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Note to Readers:

The reports in this document have not been peer reviewed.

TABLE OF CONTENTS

Section 1: <i>Xylella fastidiosa</i> and Pierce's Disease
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REPORTS

- **Assessing Pierce's Disease Spread in Grape Lines with Novel Defensive Traits**
Rodrigo Almeida 3
- **Evaluating Potential Shifts in Pierce's Disease Epidemiology**
Rodrigo Almeida 7
- **Exploiting a Chitinase to Suppress *Xylella fastidiosa* Colonization of Plants and Insects**
Rodrigo Almeida 13
- **Development of a Biological Control for Pierce's Disease**
Thomas J. Burr, Patricia Mowery, Luciana Cursino, and Lingyun Hao 15
- **Defining the Role of Secreted Virulence Proteins LesA and PrtA in the Pathobiology of *Xylella* and in the Development of Pierce's Disease**
Abhaya M. Dandekar 25
- **Field Testing Transgenic Grapevine Rootstocks Expressing Chimeric Antimicrobial Protein and Polygalacturonase-Inhibiting Protein**
Abhaya M. Dandekar 35
- **High Throughput Live Cell Screen for Small Molecules Targeting the TolC Efflux Pump of *Xylella fastidiosa***
Dean W. Gabriel 43
- **Evaluation of Pierce's Disease Resistance in Transgenic *Vitis vinifera* Grapevines Expressing *Xylella fastidiosa* Hemagglutinin Protein**
David G. Gilchrist (for Bruce Kirkpatrick, deceased) 49
- **Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual and Single DNA Constructs**
David G. Gilchrist and Abhaya M. Dandekar 54
- **Transgenic Rootstock-Mediated Protection of Grapevine Scions by Introduced Single and Dual Stacked DNA Constructs**
David G. Gilchrist, James E. Lincoln, Abhaya M. Dandekar, Steven Lindow, and David M. Tricoli 59
- **Conducting Pierce's Disease Symptom Evaluations at the Solano County Field Trial Research Block**
Deborah A. Golino 67
- **Monitoring Glassy-winged Sharpshooter and Pierce's Disease in Kern County, California**
David Haviland and Beth Stone-Smith 75

- **Comparison and Optimization of Different Methods to Alter Diffusible Signal Factor Mediated Signaling in *Xylella fastidiosa* in Plants to Achieve Pierce’s Disease Control**
Steven Lindow 81
- **Continued Field Evaluation of Diffusible Signal Factor Producing Grape for Control of Pierce’s Disease**
Steven Lindow 94
- **Field Evaluations of Grafted Grape Lines Expressing Polygalacturonase-Inhibiting Proteins**
Ann L.T. Powell and John M. Labavitch..... 109
- **Genome Editing of *TAS4*, *MIR828*, and Targets *MYBA6/A7*: A Critical Test of *Xylella fastidiosa* Infection and Spreading Mechanisms in Pierce’s Disease**
Chris Rock, Sunitha Sukumaran, Md. Fakhrul Azad, and Sy Mamadou Traore..... 121
- **Greenhouse Evaluation of Grapevine Microbial Endophytes and Fungal Natural Products for Control of Pierce’s Disease**
Philippe Rolshausen, Caroline Roper, and Katherine Maloney 130
- **Characterization of the Lipopolysaccharide-Mediated Response to *Xylella fastidiosa* Infection in Grapevine**
Caroline Roper and Dario Cantu 139
- **Characterization of *Xylella fastidiosa* Plant Cell Wall Degradation and Inhibition of the Type II Secretion Machinery**
Caroline Roper, Dario Cantu, Andrew McElrone, and Qiang Sun 152
- **Expanding the Range of Grape Rootstock and Scion Genotypes That Can Be Genetically Modified for Use in Research and Product Development**
David M. Tricoli..... 158
- **Breeding Pierce’s Disease Resistant Winegrapes**
Andrew Walker 167
- **Molecular Breeding Support for the Development of Pierce’s Disease Resistant Winegrapes**
Andrew Walker and Dario Cantu 178

ABSTRACTS

- **Plasmid Transfer by Conjugation as a Possible Route of Horizontal Gene Transfer and Recombination in *Xylella fastidiosa***
Lindsey P. Burbank and Drake C. Stenger 188
- **Study of the Taxonomy of *Xylella* Based on Whole Genome Sequences**
Jianchi Chen..... 189
- **The Diversity of *Xylella fastidiosa* in France**
Marie-Agnès Jacques and Claire de Boisséson 190
- **Functional Characterization of the Role of *RPFA* in *Xylella fastidiosa***
Hong Lin 191
- **Modeling Deployment of Pierce’s Disease Resistant Grapevines**
Mark Sisterson and Drake Stenger 192

<p>Section 2: Glassy-winged Sharpshooter</p>

- **The Riverside County Glassy-winged Sharpshooter Program in the Temecula Valley**
Matt Daugherty and Diane Soto 195
- **RNA Interference and Control of the Glassy-winged Sharpshooter and Other Leafhopper Vectors of *Xylella fastidiosa***
Bryce W. Falk 199
- **Selective Disruption of Glassy-winged Sharpshooter Maturation and Reproduction by RNA Interference**
Shizuo George Kamita 205
- **Substrate-Borne Vibrational Signals in Intraspecific Communication of the Glassy-winged Sharpshooter**
Rodrigo Krugner, Valerio Mazzoni, Shira D. Gordon, and Rachele Nieri..... 212
- **Monitoring for Insecticide Resistance in the Glassy-winged Sharpshooter in California**
Thomas M. Perring and Nilima Prabhaker 221
- **Management of Insecticide Resistance in Glassy-winged Sharpshooter Populations Using Toxicological, Biochemical, and Genomic Tools**
Richard Redak, Bradley White, and Frank Byrne..... 230

ABSTRACTS

- **Association of the Sharpshooter X Wave with Xylem Inoculation of *Xylella fastidiosa* Leading to Systemic, Symptomatic Pierce’s Disease Infection in Grape**
Elaine A. Backus, Holly Shugart, and Felix Cervantes..... 237
- **Grapevines Respond to Glassy-winged Sharpshooter Oviposition by Increasing Local and Systemic Terpenoid Levels**
Christopher M. Wallis and Rodrigo Krugner..... 238

<p>Section 3: Other Pests and Diseases of Winegrapes</p>

INSECTS

- **Evaluation of Commercial Ant Baits as a Component of an Integrated Pest Management Program for Vine Mealybug**
Monica L. Cooper and Lucia G. Varela..... 241
- **Improving Vine Mealybug Winter and Spring Controls: I. Bioassays. II. Using High Pressure Liquid Chromatography to Follow Insecticide Movement in the Vine**
Kent Daane, Sonet Van Zyl, Monica L. Cooper, Valeria Hochman Adler, and Geoffrey Dervishian 247

- **Brown Marmorated Stink Bug Risk and Impacts in Western Vineyards**
Vaughn Walton, Nik Wiman, Kent Daane, Frank Zalom, Monica L. Cooper, and Lucia G. Varela 257

VIRUSES

- **Searching for Potential Vectors of Grapevine Red Blotch-Associated Virus**
Kent Daane, Rodrigo Almeida, Monica L. Cooper, Deborah A. Golino, Houston Wilson, and Kai Blaisdell..... 267
- **Biology and Spread of Grapevine Red Blotch-Associated Virus**
Marc Fuchs, Keith Perry, and Deborah A. Golino..... 277
- **Timing of Field Transmission of Grapevine Red Blotch-Associated Virus**
Robert R. Martin 285
- **Education and Outreach for the California Grapevine Registration and Certification Program, and an Assessment of Recently Established Production Vines from Increase Blocks**
Neil McRoberts 288

AUTHOR INDEX..... 291

Section 1:

Xylella fastidiosa **and** **Pierce's Disease**

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 2016 to October 2016.

ABSTRACT

The Pierce's disease research community has developed grapevines that exhibit novel and promising defenses against *Xylella fastidiosa* (*Xf*) 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 the large-scale spread of Pierce's disease within and among vineyards, which is a critical dimension of sustainable disease management. We are conducting transmission experiments with important insect vectors of *Xf* and using the data from these experiments to explore pathogen spread using mathematical models. We are assessing the efficacy of defenses by comparing simulated spread in defended and susceptible vineyards and using this data to inform vineyard managers of how to minimize disease outbreaks across California.

LAYPERSON SUMMARY

The Pierce's disease research community has developed grapevines that exhibit novel and promising defenses against *Xylella fastidiosa* (*Xf*) 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 are conducting transmission experiments with important insect vectors of *Xf* and using the data from these experiments to explore pathogen spread using mathematical models. We are assessing the efficacy of defenses by comparing simulated spread in defended and susceptible vineyards and using this data to inform vineyard managers of how to minimize disease outbreaks across California. So far, our results suggest that the blue-green sharpshooter (*Graphocephala atropunctata*), an important insect vector, is capable of acquiring and transmitting *Xf* from transgenic resistant grapevines and conventional susceptible grapevines at similar rates.

INTRODUCTION

This proposal expands on previous work funded by this program to develop Pierce's disease resistant grape lines. Previous projects have successfully developed grapevine lines with promising traits conferring resistance against *Xylella fastidiosa* (*Xf*), including plants expressing the *rpjF* gene, the *PdR1* major locus, and the HxfB protein (Meredith et al., 2000; Walker and Tenschler, 2014; Lindow et al., 2014). All these grape lines exhibit low symptom severity when mechanically inoculated with *Xf*. We propose to expand upon previous work by testing the potential of Pierce's disease defended grapevine lines to reduce the spread of *Xf* 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.

OBJECTIVES

The overall goal of this project is to assess the potential for novel defensive traits in grapevine lines to reduce the transmission of *Xf* 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 grapevine lines expressing the *rpjF* gene (Lindow et al., 2014) and conventionally bred grapevine lines with the *PdR1* dominant locus (Walker and Tenschler, 2014). The research consists of three specific objectives:

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

RESULTS AND DISCUSSION

Objective 1. Estimate transmission of *Xf* and vector feeding behavior on novel Pierce's disease-defended grapevine lines.

As reported in our July 2016 Interim Progress Report, we are investigating the influence of the PdR1 major locus on vector feeding behavior and transmission. Sharpshooter vectors, when given a choice, avoid feeding on Pierce's disease symptomatic plants. We are using measures of preference and transmission rates of the blue-green sharpshooter (*Graphocephala atropunctata*; BGSS) to understand the progression of infectiousness, disease symptoms, and ultimately transmission rates between PdR1 resistant plants and near-isogenic susceptible plants.

We inoculated "resistant" plants that expressed the PdR1 allele and "susceptible" plants that were near-isogenic but did not have the PdR1 resistance allele. We then placed eight *Xf*-free BGSS in a cage with two plants to choose from: an *Xf*-free susceptible test plant and an *Xf*-infected source plant either of the resistant or susceptible genotype. We included eight replicates of each of the two treatments and repeated the experiment three weeks, eight weeks, and 12 weeks after inoculating the source plants. We recorded which plant the vectors were feeding on at regular intervals over an eight-day period, estimated *Xf* populations in the source plants, and assessed Pierce's disease symptoms in the source plants. We are currently also assessing the infection status of the test plants and estimating *Xf* populations in the vectors.

Xf populations in the source plants increased over time and increased much more dramatically in the susceptible plants than in the resistant plants (**Figure 1A**). Mean *Xf* population did not differ significantly between grape genotypes over all sampling periods ($t = -0.64$, $P = 0.523$) and nor were populations among sampling periods significantly different ($t = 0.47$, $P = 0.638$). However, the interaction between genotype and sampling period was significant ($t = 2.52$, $P = 0.015$); populations increased over time significantly more in the susceptible plants than in the resistant plants (**Figure 1A**).

Similarly, there was no statistically significant difference in the proportion of vectors feeding on the infected resistant plants compared to the infected susceptible plants ($t = 0.96$, $P = 0.344$), and the difference between sampling periods was only marginally significant ($t = 1.71$, $P = 0.094$). However, the interaction between sampling period and genotype was once again significant ($t = -2.15$, $P = 0.0367$). Early after inoculation, vectors showed similar feeding preference for the inoculated resistant and susceptible source plants (**Figure 1B**). Then, later as the infection progressed, vectors showed a significantly greater preference for resistant infected plants compared to susceptible infected plants (**Figure 1B**). Importantly, the proportion of vectors feeding on any of the source plants was not significantly different than 0.5, indicating that they did not show a significant preference or avoidance of the source plants relative to the *Xf*-free test plants (statistics not shown). As we finish collecting data, we will be analyzing whether the populations in the source plants and any differences in vector feeding preference influence acquisition and transmission rates.

We are also investigating the transmission biology of diffusible signal factor (DSF)-producing *rpfF* transgenic grapevines with the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). We caged four GWSS adults individually on *Xf*-inoculated *rpfF* and conventional wild-type plants for a four-day acquisition access period (AAP); we then allowed them to feed on a wild-type plant for a four-day inoculation access period (IAP). The four vectors were caged on different plant tissues (stem, petiole) and at differing distances from the point of inoculation: near the point of inoculation (< 45 cm up the stem), and far from the point of inoculation (> 45 cm up the stem) in a paired-factorial design. We will be estimating the populations of *Xf* in the vectors and the infection status of test plants. In the meantime, we have estimated populations of *Xf* in the source plant tissues from which vectors fed.

Mean *Xf* populations were similar across grape genotypes, plant tissues, and distance from point of inoculation (**Figure 2**). There were no statistically significant differences in the population sizes across experimental factors or interactions (results not shown). We will be analyzing whether the similarity in source plant populations resulted in similar acquisition and transmission rates from our different experimental factors.

Objective 2. Assess large-scale and long-term Pierce's disease prevalence in defended grape vineyards.

As described in our previous report, our work to develop spatially-explicit epidemic models is ongoing. Based on previous results, we are focusing our modeling efforts on the interactive effects of spatial distribution of *Xf* and

vector feeding preference based on plant infection status. We are leveraging the modeling approaches of Webb et al. (2007) and Chiyaka et al. (2012) to model within- and among-plant pathogen distribution, and the approach of Zeilinger and Daugherty (2014) to model vector feeding preference. Model development is still in progress.

Objective 3. Inform vineyard managers on the efficacy of novel Pierce's disease defenses.

We will begin work on objective 3 when we have more results from objectives 1 and 2.

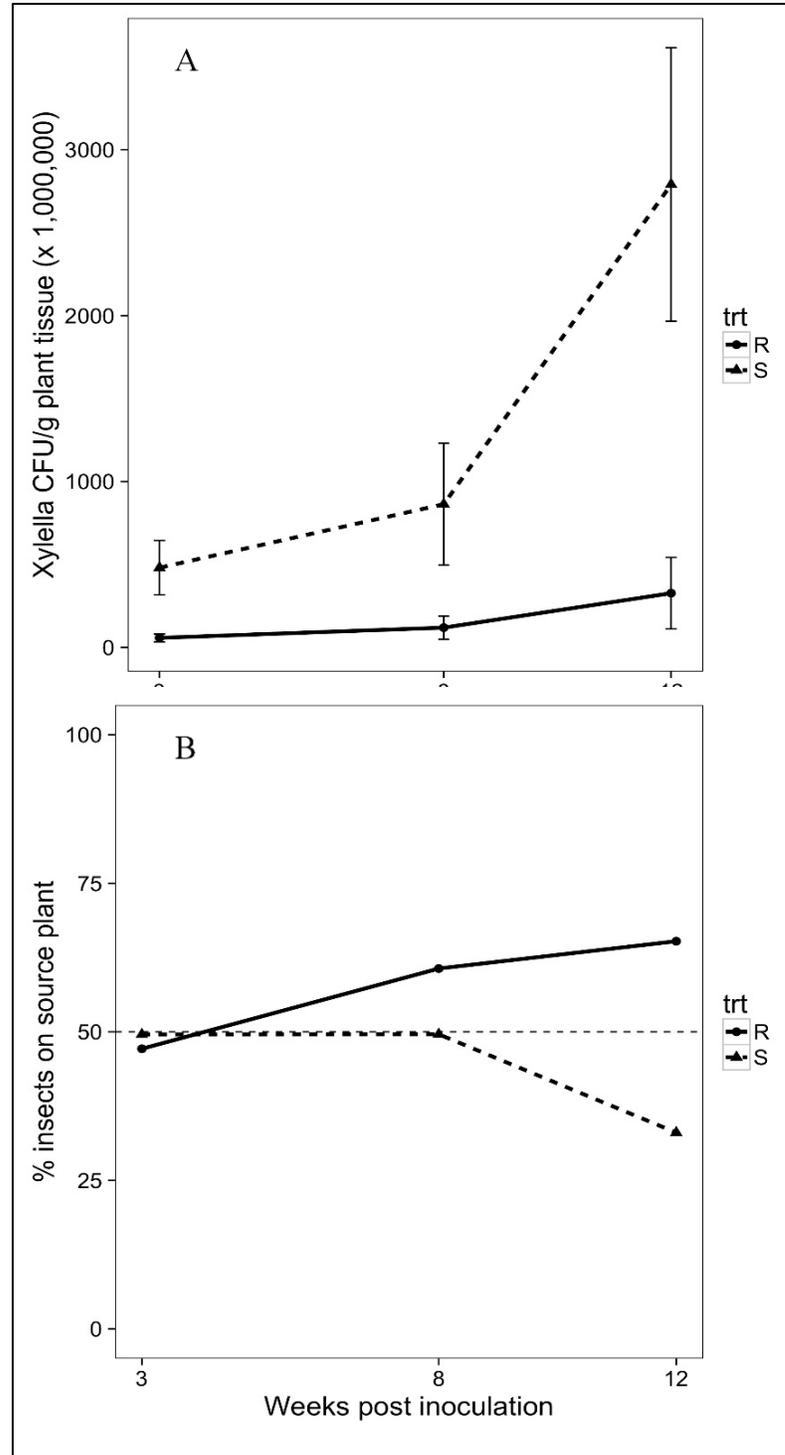


Figure 1. Preliminary results from PdR1 vector transmission and preference trials. (A) Mean *Xf* populations in petioles of source plants of either the resistant (R, solid line) or susceptible (S, dashed line) genotype. (B) Percent of vectors found on source plants of either the resistant (R, solid line) or susceptible (S, dashed line).

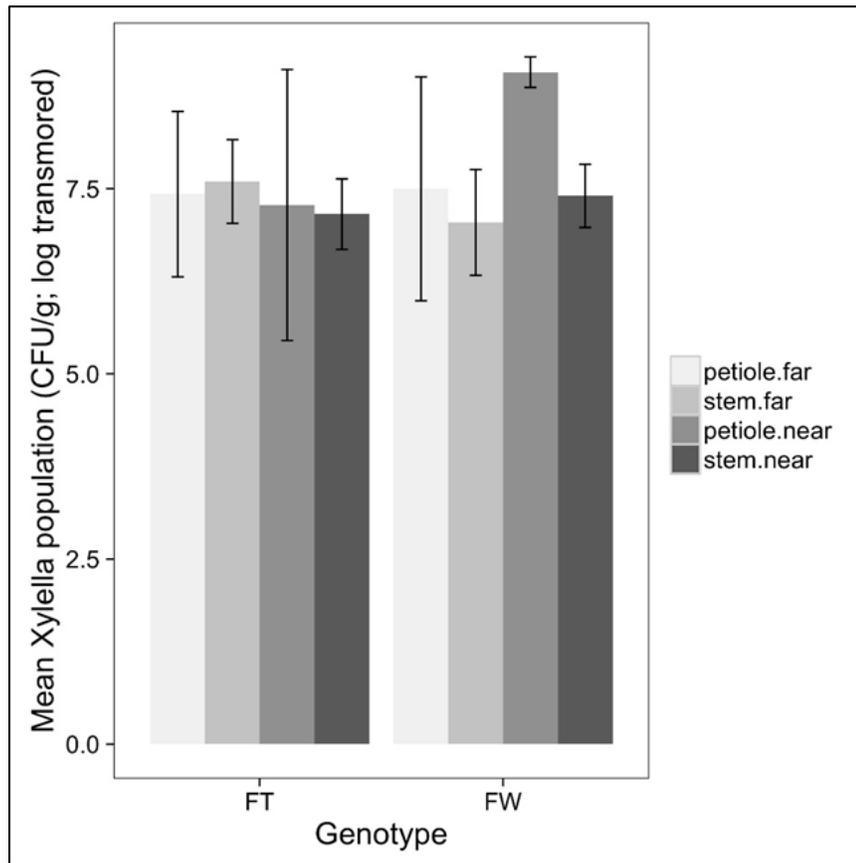


Figure 2. Mean populations of *Xf* in source plants of either DSF-producing transgenic (FT) or conventional wild-type (FW) genotypes. Cages with vectors were placed on either stems or petioles and either near (< 45 cm) or far (> 45 cm) from the point of inoculation. Error bars represent SEM.

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EVALUATING POTENTIAL SHIFTS IN PIERCE'S DISEASE EPIDEMIOLOGY

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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. In summer 2015, the project team held a series of joint meetings / field visits with the Farm Advisors. Two observations have been made that raised our concern about the problem. First, the prevalence of Pierce's disease in the North Coast is usually below 1-2% per vineyard, but several vineyards visited had over 25% of vines symptomatic. Second, historically Pierce's disease is closely associated with riparian zones in the North Coast, but we have visited several vineyards where Pierce's disease does not appear to be associated with riparian zones. We have observed these greater rates of disease incidence and dissociation from riparian areas throughout Napa and Sonoma counties; they are not district specific. 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.

OBJECTIVES

We 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* (*Xf*) colonization of grapevines and the role of overwinter recovery in Pierce's disease epidemiology.
3. Determine the role of spittlebug insects as vectors of *Xf*.
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 *Xf* website, the main online resource for Pierce's disease information.

RESULTS AND DISCUSSION

A previous report provided a summary of activities for each objective. Here we focus on the activities part of objective 1, the main component of this project.

Sixteen vineyard blocks in Napa County and 16 vineyard blocks in Sonoma County were selected as study sites (**Table 1**; total of 138 acres). Beginning in late February 2016, yellow sticky traps were deployed in either a transect or grid pattern in each study block such that between four and 12 traps were deployed at each location. In addition, between one and three vegetation traps were deployed at each location adjacent to the putative source of blue-green sharpshooters (*Graphocephala atropunctata*; BGSS; mostly riparian vegetation). Insect vectors were also monitored with the use of insect sweep nets; sweeping of the vine canopies and vegetation between the vine rows (middles) began in August and continued every two weeks. BGSS were collected from the canopy and green (*Draeculacephala minerva*) and red-headed (*Xyphon fulgida*) sharpshooters from grasses in the middles. Traps were checked every 14 days, and presence of vectors (mainly BGSS) was recorded.

Beginning in late August and continuing through mid-September 2016, the incidence of Pierce's disease was recorded for each vine in all study blocks. Disease incidence was based on the occurrence of a combination of the common visual symptoms of Pierce's disease, including leaf scorching, uneven lignification of shoots, matchstick petioles, and stunted growth (**Figure 1**). Two hundred samples were collected from Napa and Sonoma valleys, respectively (n = 400), to correlate visual assignment of vines as Pierce's disease-positive with *Xf* PCR-based detection in the laboratory (ongoing). Researchers walked every row of each block and recorded the incidence of Pierce's disease symptoms for individual vines on vineyard block maps. These maps were generated using Geographic Information Systems (GIS) to digitize a matrix of points in a spatial environment, where each point represents a vine in its exact geographic location. A customized geoprocessing tool was created to generate detailed vine-by-vine GIS files with accurate row and vine spacing. Based on the inputs, the tool generates a new GIS point shapefile representing vine locations within a vineyard block. Data on disease incidence was recorded in the vineyard on these maps. Once digitized, the georeferenced data on Pierce's disease incidence and trap captures of BGSS (similar to sample map, **Figure 2**) will be subjected to spatial statistics.



Figure 1. Common visual symptoms of Pierce's disease include stunting, uneven lignification of shoots, leaf scorching, and "matchstick" petioles. Photos courtesy D. Fletcher

Table 1. Description of study sites.

County	Vineyard	Variety	Rootstock	Planting date	Size (acres)
Napa	CL	Merlot	101-14	1997	3.03
	CV	Cabernet Franc	3309C	1999	6.37
	C	Cabernet Sauvignon	3309C, St. George	2004, 2005	7.44
	DC	Cabernet Sauvignon	110R	2000	1.92
	E2	Petit Verdot	110R	2004	0.89
	E3	Cabernet Sauvignon	101-14	2004	2.84
	F	Cabernet Sauvignon	O39-16	2014	9.07
	I	Cabernet Franc	O39-16	2002	2.5
	JP	Cabernet Sauvignon	101-14	2004	4.88
	RJ	Chardonnay	101-14	2000	4.6
	R	Malbec	420A	2004	1.69
	SF	Cabernet Sauvignon	Riparia Gloire, 420A, 101-14, 3309C	2000, 1994, 1993, 1990	7.33
	S	Cabernet Sauvignon	3309C, 1103P, 110R	1991, 1993	4.42
	T	Chardonnay	1103P	2008	2.6
Sonoma	V	Cabernet Sauvignon	101-14	2013	5
	WH	Chardonnay	101-14	2010	5
	1	Chardonnay	5C	1998	5.1
	2	Chardonnay	039-16	2011	5.45
	3	Zinfandel	110R	2001	1.1
	4	Zinfandel	039-16,110R	NA	4.3
	5	Chardonnay	1103P	2001	3.8
	6	Gruener Veltliner	101-14	NA	4.93
	7	Merlot, Cabernet Sauvignon	NA	NA	5.8
	8	Pinot Noir	101-14		3.97
	9	Merlot	5C	1996	3.8
	10	Chardonnay	5C	2001	7.5
	11	Malbec	039-16	2008	4.25
	12	Chardonnay	3309C	2008	2.25
	13	Chardonnay	AXR, 5C	1986, 1992, 1993, 1994	5.0
	14	Chardonnay	101-14	2003	4.2
15	Chardonnay	101-14	2003, 2005	4.1	
16	Chardonnay	S04	2007	2.8	

Plant communities adjacent to surveyed vineyard blocks

To test the hypothesis that vegetation composition of the areas surrounding vineyards may be influencing the abundance of *Xf* vectors (sharpshooters and spittlebugs) and the prevalence of Pierce's disease in the vineyards, we designed the following protocol. We surveyed the species richness and relative abundance of all vascular plants found at each vineyard site (N = 32 sites) in Napa and Sonoma counties. Each site included one to three lines of insect traps extending from the center of the vineyard towards the edges. Our vegetation surveys extended these lines for 50 meters in the same cardinal direction, sampling the vegetation in the bordering areas surrounding the vineyards. In most cases these areas were riparian communities with mixes of native and non-native vegetation; however, in some cases these areas were cleared or managed lands with plantings. In total, we surveyed 71 geo-referenced 50-meter transects at the 32 sites (one to three transects per site), and sampled a total of 154 different vascular plant species. Our objective was to characterize the vegetation structure as well as sample the plant diversity at each vineyard, so we sampled ground cover as well as all woody stems greater than one cm in diameter. For ground cover, we calculated the percent cover of different species of herbs, grasses, woody stems, and bare ground for two meters x 50 meters (0.01 hectare) in each transect. For woody stems, we counted and identified each stem greater than one cm in diameter in the same two meter x 50 meter area as the groundcover transect. For large trees, we counted and identified each stem greater than 10 cm in diameter for 10 meters x 50 meters (0.1 hectare) in each transect. In total, we counted and identified 3,935 stems > 1 cm < 10 cm, and 1,208 trees greater than 10 cm in diameter. We will use this vegetation structure data, percent cover data, and

species composition and relative abundance data to correlate with the insect trap vector relative abundance and Xf prevalence data when it is available.

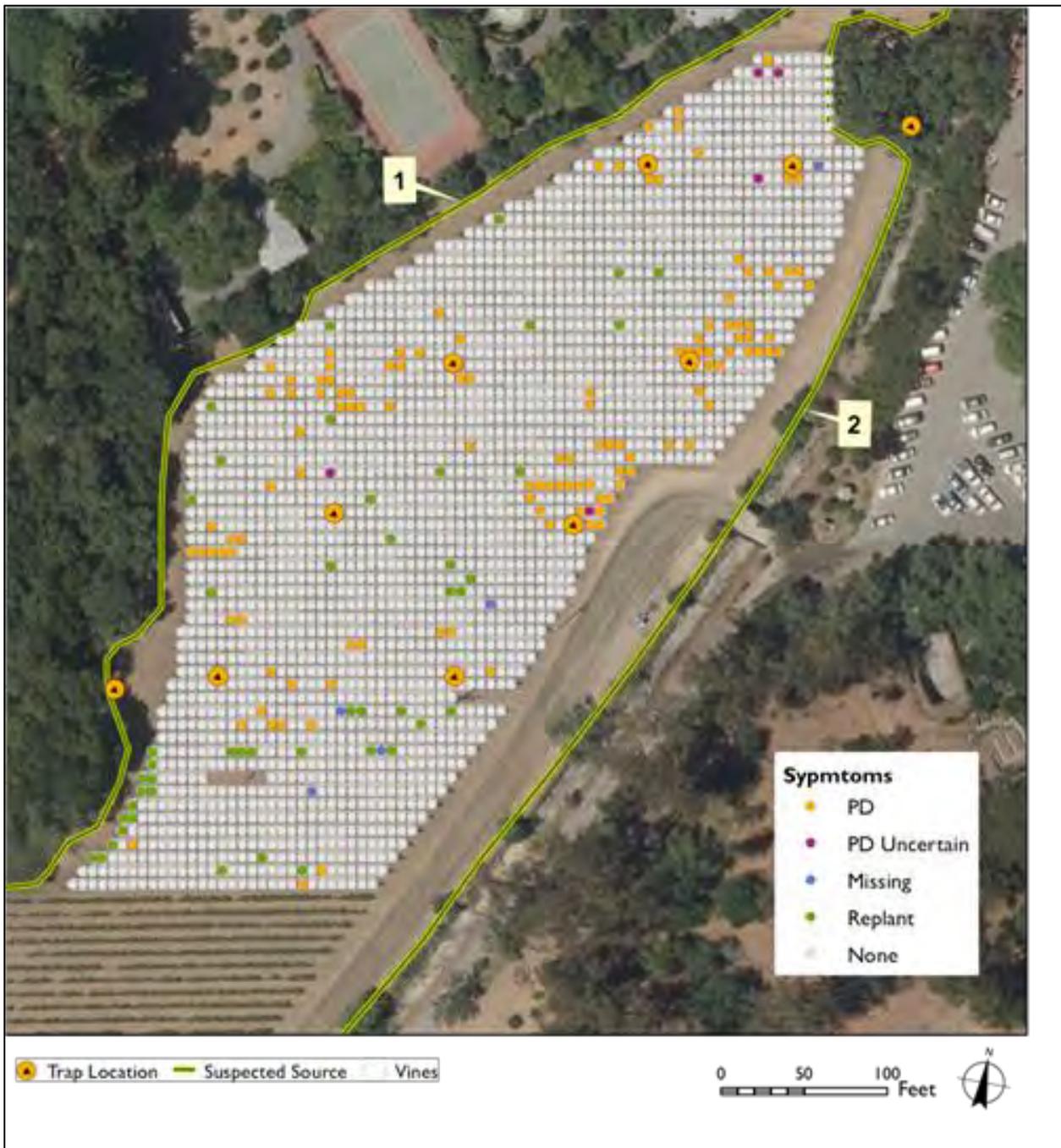


Figure 2. Sample map for Napa-block I showing georeferenced vineyard and vegetation trap locations and Pierce's disease incidence (2015).

Blue-green sharpshooter monitoring.

So far this season, the BGSS monitoring program indicates vector populations in vineyards are low. For example, although the grower-generated monitoring data showed up to an average of two BGSS per trap in late April, approximately 70% of vineyard block censuses found no BGSS on that date (**Figure 3A**). The researcher-generated monitoring data showed similarly low BGSS densities, with the highest densities primarily in some Sonoma County vineyard blocks between the end of March and mid-May, but with most vineyard censuses finding no BGSS on that date (**Figure 3B**).

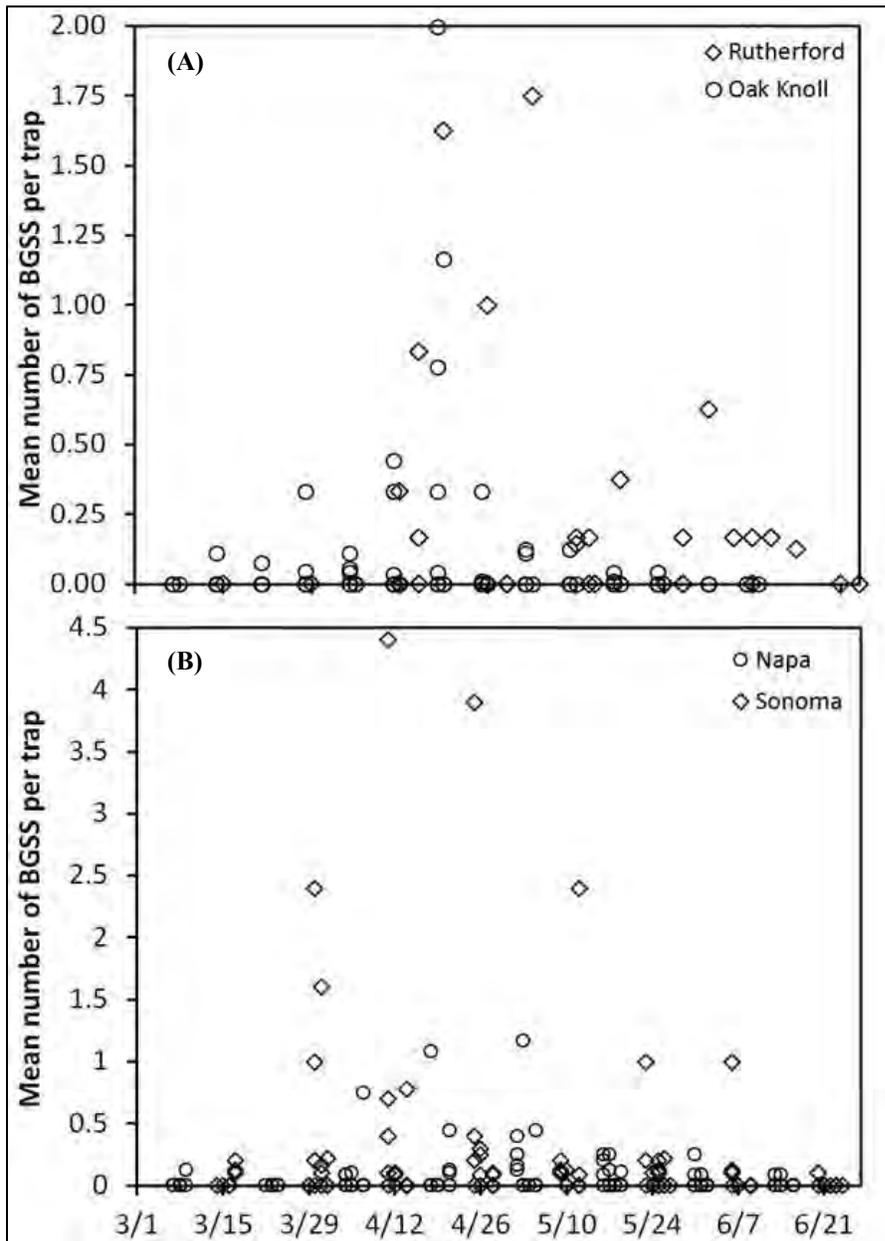


Figure 3. Number of BGSS caught over spring 2016 in parallel trapping programs (A) conducted by grape growers in two regions of Napa County, and (B) by researchers (our team) at sites throughout Napa and Sonoma counties.

Climatic effects on disease incidence

As a first step towards understanding whether climatic conditions in recent years have contributed to the ongoing Pierce’s disease resurgence in the North Coast, we have started to compare recent versus historic climate data. Thus far we have collated climate data from more than a dozen weather stations in the region, with some having temperature data going back more than 70 years.

All else being equal, a lack of cold conditions over the winter and early spring should contribute to Pierce’s disease incidence by reducing the fraction of vines recovering from infection. To address this prediction, we’ve started to compare two metrics of dormant season climate – the mean daily minimum temperature, and the number of days where minimum temperatures were below 40 °F – for recent seasons compared to historic values. Thus far, for two sites in Napa and Sonoma counties (i.e., Oakville and Healdsburg, respectively), temperature data over the previous five seasons do not stand out as being warm by historic standards. Indeed, two to three of

the previous five seasons have somewhat higher numbers of cold days (i.e., <40 °F) and lower mean minimum temperatures compared to historic averages.

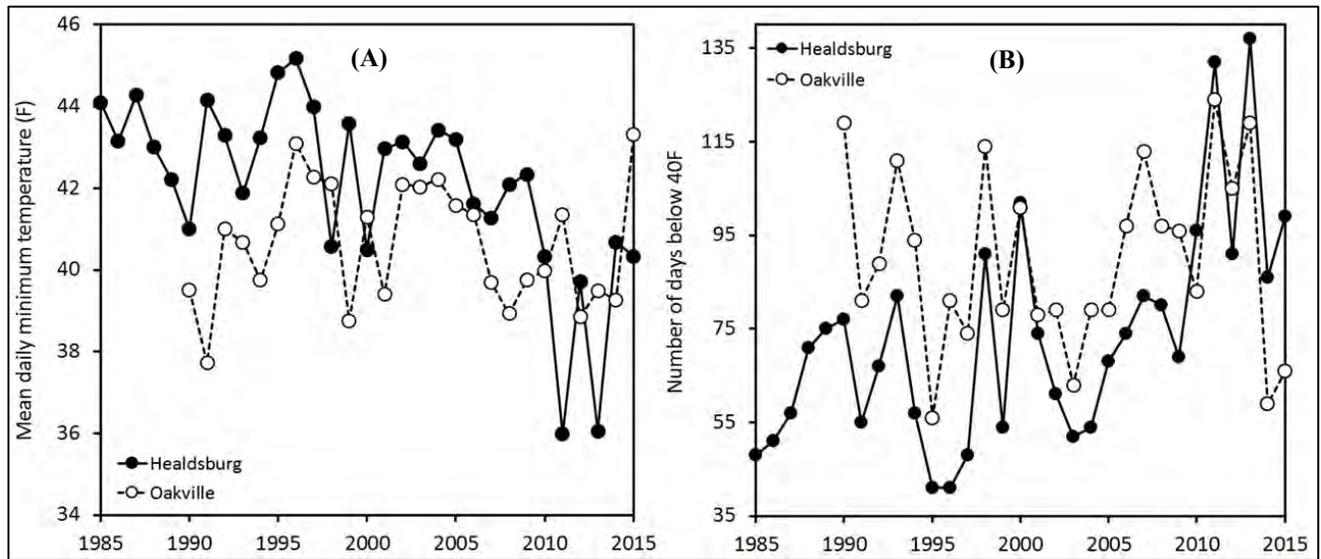


Figure 4. Comparison of (A) daily minimum temperature and (B) number days with minimum temperatures below 40°F over the dormant season (October to April) for the last 30 years at two sites in Napa and Sonoma counties.

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 August 2016 to October 2016.

ABSTRACT

Previous research showed that *Xylella fastidiosa* (*Xf*) 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 *Xf* interactions with both plant and insect hosts. This report summarizes recent efforts aimed at experimentally determining carbon sources that can be used by *Xf* in this context; previous reports discussed other aspects of the project.

LAYPERSON SUMMARY

The previously identified *Xylella fastidiosa* (*Xf*) chitinase (ChiA) represents a unique opportunity to try to disrupt *Xf* 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

This project has three objectives:

1. Identify *Xf* proteins or protein complexes that bind to ChiA and are required for its activity.
2. Screen potential substrates cleaved by ChiA.
3. Functionally demonstrate the role of ChiA partners during insect and plant colonization.

Efforts during the report period focused on experimentally determining ChiA substrates in plants.

RESULTS AND DISCUSSION

Chitinase substrates.

Three different media containing 1.5 g/L K₂HPO₄, 1g/L KH₂PO₄, 1g/L MgSO₄·7H₂O, 10 mL/L of a 0.2% phenol red solution, 10 mL/L of a Hemin chloride (0.1% in 0.05% NaOH) solution, 10g/L of gelrite, 3g/L of BSA, and 0.2% of methylcellulose or xylan from oat spelts or 0.1% of pectin from apple were prepared. Eight 10 µL droplets of the wild-type, chitinase mutant, or the chitinase complemented strain (OD₆₀₀ = 1.4 - 1.5) were spotted on each medium. After six days of incubation at 28 °C the cellulose and xylan plates were flooded with 1 mg/mL of Congo red for 15 min. The Congo red was then poured off and a solution containing 1M NaCl was added for an additional 15 min. 1 M HCl was finally poured on the plates for longer visualization of the hydrolysis zone (Teather et al., 1982). The pectin plates were flooded with 1% cetrinide solution for three days after a six-day incubation at 28 °C (Beg et al., 2000). The experiment was repeated three times per medium and per strain. Halos were observed for the wild-type, the chitinase mutant, and the chitinase complemented strains for the three different plant polysaccharides (cellulose, xylan and pectin) tested (**Figure 1**).

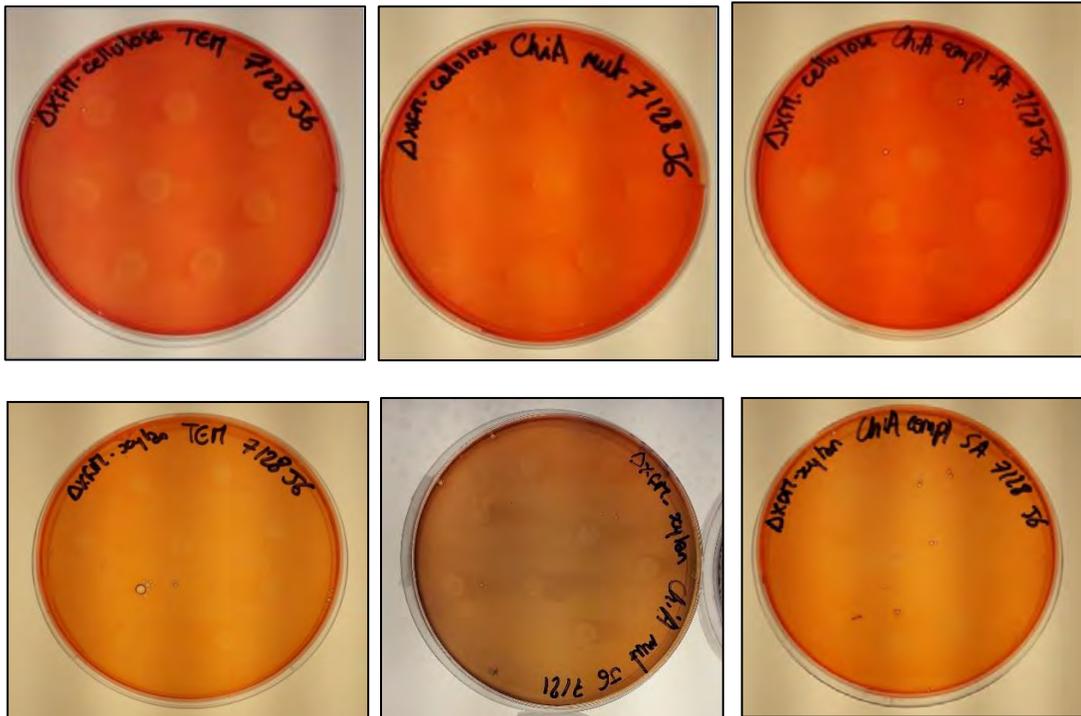


Figure 1. From left to right, test of the ability of the wild-type strain, the ChiA mutant strain, and the ChiA complemented strain to degrade cellulose (above) and xylan (below). The pictures were taken after Congo red staining.

CONCLUSIONS

Earlier research has identified a series of new carbon sources that may be utilized by *Xf*. Efforts are now focusing on experimentally confirming these results. Some appear to not be used as carbon sources by the *chiA* mutant strain. The results presented here are examples of ongoing research addressing this question.

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FUNDING AGENCIES

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

DEVELOPMENT OF A BIOLOGICAL CONTROL FOR PIERCE'S DISEASE

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ABSTRACT

Xylella fastidiosa (*Xf*) is a serious pathogen that infects a number of important crops including citrus, almonds, and coffee. The *Xf* Temecula strain infects grapevines and induces Pierce's disease (PD). In efforts to understand infection better, we deleted the *Xf* 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 *Xf* 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 Δ PD1311 strain may suppress disease when applied prior to a virulent strain and thereby acting as a biocontrol for management of Pierce's disease, as it significantly reduces the symptoms when inoculated prior to wild-type *Xf* Temecula 1 (TM1).

LAYPERSON SUMMARY

We discovered that deleting the *Xylella fastidiosa* (*Xf*) Temecula 1 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 suppress disease and function as a biocontrol. When inoculated prior to wild-type *Xf*, disease incidence was significantly lower. Since the options for managing Pierce's disease are limited, developing alternative control strategies to be integrated into existing ones is 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 (*Xf*) is a Gram-negative, xylem-limited bacterium that causes Pierce's disease of grapevines (Chatterjee et al., 2008). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilm that clogs the vessels leading to Pierce's disease. Recently, secreted toxins and effectors have been identified as also playing roles in Pierce's disease development (Matsumoto et al., 2012; Nasci et al., 2014; Nascimento et al., 2016; Zhang et al., 2015). We, and others, have studied *Xf* proteins and genetic mechanisms involved in these steps (Guilhbert 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 virulence and for development of prevention strategies.

We deleted the *Xf* PD1311 gene (Δ PD1311), a putative acyl-CoA synthetase (ACS), as we were interested in genes potentially 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 recently published this work, which includes showing it as having potential function as a biocontrol (Hao et al., 2016).

We found that PD1311 is a functional enzyme that has the conserved domains found in acyl-coA synthetase (data not shown), and that Δ PD1311 grows in PD2 and *Vitis vinifera* sap (**Figure 1**) (Hao et al., 2016). Additionally,

motility, aggregation, and biofilm production are key behaviors of *Xf* that are associated with Pierce's disease (Chatterjee et al., 2008). The Δ PD1311 strain is reduced in type IV pili-mediated motility on periwinkle wilt (PW) plates minus bovine serum albumin (BSA) and is non-motile on sap agar (Figure 2) (Hao et al., 2016). In comparison to TM1, Δ PD1311 is reduced in aggregation and biofilm production. Therefore, we hypothesized that Δ PD1311 is less virulent in plants, as mutants with similar phenotypes have been shown to have reduced virulence or be avirulent (Cursino et al., 2009; Cursino et al., 2011; Guilhabert and Kirkpatrick, 2005; Killiny et al., 2013). We found that Δ PD1311 was avirulent and showed no Pierce's disease, even at 24 weeks post-inoculation (Figure 3).

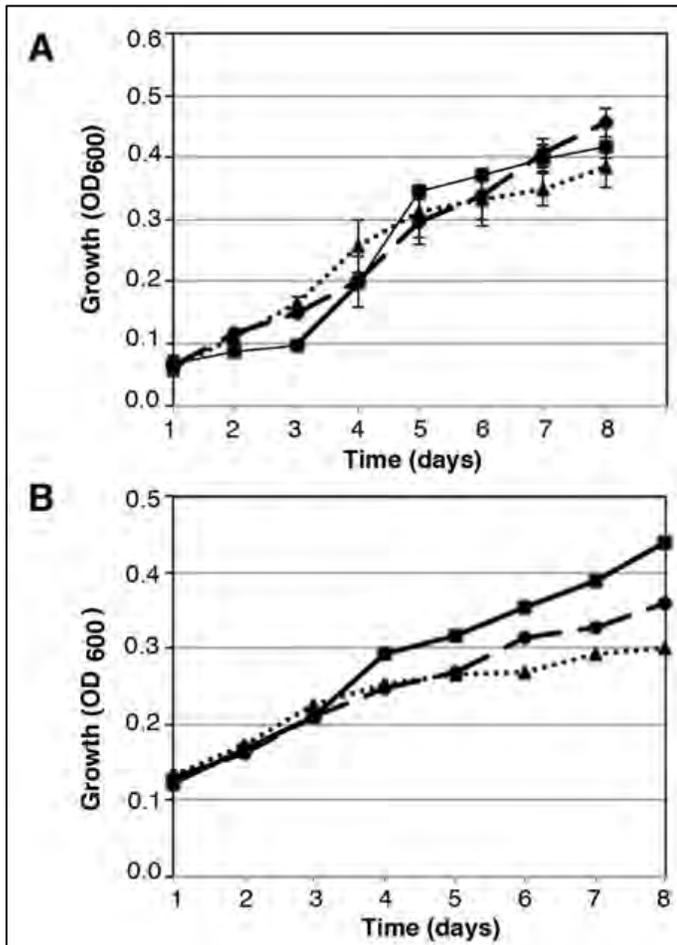


Figure 1. Δ PD1311 growth and survival in grape sap. Shown are growth curves of TM1 (solid line, square), Δ PD1311 (dotted line, triangle), and C- Δ PD1311 (dashed line, circle) in PD2 broth (A) and 100% Chardonnay sap (B). Six replicates were included for each experiment and the assays were repeated three times. Error bars represent standard deviations. Three replicates were included for each experiment and the assay was repeated twice. TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene, C- Δ PD1311 = Δ PD1311 complement strain.

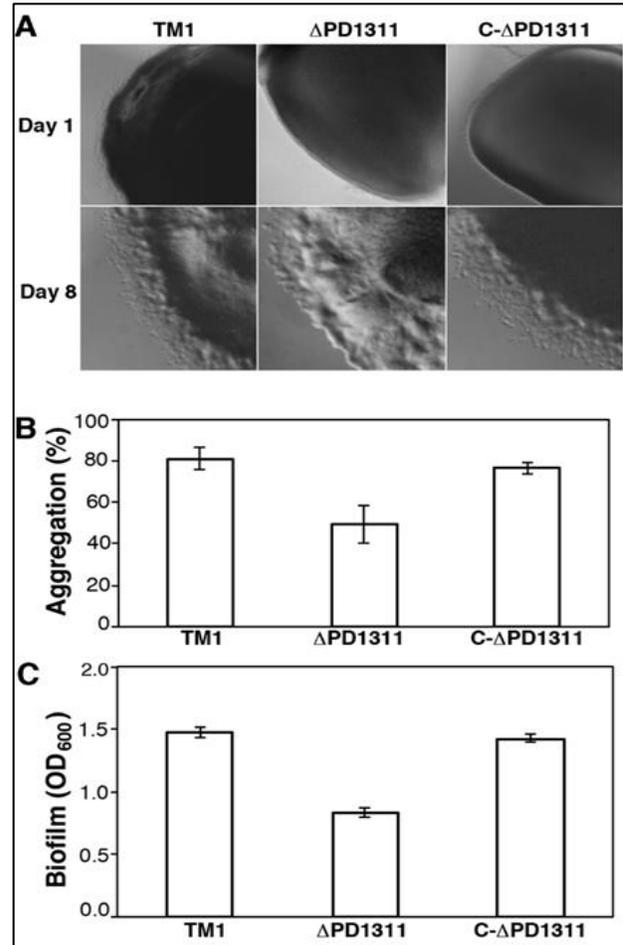


Figure 2. Δ PD1311 was defective in motility, aggregation, and biofilm. A) Representative images of colony fringes of TM1, Δ PD1311, and C- Δ PD1311 on PW-BSA plates at day 1 (top) and 8 (bottom) post-inoculation (p.i.). B) Mean percentage of aggregation and (C) biofilm quantification of wild-type, Δ PD1311, and C- Δ PD1311 strain in PD2 broth 5 d.p.i. Error bars represent standard error. Twenty-four replicates were included for each experiment and the assay was repeated three times. * represents a significant difference of $p < 0.01$. TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene, and C- Δ PD1311 = Δ PD1311 complement strain.

The weakly virulent *Xf* 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 and scion varieties (Cousins and Goolsby, 2011; Walker, 2015) and transgenic vines (Dandekar, 2014; Gilchrist et al.,

2014; Gilchrist and Lincoln, 2014; Kirkpatrick, 2014; Lindow, 2014; Powell and Labavitch, 2014). However, continued research of Pierce's disease controls is warranted. We had results that Δ PD1311 lowers the incidence of wild-type-induced Pierce's disease. Given the avirulent phenotype of Δ PD1311 and its ability to limit wild-type induced Pierce's disease, this strain provides new potential for a commercialized biological control.

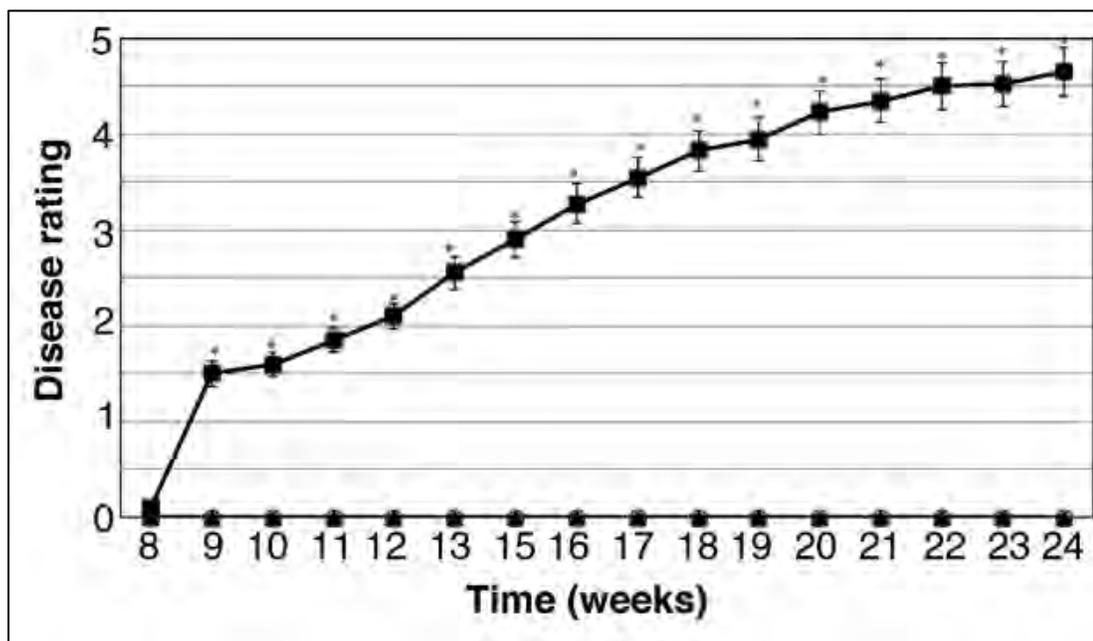


Figure 3. Δ PD1311 is avirulent on grapevines. Shown are weekly mean disease ratings of vines inoculated with TM1 (solid line with squares), Δ PD1311 (triangles), C- Δ PD1311 (open circles), and buffer (dotted line on x-axis) respectively. Error bars represent standard errors. Ten plants were included for each experiment and the assay was repeated twice. * represents a significant difference of $p < 0.01$. TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene, C- Δ PD1311 = Δ PD1311 complement strain.

OBJECTIVES

The overall goal is to optimize Δ PD1311 as a biological control for Pierce's disease and to understand the mechanisms of disease inhibition that will facilitate future application.

1. Examine aspects of the Δ PD1311 Temecula strain as a biological control of Pierce's disease.
 - a. Optimize application timing and conditions for the Δ PD1311 strain.
 - b. Determine if overwintered Δ PD1311-inoculated plants maintain Pierce's disease resistance.
 - c. Explore leafhopper transmission of the Δ PD1311 strain.
 - d. Develop a clean deletion strain of Δ PD1311 that would be suitable for commercialization.
2. Determine the function of the PD1311 protein and the mechanism by which Δ PD1311 acts as a biological control.
 - a. Elucidate the role of the PD1311 protein.
 - b. Examine the impact of the Δ PD1311 strain on wild-type *Xf* *in vitro* and *in planta*.

RESULTS AND DISCUSSION

Objective 1. Examine aspects of the Δ PD1311 Temecula strain as a biological control of Pierce's disease.

Objective 1a. Optimize application timing and conditions for the Δ PD1311 strain.

To examine if the *Xf* Δ PD1311 Temecula 1 strain could act as a potential biocontrol, we inoculated *V. vinifera* cv. Cabernet Sauvignon vines per standard procedures (Cursino et al., 2011) and recorded development of Pierce's disease using the five-scale assessment (Guilhabert and Kirkpatrick, 2005). We created three different inoculation conditions: i) TM1 after a two-week pre-treatment with Δ PD1311 [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins, 2005)], ii) TM1 and Δ PD1311 co-inoculated, and iii) controls (TM1-only, Δ PD1311-only, buffer). We previously found that inoculating Δ PD1311 after a two-week pre-treatment with TM1 did not limit Pierce's disease (data not shown). Our controls included vines inoculated with TM1,

Δ PD1311, or buffer (Hopkins, 1984). Our results for 2015 indicated that pre-treatment with Δ PD1311 could inhibit Pierce's disease in a significant proportion of TM1-inoculated vines, while co-inoculation does not alter disease development (**Figure 4**) (Hao et al., 2016).

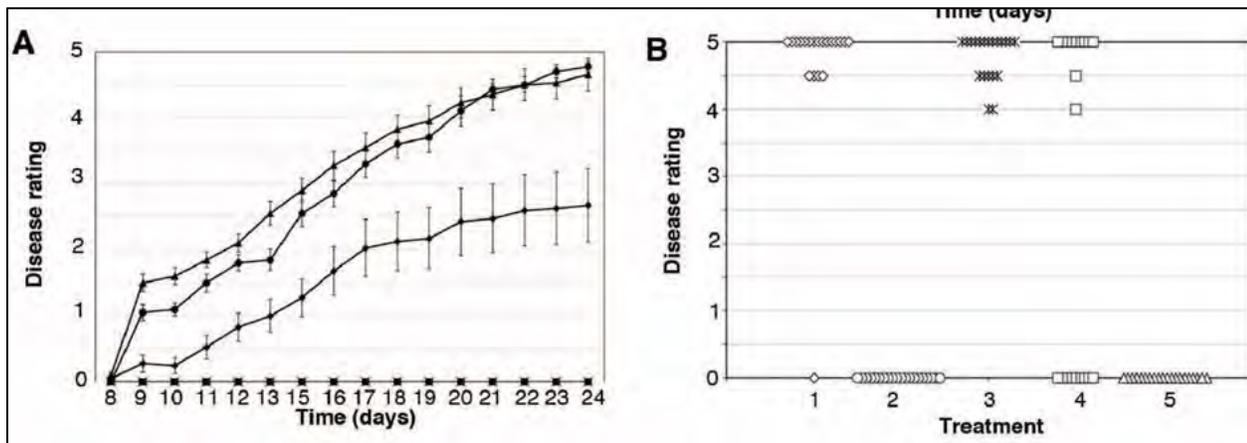


Figure 4. Δ PD1311 inoculation to grape prior to TM1 suppressed Pierce's disease development. A) Weekly mean disease ratings of vines inoculated with TM1-only (triangles), TM1 and Δ PD1311 simultaneously (circles), Δ PD1311 two weeks prior to TM1 (diamonds), Δ PD1311-only (squares), and buffer (x marks), respectively. Error bars represent standard errors. Ten plants were included for each experiment and the assay was repeated twice. B) Disease rating for each vine at 24 w.p.i. 1 = TM1-only, 2 = Δ PD1311-only, 3 = co-inoculation with TM1 and Δ PD1311 simultaneously, 4 = Δ PD1311 two weeks before TM1, and 5 = buffer. TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene.

Objective 1b. Determine if overwintered Δ PD1311-inoculated plants maintain Pierce's disease resistance.

In 2014 we had *V. vinifera* plants infected with TM1 or Δ PD1311 two weeks prior to TM1. These vines were cut back and placed in nursery storage for the 2015 winter. The plants were then grown in the greenhouse in Spring 2015 to follow potential Pierce's disease symptoms. Our preliminary findings showed that TM1 could overwinter and cause Pierce's disease in the following year. Plants treated with Δ PD1311 followed by TM1 did not show symptoms either year and ELISA did not detect *Xf* (TM1 or Δ PD1311) in year 2 (**Table 1**). This data suggests that Δ PD1311 protection may last overwintering. We are currently exploring whether biocontrol treatment in year 1 protects against a fresh wild-type inoculation in year 2. If overwintering protection is found, this result would indicate that the Δ PD1311 biocontrol may have long-lasting protection in the field. If symptoms do develop in year 2 in the Δ PD1311-treated plants, this result will indicate that reapplication of the biocontrol will be necessary to maintain Pierce's disease suppression.

Table 1. *Xf* ELISA results overwintered plants.^a

Treatment Year 1 ^b	Symptoms Year 1 ^c	Symptoms Year 2	0 cm ^d	30 cm ^d	150 cm ^d
TM1 ^f	+	+	+/1 ^e	+/1	+/1
		-	-/3	-/3	-/3
Δ PD1311 then TM1	-	-	-/2	-/2	-/2

^a Plants overwintered in cold storage between year 1 and 2.

^b Plants were given no further inoculations in year 2.

^c “+” = Pierce's disease symptoms; “-” = no Pierce's disease symptoms.

^d Sample distance up from inoculation point in year 2.

^e “+” or “-” indicated positive or negative for *Xf*, respectively / “number” is the number of plants tested by ELISA in year 2.

^f TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene.

Objective 1c. Explore leafhopper transmission of the Δ PD1311 strain.

Xylem-sap feeding leafhopper vectors transmit *Xf* 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 become 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 *Xf* to accomplish its life cycle. For development of Δ PD1311 as a commercially viable biological control agent and for future field studies, it will be necessary to understand its insect transmissibility. Because Δ PD1311 has reduced aggregation and biofilm (**Figure 2**), we hypothesize that Δ PD1311 is altered in its ability to be insect vectored. 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 owing to the similar chitinous nature of the cuticles (Killiny et al., 2010). We have preliminary data to show that Δ PD1311 attached to insect wings at a level similar to the wild-type strain (**Figure 5**).

Objective 1d. Develop a clean deletion strain of Δ PD1311 that would be suitable for commercialization.

Δ PD1311 was created via site-specific recombination of a kanamycin cassette into the *Xf* 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 attempt. We anticipate beginning this work after we complete data collection from objective 1a to confirm the biological control function of Δ PD1311 with optimized application conditions.

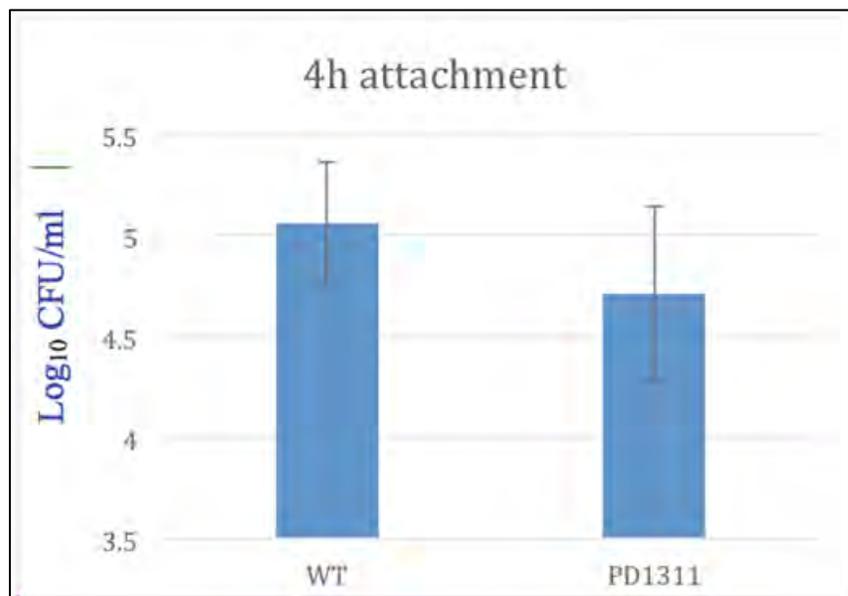


Figure 5. The Δ PD1311 strain attached to leafhopper hindwings similar to the wild-type strain. The attachment assay was performed as described previously (Baccari et al., 2014). The experiment was performed once with eight replicates included for each strain.

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

Objective 2a. Elucidate the role of the PD1311 protein.

The *Xf*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 β -oxidation and phospholipid biosynthesis, and 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).

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 is converted to AMP + pyrophosphate + acetyl-CoA (data not shown). Therefore we confirmed that the protein is functional.

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 (DSF) 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 *Xf*, and changes in LPS integrity renders bacteria more susceptible to environmental stress and defective in virulence (Clifford et al., 2013).

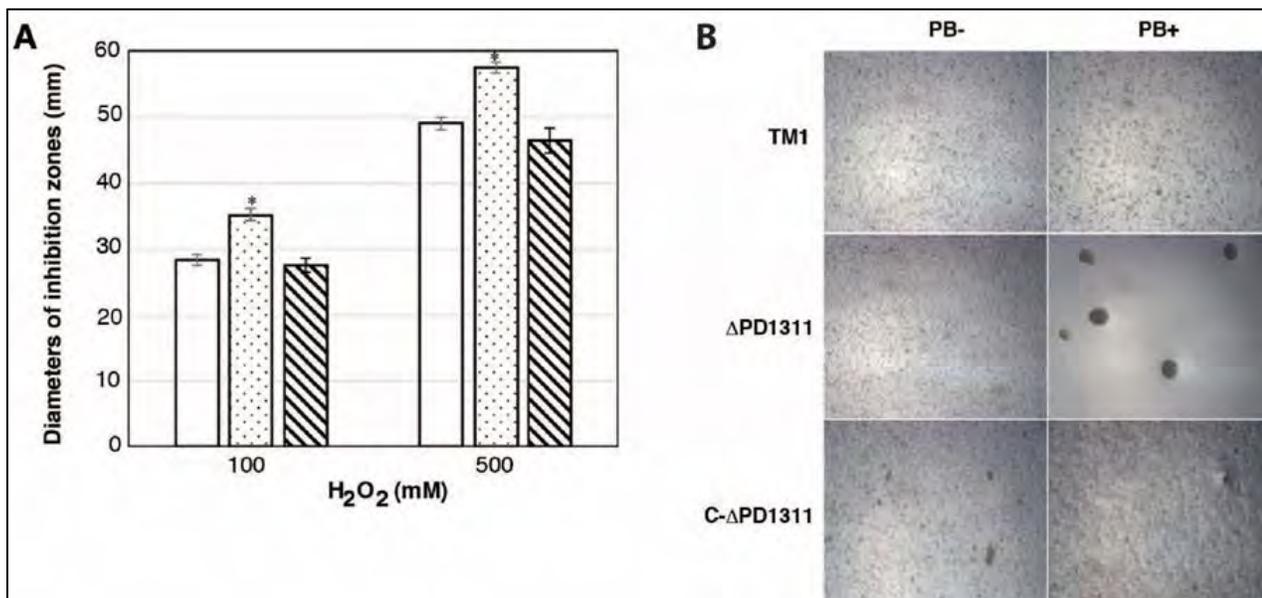


Figure 6. Relative sensitivity of Δ PD1311 to H₂O₂ and polymyxin B (PB). A) Mean diameters of inhibition zones of TM1 (empty bars), Δ PD1311 (dotted bars), and C- Δ PD1311 (dashed bars) exposed to 100 or 500 mM of H₂O₂ on PD2 agar plates. Error bars represent standard deviations. Three replicates were included for each experiment and the assay was repeated twice. * represents a significant difference of p<0.01. B) Growth of TM1 and C- Δ PD1311 on PD2 plates amended with 16 μ g/mL PB and growth of Δ PD1311 on PD2 plates with 1 μ g/mL PB. Images were taken under a Stemi-2000C dissecting microscope with a magnification of 3.2X. The assay was repeated at least four times with similar observations. TM1 = wild-type *Xf* Temecula 1, Δ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene, C- Δ PD1311 = Δ PD1311 complement strain.

Considering the avirulent phenotype of Δ PD1311 on grapevines, PD1311 may be involved in lipid A biosynthesis or membrane production. Therefore, the Δ PD1311 cells may be more sensitive to environmental stresses such as oxidative stress and cationic antimicrobial peptide polymyxin B (PB). When TM1 and Δ PD1311 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 6A**) (Hao et al., 2016). In addition, Δ PD1311 cells were more sensitive to PB than wild-type or Δ PD1311 complement cells. While both TM1 and Δ PD1311 complement cells grew on plates supplemented with 16 μ g/mL PB, almost all Δ PD1311 cells were killed when plated on PD2 agar supplemented with 1 μ g/mL PB (**Figure 6B**).

Table 2. Wild-type *Xf* detection by ELISA in petioles 24 w.p.i.^a

Treatment	PD Symptom	Trial	Distance above inoculation point (cm)		
			0	30	150
ΔPD1311 then TM1 ^b	-	1	- ^c /3 ^d	-/3	-/3
		2	-/5	-/5	-/5
	+	1	n.d. ^e	n.d.	+/6
		2	n.d.	n.d.	+/4
TM1 + ΔPD1311	+	1	n.d.	n.d.	+/5
		2	n.d.	n.d.	+/4
TM1 only	+	1	n.d.	n.d.	+/5
		2	n.d.	n.d.	+/4

Shown are results of TM1 detection in petioles by ELISA 24 weeks post-inoculation. Each trial contained 10 plants total of which a subset was tested.

^a w.p.i. = weeks post-inoculation.

^b TM1 = wild-type; TM1 was inoculated two weeks after ΔPD1311.

^c “+” or “-” indicates positive or negative for *Xf*, respectively.

^d Number is the number of plants tested by ELISA.

^e n.d. = not assessed as no petioles left due to disease.

Objective 2b. Examine the impact of the ΔPD1311 strain on wild-type *Xf* *in vitro* and *in planta*.

To have better grounding on the potential of PD1311 for suppressing Pierce’s disease and how it may function as a biocontrol we need to explore the mechanism by which the mutant strain impacts wild-type cells. We have results showing that the TM1-induced disease can be limited only when ΔPD1311 was inoculated two weeks before the pathogen (**Figure 4**). Therefore, we would like to know how the two strains spread through the plant when both are inoculated. ΔPD1311 does not secrete a toxin that affects wild-type populations (**Table 2**), as we grew TM1 cells in supernatant from ΔPD1311 cells and found no growth changes (data not shown). Understanding how the mutant cells impact TM1 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

Concerning objective 1, we confirmed that ΔPD1311 is avirulent, and we found that it can significantly reduce Pierce’s disease development by TM1. Preliminary data suggests that ΔPD1311 attaches to insect hindwings equal to TM1 cells and therefore could possibly be distributed by the vector. We are completing the overwintering studies in objective 1b, which we hope will provide insights into the lasting impact of the ΔPD1311 biocontrol. For objective 2, our preliminary results show that the mutant has greater sensitivity to chemical environments (hydrogen peroxide, antimicrobial peptides), which may contribute to its avirulent phenotype and help explain the role of the protein in the bacterium. Overall, this work explores a potentially new biocontrol for limiting Pierce’s disease.

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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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ABSTRACT

The goal of this research was to understand the relationship between the expression of secreted virulence proteins by *Xylella fastidiosa* (*Xf*) and the leaf scorching symptoms observed during the development of Pierce's disease, and to exploit this information to develop new strategies to control Pierce's disease in grapevines. The analysis of *Xf* 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 *Xf* that are defective in their capacity to secrete either of these two proteins and individually these mutants displayed unique alterations in growth and disease phenotype. The mutant lesA1 that does not make LesA protein is less virulent, therefore we conclude that LesA is a 'virulence factor,' while prtA1 that does not make PrtA protein is more virulent, therefore PrtA is an 'anti-virulence factor.' LesA protein displays lipase/esterase activities and is the most abundant secreted protein, with structural similarity to two less abundant secreted proteins LesB and LesC. LesA, B, and C proteins produced individually in *Escherichia coli* were infiltrated into grapevine and walnut leaf tissues and were able to induce scorching symptoms in leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. We have analyzed the microbiome of *Xf*-infected plants and compared these with uninfected plants and we observed a striking variation in the alpha diversity of the microbial community. Different parts of the plant along the axis of infection differ in their alpha diversity. We compared bottom and top stems and roots. Infection with *Xf* leads to a loss of diversity indicating that *Xf* is able to colonize and displace or outcompete existing microbial communities in the xylem. More virulent strains like prtA1 are able to more quickly colonize as compared to lesA1 that was slower than the wild-type strains. Since PrtA was an anti-virulence factor and possibly has a role to play in the biofilm we expressed PrtA as a transgene in transgenic tobacco. Two of the eight transgenic plants displayed some anti-virulence activity and showed a reduction of symptoms when infected with *Xf*. A deeper understanding of how these two secreted proteins LesA and PrtA function and their associated pathobiology has provided new insights into this disease and provided a new avenue for therapy against Pierce's disease.

LAYPERSON SUMMARY

Pierce's disease of grapevines is caused by the bacterium *Xylella fastidiosa* (*Xf*), 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 by growth of *Xf* biofilm leading to an interference with *in planta* water transport have been posited to be the main cause of Pierce's disease symptom development. This research has developed an

alternative hypothesis for disease symptom development. Our analysis of *Xf* secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA, that play a role in the development of Pierce's disease symptoms. We generated mutant *Xf* that are defective in the secretion of either of these two proteins that show alterations in bacterial physiology and plant disease phenotype. Mutant bacteria defective in secreting LesA were less virulent and displayed a biofilm behavior in culture, while the bacteria defective in the secretion of PrtA were the opposite; i.e., they were highly virulent and correspondingly displayed a planktonic growth in culture. Our experiments showed that these two proteins play a role in disease progression. We have also examined the role of these secreted proteins with respect to colonization of the xylem in different parts of grapevines and investigated the influence that these bacteria have on the resident microbial communities that inhabit these locations. We observed that more virulent strains are able to more rapidly colonize the grapevine and change the diversity of the microbial community. It is possible that these changes in community are influenced also by these secreted virulence factors, specifically LesA. One of these factors that we have proposed as an anti-virulence factor (PrtA) is able to control symptoms when expressed in tobacco plants. An understanding of how these two proteins work has provided new insights into this disease and indicate new avenues of therapy.

INTRODUCTION

Xylella fastidiosa (*Xf*) 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, *Xf* is vector-transmitted by various xylem sap-feeding sharpshooter insects (Purcell and Hopkins, 1996; Redak et al., 2004). The *Xf* subspecies *fastidiosa* (*Xff*), as exemplified by the California strain Temecula 1, causes Pierce's disease in grapevine. The *Xf* life cycle and virulence mechanism are not entirely understood (Chatterjee et al., 2008). This research focused on understanding the pathobiology of *Xf* that leads to disease; specifically, the underlying mechanism that leads to leaf scorching symptoms. Understanding the pathobiology could lead to the development of new strategies to control Pierce's disease in grapevines in California. The secretion of virulence factors by pathogens is an important tool that is used to trigger plant disease. Unlike closely-related pathogens from the genus *Xanthomonas*, *Xf* does not possess the type III secretion system (T3SS) that is used to inject effector proteins into plant cells (Van Sluys et al., 2002). However, *Xanthomonas* and *Xf* have in common a similar type II secretion system (T2SS) that is used to secrete a battery of important extracellular enzymes that are responsible for virulence (Ray et al., 2000). In *Xf*, 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 *Xf* migration inside xylem vessels by degrading the pit membrane and also help release the carbohydrates necessary for bacterial nutrition and survival. One T2SS virulence factors, a polygalacturonase encoded by *pglA*, lost pathogenicity when it was mutated and resulted in *Xf* that was unable to colonize grapevine (Roper et al., 2007). This confirmed an earlier finding that expression of a polygalacturonase inhibitory protein that possibly blocked the action of the virulence factor *PglA* provided resistance to Pierce's disease (Aguero et al., 2005). Cell wall degradation by CWDEs also releases oligosaccharides as products, which can induce potent innate immune responses from plants. The plant immune 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).

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.

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, and 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 *Xf* Temecula 1. Of these, we have characterized two proteins, LesA and PrtA. A proteomic analysis of infected leaf tissues revealed five of the 24 secreted *Xf*

proteins, the most abundant of which is LesA. To further characterize the role of these proteins, we used insertional mutagenesis of *Xf* 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 most abundant *Xf* protein in infected grapevine leaves displaying Pierce's disease symptoms was an uncharacterized *Xf* protein that we have designated LesA. 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 *Xf* cells, confirming that LesA is a secreted protein (**Figure 1**). We compared the structure of lesA to proteins in the Protein Data Bank (PDB) and found a close structural similarity to a *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) LipA that has lipase and esterase activity (**Figure 2**; Aparna et al., 2009). Lipase activity was confirmed by growing *Xf* cultures on plates containing tributyrin, a triacylglyceride of butyrate. Zones of clearance were clearly visible surrounding the colonies, indicating lipase activity (**Figure 1**).

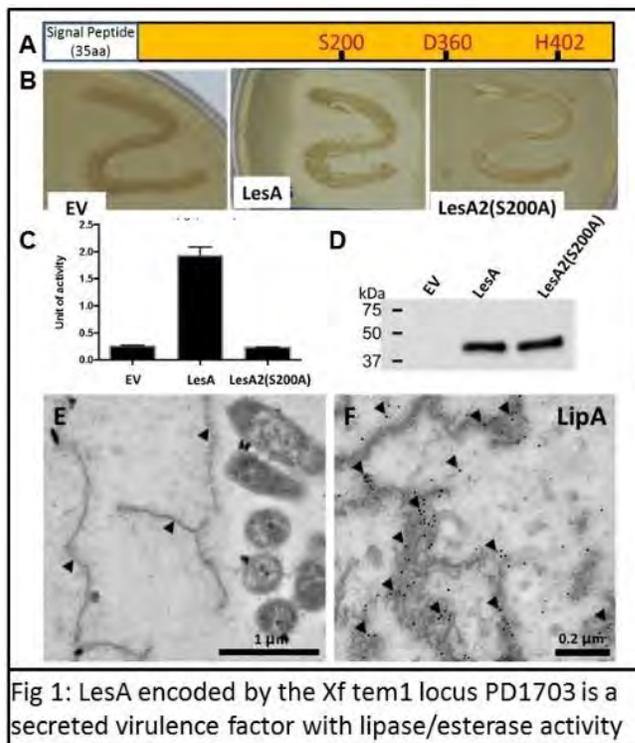


Fig 1: LesA encoded by the *Xf* tem1 locus PD1703 is a secreted virulence factor with lipase/esterase activity

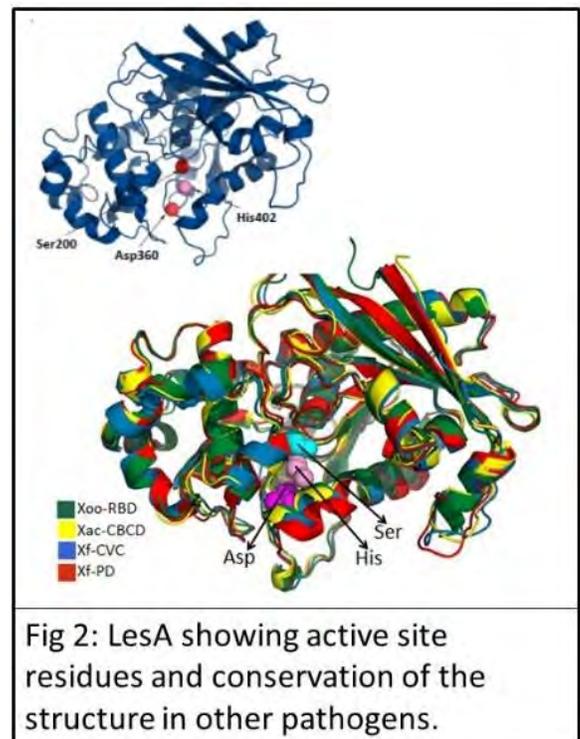
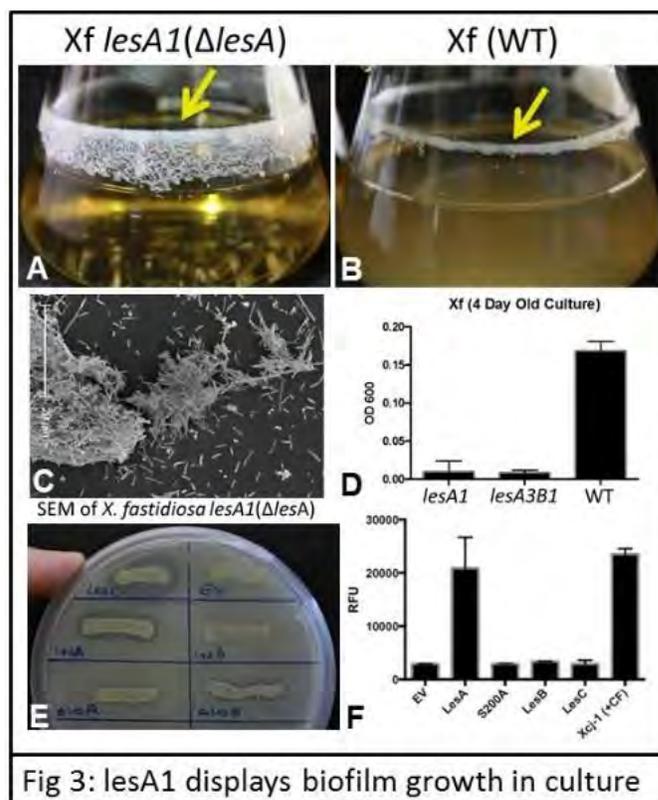


Fig 2: LesA showing active site residues and conservation of the structure in other pathogens.

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 (**Figure 2**). Additionally, LesA was found to be highly conserved among both *Xylella* and *Xanthomonas* strains (**Figure 2**). 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 tributyrin 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 (**Figures 1 and 3**). 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 (**Figures 1 and 3**). In addition, LesA protein was detected on western blots from cultures expressing both LesA and LesA2 (**Figure 1**). 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 *Xf* 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 LesA in LesB and C. LesB is located adjacent to the *lesA* on the *Xf* 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 confirmed by PCR comparison of wild-type *Xf* with *lesA1* and *lesA3B1* that we had knocked out one and both genes. *lesA1* and *lesA3B1* show less lipase and esterase activities (**Figure 3**). We expressed LesA, B, and C in *E. coli* so we could study the lipase/esterase activities they possessed. We observed some differences among the activities of LesA, B and C. LesA has both lipase and esterase activities, LesB has neither lipase nor esterase activity for the substrates that we tested, and LesC has lipase activity similar to LesA but no corresponding esterase activity directed to butyrate substrates (**Figure 3**). *lesA1* and *lesA3B1* cultures displayed increased aggregation, in contrast to wild-type *Xf* 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* (**Figure 3**). We further confirmed this observation using scanning electron microscopy, where *lesA1* showed marked aggregation of cells (**Figure 3**). To investigate the role of *lesA* in the virulence response and Pierce's disease development, 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 any symptoms at this time point (**Figure 4**). 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 (**Figure 4**). These results clearly show that the presence of the secreted LesA protein is related to leaf scorching and that the activity of the lipase/esterase is necessary for the observed symptoms. The *in planta* testing in grapevine leaves via syringe infiltration is difficult, however, we were also able to use walnut leaves and obtain cell death symptoms. In walnut leaves, LesA, LesB, and LesC were capable of causing lesions while just phosphate buffered saline (PBS) or LesA2 (functional mutant: S200A) protein and empty vector (data not shown) displayed no symptoms.



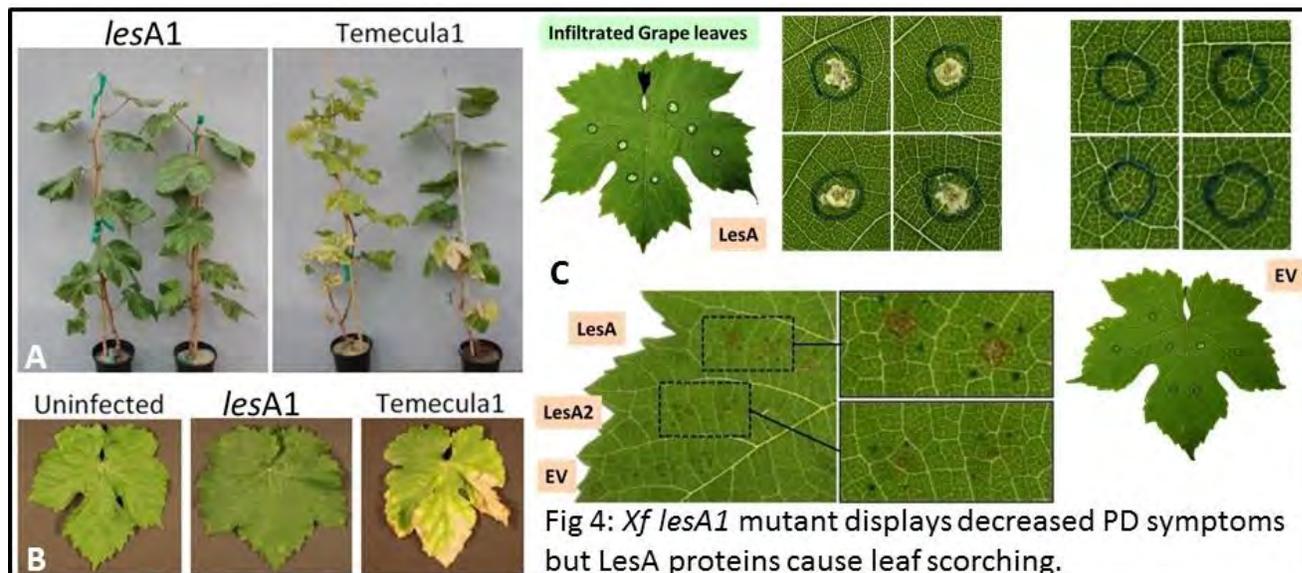


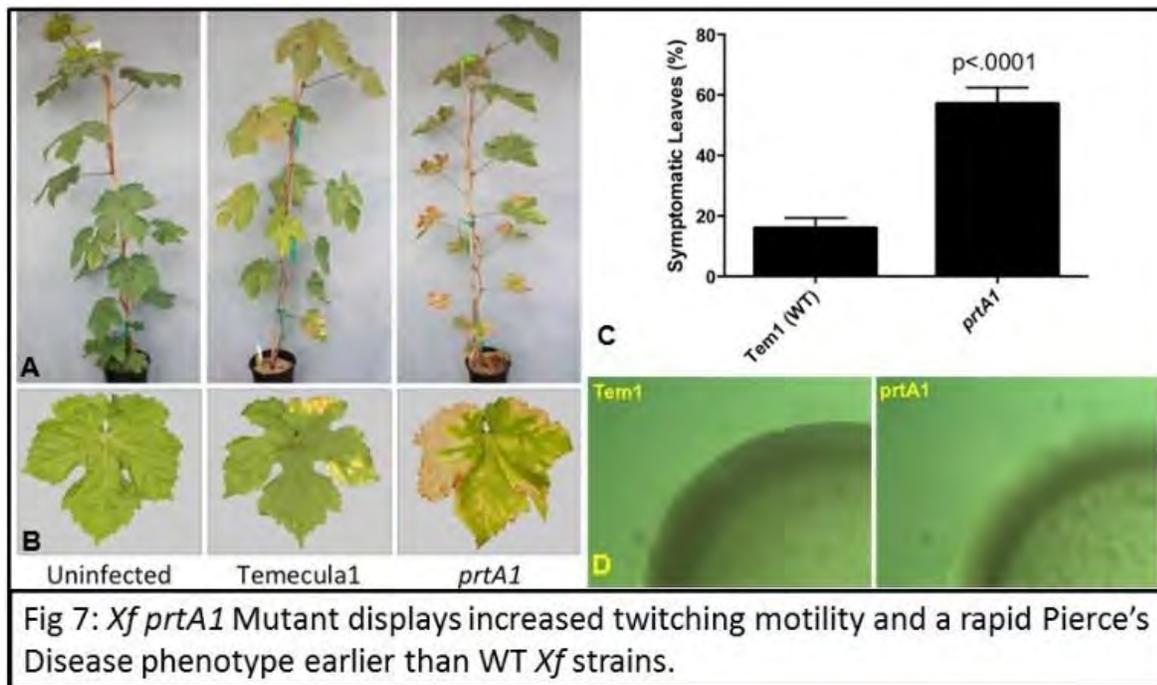
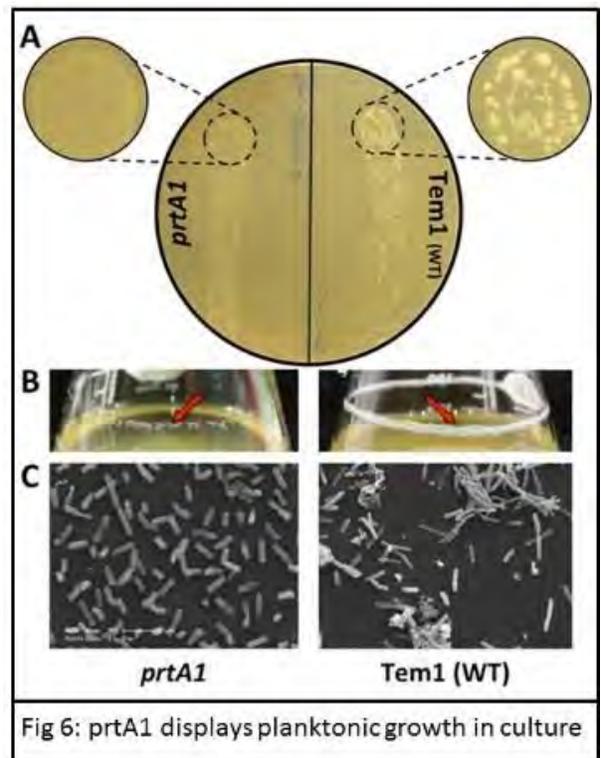
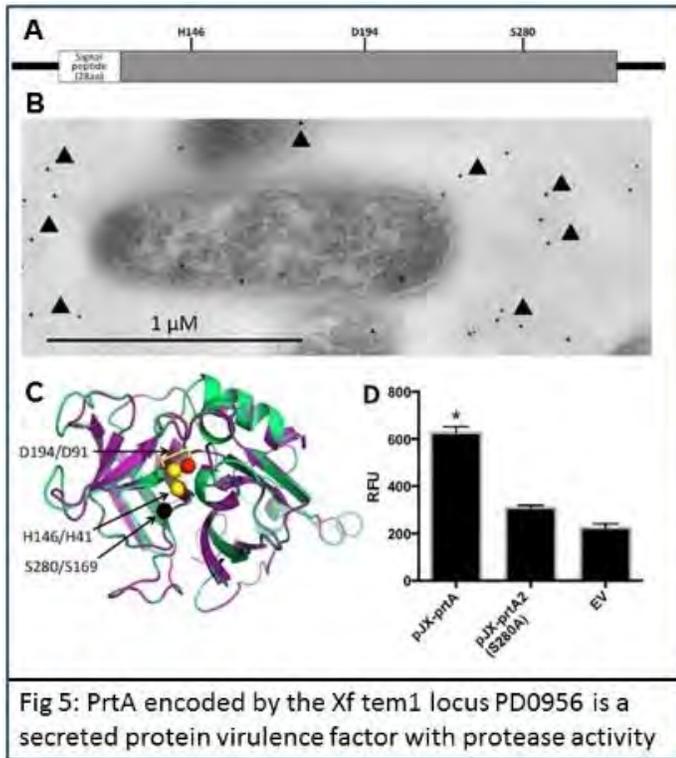
Fig 4: *Xf lesA1* mutant displays decreased PD symptoms but LesA proteins cause leaf scorching.

