-well plate format was achieved using 150 µl volumes, with or without Silwet. In the absence of chemical treatment, 150 µl volumes of Temecula displayed ca. a 10.5 x increase in bacterial density and an 11.7 x increase in GFP fluorescence, with or without Silwet L-77. Not surprisingly, under similar experimental conditions, M1 (*tolC*$^{-}$) failed to grow significantly. Experiments are underway to assess cell viability and titer at the end of the culture period, as well as quantification of cellular adenosine triphosphate (ATP) content. Two Prestwick chemical libraries have been acquired, and the primary high-throughput screens are being planned.

LAYPERSON SUMMARY
Xylella fastidiosa survival in grapevine and in many culture conditions depends on a Type I multidrug resistance (MDR) efflux pump system, which plays a critical function in pumping out environmental toxins and host antimicrobial compounds and antibiotics that leak into the bacterial cell and would otherwise kill *X. fastidiosa*. Any method that could block or disrupt specific components of this system would likely result in both control of Pierce's disease and elimination of *X. fastidiosa* from infected plants. Portions of the outermost efflux pump protein, TolC, are embedded in the protective outer membrane (OM) of the bacterium and form the exit portal of the efflux pump. Both the OM and TolC are exposed at the *X. fastidiosa* cell surface, making small molecule chemical treatments that target TolC or even the OM barrier attractive chemical targets. Many small molecule combinatorial libraries are commercially available for screening, some including synthetic and exotic chemicals that would likely require considerable testing to meet the high bar set for food safety and agricultural use. Also available are the highly diverse and complementary Prestwick natural phytochemical library and the Prestwick Food and Drug Administration (FDA) approved drug combinatorial library, together representing 1,600 different small molecules in total. This proposal is to adapt an existing and tested live bacterial cell count assay originally tested using mutants affecting Type I efflux in another bacterium to enable high-throughput screening of the two Prestwick libraries and also fractionated extracts of *X. fastidiosa* resistant. The result could be an immediately applicable phytochemical spray to control Pierce's disease or one requiring a minimum of regulatory approval for use on *Vitis vinifera* grapevines.

INTRODUCTION
This is a new project that is based on two discoveries made during the course of two earlier funded projects. The first discovery is our demonstration that the Type I multidrug resistance (MDR) efflux system of *Xylella fastidiosa* is absolutely required for both pathogenicity and even brief survival of the Pierce's disease pathogen in grape (Reddy et al., 2007). Knockout mutations of either *tolC* or *acrF* (manuscript in preparation) render *X. fastidiosa* nonpathogenic, and in addition, the *tolC* mutants were so highly sensitive to grape chemicals that the mutants are not recovered after inoculation. Inoculation of very high titers of *X. fastidiosa* strain Temecula *tolC*$^{-}$

mutants in grape results in rapid, 100% killing of inoculated bacteria. These results demonstrated a critical role for Type I efflux in general and TolC and AcrF in particular for defensive efflux by *X. fastidiosa* of plant antimicrobial compounds, such as phytoalexins.

In the process of investigating the increased sensitivity of the MDR efflux mutants to plant-derived antimicrobial chemicals, we also discovered that even wild-type *X. fastidiosa*, with its lone MDR efflux system, is much more sensitive to plant-derived antimicrobial chemicals than most other plant pathogens, which carry multiple efflux systems. Both *tolC* (encoding the outer membrane and periplasmic tunnel component of Type I secretion) and *acrF* (encoding the inner membrane pump component of Type I secretion) are essential for MDR efflux in *X. fastidiosa,* which has only one copy of each gene and only one such MDR efflux system. By contrast, most plant pathogens have redundant MDR efflux systems and multiple *tolC* genes. These results suggest that *X. fastidiosa* should be much more vulnerable to treatments affecting Type I efflux than other bacterial plant pathogens.

MDR efflux mutants in other systems have provided proven, highly sensitive and quantitative screening methods for antimicrobial chemicals (Tegos et al., 2002). The goal of this new project is to exploit the increased vulnerability of *X. fastidiosa* and our knowledge of particular chemicals that require efflux in a high-throughput assay that screens small molecule combinatorial libraries and *X. fastidiosa* resistant grapevines for chemicals that may disable Type I secretion directly or indirectly. Since (1) there is only a single *tolC* gene (and TolC is the sole Type I secretion system outer membrane component) in *X. fastidiosa* strains, and (2) Pierce's disease strains are clonal and there is little variation in TolC among Pierce's disease strains, this makes TolC a particularly attractive molecular target. This should make Pierce's disease strains highly vulnerable to any blocking agent, including small molecules, that specifically targets TolC or AcrF, disrupts the TolC / AcrF interaction or generally affecting Type I efflux. A highly sensitive live cell assay that is well suited for high-throughput screening was developed and is proposed for use for such a screen.

OBJECTIVES

The specific objectives of this one-year proposal are:

1. Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from *X. fastidiosa*.
2. Screen sap and crude extracts from *Vitis vinifera* grape plants subjected to freezing treatments (sufficient to cure Pierce's disease) for potential effects on Type I efflux from *X. fastidiosa*.
3. Determine if sap and crude extracts from Pierce's disease resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from *X. fastidiosa* than susceptible *V. vinifera* plants.

RESULTS AND DISCUSSION

Objective 1. Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from *X. fastidiosa*.

The P_{trp}-*gfp* cassette was PCR amplified from pUFZ75 (Zhang et al., 2009) and subcloned in shuttle plasmid pBBR1MCS-5 to generate pSZ90. *X. fastidiosa* Pierce's disease strain Temecula (PDT) and *tolC* mutant cells were transformed with pSZ90 as described by Zhang et al. (2015). To determine the bacterial growth pattern in flat-bottom 96-well plates, 100 µl and 150 µl PD3 medium in presence and absence of 20 ppm Silwet L-77 with different initial inoculum densities. Optical density (OD_{600}) (**Figure 1**) and green fluorescent protein (GFP) fluorescence (excitation at 485/20 nm and emission at 528/20 nm; **Figure 2**) data were recorded for six days using BioTek SynergyHTX multi-mode reader. Volumes of 100 µl, 150 µl, and 300 µl culture suspensions were evaluated; the 300 µl volumes were overly sensitive to contamination problems and were dropped from consideration. From **Figures 1 and 2**, it may be seen that wild-type Temecula cells grew best at 150 µl volumes, both with and without Silwet L-77, and additionally gave the highest reliable fluorescence signal per well under test conditions, over a period of six days; bacterial densities increased ca 10.5 x and fluorescence increased ca. 11.7 x over six days. The M1 (*tolC*$^-$) mutant strain failed to grow or to accumulate GFP, indicating bacterial stasis under these static growth conditions, even without Silwet L-77. Experiments are underway to assess the cell viability at the end of culture period for determination of cfu/ml as well as quantification of cellular adenosine triphosphate (ATP) content using the BacTiter-Glo reagent (Promega). Two Prestwick chemical libraries were received. Primary high-thorough screen of chemical are planned.

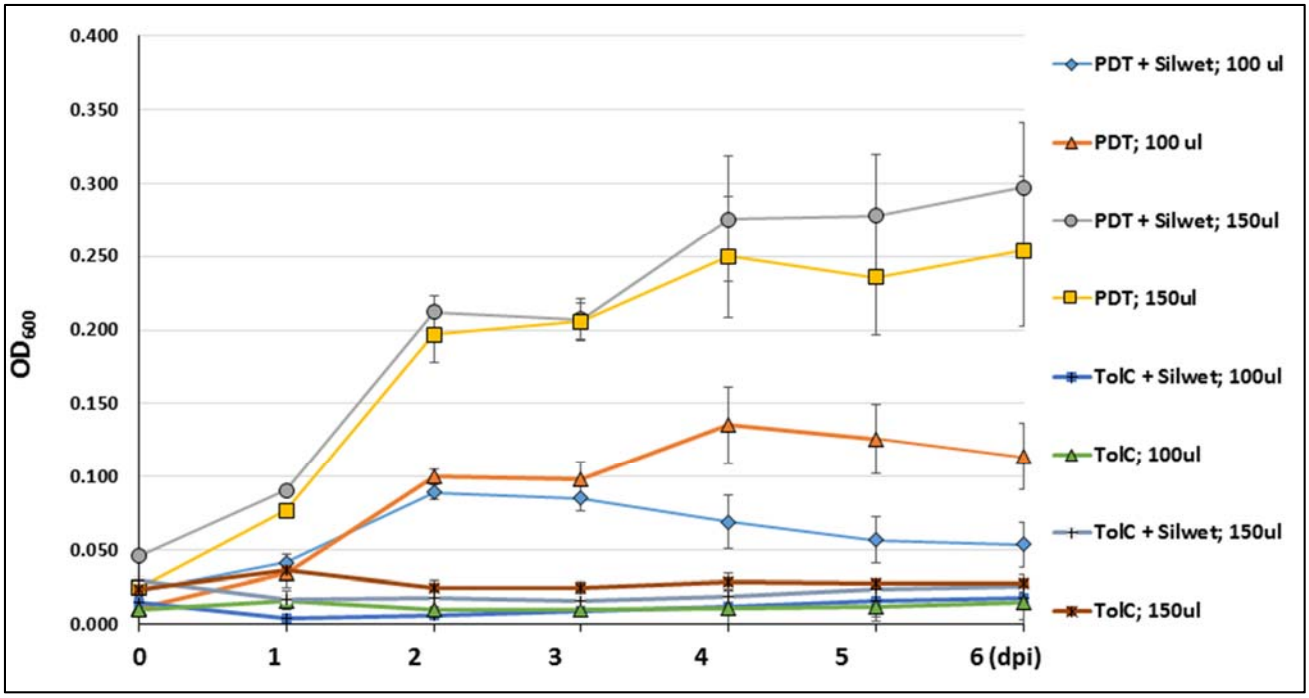

Figure 1. Growth curves of *X. fastidiosa* Pierce's disease strains Temecula (PDT) and *tolC*⁻ mutant M1 (TolC). Silwet L-77 (Silwet) was added in the medium of both strains as indicated at a concentration of 20 ppm. OD600 was read for six days post-inoculation (dpi).

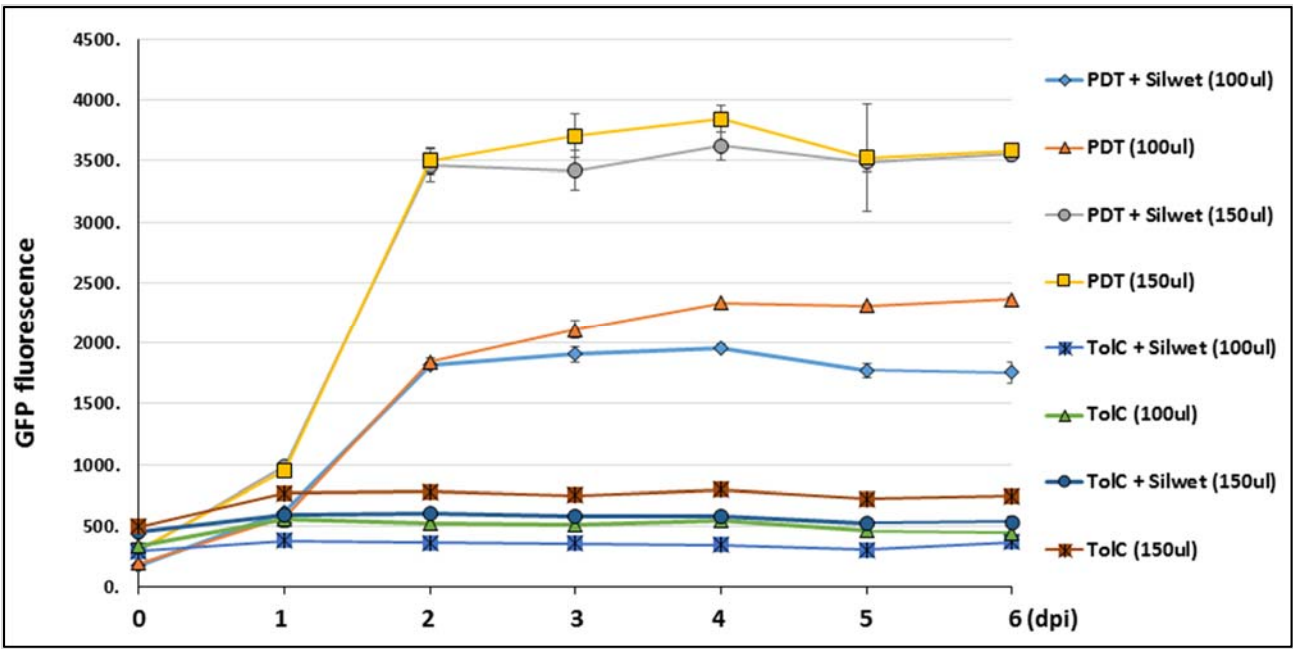

Figure 2. GFP fluorescence reads of *X. fastidiosa* Pierce's disease strains Temecula (PDT) and *tolC*⁻ mutant M1 (TolC). Silwet L-77 (Silwet) was added in the medium of both strains as indicated at a concentration of 20 ppm. GFP fluorescence was determined daily for six days post-inoculation (dpi).

Objective 2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments (sufficient to cure Pierce's disease) for potential effect on Type I efflux from *X. fastidiosa*.

V. vinifera grape plants have been started in the greenhouse.

Objective 3. Determine if sap and crude extracts from Pierce's disease resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from *X. fastidiosa* than susceptible *V. vinifera* plants.

Muscadinia rotundifolia grapevines are being procured.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

FIELD EVALUATION OF GRAPE PLANTS EXPRESSING POTENTIAL PROTECTIVE DNA SEQUENCES EFFECTIVE AGAINST PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2014 to October 2015.

ABSTRACT
The objective is to evaluate transgenic grape and grape rootstocks expressing various genes from different constructs in a field site in Solano County for resistance to *Xylella fastidiosa* (Pierce's disease strain) following mechanical injections of *X. fastidiosa* into the plant stems. Over the course of the multi-year field evaluation, test plants will include ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from Dandekar, Powell, Lindow, and Gilchrist projects and, as plant material availability permits, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were introduced in 2011 and 2012. All plants are located in a USDA APHIS approved field area with no risk of pollen or seed dispersal. The area is adjacent to experimental grape plantings that have been infected with Pierce's disease for the past two decades with no evidence of spread of the bacteria to uninfected susceptible grape plantings within the same field plot. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding by soil-borne diseases, including nematodes. Over the current five-year course of this field experiment there is no evidence of spread of the bacteria to uninfected susceptible grape plantings.

LAYPERSON SUMMARY
The purpose of the field planting is to evaluate grape and grape rootstocks expressing several transgenes from several investigators, with differing putative modes of action against *Xylella fastidiosa,* under natural field conditions for efficiency in providing protection against Pierce's disease. The site in Solano County was selected and approved by USDA APHIS to enable controlled inoculation and close monitoring of the host response in terms of symptoms, bacterial behavior, and plant morphology. Over the course of the multi-year field evaluation, test plants included ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from investigators Dandekar, Labavitch, Lindow, and Gilchrist and later transgenic rootstocks expressing some of the test genes were grafted to untransformed Pierce's disease susceptible scions to assess potential for disease suppression in an untransformed scion from signals originating in the transformed rootstocks. We will continue maintaining and collecting data from this site for the coming season through June 30, 2016. This time period matches the time extension proposed by Dr. Dandekar, who has now assumed responsibility for the USDA APHIS permit. Dr. Gilchrist will continue to manage the field operations at this site. The USDA APHIS permit specifies that the plants are to be removed, burned on site and the field monitored as fallow for an additional year. This latter step may be modified if there is additional planting at this site. Additional space has been set aside to enable doubling of the current work area with a modification of the current permit.

INTRODUCTION
Land preparation, planting, and management of the experimental resources to accommodate 500 plants. Plants occur with a row spacing of 15 feet between rows and four feet between plants in a row. There is a 50-foot open space buffer area surrounding the field, which is fenced to protect against rabbits. Each row is staked with seven-foot grape stakes supporting 13 gauge wire in two wire trellis system with a stake at each plant site. Wires are stretched and anchored by seven-foot pressure-treated posts at the end of each row. The plants are irrigated by surface furrow in accordance with standard practices for maintaining grapes for experimental purposes at this site.

Although a drip irrigation system was installed in 2014 and will be used in all future plantings, furrow irrigation will be continued on the existing plots to avoid confounding of different water application methods in this experiment. Irrigation and pest management, primarily for powdery mildew, weeds, and insects, is coordinated by PI Gilchrist and conducted by Mike Eldridge, the Field Superintendent employed by the Department of Plant Pathology who has 20 years of experience working locally with grapes and other perennial crops. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices.

OBJECTIVES

1. Principal Investigators, with assistance from contract field crews, are responsible for pruning in the spring of each year and within the season as needed to maintain a reasonable canopy permitting sun exposure to leaves on inoculated canes. Periodic trimming is necessary, given that the transgenic plants are derived from Freedom (a common rootstock) and Thompson Seedless, both of which exhibit tremendous vegetative growth during the season. In addition, annual pruning deviates from conventional practice in that multiple cordons have been established with a separate new cordon retained from each successive inoculation. This enables differential experimental materials for evaluation and sampling in the form of seasonal canes associated each succeeding annual inoculation. The objective is to provide sufficient inoculated and control material for destructive sampling over years to assess both timing of symptom development after successive inoculations and to assess bacterial presence and movement over time.
2. Plants will be and have been inoculated annually since 2011. *Xylella fastidiosa* was mechanically inoculated again in 2015. Sampling of inoculated stems from 2015 and previous years confirmed the presence of the bacteria and symptoms of Pierce's disease later in the season.

RESULTS AND DISCUSSION

The objectives set out for the establishment and management of this field planting were completed and the first planting occurred July 12, 2010 (**Figure 1**), with all plants surviving the winter as shown in **Figures 2 and 3**. The second phase of the planting, including grafted transgenics, was completed in May 2011 and June 2012.

Extensive polish trimming during the season is necessary to manage the Freedom and Thompson Seedless plants in a fashion to allow ease of mechanical inoculation and recovery of experimental samples (**Figure 4**).

From planting through the 2015 growing season, the individual transgenic plants all exhibited a normal phenotype, true to the untransformed control plants of each parental genotype (**Figure 4**). Symptoms of Pierce's disease did not appear until two years after the first inoculation. Evaluations in the summer of 2014 and 2015 indicated that all inoculated controls and some transgenic plants showed symptoms of Pierce's disease. It is clear that this field planting will provide important data on the effectiveness of any of the transgenic strategies employed by the respective researchers.

As of March 2014, many inoculated canes on control plants and some transgenics did not survive the winter but the uninoculated canes on these plants still appear healthy. Visual observation and destructive sampling of inoculated canes indicates that mechanical inoculation was successful in infecting inoculated canes (**Figure 5**). As of July 2015, several uninoculated canes adjacent to inoculated canes show foliar symptoms indicating that the bacteria have moved systemically through the plants and, in the case of some non-transformed control plants, the entire plant is now dead.

There are two points to be made regarding the appearance of symptoms. First, plant turgor has been maintained throughout the growing season with timely irrigation and there has been no evidence of wilt or epinasty symptoms prior to appearance of classic foliar symptoms (**Figure 6**) or even death of inoculated control susceptible canes. Symptomatic leaves occur on inoculated canes without the appearance of water stress (**Figure 6**). This belies the long-held anecdotal effect of vascular plugging leading to the classic foliar symptoms of sectored death within green areas of leaves. Second, excellent symptoms associated with the presence of the pathogenic bacteria are readily seen in the spring of each year from buds emerging on inoculated canes. Buds break, push tiny leaves, and then die in tissues confirmed in the laboratory to harbor bacteria from inoculations that occurred one to two years prior.

As of September 2014, it is clear that there is a rich source of additional data to be collected from this field experiment. There are now substantial differences between inoculated control plants compared with plants

expressing some of the transgenes. There is no evidence of any spread of the bacteria from inoculated to uninoculated control plants but there is now evidence of systemic spread within some of the plants representing different genetic composition (different transgenes). The positive result of effective mechanical inoculation over time suggests that plants consisting of transgenic rootstocks grafted to non-transgenic scions will enable experimental assessment of cross-graft protection. Field data over the course of this experiment has been collected by all investigators and can be found in their individual reports from the *Proceedings of the 2014 Pierce's Disease Research Symposium*.

As of March 15, 2015, all plants have been pruned to remove excess growth from the past year but to retain all inoculated wood (**Figure 5**). Spurs on old inoculated cordons were pruned to two-three buds while the 2014 inoculated branches were trimmed to retain up to 10 buds for data collection to include live/dead bud counting and destructive sampling for bacterial counts. Inoculation was done in May 2015 (**Figure 6**) and symptoms were observed on leaves from canes inoculated in 2014 (**Figure 7**).

We are now approved and funded to continue maintenance and data collection from this site through June 30, 2016. This time period matches the time extension proposed by Dr. Dandekar, who has now assumed responsibility for the USDA APHIS permit. Dr. Gilchrist will continue to manage the field operations at this site.

Solano County Pierce's Disease Field Work, March to August 2015: All field activities are conducted or coordinated by field superintendent Mike Eldridge. Regular tilling and hand weeding maintained a weed-free planting area. Plants were pruned carefully in March, leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA APHIS permit. Frequent trimming of the plants was done to ensure that leaves on inoculated canes were exposed to sunlight, and shading of the associated leaves was avoided. Surface irrigation was applied as needed to maintain the soil at field capacity and turgor in the plants. Application of the fungicides Luna Experience and Inspire were alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites were treated with insecticides when needed. Neither powdery mildew nor insect pressure was noted throughout the growing season. The same maintenance program is following the same general format for 2015 with timely pruning, rototilling weeds, and applying fungicides on a monthly basis to protect the vines against powdery mildew. Irrigation was applied at monthly intervals beginning in June. Pierce's disease symptoms were visible at bud emergence and later with foliar symptoms in late June and continuing to develop into late August.

CONCLUSIONS

The results to date of this field experiment indicate that the mechanical inoculations successfully introduced the bacteria into the plants with subsequent appearance of foliar symptoms and cane death. There are transgenes from each of the investigators that appear to be suppressing the symptoms of Pierce's disease inoculated vines.

The images below (**Figures 1-7**) illustrate the status of the field experiment from planting in 2010 to the summer of 2015. The caption to each figure indicates when the image was obtained, and together the images represent both the asymptomatic inoculated transgenic and symptomatic inoculated non-transgenic control plants at the Solano County site.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the Regents of the University of California.

Figure 1. July 2010. Solano planting.

Figure 2. July 2010. Solano planting.

Figure 3. April 2012. Solano planting.

Figure 4. July 2013. Solano planting.

Figure 5. Images of Solano County plants at the time of pruning in March 2015. Colored tags indicate sites and dates of inoculations in 2011-2014.

Figure 6. May 2012. Solano County field trial site at the time of inoculation of the field plots with *X. fastidiosa.*

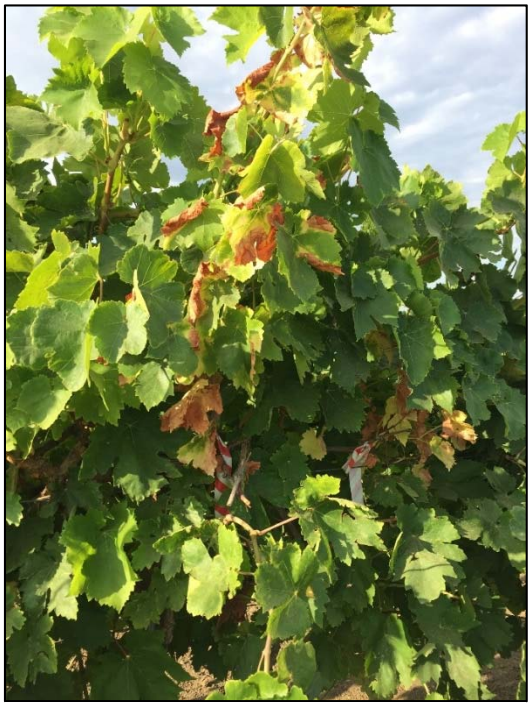

Figure 7. July 2015. Symptoms of Pierce's disease recorded July 21, 2015 on branches inoculated May 27, 2014.

FIELD EVALUATION OF GRAPE PLANTS EXPRESSING PR1 AND UT456 TRANSGENIC DNA SEQUENCES FOR PROTECTION AGAINST PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2014 to October 2015.

ABSTRACT
The objective of this field experiment is to evaluate transgenic grape and grape rootstocks expressing various transgenes for protection against *Xylella fastidiosa* (Pierce's disease strain) in a field site in Solano County. The pathogen is introduced into individual vines by mechanical injections of *X. fastidiosa* into the grape stems of transgenic and non-transgenic control plants. The experiment is now in the fifth year after inoculations were initiated. Test plants include own-rooted transgenic and non-transgenic plants and grafted plants with non-transgenic scions of a Pierce's disease susceptible variety grafted to rootstocks bearing transgenes. The plants have been maintained under optimum field conditions with respect to water management, powdery mildew, and insect control. Following the third (2013) and fourth (2014) years after inoculations began, control plants are showing clear symptoms of Pierce's disease and many inoculated canes are dying or dead. These results were extended with a new inoculation in 2015 with comparable results as in 2014 but at this point most all of the control plants were dead in contrast to a number of the transgenic plants that remained healthy. The conclusion at this point is that several lines bearing the PR1 and UT456 DNA sequences effectively suppress Pierce's disease symptoms, including some of the grafted plants. These two sequences are now moving forward into the dual construct project described in the report titled, "Transgenic Rootstock-Mediated Protection of Grapevine Scion by Single and Stacked DNA Constructs."

LAYPERSON SUMMARY
Previously, we identified novel genes that suppress Pierce's disease symptoms by blocking programmed cell death (PCD), elicited by *Xylella fastidiosa* through use of a functional screen from cDNA libraries of grape and tomato. Two of these sequences (PR1 and UT456) expressed as transgenes in grape suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse conditions. Field experiments underway in Solano County, conducted with a USDA APHIS permit, are designed to evaluate clonal copies of several of these transgenic lines under field conditions for resistance to Pierce's disease. The field evaluation includes mechanical inoculation with *X. fastidiosa* in Solano County. Data sets include visual monitoring of plant morphology, Pierce's disease symptoms, and bacteria titer by quantitative polymerase chain reaction (qPCR) assays. To date, PCR data and plating assays confirm the presence of *X. fastidiosa* in the plants. Inoculated untransformed plants are now showing typical symptoms of Pierce's disease. Bacteria are present in inoculated plants at the Solano County site and there is definitive evidence of symptom differences between several of the transgenic lines compared with the non-transgenic control. Evaluations at the Solano County site are ongoing and inoculations continued in 2015.

INTRODUCTION
Field experiments were initiated in Solano County to evaluate transgenic grape plants and grape rootstocks expressing two DNA constructs, PR1 and UT456, in several different transgenic lines of each construct for resistance to the Pierce's disease strain of *Xylella fastidiosa*. Mechanical inoculation of *X. fastidiosa* was employed at the Solano County site. The Solano County field experiment was conducted in two phases. The first phase of the field studies started in 2010 to evaluate clonal copies of the fully transformed own-rooted plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers in greenhouse assays (1). The second phase began in 2011 with planting of the untransformed Thompson Seedless scions grafted onto PR1 and UT456 primary transformants as rootstocks, including Freedom and Thompson Seedless. Data collected in 2012-15 indicate that the bacteria are present in the mechanically-inoculated canes on plants at the Solano County site (2, 3). Results indicate that both PR1 and UT456 transgenes provide protection against Pierce's disease, while the level of protection varies between individual transgenic lines. Field symptom data was collected in the fall of 2014. As of March 15, 2015, the plants were pruned to remove excess dormant branches while leaving portions of

previously inoculated cordon-like branches dating back to 2011and last year's inoculated canes (**Figure 1**). In the latter case, up to 10 buds were preserved on each inoculated cane as data-rich resources for scoring dead versus live buds on control and transgenic plants as new shoots begin to emerge in the spring of 2015 (**Figures 2, 3, and 4**). The 2014 inoculated canes were destructively sampled to determine the presence and concentration of *X. fastidiosa* in the tissues after scoring the new buds (**Figure 5**).

OBJECTIVES

The overall objective is to continue to evaluate several lines of transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 for resistance to the Pierce's disease strain of *X. fastidiosa* at a site in Solano County. Controlled mechanical inoculation of *X. fastidiosa* is used at the Solano County site. The background research on selected transgenic lines leading to these field trials is from four controlled inoculation experiments in a greenhouse over a two-year period, involving more than 300 transgenic plants of five lines derived from independent transformation events bearing PR1and UT456. Each of these transgenes in several lines suppressed Pierce's disease symptoms and reduced bacterial titer compared with untransformed controls of the same genotype. A positive correlation between the P14 and UT456 message level, suppression of bacterial titer, and absence of Pierce's disease symptoms was established using qPCR to measure both the message and the bacteria titer.

A first planting of fully transformed plants was established in 2010 and a second set of plants consisting of rootstocks transformed with PR1 and UT456 genes grafted to untransformed Pierce's disease susceptible Thompson Seedless scions. The grafted plants are designed to assess the potential for trans-graft protection against Pierce's disease.

RESULTS AND DISCUSSION

Plant phenotypes.

There were no distinguishable morphological differences in the control plants compared with any of the transgenic lines using criteria of descriptors described by the International Organization of Vine and Wine. All plants have a normal phenotype, true to the untransformed control plants of each parental genotype and all produced abundant fruit. The Thompson Seedless transgenic plants are fully fruited with no visually distinguishable differences in fruit set, fruit size, or maturity from the untransformed control plants. The field map in **Figure 6** shows the genotypes and colored bars indicating the various inoculation dates and bacterial populations introduced at each inoculation date. By late June of 2014 all the inoculated untransformed control plants showed foliar symptoms of Pierce's disease, along with some of the experimental plants. Uninoculated control plants appear healthy in all cases indicating no spread of disease from inoculated to uninoculated untransformed susceptible plants.

X. fastidiosa titers by qPCR.

Inoculated plants were confirmed to have been successfully infected in the 2011, 2012, 2013, and 2014 inoculations by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations. Bacterial numbers from inoculated plants not showing symptoms varied from 500-1,500 cells per 1 cm of inoculated stem tissue. The inoculations on non-transgenic plants showing symptoms ranged from 10^4-10^6 cells per 1 cm of inoculated stem tissue. Example data from the untransformed Thompson Seedless scions grafted to the transformed rootstocks bearing either PR1 or UT 456 transgenic DNA sequences is presented in **Figure 5**.

Disease ratings in 2014 and 2015.

By late June of 2015 all the inoculated untransformed control plants showed foliar symptoms of Pierce's disease, along with some of the experimental plants (**Figures 6 and 7**). Uninoculated control plants appeared healthy in all cases. There is no evidence of plant-to-plant spread and only limited movement of bacteria from an inoculated cordon to uninoculated adjacent cordons or canes. The young canes of untransformed scions grafted to transgenic rootstocks, inoculated in May 2014, began to show Pierce's disease symptoms within 90 days. Eight leaves from the point of inoculation were rated for foliar symptoms at 120 days and revealed significant differences in Pierce's disease symptoms between control and transgenic rootstocks.

As of March 15, 2015, the plants were pruned to remove excess dormant branches (**Figure 1**) while leaving portions of previously inoculated cordon-like branches dating back to 2011and last year's inoculated canes. In the latter case, up to 10 buds were preserved on each inoculated cane as data rich resources for scoring dead versus

live buds on control and transgenic plants as new shoots begin to emerge in the spring of 2015 (**Figures 2, 3, and 4**). The 2014 inoculated canes were destructively sampled to determine the presence and concentration of *X. fastidiosa* in the tissues after scoring the new buds on the grafted plants (**Figure 5**).

CONCLUSIONS

X. fastidiosa induces Pierce's disease symptoms that result from activation of a genetically regulated process of programmed cell death. We identified two plant DNA sequence, designated PR1 and UT456, from a cDNA library screen, which, when constitutively expressed in transgenic grapes suppressed the death-dependent symptoms of Pierce's disease and reduced the bacterial titer to a level found in Pierce's disease resistant wild grapes in controlled greenhouse studies. Similar results are being seen under field conditions. The objective of the current experiment is to evaluate transgenic grape and grape rootstocks expressing these transgenes in individually derived transgenic lines in a field site in Solano County for protection against *X. fastidiosa* (Pierce's disease strain) following mechanical injections of the bacteria into the grape canes of both transgenic and co-planted non-transgenic control plants. Current data from the Solano County site suggests that protective sequences may function across a graft union to protect an untransformed and susceptible wild-type scion, although this data is preliminary. Both the PR1 and UT456 expressing plants show suppression of symptoms and reduced bacterial counts. Individual plants within several of the UT456 and PR1 lines have remained asymptomatic. While some lines are less suppressive, all lines are rated more suppressive of Pierce's disease than the controls. This project has identified a basis for Pierce's disease symptoms and a genetic mechanism to suppress symptoms and bacterial growth within an infected plant. These two sequences are now moving forward into the dual construct project described in the report titled, "Transgenic Rootstock-Mediated Protection of Grapevine Scion by Single and Stacked DNA Constructs."

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the Regents of the University of California.

Figure 1. Vine pruning. As of March 15th, 2015, all plants were pruned to remove excess growth from the past year but to retain all inoculated wood. Spurs on old inoculated cordons were pruned to 2-3 buds while the 2014 inoculated branches were trimmed to retain up to 10 buds for data collection to include live/dead bud counting and destructive sampling for bacterial counts.

Figure 2. Example of inoculated non-transgenic Thompson Seedless control plants with death of buds as they emerge in spring 2015. Plant was subjected to successive inoculations in 2011-2014. *Xylella* infection confirmed by PCR. Photo taken April 2, 2015. Colored tags visible on branches reflect the year and date of inoculation.

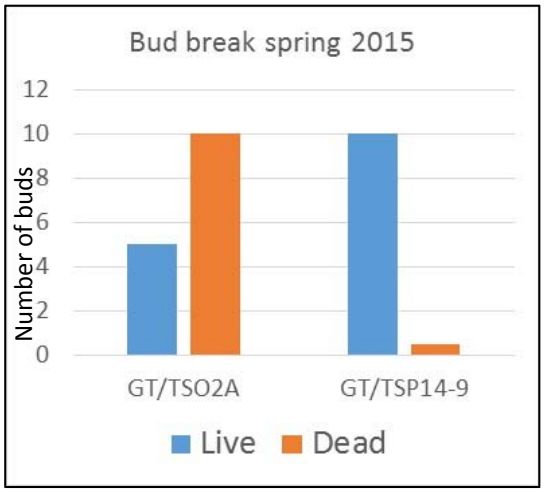

Figure 3. Live/dead analysis at bud break on grafted plants. The graph indicates the number of buds from the designated genotypes on canes inoculated in spring 2014. All grafted plants have untransformed Thompson Seedless as scions. GT/TSO2A is Thompson Seedless untransformed as a rootstock. GT/TSP14-9 is Thompson Seedless expressing P14 as a rootstock.

Figure 4. Close-up of non-transgenic control plant showing shoot dying shortly after emergence on inoculated canes in spring 2015. This cane was inoculated in 2013. Photo taken April 2, 2015.

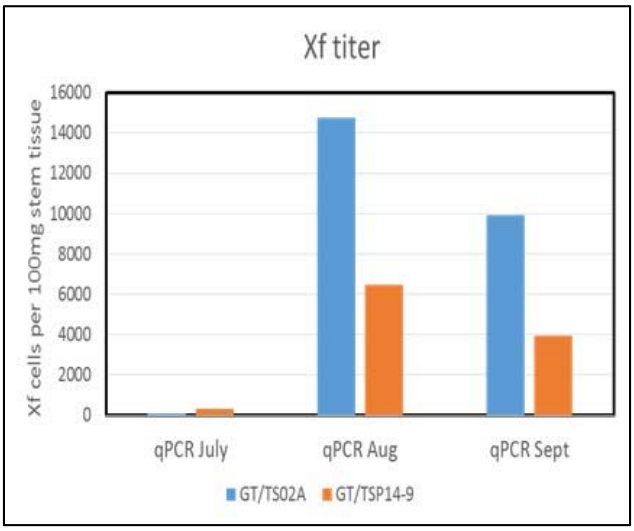

Figure 5. qPCR assessment of *X. fastidiosa* in untransformed Thompson Seedless scions grafted to untransformed Thompson Seedless rootstocks or transformed rootstocks. GT/TSO2A is Thompson Seedless untransformed as a rootstock. GT/TSP14-9 is Thompson Seedless expressing P14 as a rootstock. The respective untransformed Thompson Seedless scions were inoculated with *X. fastidiosa*.

Figure 6. Inoculated non-transgenic Thompson Seedless foreground; essentially dead. Inoculated transgenic Thompson Seedless in the rear; asymptomatic. Photo taken in May 2015. Tags indicate sites of inoculation.

Figure 7. Inoculated transgenic and non-transgenic Thompson Seedless seen from a distance. Asymptomatic and dying plants can be seen in the center row (arrow). May 2015.

TRANSGENIC ROOTSTOCK-MEDIATED PROTECTION OF GRAPEVINE SCIONS BY SINGLE AND STACKED DNA CONSTRUCTS

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ABSTRACT

Xylella fastidiosa is the causative agent of Pierce's disease. Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) has identified or constructed and advanced the evaluation of five novel genes (DNA constructs; **Table 1**) that, when engineered into grapevines, suppress symptoms of Pierce's disease by reducing the titer of *X. fastidiosa* in the plant, reducing its systemic spread in the plant, or blocking *X. fastidiosa*'s ability to trigger Pierce's disease symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of Pierce's disease resistance under field conditions where current data indicate that each of the five transgenes effectively reduces the disease levels under field conditions. The current plants are mechanically inoculated and monitored for the amount and movement of the bacteria and for expression of typical Pierce's disease symptoms. Symptom assessment is conducted by the principal investigators individually and by an independent team of evaluators lead by Deborah Golino. These existing field trials will continue through 2016. Importantly, each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scion, with obvious benefit that any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. Under Objective 1, the ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, will be tested. Objective 2 addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *X. fastidiosa* pathogen, a critical factor for a long-lived perennial crop such as grapevine. The approach under Objective 2 is "stacking," the combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against Pierce's disease. The first of the stacked transgene rootstock lines are beginning to be released from transformation and are undergoing the first stage of RNA evaluation. It is anticipated that the first wave of transgenic rootstocks will be ready for field planting in 2017 and the final wave by 2018.

LAYPERSON SUMMARY

Xylella fastidiosa is the causative agent of Pierce's disease. Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) has identified or constructed and advanced the evaluation of five novel genes (DNA constructs; **Table 1**) that, when engineered into grapevines, suppress symptoms of Pierce's disease by reducing the titer of *X. fastidiosa* in the plant, reducing its systemic spread in the plant, or blocking *X. fastidiosa*'s ability to trigger Pierce's disease symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of Pierce's disease resistance under field conditions where current data indicate that each of the five transgenes, introduced as single constructs, reduces the disease levels under field conditions. Importantly, preliminary data indicates that each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scion, with obvious benefit: any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, is currently being tested. The objective described herein addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *X. fastidiosa* pathogen, a critical factor for a long-lived perennial crop such as grapevine. This approach involves "stacking," a combination of

distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against Pierce's disease. The stacking of genes is the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in 2016 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing, to be initiated by 2017. The proposed changes are the next logical step toward achieving commercialization of transgenic resistance.

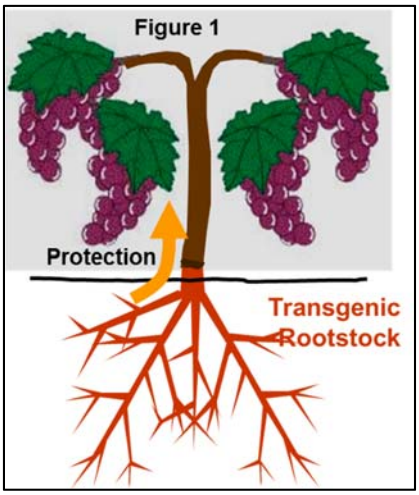

Figure 1. Example scenario whereby a transgenic rootstock is being tested for its ability to protect an untransformed scion from Pierce's disease.

OBJECTIVES

1. Introduce pairs of protective constructs into adapted grapevine rootstocks 1103 and 101-14.
2. The resulting lines will be tested for efficacy by inoculation with *X. fastidiosa* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes.

The primary motive for expressing genes in combination is to create durable resistance, resistance to *X. fastidiosa* that will last the life of the vine. Since at least several of the five DNA constructs (**Table 1**) have biochemically distinct mechanisms of action, having two or more such distinctly acting DNA constructs "stacked" in the rootstock should drastically reduce the probability of *X. fastidiosa* overcoming the resistance. With multiple, distinct transgenes, *X. fastidiosa* would be required to evolve simultaneously multiple genetic changes in order to overcome the two distinct resistance mechanisms.

Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating synergism in other crops. For example, the paper, "Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus" (Tricoli et al., 1995), describes the stacking of several genes for virus resistance in squash. (Note: David Tricoli, the lead author in this paper, will be doing the stacking transformations in this proposal.) Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall (Escobar et al., 2001). Experiments proposed here will evaluate potential synergism in suppression of Pierce's disease symptoms and in reducing *X. fastidiosa* titer for inoculations distant from the graft union. Briefly, we describe information on the history and impact of the genes deployed as single transgenes currently in USDA APHIS approved field trials. The subjects of this project are five specific DNA constructs (**Table 1**) that have shown to be effective in Pierce's disease suppression under field conditions as single gene constructs and also appear to have potential in cross-graft-union protection as described by Lindow, Dandekar, and Gilchrist in previous reports (provided in the list of references).

Table 1. Genes selected to evaluate as dual genes in the second generation field evaluation for suppression of Pierce's disease in grape. The table lists gene names and presumed functions.

Gene	Function
CAP	*X. fastidiosa* clearing/antimicrobial
PR1	grape cell anti-death
rpfF	changing quorum sensing of *X. fastidiosa* (DSF)
UT456	non-coding microRNA activates PR1 translation
PGIP	inhibits polygalacturonase/ suppressing *X. fastidiosa* movement

CAP and PGIP (Abhaya Dandekar)

The Dandekar lab has successfully participated in the two field plantings to investigate two greenhouse-tested strategies to control the movement and to improve clearance of *X. fastidiosa*, the xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease in grapevine (Dandekar, 2013). A key virulence feature of *X. fastidiosa* resides in its ability to digest pectin-rich pit pore membranes that connect adjoining xylem elements, enhancing long-distance movement and vector transmission. The first strategy tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to inhibit the *X. fastidiosa* polygalacturonase activity necessary for long distance movement (Aguero et al., 2006). The second strategy enhances clearance of bacteria from *X. fastidiosa* infected xylem tissues by expressing a chimeric antimicrobial protein (CAP), that consists of a surface binding domain that is linked to a lytic domain. The composition and activity of these two protein components have been described earlier (Dandekar et al., 2012).

rpfF, DSF (Steven Lindow)

The Lindow lab has shown that *X. fastidiosa* uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants (Lindow, 2013). Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant (Chatterjee et al., 2008). Accumulation of DSF in *X. fastidiosa* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant.

PR1 and microRNA UT456 (David Gilchrist)

The Gilchrist lab is focused on the host response to *X. fastidiosa* through identifying plant genes that block a critical aspect of grape susceptibility to *X. fastidiosa,* namely the inappropriate activation of a genetically conserved process of programmed cell death (PCD) that is common to many, if not all, plant diseases in which cell death is the visible symptom of disease. Blocking PCD, either genetically or chemically, suppresses disease symptoms and bacterial pathogen growth in several plant-bacterial diseases (Richael et al., 2001; Lincoln et al., 2002; Harvey et al., 2007). In the current project with Pierce's disease, a functional genetic screen identified novel anti-PCD genes from cDNA libraries of grape and tomato (Gilchrist and Lincoln, 2011). Two of these grape sequences (PR1 and UT456), when expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse and field conditions. Assays with various chemical and bacterial inducers of PCD confirmed that the PR1 was capable of blocking PCD in transgenic plant cells (Sanchez et al., 2015a). It was then discovered that the mechanism blocking PR1 translation is due to the ability of the PR1's 3'UTR to bind to a region in the PR1 coding sequence to prevent translation. Sequence analysis of UT456 revealed a strong sequence complementarity to a region in the PR1 3'UTR that released the translational block of PR1 translation. Hence, the mechanism of suppression of Pierce's disease symptoms depends on translation of either the transgenic or the endogenous PR1 message in the face of *X. fastidiosa* trigger cell stress (Sanchez et al., 2015b).

RESULTS AND DISCUSSION

Construction of dual gene expression binaries.

The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single

plasmid with a single selectable marker. The binary backbone is based on pCAMBIA1300 (Hajdukiewicz et al., 1994). Binaries were constructed to express two genes from two 35S promoters (**Figure 2**). The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites for ligation into the backbone. The nt-PGIP used in these constructs is a modified version of the Labavitch PGIP that was modified in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al., 2006).

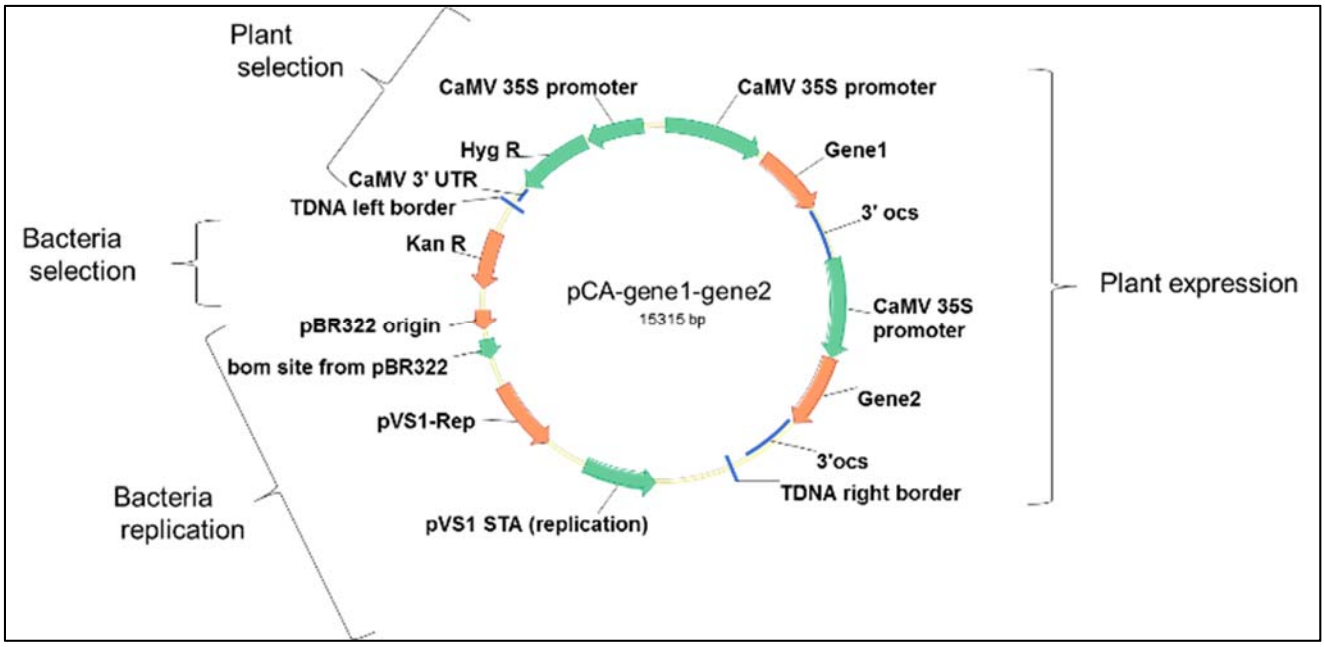

Figure 2. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

Binary plasmids capable of expressing two genes from the same TDNA (dual expressers) were constructed by Dr. Lincoln and are of the general form shown in **Figure 2**. All plasmids were transformed into *Agrobacterium* strain EHA105, the transformation strain for grape plant transgenics. As a check on stability of the dual expresser binary plasmid, the plasmid was isolated from two *Agrobacterium* colonies for each construct and the plasmid was used to transform *Escherichia coli*. Six *E. coli* colonies from each *Agrobacterium* isolated plasmid (for a total of 12 for each construct) were analyzed by restriction digest to confirm that the plasmid in *Agrobacterium* is not rearranged. **Table 2** shows when transformations were started by the UC Davis Plant Transformation Facility. To ensure optimum recovery of the transgenic embryos, two versions of the plasmid with different antibiotic selectable markers were prepared. Hence, the dual inserts can now be subjected to two different selections that enables transformation to move forward in the fastest manner depending on which marker works best for each dual or each rootstock background.

Each dual protective gene plasmid will be introduced into embryogenic grapevine culture in a single transformation, i.e., conventional grapevine transformation in the UC Davis Plant Transformation Facility. The progress for each line is shown in **Table 2**.

Table 2. The current status of grape transformations into rootstocks 1103 and 101-14.

October 26, 2015 PD Transformation update

						Months																
Genotype	**Select**	**PTF Code**	**PI**	**Start Date**		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1103	hygro	AT14119	Gilchrist	10/15/14	pCA-5fCAP-5oP14HT												6	3				
1103	hygro	AT14120	Gilchrist	10/15/14	pCA-5fCAP-5oUT456												15	2				
1103	hygro	AT14121	Gilchrist	10/15/14	pCA-5PGIP-5oUT456																	
1103	hygro	AT15007	Gilchrist	1/9/15	pCA-5PGIP-5oP14HT											3						
1103	hygro	AT15008	Gilchrist	1/9/15	pCA-5PGIP-5FCAP											14						
1103	hygro	AT15009	Gilchrist	1/9/15	pCA-5oP14HT-5oUT456											20						
1103	hygro	AT15039	Gilchrist	5/26/15	pCA-5oP14HT-5orpfF																	
1103	hygro	AT15040	Gilchrist	5/26/15	pCA-5oUT456-5orpfF																	
1103	hygro	AT15041	Gilchrist	5/26/15	pCA-5fCAP-5orpfF																	
1103	hygro	AT15042	Gilchrist	5/26/15	pCA-5PGIP-5orpfF																	
1103	kan	AT15100	Gilchrist	8/28/15	pCA-5oP14HT-5orpfF																	
1103	kan	AT15001	Gilchrist	8/28/15	pCA-5oUT456-5orpfF																	
1103	kan	AT15002	Gilchrist	8/28/15	pCA-5fCAP-5orpfF																	
1103	kan	AT15003	Gilchrist	8/28/15	pCA-5PGIP-5orpfF																	
TS	kan	AT15050	GilChrist	4/17/15	pCB5o-UPUN							24										
101-14	kan	AT15107	Gilchrist	10/1/15	pCK-5oP14HT-5oUT456																	
101-14	kan	AT15108	Gilchrist	10/1/15	pCK-5fCAP-5oP14LD																	
101-14	kan	AT15109	Gilchrist	10/1/15	pCK-5PGIP-5oP14LD																	
101-14	kan	AT15110	Gilchrist	10/1/15	pCK-5ofCAP-5oUT456																	
101-14	kan	AT15111	Gilchrist	10/1/15	pCK-5PGIP-5oUT456																	
101-14	kan	AT15112	Gilchrist	10/1/15	pCK-5PGIP-5FCAP																	
101-14	kan	AT15113	Gilchrist	10/1/15	pCK-5oP14LD-5orpfF																	
101-14	kan	AT15114	Gilchrist	10/1/15	pCK-5oUT456-5orpfF																	
101-14	kan	AT15115	Gilchrist	10/1/15	pCK-5ofCAP-5orpfF																	
101-14	kan	AT15116	Gilchrist	10/1/15	pCK-5PGIP-5orpfF																	

Key

- Embryos harvested, embryos to plants takes 3-9 months
- Plants rooted in vitro- Once plants are rooted in vitro, it takes 8 wks to transfer to soil and 2 wks to acclimate to soil
- ** Plants delivered

The following images (**Figure 3**) illustrate the development of transgenic embryos, the initiation of roots and shoots from the transgenic embryo, and finally, the fully developed transgenic rootstock containing two of the transgenes.

Figure 3. Rootstock 1103 embryos and developing plantlets with pCA-5fCAP-5oP14HT AT14119 transgenes inserted and the developed transgenic plant ready for RNA analysis and pathogenicity testing for response to *X. fastidiosa*.

CONCLUSIONS

Our capacity to achieve all the objectives is essentially assured based on prior accomplishments. All techniques and resources are available in the lab and proven reliable, informative, and reproducible. This project will bring together a full-time research commitment for this team of experienced scientists to Pierce's disease. Each of the senior personnel, including Dr. Lincoln, have been with this project since 2007 and have different skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation, and analysis of transgenic plants. This includes both greenhouse and field evaluation of protection against Pierce's disease. Commercialization of the currently effective anti-Pierce's disease containing vines and/or rootstocks could involve partnerships between UC Foundation Plant Services, nurseries, and potentially, with a private biotechnology company. The dual constructs have been assembled and forwarded to David Tricoli at the UC Davis Plant Transformation Facility. The first transgenic plants have been delivered to Dr. Lincoln, who has been begun the RNA analysis to verify that each plant contains both of the intended constructs. The timeline shown in **Figure 4** is on track.

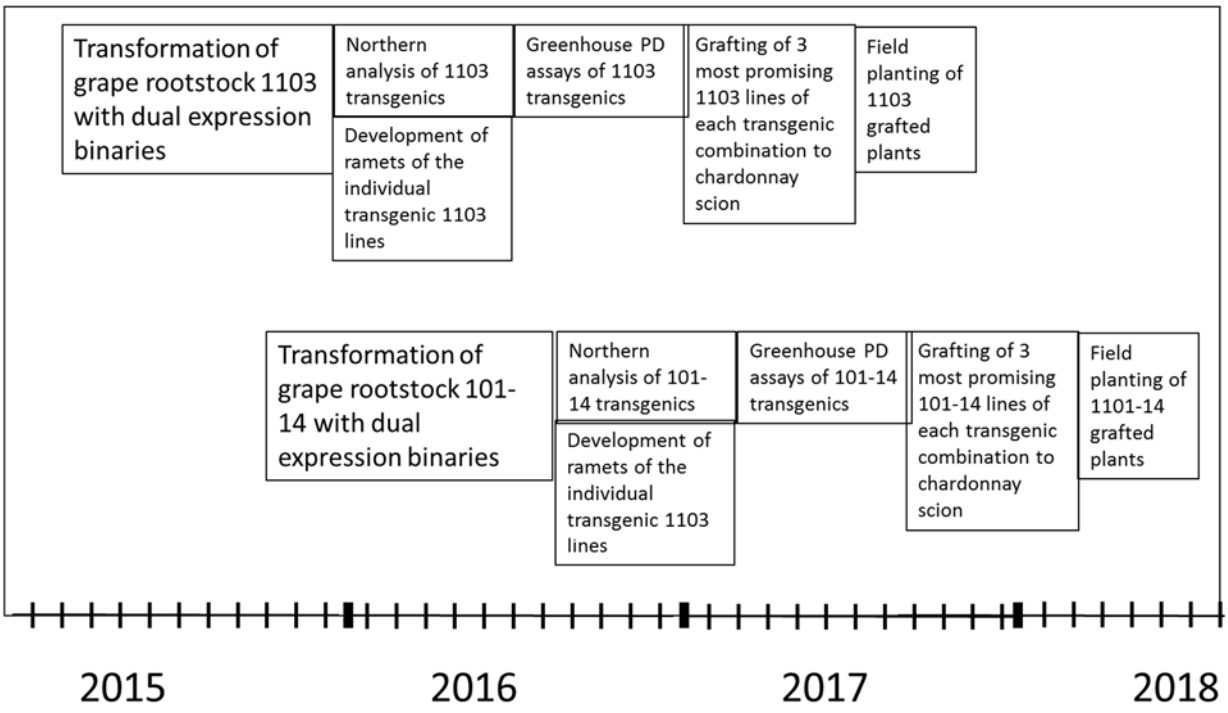

Figure 4. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

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BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPEVINE IN THE UC RIVERSIDE VINEYARD WITH A BENIGN STRAIN OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted March 2011 through October 2015.

ABSTRACT
To evaluate *Xylella fastidiosa* strain EB92-1 for the biocontrol of Pierce's disease in Southern California, a test site was established at the University of California, Riverside vineyard in October 2011. Treated and untreated Cabernet Sauvignon and Pinot Noir vines were transplanted in mid-October. This was an excellent test site to evaluate Pierce's disease control because of the heavy disease pressure. Symptoms began to develop in the first year of the trials. There were other abiotic and biotic stresses on the vines, but Pierce's disease seemed to be responsible for most of the symptoms. As a result of the heavy disease pressure and other stresses in this vineyard, four other test plots in the vineyard were abandoned in 2015. However, in our biocontrol plots, only 16% of the treated Cabernet Sauvignon and 29% of the treated Pinot Noir vines had Pierce's disease symptoms in 2015, and no vines had died. One treated Cabernet Sauvignon vine and three Pinot Noir vines had severe symptoms and were not producing fruit. In both plots, vigor had remained strong throughout the four years of the trial and fruit production was good. Under heavy disease pressure, EB92-1 has provided control of Pierce's disease for four years in Cabernet Sauvignon and Pinot Noir in the University of California, Riverside vineyard.

LAYPERSON SUMMARY
Since an earlier trial in Temecula was terminated, trials to evaluate the biocontrol of Pierce's disease in Cabernet Sauvignon and Pinot Noir were established in the University of California, Riverside vineyard in 2011. The vines were transplanted in October. The University of California, Riverside vineyard proved to be an excellent site to evaluate the control of Pierce's disease under strong disease pressure. Glassy-winged sharpshooter (*Homalodisca vitripennis*) vectors of Pierce's disease were abundant. Nutrient deficiencies, boron toxicity, nematodes, and powdery mildew increased the susceptibility of the vines to Pierce's disease, but it is not unusual to have other stresses on the vines and an effective Pierce's disease control should still work under these conditions. In our plots evaluating the biocontrol of Pierce's disease with a benign strain of *Xylella fastidiosa* (EB92-1), the biocontrol strain provided excellent control of Pierce's disease in Cabernet Sauvignon and Pinot Noir through 2015. Over the four years of the trials, no vines have died; one treated Cabernet Sauvignon vine and three treated Pinot Noir vines had severe symptoms and would be removed from a commercial vineyard. In both cultivars, plant vigor and fruit production have remained very good. Four nearby plots were abandoned in 2015 because of plant loss. Under severe Pierce's disease pressure, the biocontrol strain has proven to be effective over four seasons in the University of California, Riverside vineyard.

INTRODUCTION
Pierce's disease of grapevine is an endemic, chronic problem in the southeastern USA where it is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.) (Hopkins and Purcell, 2002). Pierce's disease is also endemic in California and became more of a threat to the California grape industry with the introduction of the glassy-winged sharpshooter (*Homalodisca vitripennis*). While vector control has been effective for Pierce's disease control in some situations, the only long-term, feasible control for Pierce's disease has been resistance. Over 20 years of research on the biological control of Pierce's disease of grapevine by cross protection with weakly virulent strains of *Xylella fastidiosa* has demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). One strain of *X. fastidiosa*

that was able to control Pierce's disease in *V. vinifera* for 14 years in Central Florida has been identified. We have successfully tested this strain in commercial vineyards in several states and the strain should soon be ready for commercial use.

In 2008 and 2009, field trials to evaluate the biological control of Pierce's disease in California with strain EB92-1 were established in Temecula, Napa, and Sonoma. The trials in Temecula were terminated in 2010, since many of the young vines had died from extreme water stress and poor fertility. At the end of the project in 2013, there was a very low level of Pierce's disease in the vineyard in Napa. In Sonoma, there was significant Pierce's disease in Voignier (64% in 2012 and 45% in 2013) with no significant differences between the treated and untreated vines. The tests in Napa and Sonoma were conducted with new plants replacing mature vines that had died. None of the tests had produced sufficient data to tell us whether or not the biocontrol strain is effective in California. To replace the lost trials in Temecula, trials were established in 2011 in the University of California, Riverside vineyard. Our evaluation of EB92-1 for the control of Pierce's disease was included in a trial of alternate strategies for the control of Pierce's disease. Four different transgenic constructs were also in the trial. The overall goal of this project is to develop a biological control system for Pierce's disease of grapevine that would control the disease in California and other areas where Pierce's disease and the glassy-winged sharpshooter are endemic.

OBJECTIVES

To evaluate strain EB92-1 of *X. fastidiosa* for the biological control of Pierce's disease of grapevine in new plantings in the University of California, Riverside vineyard in California.

RESULTS AND DISCUSSION

To replace the lost tests in southern California, a replacement test was established in 2011 at the University of California, Riverside. For transplanting into the University of California, Riverside vineyard, 100 Cabernet Sauvignon plants and 100 Pinot Noir plants were obtained from Sunridge Nursery in March 2011 and maintained in a University of California, Davis greenhouse. Fifty Cabernet Sauvignon and 50 Pinot Noir vines were inoculated with EB92-1 in July 2011 and 50 plants of each cultivar were kept as untreated controls. These plants were maintained in the greenhouse for six weeks and then moved outside to harden them off. The plants were transported to Riverside in mid-October and transplanted into the plots at the University of California, Riverside. This planting time reduced heat stress on the transplants and gave them the fall season to establish a strong root system. This resulted in vigorous plants in the spring of 2012 for inoculation with the Pierce's disease strain of *X. fastidiosa* by resident glassy-winged sharpshooters (*Homalodisca vitripennis*) throughout the season.

Vigorous growth has occurred in the EB92-1 biological control plot through the 2015 season (**Figure 5**). This plant vigor has continued in spite of various abiotic and biotic stresses on the vines that were reported in 2014 (Rolshausen et al, 2014). Among the abiotic stresses that were identified included a slightly alkaline and nutrient deficient soil (i.e., nitrogen, magnesium, boron, and phosphorous). There were also deficiencies in the plant tissue, as well as boron toxicity. These abiotic stresses occurred across four nearby trials and our biological control trial, which was located approximately 100 feet from the nearest other trial. Improper pruning resulted in the additional stress of a heavy crop load only on the Pinot Noir in the EB92-1 trial (**Figure 1B** in Rolshausen et al., 2014). In addition to Pierce's disease, biotic stresses from nematodes in a nearby plot and from powdery mildew in the EB92-1 biological control plot were observed (**Figure 1A** in Rolshausen et al., 2014). All of these stresses could have resulted in decline of the vines or added to the effect of Pierce's disease. Pierce's disease was observed in all the plots.

Sharpshooters were monitored at the experimental site in all five plots. Double-sided yellow sticky traps (6" x 9") were placed randomly throughout the plots. Traps were mounted on wooden stakes slightly above the vine canopy. These traps were collected every month and returned to the laboratory to identify, under the stereomicroscope, the number of glassy-winged sharpshooters. Significant populations of sharpshooters were captured in the traps every year. For example, in 2014 and 2015, low insect vector populations were recorded early in the season, but increased rapidly over the summer (**Figure 1**).

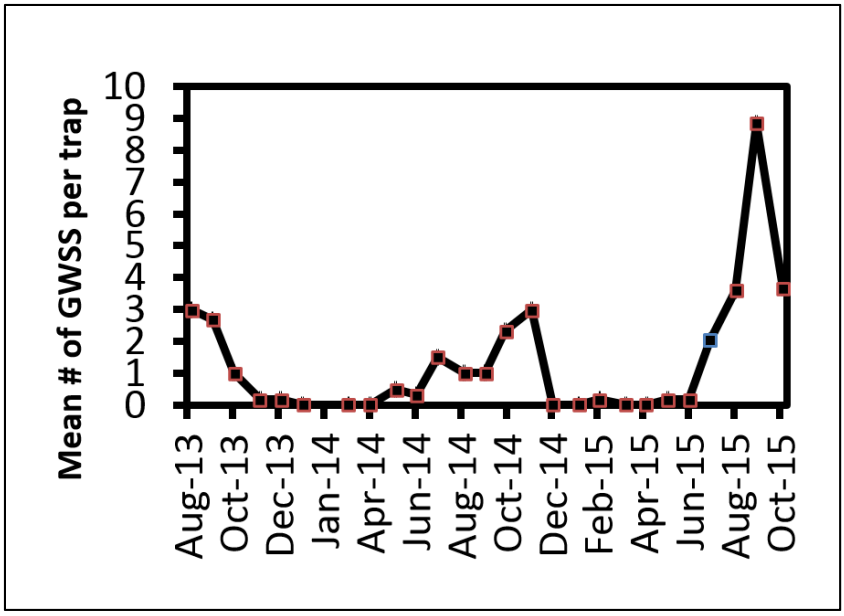

Figure 1. Mean number of glassy-winged sharpshooter insect vectors per trap captured on yellow sticky traps from the biocontrol plots (based on six traps).

Pinot Noir trial

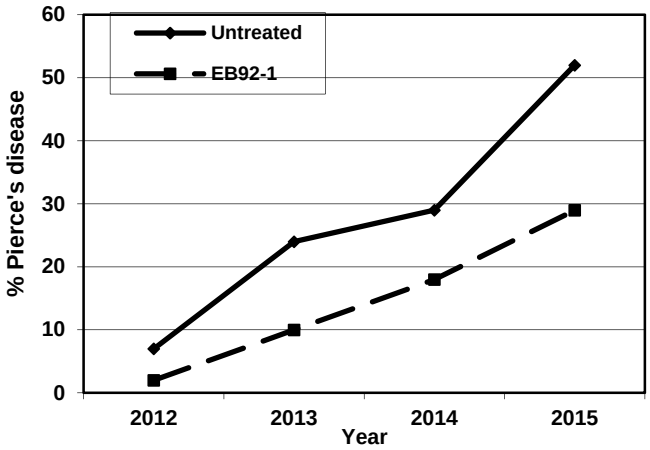

Figure 2. Development of Pierce's disease in treated and untreated grapevine cv. Pinot Noir in the UC Riverside vineyard.

Figure 3. Good fruit load on treated grapevine cv. Pinot Noir in the UC Riverside vineyard.

After one year, symptoms had developed in a few vines (**Figure 2**). Pierce's disease has increased over the four years of the trial and has developed more rapidly in the untreated vines. It is important to note that none of the vines have died from Pierce's disease (two untreated vines died in the first year from the stress of transplanting). However, a few vines have severe Pierce's disease symptoms with a rating of three on the three-five scale; this includes seven vines in the untreated and three in the treated. Many of the symptomatic vines have had a rating of one or two for two-three years and continue to grow and produce fruit. In spite of severe powdery mildew and the stress of heavy fruit loads in 2014 (Rolshausen et al., 2014), fruit production was very good on most of the vines, especially in the treated vines (**Figure 3**). Since abiotic stresses and Pierce's disease resulted in the abandonment of the four other plots, it is most surprising that almost 50% of the untreated vines in our biological control plots were still healthy after four years. Perhaps this resulted from lower disease pressure because of the presence of the treated vines. If we were removing severely infected vines (rating of three-five), we would have had to replant only seven of 42 untreated Pinot Noir vines and three of 49 treated vines over the four years.

Pierce's disease symptoms observed in the Pinot Noir vines were typical. They included leaf scorch, leaf drop with petioles remaining attached, dieback of branches, and new growth of shoots from the trunk of the vines that died back. Some of the vines with dieback actually produced fruit on the new growth, but it would have been advantageous to replace these vines.

Cabernet Sauvignon Trial

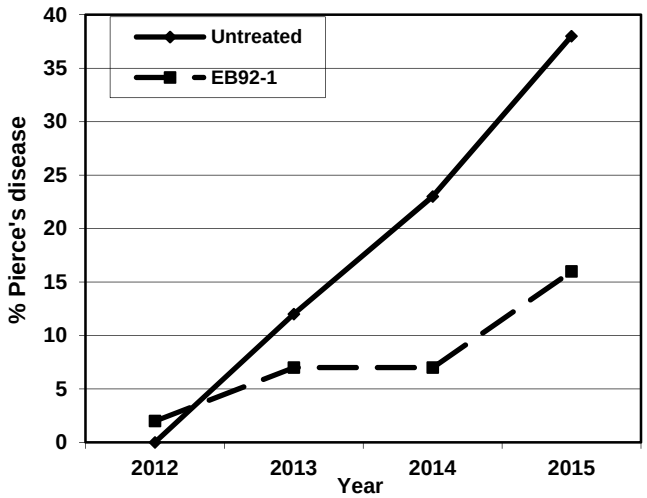

Figure 4. Development of Pierce's disease in treated and untreated grapevine cv. Cabernet Sauvignon in the UC Riverside vineyard.

Figure 5. Excellent vigor in grapevine plots in July 2015 in the UC Riverside vineyard after severe abiotic and biotic stresses in the 2014 season.

The Cabernet Sauvignon vines appeared less susceptible to Pierce's disease than Pinot Noir did in this trial. In the first season, only two treated vines developed symptoms (**Figure 4**). During the next three seasons, Pierce's disease developed much more rapidly in the untreated vines than in the treated. Symptoms were as observed in Pinot Noir. As with the Pinot Noir, no Cabernet Sauvignon vines have died of Pierce's disease, and only one untreated vine died from transplanting. Only three of 26 untreated vines and one of 44 treated vines had a severity rating of three after the years. Only one treated vine would have needed to be replaced over the four years. Vine vigor was excellent through 2015 and fruit production good (**Figure 5**).

CONCLUSIONS

The University of California, Riverside vineyard was an outstanding site for the evaluation of Pierce's disease control. Present at this site were natural inoculum sources as well as the glassy-winged sharpshooter. There were abiotic and biotic stresses on the vines that may have increased the rate and severity of disease development. However, I believe that Pierce's disease was primarily responsible for the decline and death of vines in the trials. The development of Pierce's disease was very similar in this vineyard to what we observe in Florida. Vineyards are not always stress-free, so controls must be effective in spite of other stresses.

Biological control of Pierce's disease in Pinot Noir and Cabernet Sauvignon by *X. fastidiosa* strain EB92-1 was excellent in this trial. No vines died from Pierce's disease during the four years of the test. Fruit production has been good in most treated vines throughout the test and vine vigor has been maintained. Interestingly, treatment of half the vines in the plots also resulted in many healthy, untreated vines. This was probably due to reduced disease pressure on the untreated vines.

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SELECTIVE DISRUPTION OF GLASSY-WINGED SHARPSHOOTER MATURATION AND REPRODUCTION BY RNA INTERFERENCE

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Reporting Period: The results reported here are from work conducted November 2014 to October 2015.

ABSTRACT
The overall goal of this project is to develop an RNA interference (RNAi) mediated system to inhibit maturation and reproduction of the glassy-winged sharpshooter (*Homalodisca vitripennis*). The initial target for RNAi will be glassy-winged sharpshooter *jheh* (also known as *hovi-meh1*), the gene that encodes juvenile hormone epoxide hydrolase (JHEH). Glassy-winged sharpshooter *jheh* will be used as a model gene target to establish an efficient expression and screening system for characterizing RNAi effectors. This system will then be used to evaluate other juvenile hormone metabolic genes including those that encode juvenile hormone esterase, juvenile hormone acid methyl transferase, and other identified genes as targets for RNAi. These gene sequences will be mined from the recently determined transcriptome sequence of the glassy-winged sharpshooter. Finally, plant virus- or insect virus-based systems for expression and delivery of the RNAi effectors in insects will be developed.

LAYPERSON SUMMARY
A natural process called RNA interference (RNAi) is used by a wide range of organisms to regulate normal gene function and defend against viruses. This process can be artificially manipulated and potentially used as a "gene based" insect control tactic. Two critical keys for developing an RNAi-based control tactic are (1) the identification of a selective target gene, and (2) the development of a system to produce and deliver RNAi effectors in whole insects. In this project, we are identifying genes that are found in endocrine system of the glassy-winged sharpshooter (*Homalodisca vitripennis*) as targets for RNAi. A field-applicable delivery system for inducing RNAi against these targets will also be tested.

INTRODUCTION
In California, the control of the glassy-winged sharpshooter (*Homalodisca vitripennis*) relies primarily on the use of neonicotinoid insecticides such as imidacloprid and to a lesser extent on biological control using parasitic wasps and other classes of chemical insecticides. Both metabolic and target site resistance to neonicotinoids are found in hemipterans and other insects [1]. The effectiveness of imidacloprid treatment against the glassy-winged sharpshooter also appears to be on the decline in California [2]. Furthermore, neonicotinoids have been linked to negative off-target effects such as colony collapse disorder in honeybees resulting in restrictions in their use in the European Union. The registration of several neonicotinoids is also under re-review by the US Environmental Protection Agency. Because of the potential loss of imidacloprid both in terms of its efficacy and availability (due to regulatory restrictions), alternative technologies to control the glassy-winged sharpshooter should be considered.

RNA interference (RNAi)-based technologies [3,4] that selectively target the glassy-winged sharpshooter's endocrine system is a potential alternative tactic for controlling glassy-winged sharpshooter and the diseases that it transmits. RNAi is a natural process found in a wide range of organisms that regulates gene function and protects against viruses. The natural RNAi process can be artificially induced in insects by the introduction of an RNAi effector, i.e., double-stranded RNA (dsRNA) or small interfering RNA (siRNA) that targets a specific messenger RNA. This technology has been shown to work in insects that feed on artificial diet infused with dsRNA or siRNA as well as on transgenic plants that express dsRNA. Two critical keys for developing an RNAi-based control tactic are (1) the identification of a highly selective and effective gene target, and (2) the availability of a system to produce and deliver the RNAi effector in whole insects. In this project, genes that are found in the glassy-winged sharpshooter's endocrine system are being identified and developed as targets for RNAi. Genes in the insect's endocrine system are ideal targets for knockdown because they are part of an essential and highly sensitive developmental pathway that is only found in arthropods.

OBJECTIVES

1. Develop *jheh* as a model target for RNAi-based control of glassy-winged sharpshooter maturation.
2. Mine the glassy-winged sharpshooter transcriptome for other RNAi targets.
3. Develop virus-based dsRNA production and delivery systems for controlling the glassy-winged sharpshooter.

RESULTS AND DISCUSSION

Objective 1. Develop *jheh* as a model target for RNAi-based control of glassy-winged sharpshooter maturation.

Juvenile hormones (JHs) and molting hormones (ecdysones) are key components of the insect endocrine system that help to regulate insect development. JHs also regulate other important biological actions such as reproduction, mating behavior, feeding induction, and diapause (reviewed in [5]). The level of JH within an insect is determined by a combination of its biosynthesis and degradation. JH acid methyl transferase (JHAMT) is the enzyme that catalyzes the final step of JH biosynthesis. On the other hand, JH degradation occurs through the action of two hydrolytic enzymes called JH epoxide hydrolase (JHEH) and JH esterase (JHE). JHEH and JHE metabolize the epoxide and ester moieties that are found on all JH molecules resulting in the formation of JH diol and JH acid, respectively (**Figure 1**). Minor changes in normal JH levels through alteration in the action (or lack of action) of JHEH, JHEH, and/or JHAMT are hypothesized to cause dramatic changes in insect development and/or death. The sensitivity of the insect endocrine system to minor changes is a critical factor in the success of JH analog insecticides such as pyriproxyfen and methoprene.

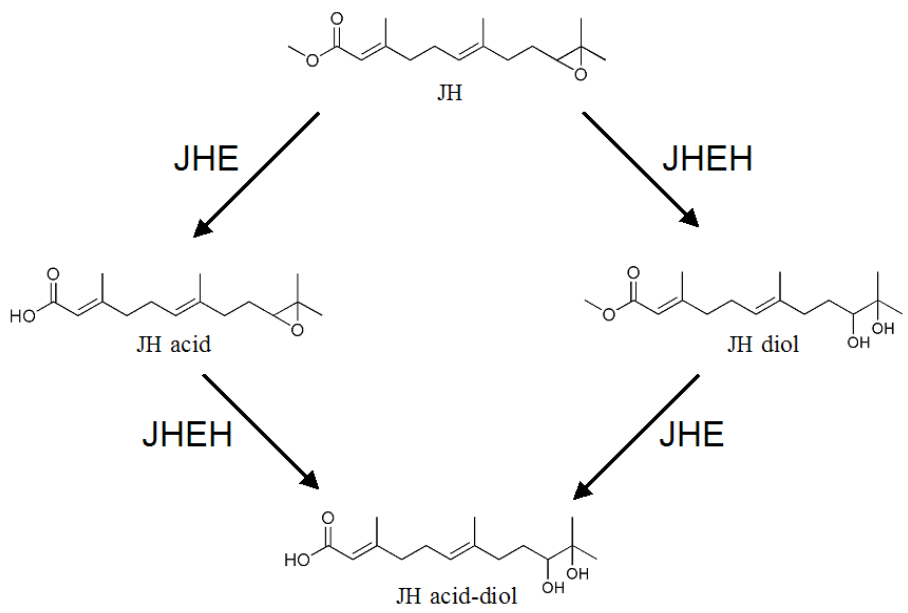
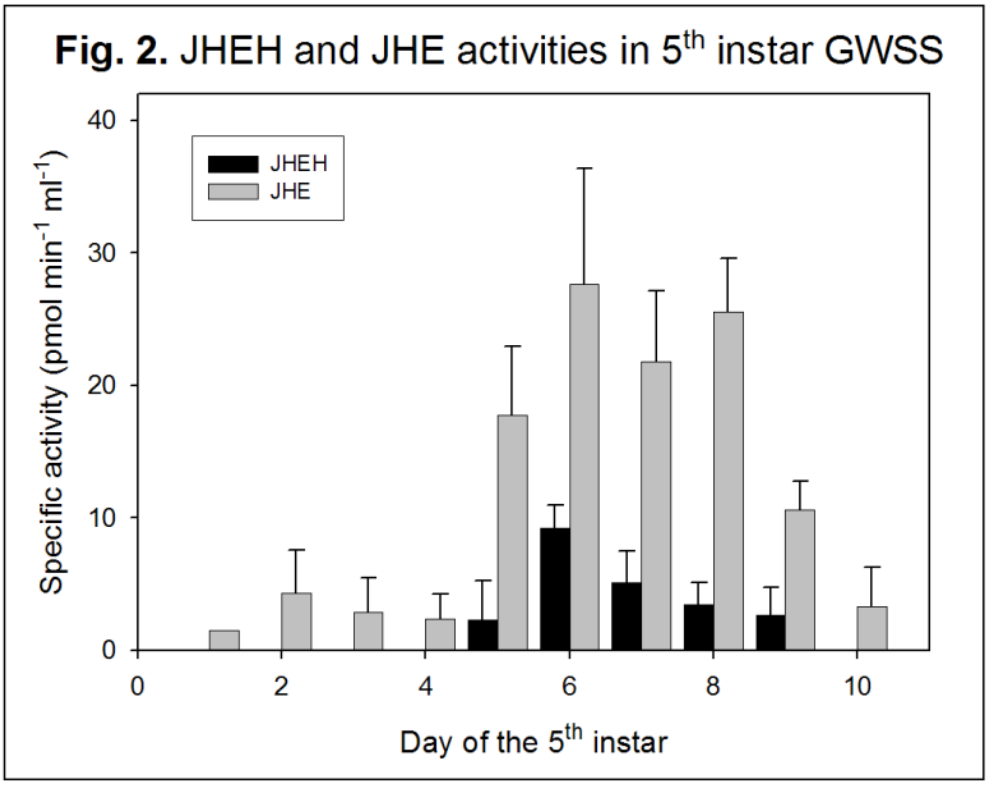


Fig. 1. Structure of juvenile hormone III (JH) and metabolism of JH III by JH esterase (JHE) and JH epoxide hydrolase (JHEH).

The coding sequence of the *jheh* gene of the glassy-winged sharpshooter has been identified and confirmed to encode a biologically active JHEH in a previous project [6]. This gene is now being developed as a target for RNAi in the glassy-winged sharpshooter. Plasmid constructs for the expression of full-length dsRNAs corresponding to *jheh* of the glassy-winged sharpshooter have been designed. The baseline levels of JHEH and JHE activities in control fifth instar glassy-winged sharpshooters has been quantified (**Figure 2**). Detailed information of these enzyme activities is needed to quantify the efficacy and selectivity of the RNAi against the *jheh* and *jhe* genes. During the first four days of the fifth instar, JHE activity was relatively low (1.5 to 4.4 pmol of JH acid formed min^{-1} ml^{-1} of hemolymph) and found at relatively constant levels. JHE activity dramatically increased (by about seven-fold) on the fifth day of the fifth instar. JHE activity remained high (9- to11-fold higher

than that found on the fourth day of the fifth instar) on the sixth, sevent, and eighth days of the fifth instar, then started to decline on the ninth day of the fifth instar. The dramatic increase in JHE activity during the second half of the fifth instar is predicted to remove residual JH from the hemolymph so that (in conjunction with small spikes of ecdysteroids) the juvenile insect undergoes a nymph-to-adult molt instead of a nymph-to-nymph molt. JHEH activity was lower than JHE activity during all of the time points tested. JHEH activity increased by about four-fold on the sixth day of the fifth instar, a delay of about one day in comparison to the spike in JHE activity. These findings suggest that JHE may play a more predominant role than JHEH in JH metabolism in the glassy-winged sharpshooter.



Objective 2. Mine the glassy-winged sharpshooter transcriptome for other RNAi targets.
A transcriptome is defined as a set of all of the RNA molecules that are found in a specific set of cells at a particular moment in time. The cooperator Professor Bryce Falk's laboratory has recently determined the sequence of the transcriptome of fifth instar glassy-winged sharpshooters [7]. By computer software-based screening of the glassy-winged sharpshooter transcriptome, multiple *jhe*-like coding sequences were identified. These potential JHE encoding sequences were manually analyzed (24 deduced amino acid sequences during the initial screening) for the presence of conserved motifs (see [8]) that are found in biologically active JHEs. A rank order of the potential of these sequences to encode a biologically active JHE was determined and primer sequences were designed for the amplification of the full-length sequences of the top three candidates. In order to mine the full-length JHE sequence from the glassy-winged sharpshooter, double-stranded cloned DNA (ds cDNA) libraries were generated from a developmentally mixed population of fifth instar glassy-winged sharpshooters (30 individuals) as well as individual glassy-winged sharpshooters at day seven, eight, and nine of the fifth instar. The ds cDNAs were used as template sequences for 3'- and 5'-random amplification of cDNA ends (RACE) procedures to generate full-length gene coding sequences.

A.

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agggcaccttggacagacccctcgctacacacaaaaagatggcagcctgtccacaatca
 R  A  P  W  T  D  P  R  Y  T  Q  K  D  G  P  A  C  P  Q  S  100
agcatggcgtatgatgaagactgtttgtacttaaatgtgttcacaccaatgaatgcatct
 S  M  A  Y  D  E  D  C  L  Y  L  N  V  F  T  P  M  N  A  S  120
gctactcacggaattttacctgtaatggtgttcattcatggaagtggctttctcagtagc
 A  T  H  G  I  L  P  V  M  V  F  I  H  G  S  G  F  L  S  S  140
tctagtaattcacattggattggaccagacttcttgatacctgaacatgtcattctagtt
 S  S  N  S  H  W  I  G  P  D  F  L  I  P  E  H  V  I  L  V  160
gctatgaactatcgtctaggagcaccaggtttcctcactttgggttctaagatagcacct
 A  M  N  Y  R  L  G  A  P  G  F  L  T  L  G  S  K  I  A  P  180
ggcaacttgggtttgcatgatacaagattagctctggaatgggtcagagacgagatctca
 G  N  L  G  L  H  D  T  R  L  A  L  E  W  V  R  D  E  I  S  200
gtattggaggagatcctacacaagtgactctgtttggtcagagtgctggttcagctatg
 V  F  G  G  D  P  T  Q  V  T  L  F  G  Q  S  A  G  S  A  M  220
actcaatttcactacatctcatctctatcctcagatctgttccaaagagctattggacat
 T  Q  F  H  Y  I  S  S  L  S  S  D  L  F  Q  R  A  I  G  H  240
agtggatctgcgttggcaggctggagctcttactcactttctgagggcgtgcacagagcc
 S  G  S  A  L  A  G  W  S  S  Y  S  L  S  E  G  V  H  R  A  260
aggcttcttgctgaaagtctaaaatgcaacatgacgcagaacgacacaatgcttctggat
 R  L  L  A  E  S  L  K  C  N  M  T  Q  N  D  T  M  L  L  D  280
tgtatgcagaaagctgacatcaaagatgtcatctctaatcaatatgtacaactgtcgtat
 C  M  Q  K  A  D  I  K  D  V  I  S  N  Q  Y  V  Q  L  S  Y  300
gatgattatactgtagggagcagtttccatttctgcctgtgcttgatacatttgagaca
 D  D  Y  T  V  G  S  S  F  P  F  L  P  V  L  D  T  F  E  T  320
tcggacacaccattctttaatgatacatccatagacaacatgatgcagcaagctctcttg
 S  D  T  P  F  F  N  D  T  S  I  D  N  M  M  Q  Q  A  L  L  340
cgggccaaaccactcatcacagggttcacaactgatgaggggattcttaagttcatggat
 R  A  K  P  L  I  T  G  F  T  T  D  E  G  I  L  K  F  M  D  360
aaggggtggcaaatggctgaaggcaacttaggagctttatccctccaaagatcagagat
 K  G  W  Q  M  A  E  G  N  L  G  A  F  I  P  K  I  R  D  380
agcgtcagtaaagctgagagatcgagtcttgccgatactatcaagagtcggtactaccct
 S  V  S  K  A  E  R  S  S  L  A  D  T  I  K  S  R  Y  Y  P  400
gactctgttgatgagaacaaaattgagagtgctgtcaggatatacaccgatgccatgttc
 D  S  V  D  E  N  K  I  E  S  A  V  R  I  Y  T  D  A  M  F  420
tcatatccttctctacaagtgactcggtactttgctaacctgacctacggttatctattt
 S  Y  P  S  L  Q  V  T  R  Y  F  A  N  L  T  Y  G  Y  L  F  440
gcatacaatggtgcatgggcaggaccgcctcttcctctctgtctacaaaatgactgga
 A  Y  N  G  A  W  A  G  P  P  S  S  F  S  V  Y  K  M  T  G  460
gtcggtcatggggcagacttgtactacttgctatatgtaaacggcagttcacagtatgtg
 V  G  H  G  A  D  L  Y  Y  L  L  Y  V  N  G  S  S  Q  Y  V  480
gacacctgcacacccaacttgcccaatcttcaaatgaaggaccaaatggtcaaatggtgg
 D  T  C  T  P  N  L  P  N  L  Q  M  K  D  Q  M  V  K  W  W  500
acatcgtttgcaaagaatggtgttcctggtctgccttgggagacaatatctgagggaggc
 T  S  F  A  K  N  G  V  P  G  L  P  W  E  T  I  S  E  G  G  520
tacctataatagatggctctgatccttctaagatgaacactacagaatttgagagtcaa
 Y  L  I  I  D  G  S  D  P  S  K  M  N  T  T  E  F  E  S  Q  540
ttctatgacttctgggcaaatatgaagccacaaagtggtaattcggctgatcctctcggc
 F  Y  D  F  W  A  N  M  K  P  Q  S  G  N  S  A  D  P  L  G  560
ctcagatttttttcatcaaagttgctctgttaagtttattccatcatattttaaagtc
 L  R  F  F  F  I  K  V  A  L  L  S  L  F  H  H  I  F  K  V  580
tgattttgtcaaattaaaagtttattttagatgc
 *
```

D.

```
GQSAG2      -------MSWYLSVGLVSA---ILAAASSEFVDLPVLVTKKGPIIGLRVDPNPATNISYD
CqJHE       MCELKKMLVLFILLCHVFAHETQRLGTEENTLQNPRVCIDDGCLRGKTM--EGYQAGPFN
GNSAG1      ---MKNINLQFILLTVLNAY------IAADAHSSLVVDSSYGSLRGKWM--TSRGGRQFA
GQSAG1      ------------------------------MLDEIEISTKQGRLRGLVKKSIGRSNKLYY
                                                .   :  . * : *              :

GQSAG2      AYIGIPFGQIPG---RFQVALPRAPWTDP--RYTQKDGPACPQS-------SMAYDEDCL
CqJHE       AFVGIPYAKPPTEELRFSNPVRNEPWKRGIVYNATEDKPMCVQKNDLLPNAKVSGDEDCL
GNSAG1      TFLGIPYALPPTGHLRFLPPSPPLKWNGT--RDATVEGKACVQR-------EVRGDEDCL
GQSAG1      SFQGIPYAKPPVGNLRFKEPEPYGHWEDT--RDATKEGGDSLQEHIIF--LNIHGGEDCL
            :: ***:.  *    **        *       :  :   . *        .:   ****

GQSAG2      YLNVFTPMNASATHGILPVMVFIHGSGFLSSSSNSHWIGPDFLIPE-HVILVAMNYRLGA
CqJHE       YLNVYQPMVHSSSKTPLPVMVYIHGGGFFAGGASPSIIGPEYFMDTRRVILVTFQYRLGV
GNSAG1      YLNVFTHSTNNS-GQTSPVMVYIHGGGFYGGSSSLGMYGPEYLLDR-NIVLVTLQYRLGV
GQSAG1      YLNVYTTKTGEQ-GDRKAVMVWIHGGGFFRGSGSTEIYGPDFLIEE-DIVLVTINYRLGV
            ****:     .        ***:***.**  ....    **::::    ::**:::****.

GQSAG2      PGFLTLGSKIAPGNLGLHDTRLALEWVRDEISVFGGDPTQVTLFGQSAGSAMTQFHYISS
CqJHE       FGFLSTGDEVAPGNFGLKDQVMALRWVKHNIAYFGGNPDLVTIFGQSAGGASVHMHMISP
GNSAG1      FGFLSTEDSIIPGNMGLKDQTIALQWVQENIKVFGGDASKVTIFGNSAGSASVHLHMLSP
GQSAG1      LGFLSLENEKLPGNLGLKDQVLALKWVRDNIDSFGGDPNNVTIFGQSAGGASVHYHLLSP
             ***:  ..  ***:**:*  :**.**:..:*  ***:   **:**:***.* .: * :*

GQSAG2      LSSDLFQRAIGHSGSALAGWSSYSLSEGVHRARLLAESLKCNM--TQNDTMLLDCMQKAD
CqJHE       MSDGLFSRAIVMSGNAIAPWNIPTEDPL-SLAQRQAEAVGITQVDTLSSKQLVDALRNVD
GNSAG1      GSRRLFSKAISQSGTALSAFAMIGRGTSRNITFQLARSLNC---STDSSYQILNCLQNKT
GQSAG1      LSKGLFHKAILQSGTPMCQWAFQDK--PREKTFLLAKELGC---NSQDPDTVLEFLMNVP
             *  ** :**  **. :. :           :   *. :      : .   ::: : :

GQSAG2      IKDVISNQYVQL--SYDDYTVGSSFPFLPVLDTFETSDTPFFNDTSIDNMMQQALLRAKP
CqJHE       ANVLSGSI-----DELKFWSIDPLTLYRPVVEPLCSSNESFLIEDPR-ISWRKGSYQKIP
GNSAG1      STDVQKKY----SSLQDTKYEIKKVLFRPIVEE--ESENAFLTSNPL-HIHT-----DKP
GQSAG1      CLDILKAQERQTIRTEKEKIQKSTLLFLPCVEV--SGDAPFLPDYPR-KMMEKGEFSKVP
               :            .         : * ::    .:  *: .                *

GQSAG2      LITGFTTGEGILKFMDKG-----WQMAEGNLGAFIPPKIRDSVSKAERSSLADTIKSRYY
CqJHE       WMTGYLPNDGAVRAIAITSNEKLLNELNANISYILPMLLE-KPSSQELM---KVLKLRYF
GNSAG1      WLVGINQNEGLFKI-SLKHLNETIHLIKTEFDQFGPAVLFFEDTCSKPVEM-AGLIYNFY
GQSAG1      IIMGLTDQEGLSAL-AHGEVK--CEKINENLSVLVPHDLAITPDSEEELRK-GKEILQFY
             : *   ::*             .  : ::  : *  :       :         .::

GQSAG2      PDSVDENKI-----ESAVRIYTDAMFS----YPSLQVTR-------YFANLTYGYLFAYN
CqJHE       NDSTDEKWITTENEQRLVDLYTEAAFL----YPIQSAVKQHVTSADTKLAPVSIYKFSFK
GNSAG1      FKNDSTKNDMIV---SIERVISDSWFL----WPTMQSIG-------NHNGTLYCYLFNHT
GQSAG1      TNTDSLSWDVVP---QYVDYMSDIAFANAEEFSRKCFLK-------HHTAPVYNYLFTYF
             .. . .              ::  *     :                        * * .