The secreted protein PrtA was previously annotated also as an uncharacterized protein. We analyzed the structure of PrtA, comparing it with proteins in the Protein Data Bank, 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 (Figure 5). Also, prtA is highly conserved among various *Xylella* strains, but interestingly not among *Xanthomonas* strains like LesA is.

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 (Figure 5). 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 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 *Xf* Tem1 strain (Figure 6). Growth on plates showed less aggregation and when grown in flasks, a clear biofilm ring was formed by wild-type but not prtA1 cultures (Figure 6). We used scanning electron microscopy to confirm that wild-type cultures showed marked aggregation whereas prtA1 appeared to be exclusively planktonic (Figure 6). 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 to that observed with the wild type (Figure 7). 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 (Figure 7).

Activity 2. Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, and C, and PrtA.

Because the secreted proteins may influence the grapevine microbiota and indirectly that interaction could influence the disease outcome, we investigated the microbial communities in the xylem of grapevine. Since there is not much information available on the microbial communities in grapevine we investigated a comparison of Thompson Seedless (TS) samples infected with different *Xf* strains; one unable to make PrtA (prtA1), wild-type *Xf* (Tem1), and uninfected tissue. Grapevine stem and root tissues were investigated to determine the alpha diversity of the xylem microbial communities along the axis of infection. DNA was extracted and the V4 region of the 16S rRNA gene was amplified using region-specific primers and sequenced using standard protocols as agreed upon in the Earth Microbiome Project (<http://www.earthmicrobiome.org/emp-standard-protocols/>) to reveal the composition of resident microbial communities (Caporaso et al., 2012).



Initial extractions of DNA from grapevine tissues revealed a high proportion of host chloroplast DNA that was abundantly extracted and that greatly diminished the sequence depth needed to analyze the composition of resident microbial communities. We employed the use of specific PCR blockers to selectively inhibit the amplification of grapevine chloroplast sequences (Orum, 2000). This was successful and rarefaction plots of the samples extracted in our infection study showed that novel operational taxonomic units (OTUs) are indeed extracted and that they plateau upon increasing sequence depth, indicating that we sampled a majority of the resident microbial community (Figures 8 and 9). An analysis of the alpha diversity in the different tissue samples revealed that the top and bottom stem tissues clearly separate from root tissues (Figure 10). This observed

difference in alpha diversity, shown in **Figure 10**, allowed us to compare the diversity of the resident microbial communities after challenge with wild-type *Xf* and also *lesA1* and *prtA1* mutant bacteria. We chose to compare just the stem segments and the results showed not only a clear separation based on infected and non-infected stem segments but also a clustering based on the degree of virulence (**Figure 11**). The mutant *lesA1* that we show in activity 1 to be less virulent clusters more closely to uninfected, whereas those sample tissues obtained from plants infected with the highly virulent *prtA1* strain show the least similarity (**Figure 11**). Next we investigated what was driving the separation between infected and uninfected samples. To do so, we began by looking at the alpha diversity (within sample diversity) of each sample and the variance of these samples. In both “Top” and “Bottom” tissue we observed a clear loss of diversity over time, which was clearly visible with both Shannon (**Figure 12**) and Observed OTU Alpha Diversity measures (data not shown). At pre-infection the alpha diversity of top and bottom tissue was similar, though bottom tissue was significantly more diverse. This was attributed to the bottom tissue being older and closer to the soil. Alpha diversity is lost initially near the point of infection (bottom tissue) as seen at two weeks post-infection (2WPI; **Figure 12**). As the infection spreads over time we observed changes in microbial diversity near the top part of the vine, which was first noted at six weeks post-infection (6WPI; **Figure 12**). Both top and bottom tissues showed significant decreases in alpha diversity when compared to uninfected tissue.

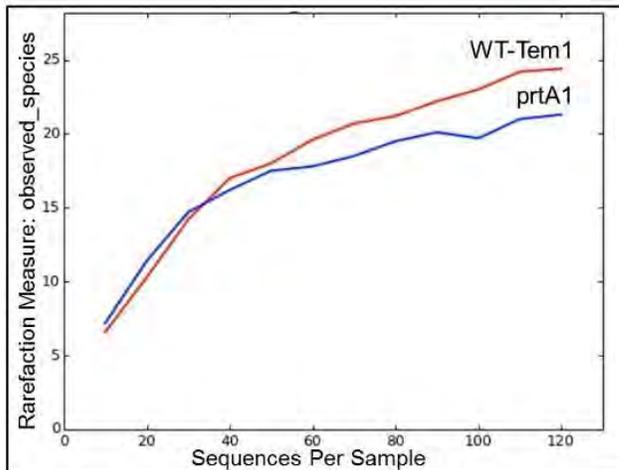


Fig 8: Rarefaction plots show how diversity varies in a sample with the number of sequences sampled.

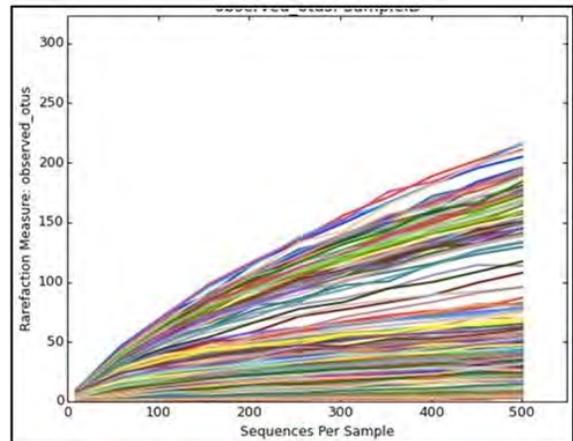


Fig 9: Rarefaction plot showing the sequencing depth of samples as it pertains to new microbial OTU discovery in the *Xf*. infection experiments.

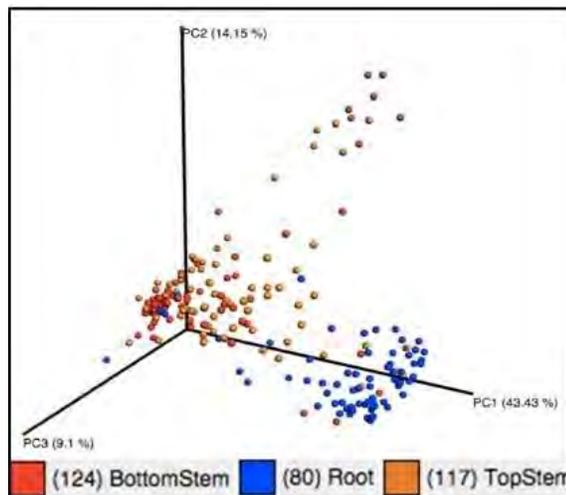


Fig. 10: PCoA plot of Weighted Unifrac Values. Data shows a difference between root tissue (blue) and stem (red and orange). Bottom Stem tissue is not highly differentiable from Top Stem tissue.

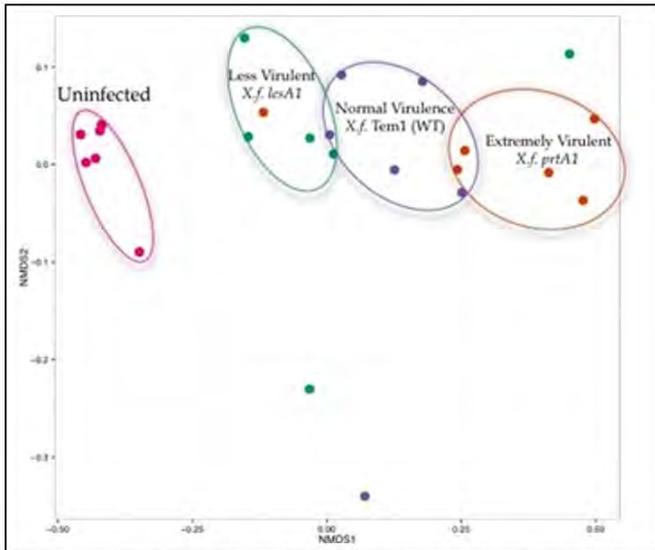


Figure 11: NMSD of non-leaf tissue between nodes 10 and 11 above infection point. Separation between uninfected and those infected with wild type compared to mutants differing in virulence

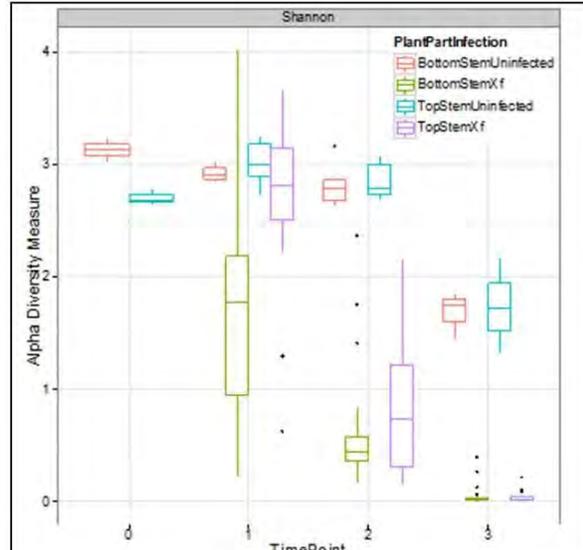


Fig. 12: Shannon Alpha Diversity measure showing "Top" and "Bottom" aerial tissue. Uninfected and Xf infected compared at four time points. Key: 0=Pre-infection, 1= 2 WPI, 2=6 WPI 3=10 WPI.

From pre-infection to six weeks post-infection we also see the composition of the microbiome change dramatically. **Figure 13** shows the taxonomic composition of the microbiome broken down by family for vines infected with *Xf* Tem1 (wild-type) over time. After *Xf* infection, the composition of the microbiome quickly becomes completely dominated by the family *Xanthomonadaceae*. This corresponds to the decrease in alpha diversity observed in **Figure 12**.

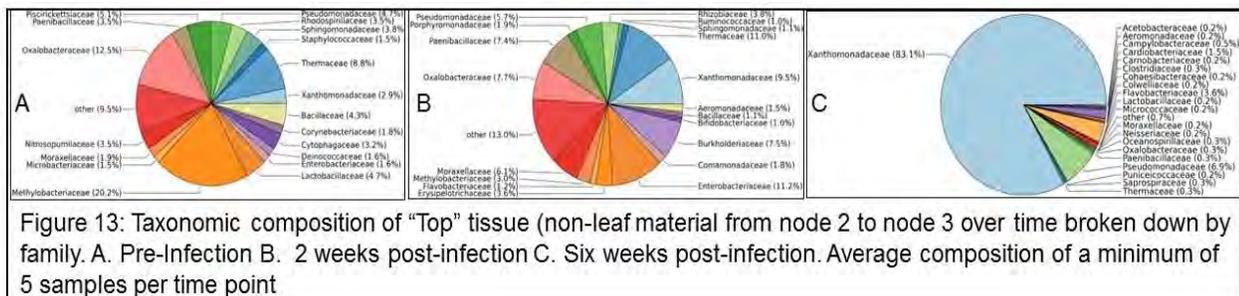
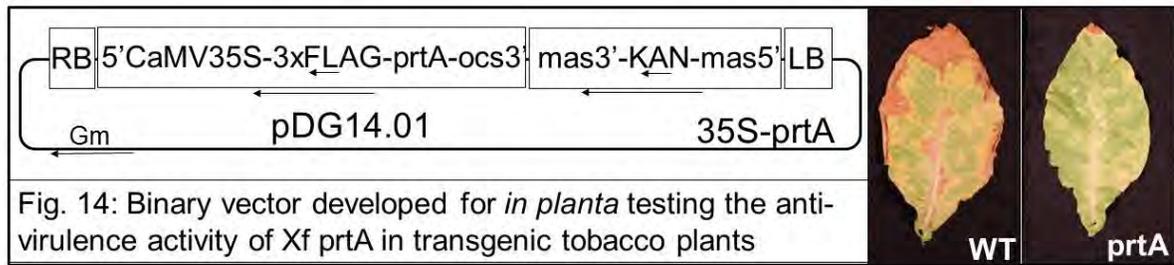


Figure 13: Taxonomic composition of "Top" tissue (non-leaf material from node 2 to node 3) over time broken down by family. A. Pre-Infection B. 2 weeks post-infection C. Six weeks post-infection. Average composition of a minimum of 5 samples per time point

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 14**). The binary vector construct was introduced into a disabled 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 Plant Transformation Facility. We have screened eight of these plants and they are all positive for the presence of the kan genes and express PrtA as detected using an anti-FLAG antibody. We did not detect the protein using an anti-PrtA antibody. The resulting plants were tested for their susceptibility to *Xf* infection using a previously established technique and two of the eight independent transgenic tobacco lines tested showed some level of tolerance (**Figure 14**; Francis et al., 2008). These results show that PrtA holds some promise as an anti-virulence factor. This observation needs to be confirmed in transgenic grapevines.



CONCLUSIONS

The goal of this project is to understand the virulence mechanisms of *Xylella fastidiosa* (*Xf*) that lead to 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 *Xf* is regarded to be the main cause of Pierce's disease symptom development. The analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins: LesA and PrtA. We generated mutant *Xf* 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 *Xf*. Expression of LesA, B, and C individually in *E. coli* indicates 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 *Xf*-prtA1 mutants are highly virulent, suggesting that this protein may somehow block disease. We have investigated the 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 reveals that two of the eight lines tested show evidence of tolerance/resistance to infection. The understanding of how these two proteins work has shown that LesA is a good diagnostic for Pierce's disease infection and that expression of PrtA in tobacco shows promising results. PrtA needs to be tested in grapevines to determine if it can provide a new avenue of therapy against Pierce's disease.

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FIELD TESTING TRANSGENIC GRAPEVINE ROOTSTOCKS EXPRESSING CHIMERIC ANTIMICROBIAL PROTEIN AND POLYGALACTURONASE-INHIBITING PROTEIN

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Reporting Period: The results reported here are from work conducted April 2016 to September 2016.

ABSTRACT

This research is a continuation of the field evaluation of chimeric antimicrobial protein (CAP) and polygalacturonase-inhibiting protein (PGIP) expressing rootstocks that enable trans-graft protection of scion varieties of grapevine from developing Pierce's disease after infection with *Xylella fastidiosa* (*Xf*). The research has two activities. The first is to conclude the field testing of Thompson Seedless expressing CAP or PGIP as a rootstock. The second is to conduct greenhouse and field evaluations of commercially relevant rootstocks expressing CAP constructs. Grapevine survival of grafted transgenic grapevines, inoculated in 2014/2015 was assessed on June 14, 2016. The data showed that the survival rate for most grafted inoculated transgenic Thompson Seedless lines expressing either CAP or PGIP was higher than those grafted to the untransformed control. The lines expressing CAP showed the highest efficacy in protecting grafted transgenic grapevines from developing Pierce's disease. Severity or absence of Pierce's disease symptoms for all Solano County field trial grafted transgenic grapevines inoculated from 2012 to 2015 was assessed in the 2015 fall season and the Pierce's disease symptom severity score was lower in most of the grafted inoculated transgenic lines from each strategy but higher in grafted untransformed control vines. *Xf* was detected in grafted transgenic vines, but *Xf* titer was lower in the transgenic as compared to grafted control grapevine rootstocks. The second activity of this project focuses on the field evaluation of CAP constructs in commercially relevant rootstocks (110-14/1103). Five CAP constructs that vary in the source and type of components to create each of the CAPs have been transformed into both rootstock backgrounds, and as transgenic plants emerge they are propagated for greenhouse and field evaluations. A two-cane disease evaluation pipeline was developed to test the transgenic rootstocks for resistance to Pierce's disease. This pipeline was successfully tested for evaluating 33 CAP-1 expressing rootstocks, with 30 in the 101-14 and three in the 1103 rootstock background. Of these 33, six displayed good efficacy and have been propagated to initiate field efficacy testing for protecting the sensitive Chardonnay grapevine variety from developing Pierce's disease.

LAYPERSON SUMMARY

This project is a continuation to evaluate the field efficacy of transgenic grapevine rootstocks expressing a chimeric antimicrobial protein (CAP) or a polygalacturonase-inhibiting protein (PGIP) to provide protection to the grafted scion variety from developing Pierce's disease. We are concluding the current field evaluation where four CAP and four PGIP expressing Thompson Seedless were tested as rootstocks to protect grafted wild-type Thompson Seedless scions. These plants were infected with *Xylella fastidiosa* (*Xf*) in 2012, 2013, 2014, and 2015 and evaluated each year for their ability to provide resistance to Pierce's disease. Our conclusion is that the transgenic rootstocks were able to provide transgraft protection to the scion; they showed less symptoms and harbored a lower titer of the pathogen. Since Thompson Seedless is not a commercially relevant rootstock we have now begun testing the field efficacy of this strategy by expressing different CAP proteins in the commercially relevant rootstocks 110-14 and 1103. The technology to transform these two rootstocks developed in an earlier project is being implemented to develop transgenic 110-14 and 1103 rootstocks expressing different versions of the CAP protein. We have implemented a two-cane Pierce's disease screen to test these transgenic rootstocks. We evaluated 33 transgenic rootstock lines expressing CAP-1 and were able to identify six good lines that we will test in the field for their ability to protect the sensitive scion cultivar Chardonnay from developing Pierce's disease. More transgenic rootstock lines are being developed, and as they emerge they will be tested in the greenhouse and field, a process that is currently ongoing. Elite rootstock lines identified in this project will be good candidates for commercialization.

INTRODUCTION

The focus of this study is to evaluate the rootstock-based expression of chimeric antimicrobial protein (CAP; Dandekar et al., 2012a) and polygalacturonase-inhibiting protein (PGIP; Agüero et al., 2005, 2006) to provide transgraft protection of the scion grapevine variety against Pierce's disease. Rootstocks (Thompson Seedless) expressing these proteins individually are currently being evaluated in the field. This part of the study will be concluded this year. Thompson Seedless rootstock lines expressing either CAP or PGIP show promise in their ability to transgraft protect a scion variety (also Thompson Seedless) against Pierce's disease, which is being validated with in-field inoculations. Since Thompson Seedless is not a rootstock these genes must be tested in a commercially relevant rootstock. Methods to successfully transform two commercially relevant rootstocks (101-14 and 1103) (Christensen, 2003) was successfully developed (Dandekar et al., 2011; 2012b) and the method was further improved by David Tricoli in the Plant Transformation Facility at UC Davis. The original neutrophil elastase - cecropin B (NE-CB) CAP construct (Dandekar, 2012a) was improved by identifying grapevine-derived components (Chakraborty et al., 2013; 2014b). The surface binding neutrophil elastase component was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease activity (Chakraborty et al., 2013; Dandekar et al., 2012c; 2013). The antimicrobial component cecropin B was replaced with HAT52 and/or PPC20 that were identified using novel bioinformatics tools developed by us (Chakraborty et al., 2013; 2014a) and the efficacy of the selected peptides were verified for their ability to kill *Xylella fastidiosa* (*Xf*) cells (Chakraborty et al., 2014b). In addition to the original NE-CB CAP (CAP-1) four additional CAP constructs were developed that contained VsP14a (CAP-2); VsP14a-CB (CAP-3); VsP14a-HAT52 (CAP-4) and VsP14a-PPC20 (Dandekar et al., 2012c; 2013; 2014). These transgenic CAP-expressing rootstocks will be tested in the greenhouse and field starting in fall 2016. These additional CAP constructs that will be tested here are aimed to address the concern that the protein components of the present CAP-1 have a non-plant origin. Transformation of these five CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2015 and will be ready for greenhouse and field testing in 2017 onward. The field testing of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting the grafted, sensitive scion cultivar Chardonnay from developing Pierce's disease.

OBJECTIVES

1. Complete the efficacy of the current round of *in planta* expressed chimeric NE-CB and PGIP proteins to inhibit and clear *Xf* infection in xylem tissue and through the graft union in grapevines grown under field conditions.
Activity 1. Complete and conclude testing of the current round of plants in the field.
Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 110-14 and 1103 rootstocks.

RESULTS AND DISCUSSION

Activity 1. Complete and conclude testing of the current round of plants in the field.

At the Solano County field trial site (**Figure 1**) half of the non-grafted transgenic lines were manually inoculated as described (Almeida et al., 2003) on July 13, 2011, and the rest on May 29, 2012. Half of the grafted transgenic lines were also manually inoculated on the latter date. Non-grafted and grafted grapevines at the Solano County site that were not previously inoculated were manually inoculated on June 17, 2013, completing the inoculations of all grapevines at this location. On May 27, 2014 and May 27, 2015, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease and Glassy-winged Sharpshooter Board, at least four new canes per year from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf*. Inoculation dates from 2011 to 2015 are shown in a color-coded map (**Table 1**).

On July 22, 2014 and September 15, 2015, one 2014-inoculated cane from each grafted transgenic plant was harvested for quantification of *Xf* by quantitative polymerase chain reaction (qPCR) using an Applied Biosystems SYBR green fluorescence detection system. *Xf* DNA was extracted using a modified CTAB (hexadecyltrimethylammonium-bromide) method that allowed us to obtain DNA of a quantity and quality suitable for qPCR. The *Xf* 16s primer pair (forward 5'-AATAAATCATAAAAAAATCGCCAACATAAACCA-3' and (reverse 5'-AATAAATCATAACCAGGCGTCCTCACAAGTTAC-3') was used for *Xf* quantification. qPCR standard curves were obtained using concentrations of *Xf* ranging from 10^2 to 10^6 cells per 0.1 g tissue. *Xf* was detected in grafted transgenic vines, but at *Xf* counts that were lower than in grafted control grapevines (**Figure 2**).



Figure 1. Solano County field trial grafted transgenic grapevines inoculated in spring 2014 and spring 2015. Photo taken in fall 2016

Table 1. Solano County grape field trial map, color-coded by *Xf* 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																									
Row 8																									
Row 7																									
Row 6																									
Row 5																									
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

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

North

Severity or absence of Pierce's disease symptoms was assessed for all Solano County field trial grafted transgenic grapevines inoculated from 2012 to 2015 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 3**). Pierce's disease symptom severity scores were lower in most grafted inoculated transgenic lines from each strategy (CAP or PGIP) than in grafted untransformed controls.

Grapevine survival of grafted transgenic grapevines that were inoculated in 2014/2015 was assessed on October 6, 2016 using a 1 to 5 score, where 1 = very healthy and vigorous grapevine; 2 = healthy grapevine and slightly reduced vigor; 3 = slightly reduced spring growth; 4 = much reduced spring growth; and 5 = dead grapevine (**Figure 4**). The grapevine survival rate was greater in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls, with the greater efficacy seen in CAP lines.

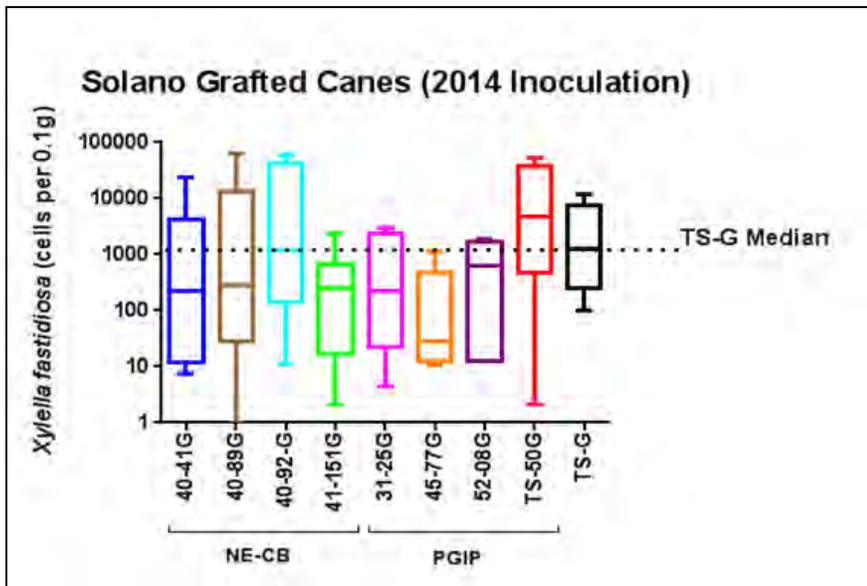


Figure 2. *Xf* quantification by qPCR of Solano County field trial grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014 and fall 2015.

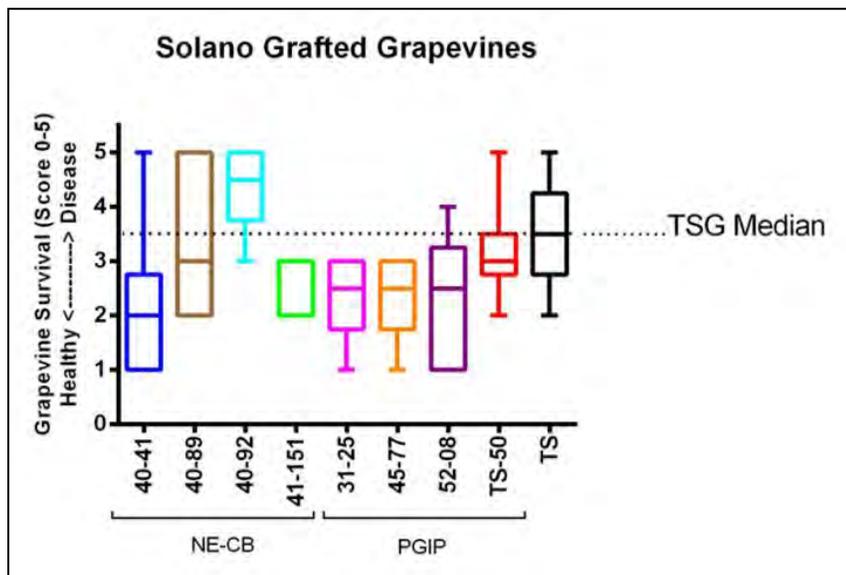


Figure 3. Severity or absence of Pierce’s disease symptoms for all Solano County field trial grafted inoculated grapevines in fall 2015.

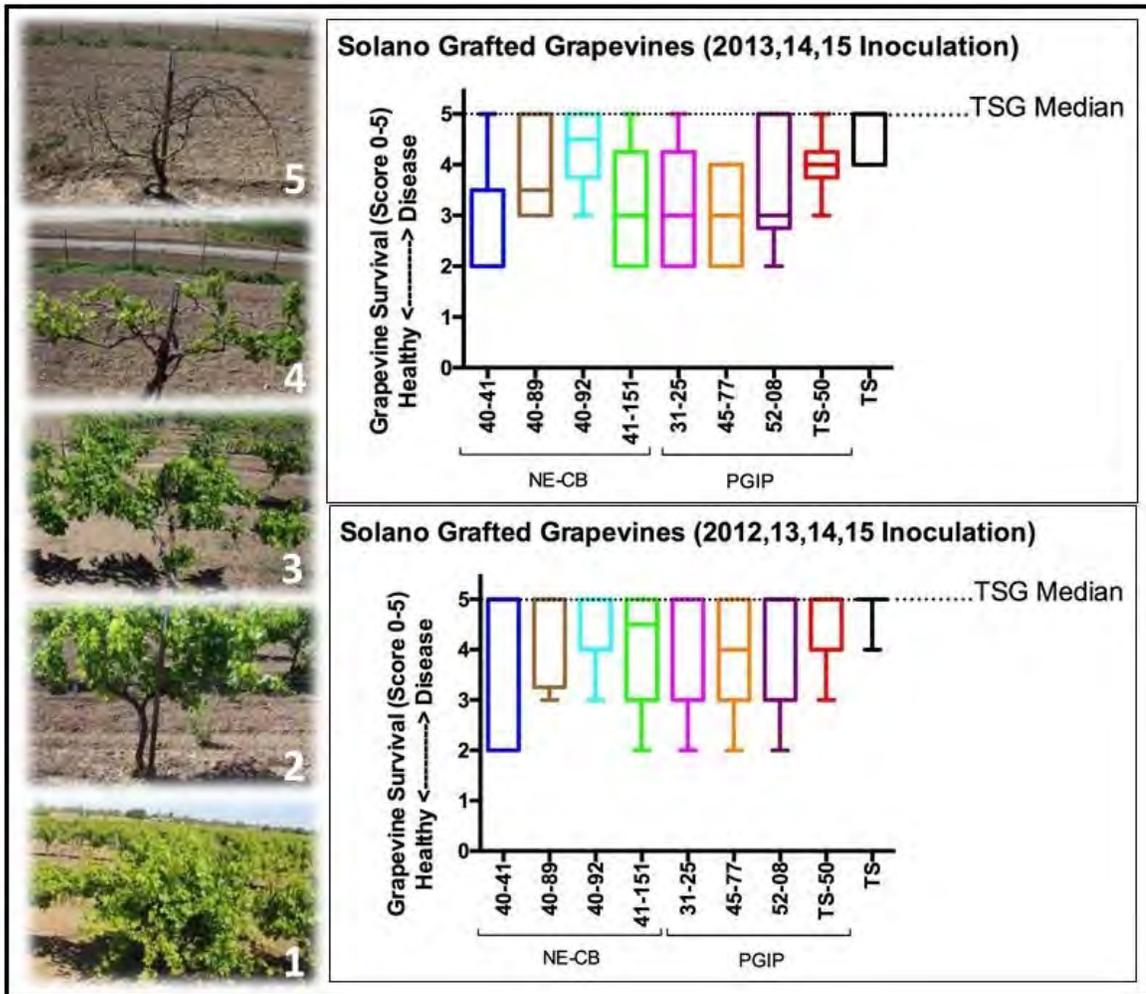


Figure 4. Grapevine survival of Solano County field trial grafted transgenic grapevines inoculated in 2013-2015 (upper right) and all inoculated grafted transgenic grapevines (lower right), scored in fall 2016 using a scale of 1 to 5 (left).

Activity 2. Conduct greenhouse and field evaluation of CAP-expressing 101-14 and 1103 rootstocks.

This activity focused on greenhouse and field testing of five vector constructs that are in the plant transformation pipeline on two commercially relevant rootstocks, 101-14 and 1103 (Christensen, 2003). The components present in these constructs are shown in **Figure 5** below. The construction of CAP-1 was described earlier (Dandekar et al., 2012a) and the components, mostly from grapevine, and construction of CAP-2, CAP-3, CAP-4 and CAP-5 shown in **Figure 5**, have been previously described (Chakraborty et al., 2014b; Dandekar et al., 2012c; Dandekar et al., 2013; Dandekar et al., 2014a). The grapevine transformation methods for the 101-14 and 1103 rootstocks have been described previously (Dandekar et al., 2011; Dandekar et al., 2012b) but were further improved by David Tricoli in the UC Davis Plant Transformation Facility who did the transformation of all of the binary vector constructs shown in **Figure 5**. The transgenic plants obtained from the facility propagated for testing are described in detail below. The transformation of the two rootstock species with all five CAP constructs was initiated in 2014 and the selection and regeneration of plants is ongoing. As plants emerge they are propagated for greenhouse and field testing.

Transformation of the first construct (CAP-1) yielded thirty 101-14 and three 1103 derived transgenic lines. The most progress was made in the analysis (described below) of these CAP-1 lines this summer, as can be seen in **Table 2**. Since the yield for 1103 lines transformed with CAP-1 was low, a new transformation was initiated back in August 2015. Also, this summer we began receiving 110-14 and 1103 lines transformed with the other constructs (CAP-2 to 5) and the numbers and distribution of these lines is indicated in **Table 2**.

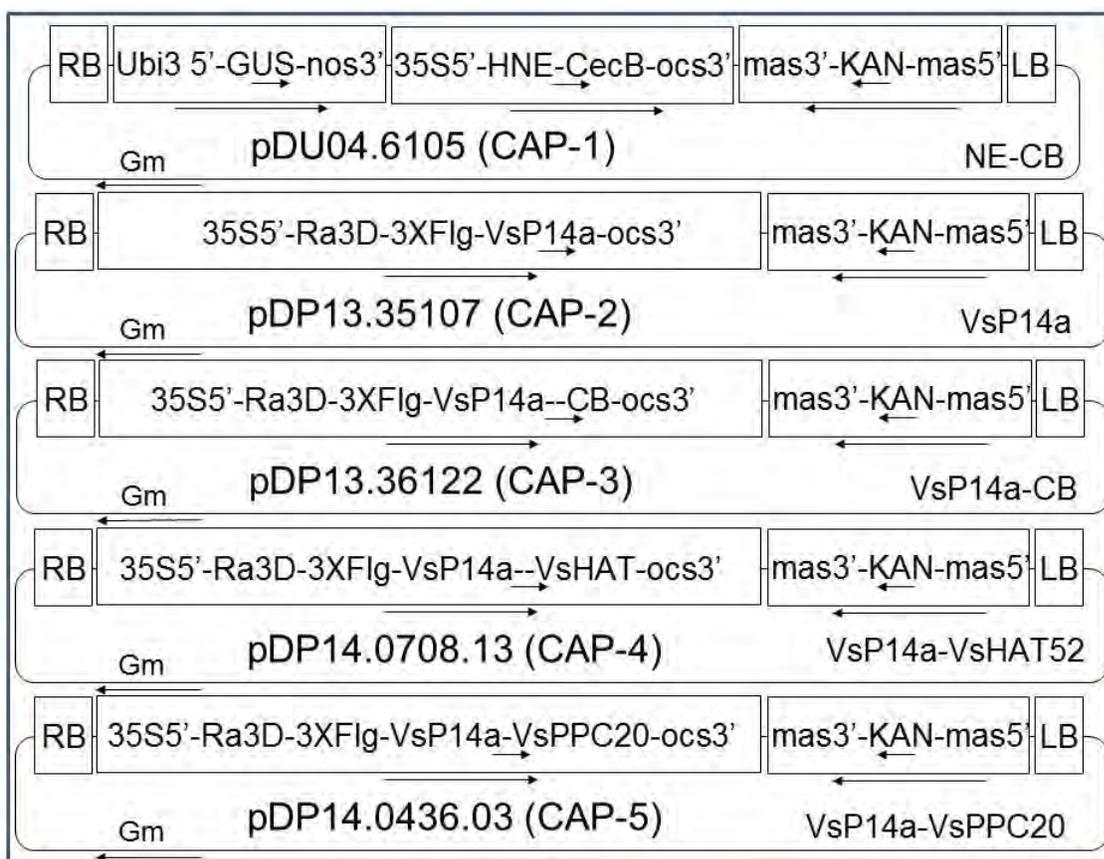


Figure 5. CAP vectors testing the original and grapevine components, used to create transgenic 101-14 and 1103 rootstocks that will be verified in greenhouse and field.

Table 2. Progress on the analysis of transgenic lines obtained from different CAP constructs in the two rootstock species.

CAP Designation	Binary Vector	Greenhouse propagation		Greenhouse Testing		Field Testing	
		101-14	1103	101-14	1103	101-14	1103
CAP-1	pDU04.6105	30	4	30	4	6	0
CAP-2	pDP13.35107	3	2				
CAP-3	pDP13.36122	3	1				
CAP-4	pDP14.0708.	8	5				
CAP-5	pDP14.0436.03	8	5				

A propagation/testing pipeline has been successfully developed to test the efficacy of both 101-14 and 1103 grapevines, and the transgenic lines will be tested for Pierce's disease resistance in the greenhouse as they emerge from the transformation and after propagation. The testing of the 101-14 and 1103 transformed rootstocks transformed with CAP-1 has already been completed in the greenhouse and field testing of the promising lines will be initiated in the fall of 2016. The field introduction of these rootstocks is aimed at evaluating their efficacy in protecting the grafted sensitive Chardonnay grapevine variety from developing Pierce's disease.

The 101-14 and 1103 transgenic rootstocks lines are first screened for the presence of CAP transgene using PCR. Those 101-14 and 1103 plants that are PCR-positive are clonally propagated for greenhouse testing. The clones are trained into a two-cane system and inoculated on one of the canes with *Xf*. Plants are inoculated with 20 uL of *Xf* at a site roughly three nodes above the fork in the canes and eight leaves below the top of the cane, then it is turned over and inoculated with another 20 uL of *Xf* directly behind the first inoculation. The *Xf* inoculum is prepared as described earlier (Dandekar et al., 2012a).

The transgenic rootstocks successfully inoculated as described above are evaluated for Pierce's disease symptoms 12 weeks post-inoculation when the first disease symptoms appear, and subsequently every two weeks thereafter until 18 weeks post-inoculation. A scoring system of 1 to 5 was used with values of 1 = no visible disease symptoms (good); 2 = disease symptoms on less than four leaves (good/ok), 3 = disease symptoms exhibited on 50 percent of the cane (four leaves, ok); 4 = disease symptoms exhibited on 75 percent of the cane (six leaves, ok/bad); and 5 = symptoms stretching the entire length of the inoculated cane (eight leaves, bad).

All 33 CAP transgenic lines have been analyzed. Of these, six have been identified for field testing. All six were 110-14 transgenics. Of the six 110-14 transgenics selected, one was an elite line and presented no Pierce's disease symptoms and got a score of 1. The remaining five 101-14 plant lines got a score of 2; they look very promising and were considerably less sick than the untransformed 101-14 control, which was scored a 5 (**Figure 6**). All lines from 1103 scored bad and received a score of 5. The six 101-14 transgenic rootstocks expressing CAP-1 that scored a 1 or 2 have been clonally propagated from the uninfected mother plants. Well-established 101-14 transgenic rootstock plants will be planted in the field in the fall of 2016 and grafted with the scion Chardonnay in the spring of 2017.



Figure 6. Two-cane vines with the left uninfected and the right infected. (A) Wild-type 101-14 grapevines with disease symptoms running the entire length of the infected cane. (B) The elite CAP-1 transgenic line of 110-14 that showed no symptoms 18 weeks post-inoculation.

CONCLUSIONS

We have successfully concluded field-testing of Thompson Seedless as a rootstock expressing CAP or PGIP. Grapevine survival of grafted transgenic grapevines inoculated in 2013 to 2015 was assessed. The survival rate of most grafted inoculated transgenic Thompson Seedless lines using both strategies was greater than in untransformed controls, with the CAP lines most efficient in protecting against Pierce's disease. The phenotypic disease data corresponded to the bacterial titer estimations using qPCR, which revealed lower bacterial titers in transgenic plants as compared to the wild-type susceptible Thompson Seedless plants. Severity or absence of Pierce's disease symptoms on all Solano County field trial grafted transgenic grapevines inoculated between 2012 and 2015 was also assessed, and Pierce's disease symptom severity scores were lower in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. The field-testing data confirm that Thompson Seedless rootstock lines expressing either CAP or PGIP are able to provide protection against Pierce's disease. We have developed a successful propagation and two-cane testing pipeline to evaluate 101-14 and 1103 transgenic rootstocks expressing various CAP constructs. We have successfully tested this pipeline by evaluating 33 transgenic lines expressing CAP-1. Of the 33 CAP-1 expressing rootstocks, 30 were in the 101-14 and three in the 1103 rootstock background. Of these 33 we have identified six lines that show promise based on two-cane greenhouse testing for resistance to Pierce's disease. Field planting of these six lines has been initiated to conduct efficacy testing in the field for transgraft protection of the sensitive Chardonnay grapevine variety from developing Pierce's disease. Thirty-five lines corresponding to the rest of the CAP constructs are in the greenhouse to initiate testing, and more will be included as they emerge from the transformation facility.

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FUNDING AGENCIES

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

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 November 1, 2015 to September 30, 2016.

ABSTRACT

Type I secretion (T1S) by *Xylella fastidiosa* (*Xf*) is required for multidrug efflux, a pump critical for survival of *Xf* in grapevines. In *Xf*, T1S depends on a very limited number of genes, possibly making this system more vulnerable to inhibition by small molecule treatments than T1S found in most bacterial pathogens, which typically carry redundant T1S systems. *Xf* single gene mutations in the T1S system are much more sensitive to the surfactant Silwet L-77 than wild-type *Xf*. High throughput screening assays of *Xf* cell viability were developed using fluorescence and optical density measurements both with and without 200 ppm Silwet L-77. Green fluorescent protein marked *Xf* strain Temecula-1 was used to screen two Prestwick combinatorial small molecule libraries (phytochemical and FDA approved drugs; 1,600 chemicals in total) for *Xf* cell growth inhibition. Significant (>50%) inhibition of Temecula-1 growth was observed in the presence of 50 μ M of 215 different chemicals, six of which exhibited even higher (24% to 40%) stronger inhibition in the presence of Silwet L-77, indicating these six chemicals possibly target T1S efflux. Forty-six chemicals reduced growth >100%, indicating *Xf* cell lysis. Seven chemicals, including four phytochemicals, reproducibly lysed *Xf* at 25 μ M levels. Four of these chemicals were eliminated from further consideration because they have pharmaceutical uses and would likely face severe regulatory hurdles. Three chemicals are being further evaluated as potential treatments for Pierce's disease as both soil drench and spray applications, and for phytotoxicity to grape and tobacco leaves. One is strongly phytotoxic to grape leaves at 25 mM levels and may be eliminated from further consideration, while another appears only slightly toxic at 50 mM levels, based on chlorophyll degradation assays.

LAYPERSON SUMMARY

Xylella fastidiosa's (*Xf*'s) survival in grapevine and in many culture conditions depends on a Type I multidrug resistance 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 *Xf*. 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 *Xf* from infected plants. Portions of the outermost efflux pump protein, TolC, are embedded in the protective outer membrane of the bacterium and form the exit portal of the efflux pump. Both the outer membrane and TolC are exposed at the *Xf* cell surface, making small molecule chemical treatments that target TolC or even the outer membrane barrier attractive chemical targets. Several 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 FDA approved drug combinatorial library, together representing 1,600 different small molecules in total. Seven chemicals have been identified from the Prestwick libraries that appear to lyse *Xf* cells at low concentrations, and three are likely to pass environmental and regulatory safety tests. One appears strongly phytotoxic to grape, but two others are being evaluated for control of Pierce's disease in infected *Vitis vinifera* grapevines and may be economical for use in controlling Pierce's disease of grape.

INTRODUCTION

This is a new project that is based on two discoveries made during the course of two earlier CDFA-funded projects. The first discovery is our demonstration that the Type I multidrug resistance (MDR) efflux system of *Xylella fastidiosa* (*Xf*) 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 *Xf* 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 *Xf* 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 *Xf* 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 *Xf*, 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 *Xf*, 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 *Xf* should be much more vulnerable to chemical 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 project is to exploit the increased vulnerability of *Xf* and our knowledge of particular chemicals that require efflux in a high throughput assay that screens small molecule combinatorial libraries and *Xf*-resistant grapevines for chemicals that may disable Type I secretion directly or indirectly. A highly sensitive live cell assay that is well suited for high throughput screening was developed and used for this screening.

OBJECTIVES

The specific objectives of this one-year project are:

1. Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from *Xf*.
2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments sufficient to cure Pierce's disease for potential effects on Type I efflux from *Xf*.
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 *Xf* than susceptible *V. vinifera* plants.

RESULTS AND DISCUSSION

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

Initial experiments focused on *Xf* culturing conditions (starting optical density and cell volumes) that would be adequate to obtain reproducible results in a chemical screen for *Xf* growth using a 96-well microtiter plate format. Two day-old cultures of green fluorescent protein (GFP)-marked Temecula-1 cells (optical density at 600 nm (OD₆₀₀) = 0.25) were diluted to starting OD = 0.05 and used for seeding 96-well microtiter plates for high throughput screening of the chemical libraries. Cell volumes of 100, 150, and 200 μ l/well were tested at 28°C. Overall, 150 μ l/well volumes were determined to be practical and reproducible for observing growth, both as measured by OD and GFP fluorescence (**Figure 1**).

As can be observed from **Figure 1**, maximum growth and fluorescence emission was observed at 48 hours after seeding the plates using 150 μ l volumes. Therefore, chemical treatments were added at the time of plate seeding and effects of the treatments were evaluated 48 hours later. Silwet L77 at 200 ppm had no effect on growth of the wild-type strain Temecula-1.

For the primary chemical screens plates were preloaded with Temecula-1 cells with or without 200 ppm Silwet L-77 and with each tested chemical loaded at a concentration of 50 μ M. Each chemical in the Prestwick Phytochemical and Chemical libraries was screened in two separate experiments per library. The statistical parameter (*Z'*) was used to evaluate the quality of the assays exactly as described (Zhang et al., 1999). The overall *Z'* value for the Prestwick Phytochemical library was 0.76 and the overall *Z'* for the Prestwick Chemical library was 0.78; these values are within the statistically "excellent" reproducibility range (*Z'* > 0.75; Zhang et al., 1999).

Significant growth inhibition (>50%) of Temecula-1 was observed with 22 phytochemicals (**Figure 2**), eight of which exhibited strongly significant growth inhibition (>90%). Greater than 100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (**Figure 3**) was reduced to below that of the starting cell values, and indicated lysis. None of the 320 phytochemical library compounds was found to enhance growth. None of the 320 phytochemical library compounds exhibited enhanced inhibition in the presence of 200 ppm

Silwet L-77, indicating that none of these compounds directly affected T1S. Eleven phytochemicals, including some natural antibiotics, were identified as strongly inhibitory (> 80%) at 50 μ M, including the phytoalexin gossypol and the alkaloids remerine and olivicine.

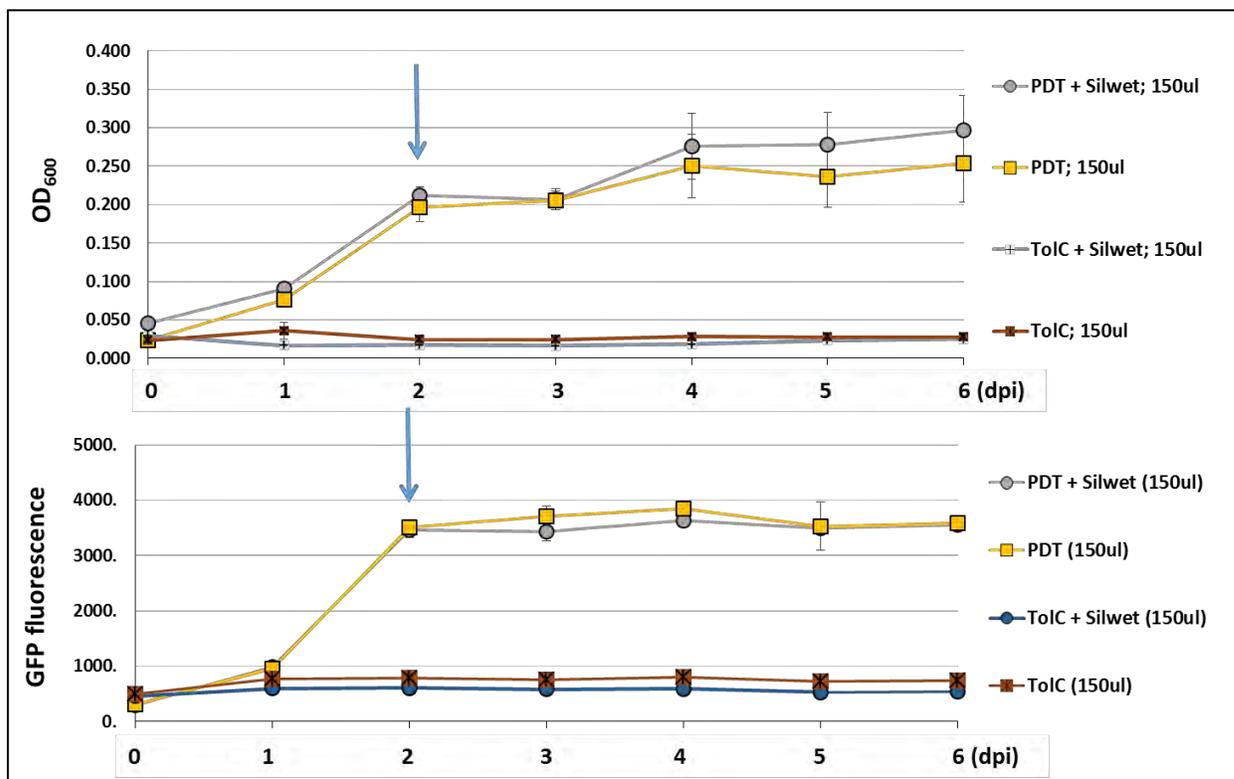


Figure 1. Growth of *Xf* cells at a cell volume of 150 μ l/well in a 96-well format. PDT, wild-type Pierce's disease strain Temecula-1. ToIC, a *tolC* mutant of PDT. Silwet L77 (Silwet) was added at 200 ppm to both PDT and ToIC for evaluation purposes.

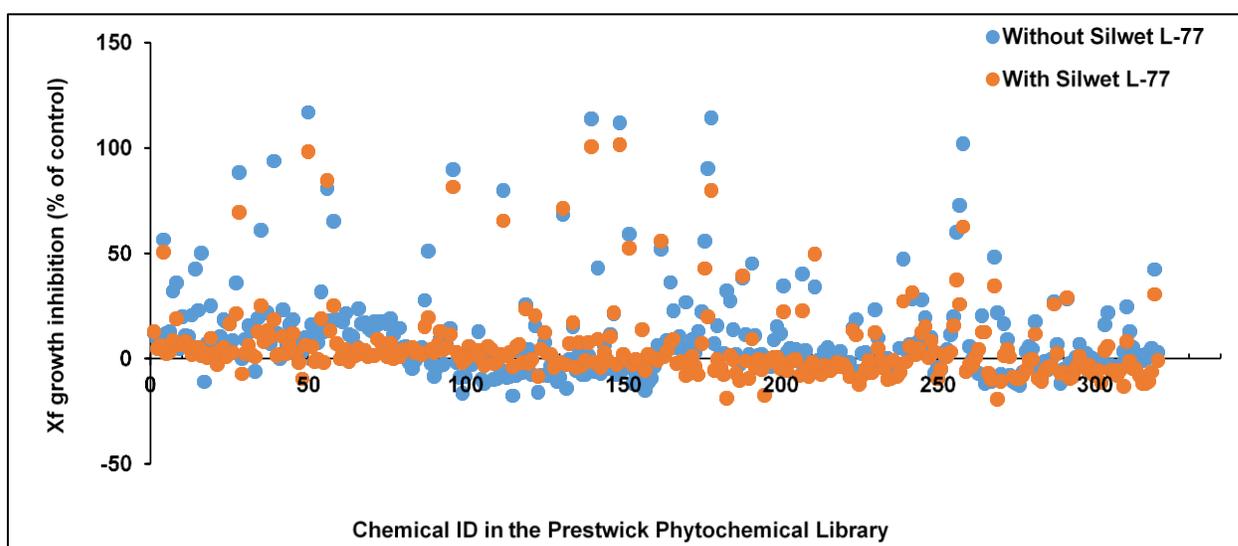


Figure 2. Screening of the Prestwick Phytochemical Library of 320 compounds for growth inhibition of *Xf*, both with and without Silwet L-77. Growth of PDT in the presence of 320 chemicals (numbered along the horizontal axis) both with (orange dots) and without (blue dots) 200 ppm Silwet L-77. Both OD and GFP fluorescence were measured. Plates were incubated at 28 $^{\circ}$ C for two days, and both OD and GFP fluorescence again measured. Growth inhibition was calculated as the difference between the change in OD (not shown) or GFP fluorescence between treatments and the respective untreated control. Chemicals exhibiting at least 50% of growth inhibition relative to the respective untreated control were selected for additional screening at different concentrations (dose effect).

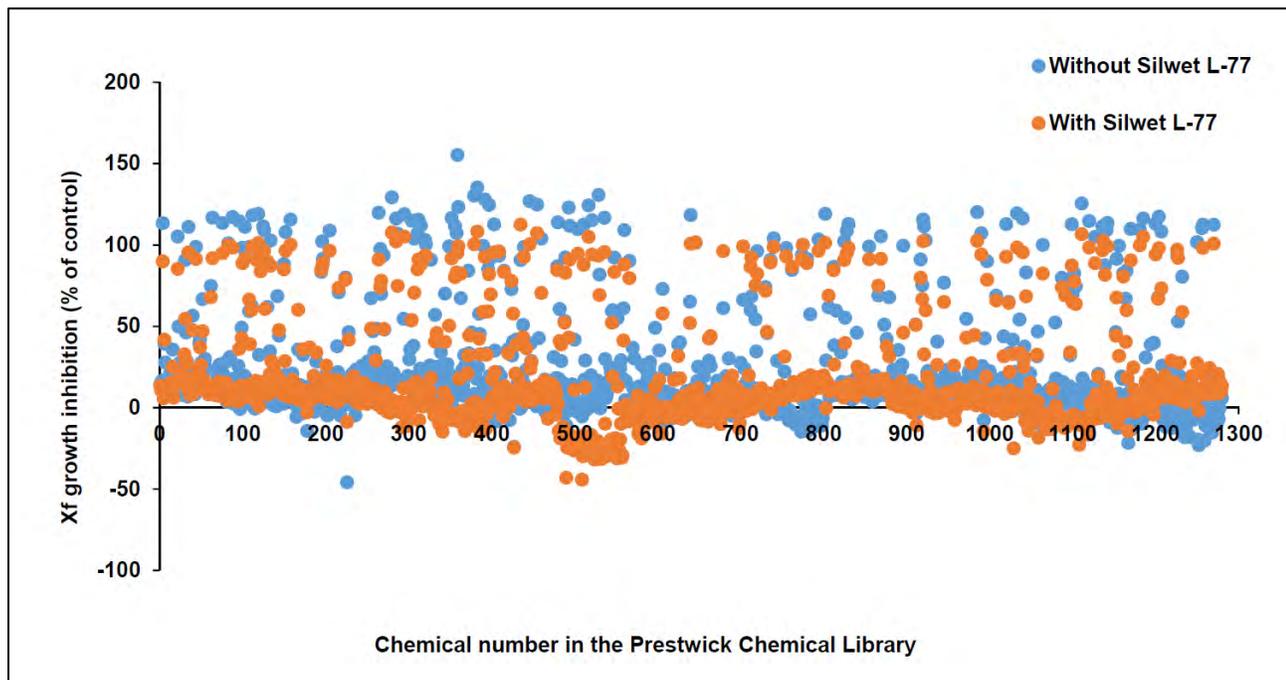


Figure 3. Screening of the Prestwick Chemical Library of 1,280 compounds for growth inhibition of *Xf*, both with and without Silwet L-77. Legend as in **Figure 2**.

Significant growth inhibition (>50%) of Temecula-1 was observed with 193 chemicals from the Prestwick Chemical library (**Figure 3**), 121 of which exhibited strongly significant growth inhibition (>90%). Greater than 100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (shown in **Figure 3**) was reduced to below that of the starting cell values, and indicated lysis. Notably, six chemicals exhibited not only direct growth inhibition (ranging from 53% to 90%) but this inhibition was enhanced (>24% more) by Silwet L-77, indicating that these chemicals possibly target T1S efflux. These chemicals include a thiazolidine antiparasitic agent, several antibiotics, and a calcium antagonist.

Following the primary screen at 50 μM , the effect of different dose levels (25 μM , 50 μM , and 100 μM) were evaluated using three replications of each level, in each case with and without Silwet L-77. This evaluation was performed both for confirmation purposes and to determine if a threshold level effect was present for some chemicals. No threshold effects were observed; initial results were confirmed at all dose levels. Silwet L-77 had no effect on any of the phytochemicals. However, Silwet enhanced the inhibition of six compounds from the Prestwick chemical library in the primary screen. At different dose levels only one compound consistently inhibited *Xf* growth more strongly in the presence of Silwet, and at all three treatment levels, indicating an effect of the chemical on multidrug efflux (Type I secretion).

Over 120 chemicals have been identified that inhibited growth of *Xf* by >90% at 50 μM , including 46 chemicals that appeared to lyse *Xf* cells. Seven chemicals proved to lyse *Xf* cells at 25 μM , including four phytochemicals. Four of these chemicals were eliminated from further consideration because they have pharmaceutical uses and would likely face severe regulatory hurdles, and one was eliminated due to cost considerations. Two chemicals are being further evaluated as potential treatments for Pierce's disease as both soil drench and spray applications. Phytotoxicity to grape and tobacco leaves has now been evaluated using chlorophyll loss as a sensitive indicator of phytotoxicity (for example, refer to Jain et al., 2012). Leaf disc punches from young fully expanded leaves were floated in water containing different levels of each phytochemical evaluated, both vacuum infiltrated and uninfiltrated (**Figure 4**). Chlorophyll content in both grape and tobacco leaf discs was estimated after three days extracting overnight in 80% acetone and quantified spectrophotometrically according to the procedure of Arnon (1949). The results are presented in **Figure 5**, below.

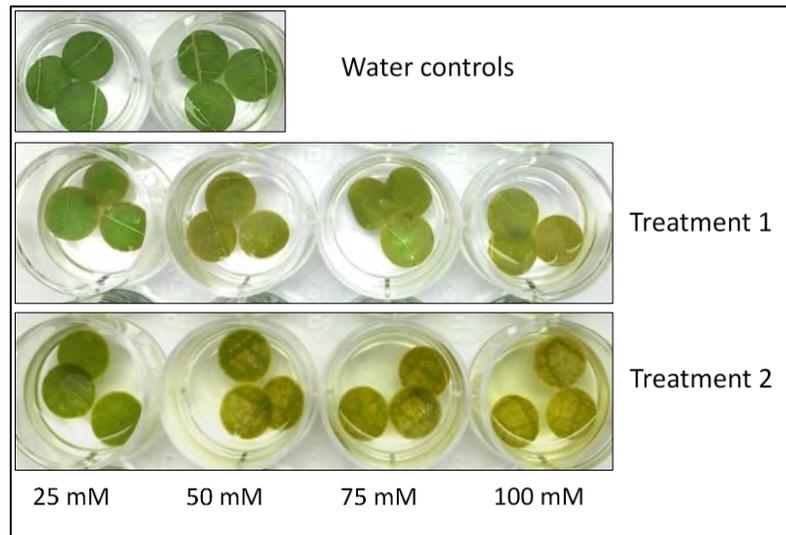


Figure 4. Effect of two selected Prestwick chemical treatments at the indicated concentrations on chlorophyll degradation in grape leaf discs (photo taken after 18 hours).

Clearly, treatment 1 was strongly phytotoxic to grape leaves at 25 mM levels and somewhat to tobacco leaves at the same level, whereas treatment 2 was phytotoxic to tobacco leaves at 25 mM but not to grape leaves until ca. 50 mM levels were used.

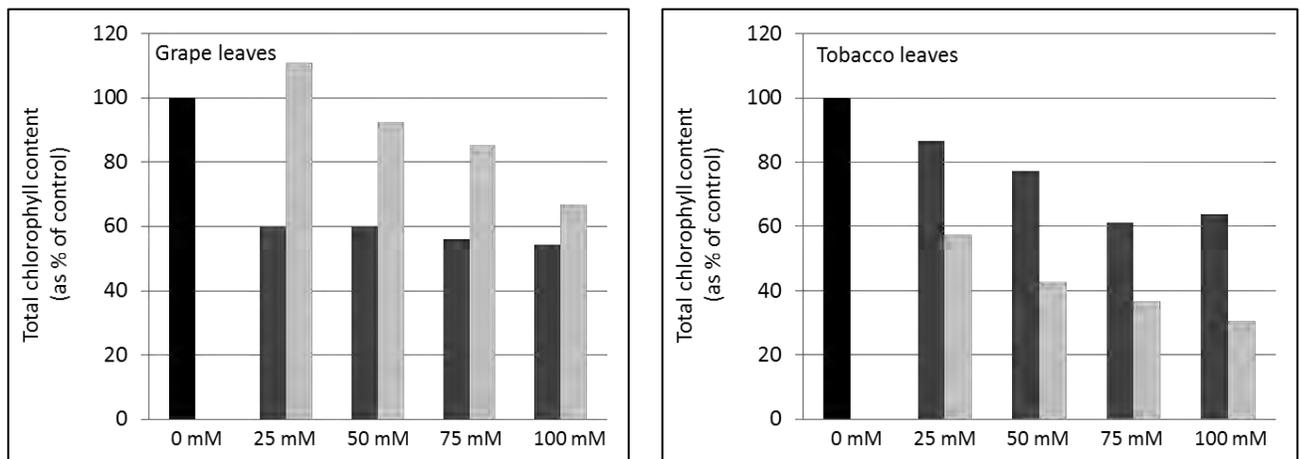


Figure 5. Three leaf discs (10 mm diameter) were floated in water for 18 hours (for grape leaves) and 48 hours (for tobacco leaves) containing two treatments (black bars for Treatment 1 and gray bars for Treatment 2, as indicated). Chlorophyll was extracted overnight in 80% acetone and quantified.

Both chemical treatments are currently being evaluated in Pierce's disease inoculated *Vitis vinifera* grapevines by soil drench and spray inoculations. These inoculations require multiple plants, uniformly inoculated and of similar age and size.

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 *Xf*.

V. vinifera grape plants are being cold treated. An earlier experiment failed due to over-treatment.

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 *Xf* than susceptible *V. vinifera* plants.

Muscadinia rotundifolia grapevines are being cold treated.

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FUNDING AGENCIES

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

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ABSTRACT

Previous research in B. Kirkpatrick's lab identified two hypervirulent mutants of *Xylella fastidiosa* (*Xf*). These mutations were in large hemagglutinin genes called *HxfA* and *HxfB*. These *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. B. Kirkpatrick hypothesized that if the Hxf protein, or a portion of the Hxf protein, is expressed in the xylem fluid of transgenic grapevines the *Xf* cells would clump together, remain at the inoculation site and be unable to colonize the plant. Transgenic Hxf-expressing grapevine lines were produced and mechanically inoculated with *Xf* cells in the greenhouse. These transgenic lines were transplanted to the USDA Animal and Plant Health (APHIS) permitted field site for transgenic plants coordinated by D. Gilchrist in Solano County in the spring of 2013. These vines grew well and were trained as conventional bilateral cordon vines. The shoots were cut back to two buds and then four shoots per vine were mechanically inoculated with a mixture of Temecula and Stags' Leap *Xf* strains in April 2014. Over 95% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014 with at least two Pierce's disease symptomatic canes present on all inoculated vines, indicating that the inoculations were successful. In January 2015 the shoots were trimmed to two buds, and in August 2015 the emerging shoots and the entire vines were rated for Pierce's disease symptoms. In three out of the five lines expressing the Hxf adhesion domain the majority of the vines showed no Pierce's disease symptoms. However, Pierce's disease symptoms were evident on the canes of other adhesion domain transgenic plants. The final conclusion is that the *Hxf* transgenes appeared to retard the progression of Pierce's disease symptoms initially, but eventually all plants expressed Pierce's disease symptoms and the Pierce's disease suppressive effect of the transgenes was no longer evident. In summary, the *Hxf* gene, expressed transgenically as the full-length gene or the adhesion domain, does not provide long-term protection.

LAYPERSON SUMMARY

B. Kirkpatrick invested more than 10 years investigating the role of *Xylella* hemagglutinins (Hxfs), large proteins that mediate the attachment of bacteria to themselves and to various substrates, and how these proteins may be involved in Pierce's disease pathogenicity and insect transmission. Early work showed that Hxf mutants were hypervirulent; i.e., they caused more severe symptoms and killed vines faster than did wild-type *Xylella fastidiosa* (*Xf*) cells (Guilhabert and Kirkpatrick, 2005). Hxf mutants no longer clumped together in liquid cultures like wild-type cells, indicating that cell adhesion molecules were important in establishing a pathogenic population of bacteria in the grape xylem. This information is of fundamental importance in understanding a genetic mechanism regulating spread of *Xf* in grapevines. The next logical step was to try to block this behavior transgenically as is reported herein. The current project tested the hypothesis that Hxfs expressed in the xylem sap of transgenic grapevines may act as a "molecular glue" that would aggregate and thus slow the movement of *Xf* cells introduced into grapevines. Transgenic lines expressing various constructs were moved to the field in the spring of 2013. The vines grew well and were trained up to the wire and established as conventional bilateral cordon vines with two-bud spurs, and then four shoots per vine were inoculated with *Xf* in April 2014. Pierce's disease symptoms were rated in September 2014 on the inoculated shoots, including whether the bacteria had moved to adjacent non-inoculated shoots and were expressing Pierce's disease symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014, indicating that the inoculations were successful. There was no evidence of plant-to-plant spread. Uninoculated controls remained disease free throughout the experiment. The summary observation is that Pierce's disease symptom severity was lower in the inoculated Hxf-transgenic grapevines than the *Xf*-inoculated non-transgenic controls in the first year following inoculation and establishment of infection. The pruning and inoculation programs were repeated beginning in January 2015 and

the shoots were rated for Pierce's disease symptoms in August 2015. The results continued to be encouraging in three out of the five independently transformed lines expressing the Hxf adhesion domain, wherein the majority of the vines showed no Pierce's disease symptoms. In the three full-length Hxf gene construct lines the majority of the vines were healthy, with no Pierce's disease symptoms. These initially encouraging results, however, were not borne out by the evaluations conducted in 2016. The final conclusion is that the Hxf transgenes appeared to retard the progression of Pierce's disease symptoms initially, but eventually all plants expressed Pierce's disease symptoms and the effect of the transgenes was no longer evident. Thus, the genes expressed as transgenes did not provide long-term protection.

INTRODUCTION¹

The bacterium *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease of grapes, is confined to the xylem, and is spread from plant to plant by xylem-feeding insects. *Xf* cell-cell attachment is an important virulence determinant in Pierce's disease as shown by previous research. Two secreted hemagglutinin (HA) genes named *HxfA* and *HxfB* are required for adhesion, and if either is mutated, *Xf* cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both 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 *Xf*. If either *HxfA* or *HxfB* is individually knocked out there is no cell-cell attachment, which suggests that both Hxf genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in *Xf*-plant interactions. Research by other Pierce's disease researchers has shown that *Hxf*s were regulated by an *Xf*-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 *Hxf*s essentially act as a "molecular glue" that is essential for cell-cell attachment, likely plays a role in *Xf* attachment to xylem cell walls, and contributes to the formation of *Xf* biofilms.

The field evaluation experiments described herein follow a series of greenhouse pathogenicity evaluations of two versions of Hxf-transgenic lines. In the preceding greenhouse studies, the results indicated that eight independent lines had disease severity ratings that were considerably less in the transgenic lines compared to the non-transgenic controls. Three are full length Hxf transgenes (PGIP220-) and five are just adhesion domains 1 through 3 transgenes (SPAD1-). The field planting of the Hxf transgenic vines occurred in April 2013 in the same location where other Pierce's disease related transgenic grapes are being grown under a USDA Animal and Plant Health Inspection Service (APHIS) permit that had been established previously for transgenic grapes.

OBJECTIVES

1. Plant transgenic vines in the APHIS permitted field in Solano County and train them into traditional bilateral cordon arrangement.
2. Inoculate four canes on each Hxf-transgenic field vine with wild-type infectious *Xf* in spring 2014. Rate Pierce's disease symptoms in September 2014 on inoculated canes. Take samples for quantitative polymerase chain reaction (qPCR) to confirm bacterial presence.
3. Cut back all canes to two buds and rate the cane growth in the spring of 2015, and rate for Pierce's disease symptoms in September 2015 and the spring of 2016 to determine if the expression of Hxf in the transgenic vines affected the movement of the inoculated *Xf* into the cordons, resulting in systemic protection against Pierce's disease.

RESULTS AND DISCUSSION

Forty Hxf-transgenic vines representing all the transgenic lines previously evaluated in the greenhouse were planted in the field in April 2013 and trained as bilateral cordons as shown in **Figure 1**. The vines were inoculated with *Xf* in the summer of 2014. Pierce's disease symptoms were observed on the non-transgenic, *Xf*-inoculated control plants and Hxf-transgenic plants in September 2014. The vines were then pruned to two buds and Pierce's disease symptoms on the vines were evaluated in the spring of 2015 (**Table 1**).

¹ Note: Bruce Kirkpatrick was the original Principal Investigator on this project. This final report was prepared by David Gilchrist, who accepted the responsibility of completing the data collection on this project following the death of B. Kirkpatrick and submitting the final report.



Figure 1. Hxf-transgenic and non-transgenic control vines planted in the field. (Photo August 2014.)

A combination of *Xf* Temecula and Stags' Leap strains were grown on solid PD3 medium and harvested cells were then suspended in phosphate buffered saline to a concentration of 108 cells/ml. Four canes on replicates of each transgenic line were labelled and then mechanically inoculated by the standard needle prick method with a 20 ul drop of *Xf* cell suspension containing 2×10^6 bacterial cells. Inoculations were done in mid-May 2014 and inoculum droplets were quickly taken up by the transpiring canes under negative pressure. The inoculations were successful in establishing infection of the plants, as evidenced by the Pierce's disease symptoms and by PCR assessment of isolated cane DNA from the inoculated canes using *Xf*-specific probes.

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 2015 (**Table 1**). Ninety-five percent of the inoculated canes had some level of leaf scorching, which indicated that the inoculation procedure was successful, as shown in **Figure 2**.

Table 1. Pierce's disease symptom ratings of Hxf-transgenic grapevines in August 2015.

Transgenic Lines	# Inoculated Vines	# of PD Rated Canes	Mean Plant Disease Rating (cane ratings)
HA Adhesion Domain only			
SPAD 1-6	3	10	0.7 (5 as 0; 4 as 1; 2 as 2)
SPAD 1-7	4	15	0.9 (7 as 0; 2 as 1; 6 as 2)
SPAD 1-8	5	20	1.6 (2 as 0; 5 as 1; 12 as 2; 1 as 3)
SPAD 1-10	3	10	1.7 (1 as 0; 2 as 1; 6 as 2; 3 as 1)
SPAD 1-12	5	19	1.2 (5 as 0; 5 as 1; 9 as 2)
HA Gene Full Coding Sequence			
PGIP 220-1	4	10	1.6 (4 as 0; 1 as 1; 6 as 2; 1 as 3)
PGIP 220-3	3	12	1.3 (4 as 0; 1 as 1; 6 as 2; 1 as 3)
PGIP 220-11	3	10	0.3 (8 as 0; 1 as 1; 1 as 2)
Control	3	12	6.9 (13 as 5; 3 as 4; 2 as 3)

Note: Pierce's disease symptoms of inoculated transgenic canes were rated August 2015. Symptom ratings of individual canes were as follows:

- 0 is no symptoms of Pierce's disease, i.e., no scorched leaves on cane;
- 1 is 2 to <10% scorched leaves on cane;
- 2 is >10% to <75% scorched leaves on cane;
- 3 is all leaves showing Pierce's disease scorch symptoms, no cane dieback observed;
- 4 is cane dieback, cane still alive; and
- 5 is dead cane.

Cane ratings are of the form [# of canes] as [rating].



Figure 2. Hxf adhesion domain transgenic Thompson Seedless vine 11 months following inoculation with *Xf* in 2014 showing the state of the vine, which was defoliated and dead from Pierce’s disease in June 2015.

In 2015, three out of the five lines expressing the Hxf adhesion domains only showed no Pierce’s disease symptoms. In the two other adhesion domain lines the majority of the inoculated canes were dead or had severe Pierce’s disease symptoms. In the three full-length Hxf gene construct lines the majority of all the canes were healthy, with no Pierce’s disease symptoms. These initial results were encouraging and were consistent with the greenhouse results in terms of occurrence of Pierce’s disease symptoms in relation to inoculation.

These results were similar to what was observed in the greenhouse inoculations. However, 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.

The results obtained in the spring of 2016, are summarized in **Figure 3**. None of the transgenic plants were free of Pierce’s disease symptoms, although all were slightly less than the non-transgenic control plants. Furthermore, there was no indication that the bacteria were suppressed in movement from the site of inoculation.

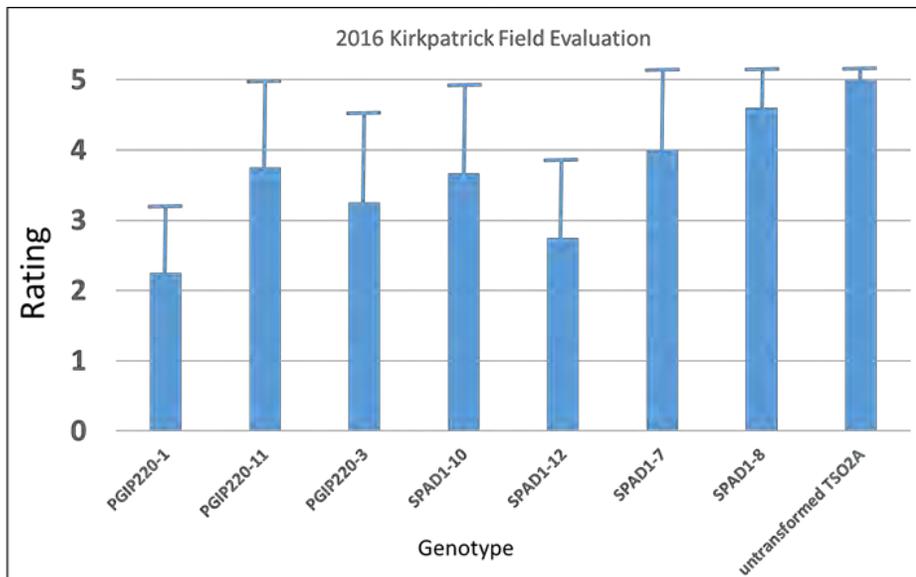


Figure 3. Solano County field trial ratings of transgenic Thompson Seedless vines expressing two versions of the hemagglutinin gene from *Xf*. Graph shows the mean disease ratings from 1 to 5 (1 is healthy and 5 is dead) of Pierce’s disease symptoms in June 2016. Plants were inoculated with *Xf* in June 2014. PGIP220 (full Hxf) and SPAD (HA subdomain) transgenics are compared to TSO2A (untransformed controls). Data are the average and standard deviation from four plants for each genotype.

CONCLUSIONS

Eight Hxf-transgenic lines were shown by qRT-PCR to express Hxf mRNA. Greenhouse inoculations of the eight Hxf-transgenic Thompson Seedless grapes with cultured *Xf* 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 controls were planted in the field in the spring of 2013. The vines grew well and were trained as bilateral cordons. Four shoots on each vine were mechanically inoculated with wild-type *Xf* in May 2014. Pierce's disease symptoms on inoculated and non-inoculated shoots were evaluated in September 2014. A high percentage of the inoculated shoots developed scorch leaves typical of Pierce's disease symptoms, indicating the needle inoculation technique was successful. Pierce's disease symptom severity ratings were lower among Hxf-transgenic lines than inoculated non-transgenic grapevine controls in the first year following inoculation. Canes from transgenic and non-transgenic vines were collected to determine the presence of the bacteria 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 *Xf* infections overwintered and formed systemically-infected vines. Most of the adhesion domain vines and full-length Hxf gene transformants had some vines that appeared Pierce's disease free. However, with other replicates of the transgenic lines some replicates were either dead or had Pierce's disease symptoms on inoculated canes that varied in severity.

Evaluation of the inoculated vines in June 2016 (**Figure 2**) indicated the bacteria had now gone systemic and nearly all the transgenic plants were dead or clearly dying. There were no significant differences in disease severity between the transgenic plants and the non-transgenic controls two years after inoculation.

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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.

FIELD EVALUATION OF CROSS-GRAFT PROTECTION EFFECTIVE AGAINST PIERCE'S DISEASE BY DUAL AND SINGLE DNA CONSTRUCTS

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Reporting Period: The results reported here are from work conducted October 2015 to September 30, 2016.

ABSTRACT

This field project began in 2010 to evaluate grapevines expressing potential Pierce's disease suppressive transgenes under field conditions. All plants are located in a secured, USDA Animal and Plant Health Inspection Services (APHIS)-approved, area in Solano County. The disease was successfully introduced into the cordon-trained plants by mechanical injection of *Xylella fastidiosa* into stems over the past four years. The plants were monitored regularly for quantity and movement of the bacteria, along with symptoms of Pierce's disease. Test plants included transgenic plants expressing genes from the Dandekar, Powell, Lindow, Gilchrist, and Kirkpatrick projects. The transgenic plants were compared with non-transgenic Pierce's disease susceptible Thompson Seedless and Freedom rootstock plants as controls. In addition, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were introduced in 2011 and 2012. The results to date indicate that the mechanical inoculations introduced the bacteria into the plants with subsequent appearance of classic foliar symptoms and cane death within 24 months in susceptible controls. There is no evidence of spread of the bacteria to uninoculated and uninfected susceptible grape plants adjacent to infected plants, confirming tight experimental control on the pathogen and symptoms. Each of the transgenes tested suppress the symptoms of Pierce's disease inoculated vines to varying degrees, including protection of untransformed scions grafted to a transformed rootstock. The field evaluation is being continued with transgenic rootstocks expressing two "stacked" genes grafted to a non-transgenic Pierce's disease susceptible scion to assess the potential for cross-graft protection of the scion.

LAYPERSON SUMMARY

This field project began in 2010 to evaluate grapevines expressing potential Pierce's disease suppressive transgenes under field conditions. This field experiment will continue evaluation of resistance to Pierce's disease in transgenic grape and grape rootstocks by expressing dual combinations of five unique transgenes under field conditions. The evaluation continues in a USDA Animal and Plant Health Inspection Service (APHIS) regulated Solano County site where the plants are mechanically injected with *Xylella fastidiosa*. Pierce's disease symptoms including classical foliar symptoms and cane death occur within 24 months. The current field tests have shown positive protection against Pierce's disease by five different DNA constructs. A new planting is in progress that will consist of untransformed Pierce's disease susceptible scions grafted to transgenic rootstocks (1103 and 110-14) expressing the paired constructs of the five genes to assess cross-graft protection of a non-transformed scion that is otherwise highly susceptible to Pierce's disease.

INTRODUCTION

This field project began in 2010 to evaluate grapevines expressing potential Pierce's disease suppressive transgenes under field conditions. All plants are located in a secured, USDA Animal and Plant Health Inspection Service (APHIS) approved area in Solano County. The disease was successfully introduced into the cordon-trained plants by mechanical injection of *Xylella fastidiosa* into stems over the past five years. The plants were monitored regularly for quantity and movement of the bacteria along with symptoms of Pierce's disease. Test

plants included transgenic plants expressing genes from the Dandekar, Powell, Lindow, Gilchrist, and Kirkpatrick projects compared with non-transgenic Pierce's disease susceptible Thompson Seedless and Freedom rootstock plants as controls. In addition, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were introduced in 2011 and 2012. The results to date indicate that the mechanical inoculations introduced the bacteria into the plants with subsequent appearance of classic foliar symptoms and cane death within 24 months in susceptible controls. There is no evidence of spread of the bacteria to uninoculated and uninfected susceptible grape plants adjacent to infected plants, confirming tight experimental control on the pathogen and symptoms. Each of the transgenes tested suppress the symptoms of Pierce's disease inoculated vines to varying degrees, including protection of untransformed scions on the grafted plants. This field research is moving forward with the generation of new transgenic rootstocks expressing pairs of the disease-suppressive genes in a gene stacking approach with the genes paired together by differential molecular function. The new rootstocks with two transgenes each will be evaluated first in the laboratory and then the greenhouse before moving to the field. The highest-expressing rootstocks will be grafted to susceptible non-transgenic scions to assess potential cross-graft protection against Pierce's disease.

OBJECTIVES

There are three principal objectives:

1. Complete the current field evaluation of transgenic grape and grape rootstocks expressing Pierce's disease suppressive DNA constructs in the APHIS-regulated field site in Solano County through the spring of 2016.
2. Remove the current planting per the APHIS agreement by dismantling trellising, uprooting the plants, and burning all grape plant material on site in the fall of 2016, followed by cultivation and fumigation to ensure no living grape vegetative material remains.
3. Establish a new planting area within the current APHIS-approved site (**Figure 3**) to contain a new set of lines bearing paired (i.e., Pierce's disease suppressive DNA constructs, referred to as stacked genes). The stacked genes will be transferred to two adapted rootstocks (1103 and 101-14). These rootstocks will be grafted to a Pierce's disease susceptible Chardonnay scion prior to field planting. The goal is to assess the potential for achieving cross-graft protection of a non-transgenic scion against Pierce's disease. Planting is to begin in 2016 and be completed by 2018.

In conjunction with the investigators, the Product Development Committee of the Pierce's Disease and Glassy-winged Sharpshooter (PD/GWSS) Board in October 2015 approved the decision to terminate the field evaluation of current transgenics as originally planned and move to the second phase of transgenic Pierce's disease resistance evaluation. Field data over the course of this experiment has been collected by all investigators and can be found in their individual project reports, which are available in the annual Pierce's Disease Symposium Proceedings and report compilations from 2012 to 2016.

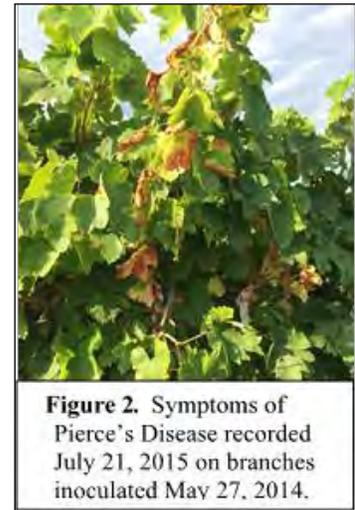
The field experiment will be terminated under objectives 1 and 2 of this proposal according to the regulations specified in the APHIS permit. This will be followed by establishment of the second phase approved by the Product Development Committee to develop transgenic rootstocks incorporating stacked genes (dual constructs) to be grafted to non-transformed Pierce's disease susceptible Chardonnay scions to test for potential cross-graft protection against Pierce's disease (objective 3). The development of the stacked gene rootstock transgenics is in progress, including molecular analysis of several lines released by the UC Davis Plant Transformation Facility. The second phase also involves limited planting and inoculation of additional single DNA constructs not previously tested. The second phase planting and inoculation will begin in 2016 and will be concluded in 2018. All field activities described in the section "Methodology to accomplish objectives" will be coordinated by principal investigator Gilchrist through field superintendent Bryan Pellissier.

Methodology to accomplish objectives.

1. Destruction of existing planting and fumigation of the area to permit future use will first involve removal of all stakes and trellises, followed by cutting and stacking the above-ground portions of the plants. Mechanical undercutting of the base of the plants and roots will complete the plant removal. The stacked plants will be burned on the site inside the APHIS-permitted area. Following burning the ashes will be scattered and the entire area rototilled prior to fumigation to complete the APHIS requirements for removal and destruction of all transgenic material.

2. Establishment and management of new planting: Mark Greenspan (PD/GWSS Board viticulture consultant) will work with principal investigator Gilchrist to develop the following approach for trellising and plant management to reflect commercial standards and to enable the experimental inoculations and pathogen and disease assessments, as well as grape yield. Land preparation and planting of the experimental area will be sufficient to accommodate and manage 900 new plants. Row spacing will be nine feet between rows with six feet between plants. This spacing permits 32 rows of 28 plants each (up to 896 plants total) and includes a 50-foot open space around the planted area as required by the APHIS permit. The planting pattern will permit a two-bud pruned bilateral cordon system of sufficient lengths for inoculation, real-time sampling of inoculated tissue, and determination of the fruit yield by the untransformed Chardonnay scions. The total fenced area occupied by plants and buffer zones as required by the APHIS permit will be approximately 3.4 acres. All plants will be maintained under a drip irrigation system that was installed in 2014.
- a. Experimental design will be a complete randomized block with eight plants per each of five entries (replications), including all controls. Each plant will be trained as a single trunk up the wood stake as with the existing planting. When the shoot tip reaches about 12 inches past the cordon wire it will be topped to just above a node that is about two to three inches below the wire. Then, the laterals that push will be used to establish the bilateral cordons. Following Mark's advice, the best practice is to let them grow vertically or close to vertical rather than tying them while green, which reduces their elongation and tends to force more lateral growth. Metal nine-foot highway stakes inserted three feet into the ground every 18 feet will support the wires, including catch wires. A single 11-gauge wire will be used for the cordons and 13-gauge for the catch wires. Two pairs of moveable catch wires will be installed to tuck and position the shoots vertically for optimizing bacterial inoculation, bacterial analysis, and fruit production. The catch wires will be installed initially or after the first year of growth using 13-gauge wire to support the drip irrigation wire, about 18 inches off the ground.
 - b. After the first year, the canes will be tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8-inch in diameter. The shoots that push will be suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about four to five inches between them.
 - c. Grape fruit yield will be measured after the second or third year, depending on the fruit set.
 - d. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's disease infection, and the presence of the bacteria will follow past protocol. Each parameter will be determined over time by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative polymerase chain reaction (qPCR) assays. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done with the current planting. A comparative quantitative determination by qPCR of the presence of *Xylella* in non-transgenic scions and grape rootstocks will be compared with conventional grape and grape rootstocks.
 - e. Both symptom expression and behavior of the inoculated bacteria will provide an indication of the level of resistance to Pierce's disease infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area.
 - f. 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 experiment. Hence, there is a documented historical precedent for the lack of spread of the bacteria from inoculated to non-inoculated plants, an important consideration for the experiments carried out for this project and for the granting of the APHIS permit. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding by soil borne diseases, including nematodes.
 - g. Irrigation and pest management, primarily for powdery mildew, weeds and insects, will be coordinated by principal investigator Gilchrist and conducted by Bryan Pellissier, the field superintendent employed by the Department of Plant Pathology. The field crew works closely with principal investigator Gilchrist to determine the timing and need for each of the management practices, including pruning and thinning of vegetative overgrowth as necessary.
 - h. Regular tilling and hand weeding will maintain 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 pruned material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the APHIS permit.

- i. Application of the fungicides Luna Experience and Inspire will be alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites will be treated with insecticides when needed. Neither powdery mildew nor insect pressure have been observed with these ongoing practices throughout the past five growing seasons.



RESULTS AND DISCUSSION

The initial plantings will be in the spring of 2016, followed by additional plantings as experimental plants become available in the second and third years. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2019 funding cycle and will depend on the results of the field evaluation up to that point. The field area has been designated legally available for planting with the specified transgenic grapes by USDA APHIS under permit number 7CFRE340 that is held by Professor Abhaya Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have been used successfully over the past five years (Gilchrist, 2015a). These protocols include plant management, inoculation with *Xylella fastidiosa*, development of classical symptoms of Pierce's disease exhibiting the range from foliar symptoms to plant death, and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's disease and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant.

CONCLUSIONS

The current planting of transgenic grapes will be terminated and the plants removed in the fall of 2016. Removal of the current planting will be done per the APHIS agreement by dismantling trellising, uprooting the plants, and burning all grape plant material onsite in the fall of 2016, followed by cultivation and fumigation to ensure no living grape vegetative material remains.

The field research using Pierce's disease suppressive transgenes is moving forward with the generation of new transgenic rootstocks expressing pairs of the disease-suppressive genes in a gene stacking approach with the genes paired together by differential molecular function. The new rootstocks with two transgenes each will be evaluated first in the laboratory and then the greenhouse before moving to the field. The highest expressing rootstocks will be grafted to susceptible non-transgenic scions to assess potential cross-graft protection against Pierce's disease. The field area has been permitted by the USDA APHIS for this experiment. The protocol for planting and management of the vines is in place and is coordinated with Mark Greenspan (PD/GWSS Board viticulture consultant). Initial plantings will be done in the spring of 2016 and followed by additional plantings as experimental plants become available in the second and third years. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2019 funding cycle and will depend on the results of the field evaluation up to that point.



Figure 3. Solano County planting area. Future area (green) available to plant the next generation of transgenic plants expressing the dual constructs or new single genes: This area is 300 x 470 feet for planting, which equals 1.8 acres accommodating up to 38 new rows (excluding the 50-foot buffer areas surrounding the plots. The new area will accommodate approximately 900 new plants in 2016-18. Current area (rows) now planted to grapes: 300 x 370 feet equaling 1.6 acres, including the 50-foot buffer areas surrounding the plots. There are currently 625 plants that have been evaluated since 2010. These plants will be removed in 2016. After the plants are removed and destroyed by burning the area will be fumigated and available for additional new plantings or a rootstock nursery by 2018.

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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.

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

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Reporting Period: The results reported here are from work conducted October 2015 to September 30, 2016.