GQSAG2      GAWAGPP---------SSFSVYK----MTGVGHGADLYYLLYVNGSSQYVDTCTPNLPNL
CqJHE       GPYSYSF-------------LYFTFHQDFGVVHCDELIYLFRSPA---LFPDFPHKSKEA
GNSAG1      GE--------------HSVTQFNGGPQHFGVSHMDELHYLFSRKR---IIP-NGLNKVDE
GQSAG1      SPRAFSAKMIQMAYPDHEADQFI----GCGASHADELIHLFKTNI--DKIPFTSPTDDDQ
            .                       :       *. *  :* :*:        .     .  :

GQSAG2      QMKDQMVKWWTSFAKNGVPGLPWET--ISEGG------YLIIDGSDPSKMNTTEFE----
CqJHE       RMSHHFVEFFINFAINGVATPLKPY----RGCNNDNEVYQSMDCDVLEFINSSEPGKPFE
GNSAG1      NVSKMLIDLWVNFAEETNPTPDSISSNPQQRGTNNSITWESSNSADPKSL---LIETNML
GQSAG1      ALMNKLIKAWTAFAKTGNPNCVE-----------LNVIWKEDTIQNPCFM---EIGKVWK
             : . ::. :  **                           :          :

GQSAG2      --------SQFYDFWANMKPQSGNSADPLGLRFFFIKVALLSLFHHIFKV
CqJHE       VRVSNGRNEDLFSFWRKFY-----------------------------
GNSAG1      S-MVENVFKTRMEFWKKLSVRDKIV-------------------------
GQSAG1      S-TDGILFPERIEFWNKIYEKYSYLY------------------------
                          .** ::
```

Figure 3. JHE-like nucleotide and deduced amino acid sequences from nymphal glassy-winged sharpshooters.

Figure 3 legend. Three full-length cDNA sequences (named *gnsag1* (A), *gqsag1* (B), and *gqsag2* (C)) are shown. The open reading frames of *gnsag1*, *gqsag1*, and *gqsag2* encode putative proteins of 550, 547, and 580 amino acid residues. The asterisk indicates a stop codon (TAG or TGA). Seven amino acid sequence motifs (RF, DQ, GQSAG, E, GxxHxxD/E, R/$Kx_{(6)}$R/KxxxR, and T) that are found in biologically active JHEs are highly conserved in the deduced amino acid sequences of *gnsag1*, *gqsag1*, and *gqsag2* (shown in bold-underlined or bold-italic text). A comparison of these conserved motifs with those found in a known JHE (CqJHE) is shown in panel D (putative catalytic site residues are shown within the boxes). Putative signal peptide sequences in the deduced amino acid sequence of *gnsag1* (N-terminal 22 amino acid residues) and *gqsag2* (amino acid residues 12-30, assuming translation begins at the third ATG) are shown in italic text. A putative signal sequence was not predicted in the amino acid sequence of *gqsag1*. Amino acid residue positions are indicated to the right.

The RACE procedures identified three full-length JHE coding sequence (*gnsag1*, *gqsag1*, and *gqsag2*; **Figure 3**) from the ds cDNA library generated from a mixed population of fifth instar glassy-winged sharpshooters. The *gqsag1* sequence was identified and characterized during the current reporting period. *Gnsag1*, *gqsag1*,and *gqsag2* encode open reading frames of 550, 547, and 580 amino acid residues, respectively. Seven amino acid sequence motifs that are found in known biologically active JHEs were highly conserved in the deduced amino acid sequences of *gnsag1*, *gqsag1*, and *gqsag2*, i.e., GNSAG1, GQSAG1, and GQSAG2 (**Figure 3**). A signal peptide sequence that is found in all known biologically active JHEs was predicted in GNSAG1 but not GQSAG1 or GQSAG2. Interestingly, two additional methionine codons were found within the N-terminal 12 amino acid residues of GQSAG2. Should translation initiation start from either of these ATG codons, a signal peptide sequence was predicted. Phylogenetic analysis placed GNSAG1 and GQSAG1 in the same clade (**Figure 4A**). GQSAG2, however, was found in a clade that was separate from that of GNSAG1 and GQSAG1, and that of known JHEs from lepidopteran insects. Surprisingly, GNSAG1, GQSAG1, and GQSAG2 did not align with NlJHE, a JHE from the hemipteran *Nilaparvata lugens*.

In order to determine if GNSAG1, GQSAG1, and GQSAG1 are able to hydrolyze JH at a rate that is consistent with known JHEs, the sequences encoding these proteins were subcloned into a baculovirus transfer vector, and the resulting recombinant transfer vectors were used to generate recombinant baculovirus expression vectors for recombinant protein expression. Recombinant GNSAG1 and GQSAG1 were unable to hydrolyze JH III at an appreciable rate (**Figure 4B**). Experiments to characterize the ability of recombinant GQSAG2 to hydrolyze JH III are ongoing.

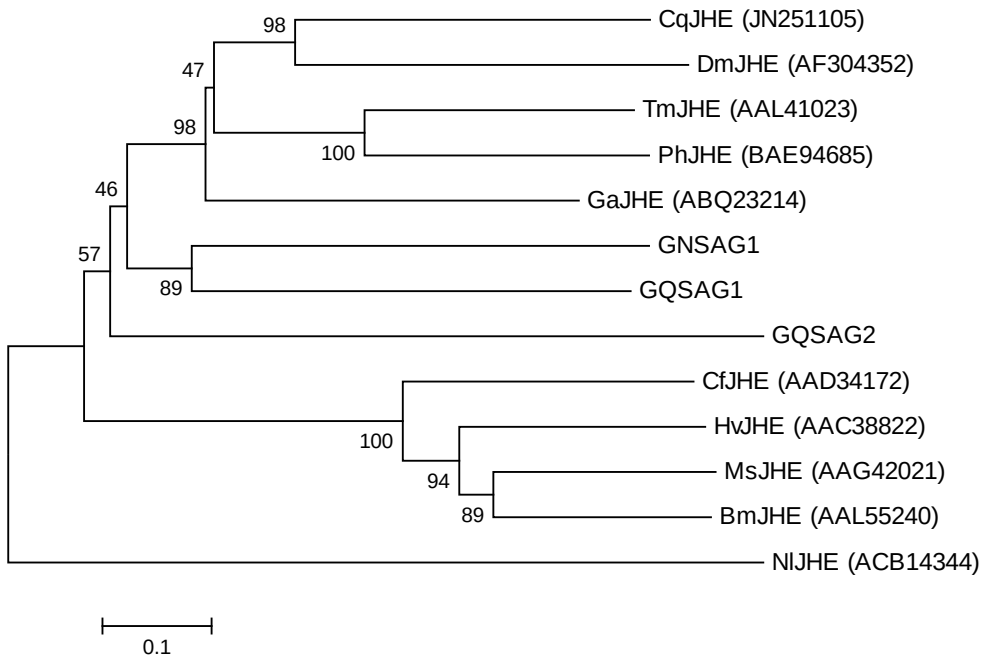
Objective 3. Develop virus-based dsRNA production and delivery systems for controlling the glassy-winged sharpshooter.

Insect viruses are used as highly effective biological insecticides to protect against pest insect of forests and agricultural planting such as soybean. Insect viruses have been genetically modified to further improve their efficacy for crop protection. For example, leaf damage caused by the tobacco budworm in tomato plants can be reduced by up to 45% when they are infected with a genetically modified virus that expresses a *jhe* gene [9]. Two viruses from the glassy-winged sharpshooter, *Homalodisca coagulata* virus-1 (HoCV-1) [10] and *H. vitripennis* reovirus (HoVRV) [11], are well-characterized. HoCV-1 and HoVRV are naturally found in glassy-winged sharpshooter populations in the field but they are not severely pathogenic against the glassy-winged sharpshooter. Recently, an *in vitro* system (e.g., a continuous cell line) that appears to support the replication of HoCV-1 has been identified [12]. The availability of an *in vitro* system (e.g., [12,13]) is a critical tool for the genetic modification of a virus that is highly pathogenic in the glassy-winged sharpshooter. The primary goal of Objective 3 is to identify new, highly pathogenic glassy-winged sharpshooter viruses that are supported by a robust *in vitro* system that can be used in the genetic modification of these viruses. The experiments to accomplish these goals are ongoing.

CONCLUSIONS

Thus far we have determined the baseline levels of JHE and JHEH activities in fifth instar glassy-winged sharpshooter nymphs. We have cloned and sequenced the complete coding sequence of three esterase-encoding cDNAs from fifth instar nymphs. We have expressed recombinant proteins from the major open reading frame of each of these cDNAs. We have shown that two of the cDNAs do not encode a protein with JHE activity. We are in the process of confirming the biological activity of the third cDNA. Experiments to develop a production and delivery system for RNAi effectors that target the *jheh* or *jhe* gene are ongoing.

A.



B.

Specific activity of GNSAG1, GQSAG1, GQSAG2, and CqJHE for JH III

Protein[1]	Specific Activity (nmol JH III acid/min/mg)
GNSAG1	not detected[2]
GQSAG1	not detected[2]
GQSAG2	not yet determined
CqJHE	600 ± 26

[1]GNSAG1 and GQSAG1 were crude enzyme preparations, whereas CqJHE was >90% pure.
[2]No activity was detected under assay conditions that could detect 3 nmol of JH III acid formed per minute per mg of enzyme.

Figure 4. Phylogenetic relatedness GNSAG1, GQSAG1, and GQSAG2 with known JHEs and their hydrolytic activity for JH III. A. Phylogenetic analysis was performed using MEGA version 6. The tree was generated by the Neighbor-Joining method using a ClustalW generated alignment of 10 known JHE sequences (GenBank accession numbers are shown within the parentheses). The percentage of replicate trees in which the sequences clustered together in the bootstrap analysis (1000 replicates) is shown at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The five insect orders from which the sequences are derived are: Coleoptera: TmJHE and PhJHE; Diptera: CqJHE and DmJHE; Hemiptera: GNSAG1, GQSAG1, GQSAG2, and NlJHE; Lepidoptera: CfJHE, HvJHE, MsJHE, and BmJHE; and Orthoptera: GaJHE. B. The specific activity of GNSAG1 and GQSAG1 was determined in 100 mM sodium phosphate buffer, pH 8, containing 1 mg/ml BSA, and 5 µM JH III. The reaction was allowed to proceed at 30ºC for 15 minutes. The ability of CqJHE, a known JHE from the mosquito *Culex quinquefasciatus*, to hydrolyze JH III under the same conditions was used as a positive control.

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CHARACTERIZATION OF THE *XYLELLA FASTIDIOSA* PHOP/Q AND COLR/COLS TWO-COMPONENT REGULATORY SYSTEM

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Reporting Period: The results reported here are from work conducted July 2014 through October 2015.

ABSTRACT
Xylella fastidiosa is a gram-negative, xylem-limited plant pathogenic bacterium that causes disease in a variety of economically important agricultural crops, including Pierce's disease of grapevine. *X. fastidiosa* biofilms formed in the xylem vessels of plants play a key role in early colonization and pathogenicity by providing a protected niche and enhanced cell survival. Biofilm formation is induced by the process of quorum sensing and may be mediated by two-component regulatory systems. Like many other bacteria, *X. fastidiosa* possesses homologs to the two component regulatory system PhoP/Q. PhoP/Q differentially regulates genes in response to divalent periplasmic cation concentration and other environmental stimuli. Grapevine pathogenicity assays showed *phoP/Q* mutants that are non-pathogenic and significantly hindered in colonization or movement within the xylem vessels. We identified another two-component system, ColR/ColS, in the *X. fastidiosa* genome that has been reported as important regulator of virulence and biofilm formation in other systems but not yet studied in *X. fastidiosa.* The purpose of this research is to further our understanding of the PhoP/Q and ColR/S regulons in order to understand essential processes responsible for survival of *X. fastidiosa* in *Vitis vinifera* grapevines.

LAYPERSON SUMMARY
Xylella fastidiosa, the causal agent of Pierce's disease of grapevine, possesses many highly conserved bacterial regulatory systems, including the PhoP/Q system. This system has been shown in other bacteria to play an important role in survival and pathogenicity. In the case of *X. fastidiosa*, we have previously shown that the PhoP/Q system is required for *X. fastidiosa* to survive in the plant, rendering *X. fastidiosa* unable to move or cause disease if PhoP or PhoQ are knocked out. We propose to further characterize this system and a second regulatory system, ColR/ColS, using next generation molecular tools, such as RNAseq. This will allow us to identify *X. fastidiosa* genes that are regulated by PhoP/Q and ColR/S to give us further insight into the processes essential for the pathogen to survive in grapevines and identify potentially novel disease control targets.

INTRODUCTION
Xylella fastidiosa is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease of grapevine (Wells et al., 1981). *X. fastidiosa* forms aggregates in xylem vessels, which leads to the blockage of xylem sap movement. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). Biofilm formation induced by QS is essential for survival and pathogenicity and may be regulated through a two-component regulatory system (TCS). TCS's are signal transduction systems through which bacteria are able to respond to environmental stimuli (Hoch, 2000). The TCS is comprised of a histidine kinase, responsible for sensing stimuli, and the response regulator, responsible for mediating gene expression (Charles et al, 1992).

The PhoP/Q TCS is a well-studied and highly conserved TCS responsible for regulation of genes involved in virulence, adaptation to environments with limiting Mg^{2+} and Ca^{2+}, and regulation of other genes. PhoQ is a transmembrane histidine kinase protein with a long C-terminal tail residing in the cytoplasm. The periplasmic domain of PhoQ is involved in sensing of Mg^{2+}, Ca^{2+}, and antimicrobial peptides. The cytoplasmic domain contains a histidine residue that is phosphorylated when physiological signals are detected in the periplasm. The PhoP/Q TCS is a phosphotransfer signal transduction system and upon activation by environmental stimuli, PhoQ phosphorylates the corresponding response regulator PhoP. In most bacteria, environments high in Mg^{2+} inhibit the PhoP/Q system through dephosphorylation of PhoP (Groisman, 2001). *X. fastidiosa* contains homologs of the PhoP/Q system (Simpson et al. 2000). We have previously shown that the PhoP/Q system is essential for

X. fastidiosa survival *in planta* and plays a role in regulation of biofilm formation and cell-cell aggregation. We identified a second two component regulatory system, ColR/ColS, that is implicated in virulence, biofilm formation and environmental stress response in the plant pathogenic bacterium *Xanthomonas citri* subsp. *citri* (Yan and Wang, 2011). The current aim of our research is to understand the PhoP/Q and ColR/S regulatory networks in *X. fastidiosa*. Furthermore, we aimed to characterize the biological function of ColR and ColS. We are also investigating factors involved in induction or repression of the PhoP/Q system such as pH, cation concentration and peptides.

OBJECTIVES

1. Characterization of factors involved in induction and/or repression of the PhoP/Q system.
2. Identification and characterization of genes regulated by PhoP/PhoQ.
3. Determine if peptides in a library provided by Professor Carlos Gonzalez are able to bind to *X. fastidiosa* PhoQ and inhibit activation of PhoP.
4. Characterization of the ColR/ColS two-component regulatory system.

RESULTS AND DISCUSSION

Objective 1. Characterization of factors involved in induction and/or repression of the PhoP/Q system.

We are currently working to further our understanding of factors that influence the PhoP/Q system in *X. fastidiosa*. So far we have found that *XfΔphoP* and *XfΔphoQ* mutants show inhibited growth in Pim6 media containing 50 µM Mg^{2+} when compared to wild-type *X. fastidiosa*. We also see a reduction in growth among the mutants compared to wild-type at a lowered pH of 5.0 (instead of pH 7.0) when the media contains 500 µM Mg^{2+}.

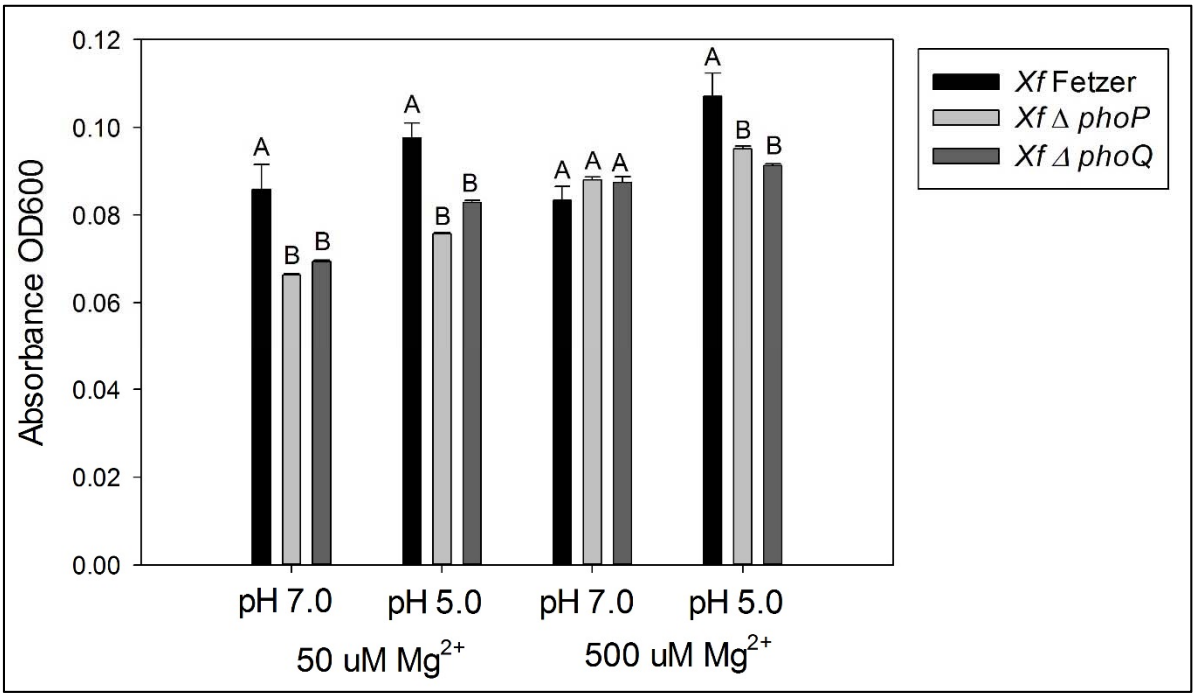

Figure 1. Absorbance OD600 of *Xf* Fetzer, *XfΔphoP,* and *XfΔphoQ* in Pim6 media containing either 50 µM or 500 µM Mg^{2+} at a pH of 5.0 or 7.0. Absorbance was measured after five days growth at 28° C.

Objective 2. Identification and characterization of genes regulated by PhoP/PhoQ.

We conducted RNAseq analysis on *Xf* Fetzer, *XfΔphoP,* and *XfΔphoQ* after incubation for one hour in PD3 media or xylem sap. Xylem sap was isolated from Thompson Seedless grapevines using a pressure bomb. For *XfΔphoP* incubated in PD3 media, we identified 111 differentially expressed genes; 51 down regulated and 61 up regulated. **Figure 2** illustrates the distribution of differentially expressed genes based on the functional category of the gene.

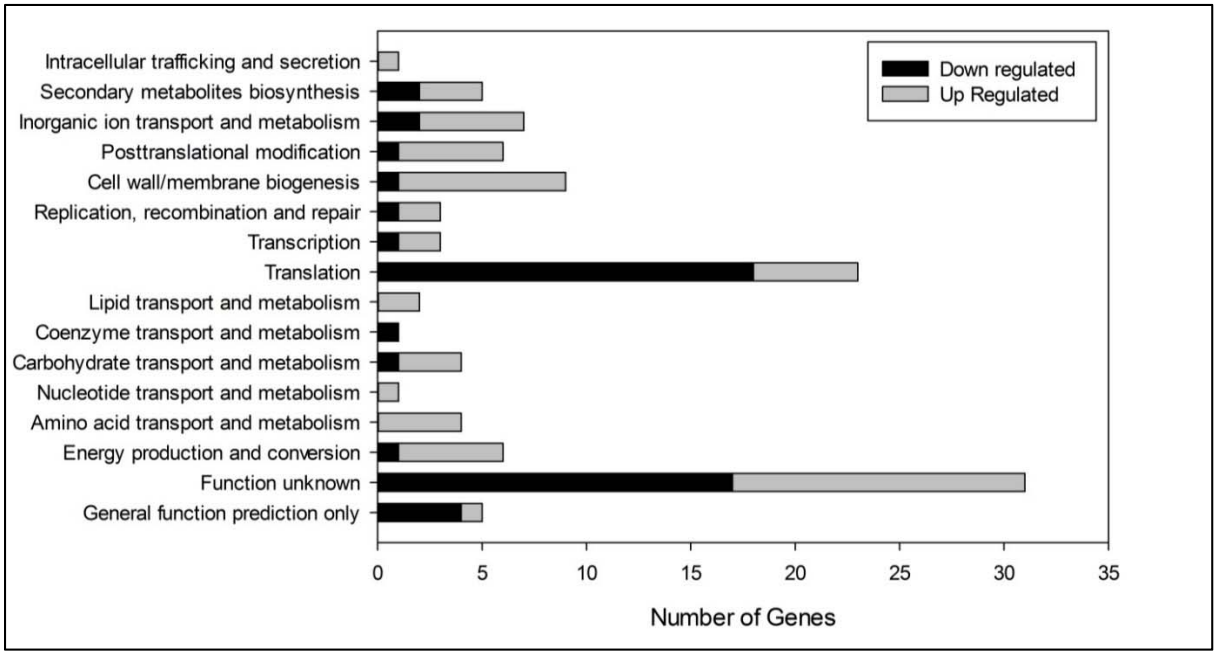

Figure 2. Genes differentially expressed in *Xf* Δ*phoP* compared to wild-type *X. fastidiosa* after one hour incubation in liquid PD3 media. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of q<0.01, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

After incubation in xylem sap, *XfΔphoP* exhibited a total of 32 differentially expressed genes; 19 of which were down regulated and 13 were up regulated.

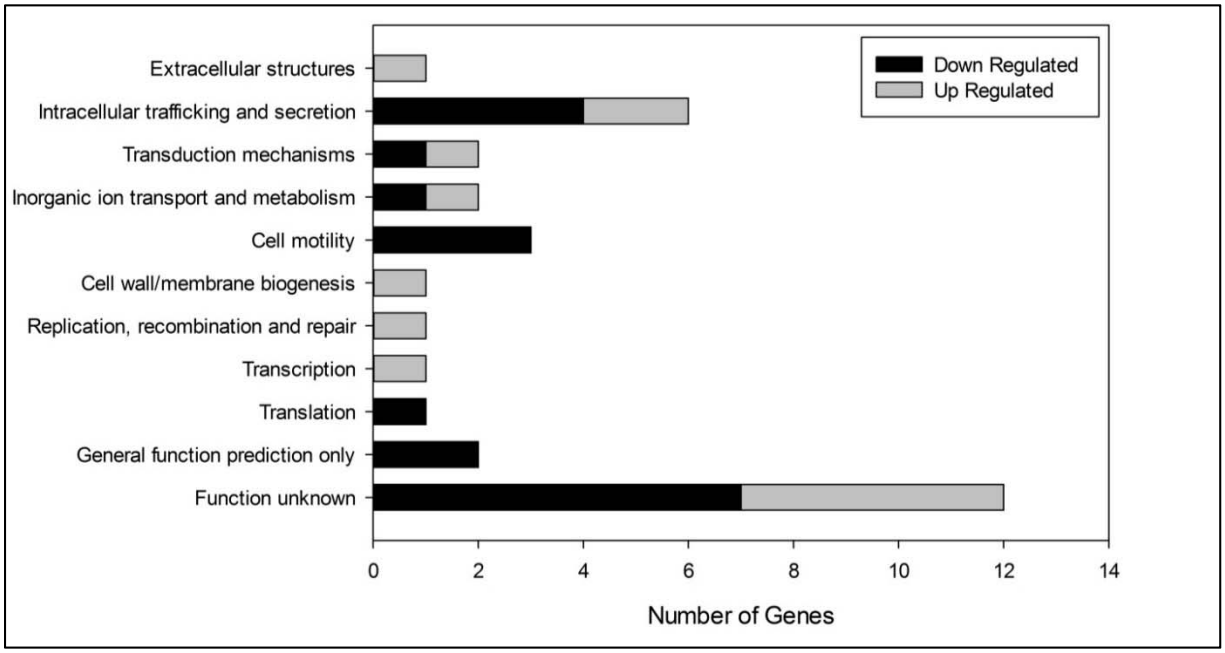

Figure 3. Genes differentially expressed in *Xf*Δ*phoP* compared to wild-type *X. fastidiosa* after one hour incubation in xylem sap. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of q<0.01, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

RNAseq analysis on *XfΔphoQ* compared to wild-type *Xf* Fetzer grown in PD3 media found a total of 143 differentially expressed genes; 52 down regulated and 91 up regulated genes.

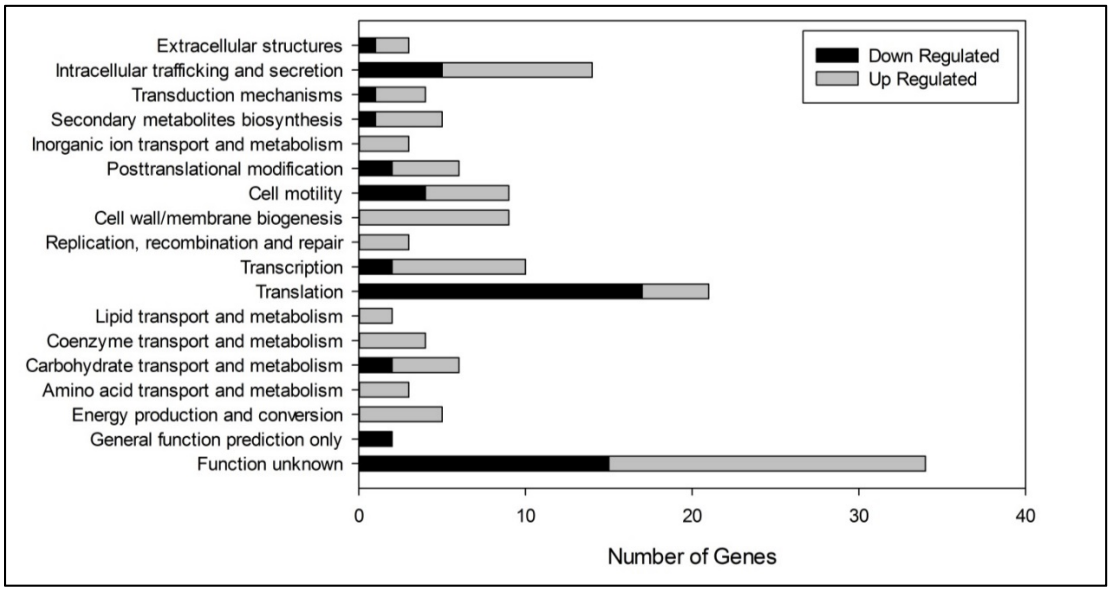

Figure 4. Genes differentially expressed in *Xf* Δ*phoQ* compared to wild-type *X. fastidiosa* after one hour incubation in liquid PD3 media. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of q<0.01, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

When grown in xylem sap, *XfΔphoQ* had a total of 108 differentially expressed genes; 58 down regulated and 50 up regulated.

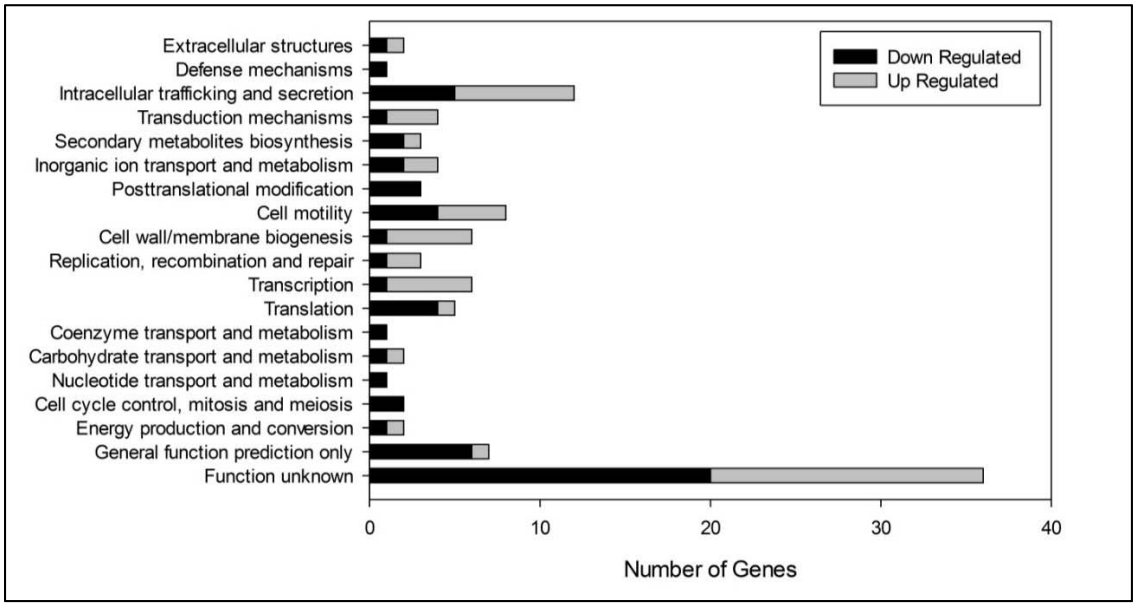

Figure 5. Genes differentially expressed in *Xf* Δ*phoQ* compared to wild-type *X. fastidiosa* after one hour incubation in xylem sap. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of q<0.01, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

We found PhoP/PhoQ is involved in regulation of iron uptake, acriflavine resistance proteins, and colicin V, but is not involved in regulation of magnesium or calcium transport genes during early adaptation to xylem sap. We also found the PhoP/PhoQ two-component regulatory system is involved in regulation of cell motility genes including Type IV pili and fimbrae genes. We also saw significant upregulation of eight gum genes in *XfΔphoQ* incubated in xylem sap while in PD3 we observed upregulation of two *hsf* genes. Lastly, the RNAseq results suggest that PhoP/PhoQ in *X. fastidiosa* is similar to other systems in that it is involved in regulation of other signal transduction regulatory systems.

Objective 3. Determine if peptides in a library provided by Professor Carlos Gonzalez are able to bind to *X. fastidiosa* PhoQ and inhibit activation of PhoP.

We have begun work on objective three, investigating whether two potential peptides, kindly provided by Professor Carlos Gonzalez, have an inhibitory effect on *X. fastidiosa*. The two peptides tested are 66-10D: FRLKFH and 77-12D: FRLKFHI (Reed et al., 1997) We found the peptides have an inhibitory effect on *Xf* Fetzer when grown for five days in Pim6 media (Michele Igo, personal communication) modified to contain 10 uM Mg^{2+}. *X. fastidiosa* was grown in the presence of the peptides at varying concentrations, with an inhibitory effect observed at peptide concentrations as low as 10 ug/ml (**Figure 2**).

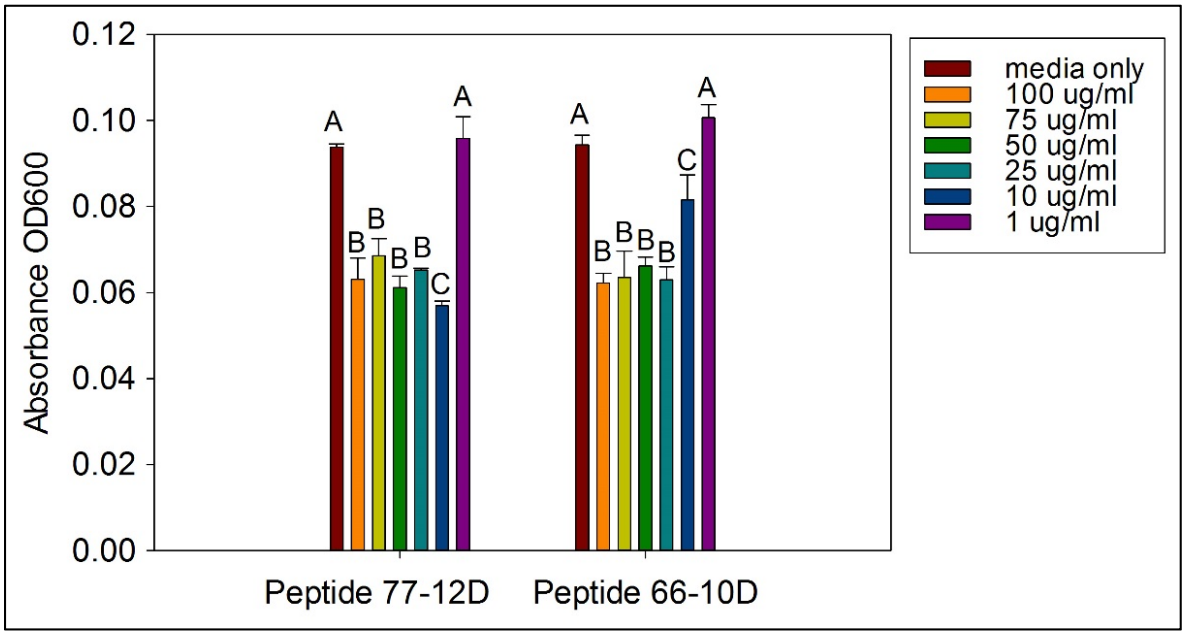

Figure 6. Absorbance OD600 of *Xf* Fetzer cells grown for five days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM Mg^{2+} and varying concentrations of 77-12D and 66-10D peptides. Different letters indicate significance ($P < 0.05$) as determined by the Tukey test.

We have observed a further reduction in growth (greater inhibitory effect) of these peptides on our *XfΔphoP* and *XfΔphoQ* mutants when grown under the same conditions as above (**Figure 3**).

We also tested the effect of these peptides on *Xf* Fetzer when *X. fastidiosa* was incubated in the presence of the peptide for one hour in Pim6 media containing 10 µM Mg^{2+}. After the incubation period, 20 µl aliquots were plated onto solid PD3 media and growth was evaluated after seven days incubation at 28° C. We found that only peptide 77-12D was able to inhibit *X. fastidiosa* growth after the one-hour incubation period (**Table 1**).

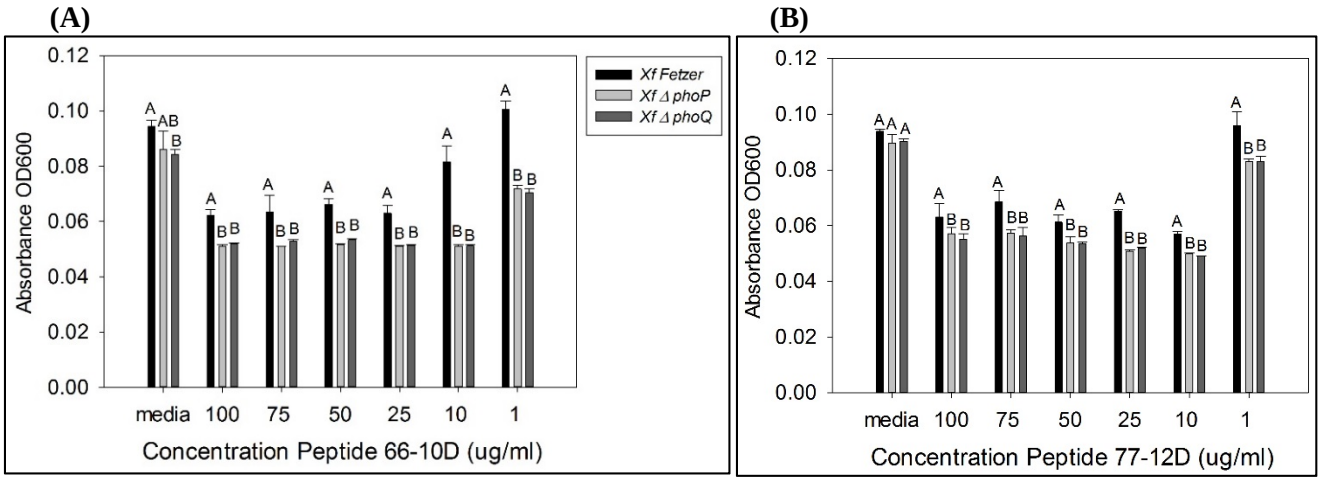

Figure 7. Absorbance OD600 of *Xf* Fetzer, *XfΔphoP,* and *XfΔphoQ* cells grown for five days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM Mg^{2+} and varying concentrations of (A) Peptide 66-10D or (B) Peptide 77-12D. Different letters indicate significance (P < 0.05) as determined by the Tukey test.

Table 1. Evaluation of *Xf* Fetzer growth after one hour incubation with peptide 77-12D or 66-10D in Pim6 media containing 10 µM Mg^{2+}.

Peptide concentration	77-12D	66-10D
100 µg/ml	No growth	Growth
75 µg/ml	No growth	Growth
50 µg/ml	No growth	Growth
25 µg/ml	No growth	Growth
10 µg/ml	No growth	Growth
1 µg/ml	Growth	Growth

Objective 4. Characterization of the ColR/ColS two-component regulatory system.
We made mutants in *XfΔcolS* and *XfΔcolR*, but were unable to obtain a viable *XfΔcolR* mutant. We conducted *in vitro* biofilm formation and cell-cell aggregation and found a significant reduction of biofilm formation and cell aggregation among *XfΔcolS* mutants (**Figure 8**). We also conducted pathogenicity assays on *XfΔcolS* mutants and found the mutant was unable to induce wild-type levels of disease symptoms and was only isolated from two (out of thirty) grapevines (**Figure 9**)

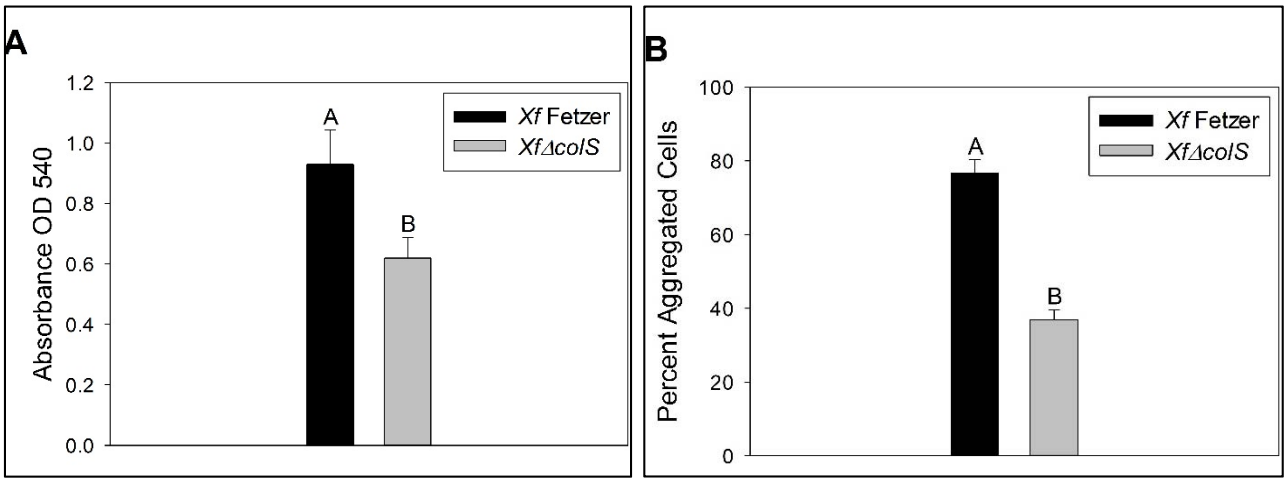

Figure 8. Evaluation of biofilm formation and cell-cell aggregation in *XfΔcolS.* Comparison of biofilm formation (A) and cell-cell aggregation (B) by wild-type *Xf* Fetzer and *XfΔcolS* after 10 days growth in static liquid culture. Values shown are mean of 10 samples +/- standard error. The assay was repeated twice and data shown are representative of both assays. Different letters indicate significance (P < 0.05) determined by the Tukey test.

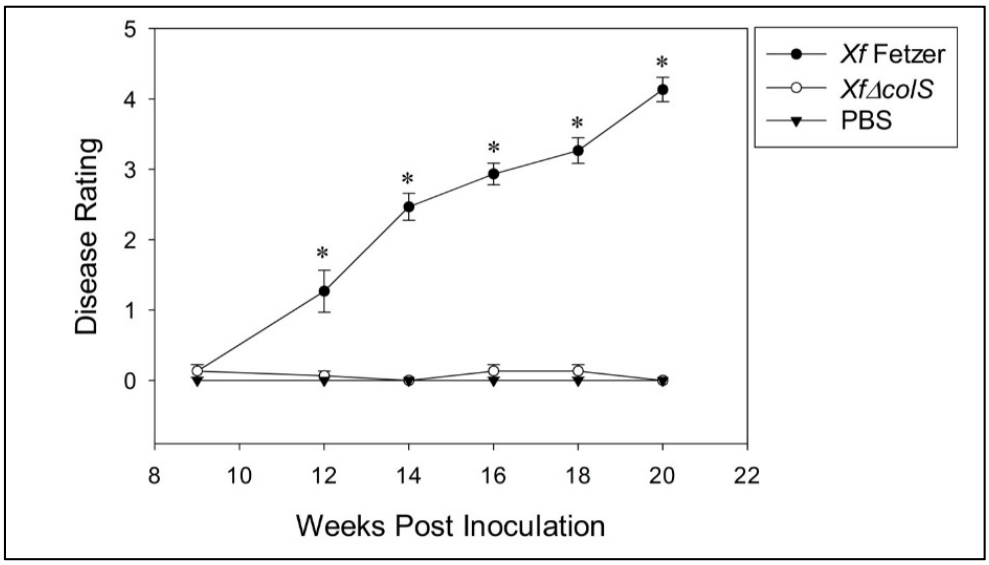

Figure 9. Evaluation of pathogenicity of *Xf* Δ*colS* on *Vitis vinifera* grapevines. Disease ratings of Thompson Seedless grapevines inoculated with wild-type *Xf* Fetzer, *Xf*Δ*colS,* and PBS (negative control) 20 weeks post-inoculation. Values shown are mean +/- standard error. The assay was repeated twice, data shown are representative of both assays. Asterisk indicates significance (P<0.001) determined by the Tukey test.

We conducted RNAseq analysis on *XfΔcolS* and wild-type *Xf* Fetzer after incubation for one hour in either xylem sap or PD3 media. After incubation in PD3 media, we identified 192 genes significantly up regulated and 23 genes significantly down regulated in *XfΔcolS* compared to wild-type *X. fastidiosa* for a total of 215 differentially expressed genes (**Figure 10**). The differentially expressed genes included those involved in cell-cell aggregation, such as six of the nine *gum* genes, and a predicted hemagglutinin-like protein. We also observed differential expression of a number of genes involved in cell motility such as fimbrial assembly proteins.

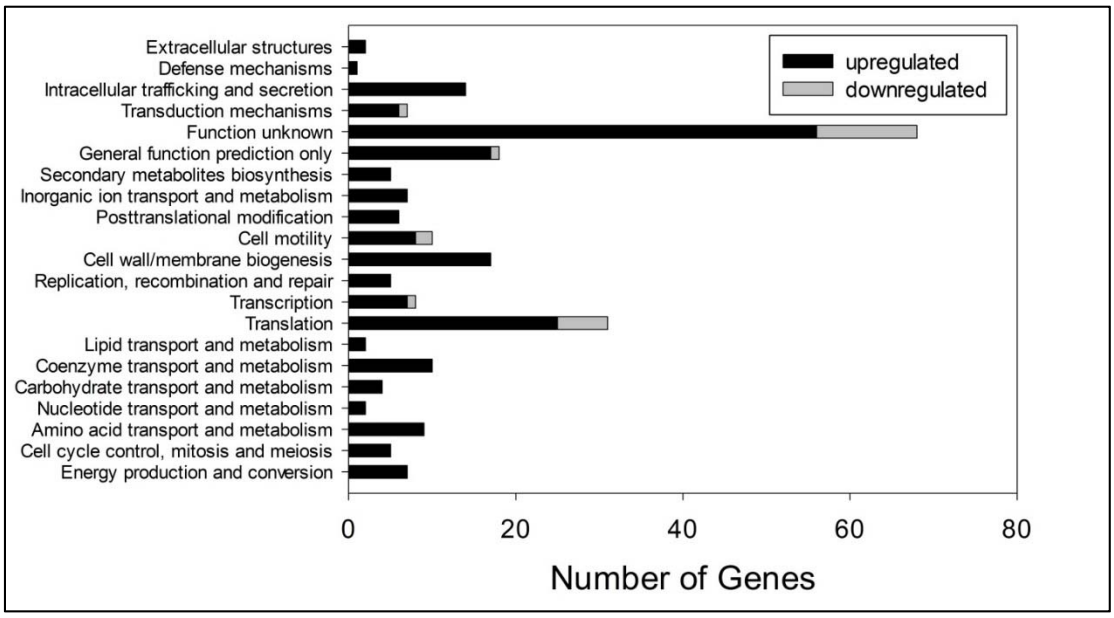

Figure 10. Functional categorization of differentially expressed genes in *Xf*Δ*colS* compared to wild-type *X. fastidiosa* after one hour growth in PD3 media. Genes differentially expressed in *Xf*Δ*colS* compared to wild-type *X. fastidiosa* after one hour incubation in liquid PD3 media. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of q<0.01, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

After incubation in xylem sap, twenty genes were significantly up regulated and 47 genes down regulated in *XfΔcolS* compared to wild-type *X. fastidiosa* for a total of 67 differentially expressed genes (**Figure 11**). We did not see differential expression of *gum* genes in xylem sap, but we did see differential expression of some cell motility genes. In both xylem sap and PD3 media, we saw differential expression of ion transport genes such as *mntH, cutC,* and iron transport genes *bfeA* and PD1711. Last, we observed differential expression of other two-component regulatory systems including the *phop* response regulator and *gacA*. Under xylem sap conditions, both *rpfG* and *colR* are up regulated. This result indicates that *cols* is acting as a negative inhibitor of *colr* under xylem sap conditions.

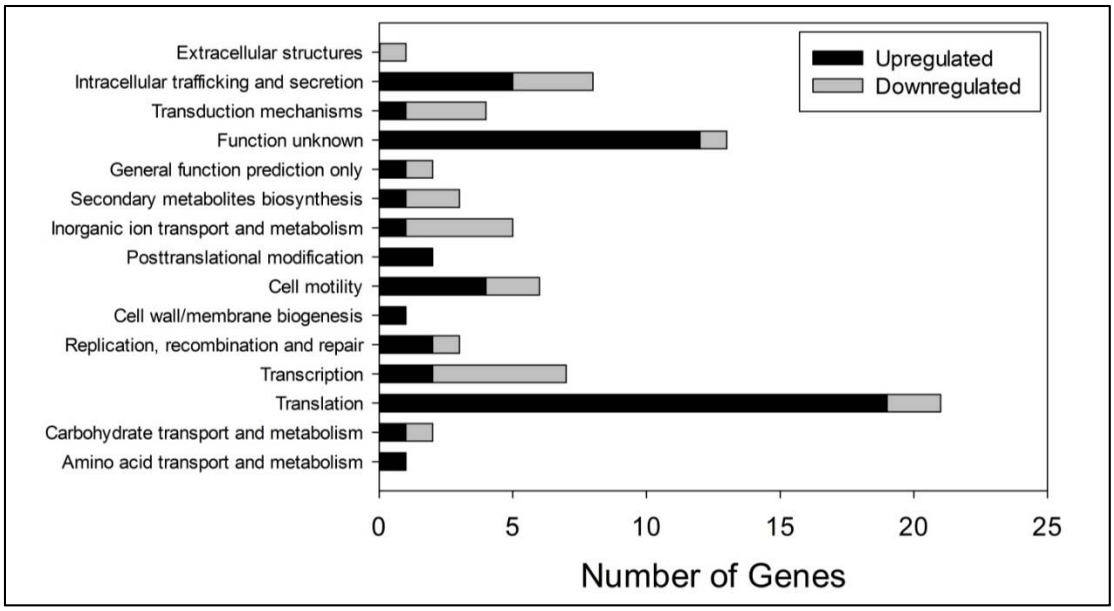

Figure 11. Functional categorization of differentially expressed genes in *XfΔcolS* compared to wild-type *X. fastidiosa* after one hour growth in xylem sap. Genes differentially expressed in *XfΔcolS* compared to wild-type *X. fastidiosa* after one hour incubation in xylem sap. Differential gene expression as determined by RNAseq analysis on an Illumina HiSeq 3000 with single-end 50bp reads and analyzed using Rockhopper version 2.03 and categorized by functional classification. Significance determined by a minimum two-fold change in gene expression and q-value of $q<0.01$, as determined by Rockhopper. The q-value reported by Rockhopper is an adjusted p-value using the Benjamini-Hochberg procedure to control the false discovery rate. A q-value of less than 0.01 is considered significant.

CONCLUSIONS

We have characterized the PhoP/PhoQ and ColR/ColS two-component regulatory systems during the initial steps of adaptation to xylem sap and compared this to a high-nutrient environment (PD3). The information we have gathered is interesting and furthers our understanding of these regulatory networks but further work is required to understand more of these processes. The two peptides, 77-12D and 66-10D, provided interesting results that we hope to explore further through qRT-PCR. The fact that peptide 77-12D can induce an inhibitory effect even after a one-hour incubation with *X. fastidiosa* indicates it is likely binding to essential *X. fastidiosa* gene products. *XfΔcolS* is a conserved two-component sensor kinase with limited studies conducted on it, thus this research provides a large insight into the role this system may be playing for *X. fastidiosa*.

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FUNDING AGENCIES

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ACKNOWLEDGEMENTS

We would like to thank Professor Carlos Gonzalez for donating the peptides used in objective three.

EVALUATION OF PIERCE'S DISEASE RESISTANCE IN TRANSGENIC *VITIS VINIFERA* GRAPEVINES EXPRESSING *XYLELLA FASTIDIOSA* HEMAGGLUTININ PROTEIN

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Reporting Period: The results reported here are from work conducted October 1, 2014 to October 1, 2015.

ABSTRACT

Previous research in our lab identified two hypervirulent mutants of *Xylella fastidiosa*. These mutations were in large hemagglutinin (HA) adhesion genes that we named *Hfx*A and *Hfx*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. We hypothesize that if Hxf protein, or a portion of the Hxf protein that mediates adhesion, could be expressed in the xylem fluid of transgenic grapevines then perhaps insect-inoculated *X. fastidiosa* cells would clump together and be less capable of colonizing grapevines. During the past five years we produced transgenic HA-expressing tobacco and grapevine lines; these transgenic lines, grown in the greenhouse, exhibited less severe symptoms of Pierce's disease following mechanical inoculation of *X. fastidiosa* cells. With the assistance of the Public Intellectual Property for Agriculture (PIPRA) we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines. We cut back the shoots to two buds and then mechanically inoculated four shoots/vine with a mixture of Temecula and Stag's Leap *X. fastidiosa* strains in April 2014, the same time frame that other Pierce's disease workers inoculated their transgenic vines a couple of years ago. Pierce's disease symptoms were rated in September 2014 on the inoculated shoots and we noted whether adjacent uninoculated shoots developed Pierce's disease symptoms. Over 95% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014, indicating that our inoculations were successful -- at least two Pierce's disease symptomatic canes were present on all inoculated vines. In only one instance did we find Pierce's disease symptoms on an adjacent, uninoculated shoot.

In January 2015 the shoots were trimmed to two buds and the emerging shoots and the entire vine were rated for Pierce's disease symptoms in August 2015. In three out of the five lines expressing the HA adhesion domain the majority of the vines showed no Pierce's disease symptoms; however, Pierce's disease symptoms were evident on the canes only in the other adhesion domain vines. In the two other adhesion domain lines, the majority of the vines were dead or had severe Pierce's disease symptoms on the canes. In the three full-length HA gene construct lines the majority of all the vines were healthy with no Pierce's disease symptoms. However, one line had one dead vine and Pierce's disease symptoms were observed on two vines from the two other lines. These are initially encouraging results that reflect the results of greenhouse testing and the occurrence of Pierce's disease symptoms on the canes of field vines following inoculation in 2014.

If the USDA permit can be extended we would like to re-inoculate the vines that were disease free in 2015. A final round of inoculations should determine whether these vines are indeed functionally resistant or tolerant to Pierce's disease.

LAYPERSON SUMMARY

Our 10+ year research effort on the role hemagglutinins (HAs), large proteins that mediate the attachment of bacteria to themselves and to various substrates, play in Pierce's disease pathogenicity and insect transmission has been very fruitful. Our early work showed that HA mutants were hypervirulent, i.e., they caused more severe symptoms and killed vines faster than vines inoculated with wild-type *Xylella fastidiosa* cells (Guilhabert and Kirkpatrick, 2005). HA mutants no longer clumped together in liquid cultures like wild-type cells, nor did HA mutants attach to inert substrates like glass or polystyrene when grown in liquid culture. ALL of these properties show that HAs are very important cell adhesion molecules. Research conducted in the Almeida lab also showed that HA mutants were transmitted at lower efficiencies than wild-type cells and they were compromised in binding to chitin and sharpshooter tissues compared to wild-type cells (Killany and Almeida, 2009). Thus they have a very important role in insect transmission. Dr. Steve Lindow's lab (UC Berkeley) showed that diffusible signal factor (DSF) mutants, which are also hypervirulent, produced much less HAs than wild-type cells, thus

providing another line of evidence regarding the importance of these proteins in *X. fastidiosa* pathogenesis and insect transmission.

We are now evaluating our hypothesis that HAs expressed in transgenic grapevine xylem sap may act as a "molecular glue" that would aggregate and thus slow the movement of wild-type *X. fastidiosa* cells introduced into grapevines by an infectious insect vector. If this happens, then it is possible that HA-aggregated *X. fastidiosa* cells would remain close to the site of inoculation, and if that site is in the terminal portion of a cane, which is where *X. fastidiosa* is introduced by our native blue-green, green, and red-headed sharpshooters, then that cane would likely be pruned off in the winter and the infection removed from the vine. Our most optimistic hope is that HAs could be expressed in transgenic rootstocks and the HAs would be translocated into a non-genetically modified fruiting scion and afford similar levels of functional Pierce's disease resistance. We finished a greenhouse Pierce's disease severity screening of the eight HA transgenic lines that were produced. The results were encouraging in that all of the HA-transgenic lines had lower disease ratings than non-transgenic controls.

With the assistance of the Public Intellectual Property for Research (PIPRA) we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as conventional bilateral cordon vines. We cut back the shoots to two buds and then inoculated four shoots/vine with the Fetzer strain of *X. fastidiosa* in April 2014, the same timeframe that other Pierce's disease workers inoculated their transgenic vines a couple years ago. Pierce's disease symptoms were rated in September 2014 on the inoculated shoots and we noted whether adjacent uninoculated shoots developed Pierce's disease symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014, indicating that our inoculations were successful. In only one instance did we find Pierce's disease symptoms on an adjacent, uninoculated shoot. Pierce's disease symptom severity was lower in the inoculated HA-transgenic grapevines than *X. fastidiosa* inoculated non-transgenic controls.

In January 2015 the shoots were trimmed to two buds and in August 2015 the emerging shoots were rated for Pierce's disease symptoms. In three out of the five lines expressing the HA adhesion domain the majority of the vines showed no Pierce's disease symptoms. However, Pierce's disease symptoms were evident on the canes only in the other adhesion domain vines. In the two other adhesion domain lines the majority of the vines were dead or had severe Pierce's disease symptoms on the canes. In the three full length HA gene construct lines the majority of the vines were healthy, with no Pierce's disease symptoms. However one line had one dead vine and Pierce's disease symptoms were observed on two vines from the two other lines. These are initially encouraging results that reflect the results of greenhouse testing and the occurrence of Pierce's disease symptoms on the canes of field vines following inoculation in 2014.

In winter 2015 the canes on healthy-appearing vines will be cut back again to two spurs and canes that emerge from the spurs will be inoculated in May 2016 with *X. fastidiosa*, as was previously done in May 2014. All canes will be cut back in winter 2016-17 and the vines will undergo a final Pierce's disease rating in August 2017. Canes from healthy-appearing vines will be rooted and planted in the field in April 2016 as back-ups that might need additional evaluation in the future.

INTRODUCTION

Xylella fastidiosa cell-cell attachment is an important virulence determinant in Pierce's disease. Our previous research has shown that if two secreted hemagglutinin (HA) genes which we have named HxfA and HxfB are mutated, *X. fastidiosa* cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both of these mutants are hypervirulent when mechanically inoculated into grapevines, i.e., they colonize faster, cause more severe disease symptoms, and kill vines faster than wild-type *X. fastidiosa*. If either HxfA OR HxfB is individually knocked out there is no cell-cell attachment, which suggests that BOTH HA genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in *X. fastidiosa* plant interactions. Research by other Pierce's disease researchers have shown that *Hxf*s were regulated by an *X. fastidiosa*-produced compound known as diffusible signal factor (DSF; Newman et al., 2004) and that they were important factors in insect transmission (Killiny and Almeida, 2009). The *X. fastidiosa* HA essentially acts as a "molecular glue" that is essential for cell-cell attachment and likely plays a role in *X. fastidiosa* attachment to xylem cell walls and contributes to the formation of *X. fastidiosa* biofilms.

Our initial objectives proposed to further characterize these HAs using some of the techniques that were used to identify active HA binding domains in *Bordetella pertussis*, the bacterial pathogen that causes whooping cough in humans. *B. pertussis* HA was shown to be the most important protein that mediates cell attachment of this pathogen to epithelial host cells (Liu, et al., 1997; Keil, et al., 1999). In the first two years of research we identified the specific HA domain (s) that mediate *X. fastidiosa* cell-cell attachment and determined the native size and cellular location of *X. fastidiosa* HAs (Voegel and Kirkpatrick, 2010). In the third year we identified a two-component transport system that mediates the secretion of *X. fastidiosa* HAs. In the final years of the initial project we expended considerable time and effort in constructing transgenic tobacco and grapevines that expressed HA. We conducted pathogenicity evaluations of our nine HA-transgenic lines. Disease severity ratings in greenhouse-grown vines were considerably less in the transgenic lines than the non-transgenic controls. Permits to establish a field planting of the HA vines were obtained with the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) and a field trial was established in April 2013. The vines were inoculated with *X. fastidiosa* in spring 2014 and Pierce's disease symptoms of HA-transgenics were compared to non-transgenic, *X. fastidiosa* inoculated controls in September 2014. Vine were then pruned back to two buds and allowed to go through the winter. Pierce's disease symptoms on the vines were rated in September 2015. In three out of the five lines expressing the HA adhesion domain the majority of the vines showed no Pierce's disease symptoms. However, Pierce's disease symptoms were evident on the canes only in the other adhesion domain vines. In the two other adhesion domain lines the majority of the vines were dead or had severe Pierce's disease symptoms on the canes. In the three full-length HA gene construct lines the majority of all the vines were healthy with no Pierce's disease symptoms. However, one line had one dead vine and Pierce's disease symptoms were observed on two vines from the two other lines. These are initially encouraging results that reflect the results of greenhouse testing and the occurrence of Pierce's disease symptoms on the canes of field vines following inoculation in 2014.

OBJECTIVES

(Note: The objectives for this project were revised per instructions of the 2013 Panel Review Committee.)

1. Complete the characterization of grape transgenic plants over-expressing *X. fastidiosa* hemagglutinin (Hxf) protein.
2. Mechanically inoculate HA-transgenic grapevines growing in the greenhouse with wild-type *X. fastidiosa* and evaluate the effect on Pierce's disease symptom expression and movement in the xylem by culture and quantitative polymerase chain reaction (qPCR).
3. Secure permits to plant HA transgenic lines in the field in Solano County. Plant transgenic vines in the field and train them into a traditional bilateral cordon.
4. Inoculate four canes on each HA-transgenic field vine with wild-type *X. fastidiosa* in spring 2014. Rate Pierce's disease symptoms in September 2014 on inoculated canes. Take samples for qPCR.
5. Cut back all canes to two buds and rate cane growth in Spring 2015 and Pierce's disease symptoms in September 2015 to determine if the expression of *X. fastidiosa* HA in the transgenic vines retarded or prevented movement of the inoculated *X. fastidiosa* into the cordons, which typically results in systemic Pierce's disease.

RESULTS AND DISCUSSION

(Note: Results described below for Objectives 1, 2, and 3 were reported in the *Proceedings of the 2013 Pierce's Disease Research Symposium*. I have included them for the reader's information only. Objective 4 and 5 results represent new data obtained in 2014 and 2015.)

Objective 1. Complete the characterization of grape transgenic plants over-expressing *X. fastidiosa* hemagglutinin (Hxf) protein.

Twenty-one transgenic Thompson Seedless grape plants that potentially over-expressed the Hxf protein in the xylem using a binary plasmid with a polygalacturonase secretory leader sequence were obtained from the UC Davis Plant Transformation Facility in September 2010. These were initially obtained as small green three-inch plants that needed to be grown in growth chambers and later in the greenhouse to produce hardened woody shoots that could be vegetatively propagated. It took approximately four months for each of the propagated shoots to grow up sufficiently to allow them to be further propagated or inoculated with *X. fastidiosa*. By July 2011 we had propagated sufficient numbers of transgenic grapevines that we could begin analyzing them for HA expression. Analysis by **standard and qPCR** for the presence of the hemagglutinin transgene in genomic grapevine DNA from each of the 22 lines showed that five of nine transgenic lines containing *X. fastidiosa* HA adhesion domains

(AD1-3, labeled as SPAD1) and three of 12 transgenic lines of the full-length HA (labeled PGIP220 in **Table 1** below), had the HA gene inserted into the grapevine chromosome.

Table 1. Results of PCR testing of transgenic grapevines for the presence of full-length (PGIP 220) of the AD1-3 fragment of *X. fastidiosa* hemagglutinin genes in grape chromosome.

DNA ID#	Genotype	Standard PCR	qPCR
1	PGIP 220-E	—	—
2	PGIP 220-5	—	—
3	PGIP 220-11	†	†
4	PGIP 220-1	—	†
5	PGIP 220-9	—	—
6	PGIP 220-14	—	—
7	PGIP 220-3	†	†
8	PGIP 220-13	—	—
9	PGIP 220-A	—	—
10	PGIP 220-D	—	—
11	SPAD1-4	NT	NT
12	SPAD1-10	†	†
13	SPAD1-6	—	†
14	SPAD1-7	†	†
15	PGIP 220-42A	†	—
16	SPAD1-I	†	†
17	SPAD1-B	†	†
18	SPAD1-8	†	—
19	SPAD1-12	†	†
20	SPAD1-1A	†	†
21	PGIP 220-15	—	—
22	SPAD1-2	—	—

Notes
Transgenic lines highlighted in tan color are the three full-length transgenic lines while lines highlighted in purple contain the AD1-3 HA fragment.
† = this line tested positive for a *X. fastidiosa* hemagglutinin insert by standard and/or qPCR.
— = this transgenic line tested negatively for a *X. fastidiosa* hemagglutinin insert by PCR.
NT = not tested by PCR for presence of hemagglutinin gene.

The construct used to transform grapevines which was recommended by the UC Davis Plant Transformation Facility contained two copies of the 35S promoter flanking the HA construct. We hypothesize that recombination occurred within the *Agrobacterium* plasmid that allowed the HA insert to be deleted but the kanamycin selection marker was still inserted into the grape genome. This would explain why a number of the kanamycin resistant transgenics did not actually have the truncated or full-length form of *X. fastidiosa* HA inserted into the grape chromosome.

RT-qPCR analysis on mRNA isolated from these lines confirmed the presence of AD1-3 or full-length HA mRNA in the lines that tested positive by standard or qPCR PCR, thus the HAs inserted into the grape genome are being expressed (**Table 2**).

Table 2. RNA RT-qPCR of Thompson Seedless HA transgenic lines; RNA analysis of HA expressing grapevines. Total RNA was isolated from leaves of transgenic grape plants, converted to cDNA by reverse transcriptase, and quantified by qPCR with HA specific primers. SPAD1 lines express short constructs and PGIP220 lines express long constructs. The higher the number the higher the RNA level in the leaves.

LINE ID	Relative Transgenic *Hxf* RNA Level
SPAD1-B	28.9
SPAD1-10	28.1
PGIP 220-01	27.9
PGIP 220-11	26.6
SPAD1-07	25.8
PGIP 220-03	19.8
SPAD1-08	19
SPAD1-12	14.7
Untransformed Thompson Seedless	0

Objective 2. Mechanically inoculate transgenic grapevines growing in the greenhouse with wild-type *X. fastidiosa* cells. Compare disease progression and severity in transgenic grapevines with non-transgenic controls.

We went through five rounds of vegetatively propagating the lignified transgenic grapevine lines. We attempted to propagate green shoots but only 10-15% of the green shoots became established, thus we are now propagating only lignified wood.

We were very interested in determining whether any of these lines possessed Pierce's disease resistance by testing the lines in the greenhouse as soon as we had sufficient plants, rather than waiting for the results of extensive ELISA and Western blot analysis of transgenics to determine if HA protein could be detected in grapevine xylem sap. On December 8 and 9 of 2011 we inoculated 10 reps of each of the nine PCR-positive transgenic lines with 40 ul of a 10^8 suspension of *X. fastidiosa* Fetzer in PBS, typically done as two separate 20 ul inoculations on each vine, an amount of inoculum that would be far greater than what a sharpshooter injects into a vine.

We also inoculated untransformed Thompson Seedless and two transgenic lines that did not contain HA inserts by PCR analysis, shown as Transformed Non-transgenic TS in **Figure 1**, as positive controls. **Figure 1** shows the results of disease severity in transgenic and non-transgenic control 16 weeks post-inoculation with *X. fastidiosa*. The TS control, inoculated at the same time as the transgenic vines, had a mean disease rating of 3.65 while two of the lines, one containing the truncated HA fragment AD1-3 and one line containing the full-length native HA protein, had the lowest disease ratings of 1.5. Most of the other lines had mean disease severity ratings below 2.0 and the average disease ratings for all of the lines representing the two HA constructs had disease ratings below 2.0. Considering the large amount of inoculum that was used, we are pleased with this promising preliminary result. We will soon be quantifying by culture and qPCR the amount of *X. fastidiosa* in each of these lines. While clearly some disease symptoms were evident, the severity was much less than the control and this could very well reflect lower *X. fastidiosa* populations in the transgenic lines. If this does indeed turn out to be true then we might have produced a moderately resistant grapevine that could very well end up being like a Muscadine grapevine, i.e., they can be infected with *X. fastidiosa* but populations are not high enough to compromise fruit quality. The original hypothesis was that transgenic vines producing HA in the xylem sap might facilitate clumping of *X. fastidiosa* cells and slow their ability to colonize a mature vine during a growing season such that the incipient infection might very well be pruned off in the dormant season. It will take a couple of years to plant and train to a cordon system that would be then mechanically inoculated, or hopefully with the assistance of the Almeida lab insect inoculated with *X. fastidiosa*. These initial greenhouse results with young vines certainly warrant further evaluations.

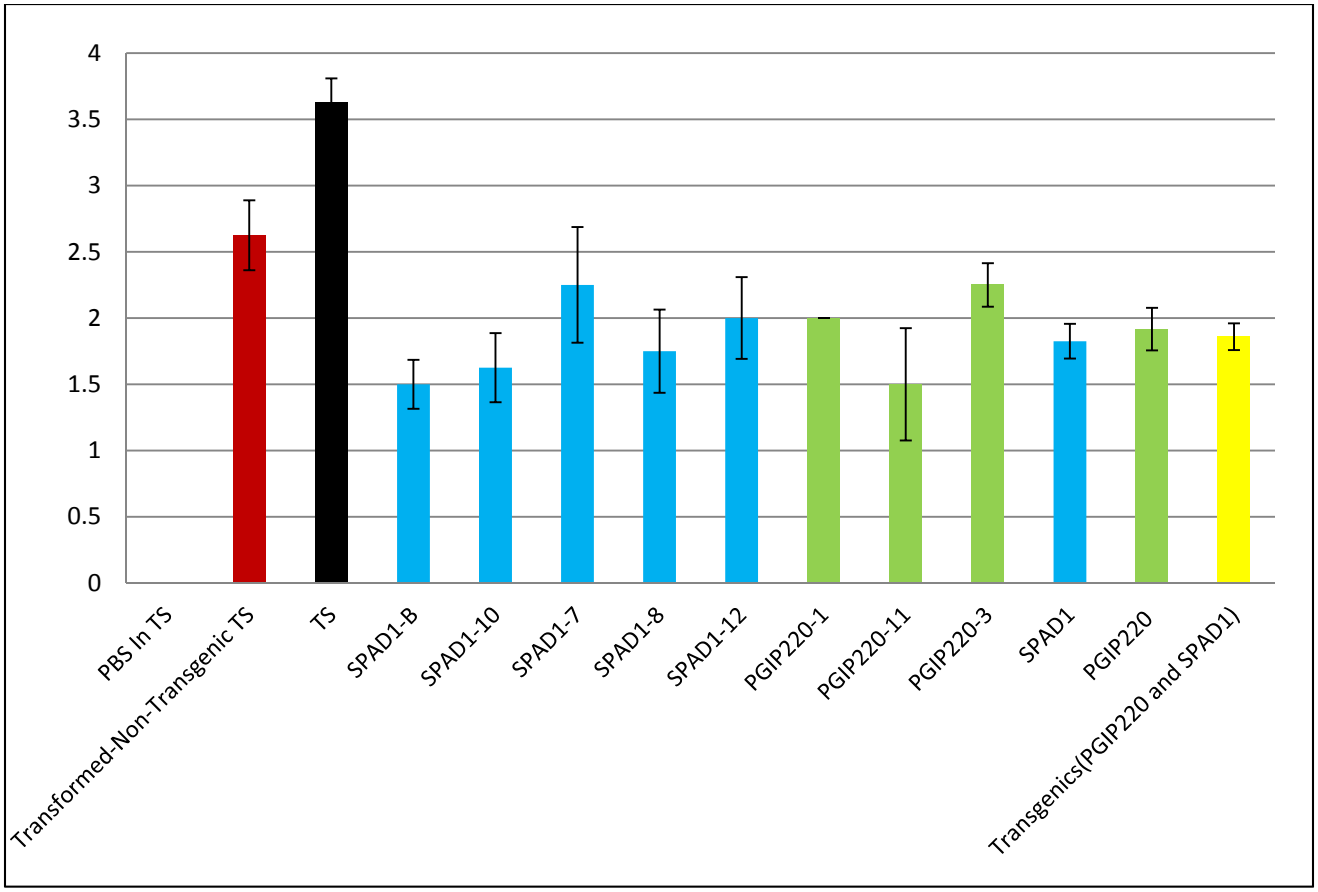

Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy, 5 is dead) of Pierce's disease symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *X. fastidiosa* Fetzer at 16 weeks post-inoculation, except for the Transformed-Non-Transgenic TS, which was inoculated four weeks later and its disease rating is for 12 weeks post-inoculation. We anticipated these vines will have disease ratings similar to the TS control at 16 weeks post-inoculation. The last three columns are the averages of all inoculated vines of the specified type of construct used, either transformed with AD1-3 (SPAD1) or the full-length native HA (PGIP220). Error bars are the standard error of the 10 reps. All PGIP220-1 vines had the same disease rating.

Objective 3a. Secure permits to plant HA-transgenic lines in the field in Solano County.
This objective was completed with the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA).

Objective 3b. Plant transgenic vines in the field.
Approximately 50 HA-transgenic vines representing all the transgenic lines that were produced were planted in the field in April 2013 and trained as bilateral cordons (**Figures 2**).

Figure 2. HA-transgenic and non-transgenic control vines planted in the field.

Objective 4. Inoculate four canes on each HA-transgenic field vine with wild-type *X. fastidiosa* in spring 2014. Rate Pierce's disease symptoms in September 2014 on each inoculated cane. Inoculate non-transgenic Thompson Seedless canes as positive controls, leave two vines of each transgenic line as uninoculated controls.

A combination of *X. fastidiosa* Temecula and Stags Leap strains were grown on solid PD3 medium and the cells were harvested and suspended in 1XPBS to a concentration of 10 X 8th. Four canes on replicates of each transgenic line were labeled and then mechanically inoculated 1X with a 20 ul drop of *X. fastidiosa* cell suspension. Inoculations were done in mid-May 2014 and inoculum droplets were quickly taken up by the transpiring canes.

Overall success in inoculating canes in transgenic and non-transgenic vines was very high. In some cases the tags marking inoculated canes in HA-transgenic vines were missing so no rating was made. 0 ratings of canes on HA-transgenic canes occurred on vines where at least two of the other canes on that vine expressed some Pierce's disease symptoms; thus we believe the inoculum that was used to inoculate 0 scoring canes was viable. However, it is certainly possible that the inoculum was not taken into actively transpiring xylem vessels which could result in an unsuccessful inoculation.

Overall Pierce's disease symptoms severity was higher in the non-transgenic positive controls than in the HA-transgenic vines, results that were similar to what we observed in the greenhouse inoculations. It is also clear from the field inoculations that none of the transgenic lines completely prevented the onset of Pierce's disease symptoms in INOCULATED CANES in 9/14, again results that were observed in greenhouse trials.

Cane samples were collected from all *X. fastidiosa* inoculated lines for testing by qPCR. This should give us some information concerning the relative *X. fastidiosa* titers in transgenic vs. non-transgenic inoculated vines.

Table 3. Pierce's disease symptom ratings of HA-transgenic grapevines.

Transgenic Lines	# Inoculated Vines	# of PD-Rated Canes	Mean Disease Rating	
			Ratings of individual canes	
Adhesion Domain Lines				
AD 6	3	10	0=5; 1=4; 2=2	**0.7**
AD 7	4	15	0=7; 1=2; 2=6	**0.9**
AD 8	5	20	0=2; 1=5; 2=12; 3=1	**1.6**
AD 10	3	10	0=1; 1=2; 2=6; 3=1	**1.7**
AD 12	5	19	0=5; 1=5; 2=9	**1.2**
Complete HA Gene				
220-1	4	10	0=4; 1=1; 2=6; 3=1	**1.6**
220-3	3	12	0=4; 1=1; 2=6; 3= 1	**1.3**
220-11	3	10	0=8; 1=1; 2=1	**0.3**

Note: Pierce's disease symptoms of inoculated transgenic canes were made by Kirkpatrick on 9/14/14. Symptoms ratings of individual canes were as follows:
0 = no symptoms of Pierce's disease, i.e. no scorched leaves on cane.
1 = 2 to <10% scorched leaves on cane.
2 = >10% to <75% scorched leaves on cane.
3 = all leaves showing Pierce's disease scorch symptoms, no cane dieback observed.
4 = cane dieback, cane still alive.
5 = dead cane.

Rating of inoculated NON-transgenic Thompson Seedless canes
Ratings of 18 inoculated canes on NON-transgenic Thompson Seedless vines were made by Lincoln and Gilchrist on 9/12/14. Symptoms of canes were rated as follows:

0 = no symptoms of Pierce's disease, i.e. no scorched leaves on cane	NO canes were rated 0.
1 = 2 to <25% of leaves with scorched leaves	NO canes were rated 1.
2 = 25% to 50% of leaves with scorched leaves	NO canes were rated 2.
3 = all leaves on cane were showing Pierce's disease scorch symptoms	2 canes were rated 3.
4 = all leaves scorched and some terminal cane dieback	3 canes were rated 4.
5 = cane near death or dead	13 canes were rated 5.

Objective 5. Cut back all canes to two buds and rate cane growth in Spring 2015 and Pierce's disease symptoms in September 2015 to determine if expression of *X. fastidiosa* HA in the transgenic vines retarded or prevented movement of the inoculated *X. fastidiosa* into the cordons, thus causing systemic Pierce's disease.

Canes were cut back to two buds once vines were completely dormant in January/February 2015. The vines were rated for Pierce's disease symptoms in late August 2014. 95% of the inoculated canes had some level of leaf scorching which indicated our inoculation procedure was successful. The severity of the Pierce's disease symptoms on the inoculated canes were rated on a 0 to 5 scale similar to the scale we have used in our previous disease evaluation: 0 = heathy, no symptoms; 1 = 1 to 10% of leaves had scorch symptoms; 2 = 25% to 75% of the leaves on the canes were scorched; 3 = all leaves on the cane had scorch symptoms; 4 = cane dieback; 5 = cane was dead. The following table summarizes the results of the various transgenic lines developing Pierce's disease symptoms.

Table 4.

Transgenic Line	# Inoculated Vines	# Canes Inoculated	Average PD Rating for Inoculated Canes
Adhesion Domain Constructs			
SPAD 6	3	10	0.7
SPAD 7	4	15	0.9
SPAD 8	5	20	1.4
SPAD 10	3	10	1.7
SPAD 12	5	19	1.2
	AVERAGE OF SPAD LINES PD RATINGS = 1.22		
Full-length HA Gene			
220-1	4	10	1.6
220-3	3	12	1.3
220-11	3	10	0.3
	AVERAGE OF 220 LINE PD RATINGS = 1.1		

Eighteen non-transgenic control canes were inoculated with *X. fastidiosa* by Dr. Jim Lincoln. The Pierce's disease severity ratings for those canes were as follows: 13 canes = 5; 3 canes = 4; 2 canes = 3.

These initial results support conclusions of greenhouse testing; i.e., HA transgenics had less severe Pierce's disease symptoms than non-transgenic vines.

All spurs were cut back to approximately two buds in January 2015. Initial growth of inoculated canes positions were similar to uninoculated canes. However, there was some death of cut-back *X. fastidiosa* inoculated spur positions on some transgenic vines.

Pierce's disease ratings of inoculated whole transgenic vines and canes were made in August 2015. The following **Table 5** summarizes those ratings.

Table 5.

Transgenic Line	# Inoculated Vines	2014 Average PD Rating of Inoculated Canes	2015 Vine/Cane Ratings
Adhesion Domain Constructs			
SPAD 6*	3	0.7	healthy; healthy; 1.5 canes
SPAD 7	4	0.9	3 dead vines; 1 healthy
SPAD 8*?	5	1.4	2 healthy; #3 and 5 canes
SPAD 10	3	1.7	1 dead; #4 canes; 1 healthy
SPAD 12*	5	1.2	3 healthy vines; #2 canes
AVERAGE PD RATING OF SPAD LINE CANES = 1.22			
Full-length HA Gene			
220-1*	4	1.6	3 healthy vines; #1 cane only
220-3*	3	1.3	2 healthy vines; #2 cane only
220-11*	3	0.3	2 healthy vines; 1 dead vine
AVERAGE PD RATING OF 220 LINE CANES = 1.1			

Figure 3A. Dead adhesion domain vine that collapsed in June 2015.

Figure 3B. Full-length transgenic cane rated #2 in August 2015.

Figure 3C. Healthy appearing full length transgenic vine, August 2015.

If the USDA permit can be extended two more years I would like to re-inoculate the vines marked with an * in **Table 5** in spring 2016, rate inoculated canes and vines in late August 2016, cut back canes in winter 2017, and do a final Pierce's disease rating of inoculated vines in August 2017. The vines in the transgenic lines that are marked with an * were mostly healthy or had mild cane symptoms, and would be worth re-evaluating if possible.

CONCLUSIONS

Eight HA-trangenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the eight HA-transgenic Thompson Seedless grapes with cultured *X. fastidiosa* cells showed all lines expressed less severe symptoms of Pierce's disease than inoculated, non-transgenic controls. All transgenic lines as well as non-transgenic Thompson Seedless vines that were used as positive and negative controls were planted in the field in spring 2013; the vines grew well and were trained as bilateral cordons. Four shoots on each vine were mechanically inoculated with wild-type *X. fastidiosa* in May 2014. Pierce's disease symptoms on inoculated and uninoculated shoots were evaluated in September 2014. A high percentage of the inoculated shoots developed scorched leaves typical of Pierce's disease symptoms, indicating our needle inoculation technique was successful. Pierce's disease symptom severity ratings were lower among HA-transgenic lines than inoculated non-transgenic grapevine controls. Canes from transgenic and non-transgenic vines were collected to determine *X. fastidiosa* titers by qPCR. All shoots were pruned back to two buds in January / February 2015 and allowed to push during the 2015 growing season. Spring shoot growth and Pierce's disease symptoms were recorded in September 2015 to determine if the *X. fastidiosa* infections overwintered and formed systemically-infected vines. Most of the adhesion domain vines and full length HA gene transformants had some vines that appeared Pierce's disease free. However, with other reps of the transformant lines some reps were either dead or had Pierce's disease symptoms on inoculated canes that varied in severity. If the USDA permit can be extended for another two years we would like to re-inoculate vines that appear to be Pierce's disease free. Final disease evaluations in September 2017 should indicate whether the transgenic vines are indeed Pierce's disease resistant or tolerant.

If *X. fastidiosa* populations in HA-transgenic lines can be kept low enough to prevent fruit symptoms and vine dieback, we may have produced transgenic vines that are functionally tolerant of *X. fastidiosa* infection. Their possible use as rootstocks grafted with non-transgenic scions will be evaluated in the coming years.

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SUBSTRATE-BORNE VIBRATIONAL SIGNALS IN INTRASPECIFIC COMMUNICATION OF THE GLASSY-WINGED SHARPSHOOTER

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LAYPERSON SUMMARY

Leafhoppers and sharpshooters communicate via vibrational signals transmitted through the plant. Signals are very low frequency and intensity "sound" waves that could be the key to a novel control method that may be incorporated in an integrated pest management strategy. A laser-Doppler vibrometer is being used to identify and describe signals used by the glassy-winged sharpshooter (*Homalodisca vitripennis*) to communicate. The glassy-winged sharpshooter uses signals in intra- and inter-gender communication with specific signals required to achieve mating. Bioassays using paired virgin males and females on plants revealed that glassy-winged sharpshooter males search for females on plants while females wait for males to approach. Visual signals, physical contact, and specific vibrational signals are used by the glassy-winged sharpshooter to establish male and female hierarchy and preferential access to mates. However, glassy-winged sharpshooter females also can be "choosy," suggesting that both intrasexual (male-male combat) and intersexual (mate choice) selections may occur. While glassy-winged sharpshooter rivalry calls negatively impact courtship behaviors, it is not known if the overall reproductive success of individuals can be artificially affected by signal playbacks.

Since what they "say" to each other has a big effect on behaviors, signals may be exploited as an attractant, repellent, and/or disruptive signal which could be a useful, non-chemical control method for suppressing glassy-winged sharpshooter populations. Can these sounds be reproduced to manipulate glassy-winged sharpshooter behaviors? The answer is "Yes." In the laboratory, mini-shakers and speakers deliver pre-recorded natural sounds or synthetic sounds to plants through a trellis wire, thereby artificially stimulating individuals to produce natural responses to playback signals. In preliminary trials, communication (duets, trios, and quartets) with glassy-winged sharpshooter males and females has been established using pre-recorded calls. The ability to establish a communication channel and elicit glassy-winged sharpshooter response to select signals represents an important step towards the next goal: identification of signals capable of influencing glassy-winged sharpshooter behavior for disruption of mating.

INTRODUCTION

Epidemiological models suggest that vector transmission efficiency, vector population density, and the number of plants visited per vector per unit time are key factors affecting rates of pathogen spread (Jeger et al., 1998). Measures to reduce glassy-winged sharpshooter (*Homalodisca vitripennis*) population densities in California include an area-wide insecticide application program and release of natural enemies. Despite such efforts, geographic distribution and population densities of the glassy-winged sharpshooter continue to expand. Chemical control of the glassy-winged sharpshooter in urban areas, organic farms, and crops under integrated pest management programs is problematic because insecticides are ineffective, not used, or incompatible with existing practices, respectively. The near-zero tolerance for glassy-winged sharpshooters in vineyards, particularly in areas where Pierce's disease is endemic, poses a constant challenge for growers and agencies involved in the area-wide program. Thus, long-term suppression of glassy-winged sharpshooter populations will rely heavily on novel methods.

Vibrational communication is a widespread form of communication in invertebrate and vertebrate animals including fish, amphibians, reptiles, birds, and mammals (Cocroft et al., 2014). Arthropods emit vibratory signals in connection with aggression, distress, calling, courtship, rivalry, searching, and other behaviors associated with finding conspecifics and avoiding predation (Čokl and Virant-Doberlet, 2003). In leafhoppers, mate recognition and localization are mediated exclusively via substrate-borne vibrational signals transmitted through the plant. Vibrational signals in leafhoppers are low-frequency bending (or flexural) waves produced by the abdomen. Signals are transmitted through the legs to the substrate and travel at a speed of about 100 m/s. Signals are detected by the receiver presumably by subgenual and joint chordotonal organs located in the legs (Čokl and Virant-Doberlet, 2003).

Exploitation of attractive vibrational signals for trapping leafhoppers or disrupting mating, as well as for excluding pests via emission of repellent signals, have been considered but not yet implemented in commercial agricultural landscapes (Polajnar et al., 2014). In Florida, an experimental prototype of a microcontroller-buzzer system attracted the Asian citrus psyllid, *Diaphorina citri*, to branches of citrus trees by playback of insect vibrational signals (Mankin et al., 2013). Recently, small-scale field studies on mating disruption of leafhoppers via playback of vibrational signals through grapevines have demonstrated promising results. Specifically, electromagnetic shakers attached to wires used in vineyard trellis successfully disrupted mating of *Scaphoideus titanus*, vector of a phytoplasma that causes the grapevine disease Flavescence dorée in Europe (Eriksson et al., 2012). Exploitation of disruptive, attractive, and/or repellent signals for suppressing glassy-winged sharpshooter populations could prove to be a useful tool. However, existing knowledge on glassy-winged sharpshooter vibrational communication is insufficient to implement a management program for this pest in California.

OBJECTIVES

1. Identify and describe substrate-borne signals associated with intraspecific communication of the glassy-winged sharpshooter in the context of mating behavior.

RESULTS AND DISCUSSION

When placed alone on plants, males were less likely to signal than females, with 19% of the animals signaling (n=21) compared with 79% of females (n=25) (G=15,61, p<0,001). The latency to begin calling was longer for males (F=7.715, p=.012) than females (**Figure 1A**). Within the females, there was a large variation in the latency to begin calling as well as the calling rate. The calling rate varied from 0 to 143 calls during a 45-min recording. When normalized on a log scale, the females showed a shorter latency to calling when they had a faster calling rate (F=6.45, p=.023; **Figure 1B**). The Female Call (FC) was a broadband signal with its peak in relative amplitude in the middle of the call at 93.07 ± 17.15 Hz; the FC increased in dominant frequency across the duration of the call with a 16.25 ± 25.34 Hz frequency range (n = 50 calls among five individual; **Figure 2**). The female can modulate its signal in length and in structure. The faster the rate of female calling the longer her maximum call was (F=9.56, p=.010), though did not affect the mean or minimum call length (Mean: F=0.83, p=.38; Min: F=0.92, p=.36). The maximum call duration recorded was 3.92s.

Recordings of a virgin male and female placed together on the plant revealed a complex series of behaviors linked to vibrational signals that lead to mating, or not. When paired with a female, males exhibit the standard MC that has two components: a harmonic part (MCs2) followed by pulses (MCs3) or a courtship version MCcrs containing a strong broadband signal at the beginning (**Figure 3**). Male-female glassy-winged sharpshooter communication can be divided into three stages: 1) duet, 2) courtship and location, and 3) precopula. In Stage 1, females initiated the duet in 15 of 21 cases. After the initial duet lead by the female, the behavior was reverted with the male leading duets. During the location phase (Stage 2), the female remained on the same position on the plant as the male searched and approached her. During Stage 2 the male added another component to the beginning of the signal, quivering (MCs1), which was alternated with Mcrs and MC. At the moment, it is not known whether glassy-winged sharpshooter female vibrational signals alone provided directionality to searching males or if there was a random component in mate search by glassy-winged sharpshooter males. However, males searched for the female on the plant by alternating a walking behavior and short stops to emit additional signals, likely to maintain communication with the female. The length of the duet was variable from as few as two calls each to over 10 calls each and does not appear to be correlated with the final outcome of mating. Mating success occurred in 55% of the animals (n=20), with one successful pair not dueting. In many cases, females were not receptive for mating despite long duets with males. To avoid copulation, females lifted the posterior part of the body and stretched the hind legs to keep males away. Unsuccessful courtship interactions only entered the second

stage of communication in 55% of the cases (n=5/9), while successful courtship entered the second stage 100% of the cases (n=12/12). When mating was successful, the couple remained in copula for more than six hours. Females were able to feed during copula. All mating pairs produced viable progeny.

Analysis of the call structure across the different stages of communication revealed that the length of FC was shorter in Stage 3 (average 0.70 ± 0.49 s, n=8) than in other stages. In addition, the dominant frequency of FC reduced from 93 to 68 Hz. Similarly, the dominant frequency of male calls reduced from 100 to 89 Hz between stages 1 and 2 (**Table 1**).

Table 1. Description of substrate-borne signals produced during three stages of glassy-winged sharpshooter mate selection behavior.

Signal	Parameter	Stage 1				Stage 2		Stage 3		Chi^2	P value
		First duet		Second duet							
		N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD		
Mc	Length (s)	10	1.82 ± 0.53	13	2.15 ± 0.74	9	2.18 ± 0.82		-	2.36	0.31
	Df (Hz)	10	100.65 ± 57.85a	13	83.18 ± 8.25ab	9	89.91 ± 50.54b		-	9.76	**0.007**
Mcrs	Length (s)		-	12	3.32 ± 0.68	12	3.55 ± 0.56	11	3.46 ± 0.58	2.54	0.28
	Df (Hz)		-	12	73.22 ± 37.07	12	61.47 ± 42.55	11	59.76 ± 35.20	1.39	0.49
Quivering	Length (s)		-		-	12	14.82 ± 9.08	9	20.41 ± 13.16	1.35	0.17
	n pulses		-		-	12	61.71 ± 35.4	9	88.15 ± 57.94	1.35	0.17
	PRT (S)		-		-	12	0.23 ± 0.03	9	0.23 ± 0.05	0.22	0.82
	Df (Hz)		-		-	12	55.2 ± 22.29	9	51.7 ± 21.13	0.53	0.59
Fc	Length (s)	10	2.24 ± 0.62a	14	1.83 ± 0.56a	7	1.14 ± 0.73b	8	0.7 ± 0.49b	42.64	**<0.001**
	Df (Hz)	10	93.12 ± 8.05a	14	91.01 ± 7.97a	7	81.24 ± 13.58b	8	68.04 ± 17.69c	38.5	**<0.001**

Mc = male call, Mcrs = courtship version of Mc, Quivering = train of pulses (see **Figure 3**), and Fc = female call.
Df = dominant frequency, PRT = pulse repetition time.
For Mc, Mcrs, and Fc the non-parametric Kruskall-Wallis statistical test, followed by the Steel-Dwass pairwise multiple comparison test, were conducted within rows. For Quivering the Mann-Whitney statistical test were conducted within rows.
(-) signal was not observed.

Glassy-winged sharpshooter male-male rivalry signals were recorded during establishment of dominance and subordination between males competing for mates. Different rivalry signals are currently being characterized (see one example in **Figure 5** and preliminary analysis in **Table 2**). Preliminary data show that visual signals, physical contact, and specific vibrational signals may be used by the glassy-winged sharpshooter to establish male hierarchy and thus, preferential access to mates. In relatively more aggressive situations, a male bent the body by lowering the posterior part of the abdomen, forming an arc. Male rivalry signals were observed after Stages 1 and 2 of male-female communication. In cases where male rivalry was observed, mating occurred in only four of 12 trials. However, glassy-winged sharpshooter females can also be “choosy,” which suggests that both intrasexual (male-male combat) and intersexual (mate choice) selection may occur in the glassy-winged sharpshooter. While glassy-winged sharpshooter male rivalry appears to negatively impact courtship behaviors, it is not known if the overall reproductive success of a male can be artificially affected by playbacks.

In conclusion, the project is providing a detailed description of vibrational communication signals that are key for understanding fundamental behaviors of the glassy-winged sharpshooter for mating success. Within the next months, a “library” of glassy-winged sharpshooter signals will be finalized, including the identification and characterization of signals produced by individuals under different conditions (insects alone and in groups on the plant). Our work has shown that 1) the glassy-winged sharpshooter uses substrate-borne vibrational signals in intra- and inter-gender communication, and 2) specific signals are required for the glassy-winged sharpshooter to achieve mating. Although the role of some signals reported here could be inferred from observations, the role and relevance of individual signals to insect behaviors can be ultimately determined only when insects are stimulated via playback of select signals. Pre-recorded glassy-winged sharpshooter signals were used to perform preliminary playback experiments, where males and females were artificially stimulated to produce natural responses to

signals transmitted to host plants. In these preliminary trials, communication with glassy-winged sharpshooter males and females was established using pre-recorded female and male calls, respectively. The ability to establish a communication channel and elicit glassy-winged sharpshooter response to select signals represents an important step towards the next goal, which is to determine the role of specific signals in glassy-winged sharpshooter communication and identify signals capable of influencing glassy-winged sharpshooter behavior for applicative purposes (e.g., disruption of mating communication, attraction).

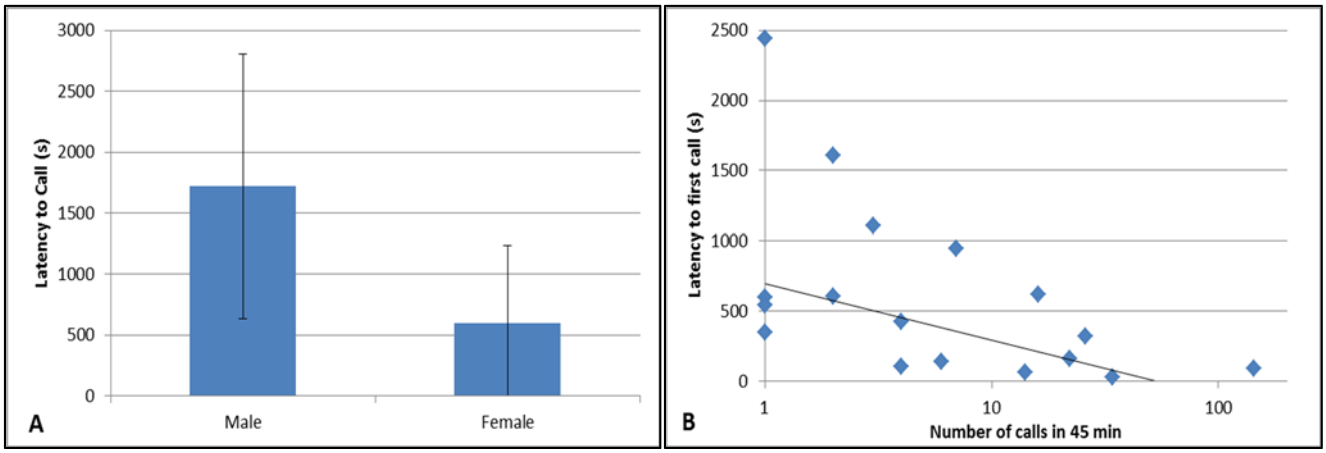

Figure 1. Mean time taken for isolated glassy-winged sharpshooter individuals to begin calling (i.e., latency to call). A) Average male (n=4) and female (n=18) latency to call. B) Female latency to call based on calling rate over a 45 min trial.

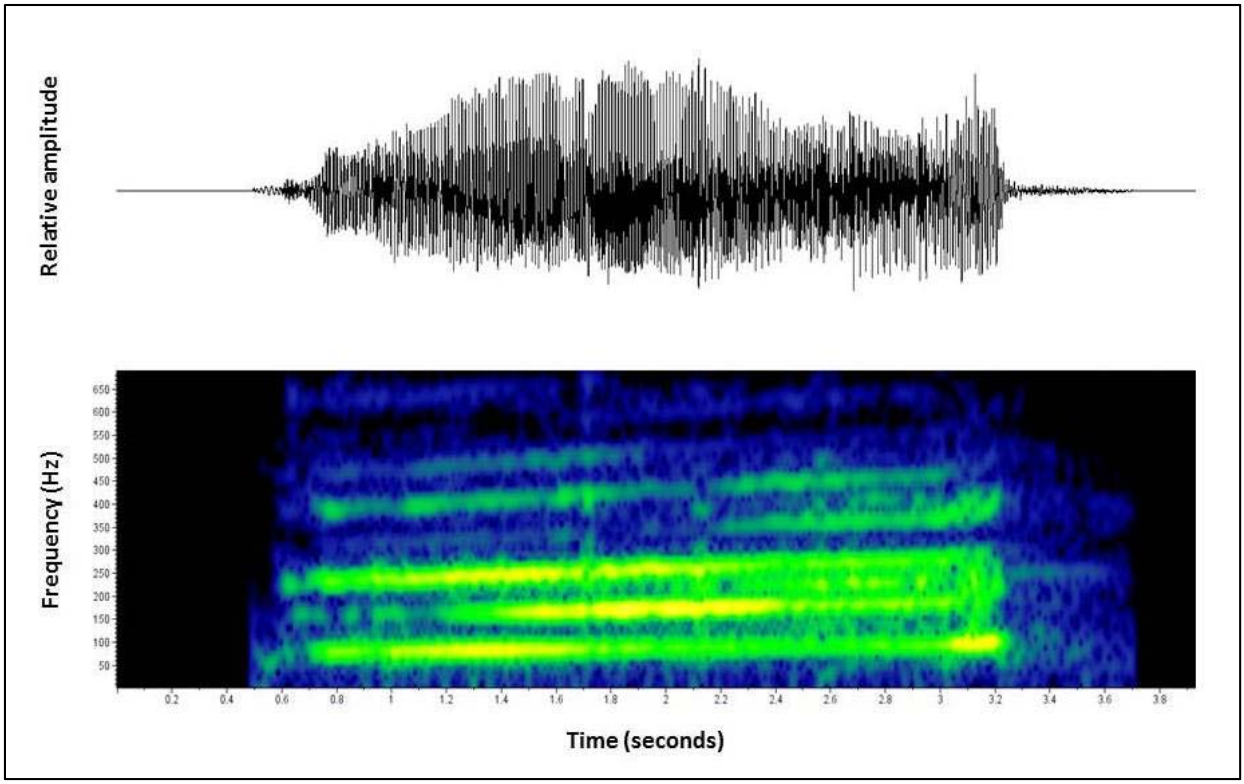

Figure 2. Oscillogram (above) and spectrogram (below) of a glassy-winged sharpshooter female call (FC).

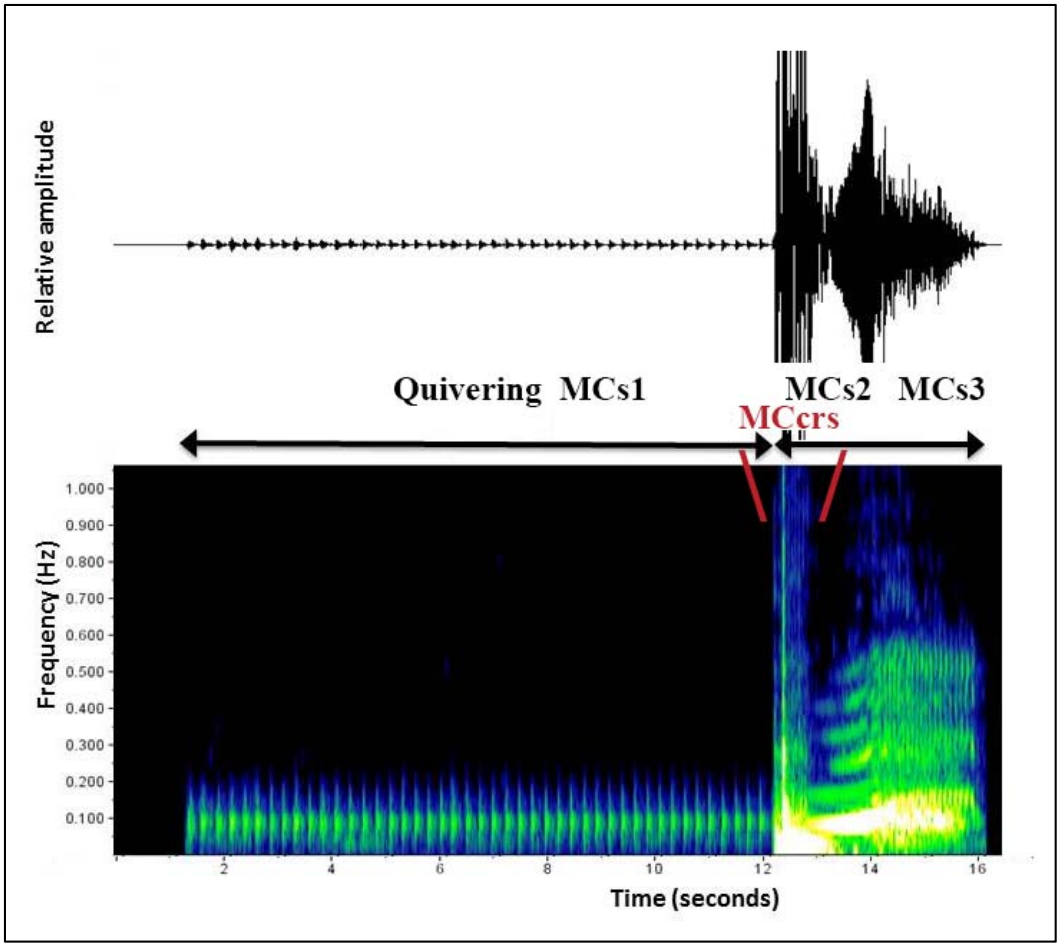

Figure 3. Oscillogram (above) and spectrogram (below) of a glassy-winged sharpshooter male call (MC2). Three different sections of MC2 are indicated by MCs1, MCs2, and MCs3.

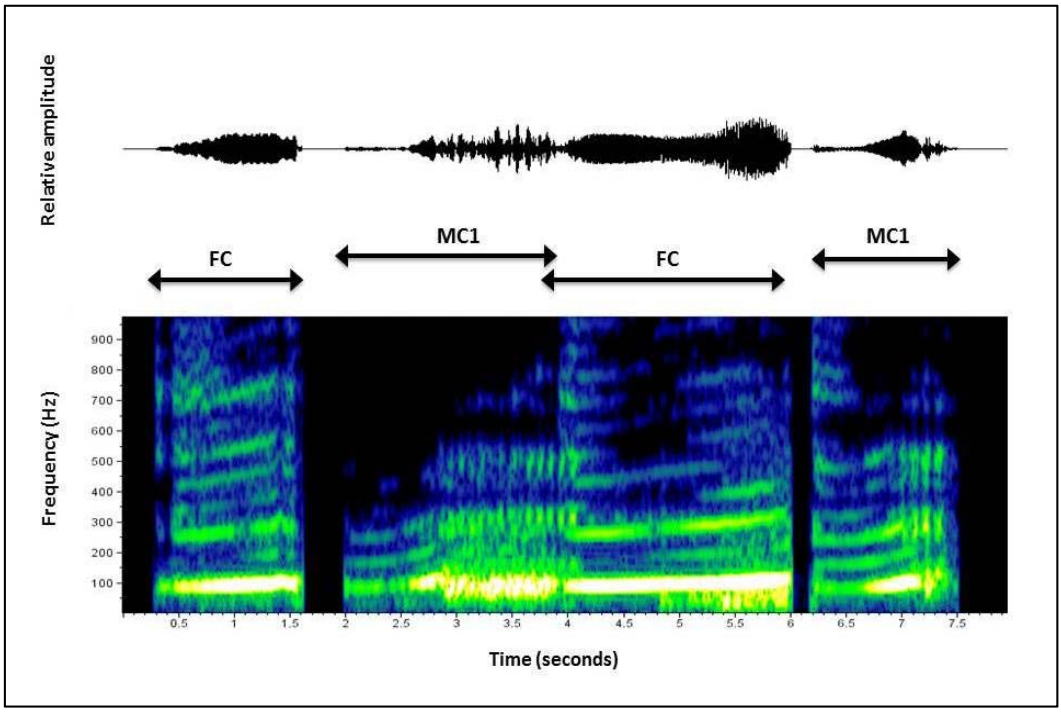

Figure 4. Oscillogram (above) and spectrogram (below) of a duet between a male and female glassy-winged sharpshooter. FC is a female call and MC1 is a male call.

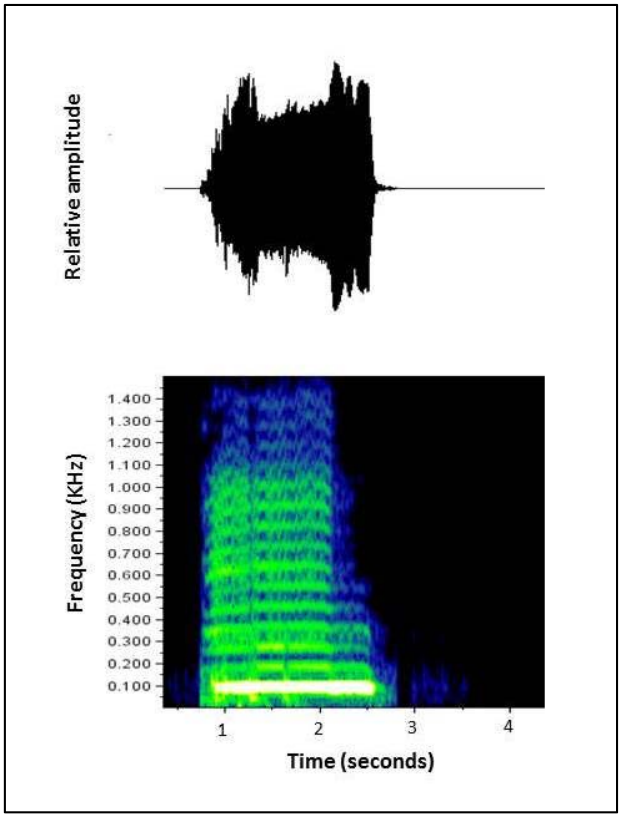

Figure 5. Oscillogram (above) and spectrogram (below) of an aggressive signal of a male glassy-winged sharpshooter produced during a male-male competition for a female.

Table 2. Analysis of spectral and temporal parameters of a glassy-winged sharpshooter aggressive signal. N = number of individuals analyzed, n = number of signals analyzed per individual, and Df = dominant frequency. Data are expressed as mean ± sd.

N/n	Length(s)	Df (Hz)
12/4	2.17 ± 0.54	98.40 ± 31.62

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COMPARISON AND OPTIMIZATION OF DIFFERENT METHODS TO ALTER DIFFUSIBLE SIGNAL FACTOR MEDIATED SIGNALING IN *XYLELLA FASTIDIOSA* IN PLANTS TO ACHIEVE PIERCE'S DISEASE CONTROL

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ABSTRACT

Xylella fastidiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor (DSF) molecule which acts to suppress its virulence in plants. Artificially increasing DSF levels in transgenic grape greatly reduced disease severity in both greenhouse and field trials. We are investigating DSF production in additional transgenic grape varieties to determine the robustness of this strategy of disease control. *X. fastidiosa* is relatively promiscuous in its production and perception of various unsaturated fatty acids as DSF signal molecules and we will explore ways to introduce the common, inexpensive fatty acid palmitoleic acid and other DSF homologs into plants following direct application. Improved DSF biosensors that we have developed will enable us to monitor the uptake and redistribution of such molecules in plants. Initial results suggest that the use of penetrating surfactants introduces sufficient amounts of this DSF-like molecule to alter behavior of *X. fastidiosa* in plants. A naturally-occurring *Burkholderia* strain capable of DSF production that is also capable of growth and movement within grape has been found that can confer increased resistance to Pierce's disease. We are exploring the biological control of disease using this strain. The movement of *X. fastidiosa* within plants and disease symptoms are greatly reduced in plants in which this *Burkholderia* strain was inoculated either simultaneously with or prior to that of *X. fastidiosa*. These results are quite exciting in that they reveal that biological control of Pierce's disease using *B. phytofirmans* is both robust and may be relatively easy to apply by various ways of inoculation.

LAYPERSON SUMMARY

Xylella fastidiosa produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF). Accumulation of DSF in *X. fastidiosa* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. We have investigated DSF-mediated cell-cell signaling in *X. fastdiosa* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants artificially reduces its movement in the plant. We will be introducing the gene conferring DSF production into a variety of different grape cultivars to determine if they also will exhibit high levels of disease resistant as did the Freedom cultivar previously constructed. Topical application of commercially available unsaturated fatty acids capable of altering gene expression in *X. fastidiosa* with penetrating surfactants can introduce sufficient amounts of these materials to reduce the virulence of the pathogen. A naturally occurring *Burkholderia* strain reduces the movement of *X. fastidiosa* and thereby its virulence in plants when inoculated prior to or simultaneously with *X. fastidiosa*. By comparing disease control by these three methods the most efficacious and practical means of control can be identified.

OBJECTIVES

1. Compare DSF production and level of disease control conferred by transformation of *X. fastidiosa* RpfF into several different grape cultivars.

2. Evaluate the efficacy of direct applications of palmitoleic acid, C16-cis, and related DSF homologs to grape in various ways to achieve disease control.
3. Evaluate the potential for *Burkholderia phytofirmans* to multiply, move, and produce DSF in grape plants to achieve Pierce's disease control.

RESULTS AND DISCUSSION

Introduction

Our work has shown that *X. fastidiosa* uses DSF perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape in various ways to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels – "pathogen confusion." Transgenic 'Freedom' grape expressing the DSF synthase RpfF from *X. fastidiosa* are much more resistant to disease than the wild-type plants in both greenhouse and field trials. Our work has shown, however, that RpfF is rather promiscuous and *X. fasti*diosa can both produce and respond to a variety of unsaturated fatty acids, and the DSF species produced is influenced apparently by the particular substrates available within cells. It is possible that grape varieties might differ in their ability to produce DSF molecules perceived by *X. fastidiosa*. It will be important, therefore, to determine whether commercial grape cultivars can all produce DSF species capable of altering pathogen behavior in high amounts if transformed with the DSF synthase. Non-transgenic strategies of achieving pathogen confusion might be preferred by the industry. While endophytic bacteria capable of producing DSF species is an attractive strategy, until recently, strains capable of growth and movement within grape could not be found. However, we have now found a *Burkholderia* strain that both colonizes grape and has conferred substantial disease control in preliminary studies. We will investigate the interactions of this endophyte with grape to optimize disease control and determine practical methods of its application. We have found that *X. fastidiosa* produces additional DSF species, including 2-Z-hexadecenoic acid (C16-cis), that are much more active than C14-cis previously found, and the common, inexpensive, unsaturated fatty acid palmitoleic acid is also reasonably active as a signal molecule in *X. fastidiosa*. Using a new *X. fastidiosa* biosensor for DSF in conjunction with such an abundant, inexpensive molecule we can now thoroughly investigate methods by which such a molecule can be directly applied to plants to achieve concentrations sufficiently high in the xylem to alter pathogen behavior and thus achieve disease control.

Objective 1. Production of DSF in a variety of grape cultivars.

While Freedom grape transformed with the *X. fastidiosa rpfF* gene encoding the DSF synthase produced DSF species to which *X. fastidiosa* was responsive, considerable evidence has been accumulated that RpfF is a rather promiscuous enzyme capable of producing a variety of DSF-like molecules. For example, we detected the production of C14-cis (*Xf*DSF1), C16-cis (*Xf*DSF2) and surprisingly, even DSF (normally produced only by *Xanthomonas* species) in transgenic RpfF-expressing Freedom grape. Likewise, introduction of *X. fastidiosa* RpfF into *Erwinia herbicola* yielded the production not only of *Xf*DSF1 and *Xf*DSF2, but other apparently related enoic acids not seen in *X. fastidiosa* itself (data not shown). The enzymatic activity of Bcam0581, a protein highly homologous to *X. fastidiosa* RpfF that mediates biosynthesis of DSF in *Burkholderia cenocepacia,* was recently shown to both catalyze the dehydration of 3-hydroxydodecanoyl-ACP to cis-2-dodecenoyl-ACP as well as to cleave the thioester bond to yield the corresponding free acid. We presume that *X. fastidiosa* RpfF also possesses these same features, although it probably shows a preference for longer chain 3-hydroxyacyl-ACPs since the DSF species produced by of *X. fastidiosa* include 2-*Z*-tetradecenoic acid. The various enoic acids that can be produced by RpfF differed substantially in their ability to induce gene expression in *X. fastidiosa*, with those of longer chain lengths such as C16-cis being much more active than those of shorter chain lengths. We have also observed that DSF-mediated signaling in *X. fastidiosa* by active DSF species such as C16-cis can be blocked in the presence of certain other *trans* unsaturated fatty acids. It is therefore possible that in some plants other fatty acid species indigenous to the plant or induced upon transformation of RpfF might interfere with signaling that would otherwise be conferred by the production of C16-cis and other "active" DSF species. To verify that the strategy of production of DSF in RpfF-containing transgenic grape is a robust one, widely applicable in a variety of grape cultivars, we are comparing the production of DSF species in such a variety of grape cultivars. In addition, it seems likely that targeting RpfF to cellular compartments where the substrates for DSF synthesis may be more abundant could lead to enhanced production of this signal molecule. We thus are comparing the amount and types