ABSTRACT

Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) identified, constructed, and advanced to field evaluation five novel DNA constructs (**Table 1**) that, when engineered into grapevines, suppress symptoms of Pierce's disease by (a) reducing the titer of *Xylella fastidiosa* (*Xf*) in the plant, (b) reducing systemic spread of the bacteria, or (c) blocking *Xf*'s ability to trigger Pierce's disease symptoms. The continuation of the basic research and the field trial results indicate that several of the five DNA constructs, when incorporated into transgenic rootstock, show potential for protecting non-transformed scions across a graft union (**Figure 1**). The present field trial consisting of single gene constructs will be discontinued at the end of the 2016 growing season to be replaced with a second field trial designed to evaluate rootstocks bearing paired combinations of the five constructs. If successful, the obvious benefit would be that any unmodified (non-transgenic) varietal wine grape scion could be grafted to and be protected by transformed rootstock lines. 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 from Pierce's disease. Stacked transgene rootstock lines are now being received for greenhouse whole-plant Pierce's disease evaluation, followed by grafting and more Pierce's disease evaluation under controlled greenhouse conditions. Ramets of the most suppressive transgenic rootstock lines will then be produced for field evaluation beginning in 2017.

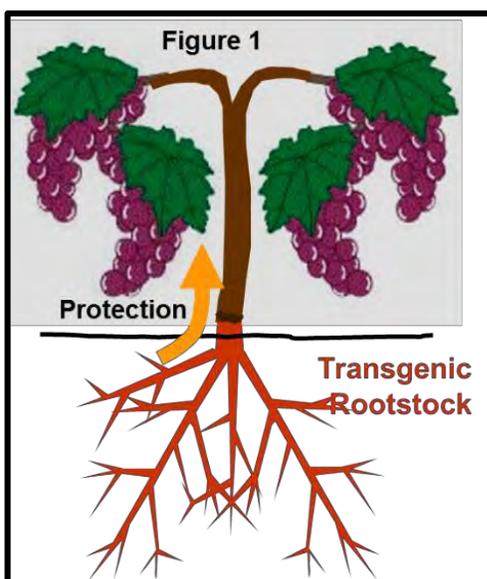


Figure 1. Example scenario whereby a transgenic rootstock is being tested for its ability to protect an untransformed scion from Pierce's disease.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) 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 (**Table 1**) novel genes (DNA constructs) that, when engineered into grapevines, suppress symptoms of Pierce's disease by reducing the titer of *Xylella fastidiosa* (*Xf*) in the plant, reducing its systemic spread in the plant, or blocking *Xf*'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 with mechanical inoculation of the test plants with pathogenic strains of *Xf*. Current data from the field experiment indicates that each of the five transgenes, introduced as single constructs, reduced the disease levels under field conditions. In addition, 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 in that any unmodified varietal scions can be grafted to and be protected by transformed rootstock lines. The objectives described herein address the issue of durability of genetic resistance to avoid being overcome by evolving virulent versions of the *Xf* 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 to assess 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.

INTRODUCTION

Briefly, we describe information on the history and impact of the genes deployed as single transgenes currently in USDA Animal and Plant Health Inspection Service (APHIS) approved field trials where test plants are mechanically inoculated with *Xylella fastidiosa* (*Xf*). 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 described by Lindow, Dandekar, and Gilchrist in previous reports and noted in the 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	<i>Xf</i> clearing; antimicrobial
PR1	grape cell anti-cell-death
rpfF	changes quorum sensing of <i>Xf</i> (DSF)
UT456	non-coding microRNA; activates PR1 translation
PGIP	inhibits polygalacturonase; suppresses <i>Xf</i> movement

Polygalacturonase-inhibiting protein and chimeric antimicrobial protein (Abhaya Dandekar).

The Dandekar lab has genetic strategies to control the movement and to improve clearance of *Xf*, the xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease in grapevine (Dandekar, 2013). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit membrane pores 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 *Xf* polygalacturonase activity necessary for long distance movement (Aguero et al., 2006). The second strategy enhances clearance of bacteria from *Xf*-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 and diffusible signal factor (Steven Lindow).

The Lindow lab has shown that *Xf* 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. Instead, they actively express extracellular enzymes and retractile pili needed for movement through the plant (Chatterjee et al., 2008). Accumulation of DSF in *Xf* 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 *Xf* through identifying plant genes that block a critical aspect of grape susceptibility to *Xf*, namely the inappropriate activation of a genetically conserved process of programmed cell death (PCD) that is common to many, if not all, plant diseases. 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 when translated in the presence of a bacterial secreted death signal. 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 *Xf* trigger cell stress.

OBJECTIVES

1. Introduce pairs of protective constructs into adapted grapevine rootstocks 1103 and 101-14.
2. Analyze each transgenic line to confirm correct insertion of the gene pairs and their expression in the respective rootstock.
3. Test the resulting lines for efficacy by inoculating with *Xf* in a preliminary greenhouse experiment to identify, based on symptom expression, the most protective lines from each combination of genes, followed by quantitative measurement of the presence and movement of the bacteria.

The primary motive for expressing genes in combination is to create durable resistance, i.e., resistance to *Xf* 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 *Xf* overcoming the resistance. With multiple, distinct transgenes, *Xf* 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 between multiple transgenes 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 that David Tricoli, the lead author in this paper, is doing the stacking transformations in this project.) Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall (Escobar et al., 2001). Experiments being conducted here will evaluate potential synergism in suppression of Pierce's disease symptoms and reducing *Xf* titer distant from the graft union.

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 constructed in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al., 2006).

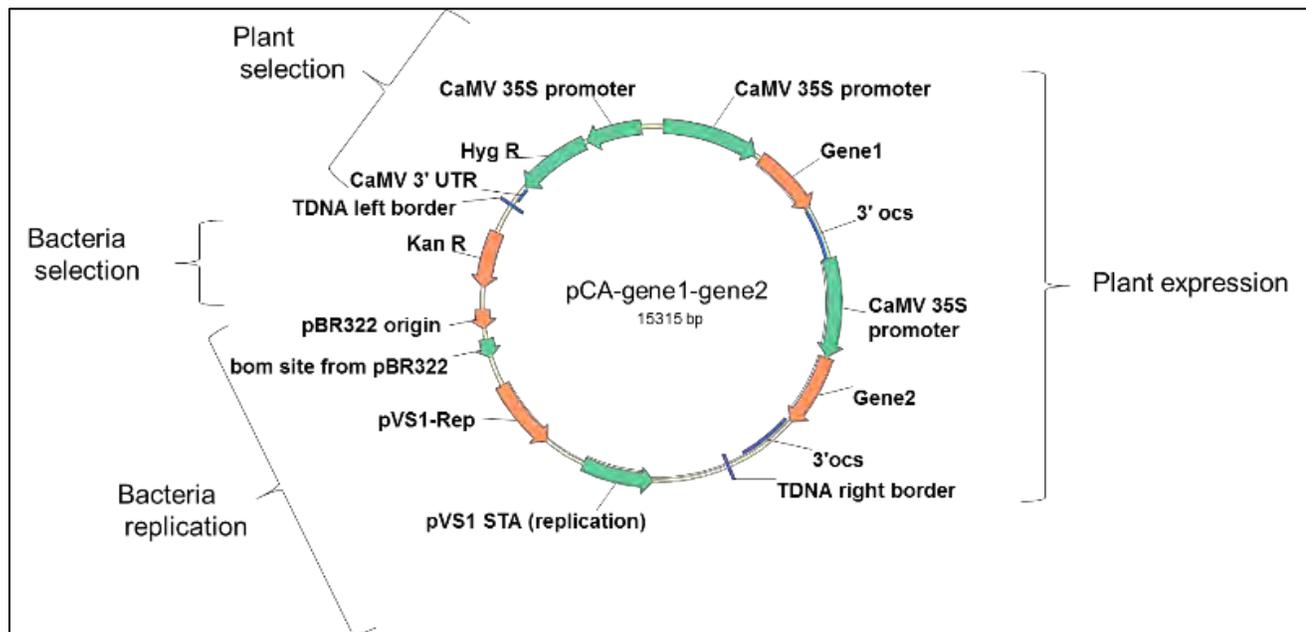


Figure 2. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

Binary plasmids capable of expressing two genes from the same TDNA (dual expressers) were constructed by J. 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 enable transformation to move forward in the fastest manner depending on which marker works best for each dual or each rootstock. Each plasmid containing the dual protective DNA sequences is being introduced into embryogenic grapevine culture in a single transformation event rather than sequentially as would normally be the conventional strategy at the UC Davis Plant Transformation Facility. The progress for each line is shown in **Table 2**.

Table 2. Progress in generation of the dual construct transformed transgenic rootstocks. The current status of grape transformations into the rootstocks 1103 and 101-14.

Genotype	Selection	PI	Start Date	construct	Months																		Comment			
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		19	20	21
1103	hygro	Gilchrist	10/15/14	pCA-5fCAP-5oP14HT																			15	Complete		
1103	hygro	Gilchrist	10/15/14	pCA-5fCAP-5oUT456																				12	Complete	
1103	hygro	Gilchrist	10/15/14	pCA-5PGIP-5oUT456																						
1103	hygro	Gilchrist	1/9/15	pCA-5PGIP-5oP14HT																				11	Complete	
1103	hygro	Gilchrist	1/9/15	pCA-5PGIP-5FCAP																				7		
1103	hygro	Gilchrist	1/9/15	pCA-5oP14HT-5oUT456																				10	Complete	
1103	hygro	Gilchrist	5/26/15	pCA-5oP14HT-5orpFF																						
1103	hygro	Gilchrist	5/26/15	pCA-5oUT456-5orpFF																				1		
1103	hygro	Gilchrist	5/26/15	pCA-5fCAP-5orpFF																				6		
1103	hygro	Gilchrist	5/26/15	pCA-5PGIP-5orpFF																				13	Complete	
1103	kan	Gilchrist	8/28/15	pCK-rpFF-P14LD																						
1103	kan	Gilchrist	8/28/15	pCK-rpFF-456																						
1103	kan	Gilchrist	8/28/15	pCK-rpFF-PGIP																						
1103	kan	Gilchrist	8/28/15	pCK-rpFF-CAP																						
101-14	kan	Gilchrist	10/1/15	pCK-5oP14HT-5oUT456																				1		
101-14	kan	Gilchrist	10/1/15	pCK-5fCAP-5oP14LD																					2	
101-14	kan	Gilchrist	10/1/15	pCK-5PGIP-5oP14LD																					2	
101-14	kan	Gilchrist	10/1/15	pCK-5ofCAP-5oUT456																					1	
101-14	kan	Gilchrist	10/1/15	pCK-5PGIP-5oUT456																						
101-14	kan	Gilchrist	10/1/15	pCK-5PGIP-5FCAP																					12	
101-14	kan	Gilchrist	10/1/15	pCK-5oP14LD-5orpFF																						
101-14	kan	Gilchrist	10/1/15	pCK-5oUT456-5orpFF																					7	
101-14	kan	Gilchrist	10/1/15	pCK-5ofCAP-5orpFF																					3	
101-14	kan	Gilchrist	10/1/15	pCK-5PGIP-5orpFF																					9	
TSPiA-9	hygro	GilChrist	11/3/15	pCA5o-456																					13	Complete

Key	
	Transformation initiated
	Embryos harvested
	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. The quantitative analysis of the transgenic rootstocks has begun, as illustrated in **Figure 4**.



Figure 3. Rootstock 1103 embryos and developing plantlets with CAP and PR1 transgenes inserted, and the developed transgenic plant ready for RNA analysis and pathogenicity testing for response to *Xf*.

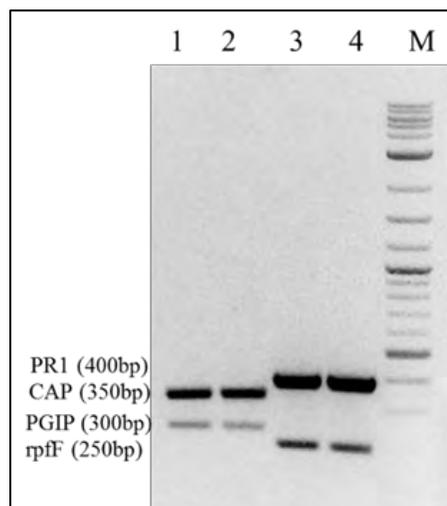


Figure 4. Leaf RNA analysis of four independent transgenic grape lines. Lanes 1 and 2 are putative CAP and PGIP dual expression lines. Lanes 3 and 4 are putative PR1 and rpfF dual expression lines. Sizes of the expected products are shown.

Analysis of the transgenic rootstocks to confirm dual insertions.

RNA from transgenic grape leaves is purified by a modification of a cetyl trimethylammonium bromide (CTAB) protocol and includes LiCl precipitation. The RNA is converted to cDNA by oligo dT priming and reverse transcriptase. PCR reactions are set up using the synthesized cDNA as template and specific pairs of primers designed against each of the five putative transgenes. The resulting products are separated by agarose gel electrophoresis (**Figure 4**). In this figure the bands shown correspond to two amplification targets in each transgenic plant. This technique, adapted by J. Lincoln to uniquely address this analysis, is referred to as multiplex analysis of each transgenic pair combination and allows for robust and rapid confirmation of the fidelity of the paired insertions. Progress on the RNA verification is shown in **Table 3**.

Table 3. Progress in RNA analysis of the dual construct transformed transgenic rootstocks. The current status of verification of transgenic RNA from transgenic rootstocks 1103 and 101-14.

Grapevine Genotype	Construct	Verified RNA	Ramets Started
1103	CAP-PR1	10	X
	PGIP-456		
	CAP-456	10	X
	PGIP-PR1	10	X
	PGIP-CAP	5	
	PR1-456	10	X
	rpfF-PR1		
	CAP-rpfF	4	
	PGIP-rpfF	10	X
	rpfF-456	1	
101-14	CAP-PR1	2	
	PGIP-456		
	CAP-456		
	PGIP-PR1	2	
	PGIP-CAP	3	
	PR1-456		
	rpfF-PR1		
	CAP-rpfF		
	PGIP-rpfF	5	
	rpfF-456		

The timeline for completing the delivery of the transgenic rootstock plants, the greenhouse and laboratory analyses, and the field planting of the selected rootstocks grafted to the non-transgenic Chardonnay scions is presented in **Figure 5**.

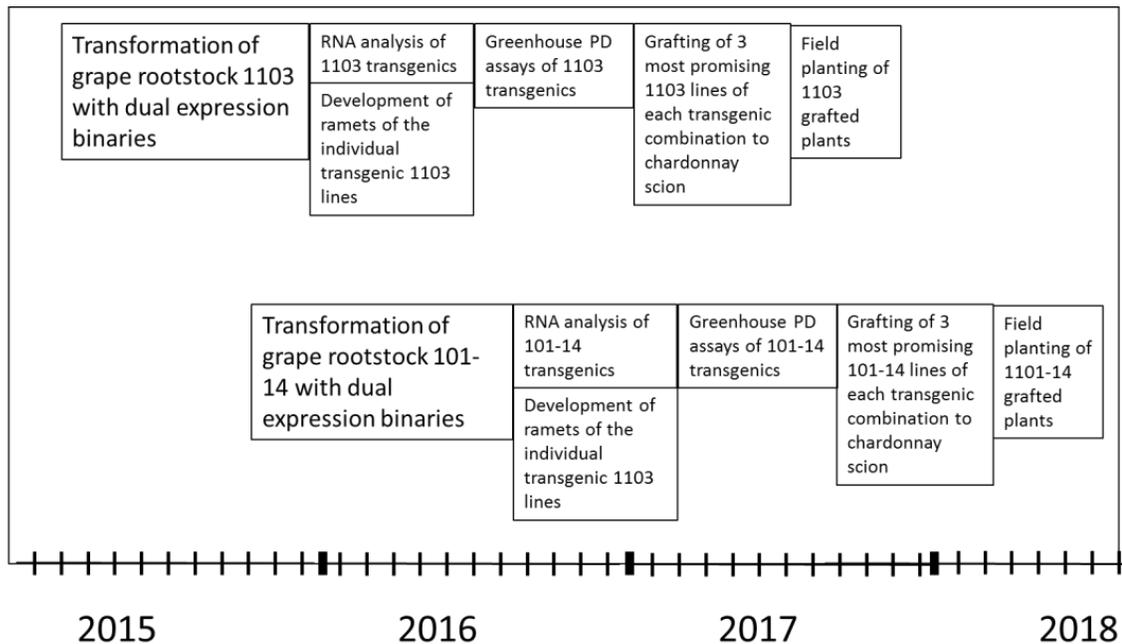


Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

CONCLUSIONS

Our capacity to achieve all the objectives is essentially assured based on prior accomplishments and the fact that we are exactly where we are projected to be within the timeline indicated in **Figure 5**. All techniques and resources are available in the lab and have proven reliable, informative, and reproducible. This project has consolidated a full-time research commitment for this team of experienced scientists to Pierce's disease. Each of the senior personnel, including J. Lincoln, have been with this project since 2007. Collectively the team brings a full range of skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation, and analysis of transgenic plants.

The scope of research includes both greenhouse and field evaluation of the transgenic rootstocks for relative suppression of Pierce's disease in the non-transgenic scions. Commercialization of the currently effective anti-Pierce's disease containing vines and/or rootstocks could involve partnerships between the UC Foundation Plant Services, nurseries, and, potentially, with a private biotechnology company. As indicated above, the dual constructs have been assembled and forwarded to D. Tricoli at the UC Davis Plant Transformation Facility. The transgenic plants are being delivered to J. Lincoln as indicated in **Table 2** and evaluations have begun as indicated in **Table 3** and **Figure 4**. The first step in the analysis of the transcribed RNA is to verify that each plant contains both of the intended constructs. The timeline shown in **Figure 5** for both transformation and analysis is on track.

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CONDUCTING PIERCE'S DISEASE SYMPTOM EVALUATIONS AT THE SOLANO COUNTY FIELD TRIAL RESEARCH BLOCK

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Reporting Period: The results reported here are from work conducted September 1, 2014 through June 30, 2016.

ABSTRACT

In September 2014, September 2015, and May 2016 the principal investigator and a team of grapevine pathologists scored Pierce's disease symptom severity in a Solano County research block planted with transgenic grapevines that had been mechanically injected with a Pierce's disease strain of *Xylella fastidiosa*. Analysis of the variation in the data overall and among individuals indicated that, regardless of when vines were scored, all scores agreed for greater than 50% of the vines and the majority of scores agreed for at least 96.5% of the vines. This indicates that the rating system was well understood by team members and provided a relatively uniform measure of Pierce's disease symptoms. Score variation was highest in May 2016, suggesting that Pierce's disease symptoms might be more variable in the spring and result in a less uniform measure of disease. In addition, the percent agreement of scores for vines with less severe symptoms was lower, suggesting that raters have more difficulty scoring these vines.

LAYPERSON SUMMARY

In September 2014, September 2015, and May 2016 the principal investigator and a team of grapevine pathologists scored Pierce's disease symptom severity in a Solano County research block planted with transgenic grapevines that had been mechanically injected with a Pierce's disease strain of *Xylella fastidiosa*. Analysis of the variation in the data overall and among individuals indicated that, regardless of when vines were scored, all scores agreed for greater than 50% of the vines and the majority of scores agreed for at least 96.5% of the vines. This indicates that the rating system was well understood by team members and provided a relatively uniform measure of Pierce's disease symptoms. Score variation was highest in May 2016, suggesting that Pierce's disease symptoms might be more variable in the spring and result in a less uniform measure of disease. In addition, the percent agreement of scores for vines with less severe symptoms was lower, suggesting that raters have more difficulty scoring these vines.

INTRODUCTION

The Product Development Committee of the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board requested research into uniform evaluation of Pierce's disease symptoms exhibited by grapevines developed by four principal investigators as part of the Board's research portfolio. These vines are planted in a single research block in Solano County.

Principal investigator Golino and Foundation Plant Services plant pathologists with multiple years of grape disease experience made up the core evaluation team. Several plant pathology PhD graduate students with grape pathology thesis research were also invited to participate. Each individual participated in training in evaluating Pierce's disease symptoms according to the scoring system below. That training included 'calibration' by examining a subset of vines including healthy and Pierce's disease-inoculated controls to ensure that ratings were as uniform as possible. Vines were evaluated twice in mid-September and once in mid-May.

Scoring technique.

A visual rating system on a scale of 1-5 was used by each member of the team to rate every vine individually. All vines were labeled by row and vine number. Data was collected by row and vine number without any information about the particular treatment that the vine received. This is a slightly modified version of the rating system used by the Kirkpatrick lab.

Golino/Gilchrist simplified rating system.

- 0: Healthy vine. All leaves green with no scorching, good cane growth, no cordon dieback or failure to push canes at bud positions. Dry or yellowing leaves may be present but do not show characteristic *Xylella* symptoms.
- 1: Leaves on one or two canes showing characteristic *Xylella* scorched leaf symptoms. No evidence of physical damage to leaf petiole(s) or cane(s). On cane in question, at least TWO leaves are symptomatic; one single leaf is NOT enough to warrant a rating of 1.
- 2: More than two canes possess multiple scorched leaves. HOWEVER, canes with symptomatic leaves are still confined to just one area of the vine.
- 3: Canes with clearly scorched leaves are found on several canes, including canes which have not been inoculated.
- 4: Ends of cane(s) begin dying back; some canes failed to push in the spring. Vine is clearly symptomatic on all or nearly all surviving canes. Main point is that the vine is NOT yet dead but is clearly facing a terminal fate.
- 5: Dead vine or a vine that had a few canes weakly push in the spring but those canes later died with onset of hot temperatures in July or August. There are NO visible signs of other potential problems such as gophers, crown gall, *Phytophthora*, or *Eutypa/Botrytis* dieback of cordons.

If a vine appeared to have died for reasons other than Pierce's disease, that was entered in the comments field for that vine and no score was entered in the rating field.

OBJECTIVES

The objectives of this project were to:

1. Train individuals to evaluate Pierce's disease symptoms according to the above scoring system.
2. Score the grapevines during the fall and spring.
3. Evaluate the extent to which the scores for any given vine agreed.

RESULTS AND DISCUSSION

In September 2014, nine members of the evaluation team scored 616 vines and the data was analyzed with the purpose of determining the extent to which the scores for any given vine agreed. Scores for a vine were counted as "in agreement" if they equaled one of the integers above or below the mean. Although mode and frequency are typically used for analyzing ordinal data, the scores in the rating system are quantitative in the sense that they follow a logical sense of order and the difference between the scores is roughly equivalent. Therefore, we felt that using the mean as a measure of central tendency was justified. The purpose of the interval was to accommodate integer data and, in practice, allows scores to vary by one integer and still be counted as "in agreement."

The percent agreement of scores for individual vines is shown in **Figure 1**. Cells of varying shades of green represent vines where at least five out of nine scores agreed.

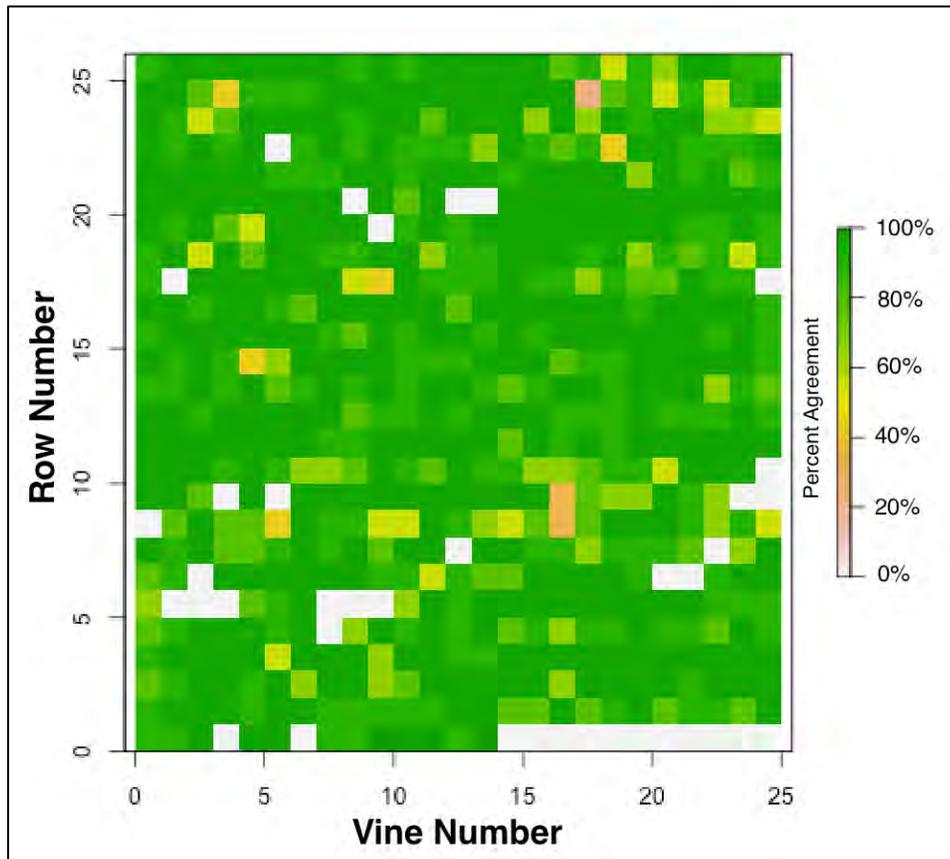


Figure 1. Cell plot of the 616 vines that were rated in September 2014. The colors indicate the percent agreement among scores of individual raters. Scores are counted as “in agreement” if they equal the integer above or below the mean for any given vine. Gray areas indicate missing vines.

The number and percent of vines in each agreement category for the first scoring in September 2014 is shown in **Table 1**. Adding columns “56%” through “100%” indicates that for 97.4% of the vines, at least five of the nine scores agreed. For 51.0% of the vines all nine scores agreed, i.e., they were within one integer above or below the mean.

Table 1. The number and percent of vines in each of the ten agreement categories in September 2014.

Percent Agreement Sept. 2014	0% (0/9)	11% (1/9)	22% (2/9)	33% (3/9)	44% (4/9)	56% (5/9)	67% (6/9)	78% (7/9)	89% (8/9)	100% (9/9)
Number of Vines	4	0	1	3	8	19	41	62	164	314
Percent of Vines	0.60	0.00	0.16	0.49	1.30	3.08	6.66	10.1	26.6	51.0

The vines were scored again in September 2015 by ten people. The percent agreement of scores for individual vines is shown in **Figure 2**.

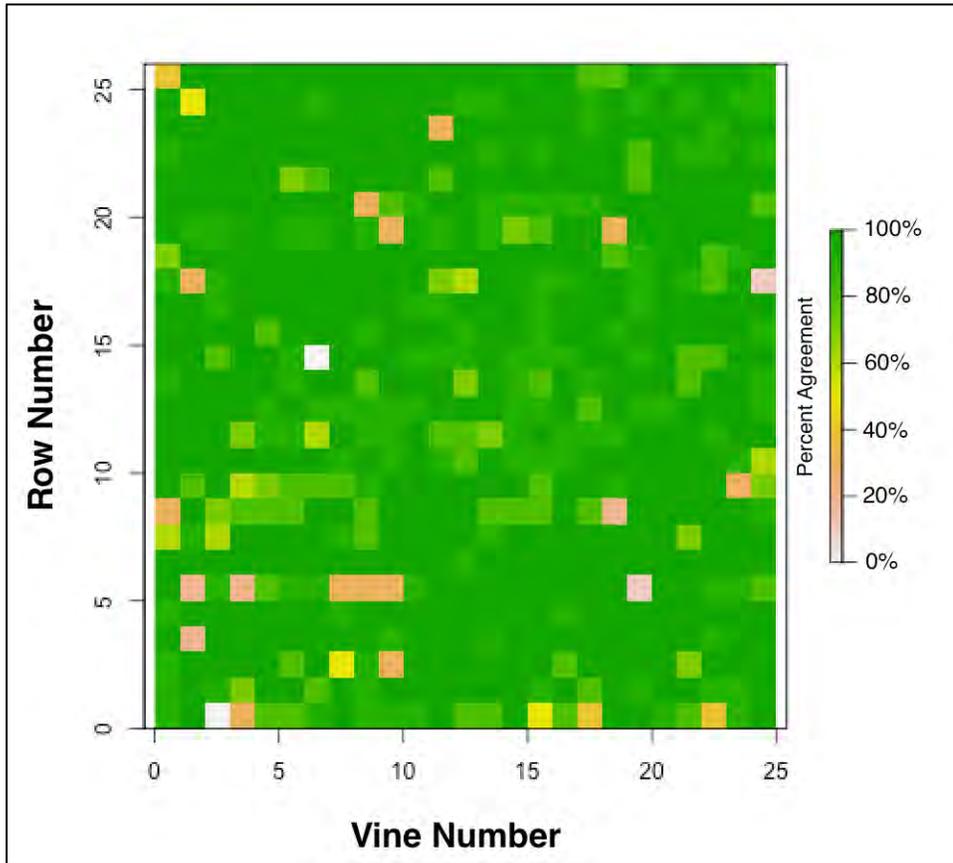


Figure 2. Cell plot of the 650 vines that were rated in September 2015. The colors indicate the percent agreement among scores of individual raters. Scores are counted as “in agreement” if they equal the integer above or below the mean for any given vine. Gray areas indicate missing vines.

The number and percent of vines in each agreement category is shown in **Table 2**. Adding columns “50%” through “100%” indicates that for 96.5% of the vines, at least five of the ten scores agreed. For 66.5% of the vines all ten scores agreed, i.e., they were within one integer above or below the mean.

Table 2. The number and percent of vines in each of the eleven agreement categories in September 2015.

Percent Agreement Sept. 2015	0% (0/10)	10% (1/10)	20% (2/10)	30% (3/10)	40% (4/10)	50% (5/10)	60% (6/10)	70% (7/10)	80% (8/10)	90% (9/10)	100% (10/10)
Number of Vines	2	2	4	12	3	3	6	13	49	124	432
Percent of Vines	0.31	0.31	0.62	1.85	0.46	0.46	0.92	2.00	7.54	19.08	66.46

The per vine change in percent agreement between 2014 and 2015 is illustrated in **Figure 3**. For 290 and 251 vines, respectively, the percent agreement increased or stayed the same. For 109 vines the percent agreement decreased in 2015. In some cases these latter vines appear to be clustered, indicating that some treatments were possibly more difficult to rate. However, percent agreement for most of these vines was still greater than 50% (data not shown). Vines with less than 50% agreement were scattered throughout the plot, indicating problems with individual vines and not entire treatments.

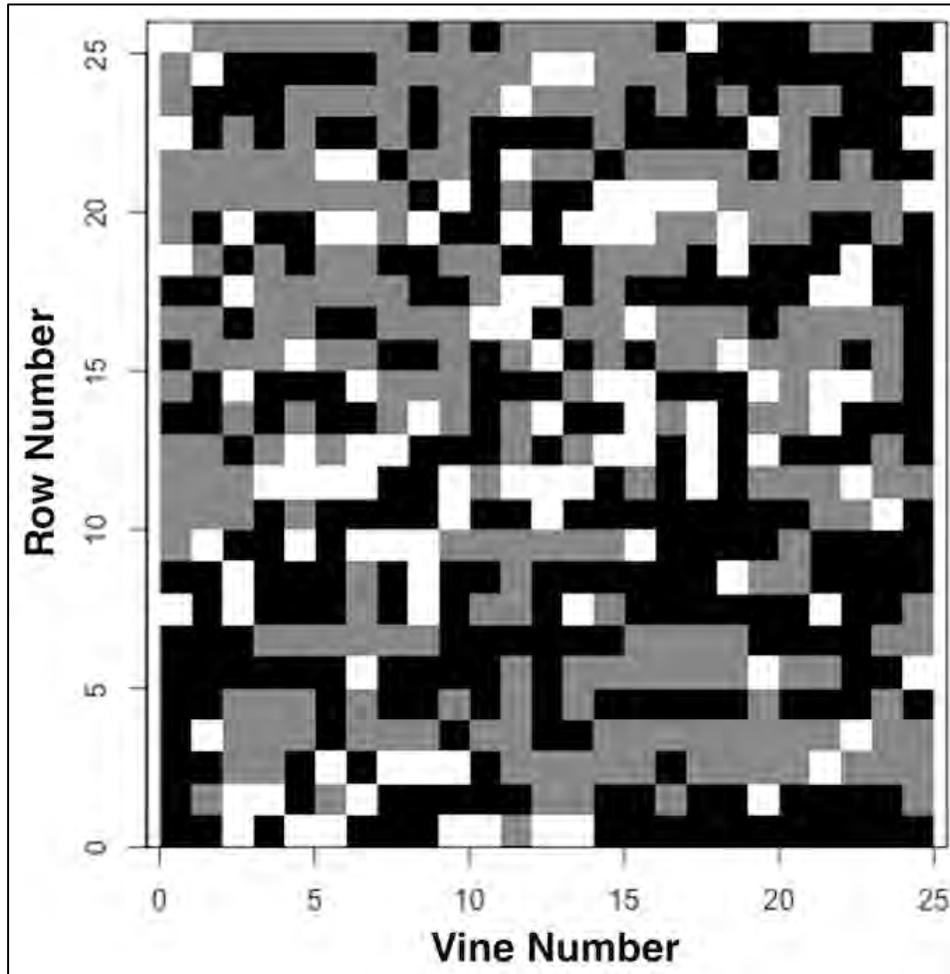


Figure 3. Cell plot representing individual vines and the change in percent agreement between September 2014 and 2015. Colors indicate the level of change, with black = increase in percent agreement, gray = no change, and white = decrease.

In May 2016 eleven members of the evaluation team scored 622 vines and the data was analyzed. The percent agreement of scores for individual vines is shown in **Figure 4**. Cells of varying shades of green represent vines where at least six out of eleven scores agreed.

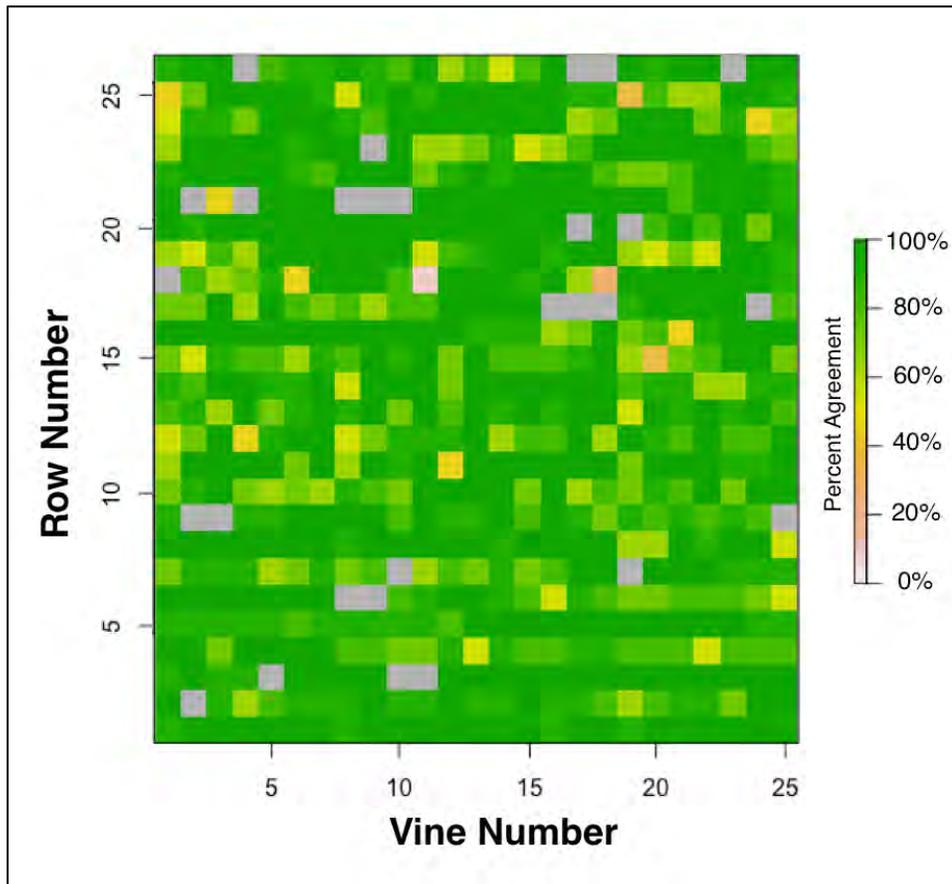


Figure 4. Cell plot of the 622 vines that were rated in May 2016. The colors indicate the percent agreement among scores of individual raters. Scores are counted as “in agreement” if they equal the integer above or below the mean for any given vine. Gray areas indicate the 28 vines that had fewer than eleven scores and were not included in the analyses. Usually this was because a vine was missing and so was not scored.

The number and percent of vines in each agreement category is shown in **Table 3**. Adding columns “55%” through “100%” indicates that for 98.2% of the vines, at least six of the eleven scores agreed. For 50.6% of the vines all eleven scores agreed, i.e., they were within one integer above or below the mean.

Table 3. The number and percent of 622 vines in each of the twelve agreement categories from May 2016.

Percent Agreement May 2016	0% (0/11)	9% (1/11)	18% (2/11)	27% (3/11)	36% (4/11)	45% (5/11)	55% (6/11)	64% (7/11)	73% (8/11)	82% (9/11)	91% (10/11)	100% (11/11)
Number of Vines	0	1	0	1	2	7	18	35	48	73	122	315
Percent of Vines	0.00	0.16	0.00	0.16	0.32	1.13	2.89	5.63	7.72	11.74	19.61	50.64

The May 2016 scores were compared with those from September 2015 to determine if there was a significant difference in score agreement when vines were rated at a different time of the year. The September 2015 scores are shown again below.

Table 2. The number and percent of vines in each of the eleven agreement categories from September 2015.

Percent Agreement Sept., 2015	0% (0/10)	10% (1/10)	20% (2/10)	30% (3/10)	40% (4/10)	50% (5/10)	60% (6/10)	70% (7/10)	80% (8/10)	90% (9/10)	100% (10/10)
Number of Vines	2	2	4	12	3	3	6	13	49	124	432
Percent of Vines	0.31	0.31	0.62	1.85	0.46	0.46	0.92	2.00	7.54	19.08	66.46

There are two notable differences in the level of score agreement between September 2015 and May 2016. First, the percentage of vines where the majority of scores (i.e., at least 50% of the scores) agree increases from 96.5% in September 2015 to 98.2% in May 2016. Second, the percentage of vines where 100% of the scores agree decreases from 66.5% in September 2015 to 50.6% in May 2016. Therefore, while the percentage of vines where the majority of scores agree increases slightly in May 2016, the “strength” of the agreement decreases.

The per vine change in percent agreement between September 2015 and May 2016 is illustrated in **Figure 5**. In May 2016 the percent agreement increased or stayed the same for 383 and 97 vines, respectively. The percent agreement decreased for 142 vines in May 2016. In some cases these latter vines are clustered, indicating that some treatments were possibly more difficult to rate. However, percent agreement for most of these vines was still greater than 50% (data not shown).

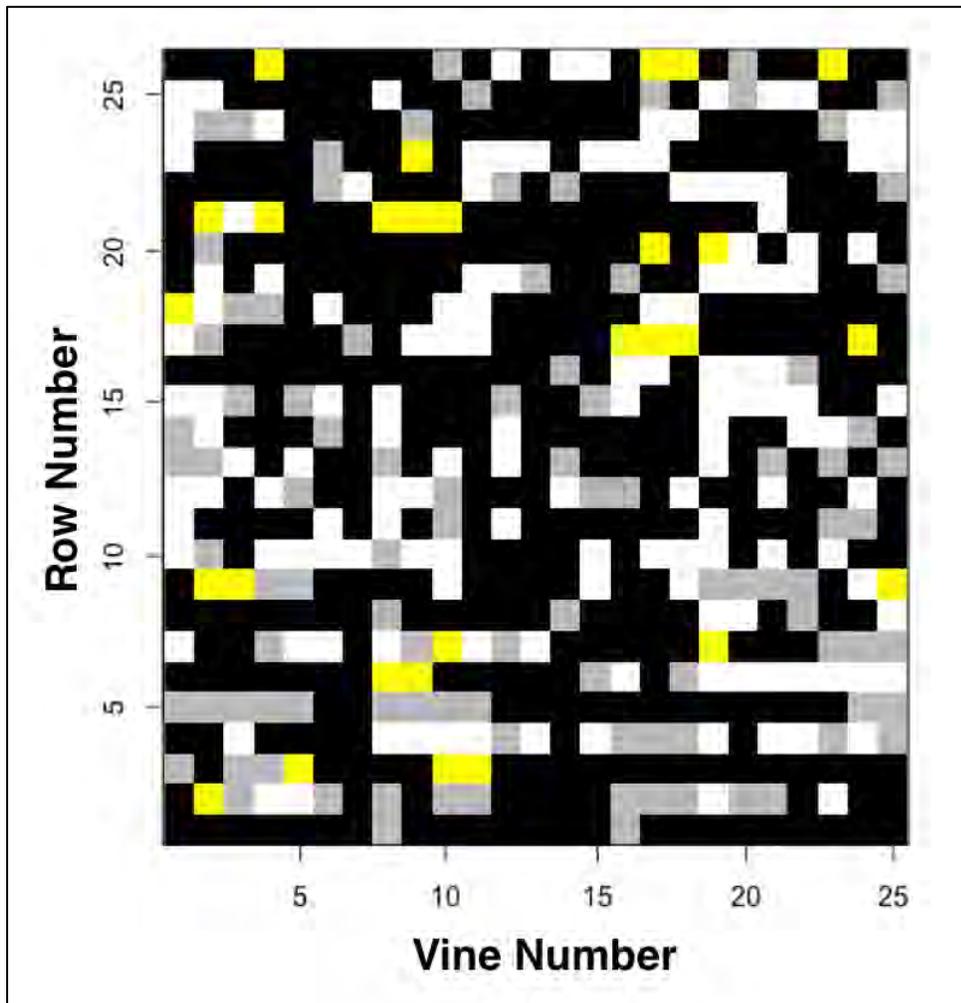


Figure 5. Cell plot representing individual vines and the change in percent agreement between September 2015 and May 2016. Colors indicate the level of change, with black = increase in percent agreement, gray = no change, and white = decrease. Yellow = vines eliminated from analyses due to fewer than eleven scores.

To determine if the percent agreement for the May 2016 data varied by score we mapped mean score against percent agreement for individual vines (**Figure 6**). The “V” shaped scatterplot indicates that there is an agreement bias for low and high scores, i.e., vines that are not very symptomatic or are showing severe symptoms have scores that are in higher agreement. This is especially true for severely symptomatic vines. Of the 315 vines that had scores 100% in agreement (**Table 3**), 193 or 61.3% had a mean score of 5.

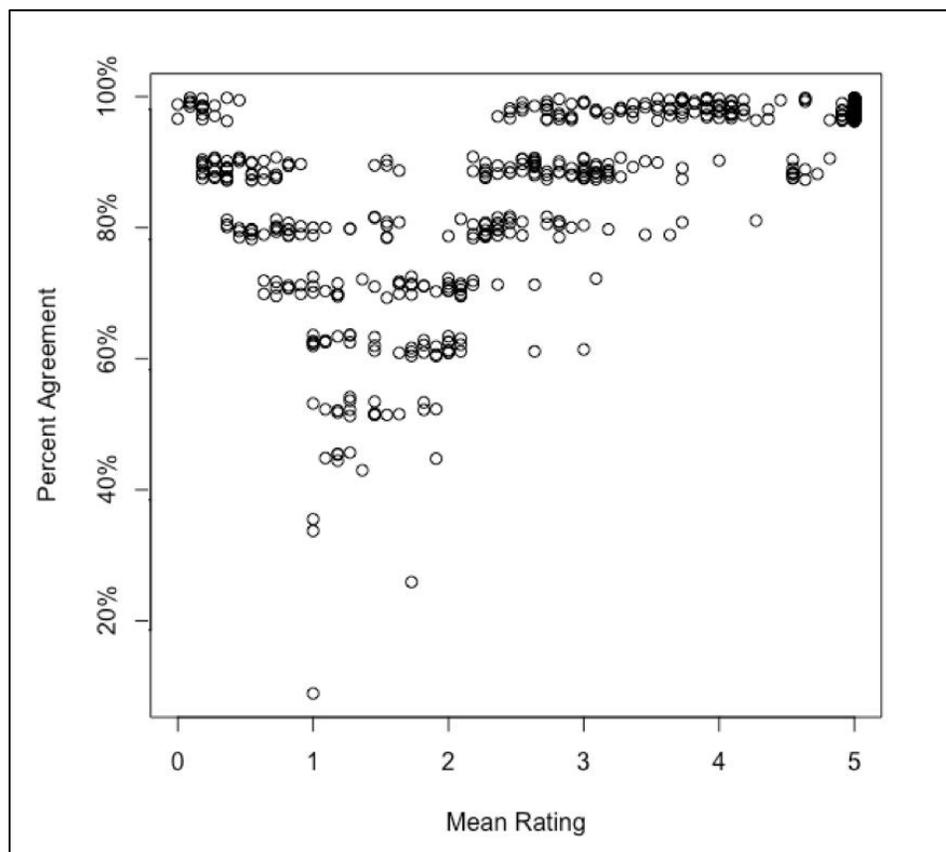


Figure 6. Scatterplot showing mean score plotted against percent agreement. Each circle represents one vine.

CONCLUSIONS

In conclusion, review of the data from the September 2014 and 2015 ratings indicates that for approximately 97% of the vines the majority of team members scored the vines within one integer above or below the mean. In 2015 the percentage of vines where all scores agreed increased from 51.0% to 66.5%. Overall, this demonstrates that the rating system was well understood by team members and provides a relatively uniform measure of Pierce’s disease symptoms that can be used to describe the vines in this experiment.

The percentage of vines where the majority of scores agreed increased by 1.7% in May 2016 compared to September 2015. However, there was a change in the extent to which scores agreed, with a 16% decrease in the percentage of vines where all scores agreed. This suggests that Pierce’s disease symptoms may be more variable in the spring and that rating vines at this time results in a less uniform measure of disease. In addition, the percent agreement of scores for vines with less severe symptoms is lower, suggesting that raters have more difficulty scoring these vines.

FUNDING AGENCIES

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

MONITORING GLASSY-WINGED SHARPSHOOTER AND PIERCE'S DISEASE IN KERN COUNTY, CALIFORNIA

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Reporting Period: The results reported here are from work conducted through the end of 2015.

ABSTRACT

For more than a decade area-wide treatment programs have been in place to reduce populations of the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) within the General Beale region of Kern County. These programs, which involve treating citrus (where GWSS overwinter), coupled with efforts by grape growers to control GWSS and remove vines that have Pierce's disease, have been the foundation of management efforts. In the early 2000s area-wide treatment programs in the General Beale area provided significant reductions in GWSS populations, often with only one treatment applied every three years. From 2009 to 2011 GWSS populations rebounded, and treatment programs were put in place every year. Then, more recently, despite annual area-wide treatments, populations of GWSS returned to levels that are of concern. Concurrent with the increase in GWSS has been a resurgence of Pierce's disease within vineyards. For example, in 2015 survey efforts identified 24 vineyards where Pierce's disease was present, including 10 vineyards with over 1% infection and four vineyards with more than 15% of the vines positive for the disease. Over the past few years several vineyards within the region have been removed specifically because of Pierce's disease, with others slated for removal following the 2016 season. Efforts are underway to determine how to maintain efficacy within area-wide treatment programs as well as to help growers identify and remove infected vines so that overall disease incidence can be brought back down to the very low levels achieved during the early 2000s.

LAYPERSON SUMMARY

Successful management of Pierce's disease requires diligent efforts to control both the disease and its vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). The theory is that if there are no GWSS, the disease cannot spread. Likewise, if there is no disease, a few GWSS are not of concern. Management programs based on this philosophy have historically been very successful, because this two-tiered checks and balances situation still provided protection when populations of either the disease or the vector temporarily increased. Unfortunately, within the past few years populations of GWSS and Pierce's disease have both simultaneously been on the rise in the General Beale region of Kern County, such that Pierce's disease is now causing economic losses in table grape vineyards in the region. GWSS populations have been on the rise due to changes in winter weather which have increased the overwintering survival of GWSS, at the same time that it is suspected that insecticides may not be working as well as they used to. Increased disease incidence is partially due to increased GWSS numbers, but also due to relaxed efforts to look for and remove infected vines during the years of excellent GWSS control and shortly thereafter. The situation has now reached a point where a new Pierce's disease epidemic has occurred, and solving the situation will require coordinated, diligent efforts on the part of individuals and organizations at the industry and government levels to turn the corner on the situation and restore the checks and balances afforded when populations of GWSS and Pierce's disease are both very low.

INTRODUCTION

Since the late 1990s Kern County table grape growers have been entrenched in a battle against the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) and Pierce's disease. Prior to the introduction of GWSS, Pierce's disease was irrelevant to grape growers in the region. The disease was rarely seen due to a very low amount of bacteria in the environment and the scarcity of native sharpshooters that could transmit the disease. However, this all changed when GWSS became established. High vector populations allowed the very small amount of Pierce's disease to be spread, and increases in the number of infected vines in combination with continually high GWSS populations allowed the situation to reach epidemic proportions. The situation reached the point where individual farmers, on their own, could not control the situation and a public-private partnership began to work jointly to protect the region's grape industry.

The first coordinated responses against GWSS and Pierce's disease in Kern County occurred in 2001 when the United States Department of Agriculture (USDA) initiated an area-wide monitoring and treatment program in conjunction with growers and other government organizations. The goal was to reduce populations of GWSS and disease. The decision was to use federal funds to treat GWSS in the citrus orchards where they spend the winter in an effort to prevent them from moving to grape vineyards. Grape growers would also treat their vineyards at their own expense to make sure that any overwintering GWSS survivors would die if they arrived in a vineyard.

The success of the plan also required an aggressive approach to managing the amount of Pierce's disease in the area. At the initiation of the area-wide treatment program there was a one-time government buyout program that subsidized the cost to growers of removing heavily infected vineyards. Since the expiration of that program it has been the responsibility of individual growers to identify and remove individual vines each year that were positive for the disease. The goal was that the combined effects of controlling overwintering GWSS in citrus as well as in grapes, combined with efforts to remove vines with the disease, would mitigate the threat this combination posed to the grape industry.

OBJECTIVES

1. Monitor Kern County vineyards for the glassy-winged sharpshooter.
2. Monitor Kern County table grape vineyards for Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Monitoring for GWSS.

For more than a decade the success of area-wide treatment programs in Kern County has been evaluated by monitoring populations of the vector and the disease. Monitoring for GWSS has been done through the joint efforts of the United States Department of Agriculture (USDA), California Department of Food and Agriculture (CDFA), and County Departments of Agriculture. This program is very visible due to the yellow sticky cards that growers are accustomed to seeing in the corners of all of their vineyards. These traps are collected every one to four weeks (depending on the time of year and area) to determine the location and populations of GWSS. Results of these trap captures are freely available to the public and maps of trapping results during the past 12 months are available online at https://www.cdfa.ca.gov/pdcp/map_index.html by clicking on the link for "Area-wide Trapping."

The cumulative captures from these traps in Kern County for the past 15 years are shown in **Figure 1**. During the first year of the area-wide treatment program there were more than 140,000 sharpshooters caught in traps in Kern County. This included GWSS from citrus and vineyards before and after the first treatments were made. The figures for the next two years (2002 and 2003) represent the total number of GWSS captured as area-wide treatment programs that started in the General Beale area were expanded to other portions of the county. By 2004 all areas in Kern County where GWSS had been found had participated in area-wide treatment programs. From 2004 until 2008 these programs were highly successful. During those years, once an area had participated in an area-wide treatment program, follow-up treatments to the entire area were only needed every two to three years, or not at all (i.e., Western Zone) and GWSS populations could be maintained at low levels with localized spot treatments as needed based on trap captures.

The success of the area-wide treatment programs within the General Beale area began to slide in 2009, while GWSS population levels in most other regions of Kern County remain very low,. During that year GWSS captures in Kern County increased to nearly 40,000. As a result, the aggressiveness of area-wide treatment programs was increased, particularly within the General Beale area where most of the captures were made. Treatment programs continued to result in significant reductions in GWSS compared to prior to the initiation of the program, but annual captures remained at levels between 35,000 to 40,000 per year, well above the lows seen from 2004 until 2008.

During the early 2010s the hope was that increased GWSS populations during 2009 until 2011 were just an anomaly, and that the aggressiveness of treatment programs would bring populations down to historic lows. Unfortunately, beginning in 2012 the opposite has been true, and over 100,000 GWSS have been captured each of those years. It is important to note that this does not mean there are more GWSS now than prior to the program

(comparing 2012 or 2015 to 2011), because there are more traps in the county now than there were back then. However, the fact that we have returned to more than 100,000 captures per year is alarming.

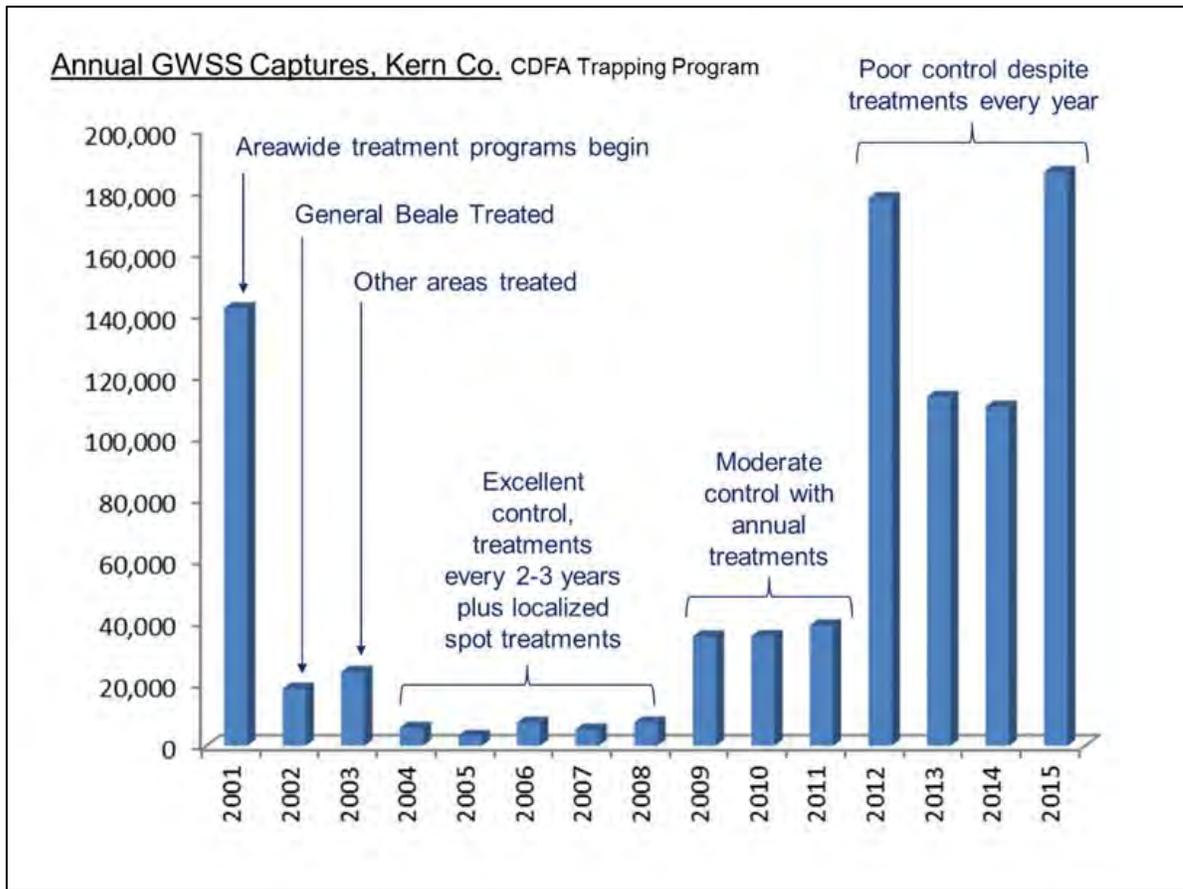


Figure 1. Number of GWSS trapped each year in Kern County in area-wide traps during 2001-2015.

There are several theories about why GWSS populations have increased over the past few years. The two most prevalent theories are climate change and pesticide resistance. With regards to climate change, overwintering GWSS can tolerate very cold temperatures but require a minimum temperature in order to feed. Historically it was commonplace to have thick fog for long periods of time such that GWSS were unable to feed. This would cause them to desiccate and die. However, for at least six or seven years we have not had the rain and inversion conditions required to have thick fog events. As a result, overwintering survival of GWSS has been excellent and the buildup of GWSS populations each year has had a carryover effect into the next year. There is also evidence that warmer winter weather may allow for GWSS reproduction during the winter instead of in the spring. If this scenario is true it means that we are no longer getting the help we need from colder weather during the winter to help reduce GWSS populations between one year and the next.

The second theory is that GWSS are becoming resistant to the insecticides that are being used against them, particularly neonicotinoids. For fifteen years most citrus and nearly all grapes have been treated for GWSS, vine mealybug, scale, or other pests with one or more of the following neonicotinoids: imidacloprid (Admire and others), acetamiprid (Assail), clothianidin (Belay), dinotefuran (Venom), or thiamethoxam (Actara). Investigations by two teams of researchers are underway to determine the status of susceptibility of GWSS to several of these insecticides.

In response to increased GWSS captures during the past four years, managers of the area-wide treatment programs are taking additional steps to reduce GWSS populations. The current approach is a multi-spray program that includes area-wide treatments to citrus that were initiated in December 2015, coupled with a second treatment around February, with the possibility of a third systemic treatment after petal fall. These treatments are being put

on for GWSS control, but are also being coordinated in a way that makes sense to citrus growers as they begin efforts to control their own new invasive pest, the Asian citrus psyllid. The hope is that this aggressive approach to controlling GWSS in overwintering citrus, coupled with aggressive efforts on the part of local grape growers to treat their own vineyards, will successfully reduce GWSS populations.

Objective 2. Monitoring for Pierce's disease.

For nearly a decade monitoring for GWSS has been accompanied by monitoring for Pierce's disease. Surveys have been done by researchers at the University of California Cooperative Extension (UCCE) office in Kern County with funding provided by table grape growers through the Consolidated Central Valley Table Grape Pest and Disease Control District. The monitoring program was initially proposed and coordinated by UCCE viticulture farm advisor Jennifer Hashim-Buckey. Later it was temporarily managed by emeritus viticulture advisor Don Luvisi and is now managed by entomology advisor David Haviland.

Each year surveys were done using a four-wheeler based on visual symptoms. Surveyors looked for vines that expressed Pierce's disease symptoms such as stunted shoot growth, leaf scorch, persistent petioles, irregular cane maturity, and shriveled fruit. In vineyards with low incidence of Pierce's disease samples were collected from all symptomatic vines and each vine was recorded by a unique block ID row number and vine number in a standardized mapping system. Samples were sent to the CDFA Plant Pest Diagnostics Lab to confirm presence of *Xylella fastidiosa* using an enzyme-linked immunosorbent assay (ELISA). Results and locations of confirmed Pierce's disease positive vines were returned to cooperating growers and/or their pest control advisers. Data that are reported in this report consist of the percentage of symptomatic vines found in a vineyard multiplied by the percentage of samples that were confirmed to be positive for Pierce's disease in samples sent to CDFA.

During 2015 personnel with the UCCE office in Kern County monitored approximately 122,000 grapevines from 39 vineyards from late July through November. Vineyards were chosen based on our past history of survey sites, knowledge of Pierce's disease distribution, and trap catches of GWSS. Samples were collected at 31 of the 39 sites; the other sites were mostly vineyards sampled in previous years that were recently removed and/or replanted.

Pierce's disease incidence at the 31 sites ranged from 0.0% to 33.2% (**Table 1**). This included nine sites (29%) with no Pierce's disease, 12 sites (39%) with Pierce's disease present in less than 1% of the vines, five sites (16%) with 1-5% infected vines, one site (3%) with 5-15% infected vines, and four sites (13%) with more than 15% positive vines.

Considering that we only sampled a portion of many vineyards we attempted to estimate the total number of vines infected with Pierce's disease within vineyards that we sampled. This was done by multiplying the percentage infected vines from our sample area at each site by the total acres of the vineyard at that site and converting the result to number of vines by multiplying by 518 (the number of vines per acre on a standard 7 foot by 12 foot spacing). In cases where we have not yet determined the total vineyard size (mostly new sites in 2015) we made the calculation based on the acres we sampled instead of the total vineyard size. After making the calculation the cumulative number of Pierce's disease positive vines was estimated to be approximately 7,500. Approximately 6,500 of those vines are located in the five most infected sites (Sites 10, 27, 29, 30, and 42). The other approximately 1,000 infected vines were spread out across the other 17 sites that had at least one positive vine.

Over the past seven years there has been a consistent increase in the amount of Pierce's disease present in the General Beale and Edison regions of Kern County. At present we are aware of 25 vineyards that currently have Pierce's disease or that were removed within the past year because of Pierce's disease. Included within this list are four to five vineyards that should be removed following the 2015 season.

Distribution of Pierce's disease has also continued to increase. During surveys from 2009 until about 2013 almost all Pierce's disease was found within two epicenters of what we call the 'core' region of the General Beale area. During the past two years we have seen significant spread outward to the periphery of the General Beale area and in 2015 for the first time we found significant amounts of Pierce's disease in isolated vineyards north of Highway 58 in the Edison region.

Table 1. Results of Pierce's disease surveys from 39 vineyards in the General Beale and Edison regions of Kern County, CA from 2009 to 2015.

Site	Variety	Year Planted	Acres	Acres surveyed	Percentage vines infected with Pierce's disease							
					2009	2010	2011	2012	2013	2014	2015	
1	RG, Sugra	1994/14	31	0	0.0	0.0	0.0	0.2	8.3	R	P	
2	RG, Flames	1997	20	0	0.0	0.0	0.0	0.0	0.0	0.1	P	
3	Flame	1982	19	19	0.0	0.0	0.0	0.0	0.0	0.0	0.01	
4	Flame, SumRoy	unk./14	19	5			0.0	0.0	0.0	P	1.0	
5	Flame	1994	19	5	0.0	0.1	0.3	0.1	0.2	-	3.0	
6	AR, Flame	1994/11	13	13			G	0.0	0.0	0.2	0.03	
7	Flame	2001	17	17		0.0	0.0	0.0	0.0	0.0	0.0	
8	Thom	1994	9	9			0.0	0.0	0.0	0.0	0.0	
9	Crim, ScarRoy	2002/12	38	5	0.0	0.0	0.0	G	0.7	1.8	1.7	
10	Thom	1992	17	6	0.0	0.0	0.0	0.0	0.0	0.2	33.2	
11	Thom	1992	6	6			0.0	-	0.0	0.0	0.0	
12	E.Sweet	2005	-	0	0.0	0.0	0.0	0.0	0.1	0.0	R	
13	Flame	2001	-	0	0.0	0.0	0.0	0.0	0.0	R	-	
14	Flame	1994	28	5	0.0	0.0	0.1	0.0	-	-	1.2	
15	RG, Flame	1982/13	19	19			0.8	100	R, P	0.5	0.03	
19	S. Celeb.	2012	-	6.3				P	1.5	0.0	0.0	
22	Flame	1993	8.8	0			0.0	0.0	0.0	0.0	R	
23	Flame	2009	4.7	4.7			0.0	0.0	0.0	0.0	0.16	
24	RG	1994	31	0				0.2	61.1	9.2	R	
24a	Sugra	2013	8.5	8.5					P	0.0	0.0	
24b	AR	Unk	5.2	0						25.6	-, R	
25	RG	1994	15	15					47.3	9.8	1.1	
26	Flame	2012	7.6	7.6				P	15.5	6.1	0.2	
27	ScarRoy	2011	12	12				P	-	46.0	18.6	15.7
28	Flame	1994	8.8	0						2.0	R	
29	AutRoy	1998	-	12			0.0	-	-	0.7	6.9	
30	ScarRoy	2013	12	1.2					P	0.2	26.8	
31	ScarRoy	2008	6.3	6.3						1.4	0.8	
34	ScarRoy	2011	-	5						0.0	0.04	
35	Timco	2011	-	5						0.4	0.0	
36	RG	1994	7	7						0.0	0.0	
37	Flame	-	-	5							0.1	
38	Magenta	2014	19	5						P	0.04	
39	Magenta	-	-	5							0.7	
40	Sugra	-	-	5							0.0	
41	Sugra	-	-	5							0.0	
42	Thom	1994	10	1.1							17.4	
43	Flame	-	-	5							0.2	
44	Thom	-	-	5							0.1	

R = removed; P = planted; G = grafted onto existing rootstock; - indicates that data are not available or were not collected; RG = Redglobe; Sugra = Sugraone; Flame = Flame Seedless; SumRoy = Summer Royal; AR = Autumn Royal; Thom = Thompson Seedless; Crim = Crimson Seedless; ScarRoy = Scarlet Royal; E Sweet = Early Sweet; S. Celeb = Sweet Celebration.

There is currently no way to cure a vine infected with Pierce's disease. For that reason, all vines that are infected should be completely removed from the vineyard. If complete removal is not possible, at minimum the vine should be cut off at the base and treated (chemically or otherwise) to ensure that the root system is dead and does not regrow. In cases where vineyards have elevated levels of Pierce's disease it is recommended that the entire vineyard be removed. This is because vine death or removal due to Pierce's disease decreases the economic viability of a vineyard, but also because not all symptomatic vines can be identified during any one survey. This is particularly true in mature vineyards where it is common for vines that become infected during the current year to appear healthy until the following year. Therefore, any time symptomatic vines are found in a vineyard it should be assumed that additional vines are infected but not yet symptomatic. These symptomless vines can still serve as a host for Pierce's disease the following year.

ACKNOWLEDGEMENTS

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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 (*Xf*) 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. *Xf* 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 the behavior of *Xf* in plants. Saponified macadamia nut oil, rich in palmitoleic acid, also appears attractive as an inexpensive source of exogenously applied signal molecule. 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 *Xf* within plants and disease symptoms are greatly reduced in plants in which this *Burkholderia* strain was inoculated either simultaneously with, prior to, or even after that of *Xf*. The biological control agent can be applied either by direct introduction into the xylem by droplet puncture or by spray application to foliage using a penetrating surfactant. 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 employ by various ways of inoculation.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) produces a mixture of unsaturated fatty acid signal molecules called diffusible signal factor (DSF). Accumulation of DSF in *Xf* 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 *Xf* 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 resistance as did the Freedom cultivar previously constructed. Topical application of commercially available unsaturated fatty acids capable of altering gene expression in *Xf* 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 *Xf* and thereby its virulence in plants when inoculated prior to or simultaneously with *Xf*. By comparing disease control by these three methods the most efficacious and practical means of control can be identified.

INTRODUCTION

Our work has shown that *Xylella fastidiosa* (*Xf*) 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 they do not express adhesins that would hinder their movement through the plant but which are required for vector acquisition. Instead, they 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, thereby causing “pathogen confusion.” Transgenic Freedom grape expressing the DSF synthase RpfF from *Xf* are much more resistant to disease than the wild-type plants in both greenhouse and field trials. It is possible that grape varieties might differ in their ability to produce DSF molecules perceived by *Xf*. 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. Our work has shown that RpfF is rather promiscuous and that *Xf* can both produce and respond to a variety of unsaturated fatty acids including the common, inexpensive unsaturated fatty acid palmitoleic acid. We thus are addressing practical issues about how such molecules might be applied to plants for disease control. Using a new *Xf* 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. 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 are investigating the interactions of this endophyte with grape to understand how it is conferring disease control and determine practical methods for its exploitation.

OBJECTIVES

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

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

While Freedom grape transformed with the *Xf rpfF* gene encoding the DSF synthase produced DSF species to which *Xf* 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. The various enoic acids that can be produced by RpfF differed substantially in their ability to induce gene expression in *Xf*, 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 *Xf* 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, we are comparing the production of DSF species in 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 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 biphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. 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.

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**. Transformation of the various varieties is underway with many 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 is taking longer than the other cultivars. Transformation with the kanamycin-marked, chloroplast-targeted *rpfF* gene is also taking longer than other constructs. Between five and ten individual transformants will be tested 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 levels of expression of *rpjF* and production of DSF the expression of *rpjF* is being assessed by quantitative reverse transcription polymerase chain reaction (RT-PCR) of RNA isolated from individual leaves of the transformed plants after they are grown to a height of approximately 40 cm.

Table 1. Grape lines being produced and tested.

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

The composition of DSF species present in xylem sap and their aggregate signaling activity will also 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, *Xf*DSF2, 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.

The initial transformed plants received have been grown to a sufficiently large size in a greenhouse to make green cuttings that have now been rooted and inoculated with *Xf* to assess their disease susceptibility compared to transformed lines. Initial disease assessments are now being performed on the first of these propagated, transformed plants. We expect that it will take at least another six months to both produce and test rooted cuttings of other transformed plants.

Objective 2. Direct application of DSF to plants.

Several recent findings in our laboratory 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 *Xf* reveal that it is quite responsive to the cheap, commercially available, enoic acid palmitoleic acid (**Figure 1**).

While about eight-fold more palmitoleic acid is required to induce gene expression in *Xf* than *Xf*DSF2, it is much more active than *Xf*DSF1 itself. We therefore have conducted a variety of studies to address how such molecules could be introduced into plants in different ways to achieve pathogen confusion. 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 *Xf*. 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. We find that this fatty acid mixture has DSF signaling activity. Alkaline phosphatase activity exhibited by the *Xf Xf:phoA* biosensor increased with increasing concentrations of the mixture of fatty acids in the soap prepared from the saponified macadamia nut oil (**Figure 2**). 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 an inexpensive source of DSF homologs that could be directly applied to grape.

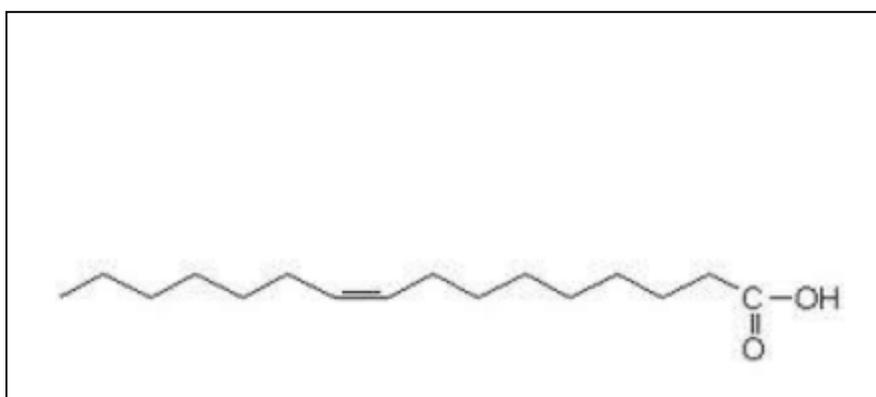
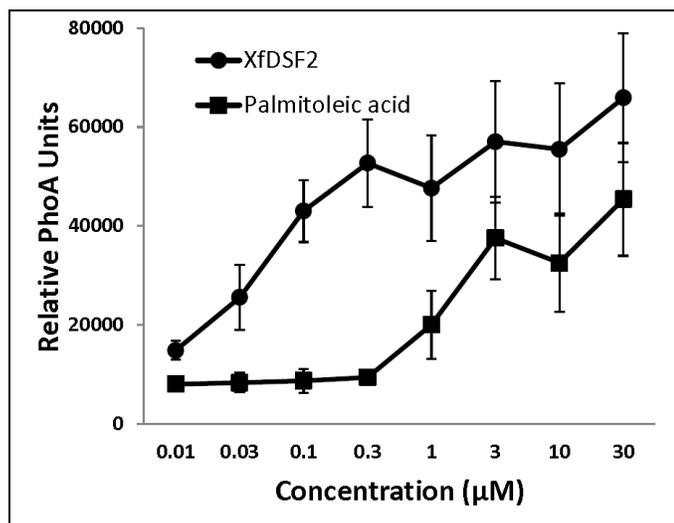
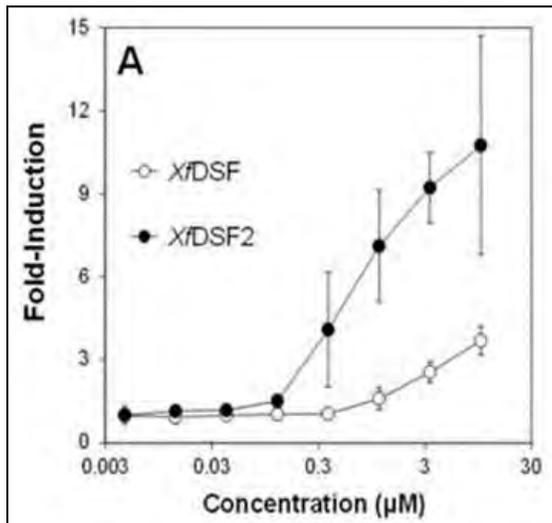


Figure 1. Responsiveness of a PhoA-based *Xf*DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

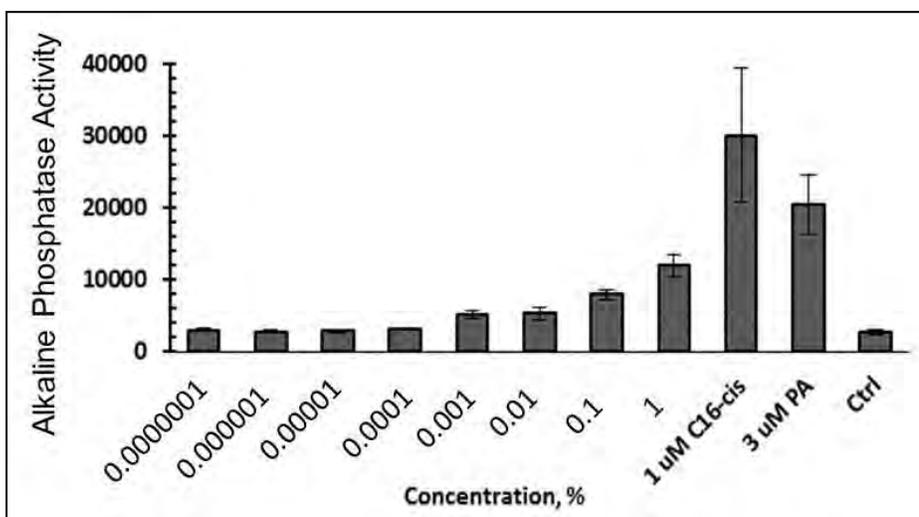


Figure 2. Alkaline phosphatase activity exhibited by the *Xf Xf:phoA* biosensor exposed to increasing concentrations of saponified macadamia nut oil as well as 1 µM *Xf*DSF2, 3 µM palmitoleic acid, or a negative control with no added DSF.

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 *Xf* 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 *Xf* in plants we have been assessing DSF levels in xylem sap of plants treated in different ways using a *PhoA*-based *Xf* biosensor. As DSF species are somewhat hydrophobic, a variety of adjuvants have been tested for their effects on enhancing their introduction into plants. For example, detergents and solubilizing materials such as Solutol HS15, Break-thru, Triton X-100, and dimethyl sulfoxide (DMSO) and Solutol increase the apparent penetration and dispersal of DSF and its analogs. Solutol and DMSO proved to be rather phytotoxic and therefore were not practical solutions for the production of signaling molecules. The organo-silicon surfactant Break-thru having very low surface tension and allowing spontaneous stomatal infiltration of solutions into leaves not only was not phytotoxic, but it also appeared to be superior to the other agents aiding the entry of signaling molecules. Considerable results have been obtained on the ability of topically-applied palmitoleic acid and macadamia nut oil saponification solutions to enter into the plants. Apparent DSF signaling activity was measured using the *Xf:phoA*-based alkaline phosphatase biosensors noted above. 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 *Xf:phoA* biosensor. These studies reveal that detectable amounts of signaling molecules could be introduced into grape leaves when applied as a foliar spray with 0.2% Break-thru (**Figure 3**). Lesser amounts could be introduced with foliar sprays without this adjuvant. As a registered surfactant for use in agriculture, Break-thru has the potential to be a practical delivery agent. Thus, solutions of fatty acid supplied with this concentration of surfactant appear to bypass the cuticular surface as a means to enter 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.

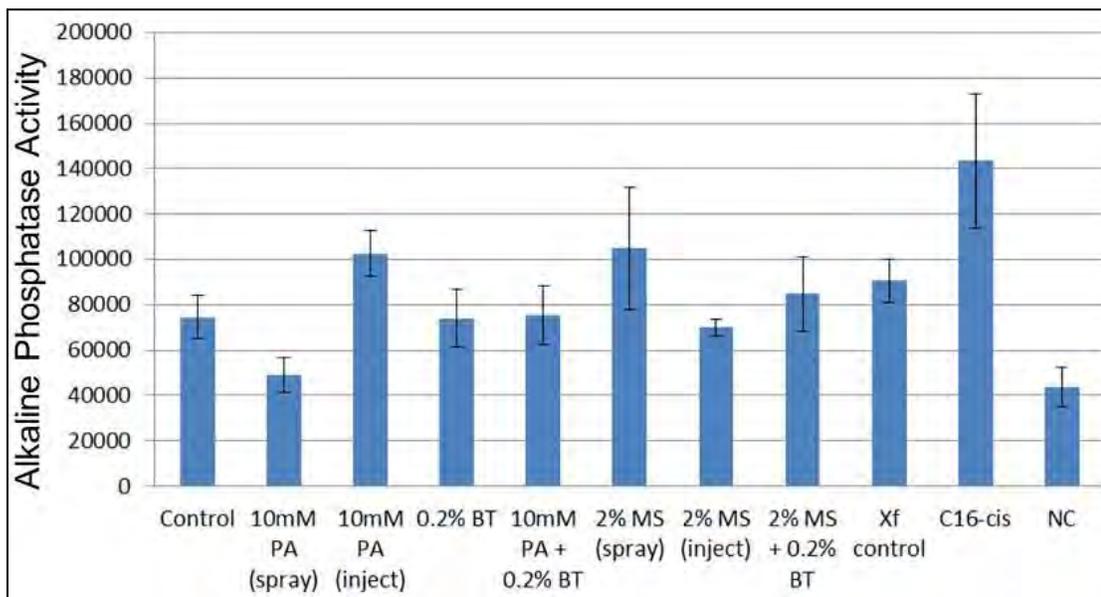


Figure 3. Alkaline phosphatase activity exhibited by 10 μ l aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Break-thru (BT) or without a surfactant as a foliar spray (spray) or a stem injection (inject).

These most promising treatments were also applied to grape plants to evaluate their efficacy in reducing the symptoms of Pierce's disease. Palmitoleic acid or macadamia oil soap was applied with various adjuvants two weeks before inoculation with *Xf* and at monthly intervals after inoculation with the pathogen. The severity of Pierce's disease was reduced on plants sprayed with a solution of 10 mM palmitoleic acid as well as on plants in which this fatty acid was injected into the stem. The disease control conferred by a 2% solution of saponified macadamia nut oil was as great as that conferred by purified palmitoleic acid. The promising results using saponified plant oils are being further pursued as this not only is a very practical but quite inexpensive strategy to

achieve disease control. Given that the efficacy of saponified plant oils applied without an adjuvant seem to be as great as when applied with a surfactant, the cost and convenience of using such treatment seems particularly good.

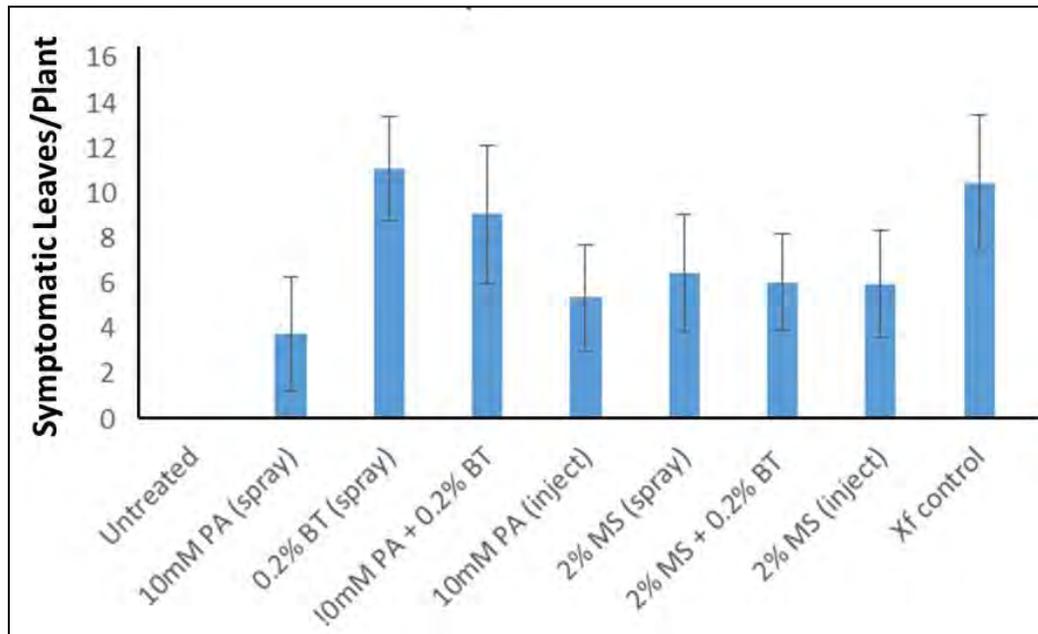


Figure 4. Symptoms of Pierce’s disease exhibited by Cabernet Sauvignon seedlings treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Break-thru (BT) or without a surfactant as a foliar spray (spray) or a stem injection (inject).

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

While the biological control of Pierce’s disease with endophytic bacteria that would grow within grapevines and produce DSF has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grapevines. All of hundreds of strains isolated from within grapevines by our group as well as that of B. Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently, however, found that *Burkholderia 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 *Xf* itself, suggesting that it is an excellent grapevine 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 *Xf rpfF*. While we have no evidence for its production of a DSF species to which *Xf* could respond, the promiscuous nature of RpfF in *Xf* and other species suggested that it might make DSF species to which *Xf* would respond under some circumstances, such as when growing within plants. Preliminary results suggest that co-inoculation of *Xf* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *Xf* alone: whereas the number of infected leaves of plants inoculated with *Xf* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *Xf* and *B. phytofirmans* (**Figure 5**).

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 0.2% Break-thru, 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 Break-thru (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 Break-thru (**Figure 6**). Very few cells were present within 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.

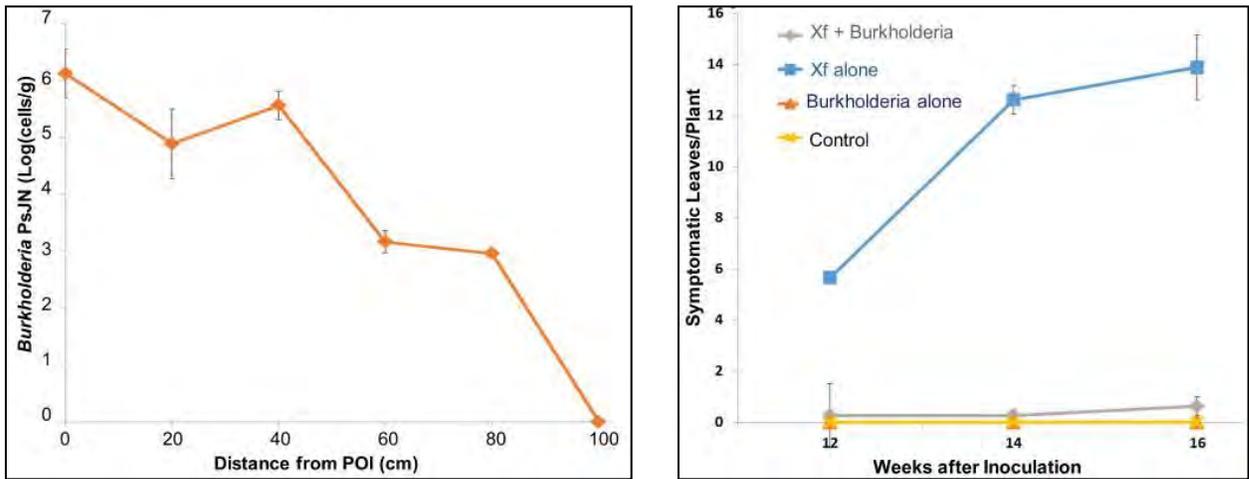


Figure 5. Left: Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after six weeks incubation. Right: Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *Xf* alone (blue) or when co-inoculated with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

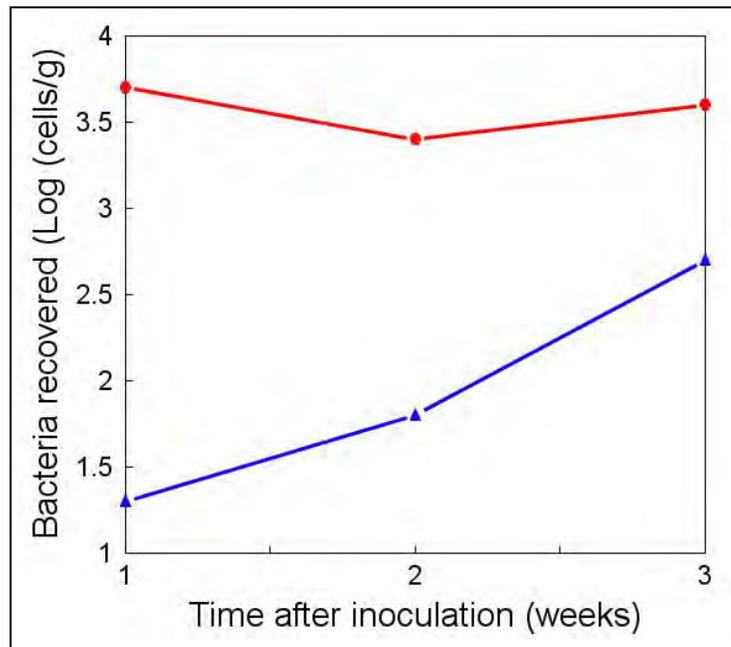


Figure 6. Population size of *Burkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Break-thru (red line).

Given the promising results of the reduction of severity of Pierce's disease in grape treated with *B. phytofirmans*, we performed additional experiments in which *Xf* was co-inoculated with *B. phytofirmans* as well as when *B. phytofirmans* both preceded or followed inoculation of plants with *Xf* by 30 days. As observed before, the severity of Pierce's disease of plants co-inoculated with *B. phytofirmans* and *Xf* was greatly reduced at all times after inoculation compared to that on plants inoculated with the pathogen alone (**Figure 7**). 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 (**Figure 7**). Almost no disease was observed on plants inoculated with *B. phytofirmans* 30 days after inoculation with the pathogen (**Figure 7**). 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 *Xf*. It might have been anticipated that pre-inoculation of plants with *B. phytofirmans* would have yielded the largest degree of disease resistance. However, this and other studies have shown that disease incidence and severity is reduced whenever *B. phytofirmans* and *Xf* are present together in the plant. Inoculation of plants with *B. phytofirmans* after that of the pathogen would, by definition, place them both in the plant together, while pre-inoculation could result in a situation where the biological control agent may not be present in a plant, particularly if it did not continuously colonize the plant.

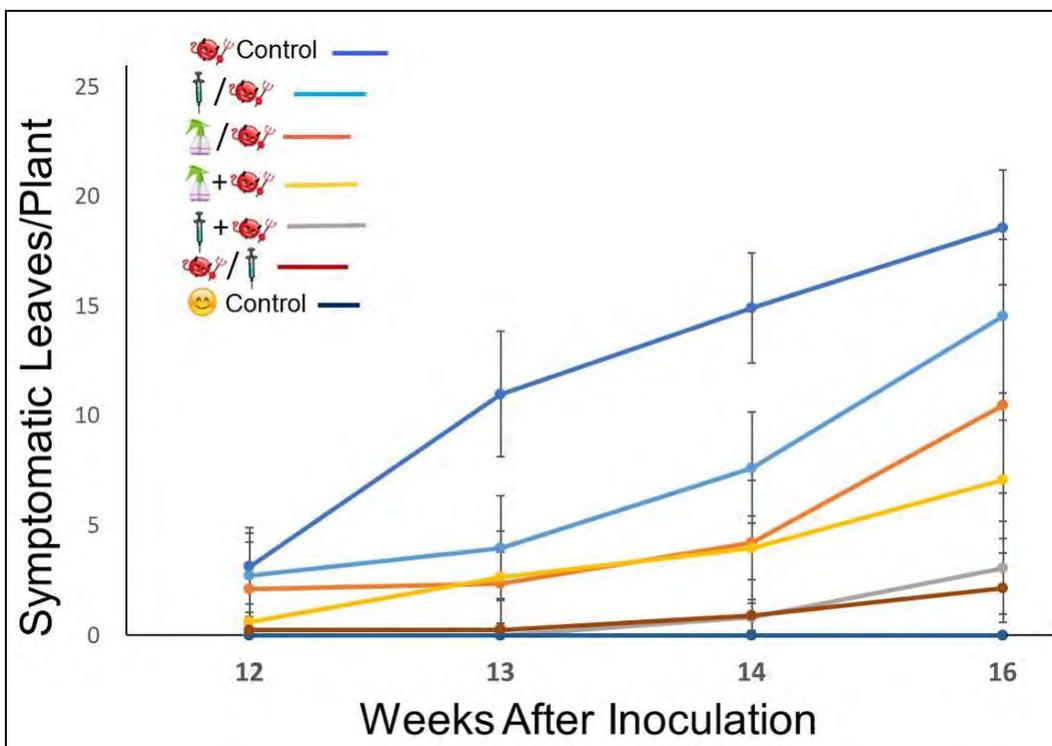


Figure 7. Severity of Pierce’s disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants needle-inoculated only with *B. phytofirmans* (dark blue line), only with *Xf* (medium blue line), or co-inoculated with *Xf* and *B. phytofirmans* (yellow line). Also shown is disease severity on plants needle-inoculated with *B. phytofirmans* 30 days before inoculation with *Xf* (light blue line) or sprayed with *B. phytofirmans* in a solution of 0.2% Break-thru 30 days before inoculation with *Xf* (orange line), as well as on plants needle-inoculated with *Xf* 30 days after inoculation with *B. phytofirmans* (maroon line). The vertical bars represent the standard error of the determination mean disease severity.