of DSF produced, and disease susceptibility, in transgenic plants in which RpfF is targeted to plastids and in plants in which it is not targeted.

RpfF was initially introduced only into Freedom grape, because it was the only variety for which transformation was feasible at that time. Continuing work by David Tricoli at the UC Davis Plant Transformation Facility has now made it possible to transform Thompson Seedless as well as the wine grapes Chardonnay and Cabernet Sauvignon and the advanced rootstock varieties 1103 and 101-14. In addition to untargeted expression of RpfF, we have produced constructs which target RpfF to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. Transformation of the various grape varieties is being conducted at the UC Davis Plant Transformation Facility. The lines being produced and tested are shown in **Table 1**.

Table 1.

Variety	**Gene Introduced**	
	Untargeted RpfF	**Chloroplast-targeted RpfF**
Thompson Seedless	+	+
Chardonnay	+	+
1103	+	+
101-14	+	+
Richter 110	+	+
Freedom	done	+

Transformation of the various varieties is underway, with some transformed plants already delivered, but we expect that it will take at least an additional six months to produce the remaining plants. There has been little experience in transformation of Richter 110 and Chardonnay, and so their successful transformation may take longer than the other cultivars. Between five and 10 individual transformants will be produced for each variety / construct combination. Because the expression of *rpfF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA, it will be necessary to identify those lines with the highest levels of expression. To most rapidly identify those transformants with high level expression of *rpfF* and production of DSF, three assays that can be rapidly employed on seedling plants will be conducted to identify the most promising transformants:

1) The expression of *rpfF* will be assessed by quantitative RT-PCR of RNA isolated from individual leaves of the transformed plant after they are grown to a height of approximately 40 cm.
2) The distal 20 cm of each 40-cm high plant will be excised, placed in a pressure bomb, and xylem sap extruded under pressure. The xylem sap collected from each plant by this method will be assessed for the presence of DSF species capable of inducing gene expression in *X. fastidiosa* by adding it to micro-cultures of a *phoA* mutant of *X. fastidiosa* harboring a *hxfA:phoA* reporter gene fusion. The alkaline phosphatase activity of the cells of this DSF biosensor is proportional to the concentration of various DSF species. This assay will not only identify those transformants within a given variety that maximally express the introduced *rpfF* gene, but will provide early evidence of those species capable of producing DSF species to which *X. fastidiosa* is maximally responsive.
3) A functional "cell release" assay to determine those transformed lines in which *X. fastidiosa* exhibits the highest adhesiveness, (expected of DSF-producing lines) will also be performed on the decapitated plant after extraction of xylem sap. The proportion of cells released from plants in such an assay is inversely proportional to the concentration of DSF in those plants (DSF-producing plants induce stickiness of *X. fastidiosa* and they are thus not released).

The composition of DSF species present in xylem sap and their aggregate signaling activity will be assessed by extracting xylem sap from mature plants of each of the two best transformed lines of a given variety / construct forwarded for further analysis. Mass spectrometry analysis of the plant xylem sap-extracts will be performed using an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization (ESI) source. DSF species will be identified by their m/Z ratio, with *Xf*DSF, XfDSF2, and DSF (having m/Z ratios of 225.18, 253.22, and

211.17, respectively) being readily distinguished in xylem sap of RpfF-expressing Freedom. We will also resolve other chemical species found in RpfF-expressing lines that are not found in control plants by a similar procedure. We expect that more than one enoic acid will be produced in a given line expressing RpfF.

Objective 2. Direct application of DSF to plants.
Several recent findings in our laboratory of the process of DSF-mediated signaling in *X. fastdiosa* suggest that Pierce's disease control by direct application of DSF to plant surfaces is both feasible and practical. Studies of the context-dependent production of DSF reveals that DSF species such as *Xf*DSF2 are far more active than *Xf*DSF1 which was originally described (Figure 1). While topical applications of *Xf*DSF1 to grape provided modest reductions in disease severity, applications of *Xf*DSF2 should be far more efficacious. Studies of applications of *Xf*DSF2 were hindered by a limitation of the amount of this material that we could chemically synthesize. Fortunately, our studies of the promiscuity of DSF signaling in *X. fastidiosa* reveal that it is quite responsive to the cheap, commercially available, enoic acid palmitoleic acid (Figure 1).

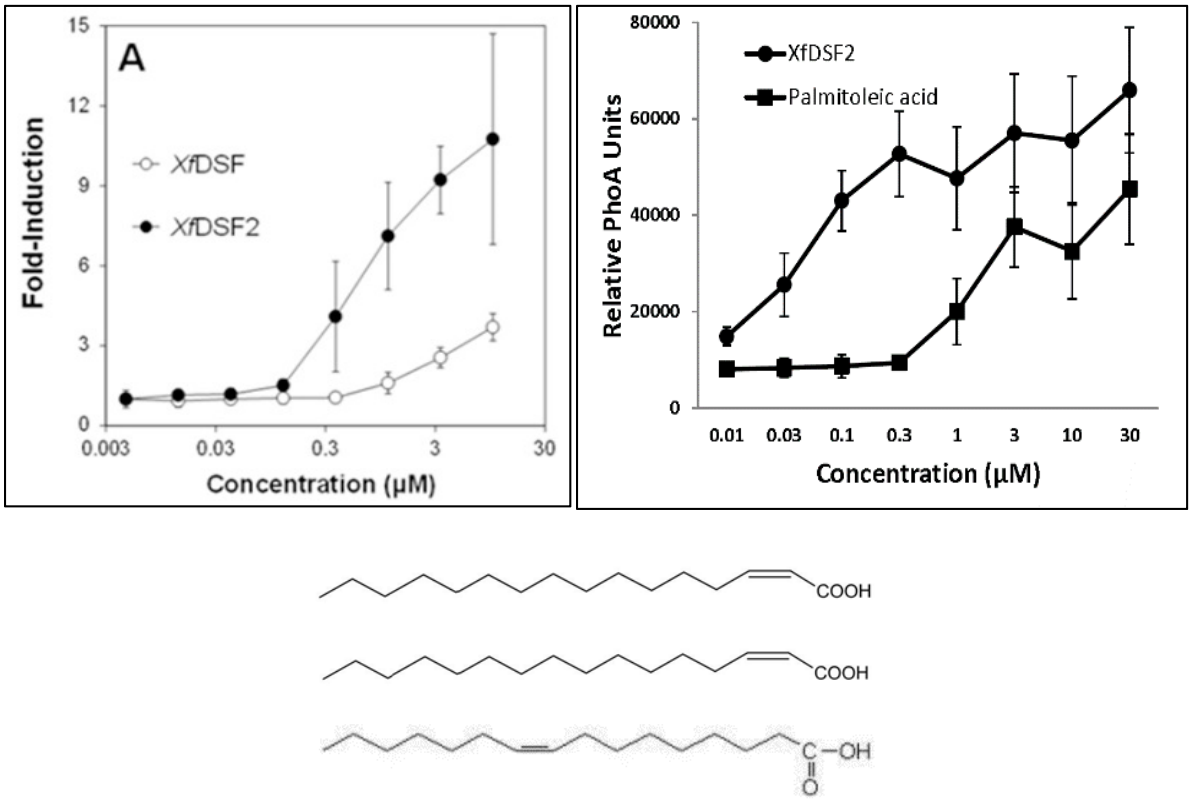

Figure 1. Responsiveness of a PhoA-based *X. fastidiosa* DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

While about eight-fold more palmitoleic acid is required to induce gene expression in *X. fastidiosa* than *Xf*DSF2, it is much more active than *Xf*DSF1 itself. We therefore will conduct a variety of studies to address how such molecules could be introduced into plants in different ways to achieve pathogen confusion. While most studies will use palmitoleic acid, we also will conduct comparative studies using synthetic *Xf*DSF2 and *Xf*DSF1.

We are investigating several strategies by which direct application of DSF molecules can reduce Pierce's disease. While we will determine the effects of application of DSF homologs on disease severity of plants inoculated with *X. fastidiosa* in some studies, direct monitoring of DSF levels in treated plants is a MUCH more rapid and interpretable strategy of assessing this strategy of disease control. As DSF must enter the xylem fluid in order to interact with the xylem-limited *X. fastidiosa* in plants, we have been assessing DSF levels in xylem sap of plants treated in different ways using the PhoA-based *X. fastidiosa* biosensor as described above. We are addressing four main issues that we hypothesize to limit the direct introduction of DSF into plants:

1) The penetration of DSF through leaves and other plant tissues may be slow or inefficient;

2) DSF may readily enter plant tissues but only slowly enter the xylem sap;
3) DSF may be degraded after introduction into plants; and
4) DSF may enter plants more readily via certain tissues than others (e.g., it may readily be taken up via the roots but more slowly from leaves).

We thus are measuring DSF species levels in 1) xylem sap, as well as in 2) leaf, stem, and root tissue after removal of xylem sap after applying synthetic DSF to (A) foliage, (B) direct injection into stems, and (C) application to roots as a drench.

As DSF species are somewhat hydrophobic, a variety of adjuvants are being tested for their effects on enhancing their introduction into plants. For example, detergents and solubilizing materials such as Solutol HS15 may greatly increase the penetration and dispersal of DSF and its analogs. We thus are suspending the hydrophobic materials in such carriers prior to foliar sprays or stem injections. We also are assessing the efficacy of applying DSF molecules with surfactants such as Breakthru that have very low surface tension. Solutions in such organo-silicon surfactants have sufficiently low surface tension that they spontaneously infiltrate leaves through stomatal openings. Considerable preliminary results have already been obtained on the ability of such topically-applied palmitoleic acid solutions to enter into the plants. Apparent DSF signaling activity was measured using the biosensors noted above. We inoculated grape with solutions of palmitoleic acid with different concentrations of the surfactants Breakthru and Triton X-100 as well as the solubilizing agents DMSO and Solutol. Palmitoleic acid was applied at a concentration of 10 mM to plants both as a foliar spray and a stem injection. While high concentrations of several of these detergents or solubilizing agents caused phytotoxicity, no or limited cytotoxicity was observed at a concentration of less than 0.2% Breakthru, 0.2% Triton X-100, 1% DMSO, or 1% Solutol. The effectiveness of these agents in introducing palmitoleic acid into grape tissue was assessed by assessing the ability of sap extracted from individual leaves using a pressure bomb to induce the expression of alkaline phosphatase activity in the *X. fastidiosa Xf:phoA* biosensor. The initial results of these studies reveal that substantial amounts of palmitoleic acid could be introduced into grape leaves applied as a foliar spray with 0.2% Breakthru (**Figure 2**). Lesser amounts could be introduced with foliar sprays including Solutol and DMSO. As a registered surfactant for use in agriculture, Breakthru has the potential to be a practical delivery agent. The efficacy of this material is probably associated with its extraordinarily low surface tension that enables spontaneous stomatal infiltration of leaves with aqueous solutions containing 0.2% of this detergent. Thus, solutions of fatty acid supplied with this concentration of surfactant appear to bypass the cuticular surface as a means of entering the intercellular spaces and presumably also the vascular tissue. These results using penetrating surfactants are very promising and will be a focus of continuing work.

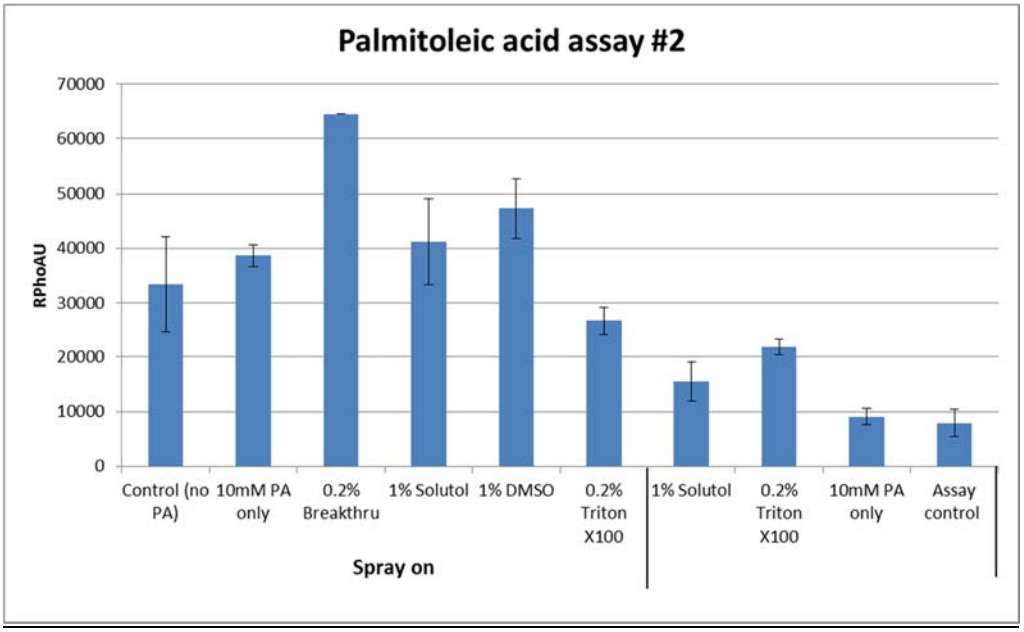

Figure 2. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid with the various surfactants noted when applied as a foliar spray (left) or a stem injection (right).

These most promising treatments were also applied to grape plants to evaluate their efficacy in reducing the symptoms of Pierce's disease. Initial application of palmitoleic acid was followed two weeks later by inoculation with *X. fastidiosa*. The palmitoleic acid treatments were re-applied every three weeks until nine weeks. Disease assessments of plants treated with palmitoleic acid in various ways are being assessed, with final results expected by the end of October 2015.

In addition to directly assessing DSF levels within plants as above, the adhesiveness of *X. fastidiosa* cells inoculated into treated plants are also being determined using the cell release assay described above. Since the virulence of various *X. fastidiosa* mutants is inversely related to their release efficiency, and cells are released at a much lower rate from transgenic RpfF-expressing grape that produce DSF that are resistant to disease, we expect that treatments with exogenous DSF that reduce the release efficiency of *X. fastidiosa* cells when measured two weeks or more after inoculation will also be most resistant to disease. This assay is far quicker than assays in which disease symptoms must be scored after several months of incubation, and will be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

In addition to the use of purified fatty acids we also are evaluating mixtures of fatty acids for their ability to alter the behavior of *X. fastidiosa*. Macadamia nut oil contains a very high concentration of palmitoleic acid (23%). We have saponified macadamia nut oil by treatment with sodium hydroxide to yield the sodium salts of the constituent fatty acids. In promising preliminary results, we find that this fatty acid mixture has DSF signaling activity. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor increased with increasing concentrations of the mixture of fatty acids in the soap prepared from the saponified macadamia nut oil (**Figure 3**). Apparently the other saturated fatty acids that would be found in the lipids of macadamia oil do not strongly interfere with DSF signaling of the palmitoleic acid in this soap. This saponified plant oil is thus very attractive as inexpensive sources of DSF homologs that could be directly applied to grape. We will focus continuing studies on the assessment of saponified plant oils as foliar or soil applied treatments to manage *X. fastidiosa.*

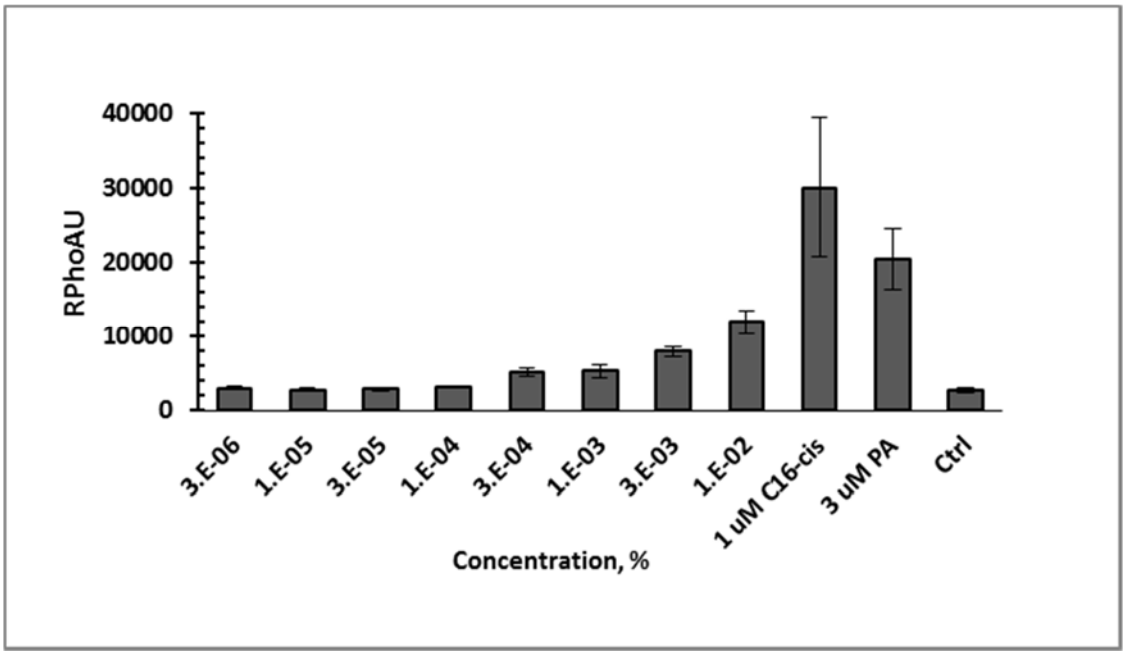

Figure 3. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to increasing concentrations of saponified macadamia nut oil as well as one uM *Xf*DSF2, three uM palmitoleic acid, or a negative control with no added DSF.

Studies were also conducted to determine the process by which *X. fastidiosa* perceives DSF so as to better understand how to supply DSF molecules to the plants to alter the behavior of this pathogen. Most DSF molecules, especially those having relatively long acyl chain lengths such as C16-cis, are expected to have relatively low water solubility. It is clear that DSF can be acquired by *X. fastidiosa* from water solutions since changes in gene expression are observed when DSF is applied to cultures. However, because of their low water

solubility, cells of *X. fastidiosa* may also acquire such extracellular molecules via mechanisms that are not dependent on the solubility of these signal molecules in water. To test this, the responsiveness of the *X. fastidiosa Xf:phoA* biosensor to DSF recovered at various times from cultures of *X. fastidiosa* in different ways was assessed. In preliminary experiments, substantial signaling activity was recovered from entire broth cultures (containing both cells and cell-free supernatants) when ethyl acetate extracts were exposed to the *X. fastidiosa Xf:phoA* biosensor (**Figure 4**). Much less signaling activity was recovered from ethyl acetate extracts of cell-free culture supernatants of these same cultures (**Figure 4**). Interestingly, substantial signaling activity was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to culture supernatants themselves (prior to extraction with ethyl acetate; **Figure 4**). These results suggest that at least a portion, and perhaps a large portion, of the DSF and cultures of *X. fastidiosa* are associated with particulate material, probably either cells themselves or in outer membrane vesicles produced by *X. fastidiosa*. Furthermore, these results also suggest that *X. fastidiosa* efficiently perceives DSF supplied by particulate material. The most parsimonious explanation for these preliminary results is that DSF occurs both in a water soluble form and also associated with hydrophobic particles such as membrane vesicles, and that membrane vesicles might serve as a conduit by which DSF transits between cells of *X. fastidiosa*. While further studies are underway to better understand the apparent role of particulate material in the cell-cell signaling of *X. fastidiosa*, these results suggest that delivery of DSF to plants in a manner that would maximize its ability to alter the behavior of *X. fastidiosa* could be facilitated by providing lipophilic carriers. The observation that the maximal detection of DSF when delivered to plants using detergents such as Breakthru and Triton X-100 might be partially explained by the fact that such detergents could form micels capable of transiting DSF into *X. fastidiosa* cells. This important delivery will guide our continued studies to formulate DSF in a way that it can both enter plants and be readily acquired by *X. fastidiosa*. We will explore various detergents and oil emulsions for their ability to maximize perception of DSF by cells of *X. fastidiosa.*

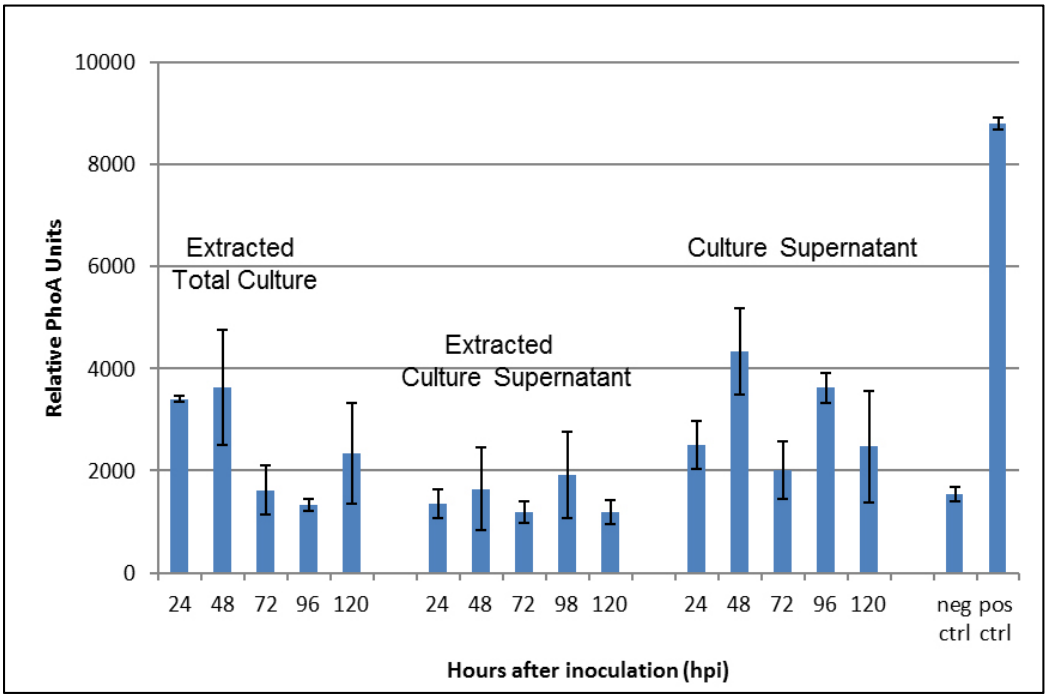

Figure 4. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to ethyl acetate extracts of total broth cultures of *X. fastidiosa* harvested at various times (left), of ethyl acetate extracts of cell-free culture supernatants from cultures harvested at various times (middle), and of cell-free culture supernatants from cultures harvested at various times (but not extracted with ethyl acetate) (right), as well as assays with no added DSF (neg. control) and assay with three uM XfDSF2 (pos. control).

Extensive studies are under way in which various concentrations of palmitoleic acid as well as different concentrations of sodium salts of saponified macadamia nut oil are being applied to the foliage of Cabernet Sauvignon grape with various concentrations of the penetrating surfactant Breakthru to alter the behavior of *X. fastidiosa*. Individual leaves are being assayed weekly on treated vines and the xylem sap expressed under pressure, and the small volume of xylem sap being assayed for DSF activity using the *X. fastidiosa Xf:phoA*

biosensor. In addition, some of these plants were also inoculated with *X. fastidiosa* both before and after application of the fatty acids, and disease severity is being measured weekly. As these studies were initiated in mid-summer, disease symptoms are just now starting to appear, and so a full report on the efficacy of these fatty acids in conferring resistance to Pierce's disease will be available by late October 2015.

Objective 3. Biological control with *Burkholderia phytofirmans* PsJN.

While the biological control of Pierce's disease with endophytic bacteria that would grow within grape and produce DSF has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grape. All of hundreds of strains isolated from within grape by our group as well as that of Dr. Bruce Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently, however, found that *B. phytofirmans* stain PsJN which had been suggested to be an endophyte of grape seedlings multiplied and moved extensively in mature grape plants (**Figure 5**). Its population size and spatial distribution in grape within six weeks of inoculation was similar to that of *X. fastidiosa* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Burkholderia* species and the genome sequence of *B. phytofirmans* revealed that it has a homologue of *X. fastidiosa rpfF*. While we have no evidence for its production of a DSF species to which *X. fastidiosa* could respond, the promiscuous nature of RpfF in *X. fastidiosa* and other species suggested that it might make DSF species to which *X. fastidiosa* would respond under some circumstances, such as one growing within plants. Preliminary results suggest that co-inoculation of *X. fastidiosa* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *X. fastidiosa* alone; whereas the number of infected leaders of plants inoculated with *X. fastidiosa* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *X. fastidiosa* and *B. phytofirmans* (**Figure 5**).

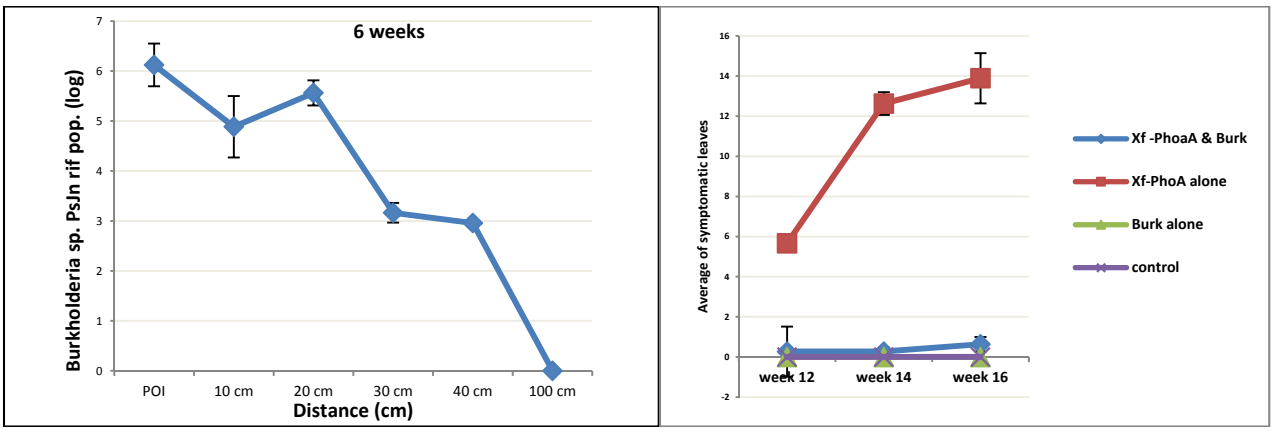

Figure 5. Left: Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after six weeks incubation. Right: Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (red) or when co-inoculated with *B. phytofirmans* (blue).

Given the promising results of the reduction of severity of Pierce's disease in grapes treated with *B. phytofirmans* we performed additional experiments in which *X. fastidiosa* was co-inoculated with *B. phytofirmans,* as well as when *B. phytofirmans* both preceded or followed inoculation of plants with *X. fastidiosa* by 30 days. As observed before, the severity of Pierce's disease of plants co-inoculated with *B. phytofirmans* and *X. fastidiosa* averages less than one leaf per vine compared to over nine leaves per vine on plants inoculated with the pathogen alone (**Figure 6**). Importantly, the severity of Pierce's disease was also substantially less on plants in which inoculation with *B. phytofirmans* followed inoculation with the pathogen by 30 days than on control plants inoculated only with the pathogen (3.5 leaves per vine compared to nine leaves per vine, respectively; **Figure 6**). Almost no disease was observed on plants inoculated with *B. phytofirmans* 30 days prior to inoculation with the pathogen (**Figure 6**), although one must recognize that these plants did not have as long a time period in which disease could develop since they were inoculated with the pathogen 30 days later then control plants inoculated only with the pathogen; we are continuing to assess the development of disease in these plants. These results are quite exciting and confirmed that *B. phytofirmans* can confer high levels of disease resistance in grape, both when co-inoculated with the pathogen and also when inoculated into plants already infected with *X. fastidiosa*. It might be anticipated that pre-inoculation of plants with *B. phytofirmans* will yield the largest degree of disease resistance.

The initial studies obtained here confirm such an expectation although repeated experiments underway are designed to confirm that disease will not eventually occur in plants pretreated with *B. phytofirmans*.

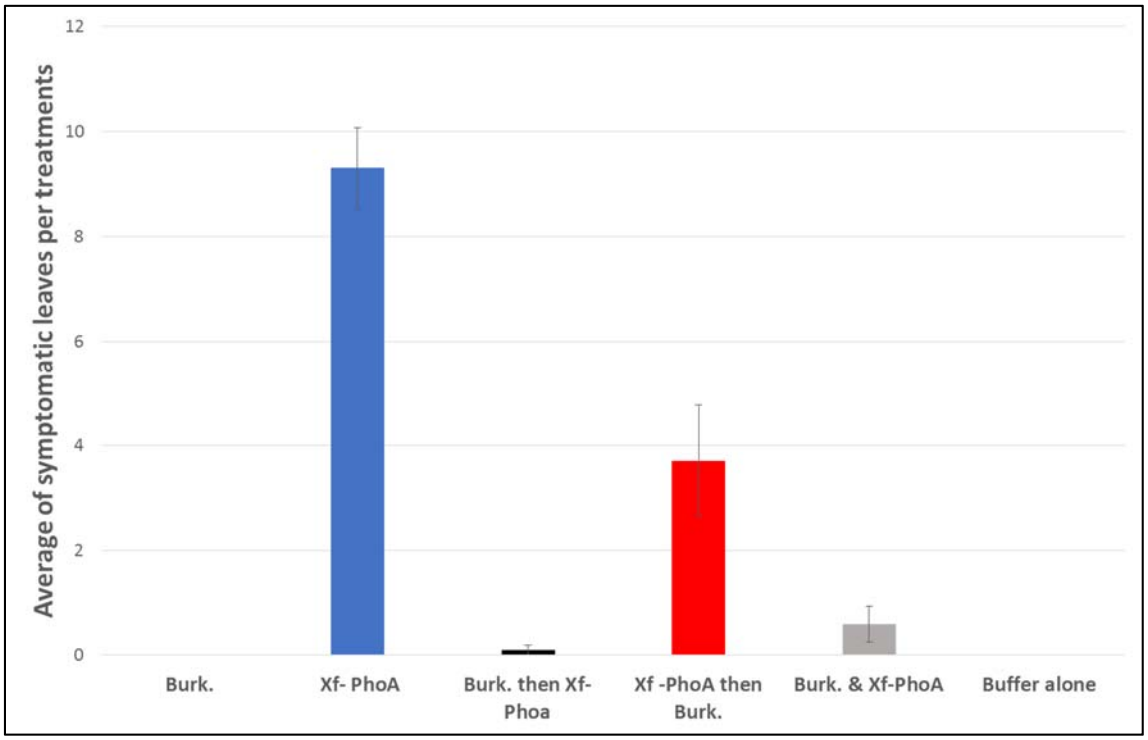

Figure 6. Severity of Pierce's disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants inoculated only with *B. phytofirmans*, only with *X. fastidiosa* (blue bar), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (gray bar). Also shown is disease severity on plants inoculated with *B. phytofirmans* 30 days before inoculation with *X. fastidiosa* (black bar) as well as on plants inoculated with *X. fastidiosa* 30 days after inoculation with *B. phytofirmans* (red bar). The vertical bars represent the standard error of the determination mean disease severity.

While the mechanism by which *B. phytofirmans* reduces the severity of Pierce's disease remains somewhat unclear, the biological control activity conferred by this bacterium is associated with its ability to reduce the population size of *X. fastidiosa* in inoculated plants. The population size of *X. fastidiosa* at various locations along the stem from the point of inoculation in plants inoculated only with the pathogen or inoculated both with the pathogen and *B. phytofirmans* in the experiment described in **Figure 6** were assessed. Relatively high population sizes of *X. fastidiosa* were recovered from stem segments collected from 30 to 300 cm away from the point of inoculation in plants inoculated only with the pathogen (**Figure 7**). As expected, the highest population sizes were seen within the first 120 cm, but population sizes greater than 100 cells per gram were observed as much as 200 cm away from the point of inoculation. In contrast, the population size of *X. fastidiosa* was much lower at a given distance away from the point of inoculation in plants co-inoculated with *X. fastidiosa* and *B. phytofirmans* (**Figure 8**). Whereas population sizes of the pathogen were usually in excess of 10^4 cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated with the pathogen alone, the pathogen population sizes were much lower, decreasing from a high of $10^{2.5}$ to less than 10 cells per gram in plants co-inoculated with *B. phytofirmans* (**Figure 8**). Consistent with the somewhat lower ability to produce severity of Pierce's disease, the reduction in population sizes of *X. fastidiosa* conferred by inoculation of plants with *B. phytofirmans* 30 days after that of the pathogen were somewhat less than that conferred by co-inoculation (**Figure 9**). While population sizes of *X. fastidiosa* were generally in excess of 10^4 cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated only with the pathogen, population sizes were generally less than about 100 cells/g in plants inoculated with *B. phytofirmans* 30 days after inoculation with the pathogen (**Figure 9**). When considered over all treatments, there was a clear relationship between the population size of *X. fastidiosa* in the stems of plants treated with *B. phytofirmans* or not and the severity of Pierce's disease (**Figure 10**). Surprisingly, when assessed 12 weeks after inoculation, the population sizes of *B. phytofirmans* in inoculated plants, irrespective of whether *X. fastidiosa* was also inoculated into the grape plants, were quite low,

but disease severity was lowest in those plants in which *B. phytofirmans* populations were the highest (**Figure 11**). Given that previous experiments had shown that *B. phytofirmans* had rapidly colonized grape and had achieved relatively large population sizes at considerable distances away from the point of inoculation within six weeks (**Figure 5**), it appears that viable cells did not persist in plants for as much as 12 weeks. These results suggest that the interactions of *B. phytofirmans* with either the plant or *X. fastidiosa* occur early in the infection process. The fact that the inoculation of plants with *B. phytofirmans* reduces population sizes of *X. fastidiosa* most at sites distal to the point of inoculation suggests that it had reduced the motility of the pathogen. Such an effect would be expected if it stimulated DSF-mediated quorum sensing. That is, the behavior of *X. fastidiosa* in plants treated with *B. phytofirmans* was similar to that seen in transgenic plants harboring *X. fastidiosa rpfF* that produce DSF.

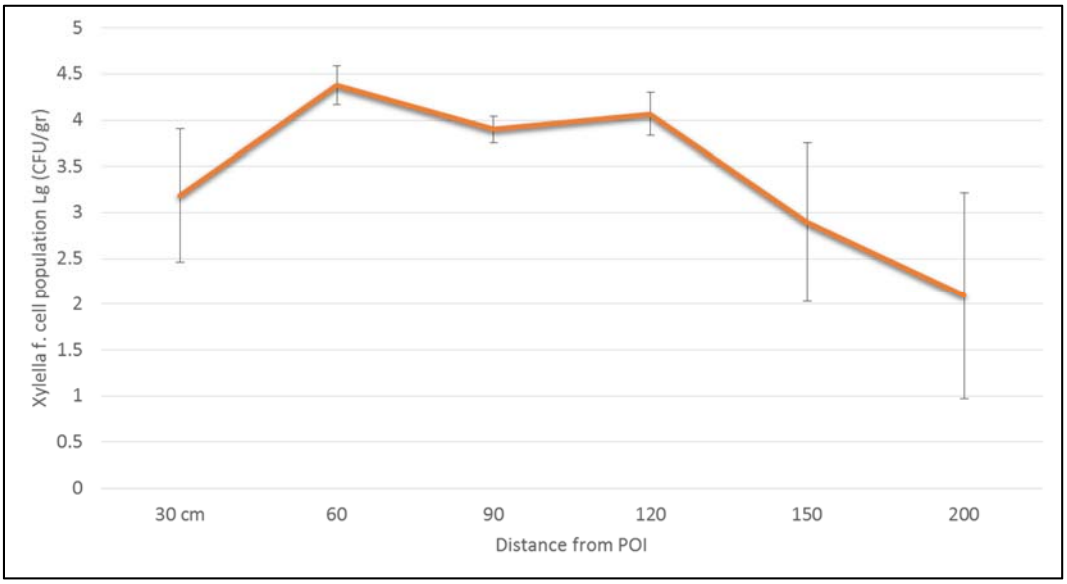

Figure 7. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. The vertical bars represent the standard error of the mean population size/g.

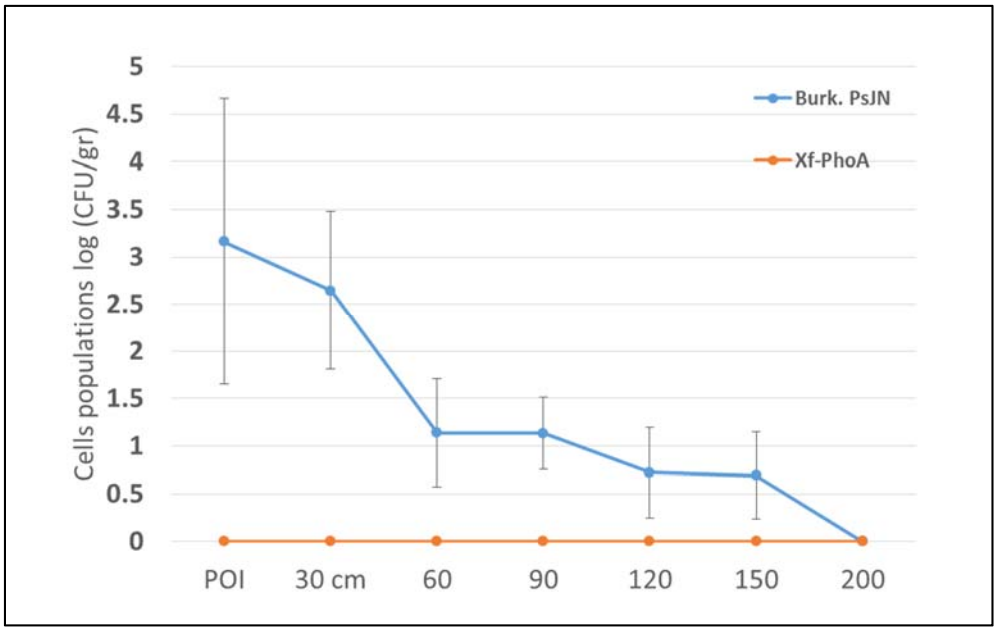

Figure 8. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen when co-inoculated with *B. phytofirmans* (blue) or populations of *B. phytofirmans* (orange). The vertical bars represent the standard error of the mean population size/g.

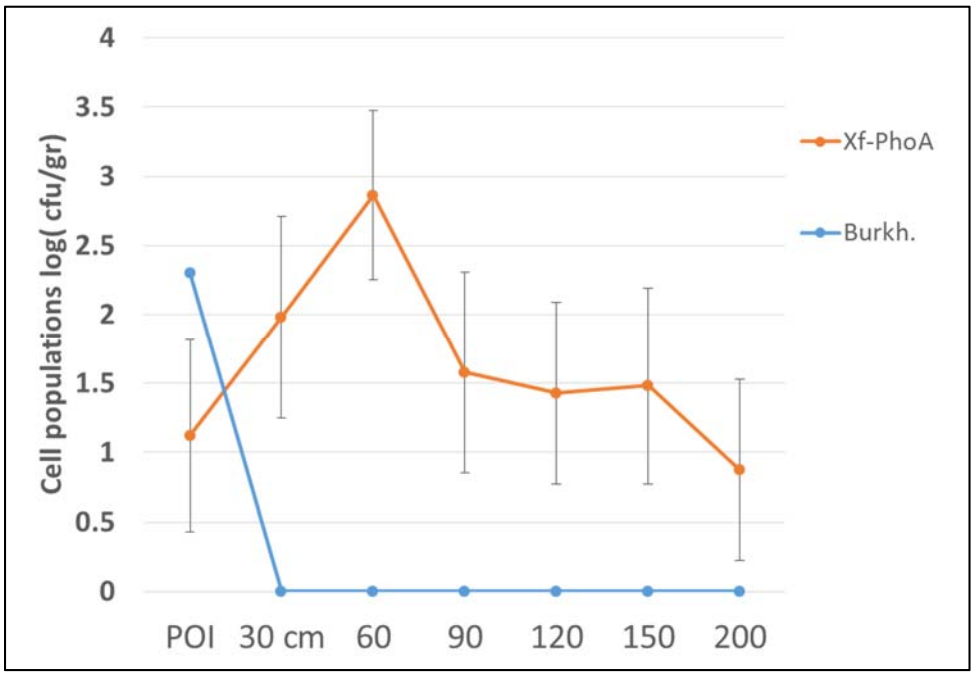

Figure 9. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen when inoculated with *B. phytofirmans* 30 days after the pathogen (orange) or *B. phytofirmans* (blue). The vertical bars represent the standard error of the mean population size/g.

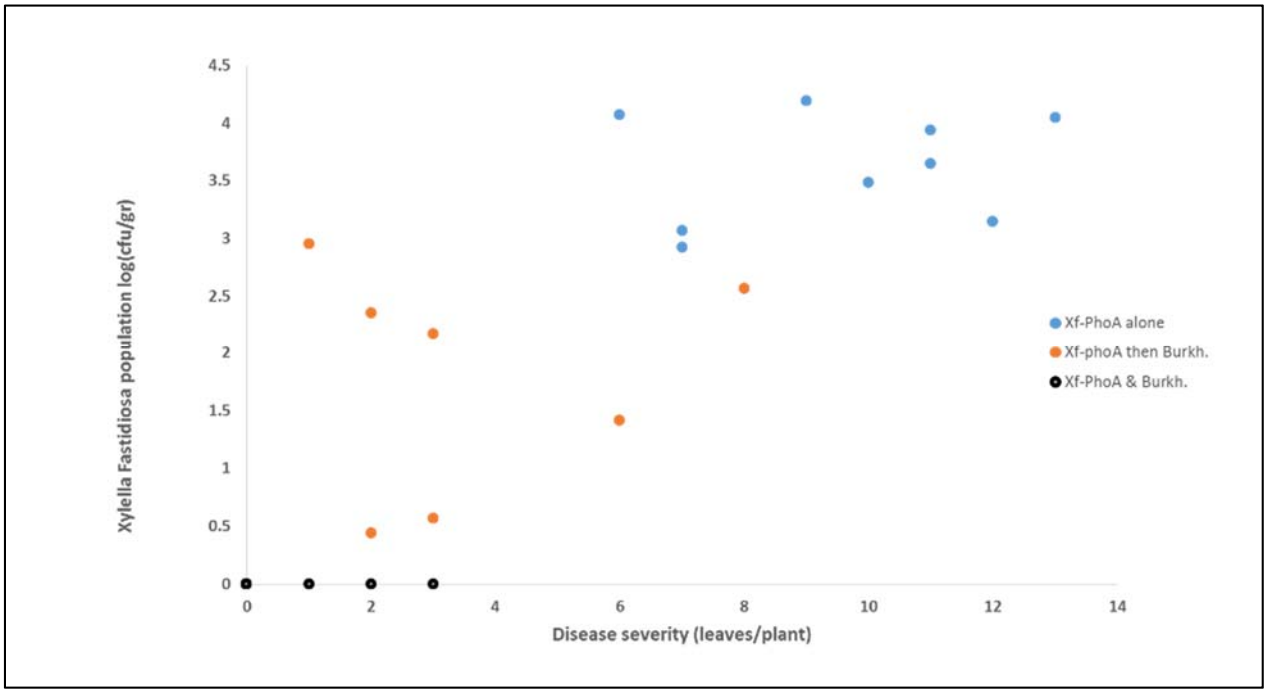

Figure 10. Relationship between mean population size of *X. fastidiosa* recovered from plants 12 weeks after inoculation of plants with only the pathogen (blue symbols), inoculated with *B. phytofirmans* 30 days after the pathogen (orange symbols), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (black symbols). Each symbol represents mean population sizes achieved in a given plant.

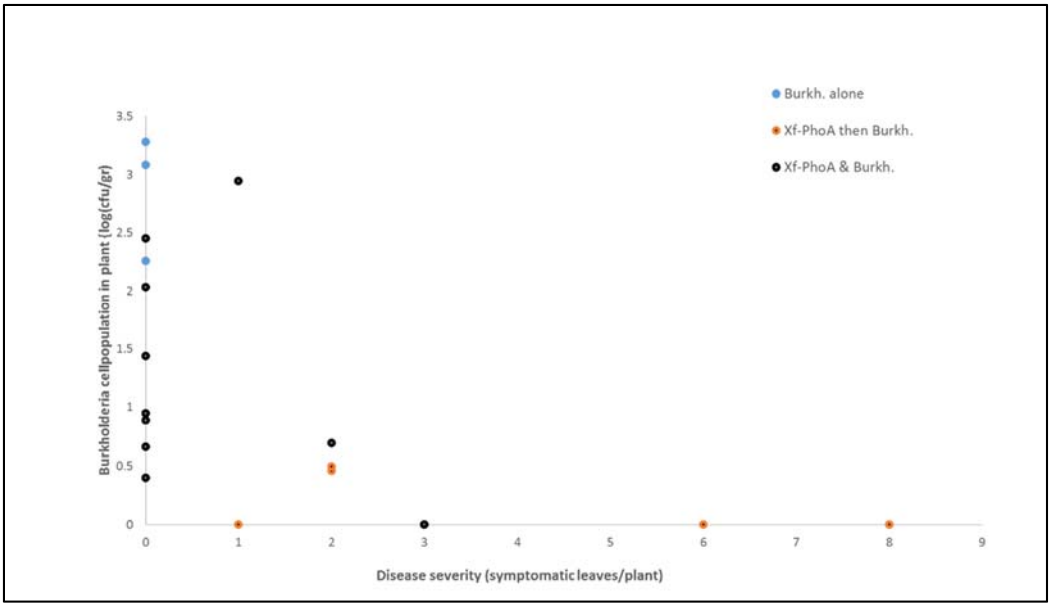

Figure 11. Relationship between mean population size of *B. phytofirmans* recovered from plants 12 weeks after inoculation of plants with *B. phytofirmans* only (blue symbols), inoculated with *B. phytofirmans* 30 days after the pathogen (orange symbols), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (black symbols). Each symbol represents mean population sizes achieved in a given plant.

While the droplet puncture method used in **Figure 5** to introduce *B. phytofirmans* is an effective way to introduce bacteria into the xylem, we have investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.05% Silwet L77, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *B. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in L77 (10^8 cells/) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *B. phytofirmans* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L77 or Breakthru (**Figure 12**). Very few cells were present in petioles when the bacterium was applied without a penetrating surfactant. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

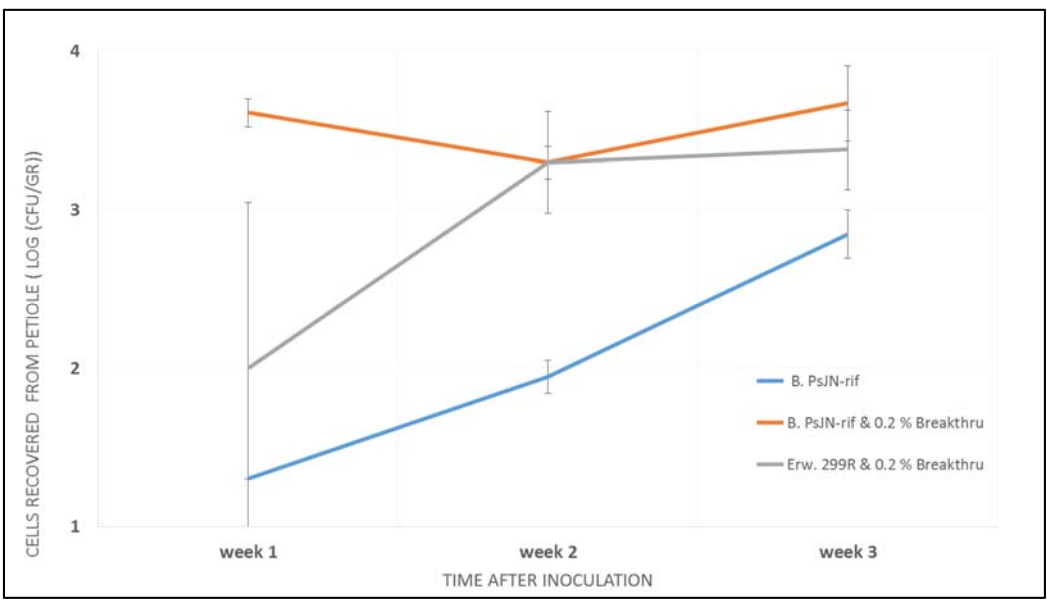

Figure 12. Population size of *B. phytofirmans* in petioles of Cabernet Sauvignon plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line), or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent the mean of the log population size at a given sampling time.

The ability of *B. phytofirmans* to achieve biological control of Pierce's disease when co-inoculated with *X. fastidiosa* and when applied at various times prior to that of the pathogen was further assessed by measuring the population sizes of both *X. fastidiosa* and *B. phytofirmans* at various points distal to the point of pathogen inoculation, as well as of disease symptoms at weekly intervals as above. As was observed previously, *B. phytofirmans* cells moved well beyond the point of inoculation and reached relatively high population sizes either four weeks after inoculation (data not shown), or eight weeks after inoculation (**Figure 13**). Detectable populations of *B. phytofirmans* could be found as much as 120 cm away from the point of inoculation within eight weeks of inoculation. Importantly, while high populations of *X. fastidiosa* were found throughout plants, to a distance of at least 120 cm from the point of inoculation when measured eight weeks after inoculation, no detectable cells of the pathogen could be found at this time in plants co-inoculated with *B. phytofirmans* (**Figure 13**).

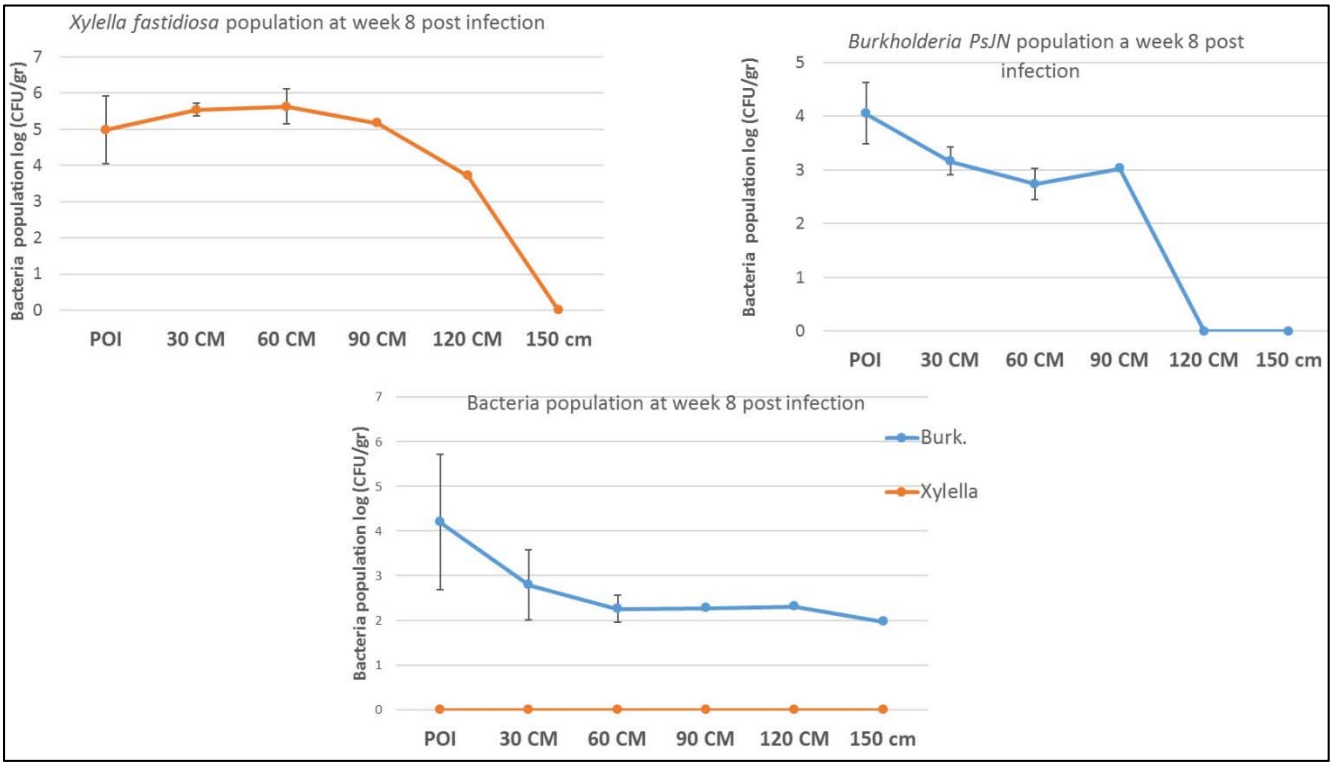

Figure 13. Population size of *X. fastidiosa* in various locations from the point of inoculation in stems of Cabernet Sauvignon plants inoculated only with the pathogen (top left), or in plants co-inoculated with *B. phytofirmans* (orange symbols, bottom). Also shown is a population size of *B. phytofirmans* in plants inoculated only with this biological control agent (top right), or in plants co-inoculated with *X. fastidiosa* (blue line, bottom). The vertical bars represent the standard error of the determination of the mean population size normalized per gram of tissue.

Assessment of disease severity in Cabernet Sauvignon plants treated with *B. phytofirmans* in various ways both before and after inoculation with *X. fastidiosa* revealed that dramatic reductions in disease occurred, irrespective of the matter in which the biological control agent was applied (**Figure 14**). As observed before, no disease symptoms were observed when *B. phytofirmans* was co-inoculated with *X. fastidiosa* by needle puncture, consistent with the lack of cells of the pathogen remaining in plants co-inoculated with this biological control agent (**Figures 13 and 14**). In very exciting results we noted that disease severity was greatly reduced on plants in which *B. phytofirmans* was applied by spray inoculation shortly before that of the pathogen (**Figure 14**). Likewise, inoculation of *B. phytofirmans* even 30 days after that of the pathogen led to a great reduction in disease severity (**Figure 14**). Additional experiments are underway to determine to what extent the reduction in populations of the pathogen in plants inoculated with *B. phytofirmans* require a coincidence of the pathogen and the biological control agent to be mediated, and the extent to which the suppression of the pathogen and hence disease symptoms might be conferred by an effect of *B. phytofirmans* on the plant itself, such as by inducing some form of host defense. These results, however, are quite exciting in that they reveal that biological control of Pierce's disease using *B. phytofirmans* is both robust and may be relatively easy to apply by various ways of inoculation.

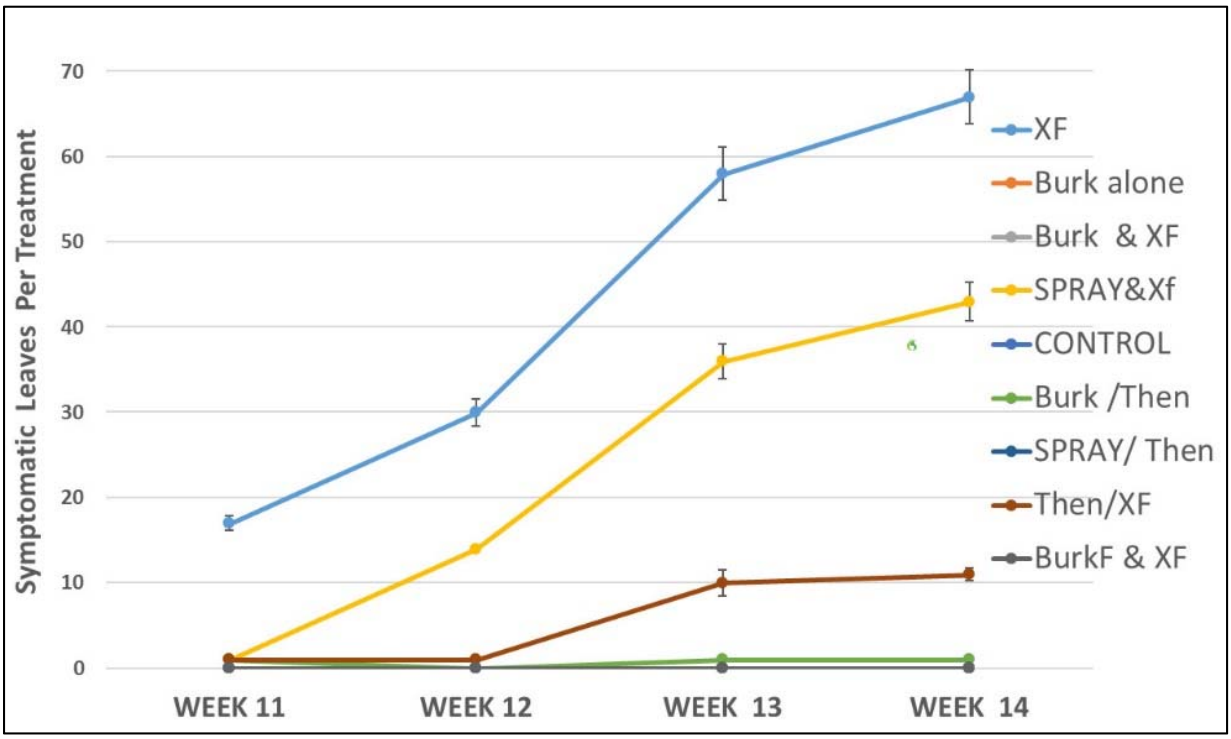

Figure 14. Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (light blue); inoculation of *X. fastidiosa* to plants in which *B. phytofirmans* had been applied by spray inoculation with 0.2% Breakthru (yellow); co-inoculation of *X. fastidiosa* and *B. phytofirmans* by needle (gray); *B. phytofirmans* alone needle-inoculated (orange); co-inoculation of *X. fastidiosa* and a *B. phytofirmans* rpf mutant by needle inoculation (black); mock inoculation with buffer only (dark blue); inoculation of *B. phytofirmans* by droplet puncture 30 days prior to that of *X. fastidiosa* (green); inoculation of *B. phytofirmans* by spray application with 0.2% Breakthru 30 days before inoculation with *X. fastidiosa* (purple); inoculation with *X. fastidiosa* 30 days before inoculation with *B. phytofirmans* (red); and mock inoculation with buffer (dark blue). The vertical bars represent the standard error of the determination of the mean number of symptomatic leaves measured at a given time.

Considerable effort has been made during this reporting period to better understand the mechanisms by which *B. phytofirmans* alters the behavior of *X. fastidiosa* in plants. DSF production has been described in other *Burkholderia* species including *B. ceonocepacia*. Furthermore, the genome sequence of *B. phytofirmans* PsJN has been determined, allowing us to putatively identify a gene with some homology to *X. fastidiosa* and *Xanthomonas campestris rpfF*, that thus might be expected to lead to the production of fatty acids capable of conferring signaling activity like that of DSF species. We therefore made a site-directed deletion mutant of the putative *rpfF* gene in *B. phytofirmans*. We subsequently investigated whether ethyl acetate extracts of wild-type *B. phytofirmans* culture supernatants or *rpfF* mutants of *B. phytofirmans* could alter the expression of genes in either *X. campestris* or *X. fastidiosa* that were known to be regulated by the presence of various DSF species. Interestingly, relatively strong induction of the *eng:gfp* reporter gene fusion in *X. campestris* was observed when the biosensor was exposed to extracts of both the wild-type and *rpfF* mutant of *B. phytofirmans* (**Figures 15 and 16**). These results suggest that, indeed, *B. phytofirmans* was capable of producing a DSF-like molecule that *X. campestris* could respond to. It also suggested, however, that the putative *rpfF* gene that we had removed was not responsible for producing the putative signal molecule. In contrast to the results that revealed that *X. campestris* could respond to the putative signal molecule from *B. phytofirmans,* little or no change in expression of the *phoA* reporter gene was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to ethyl acetate extracts of either the wild-type or *rpfF* mutant of *B. phytofirmans* (data not shown). Given that *X. fastidiosa* and *X. campestris* respond to different DSF species, it was not unexpected that they might differentially respond to the signal molecule apparently made by *B. phytofirmans*.

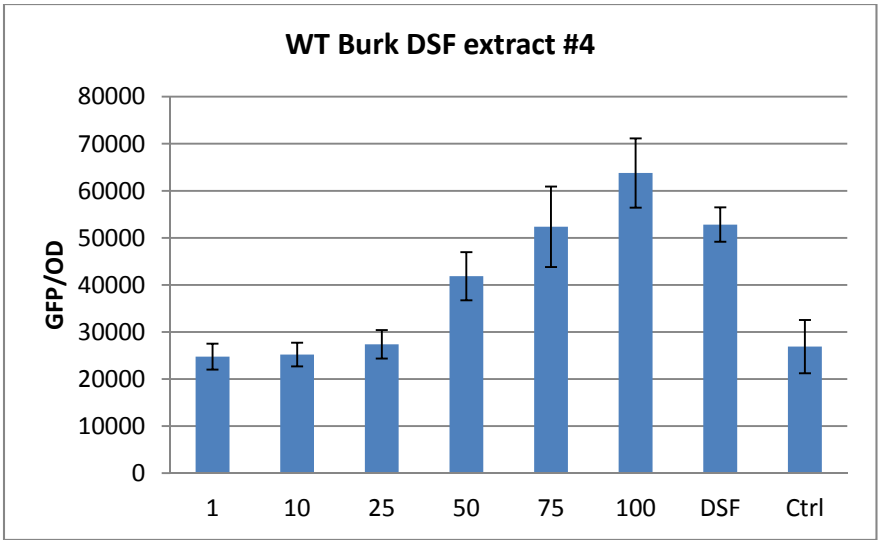

Figure 15. Normalized GFP fluorescense exhibited by the *X. campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild-type *B. phytofirmans* strain. Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, or to no added material (ctrl).

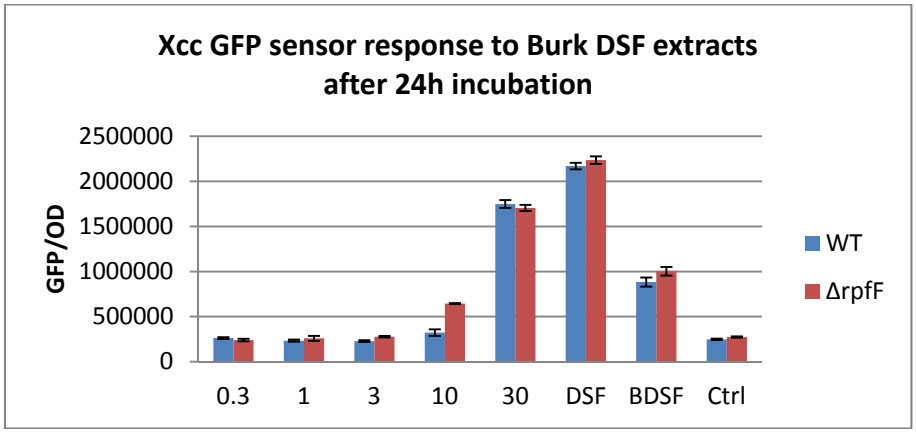

Figure 16. Normalized green fluorescent protein (GFP) fluorescense exhibited by the *X. campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild-type *B. phytofirmans* (blue bars) or an *rpfF* mutant (red bars). Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, 1 uM *Burkholderia* diffusible signal factor (BDSF), or to no added material (ctrl).

While we did not detect a change in apparent expression of the *hxfA* promoter linked to the *phoA* reporter gene in the *X. fastidiosa Xf:phoA* biosensor when it was exposed to either ethyl acetate extracts of culture supernatants of *B. phytofirmans* or small amounts of culture supernatant themselves, we observed that the biofilm formation (apparent adhesiveness) of *X. fastidiosa* was dramatically higher when either ethyl acetate extracts of culture supernatant or culture supernatant itself from *B. phytofirmans* was added to cultures of either wild-type or rpfF* mutants of *X. fastidiosa* (**Figure 17**). Not only was the amount of bacterial biomass that accumulated in the "ring" which formed at the media/air interface and shake cultures greater, but more importantly, substantial numbers of cells of *X. fastidiosa* adhered to the walls of class culture flasks below the ring, in the area exposed to turbulent mixing of the culture during shaking (**Figure 15**). These results suggested that the adhesiveness of *X. fastidiosa* was dramatically higher in the presence of some component of the culture supernatant of *B. phytofirmans.* Furthermore, the fact that biofilm formation was by extracts of both the wild-type and putative *rpfF* mutant of *B. phytofirmans* suggested that the putative *rpfF* gene of *B. phytofirmans* was not involved in production of the

signal molecule that induced biofilm formation. The enhanced biofilm formation conferred by culture supernatants of *B. phytofirmans* was readily quantified by determining the biomass of *X. fastidiosa* cells by crystal violet staining (**Figure 18**). The biomass of *X. fastidiosa* cells in biofilms was over two-fold higher in the presence of culture supernatant of either a wild-type or rpfF mutant of *B. phytofirmans* (**Figure 18**).

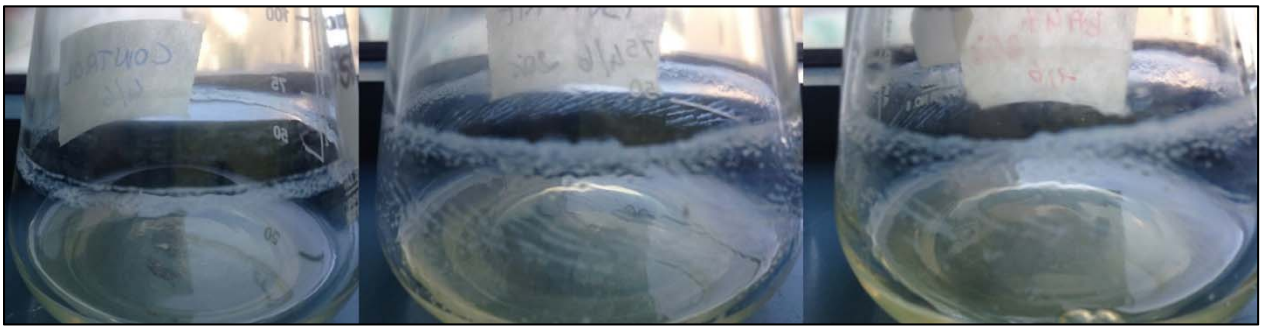

Figure 17. Biofilm formation of wild-type *X. fastidiosa* grown in PD three media alone (left), or in media containing 20%v/v of culture supernatant of wild-type *B. phytofirmans* (center) or a putative *rpfF* mutant of *B. phytofirmans* (right).

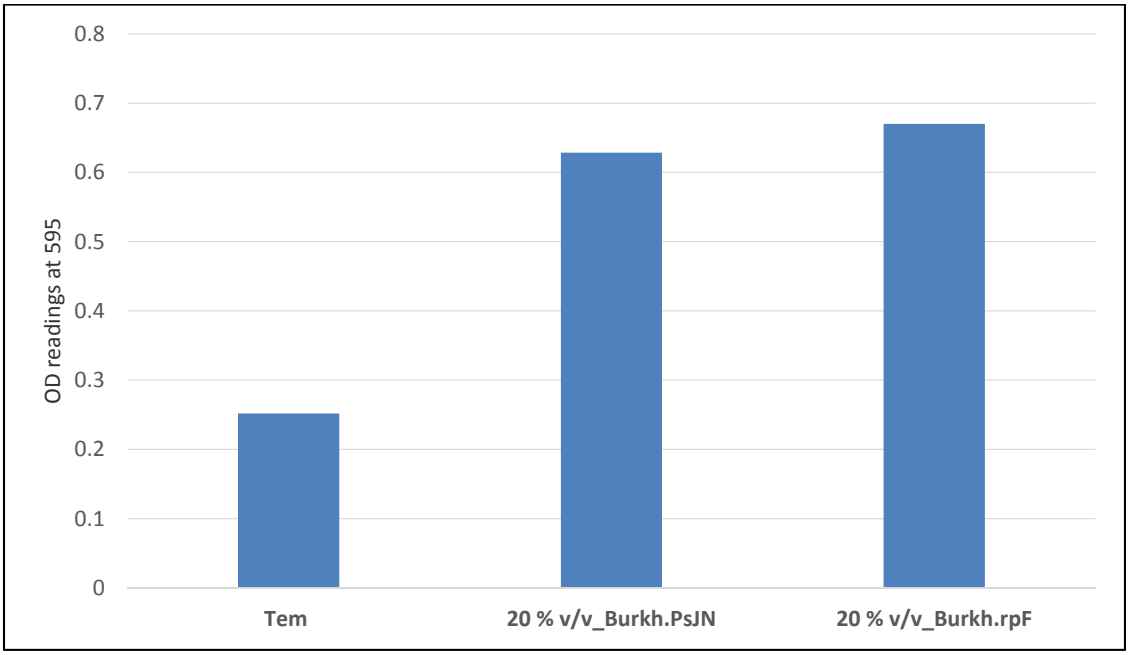

Figure 18. Optical density measured at 595 nm of ethanol extracts of biofilms of *X. fastidiosa* recovered from shake cultures as in **Figure 15** after staining with crystal violet.

Interestingly, a large increase in biofilm formation could be conferred by relatively small amounts of extracts of either wild-type or the *rpfF* mutant of *B. phytofirmans*, while higher concentrations appeared to lead to some inhibition of *X. fastidiosa* growth, and hence biofilm formation (**Figure 19**). These results are quite exciting in that they suggest strongly that *B. phytofirmans* produces a signal molecule to which *X. fastidiosa* responds, leading to its increased adhesiveness. It is unclear whether the signal molecule is a fatty acid related to DSF. It is quite possible that *X. fastidiosa* can perceive the putative signal molecule of *B. phytofirmans* using receptors different from those used to detect DSF itself, and that detection of the putative signal molecule of *B. phytofirmans* might lead to expression of somewhat different genes than of DSF itself. Work to determine the identity of the signal molecule is underway. The ability of this putative signal molecule to increase the apparent adhesiveness of *X. fastidiosa* is sufficient to explain the relatively dramatic biological control conferred by co-inoculation or pre-inoculation of plants with *B. phytofirmans.* As with DSF itself, increasing the adhesiveness of *X. fastidiosa* would restrict its ability to move within the plant. Given that the putative signal molecule made by *B. phytofir-*

mans is both a small molecule and active at quite low concentrations, it suggests that it might be readily diffusible throughout the plant, again explaining why biological control conferred by *B. phytofirmans* appears to be so robust.

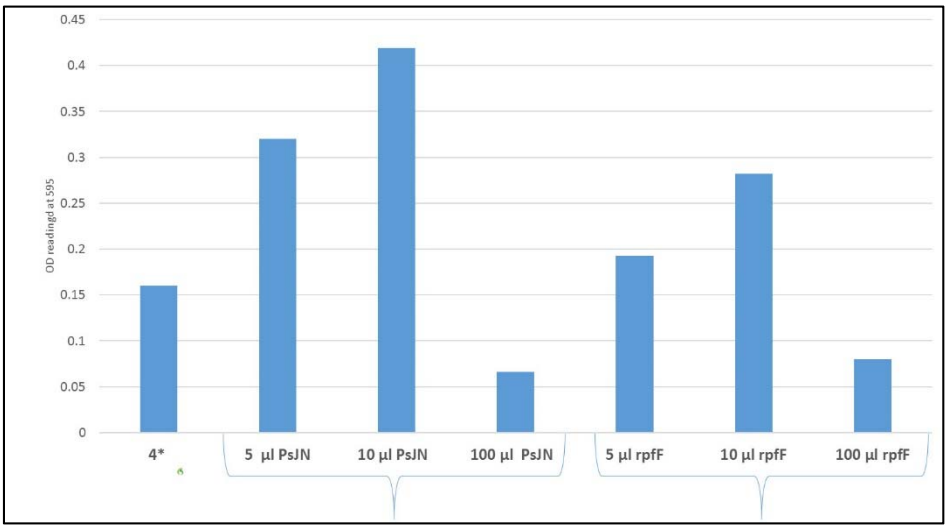

Figure 19. Optical density measured at 595 nm of ethanol extracts of biofilms of an rpfF* mutant of *X. fastidiosa* grown with either no (4*), five, 10, or 100 µL of an ethyl acetate extract ((100 ml of supernatant extracted into 1 ml of solvent) of a wild-type *B. phytofirmans* strain (left), or putative rpfF mutant of *B. phytofirmans* (right), added to cultures when cells were recovered from shake cultures as in **Figure 15** after staining with crystal violet.

CONCLUSIONS

Experimentation is well underway in producing a variety of additional DSF-producing grape varieties. Some of the plants have already been delivered, while the remainder should be delivered within the next few months. Considerable additional work will be needed to assess their production of DSF and disease resistance, but we are optimistic that they also will show at least as high a level of disease resistance as seen in earlier studies with Freedom grapes. Preliminary results using penetrating surfactants to introduce commercially-available fatty acids capable of inducing signaling in *X. fastidiosa* are quite promising, and we feel that this strategy of conferring disease resistance by direct introduction of the signal molecule can be better optimized by further attention on different formulations and delivery mechanisms. We are particularly excited about the opportunities for biological control of Pierce's disease using the endophytic bacterium *Burkholderia phytofirmans*. Not only is the bacterium the first that we have ever found that readily colonizes grape, but we continue to see very dramatically-lower disease severity on plants treated with this bacterium both before and after inoculation with *X. fastidiosa*. These results are quite exciting in that they reveal that biological control of Pierce's disease using *B. phytofirmans* is both robust and may be relatively easy to apply by various ways of inoculation.

FUNDING AGENCIES

Finding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

CONTINUED FIELD EVALUATION OF DIFFUSIBLE SIGNAL FACTOR PRODUCING GRAPE FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted July 2011 to October 2015.

ABSTRACT

A cell density-dependent gene expression system in *Xylella fastidiosa* mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis) controls the behavior of *X. fastidiosa*. The accumulation of DSF attenuates the virulence of *X. fastidiosa* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, XadA, and FimA which make cells sticky and hence suppress its movement in the plant while down-regulating the production of secreted enzymes such as polygalacturonase and endogluconase which are required for digestion of pit membranes and thus for movement through the plant. Artificially increasing DSF levels in plants in various ways increases the resistance of these plants to Pierce's disease. Disease control in the greenhouse can be conferred by production of DSF in transgenic plants expressing the gene for the DSF synthase from *X. fastidiosa*; such plants exhibit high levels of disease resistance when used as scions and confer at least partial control of disease when used as rootstocks. This project is designed to test the robustness of disease control by pathogen confusion under field conditions where plants will be exposed to realistic conditions in the field and especially under conditions of natural inoculation with insect vectors. We are testing two different lineages of DSF-producing plants, both as own-rooted plants as well as rootstocks for susceptible grape varieties in two field sites. Plants were established in one field site in Solano County on August 2, 2010. Plants were planted at a Riverside County site on April 26, 2011. The incidence of infection of inoculated vines has consistently been reduced about three-fold. Disease is observed only near the point of inoculation in transgenic Freedom, but spreads extensively in wild-type Freedom grape. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks. Plants at the Riverside County plot were subject to high levels of natural infection in 2012. The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape, while the number of infected leaves per vine was about five-fold less, suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plants grafted onto wild-type Thompson Seedless rootstocks. Similar levels of resistance of the *rpfF*-expressing Freedom grape relative to wild-type Freedom have been seen in continuing evaluations in 2013, 2014, and 2015.

LAYPERSON SUMMARY

Xylella fastidiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor (DSF) molecule which acts to suppress its virulence in plants. Artificially increasing DSF levels in grape by introducing the *rpfF* gene which encodes a DSF synthase reduces disease severity in greenhouse trials. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for

susceptible grape varieties. Plots in both Solano and Riverside counties reveal that DSF-producing Freedom grape, which was highly resistant to Pierce's disease in greenhouse trials, is also much less susceptible to disease in field trials, especially in plants naturally infected by sharpshooter vectors.

INTRODUCTION

Our work has shown that *Xylella fastidiosa* uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence cells do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition), but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly-colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion" in the greenhouse, more work is needed to understand how well this will translate into disease control under field conditions. That is, the methods of inoculation of plants in the greenhouse may be considered quite aggressive compared to the low levels of inoculum that might be delivered by insect vectors. Likewise, plants in the greenhouse have undetermined levels of stress that might contribute to Pierce's disease symptoms compared to that in the field. Thus, we need to test the relative susceptibility of DSF-producing plants in the field both under conditions where they will be inoculated with the pathogen as well as receive "natural" inoculation with infected sharpshooter vectors.

OBJECTIVES

1. Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2. Determine population size of the pathogen in DSF-producing plants under field conditions.
3. Determine the levels of DSF in transgenic *rpfF*-expressing grape under field conditions as a means of determining their susceptibility to Pierce's disease.

RESULTS AND DISCUSSION

Disease susceptibility of transgenic DSF-producing grape in field trials.

Field tests are being performed with two different genetic constructs of the *rpfF* gene in grape and assessed in two different plant contexts. The *rpfF* has been introduced into Freedom (a rootstock variety) in a way that does not cause it to be directed to any subcellular location (non-targeted). The *rpfF* gene has also been modified to harbor a 5' sequence encoding the leader peptide introduced into grape (Thompson Seedless) as a translational fusion protein with a small peptide sequence from RuBisCO that presumably causes this RpfF fusion gene product to be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). These two transgenic grape varieties are thus being tested as both own-rooted plants as well as rootstocks to which susceptible grape varieties will be grafted. The following treatments are thus being examined in field trials:

Table 1.

Treatment		
1	FT	Non-targeted RpfF Freedom
2	TT	Chloroplast-targeted RpfF Thompson
3	FW	Non-targeted RpfF Freedom as rootstock with normal Thompson scion
4	TTG	Chloroplast-targeted RpfF Thompson as rootstock with normal Thompson scion
5	FWG	Normal Freedom rootstock with normal Thompson scion
6	TWG	Normal Thompson rootstock with normal Thompson scion
7	FW	Normal Freedom
8	TW	Normal Thompson

Treatments 5-8 serve as appropriate controls to allow direct assessment of the effect of DSF expression on disease in own-rooted plants as well as to account for the effects of grafting per se on disease susceptibility of the scions grafted onto DSF-producing rootstocks. One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in a randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about three cm long) from mature vines of plants grown in the greenhouse at

UC Berkeley. The plants were inoculated in May 2012 (no natural inoculum of *X. fastidiosa* occurs in this plot area and so manual inoculation of the vines with the pathogen was performed by needle-inoculation with a suspension of *X. fastidiosa*). At least four vines per plant were inoculated. Each inoculation site received a 20 ul droplet of *X. fastidiosa* containing about 10^6 cells of *X. fastidiosa*. The incidence of infection of the inoculated vines at the Solano County trial was reduced about three-fold in assessments made in August and September (**Figure 1**). Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. Because of the shading of the inoculated vines by subsequent growth of uninoculated vines of the same plant, many of the older leaves had died or had fallen from the plant, especially by the September rating, making it difficult to quantify the number of infected leaves per vine. In August, however, we found that there were about three times as many symptomatic leaves on each inoculated vine of wild-type Freedom than on DSF-producing transgenic Freedom (**Figure 2**). Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The severity of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plants grafted onto wild-type Thompson Seedless rootstocks (**Figure 3**).

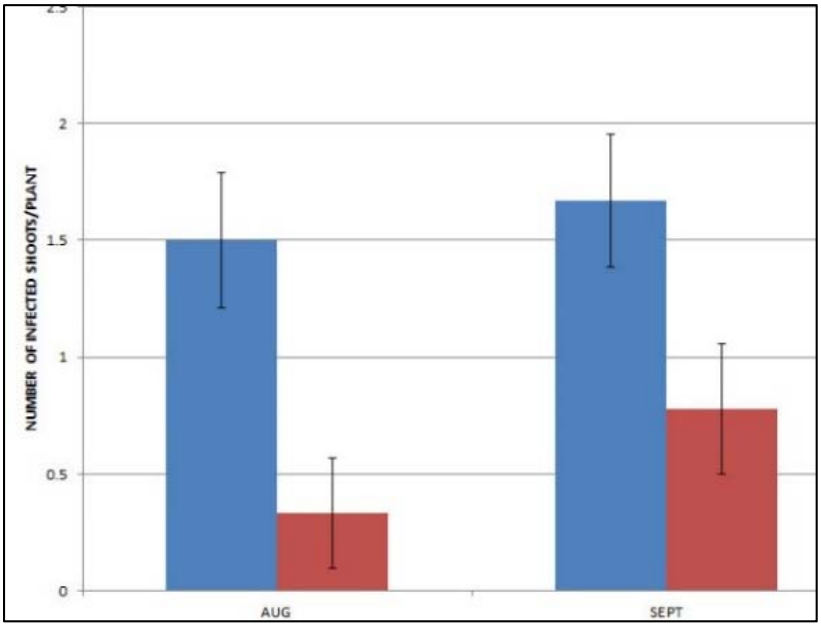


Figure 1. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild-type Freedom having any symptoms of Pierce's disease when rated in August or September 2012. A total of three vines per plant were assessed. The vertical bars represent the standard error of the mean.

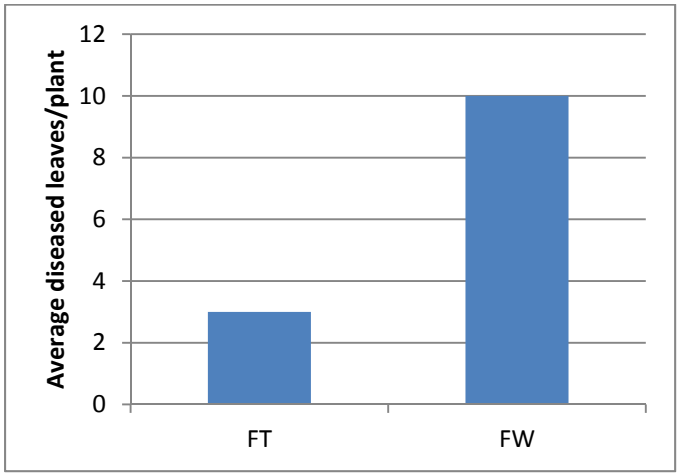


Figure 2. Severity of Pierce's disease on transgenic Freedom grape (FT) and on wild-type Freedom grape assessed in August 2012 in the Solano County trial.

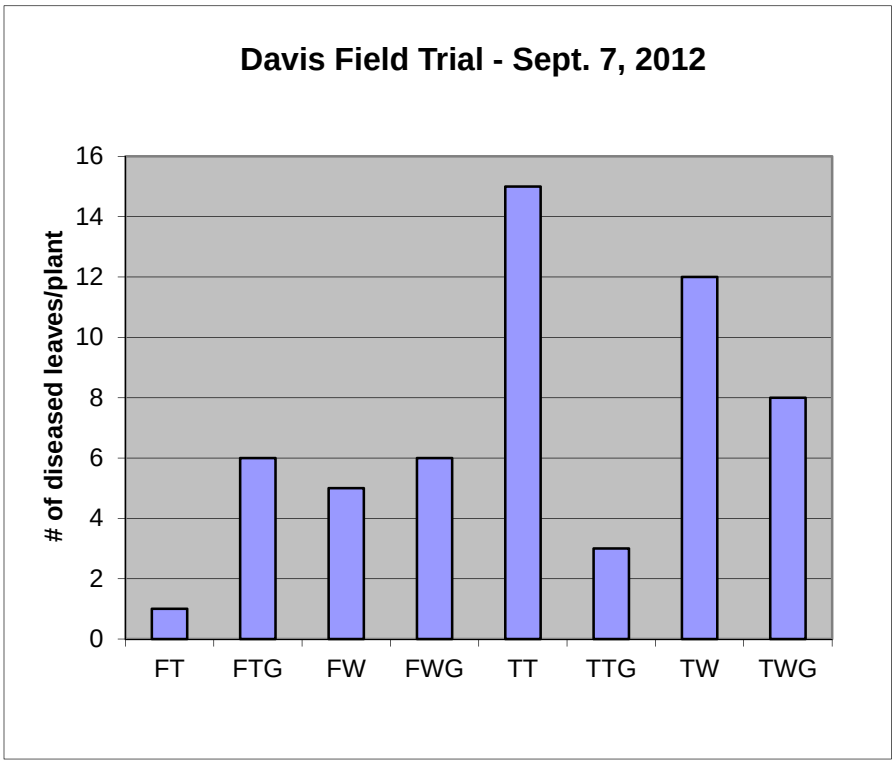


Figure 3. Severity of Pierce's disease on grape assessed in September 2012 in the Solano County trial. See treatment codes above for treatment comparisons.

The plants for the Riverside County trial were planted on April 26, 2011 (**Figure 5**) and have exhibited much less growth than those at the Solano County trial (**Figure 4**). The plants at the Riverside County trial were subjected to natural infection from infested sharpshooter vectors having access to *X. fastidiosa* from surrounding infected grapevines. Very high levels of Pierce's disease were seen in the summer of 2012, although much less symptoms were seen on the transgenic DSF-producing Freedom grape compared to other plants (**Figure 5**).

Figure 4. Establishment of grape trial in Riverside County in April 2010 (left) and photo of plot in October 2012 (right).

Figure 5. Pierce's disease symptoms on transgenic DSF-producing Freedom grape (left) and wild-type Freedom grape (right) on October 4, 2012.

The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape (**Figure 6**), while the number of infected leaves per vine was about five-fold less (**Figure 9**), suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom (**Figure 7**). The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless (**Figure 8**), while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plants grafted onto wild-type Thompson Seedless rootstocks (**Figure 9**). The effectiveness of transgenic Thompson Seedless rootstocks in reducing Pierce's disease was surprising, given that the transgenic Thompson Seedless scions were similar in susceptibility to that of the normal Thompson Seedless scions. We have seen evidence that in addition to DSF chemical species that serve as agonists of cell-cell signaling in *X. fastidiosa*, that transgenic Thompson Seedless may also produce chemical antagonists of cell-cell signaling. It is possible that the DSF agonist is more readily transported into the scion than any antagonists, and thus that DSF-mediated inhibition of pathogen mobility can be conferred by grafted DSF-producing rootstocks.

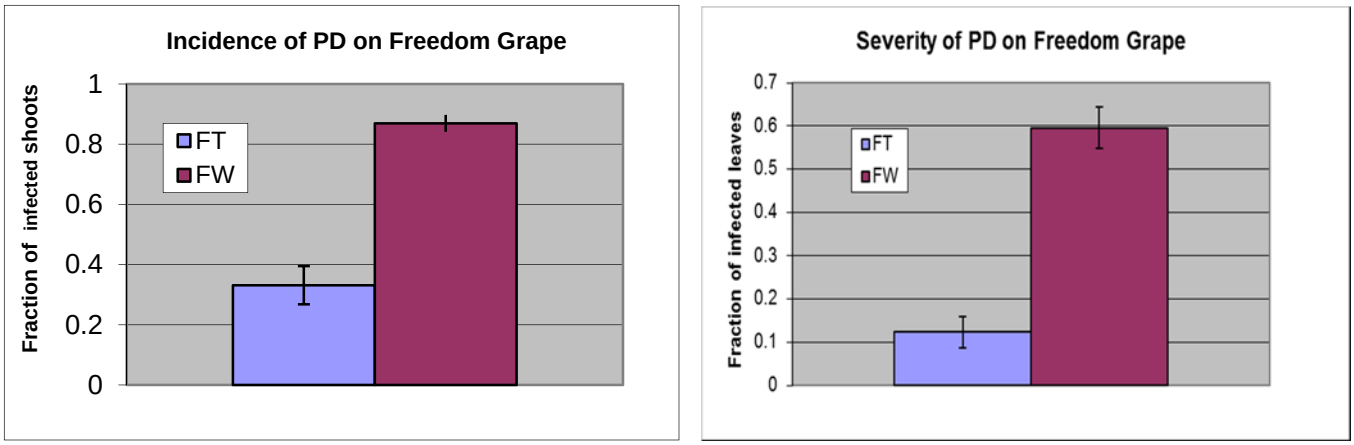
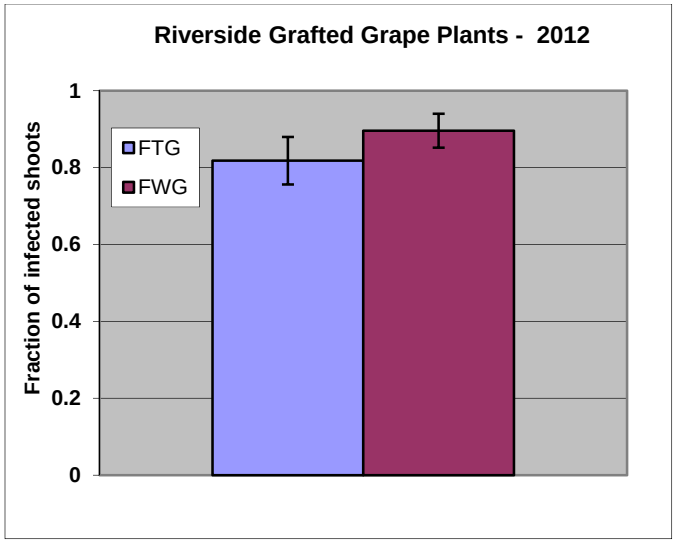


Figure 6. Incidence of Pierce's disease in transgenic DSF-producing Freedom grape (blue bars) or wild-type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.



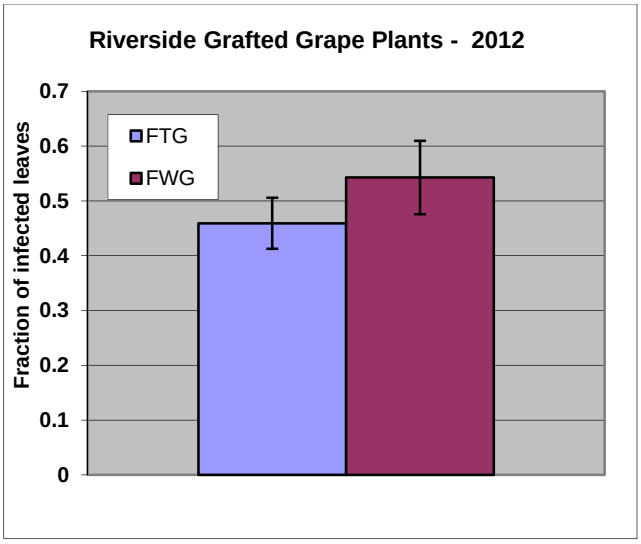
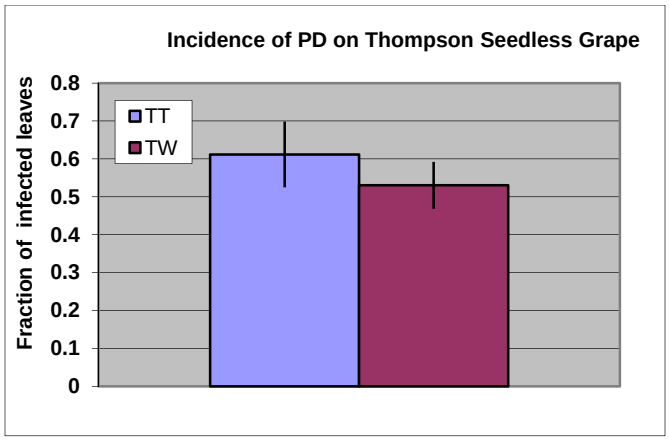


Figure 7. Incidence of Pierce's disease in normal Thompson Seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild-type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.



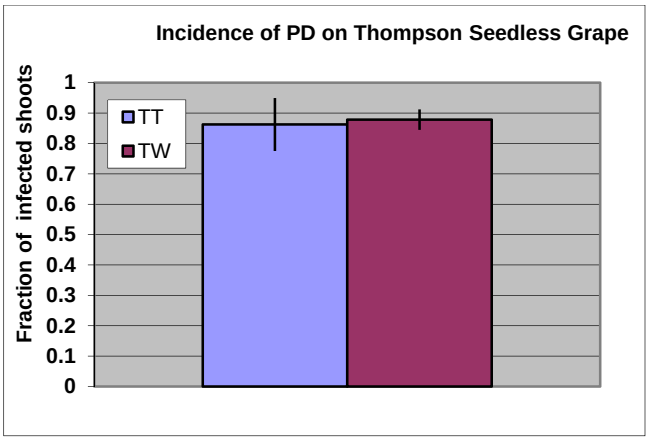
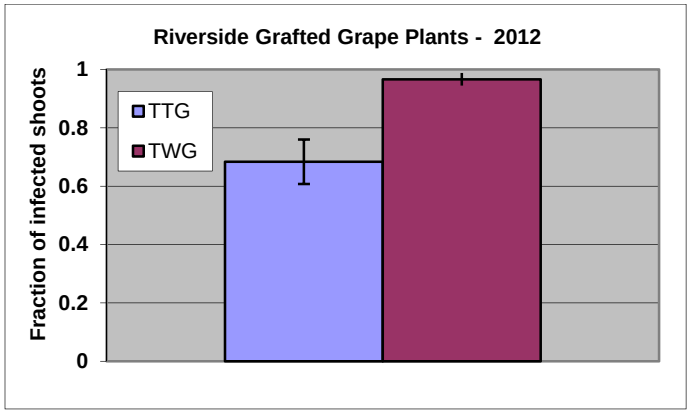


Figure 8. Incidence of Pierce's disease in transgenic DSF-producing Thompson Seedless grape (blue bars) or wild-type Thompson Seedless (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.



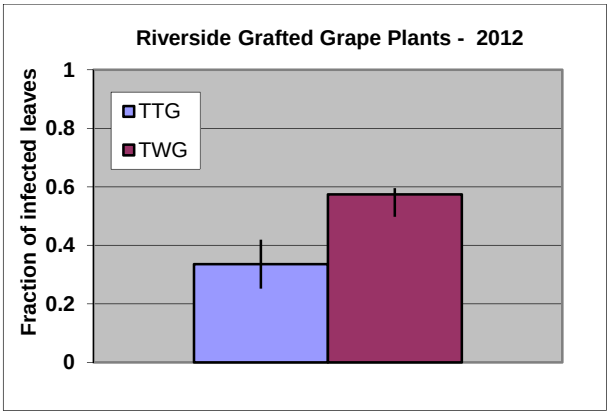


Figure 9. Incidence of Pierce's disease in normal Thompson Seedless grape grafted onto transgenic DSF-producing Thompson Seedless grape rootstocks (blue bars) or wild-type Thompson Seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

On May 15, 2013, plants at the Solano County field trial were evaluated for both the incidence of survival over winter, as well as any symptoms of Pierce's disease that were apparent at this early date. Vines that had been inoculated in 2012 had been marked with a plastic tie. The vines were pruned during the winter of 2012/2013 in a way that retained the inoculation sites and the plastic markers for each of the vines inoculated in 2012. Thus, in May 2013 the return growth on those inoculated, but pruned, vines was assessed. One or more new shoots had emerged from such vines, and the incidence as to whether at least one new shoot had emerged was assessed (**Figure 10**). Nearly all of the inoculated vines from both Freedom and transgenic DSF-producing Freedom gave rise to new shoots as of May 2013 (**Figure 10**). In contrast, many vines of Thompson Seedless inoculated in 2012 were dead, and no shoots emerged in 2013. While most new shoots emerging in 2013 appeared asymptomatic at the time of assessment in May, a few exhibited discoloration, possibly indicating early stages of Pierce's disease. A separate assessment of such possibly symptomatic shoots from that of completely asymptomatic shoots was made (**Figure 11**). It is noteworthy that no symptomatic new shoots were observed on transgenic Freedom, while about 10% of the new shoots emerging from vines of wild-type Freedom exhibited some symptoms (**Figure 11**). It was also noteworthy that a much higher proportion of the vines from Thompson Seedless scions grafted onto a transgenic Freedom rootstock gave rise to new shoots in 2013 compared to those on Freedom rootstocks (**Figures 10 and 11**). Likewise, a higher proportion of vines from Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks gave rise to new shoots in 2013 compared to scions grafted onto normal Thompson Seedless rootstocks (**Figures 10 and 11**). Thus, infection of Thompson Seedless vines by inoculation in 2012 had led to some morbidity of those vines (and even of the cordon on which they were attached in some cases), but Thompson Seedless when grafted onto either transgenic DSF-producing Freedom or transgenic DSF-producing Thompson Seedless rootstocks had a higher likelihood of surviving inoculation in 2012. Continued assessments of disease severity of those new shoots emerging on vines inoculated in 2012 were made in early October 2013, but the data was not fully analyzed at the time of preparation of this report.

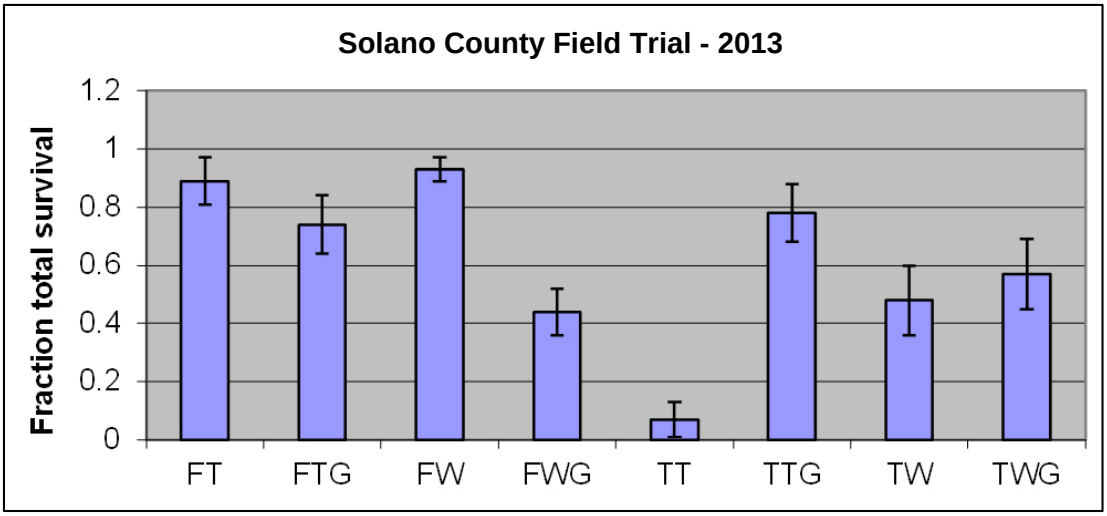


Figure 10. The fraction of vines in the Solano County field trial inoculated in 2012 with *X. fastidiosa* that gave rise to at least one new shoot by May 2013. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean fraction of inoculated vines that gave rise to new shoots in 2013.

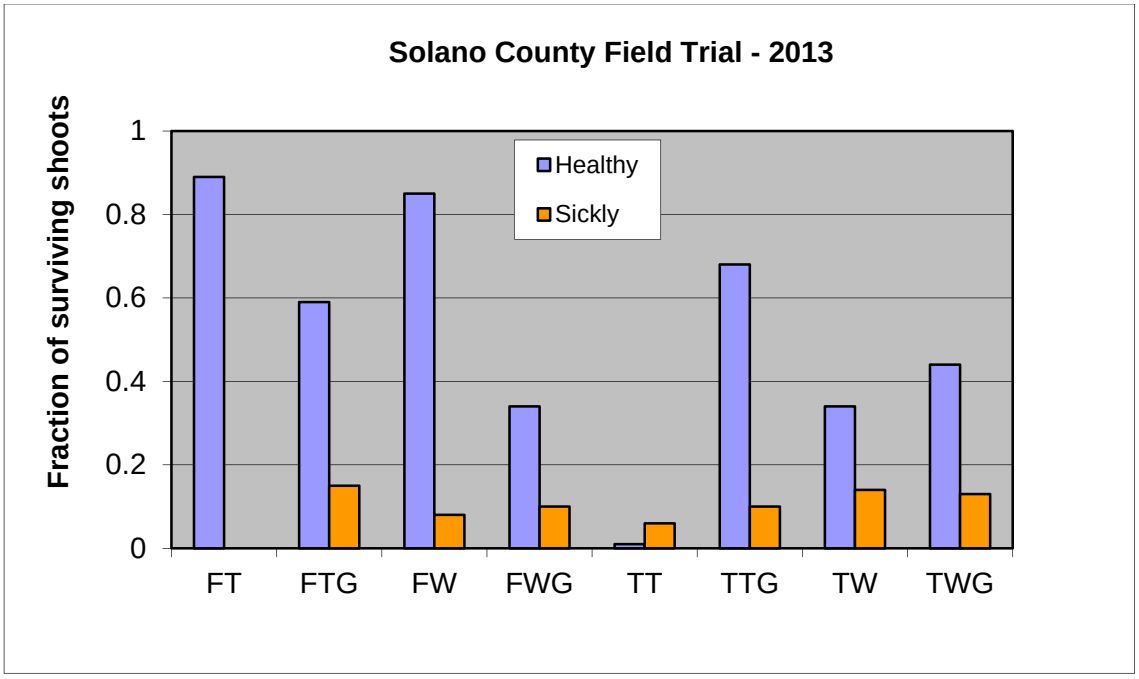


Figure 11. The fraction of vines in the Solano County field trial inoculated in 2012 with *X. fastidiosa* that gave rise to at least one new shoot by May 2013 that exhibited some abnormalities possibly indicative of early stages of Pierce's disease infection (orange bars). Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG).

Vines of transgenic and wild-type Freedom, as well as wild-type and transgenic Thompson Seedless, and Thompson Seedless scions grafted onto the various transgenic or wild-type rootstocks that were apparently healthy and derived from cordons not showing disease in 2013 were again inoculated with *X. fastidiosa* at the Solano County trial on May 28, 2014. The goal of these continuing experiments is to verify the enhanced disease resistance exhibited by transgenic Freedom, and to further quantify the differential susceptibility of Thompson Seedless scions grafted onto various transgenic rootstocks. Disease severity was assessed on August 8 and September 15. In addition, disease incidence and severity that developed in 2014 from vines inoculated in previous years was measured. A uniform rating scale for rating of all vines in both the Solano and Riverside county trials was developed by Lindow and Kirkpatrick. This rating scale will allow the severity of disease on inoculated vines in the year of inoculation to be assessed as the fraction of leaves on a given inoculated vine that are symptomatic. Furthermore, on vines that have been infected for more than one year, this new 0-5 rating scale accounts for return growth and vigor of growth of vines in years subsequent to that year in which it was originally inoculated.

Disease incidence and severity on plants was rated on both August 8 and September 15, 2014. No symptoms were apparent on inoculated vines of either wild-type or transgenic Freedom plants. However, symptoms were apparent on Thompson Seedless vines that had been inoculated earlier in the season. A lower incidence of symptomatic leaves were found on Thompson Seedless vine grafted onto transgenic Freedom rootstocks compared to those on wild-type Freedom rootstocks (**Figure 12**). The incidence of symptomatic leaves on Thompson Seedless vines grafted onto wild-type Thompson Seedless rootstocks did not differ from that on transgenic Thompson Seedless rootstocks. Similarly, the incidence of symptomatic leaves was similar on own rooted Thompson Seedless plants compared to that on transgenic Thompson Seedless plants (**Figure 12**). The overall vigor of Thompson Seedless scions grafted onto transgenic Freedom rootstocks was similar to those grafted onto wild-type Thompson Seedless rootstocks (**Figure 13**). The overall disease severity exhibited by wild-type and transgenic Thompson Seedless plants was also similar, and disease severity on Thompson Seedless scions grafted onto either wild-type or transgenic Thompson Seedless rootstocks also did not differ (**Figure 13**). Thus, some evidence for protection of scions grafted onto RpfF-expressing Freedom rootstocks was again seen in 2014 as in earlier years.

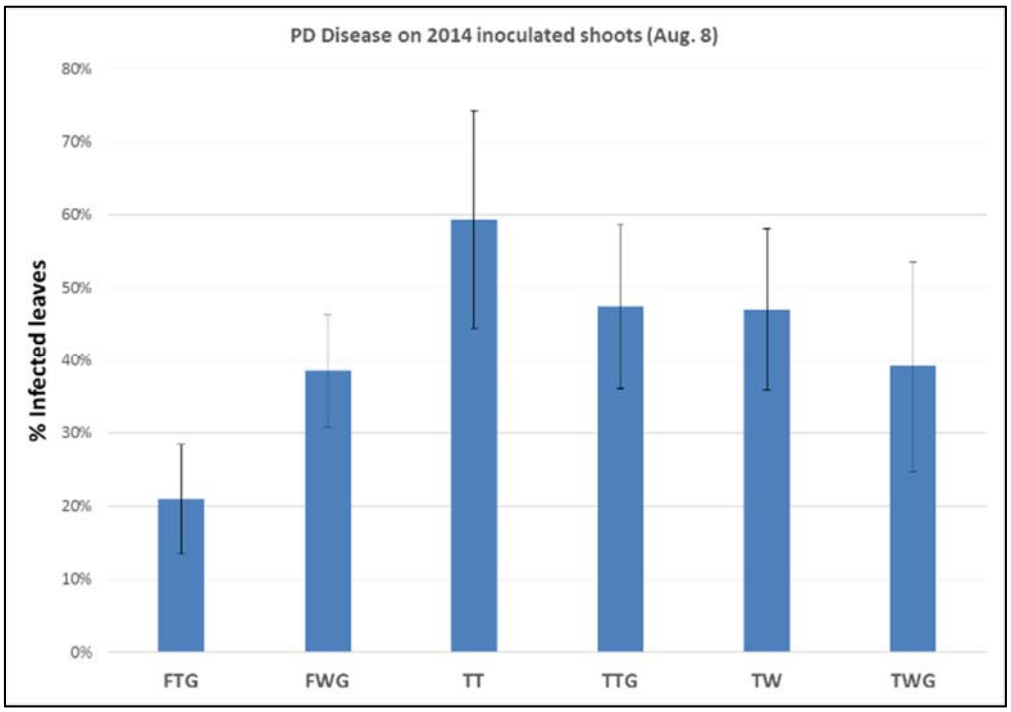


Figure 12. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *X. fastidiosa* that exhibited symptoms of Pierce's disease on August 8, 2014. Treatments include: Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

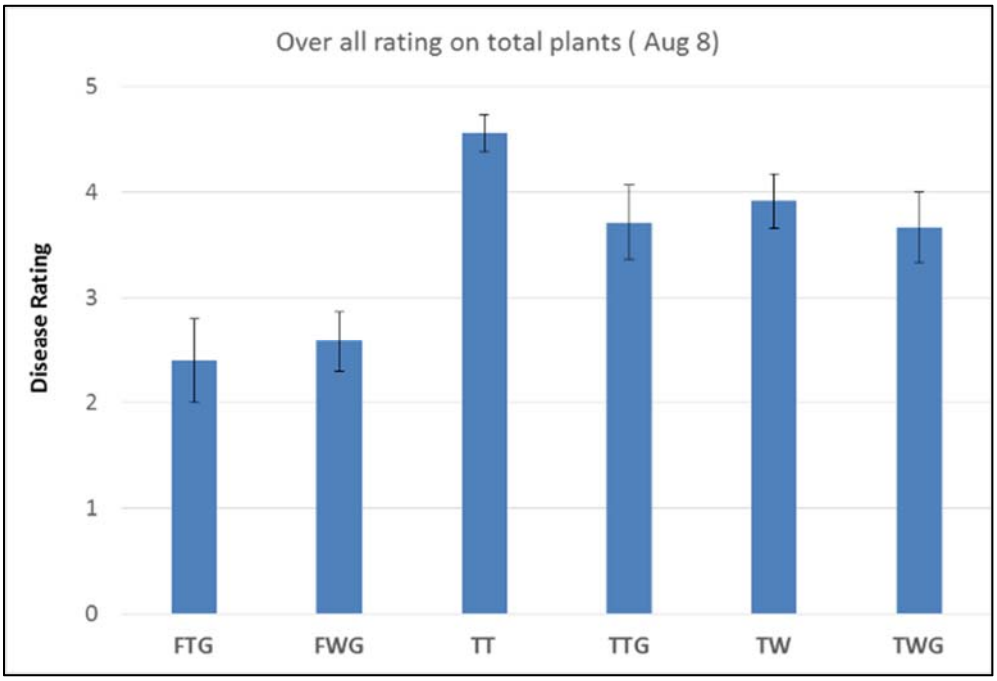


Figure 13. The overall disease rating of vines in the Solano County field trial when assessed on August 8, 2014. Treatments include: Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

The incidence of symptomatic leaves had increased by September 15 from the low levels seen in August. A dramatic difference in the incidence of symptomatic leaves was observed between wild-type and RpfF-expressing Freedom grape. While no symptomatic leaves were observed on the transgenic Freedom plants, over 15% of the leaves on the vines of wild-type Freedom plants that had been inoculated in May were showing symptoms of Pierce's disease (**Figure 14**). As observed in the August evaluation, the incidence of leaves on Thompson Seedless vines grafted to a transgenic Freedom rootstock was lower than that on Thompson Seedless vines grafted onto a wild-type Freedom rootstock (**Figure 14**). An assessment was also made in September of the overall appearance of plants. The disease rating for transgenic Freedom plants was significantly lower than that for wild-type Freedom plants (**Figure 15**). In contrast, while numerically lower, the severity of Thompson Seedless scions grafted onto transgenic Freedom rootstocks did not differ from that of Thompson Seedless scions grafted onto wild-type Freedom rootstocks (**Figure 15**). Thus, the transgenic RpfF-expressing Freedom plants continue to show relatively high resistance to Pierce's disease both in the same season that they are inoculated as well as over several years compared to the wild-type Freedom plants.

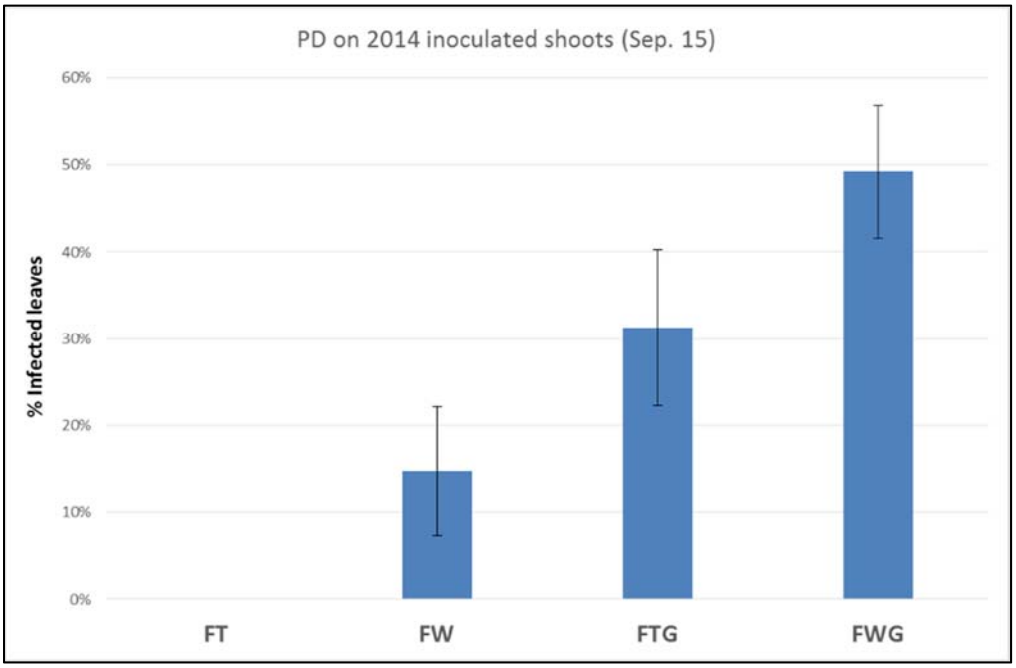


Figure 14. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *X. fastidiosa* that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

Disease was assessed in early October 2014 at the Riverside County trial. In general, the plants had not grown well, with very little new growth, even on plants that were not infected. Overall, the plants did not look thrifty, and appeared to be suffering from other growth limitations such as nematode damage. In many cases, vines did not emerge from a given cordon. The overall disease severity of these plants was high and similar between all treatments (**Figure 17**). Because Freedom plants tend to have many shoots arising from a given cordon, we assessed the disease state of each shoot arising from a given cordon to yield an overall disease severity estimate for these plants. That is, if a given cordon had 10 shoots, two of which had symptoms of Pierce's disease, disease incidence would have been assessed as 20%. While most of the shoots on some plants were healthy, on other plants most of the shoots from a given cordon were infected. Overall, the disease incidence of plants of different treatments were similar, although the incidence of infection of shoots emerging from plants grafted onto transgenic Freedom were somewhat lower than those on plants grafted onto wild-type Freedom rootstocks, as has been observed in ratings in previous years (**Figure 16**).

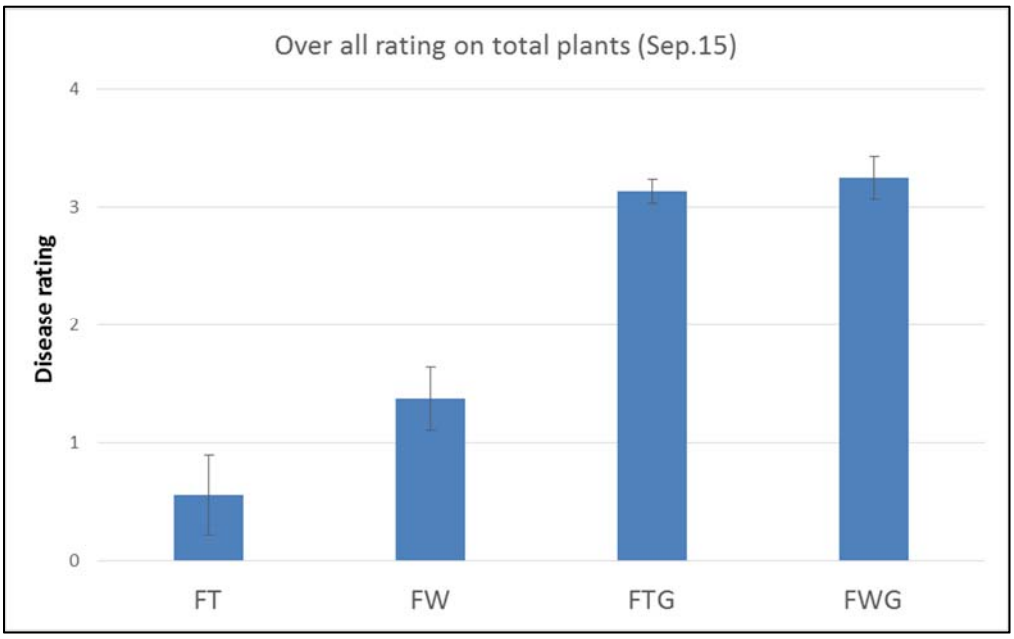


Figure 15. The overall disease rating of vines in the Solano County field trial that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

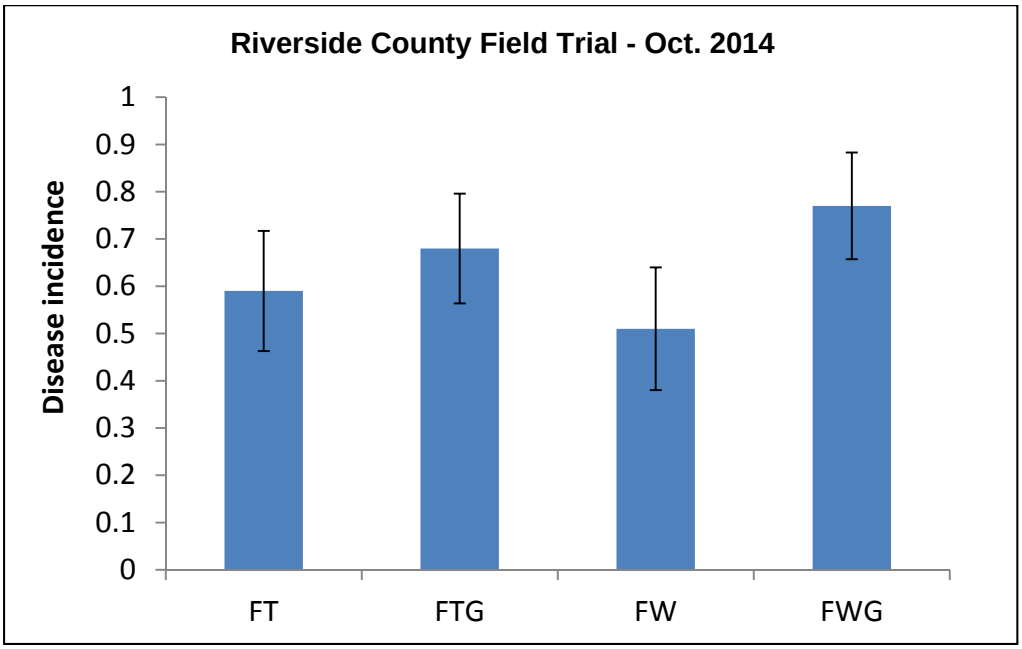


Figure 16. The percentage of vines in the Riverside County field trial that exhibited symptoms of Pierce's disease on October 6, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

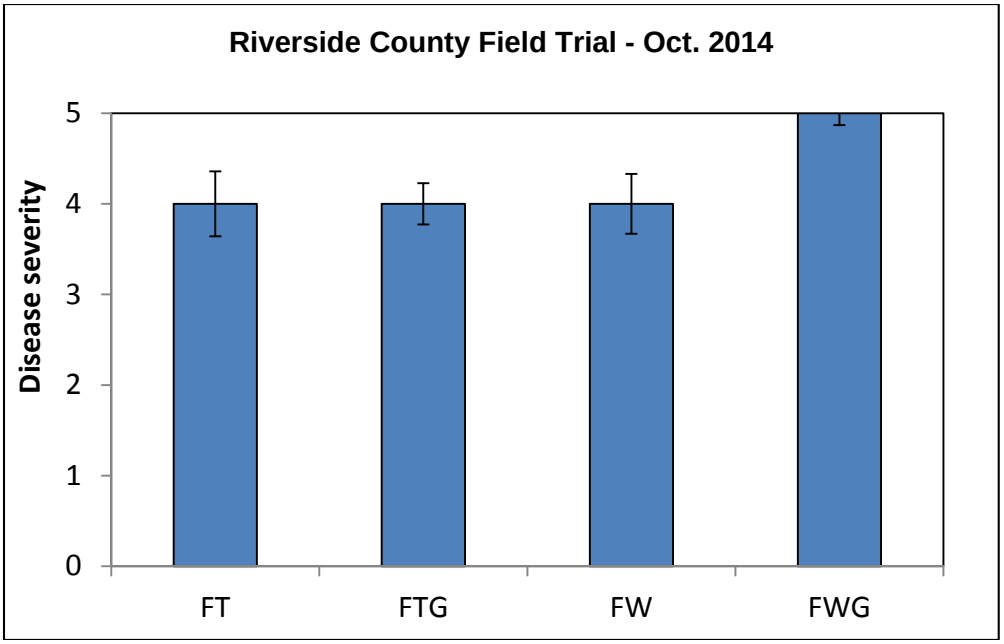


Figure 17. The overall disease rating of vines in the Riverside County field trial that exhibited symptoms of Pierce's disease on October 6, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

All plants at the Solano County trial were evaluated for the incidence and severity of Pierce's disease on May 27, 2015. By this time, all plants that remained alive had generated new shoots. It was apparent that the transgenic Freedom plants had a much lower incidence and severity of symptoms compared to wild-type Freedom own-rooted plants. While virtually no symptoms were observed on the transgenic Freedom (**Figure 18**), all wild-type Freedom exhibited substantial incidence of leaf scorching and stunting associated with Pierce's disease (**Figure 19**). While some plants were dead, others remained alive yet many or most of the cordons were dead or had given rise to only a few leaves or stunted shoots (**Figure 19**).

Figure 18. Images of two separate transgenic Freedom grape plants at the Solano County trial transformed with the *rpfF* gene encoding DSF synthesis from *X. fastidiosa*. These plants are typical of all plants in this treatment in that they show little or no symptoms of Pierce's disease despite the fact that they had been inoculated repeatedly in previous years.

Figure 19. Images of two wild-type Freedom grape plants at the Solano County trial. These plants are typical of all plants in this treatment in that they all showed considerable symptoms of Pierce's disease, ranging from several dead cordons and some stunted growth (left) or severe symptoms or death of most or all cordons on these plants that had been inoculated repeatedly in previous years.

Disease severity of these plants, which had been previously inoculated for each of the previous four years, was quantified by two different scales. In one scale, the incidence of any disease and the severity of disease between cordons is integrated to yield a range from zero (no symptoms) to five (all cordons and shoots dead) was utilized. This rating scale was developed for use by all of the participants in the Solano County trial and has been deemed the "PIPRA" (Public Intellectual Property Resource for Agriculture) rating scale. However, because the vigor of wild-type and transgenic plants obviously differed even though they did not show any disease symptoms, we also rated the plants separately using a different rating scale from zero to five, where zero indicated plants that were quite stunted or dead as of May 2015, and five indicated plants that were quite vigorous and showing no symptoms, and the new growth was as large as the largest plants in the trial. We deem this the "happiness" index of the plants. The plants depicted in **Figure 18** all would have received a rating of five in the scale, while the plants in the left-hand image of **Figure 19** would have received a rating of two and that of the right hand image, a rating of zero. Large quantitative differences were seen in both disease severity and the overall appearance of the wild-type Freedom compared to the transgenic DSF-producing Freedom at this rating time (**Figure 20**). Whereas no disease symptoms were observed on transgenic Freedom, a significantly higher disease rating was observed on the wild-type Freedom (**Figure 20**). Likewise, the overall vigor of those plants that still exhibited green tissue was much greater in the case of transgenic Freedom compared to the wild-type Freedom (**Figure 20**).

As we had observed in previous years, the incidence of Pierce's disease on Thompson Seedless scions grafted to transgenic Freedom rootstocks was significantly less than that grafted to Freedom wild-type rootstocks (**Figure 20**). Likewise, Thompson Seedless scions exhibited much more growth when grafted onto the transgenic Freedom rootstocks compared to that of the wild-type Freedom rootstocks (**Figure 20**). Also, as observed in previous years, the incidence of disease and vigor of Thompson Seedless plants grown as own-rooted plants or as scions onto either wild-type Thompson rootstocks or a Thompson rootstock transformed with the chloroplast targeted *rpfF* gene did not differ (**Figure 20**). Thus, the introduction of the *rpfF* gene into Freedom plants to compare the production of DSF continued to increase the resistance of these plants to symptoms of Pierce's disease despite the fact that they had been inoculated several times before May 2015. These transgenic plants are quite attractive both as an own-rooted plant, and also as a rootstock for more susceptible scions.

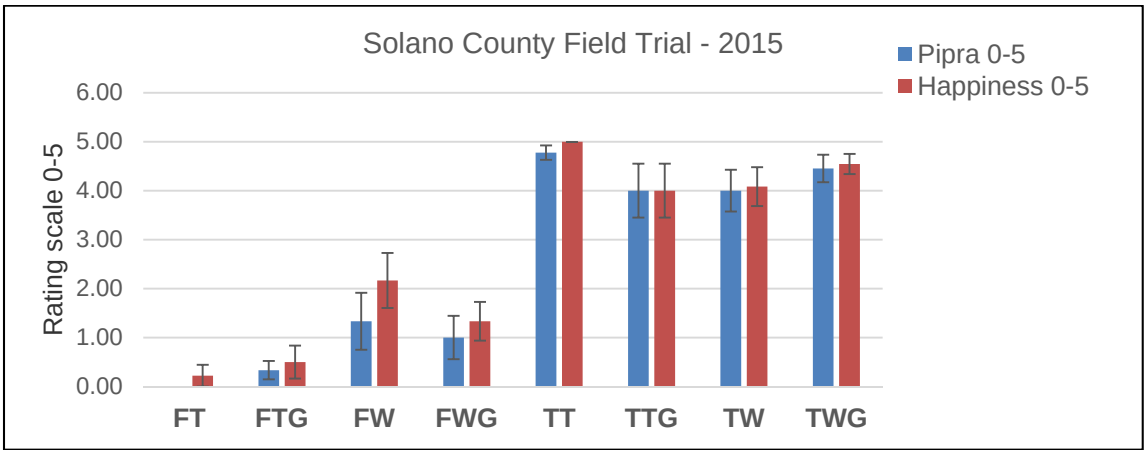


Figure 20. Severity of Pierce's disease symptoms (blue bars) rated on a scale that accounts for both the incidence and severity of disease between cordon that is integrated to yield a range from zero (no symptoms) to 5 (all cordons and shoots dead) when rated on May, 2015. Also shown is the overall vigor of the plant (red) rated from five (extremely vigorous) to 0 (dead). Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG).The vertical bars represent the standard error of the determination of the mean rating.

When rated in early October 2015, transgenic Freedom as a scion continued to exhibit much higher resistance to Pierce's disease than untransformed Freedom. The incidence of symptomatic leaves on plants inoculated in May were reduced over five-fold compared to untransformed plants (**Figure 22**). The overall vigor and incidence and severity of Pierce's disease as assessed on a 0 to 5 scale as discussed above was also much lower on the transgenic Freedom compared to wild-type Freedom (**Figure 21**).

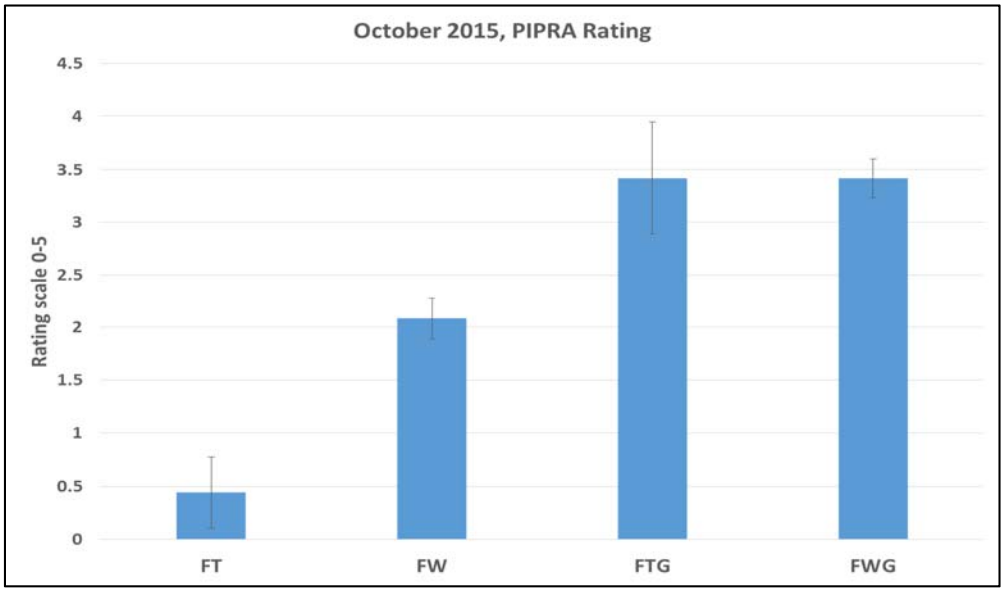


Figure 21. Severity of Pierce's disease symptoms rated on a scale that accounts for both the incidence and severity of disease between cordons that is integrated to yield a range from zero (no symptoms) to five (all cordons and shoots dead). Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG).

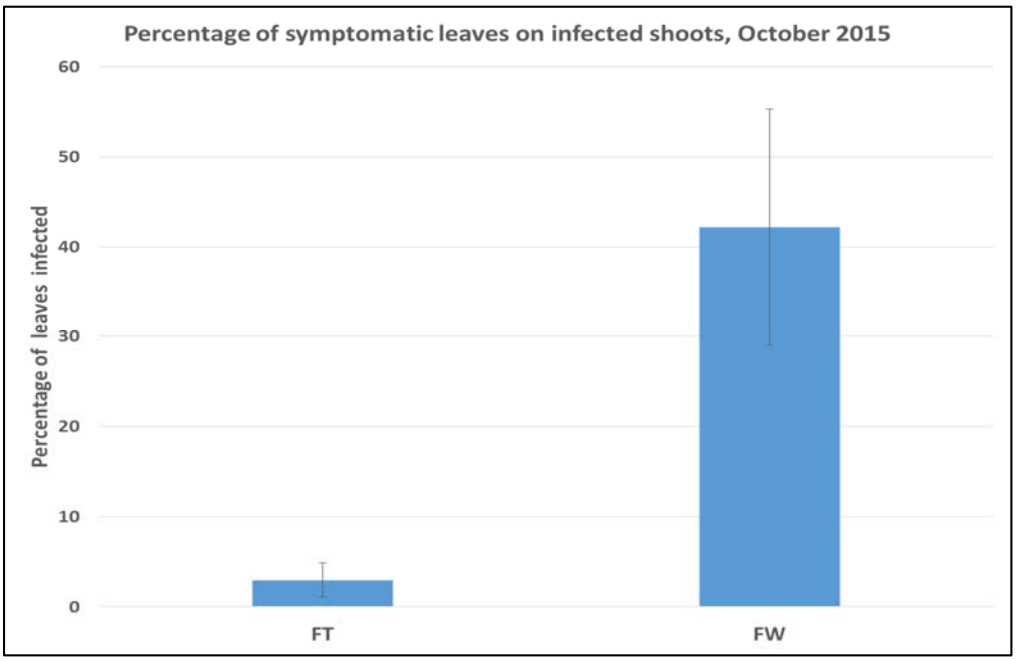


Figure 22. Percentage of symptomatic leaves on inoculated shoots in transgenic DSF-producing Freedom as an own-rooted plant (FT) and wild-type Freedom as an own-rooted plant (FW).

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *X. fastidiosa* to change its gene expression patterns, and since DSF-mediated changes all lead to a reduction in virulence in this pathogen, we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion." These field trials are direct demonstration projects to test the field efficacy of plants producing DSF to alter pathogen behavior in a way that symptom development is minimized. Results at both the Solano County and Riverside County trials provide solid evidence that pathogen confusion can confer high levels of disease control, both to plants artificially inoculated at Solano County and especially to plants infected naturally with infected sharpshooter vectors. The work therefore has provided solid evidence that this strategy is a useful one for managing Pierce's disease. These results justify the further examination of this strategy in other grape varieties.

FUNDING AGENCIES

Finding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

MONITORING FOR INSECTICIDE RESISTANCE IN THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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Reporting Period: The results reported here are from work conducted July 2015 to October 2015.

ABSTRACT
A second year of insecticide bioassays was conducted on glassy-winged sharpshooter (*Homalodisca vitripennis*) populations in Kern County from July through October 2015. Collections were made in the Edison vicinity during July and August, but subsequent late-summer increases in glassy-winged sharpshooter populations in the General Beale Road area allowed testing to be extended into October. Eight insecticides overall were monitored, but with primary focus on evaluating mortality responses to imidacloprid because of its longer history of use against the glassy-winged sharpshooter. Relative susceptibility to imidacloprid declined with each sampling date and between areas sampled as lower susceptibility was recorded in populations from General Beale Road. Differences in susceptibility were also observed in two other neonicotinoid insecticides, thiamethoxam and acetamiprid, but were less pronounced than for imidacloprid. In contrast, susceptibility to the two pyrethroids bifenthrin and fenpropathrin remained consistently high across all sampling dates while response to the organophosphates chlorpyrifos and dimethoate were much more variable. Although toxicity levels as measured by LC50s of insecticides included in this study remain modest in comparison to other insect pests, the higher LC50s for imidacloprid in the General Beale Road area and in comparison to two other neonicotinoid insecticides suggest that some loss of susceptibility to imidacloprid has occurred over the 15-year period that it has been used against glassy-winged sharpshooter populations.

LAYPERSON SUMMARY
Insecticides have played a key role in suppressing glassy-winged sharpshooter (*Homalodisca vitripennis*) populations ever since 2000 when area-wide control programs were first implemented in Riverside County. Reliance on insecticides has been particularly heavy in parts of Kern County where a mixed culture of citrus and table grapes supports development of glassy-winged sharpshooter populations and promotes the spread of Pierce's disease. Spikes in glassy-winged sharpshooter populations in recent years have raised concerns that insecticide management programs are no longer as effective as before due to the development of insecticide resistance. The study presented here examined responses of glassy-winged sharpshooters from two locations in Kern County to eight insecticides in a series of laboratory bioassays that evaluated mortality in relation to insecticide concentration. Mortality levels were consistently higher from one of the locations in which collections were made from organic citrus, whereas reduced mortality was observed at the other location that has experienced heavier insecticide applications through the years. This general pattern was particularly true for the neonicotinoid insecticide imidacloprid that has been used in Kern County since 2001 to suppress the glassy-winged sharpshooter. In comparison to imidacloprid, differences were much smaller between the two locations for thiamethoxam, another neonicotinoid insecticide that has not been used much less than imidacloprid. Similarly, relatively slight differences between the two locations were seen for the pyrethroid insecticides bifenthrin and fenpropathrin. These differences suggest that some loss of susceptibility to imidacloprid has occurred in the population of glassy-winged sharpshooter from the location that has been subjected to more frequent applications of imidacloprid.

INTRODUCTION
Chemical control has been a first line of defense against glassy-winged sharpshooter (*Homalodisca vitripennis*) populations ever since 2000 when the initial area-wide treatment program was conducted in the Temecula region of Riverside County. A similar program was initiated the following year in Kern County as high populations of the glassy-winged sharpshooter represented a critical threat to table grapes growing in proximity to citrus orchards. The systemic neonicotinoid insecticide imidacloprid was selected for use in both regions and has continued to be relied upon for suppressing glassy-winged sharpshooter populations on an area-wide basis. Various attributes of imidacloprid, including its systemic activity and long persistence within plants, its

application versatility, and its semi-selective activity against sucking plant pests such as the glassy-winged sharpshooter, have combined to make imidacloprid a leader in global insecticide sales. Applications of imidacloprid that could be made through the mini-sprinkler irrigation system of citrus orchards were viewed as a positive feature that promoted the use of imidacloprid early in the area-wide programs against the glassy-winged sharpshooter and have continued to reinforce its use. Although data on the frequency of imidacloprid use since 2000 has not been compiled for the area-wide program regions of Riverside and Kern counties, there is a general impression that imidacloprid has been used to a greater extent than other insecticides for control of the glassy-winged sharpshooter. In addition, citrus growers have used imidacloprid extensively for control of red scale and other citrus pests (Grafton-Cardwell et al., 2008) and grape growers have relied upon imidacloprid for vine mealybug control (Daane et al., 2006). The combined uses of imidacloprid across citrus and grape acreages over the past 15 years has likely elevated selection pressure for resistance to imidacloprid in the glassy-winged sharpshooter and other target pests.

Concerns about the potential for insecticide resistance developing in glassy-winged sharpshooter populations have been heightened recently in areas of Kern County due to high population levels reminiscent of the early 2000s. In 2012, CDFA-monitored yellow-trap catches in the General Beale Road region east of Bakersfield matched historic levels and have again been at extreme levels in 2015. The long history of imidacloprid use in this region coupled with a resurgence in glassy-winged sharpshooter populations raise important questions about possible factors contributing to the recent outbreaks. Resistance to imidacloprid has been documented for numerous insects including other sap-feeding insects (Liu et al., 2005; Nauen and Denholm, 2005; Karunker et al., 2008). However, reports of resistance to insecticides by xylem feeding insects are rare, and to imidacloprid are unknown. In the arthropod pesticide resistance (APR) database (http://www.pesticideresistance.org/), only a single obscure record exists for a xylem feeder: a sugarcane-feeding froghopper (spittlebug) reported in a book chapter (Fewkes, 1968). Although fundamental arguments (Rosenheim et al., 1996; Gordon, 1961) for why sap feeding insects might be less prone to resistance development compared to leaf-chewing insects are supported by the APR database, the possibility of pesticide resistance development remains in any organism subjected to a specific mortality treatment over time. There are few examples, if any, where a xylem-feeding insect has been subjected to the kind of intensive management program that has targeted the glassy-winged sharpshooter over the past 15 years in Kern County. Because pesticides are an integral part of the high-yielding production agriculture in citrus and grapes, pest resistance to pesticides must be evaluated. This potential is magnified when overreliance on a few select products occurs, such as has been the case with the use of imidacloprid in the area-wide control programs and by growers and pest control advisors protecting their orchards and vineyards. The repeated use of the same product(s) for control of a pest population results in continual selection pressure, which ultimately may result in resistance development. The continued successful implementation of insecticides in management programs require that their efficacies be evaluated, especially under present circumstances where conspicuously large numbers of glassy-winged sharpshooters are potentially initiating future epidemics of Pierce's disease in table grapes in Kern County.

OBJECTIVES

1. Conduct laboratory bioassays on field-collected glassy-winged sharpshooters from Kern and Tulare counties to determine susceptibility to neonicotinoid, pyrethroid, and organophosphate insecticides.
2. Investigate the geographic variation in susceptibility of the glassy-winged sharpshooter to determine if a pattern of resistance emerges associated with insecticide use patterns.
3. Identify potential resistance evolution of the field populations of the glassy-winged sharpshooter to insecticides by comparing the LC50 values to previously established LC50s using the same methodology.
4. Evaluate the relative toxicity of new insecticides such as flupyradifurone as candidates for alternative treatments for the glassy-winged sharpshooter.

RESULTS AND DISCUSSION

Collections of glassy-winged sharpshooters were made on three dates in July and August 2015 in organic citrus groves in the Edison area, then shifted to the General Beale Road area for three more dates in September and October as a surge in glassy-winged sharpshooter numbers continued to build (http://apps4.cdfa.ca.gov/Pierces Maps/Default.aspx). Bioassay procedures included a systemic uptake bioassay and leaf dip bioassay (Prabhaker et al., 2006) that were used according to whether an insecticide was soil or foliar applied, respectively (**Table 1**). Glassy-winged sharpshooter adults were collected from citrus trees at either of the two locations and used as test subjects. Five adults per clip cage were confined to treated citrus leaves for 24 hours and then evaluated for

mortality. The dose/mortality data were subjected to probit analysis to yield LC50s and accompanying statistics that were used to evaluate relative toxicities of the eight insecticides.

Table 1. Insecticides tested in adult glassy-winged sharpshooter bioassays in 2015.

Insecticide Class	Active Ingredient	Product	Application	Manufacturer
Neonicotinoid	Imidacloprid	Admire® Pro	soil	Bayer
	Thiamethoxam	Platinum® 75 SG	soil	Syngenta
	Acetamiprid	Assail® 70 WP	foliar	United Phosphorus
Butenolide	Flupyradifurone	Sivanto™ 200 SL	foliar	Bayer
Pyrethroid	Bifenthrin	Capture® 2 EC	foliar	FMC
	Fenpropathrin	Danitol® 2.4 EC	foliar	Valent
Organophosphorus	Chlorpyrifos	Lorsban® 4E	foliar	Dow
	Dimethoate	Dimethoate® 2.67 EC	foliar	Loveland

Responses of glassy-winged sharpshooter adults varied considerably by insecticide and location. A consistent progression to higher LC50s was seen for imidacloprid that showed a 79-fold range in LC50s from the first bioassay of the season to the last (**Figure 1**). In contrast, simultaneously run bioassays for thiamethoxam showed a more modest range of responses that varied 26-fold between highest and lowest LC50s. A third neonicotinoid, acetamiprid, was tested only one time from the Edison location and two times from the General Beale Road location, but also showed the same pattern of higher LC50s from General Beale Road. Without having records of pesticide usage in the General Beale Road area, one can only guess that imidacloprid use has indeed been greater that the other two neonicotinoid insecticides. However, it is unclear why susceptibility to imidacloprid progressively declined first at the Edison location, and then at the General Beale Road location (**Figure 1**). It is conceivable that young adults in July may have been intrinsically more susceptible to imidacloprid than the older adults tested in October. It also is possible that continuous feeding on xylem fluid containing low titers of residual imidacloprid in trees from prior year treatments might have had an inducing effect leading to a higher complement of detoxification enzymes later in the season.

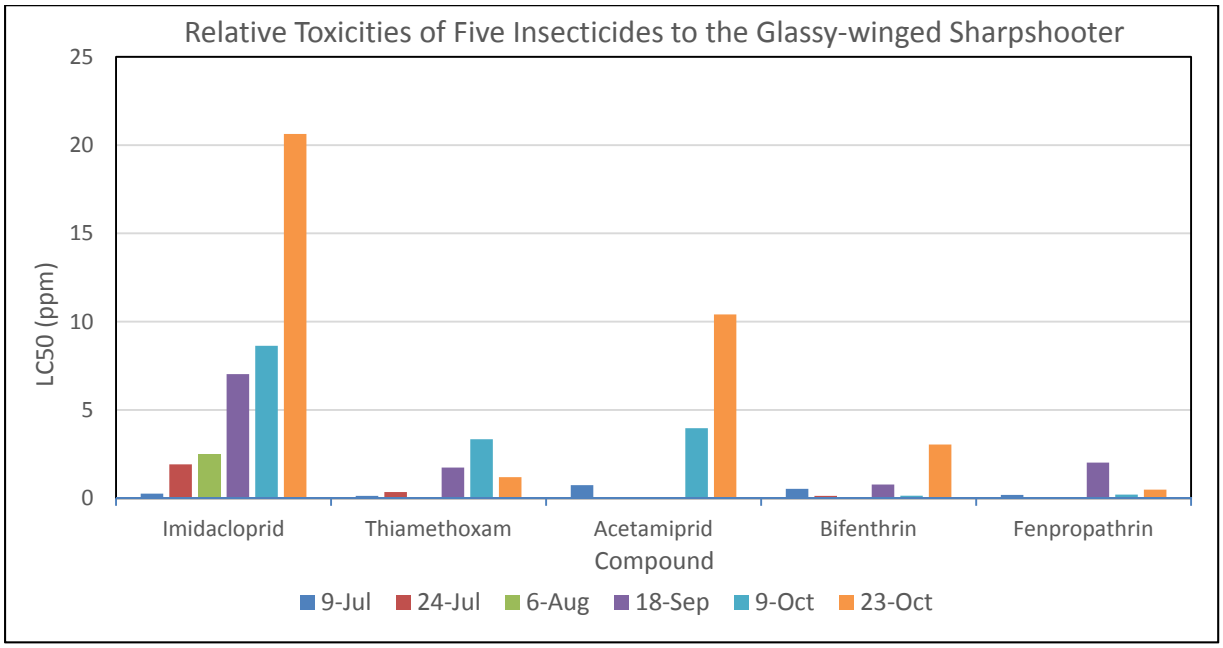

Figure 1. Chart showing LC50s for five insecticides tested over six dates between July 9 and October 23 in 2015. The first three columns of each series represent glassy-winged sharpshooter adults collected from an organic citrus field in the Edison area, whereas the second three columns represent collections from the General Beale Road area. Only three collection dates were tested against acetamiprid, and only five collection dates were tested against bifenthrin and fenpropathrin; all six collection dates were tested against imidacloprid and thiamethoxam.

Higher susceptibilities to pyrethroid and organophosphate insecticides also occurred on the first sampling dates in July compared to October (**Table 2**). The two pyrethroids bifenthrin and fenpropathrin were equivalent with one another with respect to their consistently toxic effects against the glassy-winged sharpshooter. However, the two organophosphate compounds were much less consistent in their responses that occasionally resulted in a moderate to high LC50 (**Table 2**). The recently registered butenolide insecticide flupyradifurone was tested only on the first and last dates, but also maintained the pattern of being less toxic against General Beale Road sharpshooters.

Table 2. Probit statistics for insecticides tested against glassy-winged sharpshooter adults on six dates from July to October 2015.

Location and Date	Compound	LC50	95% C.I.	Slope (± SE)	χ^2	df
Edison July 7-9 (Test 1)	Imidacloprid	0.26	0.15 – 0.46	0.86 (0.11)	31.7	38
	Acetamiprid	0.74	0.31 – 1.76	1.09 (0.16)	32.9	23
	Thiamethoxam	0.13	0.08 – 0.23	1.42 (0.20)	21.6	28
	Flupyradifurone	0.05	0.02 – 0.11	1.02 (0.18)	31.7	23
	Bifenthrin	0.53	0.15 – 1.29	1.70 (0.38)	34.9	23
	Fenpropathrin	0.19	0.08 – 0.41	0.87 (0.14)	19.6	23
	Chlorpyrifos	0.28	0.06 – 1.76	0.49 (0.11)	32.0	23
Edison July 22-24 (Test 2)	Imidacloprid	1.92	0.74 – 6.01	1.29 (0.18)	70.0	28
	Thiamethoxam	0.35	0.19 – 0.67	1.20 (0.17)	22.9	23
	Bifenthrin	0.13	0.05 – 0.30	0.79 (0.13)	13.1	23
	Fenpropathrin	0.04	0.02 – 0.09	0.76 (0.14)	14.0	23
	Chlorpyrifos	4.55	1.54 – 18.04	0.59 (0.10)	25.3	23
Edison August 4-6 (Test 3)	Imidacloprid (1)	2.5	1.24 – 4.50	0.88 (0.15)	23.0	28
	Imidacloprid (2)	8.56	4.47 – 14.56	1.72 (0.35)	24.7	28
	Dimethoate	10.99	2.68 – 118.43	0.39 (0.09)	23.0	23
Gen. Beale Rd September 16-18 (Test 4)	Imidacloprid	7.03	2.73 – 23.30	0.63 (0.11)	21.7	23
	Thiamethoxam	1.74	0.71 – 4.25	0.98 (0.14)	30.9	23
	Bifenthrin	0.78	0.25 – 2.49	0.71 (0.11)	33.0	23
	Fenpropathrin	2.02	0.86 – 5.09	0.72 (0.11)	19.0	23
	Chlorpyrifos	--	--	0.27 (0.10)	18.6	23
	Dimethoate	66.67	11.76 – 4177.39	0.37 (0.10)	13.4	22
Gen. Beale Rd. October 7-9 (Test 5)	Imidacloprid	8.63	2.63 – 29.92	0.83 (0.18)	23.4	23
	Thiamethoxam	3.34	1.25 – 7.70	1.36 (0.26)	25.4	23
	Acetamiprid	3.97	1.40 – 9.97	0.98 (0.19)	21.0	23
	Bifenthrin	0.14	0.04 – 0.40	0.57 (0.10)	15.3	23
	Fenpropathrin	0.21	0.01 – 1.42	0.39 (0.10)	29.0	21
	Chlorpyrifos	--	--	1.23 (0.46)	31.5	22
	Dimethoate	5.66	0.93 – 30.11	0.61 (0.17)	22.9	22
Gen. Beale Rd. October 21-23 (Test 6)	Imidacloprid	20.63	8.27 – 47.71	1.35 (0.33)	22.3	23
	Thiamethoxam	1.19	0.55 – 2.60	0.85 (0.12)	15.8	23
	Acetamiprid	10.41	5.18 – 22.64	1.03 (0.17)	3.3	23
	Flupyradifurone	8.00	3.79 – 19.36	0.90 (0.14)	16.6	23
	Bifenthrin	3.04	1.27 – 8.23	0.69 (0.11)	18.4	23
	Fenpropathrin	0.49	0.17 – 1.22	0.64 (0.10)	18.1	23
	Chlorpyrifos	22.19	6.93 – 96.61	0.82 (0.22)	11.6	23
	Dimethoate	0.59	0.06 – 3.96	0.33 (0.08)	24.7	23

CONCLUSIONS

Differences in mortality responses between imidacloprid and the other two neonicotinoid insecticides suggest some measure of compromised performance with imidacloprid. Additional monitoring should be conducted over the next few years to provide a more thorough evaluation of whether resistance to imidacloprid is occurring. Alternatives to imidacloprid should be considered in future treatments of citrus and grapes against the glassy-winged sharpshooter.

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FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE-INHIBITING PROTEINS

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ABSTRACT

The project was designed to establish and evaluate grapevines in typical commercial vineyard settings in order to assess whether a protein that is naturally produced in edible fruit can restrict *Xylella fastidiosa* spread and Pierce's disease symptoms without altering plant performance. Work in this project evaluates the performance and susceptibility to Pierce's disease of two varieties of grapevine that produce an introduced protein which had been selected by the Pierce's Disease Research Scientific Advisory Panel as a promising candidate to consider for advancement towards commercialization. The aim of the project is to determine whether a polygalacturonase inhibiting protein (PGIP) naturally expressed in pear fruit (pPGIP), when delivered from grafted rootstocks, can control Pierce's disease in the scion, fruit bearing parts of the grapevines. Prior to this project, transformed Thompson Seedless and Chardonnay grapevines expressing pPGIP throughout the vine showed reduced Pierce's disease incidence and symptoms after inoculation with *X. fastidiosa*, which produces a polygalacturonase that is inhibited by pPGIP (Agüero et al., 2005). Cuttings from the two varieties of grapevines that had been transformed to express pPGIP were grafted as rootstocks with non-pPGIP-producing Chardonnay or Thompson Seedless scions so that comparisons between vines producing pPGIP in their grafted rootstocks (transgrafted), those producing pPGIP throughout the vine, and vines with no pPGIP could be made. Active pPGIP protein that had been produced in transgrafted rootstocks had been detected in the xylem exudates that were collected from scions, which had not been modified to produce pPGIP (Agüero et al., 2005; Haroldsen et al., 2012). Once the vineyards were established, an objective of the project is to determine whether sufficient pPGIP that reduces Pierce's disease symptoms is delivered from rootstocks expressing pPGIP to scions, which have not been modified to produce pPGIP. Vineyards approximating commercial settings were established with own-rooted and transgrafted vines in regions of Solano and Riverside County with low and high Pierce's disease pressure, respectively. Evaluations of performance and susceptibility continue to be made that enable comparisons of scion susceptibility to Pierce's disease based on the mode of infection (introduced vs natural), varietal background (Thompson Seedless and Chardonnay), and origin of pPGIP (rootstock only vs. entire vine).

LAYPERSON SUMMARY

In order to determine whether polygalacturonase inhibiting proteins (PGIPs) have potential for commercial development and deployment to reduce Pierce's disease, vineyards were established in two locations in California. The model PGIP evaluated in this project is produced naturally in pear fruit and inhibits the polygalacturonase that *Xylella fastidiosa* produces as it spreads and causes damage in infected grapevines. Each vineyard contained Chardonnay and Thompson Seedless grapevines that were growing on their own roots (own-rooted) and others that were "transgrafted" (with rootstocks expressing pPGIP grafted to fruit-producing non-modified scions that do not themselves produce pPGIP); plantings were completed by June 2013. The genetic and varietal identities of the vines were confirmed by the end of summer 2013. The vineyards were designed to enable comparisons of plant performance and susceptibility to Pierce's disease based on mode of infection (deliberate vs. natural introductions of *X. fastidiosa*), varietal background (Thompson Seedless vs. Chardonnay), and origin of the pPGIP (transgrafted rootstock delivery to grafted non-PGIP-producing scions vs. entire plant producing PGIP). Mechanical inoculations with *X. fastidiosa* bacteria were done yearly from 2011-2015 in Solano County and, beginning with the establishment of the vineyard in June 2013, natural infections occurred in Riverside

County. Data describing the total vine and disease characteristics of the own-rooted or transgrafted vines of both varieties were collected during the 2013, 2014, and 2015 growing seasons in both locations.

INTRODUCTION

Pierce's disease, caused by *Xylella fastidiosa*, can result in the death of grapevine tissues, including scorching along leaf margins and premature abscission of infected leaves. The *X. fastidiosa* bacteria move from infection sites throughout the vine in the xylem and this spread creates systemic infections and may contribute to xylem occlusions (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2007a,b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, "filters" composed of cell wall polysaccharides whose meshwork is too small to permit movement of *X. fastidiosa* (Labavitch et al., 2004, 2007, 2009a,). However, *X. fastidiosa* produces enzymes that can digest the polysaccharides of pit membranes (Labavitch et al., 2009b), thereby opening xylem connections and permitting the spread of the bacteria from the site of introduction. Xylem-occluding tylose protrusions from adjacent xylem parenchyma cells may be another consequence of compromising pit membranes by polysaccharide-digesting enzymes produced by *X. fastidiosa*.

The *X. fastidiosa* genome encodes a polygalacturonase (*XfPG*) and several β-1,4-endo-glucanase (EGase) genes, whose predicted enzyme products could digest pectin and xyloglucan polymers in pit membranes. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of polygalacturonase and EGase into uninfected grapevines caused sufficient pit membrane breakage to allow movement of *X. fastidiosa*. Roper et al. (2007) developed an *XfPG*-deficient *X. fastidiosa* strain that did not cause Pierce's disease symptoms; *XfPG* is a Pierce's disease virulence factor.

Plant proteins that selectively inhibit pest and pathogen polygalacturonases such as polygalacturonase inhibiting proteins (PGIPs) have been found naturally in the flowers and edible fruits of many plants prior to contact with pathogens. PGIPs are induced in most plant tissues upon infection with microbial pathogens (Powell et al., 2000). PGIPs are extracellular proteins that, therefore, are available to move in the apoplastic stream as it is transported through the xylem. Grapevines, which have been modified to produce in all tissues a PGIP normally expressed in pear fruit (pPGIP), have reduced susceptibility to *X. fastidiosa*, probably because the introduced pPGIP inhibits the *XfPG* enzyme. Previous work has shown that the active pPGIP protein is transported across graft junctions from pPGIP-expressing grape and tomato rootstocks into wild-type (i.e., not expressing pPGIP) scion stem sections (Agüero et al., 2005; Haroldsen et al., 2012). Therefore, because infectious *X. fastidiosa* populates the xylem, pPGIP in the xylem coming from the roots may reduce *XfPG* activity and thereby limit spread of and damage by *X. fastidiosa*.

This project was originally designed to generate sufficient grafted and own-rooted pPGIP-expressing Thompson Seedless and Chardonnay grapevines to plant commercial-type vineyards, and to evaluate their performance and resistance to Pierce's disease. The goals of establishing and identifying the fields have been met and the plantings are now being evaluated for their responses to natural or introduced infections with *X. fastidiosa*.

OBJECTIVES

1. Scale up the number of grafted and own-rooted pPGIP-expressing grapevine plants.
2. Plant and maintain grafted and own-rooted vines in two locations with different Pierce's disease pressures.
3. Evaluate relevant agronomic traits of vines in two locations.
4. Determine Pierce's disease incidence in pPGIP-expressing grafted and own-rooted lines. Test for *X. fastidiosa* presence and determine the extent of infection.

RESULTS AND DISCUSSION

Objective 1. Generate enough grafted and own-rooted grapevines for the field trials.

Activities. This objective was completed in June 2013. Results presented in Objectives 3 and 4 show that there is vine death due to Pierce's disease and in a very small number of cases, to other causes; no plans have been made to replace the dead vines. **Table 1** shows the number of grafted and non-grafted vines of each genotype that were planted by June 2013.

Results. Sufficient plants of both the Chardonnay and Thompson Seedless varieties have been self-grafted, transgrafted, or propagated by own rooting to complete the Solano and Riverside County plots. The genotypes of the plants were verified. All of the vines have been transplanted to the sites.

Table 1. Field Inventory. Total number of grapevines planted by 2013 in Solano and Riverside County plots. Explanation of Strategy graphic: The upper portion is scion genotype, the lower is rootstock phenotype; nongrafted plants have no break. Hatched fill represents pPGIP-expressing rootstocks and/or scions; black fill is null-transformants (no pPGIP) controls; white fill is non-transformed controls. In Solano County, own-rooted vines were mechanically inoculated in the summers of 2011-2015; transgrafted vines were inoculated in 2013, 2014, and 2015. Vines planted in Riverside County had "natural" infections.

SOLANO COUNTY		Chardonnay								Thompson Seedless							
	Strategy (Scion/root)																
Own-Rooted	Inoculated (2011-2013)		17								8			9			
	Non-Inoculated		8								4			5			
Grafted	Inoculated (2013, 2014, 2015)	9		11	9	0				9		9	9				
	Non-Inoculated	4		4	4	2				4		4	4				
RIVERSIDE COUNTY																	
Own-Rooted	Natural Infections		13			11		6			9			12		6	
Grafted	Natural Infections	16		6	8		6		3	7		14	7		3		3

Objective 2. Establish field trial sites.

Activities. Field trial sites in Solano and Riverside County were established to assess the Pierce's disease resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field plans of the Powell trial plots in Solano and Riverside County are shown in **Figure 1**.The vines satisfying our initial polymerase chain reaction (PCR) analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment (**Table 1**). The young plants were placed in protective grow tubes and hand-watered every two weeks in Solano County or as needed. In Riverside County, the plants were watered by drip irrigation. In Riverside, the plot is at the bottom of a small hill and the soil is very sandy and porous; irrigation water accumulates in the lowest row (row E). At both sites, grapevines were planted approximately eight feet apart and tied to wooden stakes with trellising wires at 40 and 52 inches.

In Solano County, the vines were pruned by the principal investigator and the field crews to maximize the potential number of canes for inoculations and to establish vigorous positions for future growth. The pruning schedule and method was non-conventional but was done to try to standardize vine growth in our plots with the practices by the other principal investigators with plots in the same field and to preserve the inoculated vines. With the permit amendment granted by the USDA Animal and Plant Health Service, Biotechnology Regulatory Services in 2012, flowers and fruiting clusters were allowed to persist. Initially, all of the own-rooted Chardonnay vines were cordon trained and spur pruned and the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development in our field at the Solano County site. Subsequent prunings have not taken into account varietal differences. The vines at the Riverside County site were pruned according to the schedule established by the field site manager and varietal differences were not addressed in the prunings. The Solano County site has been observed approximately monthly for the duration of the growing season and the vines in Riverside County established themselves well and were monitored by field staff and the principal investigator twice during the season. Vines at the Riverside County site were evaluated by this group in April and October. The activities at both field sites are shown in **Table 2**.

Results. Since June 3, 2013, both the Riverside and Solano County sites have been established with all the planned plantings for this project. A consistent pruning regime remains a goal for this plot so comparisons can be made with other evaluators. In 2014, thirteen evaluations were made of the plots (10 in Solano County and three in Riverside County); nine were made by the principal investigator. In 2015, nine evaluations were made of the plots (six in Solano County and three in Riverside County); eight evaluations were made by the principal investigator.

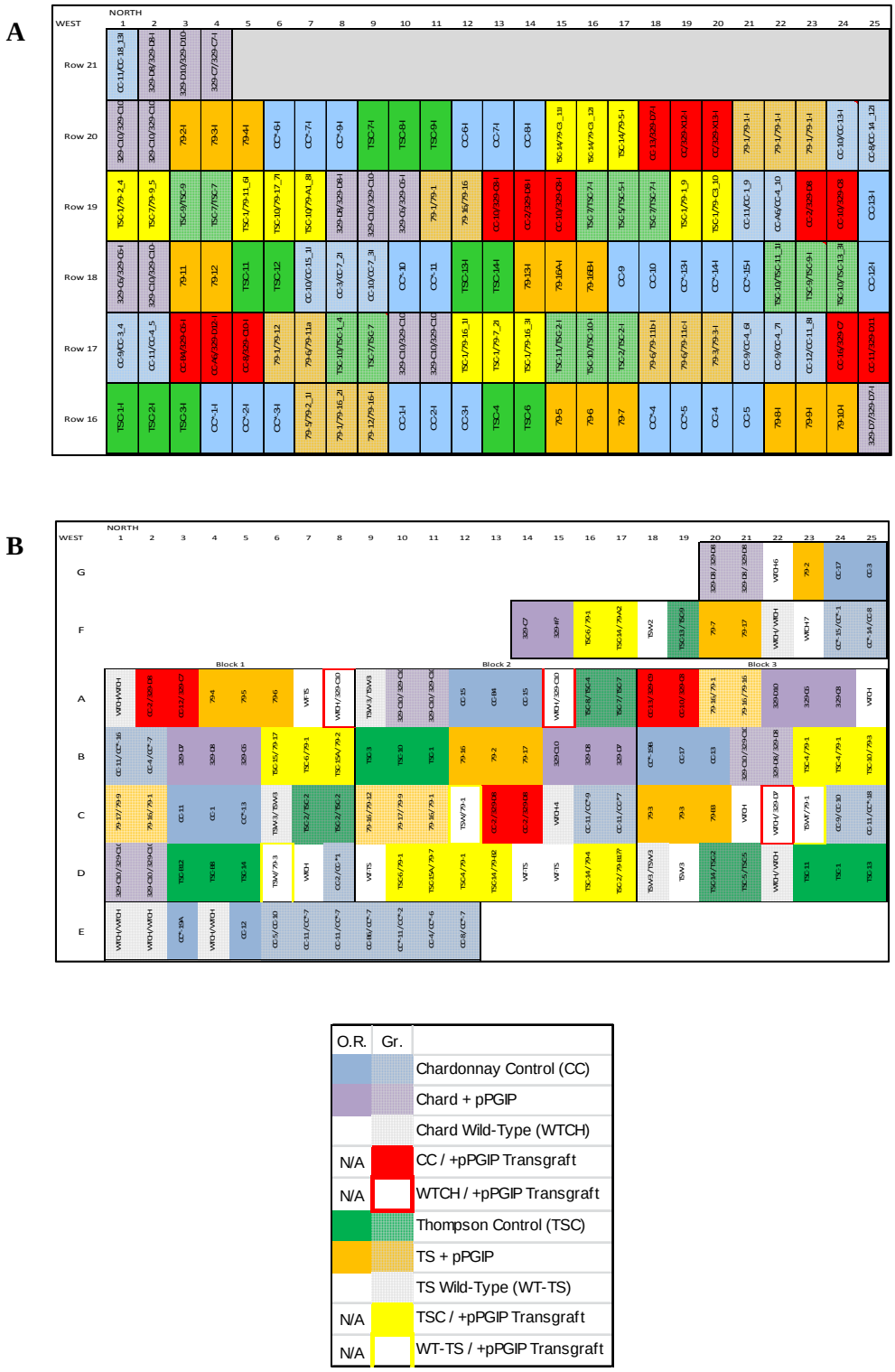

Figure 1. Field plans for Solano County (A) and Riverside County field trial sites. The color codes of the genotypes are given in the accompanying table; O.R. = own-rooted, Gr. = grafted.

Table 2. Activities at the Solano and Riverside County sites for this project through 2015.

Date	Location	Activity
14 March 2014	Solano	Visual scoring of symptoms from 2011-2013 infections at each year's inoculation site on each grafted plant
19 March 2014	Solano	Visual re-scoring of symptoms from 2011-2013 infections (see above)
20 March 2014	Solano	Photos, light pruning since vines have buds that have broken; first pruning since 2013
4 April 2014	Riverside	Disease scoring of symptoms on each plant; photos taken (CJ UCD)
28 May 2014	Solano	Inoculate ca. 4 fresh canes/grafted vine for 2014; no pruning
9 July 2014	Solano	Visit field to assess disease on each plant
27 July 2014	Solano	Take cane samples of ca. 1 cane/ genotype/plot for qPCR of canes infected in 2014; prune vines again
29 July 2014	Solano	Count scorched leaves on infected canes; photos taken
3 September 2014	Solano	Disease assessment by D. Golino (UCD)
ca. 1 October 2014	Solano	Vines pruned again
6 October 2014	Riverside	Disease scoring of all plants by P. Rolshausen (PR, UCR)
9 October 2014	Solano	Count infected leaves
24 October 2014	Riverside	Disease re-scoring of all plants, photos taken by A. Powell (AP, UCD)
15 February 2015	Solano	Prune vines assisted by M. Greenspan while other groups were also pruning (AP UCD)
25 March 2015	Solano	Score plants for scorching, late growth, death, take photos (AP, UCD)
19 May 2015	UCD	Meet with other PIs to consider future of the project
26 May 2015	Solano	Prune vines to conform with other groups (AP UCD)
27 May 2015	Solano	Inoculate at least 4 canes per grafted plant with inoculum provided by D. Gilchrist. Tag with yellow/orange pull tags (AP, BN, TL, KP UCD)
2 June 2015	Riverside	Vine assessments and photos taken with P. Rolshausen (AP UCD, PR UCR
17 June 2015	Riverside	UCR staff (Peggy Mauk) evaluated vines (PM UCR)
Late June 2015	Riverside	Plantings removed
7 August 2015	Solano	Scored for visual signs of scorching, death, photos and samples for PCR (AP UCD)
7 October 2015	Solano	Scored for visual signs of scorching, death, photos and samples for PCR (AP, JMc, JA UCD)

Objective 3. Evaluate relevant agronomic traits of vines in two locations.

Activities. Other than differences due to the variety (Chardonnay or Thompson Seedless) in general, no difference in overall growth, time to flower, fruit set, or yield was noticed between the vines expressing pPGIP and the controls. All produced buds in mid-March and flower buds broke by the end of March in 2014 and 2015. Non-grafted vines were inoculated for three years by March 2014. Numbers of bud-producing, no-bud-producing, and scorched leaves along canes inoculated in 2011, 2012, and 2013 were recorded in 2014 and 2015 and will be analyzed for further details. The data has not yet been analyzed for statistical significance or for effects due to grafting. Photos of each vine were taken throughout the 2015 growing season. Vine death was noted at the Solano County site and was monitored for each infected vine during the 2015 growing season (**Table 3**).

The principal investigator visited the Riverside County site on June 2, 2015 and scored the vines for apparent Pierce's disease damage and for herbicide damage. **Table 4** shows the damage assessments made on June 2, 2015 at the Riverside County site. Herbicide damage was independently assessed by Peggy Mauk at the Riverside County site on June 17, 2015 and the results were provided in earlier reports.

Results. By the end of the 2015 season, it was clear that vines had died in the Solano County plot. **Table 3** shows the number of dead vines of each genotype as determined by assessments in 2014 and four times in 2015. It is clear that the number of dead vines increased during the 2015 season, possibly due to the severe drought conditions but it is also clear that the plants that did not express pPGIP either in the rootstock or in the scion were far more susceptible to death under these stress conditions. The data also clearly indicate that vines that had been infected at least once were far more susceptible to death; only two uninoculated vines appeared to be dead. **Table 3** shows the number of dead plants of each genotype in the Solano County plot.

Table 4 shows the damage assessments made on June 2, 2015 at the Riverside County site. Since up to 25% of the plantings in the Riverside County plot were compromised by the herbicide drift, it was decided in late June to terminate the site with no further observations because it was not going to be possible to distinguish between damage caused by Pierce's disease and by the herbicide exposure.

Table 3. Observations of vine death at the Solano County plot from late 2014 through the 2015 growing season. wtch = Chardonnay wild-type, CC = Chardonnay control, wtTS = Thompson Seedless wild-type, and TSC = Thompson Seedless control. / denotes grafted plants with the genotypes expressed as scion/rootstock. 329 and 79 genotypes express pPGIP in Chardonnay or Thompson Seedless backgrounds, respectively.

			late 2014		25-Mar-15		27-May-15		7-Aug-15		7-Oct-15	
	Total infect-ed plants	Total unin-fected plants	Infect-ed	Not infect-ed	Infect-ed	Not infect-ed	Infect-ed	Not infect-ed	Infect-ed	Not infect-ed	Infect-ed	Not infect-ed
CC	17	8	6	0	6	0	7	0	10	0	10	1
CC/CC	9	4	2	0	2	0	2	0	3	0	7	0
329	0	2	0	0	0	0	0	0	0	0	0	0
329/329	9	4	3	0	3	0	3	0	3	0	3	0
CC/329	11	4	1	1	2	1	2	1	3	1	3	1
TSC	8	4	2	0	4	0	4	0	7	0	8	0
TSC/TSC	9	4	0	0	2	0	2	0	5	0	8	0
79	9	5	3	0	3	0	3	0	3	0	4	0
79/79	7	4	2	0	2	0	2	0	3	0	6	0
TSC/79	10	3	1	0	2	0	2	0	5	0	8	0

Objective 4. Determine Pierce's disease incidence in pPGIP-expressing grafted and own-rooted lines. Test for *X. fastidiosa* presence and determine the extent of infection.

Activities. At the Solano County plot, the leaves/petioles with evidence of Pierce's disease were counted twice during the 2015 season, including on canes which had been infected in 2011, 2012, and 2013. Infected cane material was twice collected during the summer of 2015, approximately when other groups collect their samples. The material has been kept frozen. The Powell group received separate funds for a GenoGrinder and is working on protocols to effectively grind the infected stem tissue. Tissue collected in the summer of 2014 was hand ground and frozen at -80ºC. The data analyzing the genotypes of the dead vines (shown in **Table 3**) has been preliminarily analyzed by plotting (**Figure 2**). The grafted and transgrafted vines at the Solano County site were re-inoculated along with the vines in the plots of the other principal investigators on May 28, 2014 and May 27, 2015. Up to four canes per vine were inoculated as previously with inoculum provided by D. Gilchrist. In our plot, only vines that were grafted or transgrafted were inoculated in 2015, like in 2014. Previous inoculations in 2011-2013 had included vines that were own-rooted. The extent of disease along the canes inoculated in 2014 and 2015 was measured twice during the 2015 season. Examples of the photo evidence of vine phenotypes on October 7, 2015 is included (**Figure 4**).

At the Riverside County site, vine vigor was analyzed in early June 2015. Since it was difficult to unequivocally distinguish between damage caused by natural Pierce's disease infections or by herbicide drift, the observations have not been further analyzed. The visual assessments of disease on the vines in the Riverside County plot were made in 2014 and the data are shown in **Figure 3**. To obtain the data shown, assessments of disease throughout the vines were made twice in October. Evaluators PR and AP used the same general assessment scale going from 0 (no disease) to 5 (dead) to evaluate the vines. Additionally, AP counted the total number of canes per vine and the number of canes with scorched leaves or no growth (diseased canes). The initial analyses of the results are given in **Figure 3**. In general, expression of pPGIP throughout the vine or via grafting to pPGIP-expressing rootstocks reduced slightly the disease score and reduced the number of infected canes. The data has not yet been

analyzed for statistical significance or for effects due to grafting. Examples of the photo evidence of the vine phenotypes on June 2, 2015 was collected (**Figure 4**).

Table 4. Observations of herbicide damage and vine death at the Riverside County plot on 2 June 2015. wtch = Chardonnay wild-type, CC = Chardonnay control, wtTS = Thompson Seedless wild- type, and TSC = Thompson Seedless control. / denotes grafted plants with the genotypes expressed as scion/rootstock. 329 and 79 genotypes express pPGIP in Chardonnay or Thompson Seedless backgrounds, respectively.

Genotype	Total number of vines	Severely compromised growth due to Round-up	Moderate growth due to Round-up	Minimal or slight impact on growth due to Round-up	Probably Dead	Dead
CC	13	0	1	0	0	0
CC/CC	16	4	3	3	1	0
wtch	6	0	1	0	1	0
wtch/wtch	6	3	1	0	0	0
329	11	0	2	0	0	1
329/329	7	0	1	3	1	0
cc/329	6	0	1	1	0	0
wtch/329	3	1	0	0	0	0
Total Chard.	68	8	10	7	3	1
TSC	9	1	1	0	0	4
TSC/TSC	7	2	1	0	1	2
wtTS	6	0	1	0	0	2
wtTS/wtTS	3	1	0	1	0	1
79	11	3	1	3	0	1
79/79	7	0	2	2	0	2
TSC/79	14	5	2	3	2	0
wtTS/79	3	0	0	1	0	0
Total TS	60	12	8	10	3	12
Total	128	20	18	17	6	13

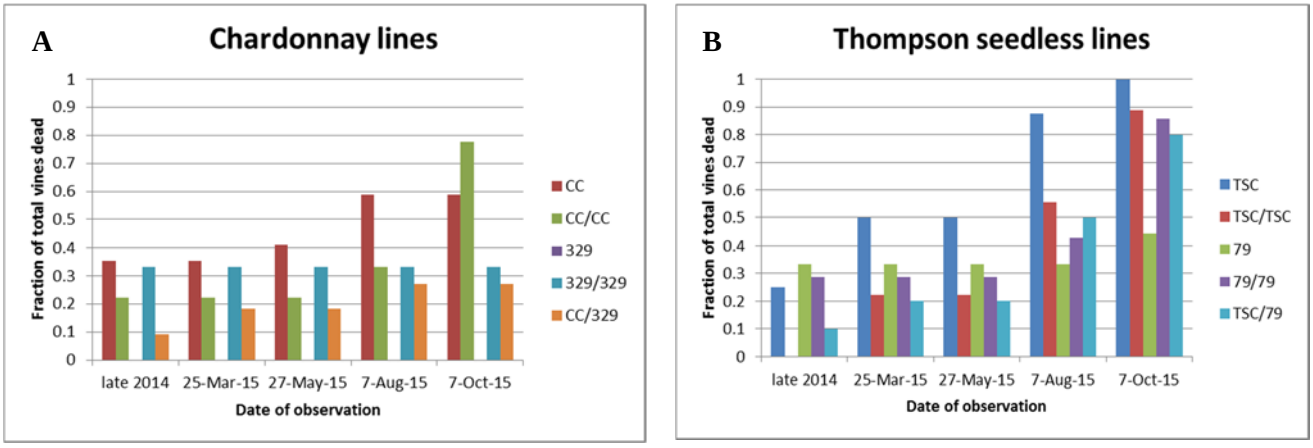

Figure 2. Vine death incidence in Solano County plot of Chardonnay and Thompson Seedless vines measured in 2014 and throughout the 2015 season. (A) Chardonnay lines; (B) Thompson Seedless lines. "/" denotes grafted plants with the genotypes expressed as scion/rootstock. 329 and 79 genotypes express pPGIP in Chardonnay (CC) or Thompson Seedless (TSC) backgrounds, respectively.

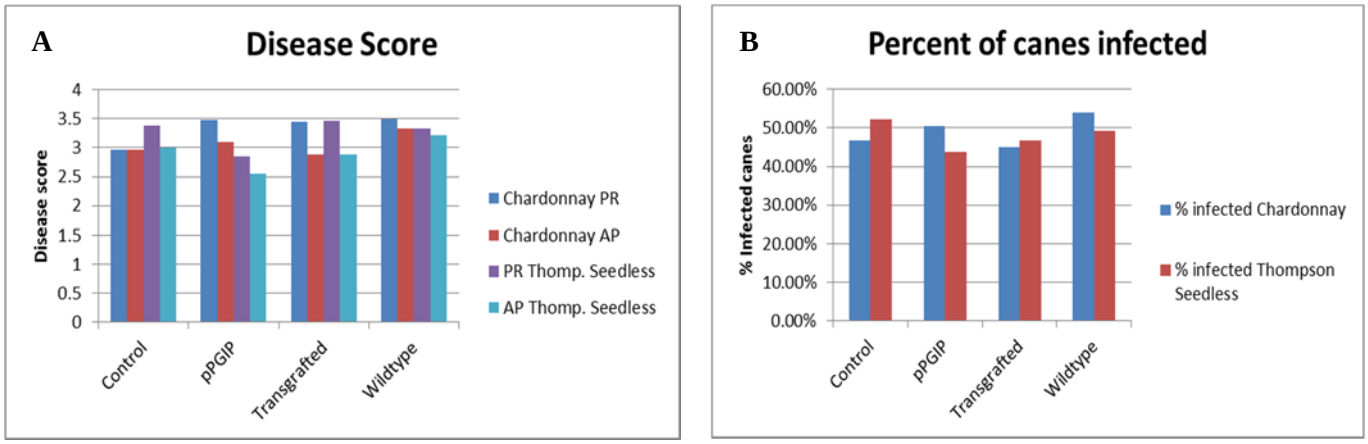

Figure 3. Disease incidence in Riverside County plot of Chardonnay and Thompson Seedless vines measured in October 2014. (A) Disease score based on 0-5 scale; (B) Percent of vine canes with symptoms or evidence of Pierce's disease.

Results. In general, the expression of pPGIP either in the scion or the rootstock or both did not impact the overall phenotype of the plant, but infected plants without pPGIP were more likely to die, especially in the Solano County site, during the 2015 season than those plants with no pPGIP.

In Solano County, analysis of the infected vines demonstrated disease progression over the summer of 2015 and suggested that pPGIP expression provided reduced disease development and ultimately vine death. The effect was clearly due to infection with *X. fastidiosa* as only two uninfected plants had died by the end of 2015. Furthermore, as was shown in the previous annual report, the number of leaves or petioles along canes infected in 2014 was greater when assessed on October 9, 2014 than on the July 29, 2014 observations. These results indicated that disease was developing in these canes. However, vines with pPGIP in the scion portion had a slower increase in disease symptoms, especially in the Chardonnay variety. Notably, vines with pPGIP in the rootstocks also showed fewer numbers of diseased leaves or petioles along the infected canes, although the increase during the 2014 season was about what was observed in the control vines that had been grafted using material that had been transformed with the empty vector construct. In all genotypes, the number of dead infected plants increased over the course of the 2015 summer but the percentage of the vines that died was clearly reduced if the infected plants were expressing pPGIP. It is possible that the severe drought heightened the effect of disease. The effects of *X. fastidiosa* infections were much more pronounced on the Thompson Seedless genotype; by the end of the 2015 season, nearly 100% of the infected Thompson Seedless vines were dead. The plants will be reanalyzed during the 2016 growing season since it is possible that parts of these plants can recover from the disease and regrow. In both varieties, rootstocks expressing pPGIP early in the season were the least likely to die, but by the end of the season plants expressing pPGIP in the scion and the rootstock or only in the rootstock were about equally likely to die. Examples of infected vines are shown in **Figure 4**. Data from the own-rooted Thompson Seedless line (79) should probably not be considered, since an equivalent Chardonnay line (329) was not infected. The conclusion is tentatively made that pPGIP expression, even in the rootstocks alone, was sufficient to delay Pierce's disease symptoms and vine death, but in Thompson Seedless lines, ultimately the plants may succumb to Pierce's disease even when pPGIP is expressed. pPGIP expression seems to offer more protection to the Chardonnay variety.

The disease scoring analyses done by PR and AP at the Riverside County site in 2014 produced approximately equivalent scores. Analysis of the actual number of infected canes generally supported the overall disease score analyses. The results, even with natural infections, suggested that some beneficial effects of pPGIP expression in rootstocks as well as in the scion portions of the vines could be seen, although the Thompson Seedless variety grown at the Riverside County site and infected naturally showed a more pronounced positive effect than the Chardonnay variety.

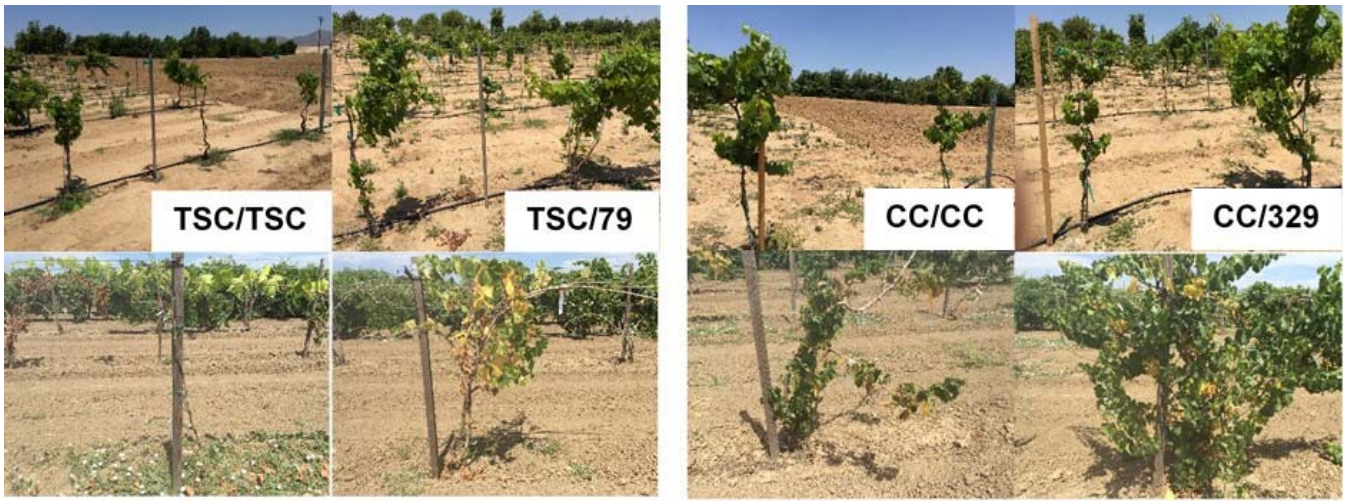

Figure 4. Examples of vines in the Riverside County (top row, Jun 2, 2015) and Solano County (bottom row, Oct 7, 2015) plots of Chardonnay and Thompson Seedless. The genotypes of the grafted or transgrafted vines are indicated.

CONCLUSIONS

All of the grafted plants necessary for the studies at both locations were generated, planted, and inoculated accordingly. The genotypes of the grafted plants were confirmed. Initial infections in 2011 of the vines in Solano County produced no visible symptoms over a year. The second set of inoculations in Year 2 resulted in detectable *X. fastidiosa* DNA in infected vines in November 2012, and visual symptoms of Pierce's disease in April 2013. Mechanical inoculations with *X. fastidiosa* bacteria in 2011 and 2012 in Solano County resulted in the accumulation of *X. fastidiosa* DNA sequences only in the inoculated, but not in the uninoculated, cane material. Symptoms of Pierce's disease infection were visible on the mechanically-inoculated vines beginning generally in the spring of the year following the introduction of *X. fastidiosa*. Inconsistent or atypical pruning schedules have made determinations of similarities of vine phenotype and vigor to commercially propagated fields difficult. However, the overall performance of the own-rooted Chardonnay and Thompson Seedless vines in the field seems to be unaffected by the expression of pPGIP either in the scion or the rootstocks unless the vines have been inoculated with *X. fastidiosa*. The evaluations of the leaf and cane phenotypes of the plants suggest that pPGIP expression improves resistance of vines to Pierce's disease, probably more in the Chardonnay vines with pPGIP which had fewer Pierce's disease symptoms than the Thompson Seedless variety when mechanically inoculated in Solano County, but more in the Thompson Seedless than in the Chardonnay variety during natural infections in Riverside County. By using varieties grown for fresh fruit and for wine production in California, we are comparing the impacts of these changes using varieties which grow with different habits and which are important to different segments of the community of California grape growers.

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MANAGEMENT OF INSECTICIDE RESISTANCE IN GLASSY-WINGED SHARPSHOOTER POPULATIONS USING TOXICOLOGICAL, BIOCHEMICAL AND GENOMIC TOOLS

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ABSTRACT

The focus of this study is to investigate the role of insecticide resistance as a contributing factor to the increased numbers of glassy-winged sharpshooters (*Homalodisca vitripennis*) that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research will be in Kern County, we broaden the scope of the project to include populations from agricultural, nursery and urban settings. This broader approach enables us to provide a more comprehensive assessment on the overall resistance status of this insect within Southern California and develop more effective resistance management plans. To date we have identified the genomic sequence data for several genes that are the putative sites for resistance to carbamate, pyrethroid, and neonicotinoid insecticides. Genomic analyses are ongoing to further elucidate the genetic basis for insecticide resistance in several populations of glassy-winged sharpshooters throughout the state. Bioassay analysis to detect insecticide resistance in the glassy-winged sharpshooter is also ongoing. Data from these analyses suggest that there has been a shift in tolerance to neonicotinoids in the Kern County area. Insects appear to be less sensitive to this class of insecticide relative to other populations that have had limited exposure. Additionally, based on very limited sampling, there does not appear to be a major shift in resistance to organophosphate, carbamate, or pyrethroid insecticides.

LAYPERSON SUMMARY

The goal of this research is to investigate the potential for the development of insecticide resistance in glassy-winged sharpshooters (*Homalodisca vitripennis*) to chemicals in the carbamate, pyrethroid, and neonicotinoid classes of insecticides, and to determine mechanisms where differences in susceptibility between populations are identified. Additionally, we wish to simultaneously evaluate the development of resistance in various populations of these insects that have been undergoing different levels of chemical control in grapes, citrus, commercial nursery, and urban environments. As of this time, we have identified several genes that are likely candidates for the development of resistance depending on the chemical class of insecticide. Additionally, we have detected the possibility that glassy-winged sharpshooters are becoming slightly resistant to neonicotinoids; however, resistance to pyrethroids or carbamates does not appear to be an issue. We caution that limited conclusions can be drawn from these very preliminary data.

INTRODUCTION

Systemic imidacloprid treatments have been the mainstay of glassy-winged sharpshooter (*Homalodisca vitripennis*) management in citrus, grapes, and commercial nursery operations. The treatments in citrus groves are generally applied post-bloom to suppress the newly emerging spring populations. The use of winter or early

spring foliar treatments of pyrethroid or carbamate treatments were introduced to the management program to suppress overwintering adults and reduce the first early season cohort of egg-laying adults. The combination of early season foliar treatments combined with the more persistent systemic treatments has effectively managed glassy-winged sharpshooter populations in Kern County for many years.

In Kern County, glassy-winged sharpshooter populations have been monitored since the area-wide treatment program was instigated by the California Department of Food and Agriculture following an upsurge in glassy-winged sharpshooter numbers and an increase in the incidence of Pierce's disease. The data shows an interesting pattern of sustained suppression of glassy-winged sharpshooter populations, following the implementation of the area-wide treatment program, until 2009 when numbers began to increase again, culminating in a dramatic flare-up in numbers in 2012. In 2012, a single foliar treatment with either Lannate® (methomyl: carbamate insecticide class), Assail® (acetamiprid: neonicotinoid insecticide class) or Baythroid® (cyfluthrin: pyrethroid insecticide class) was applied in groves in late March, while systemic treatments with imidacloprid (neonicotinoid insecticide class) were applied mid-March to early April. The application of systemic imidacloprid during 2012 mirrored the strategy used in 2001 when the imidacloprid treatments were highly effective in suppressing the glassy-winged sharpshooter populations. Despite the additional foliar treatments in 2012, the insecticide treatments failed to suppress the insect population at a level that had occurred previously. It is a worrying trend that in the two years prior to 2012, there was a steady increase in total glassy-winged sharpshooter numbers, an early indication that the predominant control strategy might be failing. The consequence of the increase in glassy-winged sharpshooter populations has been an increase in the incidence of Pierce's disease. In the Temecula area, this worrisome increase in glassy-winged sharpshooters has not occurred (yet); however the selection pressure in this area remains high as similar management approaches are in use here as in Kern County.

There is also significant concern for the development of insecticide resistance arising from the management of glassy-winged sharpshooter in commercial nursery production. The majority of commercial nurseries maintain an insect-sanitary environment primarily through the use of regular application of soil-applied imidacloprid or other related systemic neonicotinoids. For nursery materials to be shipped outside of the Southern California glassy-winged sharpshooter quarantine area, additional insecticidal applications are required. Applications of fenpropathrin (pyrethroid insecticide class) or carbaryl (carbamate insecticide class) must be applied to all nursery stock shipped out of the quarantine area. As with citrus and vineyard production, the potential for the development of insecticidal resistance in nursery populations of the glassy-winged sharpshooter to these three classes of materials (neonicotinoids, pyrethroids, and carbamates) is high.

The focus of this study is to investigate the role of insecticide resistance as a contributing factor to the increased numbers of glassy-winged sharpshooters that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research will be in Kern County, we propose broadening the scope of the project to include populations from agricultural, nursery, and urban settings. This broader approach will enable us to provide a more comprehensive report on the overall resistance status of the glassy-winged sharpshooter within Southern California and develop more effective resistance management plans.

OBJECTIVES

1. For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC50 data for current glassy-winged sharpshooter populations and compare the response to baseline susceptibility levels generated in previous studies.
2. Define diagnostic concentrations of insecticides that can be used to identify increased tolerance to insecticides in insects sampled from other locations (where numbers are relatively low).
3. Monitor populations for known molecular markers of resistance to pyrethroids.
4. Monitor populations for target-site insecticide resistance, by testing enzymatic activity against carbamates using the acetylcholinesterase biochemical assay.
5. Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of the glassy-winged sharpshooter to baseline susceptibility levels we previously recorded.
6. Develop assays for additional resistance mechanisms not previously characterized in the glassy-winged sharpshooter.

RESULTS AND DISCUSSION

Genetic Analyses

In order to identify markers that could be easily typed to determine insecticide resistance in field collected glassy-winged sharpshooter, it was first necessary to identify the genic targets of commonly used insecticides within the glassy-winged sharpshooter genome. We utilized gene sequences from housefly and mosquito to identify contigs (via Basic Local Alignment Search Tool; BLAST) within the glassy-winged sharpshooter assembly that contained genic targets of insecticides. Based on the additional analysis of the glassy-winged sharpshooter genome using housefly and yellow fever mosquito (*Aedes aegypti*) as algorithmic models, we identified nine different contigs that contained portions of the glassy-winged sharpshooter para sodium-gated channel gene, which is the molecular target of pyrethroids (HVIT018256-PA, HVIT018257-PA, HVIT018258-PA, HVIT018259-PA, HVIT018260-PA, HVIT018261-PA, HVIT018262-PA, HVIT018263-PA, and HVIT018265-PA). The exon / intron boundaries of the sodium channel gene were annotated using algorithms developed in house. To date, we now have identified two novel sodium channel gene sequences (1975 and 2121 amino acids) that are alternatively spliced. Additionally, we began identification of P450 genes, which are an extremely large gene family involved in detoxification processes, including metabolism of insecticides. Thus far, we have identified more than thirty P450 genes (e.g. HVIT028002, HVIT002146, HVIT014023, HVIT027839, HVIT014027, HVIT028006, HVIT028005, HVIT028857, HVIT002149, HVIT003205, HVIT008014, and HVIT008180). Protein products of the genes range in size from 357 to 563 amino acids. Finally, we computationally identified several new nicotinic acetylcholine receptor subunit sequences (HVIT01577, HVIT032410, and HVIT018225). To facilitate identification of specific genes in the future, we are now running an annotation program, AUGUSTUS, in order to more fully characterize the glassy-winged sharpshooter genome. The algorithm is computationally intense and was initiated in March. We expect the process to take at least four to six months to complete.

Based on the above and ongoing analyses of the glassy-winged sharpshooter genome and transcriptome, gene specific primers have been designed and synthesized to conduct potential target gene full length amplification and detect genetic variations of single nucleotide polymorphisms (SNPs) among glassy-winged sharpshooter populations located in different areas. We have extracted RNA from glassy-winged sharpshooter individuals that came from two different locations (Riverside and Corona). cDNA was synthesized from RNA and set up as the template for polymerase chain reaction (PCR). The first nicotinic acetylcholine receptor beta-like gene sequence has been identified. The open reading frame of the gene is 1593 base pairs (bp), which encodes a protein of 531 amino acids. One non-synonymous mutation and three synonymous mutations have been identified among individuals between Riverside and Corona. Sequences have been sent to I-TASSER online server to build the three dimensional structure of the protein. The protein structure and binding models with different insecticides will be studied later. At the same time, a total of 4551 bp of sodium channel gene have been confirmed, and seven synonymous mutations have been found among individuals between Riverside and Corona. The full length sequences of several genes that could be involved in insecticide resistance will be identified soon.

Imidacloprid Bioassays

Topical application bioassays were conducted during 2015 using insects collected from citrus in the General Beale Road area of Kern County, and the data compared with similar bioassays from studies conducted in 2003. In bioassays, insecticide is topically applied to the abdomen of adult glassy-winged sharpshooters and mortality is assessed at 24 and 48 hours post treatment (Byrne and Toscano, 2005). Topical application of insecticide to an individual insect ensures that the insect receives the required dose, and eliminates any behavioral factors that could occur when the insect encounters the insecticide (either through direct contact or during feeding). Imidacloprid is one of the most important insecticides used for the control of the glassy-winged sharpshooter, and this insecticide has been shown to elicit anti-feedant effects in several pest species (Nauen et al., 1998).

In 2003, the bioassays were conducted using populations from Riverside (Agricultural Operations, University of California, Riverside) and Redlands (commercial citrus grove). At the time the bioassays were conducted, the neonicotinoid insecticide imidacloprid was not being used at Agricultural Operations to control populations, and so the data from those bioassays were considered to represent baseline susceptible levels for the glassy-winged sharpshooter. The response of insects from the Redlands grove, where imidacloprid was incorporated as part of the area-wide management of the glassy-winged sharpshooter, was similar to Agricultural Operations, indicating that no tolerance to imidacloprid had arisen despite its use as part of the control program. In our view, those early data serve as a useful reference against which current populations can be compared.

In all bioassays, the Kern County populations were considerably more tolerant to imidacloprid than the reference population **(Figure 1)**. In the first bioassay, we treated insects over the range 0.25 – 10 ng imidacloprid per insect (n = 150), and there was no insecticide-related mortality at the highest concentration. A second bioassay was conducted with the same source of insects (n = 130) to check the response at 10 ng imidacloprid (using a freshly prepared stock of imidacloprid). Similar to the first bioassay, there was no imidacloprid-related mortality. Based on the reference data set, the 10 ng dose should result in *ca*. 80% mortality, so the bioassays indicated some degree of tolerance to imidacloprid in the Kern County insects. The dose range was adjusted to 1.5 – 150 ng imidacloprid per insect for the third bioassay, and a new supply of technical grade imidacloprid was used to prepare the working stock solutions. Although there was a dose response in this bioassay, complete mortality at the higher doses was not achieved, and the level of mortality at the 10 ng dose was minimal. Further bioassays are needed to obtain a full dose-response curve.

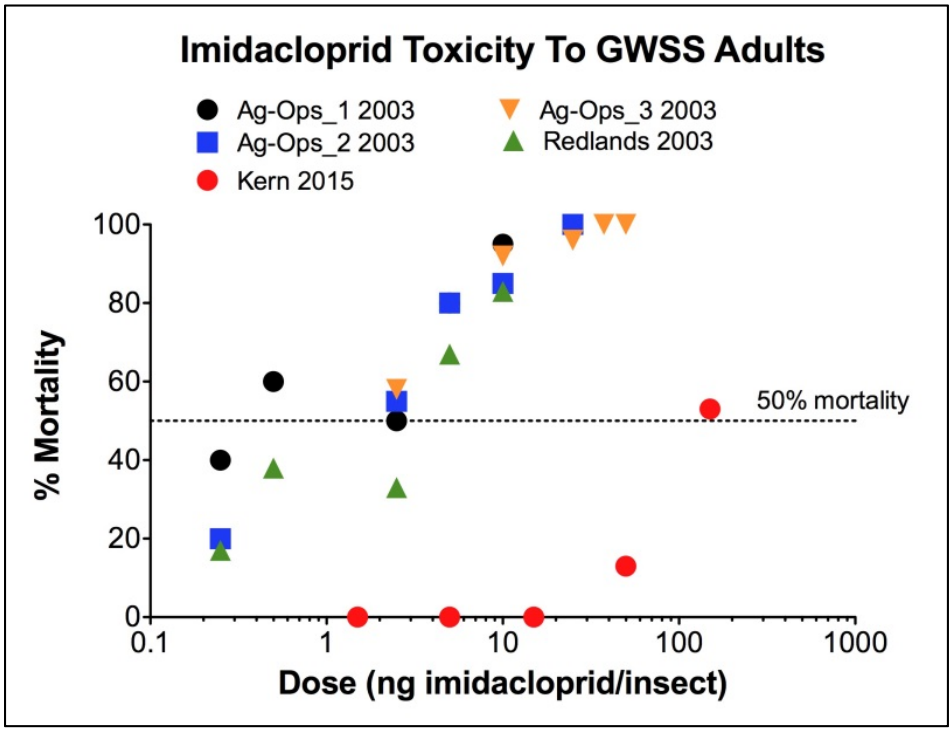

Figure 1. Dose response of glassy-winged sharpshooter adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for the Kern County population (General Beale Road) were generated in October 2015 and compared with bioassay data for insects tested in 2003 from research citrus plots at Agricultural Operations (Ag-Ops) at the University of California, Riverside, and a commercial citrus grove in Redlands.

Pyrethroid Bioassays

Bioassays were conducted at diagnostic concentrations for two pyrethroid insecticides (bifenthrin and fenpropathrin) using populations collected from citrus at General Beale Road **(Figure 2)**. Bioassay data was originally generated in 2004 and 2005 for populations sampled from citrus at Agricultural Operations, and these data are used to represent a reference susceptible.

Both pyrethroids were tested at five ng pyrethroid per insect by topical application. Bifenthin was more toxic than fenpropathrin; however, the levels of mortality were lower for both insecticides compared to the Agricultural Operations population in 2005. Despite the lower toxicity at this single dose, it will be important to develop complete dose-response curves for the two pyrethroids to determine whether these differences in toxicity indicate tolerance to the pyrethroids.

Esterase Activity

Pyrethroid insecticides are ester-based insecticides and can act as substrates for pyrethroid-hydrolyzing esterases. Total esterase activity was measured in individual glassy-winged sharpshooters using a colorimetric assay that

utilizes naphthyl ester substrates. Although the substrates are non-insecticidal, naphthyl esters can be hydrolyzed by resistance-causing esterases, and they have been used for several decades to identify pyrethroid resistance in agricultural, medical, and veterinary pests. We determined the esterase activity in glassy-winged sharpshooters collected from several citrus groves, and compared the new data with data from our studies in 2003 **(Figure 3)**. Unfortunately, we do not have esterase activity levels from Kern County populations from previous years, so we must rely on available data for Riverside and Redlands populations to make comparisons.

Large numbers of insects were collected from the General Beale Road area of Kern County, and the mean esterase activity was significantly higher than the reference value for the Riverside population (data from 2003) and a population collected in 2015 from organic citrus in Tulare County **(Figure 3)**.

During 2016, we will continue to measure the esterase activity in glassy-winged sharpshooter populations collected from various field sites in conjunction with bioassays. In that way, we will be able to better understand the impact, if any, of subtle changes in esterase activity on pyrethroid toxicity. In addition, we will measure the activity in insects that survive bioassay treatments to determine if the survivors have elevated levels of activity compared with control (untreated) insects. Individual esterases that contribute to elevated levels of total esterase activity in glassy-winged sharpshooters can be identified using polyacrylamide gel electrophoresis (Byrne and Toscano, 2005).

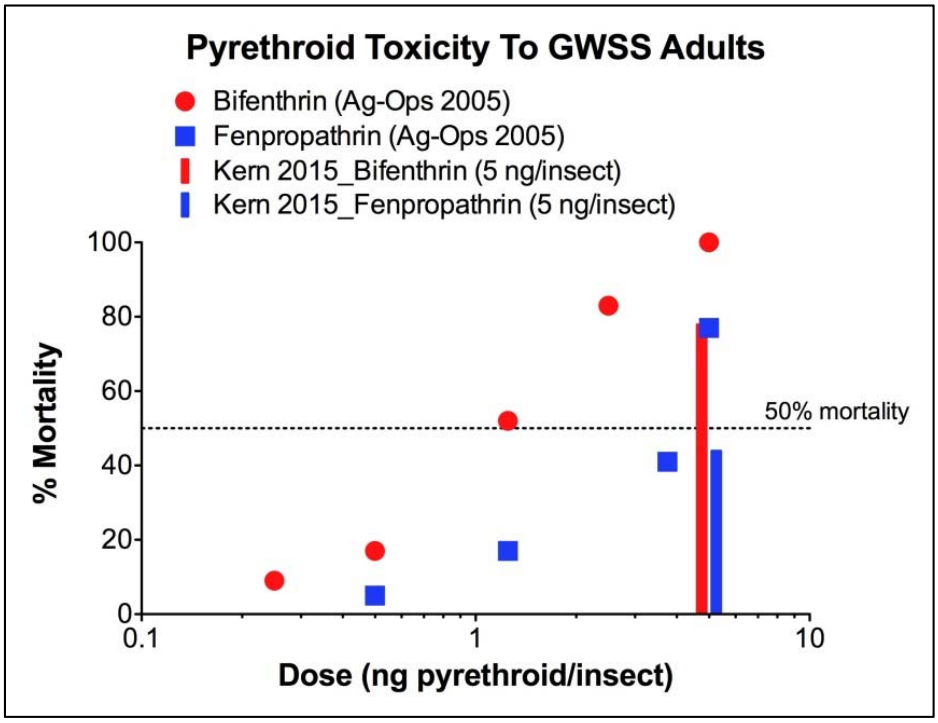

Figure 2. Toxicological response of glassy-winged sharpshooter adults to the pyrethroids bifenthrin and fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. The bars show data for the Kern County population (General Beale Road) that were generated in October 2015, using a diagnostic concentration of five ng/insecticide for both chemicals. Bioassay data for insects tested in 2005 from research citrus plots at Agricultural Operations (Ag-Ops) at UC Riverside are included for comparison.

Acetylcholinesterase Sensitivity to Paraoxon

Organophosphate (OP) and carbamate insecticides target the neurotransmitter acetylcholinesterase (AChE). Target-site resistance arises as a consequence of mutations in the enzyme that affect the binding efficiency of the insecticide. An assay was developed for glassy-winged sharpshooter that enabled the measurement of both the total esterase activity (**Figure 3)** and the sensitivity of the AChE to paraoxon in an individual insect.

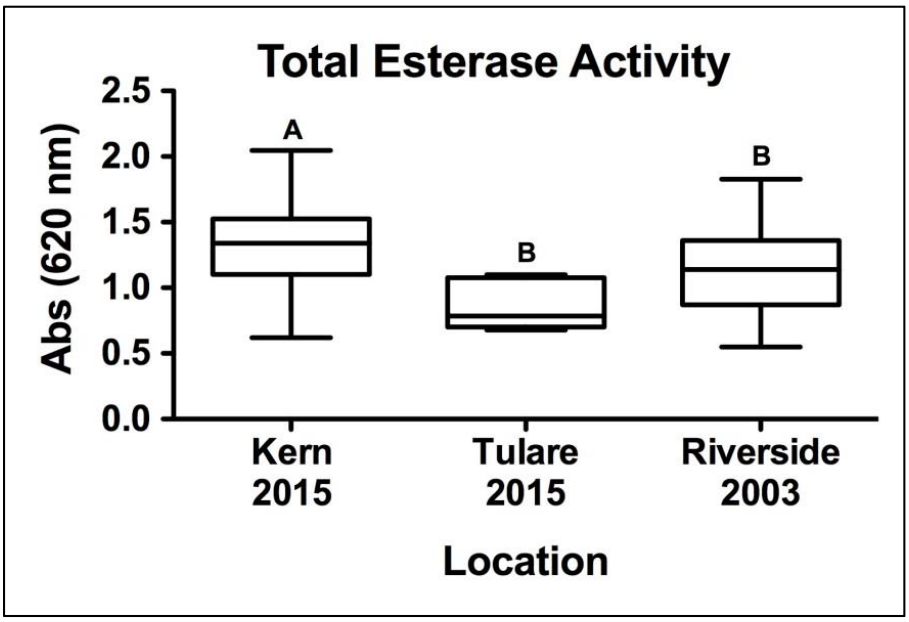

Figure 3. Total esterase activity measured in individual glassy-winged sharpshooter adults. Activity is represented as absorbance units (320 nm) measured after 30 min incubation with 0.3 mM 1-naphthyl acetate. Homogenates of individual heads were prepared in 0.1 M phosphate buffer, pH 7.5, and then an aliquot (equivalent to 0.01 head) used directly for assay. Levels not connected by the same letter (located above the box plot) are significantly different.

We tested a large number of insects from the General Beale Road area and all the insects were sensitive to the diagnostic concentration of 30 µM paraoxon **(Figure 4)**. Insects were also tested from locations in Orange County and Tulare County, and these insects were also sensitive to the OP.

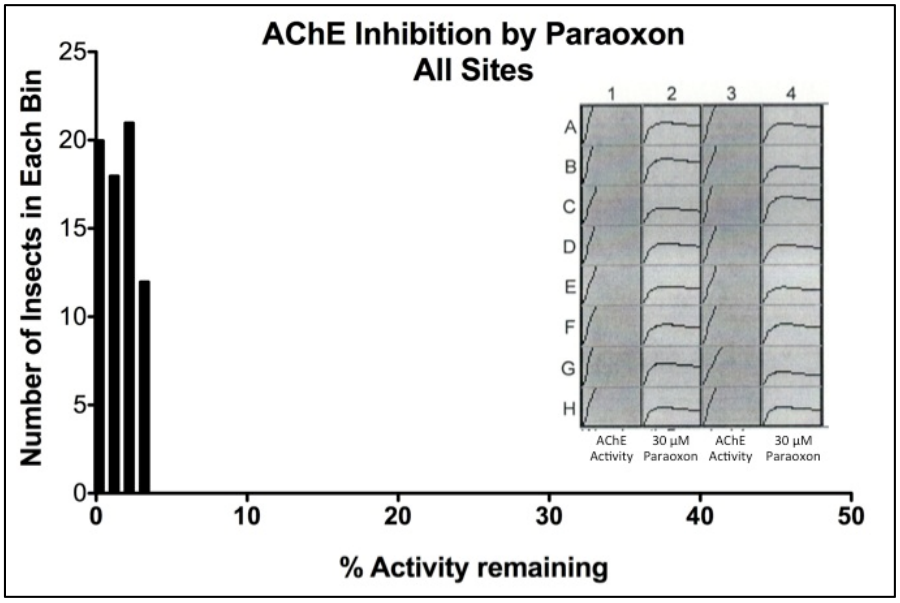

Figure 4. Sensitivity of glassy-winged sharpshooter AChEs to 30 µM paraoxon. The insert shows the computer output from an assay in which total AChE activity was measured over 20 minutes in the absence (columns 1 and 3) and presence (columns 2 and 4) of the OP. Duplicate aliquots from the same insect homogenate are assayed side-by-side in the absence and presence of the OP (thus boxes A1 and A2 represent the same insect AChE source), providing a visual display of the AChE sensitivity. The main graph shows the distribution of AChE inhibition in glassy-winged sharpshooter adults collected from multiple sites in California. All assays resulted in complete inhibition of activity within the 20 minute assay, denoting a lack of AChE insensitivity within glassy-winged sharpshooter populations.

CONCLUSIONS

We identified a shift in imidacloprid tolerance in glassy-winged sharpshooters collected from citrus in the General Beal Road area of Kern County. The shift is based on a comparison with data generated in 2003 for a population in Riverside County that we regard as a reference susceptible. An important next step in our studies will be to continue with bioassays on glassy-winged sharpshooter populations from the General Beale Road area, and to compare the data with bioassays for insects collected from other regions, particularly where control of glassy-winged sharpshooter populations is not an issue. One approach could be to compare toxicological responses of glassy-winged sharpshooters from organic and conventional sites. Gathering new data for insects in Riverside and Redlands areas will be important in order to determine whether the toxicity of imidacloprid has remained the same since the original studies were completed, or if the insects from these areas also express an increased tolerance to imidacloprid. The reference data was generated in 2003, and although the same techniques are being used in our current research, it is necessary to determine efficacy against current populations from those areas. In that way, the impacts of tolerance/resistance to field control can be more effectively evaluated.

The esterase data identified subtle, yet significant, phenotypic differences between insects collected in organic (Tulare County) and conventional (Kern County) groves. If these differences represent a pattern that exists between these two systems, it may be one that can be exploited to identify mechanisms that affect the susceptibility of the glassy-winged sharpshooter to pyrethroid insecticides. Interestingly, there was also a lower toxicity to pyrethroids in the Kern County insects, although we do not have data for the organic population to compare. Until now, we were only able to compare the Kern County data with results from earlier studies with a population collected from citrus research plots on the campus of the University of California, Riverside where pyrethroids would not have been part of a glassy-winged sharpshooter control program. The esterase data for the UC Riverside and Tulare County insects were not significantly different.

No differences in sensitivity of AChE to diagnostic concentrations of the OP paraoxon were detected in field populations of glassy-winged sharpshooter.

The genomic work is currently ongoing; however, it is too early to utilize information generated from these efforts to identify the actual genetic sites where resistance may be evolving. We are confident that with continued effort and analysis of the genome, the genetic differences between what appear to be populations with different levels of susceptibility to important insecticides will be identified.

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GENOME EDITING OF *TAS4, MIR828,* AND TARGETS *MYBA6/A7*: A CRITICAL TEST OF *XYLELLA FASTIDIOSA* INFECTION AND SPREADING MECHANISMS IN PIERCE'S DISEASE

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ABSTRACT

Manipulating gene expression is critical to exploring gene function and a useful tool for altering commercial traits. Quality improvements depend on applying new genetic insights and new technologies to accelerate breeding through improved genotyping and phenotyping methods, and by increasing the available genetic diversity in germplasm. The proposed work on fundamental processes of plant RNA interference interactions with *Xylella fastidiosa* leverages translational science from model organisms to the market, with potential for broad impacts on agriculture and understanding the mechanisms of pathogen resistance. The molecular approaches supported by this project can have significant impacts on viticulture by: (i) applying deep knowledge from model plant species to grapes; (ii) facilitating optimal selection of parents for breeding and immediate selection of elite progeny with multiple desirable traits; (iii) circumventing biological and societal limits to genetic engineering; (iv) accessing abundant genetic variation (vegetative propagation is a double-edged sword): grape varieties currently face severe pathogen pressures and long-term sustainability of industry relies on exploitation of natural genetic diversity; (v) rapid linkage disequilibrium (recombination) allowing haplotype variants to be directly associated with phenotypes, accelerating breeding programs; and (vi) understanding disease etiologies.

LAYPERSON SUMMARY

The bacterium *Xylella fastidiosa* is the cause of Pierce's disease in grapes and is a major threat to fruit, nut, and coffee groves. Obvious symptoms are anthocyanin (red pigment) accumulation in leaves and shriveling of undeveloped berries. A few studies have determined that anthocyanin compounds can reduce insect feeding. Work by the co-principal investigator shows that *X. fastidiosa* infection causes significant imbalances in leaf and xylem elemental phosphorus (P) content in grapes, tobacco, and pecan, but the bioavailable form of P underlying this phenomenon is unknown. The principal investigator has characterized in many species including grape a molecular mechanism (involving a microRNA [miRNA]) that is involved in anthocyanin and potentially disease and insect relationships. We hypothesize that these novel target genes s (VvMYBA6/A7) in grape are effectors of anthocyanin accumulation and potentially glass-winged sharpshooter feeding preference determinants important for Pierce's disease etiology, mediated through P_i and abscisic acid (ABA, a plant stress hormone) signaling crosstalk that modulates miR828 activities normally to silence target gene expression. Anthocyanins function as color and flavor enhancers and anti-oxidants in fruits, and their induction in vegetative tissues may serve as antagonists to feeding by the glassy-winged sharpshooter (*Homalodisca vitripennis*) and to colonization by *X. fastidiosa*. We propose to test the *X. fastidiosa* infection/spread hypothesis directly by "knocking out" the key genes using a new genome editing technology - Clustered Regularly Interspaced Short Palindromic Repeats (CRIPSR/Cas9) that the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board nominated as a high-priority approach to engineering Pierce's disease resistance. This technology has the capacity to produce non-"genetically modified organism" (GMO) grapevines and rootstocks (after outcrossing the vector gene by traditional breeding). The resulting stocks lacking functional endogenous effector genes will be challenged in the greenhouse with *X. fastidiosa*. This proof in principle experiment could result in a new paradigm for host-vector-pathogen interactions in Pierce's disease with potential translational benefits for other crops. This project draws on a new molecular breeding technique for deleting test genes, introducing key changes into genes (e.g., to make

them easy to study in their native state), and mitigating regulatory issues of GMOs for the advancement of the grapevine biotechnology and breeding sectors.

INTRODUCTION

A few studies have determined that some anthocyanin and derivative tannic compounds can reduce insect feeding [1], including sap-sucking insects [2, 3]. The saliva of sap-sucking insects like the glassy-winged sharpshooter (*Homalodisca vitripennis*) contains components like polyphenol oxidase and peroxidase for detoxification of plant secondary substances and break-down of plant cell walls and defense reactions [4], thought to play roles in *X. fastidiosa* migration [5] in xylem vessels and potentially insect colonization and transmission [6, 7]. *X. fastidiosa* residency in the xylem appears to be the main criteria for survival in the host. The nutritionally poor xylem does contain specific amino acids, organic acids, and inorganic ions (e.g. P_i) that are essential for the bacterium. Phenolics accumulate in xylem sap and leaves of *X. fastidiosa* infected almonds [8] and grape [9]; some cultivars (e.g. 'Rubired') induce phenolics to higher concentrations and do not develop Pierce's disease symptoms as quickly as anthocyanless cultivars such as Chardonnay or Thompson's Seedless [10, 11]. The gene expression responses of a *X. fastidiosa* resistant species, *Citrus reticulata* Blanco (Ponkan mandarin), one day after *X. fastidiosa* infection show up-regulation of genes for phenylpropanoid and flavonoids biosynthesis [12]. Significantly, low (200 μM) levels of catechin and resveratrol, the most abundant polyphenolic in xylem tissue, limit *X. fastidiosa* growth *in vitro* [13].

miRNAs and small interfering RNAs (siRNAs) derived from miRNA activities are the specificity "guide" for nucleases of the ARGONAUTE class which cleave or otherwise repress protein coding transcripts in a nucleotide sequence-specific manner [14, 15]. Because miRNAs and associated *trans-acting small interfering (TAS)* loci act ***dominantly***, they are a particularly good subject for studying the fitness landscape of interactions and genetic robustness in nature and at the bench. Microbes and viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for plant innate immunity [16-22], but the molecular mechanisms of RNA interference (RNAi) in plant-microbe interactions are poorly understood. It is quite likely that grapevine red blotch [23] and leaf-roll virus disease states involve miR828/*TAS4*: Leafroll infection increases miR828 [24], blocks berry development [25], and phenocopies symptoms of Pierce's disease [26] mediated by viral suppressor proteins that interfere with siRNA formation or activity [27]. Production of phased *trans*-acting-siRNAs (phasiRNAs) from target *MYB*s (MYeloBlastosis viral oncogene-like) has been shown to be triggered by miR828 in many species [28-35], similar to numerous miRNAs that all target the huge Nucleotide-Binding Sequence/Leucine Rich Repeat family of disease resistance genes [28, 32, 36]. The diversity and conservation of so-called "phased si-RNA-producing" loci across plant taxa [36-39] and their loss in virus- and bacteria-infected tissues that results in susceptibility [36, 40] demonstrates their broad functions as master regulators targeted by pathogens.

TAS transcripts drive antisense transcription and feedback loops that amplify the production of siRNAs that in turn negatively regulate target gene expression in *trans* [41]. Endogenous siRNAs can pass through plasmodesmata and move across graft unions to regulate gene expression by epigenetic modifications, establishing developmental gradients, or by feedback loops in adjacent cells or in separate roots and shoots [42-44]. *TAS4* is a locus discovered in *Arabidopsis* [45] that generates a ~1 kilobase long non-coding RNA spawning phasiRNAs triggered by miR828; both miR828 and derived phasiRNAs target *MYB* transcription factors that regulate anthocyanin production. Grape has one *MIR828* and three functionally conserved *TAS4* loci (*a-c*) with implications for differential *MYB* cleavage activities [46]. The high reproducibility of abundances of the *VvTAS4b* phasiRNAs support that *TAS4b* is the trigger for silencing by cleavage of *VvMYBA6/A7* targets [46].

The target genes of miR828/*TAS4* are a specific clade of MYB transcription factors shown by the principal investigator and others to be effectors of flavonol, proanthocyanidin, anthocyanin, and lignin biosynthesis pathways and associated with fruit development. Although the exact functions of the specific target MYBs are unknown, they are likely involved in phenolics metabolism because within well-studied subgroups across widely diverged species there is strong evidence they function in the same process [47].

OBJECTIVES

The general research objective (within the scope of Year 1 seed funding) is to test the hypothesis that specific trans-acting small interfering RNAs (ta-siRNAs) produced by grape are regulators of the Pierce's disease process. The long-term goal is to establish a new technology in grapes that will allow genetic manipulations that will not

carry the negative connotation of "genetically modified organism" (GMO). This is because the vector transgenes can be removed by conventional backcrosses to the transgenic lines, resulting in only mutated endogenous effector genes in progeny. We will achieve these goals in Year 1 by the following specific objectives:

1. Test the miR828, *TAS4*, and target *MYBA6/7* functions in Pierce's disease etiology and *X. fastidiosa* infection and spreading by genome editing using CRISPR/Ca9 transgenic technology.
2. Characterize tissue-specific expression patterns of *TAS4* and *MIR828* primary transcripts, sRNAs, and *MYB* targets in response to *X. fastidiosa* infections in the field.

RESULTS AND DISCUSSION

During the week of July 29, 2015, 22 samples of Pierce's disease-infected and symptomless Merlot leaves and petioles were collected from the Calle Contento vineyard in Temecula under the supervision of Matt Daugherty, University of California, Riverside Extension agent. Petiole DNA extracts will be assessed by the co-principal investigator for Pierce's disease titres, and correlated leaf materials will be extracted by the principal investigator for construction of small RNA- and transcriptome RNA-Seq libraries (Objective 2). During the first week of September 2015, the co-principal investigator collected Pierce's disease symptomatic and symptomless Chardonnay and Merlot leaf samples from the Black-stock vineyard in GA (Dahlonega, Lumpkin County) for shipment to the principal investigator's lab for molecular characterizations. Presence of *X. fastidiosa* was confirmed by culture methods, and polymerase chain reaction (PCR) confirmation is ongoing.

We have initiated methods development for quantitation of anthocyanins and polyphenolics from Pierce's disease infected leaf samples. **Figure 1** shows a chromatogram of unhydrolyzed and acid-hydrolyzed cyanidin glucoside standard as well as initial characterization of a commercial bilberry extract with validated aglycone peaks for delphinidin, cyanidin, and a combined malvidin/pelargonidin/peonidin peak (due to hydrophobic methyl groups).

The principal investigator has obtained materials from Tom Jacobs [48] and successfully engineered binary T-DNA *Agrobacterium* vectors to genome edit the grapevine *VvMIR828, VvTAS4ab*, and target *VvMYBA6 /VvMYBA7* loci; namely p201N-Cas9 (Addgene #59175) for plant expression of human codon-optimized Cas9 endonuclease from *Streptococcus pyogenes* [49] and pUC-gRNA shuttle (Addgene #47024) PCR template for engineering synthetic guide RNAs for *U6 promoter*-driven expression. Synthetic guide constructs were synthesized commercially with flanking *Spe*I and *SwaI* restriction sites and subcloned into p201N_Cas9 (**Figure 2**). The constructs have been electroporated into *Agrobacterium* strain EHA105 and will be shipped next week to Collaborator David Tricoli's lab under USDA Animal and Plant Health Inspection Service, Biotechnology Regulatory Services permit #15-231-102m (issued 10/05/15). A few additional vectors have been constructed to target the *Phytoene Desaturase* (*PDS*) gene of grapevine as a proof-in-principal test of genome editing efficiency by visual screen for photobleached sectors during regeneration. At least five regenerated independent Kan^R transformants for each of nine constructs and vector alone are anticipated in the next phase. The Collaborator (Tricoli) will transform Thompson Seedless variety with the *PDS* vectors because that variety can regenerate more quickly (estimated 4-6 months). Commercially relevant grapevine rootstocks 101-14 and/or 1103-P will be transformed with *MIR828, TAS4ab*, and *MYBA6/A7* vectors in parallel. Validation of editing events will be by PCR cloning and sequencing of target genes, and PAGE-based genotyping [50].

CONCLUSIONS

One year of funding has been provided, so the scope of work has been adjusted accordingly. We are on track to achieve our Objectives. In future applications, contingent upon satisfactory progress towards Objectives 1 and 2, we will characterize the changes in control versus edited genotypes for xylem inorganic phosphate (Pi) and polyphenolic levels of *X. fastidiosa* infected and Pi-treated stems, leaves, and berries. We will conduct *X. fastidiosa* challenge experiments with genome-edited transgenic plants. We will also endeavor to conduct insect diet preference and *X. fastidiosa* growth assays with candidate polyphenolics that arise from our results. It is noted that no host genes are yet known that normally function to *enhance* host susceptibility; knocking out any host gene (e.g. Pierce's disease resistance) may result in increased susceptibility to infections. Thus engineering Pierce's disease resistance is likely to be by incremental advances from characterizing molecular mechanisms.

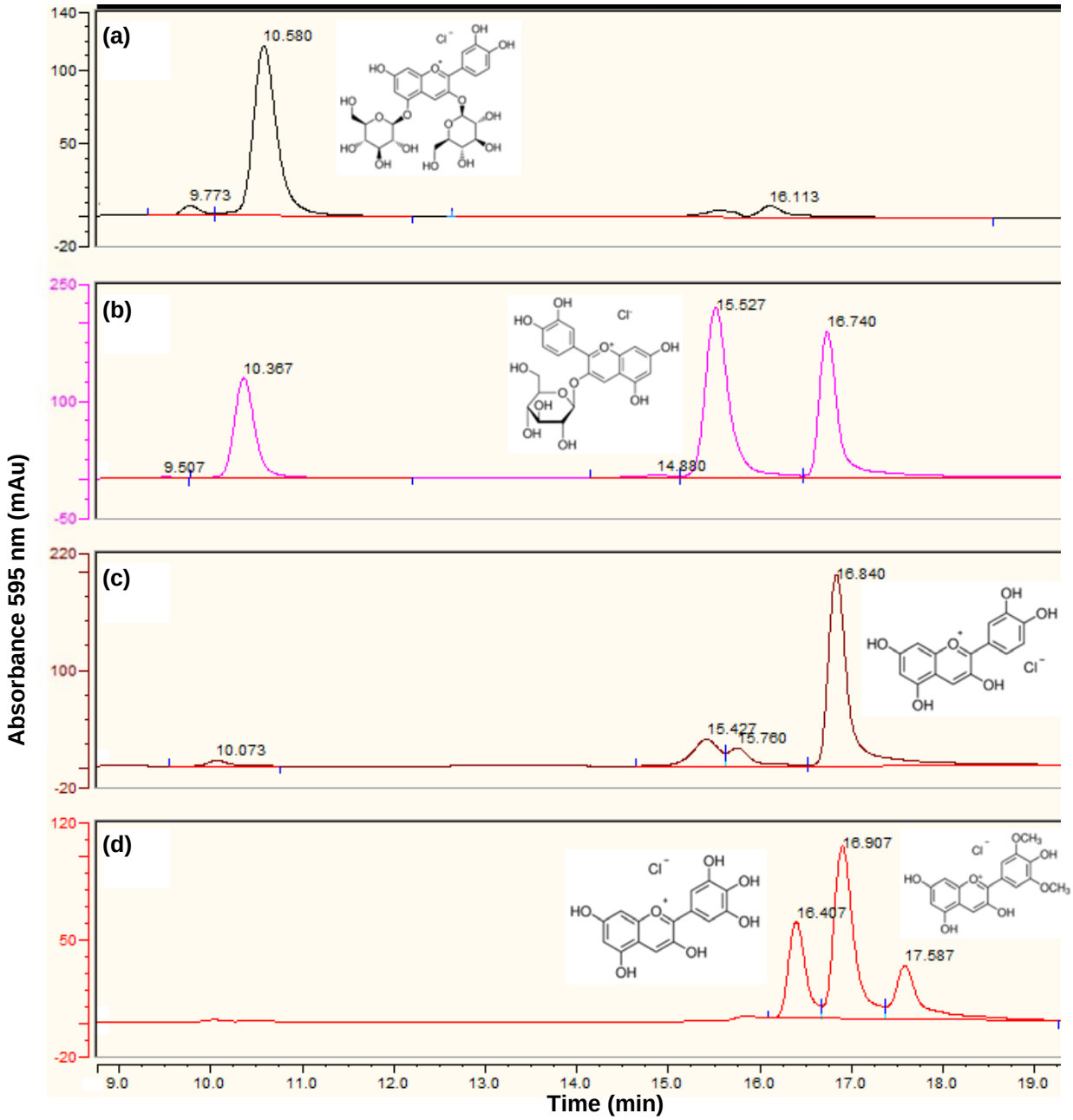

Figure1. Nano-HPLC chromatogram of timecourse hydrolysis of cyanidin-3,5-di-O-glucoside standard (a) 0'; (b) 40'; (c) 60' acid hydrolysis treatments; (d) 60' hydrolyzed commercial bilberry extract. Retention times: cyanidin-3,5-di-O-glucoside =10.3-10.6'; cyanidin-3- and -5-O-glucosides =15.4- 15.8'; cyanidin aglycone=16.8- 16.9'; delphinidin= 16.4'; malvidin= 17.6'. Samples were run on an Acclaim Pepmap RSLC 75 µm x 15 cm nanoViper C_{18} 2 µm column with 95% water:formic acid as stationary phase and 100% acetonitrile as mobile phase, linear gradient from 5-100% mobile in 30'.

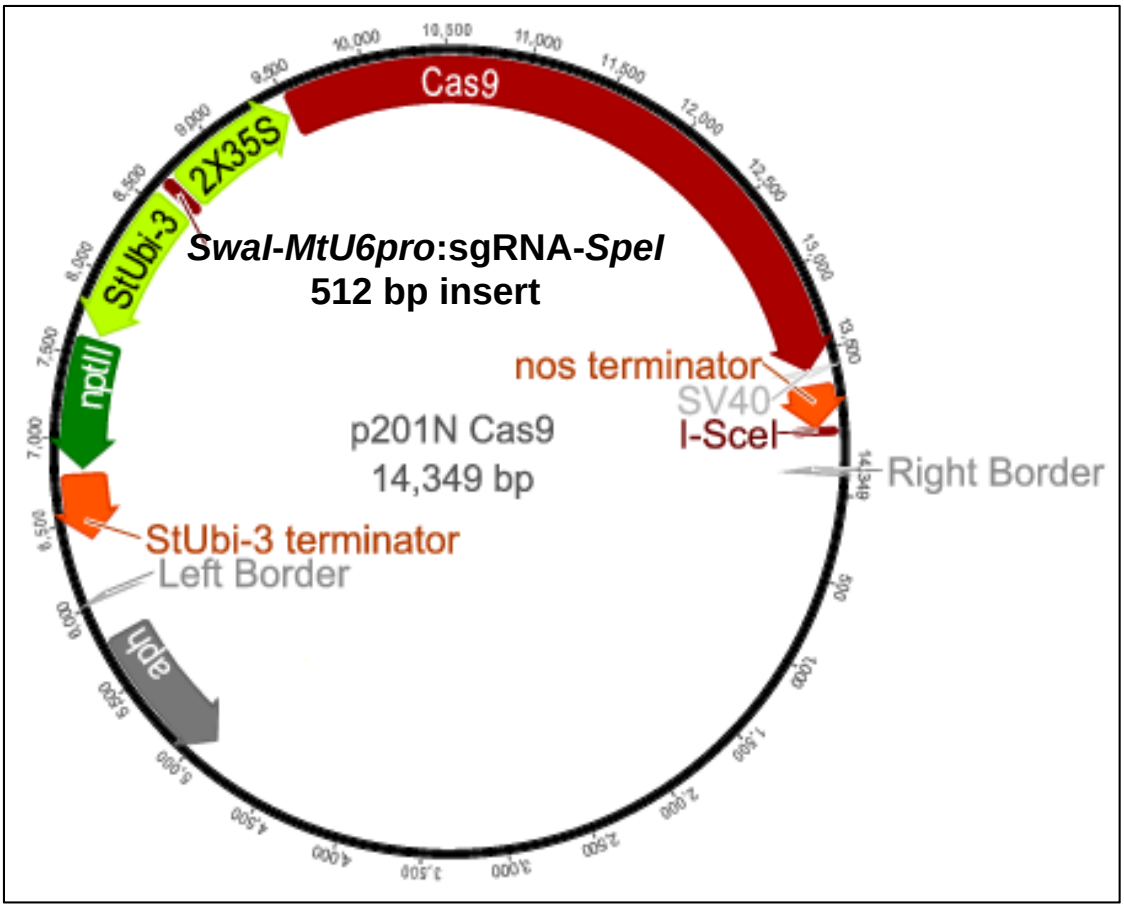

Figure2. Custom sgRNA/effector T-DNA construct p201N_Cas9 (Addgene #59175) successfully implemented for engineered *MIR828, TAS4*, and *MYB* targeting effectors. *MtU6pro*, Medicago snRNA PolIII-dependent promoter and terminator. s*gRNA*, gene-specific synthetic-guide sequence for Cas9 activity in eukaryotic cells. 2X*35S, StUbi-3,* constitutive plant promoters. SV40, in-frame nuclear localization signal. Cas9, human codon-optimized Cas9 endonuclease from *S. pyogenes*. *nos,StUbi-3 terminator*: plant transcription terminators. *aph, nptII,* kanamycin resistance genes for plant and bacterial selection, respectively.

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CONTINUATION OF THE FIELD EVALUATION OF NEW STRATEGIES FOR THE MANAGEMENT OF PIERCE'S DISEASE OF GRAPEVINE

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Reporting Period: The results reported here are from work conducted July 2014 to October 2015.

ABSTRACT

Our goal was to maintain the experimental vines evaluated as alternative strategies for management of Pierce's disease. The strategies were previously developed by project investigators (see list of collaborators) and funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board and include different plant modification approaches and use of a biocontrol agent. Here we present all the field activities that were done since July 2014 including irrigation water, soil, and plant tissue analyses. In 2014, we identified several problems that may have limited the establishment and growth of those vines after planting. Abiotic stresses that were identified include slightly alkaline soil and water, nutrient deficiencies in the soil (i.e., nitrogen, magnesium, boron and phosphorous), and in the plant (i.e., nitrogen, phosphorous), as well as boron toxicities in plant tissues. Crop load was also an issue in the Hopkins/Kirkpatrick plot. Biotic stresses other than Pierce's disease included nematode (*Tylenchulus semipenetrans*) in the Dandekar plot and powdery mildew (*Erysiphe necator*) in the Hopkins / Kirkpatrick plot. As expected, Pierce's disease pressure increased over the summer of 2014, as indicated by an increased number of glassy-winged sharpshooters (*Homalodisca vitripennis*) caught on yellow sticky traps. Pierce's disease severity was recorded in all research plots in 2014. In 2015, the Pierce's disease trials on the modified vines were terminated with herbicide sprays and thus no data on Pierce's disease severity and glassy-winged sharpshooter population were recorded. The monitoring of the biocontrol experiment from the Hopkins / Kirkpatrick plot also ended but the research was not terminated.

LAYPERSON SUMMARY

Alternative strategies for control of Pierce's diseases are currently being evaluated in the field at the Department of Agricultural Operations at the University of California, Riverside. Vines are subjected to natural disease pressure because of the presence of insect vector populations, the glassy-winged sharpshooter (*Homalodisca vitripennis*). Here we present all the field activities that were done since July 2014 including irrigation water, soil, and plant tissue analyses. In 2014, we identified several problems that may have limited the establishment and growth of those vines after planting. Abiotic stresses that were identified include slightly alkaline soil and water, nutrient deficiencies in the soil (i.e., nitrogen, magnesium, boron and phosphorous), and in the plant (i.e., nitrogen, phosphorous), as well as boron toxicities in plant tissues. Crop load was sometime an issue. Biotic stresses other than Pierce's disease included nematodes and powdery mildew. As expected, disease pressure increased over the summer, as indicated by an increased number of glassy-winged sharpshooters caught on yellow sticky traps. Pierce's disease severity was recorded in all research plots and results are presented in this report, but will be discussed in reports by individual principal investigators. In 2015, the Pierce's disease trials on the

modified vines were terminated with herbicide sprays and no data on Pierce's disease severity and glassy-winged sharpshooter population were recorded. The monitoring of the biocontrol experiment from the Hopkins / Kirkpatrick plot also ended but the research was not terminated.

INTRODUCTION