B. phytofirmans was able to inhibit Pierce’s disease development in all grape varieties in which it was evaluated. When inoculated simultaneously into different grape varieties (although not at the same location, but within about 10 cm of the site of inoculation with the pathogen) the progression of Pierce’s disease was greatly suppressed compared to that of plants inoculated with *Xf* alone (**Figure 8**). While the greatest reduction in disease severity was conferred in Cabernet Sauvignon, a variety somewhat more resistant to Pierce’s disease than either Thompson Seedless or Chardonnay, *B. phytofirmans* conferred a very high level of disease resistance (**Figure 8**). It thus appears that the beneficial effect of *B. phytofirmans* is not variety specific, and that it should confer high levels of resistance in all grape varieties.

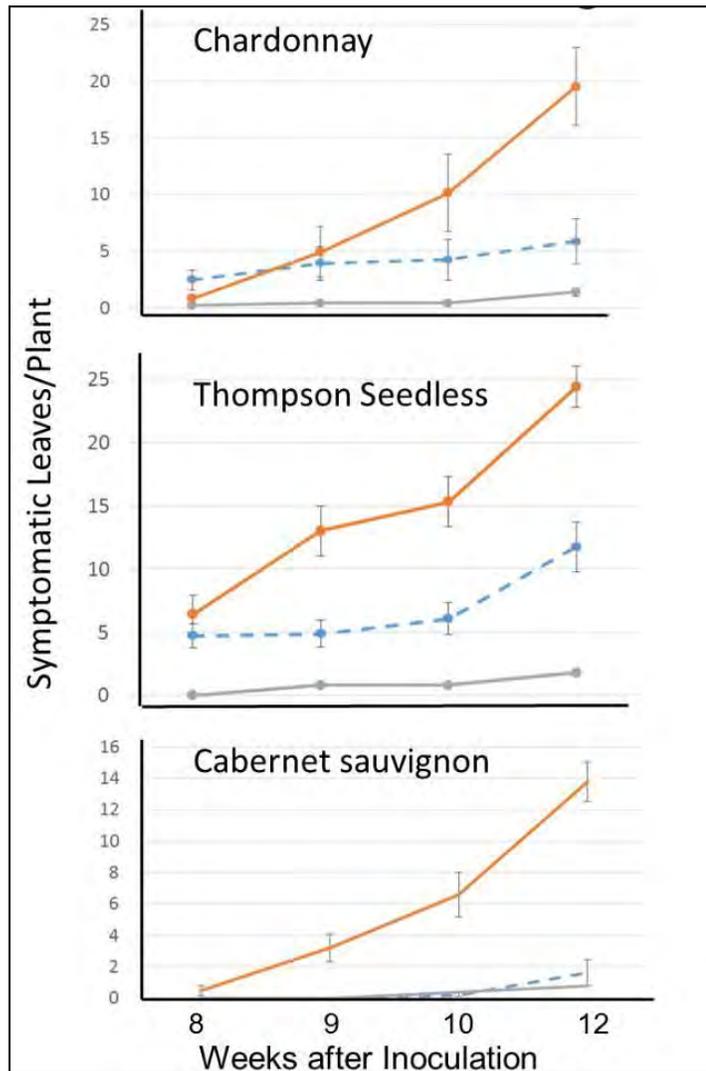


Figure 8. Severity of Pierce's disease observed in different grape varieties needle-inoculated at the same time but at different locations with *Xf* and *B. phytofirmans* (blue line), compared to that inoculated only with *Xf* (orange line) or with *B. phytofirmans* alone (gray line). The vertical bars 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 *Xf* in inoculated plants. Relatively high population sizes of *Xf* 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 9**). 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 *Xf* was much lower at a given distance away from the point of inoculation in plants co-inoculated with *Xf* and *B. phytofirmans* (**Figure 9**). 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 9**).

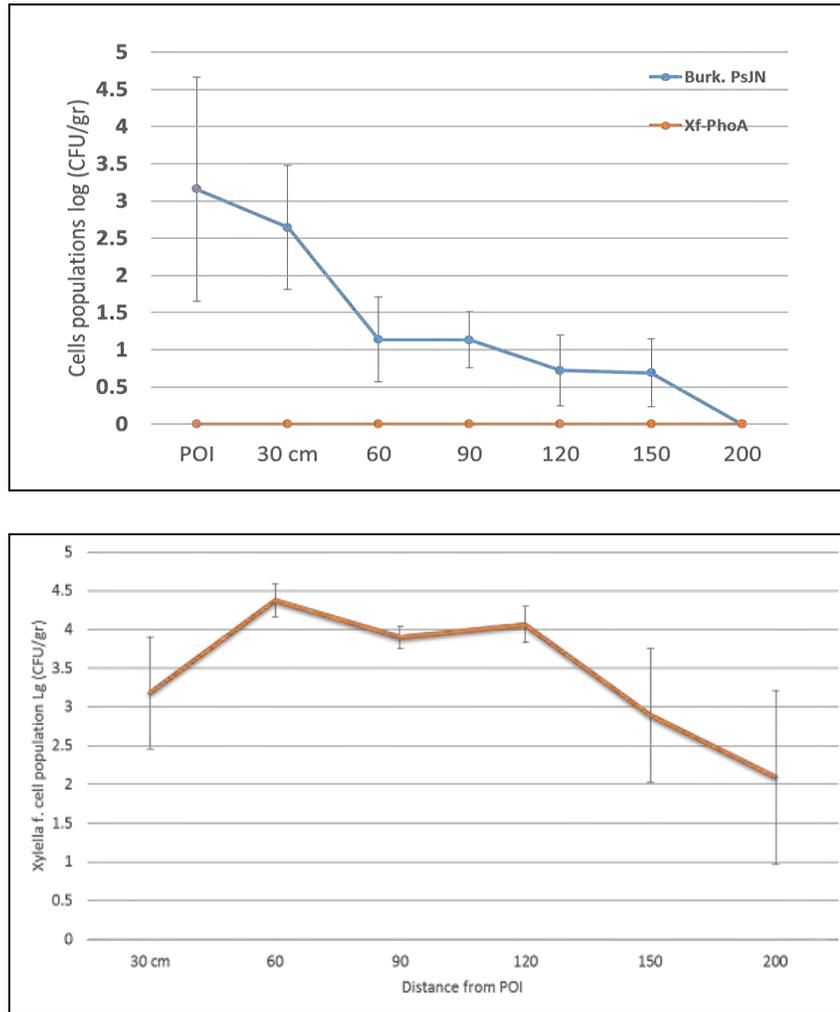


Figure 9. Top: Population size of *Xf* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. Bottom: Population size of *Xf* in the stems of grapes at various distances from the point of inoculation of the pathogen when 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.

Surprisingly, we have frequently observed that while *B. phytofirmans* rapidly achieves high population sizes and spreads extensively in plants after inoculation, when assessed several weeks after inoculation its population sizes in inoculated plants, irrespective of whether *Xf* was also inoculated into the grape plants, is often quite low. These results suggest that the interactions of *B. phytofirmans* with either the plant or *Xf* occur early in the infection process. The fact that the effect of the inoculation of plants with *B. phytofirmans* reduces population sizes of *Xf* 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 *Xf* in plants treated with *B. phytofirmans* was similar to that seen in transgenic plants harboring *Xf rpfF* that produce DSF. It is curious, however, that the population size of *Xf* is often lower even near the point of inoculation in plants also treated with *B. phytofirmans* (**Figure 10**). This suggests that in addition to any effect that *B. phytofirmans* has on changing the signaling behavior of *Xf*, possibly by altering DSF signaling, that it might also be either directly antagonistic to the pathogen in the plant or, more likely, triggering a host defensive reaction that inhibits the growth or survival of the pathogen. Experiments are underway to distinguish these different possibilities.

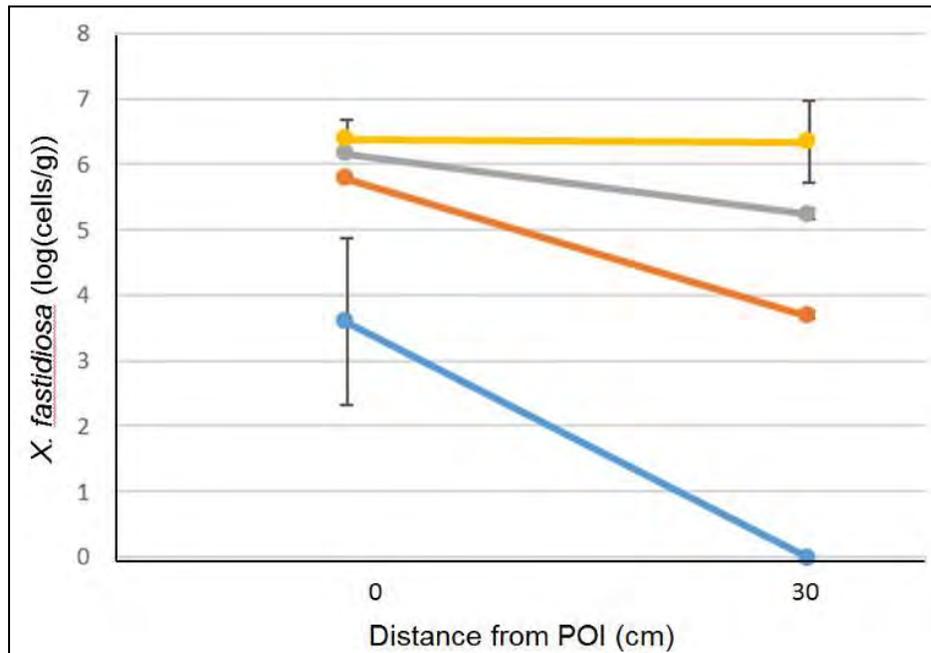


Figure 10. Population size of *Xf* three weeks after inoculation of plants with the pathogen alone (yellow line), plants sprayed with *B. phytofirmans* on the same day that it was needle-inoculated with the pathogen (gray line), plants needle-inoculated with *B. phytofirmans* on the same day that it was needle-inoculated with the pathogen at a nearby site (orange line), and plants needle-inoculated with *B. phytofirmans* three weeks prior to being needle-inoculated with the pathogen at a nearby site (blue line). The vertical bars represent the standard error of the determination of log-transformed population sizes.

Considerable effort has been made during this reporting period to better understand the mechanisms by which *B. phytofirmans* alters the behavior of *Xf* in plants. DSF production has been described in other *Burkholderia* species including *Burkholderia ceenocepacia*. Furthermore, the genome sequence of *B. phytofirmans* PSJN has been determined, allowing us to putatively identify a gene with some homology to *Xf* 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 *Xanthomonas campestris* or *Xf* 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 *Xanthomonas campestris* was observed when the biosensor was exposed to extracts of both the wild-type and *rpfF* mutant of *B. phytofirmans* (**Figure 11**). These results suggest that indeed *B. phytofirmans* was capable of producing a DSF-like molecule that *Xanthomonas 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 *Xanthomonas campestris* could respond to a putative signal molecule from *B. phytofirmans*, little or no change in expression of the *phoA* reporter gene was observed when the *Xf 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 *Xf* and *Xanthomonas campestris* respond to different DSF species, it was not unexpected that they might differentially respond to the signal molecule apparently made by *B. phytofirmans*.

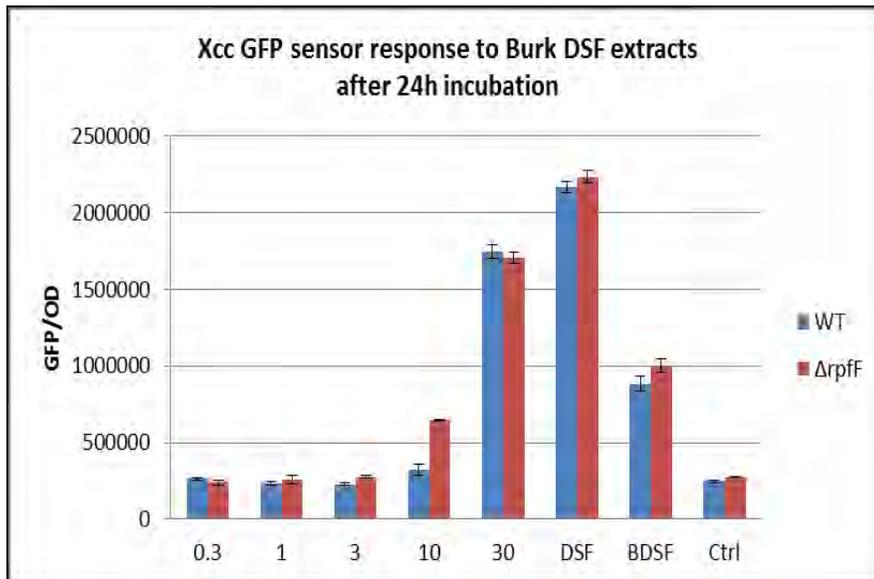


Figure 11. Normalized green fluorescent protein (GFP) fluorescence exhibited by the *Xanthomonas campestris* pv. *campestris* DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild-type *B. phytofirmans* (blue bars) or an *rpfF* mutant (red bars). Shown on the abscissa are different μ l aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 μ M DSF, 1 μ M 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 *Xf 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 *Xf* 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 *Xf* (**Figure 12**). 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 *Xf* adhered to the walls of glass culture flasks below the ring, in the area exposed to turbulent mixing of the culture during shaking (**Figure 12**). These results suggested that the adhesiveness of *Xf* 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.

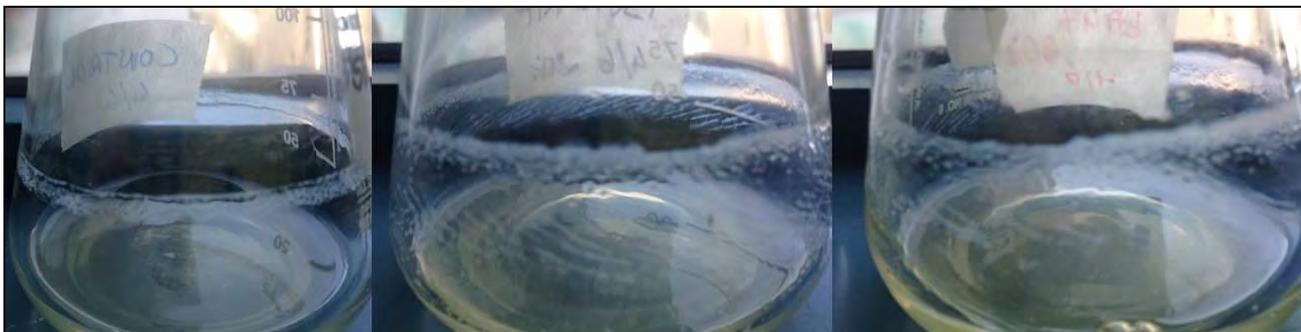


Figure 12. Biofilm formation of wild-type *Xf* grown in PD3 media alone (left), or in media containing 20% v/v of culture supernatant of wild-type *B. phytofirmans* (center), or a putative *rpfF* mutant of *B. phytofirmans* (right).

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 *Xf* growth, and hence biofilm formation. These results are quite interesting in that it suggests strongly

that *B. phytofirmans* produces a signal molecule to which *Xf* 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 *Xf* 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 *Xf* is likely contributing to the biological control of disease conferred by co-inoculation or pre- or post-inoculation of plants with *B. phytofirmans*. As with DSF itself, increasing the adhesiveness of *Xf* would restrict its ability to move within the plant. Given that the putative signal molecule made by *B. phytofirmans* 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. Experiments are underway to determine the relative importance of such putative signal molecules and possible host-mediated defenses elicited by *B. phytofirmans* in biological control.

CONCLUSIONS

Experimentation is well underway to produce a variety of additional DSF-producing grape varieties. While many of the plants have already been produced 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 in Freedom. Preliminary results using penetrating surfactants to introduce commercially available fatty acids and saponified plant oils capable of inducing signaling in *Xf* and achieving disease control 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 to different formulations and delivery mechanisms. We are particularly excited about the opportunities for biological control of Pierce's disease using the endophytic bacterium *B. phytofirmans*. Not only is this strain the first that we have ever found that readily colonizes grapevines, but we continue to see very dramatically lower disease severity on different grape varieties treated with this bacterium both before or after that of *Xf*. 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

Funding for this project was provided by the CDFR 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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ABSTRACT

A cell density-dependent gene expression system in *Xylella fastidiosa* (*Xf*) 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 *Xf*. The accumulation of DSF attenuates the virulence of *Xf* 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 endoglucanase 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 *Xf*; 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 was designed to test the robustness of disease control by pathogen confusion under field conditions where plants were exposed to realistic conditions in the field, and especially under conditions of natural inoculation with insect vectors. We tested two different lineages of DSF-producing plants, both as own-rooted plants and 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 was observed only near the point of inoculation in transgenic Freedom plants, but spread extensively in wild-type Freedom grape. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless plants grafted onto DSF-producing Freedom rootstocks compared to those grafted onto 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. 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 plants 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 onto wild-type Freedom. The incidence of infection of transgenic Thompson Seedless scions 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 *rpjF*-expressing Freedom grape relative to wild-type Freedom have been seen in continuing evaluations in 2013 through 2016.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor molecule (DSF) which acts to suppress its virulence in plants. Artificially increasing DSF levels in grapevines by introducing the *rpjF* 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 and 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* (*Xf*) 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. Instead, they actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grapevines 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 grapevines as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2. Determine the population size of the pathogen in DSF-producing plants under field conditions.
3. Determine the levels of DSF in transgenic *rpjF*-expressing grapevines under field conditions as a means of determining their susceptibility to Pierce's disease.

RESULTS AND DISCUSSION

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

Field tests are being performed with two different genetic constructs of the *rpjF* gene in grape and assessed in two different plant contexts. The *rpjF* 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 *rpjF* 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 treatments thus being examined in the field trials are presented in **Table 1**.

Table 1. Treatments examined in field trials.

Number	Code	Scion and Rootstock
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 to 8 serve as appropriate controls to allow direct assessment of the effect of DSF expression on disease in own-rooted plants, as well as 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 *Xf* occurs in this plot area, so manual inoculation of the vines with the pathogen was performed by needle-inoculation with a suspension of *Xf*). At least four vines per plant were inoculated. Each inoculation site received a 20 ul droplet of *Xf* containing about 10^6 cells of *Xf*. 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 onto wild-type Freedom. The severity of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson, 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**).

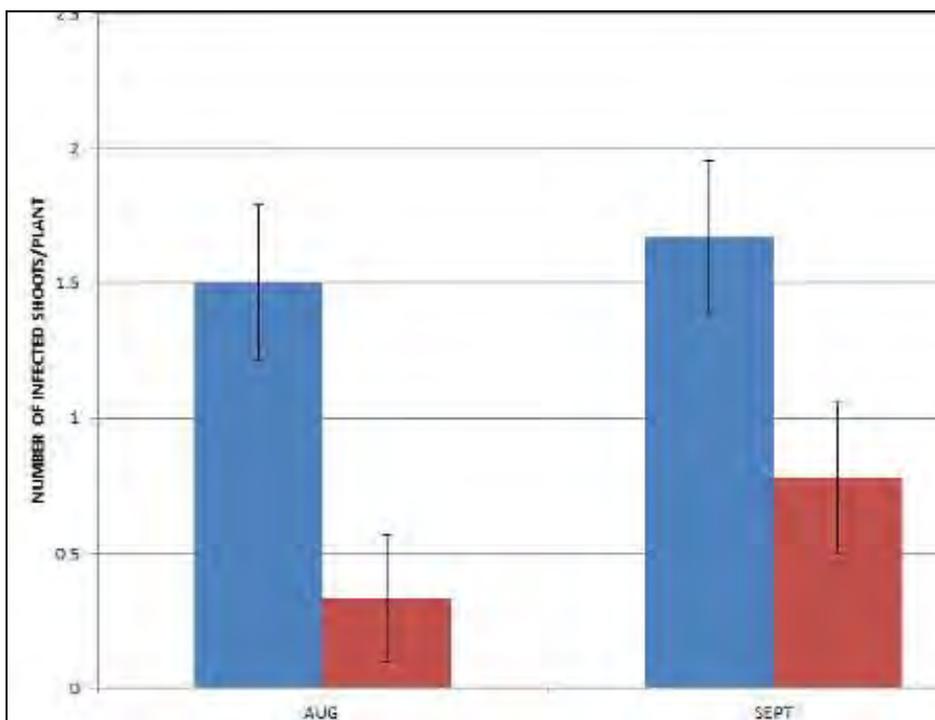


Figure 1. Incidence of vines of wild-type Freedom grape (blue) or DSF-producing transgenic Freedom grape (red) having any symptoms of Pierce's disease when rated in August or September 2012. A total of three vines per plant were assessed. The vertical bars represent the standard error of the mean.

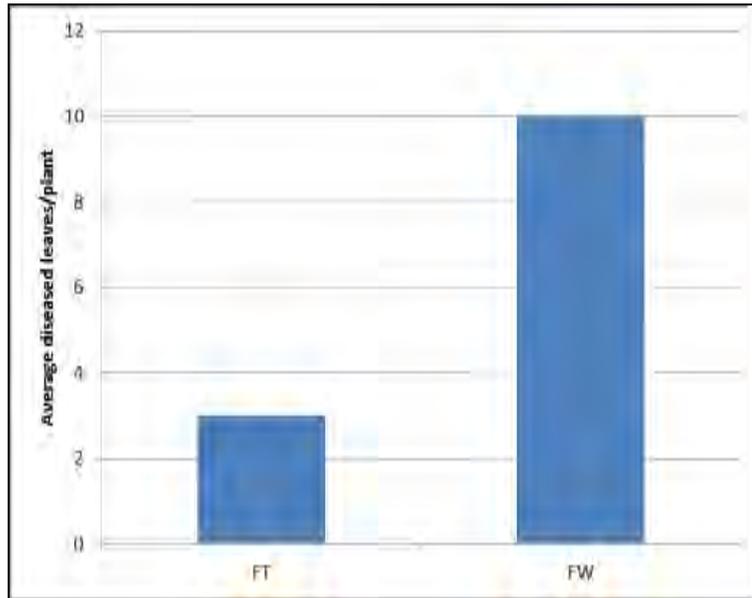


Figure 2. Severity of Pierce’s disease on transgenic Freedom grape (FT) and on wild-type Freedom grape (FW) assessed in August 2012 in the Solano County trial.

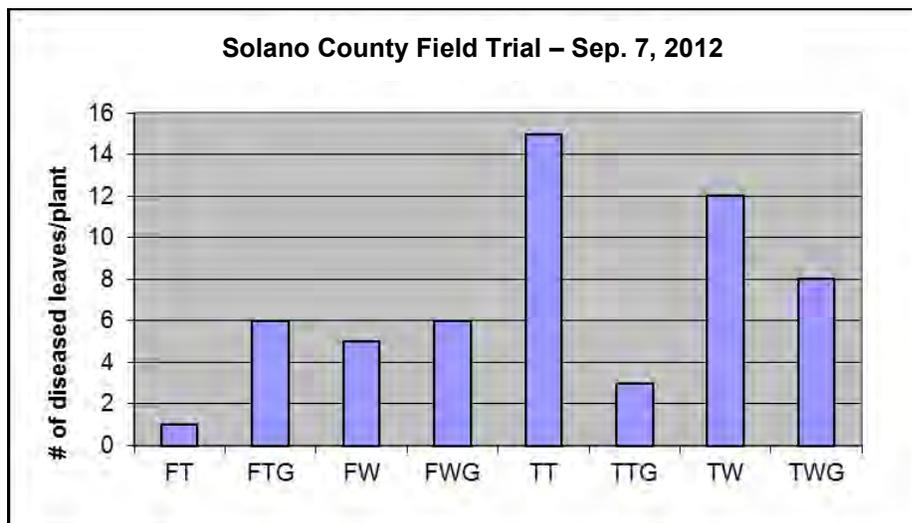


Figure 3. Severity of Pierce’s disease on grape assessed in September 2012 in the Solano County trial. See treatment codes above for treatment comparisons.

The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape (**Figure 4**), while the number of infected leaves per vine was about five-fold less (**Figure 5**), 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 5**). The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless (**Figure 6**), 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 7**). 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 *Xf*, 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 DSF-mediated inhibition of pathogen mobility can be conferred by grafted DSF-producing rootstocks.

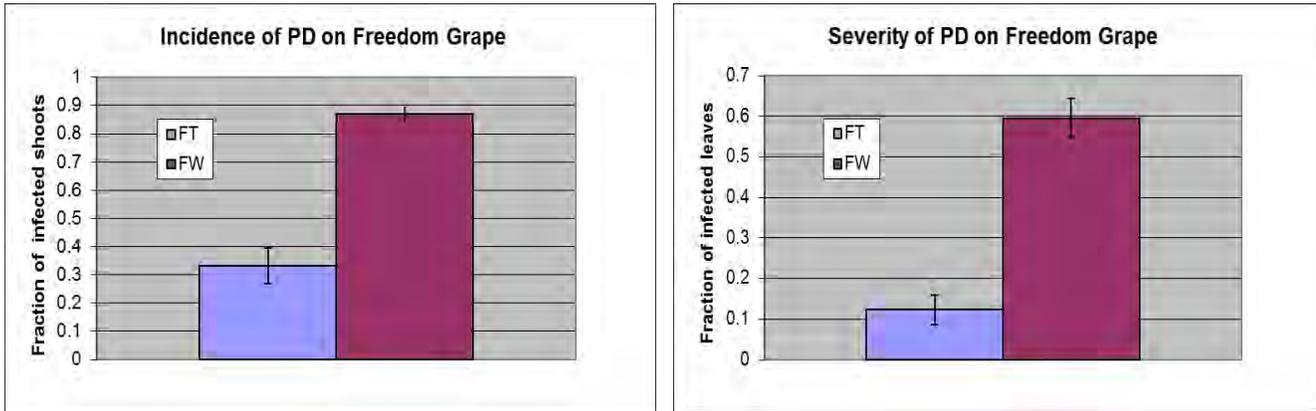


Figure 4. Incidence of Pierce’s disease of transgenic DSF-producing Freedom grape (blue bars) or wild-type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box), or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

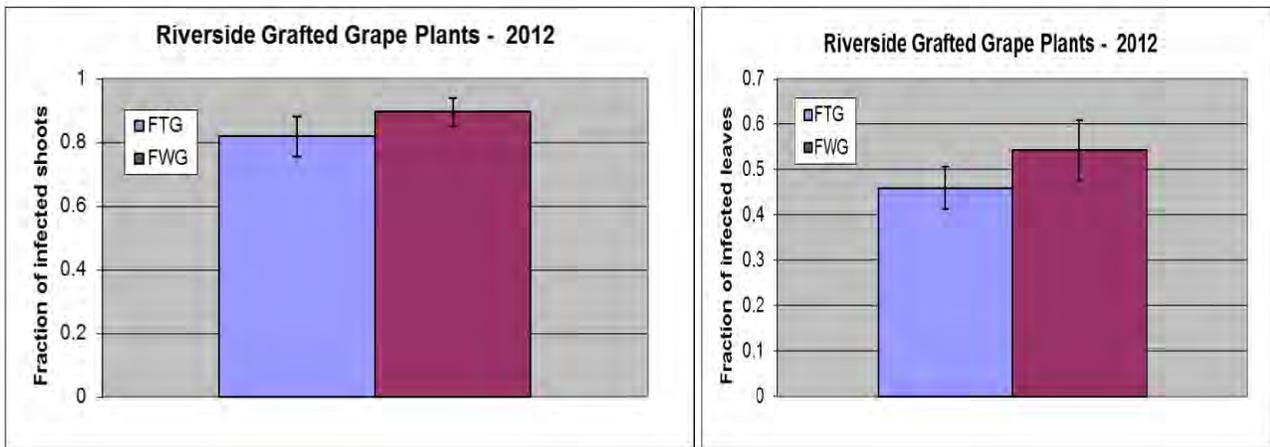


Figure 5. Incidence of Pierce’s disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild-type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box), or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

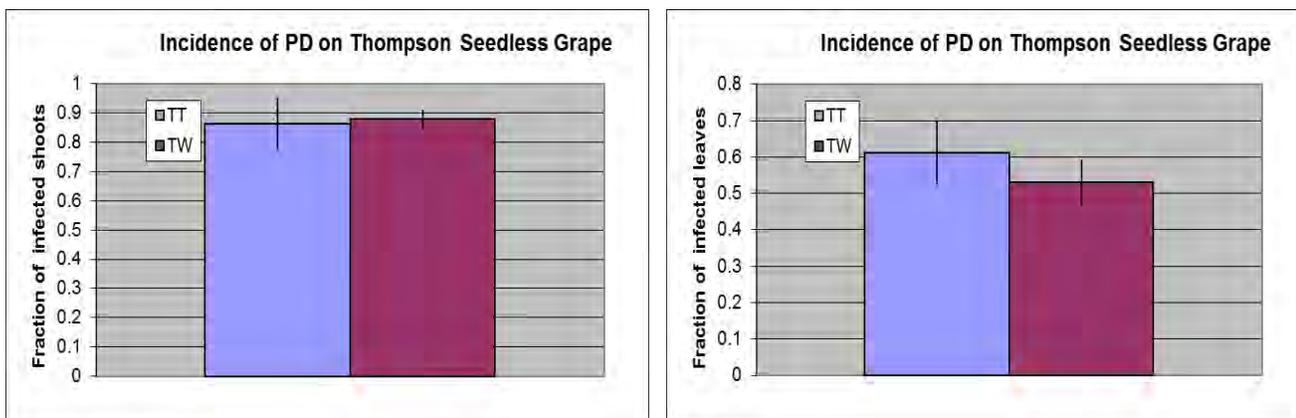


Figure 6. Incidence of Pierce’s disease of transgenic DSF-producing Thompson Seedless grape (blue bars) or wild-type Thompson Seedless (red bars) as measured as the fraction of vines with any disease symptoms (left box), or the severity of disease as measured as the fraction of leaves per shoot that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

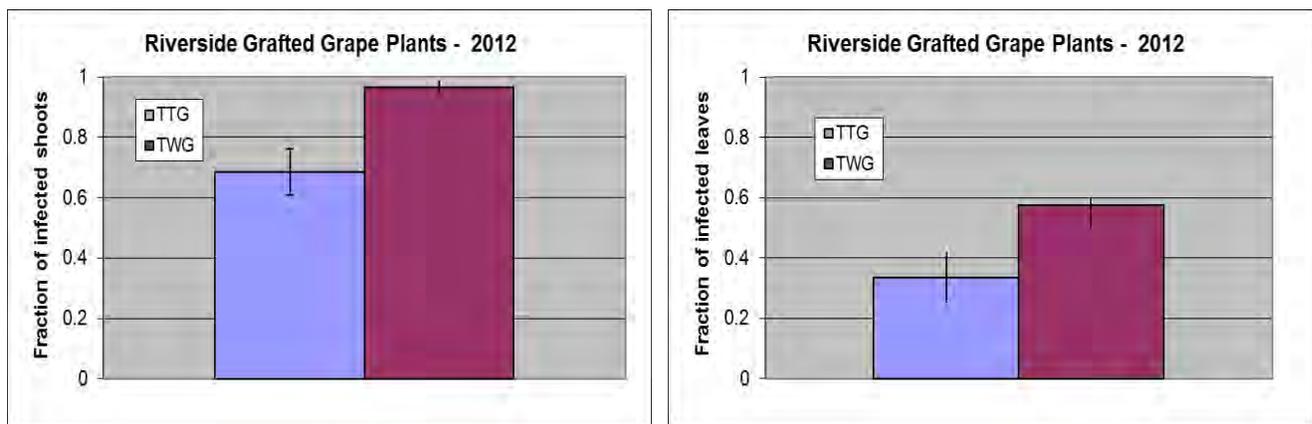


Figure 7. Incidence of Pierce's disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Thompson Seedless grape rootstocks (blue bars) or wild-type Thompson Seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity 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 survival over the winter and 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 site and the plastic marker for each of the inoculated vines. 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 8**). Nearly all of the inoculated vines from both Freedom and transgenic DSF-producing Freedom gave rise to new shoots as of May 2013 (**Figure 8**). 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 9**). 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 9**). 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 8 and 9**). 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 that of scions grafted onto normal Thompson Seedless rootstocks (**Figures 8 and 9**). 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.

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 *Xf* 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 Sept. 15, 2014. In addition, disease incidence and severity that developed in 2014 from vines inoculated in previous years was measured. A uniform rating scale for rating 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 had been infected for more than one year, this new 0 to 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.

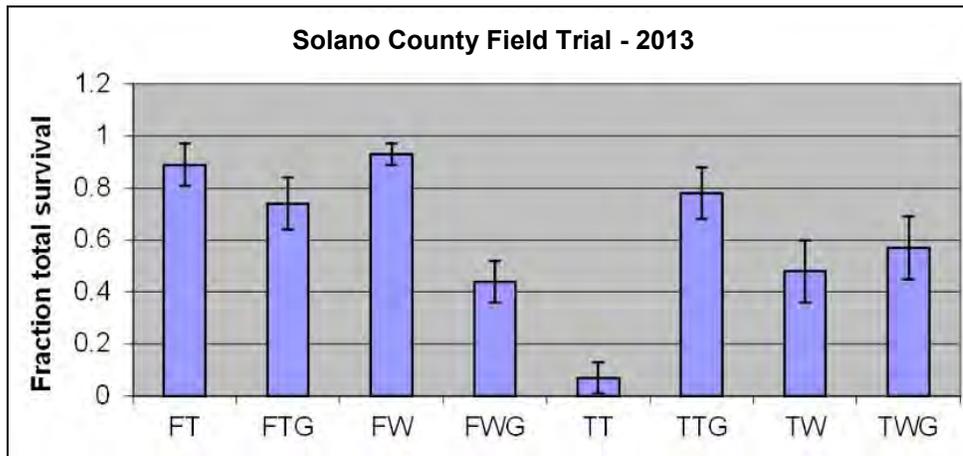


Figure 8. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), 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.

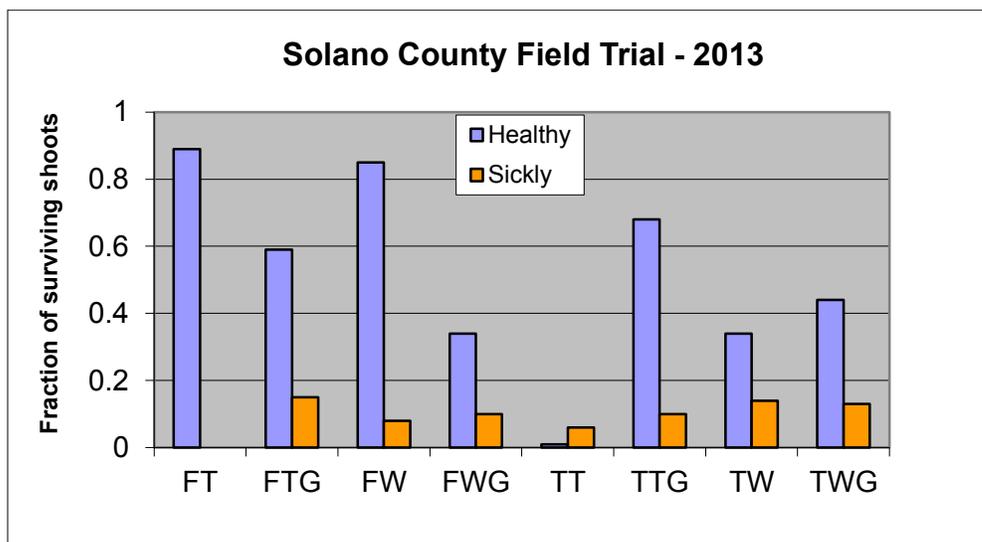


Figure 9. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013 that exhibited some abnormalities possibly indicative of early stages of Pierce's disease infection (orange bars). Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG).

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 10**). The incidence of symptomatic leaves on Thompson Seedless vines grafted onto wild-type Thompson Seedless rootstocks did not differ from Thompson Seedless vines 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 10**). The overall vigor of Thompson Seedless scions grafted onto transgenic Freedom rootstocks was similar to that of Thompson Seedless scions grafted onto wild-type Thompson Seedless rootstocks (**Figure 11**). 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 11**). Thus, some evidence for protection of scions grafted onto RpF-expressing Freedom rootstocks was again seen in 2014 as in earlier years.

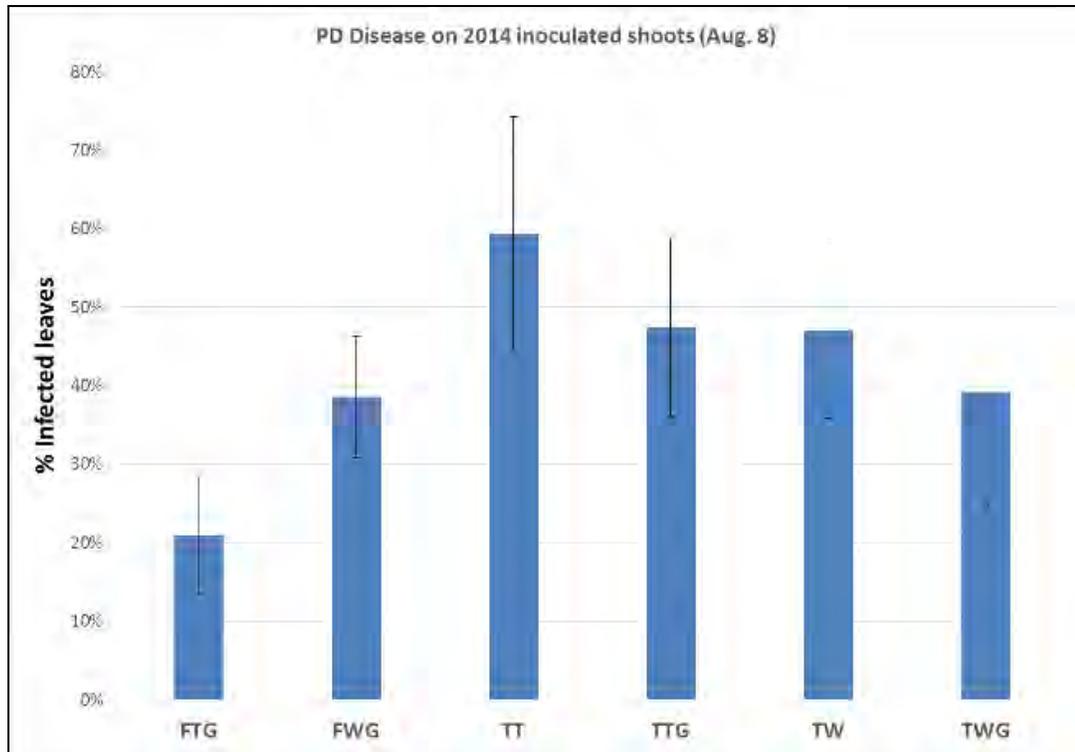


Figure 10. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *Xf* that exhibited symptoms of Pierce’s disease on August 8, 2014. Treatments include Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

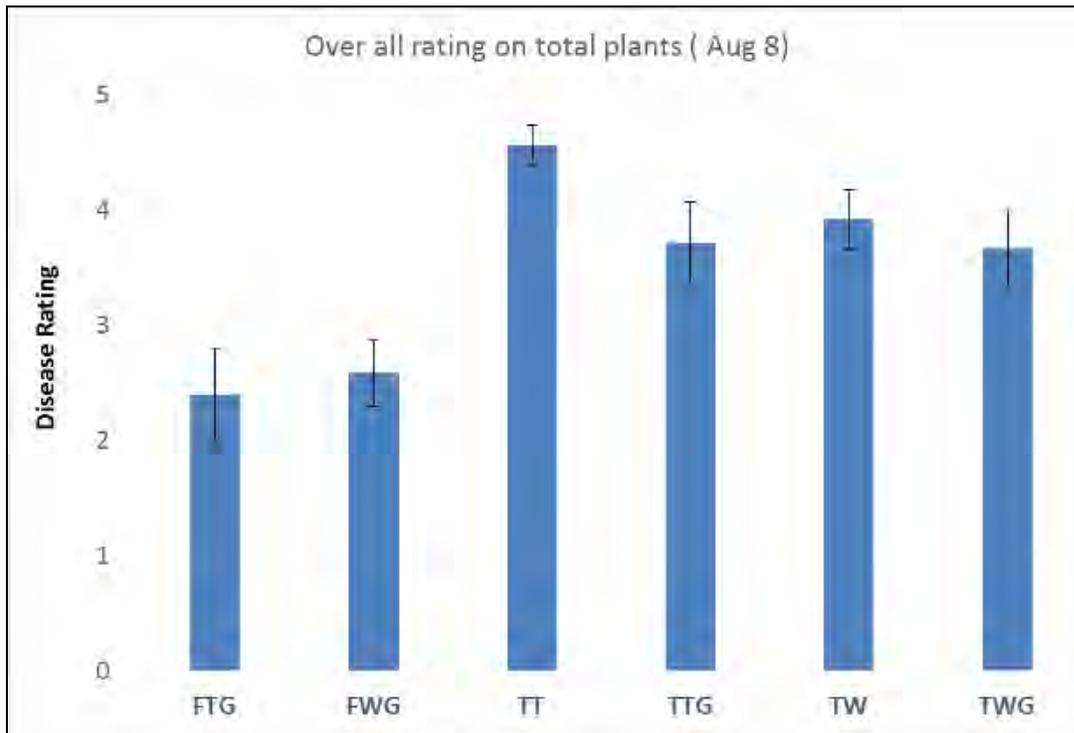


Figure 11. The overall disease rating of vines in the Solano County field trial when assessed on August 8, 2014. Treatments include Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), 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, 2014 from the low levels seen in August 2014. 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 2014 were showing symptoms of Pierce’s disease (**Figure 12**). As observed in the August 2014 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 12**). An assessment was also made in September 2014 of the overall appearance of plants. The disease rating for transgenic Freedom plants was significantly lower than that for wild-type Freedom plants (**Figure 12**). 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 13**). Thus, the transgenic RpfF-expressing Freedom plants continued to show relatively high resistance to Pierce's disease both in the same season that they were inoculated as well as over several years compared to the wild-type Freedom plants.

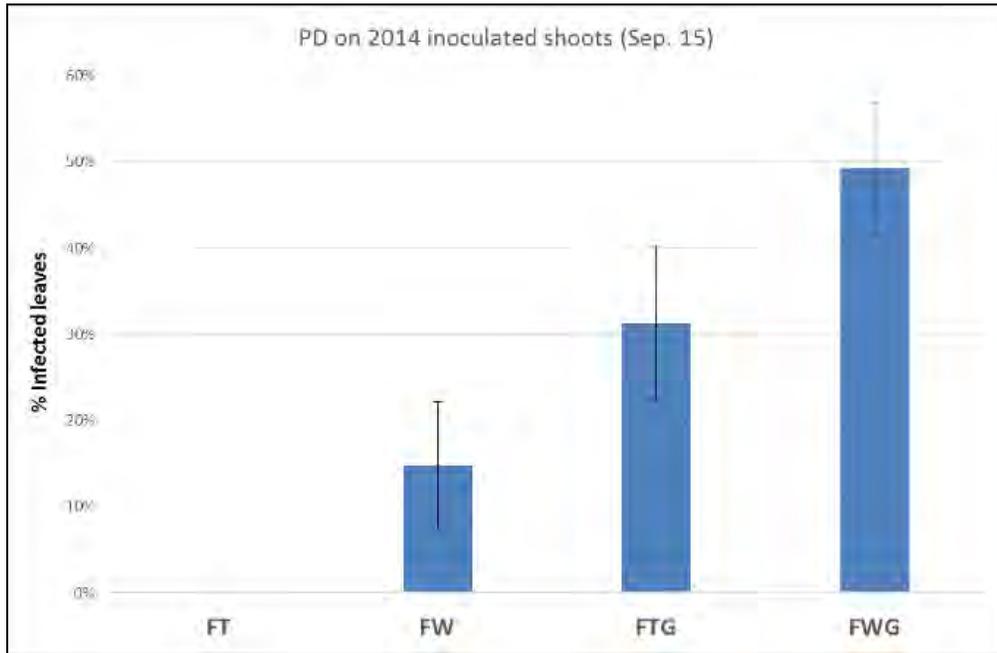


Figure 12. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *Xf* that exhibited symptoms of Pierce’s disease on September 15, 2014. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), wild-type Freedom as an own-rooted plant (FW), 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.

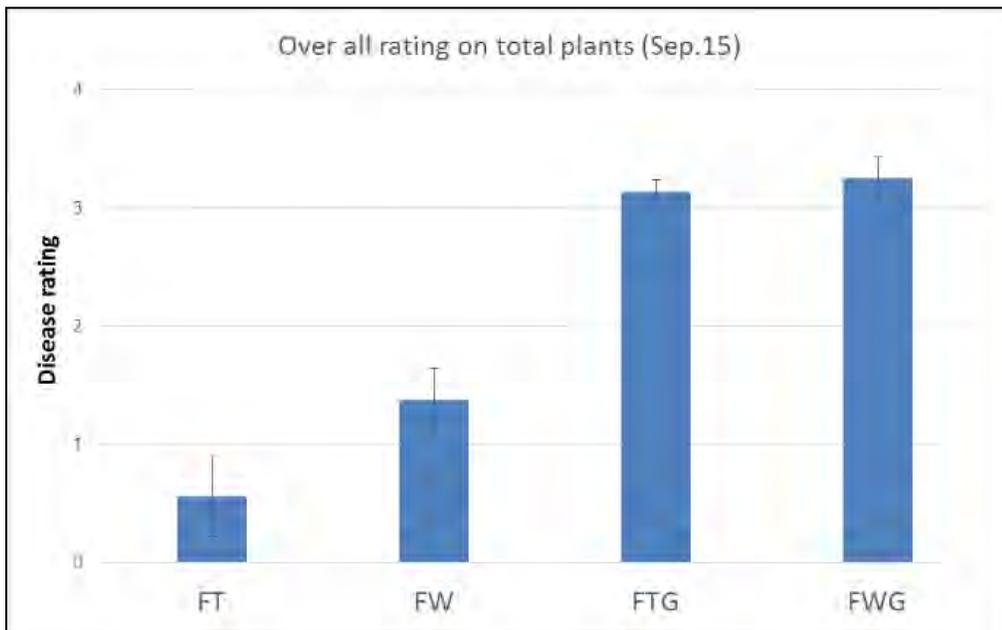


Figure 13. The overall disease rating of vines in the Solano County field trial that exhibited symptoms of Pierce’s disease on September 15, 2014. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), wild-type Freedom as an own-rooted plant (FW), Thompson Seedless scions grafted 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.

Disease was assessed in early October 2014 at the Riverside County field 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 15). 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 (i.e., 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 had been observed in ratings in previous years (Figure 14).

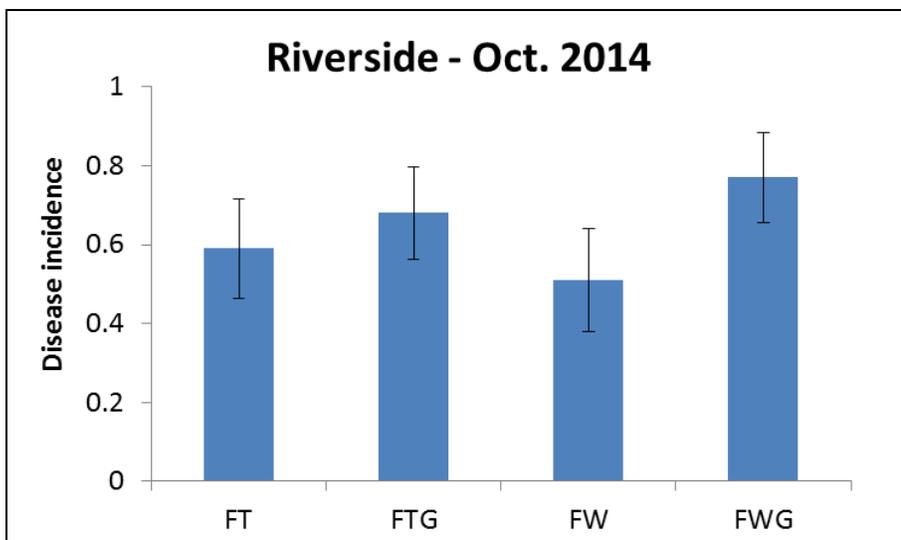


Figure 14. The percentage of vines in the Riverside County field trial that exhibited symptoms of Pierce’s disease on October 6, 2014. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

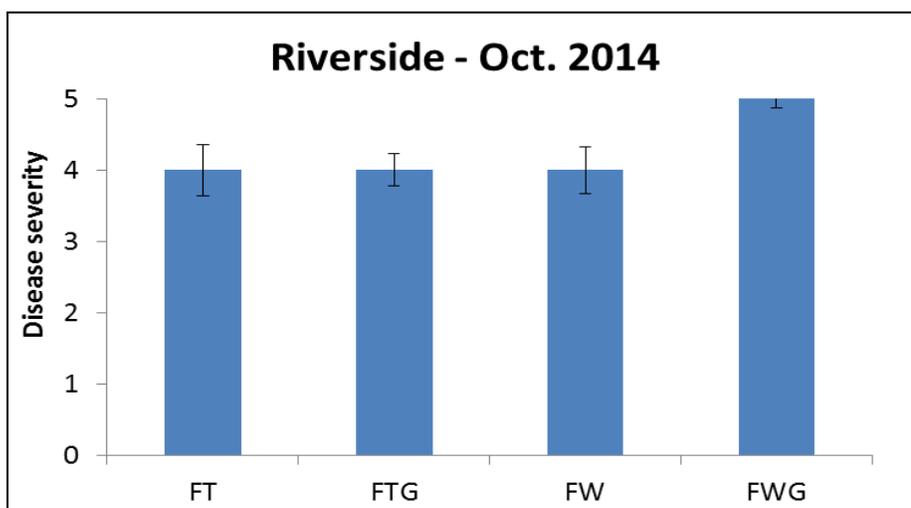


Figure 15. The overall disease rating of vines in the Riverside County field trial that exhibited symptoms of Pierce’s disease on October 6, 2014. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

All plants in the Solano County trial were evaluated for the incidence and severity of Pierce the 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 both 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 plants (**Figure 16**), all wild-type Freedom plants exhibited substantial incidence of the leaf scorching and stunting associated with Pierce's disease (**Figure 17**). Some plants were dead while others remained alive, but with many or most of the cordons being dead or giving rise to only a few leaves or stunted shoots (**Figure 17**).



Figure 16. Images of two separate transgenic Freedom grape plants at the Solano County trial transformed with the *rpfF* gene encoding DSF synthesis from *Xf*. 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 previous years.



Figure 17. 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 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, both the incidence of any disease and the severity of disease between cordons is integrated to yield a range from 0 (no symptoms) to 5 (all cordons and shoots dead). This rating scale was developed for use by all of the participants in the Solano County grapevine field trial and has

been deemed the “PIPRA” (Public Intellectual Property Resource for Agriculture) 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 0 to 5, where 0 indicated plants that were quite vigorous, showing no symptoms and having new growth that was as large as the largest plants in the trial as of May 2015, and 5 indicated that all the plants were dead. We deem this the “happiness” index of the plants. The plants depicted in **Figure 16** all would have received a rating of 0 on the scale, while the plants in the left-hand image of **Figure 17** would have received a rating of 5 and that of the right hand image, a rating of 2. Large quantitative differences were seen in both disease severity and the overall appearance of the wild-type Freedom plants compared to the transgenic DSF-producing Freedom plants at this rating time (**Figure 18**). Whereas no disease symptoms were observed on transgenic Freedom plants, a significantly higher disease rating was observed on the wild-type Freedom plants (**Figure 18**). Likewise, the overall vigor of those plants that still exhibited green tissue was much greater in the case of transgenic Freedom plants compared to the wild-type Freedom plants (**Figure 18**).

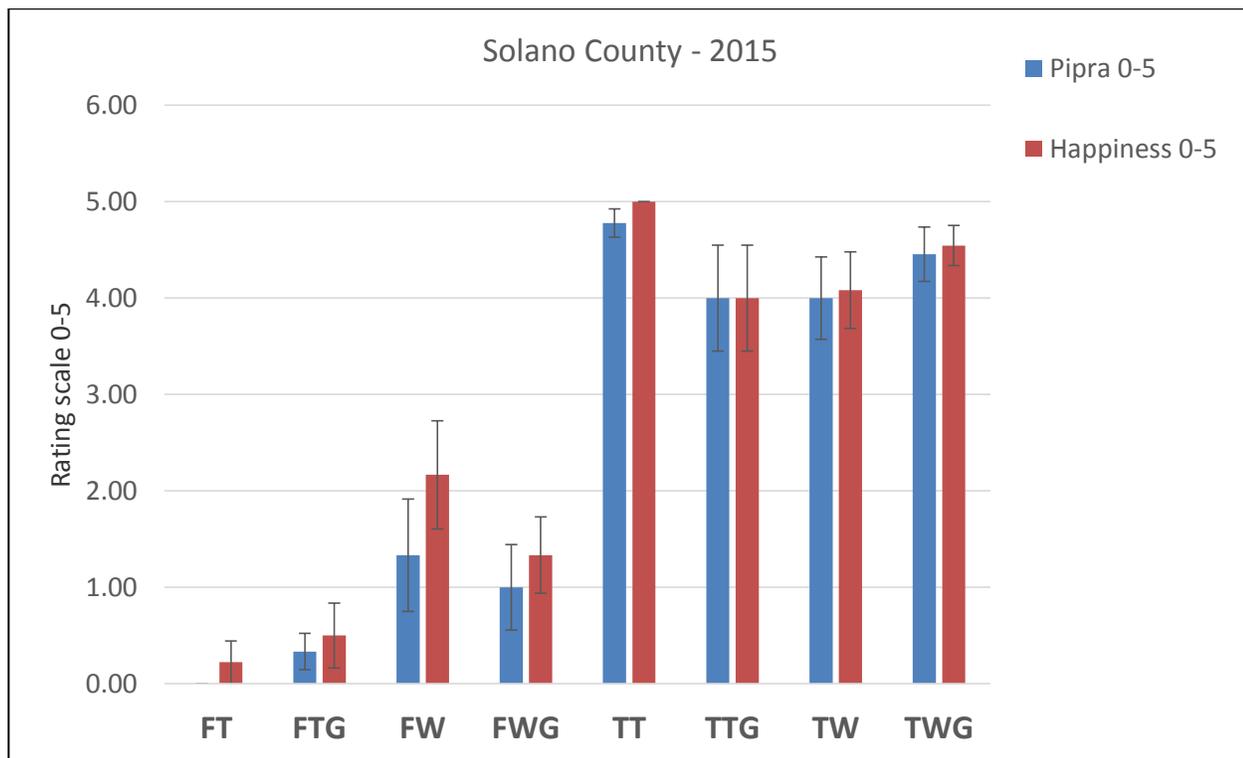


Figure 18. Severity of Pierce’s disease symptoms (blue bars) rated on a scale that accounts for both the incidence and severity of disease between cordons that is integrated to yield a range from 0 (no symptoms) to 5 (all cordons and shoots dead) when rated in May 2015. Also shown is the overall vigor of the plant (red bars) rated from 0 (extremely vigorous) to 5 (dead). Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), 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.

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 18**). 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 18**). 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 18**). 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.

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 and severity of Pierce's disease as assessed using the 0 to 5 scale discussed above was much lower on the transgenic Freedom compared to wild-type Freedom (**Figure 19**), while the incidence of symptomatic leaves on plants inoculated in May were reduced over five-fold compared to untransformed plants (**Figure 20**).

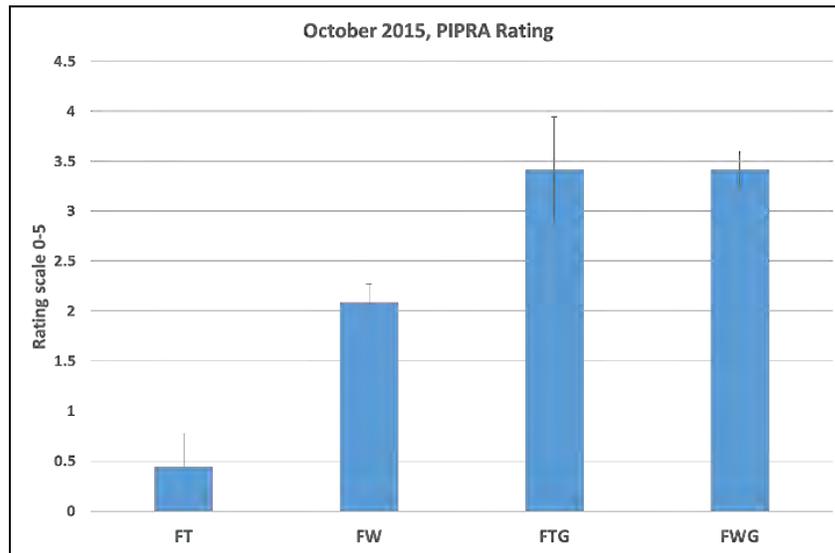


Figure 19. Severity of Pierce's disease symptoms rated on a scale that accounts for both the incidence and severity of disease between cordons that is integrated to yield a range from 0 (no symptoms) to 5 (all cordons and shoots dead). Treatments include transgenic DSF-producing Freedom as an own-rooted 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). The vertical bars represent the standard error of the determination of the mean rating.

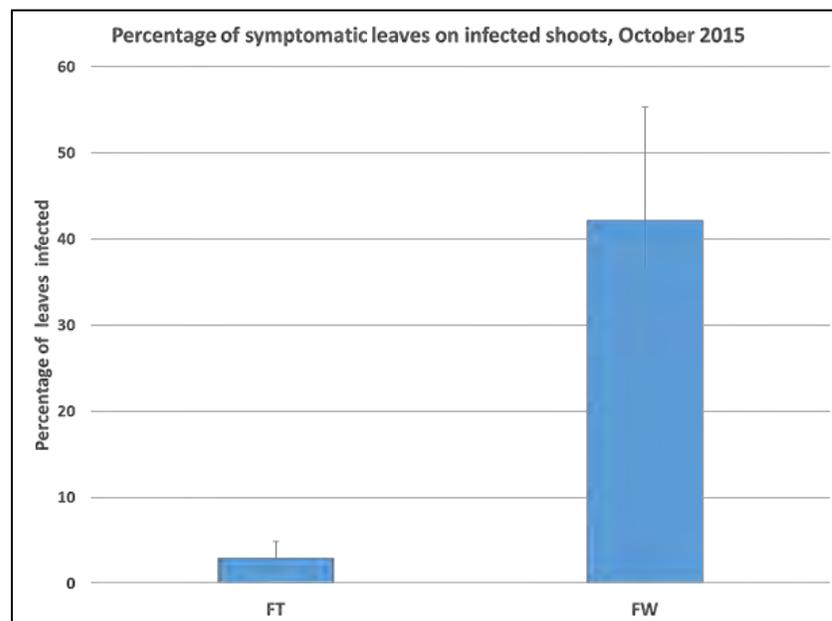


Figure 20. Percentage of symptomatic leaves on inoculated shoots in transgenic DSF-producing Freedom as an own-rooted plant (FT) and wild-type Freedom as an own-rooted plant (FW).

A final rating of the vigor of the plants at the Solano County trial was conducted in late May 2016. Plant vigor was rated on a 0 to 5 scale, with 0 representing a dead plant and 5 representing a thriving, asymptomatic plant. As in previous ratings, transgenic Freedom plants were far more vigorous than those of the non-transformed Freedom plants (**Figure 21**). The vigor of Thompson Seedless grafted onto transgenic Freedom rootstocks did not differ from that grafted onto non-transformed Freedom rootstocks. Thus the expression of DSF in the transgenic Freedom scions continued to provide a high level of disease resistance to Pierce’s disease.

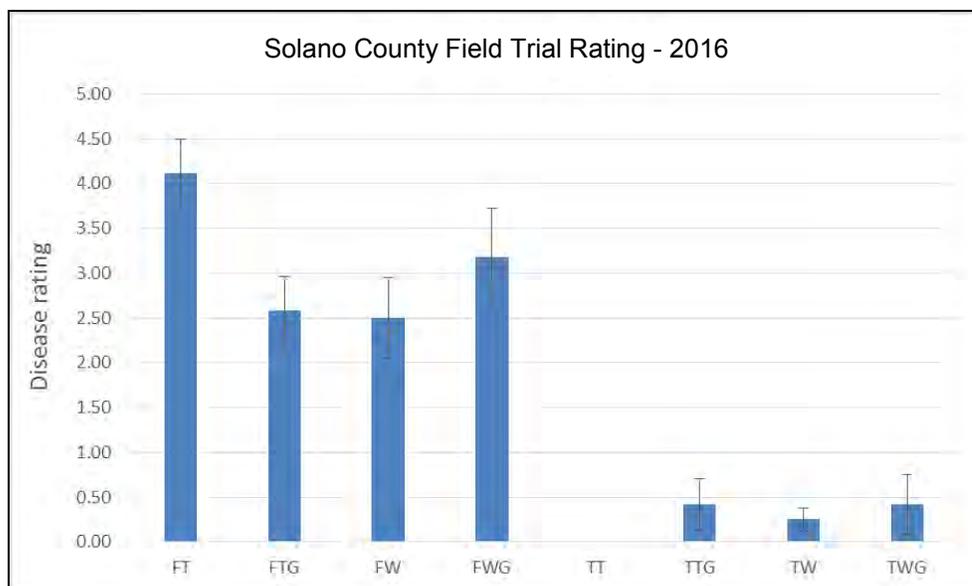


Figure 21. Vigor of plants at the Solano County field trial. Treatments include transgenic DSF-producing Freedom as an own-rooted plant (FT), Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG), wild-type Freedom as an own-rooted plant (FW), Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG), transgenic DSF-producing Thompson Seedless as own-rooted plants (TT), Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG), normal Thompson Seedless as own-rooted plants (TW), 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.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* 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 minimizes symptom development. Results from 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 and especially to plants infected naturally by 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

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

FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE-INHIBITING PROTEINS

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Reporting Period: The results reported here are from work conducted February 1, 2013 to June 30, 2016.

ABSTRACT

The aim of the project was to determine whether introduction of a plant protein that is naturally produced in edible fruit can restrict the spread of *Xylella fastidiosa* (*Xf*) and symptoms of Pierce's disease in grapevines without altering the agricultural attributes of the plants. The Pierce's Disease and Glassy-winged Sharpshooter Research Scientific Advisory Panel had identified, based on previous work, the plant protein polygalacturonase-inhibiting protein (PGIP) naturally expressed in pear fruit (pPGIP) as a promising candidate to consider for advancement towards commercialization. Prior to this project, it was known that *Xf* produces a PG that is inhibited by pPGIP (Agüero et al., 2005) and previous work had also shown that Pierce's disease incidence and symptoms decreased in Thompson Seedless and Chardonnay grapevines if the pPGIP was expressed throughout the vine. The aim of this project was to determine whether pPGIP, when delivered from grafted rootstocks, can control Pierce's disease in the fruit-bearing (scion) parts of the grapevines. Work on this project evaluated the performance of vines grown in two commercial-type vineyards and determined whether their susceptibility to Pierce's disease depended on the pPGIP protein, especially when delivered from pPGIP-expressing rootstocks. 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 to make comparisons between vines producing pPGIP in their grafted rootstocks (transgrafted), those producing pPGIP throughout the vine, and vines with no pPGIP. Once the two vineyards were established with the grafted, transgrafted, and ungrafted vines, an objective of the project was to determine whether sufficient pPGIP that reduces Pierce's disease symptoms is delivered from rootstocks expressing pPGIP to scions, which themselves did not produce pPGIP. Active pPGIP protein that had been produced in transgrafted rootstocks was detected in the xylem exudates that were collected from scions (Agüero et al., 2005; Haroldsen et al., 2012). Vineyards approximating commercial settings were established with own-rooted and transgrafted vines in locations in Solano and Riverside counties with naturally low and high Pierce's disease pressure, respectively; vines in Solano County were mechanically inoculated and disease progress was monitored on known infected vines and at known times after inoculation. Evaluations of performance and susceptibility were made for 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 the commercial development and deployment to reduce Pierce's disease (PD), two test vineyards were established in California. The model PGIP evaluated in this project is produced naturally in pear fruit (pPGIP) and inhibits the PG that *Xylella fastidiosa* (*Xf*) 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 of the same variety expressing pPGIP grafted to fruit-producing non-modified scions which do not themselves produce pPGIP). 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 *Xf*), varietal background (Thompson Seedless vs. Chardonnay), and origin of the pPGIP (delivered from transgrafted rootstock to grafted non-PGIP producing scions vs. plants expressing pPGIP in all parts). Mechanical inoculations with *Xf* bacteria were done yearly from 2011-2015 in Solano County and, beginning with the establishment of the vineyard in Riverside County in June 2013, natural infections were permitted. Data describing the total vine and disease characteristics of the own-rooted or transgrafted vines were collected during growing seasons in both locations. Since this project evaluated grape varieties grown for fresh fruit or wine production in California, we tested varieties important to most California grape growers. The Thompson Seedless and Chardonnay varieties have different growth habits and products and the project provided information for the wine and fresh product sectors of the grape industry. The initial evaluations of the symptoms, performance, and productivity suggest that pPGIP expression in a table grape variety (Thompson Seedless) or a wine grape (Chardonnay) improves resistance of vines to Pierce's disease but does not otherwise affect vine growth or berry characteristics. Eventually however, when the accumulations of inoculations were repeated and allowed to develop, Chardonnay vines benefited more from the introduction of the pPGIP than Thompson Seedless vines.

INTRODUCTION

The project was designed to establish two typical vineyard sites to assess whether polygalacturonase-inhibiting proteins (PGIPs) restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's disease symptoms, and whether expression and/or delivery of the PGIP from pear fruit (pPGIP) impacted the performance and attributes of table and wine grapevines.

This group and others had shown that the expansion of *Xf* from the infection site throughout the vine creates systemic infections that cause Pierce's disease and vine death (Krivanek and Walker, 2005; Labavitch, 2006, 2007; Lin, 2005; Lindow, 2006, 2007a, b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, pectin-rich cell wall "filters" whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2006, 2009a). *Xf* produces cell wall-degrading enzymes to digest the pit membrane polysaccharides (Labavitch et al., 2009b), opening xylem connections and permitting spread of the bacteria.

The *Xf* polygalacturonase (*XfPG*) and several β -1,4-endo-glucanases (EGases) could participate in the digestion of pectin and xyloglucan polymers in pit membranes and, thereby, facilitate Pierce's disease development as *Xf* moves within the vine xylem elements. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of PG and EGase into uninfected grapevines caused pit membrane breakage. Roper et al. (2006, 2007) developed an *XfPG*-deficient *Xf* strain and showed it was unable to cause Pierce's disease symptoms, demonstrating that *XfPG* is a Pierce's disease virulence factor, presumably because it permits *Xf* movement.

The aim of this project is to use plant PGIPs to limit *Xf* spread in grapevines. PGIPs are produced in flowers and edible fruit, are induced by contact with pathogens, and are selective inhibitors of pathogen and pest PGs (Powell et al., 2000; Shackel et al., 2005; Stotz et al., 1993, 1994). Grapevines transformed to express the pPGIP-encoding gene from pear fruit have reduced susceptibility to *Xf* and pPGIP is transported from rootstocks across the graft junction into wild-type scions (Agüero et al., 2005; Haroldsen et al., 2012).

Because the scions do not contain an introduced pPGIP gene, grafting pPGIP-producing rootstocks to non-pPGIP-expressing scions is an opportunity to deliver a beneficial plant fruit protein (i.e., pPGIP) without introducing a pPGIP gene into the part of the plant producing the berries used for produce and wine. This project was designed to generate sufficient numbers of grafted and own-rooted pPGIP-expressing grapevines, plant them in field settings comparable to commercial fields, and evaluate their agronomic performance and their resistance to Pierce's disease due to intentional inoculation or natural modes of transmission.

OBJECTIVES

1. Scale up the number of grafted and own-rooted pPGIP expressing lines.
2. Plant and maintain grafted and own-rooted lines in two locations with different Pierce's disease pressure.
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 *Xf* presence and, if present, 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. DNA was prepared from the vines used as source tissue for grafting and the genotypes were confirmed by PCR (**Figure 1**). Results (see Objectives 3 and 4 below) were that some of the vines over the past three years died due to Pierce’s disease and a few died because of other causes. After the first year, none of the dead vines were replaced. **Table 1** shows the number of grafted and non-grafted vines of each genotype that were planted at the sites by June 2013.

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

Table 1. Plant Inventory. Total numbers of grapevines planted by 2013 in Solano and Riverside counties. The upper portion of the graphic is scion genotype, the lower part of the graphic is rootstock phenotype; nongrafted plants have no break between the upper and lower parts of the graphics. 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



Figure 1. A gel used to genotype by PCR with genomic DNA from grape leaf tissue from Thompson Seedless vines expressing pPGIP and null-transformed (no pPGIP) controls used to generate transgrafted vines. A 1 kb band (arrow) indicating the pPGIP DNA sequence is expected only in samples used as rootstocks for transgrafts and pPGIP self-grafted controls. Each sample’s quality was verified by amplifying a control fragment (not shown).

Objective 2. Establish field trial sites.

Activities. Field trial sites in Solano and Riverside counties were established to assess the Pierce’s disease resistance and general agronomic characteristics of own-rooted and grafted pPGIP-expressing grapevines. The

field plans of the Powell trial plots in Solano and Riverside counties are shown in **Figure 2**. The vines satisfying our initial PCR analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment. The young plants were placed in protective grow tubes and hand-watered every two weeks in Solano County or as needed; natural rainfall accounted for most of the watering. In Riverside County, the plants were watered by drip irrigation. In Riverside, the plot was at the bottom of a small hill and the soil was very sandy and porous; irrigation water accumulated 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.

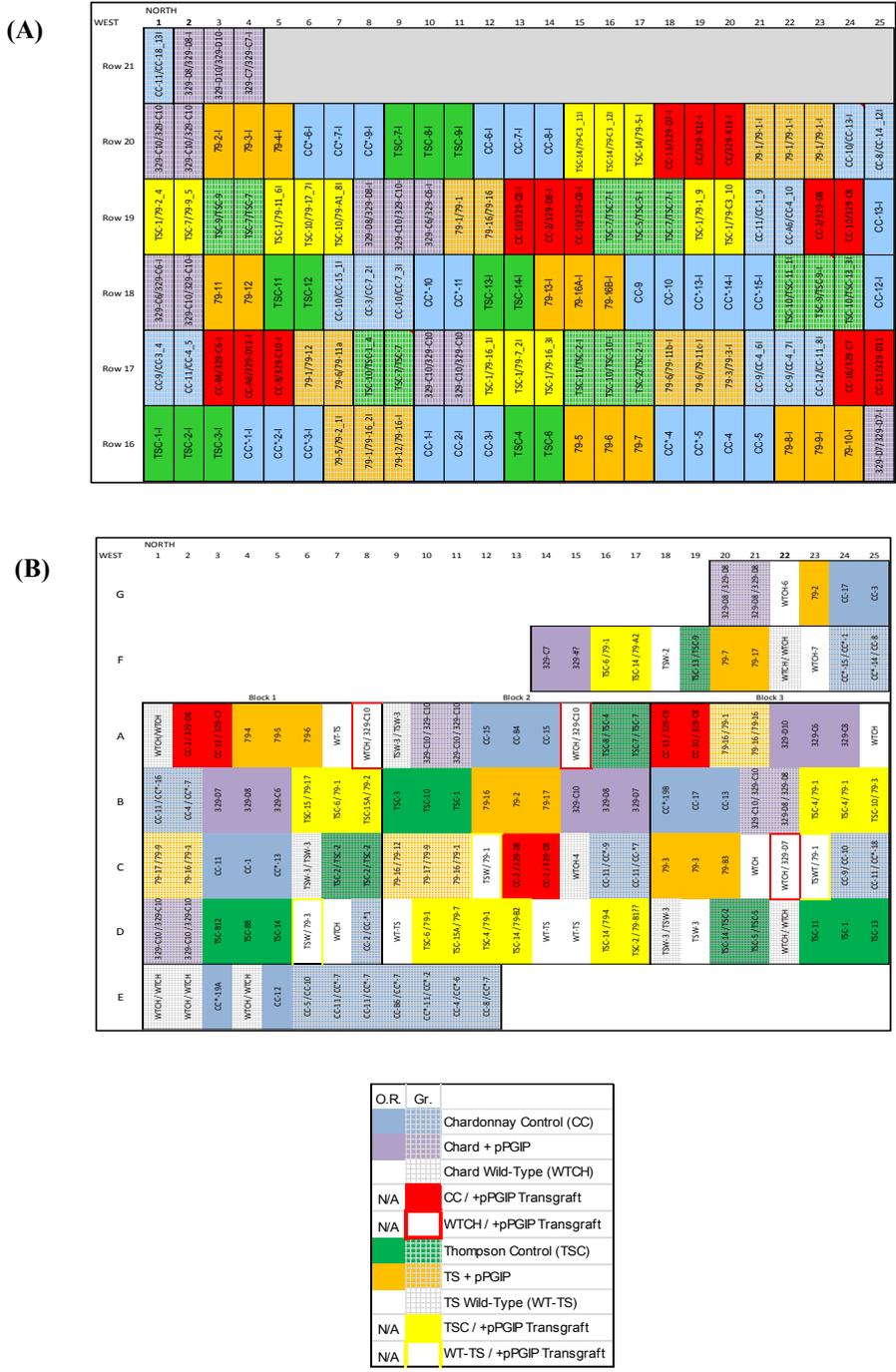


Figure 2. Field plot plans for Solano (A) and Riverside (B) county sites. The color codes of the genotypes are given in the accompanying table; O.R. = own-rooted, Gr. = grafted.

In Solano County, the vines were pruned by the PI and the field crews two to three times per year to maximize potential cane number for inoculations and to establish vigorous positions for future growth. The pruning schedule and method was non-conventional but was done in a manner to try to standardize vine growth in our plots with the practices by the other PIs with plots in the same field, and to be able to preserve the inoculated vines for observations and sampling. With the permit amendment granted by USDA Biotechnology Regulatory Services (BRS) 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 in Solano County. After 2012, pruning did not take into account varietal differences. The vines at the Riverside site were pruned according to the schedule established at UC Riverside and varietal differences were not addressed. The Solano site was observed approximately monthly in the 2014 and 2015 growing seasons and twice in 2016. The vines in Riverside County established themselves well and were monitored by UC Riverside staff and the PI twice during the 2015 season. The activities at both field sites are shown in **Table 2**.

Table 2. Activities at the Solano and Riverside county field sites for this project, through July 30, 2016.

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)
14 March 2016	Solano	Observation of field to project when pruning and assessments can be done (AP UCD)
21 April 2016	Solano	Observation of plants in the field, record dead plants (AP UCD)
27 April 2016	Solano	Confirm observations of plants in the field and record dead plants (AP UCD), field crew prunes plants.

Results. The Riverside and Solano county sites were planted by June 3, 2013 with all the vine combinations planned for this project. A consistent pruning regime was a goal for this plot so comparisons could be made with other evaluators, but pruning was variable. In 2014, thirteen evaluations were made of the plots (10 in Solano and three in Riverside); nine were made by the PI. In 2015, nine evaluations were made of the plots (six in Solano and three in Riverside); eight evaluations were made by the PI. Two evaluations of the Solano field were made in 2016. The vines at the Riverside site were removed in late 2015 because evaluations at that site had been completed and presumed herbicide drift caused unrelated vine symptoms and death.

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

Activities. Other than differences due to the variety (Chardonnay or Thompson Seedless), 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. In 2016 little sign of growth was evident on the vines on March 14, probably due to heavy rain and cool weather. The fields were saturated due to heavy rains and no weeding had been done between rows so the PI was unable to walk the field. The vines had not been pruned as of mid-March 2016. Observations of the vines were made April 21 and 27, 2016 (**Table 3**).

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. 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 site and was monitored for each infected vine during the 2015 growing season and was repeated in April 2016 (**Table 3**).

Agronomic traits such as grape cluster size, berry size, and berry and seed phenotypes were measured at the Solano site in the summer of 2013 but were not repeated. No consistent changes were observed; observations were made only for one year and are therefore not significant. On August 29, 2013, 25 berries total were collected from three plants of each own-rooted genotype and inoculation state at the Solano site; grafted plants were too juvenile to bear fruit in 2013 and were not sampled. Sample collection was randomized by choosing five berries spread across one to two clusters per plant. Clusters were chosen from inside the fruiting zone on each plant. Berries were crushed by hand and the free-run juice was combined with juice pressed from the solids, strained through cheesecloth. Sediments were precipitated overnight at 4°C and clarified juice was sampled for pH and °Brix. Soluble solids ranged from 21.7-24.4 °BRIX and pH values were 3.56-4.00. A smaller subsample was repeated on September 4, 2013 with similar results. After one week, total cluster numbers were counted and one cluster was harvested per plant. Some inoculated own-rooted vines did not bear fruit; grafted plants, with one exception, were fruitless in 2013. Cluster weight, length, and peduncle length were measured upon returning to the lab. Twenty-five berries were removed from each cluster for further analysis after counting the total number of healthy and raisined berries per cluster. Assessments of the subsamples included the weight of 25 berries, retention of pedicels, number and class of seeds (trace, rudimentary, or mature), dimensions of five berries, soluble solids, titratable acidity, and the pH of juice. Each cluster and five individual berries were photographed for assessment of cluster density and berry color and shape.

The Riverside site was visited in late summer 2014. Plant phenotypes were recorded and photographs taken. The PI visited the Riverside site on June 2, 2015 and rescored the vines for phenotypes, Pierce's disease damage, and herbicide damage. Herbicide damage was independently assessed by Peggy Mauk and Philippe Rolshausen at the Riverside site on June 17, 2015 (**Table 4**).

Results. By the end of the 2015 season, it is clear that some vines had died in the Solano County plot. **Table 3** shows the number of dead vines of each genotypes as determined in 2014, four times in 2015, and once in 2016. It is clear that the number of dead vines increased from the 2015 season through the late spring of 2016, possibly due to stress caused by 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 caused by infections with *Xf* under these stress conditions. The data 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 or were missing.

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 plot were compromised by the herbicide drift, it was decided in late June 2015 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 versus the herbicide exposure.

Table 3. Observations of vine death at the Solano County plot from late 2014 through the 2016 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.

Genotype	Total infected plants	Total uninfected plants	late 2014		25-Mar-15		27-May-15		7-Aug-15		7-Oct-15		21 April 16	
			Infect-ed	Not infect-ed	Infect-ed	Not infect-ed								
CC	17	8	6	0	6	0	7	0	10	0	10	1	11	0
CC/CC	9	4	2	0	2	0	2	0	3	0	7	0	7	0
329	0	2	0	0	0	0	0	0	0	0	0	0	0	0
329/329	9	4	3	0	3	0	3	0	3	0	3	0	3	0
CC/329	11	4	1	1	2	1	2	1	3	1	3	1	3	2
TSC	8	4	2	0	4	0	4	0	7	0	8	0	8	0
TSC/TSC	9	4	0	0	2	0	2	0	5	0	8	0	8	0
79	9	5	3	0	3	0	3	0	3	0	4	0	4	0
79/79	7	4	2	0	2	0	2	0	3	0	6	0	6	0
TSC/79	10	3	1	0	2	0	2	0	5	0	8	0	8	0

Table 4. Observations of herbicide damage and vine death at the Riverside County plot on June 2, 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

Images of the vines at the Solano and Riverside county sites are provided in **Figure 3**.

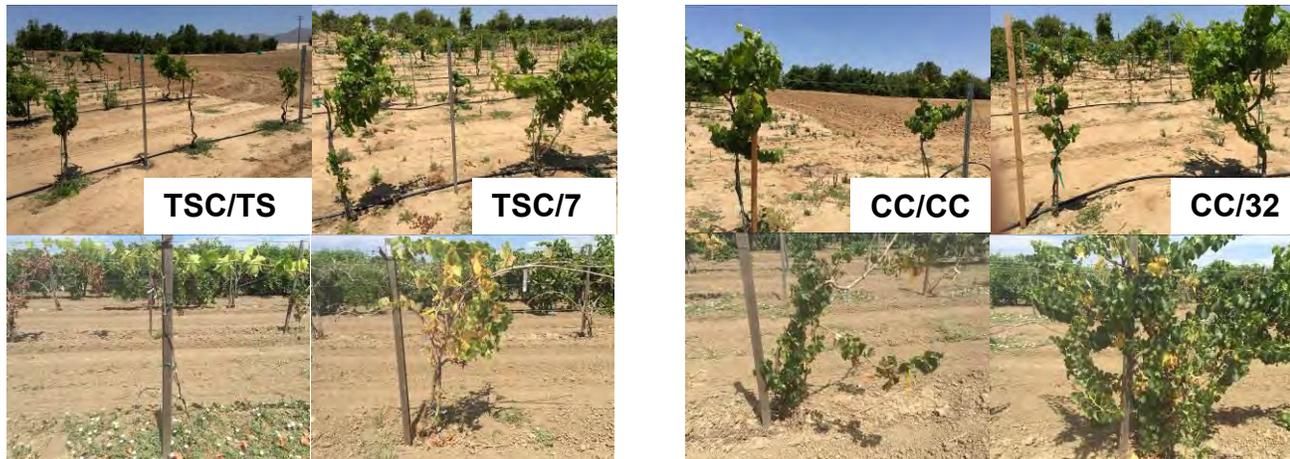


Figure 3. Examples of vines in the Riverside (top row, June 2, 2015) and Solano (bottom row, October 7, 2015) plots of Chardonnay and Thompson Seedless. The genotypes of the grafted or transgrafted vines are indicated.

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

Activities. At the Solano County plot, after a few test inoculations in 2011, 34 own-rooted vines were given mechanical inoculations on May 29, 2012 with a mixture of *Xf* Temecula and Stags’ Leap strains (3:2, v:v). Young, green tissue was chosen for inoculation with three to four canes selected per plant. In 2013-2015, mechanical inoculations were performed as in 2011 except that approximately 1.5×10^7 cells were used per inoculation. The inoculations in 2013, 2104, and 2015 were done only on grafted and transgrafted vines, although phenotype observations were made on all inoculated vines. Inoculated vines were identified by colored tags denoting the times of inoculations. Inoculations in this PI’s plot were performed simultaneously with the other field site collaborators.

The leaves/petioles with evidence of Pierce’s disease symptoms were counted twice during the 2013 season and assessments were made again in the 2015 (data not shown) season, including on canes which had been infected in 2011, 2012, and 2013 (**Table 5**). The grafted and transgrafted vines at the Solano County site were reinoculated along with the vines in the plots of the other PIs on May 28, 2014 and May 27, 2015. Up to four canes per vine were inoculated as before, with inoculum provided by D. Gilchrist. 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.

Infected cane material was twice collected during the summer of 2015, approximately when other groups collected their samples. Tissue collected in the summer of 2014 was hand-ground and frozen at -80°C . The Powell group received separate funds to purchase a GenoGrinder, similar to equipment used by the Golino group. The Powell group worked on protocols to effectively grind the frozen infected stem tissue until the machine sustained damage. Approximately six weeks were needed for repairs to be made. The group tried subsequently for several weeks to refine protocols for macerating the tissue using the machine for PCR analysis but protocols were unsuccessful.

The data analyzing the relationship between the genotypes and the appearance of dead vines were preliminarily analyzed by plotting (**Figure 4**). Examples of the photo evidence of vine phenotypes are shown in **Figure 3**. The data demonstrate that vine death increased in late 2015 and continued in spring 2016, and fewer Chardonnay lines expressing pPGIP either throughout the plant or in grafted rootstocks were dead.

At the Riverside site, vine vigor was analyzed for evidence of Pierce’s disease 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. To obtain the data for the visual assessments of disease throughout the vines, in October 2014 evaluators PR and AP used the same general assessment scale going from 0 (no disease) to 5 (dead) to assess 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 analyses of the results are given in **Figure 5**. 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 in Riverside are shown in **Figure 3**.

Results. In general, the expression of pPGIP in the scion, the rootstock, or both did not impact the overall phenotype of the plant, but infected plants without pPGIP were more likely to die, especially Chardonnay variety vines in the Solano County site, by the 2015 season than those plants with pPGIP.

In Solano County, initial analyses by PCR showed *Xf* DNA only in inoculated plants and less *Xf* DNA was detected in plants expressing pPGIP (**Figure 6**). In order to monitor earlier stages of disease development, the number of leaves or petioles along canes infected in 2013 and earlier was measured and found to be greater when assessed in the spring than in the summer of 2013 (**Table 5**). The observations of disease development along leaves and petioles was repeated in 2014. These results indicated that disease was developing in these canes. However, leaf and petiole disease symptoms developed more slowly in vines with pPGIP in the scion portion, especially in the Chardonnay variety. Notably vines with pPGIP in the rootstocks 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 controls, vines that had been grafted using material that had been transformed with the empty vector construct. Subsequent analysis of the infected vines demonstrated disease progression leading to vine death especially over the summer of 2015, leading to the conclusion that pPGIP expression provided reduced disease development and ultimately less vine death. The effect was clearly due to infection with *Xf* as only two uninfected plants had died by April 2016. It is possible that the severe drought heightened the vine-killing effects of disease. The deleterious effects of *Xf* infections were much more pronounced on the Thompson Seedless variety than the Chardonnay variety; by the end of the 2015 season, nearly 100% of the infected Thompson Seedless vines at the Solano site were dead. In both varieties, vines with rootstocks expressing pPGIP early in the season were the least likely to die compared to varieties expressing pPGIP throughout the vine, but by the end of the projects plants expressing pPGIP in the scion and the rootstock or only in the rootstock were about equally likely to die. 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 succumb to Pierce's disease due to repeated *Xf* inoculations, even when pPGIP is expressed. pPGIP expression seems to offer more protection to the Chardonnay than to the Thompson Seedless variety. The plants were reanalyzed during the 2016 growing season once the fields were accessible to see if any parts of the plants could recover from the disease and regrow but no growth was observed.

The disease scoring analyses done by PR and AP at the Riverside County site in 2014 produced approximately equivalent scores. Analysis of the counted 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 with pPGIP grown at the Riverside County site and infected naturally showed a slightly more positive effect than the Chardonnay variety.

Table 5. Observations of Pierce’s disease damage and vine responses at the Solano site in late April (spring) and late August (summer) 2013.

Genotype		Plants (#)	% Plants with Excessive Base Growth		% Plants with Marginal Leaf Necrosis on Inoculated Canes		% Plants with Marginal Leaf Necrosis on Un-inoculated Canes		% Plants with Atypical Berry Clusters (partial, aborted, or absent)	
			Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
Inoculated Thompson+pPGIP	79-I	9	77.8 (7/9)	66.7 (6/9)	0 (0/9)	33.3 (3/9)	0 (0/9)	11.1 (1/9)	-	44.4 (4/9)
Thompson+pPGIP	79	5	0 (0/5)	0 (0/5)	-	-	0 (0/5)	0 (0/5)	-	20 (1/5)
Inoculated Thompson	TSC-I	8	25 (2/8)	100 (8/8)	0 (0/8)	12.5 (1/8)	0 (0/8)	0 (0/8)	-	75 (6/8)
Thompson Control	TSC	4	0 (0/4)	50 (2/4)	-	-	0 (0/4)	0 (0/4)	-	0 (0/4)
Inoculated Chardonnay	CC-I	17	17.7 (3/17)	82.4 (14/17)	0 (0/17)	11.8 (2/17)	0 (0/17)	0 (0/17)	-	58.8 (10/17)
Chardonnay Control	CC	8	0 (0/8)	37.5 (3/8)	-	-	0	0	-	25 (2/8)

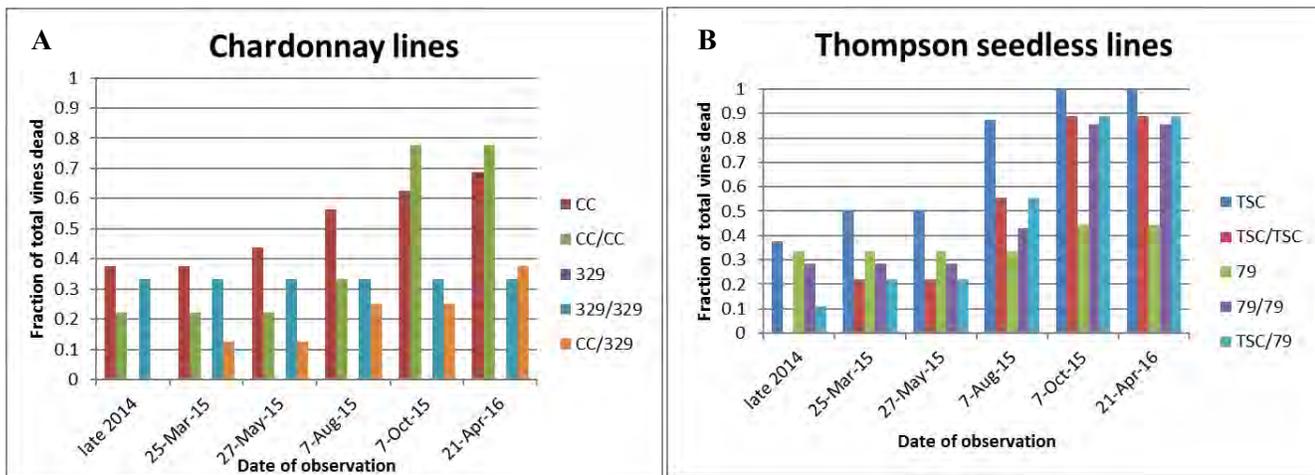


Figure 4. Vine death incidence in Solano County plot of Chardonnay and Thompson Seedless vines measured in 2014, throughout the 2015 season, and initially in spring 2016. **A.** Chardonnay lines. **B.** Thompson Seedless lines. / denotes grafted plants with the genotypes expressed as scion/rootstock. 329 and 79 genotypes express pPGIP in Chardonnay (CC) or Thompson Seedless (TSC) backgrounds, respectively.