Xylella fastidiosa is a Gram negative, xylem-limited, insect-vectored bacterium and is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002). Current Pierce's disease management strategies primarily involve vector management through the use of insecticides. Several alternative strategies are currently being evaluated in field trials. One of the field trials is located at the Department of Agricultural Operations (AgOps) at the University of California, Riverside. The experimental grapevines grown at the University of California, Riverside are subjected to natural insect populations (the glassy-winged sharpshooter; *Homalodisca vitripennis*) that vector the disease. The strategies developed by principal investigators Dandekar, Lindow, Gilchrist, Powell, and Kirkpatrick/Hopkins that are currently being evaluated include various modified grape and grape rootstocks expressing genes from different constructs as well as the use of a non-virulent *X. fastidiosa* strain as a biocontrol agent (see individual principal investigator reports for more information). Our goal is to maintain the vines growing at AgOps and record data on insect vector and disease pressure, and Pierce's disease incidence and severity in order to identify the most effective control strategy moving forward.

OBJECTIVES

1. Maintain grapevines and research plots.
2. Monitor sharpshooter populations and disease pressure.
3. Record Pierce's disease severity.

RESULTS AND DISCUSSION

Objective 1. Maintain grapevines and research plots.

Field activities since July of 2014 are reported in **Table 1**. Water, soil, and tissue samples from each experimental plot were sent to Fruit Growers Lab, Inc. for analyses (**Tables 2, 3, and 4**). Ever Green Nematode Testing Lab, Inc. also performed nematode analysis from the soil samples. For the irrigation water, no obvious problem was noticed besides a slightly alkaline pH (**Table 2**). The samples from shallow (0-25cm) and deep (25-50 cm) soils around grapevine roots as well as background soil from middle rows also showed that soils were slightly alkaline. This condition is likely affecting cation exchange capacity (CEC) as higher pH decreases cation availability (**Table 3**). Overall, deep soil seems to be more deficient in magnesium and zinc. In addition, boron and nitrate-nitrogen and phosphorous availability were limited in deep and shallow soils. Some of these carried over to tissue analyses whereby nitrogen, and sometimes phosphorous and zinc were lower than the optimum range (**Table 4**). However, toxic levels of boron were recorded in vines from all experimental plots. Mineral nutrients imbalance was also previously reported in grapevines and host plants infected with Pierce's disease (Lu et al, 2003; De La Fuente et al., 2013), but never for boron (B). Those deficiency or toxicity levels may have confounded Pierce's disease symptoms, as older leaves with B toxicity can appear scorched. Thus, improper disease severity rating may have resulted from it. Nematode analysis showed that *Tylenchulus semipenetrans* was present in Dandekar's plot (2,254 nematode per kg of soil) and they may have stressed the vines and caused them to decline (Verdejo-Lucas and McKenry, 2004). Interestingly, these nematodes were only found in Dandekar's block. Abiotic stress such as heavy crop load that was only observed on some vines in the Hopkins/Kirkpatrick plot may also have stressed the vines and caused them to decline (**Figure 1B**).

In 2015, standard viticultural practices were implemented in all research plots to correct some of the problems observed in 2014 as described above, which limited somewhat our capacity to rate Pierce's disease symptoms accurately. Grapevines were pruned and trained properly, fertilized and irrigated as needed. Clusters were dropped during the season to avoid over-cropping thereby stressing the vines. In addition, an active powdery mildew management program was implemented at the beginning of the growing season including fungicide spray and leaf removal to open the canopy. However, herbicide damages were observed on the foliage for one of the research plot (Powell) and affected about 25% of the vines (**Figure 1C**). The experiments on modified vines (Lindow, Dandekar, Gilchrist, and Powell) were terminated in July of 2015 (**Figure 1D**) and for this reason Pierce's disease severity rating and glassy-winged sharpshooter population were not monitored over the summer. The experiment on biocontrols (Hopkins/Kirkpatrick) is still ongoing and has been taken over by the principal investigators.

Table 1. Field activities for all grapevine experimental plots located at AgOps, UC Riverside.

Date	Activity
2014	
July 2	Traps collected, sharpshooters censused, new traps deployed
July 11	Rodent control
July 12	Grape tissue sampling for analysis - Fruit Growers Lab
July 17	Fungicide application (rally + stylet oil) for powdery mildew control Weed control
August 7	Traps collected, sharpshooters censused, new traps deployed
August 8	Pruning and burying grape cuttings Fungicide application (stylet oil) for powdery mildew control
August 21	Pruning and burying grape cuttings
August 22	Soil and root sampling for nematode count – Ever Green Nematodes testing Lab Soil sampling for analysis - Fruit Growers Lab
August 12-26	Weeding and vine training
August 26	Weed control
September 4	Traps collected, sharpshooters censused, new traps deployed
September 17	Water sampling from drip irrigation for analysis – Fruit Growers Lab
September 22	Pierce's disease severity rating
September 23	Weed control
September 29	Pierce's disease severity rating
October 6	Pierce's disease severity rating Sampling petioles for *X. fastidiosa* detection by qPCR
2015	
February	Grapevine pruning
April 1	Fungicide application (sulfur) for powdery mildew control
April 9	Train vines
April 17	Fungicide application (sulfur) for powdery mildew control Fertilizer application (12-26-26)
May 8	Grapevine flowers removal
May 13	Fungicide application (stylet oil) for powdery mildew control
May 29	Fungicide application (Rally) for powdery mildew control
June 10	Fungicide application (Rally) for powdery mildew control
June 18	Fungicide application (Rally) for powdery mildew control
July 7	Herbicide treatment (Garlon). Termination of PD trials on modified vines.
July 28	Herbicide treatment (Roundup). Termination of PD trials on modified vines.
August 14	Herbicide treatment (Roundup). Termination of PD trials on modified vines.
September 25	Herbicide treatment (Roundup). Termination of PD trials on modified vines.

Figure 1. (A) Grapevine cv. Pinot improperly trained, also showing powdery mildew symptoms, 2014. (B) Over-cropped grapevine cv. Pinot showing signs of stress, 2014. (C) Herbicide damage on modified vines likely caused by drift, 2015. (D) Dead modified grapevine following voluntary termination with herbicide sprays, 2015.

Table 2. Grape irrigation suitability analysis, Fruit Growers Laboratory, Inc. Values highlighted in red represent higher level than the optimal requirements.

Test Description	**Results** mg/L	Meq/L
Cations		
Calcium	50	2.5
Magnesium	10	0.82
Potassium	3	0.077
Sodium	40	1.7
Anions		
Carbonate	< 10	0
Bicarbonate	170	2.8
Sulfate	57	1.2
Chloride	29	0.82
Nitrate	16.1	0.26
Nitrate Nitrogen	3.6	
Fluoride	0.5	0.026
Minor Elements		
Boron	0.1	
Copper	0.01	
Iron	0.04	
Manganese	0.04	
Zinc	< 0.02	
TDS by Summation	376	
Other		
pH	7.6	
E.C.	0.513 dS/m	
SAR	1.4	
Crop Suitability		
No amendments	Fairly good	
With amendments	Good	
Amendments		
Gypsum requirement	0.2 Tons/AF	
Sulfuric acid (98%)	9.8 oz/1000Gal	
Leaching requirement	3.3 %	

Table 3. Soil analysis, Fruit Growers Laboratory, Inc. Soil samples representing shallow soil (SS-R) and deep soil (DS-R) of grapevine roots as well as background soil from middle row (BS), were collected from each experimental block. Values highlighted in yellow and red represent lower and higher levels than the optimal requirements, respectively.

Test Description	**Block**								
	Dandekar			Kirkpatrick / Hopkins			Lindow/Gilchrist/Powell		
	BS	SS-R	DS-R	BS	SS-R	DS-R	BS	SS-R	DS-R
Primary Nutrients									
Nitrate-Nitrogen (ppm)	11.8	1.5	2.5	7.4	1.9	1.5	111	1.4	1.4
Phosphorus-P2O5 (ppm)	48.1	25.2	45.8	20.6	25.2	18.3	38.9	20.6	29.8
Potassium-K2O *Exch* (ppm)	157	84	133	72	84	60	145	72	169
Potassium-K2O *Sol* (meq/L)	1.06	0.304	0.496	0.112	0.103	0.041	2.29	0.394	0.618
Secondary Nutrients									
Calcium *Exch* (ppm)	560	620	640	1120	1480	1140	560	580	660
Calcium *Sol* (meq/L)	3.06	2.33	1.88	3.42	5.78	3.34	26.5	4.28	1.95
Magnesium *Exch* (ppm)	78	92	84	132	174	139	79	85	96
Magnesium *Sol* (meq/L)	0.933	0.892	0.611	0.927	1.7	0.98	7.71	1.61	0.625
Sodium *Exch* (ppm)	20	40	30	40	80	50	30	30	50
Sodium *Sol* (meq/L)	0.678	4.48	2.57	2.4	7.72	4.09	5.37	4.66	3.89
Sulfate (meq/L)	0.627	3.01	1.86	1.25	7.69	3.65	6.17	2.31	2.95
Micronutrients									
Zinc (ppm)	0.8	1	0.8	0.5	0.8	0.3	0.6	0.5	0.3
Manganese (ppm)	6.4	4.1	3.2	4.2	5.4	4.1	5.3	5.3	4.8
Iron (ppm)	17.5	15.3	14.1	11.2	11.3	15	15.5	9.5	10.2
Copper (ppm)	0.6	0.7	0.6	0.7	0.9	0.5	0.6	0.6	0.5
Boron (ppm)	0.26	0.17	0.18	0.3	0.22	0.15	0.243	0.13	0.13
Chloride (meq/L)	0.45	1.65	0.69	0.68	4.59	2.6	4.04	1.54	1.72
CEC (meq/100g)	3.77	4.24	4.3	7.02	9.38	7.19	3.87	3.9	4.64
% Base Saturation									
CEC – Calcium (%)	74.3	73.1	74.4	79.8	78.8	79.3	72.4	74.4	71.1
CEC – Magnesium (%)	17	17.9	16	15.5	15.2	15.9	16.8	17.9	17
CEC – Potassium (%)	8.78	4.43	6.28	2.35	1.96	1.61	7.78	4.1	7.59
CEC – Sodium (%)	0.1	4.41	3.28	2.32	3.93	3.31	2.97	3.54	4.22
CEC – Hydrogen (%)	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Others									
pH	6.67	7.46	7.35	7.52	7.57	7.69	6.74	6.85	7.34
Soil Salinity (dS/m)	0.62	0.76	0.54	0.69	1.48	0.84	4.18	0.98	0.73
SAR	0.5	3.5	2.3	1.6	4	2.8	1.3	2.7	3.4
Limestone (%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Moisture (%)	2.7	8.1	6.5	2.8	11.4	6.5	3.5	6.2	3.7
Saturation (%)	27.2	21.6	23.4	24.5	30.7	28.6	25.2	25	26.2

Table 4. Grapevine leaf blades and petioles nutrient analyses from the three experimental blocks at AgOps, UC Riverside. Samples were collected in July of 2014 and sent to Fruit Growers Lab, CA. Values highlighted in yellow and red represent lower and higher levels than the optimal requirements, respectively.

Sample	Test Description	Block		
		Dandekar	Kirkpatrick Hopkins	Lindow Gilchrist Powell
Leaf blades	**Macronutrients**			
	Total Nitrogen (%)	3.3	2.62	2.88
	Phosphorus (%)	0.46	0.17	0.38
	Potassium (%)	1.37	0.47	1.78
	Calcium (%)	2.19	2.88	2.38
	Magnesium (%)	0.35	0.36	0.38
	Micronutrients			
	Zinc (ppm)	38.4	23.2	32.1
	Manganese (ppm)	111	100	121
	Iron (ppm)	251	290	187
	Copper (ppm)	15	8	14
	Boron (ppm)	91.3	69	102
	Sodium (%)	0.024	0.014	0.022
Petioles	**Macronutrients**			
	Total Nitrogen (%)	--	0.83	0.77
	Nitrate-Nitrogen (ppm)	--	840	710
	Phosphorus (%)	--	0.12	0.68
	Potassium (%)	--	0.98	4.30
	Calcium (%)	--	2.46	1.54
	Magnesium (%)	--	0.76	0.62
	Micronutrients			
	Zinc (ppm)	--	58.8	42.7
	Manganese (ppm)	--	223	218
	Iron (ppm)	--	47	72
	Copper (ppm)	--	7	7
	Boron (ppm)	--	34.2	42.9
	Sodium (%)	--	0.075	0.166

Objective 2. Monitor sharpshooter populations and disease pressure.
In 2014, sharpshooters were monitored at the experimental site in all three blocks (Dandekar, Gilchrist / Lindow / Powell, and Kirkpatrick/Hopkins). For each block, six 6” x 9” double-sided yellow sticky traps were placed randomly throughout the plots. Traps were mounted on wooden stakes slightly above the vine canopy. These traps were collected every month and returned to the laboratory to identify under the stereomicroscope the number of glassy-winged sharpshooters. Results (**Figure 2)** showed that a low insect vector population was recorded early in the season (March to May 2014) but the population drastically increased over the summer of 2014. No data were collected in 2015 because the Pierce’s disease modified plant material trials were terminated.

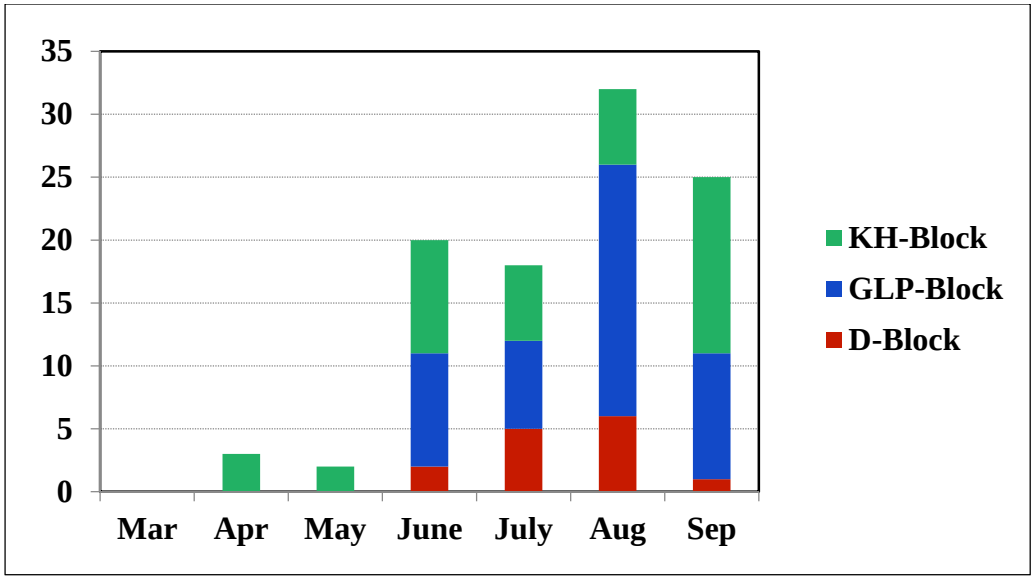

Figure 2. Total number of glassy-winged sharpshooter insect vectors captured on yellow sticky traps from all three experimental blocks (D: Dandekar; KH: Kirkpatrick/Hopkins; GLP: Gilchrist / Lindow / Powell) in 2014. Results are based on a total of 18 traps (six traps per block).

Objective 3. Record Pierce's disease severity.

The Pierce's disease rating was assessed based on a rating scale developed by Dr. Bruce Kirkpatrick. This Pierce's disease rating scale requires that vines are cordon trained. However, because grapevines at AgOps were not always trained with cordons and were sometimes pruned improperly (**Figure 1A**), it was difficult to use that disease rating scale, so it was modified (**Table 5**). Besides improper training, powdery mildew leaf symptoms (**Figure 1B**) and boron toxicity may have confounded Pierce's disease symptoms especially in the Hopkins / Kirkpatrick plot. No data were collected in 2015 because the Pierce's disease modified plant material trials were terminated.

Table 5. Rating scale use to score Pierce's disease severity on grapevines from all experimental plots at AgOps, UC Riverside.

Pierce's Disease Rating	Symptoms
0	None
1	One shoot/cane expresses PD symptoms
2	Two shoots/canes express PD symptoms
3	Less than 50% of the grapevine is symptomatic
4	More than 50% of the grapevine is symptomatic
5	Grapevine is dead

	V13	V12	V11	V10	V9	V8	V7	V6	V5	V4	V3	V2	V1
Row13				1	1	1	3	2	1	0	1	1	2
Row12	2	2	3	3	3	3	4	3	4	4	1	0	1
Row11	2	4	3	x	1	2	3	1	2	3	1	2	1
Row10	1	1	2	3	1	1	2	1	2	1	1	1	1
Row9	3	3	3	1	1	1	2	3	4	4	2	1	2
Row8	2	1	2	1	1	1	1	3	x	1	1	1	1
Row7	1	2	3	3	4	3	1	3	3	4	3	0	1
Row6	2	3	4	4	4	3	1	1	3	2	1	1	1
Row5	4	4	4	4	3	0	1	0	0	3	4	3	1
Row4	2	1	4	4	4	2	2	2	0	1	2	1	3
Row3	1	2	2	1	2	3	3	1	3	x	3	0	1
Row2	3	3	4	3	2	x	3	4	1	4	1	3	1
Row1	1	1	2	1	1	1	2	1	0	0	3	1	4

Dirt Road

Dirt Road

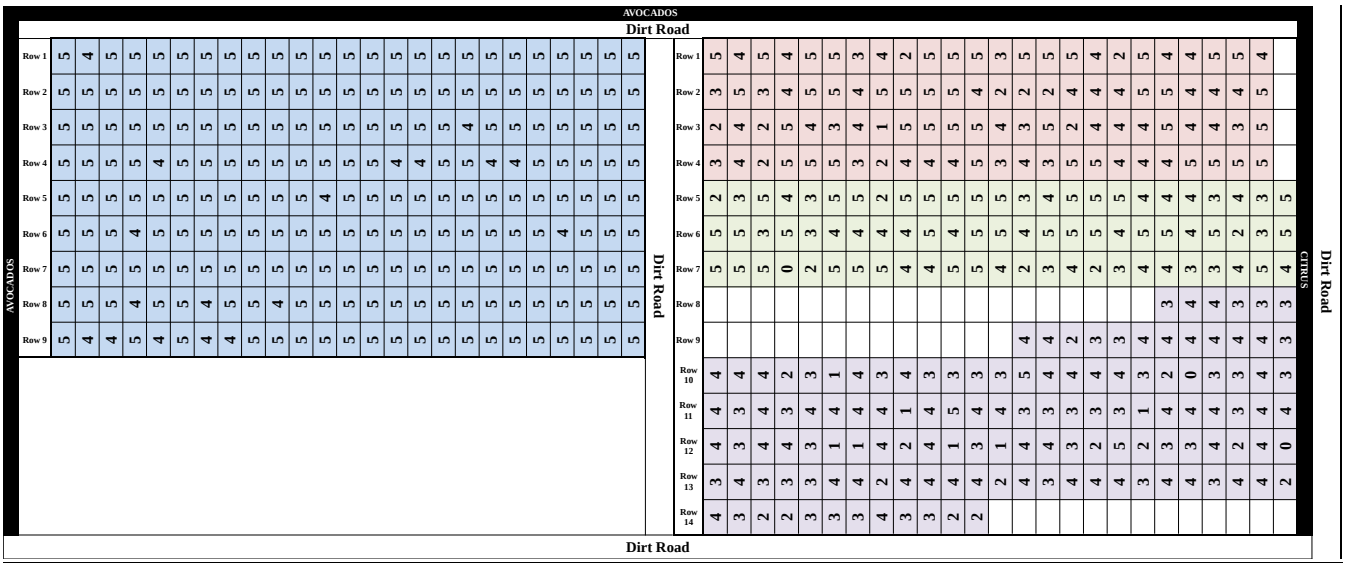

Figure 3. 2014 Pierce's disease severity rating (**Table 5**) for experimental plots including Hopkins/Kirkpatrick (gray), Dandekar (blue), Lindow (red), Gilchrist (green), and Powell (purple).

CONCLUSIONS

The experimental site at AgOps, University of California, Riverside, is the perfect site to evaluate alternative strategies for control of Pierce's disease, because of the natural presence the disease vector, the glassy-winged sharpshooter. Our 2014 observations and results indicated that the management practices at the experimental site needed to be modified in 2015 so one could fully assess the efficacy of each strategy. Nonetheless, the symptoms and decline of the grapevines that we recorded in 2014 were mostly caused by the presence of *X. fastidiosa*, although additional stressors may have caused those vines to decline faster. No data were collected in 2015 because the Pierce's disease modified plant material trials were terminated.

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EVALUATION OF NATURAL PRODUCTS ISOLATED FROM GRAPEVINE FUNGAL ENDOPHYTES FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted on July 2012 to present.

ABSTRACT
The goal of this research is to identify fungal natural products antagonistic to *Xylella fastidiosa* that could be implemented as curative treatments for Pierce's disease. We showed in *in vitro* bioassays that eight fungal endophytes isolated from grapevine wood possess anti-*X. fastidiosa* properties, due to the production of natural products. We previously purified and characterized two natural products (radicinin and molecule 'C') produced by two endophytic fungi (i.e., *Cochliobolus* sp. and *Dreschlera* sp., respectively), and demonstrated that they were effective inhibitor of *X. fastidiosa*. However, those compounds have poor solubility in water and thus were not systemic and active against *X. fastidiosa* when injected *in planta*. Recently, we showed that the fractions from the crude extracts of three additional fungal endophytes (i.e., *Eurotium*, *Geomyces*, and *Ulocladium*) also possess activity against *X. fastidiosa* in the *in vitro* bioassay. Active fractions from the crude extracts of these three fungal cultures are being examined using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) to identify their chemical structures and properties. The next step of this research is complete the identification of the newly discovered natural products and also to develop water-soluble derivatives of radicinin and molecule 'C.' We have developed a procedure to increase yield and purity of the radicinin by recrystallization instead of chromatography. Moreover, we have produced several semi synthetic molecule derivatives of radicinin with one molecule showing increased water solubility. We aim to develop a commercial product that can be applied as foliar spray, drench application, or trunk injection in vines and become active in the xylem where the bacterium resides. These molecules are currently under review for patentability by the Executive Licensing Officer in the University of California, Riverside Office of Research and, hence, their names cannot always be disclosed in this report.

LAYPERSON SUMMARY
Several management strategies for Pierce's disease are currently being deployed, but as of today successful management largely involves vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or being developed. The goal is to identify natural products of fungi associated with grapevines that are antagonistic to *Xylella fastidiosa* that could be implemented as curative treatments for Pierce's disease, either as a foliar spray, drench application, or trunk injection. We have identified eight fungi naturally inhabiting grapevines that are antagonistic to *X. fastidiosa in vitro*. We have been extracting, purifying, and characterizing these fungal compounds and have identified two molecules that are inhibitory to the bacterium in an *in vitro* bioassay. However, those molecules have poor water solubility and could not be used successfully as treatment for Pierce's disease infected grapevines. We are currently working towards increasing water solubility of these molecules by making active by-products. In addition, we recently showed that the fractions from the crude extracts of three additional fungal endophytes inhibited *X. fastidiosa* in a disc bioassay. We are now in the process of characterizing the chemical structure and property of these molecules so they can be further tested in grapevine. These natural products are currently under review for patentability by the Executive Licensing Officer in the University of California, Riverside Office of Research and, hence, their names cannot always be disclosed in this report.

INTRODUCTION.

Xylella fastidiosa is a Gram negative, xylem-limited, insect-vectored bacterium and is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002). Pierce's disease is endemic to California but the recent introduction of a more effective vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*), to Southern California shifted the epidemiology of Pierce's disease from a monocylic to a polycyclic disease. This led to a Pierce's disease epidemic with severe economic consequences for the Southern California grape industry. The potential for the glassy-winged sharpshooter to move north and become established throughout the state remains a severe threat to the other major grape-growing regions (Central and Northern California). Current Pierce's disease management strategies largely involve vector management through the use of insecticides.

Control of Pierce's disease with fungi or fungal metabolites is a largely unexplored research area. Fungi are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce, together with the fact that many fungal species remain chemically unexplored. Fungi are excellent sources of interesting novel molecules that may be candidates with potential for control of bacterial diseases. Indeed, using fungi as biocontrol agents against plant disease is an active area of research (Amna, 2010; Proksch et al., 2010; Xu et al., 2008). We first characterized the microbial diversity in grapevines that escaped Pierce's disease in natural vineyard settings, and compared this population to Pierce's disease infected grapevines with the goal of identifying fungi that are unique to Pierce's disease escaped vines. We identified eight fungal endophytes that possess anti-*X. fastidiosa* properties, likely due to the production of natural products. Our objective is to identify anti-*X. fastidiosa* fungal endophytes natural products and natural product derivatives that we could use as curative treatments for Pierce's disease.

OBJECTIVES

1. Identify fungal natural products and semisynthetic derivatives active against *X. fastidiosa*.
2. Evaluate fungal natural products and semisynthetic derivatives for their potential as curative treatments for vines already infected with Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Identify fungal natural products and semisynthetic derivatives active against *X. fastidiosa*.

The goal of this objective is to identify fungal species and fungal natural products produced by endophytes that can be used as curative treatments for control of Pierce's disease. We previously identified eight fungal specimens inhabiting grapevine tissues (xylem sap, shoot, petioles and spur) that were able to inhibit *X. fastidiosa* in a bioassay (Rolshausen and Roper, 2011). In brief, *X. fastidiosa* liquid cultures are adjusted to OD_{600nm}=0.1 (approx. 10^7 CFU/ml); 300 µl of the *X. fastidiosa* cell suspension are added to three ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture is then overlayed onto a petri plate containing PD3 medium. A sterile circle of agar is drawn from the margin of an actively growing pure fungal culture and is placed onto the plates previously inoculated with *X. fastidiosa.* Plates are incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Figure 1**).

In addition, crude extracts collected from the fungal cultures showing inhibition towards *X. fastidiosa* were collected for evaluation using a similar growth inhibition assay as described above. In brief, agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi were cultivated at room temperature on a shaker. After 10 days, each culture was filtered and further extracted with ethyl acetate, re-suspended in sterile methanol to an extract mass of 1 mg, pipetted onto sterile paper discs, and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts are placed onto the *X. fastidiosa* cultures and incubated at 28ºC for seven days. Following this, plates were observed for a halo of inhibition around the paper disc and compared to control *X. fastidiosa*-only plates and plates with paper discs treated with methanol only. Crude extracts showing inhibition were further processed to purify and identify the inhibitory molecules. Thus far, we have purified two molecules (radicinin and molecule 'C') that are active against *X. fastidiosa* growth *in vitro* and have characterized their chemical structure. Radicinin is produced by *Cochliobolus* sp. and molecule 'C' is produced by *Dreschlera* sp. These molecules are currently under review for patentability by the Executive Licensing Officer in the University of California, Riverside Office of Research and, hence, their names cannot always be disclosed in this report.

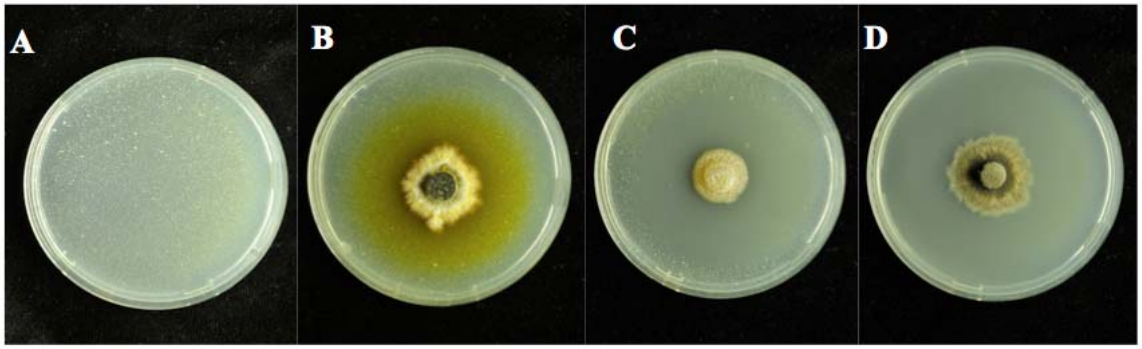


Figure 1. *In vitro* inhibition assay used to evaluate fungal activity towards *X. fastidiosa. X. fastidiosa* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after eight days of incubation at 28˚C. (A) *X. fastidiosa*-only control; (B) No *X. fastidiosa* inhibition; (C) Mild *X. fastidiosa* inhibition; (D) Total *X. fastidiosa* inhibition.

Radicinin showed great potential *in vitro* (Aldrich et al., 2015). Hence, in an *in vitro* dose response assay, where *X. fastidiosa* cells are submitted to an increasing concentration of a fungal molecule, radicinin was able to inhibit *X. fastidiosa* growth (**Figure 2**). We have developed a more efficient procedure for isolating radicinin from *Cochliobolus* sp. This is a critical step, as it will allow us to produce substantial amounts of water-soluble derivatives and further test them *in planta*. Radicinin is not commercially available, and we had been employing a multistep isolation procedure involving liquid-liquid extraction of *Cochliobolus* cultures followed by an expensive and time-consuming chromatography step to obtain pure radicinin for all our studies to date. Recently, we developed a procedure for purifying radicinin by recrystallization instead of chromatography. In this way, we were able to increase our yield of radicinin from 60.5 mg/liter of culture to 150 mg/liter of culture. This procedure also makes scaling up of the isolation for commercial use much more practical. In addition, the radicinin obtained by this new procedure is significantly more pure, as observed by nuclear magnetic resonance (NMR) spectroscopy.

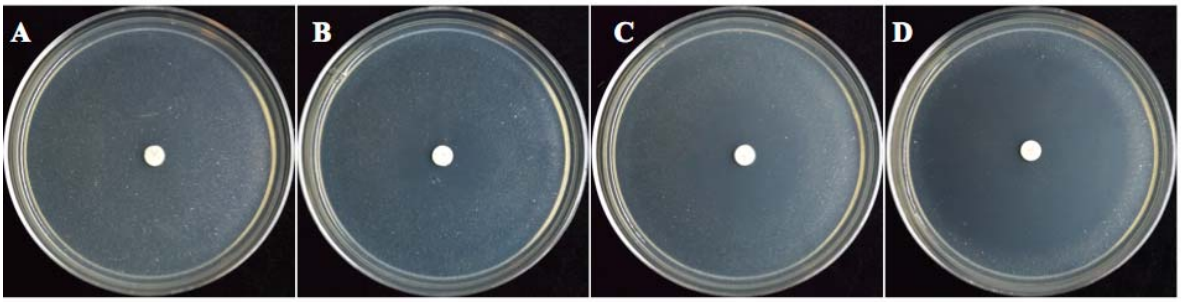
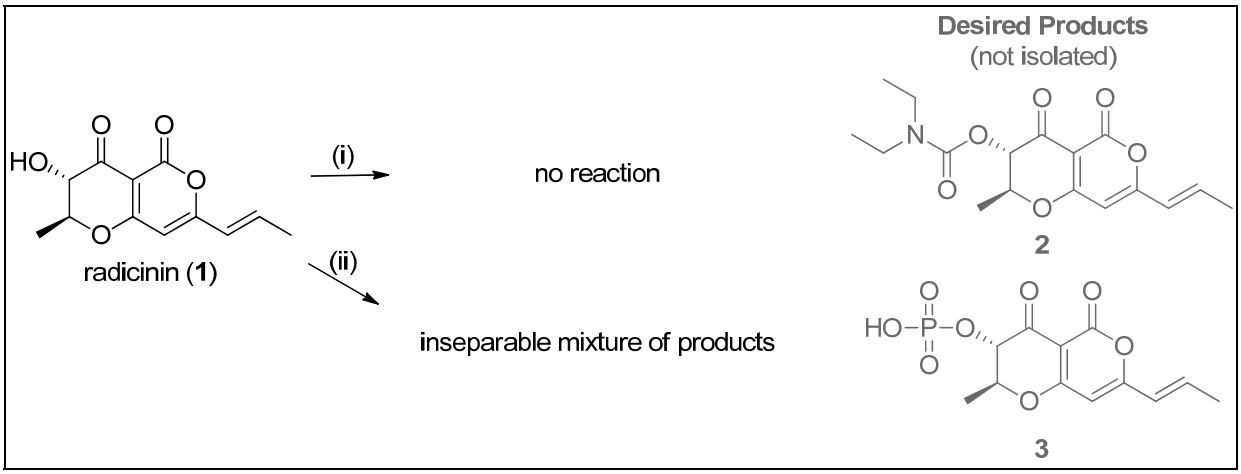


Figure 2. Dose response assay to evaluate *in vitro X. fastidiosa* inhibition at increasing concentrations of a fungal molecule. (A) 0 µg molecule R1 (control); (B) 50 µg molecule R1; (C) 100 µg molecule R1; (D) 250 µg molecule R1.

Now that we have figured out how to scale up radicinin production and purification, the next step was to prepare water-soluble semisynthetic derivatives of radicinin to facilitate testing *in planta*. We determined the solubility of radicinin in water to be 0.15 mg/mL, which is considered very slightly soluble. We have shown that acetylradicinin, which was modified at the hydroxyl group of radicinin, retains its anti-*X. fastidiosa* activity (**Figure 3**; Aldrich et al., 2015). This result suggests that modification of this position may provide a viable strategy for increasing the water-solubility of radicinin without loss of activity. Adding ionizable groups is a commonly employed strategy for improving the water-solubility of bioactive molecules (Kumar and Singh, 2013), so we had proposed to add two such groups at the hydroxyl position of radicinin (**Scheme 1**). The carbamate (2) is weakly basic and should form a water-soluble salt in low pH solutions, while the phosphate (3) is acidic and should form a water-soluble salt at high pH. Both carbamates and organophosphates are commonly found in pesticides, so we had good reason to believe that one or both of these compounds would be able to move into the

xylem of grapevines. However, attempts to prepare the weakly basic carbamate and the acidic phosphate were unsuccessful. Specifically, the reaction with diethylcarbamoyl chloride (i) did not go to completion, while the phosphate reaction (ii) gave a mixture of products that we were unable to purify.



Scheme 1. *X. fastidiosa*-inhibitory natural product radicinin (1), and semisynthetic derivatives (2-4). Reagents: (i) N,N-diethylcarbamoyl chloride, triethylamine (Vougogiannopoulou et al., 2008). (ii) 1. Cl3CCN, 2. (n-Bu)4NH2PO4, CH3CN, 3. DOWEX 50WX8, NH4HCO3 (Lira et al., 2013).

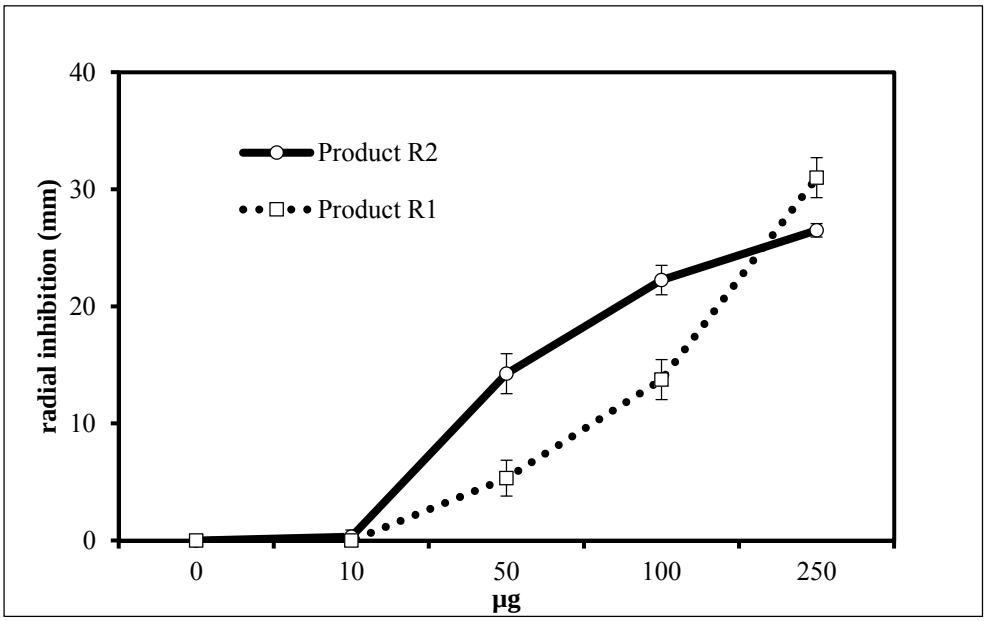
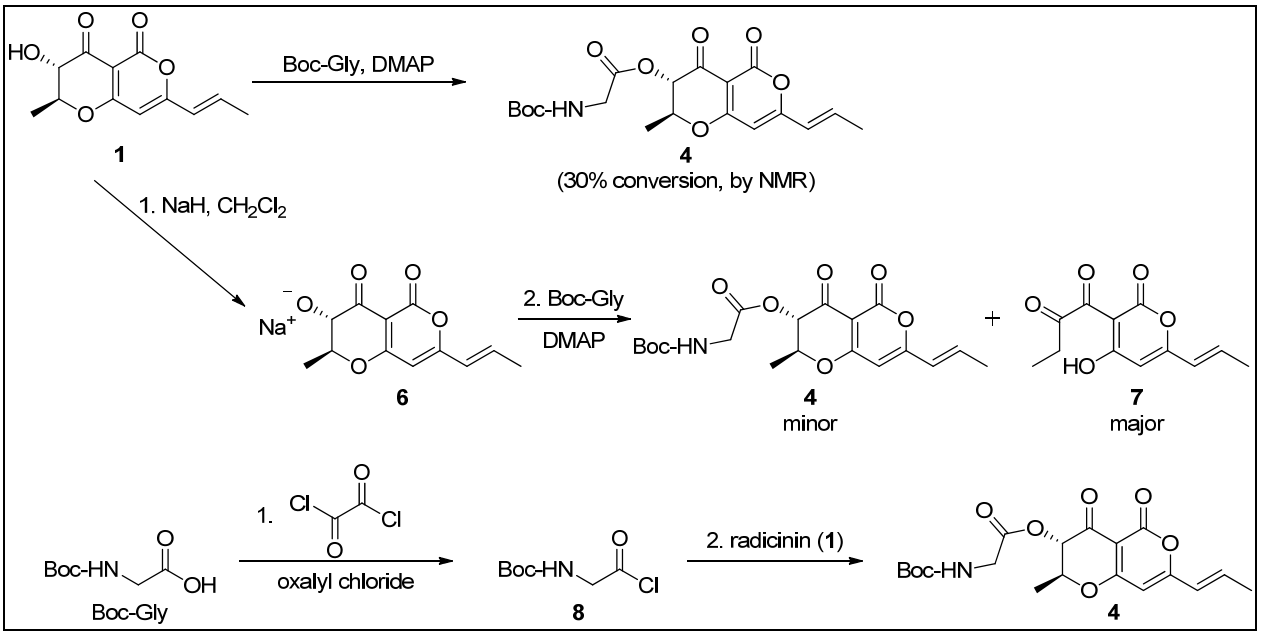


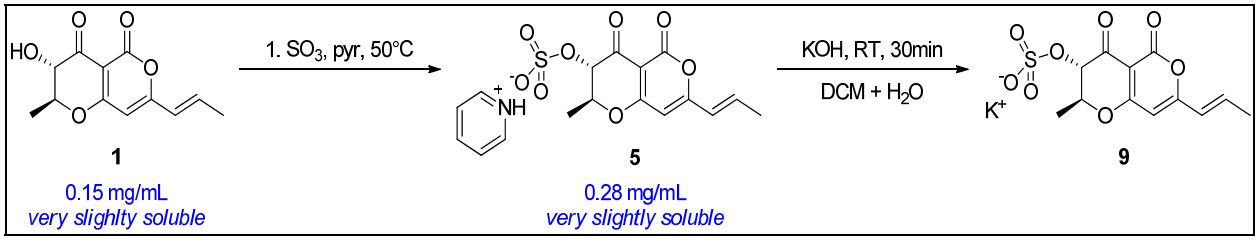
Figure 3. *In vitro* dose response assay. This lab assay quantifies inhibition of *X. fastidiosa* growth as a measure of a halo around disc (mm) containing increasing concentration of 'R1' (radicinin) and molecule derivative 'R2.'

We then attempted to make two alternate ionizable radicinin derivatives: a glycine-derivative (4, **Scheme 2**), and radicinin pyridinium sulfate (5, **Scheme 3**). The failure of reactions to form either 2 or 4 suggested that the alcohol group of radicinin is much less nucleophilic than we originally expected. We attempted to increase the nucleophilicity of this group by first deprotonating with sodium hydride to give an alkoxide (6, **Scheme 2**). We isolated 6 and found it to be more than a thousand-fold more water-soluble than radicinin, at 218 mg/mL (which is considered freely soluble). However, the high pH of the alkoxide solution leads us to be concerned about possible nonspecific toxicity. We also doubt that this high water solubility would be maintained in a cellular environment, which is buffered at neutral pH. Despite the increased nucleophilicity of 6, we never observed any

formation of carbamate 2, and observed only minimal formation of the boc-glycine derivative 4. Under the reaction conditions to form 4, radicinin appeared to undergo tautomerization and ring-opening to give isomer 7 (**Scheme 2**). We successfully prepared a sulfate of radicinin, as the pyridinium salt 5. Salt 5 maintained its activity against *X. fastidiosa* in our disc assay (**Figure 2**). This reaction proceeded to completion and the product proved easy to isolate. Unfortunately, the water solubility of 5 was only about twice that of radicinin: 0.28 mg/mL, lower than we had hoped. Recently, we were able to successfully replace the pyridinium counterion with potassium to give salt 9 (**Scheme 2**), which we hope will be more water soluble than 5, while retaining activity. We are currently in the process of bringing up more of potassium salt 9 for solubility testing.



Scheme 2. Attempts to form the Boc-Gly derivative of radicinin using traditional peptide coupling methodology (top), or deprotonating first with sodium hydride (middle) gave the desired derivative as only a minor product, along with a ring-opened isomer of radicinin (7). We next plan to try activating Boc-glycine to the acid chloride (8) using oxalyl chloride, prior to reaction with radicinin (bottom).



Scheme 3. We prepared the pyridinium sulfate of radicinin (5), which was roughly twice as water-soluble as radicinin. Recently, we were able to exchange the pyridinium counterion for a more polar potassium ion in the potassium sulfate 9.

In addition to radicinin, we had identified molecule 'C' as another natural molecule produced by *Drechslera* capable of inhibiting *X. fastidiosa* growth in our laboratory bioassay (**Figure 2**). This year, we began the bioassay-guided isolation of natural products from the remaining fungi able to inhibit *X. fastidiosa* in our lab bioassay (**Figure 1**), including *Cryptococcus* sp., *Ulocladium* sp., *Eurotium* sp., and *Geomyces* sp. Each fungus was grown for 14 days in potato dextrose broth. The cultures were extracted twice with ethyl acetate, and the organic extracts fractionated by column chromatography on silica gel to give 7-10 fractions. These fractions, along with the crude extracts, were subjected to the disc-diffusion bioassay to determine which inhibit growth of *X. fastidiosa* (**Figure 2**). Fractions from *Eurotium* strain EUR1, *Geomyces* strain GEO1, and *Ulocladium* strain ULO1 showed activity against *X. fastidiosa*. Neither the crude extract nor any of the fractions from *Cryptococcus* strain CRY1 showed activity against *X. fastidiosa*. Active fractions from EUR1, GEO1 and ULO1 were examined

using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Preliminary results indicated that; (i) one fraction from GEO1 looked like it could be a protein; (ii) another fraction from GEO1 suggested the absence of any major compound and that activity in this fraction may be due to trace amounts of a highly potent compound which will likely prove difficult to identify; (iii) a fraction of EUR1 revealed a few compounds, one of which showed an isotope pattern characteristic of a bromine atom; and (iv) a fraction of ULO1 revealed a relatively pure compound with an isotope pattern characteristic of two chlorine atoms. We are currently in the process of repeating the cultivation and extraction of ULO1 and EUR1 to obtain more material for purification by high-performance liquid chromatography (HPLC) and structure elucidation by two-dimensional NMR spectroscopy and MS.

Objective 2. Evaluate fungal natural products and semisynthetic derivatives for their potential as curative treatments for vines already infected with Pierce's disease.

The goal of this objective is to evaluate the anti-*X. fastidiosa* efficacy of fungal natural product derivatives *in planta*. Once this proof of concept is established in the greenhouse, the experiment will be carried over to the field. The deliverable for this objective is the development of a commercial product for the cure of Pierce's disease. We have currently identified two fungal natural products as an active molecule inhibitory to *X. fastidiosa* (see objective 1). We had previously developed greenhouse assays to test radicinin on Pierce's disease infected vines. However, we observe no reduction of Pierce's disease symptoms development because we established that radicinin was not water-soluble. When we have sufficient quantities of the water-soluble radicinin derivatives and/or other natural product derivatives and confirmed that they maintained the anti-*X. fastidiosa* activity, we will evaluate those products in the greenhouse assays using vascular injection techniques and spray on leaves.

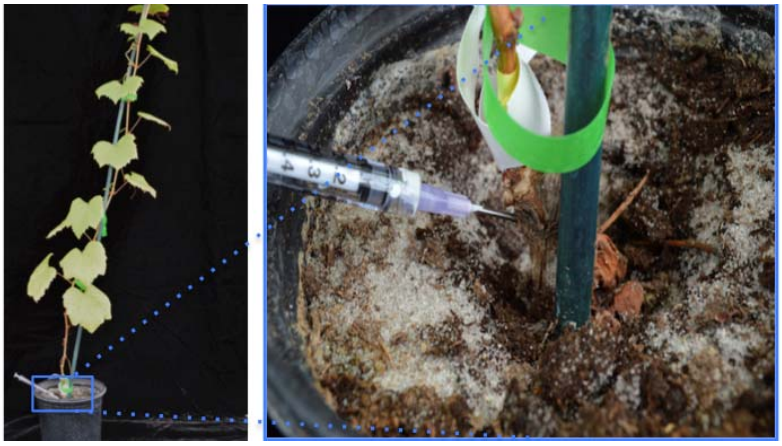


Figure 5. Needle injection of an anti-*X. fastidiosa* molecule in the xylem of Pierce's disease infected grapevine cuttings.

CONCLUSIONS

We aim to investigate curative measures for management of Pierce's disease as part of a sustainable Pierce's disease management program. Our strategy is to evaluate the use of anti-*X. fastidiosa* fungal natural products produced by grapevine endophytic fungi. The commercialization of such a product will provide a solution to growers that have vineyards already infected with Pierce's disease. We have already discovered two active anti-*X. fastidiosa* fungal natural products, radicinin and molecule 'C.' However, radicinin did not show efficacy in our greenhouse trials on Pierce's disease infected vines, likely because it is not water-soluble. Molecule 'C' has the same properties as radicinin. We are now synthesizing semi-synthetic derivative molecules to increase water-solubility of these products, which should increase their movement in the plant xylem where *X. fastidiosa* resides. In addition to these two products, we are also searching for additional active water-soluble natural anti-*X. fastidiosa* compounds. We have recently identified fractions from the crude extracts of three additional fungal endophytes that possess activity against *X. fastidiosa* in *in vitro* bioassays and we are in the process of identifying their chemical structure and properties. In the event that any of these compounds mitigate Pierce's disease in the greenhouse, we will test their efficacy in natural vineyard settings in the future.

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CHARACTERIZATION AND INHIBITION OF *XYLELLA FASTIDIOSA* PROTEINS SECRETED BY THE TYPE II SECRETION SYSTEM AND THEIR SECRETION MACHINERY

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ABSTRACT
The purpose of this study is to elucidate the contributions of host cell wall-degrading enzymes (CWDEs) produced by *Xylella fastidiosa* to systemic colonization of grapevine, as well as the role of the Type II Secretion System (T2SS) in delivering these CWDEs into the xylem. Of the CWDEs predicted to be secreted by the T2SS, this project will focus on the endoglucanases (EGases) produced by *X. fastidiosa*. We hypothesize that the T2SS secretes these EGases along with a polygalacturonase, and that these enzymes function in concert with each other to degrade the pit membranes of xylem vessels to facilitate systemic colonization of the bacterium. It has been previously reported that a purified polygalacturonase and one of the *X. fastidiosa* EGases are required to increase pore sizes of pit membranes in grapevine. Moreover, mutation of polygalacturonase results in the loss of pathogenicity and movement for *X. fastidiosa*. Similarly, we show that a loss of function in the T2SS results in a similar dramatic loss of pathogenicity. In addition, we are investigating the role of an EGase/expansin hybrid protein in pit membrane degradation. Ultimately, characterization of these EGases and the T2SS will determine if these entities will be suitable entities to target for disease management.

LAYPERSON SUMMARY
Xylella fastidiosa relies on degradation of the plant cell wall to move within the grapevine. This is accomplished by the cooperation of at least two classes of enzymes that target different components of the complex scaffold of the plant cell wall. A major goal of this research is to further elucidate the factors that lead to disassembly of the plant cell wall, thereby, allowing the bacteria to systemically colonize the plant. Systemic colonization is highly correlated with Pierce's disease development, and preventing movement of the bacteria is critical to devising successful control strategies. We propose that characterizing and inhibiting *X. fastidiosa* enzymes that facilitate movement throughout the plant and the secretion machinery responsible for delivering those *X. fastidiosa* enzymes to the plant will provide a comprehensive approach to restriction of disease development.

INTRODUCTION
Xylella fastidiosa is a xylem-limited bacterial pathogen that is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002; Chatterjee et al., 2008; Purcell and Hopkins, 1996). In order to systemically colonize the xylem, *X. fastidiosa* must be able to move efficiently from one xylem vessel element to adjacent vessels. These xylem vessels are connected by pit membranes, which are porous primary cell wall interfaces that are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000; Sun et al., 2011). The pore sizes of these pit membranes range from 5 to 20 nm, and serve to prevent the movement of air embolisms and pathogens within the xylem (Mollenhauer & Hopkins, 1974; Buchanan, 2000). Indeed, these small pore sizes do prevent the passive movement of *X. fastidiosa* between xylem vessels given that the size of the bacterium is 250-500 x 1,000-4,000 nm (Perez-Donoso et al., 2010; Mollenhauer & Hopkins, 1974). In order to move from one vessel to another, it has been shown through genomic and experimental evidence that *X. fastidiosa* utilizes Cell Wall-Degrading Enzymes (CWDEs), including a polygalacturonase and at least one β-1,4 Endoglucanase (EGase), to break down the pit membrane's network (Roper et al., 2007; Perez-Donoso et al., 2010). Furthermore, polygalacturonase is necessary for pathogenicity in grape and has become a primary target for *X. fastidiosa* inhibition studies (Roper et al, 2007). However, polygalacturonase alone is not sufficient for

pathogenicity in grape and *X. fastidiosa* requires both polygalacturonase and an EGase for pit membrane degradation (Perez-Donoso et al., 2010). Therefore, elucidating the role of EGases in pit membrane degradation is critical for understanding systemic movement within the xylem. The *X. fastidiosa* genome contains three genes that encode canonical EGases: *egl* (PD2061), *rlpA* (PD1236), and *engXCA2* (PD1851). A fourth annotated EGase, *engXCA1* (PD 1856), putatively encodes a modular hybrid protein that contains both an EGase domain and an expansin domain (Simpson et al., 2000). Expansins are primarily plant proteins that function to non-enzymatically loosen the cell wall during development (e.g., cell elongation, fruit ripening). Recently, expansins have been found in several plant-associated bacteria, most of which have a significant xylem-dwelling phase in their lifestyle (Nikolaidis et al., 2014). It is predicted that these EGases and polygalacturonase are delivered into the xylem by the Type II Secretion System (T2SS). Our data demonstrate that *X. fastidiosa* with a deficient T2SS displays a non-pathogenic phenotype similar to that of the *X. fastidiosa pglA* mutant that is deficient in production of polygalacturonase, suggesting that the T2SS is essential for *X. fastidiosa* pathogenicity. Therefore, our central hypothesis is that *X. fastidiosa* utilizes other CWDEs and an endoglucanase/expansin hybrid protein in concert with polygalacturonase to breach the pit membranes and that the majority of these are secreted by the Type II Secretion System. We are determining the role that each of these components plays in pit membrane degradation and systemic movement, and subsequently if they are good candidates for potential inhibition to limit Pierce's disease development.

OBJECTIVES

1. Characterization of *X. fastidiosa* host cell wall degrading enzymes and an endoglucanase/expansin protein.
2. Inhibition of *X. fastidiosa* endoglucanases and the endoglucanase/expansin using endoglucanase-inhibiting proteins.
3. Characterization of the *X. fastidiosa* Type II secretion system.
4. Inhibition of the *X. fastidiosa* Type II secretion system.

RESULTS AND DISCUSSION

Objective 1. Characterization of *X. fastidiosa* host cell wall degrading enzymes and an endoglucanase / expansin.

X. fastidiosa EngXCA2 is a major contributor to the pit membrane dissolution and the synergistic effects of the polygalacturonase and the EngXCA2 were sufficient to increase pit membrane pore size (Perez-Donoso et al., 2010). Indeed, recombinant EngXCA2 was capable of digesting carboxymethyl cellulose (CMC) and xyloglucan (XyG) polymers, which both contain β-1,4-linked glucan backbones and are representative of substrates *X. fastidiosa* would likely encounter in grapevine primary cell walls (Roper, 2006; Perez-Donoso et al., 2010). Given the role EngXCA2 plays in pit membrane degradation, we hypothesize that other predicted EGases produced by *X. fastidiosa* may impact pit membrane integrity as well. One of the four genes annotated as an EGase is *engXCA1*, which encodes an EGase/expansin hybrid putatively involved in plant cell wall disassembly. This is of particular interest because expansins are primarily found in the plant kingdom and are non-enzymatic proteins that function to loosen the cell wall during plant growth without enzymatic digestion of the wall (Cosgrove, 2000). Expansins facilitate cell wall loosening by binding to their target polysaccharide and disrupting the weak bonds between the cellulosic glucan and the microfibril surface, allowing turgor pressure from within the cell to expand the cell wall (Cosgrove, 2000). Expansin-like proteins with similar structure and function were later found in a few bacterial species that associate with plants likely as a result of cross-kingdom horizontal gene transfer (Nikolaidis et al., 2014). These bacterial expansins are thought to enhance the activity of bacterial CWDEs by loosening the cell wall, thereby promoting wall breakdown, colonization and virulence. Interestingly, orthologs of at least one bacterial expansin (EXLX1) are found in several plant pathogens, including *Xylella, Xanthomonas*, *Ralstonia,* and *Erwinia* species (Kerff et al., 2008; Georgelis et al., 2014). While these are phylogenetically diverse bacteria, they all share the commonality that they spend the majority of their lives in the xylem tissue of plants. It is hypothesized that they are involved in host colonization (Kerff et al., 2008). In the *X. fastidiosa* pathosystem, they could potentially weaken the wall and more readily expose carbohydrate targets for digestion by the suite of other *X. fastidiosa* CWDEs.

Characterization of the *X. fastidiosa* EGase/Expansin hybrid protein.

The gene *engXCA1* was cloned from the *X. fastidiosa* Temecula 1 genome into the pET200 Directional TOPO expression vector (**Figure 1A**). The plasmid construct (pET200::*engXCA1*) was then transformed into the *Escherichia coli* strain BL21 Star, and recombinant protein expression was induced with 1 mM IPTG for six

hours at 37°C and analyzed by Western Blot using a monoclonal α-His-tag primary antibody and a polyclonal alkaline phosphatase (AP) secondary antibody. Analysis of the soluble and insoluble lysate fractions determined that expression at 37°C did not favor soluble recombinant protein, so conditions were optimized to facilitate the presence of recombinant protein in the soluble fraction. Using the optimized induction conditions (18°C, 0.1 mM IPTG, four hours) and increasing the total volume of bacterial cells from 10 ml to 40 ml allowed for the expression of a sufficient quantity of soluble recombinant EngXCA1 protein to proceed with protein purification. The recombinant protein was purified via column chromatography using Ni-NTA resin (ThermoFisher) and will be analyzed for EGase and expansin activity. We will also assess expansin activity of the recombinant protein (i.e., its ability to promote the extension of plant tissues that are subjected to stress) in close collaboration with the Cosgrove Laboratory (Penn State University). The cell wall elongation assay will then be performed using an extensometer apparatus as described by Cosgrove, D. J. (1989), and expansin activity will be determined by measuring the extension of wall specimens over a two-hour period.

Assessment of the biological contribution of the *X. fastidiosa* EGase/Expansin and other *X. fastidiosa* endoglucanases to pathogenicity and host colonization.

To test the role of the *X. fastidiosa* EGase/expansin *in planta*, we constructed a deletion mutant (Δ*engXCA1*) in the *X. fastidiosa* Temecula 1 strain using established mutagenesis techniques and confirmed the mutant via polymerase chain reaction (PCR; Matsumoto et al., 2009). We mechanically inoculated the Temecula 1 wild-type and the Δ*engXCA1* mutant into grapevine (Cabernet Sauvignon variety) using the pin-prick method (Hill and Purcell, 1995). Grapevines inoculated with 1X phosphate buffered saline (PBS) were used as negative controls. Both the wild-type and the Δ*engXCA1* mutant were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment).

Disease ratings for all plants were recorded using a scale of 0 – 5 where 0 = healthy, 5 = dead, and 1 – 4 are increasing degrees of leaf scorching as described by Guilhabert and Kirkpatrick (2005). Interestingly, the Δ*engXCA1* mutant strain is less virulent than the wild-type parent strain (**Figure 1**). Statistical analysis using analysis of variance (ANOVA) revealed that this difference in virulence between the wild-type and mutant strains at week 14 was statistically significant ($P = 0.002$). Furthermore, the percentage of plants inoculated with the Δ*engXCA1* mutant strain rating 2 or higher on the disease index was significantly less than the percentage of plants inoculated with wild-type *X. fastidiosa* rating 2 or higher over a 14-week period (**Figure 2**). This indicates that the onset of disease in plants inoculated with the Δ*engXCA1* mutant is significantly delayed relative to plants inoculated with wild-type *X. fastidiosa*. It is very interesting that despite colonizing the plants to the same levels as wild-type *X. fastidiosa*, the onset and severity of Pierce's disease symptoms is delayed in plants inoculated with the Δ*engXCA1* mutant.

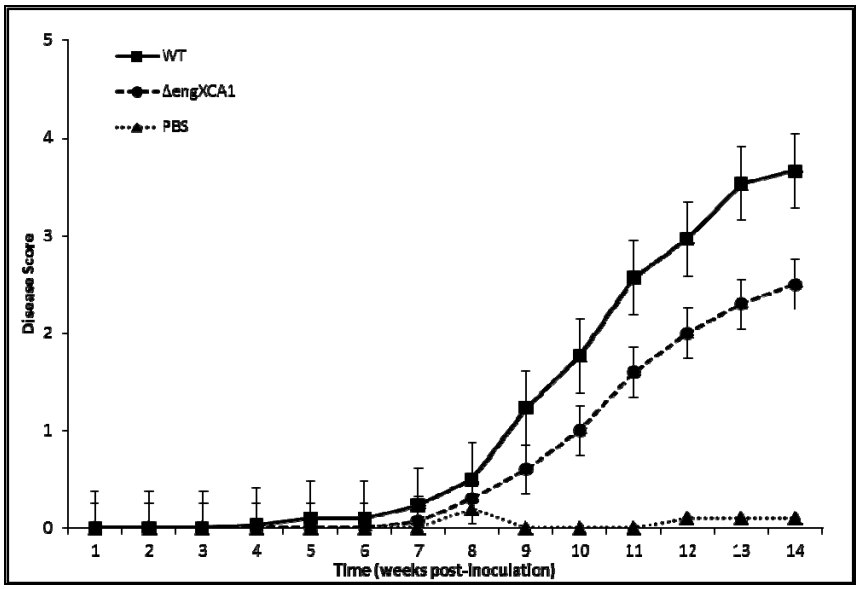


Figure 1. Disease progress of the *ΔengXCA1* mutant and the wild-type strains over 14 weeks. The *ΔengXCA1* mutant strain lags behind the wild-type strain in Pierce's disease symptom development. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

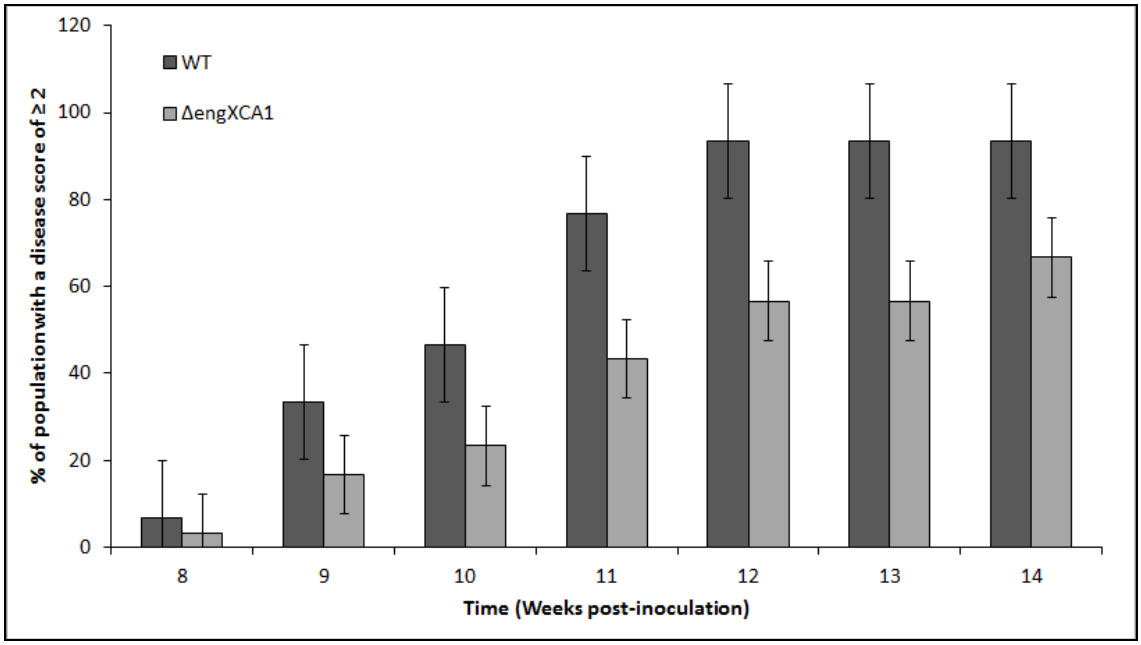


Figure 2. Percentage of plants rating a 2 or higher on the Pierce's Disease scale. The percentage of plants inoculated with the *ΔengXCA1* mutant strain that rated 2 or higher was consistently less than the percentage of plants inoculated with the wild-type strain over a period of 14 weeks. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

We quantified *X. fastidiosa* populations in the plants by isolating *X. fastidiosa* from the petioles at the point of inoculation (POI) (11 weeks post-inoculation) and ≥ 37 cm above the POI (12 weeks post-inoculation) to determine the ability of the EGase mutants to systemically colonize the host (**Figure 3**). The statistical differences between wild-type and Δ*engXCA1* mutant populations at both POI and ≥ 37 cm above the POI were determined by ANOVA. While there was a significant difference in colonization at the POI ($P = 0.027$), the difference in colonization at ≥ 37 cm above the POI was statistically insignificant ($P = 0.155$), indicating that the Δ*engXCA1*mutant is not impaired in movement 37 cm above the POI. We will test distances further from the POI this summer. A similar trend was also observed in experiments conducted with the Chardonnay variety (*data not shown*).

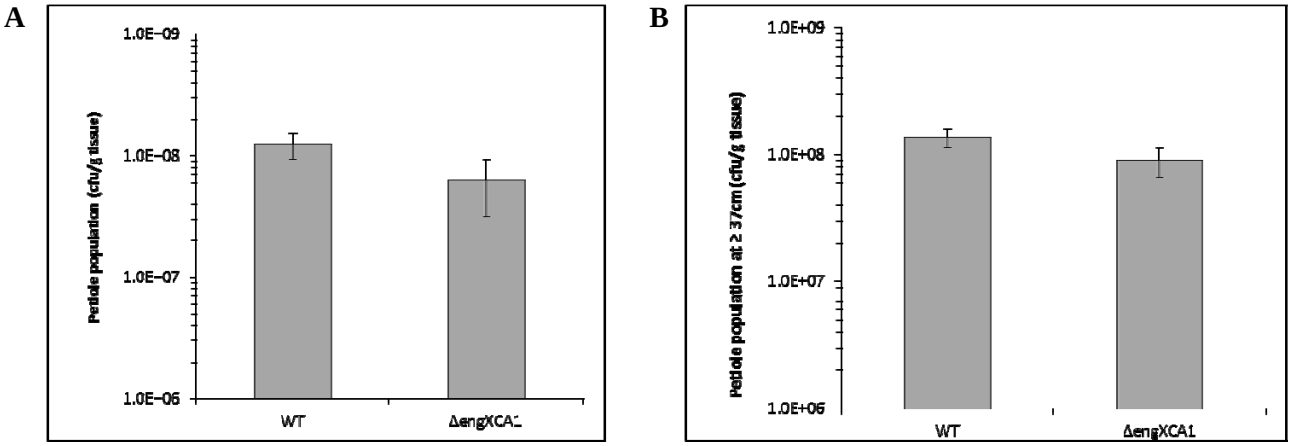


Figure 3. *In planta* populations of the Δ*engXCA1* mutant relative to the Temecula 1 wild-type strain. **(A)** *In planta* populations of the Δ*engXCA1* mutant are significantly lower than those of the wild-type strain at the point of inoculation (POI) quantified at 11 weeks post-inoculation. **(B)** *In planta* populations of the Δ*engXCA1* mutant are not significantly different from those of the wild-type strain at ≥ 37 cm above the point of inoculation at 12 weeks post-inoculation. Data are the means of three independent assays with ten replications each. Bars represent the standard error of the mean.

In these experiments, we inoculated at the base of a single shoot and assessed bacterial colonization via bacterial isolation at the petiole closest to the POI and bacterial movement via bacterial isolation at the petiole that is 37 cm above the POI (**Figure 4A**). However, in a natural scenario, sharpshooters prefer to feed on new green growth and the bacteria migrate in a basipetal direction against the flow of sap. We speculate that endoglucanases may play a role in this basipetal movement. To test this, we have developed a different strategy that allows us to assess bacterial movement in both the acropetal and basipetal directions (**Figure 4B**). In this experimental design, two shoots, an upper and a lower shoot, were allowed to grow from the same woody stem. The upper shoot was inoculated at the third internode from the base of the shoot, and bacterial colonization was assessed via bacterial isolation at the petiole closest to the POI, as done previously. Bacterial movement was assessed in the acropetal direction by isolating bacteria from the petiole at the 20th node above the POI. Bacterial movement was assessed in the basipetal direction by isolating bacteria from the petiole at the fifth node on the lower shoot.

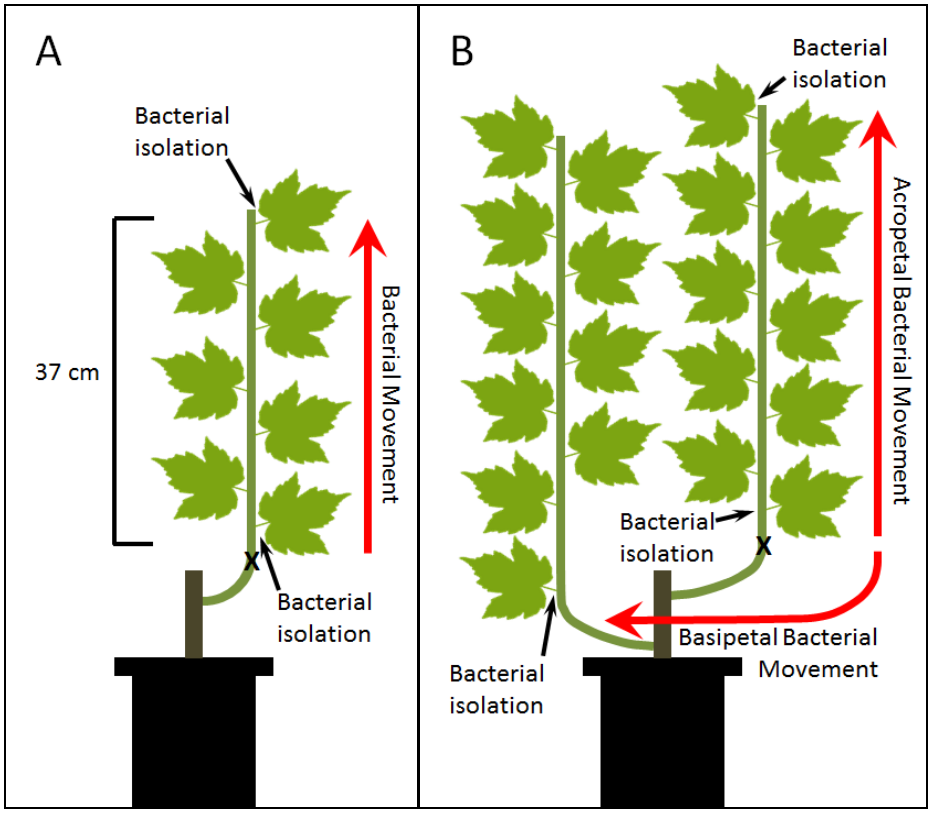


Figure 4. Experimental designs for grapevine inoculation assays. **(A)** Original experimental design where a single shoot was inoculated near the base. Bacterial isolations were performed using the petiole at the POI to assess *X. fastidiosa* colonization and using the petiole at 37 cm above the POI to assess *X. fastidiosa* movement. **(B)** Modified experimental design where two shoots are grown from the same woody stem and the upper shoot is inoculated at the third internode. Bacterial isolations were performed using the petiole at the POI to assess *X. fastidiosa* colonization, using the petiole at 20 nodes above the POI to assess *X. fastidiosa* movement in the acropetal direction, and using the petiole at the fifth node of the lower shoot to assess *X. fastidiosa* movement in the basipetal direction. The inoculation site is denoted by an "X" on the shoot.

We have constructed the *engXCA1* +/- complement by inserting the *engXCA1* gene and its native promoter into a neutral site in the *X. fastidiosa* chromosome in the *X. fastidiosa* Δ*engXCA1* mutant strain (Matsumoto et al., 2009). This complement was used in the two-shoot grapevine inoculation assay (as shown in **Figure 4B**) to show restoration of the wild-type phenotype. The Temecula 1 wild-type strain, the Δ*engXCA1* mutant strain, and the *engXCA1* +/- complement strain were mechanically inoculated into grapevine (Cabernet Sauvignon variety). Grapevines inoculated with 1X phosphate buffered saline (PBS) were used as negative controls. All treatments were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment). As before, the disease ratings were scored on the 0 – 5 Pierce's disease rating index, and normalized to the PBS negative control to account for symptoms caused by environmental conditions. While data is still being collected, the disease trend in the upper shoot is similar to the disease trend in the single shoot experiment. As of 19 weeks post-

inoculation, the Δ*engXCA1* mutant strain appears to be somewhat less virulent than the wild-type parent strain, while the virulence of the *engXCA1* +/- complement strain is similar to that of the wild-type parent strain (**Figure 5**). However, statistical analysis of the disease ratings at week 19 using the Wilcoxon rank sum with continuity correction statistical test revealed the difference between the wild-type and mutant strains was not significant ($P = 0.57$). Similarly, differences between the wild-type and complement strains ($P = 0.65$) and the mutant and complement strains ($P = 0.96$) were also not significant.

At 19 weeks post-inoculation, the wild-type parent strain and the complement strain both maintained similar disease scores while the Δ*engXCA1* mutant strain appears to be somewhat less virulent (**Figure 6**). Again, the Wilcoxon rank sum with continuity correction statistical test revealed no significant differences between the wild-type and mutant strains ($P = 0.47$), the wild-type and complement strains ($P = 0.67$), and the mutant and complement strains ($P = 0.26$). It is interesting to note that the Δ*engXCA1* mutant strain was still able to induce symptom development in the lower shoot. This indicates that the mutant strain is able to move in the basipetal direction, and likely had to cross pit membranes. While the mutant strain appears to retain movement ability in the xylem, bacterial isolations from both the upper and lower shoots will determine if there is a significant difference in bacterial titer in either shoot.

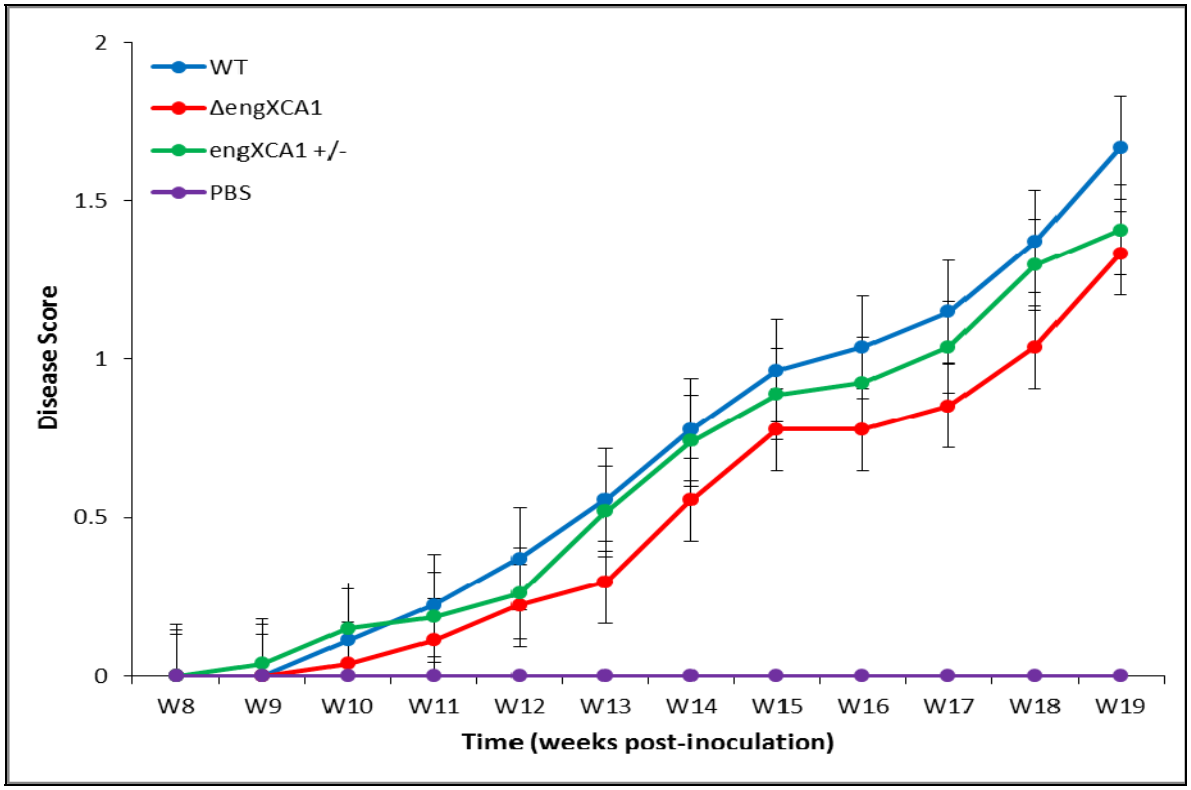


Figure 5. Pierce's disease development in the upper grapevine shoot after inoculation with either the Δ*engXCA1* mutant strain, the engXCA1 +/- complement strain, or the wild-type strain over 19 weeks. The Δ*engXCA1* mutant strain maintains a lower average disease score per week relative to both the wild-type and complement strains. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

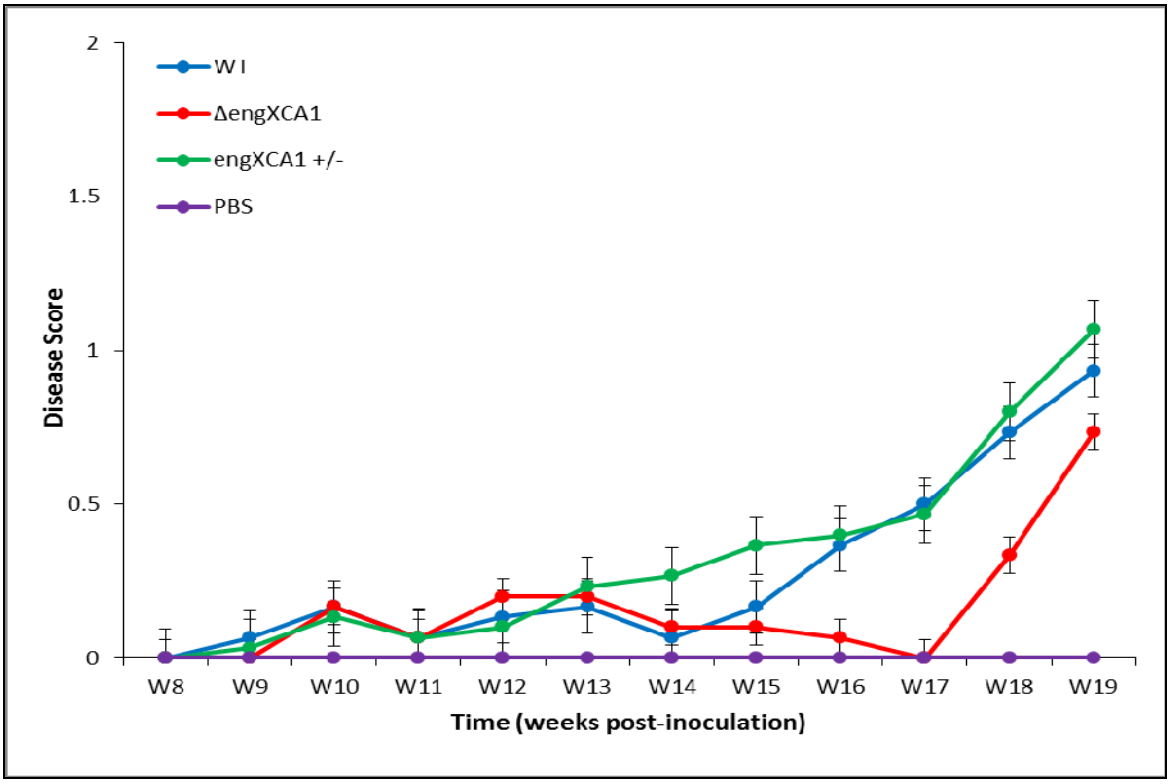


Figure 6. Pierce's disease development in the lower grapevine shoot after inoculation in the upper shoot with either the Δ*engXCA1* mutant strain, the engXCA1 +/- complement strain, or the wild-type strain over 19 weeks. While average disease score fluctuated for several weeks, a consistent trend started to emerge at week 15. Similar to the disease score trend in the upper shoot, the Δ*engXCA1* mutant strain has stated to show a consistently lower average disease score per week relative to both the wild-type and complement strains. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

A deletion mutant of another *X. fastidiosa* endoglucanase (Δ*engXCA2*) has been constructed and transformed into the *X. fastidiosa* Temecula 1 wild-type strain. EngXCA2 has previously been characterized experimentally as an endoglucanase capable of degrading both CM cellulose and xyloglucan (Roper, 2006). It has also been implicated in the degradation of pit membranes when combined with a polygalacturonase, indicating that this endoglucanase may be required for systemic colonization of the grapevine host (Perez-Donoso et al., 2010). Therefore, the *in planta* data should provide concrete evidence for the role of this endoglucanase in systemic colonization. Using the two-shoot grapevine inoculation assay, the Temecula 1 wild-type strain and the Δ*engXCA2* mutant strain were mechanically inoculated into grapevine (Cabernet Sauvignon variety). Grapevines inoculated with 1X PBS were used as negative controls. All treatments were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment), and disease ratings were normalized to the PBS negative control to account for symptoms caused by environmental conditions. While disease ratings using the Pierce's disease rating index are still being collected, the Δ*engXCA2* mutant strain already appears to be significantly less virulent than the wild-type parent strain in the upper shoot at 19 weeks post-inoculation (**Figure 7**). Indeed, statistical analysis using the Wilcoxon rank sum with continuity correction statistical test revealed the difference between the wild-type and mutant strains was statistically significant ($P = 0.0497$). Additionally, the percentage of plants inoculated with the Δ*engXCA2* mutant strain rating 2 or higher on the disease index was significantly less than the percentage of plants inoculated with the wild-type parent strain rating 2 or higher over an 19-week period (**Figure 8**). Taken together, this data set indicates that the *X. fastidiosa engXCA2* gene plays a significant role in Pierce's disease development in the grapevine host, and thus is critical to the virulence of the pathogen. However, like the Δ*engXCA1* mutant, the phenotype of the Δ*engXCA2* mutant manifests as a reduction of virulence rather than the complete loss of virulence as displayed by the Δ*pglA* mutant (Roper et al., 2007). Disease symptoms in the lower shoot are only now starting to develop, and data has only started being collected. However, the trend in the lower shoot appears to be similar to that of the upper shoot, but more data points are needed to fully make that assessment.

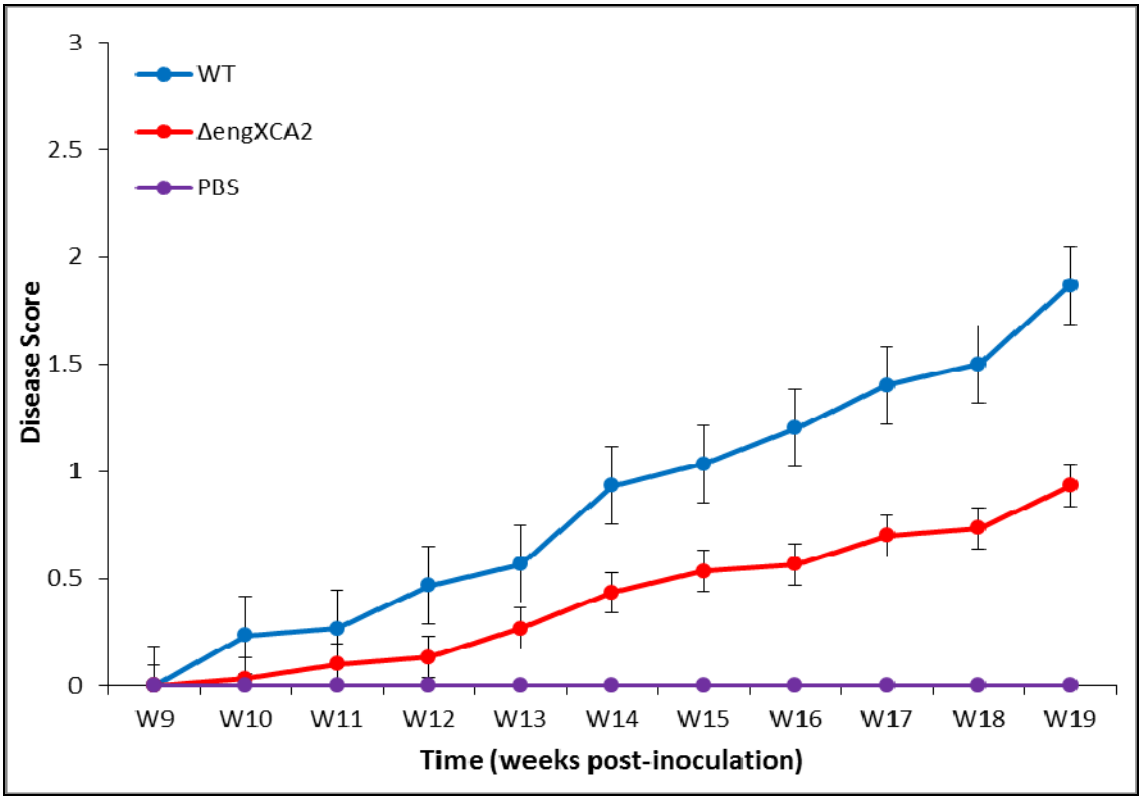


Figure 7. Pierce's disease development in the upper grapevine shoot after inoculation with either the Δ*engXCA2* mutant strain or the wild-type strain over 19 weeks. The Δ*engXCA2* mutant strain maintains a lower average disease score per week relative to the wild-type strain. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

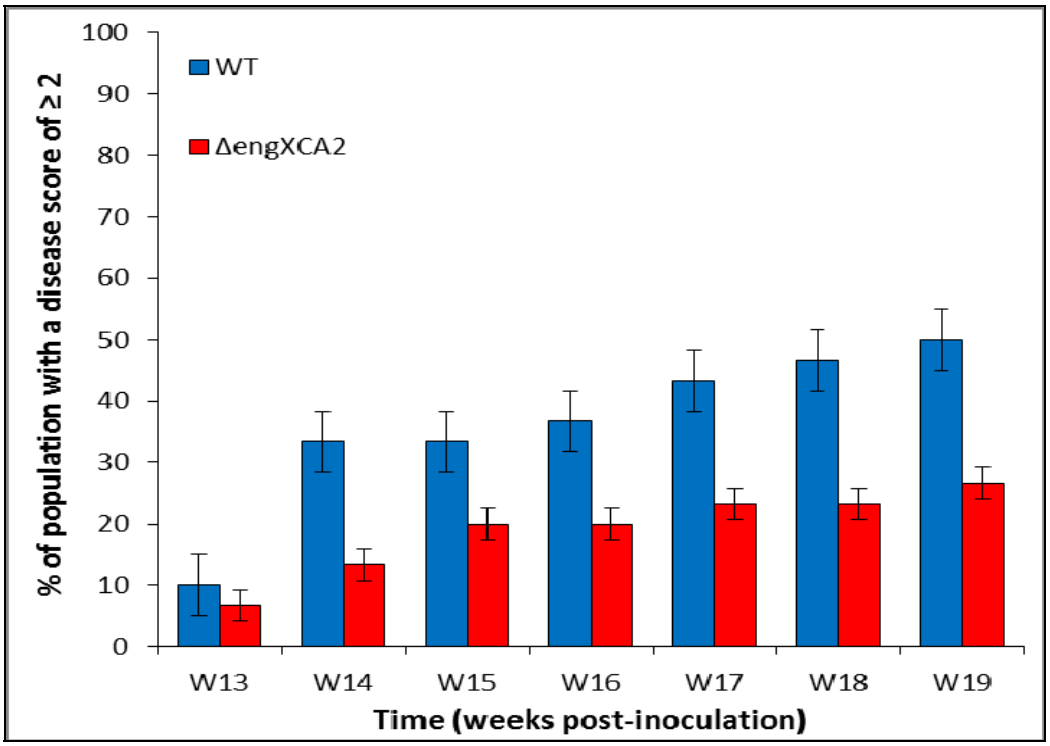


Figure 8. Percentage of plants rating a 2 or higher on the Pierce's disease scale. The percentage of plants inoculated with the Δ*engXCA2* mutant strain that rated 2 or higher was consistently less than the percentage of plants inoculated with the wild-type strain over 19 weeks. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

Both the Δ*engXCA1* and Δ*engXCA2* mutants displayed reduced virulence when assessed *in planta*, but neither displayed the complete loss of virulence. Given that *X. fastidiosa* maintains several putative EGases, it is possible the loss of one EGase is compensated for by the others. As such, we intend to create double and triple mutants to delete several of the EGase genes at one time. If our redundancy hypothesis is correct, we expect to find that a double mutant will be significantly less virulent than a single mutant, and a triple mutant will likely result in the loss of virulence.

Objective 2. Inhibition of *X. fastidiosa* endoglucanases and the endoglucanase/expansin using endoglucanase inhibiting proteins.

Because the combined action of a polygalacturonase and an *X. fastidiosa* EGase was required to digest pit membranes, both are logical targets for inhibition. Polygalacturonase is a major pathogenicity factor for *X. fastidiosa,* and grapevines expressing a pear polygalacturonase inhibiting protein were more tolerant to *X. fastidiosa* infection (Aguero et al., 2005). Several plant proteins have also been identified and characterized as xyloglucan-specific EGase inhibiting proteins (XEGIPs) that could potentially inhibit *X. fastidiosa* EGases. These include XEGIPs from tomato and tobacco (Naqvi et al., 2005; Qin et al., 2003). We propose to assess the ability of the tobacco and tomato XEGIPs to inhibit the degradative ability of the *X. fastidiosa* EGases and the EGase / expansin. Currently, we are working on expressing and purifying these *X. fastidiosa* EGases and assessing their activity as stated in Objective 1. Once these studies have been completed, we will test for inhibition using a radial diffusion assay performed in agarose containing either CMC or XyG as a substrate with increasing concentrations of each XEGIP. In addition, we will quantify the generation of reducing sugars produced by the *X. fastidiosa* EGases or EGase/expansin alone or in combination with each of the XEGIPs (Naqvi et al., 2005).

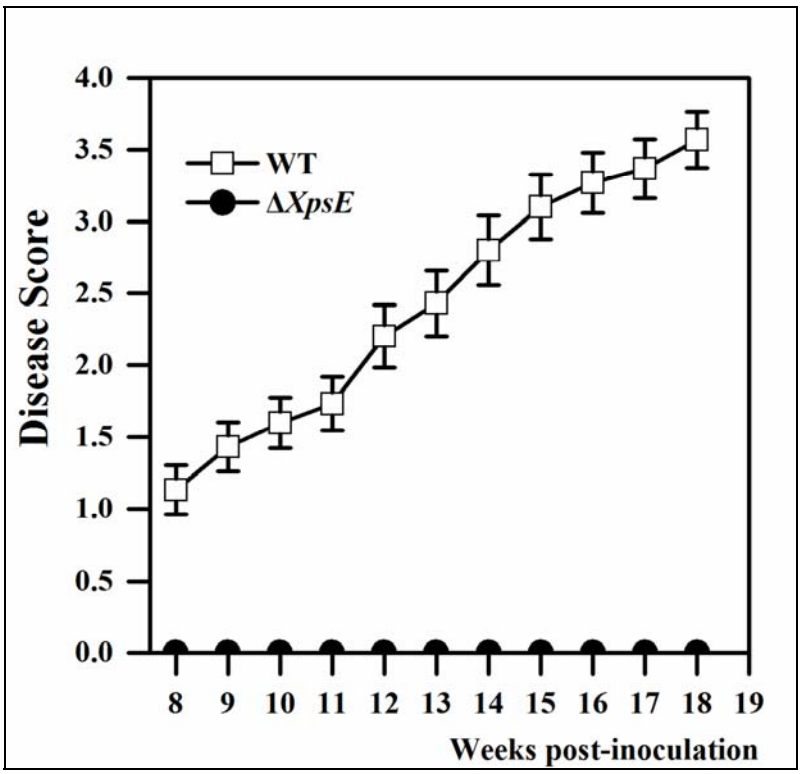
Objective 3. Characterization of the *X. fastidiosa* Type II secretion system.

The T2SS is composed of 12-15 different proteins, involved either structurally or mechanistically in the function of the T2SS, depending on the species that is being examined. These proteins are encoded in a single operon and the *X. fastidiosa* genome contains a similar operon strongly suggesting a functional T2SS (Jha et al., 2005). The T2SS can be divided into four different subassemblies that are 1) the pseudopilus; 2) the outer membrane complex; 3) the inner membrane platform and 4) the secretion ATPase. The pseudopilus is composed primarily of the major pseudopilin protein, G (XpsG), and also contains the minor pseudopilins, S, H, I, J, and K (XpsH, I, J, and K). The XpsE ATPase harnesses the energy that drives secretion through the T2SS via hydrolysis of ATP. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski, 2014). The T2SS then uses a pilus-like piston to push proteins through the T2 channel. This piston action is a function of the cyclic assembly and disassembly of pilin subunits (primarily XpsG).

We have created a mutation in the *xpsE* gene, encoding the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed Pierce's disease symptoms and remained healthy, a phenotype similar to the *X. fastidiosa pglA* mutant (**Figure 9**). Thus, we have compelling preliminary data indicating that *X. fastidiosa* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process.

We hypothesize that the non-pathogenic phenotype of the *ΔxpsE* mutant is due largely to the inability to secrete host CWDEs. Indeed, we have indirect experimental evidence that *X. fastidiosa* utilizes the T2SS to secrete polygalacturonase. This is based on an assay performed on the defined growth medium, XFM. When XFM is supplemented with pectin as the sole carbon source, this induces production of copious amounts of the carbohydrate-based exopolysaccharide (EPS) (Killiny & Almeida, 2009). Pectin is a complex carbohydrate comprised in its simplest form of repeating galacturonic acid residues. Therefore, when grown on XFM with pectin as the sole carbon source, we hypothesize that *X. fastidiosa* must first digest the pectin source utilizing its endo-polygalacturonase (Roper et al., 2007) and likely other pectin-digesting enzymes that eventually disassemble the pectin polymer into individual galacturonic acid residues that can then feed into various metabolic processes within the bacterium, such as EPS production.

A



B

Figure 9. The *X. fastidiosa* T2SS is necessary for Pierce's disease development in grapevine. **(A)** the Δ*xpsE* mutant does not incite Pierce's disease symptoms in *V. vinifera* cv. Chardonnay grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). **(B)** Representative images of plants from the virulence assay are shown here, 1 = wild-type-inoculated, 2 = Δ*xpsE*-inoculated, 3 = 1X PBS buffer-inoculated. Plants shown are 11 weeks post-inoculation.

In support of our hypothesis that polygalacturonase, and potentially other CWDEs, are secreted through the T2SS, we demonstrate that the Δ*xpsE* mutant produces visibly less EPS on XFM+pectin medium resulting in a much less mucoid phenotype (**Figure 10A**). Furthermore, when wild-type *X. fastidiosa* and Δ*xpsE* are grown on XFM+ galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer) or on XFM+glucose, both strains produce similar amounts of EPS (**Figure 10B & C**). We infer from this that, indeed, breakdown of the pectin substrate is necessary to produce EPS and when the T2SS is disrupted this prevents secretion of polygalacturonase and the subsequent breakdown of pectin.

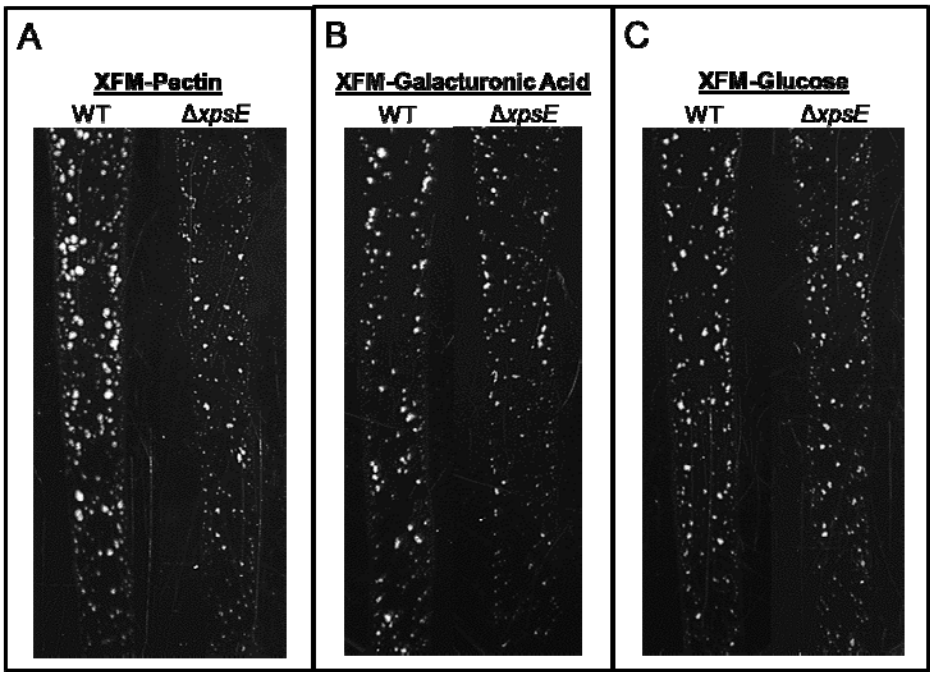


Figure 10. EPS assay with either the *X. fastidiosa* wild-type Temecula 1 strain or the *X. fastidiosa* Δ*xpsE* mutant strain grown on XFM minimal media containing a single carbon source. **(A)** XFM containing pectin as the single carbon source. The *X. fastidiosa* wild-type strain is able to produce more EPS than the Δ*xpsE* mutant strain. **(B)** XFM containing galacturonic acid as the single carbon source, and **(C)** XFM containing glucose as the single carbon source. No discernible differences in growth of the *X. fastidiosa* wild-type strain and the *X. fastidiosa* Δ*xpsE* mutant strain were detected on either of these media types.

Additionally, we will be quantitatively determining the amount of EPS produced by both the *X. fastidiosa* wild-type Temecula 1 strain and the Δ*xpsE* mutant strain via a total carbohydrate assay. Both mutant and wild-type *X. fastidiosa* will be grown on solid PD3 medium and transferred to liquid XFM minimal media containing pectin, galacturonic acid, or glucose as the only carbon source. After incubation, the bacterial cells will be centrifuged and an aliquot of the supernatant will be mixed with 95% ethanol and frozen at -80°C. The samples will be centrifuged again, and the pellet will be washed twice with 70% ethanol, and re-suspended in water. An aliquot of each sample will be added to a respective glass tube and mixed with 5% phenol and concentrated sulfuric acid. Absorbance readings at 488 nm will be taken for each sample and compared to a glucose standard curve.

Objective 4. Inhibition of the *X. fastidiosa* Type II secretion system.

Proteins destined for secretion by the T2SS are first exported to the periplasm by the Sec or Tat pathways. *X. fastidiosa* appears to only possess the Sec-dependent secretion pathway. Disruption of the T2SS by small molecule inhibitors was demonstrated in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, and could be used to inhibit the *X. fastidiosa* Sec-dependent pathway (Moir et al., 2011). A chemical compound library will be screened for Sec-inhibitory molecules, including those compounds used by Moir et al. (2011). Inhibition of the Sec-dependent pathway will be confirmed by monitoring the secretion of a CWDE using a polyclonal antibody raised against EngXCA2 and analyzed via Western Blot and ELISA.

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CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE-MEDIATED RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION IN GRAPEVINE

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ABSTRACT
Xylella fastidiosa is a gram-negative, xylem-limited bacterium that causes serious diseases in economically important crops, such as Pierce's disease of grapevine. Lipopolysaccharide (LPS) is the dominant macromolecule displayed on the bacterial cell surface. LPS acts as a selective barrier, preventing entry of toxic substances into the cell, and as an anchor for superficial structures. Finally, LPS is a well-described pathogen-associated molecular pattern (PAMP) and is known to elicit host basal defense responses in model plant systems. LPS is composed of a conserved lipid A-core oligosaccharide component and a variable O-antigen. Through mutations made in *wzy* (XP0836), which encodes an O-antigen polymerase, we have demonstrated that the *X. fastidiosa* O-antigen contributes to plant and insect colonization, and depletion of the O-antigen causes a severe reduction in overall virulence *in planta*. The goal of this project was to determine the role of the *X. fastidiosa* O-antigen in modulation of basal defense responses in grapevine. Specifically, we demonstrated that specific alterations to the LPS structure caused a change in the elicitation of the grapevine response to *X. fastidiosa*, therefore affecting critical, early stages of *X. fastidiosa* establishment *in planta*. We also demonstrated that *X. fastidiosa* LPS and O-antigen structural variants have the potential to be used as a preventative treatment for the control of Pierce's disease.

LAYPERSON SUMMARY
Xylella fastidiosa is the causal agent of Pierce's disease of grapevine and poses a serious threat to the viticulture industry. We have demonstrated that mutation of the prominent bacterial cell surface carbohydrate, lipopolysaccharide (LPS), significantly affects the pathogen's ability to colonize and cause disease in the grapevine host. Likewise, we have shown that this mutation affects pathogen interactions with the blue-green sharpshooter, a native vector in California. LPS is also a pathogen-associated molecular pattern (PAMP). Plants perceive PAMPs as "non-self" via plant host receptors, which triggers plant immune responses associated with defense. Interestingly, *X. fastidiosa* lacks the characteristic machinery (e.g. type III secreted effectors) utilized by many plant pathogenic bacteria to suppress these immune responses. Instead of using proteins to shut down the host defense response, we demonstrate that *X. fastidiosa* decorates LPS molecule to shield itself from host recognition, therefore allowing is to evade defense responses and establish itself in the plant. We are further exploring this phenomenon in the interaction between *X. fastidiosa* and the grapevine host to harness this information to develop novel preventative applications for Pierce's disease.

INTRODUCTION
Xylella fastidiosa, a gram-negative, fastidious bacterium, is the causal agent of Pierce's disease of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee et al., 2008). Pierce's disease has devastated some viticulture areas in California, and there are currently no effective control measures available to growers targeted towards the bacterium itself. Lipopolysaccharide (LPS) is a tripartite glycolipid molecule that is an integral part of the Gram-negative bacterial outer membrane. It is prominently displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell wall and its environment. LPS plays diverse roles for the bacterial cell. It provides structural integrity to the cells and can act as a permeability barrier to toxic antimicrobial substances (Raetz & Whitfield, 2002). Because of its location in the outer membrane, it is also a key contributor to the initial adhesion to a surface or host cell (Walker et al., 2004). We have been exploring the roles of LPS in the Pierce's disease cycle and in the plant-microbe-insect (PMI) interactions of *X. fastidiosa*. We targeted our studies towards the outermost exposed region of the LPS molecule, the O-antigen. By mutating a key O-antigen polymerase, *wzy* (XP0836), in the Temecula1 (Pierce's disease) isolate, we have demonstrated that severe truncation of the O-antigen alters the adhesive and aggregative properties of the cell considerably, thus causing a marked defect in biofilm formation. Furthermore, the resulting mutation of the O-antigen caused increased sensitivity of the bacterium to hydrogen peroxide stress *in vitro* and resulted in a significantly less

virulent pathogen that is severely impaired in host colonization (Clifford et al., 2013). It has long been speculated that *X. fastidiosa* surface polysaccharides play a role in the host-pathogen interaction with grapevine, and our ongoing studies confirm that LPS is a major virulence factor for this important agricultural pathogen. We have also determined that the O-antigen plays an important role in the acquisition of *X. fastidiosa* by an efficient insect vector, the blue-green sharpshooter (*Graphocephala atropunctata*). In collaboration with Dr. Thomas Perring (University of California, Riverside, Dept. of Entomology) and Dr. Elaine Backus (United States Department of Agriculture, Agricultural Research Service, Parlier, CA) we have conducted intricate acquisition assays and determined that alterations in O-antigen structure and composition, indeed, result in a change in the adhesive properties of *X. fastidiosa* within the insect as well (Rapicavoli et al., 2015).

Contrary to the role of LPS in promoting bacterial survival *in planta*, the immune systems of plants have also evolved to recognize the LPS structure and mount a basal defense response to counteract bacterial invasion (Dow et al., 2000; Newman et al., 2000). LPS is considered a pathogen-associated molecular pattern (PAMP), which are conserved molecular signatures that are often structural components of the pathogen (i.e., LPS, flagellin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of the basal defense response. This line of defense against invading pathogens is referred to as PAMP triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise et al., 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of the LPS PAMP in grapevine, particularly the *X. fastidiosa* LPS PAMP. *X. fastidiosa* is introduced by its insect vector directly into the xylem, a non-living tissue, which cannot mount a defense response on its own. However, in other systems, profound changes do occur in the adjacent living parenchyma cells upon infection, suggesting that these cells communicate with the xylem and are capable of recognizing the presence of a pathogen (Hilaire et al., 2001). The plant immune system can recognize several regions of the LPS structure, including the conserved lipid A and core polysaccharide components (Newman et al., 2007; Silipo et al., 2005). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Specifically, bacteria can display different O-antigen profiles by varying the extent of polymerization or by completely abolishing synthesis of the O-antigen depending on the environment and developmental phase of the cell (Bergman et al., 2006; Guerry et al., 2002; Lerouge & Vanderleyden, 2002). We speculate that during the interaction between *X. fastidiosa* and a susceptible grapevine host, the bacterium's long chain, rhamnose-rich O-antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert the basal defense response and establish itself in the host. A similar scenario occurs in *Escherichia coli*, where truncation of the O-antigen caused an increased sensitivity to serum, suggesting the full length O-antigen provides a masking effect towards the host immune system (Duerr et al., 2009; Guo et al., 2005).

Our main aim is to further explore the role of LPS, specifically focusing on the O-antigen moiety, in the interaction between *X. fastidiosa* and the grapevine host and to use this information to develop and evaluate an environmentally sound preventative application for Pierce's disease. In addition to its contribution to biofilm formation, we hypothesized that the LPS molecule also modulates the host's perception of *X. fastidiosa* infection. The *X. fastidiosa* O-antigen mutant we currently have provides a unique platform designed to test this hypothesis. The fundamental goal is to elucidate the mechanism(s) that *X. fastidiosa* uses to infect the grapevine host and exploit this knowledge to evaluate the use of LPS structural variants as a preventative treatment for control of Pierce's disease.

OBJECTIVES

1. Characterization of *X. fastidiosa* LPS mutants *in vitro* and *in planta*.
2. Examination of the LPS-mediated response to *X. fastidiosa* infection.
3. Evaluation of structural variants of LPS as a preventative treatment for Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Characterization of *X. fastidiosa* LPS mutants *in vitro* and *in planta*.

We have determined that the wild-type *X. fastidiosa* O-antigen is composed primarily of 2-linked rhamnose with smaller amounts of glucose, ribose, xylose, and mannose (Clifford et al, 2013). Most importantly, we demonstrated that mutation of the O-antigen polymerase, Wzy, results in a severely truncated O-antigen resulting from a depletion of the majority of the 2-linked rhamnose. This change was confirmed both electrophoretically and biochemically utilizing gas chromatography and mass spectrometry (GC/MS) techniques in collaboration

with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia. Notably, the depletion of rhamnose led to a marked reduction in virulence and host colonization (Clifford et al, 2013). This indicates that the process of rhamnose biosynthesis and its incorporation into the O-antigen is a vulnerable step in the *X. fastidiosa* LPS biosynthetic pathway that could be exploited for disease control. Therefore, in this objective we are building on our current knowledge and continuing our studies by focusing on the process of rhamnose biosynthesis in *X. fastidiosa*. We are presently creating mutants that we hypothesize will be unable to synthesize rhamnose, and we will structurally characterize the O-antigen from these mutants in collaboration with the CCRC. Following this, we will define the biological impact of these mutations by conducting virulence and colonization studies in grapevine. We will also determine the effect of these mutations *in vitro* using substrate attachment, cell-cell aggregation, and visualized biofilm studies that reflect host colonization behaviors.

Using comparative genomics, we have identified five genes with high homology to those involved in rhamnose biosynthesis in other bacterial systems. The genes are designated $rmlB_1$ (XP0208), *rmlA* (XP0209), *rmlC* (XP0210), and *rmlD* (XP0211) (in map order) that encode proteins involved in the conversion of glucose-1-phosphate into dTDP-rhamnose (Jiang et al., 1991; Koplin et al., 1993; Rahim et al., 2001). The first step is catalyzed by RmlA (dTDP-glucose synthase). Subsequent reactions are catalyzed by RmlB (dTDP-D-glucose-4,6-dehydratase), RmlC (dTDP-L-rhamnose synthetase) and RmlD (dTDP-4-dehydrorhamnose reductase) (Koplin et al., 1993). The resulting dTDP-L-rhamnose is then incorporated into the O-antigen. The *rml* genes are usually clustered within a single locus, and our *in silico* analysis demonstrates the presence of an *rml* locus in *X. fastidiosa*. We also identified an additional, unlinked copy of *rmlB*, designated $rmlB_2$ (XP1617).

Mutant construction.
We have constructed a $\Delta rmlAB_1CD$ mutant. Our next step is to make an $rmlAB_1CD/rmlB_2$ double mutant. In the event that deletion of the entire locus has pleiotropic effects (i.e., affects exopolysaccharide production), we will knock out each gene individually. We will begin with *rmlA,* which encodes the enzyme that catalyzes the first step in the pathway of rhamnose biosynthesis. We will then sequentially knock out $rmlB_1$, *rmlC,* and *rmlD* and also create a $\Delta rmlB_2$ mutant. All mutants constructed in this study will be complemented with the wild-type copy of the gene, including the native promoter, using the complementation vectors now available for *X. fastidiosa* developed by the Igo lab at the University of California, Davis (Matsumoto et al., 2009). Once we have obtained all of these mutants and their complements, we will begin biochemically characterizing their O-antigen. We predict that the O-antigen in the *rml* mutants will be completely devoid of rhamnose. We will confirm this by conducting glycosyl composition and linkage analyses in collaboration with the Complex Carbohydrate Research Center (CCRC) (University of Georgia). This Center routinely performs carbohydrate linkage analysis studies on a recharge basis.

LPS isolation and O-antigen purification.
In order to supply LPS in amounts sufficient for NMR analysis, the CCRC requested that we send cell pellets of both *X. fastidiosa* wild-type and O-antigen mutant strains (five grams per strain), instead of purified and lyophilized LPS. Wild-type and *wzy* mutant cell pellets were shipped in the fall of 2014. Prior to shipment, cell pellets were prepared in the following manner: bacterial strains were grown on solid PD3 medium (minus starch) for seven days at 28°C. Agar squares, containing *X. fastidiosa* colonies, were then cut from the plates and added to 250 mL liquid PD3 (minus starch) and placed onto a 28°C shaker at 180 rpm for an additional seven days. Following incubation, cells were spun down, treated with 1% phenol to kill cells, and then washed three times with 1x phosphate buffered saline (PBS) to remove any residual media or phenol. Pellets were stored at -80°C until shipping to the CCRC in Georgia. We recently received the final structural results for the wild-type and *wzy* O-antigen and have begun the process of analyzing the data.

Surface attachment, aggregation, and biofilm studies.
We have begun linking *X. fastidiosa* LPS structure to function using *in vitro* assays. Attachment to a surface is the first step in successful biofilm formation, and because of the location and abundance of LPS in the outer membrane, we hypothesized that LPS plays a key role in mediating initial attachment to the cellulose and chitin substrates *X. fastidiosa* encounters in the plant and insect, respectively. We previously demonstrated that a mutant in the Wzy polymerase was deficient in cell-cell aggregation and hyperattached to surfaces, which led to a defect in biofilm formation (Clifford et al., 2013). We recently tested the $\Delta rmlAB_1CD$ mutant for these behaviors to determine if the inability to synthesize rhamnose results in defective biofilm formation. We found that, similar to the *wzy* mutant phenotype, the $\Delta rmlAB_1CD$ mutant strain hyperattached to a glass surface (**Figure 1A**) but was

significantly impaired in cell-cell aggregation (**Figure 1B**). In addition, preliminary biofilm studies of the mutant strain (in which biofilm formation at the air-liquid interface was visually analyzed) indicated malformation of a mature biofilm (**Figure 2**).

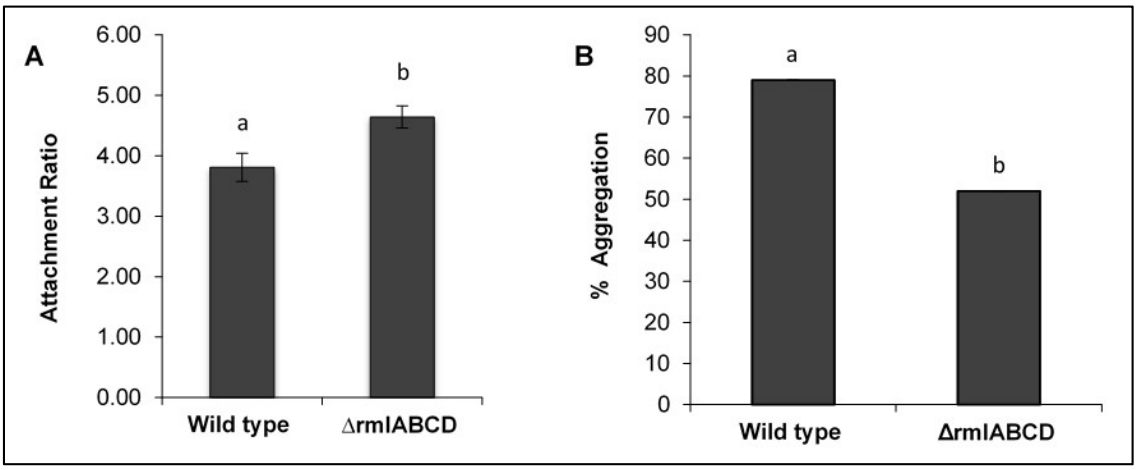

Figure 1. *In vitro* attachment and aggregation assays. Attachment ratio **(A)** and % aggregation **(B)** of *X. fastidiosa* wild-type and Δ*rmlAB1CD* mutant strains. The Δ*rmlAB1CD* mutant strain hyperattached to a glass surface ($P < 0.05$) but was significantly impaired in cell-cell aggregation ($P < 0.0001$), compared with the wild-type. Graphs represent the mean of nine samples per treatment ± standard error of the mean. Treatments with different letters on bars are statistically different.

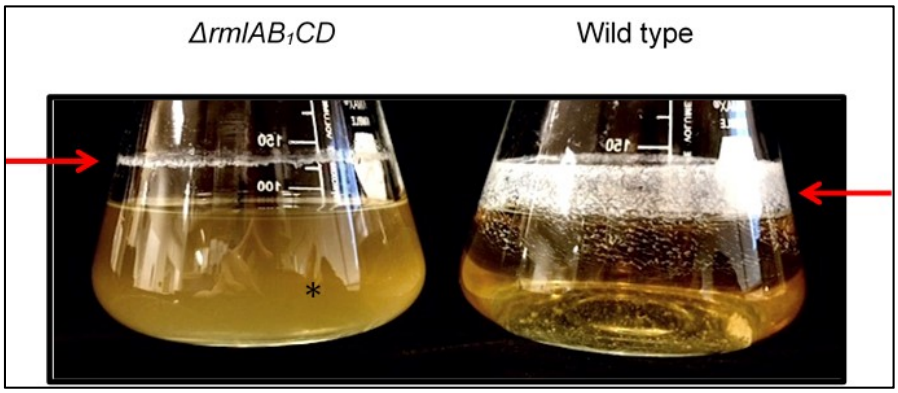

Figure 2. *In vitro* biofilm formation assay. The Δ*rmlAB1CD* mutant strain was severely impaired in building a mature biofilm. Image represents biofilm formation at four dpi. Arrows point to the formation of biofilm at the air liquid interface. *Turbidity of the media displays the defect in aggregation seen in the Δ*rmlAB1CD* mutant.

Virulence and host colonization assays.

In July, we mechanically inoculated *Vitis vinifera* Chardonnay vines with the *ΔrmlAB$_1$CD* mutant strain using the pin-prick method (Hill & Purcell, 1995). Each plant was inoculated twice with a 20 µL drop of a 10^8 CFU/mL suspension of either wild-type *X. fastidiosa*, *ΔrmlAB$_1$CD* mutant, or the *rml/rml+* complemented strain. We inoculated seven plants/strain and repeated each experiment three times. Plants inoculated with 1xPBS served as negative controls. Throughout symptom development, plants were rated weekly on a disease scale of 0-5 with 0 being healthy and 5 being dead (Guilhabert & Kirkpatrick, 2005). We have also assessed the *X. fastidiosa* populations in the plants by isolating cells from petioles at the point of inoculation (local), and 38 cm above the point of inoculation (systemic), to assess the ability of the *ΔrmlAB$_1$CD* mutant strain to systemically colonize the host. Isolations were performed from local tissue at five weeks post-inoculation and from local and systemic (38 cm) tissue at 10 weeks post-inoculation. Populations of the *ΔrmlAB$_1$CD* strain *in planta* were significantly less than populations of wild-type and the *rml/rml+* complemented strain at five weeks post-inoculation ($P < 0.05$) (data not shown). At this time, Pierce's disease symptoms were visible in wild-type and *rml/rml+*-inoculated plants, while *rmlAB$_1$CD* mutant-inoculated plants did not show any degree of leaf scorching. At 10 weeks post-

inoculation, bacterial titer of the *ΔrmlAB₁CD* strain *in planta* was consistently significantly less than *X. fastidiosa* wild-type and the *rml/rml+* complemented strain, in both local and systemic tissue (**Figure 3A**). In addition, 80% of mutant-inoculated plants still had no Pierce's disease symptoms at this time point (**Figure 3B**). **This indicates that disruption in the production of rhamnose correlates with a profound defect in *X. fastidiosa* virulence and colonization *in planta*.** Thus far, we have observed no difference in bacterial titer or disease progress between the wild-type and *rml/rml+* complemented strain, indicating full complementation *in planta*, and plants inoculated with 1xPBS buffer have not developed any Pierce's disease symptoms (**Figure 3C**).

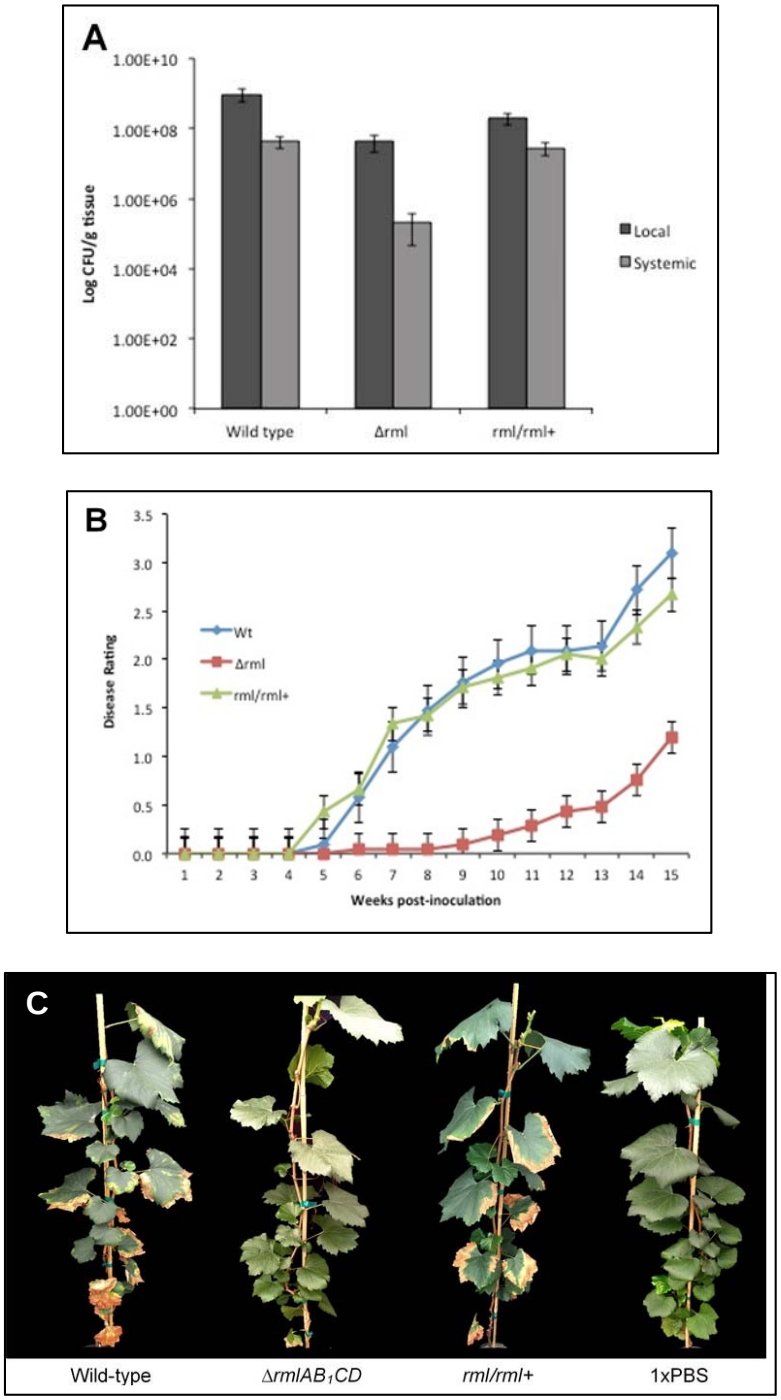

Figure 3. Pathogenicity assays *in planta*. **(A)** *In planta* populations of *X. fastidiosa* from local and systemic petioles at 10 weeks post-inoculation and **(B)** disease progress of *X. fastidiosa* wild-type and Δ*rmlAB1CD* mutant strains. The Δ*rmlAB1CD* mutant strain is significantly impaired in host colonization, as populations *in planta* are significantly less than those of the wild-type parent. In addition, Pierce's disease symptoms in Δ*rmlAB1CD*-inoculated plants are greatly attenuated, even at 14 weeks post-inoculation **(C).** Data represents the mean of 21 samples per treatment ± standard error of the mean. No *X. fastidiosa* cells were isolated from 1xPBS controls, and these plants did not exhibit Pierce's disease symptoms.

Objective 2. Examination of the LPS-mediated response to *X. fastidiosa* infection.
In grapevine, recognition of PAMPs other than LPS, such as the *Botrytis cinerea* endopolygacturonase BcPG1and β-glucans, trigger a cascade of signaling events including calcium ion influxes, reactive oxygen radical accumulations, and activation of protein kinases, that coordinate the transcriptional activation of defense genes (Aziz et al., 2003; Aziz et al., 2007; Poinssot et al., 2003). The LPS PAMP can induce similar responses in other plant species, but these studies have been performed primarily in model systems, such as *Arabidopsis thaliana* or tobacco (Desaki et al., 2006; Zeidler et al., 2004). There is limited knowledge about the grapevine response to the LPS PAMP, particularly on the transcriptional level. However, one study demonstrated that a rhamnolipid microbe-associated molecular pattern (MAMP) from *Pseudomonas aeruginosa* could induce defense-related responses in grapevine cell suspensions (Varnier et al., 2009).

The defense reactions activated upon PAMP recognition involve intricate networks of transcriptional regulators and phytohormone signaling. Genome-wide transcriptional profiling is a logical starting point to begin understanding this complex process in the *X. fastidiosa*-grape pathosystem (Jones & Dangl, 2006). We speculated that mutated *X. fastidiosa* LPS (depleted of O-antigen) recognition elicits a transcriptional response that results in the deployment of specific defense reactions in grape that results in less disease and host colonization. We hypothesized that the grapevine is recognizing the conserved core/lipid A portions of the *X. fastidiosa* LPS molecule and that the long chain O-antigen serves to camouflage the rest of the LPS PAMP (the core-lipid A complex) from being recognized by the host innate immune system. Thus, we expected an increase in expression of defense-related genes in plants inoculated with the O-antigen mutants that are depleted of O-antigen, as compared to wild-type *X. fastidiosa.* The studies detailed below were designed to test our hypothesis that loss of the rhamnose-rich O-antigen allows the grapevine to more readily perceive the *X. fastidiosa* LPS molecule and that this recognition leads to elicitation of a specific transcriptional response associated with defense.

LPS-induced oxidative burst in grapevine.
To explore the role of LPS as an elicitor of basal defense responses in grapevine, we first investigated reactive oxygen species (ROS) production in response to purified LPS using a luminol assay. Thus far, we have demonstrated that both wild-type and *wzy* mutant LPS induce an oxidative burst (of similar amplitude) in grapevine leaf disks, therefore establishing the role of *X. fastidiosa* LPS as a PAMP in grapevine. We expected that the *wzy* mutant LPS would induce a stronger oxidative burst than wild-type, but we hypothesized that the similarity was due to the increased exposure of the conserved Lipid A-Core Oligossacharide region of both molecules. Once we established that purified *X. fastidiosa* LPS induced an oxidative burst in *V. vinifera* Cabernet Sauvignon leaf disks, we then turned our attention to ROS produced in response to living *X. fastidiosa* wild-type and *wzy* mutant live culture, in which LPS is still anchored to the outer membrane. We have demonstrated that the *wzy* mutant culture induced a robust response from grapevine leaf disks. ROS production peaked at around 12 minutes and lasted nearly 100 minutes. Wild-type culture failed to produce a sharp peak, as compared with the *wzy* mutant, and ROS production plateaued around 60 minutes. **This indicates that the O-antigen does, in fact, serve to shield wild-type cells from triggering PTI responses.**

Transcriptome profiling.
In early July of 2014, individual vines of *V. vinifera* Cabernet Sauvignon were inoculated with *X. fastidiosa* wild-type or *wzy*::*kan* live culture. We inoculated nine vines for each treatment. Vines inoculated with 1xPBS buffer alone served as the negative controls for the experiment. Using the pin-prick method described previously, each vine was inoculated 2x with a 20 µL drop of a 10^8 CFU/mL suspension of either wild-type *X. fastidiosa* or the *wzy* mutant. The inoculum was immediately drawn into the petiole due to the negative pressure in the xylem. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow et al., 2000; Tao et al., 2003). Thus, petioles were harvested at the following four time points: 0, 1, 8, and 24 hours post-inoculation. To stabilize transcripts, petioles were submerged into liquid nitrogen immediately after harvesting and stored at -80°C until RNA extraction.

Sequencing libraries were generated from the polyadenylated plant messenger RNA and sequenced using an Illumina HiSeq 2500 platform. The sequencing of four HiSeq lanes generated a total of 763 million 50 base pairs (bp) single-end reads. Reads were trimmed and filtered to retain high-quality sequence information only (Q > 30). An average of 24.5 ± 3 millions of reads per sample (87.6±1.7% of the total) were unambiguously mapped on the

reference PN40024 transcriptome. Counts were normalized to control for technical variation using DESeq2, which was also used for statistical testing.

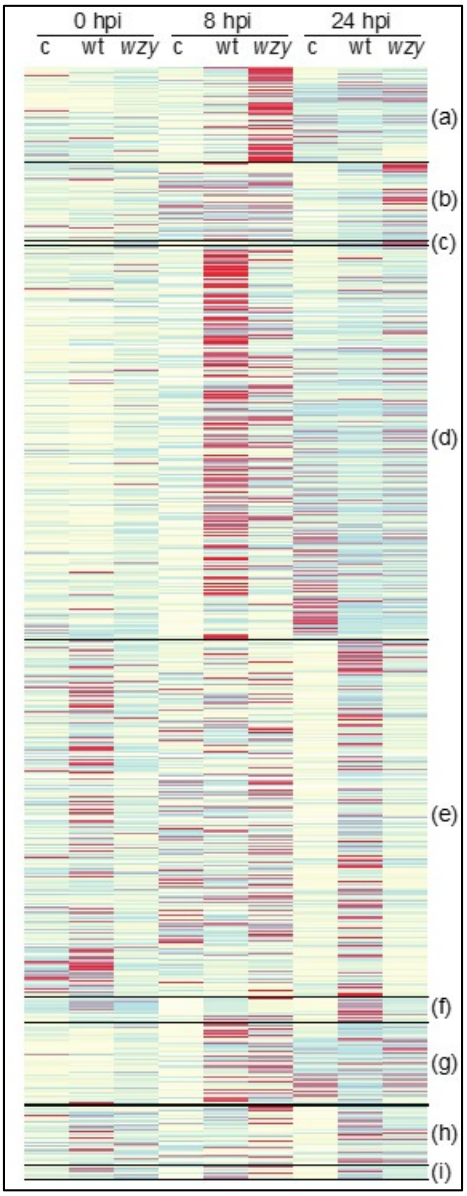

Figure 4. Heat map of the LPS-mediated response to *X. fastidiosa* infection in grapevine. Genes were grouped based on their expression pattern relative to the wounded control "c." At eight hours post-inoculation, plants inoculated with the *wzy* mutant are expressing a large number of genes belonging to group (a), which consists of genes specifically involved in immune responses (i.e., "response to oxidative stress"). At the same time point, plants inoculated with wild-type are expressing a large number of genes belonging to group (d), which consists of genes involved in responses to abiotic stress. This indicates that plants are directly sensing *wzy* mutant cells, which lack the O-antigen, and initiating PTI responses, but the wild-type cells, containing a fully polymerized O-antigen, remain seemingly undetected by the host immune system.

Our analysis focused on specific patterns of transcriptional regulation to identify genes specifically responsive to the *wzy* mutant and not to wild-type *X. fastidiosa*. 112 genes, which showed consistent up-regulation (fold change > 1.4, $P < 0.05$) in response to the *wzy* mutant at eight hours post inoculation, were either unchanged in the wounded or wild-type inoculated plants or were differentially regulated only at the later time point. The 112 genes included chitinases, endoglucanases, and other known antimicrobial peptides, typically transcriptionally induced during incompatible interactions. **Enrichment analysis of these 112 genes showed an interesting over-representation of immune response functions in these sets of genes transcriptionally responsive to *wzy***

mutant (e.g., defense responses, response to stress, oxidation-reduction process). At 24 hours, 53 genes were significantly up-regulated specifically in response to *wzy.* Overall, the RNA-seq data suggest that grapevines are activating specific defense responses (namely those involved in oxidative stress) upon recognition of *wzy* mutant infection (**Figure 4**). RT-qPCR will be performed to validate the expression of the most interesting genes. Investigation into the differential expression of genes during later stages of the *X. fastidiosa* infection process is also currently in progress. Beginning in June 2015, individual vines of *V. vinifera* Cabernet Sauvignon were inoculated with *X. fastidiosa* wild-type or *wzy*::*kan* live culture. We inoculated nine vines for each treatment. Vines inoculated with 1xPBS buffer alone served as the negative controls for the experiment. Using the pin-prick method described previously, each vine was inoculated 2x with a 20 µL drop of a 10^8 CFU/mL suspension of either wild-type *X. fastidiosa* or the *wzy* mutant. Petioles were harvested at 0, 48 hours, one week, and four weeks post-inoculation with live culture. To stabilize transcripts, petioles were submerged into liquid nitrogen immediately after harvesting and stored at -80°C until RNA extraction. All petioles were shipped to the Cantu lab at the University of California, Davis for RNA extraction and sequencing.

Objective 3. Evaluation of structural variants of LPS as a preventative treatment for Pierce's disease.
In some systems, treatment with LPS alone does not induce a measurable difference in gene expression. However, it does potentiate a more robust and measurable defense response following challenge with a pathogen. Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This defense-related "memory" is called priming and stimulates the plant to initiate a faster and/or stronger response against future invading pathogens (Conrath, 2011). Priming often results in rapid and robust activation of defense responses such as the oxidative burst, nitric oxide synthesis and expression of defense-related genes (Erbs & Newman, 2003; Newman et al., 2000). The LPS PAMP has been specifically implicated in priming in the *Xanthomonas campestris* pv. *vesicatoria* pathosystem. Pepper leaves pre-treated with LPS isolated from incompatible (non-virulent) xanthomonads had enhanced expression of several pathogenesis-related (PR) proteins after being challenged with virulent *X. campestris* pv. *vesicatoria* (Newman et al., 2000). In this objective, we hypothesized that pre-treatment with LPS isolated from *X. fastidiosa* O-antigen mutants would result in a difference in the grapevine's tolerance to *X. fastidiosa* by stimulating the host basal defense response. To determine if the primed state affects the development of Pierce's disease symptoms, we documented disease progress in plants that were pre-treated with either wild-type or *wzy* mutant LPS and then challenged with *X. fastidiosa* either four or 24 hours later.

Grapevine petioles were pre-treated with 40µL of either wild-type or *wzy* mutant LPS (50µg/mL). 1xPBS served as the negative control. After we mechanically inoculated the vines with LPS, we challenged with an inoculation of live wild-type *X. fastidiosa* cells (40 µL of a $1x10^8$ CFU/mL suspension). These inoculations were performed at four and 24 hours after the original inoculation with the LPS. These time points were established based on previously described assays (Newman et al., 2002). We inoculated 24 vines/treatment/LPS concentration/time point. Plants were rated 12 weeks later with 0 being healthy and 5 being dead or dying (Guilhabert & Kirkpatrick, 2005). As shown in **Figure 5**, plants pre-treated with either the wild-type or *wzy* mutant LPS were delayed in Pierce's disease symptom development when challenged with *X. fastidiosa* four hours later, compared to those plants that received no LPS pre-treatment. This indicates that treatment with either form of LPS (wild-type or *wzy* mutant) does elicit a defense response against *X. fastidiosa* within a four-hour time window. We reason that the purified forms of wild-type LPS and *wzy* LPS both elicit a priming response because all portions of the LPS molecule (including the conserved lipid A + core LPS) which are most often associated with activity of the LPS as a PAMP are exposed and available for recognition by the grapevine immune system. Whereas, in intact cells, the majority of the LPS molecule (lipid A and some of the core LPS) is embedded in the bacterial outer membrane and shielded from perception by the host immune system. Interestingly, two-way analysis of variance (ANOVA) analysis indicates that there is a significant interaction between time of inoculation and type of LPS applied, supporting our hypothesis that we will see large differences in the long-term defense responses elicited by *wzy* mutant LPS vs. wild-type LPS at later time points than what we have previously tested. This experiment was repeated beginning June of 2015, and results are consistent with the data presented above.

Most importantly, now that we have established that we can directly elicit an LPS-mediated defense response and also induce the primed state in grapevine, it will be important to assess how long the temporal window of the heightened defense response and primed state lasts by increasing the amount of time between the inoculation with the LPS and the challenge with live *X. fastidiosa* cells. These experiments are currently in progress.

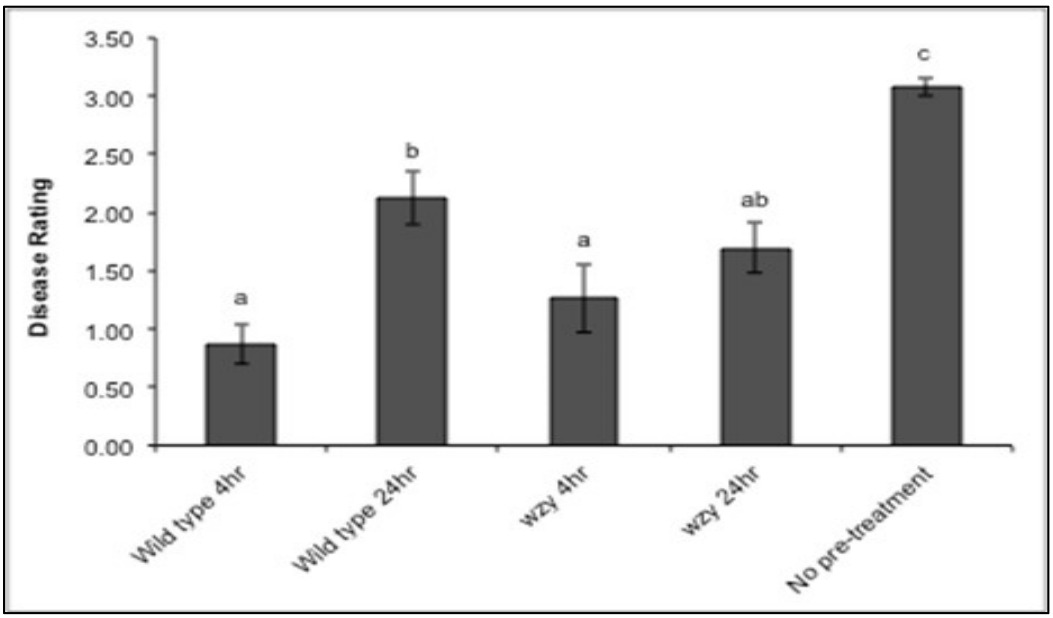

Figure 5. Pierce's disease ratings of LPS pre-treated plants. Mean disease ratings of Cabernet Sauvignon grapevines pre-treated with wild-type or *wzy* mutant LPS (50 µg/mL), then challenged at four or 24 hours post-LPS treatment. We inoculated 24 vines/treatment/time point. Disease ratings were taken at 12 weeks post-challenge. The LPS pre-treated plants are significantly delayed in symptom development, compared with plants that did not receive pre-treatment. Bars represent standard error of the mean.

CONCLUSIONS

This project has addressed key aspects of the interaction of *X. fastidiosa* with its grapevine host. In addition, the results have provided important knowledge about basal resistance to disease in grapevines and plant hosts in general. Notably, we have also tested a potential preventative measure for Pierce's disease. Information gleaned from this project could help guide traditional breeding programs aimed at disease resistance by identifying potential resistance markers. The overall outcomes have resulted in a foundation of fundamental knowledge about Pierce's disease at the molecular level that we will utilize to develop innovative and environmentally sound approaches to controlling this disease.

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DEVELOPMENT OF A GRAPE TISSUE CULTURE AND TRANSFORMATION PLATFORM FOR THE CALIFORNIA GRAPE RESEARCH COMMUNITY

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ABSTRACT

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish and maintain embryogenic cell cultures and convert cell cultures into whole plants. We are leveraging the expertise of the National Research Laboratory of Chile (INIA) and the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California, Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The two labs are sharing their latest protocol improvements for generating and increasing high quality embryogenic callus using germplasm important to their particular country. This combined effort has allowed us to make significant advances in our ability to grow and maintain embryogenic callus cultures for use in tissue culture and transformation experiments for rootstock genotypes 1103, 101-14, and the winegrapes Chardonnay and Cabernet Sauvignon. We have successfully established high quality, rapidly multiplying grape suspension stock cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay for each of the past three years by employing a modification of INIA's liquid/agar cell cycling system, allowing us to maintain a constant supply of cells needed for tissue culture and transformation studies. We have developed a method for maintaining somatic embryos for extended periods of time in a quiescent state by plating cell suspension on medium containing high concentrations of sorbitol. This system allows us to maintain a germplasm bank of embryos for numerous grape genotypes which provides a constant supply of embryos for use in tissue culture and transformation experiments. These embryos provide an excellent source of material for transformation and has allowed us to routinely transform rootstocks 1103 and 101-14. Using embryogenic suspension cultures, we are developing a high frequency transformation protocol based on direct transformation of 1103 and 101-14 suspension cultures which, when optimized, should allow us to produce transgenic rootstocks with less labor than is currently required for embryo transformation. We have also developed a sequential transformation protocol which allows stacking of multiple transgenes into grape. Although regeneration of whole plants from non-transgenic embryos of 1103 and 101-14 is routine, regeneration of transgenic embryos of 1103 and 101-14 remains inefficient and is the most significant bottleneck in grape transformation. We have made some improvements in efficiency of plant regeneration from transgenic embryos, but further improvements would greatly streamline the protocol.

LAYPERSON SUMMARY

This project is aimed at establishing an international collaboration between leading laboratories in the United States and Chile to reduce the time and cost of tissue culture and transformation for grape varieties of importance to the viticulture industries in their respective countries. The collaboration leveraged pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the Pierce's disease and glassy-winged sharpshooter research community. The two labs shared their latest protocol improvements for generating and increasing high quality embryogenic cultures using germplasm important to their particular country. Using both cell suspension cultures and bioreactors, we made significant advances in our ability to establish and increase embryogenic cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay for use in tissue culture and transformation experiments. We have developed a long-term storage medium which allows grape somatic embryos to be stored for over six months, which will allow for easy maintenance of numerous genotypes with minimal labor. Using these embryos, we achieved high transformation frequencies for 1103 and 101-14.We also demonstrated that we can directly transform grape suspension cultures, which bypasses the need to generate embryos prior to transformation. Although our results with direct transformation of grape suspensions are still inconsistent from run to run, experiments that are successful using this technique allow us to produce transgenic material with minimal input of labor. We also developed a sequential transformation protocol which will allow us to re-transform transgenic grape embryos with

a second disease gene. This will allow researchers to stack resistance genes for additional disease protection. Although regeneration from non-transgenic 1103 and 101-14 embryos is routine, regeneration of transgenic 1103 and 101-14 into whole plants remains inefficient. We made progress developing new media formulations which allow for more rapid regeneration of plantlets from non-transgenic embryos of 1103 and 101-14, which if applicable to transgenic embryos, should reduce the time required to generate transgenic grape plants for the Pierce's disease and glassy-winged sharpshooter research community.

INTRODUCTION

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish embryogenic cell cultures and convert cell cultures into whole plants. The efficiency of establishing and regenerating plants for many important grape genotypes remains very low and are not at the level required to allow for the predictable, cost effective, and timely recovery of tissue culture plants needed to successfully offer grape tissue culture and transformation through a self-sustaining service-based facility. These challenges include the successful establishment and multiplication of homogeneous embryogenic cultures, prevention of tissue necrosis caused by oxidation, conversion of embryos into true-to-type plants, transformation of embryogenic callus, and the regeneration of non-chimeric transgenic plants from embryogenic cells. The goal of this agreement is to leverage the expertise of the National Research Laboratory of Chile (INIA) and the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California, Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The proposed collaboration combines pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the Pierce's disease and glassy-winged sharpshooter research community. Results of this collaboration will accelerate the delivery of Pierce's disease and glassy-winged sharpshooter research results in genotypes that are relevant to the research community. This report outlines the progress that was made under CDFA agreement number 11-0416-SA toward achieving that goal, with concrete benefits already realized by both groups from the creation of this collaboration.

OBJECTIVES

1. Establish an international collaboration between leading laboratories in the United States and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.
    a. Adapt tissue culture and transformation methodologies developed by our Chilean partner for grape genotypes of importance to California, including 11-03, 101-14, Cabernet Sauvignon, and Chardonnay.
    b. Increase the efficiency of maintaining embryogenic cultures and reduce the time required for *in vitro* regeneration of grape plants from embryogenic cultures by adapting INIA's cell suspension technology and UCDPTF's temporary immersion system (TIS) for use in grape tissue culture and transformation.
    c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape.
2. Develop a cost-effective grape tissue culture and transformation platform for at least one priority California winegrape and one California grape rootstock, which will provide the Pierce's disease and glassy-winged sharpshooter research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.
    a. Improve grape rootstock transformation efficiency for 1103 and 101-14 using embryos harvested from robust-growing cell suspension cultures.
    b. Leverage the progress we have made in developing high quality suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by testing direct transformation of cell suspension cultures.
    c. Develop methods for transforming multiple trait genes into grape through sequential transformation using two different plant selectable marker genes.

RESULTS AND DISCUSSION

Objective 1. Establish an international collaboration between leading laboratories in the United States and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.

Objective 1a. Adapt tissue culture and transformation methodologies developed by our Chilean partner for grape genotypes of importance to California, including 11-03, 101-14, Cabernet Sauvignon, and Chardonnay.

We have established embryogenic cultures from anthers of 11-03, 101-14, Chardonnay, and Cabernet Sauvignon each year of the grant by plating them onto Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.0 mg/l benzylaminopurine (BAP) (PIV) or Murashige and Skoog minimal organics medium supplemented with 20 g/l sucrose, 1.0 mg/l naphthoxyacetic acid (NOA), and 0.2 mg/l BAP (NB medium). In 2014 we also established embryogenic callus of Richter 110.

INIA and UCDPTF explored an alternative method for generating embryogenic callus which utilizes leaf pieces from *in vitro* grown plants. Unlike generating callus from anthers which have a short window of availability in the spring of each year, leaf tissue from *in vitro* plantlets are available year-round. In addition, unlike meristem explants which are time-consuming and difficult to excise, leaf explants are easy to isolate and can be secured from known pathogen-free tissue culture plantlets. We are maintaining disease-free *in vitro* stock plants of 1103, 101-14, Chardonnay, and Cabernet Sauvignon that we received from Foundation Plant Services and used these to test the leaf embryogenic culture system. Although we successfully generated embryogenic callus from leaf explants of Thompson Seedless grape, we were not able to replicate this technique for non-table grape genotypes. Based on the advice of Andy Walker and Cecilia Aguero, we are investigating the Mezzetti et al. (2002) protocol which utilizes bulk meristems as the target tissue for transformation. We generated bulk meristem cultures from indexed tissue culture stock tissue for 11-03, 101-14, Chardonnay, and Cabernet Sauvignon (**Figure 1**). To date, scion genotypes develop bulk meristems more readily than rootstocks. Rootstock genotypes tend to produce excess callus on Mezzetti medium with 3.0 mg/l BAP.

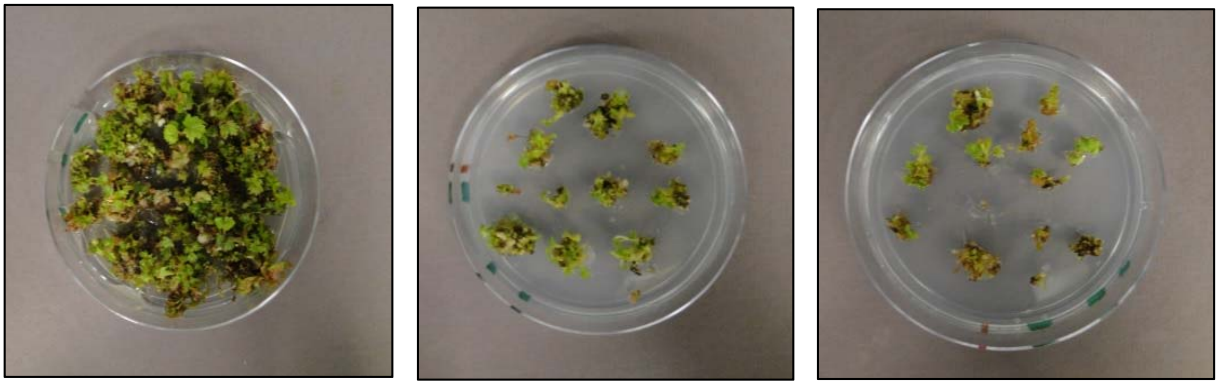

Figure 1. Bulk meristem cultures of (from left to right) Cabernet Sauvignon, Chardonnay, and 1103.

Objective 1b. Increase the efficiency of maintaining embryogenic cultures and reduce the time required for *in vitro* regeneration of grape plants from embryogenic cultures by adapting INIA's cell suspension technology and UCDPTF's temporary immersion system for use in grape tissue culture and transformation.

Using a modification of INIA's cell suspension protocol, we significantly improved the production of embryogenic grape cultures across a range of genotypes including 1103, 101-14, Cabernet Sauvignon, Chardonnay, and Richer 110. We established fresh embryogenic suspensions each year and we can routinely maintaining cell suspension cultures on liquid woody plant medium (WPM; Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1 g/liter casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2 g/l activated charcoal, 100 mg/l ascorbic acid, and 120 mg/l reduced glutathione (Pic/MTag) grown in 125 ml shake flasks on a gyratory shaker at 90 rpms in the dark. Once established, the suspensions are easily maintained by withdrawing 10 ml of the suspension each week from the flask and replacing it with 10 ml of fresh medium (**Figure 2**).

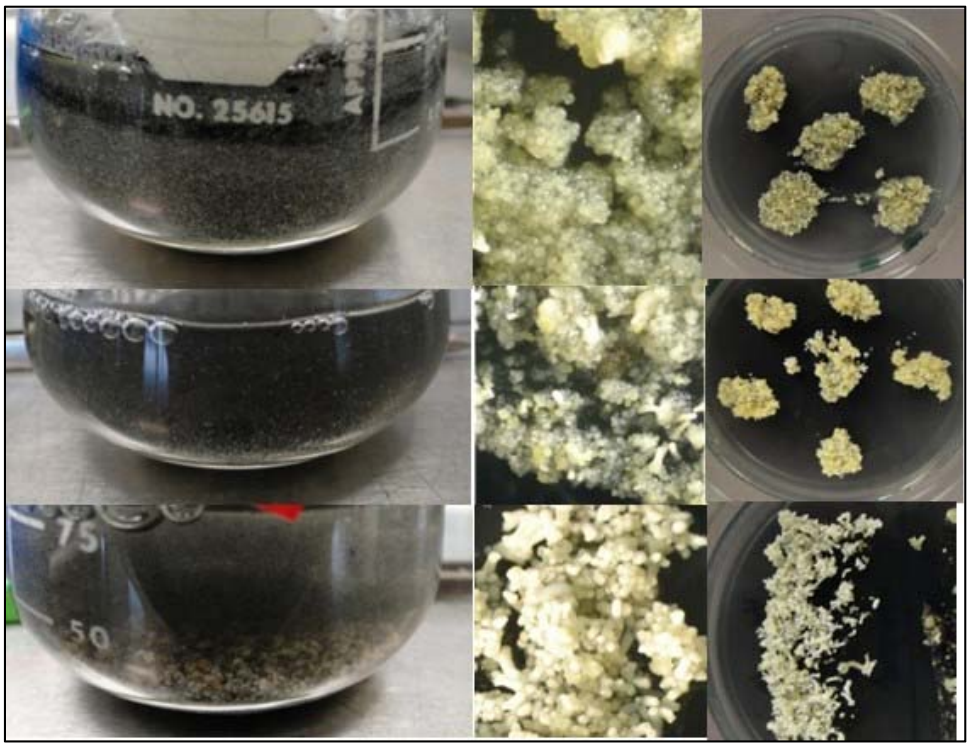

Figure 2. Production of high quality embryogenic callus from cell suspensions. Chardonnay (top), 1103 (middle), and 101-14 (bottom) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES, 1,000 mg/l activated charcoal, 10 mg/l picloram, and 2 mg/l meta-topolin (left), close-up of cell suspension cultures (middle) and embryogenic callus developing four weeks after plating 1 ml of suspen-sions on WPM medium supplemented with 1 g/l casein, 1M MES, mg/l activated charcoal, 0.5 mg/l BAP, and 0.1 mg/l NAA (right).

In addition to evaluating INIA's liquid shake flasks methodology on grape genotypes 101-14, 1103, Chardonnay, and Cabernet Sauvignon, we explored UCDPTF's temporary immersion system for use in rapidly increasing embryogenic callus. This method of increasing embryogenic callus has proven very efficient, and we have found that by adding ascorbic acid to the culture medium and growing the cultures in the dark we can significantly reduce phenolic development in the callus. Robust growth rates of 1103, 101-14, and Chardonnay callus cultures were achieved for a minimum of three months without addition of fresh medium to the bioreactor (**Figure 3**). This system is advantageous from a labor management perspective, since it allows one to maintain stock embryogenic cultures indefinitely in temporary immersion with medium exchanges occurring only once every three months.

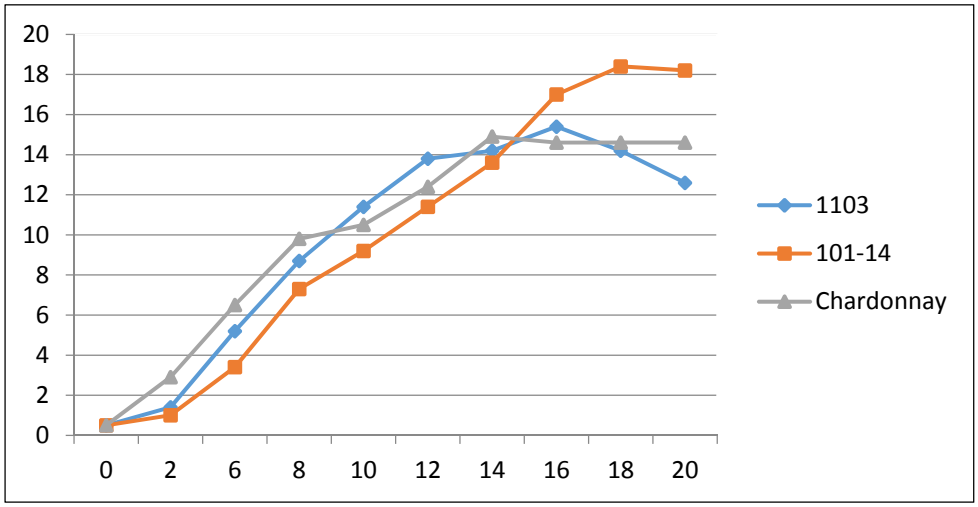

Figure 3. Embryogenic callus growth of 1103, 101-14, and Chardonnay in bioreactor, demonstrating that embryogenic callus can be maintained in bioreactors for three months with no medium additions of culture manipulations.

Objective 1c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape.
In addition to using cell suspension, temporary immersion and stir tank reactor techniques to reduce the labor associated with growing embryogenic cultures, we investigated methods for storing high quality embryogenic cultures over an extended period of time. We found that increasing the osmotic strength of the medium offers a simple solution for maintaining high quality somatic embryos over an extended period of time. One ml of embryo suspension as described above can be plated onto WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 50 g/l sorbitol, and 14 g/l agar and cultured in the dark at 26ºC. Cells plated onto this medium develop somatic embryos within approximately four to eight weeks. Embryos do not germinate into plants, but remain as quiescent somatic embryos. They can be maintained in this state for up to six months without loss of viability and upon being transferred to WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, and 0.1 mg/l BAP lacking sorbitol, they germinate into whole plants. These embryos serve as an excellent source of embryos for use in transformation (**Figure 4**). Given the high efficiency of conversion of the cell suspensions to embryos, these suspensions have utility for use in developing enhanced transformation protocols, gene editing technology, protoplast culture, and tilling populations for grape.

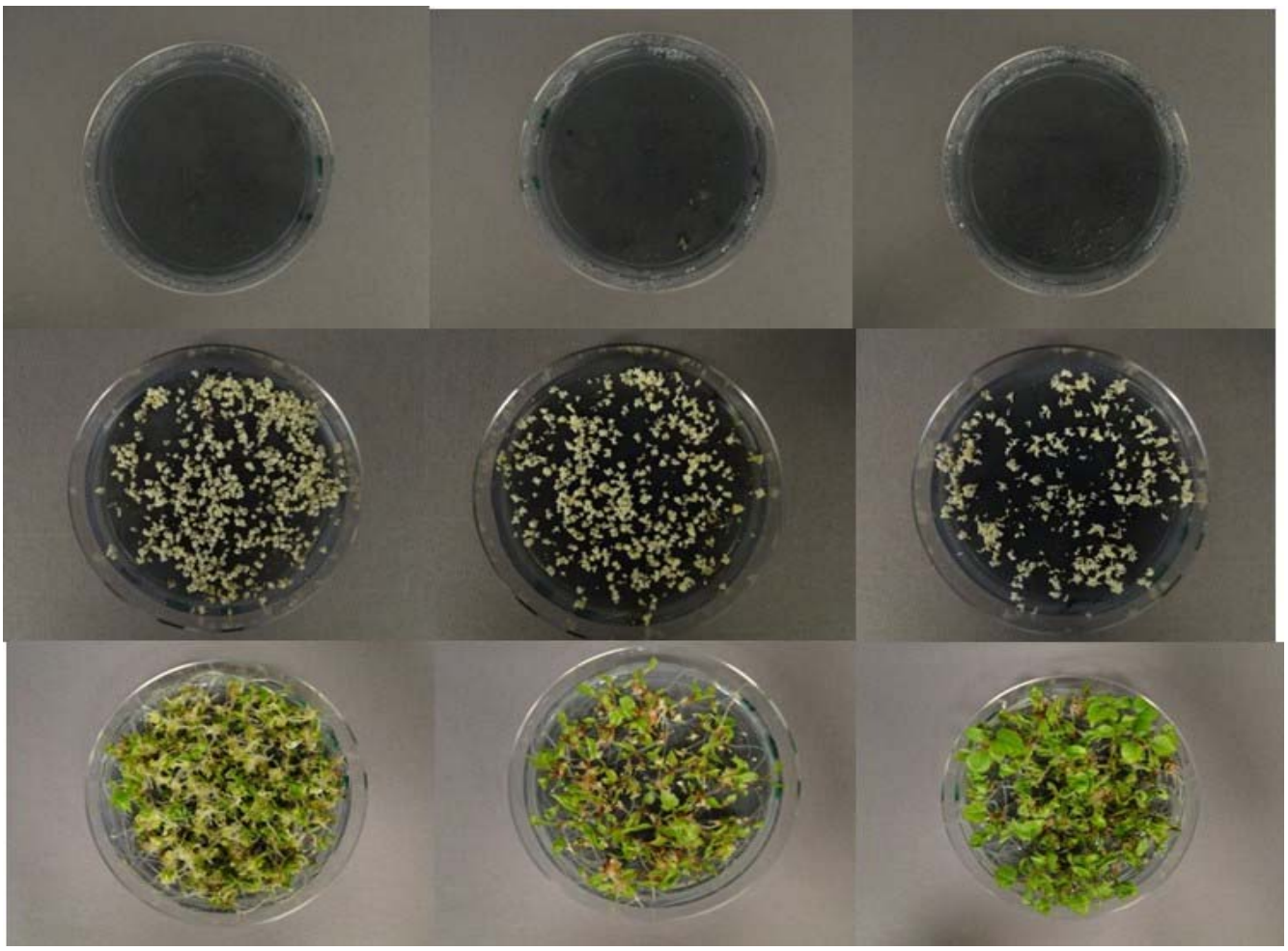

Figure 4. Long-term storage of somatic embryos of (from left to right) Cabernet Sauvignon, 1103, and Chardonnay, after plating 1 ml of cell suspensions onto WPM supplemented with 20 g/l sucrose, 1 g/l casein,1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 50 g/l sorbitol, and 14 g/l agar (top row); quiescent embryos of Cabernet Sauvignon, 1103, and Chardonnay five months after storage in the dark without sub-culturing (middle row); Cabernet Sauvignon, 1103, and Chardonnay 15 days after transferring embryos stored for five months onto WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and 8 g/l agar and cultured in the light (lower row).

Regeneration of whole plants from embryos of 1103 and 101-14 is the rate-limiting step in the production of transgenic plants. Transformation frequencies are now relatively high for these rootstocks, but regeneration of whole plants from transgenic tissue still requires many months in culture. In other species, the basal salt formulation of the tissue culture medium can have a dramatic effect on the regeneration efficiency. Therefore, we evaluated eight different salt formulations in an attempt to improve the efficiency of whole plant regeneration from embryos of 1103 and 101-14. Salt formulations tested included Andersons, Chee and Pool, DKW, Gamborg's B5, MS, WPM, SH, and X6. All media were supplemented with 1.0 g/l casein and 500 mg/l activated charcoal and 0.1 mg/l BAP. Significant differences were seen between the various salt mixtures with the best regeneration occurring on DKW, SH, and WPM. However, rootstock regeneration remains less efficient than regeneration from table grapes or winegrapes. During the period of the grant we determined that the best regeneration media for grape rootstocks was Preece's salt formulation, which is comprised of 50 % DKW salts and 50% WPM salts supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP or 0.1 mg/l Zeatin and 8 g/l agar (**Figure 5**).

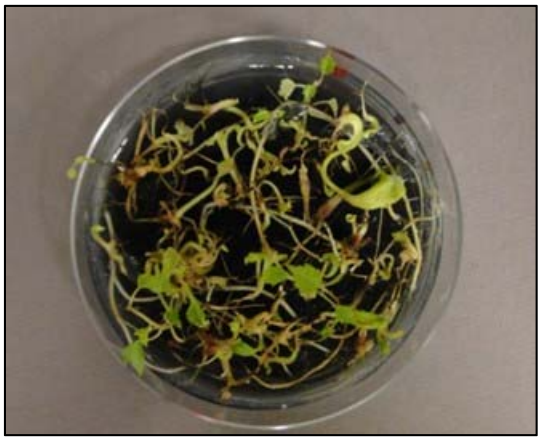

Figure 5. Regenerating transgenic shoots of 1103 developing from embryos plated on Preece medium supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and 25 mg/l hygromycin.

Objective 2. Develop a cost-effective grape tissue culture and transformation platform for at least one priority California winegrape and one California grape rootstock which will provide the Pierce's disease and glassy-winged sharpshooter research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.

Objective 2a. Improve grape rootstock transformation efficiency for 1103 and 101-14 using embryos harvested from robust-growing cell suspension cultures.

We evaluated the use of a heat shock treatment on somatic embryos prior to inoculating with *Agrobacterium tumefaciens*. Preliminary results indicate that a 10-minute heat shock treatment at 45ºC increased the transformation frequency in Thompson Seedless, 1103 (**Table 1**), and 101-14. While transformation frequencies for 101-14 and 1103 are relatively high, transformation frequencies of Cabernet Sauvignon and Chardonnay remain low and further improvements are needed.

Table 1. Transformation experiments with 1103 embryos comparing transformation efficiencies after exposure to 10 minutes of heat shock at 45ºC versus no application of heat shock prior to inoculation with *Agrobacterium tumefaciens*.

Number of Experiments	Heat Shock	Genotype	# (%) transgenic colonies
3	-	1103	17/73 (23.3)
5	+	1103	72/140 (51.4)

We developed a robust suspension system for 1103 and 101-14 which provides a continuous source of somatic embryos for transformation. Embryogenic cell suspensions are harvested from cell suspension cultures on a weekly basis as part of the process required for feeding the suspension cultures. As described above, 1 ml of the suspension can be plated on sorbitol-containing medium for regeneration of somatic embryos which enter a quiescent state and can be stored for later use for over six months without any additional manipulation. Large quantities of embryos can then be collected from the plates and transformed with *Agrobacterium* when transformations are requested. Secondary transgenic embryos arise from the epidermis of the inoculated embryos while the remainder of the inoculated embryo turns necrotic due to the selective agent (kanamycin or hygromycin) (**Figure 6**). The surviving secondary embryo can be harvested and transferred to regeneration medium for plant production. Using this system we have been able to generate transgenic embryos for both 1103 and 101-14. We are currently utilizing this technique for transformation requests for Pierce's disease and glassy-winged sharpshooter researchers (**Table 2**).

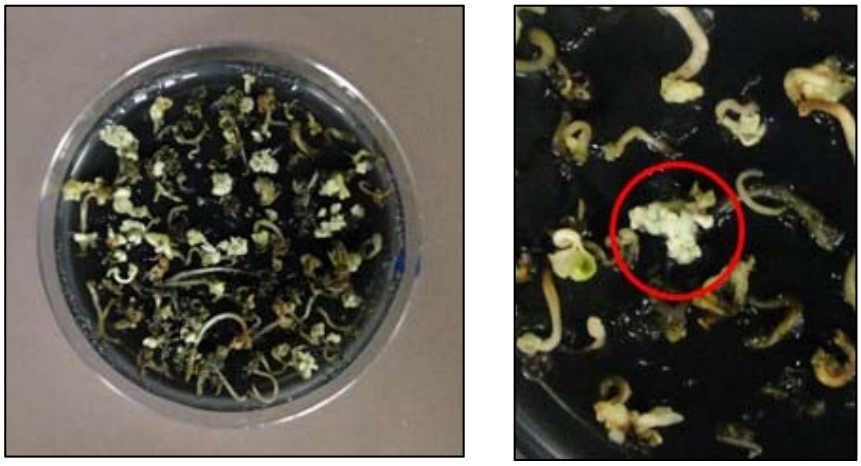

Figure 6. Clusters of transgenic secondary embryos developing from *Agrobacterium*-inoculated 1103 somatic embryos plated on 200 mg/liter kanamycin sulfate (left) and close up of one secondary embryo cluster (right).

Table 2. Inventory of transgenic 1103 and 101-14 plants generated with various genes of interest.

Genotype	Transgene	Number of Plants Produced
1103	35s HNE-CecB	15
101-14	35s HNE-CecB	25
101-14	pDU10.1818	10
101-14	HNE-CecR	6

Objective 2b. Leverage the progress we have made in developing high quality suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by testing direct transformation of cell suspension cultures.

We leveraged the progress we have made in developing high quality grape suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by developing a method for direct transformation of our grape suspension cultures. Eight to 10 ml of a grape cell suspension grown in liquid Pic/MTag medium and containing pre-embryogenic masses or small globular embryos are collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1,000 x G for three minutes. The supernatant is removed and the cells are washed by re-suspending them in WPM medium without charcoal. Cells are pelleted by centrifugation at 1,000 x G for three minutes and are washed two additional times in WPM medium. After the last wash, the cells are subjected to heat shock by placing the 15 ml conical tube in a 45ºC water bath for five minutes. After heat shock the supernatant is removed and the cells are re-suspended in five ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain EHA105 carrying the desired vector at an OD

600 of 0.1-0.2. The suspension is centrifuged at 1,000 x G for five minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes, all but 0.5-1.0 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred onto sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid is allowed to evaporate by leaving the petri dishes in the hood for a few hours. The cells are co-cultured for two to three days at 23ºC and then transferred to selection medium consisting of WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 4 ml PPM, 50 g/l sorbitol, 14 g/l agar, and 200 mg/l kanamycin, or 25 mg/l hygromycin. Sub-culturing of the plated cells is achieved by simply transferring the filter paper with the cells onto fresh medium on a biweekly basis. Within eight to 12 weeks transgenic embryos develop (**Figure 7**). Developing embryos are transferred to WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP or 0.1 mg/l zeatin, 400 mg/l carbenicillin, 150 mg/l timentin, 0 g/l sorbitol, 8 g/l agar, and 200 mg/l kanamycin or 25 mg/l hygromycin for germination. The time from inoculation to the recovery of transgenic embryos can be as short as 10 weeks. We have successfully used this technique to produce transgenic embryos of 1103 and 101-14 (**Figure 7**). The system has been employed successfully using both kanamycin and hygromycin selection. We see significant amounts of experiment-to-experiment variability in the number of transgenic embryos developing, with numerous experiments yielding no transgenic colonies and other experiments generating variable numbers of colonies (**Table 3**). However, if this protocol can be made more consistent, it represents a significant advance in our transformation system since it greatly increases transformation efficiencies while minimizing labor inputs. It appears that the plating density of the cells (too high or too low) and the quality of the suspension impacts transformation efficiency. As with our embryo-based transformation system, the limiting step in this protocol is also the regeneration of whole plants from transgenic embryos and we continue to explore media modification to enhance regeneration potential.

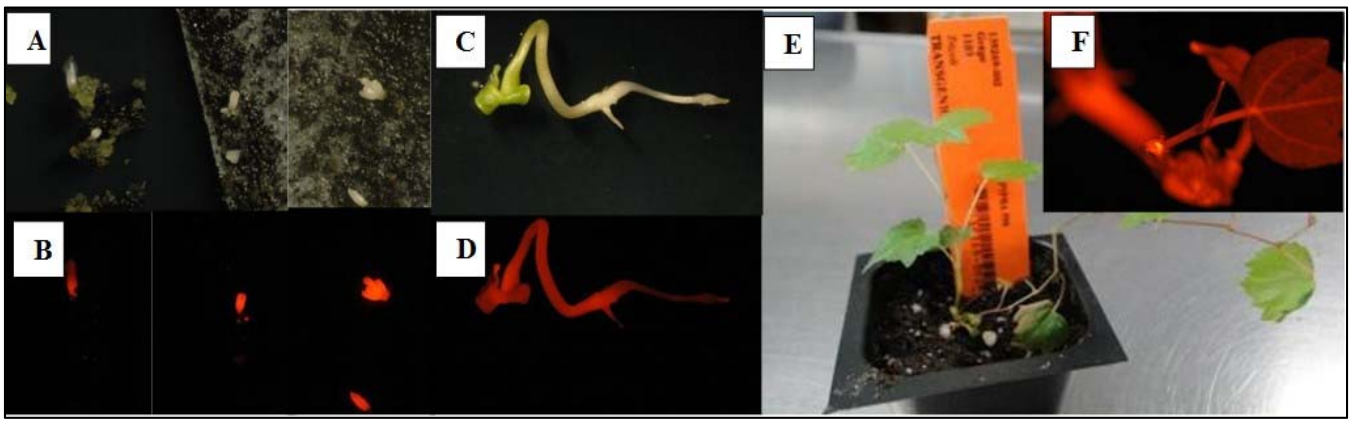

Figure 7. Transformation of suspension cultures of 1103 with the fluorescent DsRed gene plated on BN sorbitol medium supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 4 ml PPM, and 25 mg/l hygromycin. **(A)** Grape embryo expressing the DsRed gene confirming the transgenic status of the developing embryos. **(B)** Germination of transgenic embryos after transferring to WPM medium supplemented with 20 g/l sucrose, 1 g/l casein, 1mM MES, 500 mg/l charcoal, 0.1 mg/l BAP, and 8 g/l agar. Bright field **(C)** and fluorescence **(D)** regeneration of whole plant **(E)** expressing DsRed **(F)**.

Table 3. The number of transgenic embryogenic colonies forming after inoculating grape suspension cultures with *Agrobacterium* and plating onto selection medium.

Number of Experiments	germplasm	Selection	Average number of Transgenic colonies generated	Range of transgenic colonies generated per experiment
1	101-14	hygromycin	6	
4	1103	kanamycin	5	1-7
11	1103	hygromycin	10.7	1-50
4	101-14	kanamycin	11.75	3-24

Objective 2c. Develop methods for transforming multiple trait genes into grape through sequential transformation using two different plant selectable marker genes.

Since researchers expressed an interest in stacking multiple resistant strategies in a single transgenic grape line, we performed sequential transformations in which we transformed grape embryos with the first construct containing a gene of interest and the hpt plant-selectable marker gene and selected for hygromycin-resistant transgenic secondary embryos on medium containing hygromycin. Once hygromycin secondary embryos developed, they were increased. Once sufficient numbers of embryos were produced, they were re-inoculated with an *Agrobacterium* culture containing a second construct with a gene of interest and the kanamycin plant-selectable marker gene (nptii) and cultured on medium containing both kanamycin and hygromycin. Developing embryos should contain both selectable marker genes and both genes of interest. We have produced putatively sequentially transformed embryos for 101-14 and 1103 (**Figure 8**). Once plants are recovered they can be tested for the presence of both genes. Although this technique can be used to stack genes it is very inefficient and labor intensive, and stacking trait genes in a single T-DNA is recommended.

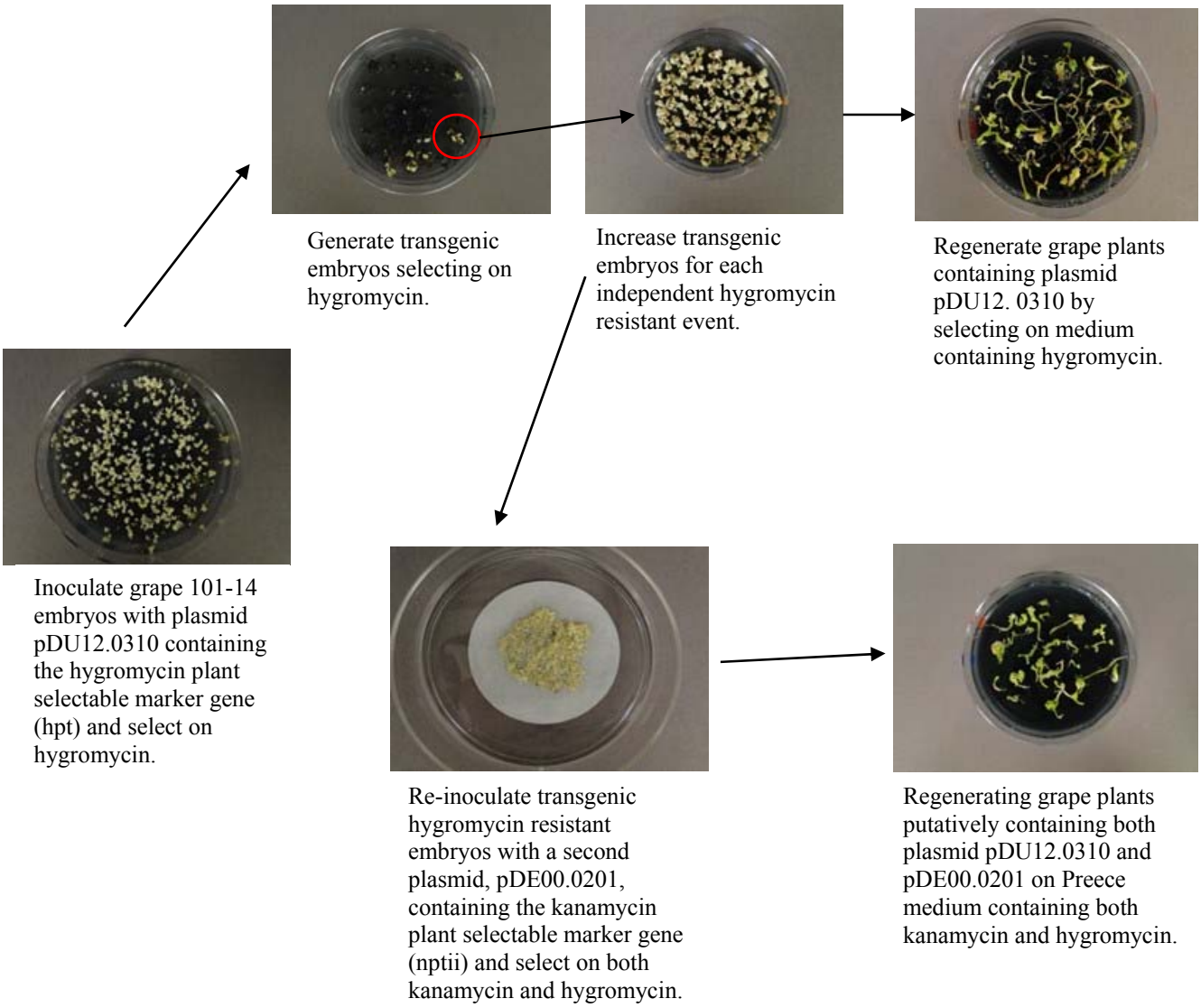

Figure 8. Sequential transformation of 101-14 somatic embryos inoculated initially with plasmid pDU12.0310 and selected on hygromycin. Some hygromycin-resistant embryos were induced to regenerate plants while a subset of embryos were re-inoculated with plasmid pDE00.0201 and double selected on hygromycin and kanamycin.

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EXPANDING THE RANGE OF GRAPE ROOTSTOCK AND SCION GENOTYPES THAT CAN BE GENETICALLY MODIFIED FOR USE IN RESEARCH AND PRODUCT DEVELOPMENT

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Reporting Period: The results reported here are from work conducted January 15, 2015 through October 2015.

ABSTRACT
The University of California, Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103P, two important grape rootstocks for the California grape industry. This technology will allow us to introduce genes useful in combating Pierce's disease into the rootstocks of grape and allow researchers to test whether a modified rootstock is capable of conferring resistance to the grafted scion. If rootstock-mediated resistant strategies are to be successful deployed throughout California, additional rootstocks will need to be modified in order to adequately address the rootstock requirements of the diverse wine growing regions in the State of California. To that end, we have cultured anthers for grape rootstocks Richter 110 (clone 01), MGT 420A (clone 04), 140Ru (clone 01), and Salt Creek (clone 08). We are also initiating embryo cultures for scion genotypes Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04). Transformation experiments using DsRed have been initiated on suspension culture and somatic embryo culture in order to access the utility of our existing transformation technologies in transforming these additional genotypes. We have also established *in vitro* shoot cultures for each of the genotypes listed above and have begun to initiate bulk meristem cultures for use in testing bulk meristem transformation methodology on these genotypes.

LAYPERSONS SUMMARY
The University of California, Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103P, two important grape rootstocks for the California grape industry. This technology will allow us to introduce genes useful in combating Pierce's disease into the rootstocks of grape and allow researchers to test whether a modified rootstock is capable of conferring resistance to the grafted scion. This strategy is commonly referred to as rootstock-mediated resistance. If rootstock-mediated resistant strategies are to be successfully deployed throughout California, additional rootstock genotypes besides 1103P and 101-14 will need to be modified in order to adequately address the rootstock requirements of the diverse wine growing regions in the State of California. We therefore are currently testing if our method for genetically modifying grape rootstocks can be used successfully on seven additional rootstock genotypes used in California winegrape production. These include 110R, 3309C, Harmony, Freedom, 420A, 140Ru, and Salt Creek. Since it is not yet known if a rootstock-mediated disease resistance strategy will confer durable, commercially viable levels of resistance to the grafted scion, we are also testing our method for modifying grapes on a select group of scions, including Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Merlot (clone 03), Pinot Noir (clone 2A), Zinfandel (clone 01), and French Colombard (clone 02). The results of this work will allow for the establishment of a self-sustaining grape tissue culture and transformation service that can be utilized by the Pierce's disease research community. It will also establish a germ bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes used in California, which can be made available to the research community.

INTRODUCTION
This project is aimed at applying the progress that has been made in grape cell biology and transformation technology of rootstock genotypes 1103P and 101-14 to additional grape rootstock genotypes in order to expand the range of genotypes amenable to transformation. The research will apply the pre-existing expertise and technical know-how developed for rootstocks 1103P and 101-14 at the University of California, Davis Plant Transformation Facility to additional rootstocks and germplasm important for the California wine industry. For this proposal, we are testing seven additional rootstocks for their amenability to transformation, including 110R, 3309C, Freedom, Harmony, 420A, 140Ru, and Salt Creek. This work will expand the range of rootstocks that can be effectively transformed, which will allow rootstock-mediated disease resistance technology to be employed

across the major wine growing regions in California. Although a rootstock-mediated resistance strategy is the preferred mechanism for achieving resistance to Pierce's Disease in grape, investing in the development of transformation technology for scions will serve an important fallback position should rootstock-mediated resistance fail to confer adequate levels of resistance to the scion and direct transformation of scion varieties be required. Therefore, in addition to testing the utility of our tissue culture and transformation protocols on seven additional grape rootstocks, we will also screen six important California scion genotypes for their amenability to transformation, including Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (2A), Merlot (clone 03), Zinfandel (clone 01A), and French Colombard (clone 02). Although it is unlikely that all seven rootstock and six scion genotypes will be amenable to transformation using our established protocols, we believe that a significant number will respond positively. The results of this work will allow for the establishment of grape tissue culture and transformation technologies that can be utilized by the Pierce's disease research community. It will also establish a germ bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes used in California, which can be made available to the research community.

OBJECTIVES

1. Develop embryogenic cultures from anthers of seven rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.
2. Develop embryogenic suspension cultures for seven rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use transformation experiments.
3. Establish a germplasm bank of somatic embryos for seven rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.
4. Test transformation efficiencies of seven rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.
5. Test direct cell suspension transformation technology on seven rootstock genotypes and six scion genotypes.
6. Establish *in vitro* shoot cultures for seven rootstock genotypes and six scion genotypes using indexed material from Foundation Plant Services (FPS) or field material from FPS and establish bulk meristem cultures for all 13 genotypes for use in transformation.
7. Test Mezzetti et al., 2002 bulk meristem transformation methodology for seven rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

RESULTS AND DISCUSSION

Objective 1. Develop embryogenic cultures from anther of seven rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.

We collected anthers of rootstock genotypes including 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, 101-14, and scion genotypes Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04) and plated them on four different embryogenic callus-inducing media. The media include Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.0 mg/l benzylaminopurine (BAP) (PIV); MS minimal organics medium supplemented with 20 g/l sucrose 1.0 mg/l 2,4-D and 0.2 mg/l BAP (MSE); MS minimal organics medium supplemented with 30 g/l sucrose 1.0 mg/l 2,4-D and 1.0 mg/l BAP (MSI); or one half strength MS minimal organics medium supplemented with 15 g/l sucrose 1.0 mg/l NOA and 0.2 mg/l BAP (NB). Anthers were collected during two separate weeks in the spring of 2015. The number of anther clusters plated for each genotype is given in **Table 1**, and the number of putative embryogenic calli or embryos developing to date is provided in **Table 2**.

Objective 2. Develop embryogenic suspension cultures for seven rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use transformation experiments.

To date, we have established suspension cultures for 1103, 101-14, Richter 110, MGT 420A (04), 140Ru, Chardonnay, and Colombard (04). We are still in the process of bulking callus cultures for rootstock genotypes 3309C (05), Freedom (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Merlot (03), Pinot Noir (clone 2A), and Zinfandel (clone 01A). Once adequate amounts of embryogenic tissue have developed, we will initiate suspension cultures for those genotypes.

Table 1. Summary of the number of plates of grape anthers explanted to four different media formulations.

Grape Anther Cultures	MSI	MSE	PIV	NB
Zinfandel 01A R5V12	4	1	3	4
Colombard 04 R1V13	3	1	4	3
Pinot Noir 02A R6V17	2	1	4	3
MGT 420A R2V3	4	2	3	4
140Ru 01 R2V7	5	2	5	4
Freedom 01 R4V6	5	2	6	5
3309C 05 R1V3	4	1	4	4
CAB 07 R2V12	4	3	3	4
Salt Creek 08 R2V3	3	0	4	3
TS PIA-9 (transgenic)	6	1	2	3
TS 02A C1V21	2	2	2	2
110R R3V3	1	0	1	1
1103 A6V2	1	1	1	0
101-14 A5V5	1	0	1	1
Total	45	17	43	41

Table 2. Number (percentage) of anther clusters forming putative embryogenic callus or embryos.

Genotype	PIV	MSE	MSI	NB
3309C (05)	1/196 (0.5)	0/196 (0)	0/196 (0)	0/196 (0)
Freedom (01)	0/294 (0)	0/49 (0)	0/147 (0)	0/245 (0)
Richter 110 (01)	0/49 (0)		0/49 (0)	2/49 (4)
MGT 420A (04)	1/147 (0.7)	1/98 (1.0)	5/196 (2.5)	1/196 (0.5)
140Ru (01)	0/49 (0)		0/49 (0)	0/49 (0)
Salt Creek (08)	5/196 (2.5)		4/147 (2.7)	1/147 (0.7)
11-03	0/49 (0)	1/49 (2.0)		8/49 (16)
101-14	0/49 (0)		0/49 (0)	0/49 (0)
Cabernet Sauvignon (07)	1/98 (1.0)	4/147 (2.7)	1/147 (0.7)	1/196 (0.5)
Cabernet Sauvignon (08)	5/539 (0.9)			
Pinot Noir (2A)	4/196 (2.0)	0/49 (0)	0/96 (0)	6/147 (4.0)
Zinfandel (01A)	2/147 (1.7)	0/49 (0)	11/196 (5.6)	0/196 (0)
Colombard (04)	7/172 (4.1)	0/49 (0)	16/123 (13.0)	2/123 (1.6)

Objective 3. Establish a germplasm bank of somatic embryos for seven rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.

We have established a germplasm bank of somatic embryos on agar-solidified woody plant media (WPM) supplemented with 20 g/liter sucrose, 1 g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 5% sorbitol, and 14 g/l phytoagar (BN-sorb) for Richter 110, 1103, 101-14, and Chardonnay. These cultures will provide a reliable source of embryos for use in transformation studies. Once rapidly dividing embryogenic suspension cultures are initiated, we will establish a germplasm bank of stored somatic embryos for rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard by plating aliquots of suspension cultures on sorbitol containing medium.

Objective 4. Test transformation efficiencies of seven rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.

Transformation experiments have been initiated using known amounts for somatic embryos as determined by fresh weight for Richter 110, 1103, 101-14, and Chardonnay using a construct containing the DsRed florescent scorable marker gene which will allow us to monitor the progress of transformation in real time without sacrificing any tissue (**Table 3**). Thompson Seedless is being included as a positive control. DsRed expression is being evaluated at 1, 2, and 3 months post-inoculation. Once germplasm banks of somatic embryos are

established we will begin testing our transformation system on somatic embryos of rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard.

Table 3. Transformation experiments to access the amenability of transformation of stored grape embryos for a range of rootstock and scion genotypes using the scorable fluorescent marker gene DsRed.

Genotype	Date	Experiment #	Tissue Weight	Percentage of DsRed embryos	Photos of DsRed Expression
TS-14 (Control)	6/26/2015	159050	0.53	10%	
	7/24/2015	159070	0.52	20%	
	8/26/2015	159096	0.92	20%	
Chardonnay	6/26/2015	159048	2.72	0%	
	7/10/2015	159064	1.12	0%	
	7/17/2015	159068	1.12	<1%	
	7/24/2015	159071	0.57	0%	
Richter	7/10/2015	159065	1.65	25%	
	7/17/2015	159069	1.83	25%	
	7/24/2015	159072	0.42	30%	
	8/26/2015	159095	0.89	5%	
1103	7/24/2015	159073	1.11	10%	
	8/26/2015	159093	1.09	10%	
101-14	7/24/2015	159074	0.86	10%	
	8/26/2015	159094	0.97	10%	

Objective 5. Test direct cell suspension transformation technology on seven rootstock genotypes and six scion genotypes.

We are trying to leverage the progress we have made in developing high quality cell suspensions that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by directly transforming our grape cell suspension cultures with the scorable marker gene DsRed. One to two ml of a grape cell suspension grown in liquid Pic/MT medium and containing pre-embryogenic masses or small globular embryos are collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1000 x G for three minutes. The cells are subjected to heat shock by placing the conical tube in a 45 degree water bath for five minutes. After heat shock the supernatant is removed and replaced with five ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain and appropriate vector at an OD 600 of 01.-0.2. The suspension is centrifuged at 1000 x G for five minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes all but 0.5 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred to sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid was carefully blotted up with a second sterile filter paper. The plates are co-cultured for two to three days at 23 degrees and then transferred to selection medium consisting of WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated

charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 50 g/ sorbitol, and 14 g/l agar. The filter paper is transferred to fresh medium every two weeks. Within eight weeks resistant embryos develop. Developing embryos are transferred to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 0 g/ sorbitol, and eight g/l agar for germination. We are currently testing this protocol on Richter 110, 1103, 101-14, and Chardonnay and using the DsRed transgene. We have observed highly variable transformation frequencies from experiment to experiment. Some experiments result in very high numbers of resistant embryos while other experiments fail to produce any embryos. The more critical issue is that transgenic embryos that do form, although normal in appearance, have been very recalcitrant to regenerate into whole plants. A few embryos develop into seedlings while the majority fail to germinate (**Figure 1**). We will continue to test additional genotypes using this system. However, if this transformation method is to serve as a viable alternative to the transformation of stored embryos, we need to achieve more consistent transformation frequencies of suspension cultures and more consistent regeneration of whole plants from the transgenic embryos. A summary of the experiments and the transformation frequency is given in **Table 4**. Once embryogenic suspension cultures are initiated for 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard, we will test direct transformation of suspension cultures using the scorable marker gene DsRed.

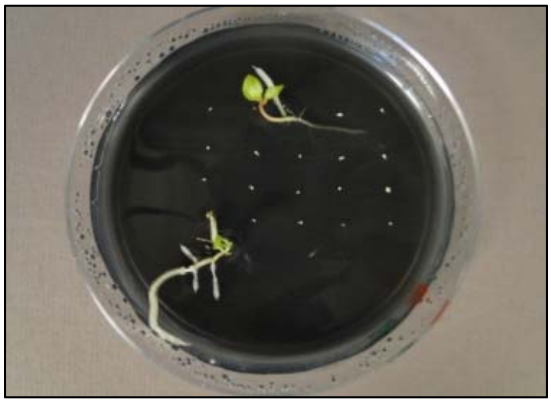

Figure 1. Germinating embryos from transformation of cell suspension cultures of 101-14 on WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA 50 g/l sorbitol, and 14 g/l agar and transfer to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and eight g/l agar for plant regeneration. Only two of the twenty-one putatively transformed embryos on this plate germinated after transfer to medium lacking sorbitol.

Table 4. Number of embryogenic colonies forming after inoculating approximately 1-2 ml of cell suspension with *Agrobacterium* and plating onto selection medium.

Genotype	Number of Experiments	Number of putative transgenic embryos/ml of plated suspension	Number of putative transgenic plants produced
101-14	17	54	2
1103	20	30	2
Richter 110	5	1	0
Chardonnay	2	0	0
Thompson Seedless	6	0	0

Objective 6. Establish *in vitro* shoot cultures for seven rootstock genotypes and six scion genotypes using material from Foundation Plant Services and establish bulk meristem cultures for all 13 genotypes for use in transformation.

We are maintaining disease-free *in vitro* stock plants of 101-14, Chardonnay, and Cabernet Sauvignon that we received as *in vitro* cultures from Foundation Plant Services (FPS). For material that was not available through FPS we have collected shoot tips from field material grown at FPS. This includes genotypes 3309C (05), Freedom

(01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04). Four-inch shoot tips were collected, cut into three-inch sections, transferred to 50 ml centrifuge tubes, and surface sterilized in 0.526% sodium hypochlorite for 15 minutes, followed by three rinses in sterile distilled water. The shoot tip was cut into nodal sections and any tissue damaged by sterilization was removed. The nodal sections were transferred onto agar-solidified Chee and Poole C2d *Vitis* medium containing 5 mg/l chlorophenol red or agar-solidified MS minimal organics medium supplemented with 1.0 mg/l BAP, 0.1 mg/l IBA, 0.1 mg/l GA3, and five mg/l chlorophenol red (**Figure 2**). The addition of chlorophenol red to the medium allows us to identify any contaminated shoots before the bacteria or fungus is visible based on the pH change of the medium. Once aseptic shoot cultures are established, we will transfer shoot meristems to medium with increasing levels of BAP in order to establish bulk meristem cultures as described by Mezzetti et al., 2002.

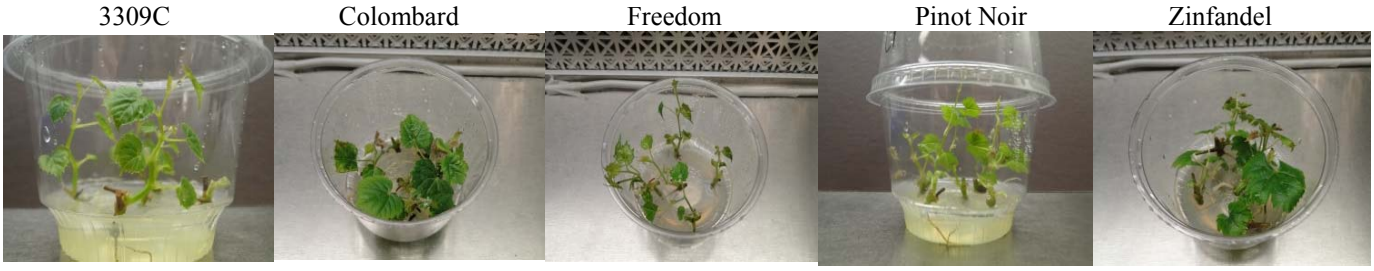

Figure 2. Established *in vitro* shoot cultures for use in generating bulk meristem cultures.

We have generated the bulk meristems 11-03, 101-14, Chardonnay, and Cabernet Sauvignon. To generate bulk meristem cultures, shoot tips are excised and transferred to Mezzetti salts modified with 1.0 mg/l BAP. After four weeks tissue was transferred to Mezzetti medium with 2.0 mg/l BAP and finally after an additional four weeks tissue was transferred to Mezzetti medium with 3.0 mg/l BAP. Bulk meristems developed readily for Thompson Seedless, Chardonnay, and Cabernet Sauvignon. We initially had established bulk meristem cultures for 1103 and 101-14, but these do not appear to be sustainable over long periods of time on the medium we are using. Repeated sub-culturing of this tissue on three mg/l BAP resulted in rapid callus growth. We also tried substituting Tridiazuron (TDZ) for BAP at three mg/l but this did not solve the re-callusing issue. Using the established *in vitro* shoot cultures of genotypes 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), and Zinfandel (clone 01A) we are excising small shoot tips and cultured them on Mezzetti salts modified with 1.0 mg/l BAP and have recently transferred them to the second step in the process: Mezzetti medium containing 2.0 mg/l BAP in order to generate bulk meristem cultures for use in testing the bulk meristem transformation system (**Figure 3**).

Figure 3. Initiation of cultures to establishment bulk meristem cultures.

Objective 7. Test Mezzetti et al., 2002 bulk meristem transformation system for seven rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

Bulk meristems of Thompson Seedless, Chardonnay, and Cabernet Sauvignon were sliced into thin, two mm slices and inoculated with *Agrobacterium* strain EHA105 and co-cultures on Mezzetti medium supplemented with three mg/l BAP at 23°C. After three days, the thin slices were transferred to Mezzetti medium supplemented with three mg/l BAP, 400 mg/l carbenicillin, 150 mg/l timentin, and 25 mg/l kanamycin sulfate. After three weeks tissue was transferred to the same medium formulation, but the kanamycin level was increased to 50 mg/l. After an additional three weeks the tissue was transferred to medium of the same formulation but the kanamycin level

was increased to 75 mg/liter. Subsequently, tissue was subcultured every three weeks on medium containing 75 mg/l kanamycin. Since the construct used to transform the bulk meristems contained the DsRed gene, we were able to monitor transformation efficiencies in real time. To date, we have only been successful producing transgenic shoots from bulk meristems of Thompson Seedless. Twenty-four of the 75 thin-slice sections of Thompson Seedless produced DsRed sectors and three of these regenerated into shoots. We were able to produce DsRed-expressing callus on Cabernet Sauvignon and Chardonnay, but none of this tissue regenerated into shoots (**Table 5**). In our hands the use of kanamycin at 75mg/l appears to be suboptimal for selection. Although we did identify a limited number of DsRed shoots for Thompson Seedless, many additional shoots which developed on selection medium containing 75 mg/l kanamycin were non-transgenic based on DsRed expression. If it were not for the use of the scorable marker DsRed, we would not be able to distinguish the true transgenic shoots from the non-transgenic escape shoots until they were transferred to rooting medium with kanamycin. We will consider using higher levels of kanamycin in the future as we test additional genotypes using this transformation methodology.

Table 5. Preliminary results of bulk meristem transformation using the scorable marker gene DsRed.

Genotype	Number (%) explants generated DsRed callus	Number (%) explants generated DsRed shoots
Cabernet Sauvignon	1/36 (3)	0/36 (0)
Chardonnay	2/38 (5)	0/38 (0)
Thompson Seedless	24/75 (32)	3/75 (4)

A summary of our progress to date for rootstock and scion genotype is listed in **Table 6**.

Table 6. Summary table providing the progress for each objective for each of the grape rootstock and scion genotype.

	Establishment of somatic embryos from anther filaments	**Establish-ment of suspension cultures**	**Establishment of a germplasm bank of somatic embryos**	**Establish-ment of shoot tip cultures**	**Establish-ment of bulk-meristem cultures**
Genotype					
1103	+	+	+	+	+
101-14	+	+	+	+	callus
110 Richter	+	+	+	+	initiated
140Ru	+	+	-	+	initiated
3309C	+	-	-	+	initiated
420A	+	+	-	-	initiated
Freedom	-	-	-	+	initiated
Harmony	-	-	-	-	-
Salt Creek.	+	-	-	+	initiated
Cabernet Sauvignon 07	+	-	-	+	+
Chardonnay 04	+	+	+	+	+
Colombard 04	+	+	-	+	initiated
Merlot 03	-	-	-	-	-
Pinot Noir 02A	+	-	-	+	initiated
Zinfandel 01	+	-	-	+	initiated

CONCLUSIONS

We established embryogenic cultures of 140Ru, 420A, 1103, 101-14, Cabernet Sauvignon, Chardonnay, Colombard 04, and Richter 110 from anther explants and initiated embryogenic suspension cultures. We have established a germplasm bank of somatic embryos for 1103, 101-14, Chardonnay, Colombard 04, and Richter 110

by plating suspensions onto high osmotic agar-solidified medium on a weekly basis. Suspension cultures of 140Ru and 420A are not yet growing quickly enough to start plating them on agar-solidified medium. We have also initiated exploratory transformation experiments to test the utility of our transformation protocol on 1103, 101-14, Chardonnay, and Richter 110. As embryogenic suspension cultures become established, we are continuing to test direct cell suspension transformation. We have established *in vitro* shoot cultures for genotypes 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04) and have excised shoot tips and begun generating bulk meristem cultures for use in transformation experiments.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT

We continue to make rapid progress breeding Pierce's disease resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. To further expedite breeding progress we are using marker-assisted selection (MAS) for the Pierce's disease resistance gene, *PdR1* (see companion report) to select resistant progeny as soon as seeds germinate. These two practices have greatly accelerated the breeding program and allowed us to produce four backcross generations with elite *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to fruit and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and other test sites. The best of these will be advanced to 100-vine commercial wine testing. the first of which was planted in Napa in June 2013. We advanced two additional selections to Foundation Plant Services (FPS) this winter to begin the certification and release process. Three Pierce's disease resistant rootstocks were previously advanced to FPS for certification. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Stacking of *PdR1b* with b42-26 Pierce's disease resistance has been advanced to the 92% *V. vinifera* level using MAS to confirm the presence of *PdR1b* and greenhouse screening to verify higher than usual levels of Pierce's disease resistance. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued but progress is limited by their multigenic resistance and the absence of corresponding genetic markers. Very small scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings in Sacramento (California Association of Winegrape Growers; CAWG) and Santa Rosa (Sonoma Winegrape Commission), Napa Valley (Napa Valley Grape Growers and Winemakers Associations), Temecula (Temecula Valley Winegrape Growers and Vintners), and Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers).

LAYPERSON SUMMARY

One of the most reliable and sustainable solutions to plant pathogen problems is to create resistant plants. We use a traditional plant breeding technique called backcrossing to bring Pierce's disease resistance from wild grape species into a diverse selection of elite winegrape backgrounds. We identified the genomic region that carries a very strong source of Pierce's disease resistance from a grape species native to Mexico and the southwestern United States (*Vitis arizonica*). Because we were able to locate this resistance gene/region - *PdR1* (Krivanek et al., 2006), we have been able to use marker-assisted selection (MAS) for markers associated with *PdR1* allowing us to select resistant progeny shortly after seeds germinate. MAS and aggressive growing of the selected seedling vines have allowed us to produce new Pierce's disease resistant high quality winegrape selections that are more than 97% *V. vinifera* in only 10 years. We have evaluated thousands of resistant seedlings for horticultural traits and fruit quality. The best of these are advanced to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at Pierce's disease hot spots around California. The best of these are advanced to 100-vine plots for commercial wine testing. We have sent 15 advanced selections to Foundation Plant Services (FPS) over the past three winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Other wild grape species are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable Pierce's disease resistance. Very small-scale wines made from our advanced *PdR1* selections have been very good, and have been received well at professional tastings throughout California.

INTRODUCTION

The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xylella fastidiosa* resistance (Buzkan et al., 2003; Buzkan et al., 2005; Krivanek et al., 2005a 2005b; Krivanek and Walker, 2005; Baumgartel, 2009), and having unique and highly resistant *Vitis rupestris* x *V. arizonica* selections, as well as an extensive collection of southwestern grape species, which allows the introduction of extremely high levels of *X. fastidiosa* resistance into commercial grapes. We genetically mapped and identified what seems to be a single dominant gene for *X. fastidiosa* resistance in *V. arizonica*/*candicans* b43-17and named it *PdR1*. This resistance has been backcrossed through four generations to elite *V. vinifera* cultivars (BC4) and we now have 97% *V. vinifera* Pierce's disease resistant material to select from. Individuals with the best fruit and vine characteristics are then tested for resistance to *X. fastidiosa* under our greenhouse screen. Only those with the highest levels of resistance are advanced to small-scale winemaking trials by grafting them onto resistant rootstocks and planting six to eight vine sets on commercial spacing and trellising at Pierce's disease hot spots around California, where they continue to thrive. We have made wine from vines that are 94% *V. vinifera* level from the same resistance background for seven years and from the 97% *V. vinifera* level for four years. They have been very good and don't have typical hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in red wines from the 87% *V. vinifera* level.

There are two forms of *PdR1* that descend from sibling progeny of b43-17 and they have different alleles of *PdR1,* designated *PdR1a* and *PdR1b*. Screening results reported previously showed no significant difference in resistance levels in genotypes with either one or both alleles. We have narrowed our focus to *PdR1b* but retain a number of selections at various backcross (BC) levels with *PdR1a* in the event that there is an as yet unknown *X. fastidiosa* strain-related resistance associated with the *PdR1* alleles. We also identified a Pierce's disease resistance locus *PdR1c* from *V. arizonica* b40-14 (*PdR1c*) that maps to the same region of LG14 as *PdR1* from b43-17. In the absence of an understanding of gene function and given the very disparate origins of the b43-17 and b40-14 resistance sources, differences in preliminary DNA sequence data between them, and differences in their Pierce's disease symptom expressions, we have continued to advance the *PdR1c* line as a future breeding resource. Our companion research project is pursuing the genetic basis of these differences between *PdR1b* and *PdR1c*. Resistance from southeastern United States (SEUS) species is being advanced in other lines. However, the resistance in these latter lines is complex (controlled by multiple genes) and markers have not yet been developed to expedite breeding. The breeding effort with alternative resistance sources and the complexing of these resistances is being done to broaden *X. fastidiosa* resistance and address *X. fastidiosa*'s potential to overcome resistance.

OBJECTIVES

1. Identify additional unique sources of *X. fastidiosa* resistance; develop breeding populations and phenotype them with our greenhouse screen to characterize their inheritance of resistance.
2. Develop ~97% *V. vinifera*-based Pierce's disease resistant lines of winegrapes utilizing diverse sources of resistance to *X. fastidiosa*, and conduct fruit and wine evaluations.
3. Utilize marker-assisted selection (MAS) to allow stacking of resistance loci, screen for resistant genotypes, and develop backcross generations by crossing resistant selections to elite *V. vinifera* varieties in order to produce high quality and Pierce's disease resistant winegrapes.
4. Develop and maintain new and existing genetic mapping populations to assist companion mapping / genetics project; begin the mapping of fruit quality traits such as color, tannin content, flavor, and productivity in Pierce's disease resistant backgrounds.

RESULTS AND DISCUSSION

To date over 293 wild accessions have been tested for Pierce's disease resistance with the greenhouse screen, mostly from the southwestern United States and Mexico (SWUS). Our goal is to identify accessions with the most unique Pierce's disease resistance mechanisms. To do so we compare genetic markers from chromosome 14 (where *PdR1* resides) to ensure that we are choosing diverse resistance sources for population development and greenhouse screening efforts. Over the last three years 15 of the most unique accessions were used to develop F1 populations with *V. vinifera* to investigate the inheritance of Pierce's disease resistance in their F1 progeny and the degree of their *X. fastidiosa* resistance. Given this extensive collection of F1 populations, with the exception of crosses to *V. caribaea* in 2015, we suspended the development of additional F1 populations following the 2014 season to focus more effort on the populations for map development. **Table 1** below shows the greenhouse screen results of our 2014 crosses.