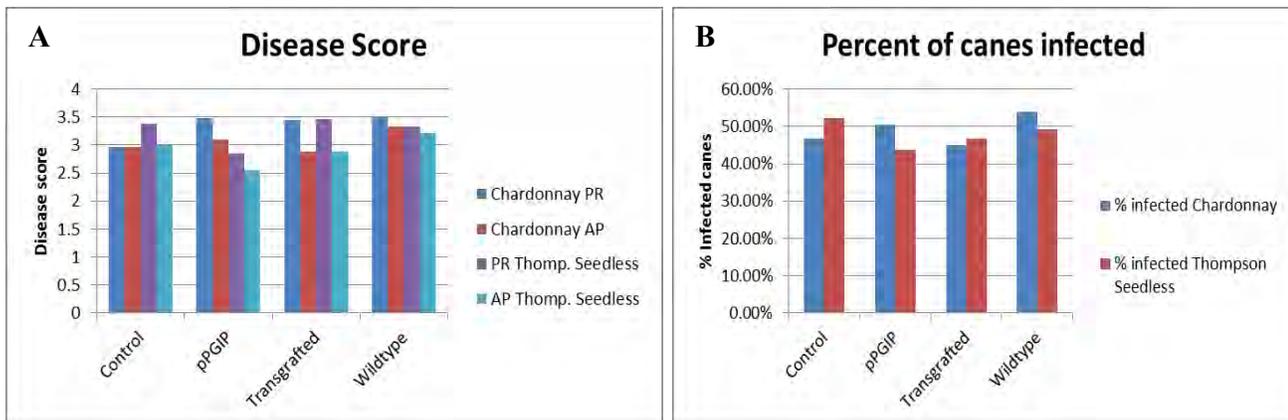


Figure 5. Evidence of disease in Riverside plot of Chardonnay and Thompson Seedless vines measured in October 2014. **A.** Disease score based on 0-5 scale. **B.** Percent of vine canes with symptoms or evidence of Pierce’s disease. PR = data collected by P. Rolshausen, AP = data collected by A. Powell.

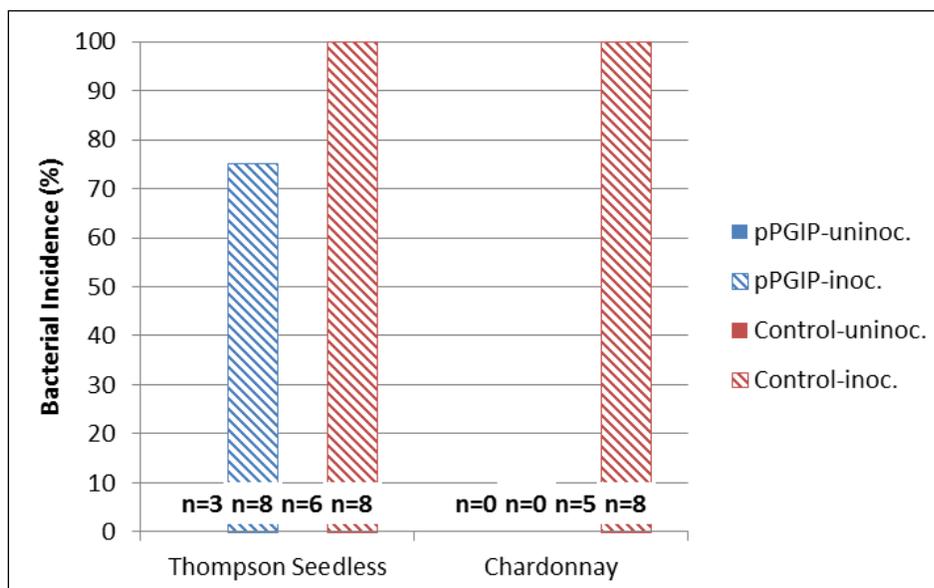


Figure 6. Results of PCR detection of *Xf* DNA sequences in inoculated vines from the Solano County site.

CONCLUSIONS

All of the grafted plants necessary for the studies at both locations were generated, planted, and inoculated with protocols similar to the other groups' procedures at the sites. The genotypes of the grafted plants were confirmed. Initial infections in 2011 of the vines in Solano County produced no visible symptoms for over a year. The second set of inoculations in Year 2 resulted in detectable *Xf* DNA in infected vines in November 2012, and visual symptoms of Pierce's disease in April 2013. Mechanical inoculations with *Xf* bacteria in 2011 and 2012 in Solano County resulted in the accumulation of *Xf* DNA sequences only in the inoculated, but not in the uninoculated, cane material, confirming the identity and history of the inoculations. 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 *Xf*. Inconsistent or atypical pruning schedules have made determinations of similarities of vine phenotype and vigor to commercially-propagated fields challenging. However, the overall performance of the Chardonnay and Thompson Seedless vines in the field seems to be unaffected by the expression of pPGIP either in the scion or the rootstock unless the vines have been inoculated with *Xf*. The evaluations of the leaf and cane phenotypes of infected 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. Only two uninoculated vines died at the Solano site. Based on counting leaves with evidence of scorching, the Chardonnay vines with pPGIP initially had fewer Pierce's disease symptoms than the Thompson Seedless variety when mechanically inoculated. By evaluating varieties grown for fresh fruit and for wine production in California, we provided information about the impacts of pPGIP and its delivery 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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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

The bacterium *Xylella fastidiosa* (*Xf*) is the cause of Pierce's disease in grapes and is a major threat to fruit, nut, olive, and coffee groves. Obvious symptoms are anthocyanin (red pigment) accumulation in leaves and shriveling of undeveloped berries. Studies have determined that anthocyanin compounds can reduce insect feeding. Work by L. De La Fuente showed that *Xf* infection causes significant imbalances in leaf and xylem elemental phosphorus content, but the bioavailable form of phosphorus underlying this phenomenon is unknown. C. Rock has characterized in many dicot species including grape a sugar-, inorganic phosphate (Pi)-, and stress hormone (abscisic acid, ABA) regulatory network controlling expression of microRNA828 (miR828), its targets MYeloBlastosis viral oncogene-like (MYB) transcription factors (a class of regulatory gene found in all animals and plants) and *Trans-Acting-Small RNA locus4* (*TAS4*) that down-regulate anthocyanin biosynthesis by targeting related MYB genes for post-transcriptional gene silencing. This regulon is very likely the mechanism by which grapevine red blotch-associated virus (GRBaV) and leaf roll-associated virus cause symptoms, because their genomes encode small RNA (sRNA) suppressor proteins. We hypothesize that these novel target MYB transcription factors (VvMYBA6/A7 and homologs) in grape are effectors of anthocyanin accumulation and potentially glassy-winged sharpshooter (GWSS) feeding preference determinants important for Pierce's disease etiology, mediated through Pi (a diffusible signal) that modulates miR828 and *TAS4* activities normally to silence target MYB expression. Anthocyanin induction in vegetative tissues may serve as antagonists to feeding by GWSS and to colonization by *Xf*. We are currently testing the *Xf* infection/spread hypothesis directly by "knocking out" the key genes using genome editing technology (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR/Cas9) that the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board nominated as a feasible, high-priority approach to engineering Pierce's disease resistance. This technology can produce non-"genetically modified organism" (GMO) grapevines and rootstocks after outcrossing the transgene locus by breeding methods. The resulting stocks lacking or overexpressing, respectively, the endogenous effector genes will be challenged in the greenhouse with *Xf*, and we will measure Pi in xylem sap and *in planta* to test whether it is involved in Pierce's disease etiology. We will test the Pi analogue phosphite for inhibitory effects on *Xf* growth and host disease development. These proof-in-principle experiments could result in a new paradigm for host-vector-pathogen interactions in Pierce's disease with potential translational benefits for other crops.

INTRODUCTION

Our working model of Pierce's disease etiology postulates miR828 and evolutionarily-related *Trans-Acting Small-interfering locus4* (*TAS4*) activities silence target VvMYBA6/A7 and other homologous MYB expression in response to *Xylella fastidiosa* (*Xf*) infection, mediated through inorganic phosphate (Pi) and plant stress hormone abscisic acid (ABA) signaling crosstalk. We are currently testing the *Xf* infection/spread hypothesis directly by "knocking out" the key hypothesized genes using a new genome editing technology (Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR/Cas9) [1, 2] that the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board nominated as a feasible, high-priority approach to engineering Pierce's disease resistance.

We are also taking a complimentary "overexpression" approach to the long-term grapevine MYB target gene knockout/editing approach to test the anthocyanins-as-*Xf*-effectors hypothesis. The surrogate tobacco *Xf* infection system developed by L. De La Fuente [3] can quickly assess susceptibility to *Xf* infection of a transgenic tobacco line [4] (Myb237) that over-expresses the *Arabidopsis* orthologue of VvMYBA6/A7: *PRODUCTION OF ANTHOCYANIN PIGMENT2/MYB90*.

OBJECTIVES

1. Demonstrate the efficacy of CRISPR/Cas9 transgenic technology for creating deletion mutants in *MIR828*, *TAS4*, and target *MYBA6/7*. When validated, future experiments will critically test these genes' functions in Pierce's disease etiology and *Xf* infection and spreading.
2. Characterize tissue-specific expression patterns of *TAS4* and *MIR828* primary transcripts, sRNAs, and *MYB* targets in response to *Xf* infections in the field, and in the greenhouse for tobacco transgenic plants overexpressing *TAS4* target gene *AtMYB90/PRODUCTION OF ANTHOCYANIN PIGMENT2*.
3. Characterize the changes in (a) xylem sap and leaf Pi, and (b) polyphenolic levels of *Xf*-infected canes and leaves. (c) Test on tobacco in the greenhouse and *Xf* growth in vitro the Pi analogue phosphite as a durable, affordable and environmentally sound protectant/safener for Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Test the *miR828*, *TAS4*, and target *MYBA6/7* functions in Pierce's disease etiology and *Xf* infection and spreading by genome editing using CRISPR/Cas9 transgenic technology.

Engineered binary T-DNA *Agrobacterium* vectors designed to genome edit the grapevine *VvMIR828*, *VvTAS4ab*, and target *VvMYBA6/VvMYBA7*, and *Phytoene Desaturase (PDS)* loci (the latter as an independent test of editing efficiency) were electroporated by C. Rock into EHA105 obtained from Stan Gelvin, Purdue University, and sent to D. Tricoli's lab under USDA APHIS Biotechnology Regulatory Services (BRS) permit # 15-231-102m in November 2015. Three independent transformation cycles for each construct were initiated in November-December 2015, and May 2016. Problems associated with both Thompson Seedless and rootstock 101-14 transformation/re-generation were described in the last progress report. Carrying out the repeat transformation of grapevine could not be initiated sooner because of a lack of sufficient numbers of stock grapevine embryogenic culture starting materials, derived from anthers of immature flowers harvested in the spring. It takes eight months to generate enough embryogenic culture materials for transformations. Ongoing grape transformations in D. Tricoli's pipeline at the Plant Transformation Facility at UC Davis have been responding as expected, discounting any issues with media or selection.

Therefore, as an independent and facile assay of the efficacy of the strain/vectors, D. Tricoli endeavored to troubleshoot the problem by using their lab stock of EHA105 containing an in-house vector to transform tobacco leaf disks in parallel with the CRISPR/Cas9 *MIR828/TAS4/MYBA6/7* vectors in the Gelvin lab-sourced EHA105 host. **Figure 1** shows the results that suggest either the strain of EHA105 used by C. Rock, or some general aspect of the vectors relating to the starting material vector Addgene #59175, p201N_Cas9, without any guide RNA effector locus, is causing the problems with grapevine transformation/regeneration. This interpretation is supported by restriction digestion of the vectors (propagated in *Escherichia coli*) with diagnostic enzymes for the specific engineered vector guide RNA sequences which show the vectors, including p201_N_Cas9 starting materials, are intact (**Figure 2**).

We hypothesize a problem with the *Agrobacterium* strain and therefore are re-electroporating vectors into the D. Tricoli lab strain of EHA105, which will be sent to UC Davis for tobacco transformation as a quick test before initiating another grapevine transformation cycle. We will also perform a Southern blot of T-DNA vectors extracted from the old and new EHA105 strains as a test, and of *Agrobacterium* strain GV2260 containing p201-N-Cas9, which we showed in the last progress report by immunoblot for Cas9 functions properly for effector expression in transient transformation assays in *Nicotiana benthamiana*. These further troubleshooting experiments should shed light on the grapevine and tobacco transformation problem and independently confirm the nature of the problem and whether it has been solved.

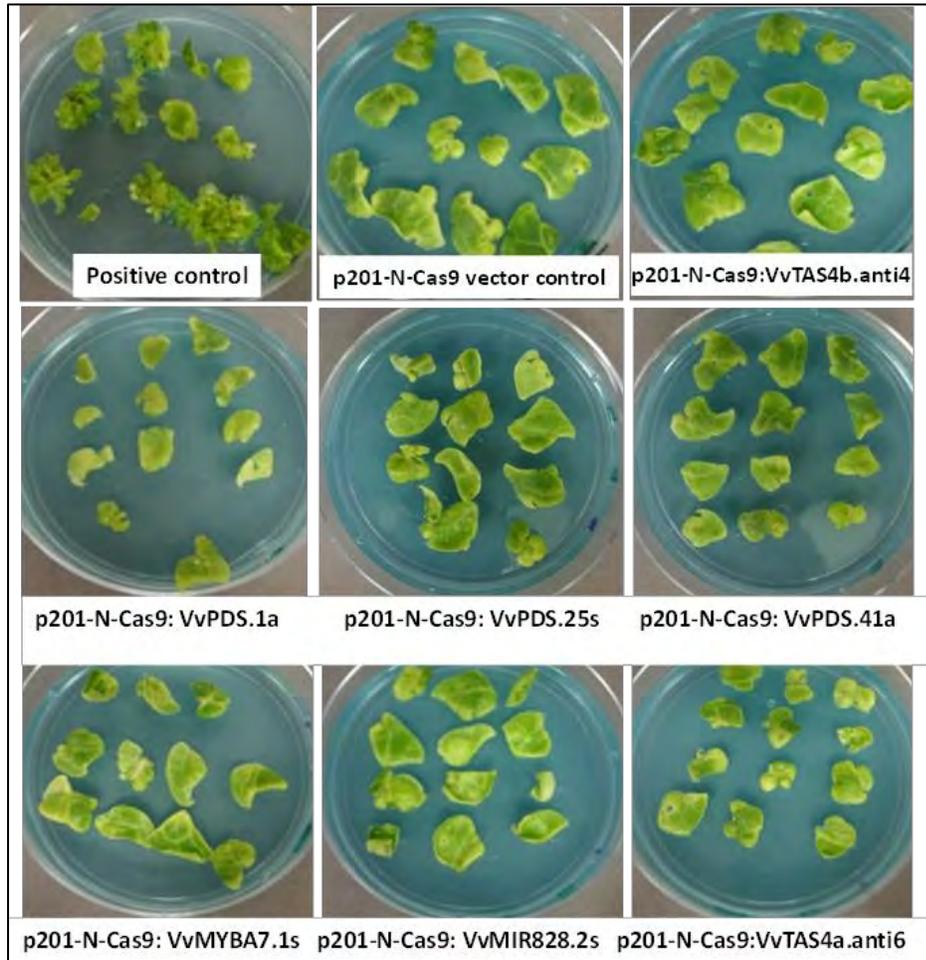


Figure 1. Tobacco transformation test of p201-N-Cas9 vector (Addgene#59175; top middle) in Gelvin-sourced EHA105 *Agrobacterium* strain and derivative constructs, showing some issue with strain and/or p201-N-Cas9 vector. Positive control (top left) used the EHA105 *Agrobacterium* strain routinely in use in the D. Tricoli lab.

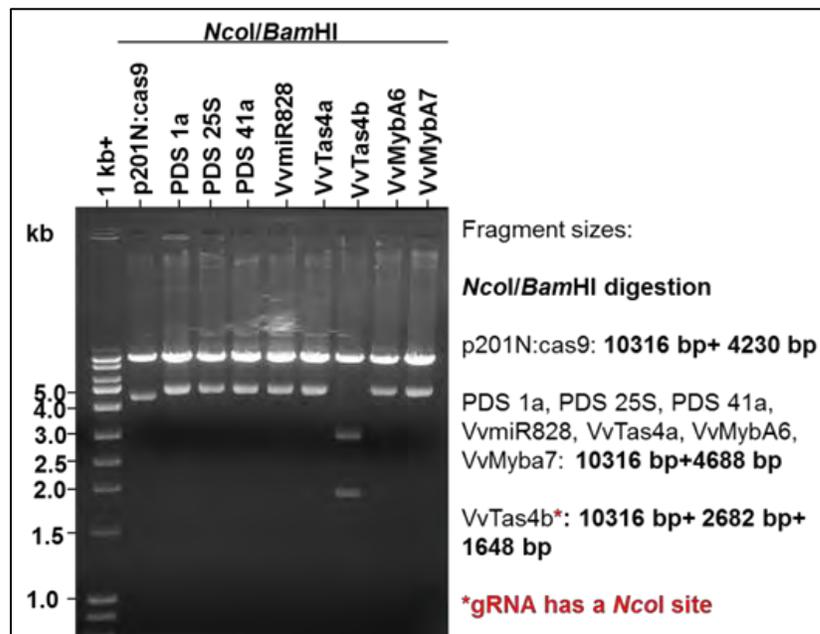


Figure 2. Restriction digestion diagnostic test of vector integrity, showing synthetic guide RNA loci are correct and overall vector size is as predicted when propagated in *E. coli* host.

Validation of editing events going forward will be by polymerase chain reaction (PCR) cloning and sequencing of target genes, and PAGE-based genotyping [5]. **Figure 3** shows the result of a mock editing assay using a known 15 nt deletion of the phytochrome *PHYD-1* gene of *Arabidopsis* ecotype Wassilewskija (Ws-2) [6] which is used to 'dope' tracer amounts of genomic DNA to the bulk wild-type *PHYD* allele from control Co-0 extracts. This allows us to create a 'needle-in-a-haystack' mock experiment for optimizing the genotyping assay which monitors heteroduplex formation and can quantify the limits of detection for editing events (and thus editing activities / efficiencies) in future experiments.

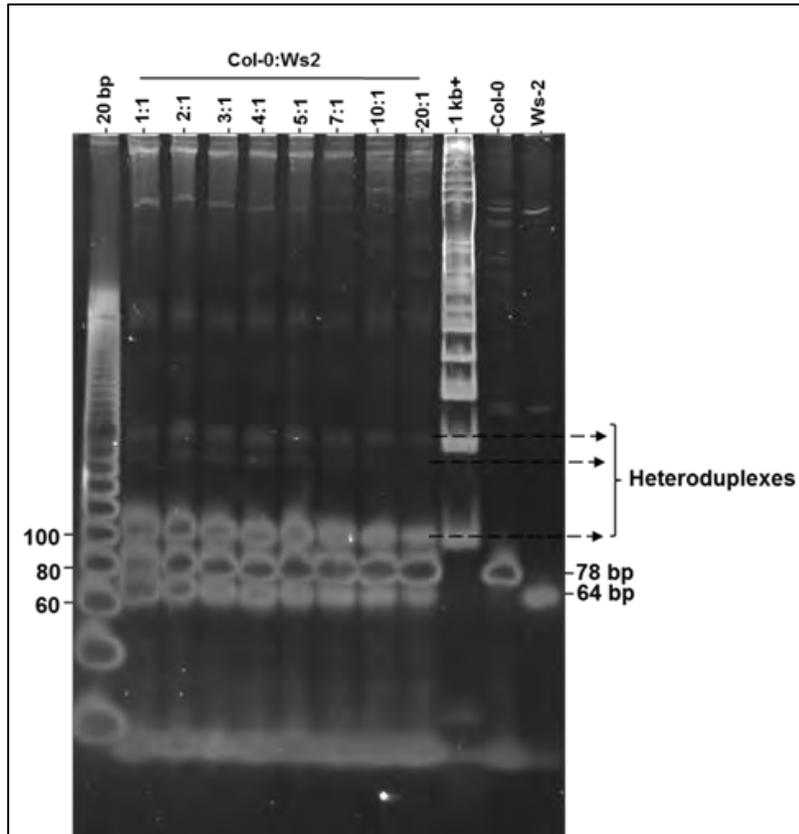


Figure 3. Mock PAGE heteroduplex genotyping assay for quantifying genome editing events.

Objective 2. Characterize tissue-specific expression patterns of *TAS4* and *MIR828* primary transcripts, sRNAs, and *MYB* targets in response to *Xf* infections in the field.

In the previous progress reports we characterized and correlated molecular phenotypes of *Xf* titres, *TAS4*-3'D4(-) small RNA abundances by RNA blot estimation, and anthocyanin quantities extracted from the transgenic tobacco line *Myb237* overexpressing *AtMYB90* challenged with *Xf* in the greenhouse, and from Pierce's disease-infected and symptomless Merlot leaves and petioles collected from the Calle Contento vineyard in Temecula, California, and the Black Stock vineyard in Dahlonega, Georgia. Those compelling results and preliminary analyses of our first Illumina small RNA libraries generated from the same tobacco- and California *Xf*-infected and control samples strongly supported our working model of *Xf* interaction with anthocyanin biosynthesis regulation by the host during Pierce's disease progression and showed significant differences in accumulation of anthocyanins in *Xf*-infected vs. control leaves from the field and greenhouse samples. L. De La Fuente and S.M. Traore are repeating the tobacco *Myb237* *Xf* challenge experiment. Symptoms are developing and the experimental material is on schedule for delivery to C. Rock for characterization in the next few weeks. The repeat *Xf* challenge experiment with the homozygous and heterozygous *AtMYB90* overexpressing transgenic tobacco lines will critically test whether previously observed significantly greater (57% of leaves) disease symptom development in homozygous (Hmo) lines five weeks after *Xf* challenge and its inverse correlation with anthocyanin accumulation in leaves of the transgenic Hmo and Hmi genotypes (more disease ~ less anthocyanins, with similar titres of *Xf* found across the experiment), is conclusive.

We previously reported strong evidence based on sRNA blots and normalized deep sequencing read counts that *Xf* infection triggers up-regulation of *TAS4* sRNAs, including in non-transgenic control tobacco plants at 1,000 x lower levels than in AtMYB90-overexpressing tobacco. Those data strongly support our working model and further provide new evidence for deep conservation of the autoregulatory loop which we showed functions in *Arabidopsis* [7], whereby target MYBs are positive regulators of *TAS4*. The feedback loop is postulated to impact endogenous *Nt-MIR828* expression when AtMYB90 is overexpressed in tobacco. Velten et al. [4] also observed that miR828 was strongly elevated in sectors of tissue from an independent transgenic tobacco line (Myb27) homozygous for MYB90 when it undergoes spontaneous transgene silencing. These data taken together are evidence for *Xf* targeting of the anthocyanin regulatory network, supported by our recent finding that miR828 is up-regulated by *Xf* in grape (**Figure 4**). Because of low abundance (see below), a Locked Nucleic Acid (LNA) probe [8] was deployed. Direct demonstration of altered expression by *Xf* of the causal effector (miR828) for the regulatory cascade under study is a significant advance that strengthens our claims, in the absence of deep sequencing proofs. We have found additional compelling evidence in the literature supporting our phosphate-regulation *Xf* etiology model: in *Arabidopsis* infected with *Xf*, genome-wide transcriptome analysis showed *TAS4* sRNA target *MYB PRODUCTION OF ANTHOCYANIN PIGMENT1/MYB75* and another phosphate-regulated locus, *At5g20150/SPX DOMAIN* which is a positive regulator of cellular responses to phosphate starvation, are both strongly down-regulated by *Xf* infection [9]. Furthermore, *SPXI* messenger RNA is mobile in the vasculature [10], which is relevant to *Xf* growth habitat. These serendipitous findings constitute 'smoking guns' supporting our working model and raise the possibility that other sRNA and/or pathogenesis regulons are also involved. The following analyses of the deep sequencing dataset from the Temecula, California vineyard libraries further substantiate these predictions.

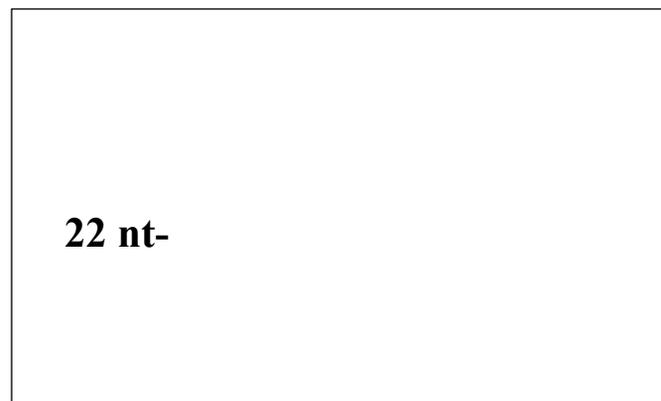


Figure 4. (left). Small RNA blot result for miR828 showing up-regulation by *Xf* infection in field samples from California used for construction of deep sequencing libraries.

We used the statistical software DESeq2 [11] for computational identification of differentially expressed clusters of small RNA-producing loci mined with ShortStack [12] from the *Xf*-infected and control sRNA Temecula, California libraries. **Table 1** shows the list of top leads that are significantly differentially expressed in *Xf*-infected leaves. Several points, both technical and biological, can be made at this juncture: (i) When biological replicate libraries are added in the near future, the statistical power to increase the number of leads by several-fold will be accomplished. DESeq2 works conservatively by assuming the variance-mean dependence estimated per gene by one condition holds well for unreplicated conditions and that most genes are not differentially expressed. (ii) Although lack of replicate libraries combined with low base mean read depths for *MIR828*, *TAS4abc*, and those MYB targets of miR828 [13] and *TAS4-3'D4(-)(MYBA6/A7)* that produce phased sRNAs [14] precludes conclusive evidence for their *significantly* different expressions in response to *Xf*, the trend is very clear and compelling: *Xf* infection in all cases results in a several-fold up-regulation of transitivity inferred to be triggered by miR828 (**Figure 4**) and/or *TAS43'-D4(-)* (data shown in last progress report). Based on strong correlations seen across other libraries previously analyzed [14] (and in process), *TAS4c* is emerging as the likely causal effector for *Xf* response. (iii) Those top-listed significantly differentially regulated genes producing sRNA clusters are known effectors (Leucine-Rich-Repeat receptors) of plant pathogen responses mediated by miRNA activities [13, 14]. (iv) There is evidence in the form of a novel transitive gene (flavonone/naringenin oxygenase, *GSVIVT0101969 5001*) that is significantly up-regulated (>100-fold) by *Xf* infection and functions in polyphenolics metabolism, a

key new piece of evidence in support of our model. We will make replicate libraries and new libraries from materials harvested from the field in July 2017 to further substantiate and extend these leads, and characterize the lead clusters of phased sRNAs to discover their trigger miRNAs and sRNAs represented in our sRNA libraries using CleaveLand software [15] in conjunction with degradome libraries [16, 17], in preparation (see below).

Table 1. Summary of *Xylella* infection-induced differentially expressed clusters of small RNA loci from Temecula, California samples reported by DESeq2.

Annotation	Mean reads	Control*	<i>Xf</i> infected*	log ₂ FC <i>Xf</i> /Con	<i>p</i> -value adj [¶]
GSVIVG01019430001; harpin-induced protein1-homology; OsRPS2/AtRPS5-like	157	472	13	-5.58	0.001
GSVIVG01004877001; TIR-NBS-LRR class homology to rice RPM1, RGA3	1690	N.D.	1033	7.37	0.009
<i>MIRNA</i> candidate; ShortStack class 11 (bulge in miR/miR* duplex)	42	159	0.2	-7.66	0.017
<i>MIRNA</i> candidate; ShortStack class 11	32	105	3	-5.40	0.017
GSVIVT01020358001. Homology to CC-NBS-LRR class; rice RPP13-L/RGA4/AtNB-ARC	68	153	N.D.	-7.25	0.050
GSVIVT01019695001. Homology to Flavanone 3-dioxygenase/naringenin 2-dioxygenase	146	N.D.	2384	7.53	0.060
Vv- <i>TAS4a</i> [^]	17071	11367	24891	0.27	1.000
Vv- <i>TAS4c</i> [^]	7668	8	357	1.58	1.000
vv- <i>TAS4b</i> [^]	214	750	2034	0.44	1.000
MYB-828 target GSVIVT01032467001	87	N.D.	11	2.00	1.000
MYB-828 target GSVIVT01006275001	30	8	36	1.00	NA
MYBA7 target of TAS4-3'D4(-) GSVIVT01030819001	12	N.D.	N.D.	0.00	NA
MYBA6 target of TAS4-3'D4(-) GSVIVT01030822001	3	N.D.	N.D.	0.00	NA
Vv- <i>MIR828</i> (genome coordinates from mirBase.org)	2	N.D.	N.D.	0.00	NA

*raw reads normalized, per 10M. N.D.: not detected. NA: not analyzed

¶ False Discovery Rate < 0.1; Benjamini & Hochberg multiple comparisons adjustment

[^] phytozome.jgi.doe.gov genome coordinates from Rock (2013) [14]

We have made Illumina TruSeq stranded mRNASeq libraries for mRNA-Seq (objective 2, method 2) and submitted them to the UC Riverside Institute for Integrative Genome Biology for sequencing, which will permit digital measurement of primary transcripts including *MIR828*, *TAS4* ncRNAs, and *MYB* targets as well as all other differentially expressed genes deranged by *Xf* in grapevine. We will next use the TruSeq kit to make degradome libraries for discovery of the sRNA triggers of transitivity discovered by Shortstack. This will leverage a systems approach building on the sRNA candidate leads to discover other etiological effectors/reporters of Pierce's disease and network analyses of gene interactions affecting primary and secondary metabolism in the process.

Objective 3. Characterize the changes in (a) xylem sap and leaf Pi, and (b) polyphenolic levels of *Xf*-infected canes and leaves. (c) Test on tobacco in the greenhouse and *Xf* growth *in vitro* the Pi analogue phosphite as a durable, affordable, and environmentally sound protectant/safener for Pierce's disease. (a) Leaf [Pi].

In early August 2016, C. Rock collected Merlot variety leaf and cane samples from the Calle Contento vineyard (Stage Ranch, Temecula, California), the same source as for the sRNA libraries made from the July 2015 samples. **Figure 5A** shows typical leaf symptoms of samples collected in August 2016 and stored for future analyses. It is noted that grapevine red blotch-associated virus (GRBaV) symptoms appeared to be present in a majority of leaves, which confounds the absolute scoring of Pierce's disease symptoms (which are also clearly manifest; see arrows). Samples from July 2015 used to make the sRNA deep sequence libraries did not show GRBaV symptoms, although some vines in the plot did manifest symptoms, verified by UC Cooperative Extension agent Matt Daugherty. Indeed, preliminary analysis of the 2015 sRNA libraries found evidence that the *Xf* Temecula1 and GRBaV sRNAs are present. Remarkably, the library from *Xf* samples showed no GRBaV reads (data not shown). This issue warrants further analysis and validation by quantitative reverse transcription (qRT) PCR-specific amplification from DNA extracts of past and current samples before conclusions are drawn regarding *Xf*-

specific effects on the hypothesized sRNA pathways, and/or antagonistic interactions with GRBaV replication *in planta*.

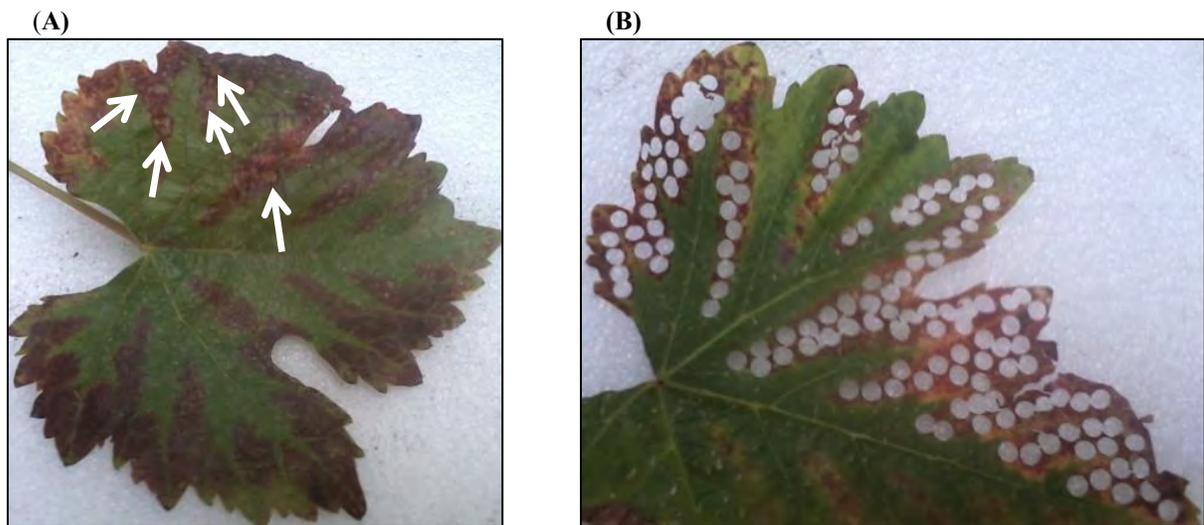


Figure 5. (A) Image of sampled leaf collected from Temecula, California vineyard in August 2016 showing both Pierce's disease (arrows) and GRBaV symptoms around the leaf margin and interveins. (B) Image of leaf showing 5 mm disks removed for packing an NMR tube for Pi quantitation.

In vivo nuclear magnetic resonance (NMR) spectroscopy permits analysis of subcellular [Pi] that can provide insight into *Xf* perturbation of host physiology. The 2016 fresh leaf samples were used for pilot experiments on NMR quantitation [18] of Pi in whole leaf disks, in collaboration with Dan Borschardt, Department of Chemistry, UC Riverside (**Figure 5B**). Preliminary results suggested there is elevated [Pi] in the GRBaV symptom samples (data not shown), but this interpretation is speculative because there are more leaf disks ($n = 160$) per unit area of symptom-expressing leaves than healthy leaves ($n = 120$) due to reduced vigor. Future experiments are planned for July 2017 with validated symptomless, Pierce's disease-, and GRBaV symptom samples to carefully quantify Pi on a per-leaf disk basis.

Xylem sap [Pi].

Prior results of L. De La Fuente [3] show strong associations of elemental P decreases with *Xf* infection in many host species. However, the biological complexities of P (e.g., phosphoproteins, lipids, nucleic acids, subcellular compartmentation, etc.) precludes conclusive interpretation of existing data. In late May 2016, C. Rock collected Cabernet Sauvignon samples from the Phelps vineyard in St. Helena, California under the supervision of UC Cooperative Extension agent Monica Cooper. Pierce's disease symptoms in May were evidenced by severe stunting and some mild leaf chlorosis. Canes from healthy and Pierce's disease vines were cut, returned to the bench, and stripped of the phloem cambium shell, for extraction of xylem wood sap by clipping ~ two-cm-long sections of cane followed by centrifugation at 10,000 x g for three minutes, and freezing of collected xylem sap exudates. Samples were quantified for phosphate, sulfate, and nitrate ions by ion chromatography-flame ionization detection (IC-FID) [19]. Quantitations by ion chromatography - flame ionization detector (IC-FID) for the St. Helena samples are presented in **Table 2**.

There is a significantly different (higher) concentration of Pi in *Xf*-infected canes, internally controlled for sulfate and nitrate concentrations. This unexpected and intriguing result warrants further study. Because we have petiole samples from each independent cane tested, we will endeavor to correlate *Xf* titers in subtending leaf petioles with phosphate concentrations in cane xylem saps. Samples will be collected in May 2017 to repeat the analysis. If the result stands, then our working hypothesis will need to be modified and measurements of leaf [Pi] brought into focus, and emphasis placed on independent tests of the hypothesis that there is an imbalance of Pi between leaf (hypothesized low) and xylem (observed high) caused by *Xf* infection.

Table 2. Quantitation of xylem sap inorganic ion concentrations from healthy and Pierce's disease symptom canes of Cabernet Sauvignon in St. Helena, California collected in May 2016.

Sample	Ion Abundance, parts per billion (+/- s.e.m.)		
	Phosphate	Sulfate	Nitrate
Healthy vines (n = 10)	394 (22)	114 (14)	8 (1)
<i>Xf</i> -infected vines (n = 7)	468 (21)	129 (20)	8 (1)
<i>p</i> -value [^]	0.03	0.52	0.73

[^] two-sided Student's t-test, equal variance assumed

(b) Polyphenolics in *Xf*-infected canes and leaves.

Analyses of xylem sap anthocyanin and *Xf* titers from petiole extracts are ongoing. Preliminary results for identification in leaf extracts of malvin and cyanin by Select Reaction Monitoring (SRM) High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) are shown in **Figure 6**. The method entails specifying the parent mass of the compound for tandem MS/MS fragmentation and then specifically monitoring for fragment ion(s) representing the aglycone species.

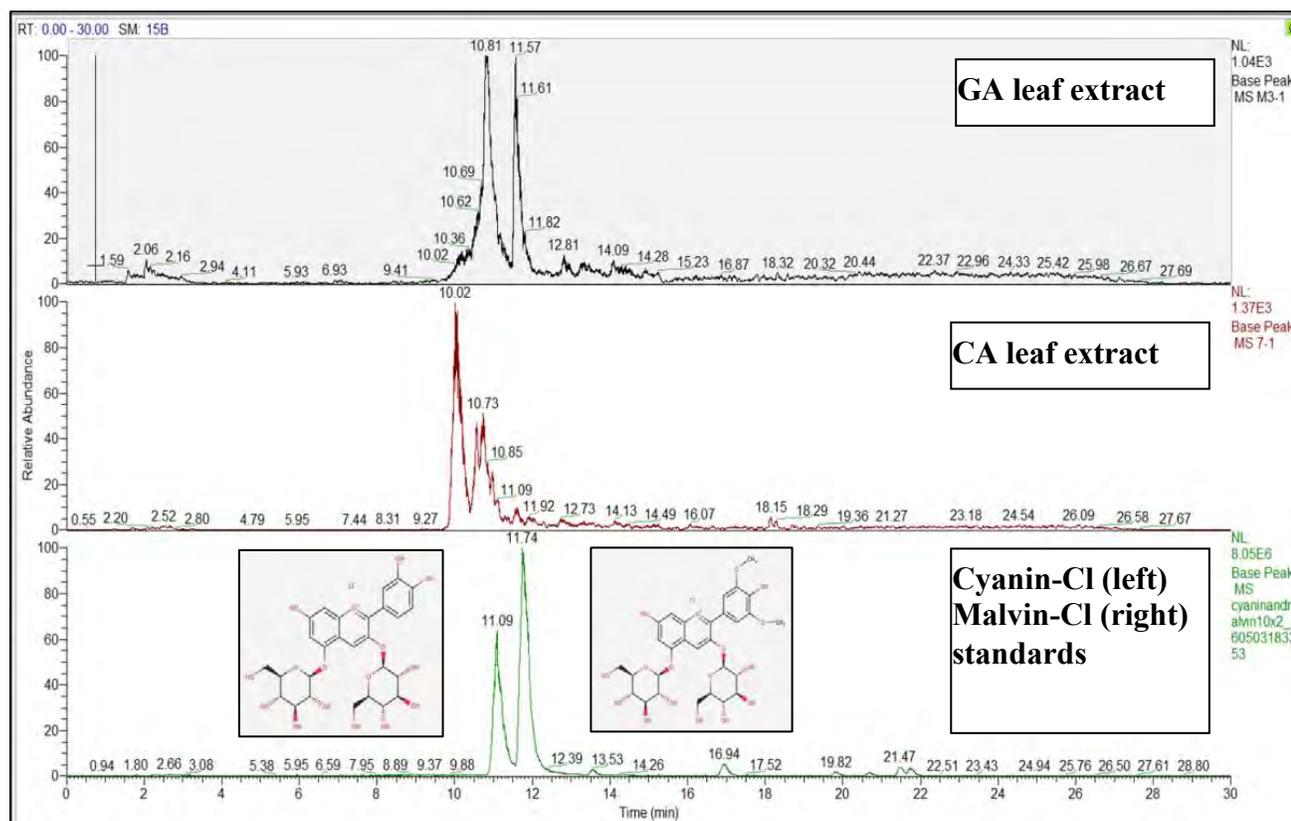


Figure 6. Preliminary analysis of anthocyanins in leaf extracts of *Xf*-infected samples by HPLC-SRM-MS. Parent ions: cyanin ($m/z = 610.99$); malvin ($m/z = 655.18$). Daughter ions: cyanin ($m/z = 449, 287$); malvin ($m/z = 493, 331$).

(c) Pi analogue phosphite as effector of *Xf* growth and safener of disease symptoms.

L. De La Fuente shipped *Xf* Temecula-1 and WM1-1 strains to C. Rock on May 4, 2016 and the C. Rock lab is currently being set up for baseline studies on greenhouse infections of grapevine and tobacco, and microbiology studies on *Xf* growth parameters. Future work will focus first on assessing phosphite effects on *Xf* growth rates in liquid culture. If results are positive, safener treatments of tobacco and grapevine under greenhouse *Xf* challenge will follow.

CONCLUSIONS

We are on track to achieve our objectives within the timeframe of one year of funding, contingent upon a final cycle of grapevine transformation being initiated within the next few months. We have identified new lead target genes and preliminary evidence for inorganic phosphate (Pi) involvement in Pierce's disease that further substantiate the working hypothesis and which will generate new knowledge about Pierce's disease etiology.

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GREENHOUSE EVALUATION OF GRAPEVINE MICROBIAL ENDOPHYTES AND FUNGAL NATURAL PRODUCTS FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT

The goal of this research is to identify biological control agents and natural products antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as prophylactic and/or curative treatments for Pierce's disease. We showed in *in vitro* bioassays that several fungal endophytes isolated from grapevine wood possess anti-*Xf* properties, due to the production of natural products. We purified radicinin produced by *Cochliobolus* sp. and demonstrated that this natural product was an effective inhibitor of *Xf*. In collaboration with the private sector, we successfully developed an emulsion of radicinin and treated vines inoculated with *Xf*. In addition, we showed that the fractions from the crude extracts of three additional fungal endophytes (i.e., *Eurotium*, *Geomyces*, and *Ulocladium*) also possess activity against *Xf* 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. We also showed that one grapevine endophytic fungus (*Cryptococcus* sp.) was able to mitigate Pierce's disease symptoms development and *Xf* bacterial titer in *in planta* bioassays and could be used as a biological control agent. Finally, using a next generation sequencing approach to study the microbiome of Pierce's disease affected and escaped grapevines we were able to identify bacteria (*Pseudomonas* sp. and *Achromobacter* sp.) as additional potential biological control agents. These are currently being evaluated in *in planta* bioassays. These molecules and formulations are currently under review for patentability by the Executive Licensing Officer in the UC Riverside Office of Research.

LAYPERSON SUMMARY

The goal of this project is to identify biological control agents and their natural products that are antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as prophylactic and curative treatments for Pierce's disease. We had previously isolated several fungi naturally inhabiting grapevines that were antagonistic to *Xf* in *in vitro* bioassays. We have been extracting, purifying, and characterizing the compounds that they produced and have identified one promising molecule (radicinin) that is strongly inhibitory to the bacterium. We have now developed an emulsion of radicinin in a concerted effort with the private sector and are currently testing the efficacy of this formulation on Pierce's disease infected grapevines in the greenhouse. In addition, we recently showed that the fractions from the crude extracts of three additional fungal endophytes inhibited *Xf* 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. Using traditional microbial techniques and novel molecular approaches we have identified one fungus (*Cryptococcus* sp.) and two bacteria (*Pseudomonas* sp. and *Achromobacter* sp.) as potential biological control agents for Pierce's disease. Those are currently being tested in the greenhouse. These natural products and formulations of these products are currently under review for patentability by the Executive Licensing Officer in the UC Riverside Office of Research and, hence, their names cannot always be disclosed in this report.

INTRODUCTION

Xylella fastidiosa (*Xf*) 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*; GWSS) to Southern California shifted the epidemiology of Pierce's disease from a monocyclic to a polycyclic disease. This led to a Pierce's disease epidemic with severe economic consequences for the Southern California grape industry. GWSS has move to the San Joaquin Valley and impacted table grape production, and it now threatens to become established in the heart of the wine grape production area including Napa and Sonoma counties. Current Pierce's disease management guidelines largely rely on vector control through the use of insecticides.

In this project we explore the use of grape endophytic microorganisms as a practical management tool for Pierce's disease. Our research adds to the ongoing integrated pest management efforts for discovery of biocontrol agents to *Xf* (Das et al., 2015; Hopkins, 2005). Our strategy is to couple culture-dependent and culture-independent approaches to identify novel biocontrol agents and active natural molecules. Control of bacterial plant diseases with commercial biological control agents has been an active area of research (Stockwell and Stack, 2007; Stockwell et al., 2010; Yuliar et al., 2015). In addition, fungi and bacteria are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce that have potential for use as antimicrobial compounds to cure plant diseases (Aldrich et al., 2015; Ben Abdallah et al., 2015). Our research team has made substantial progress in the past years and identified several potential biological control agents and natural products that could be used as prophylactic and curative treatments for Pierce's disease. Our goals are to evaluate in *in planta* bioassays those biological control agents and natural products before field testing.

OBJECTIVES

The objectives of this project are:

1. Evaluate a single organism-based approach for Pierce's disease management.
2. Evaluate natural products and derivatives for their potential as curative treatments for vines already infected with Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Evaluate a single organism-based approach for Pierce's disease management.

The goal of this objective is to evaluate individual fungal and bacterial grapevine endophytic strains for management of Pierce's disease. Pierce's disease escaped and symptomatic grapevine tissues (cane, sap, spurs) were previously sampled from several commercial vineyards in Riverside and Napa (Figure 1) counties and were analyzed by culture-dependent and culture-independent approaches. A Pierce's disease escaped vine is defined as a grapevine located in a Pierce's disease hot spot (i.e., with high disease pressure) that is infected with *Xf* but expresses no to little Pierce's disease symptoms.



Figure 1. Pierce's disease symptomatic (red arrow) and Pierce's disease escaped (blue arrow) grapevines in a vineyard located close to a riparian area in the Napa Valley, California.

Using an Illumina-based culture-independent approach, we were able to identify *Achromobacter* sp. and *Pseudomonas* sp. as the two most abundant bacteria inhabiting grapevine xylem that correlated negatively with *Xf* titer (Table 1). In other words, those two bacteria were present in higher abundance in Pierce's disease escaped than in Pierce's disease symptomatic grapevines, suggesting that those may be good biological control agent candidates. In addition, using a culture-dependent approach, we isolated eight fungi and one bacterium that showed *Xf* growth inhibition in our *in vitro* bioassay (Figure 2; Rolshausen et al., 2013). Interestingly, the bacterium isolated was identified as *Achromobacter* sp. We further evaluated those fungi and *Achromobacter* sp. in *in planta* bioassays and demonstrated that *Cryptococcus* sp. was the best biological control agent candidate, as

it mitigated Pierce's disease symptom development and *Xf* titer in grapevines and also provided some increased immunity against Pierce's disease (**Figure 3** and **Figure 4**; Rolshausen et al., 2013). *Achromobacter* sp. also reduced Pierce's disease rating and *Xf* titer, but not significantly.

Table 1. Correlations (r) between *Xf* (as expressed by the number of Illumina reads) and the abundance (%) of individual phylotype (Operational Taxonomic Units: OTU). Statistical P and FDR corrected values are presented.

OTU	P	FDR Corrected	r	Abundance %
<i>Pseudomonas</i> sp.	2.3E-18	4.4E-16	-0.83	64.3
<i>Achromobacter</i> sp.	8.4E-03	1.3E-01	-0.32	6.3

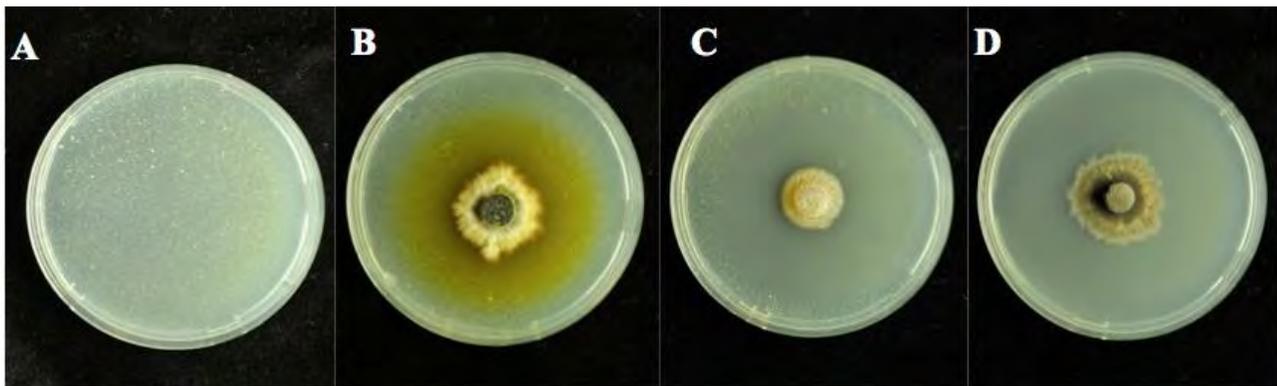


Figure 2. *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*. *Xf* cells were plated in top agar, and agar plugs containing fungi were placed on top. Inhibition was evaluated after eight days of incubation at 28°C. A) *Xf*-only control; B) No *Xf* inhibition; C) Mild *Xf* inhibition; D) Total *Xf* inhibition.



Figure 3. Greenhouse bioassay used to evaluate efficacy of biocontrol fungi and fungal natural products for control of Pierce's disease. The progression of Pierce's disease in vines infected with *Xf* is scored on a disease severity rating scale ranging from 0 (= healthy) to 5 (= dead or dying).

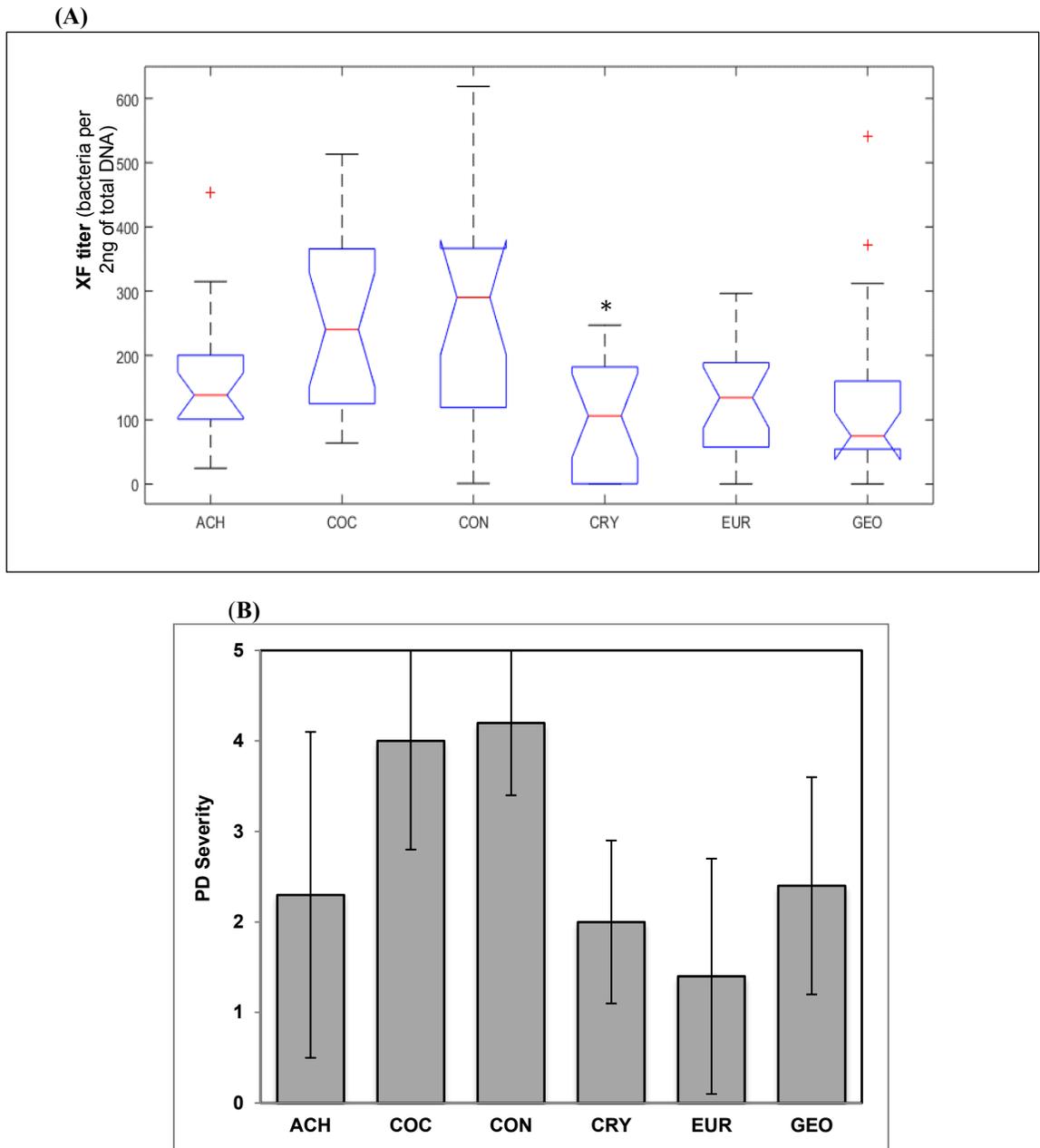


Figure 4. *Xf* titer and Pierce's disease severity in grapevines (n = 10) inoculated with five grapevine endophytes or 1X PBS alone (control) and challenged with *Xf* (ACH = *Achromobacter*; COC = *Cochliobolus*; CON = Control; CRY = *Cryptococcus*; EUR = *Eurotium*; GEO = *Geomyces*). (A) Box plots illustrate the distribution of *Xf* titer in all six treatments. Asterisks (*) indicate significance at $P < 0.05$. *Xf* titer was measured by qPCR. *Xf* titer was significantly decreased in vines that were pre-treated with *Cryptococcus* as compared to vines that were pre-treated with 1X PBS only. In addition, *Xf* titer was also decreased (just above statistical significance) in vines that were pre-treated with *Achromobacter* as compared to those inoculated with 1X PBS only. (B) Pierce's disease severity average as measured by our disease rating scale (0 to 5; **Figure 3**). Error bars represent standard deviation.

Cryptococcus is yeast commonly associated with plants and is also a known biological control agent of other plant pathogens (Schisler et al., 2014; Ulises Bautista-Rosales et al., 2014). Our Illumina sequencing results confirmed its presence in grapevine xylem although its abundance was low (below 1%) compared to both *Achromobacter* sp. and *Pseudomonas* sp. (**Table 1**). *Achromobacter* sp. is a known plant endophyte and plant growth promoting bacteria (Soares et al., 2016; Abitha et al., 2014). *Pseudomonas* sp. is both a plant growth promoting bacteria and a known biological control agent (Loper et al., 2012). These organisms are currently being tested further in *in planta* bioassays to determine which is better suited to be evaluated under natural field conditions.

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

The goal of this objective is to identify 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 *Xf* in a bioassay. Thus far, we have purified and characterized the chemical structure of two molecules (radicinin and cytochalasin) that are active against *Xf* growth *in vitro*. Radicinin is produced by *Cochliobolus* sp. and cytochalasin is produced by *Dreschlera* sp. However, cytochalasin showed to be toxic to mammals so we decided to discontinue this research axis. In addition, we pursued our efforts for the bioassay-guided isolation of natural products from the remaining fungi able to inhibit *Xf* in our lab bioassay, including *Cryptococcus* sp., *Ulocladium* sp., *Eurotium* sp., and *Geomyces* sp.

Cochliobolus natural product.

Radicinin showed great potential *in vitro* (Aldrich et al., 2015). Hence, in an *in vitro* dose response assay, where *Xf* cells are submitted to an increasing concentration of a fungal molecule, radicinin was able to inhibit *Xf* growth (Figure 5). We have now 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 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 purer, as observed by nuclear magnetic resonance (NMR) spectroscopy.

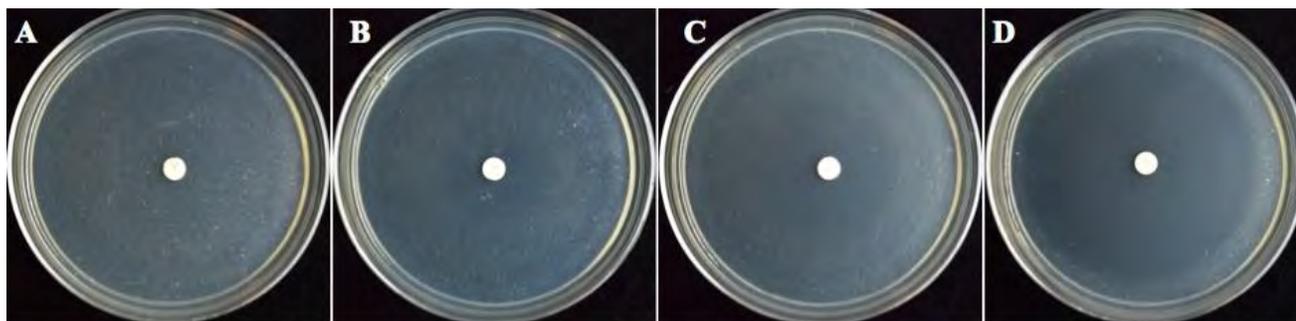
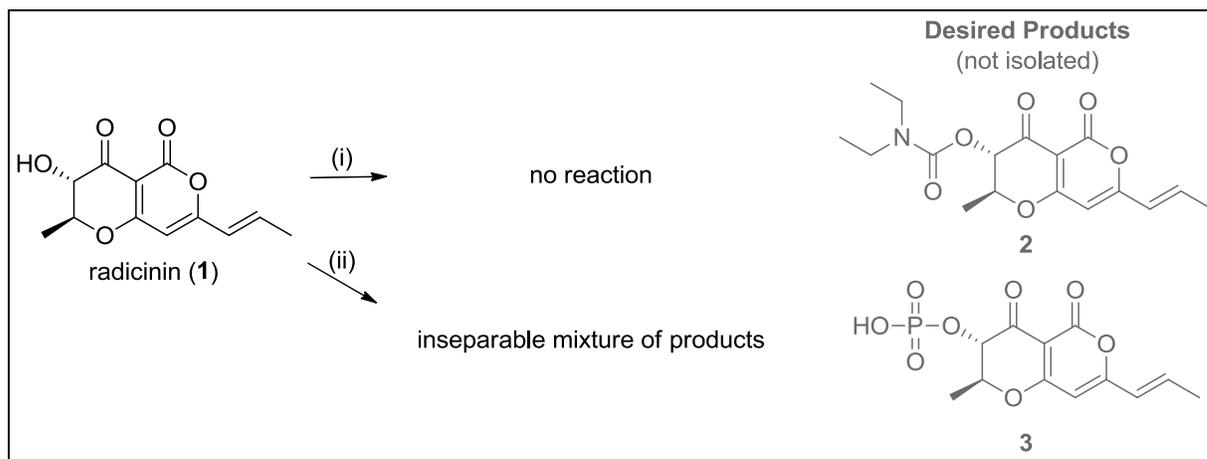


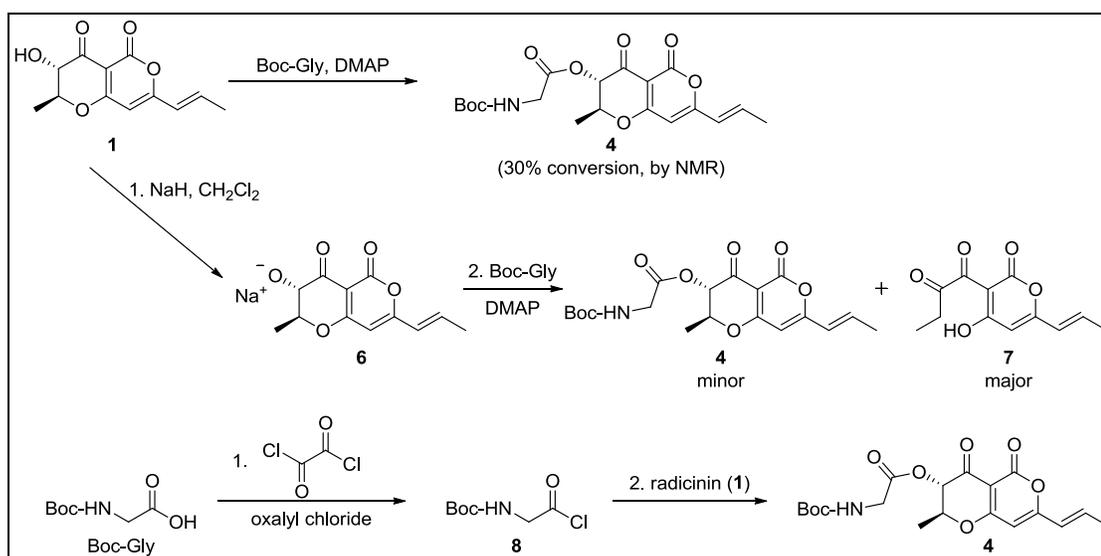
Figure 5. Dose response assay to evaluate *in vitro* *Xf* inhibition at increasing concentration of radicinin, a natural compound produced by *Cochliobolus* sp. (A) 0 µg molecule radicinin (control), (B) 50 µg molecule radicinin, (C) 100 µg molecule radicinin, and (D) 250 µg molecule radicinin (Aldrich et al., 2015).

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-*Xf* activity (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.

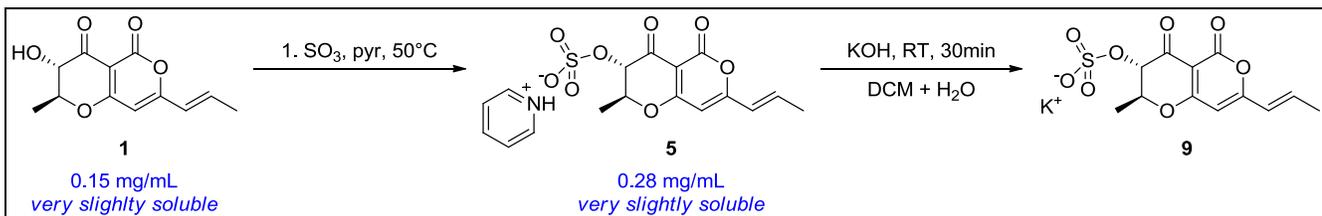


Scheme 1. *Xf*-inhibitory natural product radicinin (1), and semisynthetic derivatives (2-4). Reagents: (i) N,N-diethylcarbamoyl chloride, triethylamine (Vougogiannopoulou et al. 2008). (ii) 1. Cl₃CCN, 2. (n-Bu)₄NH₂PO₄, CH₃CN, 3. DOWEX 50WX8, NH₄HCO₃.

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 led 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 *Xf* 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.



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.

After a series of mostly unsuccessful attempts at preparing water-soluble radicinin derivatives we decided to explore another strategy for getting radicinin into grapevines, namely, using surfactants. We tested the solubility of radicinin in a variety of organic solvents that are compatible with agriculture, including o-xylene, canola oil, castor oil, mineral oil, and cyclohexanone. Radicinin was completely soluble in cyclohexanone but was not soluble in any of the other solvents. We have been working with a private company (Evonik Corporation; <http://www.break-thru.com/product/break-thru/en/Pages/default.aspx>) to help us get the radicinin in the plant. Following their recommendation we dissolved radicinin in cyclohexanone plus one of Evonik's emulsifiers to prepare a water-cyclohexanone emulsion for application on grapevine leaves. These are currently being evaluated in greenhouse bioassays.

Cryptococcus natural product.

Although live cultures of *Cryptococcus* sp. inhibited *Xf* *in vitro*, previous attempts to extract the active compound from liquid cultures failed to yield an active organic extract, either because the activity is not due to a small molecule natural product or because the particular strain of *Cryptococcus* failed to produce the compound in liquid monoculture in Potato Dextrose Broth (PDB). We tried to stimulate the production of any active metabolite(s) by growing three *Cryptococcus* strains (the original strain CRY1 along with two more recently-isolated strains, CRY3 and CRY4) in PDB, PDB with added *Vitis* sp. leaves (lyophilized and autoclaved with the media), and PD3 medium (the medium used for the *in vitro* *Xf*-inhibition assay). After 14 days of fermentation with shaking at room temperature each culture was centrifuged to separate the cell pellet from the culture broth. The broths were extracted twice with ethyl acetate and the pellets were lyophilized, ground in a mixture of 1:1 dichloromethane:methanol, and filtered to give a crude extract. Extracts were evaporated and submitted for the disc diffusion assay for activity against *Xf* (**Figure 5**). We are currently waiting for the results.

Ulocladium natural product.

We previously observed a compound in the ethyl acetate extract of *Ulocladium* sp. which high-resolution mass spectrometry revealed to have a molecular formula of $\text{C}_{10}\text{H}_8\text{Cl}_2\text{O}_4$; this compound has consistently been found in the active fractions from repeated fermentations and separations of *Ulocladium*. In an effort to produce enough of this compound we fermented 5.5 L of *Ulocladium* sp. and fractionated the organic extract by silica gel chromatography. This yielded 23.4 mg of a semi-purified fraction containing the compound of interest. This was enough material to permit collection of two-dimensional NMR spectral data (including gdcOSY, gHMBC, HSQC, and NOESY experiments) which we are in the process of analyzing. All fractions have been submitted for bioassay against *Xf*.

Characteristic ^1H NMR signals revealed the following features in the major compound from the active extracts:

- A phenol, indicated by a singlet at 12 ppm (and corroborated by the fact that the compound ionizes better in negative ionization mode on the liquid chromatography-mass spectrometry (LC-MS), and by a phenol-specific ferric chloride thin layer chromatography (TLC) stain);
- One or more pair(s) of aromatic hydrogens in an ortho- relationship (indicated by doublets in the 7-8 ppm range, with $J = 8-9$ Hz); and
- A 1,2-disubstituted cis-alkene, indicated by coupled doublets at 6.3 and 7.7 ppm ($J = 11.5$ Hz).

We recently began a time-course study to observe the appearance of the active compound over time. The results of this study will be used to optimize production of the molecule of interest.

Geomyces natural product.

Previous active fractions from *Geomyces* sp. strain GEO1 revealed weak activity and no major small molecules. However, the active fraction of a more recently isolated *Geomyces* sp. strain (GEO3) showed strong activity in the *in vitro* *Xf*-inhibition assay. We fractionated this extract by silica gel chromatography and submitted the six fractions for bioassay. We are currently waiting for the results.

CONCLUSIONS

We aim to investigate prophylactic and curative measures for management of Pierce's disease as part of a sustainable Pierce's disease management program. Our strategy is to utilize both the microbes associated with grapevines and their anti-*Xf* natural molecules. The commercialization of biological control agents and/or novel chemistries will provide a solution for the grape industry to manage Pierce's disease and if successful could also be expanded beyond grapevine. To date, we have discovered three potential biological control agents for *Xf* (*Pseudomonas*, *Achromobacter*, and *Cryptococcus*) and one active anti-*Xf* fungal natural product (radicinin). In a concerted effort with industry partners we successfully developed an emulsion of radicinin that was sprayed on Pierce's disease-infected vines and are currently waiting for the results. In addition, we are also searching for additional active natural anti-*Xf* compounds. We have recently identified fractions from the crude extracts of three additional fungal endophytes that possess activity against *Xf* in the *in vitro* bioassay and we are in the process of identifying their chemical structure and properties. The three biological control agents are also being challenged in *in planta* bioassays to ensure their ability to mitigate Pierce's disease. The next phase will be to evaluate those biological control agents and natural products under natural vineyard settings.

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

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ABSTRACT

Xylella fastidiosa (*Xf*) is a gram-negative, fastidious, xylem-limited bacterium that causes scorching diseases in many economically important plant species, like Pierce's disease of grapevine, the most valued fruit crop in the U.S. Lipopolysaccharide (LPS) covers the majority of the cell surface of Gram-negative bacteria and is a well-described pathogen-associated molecular pattern that elicits host basal defense responses in plants. In order to understand how LPS mediates host-pathogen interaction in Pierce's disease we performed transcriptional profiling and histological analysis of grapevines inoculated with either *Xf* containing a wild-type LPS molecule or *wzy* mutant containing a truncated LPS with no O antigen. Also, we investigated grapevine tolerance to *Xf* by priming plants with LPS and then challenging them with *Xf*. RNA-seq and histological analysis show the grapevine defense system is able to recognize a truncated LPS molecule, resulting in a strong oxidative burst and a small production of tyloses. In contrast, grapevines produce many tyloses, phytoalexins, and other antimicrobial compounds when inoculated with wild-type *Xf*. In addition, Pierce's disease symptoms are attenuated when grapevines are challenged with *Xf* four hours and 24 hours after LPS treatment, showing that the LPS molecule is able to prime defenses against *Xf*. Finally, we present the first evidence that the major polysaccharide present in *Xf* wild-type O antigen is a linear α 1-2 linked rhamnan.

LAYPERSON SUMMARY

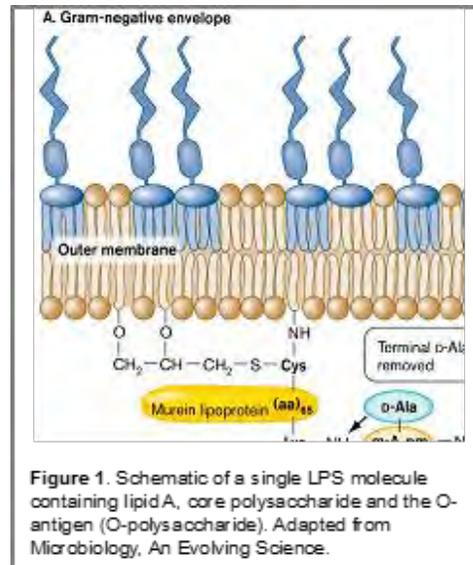
Successful plant pathogens must overcome plant immune responses to establish and cause disease. Unlike many prominent bacterial phytopathogens, *Xylella fastidiosa* (*Xf*) does not possess quintessential Type III-secreted effectors that perform this function. Although there has been extensive research identifying *Xf* virulence factors, the mechanisms utilized by this pathogen to combat plant immune responses have remained largely obscure. We demonstrate that *Xf* utilizes the prominent O antigen surface carbohydrate to shield bacterial cell surface elicitors from the grapevine immune system, effectively delaying recognition. By altering O antigen structure, we identified unique grapevine transcriptional and phenotypic responses activated during *Xf* infection. These results provide new insight into the molecular mechanisms underlying this host-pathogen interaction.

INTRODUCTION

Xylella fastidiosa (*Xf*), 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; Varela, 2001). *Xf* is limited to the xylem tissue of the plant host and is transmitted by xylem-feeding insects, mainly sharpshooters. Extensive xylem vessel blockage occurs in infected vines (Sun et al., 2013), and symptoms include leaf scorch, raisining of berries, stunting, and vine death. Pierce's disease has devastated some viticultural areas in California and there are currently no effective control measures available to growers besides roguing of infected vines and severe pruning.

Our ongoing study confirms that lipopolysaccharide (LPS) is a major virulence factor for *Xf*. LPS comprises approximately 75% of the Gram-negative bacterial cell surface, making it the most dominant macromolecule displayed on the cell surface (Caroff & Karibian, 2003; Foppen et al., 2010; Madigan, 2012). LPS is a tripartite glycolipid that is generally comprised of a highly conserved lipid A, an oligosaccharide core, and a variable O antigen polysaccharide (Whitfield, 1995) (**Figure 1**). We demonstrated that compositional alterations to the outermost portion of the LPS, the O antigen, significantly affected the adhesive properties of *Xf*, consequently affecting biofilm formation and virulence (Clifford et al., 2013). Depletion of the 2-linked rhamnose in the O antigen locks *Xf* in the initial surface attachment phase and prevents biofilm maturation (Clifford et al., 2013). In addition, we demonstrated that truncation of the LPS molecule severely compromises insect acquisition of *Xf*

(Rapicavoli et al., 2015). We coupled these studies with quantification of the electrostatic properties of the sharpshooter foregut to better understand the interface between the *Xf* cell and the insect. Our recently funded project tested our additional hypothesis that the *Xf* LPS molecule acts as a pathogen-associated molecular pattern, and the long chain O antigen serves to shield *Xf* from host recognition, thereby modulating the host's perception of *Xf* infection (Rapicavoli et al., in preparation).



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). PAMPs, also known as microbe-associated molecular patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (i.e., LPS, flagellin, fungal chitin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of basal defense responses. 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 LPS in grapevine, particularly the *Xf* LPS PAMP. *Xf* 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. Clearly, *Xf* has evolved a mechanism to circumvent the host basal defense response as it successfully colonizes and causes serious disease in grapevine. Our working hypothesis is that during the compatible interaction between *Xf* 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 basal defense responses and establish itself in the host.

To explore the role of LPS as a shield against basal defense responses in grapevine we investigated elicitation of an oxidative burst, an early marker of basal defense responses, *ex vivo* in *V. vinifera* Cabernet Sauvignon leaf disks exposed to either wild-type *Xf* or *wzy* mutant cells. When we examined reactive oxygen species (ROS) production in response to whole cells, *wzy* mutant cells (in which lipid A-core is exposed) induced a stronger and more prolonged oxidative burst in grapevine leaf disks than did wild-type *Xf*. Specifically, ROS production peaked at around 12 minutes and lasted nearly 90 minutes. Wild-type *Xf* cells (in which lipid A-core would be shielded by O antigen) failed to produce a sharp peak as compared with the *wzy* mutant, and ROS production plateaued much sooner (around 60 minutes) (data not shown). To determine where ROS production was localized

in situ, we performed DAB (3,3'-diaminobenzidine)-mediated tissue printing of grapevine petioles that were inoculated with wild-type *Xf*, *wzy* mutant, or 1x PBS buffer as a control. DAB reacts with H₂O₂, which is the major ROS associated with the oxidative burst in plants, to produce a reddish-brown color. Grapevines inoculated with the *wzy* mutant exhibited more intense H₂O₂ production prominently localized in the xylem vessels (**Figure 2A**), indicating that the *wzy* mutant elicits a more robust oxidative burst than wild-type *Xf*. Further quantitative comparison of staining intensity among the treatments, using ImageJ, indicated that, indeed, *wzy* elicits significantly more ROS in the xylem than does wild-type *Xf* (**Figure 2B**). To determine if the intensity of the *wzy*-induced ROS burst in the xylem had direct antimicrobial activity against *Xf*, we performed an H₂O₂ survival assay. Previously, we demonstrated that the *wzy* mutant was more sensitive to H₂O₂ stress, but survival rates in an oxidative environment were not quantified in that study. We chose a final concentration of 100 μM H₂O₂ based on the lower threshold of ROS detected by the DAB staining method (DAB staining detects H₂O₂ in the range of 100 μM to 10 mM). In addition, to mirror the kinetics of peak ROS production seen *in vivo*, we exposed the cells to H₂O₂ for ten minutes. Due to the increased sensitivity of the mutant cells to H₂O₂, we hypothesized that the *wzy* mutant-induced oxidative burst is lethal to *wzy* mutant cells. Indeed, only 10.06% of *wzy* mutant cells survived, compared with 50.21% of wild-type cells (**Figure 2C**).

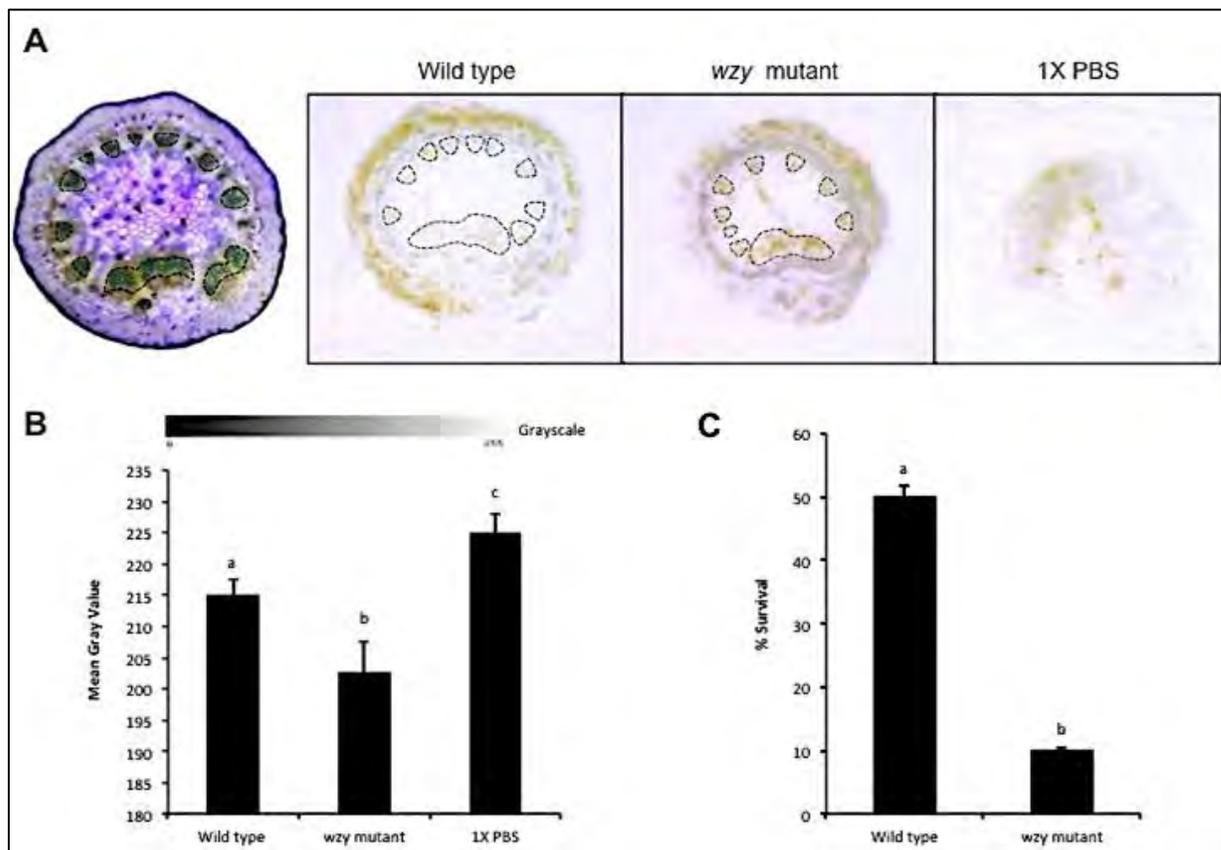


Figure 2. *In situ* localization of O antigen-modulated ROS production in the xylem. (A) DAB-mediated tissue printing at 15 minutes post-inoculation revealed a strong production of H₂O₂ specifically in the xylem vessels of grapevines inoculated with *wzy* mutant cells. (B) Mean gray value of DAB-stained images, representing differences in staining intensity. Grayscale intensities vary from 0 to 255; 0 = black, 255 = white, and the values in between make up the shades of gray. The mean gray value of DAB-stained images from *wzy* mutant-inoculated plants is significantly lower than wild-type or 1x PBS-inoculated plants, indicating a darker or more intense stain, and thus higher amounts of H₂O₂. Treatments with different letters over the bars were statistically different ($P < 0.05$). (C) Hydrogen peroxide survival assay. Suspensions of *Xf* wild-type or *wzy* mutant cells were incubated with 100 μM H₂O₂ for 10 min, followed by dilution plating and enumeration. Survival percentages of *wzy* mutant cells were significantly lower than *Xf* wild-type cells ($P < 0.0001$). Following treatment with H₂O₂, only 10.06% of *wzy* mutant cells survived compared with 50.21% of wild-type cells. Data are means of three biological replications.

Now that we have established that we can directly elicit an LPS-mediated defense response we propose to assess how long the temporal window of the heightened defense response lasts by increasing the amount of time between the inoculation with the LPS and the challenge with live *Xf* cells. In our currently funded project we are testing our working hypothesis that the grapevine is recognizing the conserved core/lipid A portions of the *Xf* 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, allowing *Xf* to circumvent the innate immune response and successfully colonize the host. We have completed the global RNA-seq-based transcriptome profiling facet of this project where we sequenced the transcriptomes of grapevines treated with wild-type, *wzy* mutant cells, or 1x PBS buffer. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow et al., 2000; Tao et al., 2003) so our initial experiments were targeted toward early time points during the infection process (0, 8, and 24 hours post-inoculation). Thus far, the RNA-seq data demonstrate that the grapevine is activating defense responses that are distinct to each treatment and time point (**Figure 3A**). For example, enrichment analysis of *wzy*-responsive genes at eight hours post-inoculation identified predominant biological processes associated with cellular responses to biotic stimulus and oxidative stress (**Figure 3B**). This included a significant increase in the production of thioredoxins, glutaredoxins, and other ROS-scavenging enzymes involved in antioxidant defense. In addition, there was high expression of genes involved in the production of phytoalexins, antimicrobial peptides, and pathogenesis-related genes. In contrast, wild-type-responsive genes at this time point were enriched primarily in responses to abiotic or general stresses (i.e., drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses (**Figure 3B**). Notably, by 24 hours post-inoculation overall transcriptional profiles of both *wzy* and wild-type-inoculated vines shifted dramatically. Grape genes in *wzy* mutant-inoculated vines were no longer enriched for immune-specific responses, and we speculate that this is due to the effective O antigen-modulated oxidative burst. In contrast, genes of wild-type-inoculated plants were strongly enriched for immune responses (**Figure 3C**). We hypothesize that at 8 hours the high molecular weight O antigen is still effectively shielding wild-type cells, therefore causing a delay in plant immune recognition. However, by 24 hours post-inoculation the production of ethylene-induced plant cell wall modifications, compounded by progressing bacterial colonization and the potential release of DAMPs via bacterial enzymatic degradation of plant cell walls, has triggered grapevine immune responses and the plant is now fighting an active infection. This indicates that the O antigen does, indeed, serve to shield the cells from host recognition, allowing them to establish an infection (Rapicavoli et al., in preparation).

Plants also modulate small RNA (sRNA) pathways based on recognition of PAMPs or pathogen effectors (Weiberg et al., 2014). sRNAs and RNA interference pathways are another important layer to the plant immune response and play a major role in the regulation of host immune responses. These sRNAs induce silencing of their target genes both at the transcriptional and post-transcriptional levels (Weiberg et al., 2014). High throughput sRNA profiling has been used to show that expression of endogenous host sRNAs are differentially regulated upon pathogen invasion in model and non-model plant systems (Weiberg et al., 2014; Katiyar-Agarwal & Jin, 2010; Seo et al., 2013). Specifically, an endogenous citrus microRNA was significantly up-regulated in trees infected with *Candidatus Liberibacter asiaticus*, causal agent of citrus huanglongbing (HLB). This sRNA was specifically found to be involved in the host phosphorus uptake pathway, and exogenous application of phosphorus reduced HLB severity (Zhao et al., 2013; Sagaram et al., 2009). sRNAs have been shown to be long range signals involved in plant defense against pathogens (Sarkies & Miska, 2014) and, in fact, can cross graft unions (Goldschmidt, 2014). We have initiated sRNA profiling in the *Xf*-grapevine interaction.

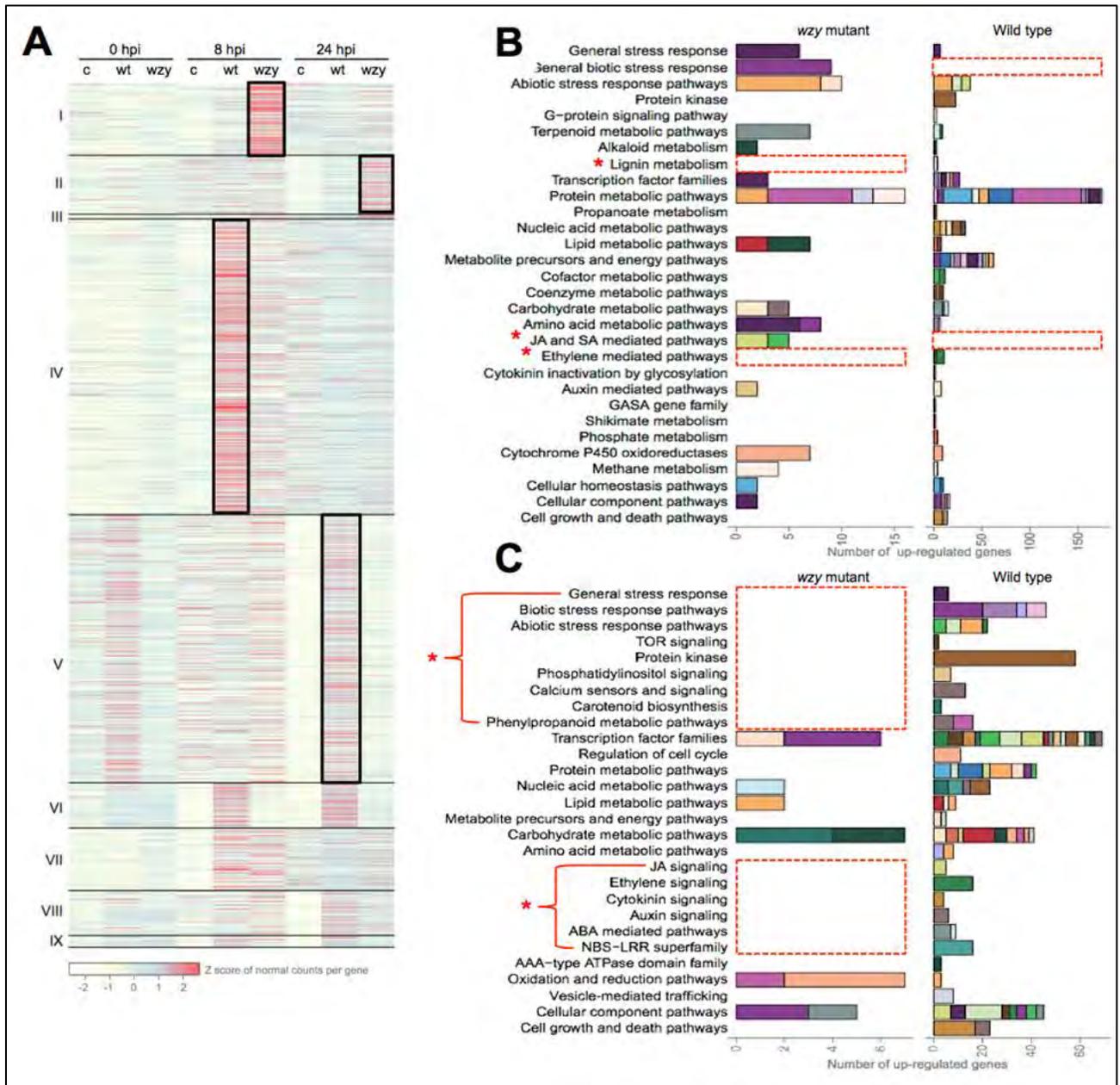


Figure 3. Grapevine responses to early infections by *wzy* mutant and wild-type *Xf*. (A) Up-regulated grape genes ($P < 0.05$) in response to *wzy* mutant (*wzy*) or wild-type (wt) bacteria at 8 and 24 hours post-inoculation (hpi) when compared to the wounded control (c). Genes are classified into nine groups (I - IX) based on their expression pattern. The colors in the heat map represent the Z score of the normal counts per gene, and black boxes represent gene groups in each treatment that exhibited the most pronounced differences in expression at each time point. (B) Enriched grape functional pathways ($P < 0.05$) among genes up-regulated during *wzy* (Group I) or wt (Group IV) infections at 8 hpi. (C) Enriched grape functional subcategories ($P < 0.05$) among genes up-regulated during *wzy* (Group II) or wt (Group V) infections at 24 hpi. Colored stacked bars represent individual pathways. Red boxes highlight functions of interest (*) that are enriched in one treatment, but not enriched in the other at each time point.

OBJECTIVES

1. Examination of the temporal response to *Xf* lipopolysaccharide.
2. Examination of *Xf* lipopolysaccharide-mediated defense priming in grapevine.
3. Linking *Xf* lipopolysaccharide structure to function.

RESULTS AND DISCUSSION

Objective 1. Examination of the temporal response to *Xf* lipopolysaccharide.

In addition to initiating PTI, PAMPs are known to induce systemic resistance (i.e., resistance in distal plant organs) (Erbs & Newman, 2003; Mishina & Zeier, 2007). Moreover, when used as a pre-treatment, LPS can systemically elevate resistance to bacterial pathogens in *Arabidopsis thaliana* (Mishina & Zeier, 2007), a phenomenon known as defense priming. It has been documented that a pathogen does not necessarily have to cause a hypersensitive response to elicit systemic resistance in the form of systemic acquired resistance (Mishina & Zeier, 2007). There is substantial experimental evidence indicating that *Xf* must achieve systemic colonization in the xylem in order to elicit Pierce's disease symptoms. In fact, mutants that stay localized at the original point of infection do not cause disease (Roper et al., 2005), and those that can move more rapidly throughout the xylem are hypervirulent (Newman et al., 2004; Guilhabert & Kirkpatrick, 2005). Because we have observed a decrease in Pierce's disease symptom severity following exposure to *Xf* LPS, we hypothesize that LPS may be involved in eliciting a downstream systemic defense response that prevents movement of *Xf* within the xylem network. This objective tests this hypothesis and further explores the spatial persistence of the observed tolerance to Pierce's disease in grapevines exposed to wild-type vs. *wzy* mutant cells using transcriptional and sRNA profiling of petioles distal to the initial inoculation site. In addition, we examined the temporal persistence of the elicited defense response by testing later time points in the infection process than in our initial study. This will provide much sought after information about which defense pathways, and possibly defense-related hormones, are induced by the *Xf* LPS PAMP in grapevine and, most importantly, may identify facets of those pathways that can be manipulated for Pierce's disease control.