Table 1. Greenhouse screening results from 2014 F1 crosses using southwestern sources of Pierce's disease resistance.

R Source	t-test	Cross	Mean No. *Xf* cells/ml	Log Values	Std. Err Mean	# Reps	Progeny Frequency Distribution
A14	a	A14 x French Colombard	31,869	10.4	0.1	29	Not Normal skewed R
SAZ7	a	F2-35 x SAZ7	36,330	10.5	0.1	52	Not Normal ~bimodal?
ANU71	b	ANU71 x Grenache blanc	74,191	11.2	0.1	30	Normal
A28	b	Rosa Minna x A28	89,720	11.4	0.1	42	Normal
C23-94	c	C23-94 x Nero d'Avola	112,050	11.6	0.1	44	Normal skewed S
DVIT2236.2	cd	F2-35 x DVIT2236.2	150,257	11.9	0.1	30	Normal
ANU67	d	F2-35 x ANU67	181,770	12.1	0.1	30	Normal

For our next mapping effort we have selected b46-43, a glabrous *V. arizonica* with some *V. monticola* characteristics from Big Bend, TX, which has the strongest resistance to *X. fastidiosa* of any species accession yet tested. All its F1 progeny were resistant by both enzyme-linked immunosorbent assay (ELISA) and lack of symptom expression. Since the progeny did vary in their resistance, we could not generate a map from the F1 population. In 2014 we made, and in 2015 we expanded, a backcross (BC) 1 mapping population to explore resistance from b46-43 and greenhouse testing of this population is underway (**Table 3 e, h**). We continue to develop small BC1 and higher BC-level breeding populations using the most highly resistant individuals chosen from various F1 populations. In addition to our initial b40-14, b42-26, and Haines City (*V. shuttleworthii*) lines, additional lines are being advanced using southwestern *Vitis* accessions ANU5, b40-29, and SC36, the latter in crosses made in 2015.

Our major breeding efforts from 2015 are summarized in **Table 2**. Our two main Pierce's disease breeding objectives in 2015 were to advance stacked *PdR1b*/b42-26 lines and stack Pierce's disease resistance with one or more powdery mildew resistance sources. Pierce's disease resistant cultivars with resistance to powdery mildew would greatly enhance the desirability of new winegrape varieties. Note that all resulting progeny in **Table 2** are above the 90% *V. vinifera* level, have an elite *V. vinifera* cultivar in their backgrounds, and advance the goal of creating highly resistant breeding lines stacked with multiple resistances. We can then cross these individuals one last time to a final elite *V. vinifera* cultivar, resulting in progeny between 96-98% *V. vinifera*. This year we took advantage of crosses made in recent years that had resulted in breeding parents with homozygous resistance at one or more resistance locus and that we had previously stacked the *PdR1b* and b42-26 Pierce's disease resistance sources. Since all progeny of homozygous crosses will carry that resistance source, we only have to screen for the integrity of the cross and any other resistance sources heterozygous in the resistant-parent. Consequently, more progeny will pass through MAS. The powdery mildew resistance sources in *Table 2* include *Ren1* found on chromosome 13 in a number of pure *V. vinifera* cultivars from Central Asia; *Ren4,* a strong and unique powdery mildew resistance locus on chromosome 18 originally discovered in the Chinese species *V. romanetii*, and *Run1,* a strong source on chromosome 12 from *Muscadinia rotundifolia*.

This spring we made F1 crosses using four *V. caribaea* selections to explore this resistance source from Costa Rica, a novel region with high Pierce's disease pressure. Crosses of the only male to *V. vinifera* produced 106 seeds, and an estimated 300 additional seeds are expected from crosses of elite *V. vinifera* wine cultivars onto the three exceptionally late ripening females. We made a cross to expand the b46-43 BC1 mapping population (F2-35 x 12305-55) that produced 896 seeds, and will allow us to expand this mapping population from our current 177 genotypes to the targeted 400 individuals. Other crosses made to advance the SC36 line to the BC1 level yielded 467 seeds. Our greenhouse screening of a 2011 BC1 cross searching for minor Pierce's disease genes in the b43-17 background absent *PdR1* revealed a single BC1 progeny that was highly resistant to Pierce's disease. A BC2 cross made this year produced 70 seeds. These will be screened in the greenhouse next year to study the inheritance of minor genes for Pierce's disease resistance.

Table 2. Number of seeds produced from Pierce's disease crosses made in 2015. *PdR1b* (F8909-08) is from Monterrey, Mexico *V. arizonica/candicans* Pierce's disease resistance b43-17; b42-26 is a Loreto, Baja California *V. arizonica/girdiana* Pierce's disease resistance source. *Ren1, Ren4* and *Run1* are powdery mildew resistance loci from *V. vinifera, V. romanetti,* and *M. rotundifolia* respectively. RR indicates one of the parents is homozygeous at the referenced resistance locus.

Resistances	Recent *V. vinifera* parents in background	Percent *V. vinifera*				No. Seeds
		92%	**93%**	**95%**	**96%**	
2a. PD - RR at *PdR1b* with b42-26	Airen, Sauvignonasse, Marsanne, Valdiguie		1,243			1,243
2b. PD - RR at *PdR1b* with b42-26. PM - *Ren4*	Alicante, Colombard, F2-35, Zinfandel			1,674		1,674
2c. PD - *PdR1b* with b42-26. PM - *Ren4* with either *Ren1* or *Run1*	Cab Sauvignon, Carignane, Grenache, Petite Sirah, Zinfandel	210		1,939		2,149
2d. PD - RR at *PdR1b*. PM -RR at *Ren4*	Airen, Cab Sauvignon, Carignane, Grenache blanc, Touriga nacional, Valdiguie				1,658	1,658
2e. PD - RR at *PdR1b*. PM - RR at *Ren4* with either *Ren1* or *Run1*	Cab Sauvignon, Carignane, Grenache, Petite Sirah, Zinfandel		1,389		4,549	5,938

Table 3 provides a list of the Pierce's disease resistance greenhouse screens analyzed, initiated, and/or completed over the last year. We are making every effort in new lines to bring minor genes along with those for which we have markers. In group A, we looked at 27 genotypes from intercrosses involving two southeastern United States resistance sources, BD5-117 and Haines City. Highly resistant progeny are completely missing from these lines at the BC2 level. In the same trial we also tested thirteen 97% *V. vinifera PdR1a* genotypes. In contrast to our *PdR1b* line, the *PdR1a* line lacks *V. rupestris* in its background. Perhaps for this reason or because of poor parental choices the recovery of highly resistant individuals was rare, with only one highly-resistant individual identified in this trial. We also tested 15 BC2 individuals in the b42-26 and none were promising. We completed testing of new southwestern United States species in group B to facilitate Pierce's disease resistance gene discovery work being done in our companion Pierce's disease mapping project. As noted previously, we have now tested almost 300 different accessions, the most resistant among them multiple times. Group C was tested to confirm previous results in the b40-14 line and to test multiple backcross levels in the same trial. Resistance was strong through the BC2 level but from a suboptimal choice of resistant parent the BC3 level only produced 20% promising progeny. Crosses to a more promising BC2 genotype were made in 2014 and 38 progeny were planted this past spring. Group C crosses also confirmed the resistance levels of the parents used for the 2014 crosses. Group D crosses continued testing of *PdR1b* selections at the 97-98% *V. vinifera* level. The special focus of these trials was on white-fruited selections and those that descend from Nero d'Avola. Results from these and earlier screens have helped us decide on the selection of the most resistant genotypes to advance to field trials and to Foundation Plant Services for certification. One promising white, a 97% *PdR1b* selection, was identified to advance to multi-vine trials. Although all five of the 2012 Dog Ridge-based rootstocks in D tested as statistically resistant, none were resistant enough to warrant advancement. At the 89% *V. vinifera* level, in the *PdR1b* x b42-26 PDR line stacked with powdery mildew resistance from *Ren4*, all eight were promising and three scored at the highest level of resistance. Similar results were seen at the 86% *V. vinifera* level. This gives us confidence that stacking these two resistance sources can be combined. The group E trial was extremely severe due to very high average temperatures and water stress caused by the belated detection of a failed cooling pump. We screened three different b46-43 BC1lines and two lines from b40-29. In the former, resistance segregated close to 1:1 R:S, which bodes well for the 14-399 b46-43 mapping population. Segregation ratios in the two b40-29 lines were closer to 1:3 or 2:3. All told, 13 promising individuals were identified from which to create the BC2 lines from these new resistance sources. Results of group F are presented in **Table 1** above. Nine promising individuals from three new lines were identified to advance additional new breeding lines. Group G tested remnants or retests promising genotypes from the various specified resistance sources. Group H examined additional progeny of the b42-26 BC1 intercross to confirm that none were as Pierce's disease resistant as the wild-type and bolster the idea that the two BC1s chosen fail to capture all the resistance loci in this multi-genic resistance source. It also included 24 genotypes of the same promising 98% *V. vinifera* level *PdR1b* x b42-26 PDR line stacked with powdery mildew (PM) resistance from *Ren4* first identified in Group D. We also examined the resistance profile of the b46-43 BC1 mapping population. Lastly, we explored the highest *V. vinifera* level *PdR1b* x b42-26 intercross by examining a 3 *PdR1b* x 3 b42-26 parental matrix comprised of 246 genotypes. Our goal is to identify extremely resistant

individuals that can either be selfed or intercrossed to create homozygous *PdR1b* parental material that can be backcrossed once to any elite *V. vinifera* wine type and produce progeny ~96% *V. vinifera,* all carrying the *PdR1b* allele and highly enriched in the additive b42-26 resistance source.

Table 3. Greenhouse Pierce's disease screens analyzed, completed, and/or initiated during the reporting period. Projected dates are in italics.

Group	Test Groups	No. of Genotypes	Inoculation Date	ELISA Sample Date	PD Resistance Source(s)
A	SEUS crosses, *PdR1a*, b42-26 BC2, SWUS Species	115	9/11/2014	12/11/2014	b40-14, Haines City, BD5-117
B	SWUS Species & Promising Genotypes	229	10/2/2014	12/18/2014	V. species, F8909-08, Ramsey
C	b40-14 F1, BC1, BC2; 2014 Cross Parents	170	9/23/2014	1/8/2015	F8909-08, b40-14, b42-26
D	*PdR1b*, 2012 PD RS, 2013 PD stacked & PD x PM stacked	117	3/17/2015	6/16/2015	F8909-08, b42-26
E	2014 Cross SWUS BC1 Seedlings	170	4/28/2015	7/28/2015	b46-43, b40-29
F	2014 Cross SWUS F1 Seedlings	265	6/4/2015	9/3/2015	A14, A28, ANU67, ANU71, C23-94, DVIT 2236.2, SAZ 7
G	b40-14, *PdR1a*, BD5-117xHC (HW)	128	8/25/2015	*11/24/2015*	b40-14, *PdR1a*, BD5-117xHC
H	b42-26^2 Inter,PdR1xb42-26xVRom Stack Promising, b46-43 BC1 map	170	9/17/2015	*12/17/2015*	*PdR1b*,b42-26
I	92% *PdR1b*xb42-26 Stack	277	*10/26/2015*	*1/28/2016*	*PdR1b*,b42-26

In our program we test selections with the potential for release multiple times in the greenhouse screen to ensure that only selections with the greatest levels of resistance are considered for release. These selections have much better resistance than two selections with long histories of field survival in the southern United States: Blanc du Bois and Lenoir (Jacquez). We want to avoid releasing selections that are tolerant to *X. fastidiosa* and therefore act as hosts for disease spread within a vineyard. This process involves passing our severe greenhouse screen multiple times. To make this list, selections must also possess desirable viticultural traits and have potential for high quality wine production. Producing small lot wines from multiple vine field trials in Davis and in Pierce's disease hot spots around California complete the evaluation process. Pierce's disease resistant scions need Pierce's disease resistant rootstocks in case low levels of the bacteria work their way into a susceptible rootstock. Three such rootstock selections were sent to FPS in spring 2013, another four from 2011 crosses are in their third round of screening, and 13 genotypes from 2012 crosses are in various stages of testing (**Table 3**).

Our first field trials were set out in California in 2001, with our first *PdR1b* lines being planted a few years later. Over that period, regardless of backcross level, our vines have continued to thrive while adjacent *V. vinifera* vines have succumbed to Pierce's disease. For many years we inoculated *PdR1* resistant selections at the Beringer / Yountville trial with no obvious detriment to the vines. We set up a trial in Temecula, CA with Ben Drake to confirm resistance in Northern California behaves the same way in Southern California. We inoculated the Temecula trial this year for the first time on September 22nd. This year and last we have added new trials and expanded some already existing (**Table 4**) and now have over 3,000 vines in the field in Pierce's disease hot spots around California.

Tables 5a through 5c detail the vine, fruit, and juice characteristics for the four 94% (those starting with 07) and eleven 97% (starting with 09 & 10) *V. vinifera PdR1b* selections used to make wine lots in 2015. In addition, we made a number of *V. vinifera* controls and Blanc du Bois and Lenoir as reference Pierce's disease resistant cultivars. All were made from Davis grown fruit.

Table 4. UC Davis field trials of advanced *PdR1* selections and selections from southeastern U.S. (SEUS) breeding programs to evaluate their resistance to Pierce's disease under California conditions.

Trial Name	Trial Location	% *V. vinifera*	No. Genotypes	Approximate No. Vines	Year(s) Planted or Budded
Beringer	Napa Valley, CA	SEUS, 50-94%	200	1,100	Various 2001-2013
Caymus	Napa Valley, CA	94-97%	2	1,500	2014, 2015
Drake	Temecula, CA	97%	8	200	2014
Mounts	Sonoma County, CA	97%	1 white	100	2010, 2015
Silverado Farming	Napa Valley, CA	97%	8	200	2014

Figure 1. The recent Caymus planting of our 94% *V. vinifera PdR1b* selection 07355-075 along the Napa River.

Table 5a. 94% (those starting with 07) and 97% (starting with 09 & 10) *V. vinifera* Pierce's disease resistant selections used in small scale winemaking in 2015: background and fruit characteristics.

Genotype	Parentage	2015 Bloom Date	2015 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1=v low, 9=v high
07355-044	U0505-01 x Petite Syrah	4/28/2015	8/11/2015	B	1.3	375	7
07355-075	U0505-01 x Petite Syrah	4/28/2015	8/11/2015	B	1.3	325	7
07370-078	F2-35 x U0502-38	4/28/2015	8/18/2015	W	1.0	151	7
07370-084	F2-35 x U0502-38	4/28/2015	8/6/2015	W	1.3	253	7
09311-160	07371-20 x Cabernet Sauvignon	5/10/2015	8/20/2015	B	1.0	185	5
09314-102	07370-028 x Cabernet Sauvignon	5/5/2015	8/13/2015	W	1.0	322	9
09330-07	07370-039 x Zinfandel	5/10/2015	8/25/2015	B	1.5	255	8
09331-047	07355-020 x Zinfandel	5/10/2015	8/20/2015	B	1.3	360	5
09331-133	07355-020 x Zinfandel	5/10/2015	8/25/2015	B	1.9	251	6
09333-111	07355-020 x Chardonnay	5/12/2015	8/18/2015	B	1.3	317	7
09333-358	07355-020 x Chardonnay	5/12/2015	8/18/2015	B	1.2	217	6
09333-370	07355-020 x Chardonnay	5/10/2015	8/25/2015	B	1.3	362	6
09338-016	07371-20 x Cabernet Sauvignon	5/12/2015	8/18/2015	W	0.8	283	6
09356-235	07371-19 x Sylvaner	5/5/2015	8/20/2015	B	1.2	236	7
10302-178	07370-028 x Riesling	5/5/2015	8/11/2015	W	0.9	133	4

Table 5b. Juice analyses of Pierce's disease resistant selections used in small-scale winemaking in 2015. Analysis courtesy of ETS Laboratories, St. Helena.

Genotype	°Brix	TA (g/L)	pH	L-malic acid (g/L)	potassium (mg/L)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
07355-044	26.1	7.1	3.43	1.5	2170	172	16	349	907
07355-075	25.4	6.3	3.41	1.5	1800	163	14	482	1640
07370-078	22.9	4.3	3.76	1.7	2000	168			
07370-084	26.9	4.8	3.88	2.6	2380	221			
09311-160	25.9	5.7	3.67	2.4	2500	193	38	381	835
09314-102	24.2	6.4	3.64	3.6	2160	223			
09330-07	24.3	5.7	3.62	1.9	2430	255	17	693	1840
09331-047	27.7	5.1	3.77	1.8	2530	235	8	421	1402
09331-133	26.0	4.5	3.71	1.3	2030	246	8	993	1212
09333-111	26.9	6.0	3.64	2.8	2310	261	15	524	946
09333-358	28.7	5.2	3.80	1.5	2860	231	147	1450	1123
09333-370	25.0	4.2	3.76	1.5	1990	225	17	721	1041
09338-016	21.3	5.7	3.50	1.3	1660	226			
09356-235	27.0	5.0	3.74	1.9	2380	189	31	407	1638
10302-178	24.3	5.1	3.62	1.1	1730	252			

Table 5c. Berry sensory analysis of Pierce's disease resistant selections used in small-scale winemaking in 2015.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity (1=low, 4=high)	Seed Color (1=gr, 4=br)	Seed Flavor	Seed Tannin Intensity (1=high, 4=low)
07355-044	pink-red	med	fresh, red fruit	plum, hay late	3	3	warm, spicy, not bitter	4
07355-075	red-orange	med	fruity, sl spice	fruity, plum,	3	4	smoky, warm	1
07370-078	green-brown	med	pear, melon	neutral, straw, vs veg?	2	4	nutty, sl chemical	3
07370-084	clear lt yellow	med	fruity, neutral, nectar	neutral, straw	1	4	warm, spicy, not bitter	3
09311-160	pink-red, sl orange	med-	plum, watermelon	fruity, berry	1	3	woody, nutty	4
09314-102	green-yellow	lt	pear, nectar, sl spice	neutral, vs fruit vs veg	1	4	buttery, sl woody	4
09330-07	red	lt-med	cherry, strawberry	neutral	1	4	woody, spicy	1
09331-047	red	med+	berry, spice, red plum	spicy, vs grass	2	3	hot, ashy, chalky, woody	2
09331-133	pink	lt	jam, plum	neutral, sl plum jam	3	4	woody, sl spice	3
09333-111	pink-orange	med	cherry, fruity	gr bell pepper, asparagus, plum	2	4	spicy, hot black pepper	2
09333-358	orange	lt	neutral, sl red fruit	fruit, plum	2	4	v hot, bitter, metallic	1
09333-370	pink-orange	lt	strawberry	fruity, sl hay	3	4	neutral	4
09338-016	green	lt	green apple	grass, veg, astringent	3	4	spicy, hot, sl bitter	2
09356-235	red	med	cherry, berry	black plum, spicy,	4	4	woody, bitter, metallic	1
10302-178	green-white	lt	spice, apple	neutral, straw, vs veg?	1	4	ashy, bitter	2

We continue to present our Pierce's disease resistant wines at the 94% and 97% *V. vinifera* levels to grower and vintner groups. Some of these tastings are at the University of California, Davis with industry and student tasters, and others are at various industry gatherings. In August 2014 we hosted about 30, including professional winemakers from Sonoma, Napa, and the Central Coast, and students and faculty. This tasting focused on our efforts with 25/75 blended wines with the 94% *V. vinifera* selections 07713-51 and 07355-75. They were well received. Tastings from the 2014 vintage began with a faculty student tasting in March 2015. Pierce's disease resistant wines were also presented to about 200 attendees at a Constellation Winery annual meeting in April. This tasting was followed by a tasting for Western Sonoma winemakers in July, and the California Alliance of Family Farms and Ramona Valley Winegrape Growers in August. In November 2015 our wines will be tasted at a meeting of Central Coast growers in Santa Maria and at the Napa Valley Grapegrowers "Rootstock" meeting. The wines have been very well received and we will prepare releases once we have final results from our repeated greenhouse tests to ensure only the most resistant selections are released to prevent them for serving as possible symptom-less hosts.

CONCLUSIONS

We continue to make rapid progress breeding Pierce's disease resistant winegrapes through aggressive vine training, marker-assisted selection, and our rapid greenhouse screen procedures. These practices have allowed us to produce four backcross generations with elite *V. vinifera* winegrape cultivars in 10 years. We have screened through thousands of seedlings that are 97% *V. vinifera* with the *PdR1* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *X. fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and in Pierce's disease hot spots around California. The best of these are advanced to 50- to 100-vine commercial scale testing, with the first selection planted in 2013. We have sent 16 advanced scion selections to FPS over the past three winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 is also being pursued, but progress and effort is limited because their resistance is controlled by multiple genes without effective resistance markers. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good, and have been received well at tastings in the campus winery and at public tastings throughout California.

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MOLECULAR BREEDING SUPPORT FOR THE DEVELOPMENT OF PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT
The main objective of this project is to identify novel Pierce's disease resistant germplasm, determine the inheritance of resistance, tag genomic regions, and develop markers capable of facilitating and accelerating the breeding of resistant winegrapes. We have completed greenhouse-based Pierce's disease resistance screening, genotyping [simple sequence repeats (SSR) and chloroplast markers], and population analysis of over 250 accessions and identified 20 new highly resistant accessions that were used to develop breeding populations in 2012, 2013, and 2014. Breeding populations were marker tested to assure correct identity. Resistance loci were identified on genetic maps, markers were developed for breeding, and physical mapping was completed for b43-17 to clone and characterize resistance genes (*PdR1a* and *PdR1b*; see earlier reports). The physical map of the *PdR1c* locus (from b40-14) is nearing completion and we are carrying out comparative sequence analysis. We have also initiated the genetic mapping of a new and very resistant source, b46-43, and marker screening is in process. We are continuously developing and expanding breeding populations from new promising resistant lines. Upstream and downstream sequences as well as gene sequences of two candidate genes ORF14 and ORF18 from *PdR1b* were verified. A large-scale multiple time point gene expression project was completed in the greenhouse and RNA extractions were completed for over 400 samples. The quantitative polymerase chain reaction (qPCR) experiments were used to test the expression of candidate genes. Cultures to generate embryogenic callus of *Vitis vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George are being maintained for use in transgenic experiments. Experiments to utilize the *PdR1* resistance gene with native promoters are underway. These efforts will help us to identify candidate resistance genes by complementation and better understand how they function. They could also lead to Pierce's disease resistance genes from grape that would be available to genetically engineer Pierce's disease resistance into *V. vinifera* cultivars. This project provides the genetic markers critical to the successful classical breeding of Pierce's disease resistant wine, table, and raisin grapes. Identification of markers for *PdR1* allowed us to reduce the seed-to-seed cycle to two years and produce selections that are Pierce's disease resistant and 97% *V. vinifera*.

LAYPERSON SUMMARY
We continue to identify and genetically characterize novel resistance sources from southwestern United States and Mexican *Vitis* species collections; use genome sequence information to identify unique resistance genes; clone and characterize these resistance genes with native promoters; and develop resistance gene constructs prior to transforming them into susceptible *V. vinifera* grapes to test their function. Creating genetic maps with DNA markers allows us to identify and validate markers that could be used for marker-assisted selection and to incorporate (stack) multiple resistance genes into a single background to create more durably resistant varieties. Genetic mapping allows us to carry out map-based positional cloning and characterization of grape Pierce's disease resistance genes under control of native promoters, which could be used to genetically engineer resistance into elite *V. vinifera* cultivars.

INTRODUCTION
A successful resistance-breeding program depends on germplasm to provide a wide genetic base for resistance. Identification, characterization, and manipulation of novel sources of resistance are prerequisites for breeding.

This evolved project continues to provide molecular support to the Pierce's disease resistance grape breeding project titled "Breeding Pierce's Disease Resistant Winegrapes" by acquiring and testing a wide range of resistant germplasm, tagging resistance regions with markers by genetic mapping, and then functionally characterizing the resistance genes from different backgrounds. In earlier versions of this project, genetic markers linked to *Xylella fastidiosa* resistance from b43-17 background were used to perform marker-assisted selection (MAS) to accelerate our Pierce's disease resistant winegrape breeding and the table and raisin grape breeding of David Ramming. Outcomes from the earlier two projects included genetic maps and bacterial artificial chromosome (BAC) libraries of the highly resistant *Vitis arizonica* accessions, b43-17 and b40-14. A physical map of the *PdR1* locus was completed and several candidate genes were identified. Five candidate genes were cloned and constructs were developed with 35S promoters to transform tobacco, Chardonnay, Thompson Seedless, and St George.

The new merged project has the following key objectives: identify novel sources of Pierce's disease resistance for use in broadening the genetic base of resistance; accelerate marker discovery and the identification of new and unique resistance genes; clone and characterize unique DNA sequences (promoters) that regulate the expression of candidate Pierce's disease resistance grape genes cloned from the *PdR1b* locus; and evaluate and compare lines transformed with native and 35S promoters. We have surveyed over 250 accessions of *Vitis* species growing in the southern United States and Mexico to identify new Pierce's disease resistant accessions. Analysis using population genetics methods allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Pierce's disease resistance in southeastern *Vitis* species seems to be different than the resistance in *Vitis* from the southwest and Mexico. We have identified new Pierce's disease resistant accessions that are genetically and phenotypically different, were collected from different geographic locations, and have different maternal inheritance. Breeding populations from new promising resistant lines were developed. These populations will be tested to study the inheritance of resistance. Then next generation sequencing will be used on the recently identified resistant accessions to expedite marker discovery and confirm that they are unique. Then genetic maps will be developed to identify genomic regions associated with resistance, and genetic markers will be used for the stacking of multiple resistance genes to breed winegrapes with durable Pierce's disease resistance.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/susceptibility in grape germplasm. In addition, these genes and their promoters can be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al., 2013). Alternatively, other well characterized *V. vinifera*-based promoters, either constitutive (Li et al., 2012) or activated by *X. fastidiosa* (Gilchrist et al., 2007) could be utilized. Development of *V. vinifera* plants transformed with grape genes and grape promoters might mitigate concerns about transgenic crops harboring genetic elements derived from different organisms that cannot be crossed by natural means. Proven resistance gene constructs could be transformed into a broad array of elite *V. vinifera* cultivars.

OBJECTIVES

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the Pierce's disease resistance-breeding program, including characterization of novel forms of resistance.
2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).
3. Employ whole genome (WG) sequencing (50X) of recently identified Pierce's disease resistant accessions and a susceptible reference accession, and use bioinformatics tools to identify resistance genes, perform comparative sequence analysis, and develop single nucleotide polymorphism (SNP) markers to be used for mapping.
4. Clone *PdR1* genes with native promoters.
5. Compare the Pierce's disease resistance of plants transformed with native vs. heterologous promoters.

RESULTS AND DISCUSSION

Objective 1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the Pierce's disease resistance breeding program, including characterization of novel forms of resistance.

Vitis species growing in Mexico and the southwestern United States have co-evolved with *X. fastidiosa* and developed natural resistance to the disease. We completed a survey of over 250 southwestern and northern Mexico *Vitis*, which included accessions collected from multiple collection trips from states bordering Mexico or

that were previously collected from Mexico. Both simple sequence repeat (SSR) and chloroplast markers were used to evaluate genetic diversity and establish relationships with known sources of resistance currently being used in the breeding program (Riaz and Walker, 2013). Some of these resistant forms may have different mechanisms of resistance that could add to the repertoire of resistance genes and loci available for the breeding program. A subset of this germplasm was greenhouse screened for Pierce's disease resistance and preliminary results identified multiple new sources of resistance. Crosses were made with five new Pierce's disease resistant *V. arizonica* accessions from the southwestern United States and Mexico to develop small breeding populations. A subset of seeds from these crosses was germinated and greenhouse screened to characterize the inheritance of their Pierce's disease resistance. Results indicated clear separation of progeny families into resistant, intermediate, and susceptible groups, and identified an unprecedented level of resistance in b46-43 based on disease phenotype and enzyme-linked immunosorbent assay (ELISA) results. More crosses were made in 2013 with five additional resistant accessions: b41-13, b43-57, b47-32, SC36, and T03-16.

We have developed F1 and backcross 1 (BC1) breeding populations using two of the resistant accessions, b46-43 and T03-16, that are geographically unique, have different maternal origin, and are genetically diverse. Greenhouse testing of the F1 population was completed and BC1 populations are under testing. Genomic DNA was isolated from 177 seedlings in the BC1 population with the b46-43 background and marker testing on a small set of seedlings and parental DNA is in process. Our objective is to employ a limited mapping strategy by focusing mapping on linked chromosomes identified from the sub-population screening and then saturate with SSR markers that reside on those chromosomes. The identification of other genomic resistance regions is critically important, since it is not genetically possible to stack more than two chromosome 14 resistance sources.

In spring 2015 we provided molecular support to the companion Pierce's disease resistance winegrape breeding project by marker testing a total of 1,237 seedlings from 17 crosses to determine Pierce's disease resistant and susceptible genotypes. Most of these crosses were designed to stack resistance from b42-26 and *PdR1b* as well as to develop advanced breeding lines with *PdR1c* (the b40-14 background).

Table 1 presents the breeding populations that were developed with new resistance sources (for details, see previous reports). In spring 2015, we completed propagation of 4-5 replicates for the subset of crosses mentioned in **Table 1**. Plants from rooted green cutting were transferred to two-inch pots first and then four-inch pots to acclimatize to greenhouse conditions. These plants were inoculated with *X. fastidiosa* at the end of August and the results of the assay will be available in December.

Table 1. Crosses that are under greenhouse testing to determine the mode of inheritance of their resistance to Pierce's disease.

Cross ID	Female Name	Male Name	Seedlings tested
14-360	F2-35	DVIT 2236.2 (*V. nesbittiana*)	90
14-367	F2-35	12340-13	50
14-321	Rosa Minna	12305-55	28
14-308	Rosa Minna	12305-55	19
14-364	Rosa Minna	A28	19
14-347	Rosa Minna	A28	23
14-322	Rosa Minna	12305-56	15
14-313	A14	Colombard	53
14-324	F2-35	12305-56	47
14-340	ANU71	Grenache blanc	38
14-303	C23-94	Nero d'Avola	64
14-362	F2-35	ANU67	31
14-363	F2-35	SAZ 7	52
14-368	F2-35	12340-14	35
14-336	F2-35	12305-83	14
Total			578

Objective 2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).

We have completed a genetic map and identified a major Pierce's disease resistance locus, *PdR1c*, on chromosome 14 from the *V. arizonica* b40-14 background (see previous reports for details). Pierce's disease resistance from b40-14 maps in the same region as *PdR1a* and *PdR1b* between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The allelic comparison of SSR markers within the 20cM region including the *PdR1c* locus revealed that the *PdR1c* locus is unique and sequences and genomic features are distinct from those in b43-17 (the sources or *PdR1a* and *PdR1b*). A total of 305 seedlings were also tested with markers to identify unique recombinants. We also developed new SSR markers using the b43-17 sequence generated in this study for comparative sequence analysis. Two of the SSR markers, SSR82-1b4 and ORF18-19-3, were tested on the combined set of recombinant plants (**Table 2**) to tighten the genetic window. We found four recombinants between Ch14-81 and VVIn64 on one side and one recombinant between the Ch14-77 and Ch14-27 markers. With the help of these markers we confined the *PdR1c* locus to 325 kb based on the sequence of b43-17.

Table 2. Positioning of genetic markers in relationship to Pierce's disease resistance in *V. arizonica* b40-14.

Genotype	14-29	14-27	VVCh14-77	SSR82-1b4	ELISA Results	ORF18 to 19-3	14-81	VVIn64	UDV025	VVIp26
09367-35	+	+	+	+	R	+	+	+	+	+
09367-37	+	+	+	+	R	+	+	+	+	+
09367-38	+	+	+	+	R	+	+	+	+	-
09367-41	-	-	-	-	S	-	-	-	+	
12325-78	+	+	+	+	R	+	+	+	-	-
12326-18	+	+	+	+	R	+	+	-	-	-
12327-54	-	-	-	-	S	-	-	+	+	+
09367-26	-	-	-	-	S	-	-	+	+	
09367-30	-	-	-	-	S	-	-	+	+	+
09367-07	-	-	+	+	R	+	+	+	+	+
09367-12	+	-	-	-	S	-	-	-	-	-
09367-40	+	+	+	+	R	+	+	+	+	+
09367-43	+	+	+	+	R	+	+	+	+	+

We developed a BAC library from b40-14 genomic DNA (see details in previous reports). BAC library screening was completed with probes that amplify a single amplicon of 600-650 base pairs (bp) using b40-14 genomic DNA. We identified 30 BAC clones by using two probes, Ch14-56 and Ch14-58. Six clones were positive with both probes. BAC clones that represent *PdR1c* were separated from the other haplotype based on the SSR markers that are polymorphic for the resistant selection b40-14. At the final stage we selected two BAC clones, VA29E9 and VA57F4, which overlap with each other and are ~ 200 kb in size. The selected BAC clones were cultured to generate a large amount of DNA, and purified DNA was sent to the University of California, Irvine Genomics High Throughput Facility for Pacific Bioscience (PacBio) RS II sequencing (see previous report).

In order to expand the region beyond probe 14-58, we selected a third BAC clone that was positive with probe 14-58 and 14-59, isolated and purified the DNA, and sent it for sequencing. The assembly of three BAC clones representing the *PdR1c* locus is presented in **Figure 1**. The assembly consisted of two contigs with no overlap. Common probes between the *PdR1c* and *PdR1b* region were used to compare and align the sequences of two backgrounds in order to determine the region that is missing in the assembly of *PdR1c* locus. Based on the comparative analysis using sequence of *PdR1b* locus, we estimate that the gap between two assembled contigs is ~50-60 kb in length. We are in process of identifying a fourth BAC that overlaps with the VA30F14 and VA57F4/VA29E9 assembly. We have designed new probes using the sequence of *PdR1c* region to test overlapping BACs. The current assembly contains a total of 363 kb of sequence, and a cluster of 18 resistance genes.

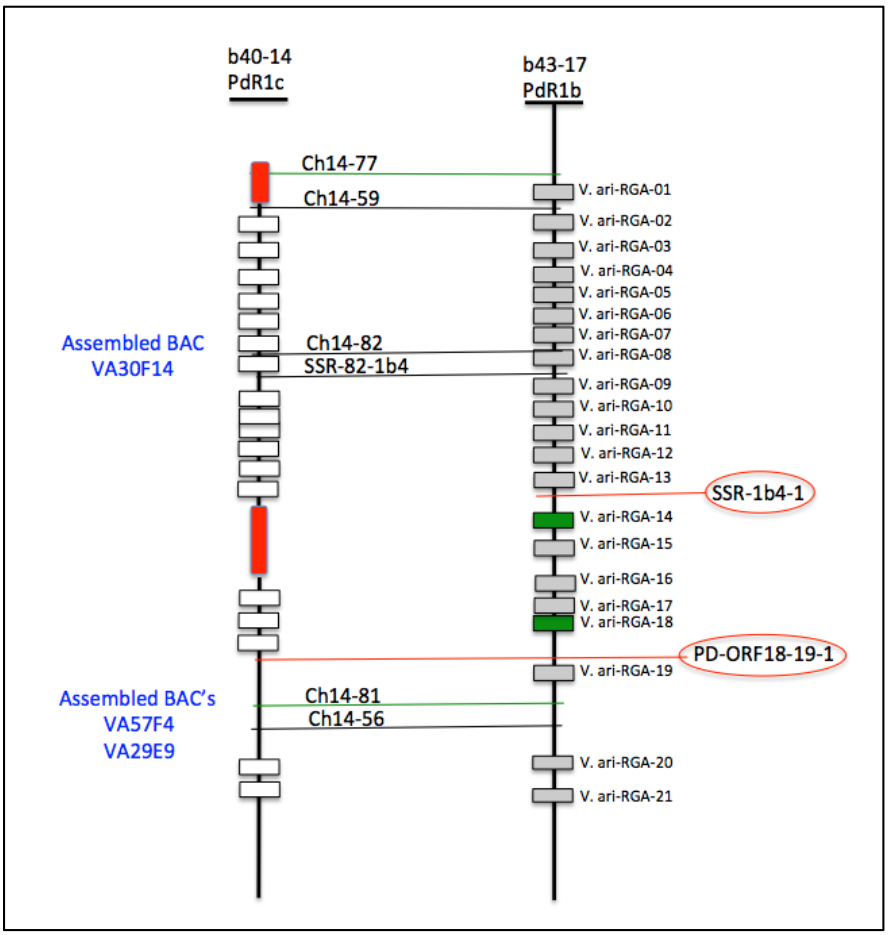

Figure 1. Sequence analysis of the *PdR1b* and *PdR1c* regions. The region between the two probes Ch14-59 and Ch14-56 was ~316 kb in PdR1b (b43-17). In *PdR1c*, the assembled BAC VA30F14 consisted of 171 kb with a cluster of 13 resistance genes. The assembled BACs VA57F4-VA29E9 consisted of 192 kb with five resistance genes. Two of the resistance genes are outside the genetic window with marker Ch14-81. The red regions represent the gaps in the assembly.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdR1a* haplotype (F8909-17) was also completed. The length of the assembled sequence was 206 kb. **Figure 2** provides details of the assembled region. The open reading frames (ORF) of the *PdR1b* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the over lapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20 and all five ORFs have identical sequences to the *PdR1b* haplotype (**Figure 2**). Based on these results, we conclude that there is complete sequence homology between haplotype *a* and *b* of the *PdR1* locus; therefore, cloning and functional characterization of genes from either haplotype will be sufficient for future work. Complete sequence homology also reflects that the parents of b43-17 must be closely related and may have a first-degree relationship and acquired resistance from shared parents. This also explains why we observed complete homozygosity of SSR markers for the *PdR1* locus in the resistant accession b43-17.

Objective 3. Employ whole genome sequencing (WGS) (50X) of recently identified Pierce's disease resistant accessions and a susceptible reference accession, and use bioinformatics tools to identify resistance genes, perform comparative sequence analysis, and develop SNP markers to be used for mapping.

The focus for WGS is on two new resistant accessions, b46-43 and T03-16. Both have been greenhouse tested to have very low bacterial levels in repeated screens. b46-43 is homozygous resistant to Pierce's disease. Crosses to develop BC1 populations were made in 2014. We have extracted DNA from the F1 population to marker verify the integrity of the cross. Our approach of traditional bi-parental mapping populations has played an important role in gene discovery and understanding of Pierce's disease resistance in North American *Vitis* species, and both bi-parental and multi-parental breeding populations remain the foundation of our breeding program. In this

project, we want to combine the traditional SSR marker system with next generation sequencing using IIlumina HiSeq and MiSeq platforms to carry out SNP discovery and identification of SNP markers linked to resistance (**Figure 1**). We will pursue the WGS approach only with those resistant lines for which we have strong greenhouse screen information, heritability of the Pierce's disease resistance, and potential screening of the population using the limited mapping strategy. The BC1 populations with b46-43 and T03-16 background are under testing and will be ready for WGS approach in winter 2015.

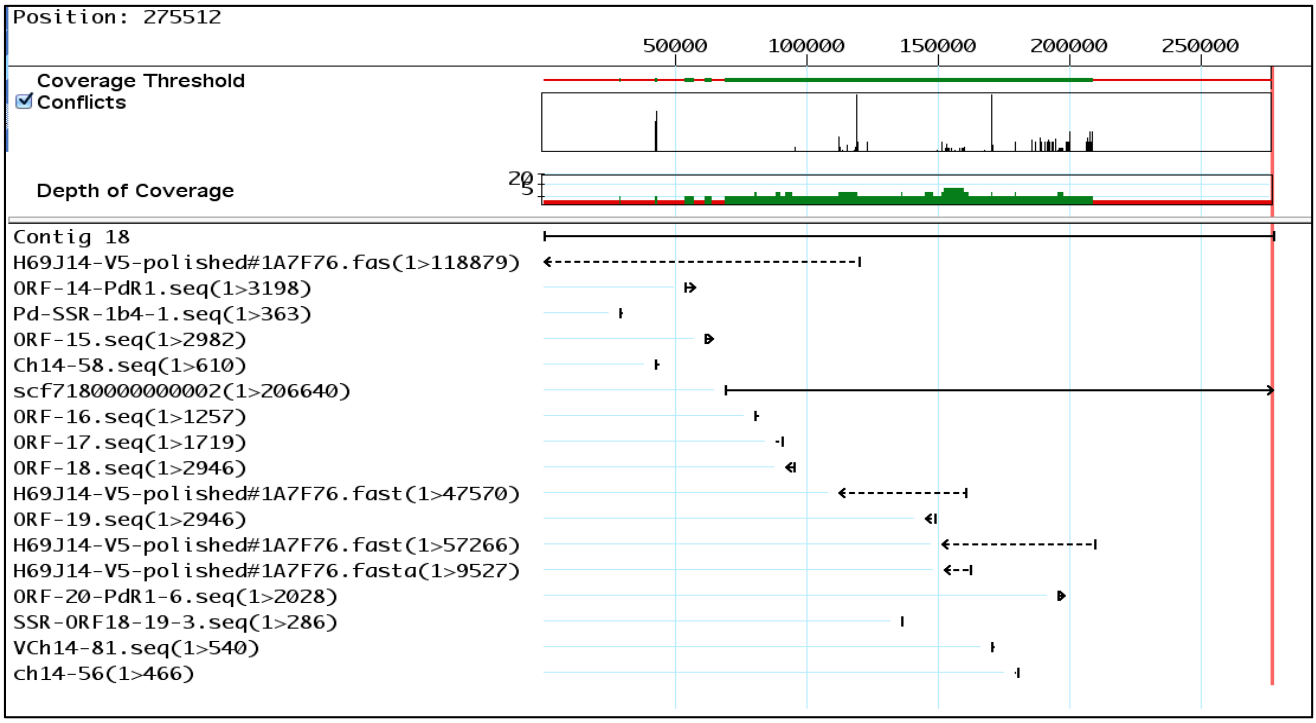

Figure 2. Assemblies of BAC clone reflecting the *PdR1a* haplotype.

Objective 4. Cloning of *PdR1* genes with native promoters.

We have completed assembly of a 604 kb region of *PdR1b* with four BAC clones (see previous reports for details). We identified multiple open reading frames (ORFs) of the Leucine-Rich Repeat Receptor Kinase gene family that regulates a wide variety of functions in plants including stem cell maintenance, hormone perception, and defense and wounding response for both host as well as non-host specific defense. With the help of molecular markers we have limited the genetic region that carries the five ORFs to 82 kb – these ORFs are associated with disease resistance and other plant functions described above (**Figure 1**). There are multiple ORFs that are outside this genetic region and have 99% sequence similarity to the candidate genes. We have also acquired binary vectors pCLB1301NH and pCLB2301NK (Feechan et al., 2013) that have been optimized to carry large DNA sequences, thus allowing us to insert candidate genes plus surrounding sequences. Two ORFs V.ari-RGA14 and V.ari-RGA18, within the resistance region boundaries, are the most likely candidates for *PdR1b*. The other three sequences, V.ari-RGA15, 16, and 17 are shorter and contain a large number of transposable elements (TE).

In order to include native promoters and terminators in constructs for future genetic transformations, we have verified sequences upstream and downstream of V.ari-RGA14 and 18, the two most likely *PdR1b* candidates. Sequence verification for V.ari-RGA14 has been completed up to 3.75 kb in the upstream region and 1 kb in the downstream. Both RGA14 and 18 are very similar in the sequence profile with the exception that RGA18 is 2946 bp in size and lacks the first 252 bp of sequence that is part of RGA14. Functional analysis of the protein sequence of both RGA revealed that RGA14 lacks a signal peptide in the initial part of the sequence. This was further verified by using 3' rapid amplification of cDNA ends (RACE) to specifically amplify RNA from grapevines transformed with V.ari-RGA14 under the 35s promoter. The results found that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence.

We have also initiated a large experiment with resistant and susceptible plants using multiple replicates, and time points for control (mock or uninoculated) and inoculated plants (see details in previous report). To date, we have completed RNA extractions from 450 samples in the above-mentioned experiment. We have also designed primers and determined primer efficiency for the gene expression studies for both RGA14 and RGA18. Two different primer pairs with an efficiency of greater than 90% were selected to carry out preliminary analysis with mock and inoculated samples of Chardonnay and F8909-17. Preliminary results with samples from six time points indicates that the expression level of both RGA14 and RGA18 in F8909-17 increases after day eight in comparison to mock inoculated, peaks at day 23, and then decreases. Mock and inoculated susceptible Chardonnay did not show any expression. Gene expression analysis will be carried out on complete data sets when we have processed all the RNA samples.

Objective 5. Comparing the Pierce's disease resistance of plants transformed with native vs. heterologous promoters.

We have established an *Agrobacterium*-mediated transformation system followed by regeneration of plants from embryogenic callus. We have streamlined the protocol to established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless (TS) and Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) (Agüero et al., 2006). In an earlier phase of this project, we have transformed these varieties with five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase (NOS) terminator, and an hptII-selectable marker gene (see previous reports for details). We completed testing five candidate genes, and the transgenic plants did not confer Pierce's disease resistance or tolerance. These results are in accordance with the latest assembly obtained using the PacBio SRII system and three additional overlapping BAC clones. They show that only one of the sequences tested, V.ari-RGA14, lays within the more refined resistance region of 82 kb defined by the two recombinants we recently obtained. The technique of 3'RACE was used to amplify RNA from V.ari-RGA14-transformed grapevines and results showed that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence.

In addition to embryogenic calli of Thompson Seedless (TS), Chardonnay (CH), and *Vitis rupestris* St. George (SG) available for transformation, we have developed meristematic bulks (MB) of these genotypes plus Cabernet Sauvignon and 101-14 Mgt for transformation via organogenesis (**Figure 3**). Slices of MB can regenerate transformed shoots in a shorter period of time than somatic embryos. We have tested different media and selective agents and established protocols for the initiation, maintenance, and genetic transformation of MB from these five genotypes (Xie et al., in preparation). MB induction in non-*V. vinifera* genotypes is less efficient but still high, with about 80% of the explants producing MB after three subcultures in medium containing increasing concentrations of cytokinins. For this reason, we have also started the production of MB of Pierce's disease susceptible genotypes selected from the 04191 population, which are 50% *V. vinifera*, 25% b43-17, and 25% *V. rupestris* A. de Serres (as in the original population used for *PdR1b* mapping). These genotypes can provide an additional genetic background for analysis of expression of *PdR1* candidate genes.

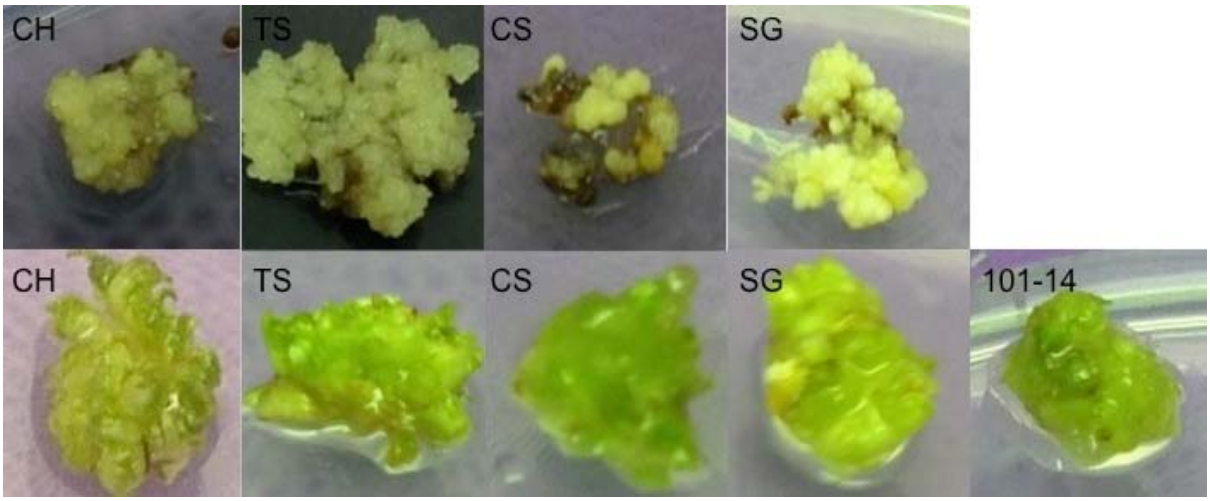

Figure 3. Embryogenic cultures (top) and meristematic bulks (bottom) of CH, TS, CS, SG, and 101-14.

In order to include native promoters and terminators in constructs for future genetic transformations, we have verified sequences upstream and downstream of V.ari-RGA14 and 18, the two strongest *PdR1b* candidates. Sequence verification has been completed up to 4-6 kb in the upstream region and 1 kb in the downstream region. *In silico* analysis of the upstream regions with PlantCare, a database of plant cis-acting regulatory elements, has shown that upstream sequences contain several motifs related to drought and defense responses.

Transformations with *Agrobacterium tumefaciens* carrying the binary plasmids pCLB1301NH and pCLB2301NK will be used to clone *PdR1b* candidate genes and their regulatory regions. These plasmids have been designed to overcome stability problems associated with the presence of large genomic fragments (Feechman et al., 2013). They carry the hygromycin (pCLB1301NH) and kanamycin (pCLB2301NK) selectable marker genes, respectively. Both plasmids also carry mGFP5-ER as a reporter gene. We have transformed MB of TS, CH, and SG with both plasmids to test the use of the hygromycin and kanamycin genes under the control of the nopaline synthase (NOS) promoter, in contrast with our previous results using the same genes under the control of the 35S promoter.

CONCLUSIONS

The genetic mapping with two new populations with b46-43 and T03-16 background is proceeding. These two accessions are geographically isolated from b43-17 and support the lowest levels of bacteria. The screening of F1 and BC1 populations with these two backgrounds in underway. Marker testing to identify polymorphic markers is initiated. The results from this work will allow us to use markers to facilitate stacking of these resistance sources with *PdR1* from b43-17. The incorporation of multiple resistance should make resistance more durable. We have completed the genetic mapping of Pierce's disease resistance from b40-14 and named it *PdR1c*. This resistance source maps within the *PdR1b* locus, and may be an alternative gene within this complex replicated locus. We are physically mapping this gene to improve our understanding of the locus. Finally, we have been sequencing the *PdR1* locus to better define the two candidate genes and prepare them for complementation tests. This effort is also identifying their promoters so that we can avoid the use of constitutive non-grape promoters like CaMV 35S.

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FUNDING AGENCIES

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STABLE PLASMID VECTORS FOR COMPLEMENTATION OF *XYLELLA FASTIDIOSA* MUTANTS *IN PLANTA*

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ABSTRACT

Current understanding of the mechanisms of Pierce's disease development has been significantly advanced by molecular genetic studies of the causal agent, *Xylella fastidiosa*. Plasmid vectors are an essential tool for studies of bacterial genetics and pathogenesis. However, most commonly used plasmids do not replicate in *X. fastidiosa*, limiting the options for complementation analysis or exogenous gene expression in this bacterium. Many of the plasmids that do replicate in *X. fastidiosa* are not stable without antibiotic selection, particularly after long periods of time. Two different plasmid vectors were created (pBBR5pemIK and pXf20pemIK) utilizing different replication origins for growth in *X. fastidiosa*. These vectors carry the PemI/PemK plasmid maintenance system for increased stability in the absence of antibiotic selection. PemK is a toxin which inhibits cell growth unless the antitoxin (PemI) is also present. Loss of the plasmid is prevented because of the need for continuous production of PemI to avoid toxic activity of PemK. Both pBBR5pemIK and pXf20pemIK are retained in *X. fastidiosa* after more than five consecutive subcultures *in vitro*, as well as after 14 weeks of growth *in planta*. Plasmid pXf20pemIK contains a high copy number pUC origin, in addition to *X. fastidiosa* replication elements, facilitating manipulation and propagation in *Escherichia coli* cloning hosts. Plasmid pBBR5pemIK is a medium-low copy number vector, but is able to replicate in a wider variety of bacterial species due to a broad host range backbone. These vectors provide a valuable tool for conducting genetic studies of *X. fastidiosa* virulence and have the potential to be used in other bacterial species as well, particularly in situations where antibiotic selection is impractical.

FUNDING AGENCIES

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WHOLE GENOME SEQUENCE ANALYSES OF *XYLELLA FASTIDIOSA* PIERCE'S DISEASE STRAINS FROM DIFFERENT GEOGRAPHICAL REGIONS

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ABSTRACT
Genome sequences were determined for two Pierce's disease-causing *Xylella fastidiosa* strains, one from Florida and one from Taiwan. The Florida strain was ATCC 35879, the type strain of the *X. fastidiosa* species used as a standard reference for related taxonomy research. By contrast, the Taiwan strain used was only recently characterized and therefore it is of importance for Pierce's disease epidemiological studies. Genome sequencing of the Taiwan Pierce's disease strain was the result of collaboration between the USDA Agricultural Research Service and National Chung Hsing University. Whole genome sequence comparison between the Taiwan strain and the strain from Florida, as well as with existing sequences from California and Texas, showed that the Taiwan Pierce's disease strain was highly similar to American Pierce's disease strains. This led in part to the classification of the Taiwan strain to *X. fastidiosa* subsp. *fastidiosa*. However, variations were found for this stain at various hypervariable loci such as those with small tandem repeats. While the biological nature of these variations remains unclear, these hypervariable loci could be used assist in strain differentiation.

FUNDING AGENCIES
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FUNCTIONAL CHARACTERIZATION OF THE ROLE OF *PILG* IN *XYLELLA FASTIDIOSA*

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ABSTRACT
Type IV pili of *Xylella fastidiosa* are regulated by *pilG*, a chemotaxis regulator in Pil-Chp operon involving signal transduction pathways. To elucidate the role of *pilG* in the twitching motility and pathogenicity of *X. fastidiosa*, phenotypes of wild-type, *pilG*-mutant and a complementary strain were characterized. While all tested strains had similar growth curves *in vitro, X. fastidiosa* wild-type and complementary *XfΔpilG*-C strain showed typical twitching motility in microfluidic flow chambers whereas mutant *XfΔpliG* exhibited a twitching defective phenotype. Greenhouse experiment further revealed that Pierce's disease symptoms were significantly reduced in grapevines inoculated with *XfΔpilG* whereas grapevines inoculated with *X. fastidiosa* wild-type and *XfΔpilG*-C developed typical Pierce's disease symptoms. These results demonstrate that *pilG* of *X. fastidiosa* is required for twitching motility and full virulence.

FUNDING AGENCIES
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CHARACTERIZATION OF DIFFERENT CALIFORNIA STRAINS OF *XYLELLA FASTIDIOSA* BY FATTY ACID METHYL ESTER ANALYSIS

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Reporting Period: The results reported here are from work conducted May 2015 to October 2015.

ABSTRACT

Detection and characterization of different subspecies of *Xylella fastidiosa* are often dependent upon genotyping (Almeida and Nunney, 2015). However, complementary methods to detect and characterize different *X. fastidiosa* strains based on phenotype are needed to confirm conclusions. Characterization of the composition of fatty acids that comprise bacterial cell membranes is one phenotyping approach to distinguish bacterial species, subspecies, and strains. Fatty acid composition of cell membranes in theory could determine interactions between bacteria and their hosts as well. Fatty acid profiling is performed by a technique involving extraction of fatty acids, converting them to methyl esters, and analyzing by gas chromatography. This study examined cell membrane fatty acid profiles of six different isolates of *X. fastidiosa*: Dixon, M12, M23, Mulberry, Olive (the 5A isolate from California), Stag's Leap (SL), and Temecula. Fatty acids were extracted and analyzed from four different cultures of each isolate. The top twelve fatty acids accounting for approximately 95% of total cell membrane fatty acids were selected for further analyses. The percent that each of these twelve comprised the fatty acid profile was then used to group each isolate via cluster analyses (both with furthest neighbor linkage using Pearson's correlations and nearest neighbor linkage using squared Euclidean distances) and principal component analysis (PCA). Results showed that M12 consistently grouped alone, M23 and SL consistently grouped together, Dixon and Olive consistently grouped together, and Mulberry and Temecula consistently grouped together. In terms of defined subspecies, M12, Dixon, and Olive are all considered ssp. *multiplex*, and grouped together consistently. However, M12 was often on a different branch than the others. The ssp. *fastidiosa* strains of M23, SL, and Temecula were on the same branch when grouped by furthest neighbor joining and closely grouped by PCA, but were on different branches when grouped by nearest neighbor joining. Mulberry, which was defined as subspecies *morus*, grouped consistently with Temecula, suggesting great similarity in fatty acid profiles. Taken together, these results generally demonstrate that fatty acid profiling can separate ssp. *multiplex* from ssp. *fastidiosa*. Additional strains and replication will be performed to verify and expand results. When completed, fatty acid profiling will provide complementary data to genotype-based studies. Commercial equipment that utilizes fatty acid methyl ester (FAME) analysis for bacterial identification could be used to detect and distinguish subspecies and even certain strains of *X. fastidiosa*.

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Section 2:

Other Designated Pests and Diseases of Winegrapes

- Brown Marmorated Stink Bug -
- Red Blotch -
- Vine Mealybug -

BROWN MARMORATED STINK BUG RISK AND IMPACTS IN WESTERN VINEYARDS

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Reporting Period: The results reported here are from work conducted July 2015 to October 2015.

ABSTRACT

In California's north coast winegrape region, Lodi-Woodbridge winegrape region, and San Joaquin Valley (Fresno County), vineyards and small vegetable farms were sampled for stink bugs and brown marmorated stink bug (*Halyomorpha halys*) in particular. No live brown marmorated stink bugs were found in the surveyed vineyards in California to date. In Fresno County Southeast Asian vegetable farms no brown marmorated stink bugs were found, but Say's stink bug (*Chlorochroa sayi*) and Bagrada bug (*Bagrada hilaris*) were collected. Brown marmorated stink bug was found in increasing numbers in Oregon vineyards as evidenced by both trap counts and online website reports.

LAYPERSON SUMMARY

Brown marmorated stink bug (*Halyomorpha halys*) has been found in large and increasing numbers in vineyards in Oregon State, but has yet to be found in any California vineyards. It appears as if increased temperatures on vines results in increased feeding activity levels. It appears as if adult life stages exposed to clusters result in elevated levels of feeding activity on clusters.

INTRODUCTION

Brown marmorated stink bug (*Halyomorpha halys*) is becoming increasingly prevalent in Oregon and is rapidly becoming an economic concern for northwestern vineyards (Oregon Department of Agriculture, 2011; Wiman et al., 2014a; CDFA PD/GWSS Board, 2015 RFA). This pest can feed on vegetative tissues and, grapes, and can potentially cause contamination of the crop that may lead to wine quality losses. Studies funded by a USDA Specialty Crop Research Initiative (SCRI) Coordinated Agricultural Project (CAP) grant confirmed spread and increased population levels of brown marmorated stink bug in important viticultural regions of Oregon (VMW et al., unpub.). Brown marmorated stink bug was first found on the west coast in 2004 in Portland, Oregon (Oregon Department of Agriculture, 2011), and the pest is now common in urban and natural areas. Found on high-value specialty crops and non-economic alternate host plants alike, brown marmorated stink bug is increasingly causing agricultural issues for growers (**Figure 1**) in Oregon. Since 2012, brown marmorated stink bug has increasingly been encountered by growers and can be found in winegrape vineyards of the Willamette Valley during the harvest period (Wiman et al., 2014a). Winemakers have recently reported finding dead brown marmorated stink bugs in fermenting wines and infestation of winery buildings by brown marmorated stink bugs.

Immature and adult brown marmorated stink bugs feed on reproductive plant structures such as fruits, and they may also feed on vegetative tissues, such as leaves and stems, sometimes piercing through bark (Martinson et al., 2013). Fruit feeding by adult brown marmorated stink bugs may cause direct crop loss due to berry necrosis (VMW, SCRI CAP grant report, 2013). Berry feeding may also result in secondary pathogen infection and provide entry points for spoilage bacteria. Vectoring and facilitation of pathogen proliferation by brown marmorated stink bugs is not unrealistic because true bugs (Heteroptera) such as brown marmorated stink bugs share feeding behaviors with homopterans implicated as disease vectors in vineyards (Cilia et al, 2012; Daugherty, 1967; Mitchell, 2004; Wiman et al., 2014a). Brown marmorated stink bug itself is a demonstrated vector of at least one phytoplasma disease in China (Hiruki, 1999; Weintraub and Beanland, 2006), while leaf-footed bugs (Heteroptera: Coreidae) and other pentatomids have also been implicated in transmission of pistachio

stigmatomycosis (Michailides et al., 1998). It is clear that brown marmorated stink bug feeding intensity is directly related to temperature (Wiman et al., 2014b), potentially making this pest more damaging in western production regions than on the east coast.

Brown marmorated stink bug can develop on a wide range of host crops, meaning that it can find refuge or reproduce on non-crop hosts and then spread to cultivated crops such as winegrapes (Nielsen et al., 2008; Nielsen and Hamilton, 2009; Leskey et al., 2012a, 2012b; Pfeiffer et al., 2012; VMW, SCRI CAP report, 2014). However, unlike other pentatomids, brown marmorated stink bugs are also capable of completing development on crop plants. As a result, crop damage from nymphs is more common than it is for other stink bugs. In the Willamette Valley, winegrapes are among the last crops to be harvested and this may increase the potential for late-season infestation and damage by brown marmorated stink bugs.

Contamination of grape clusters by brown marmorated stink bug at harvest is a major concern. Adult brown marmorated stink bugs have been observed to lodge themselves between the grapes during harvest. Work funded by the USDA Northwest Center for Small Fruits Research (NWCSFR) is evaluating physical removal of brown marmorated stink bugs from clusters, as well as removal by chemical cleanup sprays, blowers, and electronic sorters. However, some brown marmorated stink bugs may remain in grape clusters and release defensive compounds during processing, which may cause taint in finished wine (E. Tomasino, pers. comm.). These taints may persist, and may result in major market losses. Work conducted on Pinot noir has shown that trans-2-decenal, a defense compound produced by brown marmorated stink bug, is a contaminant present in wine that is processed with three brown marmorated stink bugs per cluster.

As in Oregon, many important winegrape growing regions of California are in close proximity to major urban centers where brown marmorated stink bug populations tend to increase and become sources for further spread. Little is known about brown marmorated stink bug seasonal phenology, voltinism, and distribution in these environments. Oregon research has documented rapid colonization and significant increases in populations between seasons, in part because two full generations of brown marmorated stink bug are occurring (NGW, unpublished). In Oregon, brown marmorated stink bug has dispersed from Portland to northern Willamette Valley vineyards within the last 10 years. It is important to survey the winegrape growing regions of Napa, Sonoma, and Lodi because these regions are geographically close to city centers such as Sacramento with known brown marmorated stink bug infestation (Ingels, 2014).

Feeding intensity of different life stages of brown marmorated stink bug in vineyards has not been fully determined. To date, most studies have focused on adults, even though nymphs are potentially more damaging. When brown marmorated stink bug egg masses are laid in vineyards, the nymphs are more confined to feed on the vines than the adults, which may fly back and forth between vineyards and borders. Thus, the feeding damage from nymphs may be more concentrated as the nymphs disperse from egg masses to feed on the host plant. No information is available, however, on the impact and severity of feeding by nymphs on grape berries and vines. Spatial distribution of brown marmorated stink bug in vineyards and feeding intensity may reflect environmental suitability. An observation from orchard crops is that the worst brown marmorated stink bug damage tends to occur on the borders (Joseph et al., 2014). Similarly, vineyard borders appear to be more susceptible to brown marmorated stink bug infiltration from surrounding vegetation (VMW, SCRI CAP report, 2014). Grapevines located close to vineyard borders may provide a better environment for the bugs due to microclimate effects of shading by surrounding vegetation. The observation that insect-vectored pathogen problems in vineyards also tend to begin at field margins (VMW, SCRI CAP report, 2013) suggests spatial overlap with brown marmorated stink bug and raises additional concerns.

This study aims to determine the potential for brown marmorated stink bug to cause direct damage to winegrape crops, as well as indirect damage through facilitation and vectoring of spoilage bacteria or vine diseases. Controlled damage studies to assess direct feeding damage by brown marmorated stink bug have been conducted in Oregon (Oregon State University) and New Jersey (Rutgers). These studies showed an increasing number of stylet sheaths in grape berries as the numbers of brown marmorated stink bug test populations increased. Increased numbers of stylet sheaths were associated with decreases of berry counts, premature raisining, and increased berry necrosis, but this work focused on adult feeding and was conducted for one-week periods only (VMW, SCRI CAP report, 2013). Direct crop impact may be more pronounced under more optimal temperature regimes with different varietals, and with longer feeding periods by nymphs to more realistically simulate crop

infestation by reproductive brown marmorated stink bug, as is found in vineyards in Oregon and presumably California.

OBJECTIVES

1. Survey key Oregon and California viticultural areas for brown marmorated stink bug presence.
2. Determine brown marmorated stink bug temperature-related field feeding intensity, impact, and regional risk index.
3. Provide Extension for identification, distribution, and risk of brown marmorated stink bug in western vineyards.

RESULTS AND DISCUSSION

Objective 1. Survey key Oregon and California viticultural areas for brown marmorated stink bug presence.

Surveys focused on high-risk regions containing vineyards and wineries in close proximity to high traffic areas such as highways, urban centers, throughways, and railroad lines. Initial beat sheet sampling in the aforementioned areas and in California included Sonoma, Napa, and Lodi. Pheromone-baited pyramid traps (Khrimian et al., 2014) were used in conjunction with monitoring using beat sheets. The brown marmorated stink bug pheromone traps were placed in the center of each row selected for beat sheet sampling. Brown marmorated stink bugs were additionally sampled from study vineyards using beat sheet sampling every two weeks, starting in August, from two rows, once on the vineyard edge and once in the center of the same block. Our goal was to start surveys of California vineyard regions before the reported movement of brown marmorated stink bug into commercial vineyards. The vineyard regions sampled were California's north coast winegrape region (Mendocino, Napa, and Sonoma counties), Lodi-Woodbridge winegrape region, and San Joaquin Valley (Fresno County). All vineyard surveys were conducted in concert with other ongoing studies, with outreach to participating farmers on brown marmorated stink bug description and potential presence. At each site, about 100 vines were visually sampled every two to four weeks. Specifically, in Mendocino County, six vineyard sites around Ukiah and Hopland (four Chardonnay, one Merlot, and one Grenache) were sampled as part of a leafhopper project. In Napa County, seven vineyard sites (two Cabernet Sauvignon near St. Helena, one Cabernet Sauvignon near Oakville, one Chardonnay near Yountville, one Merlot near Carneros, and one Pinot Noir and one Chardonnay near Carneros) were sampled as part of a red blotch or vine mealybug study. In Stanislaus and San Joaquin counties (Lodi-Woodbridge winegrape region), three vineyards were sampled (one Cabernet Sauvignon, one Pinot Noir, and one Chardonnay), and in Fresno County five table grape blocks (two Thompson Seedless and three flame seedless) were sampled. An additional sampling protocol was followed in three vineyard blocks in Sacramento, Yolo, and Amador counties for all hemipteran insects, but have yet to find any brown marmorated stink bugs at any of these sites. Sampling at these sites has been conducted by visual observations and sweeping of grape foliage and other vegetation present in and adjacent to the vineyards. To date, no brown marmorated stink bugs were found during these field visits in California.

Sampling in Oregon included seven vineyards in the northern Willamette Valley. There were no clear differences in between sampling sites and data from all vineyards were pooled for the 2015 season. This was the third year of sampling in these vineyards and data is presented as brown marmorated stink bugs per pyramid trap over a two-week period (**Figure 1**).

In all of the seven locations, brown marmorated stink bug was found in low numbers during the early part of summer in Oregon. The number of brown marmorated stink bugs per trap increased to ca. 30 brown marmorated stink bugs per trap per two-week period during September through October of 2014 and 2015. The total cumulative number of brown marmorated stink bugs trapped per trap during the whole season increased from 34 (2013) to 101 (2015) brown marmorated stink bugs per trap collected during the season.

In California, at the University of California, Berkeley lab (Daane laboratory) starting in October, we began monitoring the farms and gardens by utilizing traps containing aggregation pheromones, as well as sweep net collections of the landscape. In Fresno County, we have sampled five Hmong farming operations, each about three to seven acres in size. Sampling consisted of utilizing a D-Vac to collect insects from three different crops (eggplant, long beans, peppers, tomatoes, peas, bitter melon, and squash) at each site every other week. From these samples no brown marmorated stink bugs were found, but Say's stink bug (*Chlorochroa sayi*) and bagrada bug (*Bagrada hilaris*) were collected.

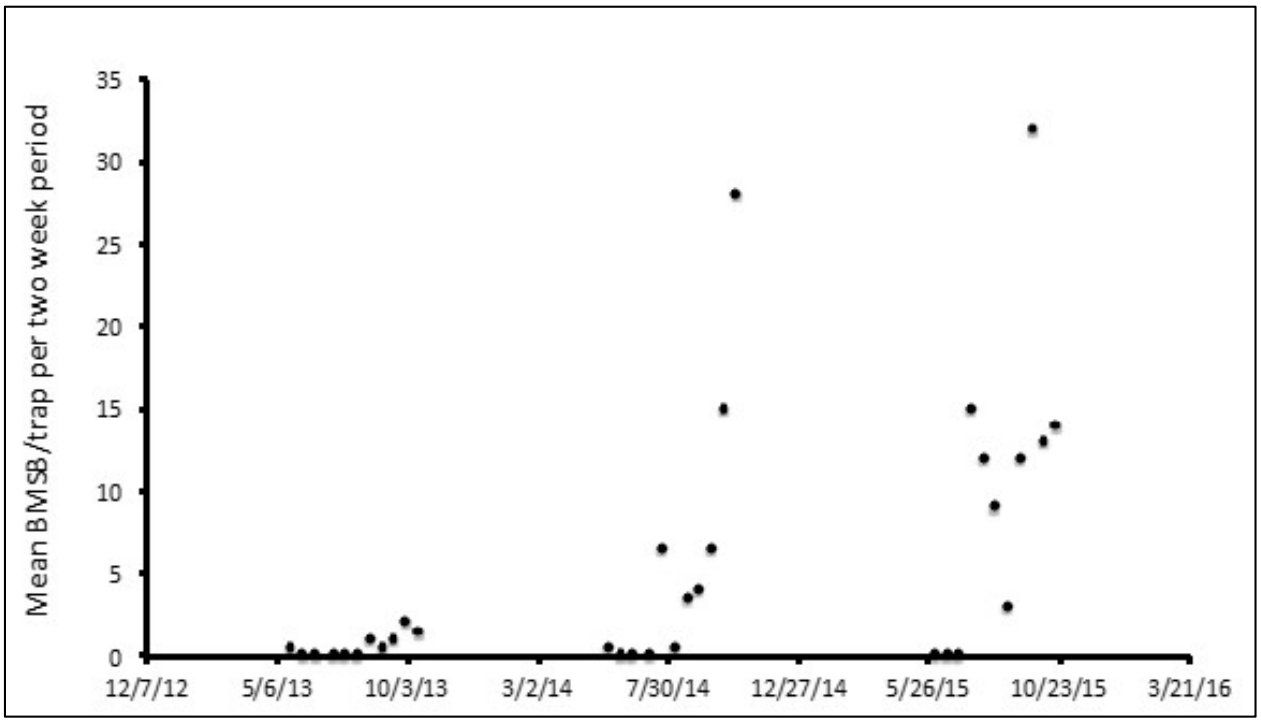

Figure 1. Number of brown marmorated stink bugs per trap per two-week period in the Willamette Valley, Oregon during 2013-2015. Traps were placed in seven vineyards in the northern Willamette Valley.

University of California, Davis (Zalom laboratory) brown marmorated stink bug sampling was initiated in fall 2015 by making visual observations and collections of stink bugs from community gardens and vineyards in Sacramento, Yolo, San Joaquin, and Amador counties. Brown marmorated stink bugs have previously been captured in the cities of Sacramento, Davis (Yolo County), and Stockton (San Joaquin County), but none have been captured in agricultural situations to date. We continued more intensive sampling of community gardens in Sacramento and Davis, and have also sampled community gardens in Galt (Sacramento County) and Lodi (San Joaquin County). Six species of stink bugs were collected from these gardens, including *Eushistus conspersus*, *Thyanta pallidovirens*, *Chlorochroa uhleri*, *Chlorochroa ligata*, *Murgantia histronica*, and *Nezara viridula*, but brown marmorated stink bug was only found in community gardens in Sacramento where it was also observed feeding on grapes that were growing there. We have yet to sample gardens elsewhere in these counties, but we have met with University of California Cooperative Extension Farm Advisor Jhalendra Rijal to discuss plans for collaboratively sampling community gardens and landscape plantings in the vicinity of previous finds in Stockton and Modesto (Stanislaus County) in the coming year. We intend to use finds of brown marmorated stink bug breeding populations at such sites as an indicator of where we might target sampling in nearby vineyards. The Zalom lab has obtained a permit to maintain a brown marmorated stink bug colony that we initiated during 2015 with bugs collected from community gardens in Sacramento, and is presently using the colony in various behavior and control studies.

Objective 2. Determine brown marmorated stink bug temperature-related field feeding intensity, impact, and regional risk index.

Feeding intensity.

In Oregon, we deployed refined portable electronic feeding monitors (Wiman et al., 2014b) August 21, 2015 for a one-month period in order to determine in-vineyard feeding intensity. Portable feeding monitors consist of an open circuit enclosed onto a section of the grapevine located within 20 meters of the pheromone traps. Four electronic feeding monitors were placed in each of the two rows in a partially shaded vineyard border and a fully sun-exposed location within the center of each vineyard. Each feeding monitor was used to determine feeding frequency, duration, and time. Each portable feeding monitor logged feeding for five individual brown marmorated stink bugs. The insects were replaced once per week. The relative risk and intensity of brown marmorated stink bug feeding damage were determined by creating a feeding index of insect-days (Ruppel, 1983) for each of the vineyard regions using standard methods as described by Wiman et al. (2014b). Additionally, these

feeding patterns were verified by counting the number of stylet sheaths and plant damage within the monitored feeding area. Data from this work is currently being analyzed.

Feeding impact.
Feeding exclusion sleeves (48.0 cm x 39.5 cm, Premier Paint Roller, Richmond Hill, NY, item 60597) were placed over winegrape clusters in a commercial vineyard with known brown marmorated stink bug infestation in the northern Willamette Valley. The trial was maintained for a four-week period from August 21 to September 21, 2015. There were four treatments: (1) no brown marmorated stink bugs; (2) a partial egg mass with 10 hatching eggs; (3) three brown marmorated stink bug nymphs; and (4) three adult brown marmorated stink bugs. All treatments were enclosed in a single sleeve on vines for four weeks. Ten replicates of each treatment were established in a randomized block design. Forty sleeves (ten of each treatment) were placed in a partially shaded vineyard border row, and forty sleeves were placed in a fully sun-exposed vineyard row in each vineyard (80 sleeves total). Brown marmorated stink bugs were exposed to clusters within a sleeve for four weeks during the period when brown marmorated stink bugs are typically found in vineyards in the Willamette Valley. Dead insects were replaced every week with brown marmorated stink bugs of the same life stage during the exposure period. At the end of the experimental period, all clusters were removed and taken to the laboratory for further inspection. Feeding activity of brown marmorated stink bug was determined by counting the number of stylet sheaths per berry. Additional key quality parameters were determined, including berry weight, pH, sugar, raisining, cracking, and presence or absence of spoilage bacteria or fungi such as *Botrytis* using the slip-skin method (Crisosto et al., 2002). These data together with weather data (five data loggers per vineyard location), feeding intensity, and direct impact on crop can be used to develop a relative risk model for brown marmorated stink bug damage in different vineyard regions (Ruppel, 1983; Froissart et al., 2010; Wiman et al., 2014a, 2014b). The key cluster data is currently being analyzed.

During 2015, there were significantly higher temperatures recorded in locations that received higher sun exposure levels (**Figures 2a** and **2b**). Mean temperatures during the experimental period ranged from 12.3-23.8°C during the experimental period. Temperature differences were as wide as 28.2 to 23.5°C on days when there was full sun exposure to virtually indistinguishable on cloudy days. During 2015 there were significantly higher levels of stylet sheaths between sunny and shady locations in vines ($F_{1, 4074} = 45.079$, $p = 0.001$; **Figure 3**), and there were higher levels of stylet sheaths in treatments with adults compared to immature brown marmorated stink bug life stages.

In order to determine if there were differences in brown marmorated stink bug feeding days (insect days, Ruppel, 1983) between sunny and shaded locations, we determined the mortality rates over the four-week period of the feeding trial. There were, however, no clear differences in cumulative mortality rates between locations where brown marmorated stink bugs were placed on vines (**Figure 4**).

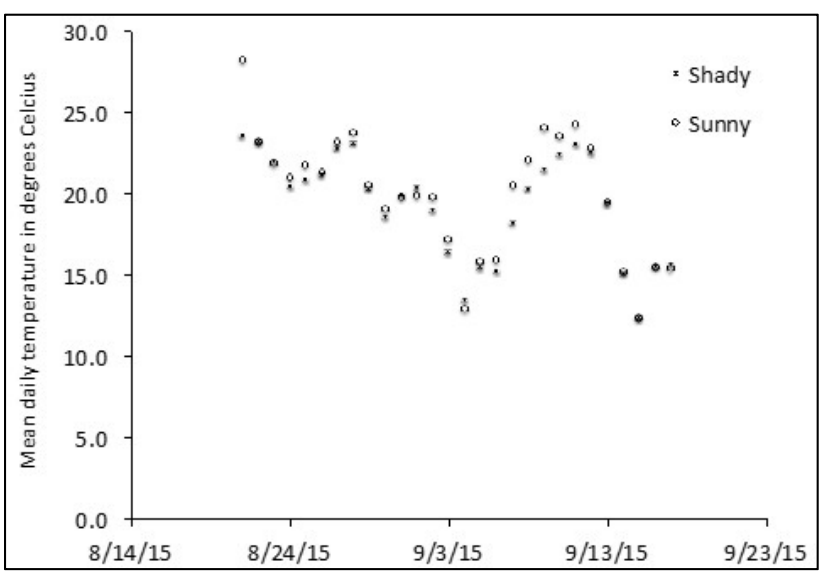

Figure 2a. Mean daily recorded temperatures from each of shady and sun-exposed locations on vines in the Willamette Valley during 2015.

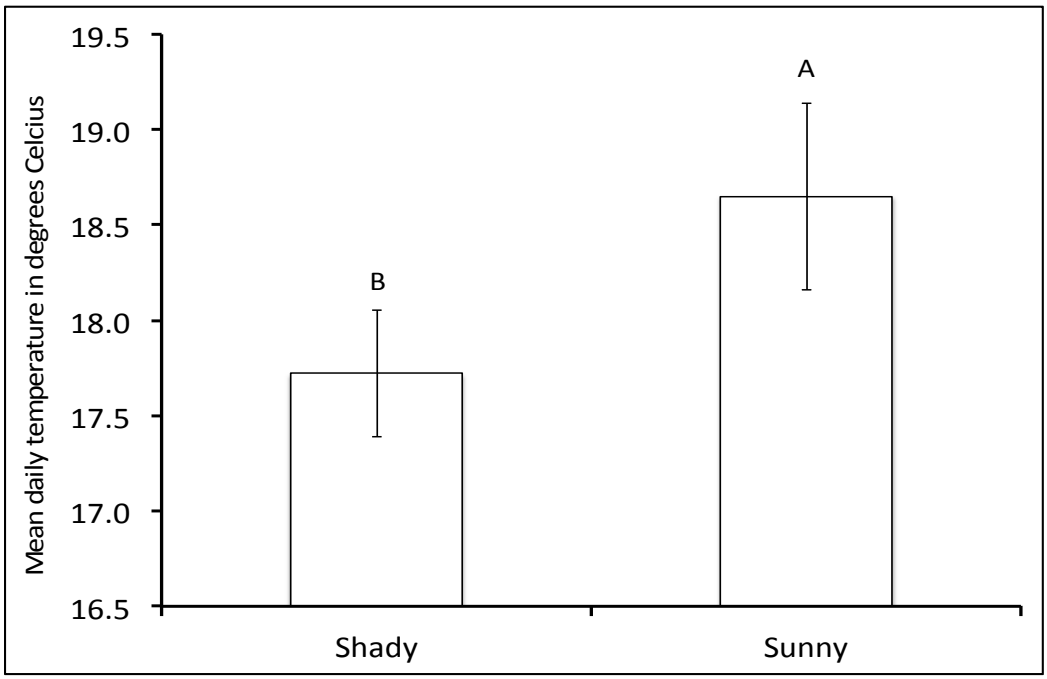

Figure 2b. Mean temperatures recorded in each of two sun-exposed locations on vines in the Willamette Valley during 2015. Significantly different letters indicates different temperatures.

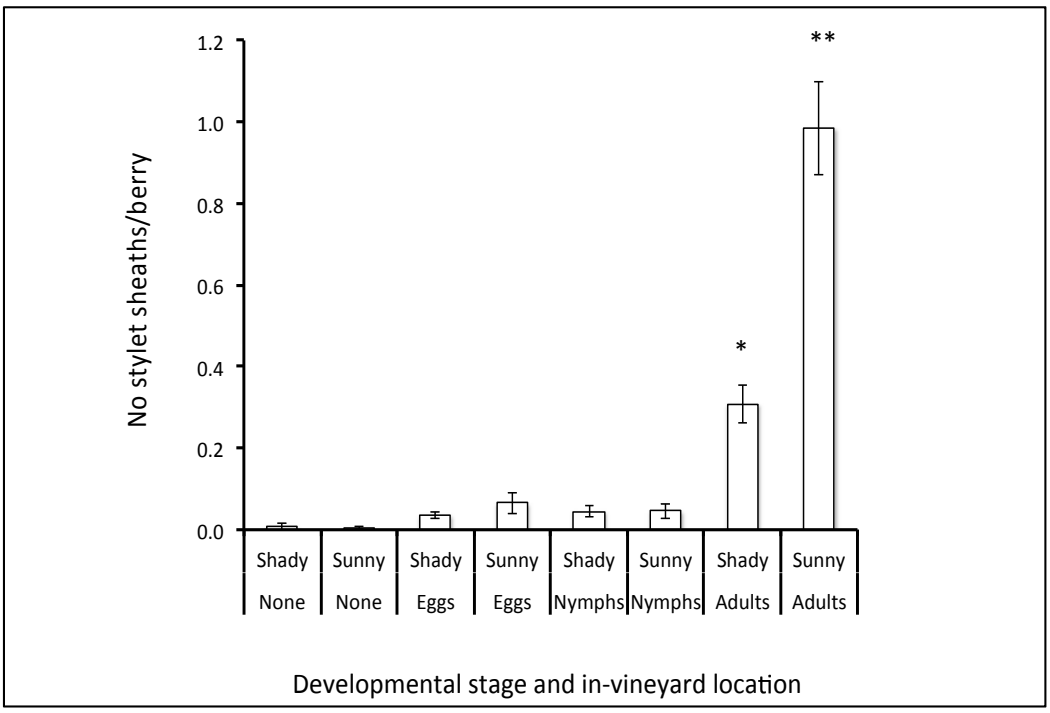

Figure 3. Number of stylet sheaths per berry in the Willamette Valley, Oregon during 2015. Bars with no, one, and two asterisks (*) are significantly different from other bars.

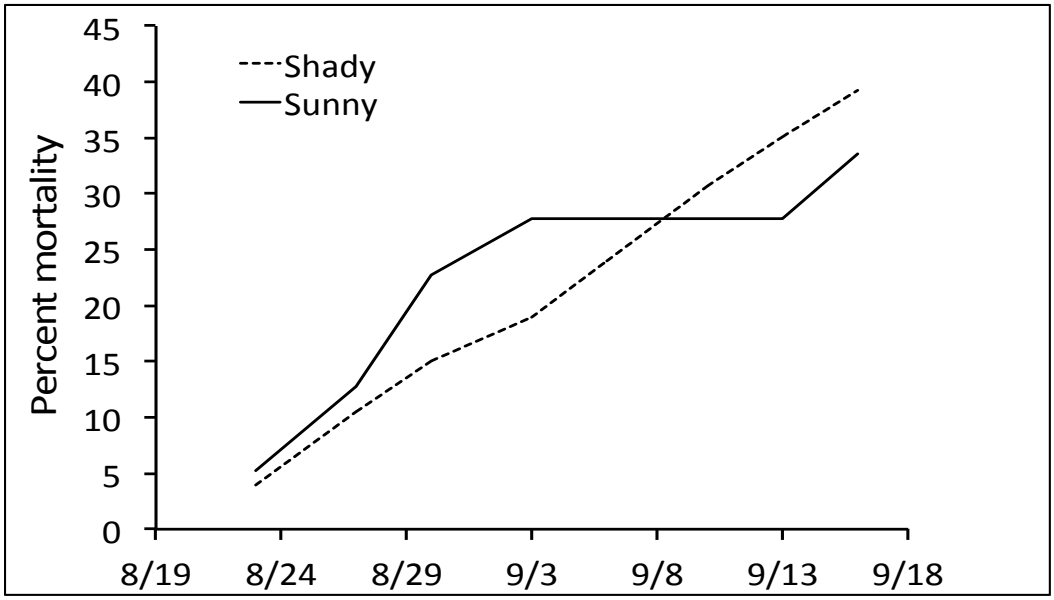

Figure 4. Percent mortality rate of brown marmorated stink bugs in sunny and shaded locations in vines in the Willamette Valley, Oregon during 2015.

3. Provide Extension for identification, distribution, and importance of brown marmorated stink bug in western vineyards.

Because brown marmorated stink bugs may first be seen in small organic gardens and ornamental trees, we also began outreach or surveys of small organic farms (Napa and Sonoma counties) and Southeast Asian vegetable farms (Fresno County). In the north coast region, we have partnered with Master Gardener groups in Napa and Sonoma to gain access to home gardens in which we may find desirable host source plants. Additionally, contacts have been made, in partnership with the Napa County Agricultural Commissioner, allowing us access to survey and sample small, diversified farms. No brown marmorated stink bugs have yet to be found at these sites. In Oregon, we presented results of earlier work for this grant to growers in five locations: McMinnville, Oregon (63 attendees), Milton Freewater, Oregon (30 attendees), Roseburg, Oregon (50 attendees), Medford, Oregon (48 attendees), and Rickreal, Oregon (211 attendees). Several extension meetings were held in the San Joaquin Valley and coastal winegrape regions as represented by the sampled regions mentioned above.

CONCLUSIONS

In California's north coast winegrape region, Lodi-Woodbridge winegrape region, and San Joaquin Valley (Fresno County) vineyards and small vegetable farms, no brown marmorated stink bugs were found. While this is only the initial study, brown marmorated stink bugs have been found in the Lodi-Woodbridge region in ornamental trees, but have yet to be found near the vineyards sampled. In Oregon, brown marmorated stink bugs were found in increasing numbers from 2013-2015 in each of the seven vineyards sampled. There were increasing website reports from winegrape growers. There is increasing concern about the higher levels of brown marmorated stink bug populations in Oregon vineyards. Our feeding trials show elevated levels of feeding in clusters that were more exposed to sun during the experimental period. Adults resulted in higher levels of stylet sheaths compared to the other life stages exposed to clusters.