Objective 1a. Transcriptome profiling.

The application of transcriptome profiling approaches using next generation RNA sequencing (RNA-seq) allows us to profile the expression of nearly all genes in a tissue simultaneously and monitor the activation or suppression of specific defense pathways at the genome scale. In this objective we shifted our focus to characterize the grapevine transcriptional response at systemic locations distal to the point of inoculation (POI) and at longer time points than our previous study, where we looked at early time points of 0, 8, and 24 hours post-inoculation. This tests our hypotheses that (i) truncated *Xf* O antigen is more readily perceived by the grapevine immune system, allowing the plant to mount an effective defense response to *Xf*, and (ii) that the initial perception of the truncated LPS belonging to the *wzy* mutant is propagated into a prolonged and systemic response.

In the summer of 2015 individual vines were inoculated with either wild-type *Xf*, the *wzy* mutant, or with 1x PBS buffer (Clifford et al., 2013). We inoculated three vines for each treatment. The cells were delivered mechanically by inoculating a 40 μ l drop of a 108 colony-forming unit (CFU)/ml bacterial cell suspension into the main stem near the base of the plant. Petioles were harvested at two different locations on the plant: at the POI (local) and five nodes above the POI (systemic). We harvested at four different time points post-inoculation: time 0 = petiole harvested just before pre-treatment, 48 hours, one week, and four weeks post-inoculation. All harvested petioles were immediately frozen in liquid nitrogen, prior to RNA extraction. RNA was extracted from the harvested petioles, and sequencing libraries were generated from the polyadenylated plant messenger RNA and sequenced using the Illumina HiSeq 2000 platform. Transcript expression levels were determined by alignment of the sequencing reads using the spliced transcripts alignment to a reference (STAR) aligner (Dobin et al., 2013) onto the PN40024 grape genome reference. Unmapped reads were *de novo* assembled using Trinity (Grabherr et al., 2011) to identify transcripts that were not present in the reference genome. Statistical inference using DESeq2 (Anders & Huber, 2010) was applied to determine with confidence the subset of genes that were up- or down-regulated by LPS treatment (Cantu et al., 2011b). Grape genes with significant differential expression were grouped into 26 clusters according to their patterns of expression across time points (**Figure 4**). Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways, specifically pectin modification, at four weeks post-inoculation (**Figure 4A**). This is a stark contrast with wild-type-inoculated vines, in which these pathways were up-regulated as early as eight hours post-inoculation. This likely explains why this pathway is not enriched in local tissue of wild-type-inoculated vines at these later time points. The induction of salicylic acid (SA)-mediated signaling pathways in *wzy*-inoculated vines was further supported by the presence of four genes, including two enhanced disease susceptibility 1 (EDS1) genes. EDS genes are known defense genes associated with the SA pathway and have been implicated in grapevine defenses against powdery mildew. The consistent enrichment and up-regulation of SA-associated genes (and thus, the maintenance of the signal), including the presence of PR-1 and other SA-responsive genes at eight hours post-inoculation, strongly suggests that the plant

is preventing the development of infections by *wzy* cells via an SA-dependent pathway. In wild-type vines consistent enrichment of jasmonic acid (JA)-associated genes was further supported by the presence of nine genes functioning in the metabolism of alpha-linolenic acid, which serves as an important precursor in the biosynthesis of JA (**Figure 4A**).

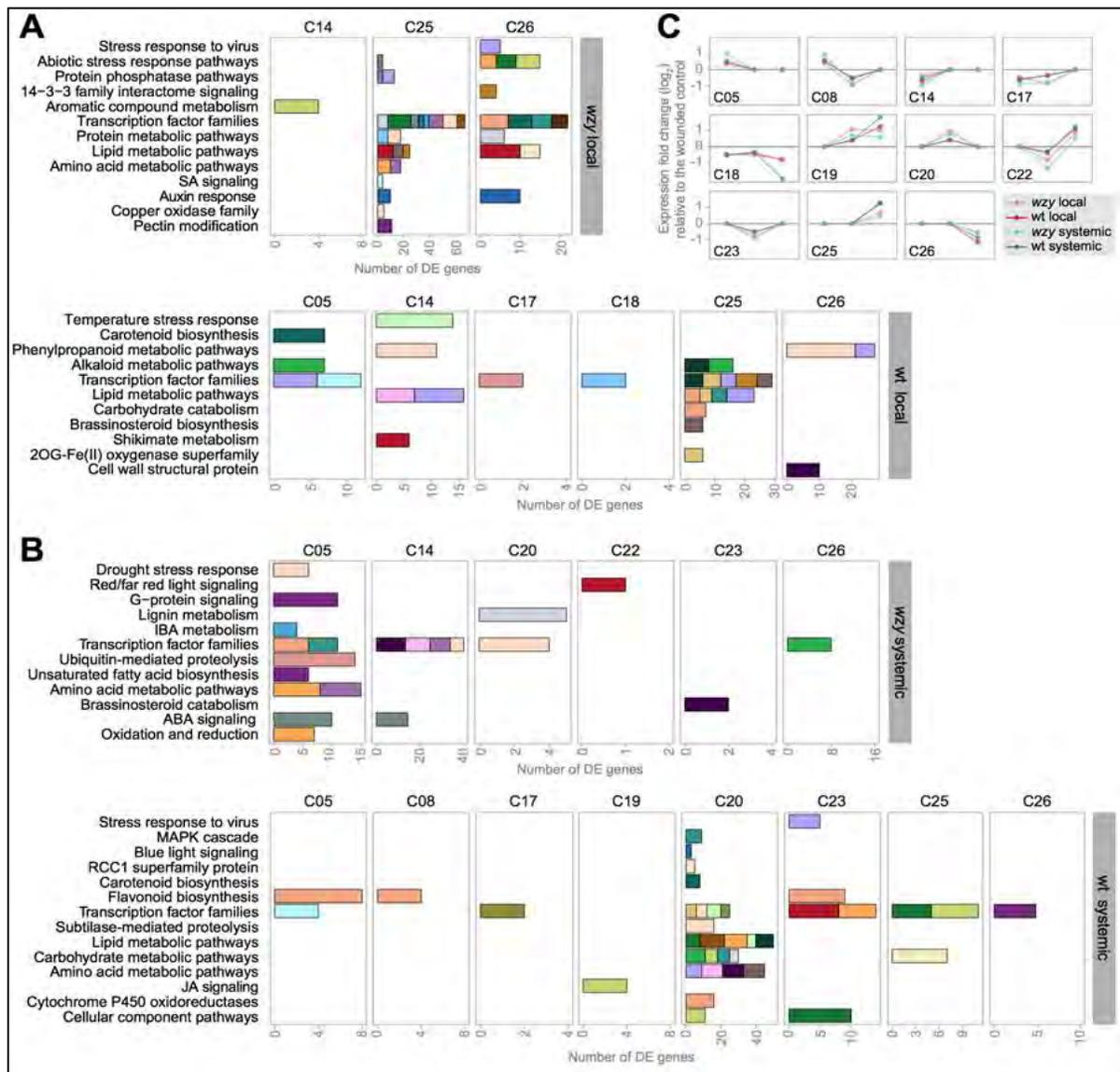


Figure 4. Transcriptomic analysis of late grapevine responses to *Xf* wild-type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ($P < 0.05$) in differentially expressed (DE) gene clusters representing local (A) or systemic (B) responses to *Xf* inoculation. Only enriched pathways related to grapevine immune responses and unique to wild-type (wt) or *wzy* mutant inoculations are depicted. Colored stacked bars represent individual pathways. (C) Patterns of expression of gene clusters enriched in functional pathways with biological relevance. Lines represent the medoids for each cluster. Dots represent expression fold changes of each medoid (\log_2) at a given time point post-inoculation (in order: 48 hours, 1 week, and 4 weeks) when compared to the wounded control.

Enrichment analyses of *wzy*-responsive genes in systemic tissue included drought stress response pathways, namely genes enriched in abscisic acid (ABA) signaling (seen at 48 hours post-inoculation) (**Figure 4B**). Subsequently at one week post-inoculation the enrichment of lignin metabolism genes is likely part of the vine's stepwise response to this abiotic stress. This is in contrast with wild-type-inoculated vines in which these pathways were enriched at eight hours post-inoculation. Enrichment analysis of wild-type-responsive genes in systemic tissue included regulation and signaling pathways, including mitogen-activated protein kinase (MAPK)

and G protein signaling (**Figure 4B**). Furthermore, genes enriched in ethylene transcription factors (ERFs) were up-regulated at four weeks post-inoculation, demonstrating that activation of ethylene-mediating signaling is perpetuated during the infection process. Notably, beginning at one week genes enriched in JA-mediated signaling pathways were up-regulated in systemic tissue and expression continued to increase at four weeks post-inoculation. This consistent enrichment and up-regulation provides further support for the role of JA in grapevine responses to wild-type *Xf*. Our findings establish that this phytohormone pathway is initiated within the first 24 hours post-inoculation and the signal is consistently maintained in both local and systemic tissue. A total of seven genes enriched in callose biosynthesis were up-regulated at four weeks post-inoculation in response to wild-type cells, which is over half of the total callose-related genes in the genome. The consistent up-regulation of these genes (beginning at 24 hours post-inoculation) establishes this structural barrier as an important plant defense response to *Xf* infection. Overall, the RNAseq data strongly indicate that during the days and weeks post-inoculation with *wzy* mutant cells grapevines are no longer fighting an active infection. We hypothesize that the intense *wzy*-induced oxidative burst during the first 24 hours post-inoculation, in combination with other pathogenesis-related responses, had a profound antimicrobial effect on invading *wzy* cells. These responses likely eliminated a large majority of *wzy* mutant populations and the plant no longer sensed these cells as a biotic threat. In contrast, following recognition of wild-type *Xf* cells at 24 hours post-inoculation, grapevines began responding to an active threat and initiated defense responses such as the production of phytoalexins and other antimicrobial compounds. Furthermore, these vines were actively trying to prevent systemic spread of the pathogen through the production of structural barriers such as tyloses and callose.

Objective 1b. Histological examination of grapevines inoculated with *Xf* wild-type or the O antigen mutant.

To corroborate the enrichment of plant cell wall metabolic pathways seen in the transcriptomic data we performed histological examination of stem tissue in grapevines inoculated with *Xf* wild-type or *wzy* mutant or 1x PBS control. Vascular occlusions are commonly produced by plants in response to infection with vascular pathogens. Tyloses are outgrowths of the xylem parenchyma cell into the vessel lumen and are abundant in Pierce's disease-susceptible grapevines. In fact, in susceptible grape genotypes tyloses can occur in over 60% of the vessels in a transverse section of vascular tissue (Sun et al., 2013). Tylose formation is considered a late response to *Xf*. Thus, we examined tylose formation in grapevines at 18 weeks post-inoculation with wild-type or *wzy* mutant *Xf* cells, compared with 1x PBS control vines. *Wzy* mutant-inoculated vines rated a 2 or below, representing a few leaves exhibiting marginal necrosis; wild-type-inoculated vines rated over 3, representing over half of the vine exhibiting foliar necrosis; and 1x PBS controls rated 0, showing no Pierce's disease symptoms (**Figure 5, panel A**). We observed pronounced differences in the abundance of tyloses in response to wild-type vs. *wzy* mutant-inoculated plants. In wild-type-inoculated vines, tyloses were present in nearly all xylem vessels (**Figure 5, panel B**), and vessels were often completely occluded with multiple tyloses (**Figure 5, panel C**). In contrast, *wzy* mutant-inoculated vines contained very few tyloses. In the case where a tylose was present it was often one large tylose that only partially occluded the vessel. All control vines, inoculated with 1x PBS, were free of occlusions. In addition to tyloses the plant vascular tissue can initiate additional reinforcement of the cell walls to limit bacterial growth in infected plants. This includes callose and suberin deposition. Light microscopy of infected stems revealed widespread deposition of callose in the phloem tissue of *Xf* wild-type-infected plants (**Figure 6, arrow**), suggesting communication between the xylem and phloem regarding the presence of *Xf*. This is the first evidence of callose production in grapevine in response to *Xf*. In addition, we also provide the first evidence of a pronounced deposition of suberin, associated specifically with tylose-occluded vessels (**Figure 6***). In contrast, *wzy* mutant-infected plants showed little to no evidence of either callose or suberin in the vascular tissue, and these plants looked similar to 1x PBS control plants.

Objective 1c. Global sRNA profiling.

This portion of the study is being conducted in close collaboration with Hailing Jin (UC Riverside), an expert in the field of plant sRNAs and their role in plant defense against pathogen attack. We propose to characterize the endogenous grapevine sRNAs that are elicited by *Xf* invasion in an LPS-mediated fashion. Our goal is to identify sRNAs in grapevines that are up-regulated during *Xf* invasion. More specifically, we are focusing our study on sRNAs that are a part of propagating the defense response elicited by the *Xf* LPS PAMP. sRNAs have been shown to be long range signals involved in plant defense against pathogens (Sarkies & Miska, 2014) and can cross graft unions (Goldschmidt, 2014). We envision that, in a future study, the identified sRNA(s) could potentially be exploited for disease control by transforming rootstocks to produce the sRNA for delivery into the scion.

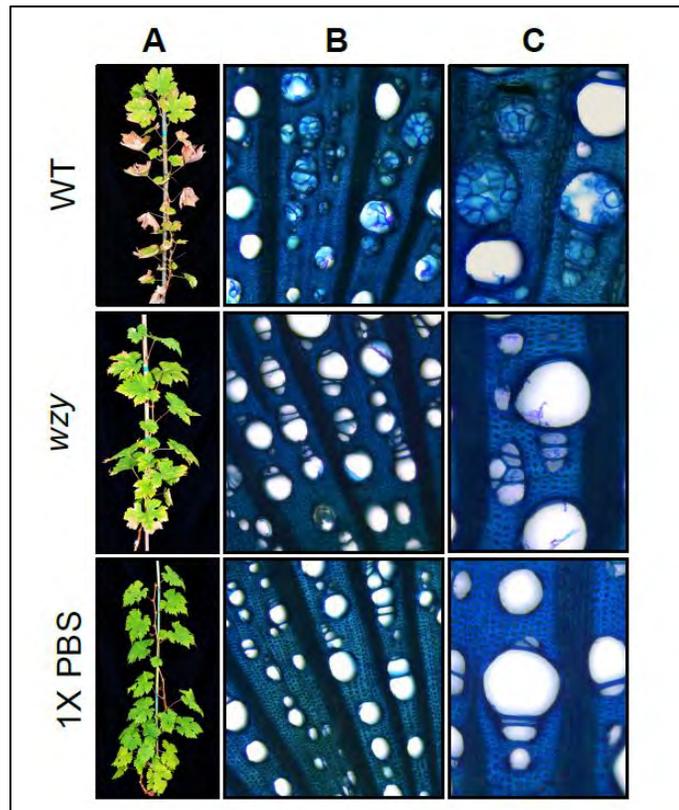


Figure 5. Tylose development in Pierce's disease-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild-type *Xf* cells, *wzy* mutant cells, or 1x PBS buffer. (A) Representative images of Pierce's disease progress prior to histological examination. (B) Micrograph showing tylose production in cross sections of grapevine xylem (brightfield Toluidine Blue O). (C) Close-up of xylem vessels showing complete occlusion with multiple tyloses (*) in wild-type-inoculated vines. A few small tyloses also occurred in these vines (closed arrowheads). Tyloses were largely absent in the xylem vessels of *wzy* mutant-inoculated vines. No tyloses were present in the stems of 1x PBS-inoculated vines.

Construction and sequencing of sRNA libraries

We have isolated sRNAs from the petioles harvested from the same plants that were inoculated in objective 1a using an optimized Trizole extraction protocol that allows for isolation of mRNA as well as of sRNAs, for RNA-seq and small RNA-seq analyses, respectively (Cantu et al., 2010). sRNA libraries were produced using the TruSeq Small RNA Sample Preparation Kit and subjected to multiplex sequencing using an Illumina HiSeq2500 platform. Adapters were trimmed using CLC Genomics Workbench. Approximately 116 million RNA reads with length ranging from 18 to 26 nt were obtained. In all samples reads showed a similar and expected pattern of size distribution, with peaks at 21 and 24 nt. These reads corresponded to an average of one million unique small RNA sequences per sample. Protein coding gene targets in the *V. vinifera* PN40024 genome could be identified unambiguously for 20% of the small RNA sequences. An average of 4,557 gene targets per sample were identified. The small RNA sequences included 134 of the known *Vitis* microRNAs. As recently reported by Kullán et al. (2015 <http://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-015-1610-5>) the vvi-miR166 family was the most abundant, representing about 94% of the total expression counts. These results show that we can successfully extract, sequence, and annotate small RNAs from grape petioles. Further work will be carried out to identify small RNAs that accumulate differentially in plants inoculated with the different *Xf* strains.

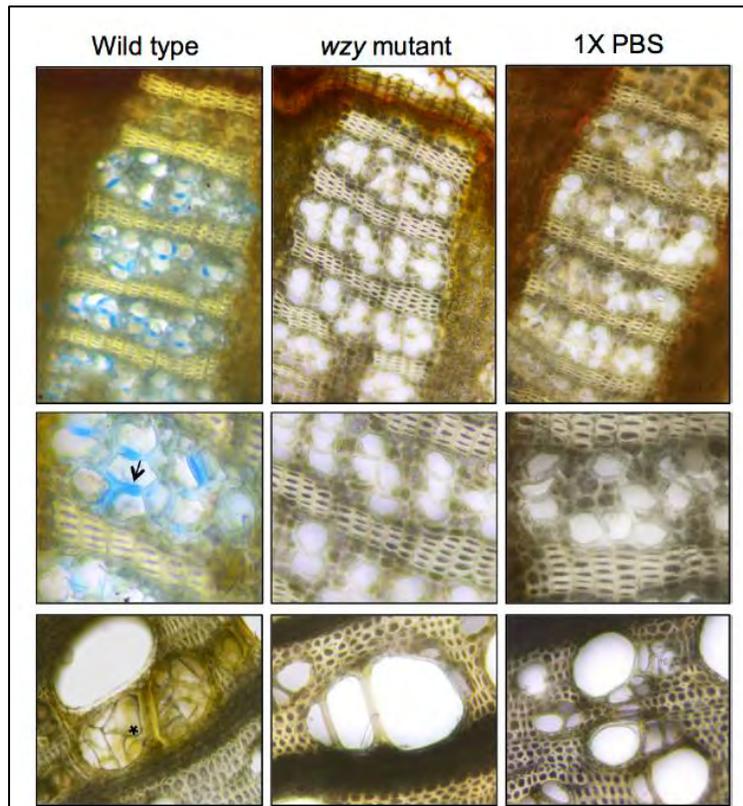


Figure 6. Callose and suberin deposition in Pierce's disease-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild-type *Xf* cells, *wzy* mutant cells, or 1x PBS buffer. Wild-type-inoculated plants exhibited widespread callose deposition in the phloem tissue (appears as blue color, indicated by arrow). In addition, there was pronounced deposition of suberin in xylem vessels (indicated by gold color), especially in vessels with multiple tyloses (*). No callose or suberin was present in the stems of 1x PBS-inoculated vines.

Objective 2. Examination of *Xf* lipopolysaccharide-mediated defense priming in grapevine.

Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This phenomenon is referred to as priming and stimulates the plant to initiate a more rapid and robust response against future invading pathogens (Conrath, 2011). In this objective we hypothesize that pre-treatment with LPS isolated from *Xf* O antigen mutants results in a difference in the grapevine's tolerance to *Xf* by stimulating the host basal defense response.

Objective 2a. Temporal persistence of LPS-mediated defense priming.

In the summer of 2015 we inoculated 20 grapevines/treatment/time point with 50 µg/ml of either wild-type or *wzy* mutant LPS re-suspended in diH₂O. Vines inoculated with diH₂O alone served as the negative controls for the experiment. Based on our previous greenhouse trials we have found that 50 µg/ml is a suitable concentration to elicit an oxidative burst and to potentiate defense priming in grapevines. This is also in agreement with studies performed in *A. thaliana* (Zeidler et al., 2004). Thus, we used the same LPS concentration for this objective. The LPS was delivered by needle-inoculating a 40 µl drop of the LPS preparation into the main stem at the base of the plant. We then challenged 15 of the vines for each treatment by inoculating 40 µl of a 10⁸ CFU/ml suspension of live wild-type *Xf* cells in 1x PBS at either 4 hours, 24 hours, 48 hours, 1 week, or 4 weeks post-LPS treatment. The remaining five vines/treatment/time point were inoculated with 1x PBS to serve as negative controls. We included the additional later time points (48 h, 1 week, and 4 weeks) because we also wanted to establish the duration of the priming effect following treatment with LPS. These inoculations were performed using the pin-prick method as previously described (Hill & Purcell, 1995). The live wild-type cells were inoculated near the point of the original LPS inoculation. Plants were visually examined for Pierce's disease symptom development throughout the infection process and rated on an arbitrary disease rating scale of 0 to 5, where 0 = healthy and 5 = dead or dying (Guilhabert & Kirkpatrick, 2005). Data was consistent with the previous year for the 4 and 24 hour time points, but we did not see significant attenuation of Pierce's disease symptoms in the remaining later

points. This indicates that the primed state may be transient, and it is possible that these plants may need repeated applications of LPS throughout the trial to help maintain the primed state. We plan to conduct a future experiment examining the efficacy of repeated applications of LPS on the development of Pierce's disease. Furthermore, enumeration of bacterial populations in both local (POI) and systemic (five nodes above POI) tissue at four weeks post-challenge with *Xf* cells was consistent with the previous year, in which titer was not significantly different between treatments. Because we do see a difference in disease progress in the earlier time points there may be differences in other host defense responses, such as the production of tyloses and other host-derived vascular obstructions. We will repeat this experiment, and in addition to evaluating titer and Pierce's disease symptom development, we will perform additional histochemical examination of tissue.

Objective 2b. Examination of persistence of defense priming through dormancy.

In the fall of 2015 we pruned back all the grapevines inoculated in this objective and allowed them to go dormant. We examined the temporal phenology of these grapevines throughout the winter months and into the spring of 2016. The premise of this part of the objective relates to the normal phenology of a grapevine which is impacted by infection with pathogens. Typically, grapevines severely infected with Pierce's disease will have abnormal leaf emergence the following spring and will remain stunted throughout the growing season. We hypothesized that the grapevines that did not receive LPS pre-treatment would have poor leaf emergence and be severely stunted. Conversely, we hypothesized that grapevines pre-treated or "primed" with LPS would have better growth and vigor as compared to those that did not receive pre-treatment. We had originally planned to score the grapevines in the spring on a scale of 1 to 3 where 1 = vigorous leaf emergence, 2 = delayed leaf emergence, and 3 = poor/no leaf emergence. Once the negative control plants (those challenged with only 1x PBS) had passed the phenological stage of leaf emergence and exhibited Pierce's disease symptoms we had planned to revert to rating them on the established Pierce's disease symptoms (described in objective 2a). While the vines produced new shoots following the winter months we did not observe the difference in leaf emergence or vigor between the treatments. It is possible that the vines were pruned too severely, removing a majority of *Xf* inoculum.

Objective 3. Linking *Xf* lipopolysaccharide structure to function.

In our currently funded proposal we endeavored to obtain structural data for both wild-type and the truncated *wzy* mutant LPS, particularly the structure of O-chain by using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. These experiments were conducted in close collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens, Georgia. Through glycosyl composition analysis (trimethylsilyl methyl glycosides-TMS, alditol acetates-AA) (York, 1985) of the LPS and composition and linkage analysis (partially methylated alditol acetates (PMAA); Ciucanu & Kerek, 1984) of O-specific polysaccharide, the CCRC has confirmed that the *Xf* wild-type high molecular weight O antigen is comprised primarily of 2-linked rhamnose, verifying previously reported *Xf* LPS compositions (Clifford et al., 2013). They have also confirmed that the *wzy* mutant LPS is lacking the high molecular weight O antigen present in wild-type cells and appears to be capped with a single rhamnose residue (**Figure 7A**). The CCRC has recently completed extensive isolation and purification of core and O-chain polysaccharides. Knowledge of the structure of the LPS is critical to understanding which portions contain the elicitor activity. The carbohydrate portion of LPS (core + O-chain) was released from lipid A by mild acid hydrolysis and the O-chain was purified by size exclusion and other chromatography techniques. A structure of the polymer was determined via NMR spectroscopy and mass spectrometry and absolute configuration of sugars (d-, l-) in the polymer was determined by GC-MS (Gerwig et al., 1978).

In order to describe structural properties of O antigen in wild-type and *wzy* mutant LPS the polysaccharide moiety (O antigen + core) was liberated from LPS (lipid A) and resolved based on molecular size. Comparative analysis of size-exclusion chromatography (SEC) profiles indicated different distributions of polysaccharides in both strains. In the wild-type strain, a majority of polysaccharide (40.8% total column load) was eluted in Fraction III (average molecular mass of approximately 10-20 kD) and a remainder (24.8% of total column load) in Fraction IV (**Figure 7B**). In contrast, a majority of *wzy* polysaccharide (55.0% total polysaccharide column load) was eluted in Fraction IV (average molecular mass below 10 kDa), which was only present in low quantity in the wild-type parent. This fraction likely represented different molecular size forms of core oligosaccharide or truncated core-O antigen polysaccharide. Fraction I that was eluted in void (Vo) column was due to traces of unhydrolyzed intact LPS. Monosaccharide analysis, including the determination of absolute configurations of O

antigen polysaccharides from the wild-type strain (SEC fraction III), confirmed the presence of L-rhamnose and D-xylose in an 8:1 molar ratio.

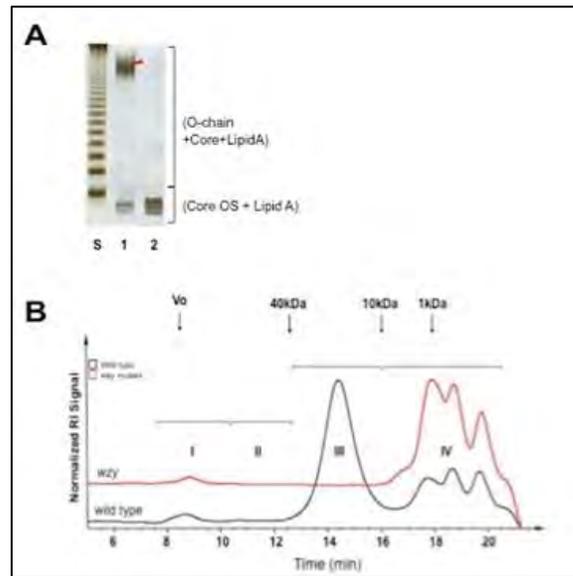


Figure 7. LPS composition and structure analysis. (A) DOC-PAGE analysis of LPS isolated from *Xf* wild-type and *wzy* mutant. Lane S = *Salmonella enterica* s. *Typhimurium*, S-type LPS; Lane 1 = wild-type; Lane 2 = *wzy* mutant. Red arrow indicates the presence of high molecular weight O antigen that is not observed in the *wzy* mutant LPS. (B) SEC chromatograms of polysaccharides liberated from LPS of *Xf* wild-type (black) and *wzy* mutant (red). Standard dextrans of 40,000, 10,000, and 1,000 Da were used for calibration of the Superose 12.

CONCLUSIONS

RNA-seq and histological analysis show the grapevine defense system is able to recognize a truncated LPS molecule, resulting in a strong oxidative burst and a small production of tyloses. Grapevines produce many tyloses, phytoalexins and other antimicrobial compounds when inoculated with *Xf* wild-type. In addition, Pierce's disease symptoms are attenuated when grapevines are challenged with *Xf* 4 hours and 24 hours after LPS treatment, showing that the LPS molecule is able to prime defenses against *Xf*. Finally, we present the first evidence that the major polysaccharide present in *Xf* wild-type O antigen is a linear α 1-2 linked rhamnan. We show *Xf* high molecular O antigen is a critical virulence factor in Pierce's disease. Our results provide unprecedented insight into the molecular mechanisms underlying host-pathogen interaction in Pierce's disease.

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CHARACTERIZATION OF *XYLELLA FASTIDIOSA* PLANT CELL WALL DEGRADATION AND INHIBITION OF THE TYPE II SECRETION MACHINERY

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ABSTRACT

Xylella fastidiosa (*Xf*) is a xylem-limited, fastidious bacterium that causes Pierce's disease in grapevine. The xylem is arranged as a series of separate vessels that are connected via paired pits. Each pit contains a pit membrane comprised of a meshwork of cellulose, hemicellulose, and pectin. *Xf* cannot passively traverse these pit membranes and must rely on its consortia of cell wall-degrading enzymes (CWDEs) to digest the membrane in order to move to the next xylem vessel. In response, the grapevine host enacts defense measures to disrupt pathogen movement in the xylem, including the production of tyloses. Indeed, there is a strong correlation between Pierce's disease severity and excessive tylose formation. The damage-associated molecular patterns (DAMPs) that trigger tylose formation are not currently understood, and we hypothesize that specific small chain oligosaccharides (OGs) generated by CWDE digestion of pit membranes may induce tylose production. Furthermore, differences in pit membrane structure and modification among *Vitis vinifera* varieties may yield particular OG profiles when degraded, and thus may account for varying degrees of tylose formation. Consequently, the induction of tylose formation by OGs may be linked to susceptibility and tolerance of *Xf* among different varieties. Accordingly, the disruption of *Xf* CWDE production could serve to limit both pathogen movement and detrimental tylose formation. Bacterial CWDEs are secreted into the environment via the Type II secretion system (T2SS). *Xf* maintains a functional T2SS and likely relies on it to secrete its many CWDEs into xylem vessels. Therefore, inhibition of the T2SS may disrupt CWDE dispersion, thus limiting *Xf* mobility in the xylem and preventing excessive xylem blockage.

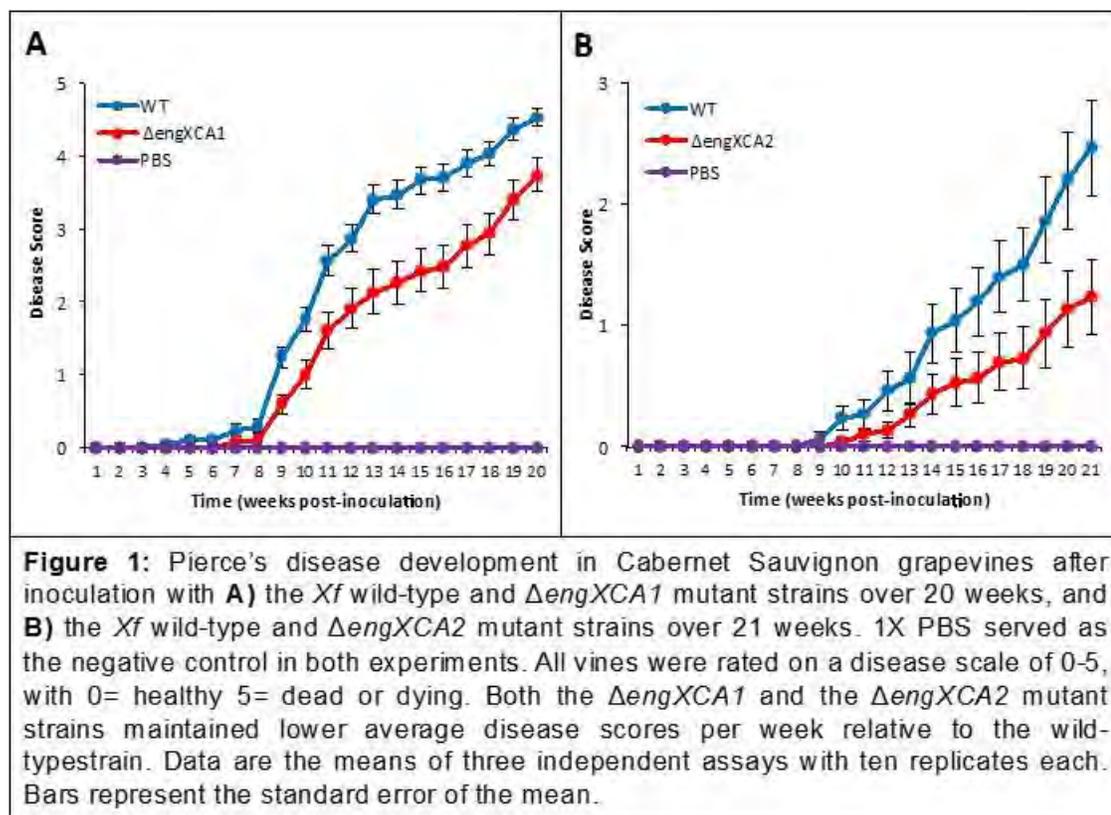
LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) relies on degradation of the plant cell wall to move within the grapevine, which occurs through cooperation between at least two classes of enzymes that target different carbohydrate components of the complex scaffold of the plant cell wall. A major goal of this project is to elucidate the mechanisms that lead to disassembly of the plant cell wall that eventually leads to systemic colonization of *Xf* in grapevines. Here we propose experiments designed to better understand what facilitates movement of the bacterium and the subsequent clogging of the water-conducting cells that worsens Pierce's disease severity. In addition, we also outline experiments that inhibit the secretion machinery responsible for delivering the *Xf* enzymes that are involved in *Xf* movement throughout the plant, thus providing a comprehensive approach to restriction of *Xf* and disease development rather than targeting individual enzymes.

INTRODUCTION

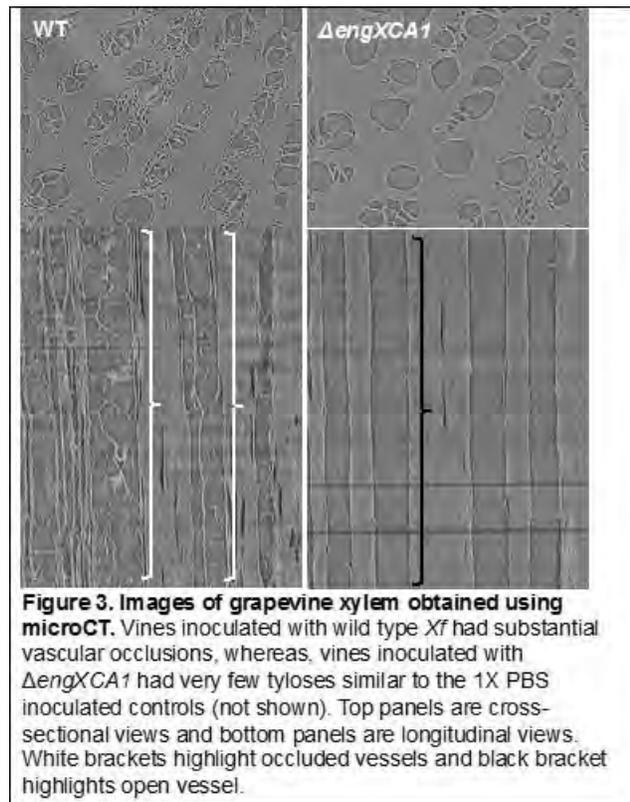
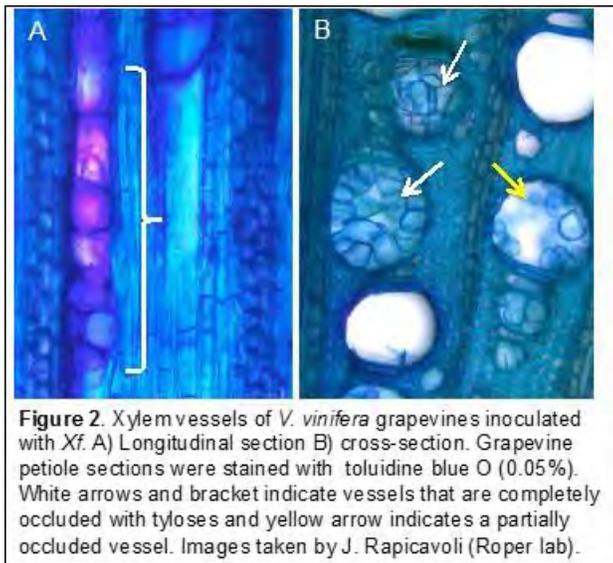
Xylella fastidiosa (*Xf*) is the causal agent of Pierce's disease of grapevine, a serious and often lethal disease (Hopkins and Purcell, 2002; Chatterjee et al., 2008; Purcell and Hopkins, 1996). This xylem-limited bacterial pathogen colonizes the xylem and in doing so must be able to move efficiently from one xylem vessel element to adjacent vessels (Roper et al., 2007). Xylem conduits are separated by pit membranes (PMs) that are composed of primary cell wall and serve to prevent movement of air embolisms and pathogens within the xylem (Buchanan, 2000). More specifically, PMs are composed of cellulose microfibrils embedded in a meshwork of pectin and

hemicellulose (Buchanan, 2000). The pore sizes within that meshwork range from 5 to 20 nM, which will not allow passive passage of *Xf* cells whose size is 250 to 500 x 1,000 to 4,000 nM (Perez-Donoso et al., 2010; Mollenhauer & Hopkins, 1974). Based on functional genomics and *in planta* experimental evidence, *Xf* utilizes cell wall-degrading enzymes (CWDEs), including three putative endoglucanases (EGases) and one polygalacturonase (PG), to actively digest the polymers within the PMs, thereby facilitating its movement throughout the xylem network (Simpson et al. 2000; Roper et al., 2007; Perez-Donoso et al., 2010). It is known that PG is a major pathogenicity factor for *Xf* (Roper et al., 2007) and that it acts in concert with at least one EGase to breach the PM barrier (Perez-Donoso et al., 2010). EGases are implicated in virulence and colonization of the xylem in other bacterial phytopathogens, such as *Pantoea stewartii* subsp. *stewartii*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *campestris* (Gough, 1988; Roberts et al., 1988; Saile et al., 1997; Mohammadi et al., 2012). In our previous study (project # 14-0144-SA) we tested the role of the *Xf* EGases *in planta* by constructing deletion mutants in two of the EGases ($\Delta engXCA1$ and $\Delta engXCA2$) and mechanically inoculating the modified *Xf* lines into *Vitis vinifera* cv. Cabernet Sauvignon and cv. Chardonnay grapevines. Interestingly, both $\Delta engXCA1$ and $\Delta engXCA2$ achieved the same titers (*data not shown*) in the Cabernet Sauvignon vines as wild-type *Xf*, yet they were significantly less virulent and elicited fewer Pierce's disease symptoms (**Figure 1A** and **1B**).



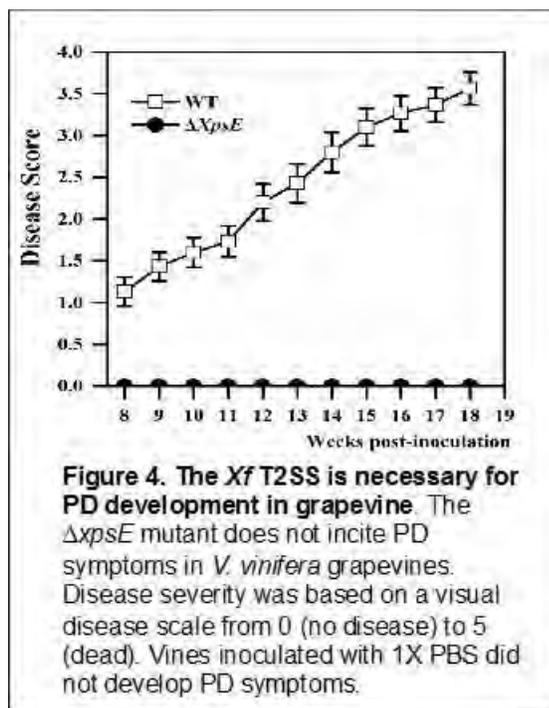
Pierce's disease symptom development is tightly correlated with the ability of *Xf* to degrade specific polysaccharides, namely fucosylated xyloglucans (part of the hemicellulosic component) and weakly esterified homogalacturonans (part of the pectin portion), that make up the intervessel PMs (Sun et al., 2011). In general, pectin is one of the first targets of cell wall digestion for invading pathogens, and the resulting oligogalacturonides (OGs), which are smaller pieces of the pectin polymer, that are released are likely used as a carbon source for the invading pathogen. In addition, specific OGs with a degree of polymerization in the size range of 10 to 15 residues can also serve as signals that trigger host defense responses (Benedetti et al., 2015). These responses include accumulation of reactive oxygen species (ROS), expression of pathogenesis-related proteins, deposition of callose, and activation of mitogen-activated protein kinases (MAPKs), among other defense related processes (Boller & Felix, 2009; Benedetti et al., 2015).

Tyloses are outgrowths of parenchyma cells that emerge through vessel-parenchyma pits into vessel lumen, and are common in a wide range of species (Bonsen and Kučera, 1990; Esau 1977; Tyree and Zimmermann, 2002). Tyloses impede fluid penetration (Parameswaran et al., 1985) and induce a permanent state of reduced hydraulic conductivity, and are triggered by abiotic and biotic stresses, such as pathogen infection (Aleemullah and Walsh 1996; Collins et al. 2009; Dimond 1955; Parke et al. 2007). Tylose formation is the predominant vascular occlusion associated with *Xf* infection (**Figure 2A and 2B**), and excessive tylose development has been linked to the extreme susceptibility of *V. vinifera* wine grapes to Pierce's disease (Fritschi et al. 2008; Sun et al. 2013). Importantly, rates of tylose development in *V. arizonica*, a resistant species, are much lower than those in *V. vinifera*, which may reflect differing innate immune responses to the presence of *Xf* in the xylem. To our knowledge no one has looked at the molecular mechanisms underlying the differences in response to *Xf* among different *V. vinifera* cultivars. Thus, we propose to better understand this difference in cultivar response to *Xf* in the context of host cell wall degradation and the elicitation of specific defense responses that lead to tylose formation in grapevines. Interestingly, a preliminary analysis of tylose formation in Cabernet Sauvignon vines inoculated with the $\Delta engXCA1$ mutant using a high resolution micro-computed tomography (microCT) technique (a kind of CAT scan) by the McElrone laboratory determined that these vines exhibited fewer tyloses than those inoculated with wild-type *Xf* (**Figure 3**). Therefore, our hypothesis is that enzymatic degradation of the plant cell wall by *Xf* CWDEs is generating cell wall fragments that elicit DAMP signaling defense pathways, which leads to downstream tylose production and Pierce's disease symptom development in certain grape cultivars.



Given that *Xf* CWDEs are important for the degradation of pit membranes (thus allowing systemic colonization), and their potential role in inducing tylose formation, it is imperative that these virulence factors are targeted for inhibition. However, inhibiting each CWDE individually as a commercial strategy for controlling *Xf* is both impractical and costly. Interestingly, these CWDEs are predicted (using SignalP software) to be secreted via the Type II secretion system (T2SS). The T2SS is a molecular nanomachine that transports pre-folded proteins from the periplasm across a dedicated channel in the outer membrane (Cianciotto, 2005; Korotkov et al., 2012). The T2SS systems of many plant and animal pathogens are either known or predicted to secrete proteins, namely polymer degrading enzymes, which are involved in nutrient acquisition (Jha et al., 2005). The *Xf* CWDEs being

studied in this proposal are predicted (using SignalP software) to be secreted through the T2SS. 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). *Xf* appears to only possess the Sec-dependent secretion pathway. Because of our interest in host CWDEs and their mechanism of secretion we created a mutation in the *xpsE* gene, which encodes 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 grapevine response to the *XfpglA* mutant (Figure 4). We hypothesize that this is due to the pathogen's inability to secrete the CWDEs necessary for xylem colonization. In addition, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. We observed that the $\Delta xpsE$ mutant produces visibly less exopolysaccharide (EPS) on XFM minimal medium containing pectin as the sole carbon source, resulting in a much less mucoid phenotype (*data not shown*). However, when wild-type *Xf* and $\Delta xpsE$ 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. 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 PG and the subsequent breakdown of pectin.



Thus, we have compelling *in planta* and *in vitro* preliminary data indicating that *Xf* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process. From this we reason that the T2SS represents an excellent target for disease control because disrupting this system would provide comprehensive inhibition of secretion of PG (the major pathogenicity factor for *Xf*) and the other auxiliary CWDEs (Roper et al., 2007, and recent results discussed above). Therefore, identifying molecules that can inhibit T2SS function is an excellent avenue of research to pursue to develop strategies that mitigate Pierce's disease by preventing pathogen ingress.

OBJECTIVES

1. Qualitative analysis of the effect of cell wall degradation on the grapevine response to *Xf*.
2. Quantitative analysis of plant defense pathways induced by *Xf* cell wall degrading enzyme activity: biochemical and transcriptional studies.
3. Inhibition of the Type II secretion system using natural products produced by grapevine microbial endophytes.

RESULTS AND DISCUSSION

In the context of plant cell wall degradation, we will examine the effects that different *Xf* mutants ($\Delta engXCA1$, $\Delta engXCA2$, *egl* (all EGases and EGase/expansin hybrid) and *pglA* (a PG)) have on integrity and carbohydrate composition of grapevine pit membranes using both microscopic and immunological techniques coupled with fluorescence (Sun et al., 2011) and/or electron (Sun et al., unpublished) microscopy. Finally, we will couple these microscopic observations with macroscopic studies of the spatial distribution of tyloses and other vascular occlusions, such as plant-derived gels and bacterial aggregates using high resolution microCT. This non-destructive method technique uses x-rays to create cross-sections of an object that can be used to recreate a virtual model (3D model). These experiments will allow us to match degradation of specific host cell wall carbohydrates with spatiotemporal patterns of production of tyloses in three dimensions. We will do these experiments in two different *V. vinifera* cultivars, Cabernet Sauvignon and Chardonnay, because of the difference in Pierce's disease severity we have observed thus far in their response to our EGase mutants in these varieties.

Wild-type *Xf*, $\Delta engXCA1$, $\Delta engXCA2$, and $\Delta pglA$ mutants have been used to inoculate Cabernet Sauvignon and Chardonnay grapevines in the greenhouse. Vines inoculated with phosphate-buffered saline (PBS) will serve as negative controls. Each *Xf* strain was inoculated into 27 plants and Pierce's disease symptoms were rated each week using a 0 to 5 Pierce's disease rating index (Guilhbert and Kirkpatrick, 2005). Vine tissue samples are currently being collected for each of the three experiments: stem and petiole tissue for RNAseq, stem tissue for microCT analysis, and stem explants for electron microscopy (EM) analysis. Samples from three biological replications (consisting of three technical replications) per treatment are being collected at each of three time points covering early, mid, and late infection based on the Pierce's disease rating index: early infection = 1 - 2, mid-infection = 2 - 3, and late infection = 4 - 5. Once all of the samples have been collected the RNAseq, microCT, and EM analyses will be completed to determine the differential responses of each variety to each of the different *Xf* strains.

CONCLUSIONS

This project was initiated in July 2016. Therefore, we do not have any conclusions at this time.

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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, 2016 through October 2016.

ABSTRACT

The UC Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103, 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 resistance strategies are to be successfully 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 California. To that end, we plated anthers from grape rootstocks 110R (clone 01), 140Ru (clone 01), 3309C, Freedom (clone 1), GRN 1(clone 1.1), Harmony, MGT 420A (clone 04), and Salt Creek, as well as scion genotypes Cabernet Sauvignon (clone 07 and 08), Chardonnay (clone 04), French Colombard (clone 04), and Merlot (clone 03). Embryogenic cultures have been generated from anther filaments for 110R, 140Ru, Freedom, GRN1, Harmony, MGT 420A, Cabernet Sauvignon, Chardonnay, French Colombard, Merlot, and Zinfandel. We have successfully established suspension and embryo cultures for grape genotypes 110R, 140Ru, Freedom, MGT 420A, Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot. Transformation experiments using DsRed have been initiated on suspension culture and stored embryo cultures in order to access the utility of our existing transformation technologies in transforming these additional genotypes. To date, we have successfully generated transgenic plants for 101-14, 110R, 1103, Chardonnay, Freedom, and French Colombard. Acclimatization of grape plantlets to soil has been problematic in the past, but altering the soil composition used to transfer plantlets to soil has significantly improved survival.

LAYPERSON SUMMARY

The UC Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103, 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 101-14 and 1103 will need to be modified in order to adequately address the rootstock requirements of the diverse wine growing regions in California. We therefore are currently testing if our method for genetically modifying grape rootstocks can be used successfully on eight additional rootstock genotypes used in California wine grape production. These include 110R, 140Ru, 3309C, Freedom, GRN1, Harmony, MGT 420A, and Salt Creek. Since it is not yet known if a rootstock-mediated disease resistance strategy will prove to 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, Chardonnay, French Colombard, Merlot, Pinot Noir, and Zinfandel. 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 101-14 and 1103 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 101-14 and 1103 at the UC Davis Plant Transformation Facility to

additional rootstock germplasm important for the California wine industry. For this project we are testing eight additional rootstocks for their amenability to transformation, including 110R, 140Ru, 3309C, Freedom, GRN1, Harmony, MGT 420A, 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, we are also screening six important California scion genotypes for their amenability to transformation, including Cabernet Sauvignon (clone 07 and 08), Chardonnay (clone 04), French Colombard (clone 02), Merlot (clone 03), Pinot Noir (2A), and Zinfandel (clone 01A). Although it is unlikely that all eight 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 eight rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.
2. Develop embryogenic suspension cultures for eight rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use in transformation experiments.
3. Establish a germplasm bank of somatic embryos for eight rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.
4. Test transformation efficiencies of eight rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.
5. Test direct cell suspension transformation technology on eight rootstock genotypes and six scion genotypes.
6. Secure *in vitro* shoot cultures for eight 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 the Mezzetti et al., 2002 bulk meristem transformation system for eight rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

RESULTS AND DISCUSSION

Objective 1. Develop embryogenic cultures from anthers of eight rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.

During the spring of 2015 we collected anthers of rootstock genotypes including 101-14, 110R (01), 140Ru (01), 1103, 3309C (05), Freedom (01), MGT 420A (04), and Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Chardonnay (clone 04), French Colombard (clone 04), Pinot Noir (clone 2A), and Zinfandel (clone 01A) and plated them on four different embryogenic callus-inducing media. The media include Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/liter sucrose, 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 mg/liter benzylaminopurine (BAP) (PIV); MS minimal organics medium supplemented with 20 g/liter sucrose, 1.0 mg/liter 2,4-D, and 0.2 mg/liter BAP (MSE); MS minimal organics medium supplemented with 30 g/liter sucrose, 1.0 mg/liter 2,4-D, and 1.0 mg/liter BAP (MS1); or one-half strength MS minimal organics medium supplemented with 15 g/liter sucrose, 1.0 mg/liter naphthoxyacetic acid (NOA), and 0.2 mg/liter BAP (NB). During spring 2016 we collected anthers of rootstock genotypes 3309C, GRN, Harmony, and Salt Creek as well as scion genotypes Cabernet Sauvignon, Merlot, and Zinfandel. To date, we have demonstrated that we can successfully establish somatic embryo cultures from anther filaments for rootstock genotypes 101-14, 110R, 140Ru, 1103, Freedom, GRN1, Harmony, and MGT 420A, and scion genotypes Cabernet Sauvignon, Chardonnay, French Colombard, Merlot, and Pinot Noir (**Table 1, Figure 1**). Embryos from Pinot Noir appear recalcitrant to generate secondary embryos on our media and tend to germinate instead. Therefore, it is not known if we will be able to generate a stably multiplying culture for this genotype. We have generated callus for Zinfandel, but it does not appear to be embryogenic.

Table 1.

Grape Anther Culture	PIV		MSI		MSE		NB	
	2015	2016	2015	2016	2015	2016	2015	2016
Rootstocks								
140Ru 01	0/245		7/196		0/98		1/196	
110R	4/438		0/49		nt		2/49	
101-14	2/539		0/49		nt		0/49	
1103	0/49		9/49		8/49		0/49	
3309C 05	1/196	0/150	0/196	0/100	0/196		0/196	0/150
MGT 420A	0/147		5/196		1/98		0/196	
Freedom 01	1/294		0/147		0/49		0/245	
Harmony		0/150		1/150		0/100		0/150
GRN-1		0/150		2/100		0/100		0/100
Scions								
Cabernet sauvignon	5/539	0/50	1/147	0/150	4/147	0/150	1/196	1/200
Chardonnay	11/539*		nt		nt		nt	
French Colombard 04	7/172		16/123		0/49		2/123	
Merlot		4/200		9/250		0/150		5/250
Pinot Noir 02A	4/196		0/96		0/49		6/147	
Salt Creek 08	5/196	0/100	4/147	0/100	0	0/100	1/147	0/150
Zinfandel 01A	147	0/150	196	2/150	49	0/50	196	0/150

*2014 data.

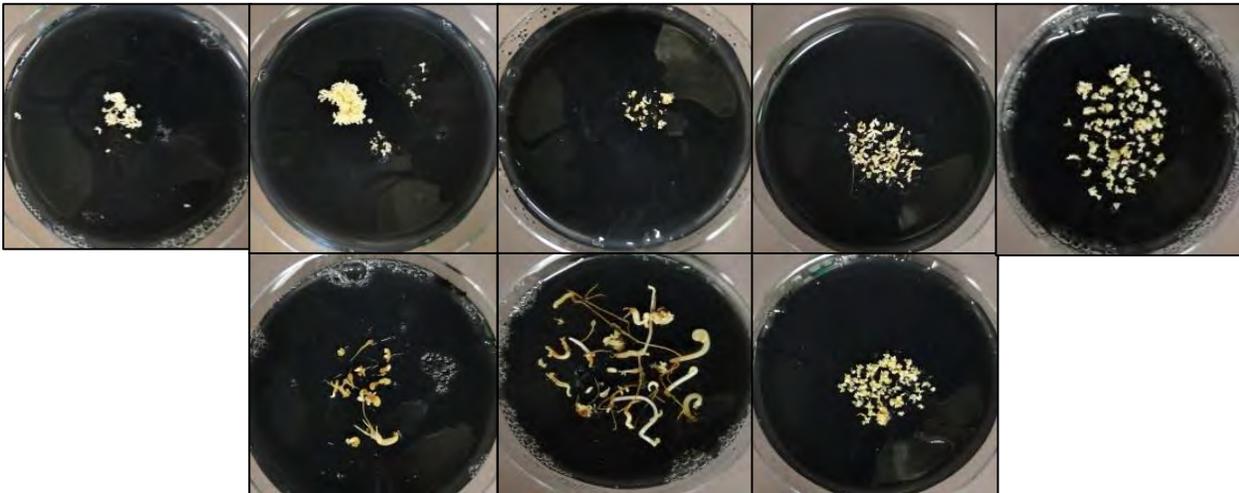


Figure 1. Somatic embryo cultures from grape genotypes for (from left to right, top row) GRN-1, Harmony, Freedom, 140Ru, and MGT 420A, and for (left to right, bottom row) Cabernet Sauvignon, Pinot noir, and Merlot, added to our collection in 2016.

Objective 2. Develop embryogenic suspension cultures for eight rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use in transformation experiments.

By transferring somatic embryos into liquid culture medium composed of woody plant media (WPM) supplemented with 20 g/liter sucrose, 1 g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/liter Picloram, and 2.0 mg/liter meta-topolin we have established suspensions for rootstock genotypes 101-14, 110R, 140Ru, 1103, Freedom, and MGT 420A, and scion genotypes Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot. Occasionally the suspensions are sieved through a 520-micron screen to eliminate large embryos and cell clusters. Alternatively, the smaller fraction of the suspension is drawn up into a wide bore 10 ml pipet and transferred to a new flask leaving the larger embryos and cell aggregates behind.

Objective 3. Establish a germplasm bank of somatic embryos for eight 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 by plating aliquots of the suspension cultures onto agar-solidified WPM supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter naphthaleneacetic acid (NAA), 5% sorbitol, and 14 g/liter phytoagar (BN-sorb). Stored embryo germplasm banks have been established for rootstock genotypes 1103, 101-14, 110R, 140Ru, Freedom, and MGT 420A, as well as scion genotypes Cabernet Sauvignon, Chardonnay, and French Colombard (**Figure 2**).

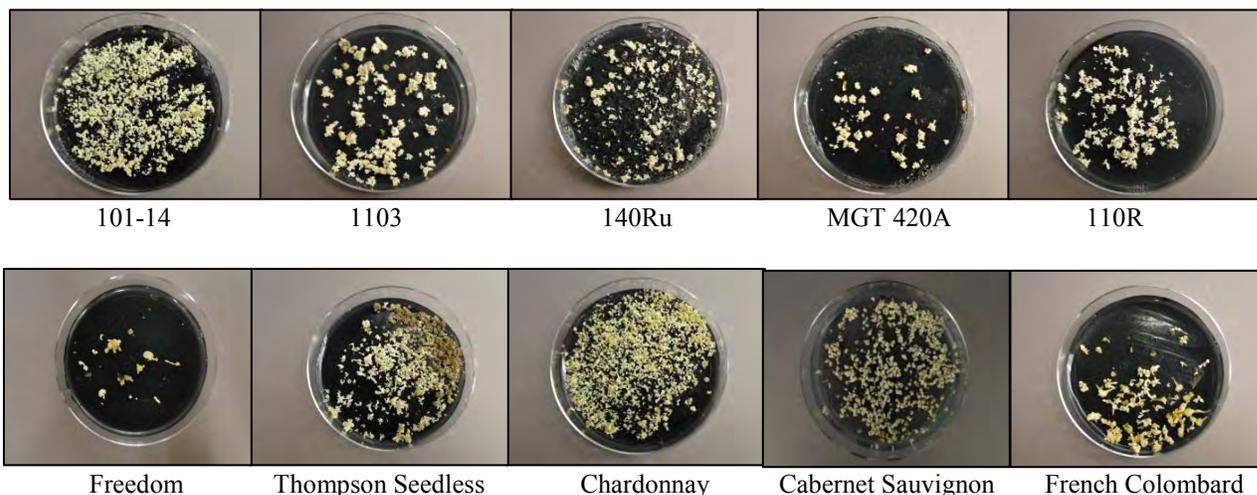


Figure 2. Germplasm bank of embryos established from grape suspension cultures plated on sorbitol containing medium.

Objective 4. Test transformation efficiencies of eight rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.

Transformation experiments continue using known amounts for somatic embryos as determined by fresh weight for 101-14, 110R, 140Ru, 1103, Chardonnay, French Colombard, and MGT 420A using a construct containing the DsRed fluorescent scorable marker gene which will allow us to monitor the progress of transformation in real time without sacrificing any tissue. Thompson Seedless is being included as a positive control. DsRed expression was scored three months post-inoculation (**Table 2, 5**) and has shown that significant numbers of transgenic somatic embryos can be generated for 101-14, 110R, 140Ru, 1103, French Colombard, and MGT 420A.

However, very little DsRed expression was seen in Chardonnay somatic embryos. The relative transformation efficiency based on recovery of whole plants is higher for 110R than that seen for 1103 and equal to or greater than that seen for 101-14. We have also demonstrated that we can generate transgenic plants for French Colombard (**Figure 2**). Based on DsRed expression we have also generated transgenic embryos for 140Ru and MGT 420A, and we are in the process of determining if we can regenerate whole plants from the embryos. Once germplasm banks of somatic embryos are established for Merlot we will begin testing our transformation system on somatic embryos.



Figure 2. Transgenic plantlets of French Colombard expressing DsRed.

Table 2. 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 tissue expressing DsRed
TS-14	05/29/2015	159030	NA	15%
	06/26/2015	159050	0.53	40%
	07/24/2015	159070	0.52	50%
	08/26/2015	159096	0.92	40%
Chardonnay	06/26/2015	159048	2.72	0%
	07/10/2015	159064	1.12	0%
	07/17/2015	159068	1.12	<1%
	07/24/2015	159071	0.57	0%
110R	06/26/2016	159049	0.49	60%
	07/10/2015	159065	1.65	60%
	07/17/2015	159069	1.83	75%
	07/24/2015	159072	0.42	80%
	08/26/2015	159095	0.89	20%
1103	07/24/2015	159073	1.11	10%
	08/26/2015	159093	1.09	5%
Colombard	12/16/2015	159150	1.96	30%
	01/15/2016	169007	0.55	20%
	02/5/2016	169029	0.76	10%
	03/2/2016	169042	0.53	25%
	05/6/2016	169049		25%
140Ru	12/16/2015	159151	1.49	20%
	01/15/2016	169008	0.92	25%
	02/05/2016	169030	1.91	25%
	03/04/2016	169043	1.44	15%
	05/06/2016	169050	nd	20%
MGT 40A	12/16/2015	159152	0.53	20%
	01/15/2016	169009	0.21	
	02/05/2016	169031	nd	25%
	03/04/2016	169044	nd	15%
	05/06/2016	169051	nd	15%
101-14	07/24/2015	159074	0.86	30
	08/26/2015	159094	0.97	20

Objective 5. Test direct cell suspension transformation technology on eight 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. Ten 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 1,000 x G for three minutes. The cells are subjected to heat shock by placing the conical tube in a 45° 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 optical density at 600 nm (OD600) of 0.1 to 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 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 in the dark for two to three days at 23° and then transferred to selection medium consisting of WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1M 2-(N-morpholino)ethanesulfonic acid (MES), 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 400 mg/liter carbenicillin, 150 mg/liter timentin, 200 mg/liter kanamycin, 50 g/liter sorbitol, and 14 g/liter 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/liter sucrose, 1 g/liter casein, 1M MES, 500 mg/liter activated charcoal, 0.1 mg/liter BAP, 400 mg/liter carbenicillin, 150 mg/liter timentin, 200 mg/liter kanamycin, 0 g/liter sorbitol, and 8 g/liter agar for germination. We are currently testing this protocol on 101-14, 110R, 140Ru, 1103, Chardonnay, French Colombard, and MGT 420A using a construct containing the DsRed transgene. We have been able to recover transgenic plants using this protocol for 1103 and 101-14 at very low frequency. For example, only two of the twenty-one putatively transformed embryos that formed from one experiment with 101-14 germinated into plants after transfer to medium lacking sorbitol. We are observing germinating embryos of MGT 420A (**Figure 3**). However, currently the transformation frequency using this protocol is too low to be practical for routine transformations. A summary of the experiments and the transformation frequency is given in **Table 3**.



Figure 4. Germinating embryos from transformation of cell suspension cultures of 101-14 (left) on WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1M MES, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 50 g/liter sorbitol, and 14 g/liter agar and transfer to WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1M MES, 500 mg/liter activated charcoal, 0.1 mg/liter BAP, and 8 g/liter agar for plant regeneration. Only two of the twenty-one putatively transformed embryos on this plate germinated after transfer to medium lacking sorbitol. DsRed expressing embryos of MGT 420A (middle and right).

Table 3. Number of embryogenic colonies forming after inoculating approximately one to two ml of cell suspension with *Agrobacterium* and plating onto selection medium.

Genotype	Number of Experiments	# Of putative transgenic embryos/ml of plated suspension	# of putative transgenic plants produced
101-14	17	54	2
1103	20	30	2
110R	5	1	0
140Ru	2	0	0
MGT 420a	2	7	4
French Colombard	2	0	0
Chardonnay	2	0	0

Objective 6. Establish *in vitro* shoot cultures for eight 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.

We are maintaining disease-free *in vitro* stock plants of 101-14, Chardonnay, and Cabernet Sauvignon that we received as *in vitro* cultures from FPS. For material that was not available through FPS we have collected shoot tips from field material grown at FPS. This includes rootstock genotypes 110R, 140Ru, 1103, 3309C, Freedom, MGT 420A, and Salt Creek, and scion genotypes Cabernet Sauvignon, French Colombard, Pinot Noir, and Zinfandel. We have collected shoot tips for three additional genotypes, Harmony, Merlot, and MGT 420A, which we were not successful in establishing shoot cultures last season. Four-inch shoot tips were collected and 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/liter chlorophenol red or agar-solidified MS minimal organics medium supplemented with 1.0 mg/liter BAP, 0.1 mg/liter IBA, 0.1 mg/liter GA3, and 5 mg/liter chlorophenol red. Aseptic shoot cultures have been established and have been plated onto Mezzetti medium with increasing levels of BAP in order to establish bulk meristem cultures (Figure 5). We are finding differences in our ability to produce bulk meristem cultures between rootstocks and scion genotypes. We have produced quality bulk meristem cultures for scion genotypes Chardonnay, French Colombard, Pinot Noir, and Zinfandel. However, rootstock genotypes do not readily produce bulk meristems in our hands but produce elongated shoots with a significant amount of non-organized callus, making it unsuitable for bulk meristem transformation (Figure 6).

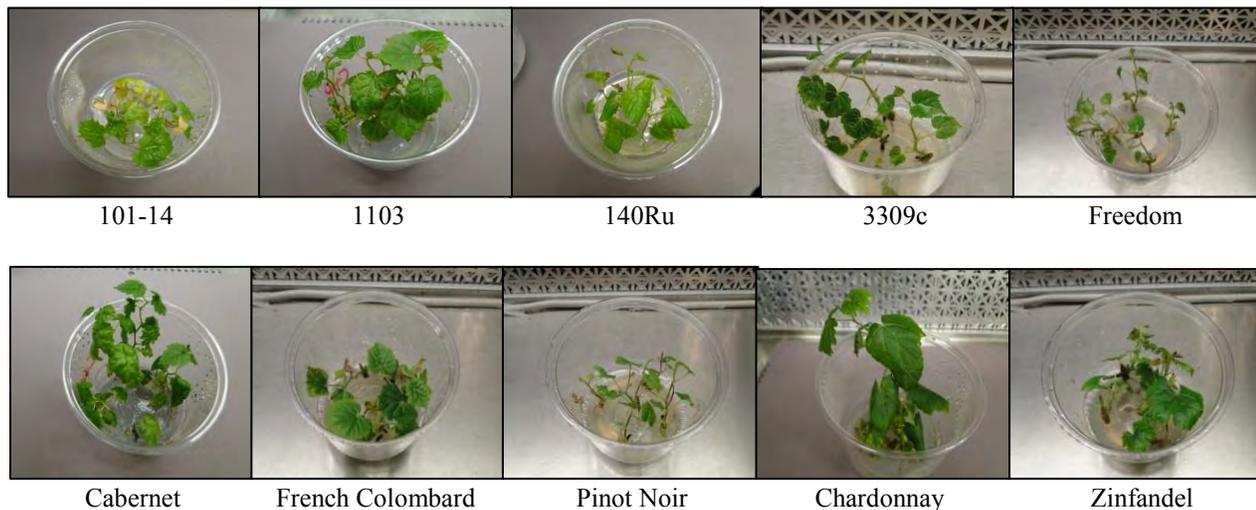


Figure 5. Shoot cultures established for rootstock and scion genotypes.

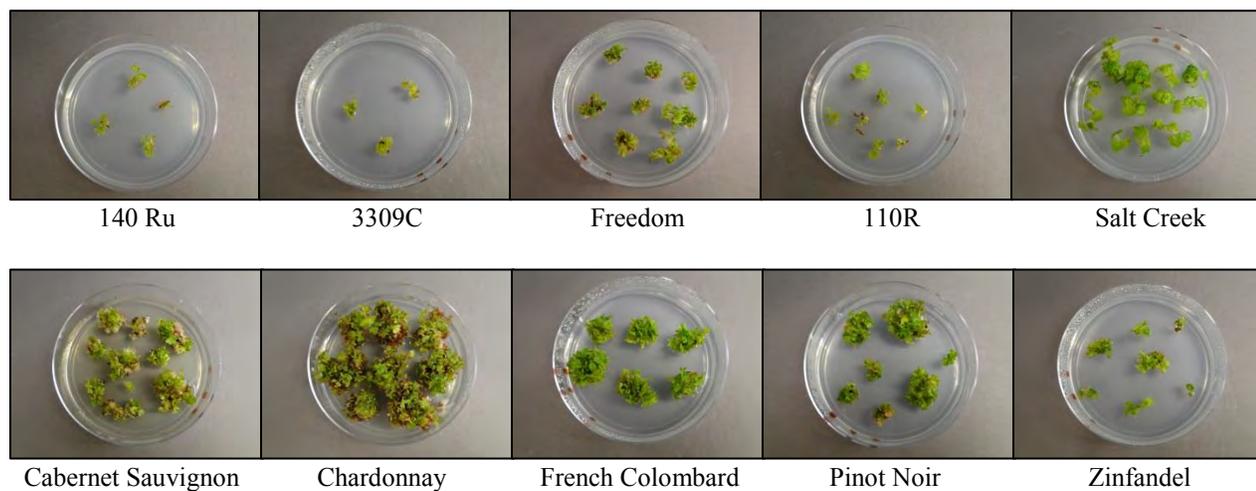


Figure 6. Initiation of bulk meristem cultures for rootstock and scion germplasm.

Objective 7. Test the Mezzetti et al., 2002 bulk meristem transformation methodology for eight rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

We have focused our efforts on studying bulk meristem transformation in scion genotypes since we have not observed good bulk meristem development on rootstock genotypes. Bulk meristems of Cabernet Sauvignon, Chardonnay, and Thompson Seedless were sliced into thin, 2 mm slices and inoculated with *Agrobacterium* strain EHA105 containing the DsRed gene and the plant selectable marker gene nptii and co-cultures on Mezzetti medium supplemented with 3 mg/liter BAP in the dark at 23° centigrade. After three days the thin slices were

transferred to Mezzetti medium supplemented with 3 mg/liter BAP, 400 mg/liter carbenicillin, 150 mg/liter timentin, and 25 mg/liter kanamycin sulfate. After three weeks tissue was transferred to the same medium formulation, but the kanamycin level was increased to 50 mg/liter. 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/liter 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. In our hands the use of kanamycin at 75mg/liter 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/liter kanamycin were non-transgenic based on DsRed expression. We repeated bulk meristem transformation experiments using higher levels of kanamycin, starting at 75 mg/liter for three subcultures and then increasing the kanamycin concentration to 150 mg/liter, but we still observed regeneration of many non-transgenic shoots and chimeric shoots, especially in Thompson Seedless. Overall, based on DsRed expression, low frequency of transformation was confirmed in Thompson Seedless; however, no transgenic shoots were recovered from Cabernet Sauvignon and Chardonnay. A summary of the bulk meristem transformation experiments initiated to date is given in **Table 4**.

Table 4. Results of bulk meristem transformation using the scorable marker gene DsRed.

Genotype	Number of experiments	Number (%) explants generated DsRed callus	Number (%) explants generated DsRed shoots
Cabernet Sauvignon	2	1/36 (3)	0/36 (0)
Chardonnay	2	2/38 (5)	0/38 (0)
Thompson Seedless	2	24/75 (32)	3/75 (4)

Table 5. Summary of the progress in adapting our existing somatic embryo-based transformation protocol for each grape rootstock and scion genotype

Genotype	Somatic embryos established from anthers	Suspensions established from somatic embryos	Establishment of stored somatic embryo cultures	Production of transgenic somatic embryos	Production of transgenic plants	Relative Transformation efficiency*
Rootstocks						
1103	+	+	+	+	+	3
101-14	+	+	+	+	+	5
110R	+	+	+	+	+	5
140Ru	+	+	+	+	-	ND**
3309C	-	-	-	-	-	ND
GRN-1	+	-	-	-	-	ND
MGT 420A	+	+	+	+	+	ND
Freedom	+	+	+	+	+	5
Harmony	+	-	-	-	-	ND
Salt Creek	-	-	-	-	-	ND
Scions						
Cabernet Sauvignon	+	+	+	-	-	0
Chardonnay	+	+	+	+	+	<1
French Colombard	+	+	+	+	+	4
Merlot	+	+	-	-	-	ND
Pinot Noir	+	-	-	-	-	ND
Thompson Seedless	+	+	+	+	+	10
Zinfandel	-	-	-	-	-	ND

* Relative transformation efficiency on a scale of 0 worst, 10 best with 10 reflecting the transformation efficiency for Thompson Seedless

** ND - not determine

CONCLUSIONS

We established embryogenic cultures of 101-14, 110R, 140Ru, 420A, 1103, Cabernet Sauvignon, Chardonnay, Freedom, French Colombard, Merlot, and Pinot Noir from anther explants and initiated embryogenic suspension cultures. We have also established a germplasm bank of somatic embryos for 101-14, 110R, 140Ru, 420A, 1103, Cabernet Sauvignon, Chardonnay, Freedom, French Colombard, and Merlot by plating suspensions onto high osmotic agar-solidified medium on a weekly basis. Suspension cultures of Merlot and Pinot Noir are not yet growing quickly enough to start plating them on agar-solidified medium. We have demonstrated that our transformation protocol established for 101-14 and 1103 is also amenable to transformation of 110R, Freedom, and French Colombard, which expands the range of rootstock and scion genotypes that can be utilized in research by the Pierce's disease research community.

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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 for the Pierce's disease resistance gene, *PdRI* (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 *PdRIb* 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 field testing with commercial-scale wine production, the first of which was planted in Napa in June 2013. We advanced three additional selections to Foundation Plant Services (FPS) last 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 *PdRIb* with b42-26 Pierce's disease resistance has been advanced to the 92% *V. vinifera* level using marker-assisted selection to confirm the presence of *PdRIb* 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 associated genetic markers. Very small scale wines from 94% and 97% *V. vinifera PdRIb* 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 (*PdRI*; Krivanek et al., 2006), we have been able to use marker-assisted selection to screen for DNA markers associated with *PdRI*, allowing us to select resistant progeny shortly after seeds germinate. Marker-assisted selection 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 field plots where commercial-scale wines can be produced. We have sent 19 advanced selections to Foundation Plant Services (FPS) over the past four 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 *PdRI* 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* (*Xf*) 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 *Xf* resistance into commercial grapes. We genetically mapped and identified what seems to be a single dominant gene for *Xf* resistance in *V. arizonica/candicans* b43-17 and 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 (PD) resistant material to select from. Individuals with the best fruit and vine characteristics are then tested for resistance to *Xf* 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 from the 94% *V. vinifera* level for eight years and from the 97% *V. vinifera* level for six 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 *Xf* 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 Chromosome 14 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 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 *Xf* resistance and address *Xf*'s potential to overcome resistance.

OBJECTIVES

1. Identify unique sources of Pierce's disease resistance with a focus on accessions collected from the southwestern United States and northern Mexico. Develop F1 and BC1 populations from the most promising new sources of resistance. Evaluate the inheritance of resistance and utilize populations from the most resistant sources to create mapping populations.
2. Provide support to the companion mapping/genetics program by establishing and maintaining mapping populations, and using the greenhouse screen to evaluate populations and selections for Pierce's disease resistance.
3. Develop advanced lines of Pierce's disease resistant winegrapes from unique resistance sources through four backcross generations to elite *V. vinifera* cultivars. Evaluate and select on fruit quality traits such as color, tannin content, flavor, and productivity. Complete wine and fruit sensory analysis of advanced selections.
4. Utilize marker-assisted selection to stack (combine) different resistance loci from the BC4 generation with advanced selections containing *PdR1*. Screen for genotypes with combined resistances, to produce new Pierce's disease resistant grapes with multiple sources of Pierce's disease resistance and high quality fruit and wine.

RESULTS AND DISCUSSION

To date over 293 wild accessions have been tested for Pierce's disease resistance with the greenhouse screen, most of which were collected from the southwestern United States and Mexico. Our goal is to identify accessions with the most unique Pierce's disease resistance mechanisms. To do so we evaluate the genetic diversity of these accessions and test them for genetic markers from chromosome 14 (where *PdR1* resides) to ensure that we are choosing genetically 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 to which they resist

Xf. We have reported previously the surprising result from our companion Pierce's disease mapping project that most of the resistance lines we have explored from the southwestern United States have Pierce's disease resistance associated with chromosome 14, the same region as our primary resistance line *PdR1b*. In **Table 1** we detail crosses made in 2016 to advance lines that preliminary screening indicates are not located on chromosome 14. Crosses in Group 1a created progeny to expand existing F1 mapping populations from the ANU67, b41-13, and T03-16 sources (all accessions from southwestern *Vitis* species). Some of the progeny from these F1 lines exhibited strong resistance, but few highly resistant progeny were detected in the T03-16 line. Crosses in Group 1b were made to examine whether complete Pierce's disease resistance in this line could be recovered through full sib crossing in the F1 generation. Two elite F1 individuals from the b41-13 line and the three most resistant F1 genotypes in the T03-16 line were backcrossed to the indicated elite *V. vinifera* parents (Group 1c) to create new breeding lines at the BC1 level. These will ultimately be combined with the b42-26 line to enhance and broaden Pierce's disease resistance in our main *PdR1b* resistance crosses.

Table 1. 2016 Crosses made to expand new Pierce's disease mapping populations and advance breeding lines to the next backcross level.

Group	Cross PDR Source	% <i>vinifera</i>	<i>vinifera</i> Parents/ Grandparents	# Crosses	Act. # Seeds
1a	ANU67	50%	F2-35	1	890
	b41-13	50%	F2-35	1	1147
	T03-16	50%	Palomino	1	47
1b	T03-16	50%	Palomino	3	160
1c	b41-13	75%	Rosa Minna, Primitivo/F2-35	2	550
	T03-16	75%	F2-35/Palomino	3	338

Our 2016 breeding crosses (**Table 2**) expand on our 2015 efforts with increased numbers and focus on parents with superior horticultural and fruit quality traits. Cross 2a in **Table 2** represents backcrosses to elite *vinifera* wine varieties to various parents from crossings of *PdR1b* x b42-26 lines at the 92% *vinifera* level. Resistant parents were selected based on the greenhouse results summarized in **Table 3**, Group 3C. Cross 2b in **Table 2** presents intercrosses among the most resistant progeny to further evaluate compatibility and resistance in this effort to stack different resistance sources. Cross 2c in **Table 2** presents the first crossing of elite *PdR1b* types to parents with three powdery mildew (PM) resistance loci to evaluate possible segregation distortion between this combination of resistance loci. Cross 2d in **Table 2** presents similar crosses although at a lower percent *vinifera* level. These crosses were created to confirm the functionality of combining two Pierce's disease resistance loci with three powdery mildew resistance loci. To increase the percentage of progeny with *PdR1b*, we cross either to a parent homozygous at *PdR1b* or have both parents carry *PdR1b* (**Table 2**, Crosses 2e, 2f, 2g). Similarly, we accomplish the same increase in percentage progeny with powdery mildew resistance markers, however, again at a slightly lower *vinifera* level as shown in **Table 2**, Crosses 2f and 2g.

Table 3 provides a list of the Pierce's disease greenhouse screens analyzed, initiated, and/or completed over the reporting period. In Group 3A we tested six BC1 and 14 BC2 progeny in the b40-14 line. Only one at the BC1 level was considered exceptionally resistant and four at the BC2 were of some interest. Six BD5-117 x Haines City intercross genotypes were tested and only two were identified as of some interest. Both BD5-117 and Haines City are Pierce's disease resistant but from the southeastern United States. This absence of highly-resistant genotypes at only a 75% *vinifera* level (BC1) again demonstrates the challenges of working with resistant species from the southeastern United States. In this same group we tested seven BC3 and 11 BC4 selections with *PdR1a* resistance with five and three genotypes of some interest, respectively. One genotype was identified with outstanding resistance at the 97% *vinifera* (BC4) level. This result was confirmed in Group 3B, and after further horticultural evaluations, this accession was advanced to multiple vine trials this spring. Of the 46 *PdR1b*

genotypes tested to confirm previous greenhouse screen results, five were classified as of interest and eight were exceptionally resistant. Following further horticultural and wine quality evaluations, decisions will be made on advancing these individuals to wine making and release.

Table 2. Pierce's disease crosses made in 2016 with percent *vinifera*, most recent elite *vinifera* parent, and number of seeds produced. The Pierce's disease resistance in *PdR1b* originated from b43-17, a Monterey, Mexico *V. arizonica/candicans*; b42-26 (*V. arizonica/girdiana*) has a multigenic form of Pierce's disease resistance from Loreto, Baja California. *Ren1*, *Ren4*, and *Run1* are powdery mildew (PM) resistance loci from *V. vinifera*, *V. romanetti*, and *M. rotundifolia*, respectively.

Cross PDR Type	Cross PM Type	% <i>vinifera</i>	<i>vinifera</i> Parents/Grandparents or .../most recent <i>vinifera</i> parents	No. Crosses	No. Seeds
2a. <i>PdR1b</i> x b42-26	none	96%	Chardonnay, Cabernet Sauvignon, F2-35, Primitivo/Chardonnay, Zinfandel	9	2,540
2b. <i>PdR1b</i> x b42-26	none	92%	Zinfandel, Chardonnay	17	7,369
2c. <i>PdR1b</i>	<i>Ren1, Ren4, Run1</i>	96%	Zinfandel/F2-35	2	136
2d. <i>PdR1b</i> x b42-26	<i>Ren1, Ren4, Run1</i>	92%	.../Grenache, Zinfandel	5	353
2e. <i>PdR1b</i> ² x b42-26	<i>Ren1, Ren4</i>	94%	.../F2-35, Grenache, Zinfandel	3	534
2f. <i>PdR1b</i> ² x b42-26	(<i>Ren1, Ren4</i>) ²	90%	.../F2-35, Karadzhandal, Zinfandel	1	797
2g. (<i>PdR1b</i> x b42-26) ²	(<i>Ren1, Ren4</i>) ²	90%	.../F2-35, Grenache, Zinfandel	4	2,506

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)
3A	b40-14, <i>PdR1a</i> , BD5-117 x Haines City	119	08/25/2015	11/24/2015	b40-14, <i>PdR1a</i> , BD5-117 x Haines City
3B	b42-26 ² Intercross, <i>PdR1b</i> x b42-26 x <i>V. romanetii</i> stack, b46-43 BC1 map	168	09/17/2015	12/17/2015	<i>PdR1b</i> , b42-26
3C	92% <i>PdR1b</i> x b42-26 stack	274	10/27/2015	01/26/2016	<i>PdR1b</i> , b42-26
3D	Additional b42-26 F1, Alternate b42-26 BC1	171	03/01/2016	05/31/2016	<i>PdR1b</i> , b42-26
3E	ANU5, b40-14, Promising selections from 2015 GH Screens, PD x PM	152	04/14/2016	07/14/2016	ANU5, b40-14, <i>PdR1b</i> , b42-26
3F	BC-UBC Irrigation Level Trial	7	04/14/2016	07/14/2016	<i>PdR1b</i> & southeast US biocontrols
3G	Mapping 14-399 b46-43 BC1	117	05/04/2016	08/09/2016	b46-43
3H	Mapping 2014 recombinants, <i>PdR1b</i> x b42-26 stack 2nd tests	170	08/11/2016	<i>11/10/2016</i>	<i>PdR1b</i> , b42-26
3I	T03-16, 2016 parents, b41-13, ANU67	259	09/13/2016	<i>12/13/2016</i>	ANU67, b41-13, b42-26, <i>PdR1b</i> , T03-16
3J	<i>PdR1b</i> x b42-26 stack & recent promising parents	115	<i>10/07/2016</i>	<i>01/06/2017</i>	<i>PdR1b</i> , b40-14, b42-26

Another 22 progeny of the 13-309 intercross of the two most highly resistant 07-344a BC1 genotypes in the b42-26 line were tested in Group 3B, making a total of 48 genotypes tested. In total, 45 had intermediate resistance, two were susceptible, and one was as resistant as the parent, b42-26. This confirms our assessment in a previous report that some important resistance factors were left behind, likely at the F1 level. Group 3B also included the first 23 genotypes in the 14-399 (b46-43 BC1) mapping population, which segregated 13:10 (R:S). Fifteen

genotypes at the 94% *vinifera* level in the *PdR1a* line were evaluated, but none were sufficiently resistant to advance. This group also included 24 genotypes at the 89% *vinifera* level that are homozygous at *PdR1b*, have some b42-26 resistance, and also carry powdery mildew resistance from *V. romanetii*. All were resistant, 12 significantly so and two exceptionally so. This cross is the first instance where such a high frequency of elevated Pierce's disease resistance has been observed at this advanced *vinifera* level and it will be further tested. Group 3B also contained a test of 10 progeny of a cross at the 87% *vinifera* level involving *PdR1b* x b42-26, but where the b42-26 was backcrossed a second time to another resistant b42-26 line genotype. All progeny were resistant, half significantly so and two exceptionally so. These results underscore the value of combining the *PdR1b* and b42-26 resistance lines.

Group 3C was an extensive test of 245 progeny from a cross at the 92% *vinifera* level involving *PdR1b* x b42-26, with results reported in **Table 4** below. This group also included testing of nine Pierce's disease resistant rootstocks. Three previously identified selections from 2011 crosses were confirmed as highly resistant and are now in multi-vine trials in Davis. This group also included five *V. tiliifolia* accessions from the Caribbean that had potential to be Pierce's disease resistant, but all proved moderately to highly susceptible to Pierce's disease.

One hundred and twenty more progeny from the b42-26 background were tested in Group 3D in an effort to improve the genetic map in this multigenic resistance background. In an attempt to identify missing resistance factors in the BC1 07-344a b42-26 line, we also tested 25 genotypes from an alternate BC1 population derived from a different highly-resistant F1 parent. As expected, we found a range of *Xf* titers from about 65,00 to 6.5 million cfu/ml. None were as resistant as b42-26. Greenhouse screen results and DNA samples were provided to our companion mapping project for bulked segregant analysis. We also tested 19 genotypes which have *PdR1b* and the *Ren1* and *Ren4* powdery mildew resistance loci. Eleven of 19 had titers lower than 500k cfu/ml with four of those less than 100k. These results suggest that we can effectively combine Pierce's disease resistance with multi-loci powdery mildew resistance.

In Group 3E we tested 42 BC1 progeny in the ANU5 (*V. arizonica* from Littlefield, AZ) line for the presence of minor resistance genes, since we now believe it to have its major source of Pierce's disease resistance on chromosome 14. Five genotypes exhibited intermediate resistance so could be of some interest. We also tested 35 genotypes at the BC2 or BC3 level in the b40-14 breeding line, the source of our *PdR1c* resistance source. Ten genotypes were rated as either highly resistant or promising and will be used to advance this resistance source to the 97% *vinifera* level. In the retest of 45 genotypes previously identified as promising, a total of 25 selections from various sources including A14, A28, b40-29, b46-43, BD5-117 x Haines City, *PdR1a*, *PdR1b*, and SAZ7 were scored as highly resistant. The two genotypes in the BD5-117 x Haines City resistance line have good fruit and yield characteristics and likely will be advanced to multi-vine trials. In addition, 22 genotypes at the 88-93% *vinifera* levels were screened. Ten were rated Pierce's disease resistant in this screen, two at the promising level, of which one was used as a parent in our 2016 Pierce's disease x powdery mildew crosses (Table 2e, 2g).

We refined our rapid greenhouse screen with an experiment in Group 3F. We have observed that expression of Pierce's disease symptoms increases when the test plants in a given trial become water stressed. In addition, in at least one trial, symptoms were dramatically diminished when excess irrigation levels were maintained. Plant water status also may impact bacterial titer. In this experiment we better defined the water status impact on Pierce's disease expression using our four *PdR1* and two southeast United States species biocontrol genotypes that range in symptom levels and *Xf* titers. From previous analysis of this trial and other experiments, we know that the bench where a test plant is located is often highly significant, based on proximity to cooling pads in the greenhouse. When genotype and bench were eliminated as variables, and when we only analyze together irrigation reps (sub-blocks on a bench) that have statistically similar means, then irrigation volume applied, at least at the +12.5% and +25% levels, had at most a weak effect relative to bench and genotype. In addition, this statistically weak effect of irrigation volume may only apply to some genotypes of intermediate resistance, as it did with U0505-35. In the other two intermediate PDR genotypes, Blanc du Bois and Roucaneuf, increased irrigation volume did result in less bacteria but it was not statistically significant.

In our companion Pierce's disease mapping project we identified a major Pierce's disease resistance locus on chromosome 14 in the b46-43 line. Our early results from the 14-399 cross tested in 3B above facilitated this discovery. Group 3G tests approximately 100 additional genotypes to check for any minor resistance loci.

Enzyme-linked immunosorbent assay (ELISA) results are pending. Recombinants from 2014 crosses in the *PdR1b* line are being tested in Group 3H to further refine its genomic location. In the same screen we are testing 127 genotypes in the 92% *PdR1b* x b42-26 stack group, 66 for the first time. Group 3I tests or retests F1 genotypes in the T03-16, b41-13, and ANU67 resistance lines, our focus now for non-chromosome 14 Pierce’s disease resistance. We also retested genotypes used as parents in 2016 crosses to confirm Pierce’s disease resistance. Group 3J continues our screening of the 92% *PdR1b* x b42-26 stack group by testing 93 genotypes for the second or third time to assure resistance. We are making strong progress evaluating the important *PdR1b* x b42-26 stacking group.

The next step in our stacking, completed this spring (**Table 2**, Cross 2b, above), was the intercrossing of numerous of the most resistant individuals descending from different parent combinations identified from this group to create breeding genotypes homozygous at *PdR1b*, enriched in b42-26 quantitative trait loci (QTLs), and showing minimal *Xf* titers by ELISA and no cane or leaf symptoms. These crosses will be followed by crossing the most promising and resistant of these elite selections to create populations that are 96% *vinifera* in which all progeny have *PdR1b*, and all should be highly Pierce’s disease resistant. The most promising selections would then be advanced to FPS for certification and eventual release as the next iteration of our Pierce’s disease resistant scion breeding efforts. In **Table 2**, Cross 2a above we also made crosses of the most resistant *PdR1b* x b42-26 line progeny directly to elite *vinifera* as baseline populations to later quantify the value of double stacking the b42-26 resistance.

As we have mentioned in previous reports, it is essential to greenhouse screen genotypes multiple times to ensure our assessment of their resistance. We usually consider three tests sufficient to designate a genotype as resistant. As detailed in **Tables 2** and **3** and discussed above, our breeding and testing efforts are currently focused on the 92% *PdR1b* x b42-26 stack group. **Table 4** summarizes the testing status. Genotypes that have not been tested are either too weak to test or failed to propagate, while those being tested a fourth time indicate an inconclusive or anomalous previous test.

Table 4. Percent of 92% *vinifera PdR1b* x b42-26 line genotypes at indicated level of screening.

Cross ID	# times tested or in testing					# Genotypes
	0	1	2	3	4	
14-309	2%	51%	28%	16%	3%	61
14-310	6%	44%	31%	13%	6%	16
14-318	0%	50%	31%	14%	5%	42
14-382	11%	64%	18%	7%	0%	28
14-383	5%	62%	22%	11%	0%	37
14-386	17%	50%	23%	10%	0%	30
14-387	7%	70%	11%	10%	1%	71
14-388	4%	64%	16%	12%	4%	25
14-389	5%	57%	33%	5%	0%	21
Total	6%	58%	22%	11%	2%	331

To determine the field resistance of our various Pierce’s disease varieties, over the last 15 years we have established field trials at various Pierce’s disease hotspots around California and in several southern states where Pierce’s disease is endemic (**Table 5**). At a site in Yountville we have inoculated with *Xf* for seven years and have also mechanically inoculated vines at a vineyard in Temecula in 2015. At the other locations we rely on natural infection. To date all of our resistant vines in these diverse settings continue to thrive. In 2013 we began noticing red blotch virus spreading rapidly through our existing trial at the Yountville site and within a year it had spread through the first 100-vine plantings of our advanced Pierce’s disease resistant vines planted earlier that year. We continue to monitor the Pierce’s disease status of the vines, but are no longer able to make wines from this site due to the virus infection.

Two of our advanced selections are planted along the Napa River (**Figure 1**). Rootstock and chip-budded 07355-075 and 09331-047 were planted and there are now 375 and 1,125 vines. Another 1,000 more may be planted. This trial will give us an excellent view of the commercial potential of these selections, as it is planted in a severe riparian Pierce's disease hot spot. **Figure 1** presents the plot as it looked last summer.

I will be going to Driftwood, Texas in November to check on our research plots and present the Pierce's disease breeding program with a talk and tasting. We have been collaborating with Jim Kamas (Texas A&M, Fredericksburg) who has planted seven of our 88% *vinifera* and four of our 94% selections in a range of sites across a severe Pierce's disease region (Fredericksburg, Leakey, Hye, and Industry, Texas). We also sent five of the U050x series to Alabama, where they have been repropagated and are now at 100 to 500 vine level trials; wines were made this year. Resistance is holding up well in all the selections, although some are more susceptible to the high limestone soils and downy mildew. We also sent 88% and 94% selections to Gainesville, Florida for testing with the University of Florida. Mercy Olmstead is directing this trial, although she is leaving soon. The vines are establishing well and showing no signs of disease.

Table 5. Numbers of grafted UC Davis Pierce's disease resistant vines, by selection, in various field trials. 05 selections are 88% *vinifera*, 07 are 94%, and 09 are 97% *vinifera*. The green shaded vines are being considered for release.

Genotype	Temecula, CA (2014)	Napa Valley, CA (2001-13)	Napa River, CA (2014-15)	Sonoma Co., CA (2012, 2015)	Napa, CA (2014)	Texas (2008)	Alabama (2011)	Florida (2016)
U0501-12						86	30	
U0502-01		6				86	30	
U0502-07						86		
U0502-10		6				86	30	
U0502-20				25			30	40
U0502-26						100		
U0502-38						100	30	
07329-37		9		25		100		
07355-075		105	375	25		100		40
07713-51		9		30		100		
07355-044								40
07338-37						100		40
07370-078								40
07370-084						100		40
09314-102	25			75	25			
09330-07	25				25			
09331-047	25		1125		25			
09331-133	25				25			
09333-178	25				25			
09333-253	25				25			
09333-331	25				25			
09333-370	25				25			
09338-016								



Figure 1. The curving rows on the levee are a 2014 planting of our 94% *vinifera PdR1b* selection 07355-075 along the Napa River.

Tables 6a through **6c** detail the vine, fruit, and juice characteristics for the two 94% (those starting with 07) and fourteen 97% (starting with 09 & 10) *vinifera PdR1b* selections used to make wine lots in 2015. In addition, we made a number of *vinifera* controls and Blanc du Bois and Lenoir as reference Pierce's disease resistant cultivars. All were made from Davis-grown fruit.

Table 6a. 94% (those starting with 07) and 97% (starting with 09 & 10) *vinifera* Pierce's disease resistant selections used in small-scale winemaking in 2016: Background and fruit characteristics.

Genotype	Parentage	2016 Bloom Date	2016 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1 = v low, 9 = v high
07355-075	U0505-01 x Petite Syrah	04/19/2016	08/11/2016	B	1	278	7
07370-084	F2-35 x U0502-38	04/26/2016	08/02/2016	W	1	151	7
09311-160	07371-20 x Cabernet Sauvignon	04/26/2016	08/18/2016	B	1	210	5
09314-102	07370-028 x Cabernet Sauvignon	04/28/2016	08/09/2016	W	1	355	9
09330-07	07370-039 x Zinfandel	04/28/2016	08/11/2016	B	1.1	336	8
09331-047	07355-020 x Zinfandel	05/03/2016	08/16/2016	B	1.3	368	5
09331-103	07355-020 x Zinfandel	05/05/2016	08/23/2016	B	1.3	370	8
09331-133	07355-020 x Zinfandel	05/03/2016	08/18/2016	B	1.7	360	6
09333-111	07355-020 x Chardonnay	04/28/2016	08/16/2016	B	1.4	280	7
09333-358	07355-020 x Chardonnay	05/03/2016	08/11/2016	B	1.2	261	6
09333-370	07355-020 x Chardonnay	05/03/2016	08/18/2016	B	1.3	317	6
09338-016	07371-20 x Cabernet Sauvignon	05/03/2016	08/09/2016	W	1.2	247	6
09356-235	07371-19 x Sylvaner	05/05/2016	08/11/2016	B	1.1	248	7
10302-178	07370-028 x Riesling	04/28/2016	08/02/2016	W	0.9	140	4
10302-293	07370-028 x Riesling	04/30/2016	08/16/2016	W	1.2	185	7
10302-309	07370-028 x Riesling	04/28/2016	08/16/2016	W	1.7	265	8

Table 6b. Juice analysis of Pierce’s disease resistant selections used in small-scale winemaking in 2016.

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 anthocyanins (mg/L)
07355-075	24.0	8.3	3.41	1.4	2,020	206	6	450	1,416
07370-084	26.1	6.6	3.72	2.2	2,230	209			
09311-160	25.0	7.6	3.63	1.8	2,090	230	19	419	1,332
09314-102	23.2	6.0	3.59	4.4	2,420	251			
09330-07	23.1	7.0	3.6	2.3	2,230	258	15	397	1,508
09331-047	27.8	8.0	3.74	1.3	2,200	270	14	556	1,493
09331-103	23.9	7.3	3.30	1.6	1,450	210	11	634	1,253
09331-133	22.5	7.2	3.5	1.7	1,690	242	<1	601	965
09333-111	26.1	6.6	3.54	2.5	2,060	232	13	357	688
09333-358	24.1	7.5	3.53	1.8	2,230	270	65	347	709
09333-370	22.3	6.9	3.57	1.8	1,860	282	6	435	845
09338-016	21.9	7.5	3.54	1.5	1,840	274			
09356-235	25.7	6.7	3.77	2.6	2,400	279	38	381	1,502
10302-178	24.1	8.5	3.49	1.5	2,140	270			
10302-293	24.5	7.7	3.53	1.5	2,160	211			
10302-309	21.8	6.6	3.36	2.2	1,830	128			

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 UC Davis with industry and student tasters, and others are at various industry gatherings. Tastings from the 2014 vintage began with a faculty student tasting in March 2015. 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. 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 tastings 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 were tasted at a meeting of Central Coast growers in Santa Maria and at the Napa Valley Grapegrowers meeting. On May 6, 2016 a tasting was held at UC Davis to evaluate 2015 vintage wines from our new Pierce’s disease resistant varieties. A total of 17 tasters comprised of winemakers, viticulturists, faculty, staff, and students rated the wines on a hedonic quality scale from 1 = poor to 5 = very good. All wines were produced from grapes grown in Davis. The tasters didn't assess the wines uniformly, however, no taster rated every wine as poor and most wines were considered “very good” or nearly so by at least one taster. Considered together, all eight of the UC Davis Pierce’s disease wines and the Chardonnay and Cabernet Sauvignon control wines were perceived as being of average quality. This is significant praise from a group of professionals familiar with evaluating some of the finest *vinifera* wines in the world, especially considering that the wines were produced from grapes grown in Davis, were made at a three to five gallon scale, were less than a year old, and had no oak treatment. Overall, wines from our new Pierce’s disease resistant varieties have been very well received. The first selections have cleared certification from FPS and we are currently working through the UC patent and release process.

Table 6c. Pierce's disease resistant selections used in small-scale winemaking in 2016: Berry sensory analysis.

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)
07355-075	pink-red	med-	fruity, berry, cherry	plum, vs cab sauv veg	2	4	ashy, warm, sl bitter	2
07370-084	clear, green-yellow	pale	melon, yellow apple	neutral, sl grass	2	4	woody, nutty, manzanita berry	4
09311-160	pink orange	med	fruity, spicy	fruity, berry	1	4	nutty, sl metallic, warm	3
09314-102	green-gold	pale	apple, spice	vs veg, hay	1	4	buttery, woody	1
09330-07	red	med	plum, red fruit, cherry	fruity, spicy	2	4	spicy, bitter	1
09331-047	pink red	med	strawberry, plum jam	plum, spice	3	3	woody, spicy, hot	1
09331-103	red, vs orange	med+	plum, fruity, spicy	chalky, vs fruity	3	4	nutty, woody, vs bitter	3
09331-133	orange, sl red	med-	cherry, apple	neutral, sl plum jam	2	3	woody, nutty, warm	3
09333-111	pink, vs brown	lt	red apple, sl cs veg	cs veg, plum	4	3	nutty, ashy, hot	1
09333-358	brown	med	spicy, hay	hay, berry	1	4	nutty, smoky	2
09333-370	orange	med-	plum jam	fruity, sl hay	2	4	woody, sl ashy, hot	1
09338-016	green	pale	green apple	neutral, sl veg	1	3	warm, woody	3
09356-235	red	med	jammy, ripe plum	black plum, chalky	3	4	woody, bitter, metallic	1
10302-178	clear, gold	pale	green apple, sl spice, vs herbal	Neutral, straw, vs veg?	2	4	spicy, hot, acrid	2
10302-293	green, white	lt-med	pear, melon	neutral, melon, hay	1	4	woody, hot	2
10302-309	green, yellow	med	spicy, floral, sl muscat	spicy, neutral	3	4	woody, med hot	2

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 *PdR1b* 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 *Xf*, 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 being planted in vineyards at 50 to 1,000 vine trials with enough fruit for commercial scale winemaking. We have sent 19 advanced scion selections to FPS over the past four 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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Reporting Period: The results reported here are from work conducted October 2015 to October 2016.

ABSTRACT

The goal of this project is to support molecular breeding of Pierce's disease resistant grapes by identifying novel resistant germplasm, determining the inheritance of resistance, and tagging genomic regions to develop markers that facilitate and accelerate breeding. A total of 250 accessions were greenhouse screened and tested with markers to determine their genetic diversity and distance from one another. Twenty resistant accessions were identified and then used to develop breeding populations from 2012 to 2015. 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, which led to the cloning and characterization of its resistance genes (*PdR1a* and *PdR1b*; see earlier reports). The physical map of the *PdR1c* locus (from *Vitis arizonica* b40-14) is complete. 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 (open reading frame) 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 *V. 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 a native promoter are underway, as standard gene promoters did not work. These efforts will help us to identify candidate resistance genes by complementation and allow us to better understand how they function. Such efforts 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% *vinifera*.

LAYPERSON SUMMARY

We continue to identify and genetically characterize novel resistance sources from southwestern United State and Mexican *Vitis* species collections; use genome sequence information to identify 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.

INTRODUCTION

A successful resistance breeding program depends on the germplasm that provides a wider genetic base for resistance. Identification, understanding, and manipulation of novel sources of resistance are prerequisites for successful breeding. This project continues to provide molecular support to the Pierce's disease resistance grape breeding project "Breeding Pierce's Disease Resistant Winegrapes" by acquiring and testing a wide range of

resistance germplasm, tagging resistant regions with markers by genetic mapping, and functionally characterizing the resistance genes from different backgrounds. In earlier versions of this project, genetic markers linked to *Xylella fastidiosa* (*Xf*) resistance from *V. arizonica* b43-17 were used to perform marker-assisted selection (MAS) to accelerate our Pierce's disease resistant winegrape program, and the table and raisin grape breeding program of David Ramming in the past. Outcomes from the earlier two projects included genetic maps, and bacterial artificial chromosome (BAC) libraries of the highly-resistant *V. arizonica* accessions b43-17 and b40-14. A physical map of the *PdRI* locus was completed and several candidate genes were identified. Five candidate genes were cloned and constructs were developed with the 35S promoter to transform tobacco, Chardonnay, Thompson Seedless, and St. George in order to test gene function.

This new project has the following key objectives: identify novel sources of Pierce's disease resistance for use in broadening the genetic base of Pierce's disease 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 resistant grape genes cloned from the *PdRIb* locus; and evaluate and compare lines transformed with *PdRI* constructs 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 tools allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Pierce's disease resistance in the southeastern United States *Vitis* species seems to be different than the resistance in *Vitis* from the southwestern United States 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 have been developed. These populations will be tested to study the inheritance of resistance. Next generation sequencing will be used on the recently identified resistant accessions to expedite marker discovery and confirm that the resistance genes are unique. Next, genetic maps will be developed to identify genomic regions associated with resistance, and genetic markers will be used to enable stacking of multiple resistance genes and breeding of 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 could 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 *vinifera*-based promoters, either constitutive (Li et al., 2012) or activated by *Xf* (Gilchrist et al., 2007) could be utilized. Development of *V. vinifera* plants transformed with our Pierce's disease resistance genes and grape promoters might work more effectively and allow us to better understand *PdRI*'s function.

OBJECTIVES

The specific objectives of this project are:

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 *PdRIc* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdRIa* and *b*).
3. Employ whole genome 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 *PdRI* genes with native promoters.
5. Compare the Pierce's disease resistance of susceptible grapevines transformed with native versus 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.

To make a new variety durably resistant to diseases it is often necessary to combine multiple sources of resistance genes into one background to obtain broad long-lasting resistance. We completed greenhouse testing 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 by Olmo. Both simple sequence repeat (SSR) and chloroplast markers were used to establish relationships with known sources of resistance currently being used in the breeding program (Riaz and Walker, 2013). We found 20 highly-resistant accessions and 15 accessions were selected to develop small breeding populations by crossing them with the highly susceptible *V. vinifera*. In spring 2016 we extracted DNA from the 704 individuals obtained from these breeding populations that were also greenhouse screened. We carried out a limited mapping strategy by utilizing markers from chromosome 14 that are linked to the *PdR1* locus (see previous reports for details of the *PdR1* locus). This strategy allowed us to identify resistance sources whose resistance is similar to *PdR1* and sources that are different among the newly identified accessions. We selected 12 SSR markers that flanked a 3.5 megabase (Mb) region around the *PdR1* locus (**Figure 1A**), which is located between markers Pd82-1b4 and open reading frame (ORF) 18-19-03. The genotypic data of all resistance accessions with 22 markers from 19 chromosomes was used to analyze how genetically distinct the resistant accessions were from each other (**Figure 1B**).

Based on the polymorphic markers for each breeding population a genetic map was created to determine the relative marker order, and then QTL analysis for each population was carried out. We were able to identify two accessions that were resistant to Pierce's disease, but none of the markers from chromosome 14 showed any association to the resistance, indicating that a distinctive resistance locus resides on a different chromosome and most likely is different from the *PdR1* locus.

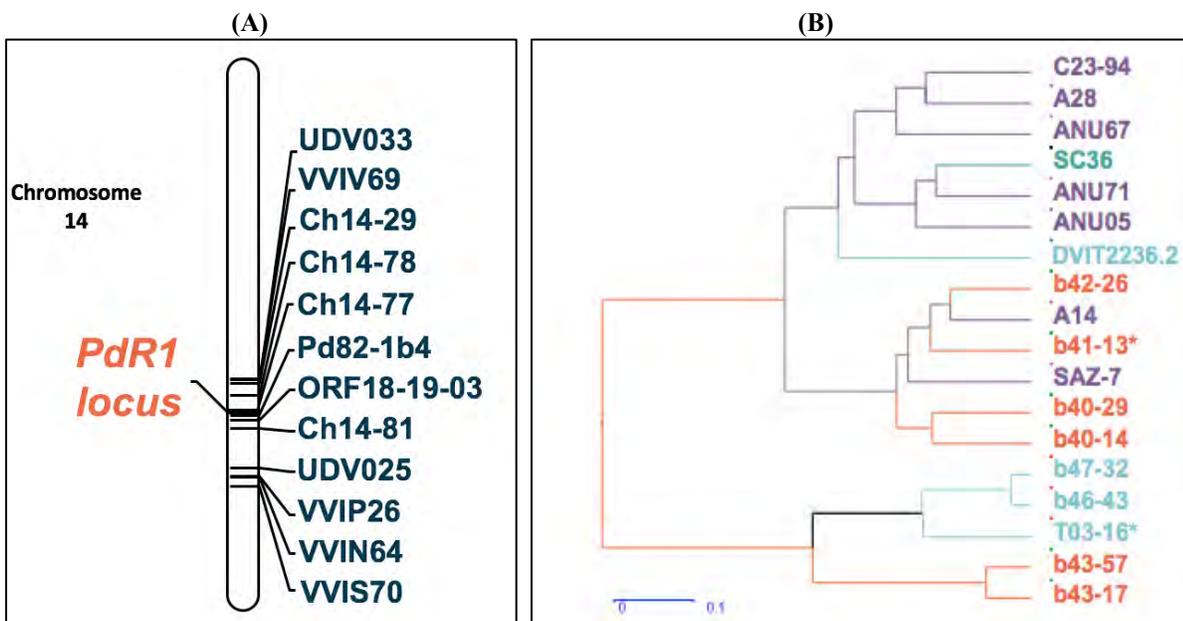


Figure 1. (A) 12 SSR markers that flank 3.5 Mb region on Chromosome 14. The *PdR1* locus resides between markers Pd82-1b4 and ORF18-19-03. (B) Dendrogram showing genetic relationship between resistant accessions; asterisks show Pierce's disease resistant accessions with different source of resistance. Purple: accessions from Arizona; Green: accessions from California; Blue: accessions from Mexico; Orange: accessions from Texas.

Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas State in Mexico are strong candidates that do not possess *PdR1*. **Figure 2** shows the location of some of the more strongly resistant accessions tested so far in this study. These accessions show great potential for use in the Pierce's disease

grapevine breeding program. In order to proceed and to identify the genomic regions in these two accessions, crosses were made in spring 2016 to expand the size of populations. In four backgrounds we were not able to determine if resistance is different than *PdRI* due to the small population size (**Table 1**). We plan to expand the number of individuals in those backgrounds, greenhouse test them for Pierce's disease resistance, and carry out analysis next year to determine if they possess *PdRI*. These results will get us one step closer to finding a new mechanism of Pierce's disease resistance that we can use for our breeding program. **Table 1** presents the breeding populations that were developed with new resistance sources (for details, see previous reports). We completed propagation of four to five replicates for the subset of crosses mentioned in **Table 1**. The rooted green cuttings were transferred to two-inch pots first and then four-inch pots to acclimatize to greenhouse conditions. These plants were inoculated with *Xf* in September and the results of the assay will be available in winter.

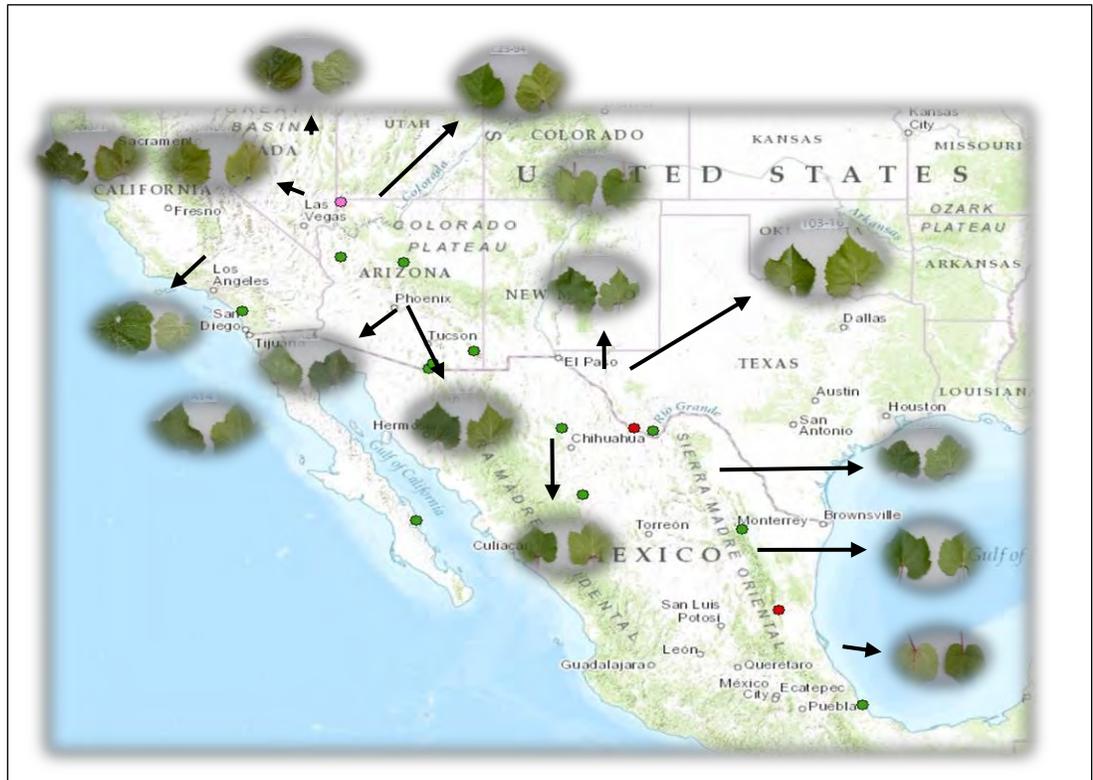


Figure 2. Geographic location of the 15 different Pierce's disease resistant accessions tested in our program.

In spring 2016 we provided molecular support to the companion Pierce's disease resistance winegrape breeding project by marker testing a total of 745 seedlings from six crosses to identify resistant and susceptible genotypes. An additional group of 1,400 more genotypes were tested for the presence of combined resistance. The objective was to stack resistance from b42-26 and *PdRIb* as well as to develop advanced breeding lines with *PdRIc* (from the b40-14 background).

Objective 2. Complete a physical map of the *PdRIc* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdRIa* and *b*).

The SSR-based framework genetic map of *V. arizonica* b40-14 was completed. Greenhouse ELISA screen data was used to carry out QTL analysis and a major Pierce's disease resistance locus, *PdRIc*, was identified on chromosome 14 (see previous reports for details). Pierce's disease resistance from b40-14 maps between flanking markers VVCh14-77 and VVIN64 within a 1.5 cM interval. The genomic location of the *PdRIc* locus is similar to the *PdRIa* and *PdRIb* loci. An additional 305 seedlings were marker tested to identify unique recombinants using new SSR markers developed from the b43-17 sequence (**Table 2**) to narrow the genetic mapping distance. Four recombinants were identified between Ch14-81 and VVIN64, and one recombinant between the Ch14-77 and Ch14-27 markers. The new markers position the *PdRIc* locus to a 325 Kb (kilobase) region based on the sequence of b43-17.

Table 1. Resistant accessions used for the 23 breeding populations. Resistant accessions with different sources of resistance are marked as Not 14 in last column. Accessions marked as LG14 possess the *PdRI* locus. Resistance affinity to Ch14 could not be determined for the accessions that are marked as ND due to small population size and less informative markers.

Resistance Source	Species Description	Populations Tested	Number of Screened Genotypes	Results of Limited Mapping Strategy
ANU5	<i>V. girdiana</i>	12-314	60	LG14
b40-29	<i>V. arizonica</i> , brushy	12-340, 12-341, 14-367, 14-368	29	LG14
b46-43	<i>V. arizonica</i> , glabrous with <i>V. monticola</i>	12-305, 14-308, 14-321, 14-322, 14-324, 14-336	159	LG14
b41-13	<i>V. arizonica-mustangensis</i> and <i>champinii</i> hybrid, red stem with hairy leaves	13-355	47	Not 14
b47-32	<i>V. arizonica</i> glabrous with <i>monticola</i> , small clusters, red stem	13-344	13	ND
SC36	<i>V. girdiana</i>	13-348	35	LG14
T03-16	<i>V. arizonica</i> glabrous	13-336	62	Not 14
A14	<i>V. arizonica</i>	14-313	25	ND
A28	<i>V. arizonica</i>	14-347, 14-364	42	LG14
ANU67	<i>V. arizonica</i> glabrous	14-362	28	ND
ANU71	<i>V. arizonica-riparia</i> hybrid	14-340	30	ND
C23-94	<i>V. arizonica</i> glabrous and brushy	14-303	44	LG14
DVIT 2236.2	<i>V. cinerea</i> like, long cordate leaves, short wide teeth, small flower cluster	14-360	30	LG14
SAZ 7	<i>V. arizonica</i>	14-363	52	LG14

A BAC library from b40-14 genomic DNA (see details in previous reports) was screened and 30 BAC clones were identified with two probes, Ch14-56 and Ch14-58. BAC clones that represent *PdRIc* were separated from the other haplotype, and two BAC clones VA29E9 and VA57F4 were selected. The DNA of the selected BAC clones was sequenced using PAC BIO RS II (see previous report).

A third BAC clone was sequenced to expand the region beyond the probe Ch14-58. The previous assembly consisted of two contigs with no overlap. Common probes between the *PdRIc* and *PdRIb* region were used to align the sequences in order to determine length of the gap in the assembly. A fourth BAC clone that overlaps with the VA30F14 and VA57F4/VA29E9 assembly was selected based on use of the new probes. Sequencing of this BAC clone was completed. New probes were designed using the sequence of *PdRIc* region to test for overlapping BACs. The assembly of four BAC clones is presented in **Figure 3**. A manuscript titled “The Genetic and Physical Map of Pierce’s Disease Resistance Locus *PdRIc*” is in preparation.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdRIa* haplotype (F8909-17) was also completed. The length of assembled sequence was 206 Kb. The ORFs of the *PdRIb* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the overlapping 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 *PdRIb* haplotype. Based on these results we conclude that there is complete sequence homology between haplotype a and b of the *PdRI* locus; therefore, cloning and functional characterization of genes from any one 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 *PdRI* locus in the resistant accession b43-17.

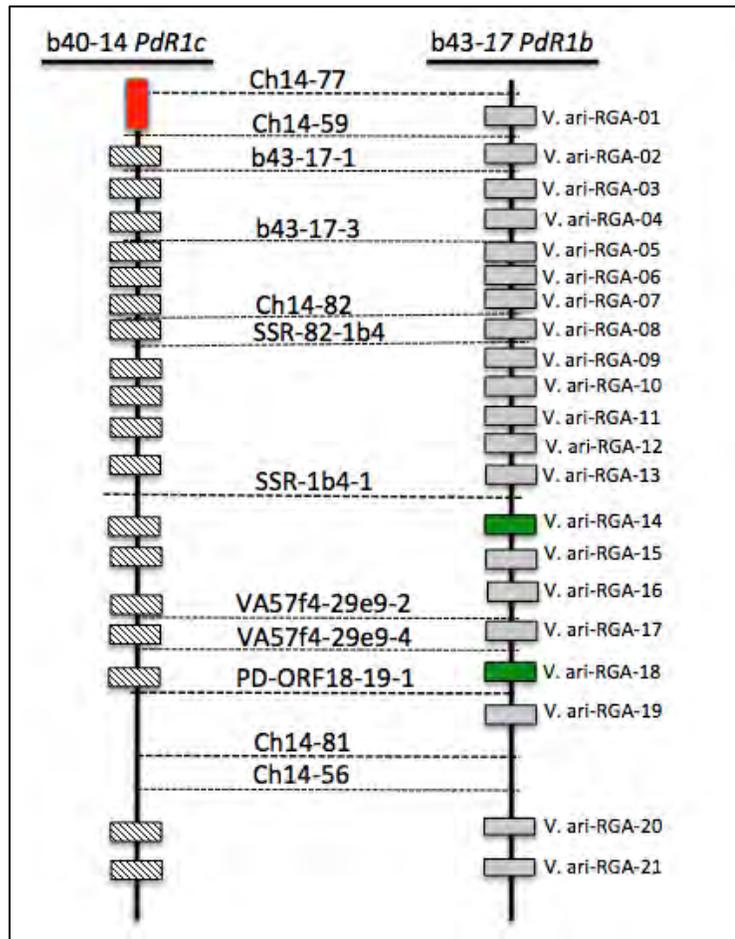


Figure 3. Sequence analysis of the *PdR1b* and *PdR1c* regions. In *PdR1c*, the assembled sequence is 426Kb. Two of the resistance genes are outside the genetic window with marker Ch14-81. The red regions represent the gap between the Ch1459 and Ch14-77 markers in the assembly.

Objective 3. Employ whole genome 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 SNP markers to be used for mapping.

In this project and as detailed in previous reports we have proposed to use whole genome sequencing to genetically map two new resistant accessions, b46-43 and T03-16, which have very strong *Xf* resistance in repeated greenhouse screens. Next generation sequencing using Illumina HiSeq and MiSeq platforms to carry out SNP discovery and identification of SNP markers linked to resistance would only be used with those resistant lines for which we have strong greenhouse screen information, information on the heritability of their Pierce’s disease resistance, and the potential to screen the population using a limited mapping strategy.

The *V. arizonica* accession b46-43 is homozygous resistant to Pierce’s disease. Multiple crosses to *V. vinifera* were made to develop BC1 populations in 2014 and 2015. Breeding populations were tested with markers to verify the integrity of the crosses. Greenhouse screening of the BC1 populations with b46-43 and other resistant sources was completed (see companion project report) and results were used in conjunction with markers from chromosome 14 to evaluate the correlations between markers and resistance. Preliminary results indicate that there is a major Pierce’s disease resistance locus on chromosome 14. However, our breeding program has already identified two other accessions that have a major Pierce’s disease resistance locus on this chromosome. In order to optimize the development of broadly resistant Pierce’s disease winegrapes we need to use Pierce’s disease resistance sources that map to different regions, so that we have the greatest chance of stacking resistance genes from multiple and diverse sources. Test results suggest that b46-43 is not a unique source of Pierce’s disease resistance since it maps to the same location as *PdR1*, although it does have very strong resistance to *Xf*. In the light of these results, we will not pursue whole genome sequencing to map in the b46-43 background. We will

finalize the map of only chromosome 14 for the BC1 mapping population and complete screening in the greenhouse (take down of experiment is in the first week of August), with analysis in fall/winter 2016.

Objective 4. Cloning of *PdRI* genes with native promoters.

The physical map of *PdR1b* using four BAC clones covers 604 Kb (see previous reports for details). Multiple ORFs of the Leucine-Rich Repeat Receptor Kinase gene family were identified. These genes regulate a wide range of functions in plants, including defense and wounding responses for both host as well as non-host specific defense. The physical distance is limited to the 82 Kb, with five ORFs associated with disease resistance and other plant functions described above. The main challenge is the high sequence similarity of the ORFs that are present outside the genetic window to the candidate ORFs.

We have acquired optimized binary vectors pCLB1301NH and pCLB2301NK (Feechan et al., 2013) which are capable of carrying large DNA sequences, thus allowing us to insert the 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).

We have verified upstream and downstream sequences of V.ari-RGA14 and 18, two more likely *PdR1b* candidates. Both RGA14 and 18 (resistance gene analogs) have a very similar 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 RGAs revealed that RGA14 lacks a signal peptide in the initial part of the sequence. This result was verified 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, thus leaving RGA18 as the strongest candidate. Sequence verification for RGA14 and RGA18 and flanking sequences was completed, and fragments that contain the entire coding region plus ~3 kb upstream and ~1 kb downstream sequences were synthesized and cloned into pCLB2301NK at Genewiz, Inc. The new plasmids, called pCLB2301NK-14 and pCLB2301NK-18, were verified by restriction analysis in our lab (**Figure 4**). Besides the corresponding 7 kb fragment, containing RGA14 or RGA18, these plasmids contain a 35S:mGFP5-ER reporter cassette and a kanamycin-selectable marker gene with the nopaline synthase (NOS) promoter.

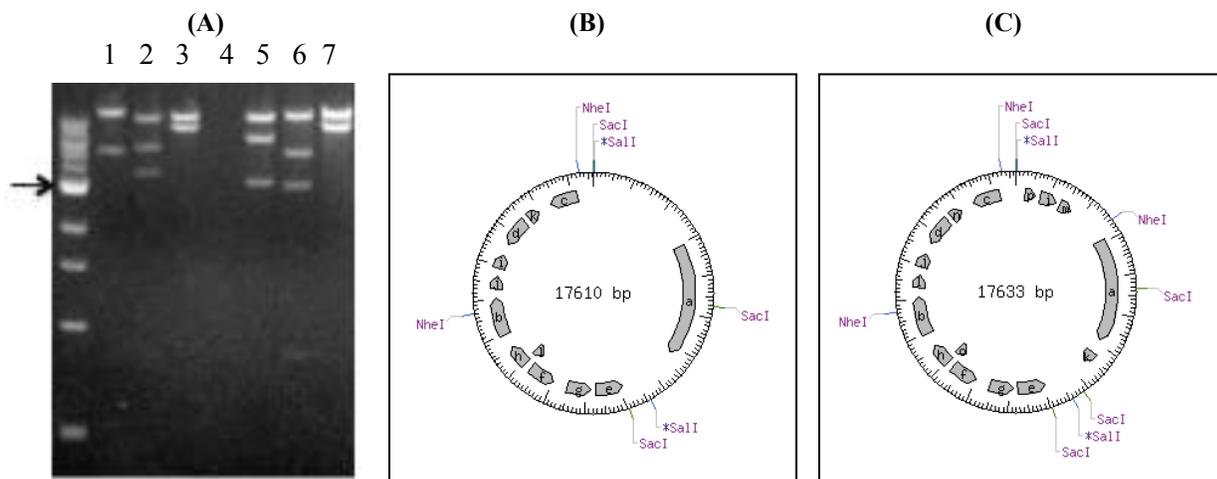


Figure 4. (A) Restriction analysis of plasmids pCLB2301NK-14 (lanes 2, 3, 4) and pCLB2301NK-18 (lanes 5, 6, 7) after digestion with NheI (lanes 2, 5), SacI (lanes 3, 6), and SalI (lanes 4, 7). Gel image includes a 1 kb ladder (lane 1) with the 3 kb fragment having increased intensity to serve as a reference band. The results on the gel match the predicted sizes inferred from the plasmid information; (B) pCLB2301NK-14 restriction map; (C) pCLB2301NK-18 restriction map.

We carried out sequence verification of genotypes U0505-22 and U0505-01, which are being used as biocontrols in our greenhouse screenings. These genotypes were originally selected for the presence of *PdR1b* markers in our

breeding program. However, U0505-22 displays Pierce's disease susceptibility despite being positive for the markers, which then offers the opportunity to explore the changes that could explain this behavior at the DNA level. Presently we are focused on RGA18 and RGA14 sequence verification, including the promoter region. Our first results have not been conclusive since direct sequencing of non-cloned PCR fragments, using primers originally designed for sequence verification of RGA14 and RGA18, produced mixed signals. Consequently, we have designed new primers to produce approx. 4.5 kb fragments that include sequences upstream and downstream of RGA14 or RGA18, in order to increase the specificity of the amplification and facilitate cloning by reducing the number of fragments to two.

A large experiment with resistant and susceptible plants using multiple replicates and time points for control (uninoculated) and inoculated plants (see details in previous report) was completed. 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 gene expression studies with both RGA14 and RGA18. Two different primer pairs with efficiency of greater than 90% were selected to carry out preliminary analysis with uninoculated and inoculated samples of Chardonnay and F8909-17 (source of *PdRI*). 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 uninoculated, peaks at day 23, and then decreases. Uninoculated and inoculated susceptible Chardonnay did not show any expression. Gene expression analysis is underway.

Objective 5. Comparing the Pierce's disease resistance of plants transformed with native versus heterologous promoters.

We have established an *Agrobacterium*-mediated transformation system followed by regeneration of plants from embryogenic callus. We have streamlined the protocol and have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless, Cabernet Sauvignon, Chardonnay, and the rootstock *V. rupestris* St. George (Agüero et al., 2006). In an earlier phase of this project we transformed these varieties with five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator, and an *hptII*-selectable marker gene (see previous reports for details). We completed testing and found that the transgenic plants did not confer Pierce's disease resistance or tolerance. These results are in accordance with the latest assembly obtained using the PAC BIO SR11 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 3'RACE technique was used to amplify RNA from V.ari-RGA14-transformed grapevines and results showed that mature mRNA does not contain the signal peptide necessary for proper membrane localization at the beginning of the sequence.

In addition to the embryogenic calli of Thompson Seedless, Chardonnay, Cabernet Sauvignon, and *V. rupestris* St. George we have available for transformation, we developed meristematic bulks of these genotypes plus 101-14 Mgt for transformation via organogenesis (**Figure 5**). Slices of meristematic bulks can regenerate transformed shoots in a much 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 meristematic bulks from these five genotypes (Xie et al., 2016). Meristematic bulks induction in non-*vinifera* genotypes is less efficient but still high, with about 80% of the explants producing meristematic bulks after three subcultures in medium containing increasing concentrations of cytokinins.

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 *PdRIb* 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.

Previous transformations with *Agrobacterium tumefaciens* carrying binary plasmids that contain hygromycin (pCLB1301NH) or kanamycin (pCLB2301NK) selectable marker genes showed that both antibiotics are effective selection agents for embryogenic calli. However, meristematic bulks regeneration has mainly occurred in selection with kanamycin, confirming our previous observation that meristematic bulks are highly sensitive to hygromycin. Thus, pCLB2301NK was chosen to carry RGA14 and RGA18 expanded sequences and named pCLB2301NK-14 and pCLB2301NK-18 thereafter.

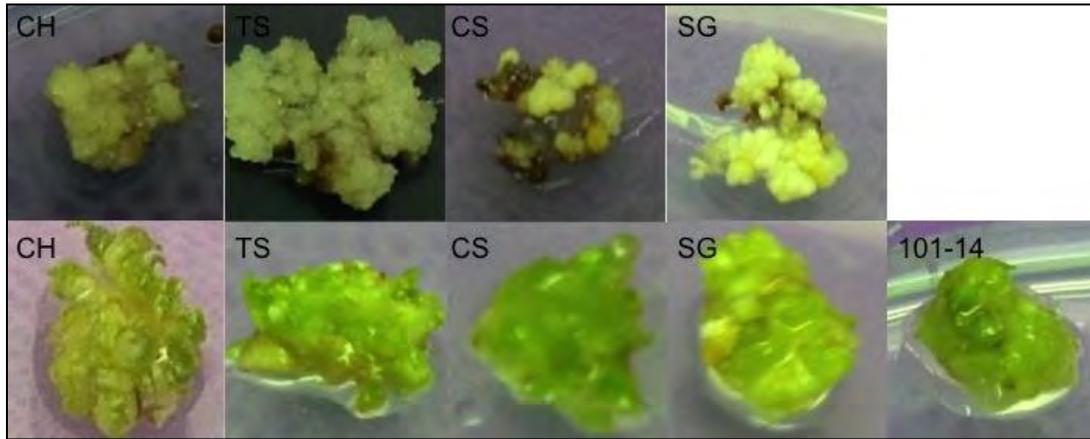


Figure 5. Embryogenic cultures (top) and meristematic bulks (bottom) of Chardonnay (CH), Thompson Seedless (TS), Cabernet Sauvignon (CS), *V. rupestris* St. George (SG), and 101-14.

Agrobacterium tumefaciens strain EHA 105 pC32 was chemically transformed with pCLB2301NK-14 or pCLB2301NK-18 and subsequently used to transform embryogenic calli of *V. vinifera* cvs. Chardonnay, Thompson Seedless, and rootstock *V. rupestris* St. George. Transformation experiments with pCLB2301NK-18 and pCLB2301NK-14 were initiated in March and July 2016, respectively, after synthesis and cloning was completed. In addition, *Agrobacterium* was used to transform meristematic bulks produced from the same genotypes. **Table 2** shows the number of inoculated explants with *Agrobacterium* carrying pCLB2301NK-18, while **Figure 6** shows the most advanced cultures growing in selection medium.

Table 2. Number of embryogenic calli (EC) and meristematic bulks (MB) inoculated with *Agrobacterium* carrying pCLB2301NK-18 or pCLB2301NK-14. The numbers in brackets represent the number of independent lines regenerating in selection medium to date.

Genotype	Explant	No. Explants pCLB2301NK-18	No. Explants pCLB2301NK-14
Chardonnay	EC	800 (10)	280
T. Seedless	EC	603 (37)	290
St. George	EC	692 (18)	401
Chardonnay	MB	70	50
T. Seedless	MB	80	120
St. George	MB	70	110

We have also started the production of meristematic bulks of Pierce's disease susceptible genotypes selected from the 04-191 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. Two of these genotypes, designated 29-42 and 47-50, exhibited great potential for the development of meristematic bulks (**Figure 6**) and will be ready for transformation with *Agrobacterium* next month.

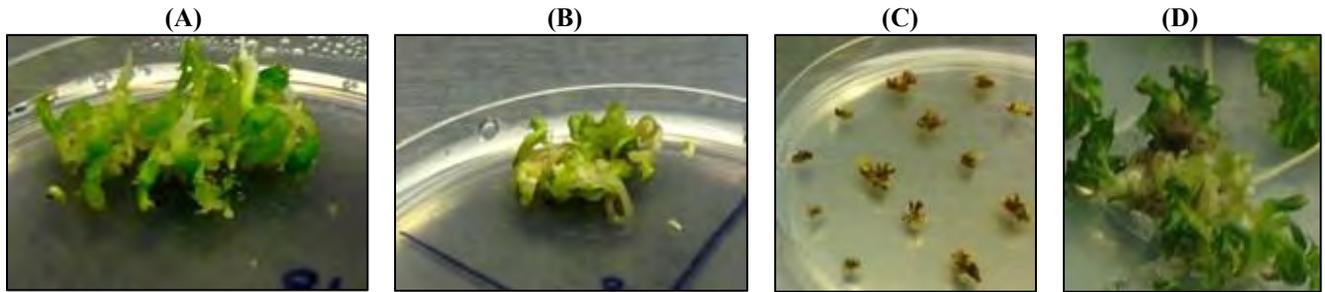


Figure 6. Embryo regeneration from embryogenic callus in (A) Thompson Seedless and (B) St. George. Shoot regeneration from meristematic bulks in (C) St. George. Meristematic bulk development in (D) genotype 47-50 from the 04191 population.

CONCLUSIONS

We completed greenhouse screening, marker testing, and QTL analysis of breeding populations from 15 new resistance sources, including b46-43 and T03-16. We identified T03-16 and b41-13 as possessing resistance on a different region than chromosome 14. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions. Our primary goal is to identify new sources of resistance whose resistance region is not on chromosome 14 so we can facilitate stacking of these resistance sources with *PdRI* from b43-17, since the incorporation of multiple resistances should make resistance more durable. We have completed the genetic and physical mapping of Pierce's disease resistance from b40-14. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. Finally, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with ORF18, and are preparing ORF14 for complementation tests. This effort is also identifying the promoters of these genes 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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PLASMID TRANSFER BY CONJUGATION AS A POSSIBLE ROUTE OF HORIZONTAL GENE TRANSFER AND RECOMBINATION IN *XYLELLA FASTIDIOSA*

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ABSTRACT

Horizontal gene transfer is an important component of evolution and adaptation of bacterial species. *Xylella fastidiosa* (*Xf*) has the ability to incorporate exogenous DNA into its genome by homologous recombination at relatively high rates. This genetic recombination is believed to play a role in adaptation of different *Xf* strains to infect different host plant species. Although in many cases exogenous DNA is taken up by natural transformation, there also is evidence that certain strains of *Xf* carry native plasmids equipped with transfer and mobilization genes, suggesting conjugation as an alternate mechanism of horizontal gene transfer in some instances. *Xf* subsp. *fastidiosa* strain M23 which causes disease in both grape and almond hosts carries a 38 kb plasmid pXFAS01. This plasmid contains two operons, *tra* and *trb*, that share homology with conjugal transfer and mating pair formation genes found in other bacterial species. A nearly identical plasmid, pXf-Riv5 was found in *Xf* subsp. *multiplex* strain Riv5 isolated from ornamental plum, suggesting plasmid transfer between *Xf* strains of different subspecies. Using M23 as the donor strain and *Xf* subsp. *fastidiosa* Temecula as the recipient strain, plasmid transfer was characterized using the mobilizable broad host range vector pBBR5pemIK. Transfer of plasmid pBBR5pemIK from M23 to Temecula was observed under *in vitro* conditions, although transfer of 38 kb pXFAS01 was not observed. The possibility of plasmid transfer by conjugation in the natural environment would have implications for horizontal gene transfer between different strains of *Xf* that may be present in the same location and/or in the same vector or host.

FUNDING AGENCIES

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STUDY OF THE TAXONOMY OF *XYLELLA* BASED ON WHOLE GENOME SEQUENCES

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ABSTRACT

Many members in the bacterial genus of *Xylella* cause disease on economically important crops, generally in the Americas, e.g. Pierce disease of grapevine in the United States and citrus variegated chlorosis disease in Brazil. In the past decade, there has been an increase of reports on *Xylella*-caused diseases from outside the Americas, e.g. pear leaf scorch disease in Taiwan and olive quick decline syndrome in Italy. Because all *Xylella* strains are nutritionally fastidious, phenotypic characterizations such as physiological and biochemical tests are highly challenging to perform and could be inaccurate, leading to the current situation of ambiguous taxonomy and difficulty in strain detection and identification. We are taking advantage of recent development of next generation sequencing technology to establish a whole genome sequence-based system to address *Xylella* taxonomy. One significant achievement is the use of average nucleotide identity (ANI) values to replace the time-consuming and technically demanding DNA-DNA hybridization relatedness, a commonly accepted gold standard for bacterial species definition. Thus far, this research has established a new species, *Xylella taiwanensis*. The ANI values between *X. taiwanensis* and *X. fastidiosa* strains were 83.4-83.9%, significantly lower than the bacterial species threshold of 95%. Interestingly, ANI analyses also drew defined lines among *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, and *X. fastidiosa* subsp. *pauca*, suggesting that ANI values, along with sequence similarity in other genes/sequences including the 16S rRNA gene and 16S-23S ITS, also could be useful for subspecies analyses.

FUNDING AGENCIES

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THE DIVERSITY OF *XYLELLA FASTIDIOSA* IN FRANCE

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ABSTRACT

Xylella fastidiosa (*Xf*) has been thought to be restricted to the Americas for a long time. Listed as a quarantine pest for Europe, this phytopathogenic bacterium is now present in Italy and France and its emergence has been evidenced through a survey of coffee plants trading from Latin America and a survey of natural settings. Since the first contaminated foci have been discovered on *Polygala myrtifolia* (myrtle-leaf milkwort) plants showing leaf scorch symptoms in France during the summer of 2015, numerous plants were screened, ending in the identification of almost 250 foci. Here we report on the diversity of *Xf* identified during the year 2015 in France in natural settings and in imported coffee plants. A multilocus sequence typing (MLST) approach revealed that several subspecies and sequence types are associated with the emergence of *Xf* in France. This includes subspecies *multiplex* (ST6 and ST7), subspecies *pauca* (ST53), and subspecies *sandyi* (ST72 and ST76). Moreover, new recombinant individuals issued from subsp. *multiplex* and *sandyi* parents, and populations living in sympatry (same plant) have been found. The genome of the first three strains of *Xf* subsp. *multiplex* that were isolated were sequenced and compared to their American relatives. Altogether, these analyses suggest that *Xf* has been introduced several times in the past into France in various plant materials from different origins.

FUNCTIONAL CHARACTERIZATION OF THE ROLE OF *RPFA* IN *XYLELLA FASTIDIOSA*

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ABSTRACT

Xylella fastidiosa (*Xf*) coordinates its virulence in grapevines via quorum sensing signal molecules that are regulated and synthesized by the *rpf* gene cluster (regulation of pathogenicity factors). *rpfA* encodes aconitate hydratase and could play a regulator role involved in virulence. To elucidate the role of *rpfA* in the pathogenicity of *Xf*, an *rpfA*-mutant (*Xf*Δ*rpfA*) and a complementary (*Xf*Δ*rpfA*-C) strain were characterized. *In vitro* studies showed that mutant *Xf*Δ*rpfA* exhibited increase in biofilm formation and cell-cell aggregation compared with wild-type. The complementary *Xf*Δ*rpfA*-C strain restored wild-type phenotypes. These data suggest that the expression of *rpfA* may negatively modulate biofilm formation or other related virulence factors. Greenhouse experiments will be conducted to further evaluate the role of *rpfA* involving Pierce's disease.

FUNDING AGENCIES

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MODELING DEPLOYMENT OF PIERCE'S DISEASE RESISTANT GRAPEVINES

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ABSTRACT

Deployment of Pierce's disease resistant grapevines is a key solution to mitigating economic losses caused by *Xylella fastidiosa* (*Xf*). While the Pierce's disease resistant grapevines under development display mild symptoms and have lower bacterial populations than susceptible varieties, all appear to remain hosts of *Xf*. Since resistant grapevines are anticipated to maintain yield after infection, resistant grapevines are less likely to be removed after infection than susceptible grapevines. Accordingly, there is a risk that vineyards planted with resistant grapevines may become sources of *Xf*. To assess the risk of resistant varieties serving as a source for pathogen spread to susceptible varieties, a coupled-differential equation model was developed. The model tracked spread of an arthropod-transmitted pathogen in a plant population consisting of a mixture of resistant and susceptible plants. To analyze the model, infection of susceptible plants was separated into two components: spread among susceptible plants and spread from resistant to susceptible plants. Analytical manipulation of the model identified a threshold acquisition rate from resistant plants that resulted in limited pathogen spread from resistant plants to susceptible plants. Acquisition rates from resistant plants that resulted in limited spread to susceptible plants depended on assumptions regarding vector abundance, vector turnover (mortality), removal of infected susceptible plants, and proportion of plants that were resistant. Thus, acquisition rates from resistant plants that result in limited spread to susceptible plants depend on management practices. Simulation of the model determined that effects of deploying a resistant variety on disease incidence in the susceptible variety depended on the extent to which pathogen spread among susceptible plants was controlled (by means other than resistance) and acquisition rates from resistant plants. Deployment of resistant plants that were poor acquisition sources generally resulted in lower disease incidence in the susceptible variety, whereas deployment of resistant plants that were good acquisition sources generally increased disease incidence in the susceptible variety. Results support quantifying acquisition from resistant varieties prior to deployment to determine if additional management is needed to limit spread to nearby vineyards cultivating susceptible varieties. As the model framework was general, additional refinement of the model to consider specific regions, grape cultivars, and vectors in California could provide additional insight.

FUNDING AGENCIES

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Section 2:

Glassy-winged Sharpshooter

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

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ABSTRACT

For approximately 15 years Temecula Valley has been part of an area-wide control program for an invasive vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). 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 GWSS control, spring applications of the systemic insecticide imidacloprid to citrus have been made in years past. As part of this treatment program there is ongoing monitoring of GWSS 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 several seasons, no Temecula Valley citrus acreage was treated specifically for GWSS, although it is likely that some treatments are occurring to target important citrus pests. Approximately 135 yellow sticky traps were inspected on a biweekly basis throughout 2016 to monitor GWSS in citrus. The results show a typical phenology for this pest in the region, with a total of approximately 650 GWSS caught during the summer peak (July through September). Overall, GWSS catch this year has been the highest observed since 2009, but is still far below the densities seen at the outset of the area-wide program.

LAYPERSON SUMMARY

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) constitutes one of the primary threats to the wine, table grape, and raisin industries in California owing to its ability to spread the pathogen that causes Pierce's disease. In the Temecula Valley an area-wide control program has been in place for more than 15 years which, until recently, relied on insecticide applications in citrus groves to control GWSS before they move into vineyards and still entails regular monitoring of GWSS populations throughout the region. This program is important for guiding management decisions for vineyards in the area. This year, GWSS catch was the highest seen in seven years. It is not yet clear whether the pattern this year indicate a resurgence in GWSS populations, as has occurred in other parts of California, or simply reflects a single, anomalous season.

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 (*Homalodisca vitripennis*; GWSS) into southern California from the southeastern United State, 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 GWSS management program initiated in the spring of 2000 saved the industry from even more dramatic losses. Since the initiation of the Temecula GWSS area-wide management program several hundred new acres of grapes have been planted and multiple new wineries have been built.

GWSS has the potential to develop high population densities in citrus. Fortunately, GWSS 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. In addition, parasitoid wasps that attack GWSS egg masses are also contributing to management in the region.

As part of the area-wide treatment program monitoring of GWSS 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 GWSS 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 GWSS populations, which already show substantial interannual variability, appear to be rebounding. This is particularly true given the notable resurgence of GWSS in other areas of the state.

OBJECTIVES

1. Monitor regularly GWSS 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 grapegrowers.
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 for adult sharpshooters in citrus. One hundred thirty-four 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 (GPS) 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 GWSS and smoketree sharpshooters (*Homalodisca liturata*) are recorded, and 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://c isr.ucr.edu/temeculagwss/>).

RESULTS AND DISCUSSION

The results for 2016 are shown in **Figure 1**. This includes monthly censuses of GWSS in citrus through April, then biweekly censuses from May through October. Census results show seasonal patterns of GWSS abundance and activity that are typical for this region. GWSS 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, GWSS populations appear to have declined substantially.

Figure 2 shows GWSS catch in 2016 relative to other years. The year 2016 has shown a qualitatively similar seasonal phenology as in other years, but with a higher overall catch compared to recent years. Indeed, the 2016 GWSS catch was the highest since 2009, but is still several times lower than at the inception of the program.

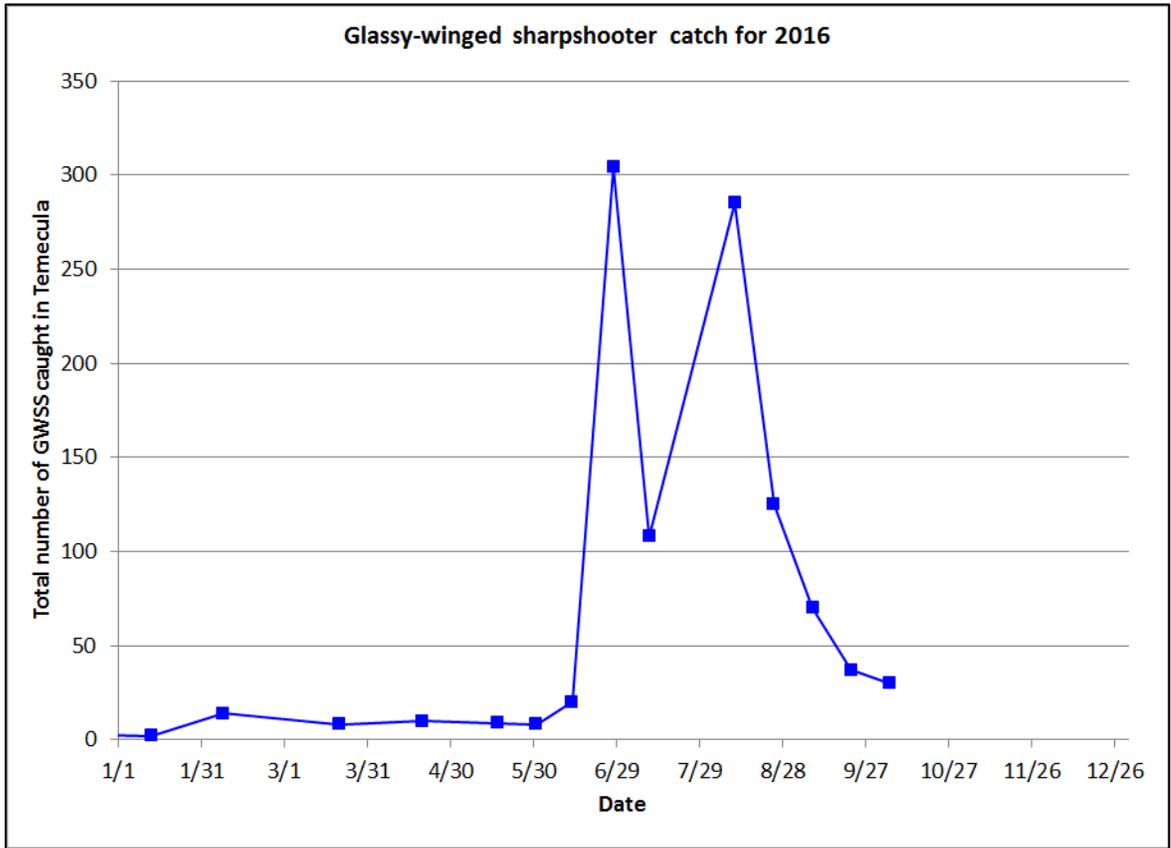


Figure 1. Seasonal total GWSS catch in 2016 for 134 traps throughout the Temecula Valley.

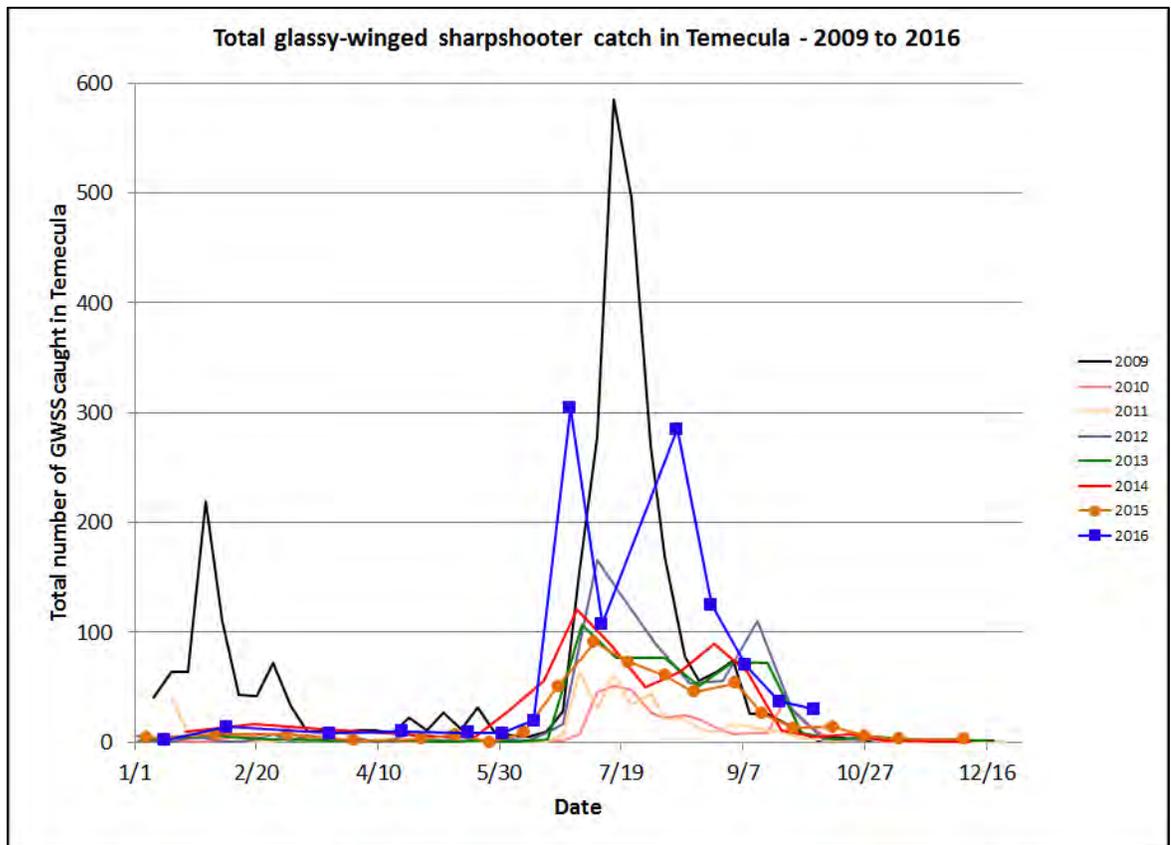


Figure 2. Seasonal total GWSS catch in Temecula Valley from 2009-2016.

CONCLUSIONS

Despite the troubling patterns observed this year there is not yet compelling evidence of a GWSS resurgence in the Temecula Valley region, as is occurring in portions of the southern Central Valley. Although the overall 2016 GWSS catch appeared earlier in the season and slightly higher (i.e., 100 to 300 GWSS) than most years, it is still lower overall compared to the 500 to 1,000+ GWSS catches in 2008 and 2009. 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 GWSS, as long as insecticide resistance does not develop, applications made for its control may aid somewhat with GWSS control. Nonetheless, in response to the relatively higher GWSS catch this year, Temecula grape growers were cautioned to remain vigilant and consider alternative steps for managing Pierce's disease pressure in their vineyards.

FUNDING AGENCIES

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

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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 evaluated several approaches in attempts to induce RNAi effects in the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), an important vector of *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevines. In order to identify promising RNAi targets we performed transcriptome and small RNA next generation sequencing of GWSS. We identified RNAi-based responses to GWSS-infecting viruses and assessed *in vitro* feeding and transgenic plant assays as a means to initiate RNAi effects against GWSS. We were able to demonstrate RNAi-induced decreased mRNA levels for specific RNA targets but we did not obtain consistent phenotypic effects on GWSS.

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 U.S., and the causal agent, *Xylella fastidiosa* (*Xf*), a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xf*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), and other sharpshooter vectors of *Xf*.

We focused our efforts towards understanding and optimizing the means to induce RNAi effects in GWSS. In this regard we evaluated specific interfering RNAs via *in vitro* assays and transgenic plant-based approaches. We also generated large scale genomic data along with transcriptome and small RNA datasets, to help us design rational and effective genetic/genomic efforts against GWSS. We achieved target mRNA reductions in some assays but did not consistently induce desired phenotypic effects in recipient GWSS.

INTRODUCTION

Our primary objectives were to evaluate and demonstrate RNA interference (RNAi) activity against the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). GWSS is an important vector of *Xylella fastidiosa* (*Xf*) and unlike other native sharpshooters, GWSS readily feeds on grapes and has the potential to move through vineyards, moving *Xf* as it feeds. New, environmentally sound approaches to target GWSS and other sharpshooter vectors of *Xf* are needed in order to help manage Pierce's disease. RNAi strategies have the potential to help in long-term, environmentally sound strategies to manage insects.

RNAi is a natural gene regulation and anti-viral defense mechanism found in insects and other organisms. RNAi was discovered in the early 1990s when studies with plants demonstrated that transgene-encoded RNAs did not accumulate in plants as expected, but were degraded in the cell cytoplasm in a sequence-specific manner (Jorgensen et al., 1996; Lindbo and Dougherty, 1992; Lindbo et al., 1993; Napoli et al., 1990). Most important from a practical sense is that these also correlated with desirable phenotypic effects in the plants. Since these initial findings, RNAi has become one of the most intensely studied areas in all of biology and the 2006 Nobel Prize in Physiology or Medicine was awarded for seminal mechanistic studies on RNAi in the nematode *Caenorhabditis elegans* (Fire et al., 1998).

Double-stranded RNAs (dsRNAs), or single-stranded RNAs (ssRNAs) with significant intramolecular base-paired regions, are recognized as powerful inducers of RNAi. These RNAs are processed by dsRNA-specific endonucleases (Dicers and/or Drosha, depending on the organism and cellular location) to yield small dsRNAs ranging from 20 – 30 bp. The resulting small dsRNAs are unwound and one strand (the guide strand) is incorporated into the Argonaute 1 (AGO1)-associated RNA-induced silencing complex (RISC). When the guide RNA searches and finds a complementary ssRNA, RNAi activity results, either mRNA degradation or interference with mRNA translation, depending on the type of guide RNA and the amount of base-pairing with the RNA target. Although RNAi processes vary in different organisms, the overall mechanisms among various eukaryotes are generally conserved (Siomi and Siomi, 2009). We use the term “siRNAs” to refer in general to the 21-25 nucleotide (nt) small RNAs generated by RNAi activity. The major value of RNAi approaches for agriculture is that they can be used to very selectively reduce specific gene expression and induce desirable phenotypes in plants, particularly to prevent pathogen or pest attack.

Transgenic RNAi-specific approaches have already been demonstrated to be very effective for many different plant viruses, and commercial RNAi-based antiviral resistance is used in U.S. papayas, squash, and recently plums (Fuchs and Gonsalves, 2007; Gonsalves, 2006; Scorza, 2013; Tricoli, 1995). Hundreds of thousands of these plants have been planted in the U.S. and the specific, RNAi-based anti-viral resistance has proven to be robust and to provide environmentally sound virus disease control with no identified negative effects. In recent years RNAi has been evaluated as a control strategy for insects, even leading to the suggestion of “insect-proof plants” (Gordon and Waterhouse, 2007). In fact, a recent issue of *Science* (16 August 2013) featured on the cover the suggestion of “smarter pest control” and a special section within that issue (pages 728 – 765) was dedicated to our “pesticide planet” (Kupferschmidt, 2013), and how new opportunities based on our understanding of RNAi could help feed the world’s growing population and how this could be done in a way that improves sustainability in agriculture by decreasing our dependence on pesticides for pest control. We took fundamental approaches to assess the potential for inducing RNAi effects in GWSS and evaluated different strategies to induce RNAi activity. We successfully demonstrated induction of RNAi effects by several approaches, but did not consistently demonstrate the ability to induce negative phenotypic effects in GWSS. However, our results provide new information that is important for assessing RNAi strategies against insect vectors of plant pathogens.

OBJECTIVES

1. Generate and evaluate transgenic potato plants for their ability to generate small RNAs capable of inducing RNAi effects in GWSS.
2. Identify GWSS-interfering RNAs for practical application.
 - a. Utilize transgenic potato plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.
 - b. Enhance production of interfering RNAs *in planta*.
3. Generate and use microRNAs from different developmental stages of GWSS insects.
4. Assess the potential of using plant viruses for delivery of small RNA effectors.

RESULTS AND DISCUSSION

Objective 1. Generate and evaluate transgenic potato plants for their ability to generate small RNAs capable of inducing RNAi effects in GWSS.

Initially, we used 14 GWSS Genbank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we showed that RNAi activity is inducible in GWSS (Rosa et al., 2010). Quantitative reverse transcription polymerase chain reaction (RT-PCR), semi-quantitative RT-PCR, and Northern blot of small and large RNA

fractions showed that RNAi was achieved in cells and insects injected with dsRNA where target mRNAs were partially degraded and specific siRNAs (short-interfering RNAs), hallmarks of RNAi, were detected (Rosa et al., 2010).

In order to generate dsRNAs that can target GWSS, target sequences were cloned into a gateway-compatible binary vector pCB2004B. The target sequences were cloned in head-to-tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *Escherichia coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform potato plants (Kennebec and Desiree varieties) against GWSS target genes Actin, Cuticle, and Chitin Deacetylase (**Table 1**). Transformation / regeneration were performed via recharge at the UC Davis Ralph M. Parsons Plant Transformation Facility (<http://ucdptf.ucdavis.edu/>) and approximately ten independent transgenic lines were obtained for each of the constructs. We performed screening of these transgenic potato plants for insert composition and generation of small RNAs (**Figure 1**). We vegetatively propagated the T₀ plants confirmed to yield the desired RNAs for use in RNAi experiments with GWSS. In addition to the transgenic plants expressing GWSS target genes under control of the 35S promoter we generated some potato plants with transgene expression under a specific xylem promoter EgCAD2, which was cloned from *Eucalyptus gunii*.

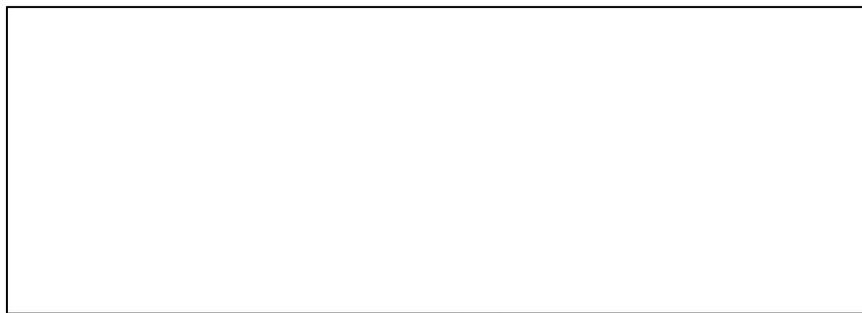


Table 1: GWSS insect sequences used for cloning and generation of po

GWSS Targets	Potato Pedigree	Potato Variety	Selection
Chitin Deacetylase	102203	Kennebec	Bas
Chitin Deacetylase	102203	Kennebec	Bas
Actin	112064	Desiree	Bas
Cuticle	102203	Desiree	Bas

We used potato cuttings with caged fourth and fifth instar GWSS nymphs. The cuttings were placed in dilute nutrient solution and GWSS remained on cuttings for approximately seven days (**Figure 3**). The GWSS nymphs were allowed to feed on the cuttings for five days at which point the insects were harvested and RNA was extracted to test for target mRNA knockdown using RT-qPCR. Unfortunately, these feeding trials did not induce a detectable phenotype or result in consistent, detectable reduced target gene expression when compared to the wild-type and green fluorescent protein (GFP) negative control plants. 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 (i.e., they must go through nymphal instar stages and molt). For GWSS 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. We also performed experiments using second and third instar nymphs and kept insects until they became adults, but no obvious effects were observable.

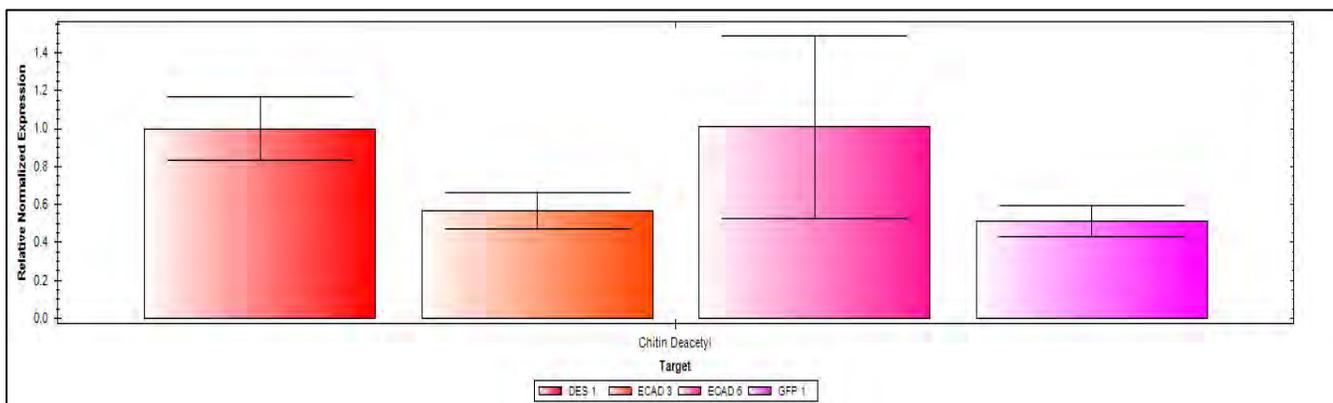


Figure 2. Relative normalized expression of the GWSS chitin deacetylase gene after GWSS feeding on wild-type and transgenic plants expressing dsRNA showing no difference in target gene expression between wild-type and transgenic plant lines. DES 1 is the wild-type potato control. ECAD 3 and ECAD 6 are separate transgenic lines expressing dsRNA for GWSS chitin deacetylase under control of the EgCAD promoter. GFP 1 is a control transgenic line expressing dsRNA for GFP. The GWSS ubiquitin gene was used as an internal control for the RT- qPCR. Error bars represent the standard error of the data.

Objective 3. Generate and use microRNAs from different developmental stages of GWSS insects.

We evaluated three approaches for expressing artificial microRNAs (amiRNAs) in plants. Our intent was two-fold: one, to use specific amiRNAs to target GWSS 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 GWSS-novel miRNAs by Illumina-based sequencing and bioinformatics analysis (see **Figure 4**). We have so far only identified miRNAs in adult GWSS, but our goals are to identify potential miRNAs that may be GWSS instar-stage specific and evaluate their potential for use in RNAi towards GWSS.

We 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 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.



Figure 3. GWSS feeding on basil stem which is submerged in a solution of double-stranded RNA.

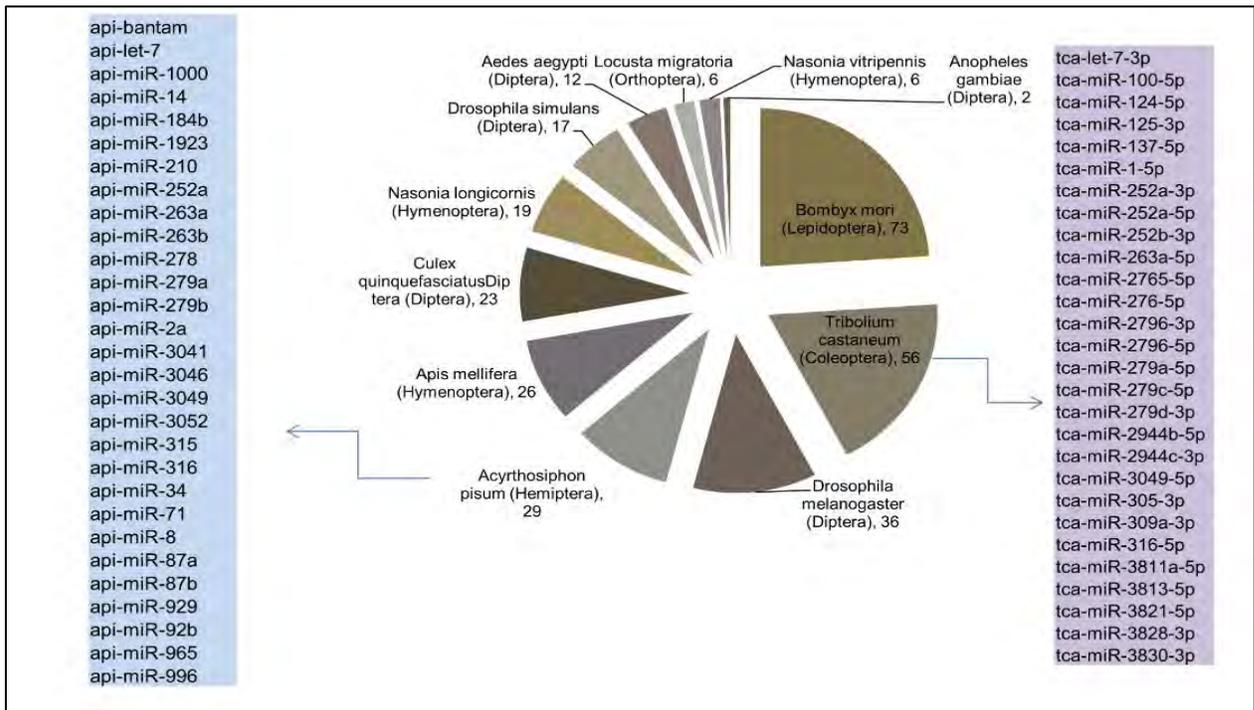


Figure 4. The microRNA profile analysis of GWSS adult insects revealed the presence of microRNAs that are conserved between different insects. GWSS adults also share some microRNA conservation with plants.

Objective 4. Assess the potential of using plant viruses for delivery of small RNA effectors.

Our efforts here were based on our previous successes using plant-infecting viruses to express interfering RNAs in plants. There we used recombinant plant viruses expressing insect RNAi inducers and were able to achieve negative phenotypes in specific phloem-feeding target insects. Here we attempted to engineer grapevine leafroll-associated virus-7 (GLRaV-7), a phloem-restricted virus from the complex family *Closteroviridae*, and grapevine red blotch-associated virus (GRBaV) for our 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), 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 were capable of expressing foreign genes/sequences for long periods of time showing a significant stability and durability. Furthermore, our own results using a DNA virus-based system (unpublished) showed that we were able to produce specific artificial micro RNAs in plants.

We have a GLRaV-7 isolate from California in culture (Genbank accession number: JN383343; Al Rawhni et al., 2012), and now have generated complete, full-length cloned cDNAs for this virus. The entire cDNA of GLRaV-7 is now cloned into a binary vector, pCAMBIA₁₃₈₀. Experiments are still ongoing to assess infectivity by using standard agroinfiltration, but we also will attempt 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 we also have been attempting to generate infectious cloned cDNA versions of GRBaV. We are making progress but this work also is ongoing.

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 GWSS and evaluated initial transgenic plants as a means to initiate RNAi effects in GWSS and other leafhopper vectors of *Xylella fastidiosa* (*Xf*). Our lack of producing a desired negative phenotype in target GWSS was due at least in part to the experimental system and biology available. As we have learned more about GWSS and RNAi application potential, it seems unlikely that the plant-based approaches used by us have good potential for helping

to manage GWSS and other sharpshooter vectors of *Xf*. This is important and thus our data are useful for long-term decisions. However, because RNAi effects can be induced in GWSS, the use of insect-infecting viruses modified to induce specific RNAi effects in sharpshooters is a potential strategy that could be considered for future experimentation.

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

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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 (GWSS; *Homalodisca vitripennis*). The initial target for RNAi will be GWSS *jheh* (also known as *hovi-meh1*), the gene that encodes juvenile hormone epoxide hydrolase (JHEH). GWSS *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 JH metabolic genes including those that encode JH esterase, JH acid methyl transferase, and other identified genes as targets for RNAi. These gene sequences will be mined from the recently determined transcriptome sequence of GWSS. 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 (GWSS; *Homalodisca vitripennis*) as targets for RNAi. A field-applicable delivery system for inducing RNAi against these targets will also be developed.

INTRODUCTION

In California, the control of the glassy-winged sharpshooter (GWSS; *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 on 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 GWSS 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 U.S. 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 GWSS should be considered.

RNA interference (RNAi)-based technologies [3, 4] that selectively target the GWSS endocrine system are a potential alternative tactic for controlling GWSS and the diseases that it transmits. RNAi is a natural process that is 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 GWSS endocrine system are being developed as targets for RNAi. Genes in the insect endocrine are ideal targets for knockdown because they are part of an essential and highly sensitive developmental pathway that is only found in arthropods.

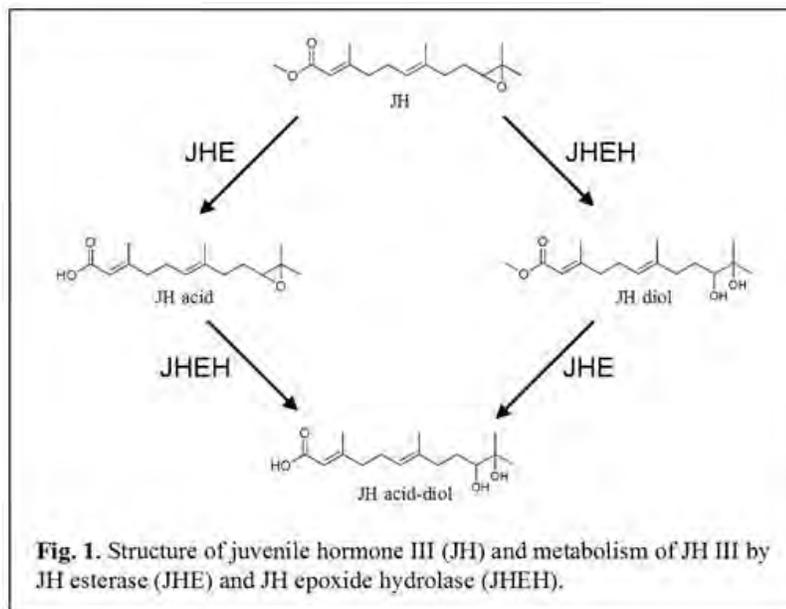
OBJECTIVES

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

RESULTS AND DISCUSSION

Objective 1. Develop *jheh* as a model target for RNAi-based control of GWSS 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. In many insects, 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**).

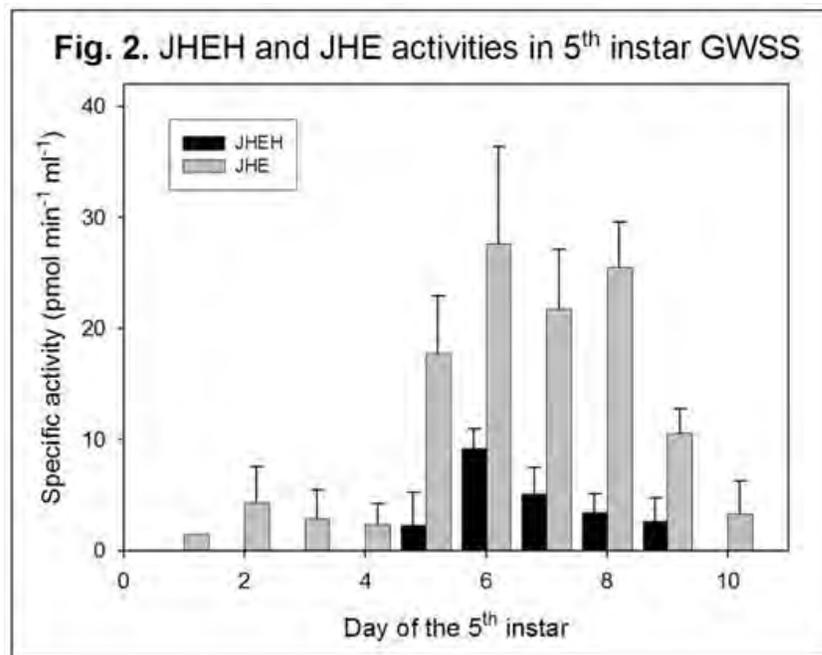


Minor changes in normal JH levels through alteration in the action (or lack of action) of JHEH, JHE, and/or JHAMT have been shown to cause dramatic changes in insect development and/or death. The sensitivity of the insect endocrine system to minor changes is a critical factor in the success of JH analog insecticides such as pyriproxyfen and methoprene.

The coding sequence of the *jheh* gene of GWSS 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 GWSS. Plasmid constructs for the expression of full-length dsRNAs corresponding to *jheh* of GWSS have been designed and are in the construction process. The baseline levels of JHEH and JHE activities in control fifth instar GWSS have been quantified (**Figure 2**).

Detailed information about 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 of GWSS, JHE activity was relatively low (1.5 to 4.4 pmol of JH acid formed $\text{min}^{-1} \text{ml}^{-1}$ 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- to 11-fold higher than that found on the fourth day of the fifth instar) on the sixth, seventh, 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 suggested that JHE may play a more predominant role than JHEH in JH metabolism in GWSS.

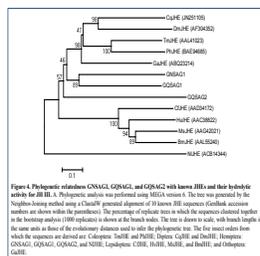


Objective 2. Mine the GWSS transcriptome for other RNAi targets.

A transcriptome is defined as a set of all of the RNA molecules that are found in a specific set of cells at particular moment in time. Cooperator Professor Bryce Falk's laboratory has recently determined the sequence of the transcriptome of fifth instar GWSS [7]. By computer software-based screening of the GWSS 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 GWSS, double-stranded cloned DNA (ds cDNA) libraries were generated from a developmentally mixed population of fifth instar GWSS (30 individuals) as well as individual GWSS at day 7, 8, and 9 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.

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 GWSS. *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. However, two additional methionine codons are 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 is predicted. Phylogenetic analysis placed GNSAG1 and GQSAG1 in the same clade (**Figure 4**). 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 NIJHE, a JHE from the hemipteran *Nilaparvata lugens*.

Figure 3 legend. JHE-like nucleotide and deduced amino acid sequences from nymphal GWSS. 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, respectively. The asterisk indicates a stop codon (TAG or TGA). Seven amino acid sequence motifs (RF, DQ, GQSAG, E, GxxHxxD/E, R/Kx₆R/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.



In order to determine if GNSAG1, GQSAG1, and GQSAG2 are able to hydrolyze JH at a rate that is consistent with known JHEs, recombinant baculoviruses expressing these proteins were generated. Initially, four constructs were generated that expressed GNSAG1, GQSAG2, and two forms of GQSAG2. Namely, constructs expressing the full-length GQSAG2 (i.e., GQSAG2L) and a slightly (11 amino acid residues) shorter version of GQSAG2 (i.e., GQSAG2S) were produced. The GQSAG2S protein initiates from the third methionine codon (see **Figure 2C**) resulting in a protein (unlike GQSAG2L) that encodes a predicted signal peptide for secretion. Unfortunately, these constructs produced recombinant proteins that showed approximately 3,000-fold or lower specific activity for JH III in comparison to a known JHE that was expressed and assayed under identical conditions (**Table 1**). In order to confirm that the cDNA insert of these recombinant baculoviruses was correct, new recombinant baculoviruses were isolated and the recombinant protein expressed by these new baculoviruses was tested for JH hydrolytic activity. These newly expressed proteins showed the same pattern of specific activity for JH III as the original constructs (**Table 1**).

Table 1. Specific activity of recombinant GNSAG1, GQSAG1, GQSAG2L, and GQSAG2S for JH III

Protein Source ¹	Specific Activity ² (nmol JH III acid/min/ml)	Total Activity (nmol JH III acid/min)
GNSAG1-A supernatant	<0.007	<0.7
GNSAG1-B supernatant	<0.003	<0.3
GQSAG1-A supernatant	0.007 ± 0.001	0.7
GQSAG1-B supernatant	0.005 ± 0.002	0.5
GQSAG2L-A supernatant	<0.007	<0.7
GQSAG2L-B supernatant	<0.003	<0.3
GQSAG2S-A supernatant	<0.007	<0.7
GQSAG2S-B supernatant	0.003 ± 0.001	0.3
CqJHE supernatant	22.1 ± 3.5	2,210
CqJHE cell lysate	1.3 ± 0.2	130

¹The culture supernatant of recombinant baculovirus-infected High Five cells was diluted 1:10 for the recombinant GWSS proteins or 1:1000 for CqJHE. The cell pellet of the CqJHE baculovirus-infected High Five cells was resuspended in the same volume of buffer (100 mM sodium phosphate buffer, pH 8) as was used for cell culture. The baculovirus-infected High Five cells and supernatant was harvested at 65 h post inoculation. The "A" and "B" notations indicate supernatant from cells that were inoculated with independently isolated recombinant baculovirus clones.

²Specific activity was determined in 100 mM sodium phosphate buffer, pH 8, containing 1 mg/ml BSA, and 5 µM JH III. The reactions were allowed to proceed at 30°C for 15 or 150 minutes. The hydrolytic activity of CqJHE, a known juvenile hormone esterase from the mosquito *Culex quinquefasciatus*, was determined under the same conditions.

Objective 3. Develop virus-based dsRNA production and delivery systems for controlling GWSS.

Insect viruses are used as highly effective biological insecticides to protect against pest insects of forests and agricultural plantings such as soybeans. 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 GWSS, *Homalodisca coagulata* virus-1 (HoCV-1) [10] and *Ho. vitripennis* reovirus (HoVRV) [11], are well-characterized. HoCV-1 and HoVRV are naturally found in GWSS populations in the field but they are not severely pathogenic against GWSS. Recently, an *in vitro* system (i.e., a continuous cell line) that appears to support the replication of HoCV-1 has been identified [12]. The availability of an *in vitro* system (i.e., [12, 13]) is a critical tool for the genetic modification of a virus that is highly pathogenic in GWSS. The primary goal of Objective 3 is to identify new, highly pathogenic GWSS 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

The overall goal of this project is to study and exploit targets within the endocrine system of GWSS that can be used to control GWSS or reduce its ability to spread Pierce's disease. The approach involves the identification and characterization of genes that are unique to the GWSS endocrine system that metabolize a key insect hormone called juvenile hormone. Once characterized the genes will be targeted for gene knockdown using a process called RNA interference. A potential outcome of this project is the development of an alternative control strategy for GWSS. Thus far we have determined the baseline levels of JHE and JHEH activities in fifth instar GWSS 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.

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

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Reporting Period: The results reported here are from work conducted July 1, 2014 to June 30, 2016.

ABSTRACT

Exploitation of vibrational signals for suppressing glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) populations in citrus orchards and vineyards could prove to be a useful tool. However, existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California. Therefore, the objective of this study was to identify and describe substrate-borne signals associated with intraspecific communication of GWSS. Recordings of GWSS placed together on plants revealed a complex series of behaviors linked to vibrational signals that lead to mating. Analysis of the spectral and temporal features of GWSS vibrational signals such as frequency span, dominant and/or fundamental frequency, intensity, and pulse repetition rate identified candidate disruptive signals (natural and synthetic) that can be reproduced in any GWSS host plant, but efficacy of such signals in interfering with GWSS communication remains to be determined.

LAYPERSON SUMMARY

Animal communication is vital to reproduction, particularly for securing a mate. Some insects, including the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), communicate by exchanging vibrational signals that are transmitted through host plants. Since GWSS mate selection behaviors rely on vibrational communication, what if signals can be interfered with to prevent communication? If animals fail to communicate, population densities are likely to reduce due to lack of fertilization. Exploitation of disruptive vibrational signals for suppressing GWSS populations in citrus orchards and vineyards could prove to be a useful tool for reducing incidence of Pierce's disease, but existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California. Using a laser-Doppler vibrometer and associated softwares, project scientists identified and described signals used by GWSS to communicate. Candidate disruptive signals (natural and synthetic) that can travel in any GWSS host plant (including citrus and grapevines) were identified, but efficacy of such signals remains to be determined.