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SEARCHING FOR POTENTIAL VECTORS OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT

At this time there is no accurate information on the epidemiology of grapevine red blotch-associated virus (GRBaV) - is it transmitted by insects, or dispersed with the movement of infected planting material? Our goal is to (1) screen possible vectors to determine if they can or cannot acquire GRBaV from infected vines and transmit GRBaV to clean vines, (2) screen uncommon organisms that feed on vines at vineyards where movement of GRBaV has been observed or reported (assumed to have happened), and (3) follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

Starting in July 2015, replicated groupings of Virginia creeper leafhopper (*Erythroneura ziczac*), vine mealybug (*Planococcus ficus*), and foliar-form grape phylloxera (*Daktulosphaira vitifoliae*) were tested for their ability to successfully transmit GRBaV to uninfected grapevines. Petiole samples from inoculated test plants were tested for the presence of GRBaV and, to date, none of the inoculated plants show symptoms of GRBaV and all petioles have tested negative. Subsamples of insects that were used in experiments were frozen and later tested for the presence of the virus, and all have also tested negative. Inoculated vines used in these trials will be held for a two-year period, during which time petioles will be tested for GRBaV approximately every four months. Additional candidate vectors will also be tested in 2016.

To determine novel organisms for evaluation, monthly samples of insects were collected from five established vineyards in Napa County where movement of GRBaV has been observed or reported. Samples were collected from both grapevines as well as ground covers and non-crop vegetation in the surrounding landscape. To date none of these organisms have tested positive for GRBaV.

Field epidemiology was monitored at six vineyard sites in Napa County. While five of these sites are in their first year of observation, one site has been monitored annually since 2009 for GLRaV as well as "red leaf symptomatic vines" that tested (polymerase chain reaction; PCR) negative for GLRaV. The red blotch infected vines were randomly distributed within the plot, indicating that infection did not spread from previously infected vines, which is often indicative of vector movement. Suspected vines are now being re-tested using new and more complete primers for both leafroll and red blotch.

LAYPERSON SUMMARY

Grapevine red blotch-associated virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other grape leafroll diseases (GLD). There has been some initial laboratory evidence that leafhoppers are a potential vector of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards. Our goal is to test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. This work must be completed to develop a control program for "red blotch" and develop accurate information on the epidemiology of this newly reported pathogen. In 2015, we tested Virginia creeper leafhopper (*Erythroneura ziczac*), vine mealybug (*Planococcus ficus*), and foliar-form grape phylloxera (*Daktulosphaira vitifoliae*). To date, none of these insects have moved the pathogen from an infected plant to a clean plant in laboratory studies. Our field studies have surveyed insects in vineyards with suspected movement of red blotch. None of the tested herbivores have been positive for the virus responsible for red blotch. We have also

surveyed vineyards where red blotch is suspected of moving and samples from these vineyards are currently being processed.

INTRODUCTION

In 2006 an increase in grapevine leafroll disease (GLD) and vines with "red leaf" symptoms was observed by growers in vineyards located within the Napa Valley, California. Symptoms were also observed at the Oakville Experimental Vineyard (OEV) by Jim Wolpert [University of California (UC), Davis Viticulture Extension Specialist], Ed Weber (UC Cooperative Extension Viticulture Farm Advisor), and Mike Anderson (UC Davis Staff Research Associate). Tissue samples were collected from symptomatic vines and tested by commercial laboratories and the UC Davis Foundation Plant Service (FPS). Test results were most often negative for grapevine leafroll-associated viruses (GLRaVs).

The increasing awareness of blocks containing vines with grapevine leafroll disease symptoms, primarily in Napa and Sonoma counties, but testing negative for grape leafroll-associated viruses (GLRaV), resulted in a renewed focus on virus species and strains causing GLD. New GLRaV-3 strains have been discovered (e.g., Sharma et al., 2011); however, this did not fully explain all of the observed symptomatic vines. In 2010, next generation sequencing analyses identified a new pathogen (Al Rwahnih et al., 2013). Soon after a circular DNA virus, similar to members of the Geminiviridae, was isolated (Krenz et al., 2012) and, concurrently, polymerase chain reaction (PCR) primers were developed (Al Rwahnih et al., 2013) for this pathogen now known as grapevine red blotch associated virus (GRBaV). GRBaV has since been isolated from vines throughout North America and in Switzerland (Krenz et al., 2014), highlighting either a rapid dissemination or, more likely, its long hidden presence (e.g., misidentified as GLD). Furthermore, an archived herbarium specimen collected in Northern California in 1940 tested positive for GRBaV (Al Rwahnih et al., 2015).

This proposal focuses on possible vectors of GRBaV. Multiple viruses in the Geminiviridae are insect transmissible (Ghanim et al., 2007; Chen and Gilbertson, 2009; Cilia et al., 2012), and there has been some initial evidence that leafhoppers may vector GRBaV (Poojari et al., 2013). However, there has been mixed evidence of GRBaV field spread in association with leafhoppers. Concern over the spread of GRBaV led to an off-cycle project in summer 2013, funded by the Napa County Winegrape Pest and Disease Control District to initiate appropriate scientific studies of possible insect vectors of GRBaV. The work was continued in 2014 with American Vineyard Foundation (AVF) and Napa County funds.

Our goal is to test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. Determining field epidemiology of GRBaV is critical in the development of a control program – whether the pathogen is moved via infected nursery material, mechanically or, as with the focus of this study, by a vector. There are ample California vineyard sites where the pathogen is present but does not appear to have moved from infected vines over a period of many years, but in a few vineyards vine-to-vine movement has been recorded. This difference – whether there is no vector movement and disease presence is exclusively from infected nursery material or there is a vector – completely changes the needed control programs.

Table 1. Arthropods targeted for GRBaV tests.

Common name	Scientific Name	Common Distribution
Western grape leafhopper	*Erythroneura elegantula*	North Coast (north of Tehachapi Mountains)
Variegated leafhopper	*Erythroneura variabilis*	Central Valley (San Joaquin Co. to southern CA)
Virginia creeper leafhopper	*Erythroneura ziczac*	Northern California
Potato leafhopper	*Empoasca* sp.	Sporadic vineyard populations
Vine mealybug	*Planococcus ficus*	California vineyards
Grape mealybug	*Pseudococcus maritimus*	North Coast and San Joaquin Valley
Obscure mealybug	*Pseudococcus viburni*	Central and North Coast
Blue-green sharpshooter	*Graphocephala atropunctata*	Northern California
European fruit Lecanium scale	*Parthenolecanium corni*	North Coast
Grape phylloxera	*Daktulosphaira vitifoliae*	North Coast, Sacramento Delta, Foothills
Grape whitefly	*Trialeurodes vittatas*	California
Mites	*Tetranychus spp.*	California

Our proposed work will screen all common vineyard arthropods, as well as the "long shots" that are potential red blotch vectors, thereby providing the proper target for control. **Table 1** provides a partial list of the common vineyard insect species that should be screened as potential vectors of GRBaV, based on their incidence and distribution in California vineyards.

Once tested, organisms are either identified as vectors or our work shows that they are either not vectors or that they are so inefficient that spray programs are not needed. This information will then be disseminated to farmers, pest control advisors, and extension personnel, thereby having a practical, direct, and immediate impact on control decisions to "spray or not to spray."

OBJECTIVES

To screen potential vectors for their ability to acquire and transmit grapevine red blotch associated virus (GRBaV) and, if a vector is discovered, to determine vector efficiency.

1. Screen common vineyard insects and mites as potential vectors of GRBaV.
2. Screen uncommon organisms that feed on vines as potential vectors of GRBaV.
3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

RESULTS AND DISCUSSION

Objective 1. Screen common vineyard insects and mites as potential vectors of GRBaV.

In 2013 and 2014, we prioritized the screening of leafhoppers (*E. elegantula* and *E. ziczac*), grape whitefly (*Trialeurodes vittatas*), and mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), because of the published work by Poojari et al. (2013), their prevalence in California vineyards, and/or their phloem feeding (this category of viruses is phloem-limited, although the biology and ecology of GRBaV is not fully understood).

In both years, canes were collected from Cabernet Sauvignon (clone 6) and Cabernet Franc (clone 04) vines in vineyard blocks where vines were known to have tested positive for GRBaV, and negative for all known GLRaVs and other known grapevine viruses. PCR test results for these vines were made and canes negative for all viruses except GRBaV and grapevine rupestris stem pitting-associated virus (RSP) (UC Berkeley and FPS test results) were transferred to UC Berkeley Oxford Tract Greenhouse and established in pots on a mist bench. Vines were maintained in the greenhouse, strictly treated to be insect and mite-free, and isolated from other vines that may have harbored viral pathogens. As indicators for these studies, we used Cabernet Sauvignon vines propagated from material provided by FPS and maintained under similar conditions.

Initial tests were conducted using the most mobile stages of key species, including adults of the *Erythroneura* (leafhopper) species and the grape whitefly, and crawlers of the vine mealybug and grape phylloxera. We employed standard transmission protocols to evaluate the potential of these insects to transmit GRBaV, as has recently been done for GLRaVs (Tsai et al., 2008; Tsai et al., 2011; Blaisdell et al., 2015) and Pierce's disease (Almeida and Purcell, 2003a, b). We used a standard acquisition access period (AAP) and inoculation access period (IAP) of 120 hours (five days) each for all tested insect species except the more delicate grape whitefly, which was allowed to feed on plants for an AAP and IAP of 48 hours (two days) each. In the "controlled trials," known infected source plants or uninfected control plants in pots (one-liter size) were inoculated with 30-50 insects for the AAP, and surviving insects were then transferred to uninfected plants for the IAP. Field-collected leafhopper adults and blue-green sharpshooter adults were taken from an insectary colony and released on plants that were placed singly in 61 x 61 x 61 cm BugDorm cages. Grape whitefly adults reared from pupae were collected in Napa County vineyards and then released into nylon bags enclosing five leaves on potted grape plants. Mealybug crawlers were moved onto individual grape leaves (three leaves per plant) using a brush, and grape leaves were then enclosed with white paper bags. Following the IAP, all vines were treated with a contact insecticide to kill any remaining insect species. All insects were collected and tested for GRBaV within 48 hours after the AAP period. Every four months thereafter, three petioles were collected from each host plant and assayed for GRBaV infection. All treatments were compared to a negative and positive control. A total of 20 test vines were inoculated for each of the above insect species in 2014 trials.

To date, results from all of these trials have not indicated that any of these insects are capable of transmitting GRBaV to uninfected grapevines. Inoculated vines from these trials are being held for a two-year period, during which petioles are tested for GRBaV every four months and vines are visually evaluated for symptoms every fall.

In 2015, protocols for these transmission experiments were modified due to concerns about (a) low virus titer levels in the potted vines grown from cuttings of GRBaV-positive vines at vineyard field sites, and (b) small numbers of insects per trial. Our concern is that candidate vector ability to transmit GRBaV is confounded by low titer levels in the GRBaV-positive vines used in previous trials and/or inadequate insect sample size.

The new approach involves using "bouquets" of mature grape leaves collected from GRBaV-positive vines at vineyard field sites. Each bouquet consists of ten mature grape leaves held in a 16 oz. plastic container that contains moist perlite. Ten leaves were collected from each of ten GRBaV-positive vines (nodes 1-5) in an established vineyard in Napa County (100 leaves total). Each bouquet consisted of one leaf from each of the ten vines, totaling ten leaves per bouquet and ten total bouquets (i.e. one bouquet per replicate). Furthermore, each trial now contains at least 100 insects/replicate and 10 replicates per treatment.

Since July 2015, we have completed trials with the bouquets on Virginia creeper leafhopper (adults), vine mealybug (crawlers), and foliar-form grape phylloxera (crawlers). Due to concerns about bouquet degradation, these experiments used an AAP of 48 hours (two days) and an IAP of 72 hours (three days). Clip-cages (7 cm diameter x 2 cm height) were used to confine 10 insects/leaf to each bouquet (100 insects/bouquet). Bouquets with insects were placed in a 61 x 61 x 61 cm BugDorm cage and there were a total of 10 replicates per treatment. After the 48 hour AAP, clean potted vines were introduced into the cages. The clip cages were then removed, thus allowing the insects to move onto the clean vine. Bouquets were also removed at this time, after ensuring that they were free of the candidate vectors. Petioles from the bouquets were then isolated for GRBaV testing as well as a sub-sample of the candidate vectors (10-50 insects per replicate). After the 72-hour IAP, another subsample of the candidate vectors were collected for testing (10-50 insects per replicate) and the potted vines were then treated with a contact insecticide to kill any remaining insects. Three petioles were sampled from each vine (nodes 1-5) for immediate testing. Vines are now being held for a two-year period and petioles tested for GRBaV every four months.

Bouquet experiments with grape phylloxera were initially unsuccessful due to their rejection of the bouquet material. Following the 48 hour AAP it was observed that none of the phylloxera crawlers had settled on the leaves and instead were mostly desiccated inside the cages. As such, we reverted to the previous experimental approach utilizing potted vines that were confirmed to be GRBaV positive. This time, two-year-old GRBaV-positive vines were used in these trials to possibly provide vines with elevated virus titer levels. Control vines were one year old. Vines were placed in 61 x 61 x 61 cm BugDorm cages and inoculated by pinning ten leaf discs containing a large number of galls (>15) on each vine. The galls on these discs had been cut open with a razor in order to encourage movement of the crawlers onto the vine. After 25 days all of the potted vines exhibited >50 galls (i.e., 25-day AAP). At this point, clean vines were introduced into the cages and sub-samples of grape phylloxera adults, eggs, and crawlers were collected for testing. Acquisition and inoculation vines remained together in the cages until the inoculation vines had >50 galls/vine, which resulted in a 38-day IAP. At this point vines were treated with both a contact and systemic insecticide. As before, vines will be held for a two-year period and tested every four months.

For all plant material, a standard DNA extraction protocol was used in order to extract DNA from grapevine petioles potentially infected with red blotch disease (Sharma et al., 2011). Three petioles were randomly selected from nodes 1-5, and 0.1 g of tissue was macerated in 1.8 ml grape enzyme-linked immunosorbent assay (ELISA) grinding buffer in Mo-Bio 2.0 ml tough tube containing a Boca chrome steel ball bearing (Sharma et al., 2011), using a Precellys 24 Tissue Homogenizer at 6,500 Hz for two 10-second cycles with a 30-second intermission between cycles. The samples were then centrifuged for 10 minutes at 13,200 rpm and 20°C. One ml of the supernatant was pipetted into 1.5 ml Eppendorf tubes and stored at -20°C until analysis by qPCR. After briefly vortexing, the DNA extracts were denatured prior to performing qPCR; eight uL of extract was denatured with 99 uL of GES Denaturing Buffer plus one ul 1% beta-mercaptoethanol, by incubating at 95°C for 10 minutes and 4°C for five minutes (Sharma et al., 2011).

The qPCR was performed using Promega GoTaq qPCR Master Mix. Two ul of each denatured sample were added to 12.5 ul Promega GoTaq master mix, 2.5 ul of 10 uM primers GVGF1 and GVGR1, or with 10 uM primers RB-F and RB-R (developed by the lab for this study), 0.25 ul CXR reference dye, and eight ul water (Al Rwahnih et al., 2013). An Applied Biosystems qPCR machine with 7500 Fast System SDS Software was used for

qPCR and to analyze the data. Thermocycling conditions include one cycle of 95°C for two minutes; forty cycles of 95°C for 15 seconds, 58°C for one minute; and one cycle of 72°C for 10 minutes, followed by a final dissociation cycle. The PCR product was analyzed by the 7500 Fast System SDS Software, taking into account the Ct values, melting temperatures, and component curves when interpreting data.

All insects used in these studies were stored at -80 ºC until testing for GRBaV. The Qiagen DNeasy Blood and Tissue Kit was used for extractions and the bench protocol was followed to prepare the insect samples for the QIAcube; 25mg of insect were used for each extraction. The New England Biolabs Phusion High Fidelity kit was used for PCR. For each sample, 10 µL 5x Phusion buffer, one µL of 10 mM dNTP, 2.5 µL of 10 uM forward primer, 2.5 µL of 10 uM reverse primer, 100 ng of DNA, and 0.5 µL of Phusion DNA polymerase were used and diluted to 50 ul total reaction volume with water. After the samples are prepared, they will be briefly centrifuged before being placed in a thermal cycler (DNA Engine Peltier, Biorad) with a heated lid. The thermal cycler conditions were as follows: (1) Initialization at 98ºC for 30 seconds, (2) Cycle of denaturing step at 98ºC for 10 seconds, annealing step at 62ºC for 30 seconds, and extension step at 72ºC for 30 seconds, repeated 30 times, and (3) Final Extension at 72ºC for 10 minutes. To visualize PCR product, a 2% agarose gel was used in 1x TAE buffer running at 85 volts. A Qiagen GelPilot100bp Plus ladder will be used. The gel was stained with ethidium bromide, and visualized under ultraviolet light using the Quantity One program.

To date, our 2015 "bouquet" trials have shown no transmission of GRBaV with the Virginia creeper leafhopper and the vine mealybug. Similarly, the trial with foliar form grape phylloxera on two-year-old GRBaV-positive vines did not show any transmission either. Testing of insect and plant material from these experiments is ongoing, and in 2016 we will continue testing candidate vectors listed in **Table 1**.

Objective 2. Screen uncommon organisms that feed on vines as potential vectors of GRBaV.
We used the same methodologies described for Objective 1 to screen lesser known vineyard organisms or unlikely vectors. Insects were collected 1x/month from five established vineyards where movement of GRBaV has been observed or reported (assumed to have happened). Samples were collected from grapevines, ground covers and non-crop vegetation in the surrounding landscape using a combination of sweep-nets (30 sweeps per sample) and a D-Vac type suction sampling machine, which consisted of a 25 cc gas blower/vacuum (Craftsman) fitted with a five-gallon (18.9 liter) bucket on the vacuum tube to create a one ft^2 (0.093 m^2) sampling cone. Each D-Vac sample consisted of five thrusts with the D-Vac running at full speed. All samples were held in a cooler and brought to the laboratory for processing. Specimens were incapacitated using CO_2 gas, sorted, and identified to species or genus, and then stored in 95% EtOH and held at -80º C until testing. So far we have collected leafhoppers in the genera *Aceratagallia* sp., *Acinopterus* sp., *Alconeura* sp., *Colladonus* sp., *Empoasca* spp., *Macrosteles* sp., *Osbornellus* sp., and *Scaphytopius* spp., as well as the species *Deltocephalus fuscinervosus*, *Dikrella californica*, and *Euscelidius schenki*. Other organisms include members of the families Acanaloniidae, Membracidae, Miridae, Lygaeidae, Psyllidae, and Tingidae.

To date none of these alternative organisms have tested positive for GRBaV, although many of the specimens collected have not yet been tested and we are still in the process of refining our laboratory techniques to better evaluate insect material.

Objective 3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.
We have been studying GLD movement at one particular site in Napa, beginning in 2009. The block is a 20 ha newly planted (in 2008) block of Cabernet Sauvignon in Napa Valley. Each year, incidence of GLRaV and more general "red leaf" symptoms were mapped in September. Locations of diseased vines were recorded with global positioning system (GPS). In 2014, 136 tested positive for red blotch, nine tested positive for leafroll, and 11 tested positive for both red blotch and leafroll. In 2015, there were about 250 "red leaf symptomatic vines" that had tested negative for GLRaV in 2014. We are currently testing these suspect vines using new and more complete primers for both leafroll and red blotch.

Additionally, in September 2015 we began to map and test for GRBaV (using the protocols described previously) at the same five established vineyards mentioned in Objective 2. At each site, an area consisting of six rows by 20 vines per row (120 vines/site total) was visually evaluated for GRBaV and petiole samples were collected from each vine for testing with the most up-to-date primers. The idea is to return to these same blocks in September of

2016 and 2017 to repeat this detailed mapping in order to evaluate if the virus appears to be spreading from vine to vine.

CONCLUSIONS

In 2015 we evaluated three candidate vectors using modified experimental protocols that were designed to overcome perceived limitations in previous transmission experiments from 2013-2014. To date, none of the candidate vectors have tested positive for GRBaV and no transmission has been observed, although we continue to test vines from previous trials and are planning to evaluate additional vectors in 2016. Many novel insects have been collected from vineyard sites where movement of GRBaV is suspected, and again to date none have tested positive for GRBaV, although many specimens are still in the process of being tested. Finally, an ongoing, long-term GLD mapping effort in Napa County is now being complemented by additional mapping of GRBaV at five additional vineyard sites. Results from the new mapping effort will not be available until follow-up mapping can take place in the fall of 2016.

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BIOLOGY AND SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT
Grapevine red blotch-associated virus (GRBaV) was isolated from table and winegrapes, as well as rootstocks, affected by red blotch, a recently recognized viral disease. Analysis of the genetic diversity among isolates of GRBaV indicated the existence of two groups (clades) of genetic variants (Krenz et al., 2014; Al Rwahnih et al., 2015). Producing a full-length infectious clone of a representative isolate of each of the two clades showed systemic GRBaV infection of healthy grapevines following agroinoculation and the manifestation of typical disease symptoms, i.e., interveinal reddening on *Vitis vinifera* cvs. Cabernet franc, Cabernet Sauvignon, Syrah, Pinot noir, and Pinot gris; and chlorotic and necrotic leaf areas on *V. vinifera* cv. Chardonnay, while infection was latent in rootstock genotype 3309C. This work revealed that GRBaV isolates of both clades cause red blotch disease. Analysis of the spatio-temporal incidence of GRBaV in a selected vineyard of Cabernet franc in California and in New York was consistent with the occurrence of virus spread in the former but not in the latter vineyard. GRBaV isolates spreading in California corresponded to phylogenetic clade 2. A survey of alternate hosts in proximity to the diseased vineyard in California showed some free-living grapevines infected with GRBaV, suggesting the existence of a hemipteran vector. Insect sticky traps placed in the section of the California vineyard with extensive clustering of diseased vines in 2014 and 2015 revealed a diversity of insect families, genera, and species that visited the vineyard, among which the majority of specimens of three species consistently tested positive for GRBaV in polymerase chain reaction (PCR). These species are investigated for their potential to transmit GRBaV in controlled conditions in the greenhouse.

LAYPERSON SUMMARY
Red blotch is a newly recognized viral disease of grapevines that is widely distributed in United States vineyards. Limited information is available on spread of its associated virus called grapevine red blotch-associated virus (GRBaV) and on the association between virus variability and pathogenicity. We showed that GRBaV isolates cause red blotch disease, regardless of their genetic makeup and variability. Studying changes in virus prevalence over time in selected vineyards of Cabernet franc in California and New York revealed an increased virus incidence in the California but not in the New York vineyard. Free-living grapevines proximal to diseased vines in the California vineyard were found infected with GRBaV, suggesting their potential role as alternate host. Among insects visiting the California vineyard three species were found to carry the virus, suggesting a role as vector. Determining the capacity of these three insect species at transmitting GRBaV is underway.

INTRODUCTION
Red blotch is a recently recognized disease of grapevines (Calvi, 2011; Sudarshana et al., 2015). It was described for the first time on Cabernet Sauvignon at the University of California Oakville Research Field Station in 2007 (Calvi, 2011). Leaves of GRBaV-infected vines of red winegrapes show red specks and blotches (**Figure 1**) first on old leaves at the bottom of the canopy in late June or July. Symptoms progressively appear upward in the shoots over time. Veins underneath the leaf blade often turn partly or fully red. For white winegrapes, foliar symptoms are less conspicuous; they correspond to localized and generalized foliar discoloration or chlorosis, sometimes combined with necrotic areas at the edge of leaf blades (**Figure 1**).

Diagnosis based on specific symptoms can be challenging because of several confounding factors, including striking similarities between foliar symptoms elicited by red blotch and leafroll. There are also similarities between foliar symptoms of red blotch and abiotic factors such as poor root health, or physical injuries due to trunk or shoot girdling, mite damage, mineral deficiencies, or even the presence of *Xylella fastidiosa* or *Agrobacterium tumefaciens* in young vines. Because symptom variation makes visual diagnosis of GRBaV-infected vines difficult, only DNA-based assays such as polymerase chain reaction (PCR) are reliable for accurate diagnosis (Sudarshana et al., 2015).

Figure 1. Foliar symptoms of red blotch on Pinot noir (top) and Chardonnay (bottom).

GRBaV was isolated from grapevines affected by red blotch disease (Sudarshana et al., 2015). GRBaV is a putative member of a new genus in the family *Geminiviridae* (Varsani et al., 2014; Sudarshana et al., 2015). It has a single-stranded DNA genome that codes for six open reading frames (Al Rwahnih et al., 2013; Krenz et al., 2012; Poojary et al., 2013; Seguin et al., 2014). The role of GRBaV in the etiology of red blotch disease was investigated using agroinoculation of tissue culture-grown grapevines with a partial dimer or bitmer construct of the GRBaV genome of a representative isolates of each of the two phylogenetic clades (See section on **Results and Discussion**). Results indicated that GRBaV is the causal agent of red blotch disease; red blotch symptoms were reproduced on red and white winegrape cultivars and the genome of the virus recovered from infected vines was nearly identical to the one used as inoculum (Fuchs and Perry, unpublished). In parallel to our efforts to fulfill Koch's postulates, we used microshoot tip culture to sanitize GRBaV-infected material. Following virus elimination treatment, some vines remained symptomatic while others were asymptomatic. Symptomatic plants were positive for GRBaV in PCR while most of the asymptomatic plants were negative. Plants were carried through one dormancy period and tested again for GRBaV. Out of 54 vines obtained after therapy, 13 tested negative and 41 tested positive for GRBaV by PCR. Plants that were PCR positive exhibited typical red blotch symptoms while PCR negative plants were asymptomatic (Fuchs and Perry, unpublished).

Analysis of the genetic diversity among isolates of GRBaV indicated the existence of two groups (clades) of genetic variants (Krenz et al., 2014). The majority of isolates belong to the predominant clade 2 and recombination is underlying some of the variation seen among GRBaV genomes within clade 1. The two groups of isolates are involved in the etiology of the disease but it is not known if mixed infections by representative isolates of each group can have a synergistic effect and exacerbate the negative impact on production.

Based on the nucleotide sequence information available, primers were designed in the coat protein and polymerase genes to amplify a specific product from nucleic acid or DNA extracts of grapevines showing red blotch symptoms by PCR in either single or multiplex assays (Krenz et al., 2014). These primers are robust for the detection of GRBaV in leaves, petioles, and bark scrapings of dormant canes (Krenz et al., 2012). Interestingly, GRBaV has been detected from almost any tissue (petiole, leaf, cluster, roots, cambium, and fruit skin and juice) and from budbreak to post-harvest although the optimal tissue and sampling time remain to be determined.

GRBaV was documented in major grape-growing U.S. States (Krenz et al., 2014). The virus was also reported in British Columbia (GenBank JX559642.1) and Ontario (Fuchs, unpublished observations) in Canada, indicating its widespread presence in North America (**Figure 2**).

Figure 2. Occurrence of GRBaV in North America. Stars represent U.S. states and Canadian provinces with documented presence of the virus in vineyards.

GRBaV was found in table grapes, winegrapes, French-American interspecific hybrids (Chambourcin and Chardonnel), and rootstocks (101-14, 420A, 5C, and 039-16). The widespread occurrence of GRBaV and its wide geographic distribution in North America suggest that propagation material has played a significant role in its dissemination.

The ziczac leafhopper (Virginia creeper; *Erythroneura ziczac)* was claimed to transmit GRBaV from vine to vine in the greenhouse (Poojari et al., 2013) but a vector of GRaBV of epidemiological significance in vineyards has yet to be identified. Tremendous progress was made in recent years on the biology of GRBaV, but there are several gaps in knowledge for which research is needed. For example, it is paramount to expand ongoing studies to determine if and how GRBaV spreads in vineyards. This research is important not only to document the occurrence and extent of vector-mediated transmission of GRBaV but also to identify insect vector candidates. This is a prerequisite for transmission assays to determine if vectors can transmit GRBaV and for developing optimal management strategies. The development of serological detection assays for GRBaV is of interest not only to complement PCRs but also to provide the wine and grape industry with cheaper tools that have the potential for more high throughput screening. Knowing that GRBaV is the causal agent of red blotch disease, it is critical to evaluate the role of environmental factors in symptom development and to assess the range of symptoms in field-grown vines experimentally inoculated with GRBaV. Similarly, it is important to develop a better understanding of interactions between GRBaV isolates from different clades in terms of synergism and increased virulence, hence, a greater impact on vineyard production. Finally, disseminating information to the industry is essential to extend research and share the latest knowledge on red blotch disease and GRBaV, its causal agent. Raising awareness on the potential threat of GRBaV to grape production and communicating on strategies to mitigate its impact are major components of this proposal.

Most vineyard managers and vintners report ripening issues with GRBaV-infected winegrapes (**Figure 3**). Reductions of 1-6°Brix have been consistently documented in fruits of infected vines, as well as lower berry

anthocyanin and skin tannins, particularly in red winegrapes such as Cabernet franc and Cabernet Sauvignon (Calvi 2011; Sudarshana et al., 2015). Based on the effect of GRBaV on fruit quality and ripening, several growers are culling infected vines and replacing them with clean, virus-tested ones.

Figure 3. Delayed ripening of infected (left) compared to healthy (right) fruits of Pinot noir.

OBJECTIVES

The overarching goal of this project is to advance our understanding of red blotch disease and its causal agent, GRBaV, with a major emphasis on horizontal spread in vineyards and optimized detection methodologies. Our specific objectives are to:

1. Investigate spread of GRBaV in selected vineyards in California and New York.
2. Improve diagnostics for GRBaV.
3. Determine if either of the two groups of GRBaV isolates show greater virulence and pose an increased threat to vineyard production.
4. Disseminate research results to farm advisors and the industry.

RESULTS AND DISCUSSION

To address objective 1 and study spread of GRBaV, two vineyards of Cabernet franc were selected, one in California and one in New York. The California and New York vineyards were planted in 2008. In 2013 and 2014, virus prevalence was determined in the two selected vineyards. This information served as a baseline to determine the spatio-temporal incidence of GRBaV. A comparative analysis of the infection rate of GRBaV as measured by the number of symptomatic vines in the selected vineyard in California between 2014 and 2015 indicated a 1.5% increase, suggesting the possibility of virus spread (**Figure 4**). In addition, an investigation of the spatial distribution of symptomatic vines through an ordinary runs analysis, a statistical test for randomness of infected plants, revealed disease clustering in the majority of rows within the selected vineyard ($-Z > 1.64$ in 32/44 rows). These data confirmed the occurrence of GRBaV spread in the California vineyard as a result of either vine-to-vine transmission within the selected vineyard or of an influx from adjacent vineyards. Characterizing 10 randomly selected GRBaV isolates in the selected Cabernet franc vineyard in California by PCR followed by sequencing indicated that they all correspond to the phylogenetic clade 2 that was previously reported (Krenz et al., 2014).

Spread of GRBaV was further studied in the vineyard area with extensive clustering of symptomatic vines (top middle area of the maps in **Figure 4**). This area consists of 10 consecutive rows of 25 vines each (**Figure 5**). Symptomatic and asymptomatic vines were mapped in this area in 2013 and 2014. In addition, the presence or absence of GRBaV was confirmed in individual vines by PCR in spring and winter 2014 by using leaf and cane material, respectively (**Figure 5**). Data showed an increase of symptomatic vines from 47% (118 of 250 vines) in 2014 to 67% (168 of 250) in 2015. These results further support the occurrence of short distance spread of GRBaV in the California vineyard. These findings are being confirmed by testing the presence or absence of GRBaV by PCR in leaf samples that were collected from each of the 250 test vines in late summer 2015.

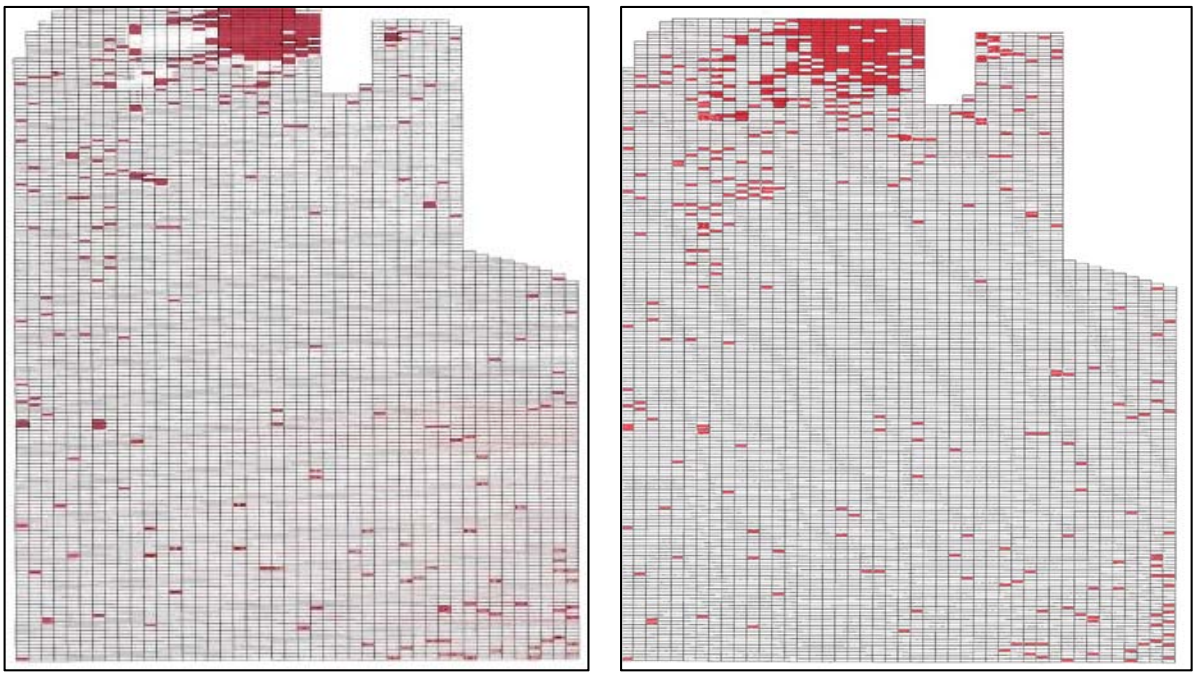

Figure 4. Spatial distribution of vines showing red blotch symptoms (in red) in a Cabernet franc vineyard in California in 2014 (left) and 2015 (right).

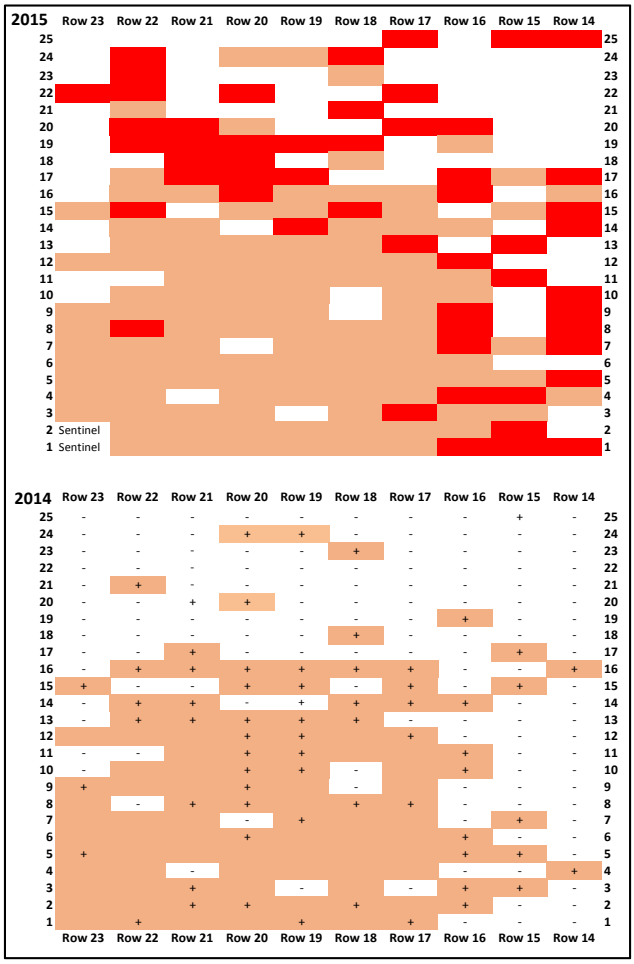

Figure 5. Clustering of symptomatic vines in 2014 (bottom map) and 2015 (top map) in a selected area of the California vineyard. Symptomatic vines in 2014 and 2015 are shown in salmon and red, respectively. Vines that tested negative and positive for GRBaV in PCR in 2014 are indicated with (-) and (+), respectively.

Close to 100 sentinel vines, i.e., healthy vines for which the mother stocks from which scion budwood and rootstock canes were collected from tested negative for GRBaV, were planted in the Cabernet franc vineyard in California in spring 2015. Some of these sentinel vines are shown at the bottom left of **Figure 5**. These vines will be used to gain direct evidence of insect-mediated GRBaV spread if they become infected. Sentinel vines replaced existing vines that were weak, regardless of their GRBaV infectious status.

The fact that extensive clustering of diseased vines occurred in one area of the selected vineyard in California (see top middle area of the maps in **Figure 4**) provided an incentive to investigate the occurrence of alternate hosts in the proximal riparian ecosystem. Samples of oak, willow, walnut, oat, vetch, and free-living grapevines were collected and tested for GRBaV in PCR. Among the samples tested, only those from free-living grapevines tested positive for GRBaV. These results suggested that free-living grapevines can potentially serve as an alternate host of GRBaV. They also supported the existence of a hemipteran vector of GRBaV as infected free-living grapevines were at least 150 away from the nearest infected vine in the Cabernet franc vineyard. Fingerprinting of the GRBaV-infected free-living grapevine samples indicated that they corresponded to hybrids of *Vitis californica* x *V. vinifera*. Characterizing the GRBaV isolates in free-living grapevines by PCR and sequencing indicated they cluster in phylogenetic clade 2, as did the isolates from the diseased Cabernet franc vineyard, further providing a link between the test vineyard and its proximal riparian area in terms of virus spread.

Insect sticky traps were placed in the area of the selected vineyard in California where clustering of diseased vines is occurring (see top middle area of the maps in **Figure 4**). Traps were placed on diseased and healthy grapevines from early April to late November in 2014 and 2015 with the goal of catching insects visiting the vineyard. Traps were rotated on a weekly basis. Each trap was analyzed for the presence of insects to establish a census population and identify them at the species level, if possible, by using morphological parameters. Then, a sub-set of each insect family, genus or species that was caught was removed from the traps and tested for the presence of GRBaV by PCR. Results indicated that specimens of three species, among more than 45 species of Diptera, Apocrita, Coleoptera, Cicadellidae, Thysanoptera, Aphidae, Fulgoroideae, Phylloxera, Aleyrodidae, Membracidae, Blissidae/Lygaeidae, Psyloidea, Psocoptera, Cixiidae, and Miridae that were caught on sticky traps, consistently carried genetic elements of GRBaV. These three species were members of the Membracidae, Cicadellidae, and Cixiidae. These findings suggest that these three species can acquire GRBaV in the vineyard. Testing the capacity of these three hemipteran insects at transmitting the virus to healthy grapevines in the greenhouse is under way.

A spatio-temporal analysis of a Cabernet franc vineyard in New York in 2013-2015 did not provide any evidence of an increased prevalence of GRBaV over time. These findings suggested that a GRBaV vector does not exist in the New York vineyard ecosystem or it exists at a very low population density or it exists but does not visit the vineyard. Alternatively, the plant protection program used by the vineyard manager in New York is effective at reducing the vector population.

To address objective 2 and improve diagnostics for GRBaV, efforts are ongoing with regard to the development of a robust real time PCR methodology and the refinement of the strategy needed to produce an antiserum. This work is critical because preliminary efforts to develop an antiserum against the structural coat protein have failed (Perry and Fuchs, unpublished).

To address objective 3 and determine if either of the two groups of GRBaV isolates show greater virulence and pose an increased threat to vineyard production, we engineered infectious clones of a representative GRBaV isolate of clade 1 and clade 2. Partial dimer constructs of the genome of GRBaV isolates NY358 and NY175 were engineered and cloned into a binary plasmid for mobilization into *Agrobacterium tumefaciens* strains LBA4404 or C58. Isolates NY175 from *V. vinifera cv.* Merlot and NY358 from *V. vinifera cv.* Cabernet franc belong to GRBaV phylogenetic clades 1 and 2, respectively (Krenz et al., 2014). These clones were used in agroinoculation experiments using healthy, tissue culture-grown vines of *V. vinifera* cvs. Cabernet Sauvignon, Cabernet franc, Syrah, Chardonnay, Pinot noir, and Pinot gris, and rootstock genotypes SO4 and 3309C that tested negative for GRBaV by PCR (Krenz et al., 2014). Tissue culture-micropropagated grapevines (30-40 per genotype) showing four to six leaves (Alzubi et al., 2012) were selected for agroinoculation experiments using vacuum-assisted infiltration. Alternatively, grapevine tissue was gently pricked with needles dipped in a solid A*grobacterium* culture grown on a Petri plate. A β-glucuronidase gene construct containing an intron was used as control to optimize conditions for agroinfiltration-mediated delivery of DNA. Constructs of both genomic RNAs of grapevine fanleaf virus (GFLV) were used as negative control in agroinfiltration experiments. Following

agroinfiltration and/or pricking, plants were maintained at 25±2°C and 33-45 $\mu Em^{-2}s^{-1}$ (16-hour photoperiod) in a tissue culture growth room for two to three months prior to establishment in a greenhouse for symptom observations and testing. The presence of GRBaV was tested by PCR in newly developed leaves of agroinoculated grapevines by using specific primers designed in the putative coat protein and replicase-associated genes, and the 16S ribosomal RNA used as a housekeeping gene (Krenz et al., 2014). Plants were tested three to ten months post-agroinfiltration and some of them were also tested after one or two dormancy periods. The full-length genomic sequence of some of the GRBaV progeny was determined in a few selected agroinfected plants by rolling circle amplification, cloning and sequencing.

A number of treated vines of Cabernet Sauvignon, Cabernet franc, Syrah, Pinot noir, Pinot gris, and Chardonnay showed red blotch-like symptoms at one to three months post-treatment. Foliar symptoms consisted of interveinal reddening in red-berried cultivars and chlorotic spots in the white-berried cultivar Chardonnay. Unlike for winegrape cultivars, agroinoculated SO4 became symptomatic (chlorosis and cupping) only after one dormancy period, whereas agroinoculated 3309C remained asymptomatic. Some of the grapevines agroinfiltrated with the NY358 construct (28-76%) tested positive for GRBaV by PCR. All the PCR-positive plants were symptomatic, while the negative plants were asymptomatic. None of the plants treated with GFP (0 of 237), GFLV-derived constructs (0 of 476), or untreated plants (0 of 56) exhibited red blotch-like symptoms, nor those that were assayed tested positive for GRBaV in PCR. The virus detected in symptomatic, agroinoculated vines was further characterized by sequencing. Sequence analysis indicated a 99.6-99.9% identify with the partial dimer construct used as inoculum in agroinfection assays, indicating that the recovered GRBaV variant is nearly-identical to the engineered inoculum. Similar results were obtained from agroinfiltration experiments with the NY175 construct. These findings were consistent with our hypotheses that GRBaV is the causal agent of red blotch disease and that GRBaV isolates from the two phylogenetic clades are equally infectious. In agroinfiltrated plants, the detection of GRBaV correlated with symptoms and virus progeny nearly identical in sequence to the inoculated partial dimer genomic construct was obtained from agroinfiltrated plants.

To address objective 4 and disseminate information to farm advisors and the industry, research results will be communicated to farm advisors, extension educators, crop consultants, researchers, vineyard managers, and regulators at winter school meetings in California and New York. Some of the targeted venues are the Cornell Recent Advances in Viticulture and Enology conference on November 4 at the Industrial and Labor Relations (ILR) Conference Center in Ithaca, New York and the Napa Continuing Education Class Series 3 on November 10 in Yountville, California.

CONCLUSIONS

Isolates of each of the two phylogenetic clades of GRBaV cause red blotch disease symptoms in healthy *V. vinifera* grapevines following agroinoculation, confirming their etiological role. Analysis of the spatio-temporal distribution of symptomatic, infected vines suggested spread of GRBaV in a vineyard of Cabernet franc in California but not in New York. Free-living grapevines proximal to the diseased vineyard in California can be infected with GRaBV. The use of insect sticky traps followed by the analysis of a subset of insect species for the presence of GRBaV enabled us to identify three candidate vectors. This work is setting the stage for the identification of a hemipteran insect vector of GRBaV.

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EVALUATION OF COMMERCIAL ANT BAITS AS A COMPONENT OF AN INTEGRATED PEST MANAGEMENT PROGRAM FOR VINE MEALYBUG

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ABSTRACT
Vine mealybug (*Planococcus ficus*) is a destructive phloem-feeding pest in California vineyards. Vine mealybug can reach very large population densities. Feeding activity can debilitate vines while excrement and the associated sooty mold can contaminate clusters, making them unsuitable for harvest. Vine mealybug's cryptic habits - populations are typically found under the bark - complicate management, particularly with contact insecticides. An integrated pest management (IPM) program that relies on several tactics (insecticides, mating disruption, and biological control) can provide sustainable control of vine mealybug populations. Argentine ants (*Linepithema humile*) may disrupt IPM programs by interfering with the activity of biological control agents. Baits are an effective means to control ant populations and minimize their disruptions. We evaluated three commercially available granular bait products in a northern California vineyard and measured their effect on Argentine ant populations. We made three bait applications: March 14 and 15; April 15 and 16; and June 15 and 16, 2015. A modified broadcast fertilizer spreader was used to apply the baits under the vines. Argentine ant populations were measured indirectly via feeding activity, assessed as the amount of nontoxic sucrose water consumed by the ants. We measured feeding activity monthly from February (pre-treatment) through September. Feeding activity was significantly reduced in only one bait treatment, and on only one monitoring date. In July and August, ant feeding was low to non-existent across all treatments. Revising our monitoring frequency to shorter intervals early in the season (March to May) may allow us to capture more clearly the potential impact of baits on Argentine ant populations.

LAYPERSON SUMMARY
Vine mealybug is a destructive pest in California vineyards. It contaminates fruit and reduces vine health and productivity. Grape growers may use multiple tactics (integrated pest management; IPM) including insecticides, mating disruption, and biological control, to achieve control of vine mealybug populations. Argentine ants are invasive insects common in coastal California vineyards. Ants disrupt IPM programs for vine mealybug because they interfere with the activity of a small parasitic wasp that attacks vine mealybug. Ant baits are an effective approach to manage ant populations while minimizing impacts on non-target organisms. We are investigating the potential of three commercial bait products to control Argentine ants in vineyards. Baits were applied under the vine with a modified broadcast spreader in mid-March, mid-April, and mid-June 2015. Ant populations were monitored monthly from February to August 2015. Only one of the bait products impacted ant populations, and only on one monitoring date. Modifications of our monitoring strategy in 2016 may allow us to capture more clearly the impact of baits on ant populations.

INTRODUCTION
The vine mealybug, *Planococcus ficus*, is a destructive vineyard pest that contaminates fruit, debilitates vines, and vectors plant pathogens such as grapevine leafroll-associated virus-3 (Daane et al., 2012). First reported from vines in the Coachella Valley (Gill, 1994), vine mealybug soon spread throughout California, likely on infested nursery stock (Haviland et al., 2005). It is currently found in most California grape-growing regions (Godfrey et al., 2002; Daane et al., 2004a, 2004b) and has the potential to spread throughout the western United States.

Management of vine mealybug populations can prove challenging and often requires the use of multiple tactics, including biological control, mating disruption, and insecticides (Daane et al., 2008b). Management can be particularly complicated in coastal winegrape growing regions where vine mealybug populations are tended by Argentine ants, *Linepithema humile*. In the presence of tending ants, biological control of mealybugs can be significantly interrupted, resulting in large vine mealybug populations that may be more easily spread to new areas. These populations also contaminate the fruit, causing yield losses and decreased fruit quality. In vineyards

where Argentine ant is prevalent, management of ant populations is a critical part of an integrated pest management (IPM) program for vine mealybug and necessary for containment of insect populations (Nyamukondiwa and Addison, 2011; Mgochecki and Addison, 2009).

Liquid ant baits adapted from the urban environment (Klotz et al., 2002) for use in vineyards (Cooper et al., 2008) significantly reduce mealybug populations in vineyards by contributing to increases in biological control (Daane et al., 2007). The costs associated with the manufacture, deployment, and maintenance of bait stations have been prohibitive to widespread adoption of Argentine ant management in vineyards, despite the benefits that could result from such programs (Nelson and Daane, 2007).

There is continued interest among coastal grape growers in the development of a simpler and more economical bait program that could be widely implemented. Granular bait that can be broadcast with a commercial spreader could be distributed more quickly and frequently over a large area, and would not require the manufacture and maintenance of bait stations. Although granular baits have been shown to effectively reduce the population of foraging Argentine ants (Krushelnycky and Reimer, 1998) and the spread of ant populations (Krushelnycky et al., 2004) in natural areas, they have not been widely tested in agricultural settings. The sustained use of the baits could lead to longer-term containment and control of Argentine ant populations (Krushelnycky et al., 2004).

Granular baits have the potential to become an alternative to the liquid bait program. We are evaluating commercially available granular ant baits that can be broadcast under vines to control populations of Argentine ant. Ant control would in turn contribute to the sustainable control of vine mealybug populations. In the absence of an economical bait program, ant suppression must be achieved with the broad-spectrum insecticide chlorpyrifos that can disrupt populations of beneficial insects and pose vertebrate health risks.

OBJECTIVE

The broad goal of this research is to increase the efficacy and adoption of integrated pest management programs for vine mealybug, a destructive pest of grapevines in California. Our specific objective is to evaluate the efficacy of commercial baits to control Argentine ant as part of an integrated pest management program for vine mealybug.

RESULTS AND DISCUSSION

A crucial factor in success of ant baits is delayed toxicity. If the ants die too quickly (acute toxicity) they do not have time to exchange bait with other foragers, larvae, and the queen(s) in the colony. Bait with delayed toxicity can be transferred to other members of the colony, with the goal of eliminating the entire colony rather than just the foragers.

Our experiment was established in two vineyard blocks in Napa, California (Carneros American Viticultural Area; AVA). Both blocks were planted in 1999 and are a mix of Chardonnay clones (17-Robert Young and 6) on SO4 rootstock (*Vitis berlandieri* x. *V. riparia*). We used a randomized complete block design, and established six, six-row replicates of each treatment. The treatments were three commercial granular bait products (**Table 1**). In March, April, and June 2015 the cooperating vineyard manager applied the bait in the vine row with a modified broadcast spreader mounted on an all-terrain vehicle (ATV) (**Figure 1**). Because Altrevin and Extinguish are formulated with a protein attractant specifically for control of red imported fire ant (*Solenopsis invicta*), we included one Altrevin treatment in which the bait was coated with powdered sugar before application to make it more attractive to Argentine ants. The spinosad bait, Seduce, is formulated with a carbohydrate attractant (sugar) specifically to target the Argentine ant. Additionally, Seduce has been approved for use in organic vineyards. Since there are a limited number of insecticides approved for vine mealybug management in organic vineyards, ant bait can be an essential component of an IPM program in these vineyards.

Ant densities were determined indirectly as a measure of feeding activity, assessed as the amount of nontoxic sucrose water removed from 50-milliliter (ml) polypropylene centrifuge tubes (Corning Inc., Corning, NY) tied to the vine trunk (Klotz et al., 2002; Daane et al., 2008a) in the center two rows of each plot. The 50-ml tubes are henceforth referred to as monitoring tubes. A two-centimeter (cm) hole was drilled in the cap, and a square of permeable plastic mesh (Weedblock, Easy Gardener Inc., Waco, TX), was placed between the cap and the filled tube, covering the hole. The mesh is fine enough to retain the liquid when the tube is inverted, but coarse enough to allow ants to remove the liquid on contact. A second lid was fixed to the original lid, and covered with a

permanent mesh to discourage feeding by honeybees and wasps. Before the tubes were deployed in the vineyard, each tube was filled to 45 ml with 25% sucrose water and the weight of each tube was recorded. Tubes were inverted on a vine trunk for four to seven days (depending on ant activity), at a density of 12 tubes per plot, or a total of 72 tubes per treatment. At the end of the monitoring period, the tubes were brought back to the laboratory and the new weights were recorded. One additional monitoring tube per plot was attached to an ant-excluded bamboo stake to measure the amount of water lost to evaporation; this amount was averaged across all plots and used to adjust the final weight.

Table 1. Ant bait products applied in trial blocks in a Napa County vineyard.

Treatment	Active ingredient (concentration)	Rate per acre	Bait applications (2015)
Altrevin	metaflumizone (0.063%)	1.5 lb.	March 14 & 15; April 15 & 16; June 15 & 16
Altrevin & powdered sugar	metaflumizone (0.063%)	1.5 lb.	
Extinguish	hydramethylnon (0.365%) & methoprene (0.25%)	1.5 lb.	
Seduce	Spinosad (0.07%)	20 lb.	
Untreated	none	none	none

A

B

C

Figure 1. (A) Modified broadcast spreader mounted on ATV. **(B)** Altrevin bait in the hopper of the spreader. **(C)** Seduce bait (reddish pellets) under the vine row. All photos taken in a Chardonnay vineyard in Carneros AVA, California.
Photo credit: **(A)** and **(B)**: K. Taylor, Constellation Brands. **(C)**: M. Cooper, UC Cooperative Extension, Napa County.

We measured ant feeding activity with monitoring tubes during six periods: February 24 to March 3 (pre-treatment); March 24 to 31; April 24 to 28; May 26 to June 3; July 2 to 7; and August 6 to 11. We conducted an additional monitoring September 18 to 25; however, bees foraging at the tubes removed large quantities of sugar water so the September data were discarded. We are reporting the amount of feeding activity by ants as grams (g) of sugar water removed from monitoring tubes per day (**Figure 2**). During the February and March monitoring periods (pre-treatment and 10 days after the first treatment, respectively) ant feeding activity was not significantly different across all treatments. This is not surprising, since we blocked for consistent ant populations prior to treatment, and baits have delayed toxicity and would not be expected to control populations so quickly (10 days) after application. During the April 24 to 28 monitoring period, feeding activity was significantly reduced in the Seduce bait treatment (Tukey's pairwise comparison, p = 0.0099); this is roughly six weeks after the first bait application and one week after the second. From May 26 to June 3, feeding activity in the Seduce treatment (-0.007 +/- 0.12 g per day) was reduced compared to other treatments (0.52 to 0.92 +/- 0.35 to 0.55 g per day), although the difference was not statistically significant due to the high variability in ant populations, particularly in the Altrevin and untreated blocks. During the July and August monitoring periods, ant feeding was low to none in all treatments. In other ant bait trials, we have detected similar feeding lulls at our monitoring tubes during the summer (Daane et al., 2006, 2008a). We did not see any differences in population suppression between the powdered sugar-coated bait and those protein-based baits without powdered sugar. Since the sugar is not an inert ingredient of the bait, it may not adhere well to the bait. It could have been removed during the application process or not durable in the field. At this point, there does not appear to be a measurable improvement in bait performance through the addition of the powdered sugar under these conditions. Adverse effects were noticed as the sugar heated (and melted) in the spreader, thereby clogging the mechanisms of the spreader that impacted application efficiency and necessitated additional disassembly/cleaning time. Overall, the collaborating vineyard manager concluded that the Seduce bait was the easiest to apply; we attributed this to weight and consistency of the bait as well as application rates (higher rates made the applied bait more visible, and therefore easier to calibrate the spreader and adjust drive speeds). This study will continue in 2016, and we will shorten our monitoring intervals to 14 days from April to June to more clearly elucidate the impacts of bait during this key ant foraging period. This is also a critical period for early-season control of vine mealybug; concurrent control of Argentine ant populations has the potential to reduce vine mealybug populations through an increase in biological control.

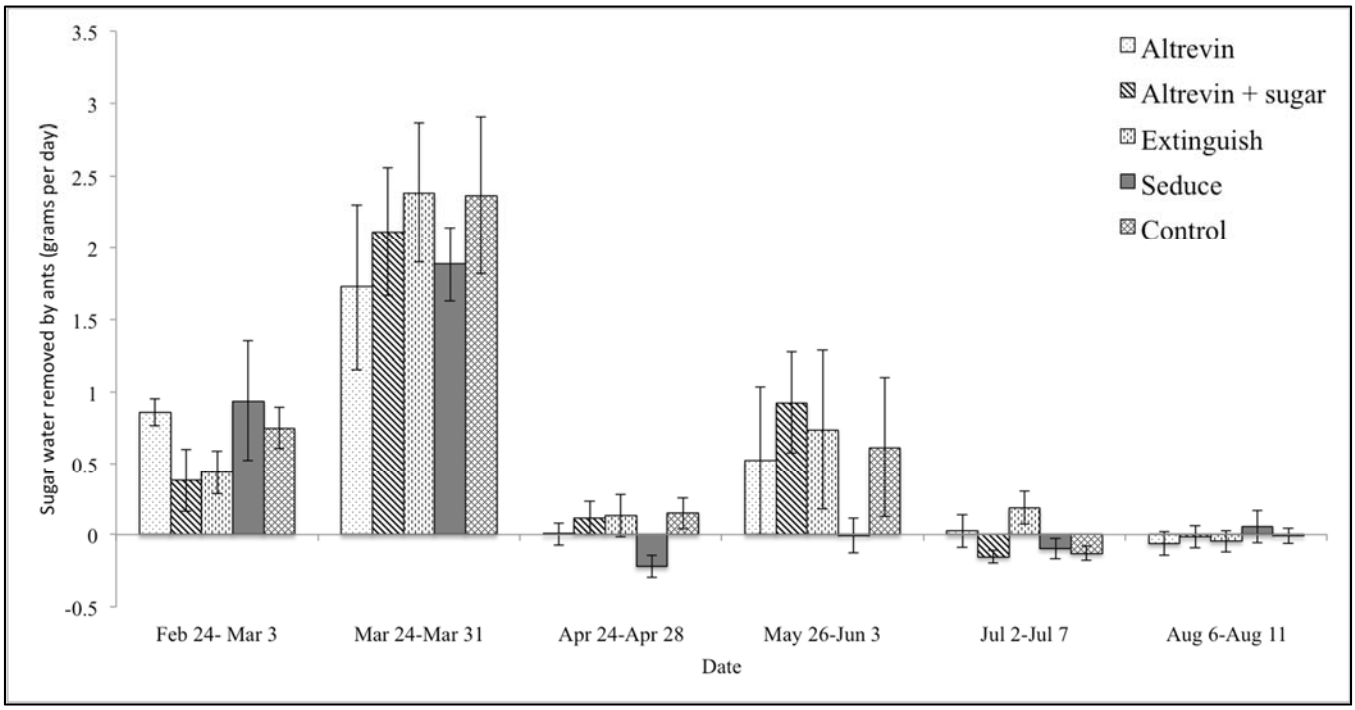

Figure 2. Average sucrose water removed (grams per day) from monitoring tubes by Argentine ants, during six monitoring periods in a Chardonnay vineyard (Carneros AVA) in 2015. Results are reported for each bait treatment and the untreated control. During the April 24 to 28 monitoring period, feeding activity was significantly reduced in the Seduce bait treatment (Tukey's pairwise comparison, p = 0.0099). On all other dates, there were no significant differences among treatments.

CONCLUSIONS

We evaluated three granular bait products for control of Argentine ant populations in a coastal California vineyard. Because Argentine ants disrupt biological control of vine mealybug by interfering with the activity of predators and parasitoids, control of Argentine ants can be an essential component of IPM programs for vine mealybug. Granular baits that can be broadcast under vines are a more manageable alternative to the current stations with liquid baits used in vineyards. Handling and distribution of granular baits is simpler and more efficient than liquid baits that must be contained within bait stations. Additionally, Argentine ant nests are typically multiple and widely dispersed throughout agricultural ecosystems in the spring, summer, and fall (Markin, 1970) so multiple point-sources make bait more accessible to all nests within an infested area (Boser et al., 2014). Our results suggest that one of the baits, Seduce, which is formulated with a sugary attractant, has the potential to control populations of Argentine ant, particularly early in the growing season (April and May). This is also a critical period to control vine mealybug because early-season population control, while the fruit is developing, can reduce pest pressure later in the season. Later-season vine mealybug populations are more likely to cause economic damage by infesting and soiling the fruit, thus subjecting it to potential rejection by the processor. In 2016 we will continue to evaluate these bait products.

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IMPROVING VINE MEALYBUG WINTER AND SPRING CONTROLS

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ABSTRACT
The vine mealybug, *Planococcus ficus,* has become one of the more important insect pests of California vineyards. Insecticides do not always provide complete control of the vine mealybug population under the bark of the trunk or on the roots. Our first objective is to improve pre- and post-harvest insecticide application to control this overwintering population. Our second objective is to develop temperature-based models to better predict the spring emergence of the mealybug crawlers in order to better time spring foliar insecticide treatments. In the first four months of this project we have made considerable progress towards our first objective, taking samples from vineyards in Napa and Lodi-Woodbridge winegrapes and San Joaquin Valley table grapes, winegrapes, and raisin grapes. At each site we have counted mealybug densities on the vine and taken vine samples (sections from the leaf, cane, and trunk) to be analyzed for the presence of the insecticide spirotetramat and its enols. These samples are being stored at -20 °C to be later processed by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (MS-GC).

LAYPERSON SUMMARY
The vine mealybug, *Planococcus ficus,* has become one of the more important insect pests of California vineyards, threatening economic production and sustainable practices in this multi-billion dollar state industry. Researchers have improved biological and chemical controls, but this pest remains in vineyards and can quickly build in numbers during the summer and damage the crop near harvest time. One reason that insecticides do not provide complete control is that a portion of the vine mealybug population remains under the bark of the trunk or on the roots and emerges from this refuge in the spring and summer. Our first objective is to improve pre- and post-harvest insecticide application to control this overwintering population. Our second objective is to develop temperature-based models to better predict the spring emergence of the mealybug crawlers in order to better time spring foliar insecticide treatments. In the first four months of this project we have made considerable progress towards our first objective, taking samples from vineyards in Napa and Lodi-Woodbridge winegrapes and San Joaquin Valley table grapes, winegrapes, and raisin grapes. At each site we have counted mealybug densities on the vine and taken vine samples (sections from the leaf, cane, and trunk) to be analyzed for the presence of the insecticide spirotetramat and its enols. These samples are being stored at -20 °C to be later processed by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (MS-GC).

INTRODUCTION
The vine mealybug, *Planococcus ficus*, has become one of the more important insect pests of California vineyards, threatening economic production and sustainable practices in this multi-billion dollar state commodity. Insecticides are the primary control tool for vine mealybug (Daane et al., 2006; Prabhaker et al., 2012; Daane et al., 2013; Bentley et al., 2014), especially when grapevine leafroll diseases (GLDs) are a concern (Daane et al., 2012). The vine mealybug population is primarily on the trunk and upper root zone near the soil line during the winter and early spring (Daane et al., 2013). This population has a refuge from natural enemies (Daane et al., 2008; Gutierrez et al., 2008) and can be the most difficult to control with insecticide applications (Daane, pers. observation). Moreover, mealybugs can remain on even the remnant pieces of vine roots after vineyard removal, hosting both pathogens and the mealybug (Bell et al., 2009).

A delayed dormant (typically in February) application of chlorpyrifos (Lorsban®) was the standard post-harvest or pre-season control (Daane et al., 2006), but more recent work suggests that a post-harvest application of spirotetramat (Movento®) provides equal or better control in some regions of the state (Haviland, pers. comm.). Still, effectiveness will depend on application timing, soil moisture, vine condition and age and commodity (for example, post-harvest application timing). Our objectives are to improve pre- or post-harvest controls that target

the winter-spring vine mealybug population and to better determine the spring emergence of vine mealybug crawlers in order to better time foliar applications.

Researchers have developed relatively good controls that target exposed vine mealybugs (i.e., those on the leaves or canes). However, controlling the more protected mealybug population found under the bark of the trunk or on the roots has been more difficult, both for biological controls and insecticides. The application of insecticides with systemic action has helped control this protected population, but their proper use appears to vary among vineyards and regions. Work in Kern County has helped provide guidelines for insecticide use in table grapes in this region (Castle, Haviland, and Prabhaker, unpubl. data). However, similar studies in the central San Joaquin Valley and north coast winegrapes should also be conducted. Typically, vineyards with mealybug damage have large over-wintering populations that are never fully regulated, and annually are the source for new generations throughout the summer that infest leaves and fruit of that vineyard and can disperse to other vineyards. Therefore it is critical to develop better control programs for this overwintering population.

Our proposed work will improve pre- and post-harvest systemic insecticide treatments and produce vine mealybug temperature development models that will better predict emergence of crawlers from these protected locations in order to time foliar insecticide treatments. This information will be disseminated to farmers, pest control advisors, and extension personnel, thereby having a practical, direct, and immediate impact on insecticide application methods. Because these are straightforward and applied objectives, we foresee the insecticide trials conducted in the two-year timeframe and the temperature model developed in the first year and field validated in the second year of the proposed project. If these results suggest other variables significantly impact insecticide effectiveness than these will be tested in a future proposal.

OBJECTIVES

The proposal seeks to develop better controls for the overwintering vine mealybug population found primarily under the bark of the trunk or on the roots at the soil line.

1. Investigate population dynamics and controls for overwintering vine mealybugs.
2. Determine the temperature relationship of vine mealybug and grape mealybug to better predict spring emergence and spray timing.

RESULTS AND DISCUSSION

Objective 1. Insecticide controls for the vine mealybug.

Commercial vineyards were selected in the central San Joaquin Valley (Fresno County) with four vineyard blocks near Fresno (one Thompson Seedless raisin grapes, one Crimson Seedless table grapes, and two Thompson Seedless table grapes); the Lodi-Woodbridge winegrape region (San Joaquin County) with three vineyards near Lodi (one Cabernet Sauvignon, one Pinot Noir, and one Chardonnay); and North Coast winegrape region (Napa County) with two vineyards at a site in the Carneros region of Napa (one Pinot Noir and one Chardonnay). We are also sampling numerous 'experimental' vineyard blocks at the Kearney Agricultural Research and Extension (KARE) Center that represent wine and table grape blocks undergoing studies for nitrogen, irrigation, and winegrape cultivars. In the first year the important aspect is to find different types of vineyards and apply the same treatments to better determine treatment effects across different vineyard ecosystems and management practices.

The treatment is different Movento application timings, as measured by calendar date as well as by weeks before or after harvest (Movento has a seven-day pre-harvest-interval). We applied Movento at the label rate and determined the percentage kill of mealybugs on different sections of the vine during the fall (completed), spring, and winter. A standardized application method will be used across all vineyards so that surfactant and application rate will not be an influence.

As this study has just begun we are currently only looking at the standard (spring-summer) treatment. There will be one to two pre-harvest applications and one to two post-harvest application timing treatments.

At each site, there are 10-15 replicates (individual vines) per treatment per vineyard, with treatments placed in a complete randomized design. Pre-treatment mealybug counts were taken using a time count (Geiger and Daane, 2001). In brief, on each sampled vine, an experienced sampler searched for mealybugs for a three-minute period. The areas of the vine searched change with the seasonal movement of the mealybug population (i.e., during the

winter the roots and lower trunk sections are the most likely regions to find vine mealybug). The pre-treatment mealybug density will then be used to block treatments against density because vineyard mealybug populations can be clumped.

Post treatment effects will be measured with winter, spring, and mid-summer three-minute timed counts. Because timed counts can be biased based on where on the vine the sampler finds mealybugs, data will also be analyzed for per capita changes in density on each section of the vine sampled (i.e., trunk vs. leaves). We have just completed a measurement of economic damage on five clusters on each vine using a zero to three scale: zero means no mealybug damage, one means honeydew present but the bunch is salvageable, two means honeydew and mealybugs present but at least part of the bunch is salvageable, and three means a total loss.

The data are still being entered (harvest evaluations are ongoing). All data will first be screened for normality and equal variance, and transformed accordingly. Analysis of variance (ANOVA), linear regression, and correlation analyses will be used to compare mealybug density between treatments. For fruit damage ratings the treatment effects were compared using contingency tables; for all experiments with three or more treatments, pairwise comparisons (treatment × damage ratings) were made for all possible treatment combinations, with an experiment-wide error rate at alpha = 0.05/*n*, where *n* is the number of possible pairwise comparisons.

The most time-consuming aspect has been the collection of tissue samples on different parts of each treatment vine. These samples will be analyzed for the amount of spirotetramat and its enol(s), as an indication of the movement of the insecticide to different parts of the vine. Since July 2015, approximately 5,000 tissue samples had been collected. Every sample is individually identified and stored at -18°C. Five portions of the vine were sampled for living tissue: leaves and petiole, trunk above and below the girdle, cane, and arm. If girdle is not applicable, a bottom and middle part of the trunk was taken. If arm was not applicable, an upper part of the trunk was taken.

Currently we are in the initial steps of analyzing the samples using the high performance liquid chromatography (HPLC) methodology. In order to calculate the concentration of spirotetramat and its enol present in each sample, the sample should be prepared following the "Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS) extraction protocol. Following this protocol, we noticed that spirotetramat (SPTA) and the enol form eluted at 27 and six minutes, respectively (**Figure 1**). As predicted, in the samples that have been analyzed until the present, SPTA enol did elute at six minutes (**Figure 2**).

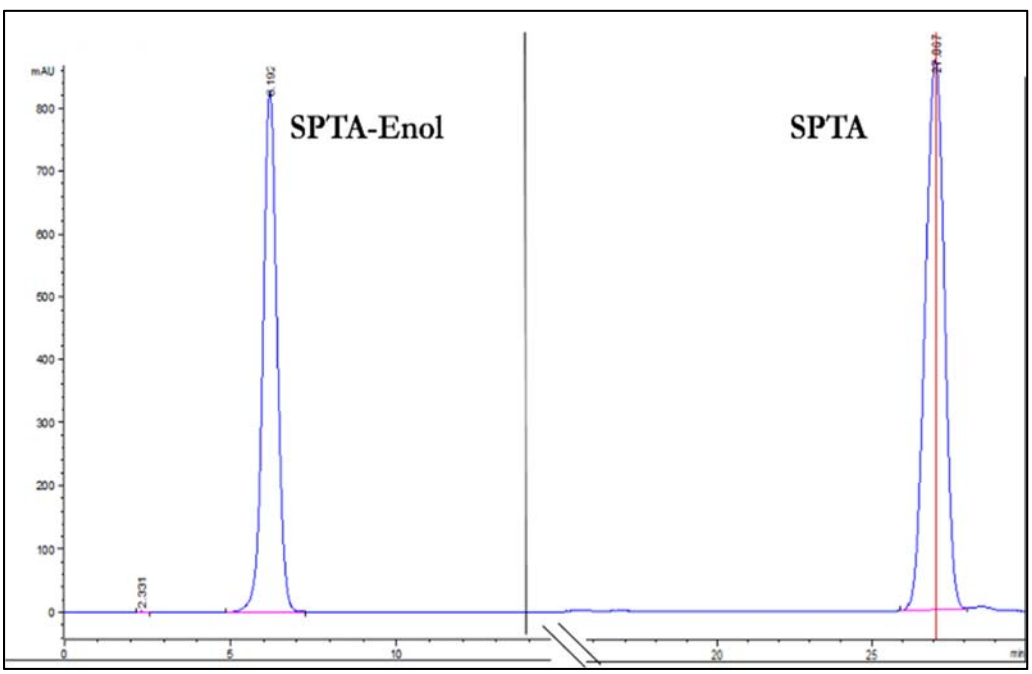

Figure 1. Spirotetramat enol (SPTA-enol) is eluted at 6.14 min and the parent compound, spirotetramat (SPTA) at 27 minutes.

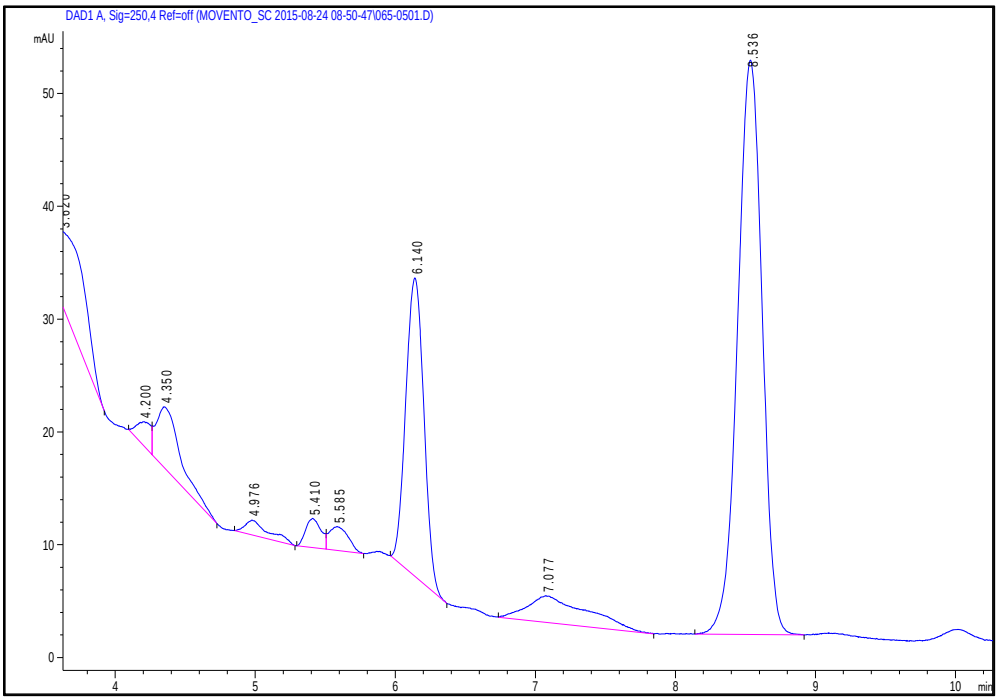

Figure 2. Example of one sample showing a spirotetramat enol peak that eluted at 6.14 minutes.

At this time, treatment vines at all sites have been sampled for vine mealybug, at least once and sometimes twice, and additional treatments of Movento have been applied for the pre-harvest treatments, with the exception of some blocks in Fresno and Napa that are still being harvested.

Taking into consideration all the samples areas, approximately 530 vines have been sample for live tissue, mealybug counts, and for cluster evaluation. Together, the treated vineyards include several factors that could be affecting the pesticide efficiency:

- Age of vineyards: 6- to 25-year-old vines
- Irrigation type: drip vs. flood
- Type of vines:
  - Table grapes (Crimson, Thompson)
  - Raisin grapes (Selma Pete, Thompson)
  - Winegrapes (Chardonnay, Pinot Noir, Cabernet Sauvignon, and multiple cultivars at KARE)
- Grafted vs. non-grafted
- Different rootstocks
- Presence of girdle
- Different pesticide application rates
- Geographical area
- Level of vine mealybug infestation and location on the vine

Mealybug counts and cluster evaluation data analyses are currently taking place.

Objective 2. Temperature development of vine mealybug.

Temperature development rates were previously determined for vine mealybug (Daane et al. unpublished), using temperature cabinets with constant temperatures at 12, 15, 18, 21, 24, 27, 30 and 32°C. During winter and spring 2015, these temperature development data will be analyzed using linear and nonlinear population growth models (Brière et al., 1999) to determine lower, optimal, and upper temperature thresholds. The information will be used to develop temperature models (e.g., University of California Integrated Pest Management website) to help determine crawler movement in spring (this is most important for the vine mealybug) and male flight periods (this is most important for the grape mealybug).

CONCLUSIONS

At this initial stage our study is on progress, with the summer-fall period used to select vineyards, collect tissue samples, and learn the protocols to determine levels of spirotetramat and its enol in the vine tissue samples. We selected sites in California's north coast winegrape region, Lodi-Woodbridge winegrape region, and San Joaquin Valley (Fresno County) vineyards and collected samples in two different periods: pre- and post-Movento applications. Additionally, we are also sampling numerous 'experimental' vineyard blocks at the Kearney Agricultural Research and Extension Center that represent wine and table grape blocks undergoing studies for nitrogen, irrigation, and winegrape cultivars. In the first year the important aspect is to find different types of vineyards and apply the same treatments to better determine treatment effects across different vineyard ecosystems and management practices.

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