INTRODUCTION

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) is a polyphagous pest that mates and lays eggs on hundreds of plant species, including grapevines (**Figures 1A** and **1B**). In laboratory conditions, highest fecundity and longevity observed for a single GWSS female were 967 eggs and 296 days, respectively (Krugner, 2010). On grapevines, GWSS reproduce from spring to fall producing at least two generations per year. During winter months, GWSS population densities decline sharply and are strictly associated with non-deciduous shrubs and trees. Population size is a result of the combined actions of births, deaths, immigration, and emigration. While products (e.g., insecticides) are available to increase mortality of insect vectors of plant pathogens, research is needed to identify methods to reduce birth (Sisterson and Stenger, 2016). Current measures to reduce GWSS population density in California include mass release of egg parasitoids and insecticide applications in urban and agricultural areas. Despite such efforts, geographic distribution and population densities of GWSS continue to expand. Chemical control of GWSS 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 GWSS in vineyards, particularly in areas where Pierce's disease is endemic, poses a constant challenge for grape growers. Thus, long-term suppression of GWSS 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, localization, and courtship occur via substrate-borne vibrational signals transmitted through the plant. Disruption of communication has been the goal of research on many harmful organisms, including *Xylella fastidiosa*. Since the early 2000s, methods have been developed to interfere with *X. fastidiosa* cell-cell communication (Lindow et al., 2000, 2014), but only recently vector mating communication became a target for controlling Pierce's disease (Krugner et al., 2014). Exploitation of disruptive, attractive, and/or repellent signals for suppressing GWSS populations in citrus orchards and vineyards could prove to be a useful tool. However, existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California.

OBJECTIVES

The objective of this research was to identify and describe substrate-borne signals associated with intraspecific communication of GWSS in the context of mating behavior.

MATERIALS AND METHODS

Insects and plants.

Late-instar (4th and 5th) GWSS nymphs obtained from colonies were separated by gender in cages to rear virgin adult individuals. After molting to the adult stage, females were transferred individually to a mesh-screen tube cage (10 cm diameter × 40 cm height) containing a cowpea plant. Reproductive maturity in about 150 individually caged females was determined by oviposition of non-fertilized eggs. Male insects used in the experiments described below were of the same age as reproductively active females. After female reproductive maturity and virginity was confirmed (oviposition of unfertilized eggs, without embryo development), test insects were used in the recording experiments described below. Each insect was tested only once.

Experimental setup.

Experiments were conducted in a transparent arena (60 cm length × 60 cm width × 80 cm height) made of 1-cm thick acrylic walls, centered inside a chamber formed by 86 cm × 86 cm × 98 cm high black fabric and sound isolating walls. The arena and chamber were placed on an active vibration isolation table (Model 20-561, Technical Manufacturing Corporation, Peabody, MA). Light-emitting diode (LED) lights were affixed to the top of the chamber. Insect behaviors were monitored via video and recorded to a computer. Vibrational signals produced by individuals were recorded using a laser Doppler vibrometer (PDV 100, Polytec, Inc., Irvine, CA) and digitized with Adobe Audition® C26 (Adobe Systems, Inc., San Jose, CA) at a 44.1 kHz sample rate and 32 bits resolution. Plants were about 30 cm in height, with two apical leaves (approximate surface of 49 cm²).

Experiment 1. Mating behavior and signal characterization.

Bioassays were conducted between 0800 and 1900 hours at 25 ± 0.5°C. Before testing, insects were allowed 15 minutes to acclimatize to ambient conditions in 130 ml plastic vials placed within the chamber housing the plant. After the acclimatization period, insects were released into the acrylic arena. Three types of trials were performed: 1) single individuals, 2) mating pairs, and 3) male rivalry for a female. In trial 1, virgin males (n = 21) and females (n = 26) were placed on plants individually to identify spontaneous signaling (i.e., calling songs) for each gender. In trial 2, mating pairs (n = 33) were monitored for their signaling by placing a female and a male on the plant. In trial 3, two males were placed on a plant and immediately after a female was added (n = 30). Trials consisted of 90-minute observations, except for trials with individual female that consisted of 45 minutes. This was because in preliminary trials we have found a higher signaling rate in females than males. When mating occurred during the trials, the pair was immediately transferred to a tube cage containing a cowpea plant and kept until copulation ended. After copulation, the female was kept individually on a plant until fertility was confirmed by deposition of fertilized eggs.

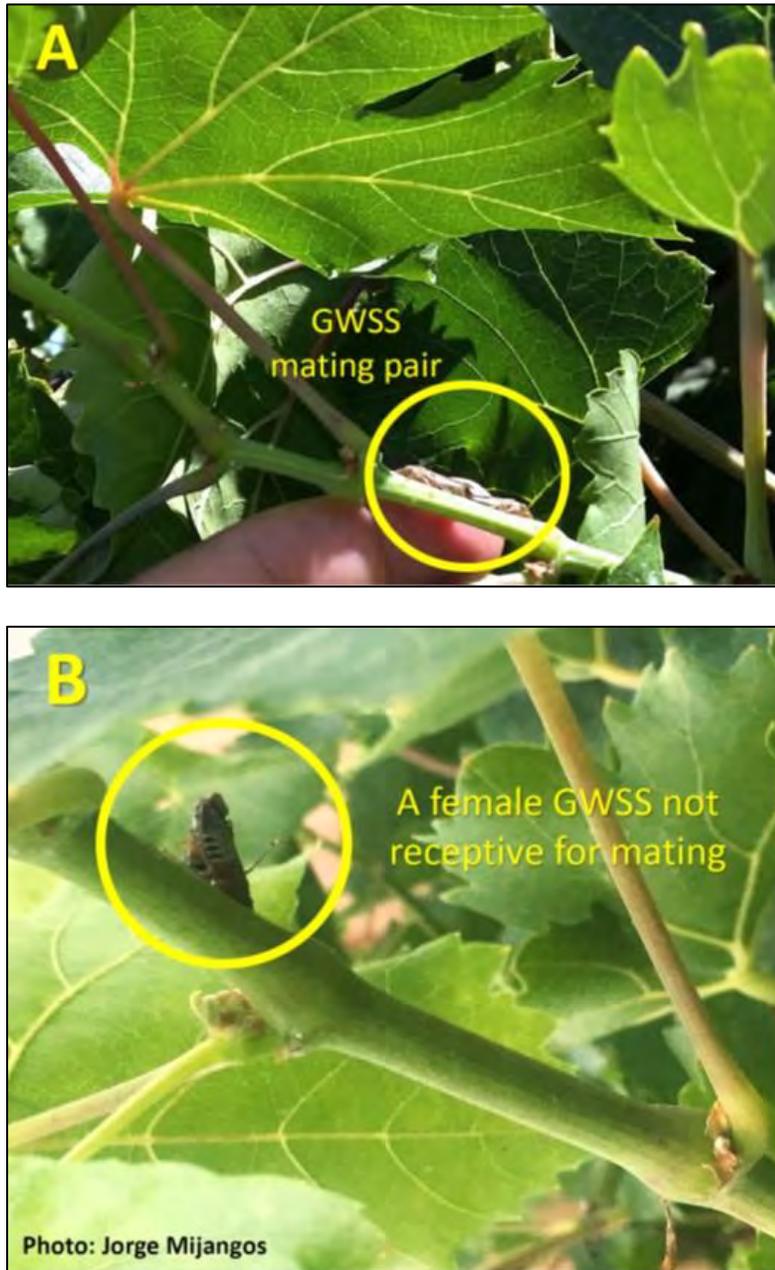


Figure 1. A) GWSS mating pair in Kern County and B) a male GWSS attempting to mate with a non-receptive female in Fresno County. The GWSS pairs in the photos are individuals from wild populations reproducing on grapevines.

Experiment 2. Validation of signal playback as a stimulus to initiate duets.

Playback trials were conducted to assess whether 1) substrate-borne vibrations alone were sufficient to elicit a conspecific response and 2) vision had a role in eliciting male signaling activity. Signals selected from those recorded in Experiment 1 were transmitted to the plant with an electrodynamic mini-shaker (Type 4810, Brüel & Kjær, Inc., Norcross, GA). Signal playback was activated two minutes after the individuals were released on the test plant. Amplitude of stimulatory playback signals were adjusted to the level of recorded natural GWSS signals as registered at the point of recording with laser vibrometer. In the first trials, a female ($n = 20$) was stimulated with a playback consisting of a repetition of two Male Signal 1 (MS1) and two Male Signal 2 (MS2) using a randomized design and signal interval of 10 seconds. In the second trial, a male ($n = 20$) was stimulated with a playback made of a repetition of two different female signals (FS1) (see results of Experiment 1 for male and female signal definitions). To assess the potential role of visual stimuli in eliciting the male mating behavior, a thawed GWSS female was affixed to the plant using an entomological pin. A male was placed on the downside of

a leaf at approximately 15 cm from the thawed female, which was placed near the shaker position on the stem plant. The male was stimulated (n = 20) or not (n = 21) with the playback.

Terminology and signal characterization.

Vibrational signals were named according to their behavioral context. Calling signals were defined as signals that are emitted spontaneously to trigger a reply from the opposite sex. Pulse was defined as a physically unitary or homogeneous sound, composed of a brief succession of sine waves. A pulse train was defined as a succession of repetitive and temporally well-distinct group of pulses. A signal, or part of it, was defined as fragmented when its emission was not continuous but characterized by regularly repeated interruptions. Spectral and temporal parameters of the recorded signals were analyzed with Raven Pro 1.5 (The Cornell Lab of Ornithology, Ithaca, NY) using Fast Fourier Transform (FFT) type Hann with window length of 8192 samples and 80% overlap. The following parameters, when applicable, were measured for each signal: duration, pulse (or fragment) repetition time (measured as the distance between the onset of two consecutive elements), percentage of male signal length made up of Section 2 (fragmented part, see below), fundamental frequency (ff), and relative amplitude measured as root mean squared (RMS). To describe the frequency trend of harmonic signals, the ff was measured at the beginning (b), at mid length (m), and at the end (e) of any signal. To determine ff rate of increase/decrease within a signal, modulation rate (MR) was calculated as follows:

$$MR_{xy} = (ff_y - ff_x) / t_{xy}$$

where x and y indicate the 0.1-sec part of the signal where the ff was sampled, and t was the time (seconds) between the sampling points x and y.

Recordings of single males (n = 5) and females (n = 10), couples that mated (n = 12), and trios that resulted in rivalry behavior (n = 17) were used to characterize the vibrational signals of GWSS. A total of 40 signals (per type, at most five samples per individual) were analyzed using t-test or One-Way ANOVA followed by Tukey's post hoc test. To eliminate biases in signal amplitude due to different distances between the emitter and the recording location (i.e., laser beam) on the plant, the Root Mean Square (RMS) was measured only from stationary individuals. To compare the RMS of male and female signals, the Friedman test (non-parametric repeated measures ANOVA) with five replications was performed followed by pairwise multiple comparisons. Amplitude was not analyzed for signals in trio trials because males involved in rivalry contests tended to keep moving along the plant. Therefore, it was not possible to assign with certainty a signal to a specific male or to record enough samples of different signals from stationary males. To determine whether the spectral and temporal features of male and female signals varied during the pair formation process, statistical analysis was conducted across the two identified behavioral phases (see results). A stepwise discriminant analysis was used to determine whether signals could be distinguished based on their temporal (duration) and spectral (ff_b , MR_{bm} and MR_{me}) profiles, and if so, which parameters were more important.

Analysis of behavioral parameters.

For insects tested individually on plants, the following parameters were measured: time from beginning of recording to first emission of a vibrational signal (call latency), number of individuals who emitted at least one signal during trials (signaling activity), and number of signals emitted in the given time. Since the number of males that spontaneously emitted signals was low, call latency and number of signals emitted were not compared between males and females. For insects tested in pairs, latency to first duet, as the first reply to a signal regardless of gender, duration of identification duet (see results), and latency to mating as the time between latency to first duet and copula were recorded. To estimate duration of copula, mating pairs were checked every 30 minutes until the couple separated or 6:00 PM, depending on which came first. For the analysis of signaling behavior, a first-order Markovian behavioral transition matrix for the pair formation process was created for each individual using data from all pairs that established a duet (n = 21). Transition probabilities were calculated from the observed frequency of a transition between two events (either a signal emission or a behavior) divided by the total number of occurrences of the first of the two events. Male signals analyzed were MS1, MS2, and Qv; female signals were FS1 and FS2 (see results). The selected behaviors were: identification duet, movement (i.e., walking), mating attempt, and copula. The expected values were calculated using the iterative proportional fitting method, then the G-test (Williams' corrected) was performed to determine the significance of the overall table and of transitions by

collapsing the table in a 2×2 matrix. Significance of each transition was calculated after sequential Bonferroni method.

Because of the unreliability in distinguishing the signals emitted by each male on trios, behavioral analysis based on Markovian transitional matrices were not performed for trios. A G-test (Williams' corrected), followed by Ryan's multiple comparison test for proportions, was performed to determine which rivalry signal transitions were most common. One tail unpaired t-test was used to compare latency to copula between pairs (no rivalry) and trios. G-test (Williams' corrected) was used to determine whether male rivalry behaviors affected the probability of accomplishing mating. In experiment 2, G-test (Williams' corrected) was used to compare the replying activity, number of individuals who emitted at least one signal when stimulated with the playback, and to compare with males in the presence of visual cue (dead female), either with playback on or off. In this case, the G-test was followed by Ryan's multiple comparison test for percentages.

RESULTS AND DISCUSSION

Experiment 1. Mating behavior and signal characterization.

Two female signals (Female Signal (FS) 1 and FS2) and six male signals (Male Signal (MS) 1, MS2, Quivering (Qv), Male Rivalry Signal (MRS) 1, MRS2, and MRS3) were identified. Emission of signals occurred concomitantly with distinct abdominal tremulations. For one signal, MS2, part of the signal involved a broad dorso-ventral movement of the abdomen and flicking of wings. Qv occurred simultaneously with a slow dorso-ventral pulsing of the abdomen. Temporal and spectral parameters of signals are reported in **Figure 2**. The process of pair formation was divided in two main phases: identification and courtship. Identification was characterized by stationary individuals that communicated exclusively using FS1 and MS1. In Courtship, males alternated signal emission with walking towards the female, which remained stationary on the plant and replied with either FS1 or FS2.

Description of signals.

- **FS1 (Figure 2A)** was the most common female signal. FS1 had clear harmonic structure and increasing ff. FS1 spectral and temporal parameters were rather variable according to the behavioral phase. During species identification it was significantly longer and with higher amplitude than in Calling, which in turn was longer than in Courtship. FS1 during species identification had constant positive slope increase of ff ($MR_{bm} = MR_{me} > 0$), whereas FS1 in Calling and Courtship had a significantly sharper increasing slope during the second half of the call ($MR_{me} > MR_{bm} > 0$). Emission of FS1 was occasionally variably fragmented for part of the signal (8% and 25% of the analyzed samples during Identification and Courtship, respectively).
- **FS2** was significantly shorter and had lower amplitude and starting frequency than FS1. The ff decreased constantly ($MR_{bm} = MR_{me} < 0$) and the signal was repeated in sequences (we counted up to 13 consecutive elements) with rather variable pulse repetition time (mean \pm SD: 0.67 ± 0.76 s). The emission of FS2 was limited to the Courtship phase.
- **MS1** was made of two distinct parts: the first part (section 1) was given by a continuous emission and characterized by significant slope increase ($MR_{me} > MR_{bm} > 0$) before the onset of the second part (section 2), which had constant frequency and was fragmented. MS1 did not significantly change across the two behavioral phases.
- **MS2 (Figures 2A and B)** was composed of sections 1 and 2 with characteristics similar to MS1, but the main feature of MS2 was a strong broadband pulse that anticipated section 1. In addition, MS2 temporal parameters (signal duration, percentage of fragmented part) and amplitude were significantly higher than MS1. In general, the spectral and temporal parameter variability of MS2 was lower than all other signals.
- **Qv (Figure 2B)** was a train of low amplitude pulses with variable duration (0.5 to 240 s) and regular pulse repetition time (0.23 ± 0.03 s). Occasionally, sudden rhythm acceleration was observed with pulses that fused in a continuous signal (max. 1.7 s), with clear harmonic structure and constant ff (approximately 75 Hz).
- **MRS1 (Figure 2D)** had clear harmonic structure with ff that significantly increased during the emission, but unlike the other signals the first half increased more than the second half ($MR_{bm} > MR_{me} > 0$). Often (80% of analyzed samples), the last part (on average $25 \pm 14\%$ of signal duration) of MRS1 was fragmented, forming a second section. However, section 2 of MRS1 was significantly shorter than in MS1 ($55 \pm 15\%$ and $53 \pm 17\%$ in identification and courtship, respectively), which in turn was significantly shorter than MS2 ($69 \pm 7\%$) ($F_{3,156} = 84.0$, $P < 0.0001$). In addition, the fragment repetition time of MRS1 (0.1 ± 0.01 s) was significantly higher than MS1 ($F_{3,156} = 7.0$, $P < 0.001$).

- **MRS2 (Figure 2D)** was significantly shorter than the other MRS and characterized by constant increase of ff ($MR_{bm} = MR_{me} > 0$).
- **MRS3 (Figure 2D)** was variable in duration, not fragmented, and with peculiar ff trend that initially increased and then, starting from approximately half of the signal length, decreased ($MR_{bm} > 0 > MR_{me}$).

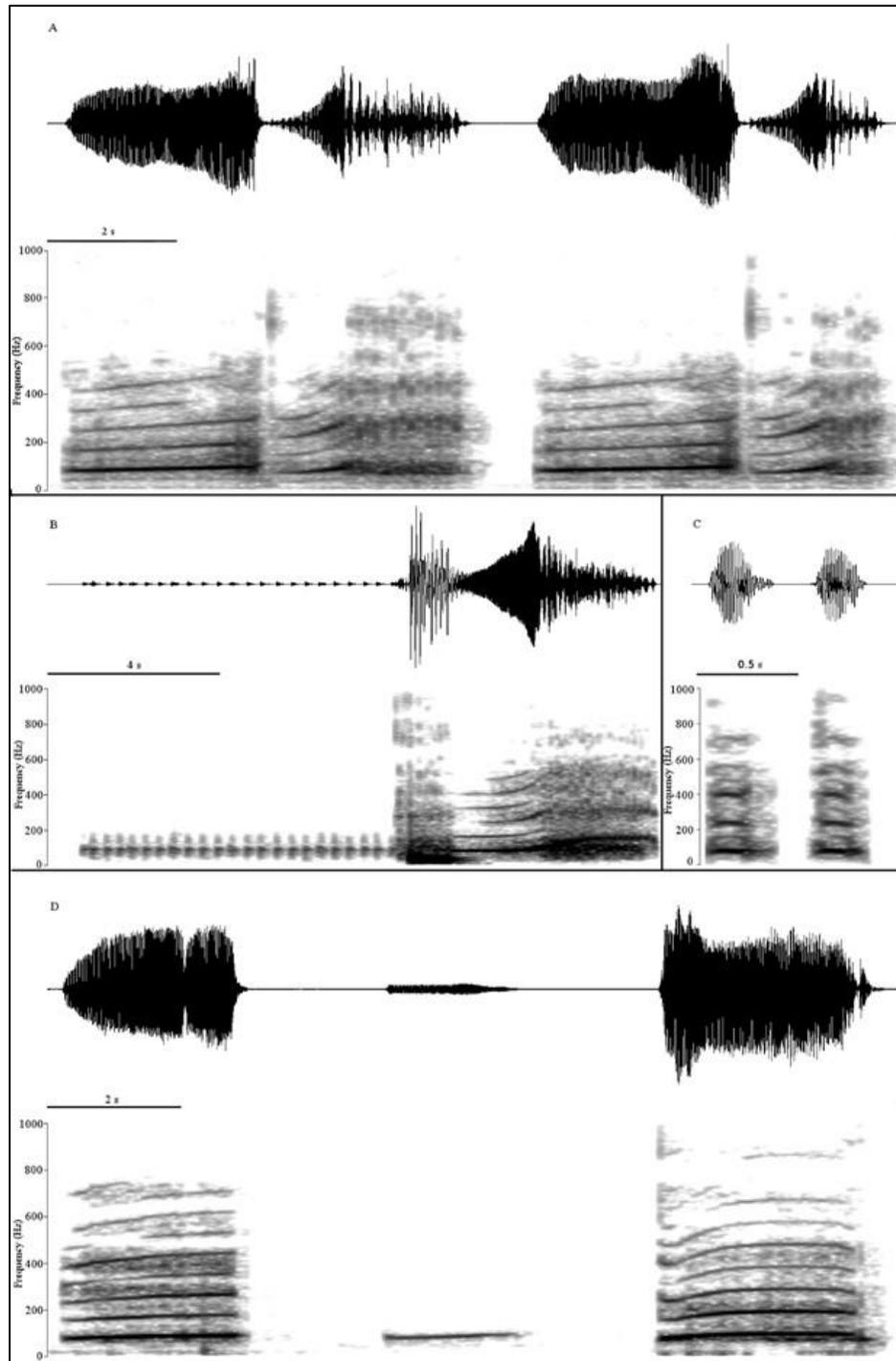


Figure 2. Oscillogram (above) and spectrogram (below) of GWSS vibrational signals. In A, the identification duet formed by two FS1 and two MS1 alternated. In B, MS2 preceded by Qv. In C, two consecutive FS2. In D, three different MRS (from left to right: MRS1, MRS2, and MRS3).

Discriminant analysis (**Figure 3**) showed that temporal and spectral parameters of signals have a role in signal specificity, although the accuracy of discrimination was not high (50.8% of the signals correctly classified). The

first two discriminant functions explained 95.8% of the variance (function 1 = 54.6%, canonical correlation = 0.85, Wilks' lambda = 0.083, Chi square = 869, $P < 0.001$; function 2 = 41.2%, canonical correlation = 0.81, Wilks' lambda = 0.294, Chi square = 427, $P < 0.001$). The plot of the first vs. the second roots of the discriminant analysis showed that male signals used during Identification and Courtship (MS1 and MS2) can be easily distinguished from female signals, while more uncertainty occurs between FS1 and male rivalry signals, in particular between FS1 (Identification) and MRS1, and FS1 (Courtship and with minor degree Calling) and MRS2. On the contrary, FS2 and MRS3 were well discriminated (accuracy > 60%).

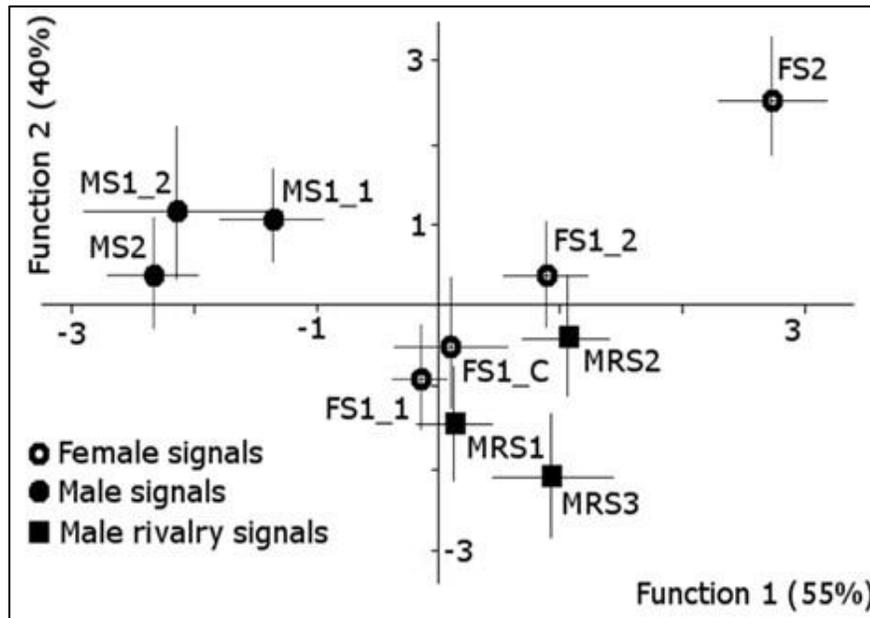


Figure 3. Combined-groups plot showing functions 1 and 2 derived from the discriminant function analysis of signal duration, starting frequency and modulation rates (MR_{bm} and MR_{mc}). Function 1 and 2 explain 55% and 40% respectively of variance, separating MS (1 and 2) from FS2 and from FS1 and MRS. Only centroids (calculated as averages (\pm SD) of canonical variables) are showed. Discrimination between FS1 and MRS is low, in particular between MRS1 and FS1_1/FS1_C (Call) and between MRS2 and FS1 and FS1_2.

Behavioral analysis

Trial 1. Single individual on plant.

When placed alone on plants, 20 of 26 females (77%) emitted FS1 and 5 of 21 males (24%) emitted MS1. Female call latency (530 ± 606 s) was quicker than males (1559 ± 843 s); the number of female signal emissions per individual (15.8 ± 31.3) was higher than males (2.8 ± 2.9).

Trial 2. Mating pairs.

A total of 21 of 33 (64%) pairs initiated the mate selection behavior during the trial. Among these, 12 of 21 (57%) mated in the given time. In six trials, the male called first, whereas in 15 trials the female called first. Latency to and length of the identification duet were variable, 1378.6 ± 1315.7 s ($n = 21$) and 64.2 ± 97.4 s ($n = 20$), respectively, containing as few as two signals each to over 10 signals each. While during identification the ratio of female:male response rate was close to 1 (1.08 ± 0.49), in Courtship the female reply rate was much lower ($1:4$) (0.26 ± 0.21). Finally, when a male arrived at a short distance (two to three body lengths) from the female, FS2 was emitted. Latency to mating was variable (391 to 2690 s). Copulation was relatively long (333.7 ± 156.9 minutes ($n = 6$)). Behavioral analysis based on the Markovian transition matrix (**Figure 4**) indicated that males started the courtship phase with MS2, Qv, or Movement (i.e. searching). After courtship was initiated, males alternated emission of MS1 and MS2, interspaced by Qv. In particular, MS1 appeared to be correlated with emission of FS1, which in turn elicited either establishment of a duet or movement of the male. MS2 and quivering significantly anticipated the emission of FS2, which was the signal that preceded male mating attempts. However, in three cases (out of 12) the mating attempt was preceded by emission of FS1, MS2, or Qv. In one trial, the male located the female and the pair mated without any female signal emission during the Courtship phase. A

vibrational signaling. The number of pairs that mated in the presence and absence of a rival contest was not significantly different (G-test, $G = 1.2$, $P = 0.27$). The time spent to achieve copula was not significantly different ($t = -1.2$, $P = 0.12$) between trials where rivalry occurred (average (\pm SD) 3120 ± 1589 s, $n = 8$, range = 494 to 5123 s) or not (2191 ± 1004 s, $n = 12$, range = 625 to 3572 s).

Experiment 2. Playback tests.

When stimulated by pre-recorded signals from the opposite gender, both females and males replied with FS1 and MS1, respectively, and in one case (out of 20), a male located the source of the signal (shaker) and walked over the metal rod connected to the shaker. Female replying rate to playback was significantly higher than the male ($G = 11.39$, $P < 0.001$). The presence of a dead female near the male, as an attempt to provide visual stimulus, did not affect the male responsiveness.

CONCLUSIONS

This project encompasses three compounding phases built on research findings of previous phases: 1) Exploratory Phase – identify and describe the substrate-borne signals associated with intraspecific communication of GWSS; 2) Developmental Phase - Identification of signals capable of influencing GWSS behavior for applicative purposes (e.g., interference with communication); and 3) Application Phase - technology transfer for implementation of a sustainable management strategy for GWSS. Phase 1 of the project was successfully completed and provided sufficient information to initiate Phase 2. If and when funds become available to complete Phase 2, an anticipated product of the research would be a method to reduce numbers of GWSS. A reduction in numbers of insect vectors is expected to result in reduced spread of *Xylella fastidiosa*. Consequently, a reduction in rates of spread of *X. fastidiosa* is expected to result in reduced incidence of Pierce's disease.

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

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ABSTRACT

Monitoring for resistance to insecticides continued in 2016 with a series of insecticide bioassays conducted on the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) in Kern County. Collections were made from table grapes adjacent to General Beale Road in July and August and from navel oranges in the same vicinity in October. Much lower population densities in 2016 compared to the previous year precluded more frequent testing. Susceptibility to six insecticides was evaluated in the initial test conducted in July, but dropped to only two or four insecticides in subsequent tests due to a paucity of GWSS adults. Relative susceptibility to imidacloprid in the first ($LC_{50} = 3.99$) and third ($LC_{50} = 7.26$) tests remained well within the range of LC_{50} s observed in 2015, but in the second test was much lower ($LC_{50} = 0.04$). Similarly, LC_{50} s recorded for two other neonicotinoids, acetamiprid and thiamethoxam, and for two pyrethroids, bifenthrin and fenpropathrin, fell within the range of LC_{50} s for each compound observed in 2015. Relatively little difference in susceptibility of these five insecticides was seen in a comparison of annual mortality curves for each compound. Although data on the timing, frequency, and location of insecticide applications against GWSS in 2016 are still being gathered, the overall drop in GWSS numbers is suggestive that more aggressive control tactics effectively impacted GWSS populations. Moreover, results of insecticide bioassays in 2016 indicate that relative susceptibility to five insecticides is essentially unchanged compared to 2015, suggesting no loss of efficacy due to resistance.

LAYPERSON SUMMARY

Insecticides have been a key component of the management program for Pierce's disease, effectively reducing glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) numbers. However, from 2012 through 2014 high population levels were present and densities in 2015 exceeded those in 2001, when the program began. In 2015 we documented lower susceptibilities to commonly used insecticides in Kern County populations of GWSS, with declining susceptibility as the season progressed. This suggested that treatment practices in the vicinity of the collection sites may have contributed to the lack of control. However, no further reduction in susceptibility was observed in the 2016 season, although fewer tests were conducted due to a decline in population densities compared to the previous year. Whether reduced GWSS numbers in 2016 were due to more aggressive insecticide applications or to natural variation is key to understanding the role that regional control programs play in GWSS management. In addition to continuing to monitor for resistance to insecticides, this project will explore the relationship between historical insecticide treatment records and current levels of susceptibility, informing how we effectively use insecticides in the future.

INTRODUCTION

The Pierce's disease area-wide management programs in California rely on insect monitoring which triggers chemical control in citrus orchards and vineyards. These programs, initiated in Riverside County in 2000 and expanded to Kern County the following year, were successful at keeping glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) densities low from 2001-2008 (**Figure 1**). From 2009-2011 control was still adequate but insect numbers increased. Despite continued insecticide usage high densities of GWSS in 2012 and

2015 surpassed the 2001 density, while levels in 2013-14 nearly attained the 2001 level (**Figure 1**). It is important to note that the GWSS densities in the last four years have occurred while under chemical management, whereas the 2001 densities occurred prior to the widespread use of insecticides. Concomitant with large GWSS densities has been a resurgence of Pierce's disease infected vines. While levels of Pierce's disease in the General Beale region of Kern County were nearly undetectable from 2002-2009, they have increased in the last five years; the number of infected vines has increased in nearly all vineyards surveyed (Haviland, 2015).

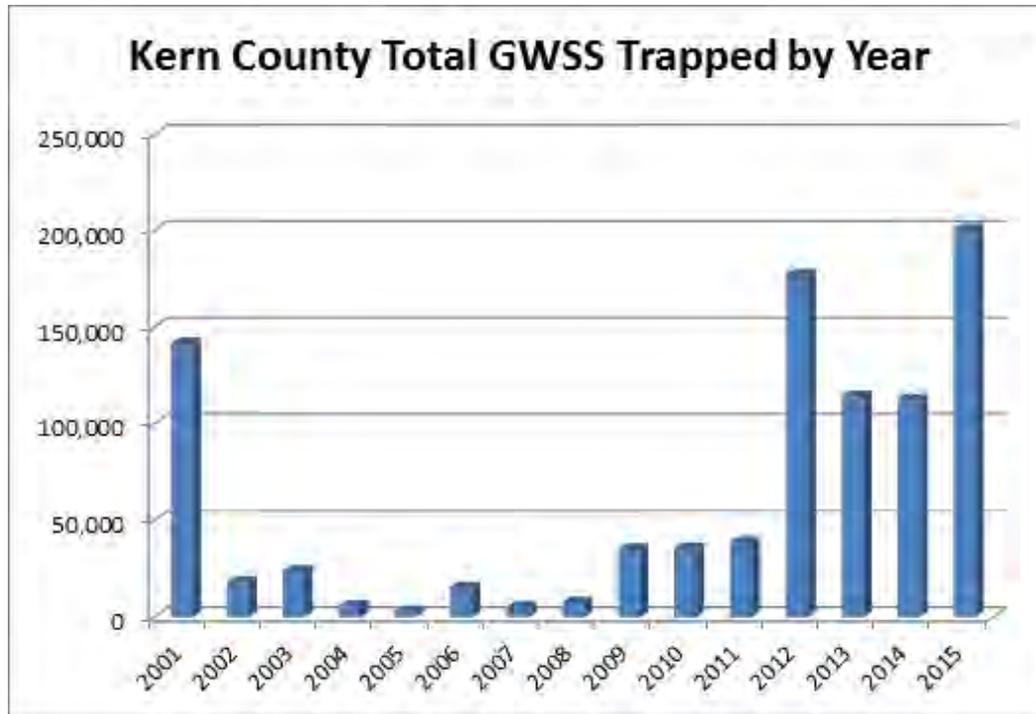


Figure 1. Total number of GWSS caught on CDFA traps in Kern County from 2001 to 2015. (From Haviland, 2015)

Due to a number of factors the systemic neonicotinoid insecticide imidacloprid has been used preferentially for GWSS suppression. Positive attributes of imidacloprid include systemic activity, persistence in treated plants, and selectivity for xylem and phloem feeding insects. Although data on the frequency of imidacloprid use since 2000 has not been compiled for the area-wide programs, it is generally believed that it has been used to a greater extent than other insecticides. 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). With the selection pressure that has resulted from the combined use of imidacloprid across citrus and grape acreages over the past 15 years there is reason to believe that the resurgence of GWSS is related to imidacloprid resistance. 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). Yet 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 record exists for a xylem feeder: a sugarcane-feeding froghopper (spittlebug) reported in a book chapter (Fewkes, 1968). Although fundamental arguments by Rosenheim et al. (1996) and 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 that is subjected to a specific mortality factor 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 GWSS over the past 15 years in Kern County. Pesticides are an integral part of the high-yielding production agriculture in citrus and grapes, and understanding the levels of resistance to insecticides is critical to the future selection of materials that are used to manage GWSS and Pierce's disease.

With this in mind, and at the request of the Consolidated Central Valley Table Grape Pest and Disease Control District, we initiated a pilot study to evaluate insecticide susceptibility of GWSS to a number of insecticides (**Table 1**). In this study we collected GWSS 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. Insects were subjected to a systemic uptake bioassay and a foliar insecticide bioassay adapted from Prabhaker et al. (2006b). From these bioassays LC₅₀ (lethal concentration that kills 50% of the population) values were calculated and compared to LC₅₀s determined in 2001 and 2002 (Prabhaker et al., 2006a).

Table 1. Insecticides tested in adult GWSS 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

The data showed that GWSS tested in 2015 were less susceptible to the tested compounds than they were in 2001 and 2002. For the neonicotinoids, the LC₅₀ values for thiamethoxam, imidacloprid, and acetamiprid were up to 1.78, 57.31, and 130 times, respectively, higher in 2015 (**Table 2**). Even larger differences existed for the pyrethroids bifenthrin (5,066 times higher), and fenpropathrin (101 times higher) and the organophosphates chlorpyrifos (22,190 times higher) and dimethoate (2,150 times higher). We believe that the extraordinary differences in the pyrethroids and the organophosphates may be the result of different research protocols used in the 2001/2002 studies and the 2015 studies. In the earlier work we used a petri dish assay which enclosed the treated leaves and insects, probably contributing to fumigation action and extremely low LC₅₀ values. In 2015 we used a screened clip cage which eliminated or greatly reduced the fumigation action of the insecticides. Even so, the data from all studies indicate that GWSS is less susceptible to most of the insecticides being used than it was 14 years ago. Similar results were obtained using topical bioassays for imidacloprid, bifenthrin, and fenpropathrin (Redak et al., 2015).

Of particular interest in our study was the fact that there was variation in the relative toxicities at different times and locations throughout the 2015 season (Perring et al., 2015). The LC₅₀s for imidacloprid increased 79-fold from the first bioassay of the season to the last (**Figure 2**). However, 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 increasing LC₅₀s from General Beale Road as the season progressed. The two pyrethroids, bifenthrin and fenpropathrin, were equivalent to one another, but higher LC₅₀s occurred on the later sampling (**Figure 2**). The two organophosphate compounds were inconsistent in their responses, with low to high LC₅₀s (data not shown). 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 later in the season (Perring et al., 2015).

Table 2. LC₅₀ values for seven insecticides evaluated on GWSS in 2001, 2002, and 2015.

Compound	2001	2002	2015
Acetamiprid	0.44 (0.18 – 0.56)	0.08 (0.02 – 0.14)	0.74 (0.31 – 1.76) – July 9 3.97 (1.40 – 9.97) – Oct 7 10.41 (5.18 – 22.64) – Oct 21
Thiamethoxam	1.87 (1.18 – 2.06)	2.12 (1.49 – 3.28)	0.13 (0.08 – 0.23) – July 9 0.35 (0.19 – 0.67) – July 22 1.74 (0.71 – 4.25) – Sept 16 3.34 (1.25 – 7.70) – Oct 7 1.19 (0.55 – 2.60) – Oct 21
Imidacloprid	1.27 (0.68 – 2.54)	0.36 (0.09 – 0.52)	0.26 (0.15 – 0.46) – July 9 1.92 (0.74 – 6.01) – July 22 8.56 (4.47 – 14.56) – Aug 3 7.03 (2.73 – 23.30) – Sept 16 8.63 (2.63 – 29.92) – Oct 7 20.63 (8.27 – 47.71) – Oct 21
Compound	2001	2002	2015
Bifenthrin	0.0006 (0.0002 – 0.004)	0.013 (0.008 – 0.035)	0.53 (0.15 – 1.29) – July 7 0.13 (0.05 – 0.30) – July 22 0.78 (0.25 – 2.49) – Sept 16 0.14 (0.04 – 0.40) – Oct 7 3.04 (1.27 – 8.23) – Oct 21
Fenpropathrin	0.063 (0.045 – 0.204)	0.020 (0.006 – 0.059)	0.19 (0.08 – 0.41) – July 7 0.04 (0.02 – 0.09) – July 22 2.02 (0.86 – 5.09) – Sept 16 0.21 (0.01 – 1.42) – Oct 7 0.49 (0.17 – 1.22) – Oct 21
Compound	2001	2002	2015
Chlorpyrifos	0.002 (0.001 – 0.003)	0.001 (0.0007 – 0.005)	0.28 (0.06 – 1.76) – July 7 4.55 (1.54 – 18.04) – July 22 22.19 (6.93 – 96.61) – Oct 21
Dimethoate	0.046 (0.029 – 0.081)	0.031 (0.024 – 0.044)	10.99 (2.68 – 118.43) – Aug 3 66.67 (11.76 – 4177) – Sept 16 5.66 (0.93 – 30.11) – Oct 7

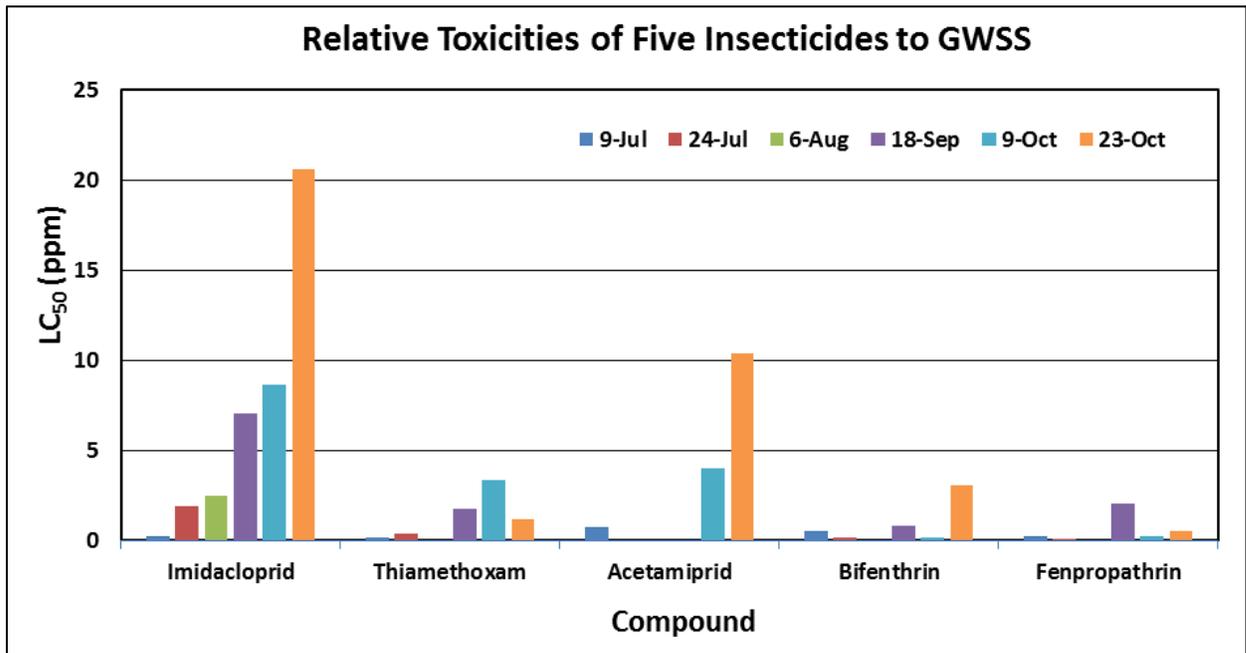


Figure 2. LC₅₀s for five insecticides tested over six dates between July 9 and October 23 in 2015. The first three columns of each series represent GWSS adults collected from an organic citrus field in the Edison area, whereas the second three columns represent collections from the General Beale Road 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. (From Perring et al., 2015)

Taken in total, our work from last year showed that GWSS was less susceptible to commonly used insecticides than it was in 2001-2002. Furthermore, the levels of susceptibility were geographically variable and dramatically declined over the course of the 2015 growing season (July to October). It is reasonable to think that consistent usage of materials over time would lead to resistance, and this is the most parsimonious explanation for the reduced toxicities measured in 2015 compared to the 2001/2002 data. However, the variation in toxicity within the 2015 season also was related to location (organic vs. conventional) and time (higher LC₅₀s later in the season). These data suggest that factors like insecticide usage in a local context may be important determinants for how effective certain insecticides are in certain areas. Understanding these dynamics will lead to more informed selection of materials in the future.

OBJECTIVES

1. Conduct laboratory bioassays on field-collected GWSS from Kern County to document the levels of resistance at the beginning of the 2016 and 2017 field seasons, and to document changes in susceptibility as each season progresses.
2. Document differences in insecticide susceptibility in GWSS collected from organic vs. non-organic vineyards (grapes) and/or orchards (citrus) and from different locations in Kern County.
3. Obtain and organize historic GWSS density and treatment records (locations, chemicals used, and timing of applications) into a geographic information system (GIS) for use in statistical analyses.
4. Determine the relationship between insecticide susceptibility of different GWSS populations and treatment history in the same geographic location and use relationships to inform future insecticide management strategies.

RESULTS AND DISCUSSION

Insecticide bioassays were conducted on GWSS adults collected in table grapes on July 26 and August 16, and in navel oranges on October 4. Over 900 adults were obtained on July 26, sufficient for testing six insecticides (**Table 3**) at five concentrations per insecticide plus an untreated control. Five replications of each insecticide concentration were used that required a total of 150 adults per insecticide. Upon returning to the same vineyard on August 16 only 300 adults were collected; that provided only enough insects for the testing of imidacloprid and

thiamethoxam. The 600+ adults collected on October 4 were highly dispersed in navel oranges and required sampling from numerous trees to collect enough insects for complete tests of four insecticides.

Bioassay procedures included a systemic uptake bioassay and leaf dip bioassay (Prabhaker et al., 2006a) that were used according to whether an insecticide was soil or foliar applied, respectively (**Table 3**). 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 for evaluating relative toxicities of the six insecticides.

Table 3. Insecticides tested in adult GWSS bioassays in 2016.

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
Pyrethroid	Bifenthrin	Capture [®] 2 EC	foliar	FMC
	Fenpropathrin	Danitol [®] 2.4 EC	foliar	Valent
Organophosphorus	Chlorpyrifos	Lorsban [®] 4E	foliar	Dow

Among the three neonicotinoid insecticides, LC₅₀s were highest for imidacloprid in Tests 1 and 3 in comparison to acetamiprid or thiamethoxam, but abnormally low in Test 2 relative to thiamethoxam (**Table 4**). The pyrethroid insecticides bifenthrin and fenpropathrin were similarly toxic to GWSS in Test 1 of 2016 as they had been in the 2015 bioassays. A second bioassay conducted with bifenthrin showed only a 2.2-fold difference in LC₅₀s between the July and October samples. The relative toxicity of chlorpyrifos (LC₅₀ = 11.49) to GWSS in Test 1 was considerably lower than for the other five insecticides, but it may be that the leaf-dip bioassay does not conform well to the toxicity profile of chlorpyrifos. Probit analyses on data from two chlorpyrifos bioassays in 2015 failed to yield an LC₅₀ value, an indication of the mortality data not fitting the probit model. Variation in mortality data from field-collected insects is not unusual and is an important reason why multiple tests are required for confident interpretation of the results. Prior exposures of insects collected in the field to various insecticides are usually unknown but could influence test results if residues are present on leaves or if contact by spray drift has occurred. Movement among crops and fields is facilitated by the strong flying capabilities of GWSS and by the demand for higher amino acid content of xylem fluid that varies among host plants (Bi et al., 2007).

Table 4. Probit statistics for insecticides tested against GWSS adults on three dates from July to October 2016.

Location and Date	Compound	LC ₅₀ (µg/ml)	95% C.I.	Slope (± SE)	χ ²	df
Gen. Beale Rd July 26-28 Table Grapes (Test 1)	Imidacloprid	3.99	2.11 – 7.83	1.18 (0.19)	17.2	23
	Acetamiprid	1.76	0.66 – 5.15	0.59 (0.10)	15.6	23
	Thiamethoxam	0.53	0.32 – 0.84	2.45 (0.51)	10.2	22
	Bifenthrin	0.70	0.38 – 1.28	1.30 (0.20)	16.0	23
	Fenpropathrin	0.59	0.29 – 1.19	1.00 (0.15)	14.6	23
	Chlorpyrifos	11.49	2.05 – 357.83	0.44 (0.09)	37.6	23
Gen. Beale Rd Aug 16-17 Table Grapes (Test 2)	Imidacloprid	0.04	0 – 0.19	0.53 (0.16)	12.5	18
	Thiamethoxam	2.87	1.02 – 7.88	0.66 (0.13)	13.2	18
Gen. Beale Rd October 4-5 Navel Oranges (Test 3)	Imidacloprid	7.26	2.81 – 24.83	0.62 (0.11)	18.9	23
	Acetamiprid	0.40	0.16 – 1.02	0.97 (0.14)	32.1	23
	Thiamethoxam	1.21	0.68 – 2.09	1.34 (0.21)	20.4	22
	Bifenthrin	1.54	0.68 – 3.65	0.97 (0.14)	27.0	23

The drop in susceptibility to imidacloprid observed at the end of the 2015 season (Perring and Prabhaker, 2015) raised real concerns that resistance to imidacloprid was present in GWSS populations in the General Beale Road vicinity of Kern County. Not only did LC_{50} s for imidacloprid trend progressively upward through the 2015 season, a substantial decrease in susceptibility to acetamiprid also was observed on the last test date of 2015. However, a comparison of composite mortality curves from the 2015 and 2016 seasons for all three neonicotinoid insecticides indicates relatively little difference in mortalities at various concentrations of each insecticide (**Figure 3A**). The only consistent difference (although not statistically) in mortality curves was for acetamiprid, to which GWSS test insects in 2016 were actually slightly more susceptible than those tested in 2015. Relative differences in susceptibility to either imidacloprid or thiamethoxam varied inconsistently by concentration between years. Comparison of 2015 to 2016 mortality curves for the pyrethroids revealed a similar pattern for each compound (**Figure 3B**). Higher mortalities were observed at lower concentrations in 2015, but then crossed over at either 10 $\mu\text{g}/\text{ml}$ for bifenthrin or 1 $\mu\text{g}/\text{ml}$ for fenpropathrin.

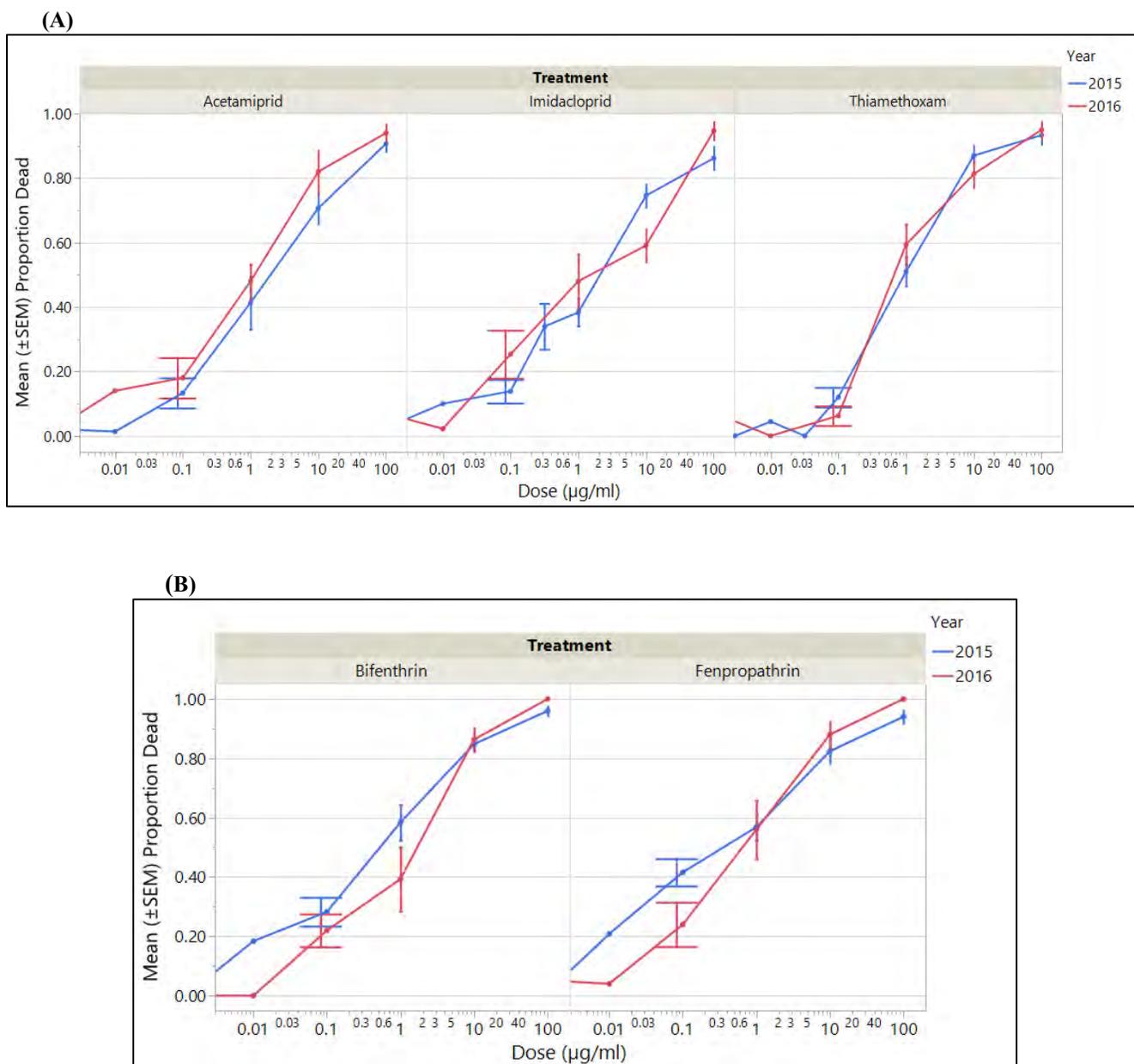


Figure 3. Composite mortality curves for (A) three neonicotinoid insecticides and (B) two pyrethroids for 2015 and 2016.

Comparison of mortality curves for all five insecticides (**Figure 3**) is rather tenuous due to the fewer number of bioassays conducted in 2016 relative to 2015. Nevertheless, identification of patterns of change to insecticide treatments in a particular population can only occur by gathering enough data points that reveal a trend up or down or lack thereof. The related issue of what happened to GWSS numbers in 2016 compared to previous years is one that should be addressed in the context of the pesticide use history in the General Beale Road area since 2001 and how it has affected annual variation in population densities. Has heavy insecticide use since 2001 caused resistance that has contributed to higher population densities over the last four to seven years, or has pesticide use slackened in recent years to allow a resurgence of GWSS? This question will be addressed as we begin to gather historical pesticide use records into a GIS platform that will enable us to relate spatial and temporal variation in pesticide use with present pesticide susceptibility.

CONCLUSIONS

Further monitoring should be conducted over the next few years to provide a more thorough evaluation of whether resistance to imidacloprid is occurring. Historical analyses of pesticide use patterns in relation to GWSS yellow sticky trap catches will provide essential information for understanding the basis of GWSS resurgence in Kern County.

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

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ABSTRACT

We can confirm that glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) in the General Beale Road citrus-growing area are exhibiting high levels of imidacloprid resistance based on data generated from topical application bioassays. In addition, we have also detected shifts in pyrethroid susceptibility. During the summer of 2016 we bioassayed adult insects collected from citrus groves in Kern (conventional and organic), Tulare (organic), and Riverside (organic) counties and compared the responses with toxicology data that were generated in 2003 for populations in Riverside County. In addition to the bioassay work we also used biochemical and molecular techniques to investigate putative resistance mechanisms to these (neonicotinoid and pyrethroid) and other (organophosphate; OP) insecticide classes. Thus far we have not identified any acetylcholinesterase insensitivity, indicating that there is no target site resistance to OPs. Esterase levels in susceptible and resistant populations are also very homogeneous, confirming that elevated esterase levels are unlikely to play a significant role in conferring pyrethroid resistance. We are using genomics tools to elucidate possible roles of cytochrome P450 enzymes in conferring imidacloprid and fenpropathrin resistance, as the biochemical assays have not been optimized for measuring these enzyme systems in GWSS. We are currently comparing cDNA sequence data for sodium channel (pyrethroid target site) and nicotinic acetylcholine receptor (neonicotinoid) genes in insects from the different populations to determine whether mutations known to confer insecticide resistance in other arthropod species occur in GWSS.

LAYPERSON SUMMARY

The goal of this research is to investigate the potential for the development of insecticide resistance in glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) 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. Using topical application bioassays we have now detected substantial differences in response to imidacloprid (neonicotinoid) and, to a lesser extent, fenpropathrin (pyrethroid) between populations collected from citrus groves in Kern, Tulare, and Riverside counties. At this time the differences appear to be related to the GWSS management program, with the highest levels of resistance occurring in populations receiving conventional insecticide treatments and no resistance in those under organic management. Biochemical and molecular tests are being used to elucidate the specific mechanisms conferring the resistance. These tests will be essential for investigating the resistance profiles of populations occurring in the nursery and urban environments where available numbers of GWSS are insufficient for conducting full scale bioassays.

INTRODUCTION

Systemic imidacloprid treatments have been the mainstay of glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) 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 GWSS populations in Kern County for many years.

In Kern County, GWSS populations have been monitored since the area-wide treatment program was initiated following an upsurge in GWSS numbers and an increase in the incidence of Pierce's disease. The data shows an interesting pattern of sustained suppression of GWSS populations throughout most of the 2000s, 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 GWSS populations. Despite the additional foliar treatments in 2012 the insecticide treatments failed to suppress the insect population to a level that had occurred previously. There were concerns that in the two years prior to 2012 there was a steady increase in total GWSS numbers, an early indication that the predominant control strategy might be failing. The consequence of the increase in GWSS populations has been an increase in the incidence of Pierce's disease. In the Temecula area this worrisome increase in GWSS has not occurred; however, the selection pressure in this area remains high as similar management approaches are in use there as in Kern County.

There is also significant concern for the development of insecticide resistance arising from the management of GWSS in commercial nursery production. The majority of commercial nurseries maintain an insect-sanitary environment primarily through the use of regular applications of soil-applied imidacloprid or other related systemic neonicotinoids. For nursery materials to be shipped outside of the southern California GWSS quarantine area additional insecticidal applications are required. Applications of fenprothrin (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 insecticide resistance in nursery populations of GWSS 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 GWSS that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research to date has been in Kern County, we will broaden the scope of our investigations to include populations from agricultural, nursery, and urban settings. This broader approach will result in a more comprehensive report on the overall resistance status of GWSS within southern California and will contribute to more effective resistance management plans.

OBJECTIVES

1. For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC₅₀ data for current GWSS 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 AChE biochemical assay
5. Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of GWSS to baseline susceptibility levels we previously recorded.
6. Develop assays for additional resistance mechanisms not previously characterized in GWSS.

RESULTS AND DISCUSSION

Imidacloprid Bioassays.

An extensive bioassay program was undertaken during 2016 to evaluate the responses of different Central Valley GWSS populations to imidacloprid. The data generated from topical application bioassays were compared with similar bioassays from studies conducted in 2003 with Riverside County populations. In bioassays, insecticide is topically applied to the abdomen of adult GWSS and mortality is assessed at 24 and 48 hours post-treatment (Byrne and Toscano, 2005). Although imidacloprid is used systemically under field conditions to target GWSS feeding on citrus and other host plants, topical application of insecticide to individual insects ensures that the insect receives a uniform dose and eliminates any behavioral factors that might 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 GWSS, and this insecticide has been shown to elicit anti-feedant effects in several pest species (Nauen et al., 1998).

In 2003, bioassays were conducted using populations from Riverside (Agricultural Operations; UC 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, so the data from those bioassays were considered to represent baseline susceptible levels for GWSS. The response of insects from the Redlands grove, where imidacloprid was incorporated as part of the area-wide management of the GWSS, 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.

During the 2015 season, bioassays were conducted with insects collected from the General Beale Road (GBR) citrus region. The insects were considerably more tolerant to imidacloprid than the reference populations (Redak et al. 2015). In bioassays conducted over the dosage range 0.25 – 150 ng imidacloprid per insect ($n = 280$), there was a dose-response, although complete mortality at the higher dose was never achieved. Based on the reference data set from 2003, a 10 ng dose should result in *ca.* 80% mortality of a susceptible insect, so the bioassays showing minimal mortality at the 15 ng dose provided the first evidence that the insects were tolerant to imidacloprid.

The situation appears to have worsened in 2016, with doses as high as 500 ng imidacloprid per insect having no effect on survivorship of the GBR population (**Figure 1**). We are continuing to test higher doses against this strain to see if we can define a dose that will kill more than 50% of these insects, but there can be little doubt that the levels of resistance are extremely high. We were able to generate a full dose-response line for the Tulare population; these insects originated from a grove under organic management and would not have been directly exposed to imidacloprid as part of the management program. Despite their origin, there was still a shift in their susceptibility compared with the 2003 Riverside County populations. The response of the HWY65 population was intermediate between the Tulare and GBR populations, and closely matched the data for the 2015 GBR population. This result highlights the dynamic nature of imidacloprid resistance in the Bakersfield area, and the likelihood that resistance was a contributing factor in the upsurge in GWSS numbers in the region and the associated increase in Pierce's disease incidence. We will have additional data to present at the Symposium in December.

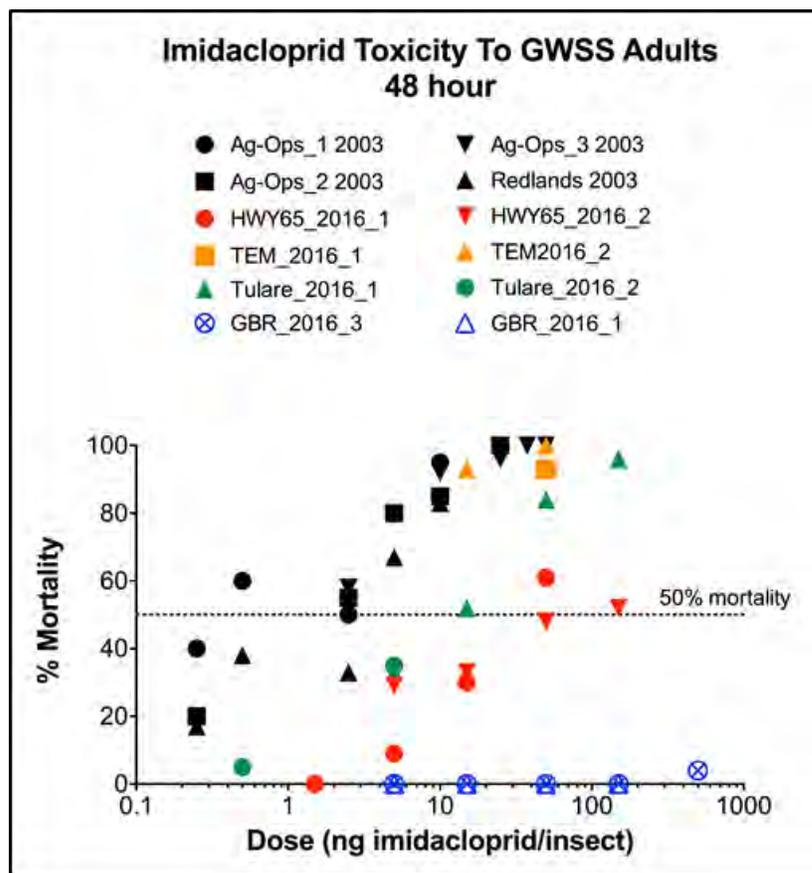


Figure 1. Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. HWY65 (red symbols) and GBR (blue symbols) are populations collected from organic and conventional groves, respectively, in Kern County. TEM (orange symbols) was collected from an organic grove in Temecula Valley. Tulare (green symbols) was collected from an organic grove in Tulare County.

Pyrethroid Bioassays.

We are currently completing bioassays (topical application) with fenpropathrin using the GBR, HWY65, and Tulare populations (**Figure 2**). Bioassay data that were originally generated in 2004 and 2005 for populations sampled from citrus at Agricultural Operations (Ag-Ops) are being used to represent a reference susceptible. Data for bioassays at two concentrations (0.5 and 5 ng pyrethroid per insect) are shown in **Figure 2**.

The levels of mortality observed in the GBR, HWY65, and Tulare populations were lower than those of the Ag-Ops population in 2005. At the 5 ng dose, 77% mortality was recorded in the Ag-Ops population, compared with 5% or less in the GBR and HWY65 populations at the same dose. Clearly, the Kern County insects are expressing resistance to the pyrethroid. The response of the Tulare population (35% at 5 ng dose) was intermediate between the Ag-Ops population and the GBR and HWY65 populations. The significance of these differences in response will be clearer when we generate full dose-response lines, but the data confirm the presence of pyrethroid resistance in Central Valley GWSS populations. We will present full dose-response data at the Symposium in December.

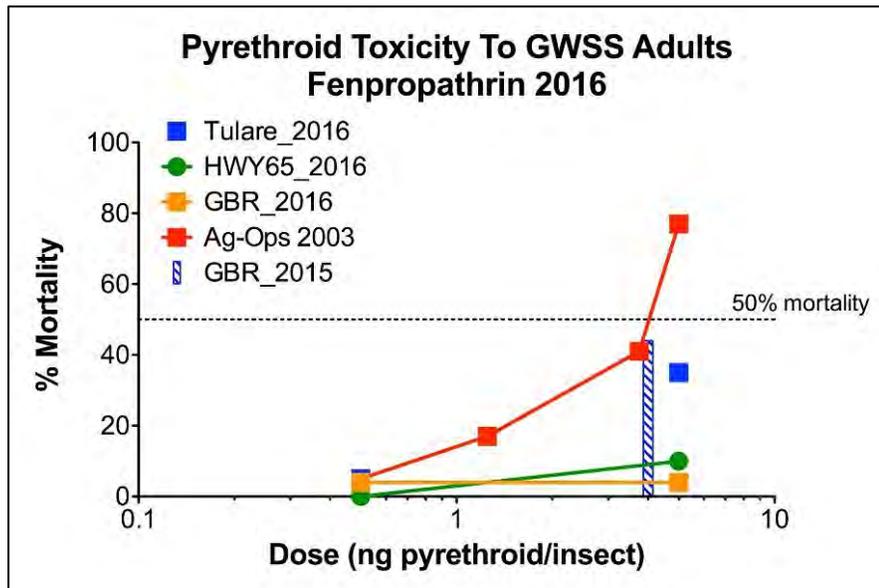


Figure 2. Toxicological response of GWSS adults to the pyrethroid fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. The blue bar shows data for GBR, a Kern County population collected from the General Beale Road area, that were generated in October 2015, using a diagnostic concentration of 5 ng/insect. Data for Ag-Ops (red symbols) were generated in 2003 and are included for comparison. HWY65 (green symbols) and GBR (orange symbols) are populations collected from organic and conventional groves, respectively, in Kern County. Tulare (blue symbols) was collected from an organic grove in Tulare County.

Esterase Activity.

Pyrethroid insecticides are ester-based insecticides and are substrates for pyrethroid-hydrolyzing esterases. Total esterase activity was measured in individual GWSS 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 GWSS collected from the Kern, Riverside, and Tulare County populations, and compared the new data with data from our studies in 2003 (Riverside County) and 2015 (Kern County) (**Figure 3**).

We found no significant differences in esterase levels between the five populations, including the 2003 Ag-Ops population, and conclude that elevated levels of esterase activity cannot be used as a marker for resistance (**Figure 3**).

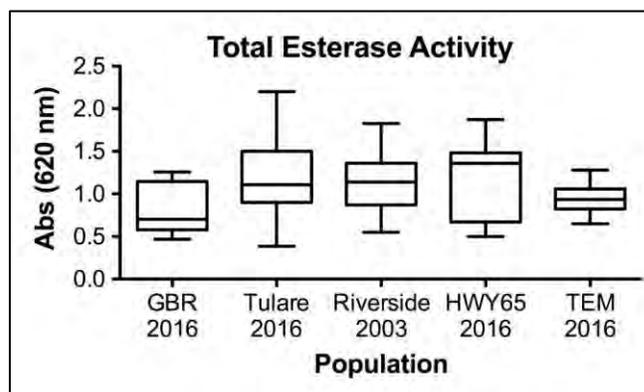


Figure 3. Total esterase activity measured in individual GWSS adults. Activity is represented as absorbance units (320 nm) measured after 30 min incubation with 0.3 mM 1-naphthyl acetate. Homogenates of individual heads were prepared in 0.1 M phosphate buffer, pH 7.5, and then an aliquot (equivalent to 0.01 head) used directly for assay.

Acetylcholinesterase (AChE) 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 GWSS that enabled the measurement of both the total esterase activity and the sensitivity of the AChE to paraoxon in an individual insect.

We compared insects from the GBR (n = 8), HWY65 (n = 14), TEM (n = 22), and Tulare (n = 27) populations, and all the insects were sensitive to the diagnostic concentration of 30 μ M paraoxon. Insects were also tested from locations in Orange County and Tulare County, and these insects were also sensitive to the OP.

Genetic Analyses.

A large number of studies have shown that decreased sensitivity of the target site gene and increased metabolic detoxification of insecticides are two major mechanisms involved in insecticide resistance. To elucidate the molecular mechanisms of resistance to imidacloprid (neonicotinoid) and fenpropathrin (pyrethroid) in GWSS, we are checking for the presence of target site mutations in sodium channel (the target site of pyrethroids) and nicotinic acetylcholine receptor (nAChR; the target site of the neonicotinoids) genes that are known to confer resistance in other pest species. We are using RNA-seq to identify potential roles for detoxification enzymes, such as cytochrome P450, Glutathione S-transferase, and ATP-binding cassette (ABC) transporters.

In our initial investigations, we have not found the classic leucine to phenylalanine (L to F) mutation in the domain II region of the sodium channel gene that confers *kdr* resistance in houseflies and other species. Furthermore, the L to F mutation was not detected in several Tulare and Kern County populations showing differential responses to fenpropathrin in bioassays (**Figure 2**). We are currently evaluating several synonymous and non-synonymous mutations that have been found in individuals from these populations to determine whether they play a significant role in conferring resistance.

Based on the study of the green peach aphid (*Myzus persicae*), the mutation (R81T) in the loop D region of the nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides. We have identified one nicotinic acetylcholine receptor beta-like gene from the GWSS, with a single open reading frame of 1587 bp that encodes a protein of 529 amino acids, a 5' untranslated region (UTR) located 337 bp upstream of the putative start codon (ATG), and a 3' UTR of 314 nucleotides that ended in a poly (A) tail. DNA has been extracted from Tulare, HWY65, and GBR GWSS. Sequence analysis revealed four synonymous mutations and one non-synonymous mutation in individuals expressing different imidacloprid resistance levels. Although the R to T mutation has not been detected in GWSS, further studies will determine whether other mutations are involved in conferring imidacloprid resistance.

We identified several cytochrome P450, glutathione S-transferase, and ABC transporter genes based on the genome database of GWSS. In order to facilitate a more comprehensive analysis of their potential involvement in conferring resistance to imidacloprid and fenpropathrin, we are conducting RNA-seq analysis to compare individuals sampled from the Riverside, Tulare, and Kern County locations where differences in response to the insecticides were measured. In addition, we are including in our RNA-seq analyses survivors from the topical application bioassays, as these individuals are more likely to express resistance-causing genes.

CONCLUSIONS

We identified resistance to imidacloprid in GWSS collected from citrus in the GBR area of Kern County. The dramatic shift in susceptibility is based on a comparison with bioassay data generated in 2003 for a population in Riverside County that we regard as a reliable reference susceptible, and a comparison with 2016 bioassay data for a population collected from an organic grove in Tulare County. In addition to imidacloprid resistance, we have also identified resistance to the pyrethroid fenpropathrin.

The esterase data for all populations included in our investigations showed no major differences that could implicate esterases in pyrethroid resistance. In addition, populations were homogeneous for a sensitive AChE.

The genomic work is becoming increasingly important as a tool for identifying resistance mechanisms. In particular, we are confident that the RNA-seq analysis of populations expressing different levels of resistance to imidacloprid and fenprothrin, will identify specific enzymes that are involved in conferring resistance.

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FUNDING AGENCIES

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

**ASSOCIATION OF THE SHARPSHOOTER X WAVE WITH XYLEM INOCULATION OF
XYLELLA FASTIDIOSA LEADING TO SYSTEMIC, SYMPTOMATIC
PIERCE'S DISEASE INFECTION IN GRAPE**

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Reporting Period: The results reported here are from work conducted May 2015 to September 2016.

ABSTRACT

Despite several decades of study, the mechanism of inoculation of *Xylella fastidiosa* (*Xf*) to grapevines by its sharpshooter vectors still is not fully understood. Recent research showed that *Xf* is inoculated into or onto artificial diets by a combination of egestion and salivation. However, the salivation-egestion mechanism has not been: (1) demonstrated in plants; (2) associated with the sharpshooter X wave (the proposed electropenetrography [EPG] waveform thought to represent salivation and egestion); nor (3) associated with systemic Pierce's disease symptoms. Herein is reported results of a preliminary experiment consistent with all three associations for the salivation-egestion hypothesis. Non-inoculative blue green sharpshooters were wired for EPG recordings, then were individually allowed one to three hours of access to diets containing *Xf* 'Temecula.' One at a time, a wired insect was removed from a diet and immediately placed on a petiole of a small (eight to ten cm tall) 'Chardonnay' grapevine leaf for EPG. Each sharpshooter was allowed to make a single, marked probe until the stylets had reached a xylem cell and produced three, consecutive X waves in that cell, whereupon the insect was immediately removed from the plant. Two control insects were not permitted to reach xylem before their probes were terminated. Probed grapevines were transferred to a greenhouse and held for five months for symptom development. Of the 26 insect-probed grapevines, 16 developed apparent Pierce's disease symptoms, and 12 of those plants were found to be polymerase chain reaction (PCR)-positive for *Xf*. Therefore, about half of the insects that fed on *Xf*-laden diets and then produced X waves on grape successfully inoculated *Xf* into xylem. Each of those single, xylem-inoculation probes later led to a systemic, symptomatic Pierce's disease infection that was confirmed by PCR. Neither of the two plants from control insects became infected. The experiment will be replicated two more times, including many more controls where insects are not permitted to reach xylem. If future tests continue to be successful, there will be conclusive evidence that the sharpshooter X wave represents *Xf* inoculation. Ultimately, this research aims to improve host plant resistance to *Xf* by using EPG of X waves to select grapevines resistant to *Xf* inoculation by the vector.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service, appropriated project 5302-22000-010D.

GRAPEVINES RESPOND TO GLASSY-WINGED SHARPSHOOTER OVIPOSITION BY INCREASING LOCAL AND SYSTEMIC TERPENOID LEVELS

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ABSTRACT

Grapevines (*Vitis vinifera*) have been observed to respond to oviposition by glassy-winged sharpshooters (*Homalodisca vitripennis*) by producing volatile compounds that attract egg parasitoids such as *Gonatocerus ashmeadi* Girault (Krugner et al., 2008). Recent work also has shown that two particular volatiles, the terpenoids β -ocimene and α -farnesene, were present in greater amounts in air space around egg mass-infested grapevines versus non-infested grapevines, and these compounds were attractive to *G. ashmeadi* in olfactometry studies (Krugner et al., 2014). However, methodologies to trap and sample volatiles from air around plants are less sensitive than determining accumulation within plant foliage. This study quantified terpenoids, which are defense-associated volatile compounds, within leaves of non-infested plants and those exposed to egg-laying female sharpshooters. Infested grapevines had leaves with and without egg masses taken to examine both localized and systemic, plant-wide changes in terpenoid levels. Total terpenoid levels were increased in leaves collected from infested plants, regardless if the leaves had egg masses present. A total of 13 monoterpenoids, including ocimene isomers, and nine sesquiterpenoids, including farnesene isomers, were present in greater amounts in leaves with egg masses than leaves without eggs. Leaves from infested plants without egg masses did not have significantly greater monoterpene levels than controls, but there was greater levels of three individual sesquiterpenoids in such leaves than controls. Of all the terpenoids, only β -ocimene appeared to also be present in greater amounts in leaves with egg masses than leaves without egg masses taken from infested plants. These results support previous findings that ocimene and farnesene produced by grapevines attract egg parasitoids (Krugner et al., 2014). However, additional volatile compounds also were upregulated and could be involved in attraction of natural predators or possess other roles in host defense against sharpshooters. Therefore, future studies should focus on observing terpenoid roles in providing grapevine resistance to sharpshooters and similar insects.

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FUNDING AGENCIES

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Section 3:

Other Pests & Diseases of Winegrapes

- Insects -**
- Viruses -**

EVALUATION OF COMMERCIAL ANT BAITS AS A COMPONENT OF AN INTEGRATED PEST MANAGEMENT PROGRAM FOR VINE MEALYBUG

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Reporting Period: The results reported here are from work conducted July 1, 2016 to September 30, 2016.

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 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 integrated pest management 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 broadcast applications of a commercial ant bait and an experimental ant bait in northern California vineyards and measured the effects on Argentine ant populations. Pre- and post-application, Argentine ant populations were measured indirectly via feeding activity, assessed as the number of ants present on cotton balls (Fisher Scientific) soaked in 25% sucrose solution. Ant activity was measured once every two weeks. Both baits reduced feeding activity, although the effect was more sustained in the experimental bait treatment, suggesting the potential of this bait to provide long-term control of Argentine ants in coastal California vineyards.

LAYPERSON SUMMARY

Vine mealybug (*Planococcus ficus*) 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) including insecticides, mating disruption, and biological control to achieve control of vine mealybug populations. Argentine ants (*Linepithema humile*) are invasive insects common in coastal California vineyards. Ants disrupt integrated pest management 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 commercial and experimental baits to control Argentine ants in vineyards. Baits were either broadcast using a fertilizer spreader or applied under the vine with a modified broadcast spreader in March, April, and May 2016. Both baits reduced ant activity in the treated areas, although the effect was more sustained with the experimental bait, suggesting its potential as a component of sustainable vine mealybug management in coastal California vineyards.

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., 2008). Management can be particularly complicated in coastal wine grape-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 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. Baits formulated as granular products or polyacrylamide gels that can be broadcast with a fertilizer spreader could be distributed more quickly and frequently over a large area, and would not require the manufacture and maintenance of bait stations. The sustained use of the granular or polyacrylamide baits could lead to longer-term containment and control of Argentine ant populations (Boser et al., 2014; Krushelnycky et al., 2004). We are evaluating granular and polyacrylamide ant baits that can be broadcast to reduce 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 affect water quality, disrupt populations of beneficial insects, and pose vertebrate health risks.

OBJECTIVES

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 two bait formulations to reduce Argentine ant populations as part of an integrated pest management program for vine mealybug.

RESULTS AND DISCUSSION

Granular bait trial.

We established this experiment in five unique vineyard blocks in the Oakville and Rutherford appellations of the Napa Valley American Viticultural Area and established split-plot designs (bait and untreated) in all blocks. In two of those blocks (designated I1 and I2), Seduce ant bait (0.07% spinosad) was applied at a rate of 20 pounds per acre on April 15 and 16. In the remaining three blocks (designated T1, T2, F1), Seduce ant bait was applied at a rate of 28 pounds per acre (slightly higher than the target rate due to challenges with calibration and the spreader equipment) on May 19 and 20; a second application at the rate of 20 pounds per acre was applied in blocks T1, T2, and F1 on June 25 and 27, 2016. Bait was applied in a strip under the vine row using a modified broadcast spreader (**Figure 1**). The cooperating vineyard managers made all the bait applications.

We monitored ant activity pre- and post-application using cotton balls (Fisher Scientific) soaked in 25% sucrose solution (**Figure 1**). Ant activity was measured once every two weeks. Forty-five or fifty vines per treatment per block were selected as monitoring vines. One saturated cotton ball was deployed on each monitoring vine, either on the ground (early season) or on the vine (after fruit set), depending on where the ants were predicted to be most active. After 2.5 to 3 hours cotton balls were retrieved from each monitoring vine, and ant activity on the cotton ball was assessed using a 0 to 3 scale where '0' equals no ants, '1' equals the presence of 1 to 10 ants, a value of '2' is assigned to cotton balls with 11 to 50 ants, and a rating of '3' assigned for the presence of greater than 50 ants.

Due to some challenges with site selection, the first bait applications in blocks T1, T2, and F1 occurred later (May 19 and May 20) than would be desired to optimize results. In blocks I1 and I2, bait applications were initiated early in the growing season (April 15 and 16), and within 14 days of the time when ants were reliably detected and temperatures were adequate for foraging to occur. On May 6, foraging activity was reduced in blocks I1 and I2 in the bait treatment (**Figure 2**). However, by June 3 (seven weeks after application) and continuing through the rest of the season there was no difference in ant activity between treatments in these blocks. In block T1, ant activity was reduced immediately after bait application; however, ants were detected in the baited treatment on June 10 (22 days post-application), although populations remained lower than in the untreated control. In T2, ants were detected in the treated plot on May 27 (seven days post-treatment) and had rebounded to levels no different from the control plot by June 10 (22 days post-treatment). These results suggest that multiple applications of Seduce may be necessary to obtain adequate control of Argentine ant populations. Alternately, it is possible that a

higher product rate may be more efficacious over a longer period. Both options should be explored in future studies.

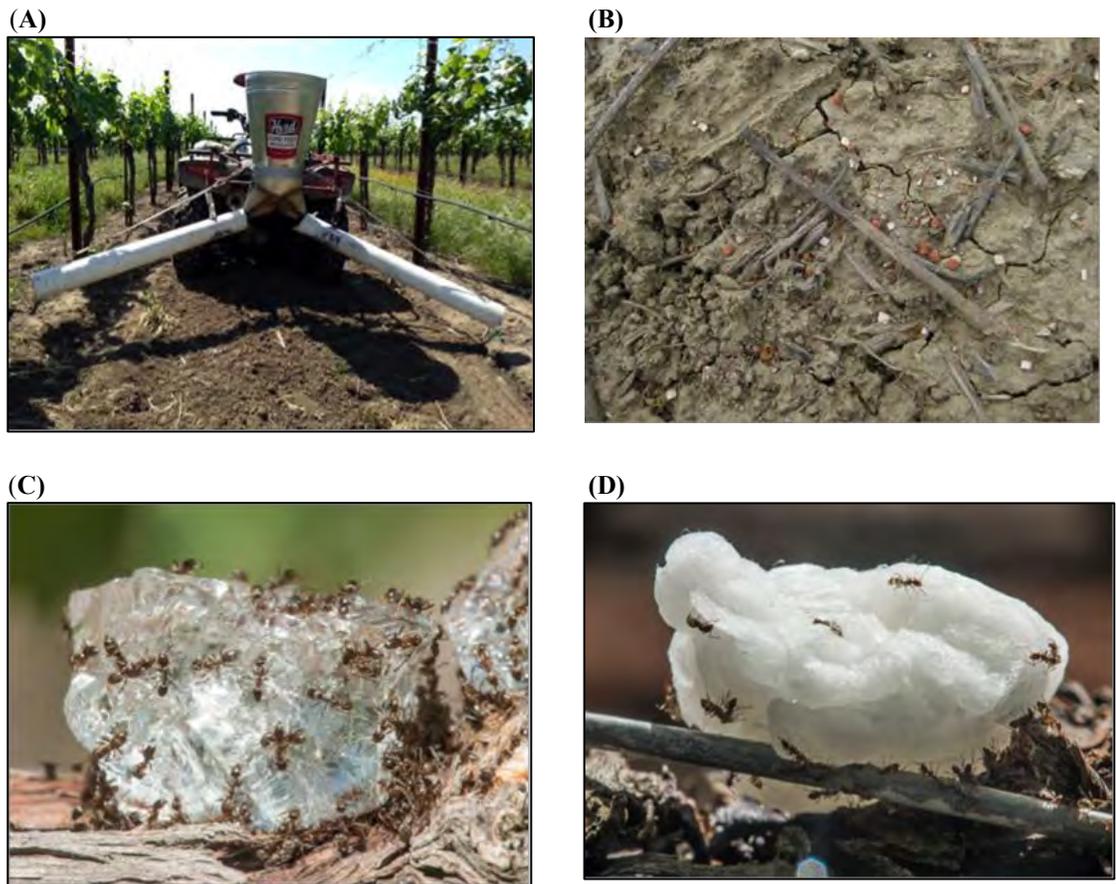


Figure 1. (A) modified broadcast spreader mounted on ATV; (B) Seduce bait (reddish pellets) under the vine row; (C) Argentine ants feeding on polyacrylamide bait; (D) Argentine ants feeding on cotton ball used for monitoring ant activity. Photo credits: (A) K. Taylor, Constellation Brands; (B) M. Cooper, UC Cooperative Extension (UCCE); (C) & (D): M. Hobbs, UCCE.

Polyacrylamide gel bait trial.

Based on a pilot study that eliminated >99% of ants from treated plots in the California Channel Islands (Boser et al., 2014) and a preliminary vineyard study conducted by the Principal Investigators in 2015, we are evaluating the efficacy of a polyacrylamide gel bait formulation in vineyards. We established three experimental blocks (split-plot design: treated and untreated treatments). Two of these blocks (designated C1 and C2) are located in the Carneros appellation (Napa Valley American Viticultural Area) and one (designated M1) is located in the St. Helena appellation. Blocks C1 and C2 are populated with the invasive vine mealybug; block M1 is populated with the native grape mealybug (*Pseudococcus maritimus*). In addition to the economic damage sustained by vine mealybug populations, the spread of grapevine leafroll-associated virus 3 (GLRaV-3) is a major concern in all of these blocks.

The bait solution consists of 0.0006% thiamethoxam (Platinum insecticide, Syngenta U.S.) in 25% sucrose solution, deployed at a rate of 10 pounds per acre in polyacrylamide Water Storing Crystals (MiracleGro®) (Figure 1). These crystals absorb water and water-soluble chemicals, and when hydrated present a thin layer of liquid bait solution on the surface for 24 to 72 hours following application. To allow sufficient time for the crystals to absorb the bait solution, they were added to the mixture 24 hours prior to the application. The hydrated crystals were deployed using an 85-pound tow spreader (Agri-Fab, model #45-0315) pulled with an all-terrain vehicle (ATV). Bait applications were initiated once foraging ants were detected at sugar-soaked cotton balls. The cooperating vineyard manager made the bait applications on March 16 and April 14 in blocks C1 and C2, and on

April 15 and May 26 in block M1. Because block M1 is in a more northerly location within Napa County, ants did not become active until later in the season (ant foraging is reduced below 60 °F (15 °C)). Ant monitoring pre- and post-application followed the method described previously, using cotton balls soaked with a 25% sucrose solution.

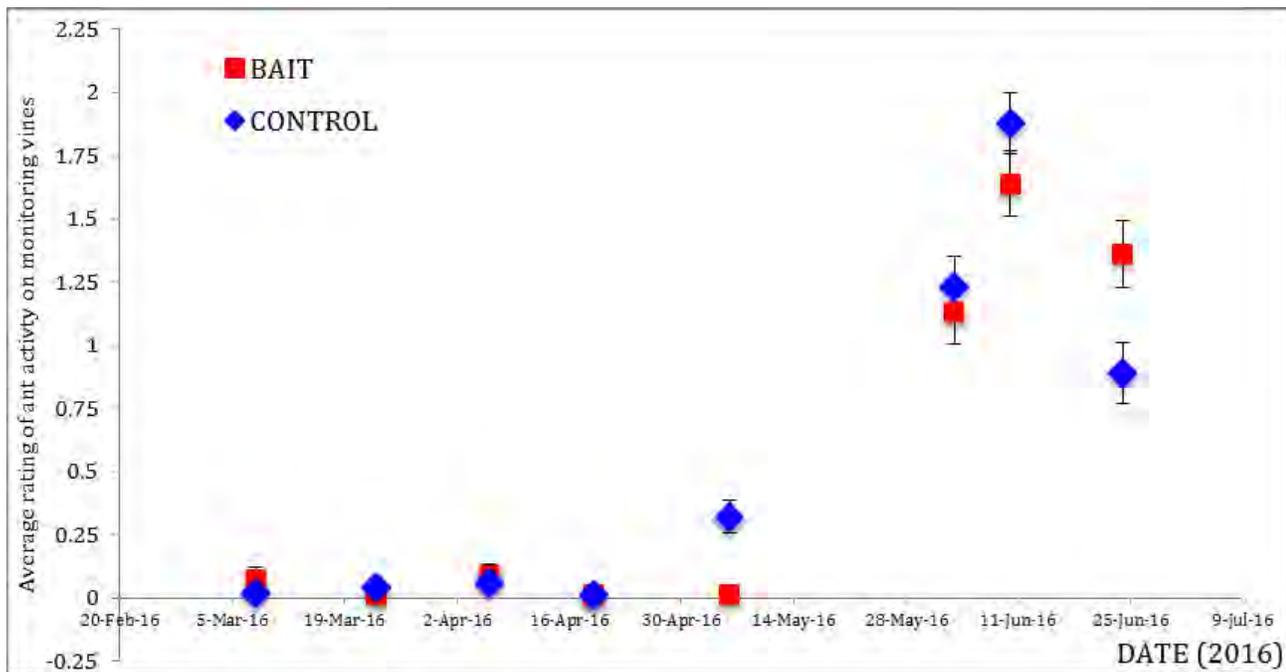


Figure 2. Ant activity (rated on a 0 to 3 scale) at 90 monitoring vines per treatment for blocks I1 and I2. Data are presented as the average of the ratings for all vines, by treatment. Seduce (0.07% spinosad) was applied on April 15 and 16, 2016 at a rate of 20 pounds per acre.

In blocks C1 and C2, ants were present in pre-treatment monitoring conducted on February 26 and March 8, so the first bait applications were made on March 16. By March 23, ant activity in the baited blocks was lower than the untreated blocks (**Figure 3**). Ant activity remained low in the baited blocks. On three monitoring dates (April 15, April 28, and May 11) we detected no ants in the baited areas. By May 30, ants had begun to reinvade the treated blocks, although populations remain much smaller than in the untreated areas. Given the invasion biology of the Argentine ant, and that each untreated area is a large, proximal source of ants, it is not surprising that they have begun re-colonizing the baited areas. Large-scale, area-wide treatments (such as those conducted in the California Channel Islands (Boser et al., 2014)) could be expected to be more successful as they would leave fewer population pockets from which ants could re-invade. Future studies should concentrate in this area.

CONCLUSIONS

We evaluated two baits (one commercial and one experimental product) to reduce 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 integrated pest management programs for vine mealybug. Handling and distribution of baits that can be broadcast 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 both the granular product (Seduce, 0.07% spinosad) and an experimental bait (0.0006% thiamethoxam in polyacrylamide crystals) have the potential to reduce populations of Argentine ant, although the effect of the polyacrylamide bait on foraging ants was more sustained than the granular bait. Our results also suggest that multiple applications of Seduce may be necessary to obtain adequate control of Argentine ant populations. Alternately, it is possible that a higher product rate may be more efficacious over a longer period. Both options should be explored in future studies. Additionally, large-scale, area-wide treatments (such as those conducted in the California Channel Islands (Boser et al., 2014)) could be expected to be more successful as they

would leave fewer population pockets from which ants could re-invade. Future studies should concentrate on this area.

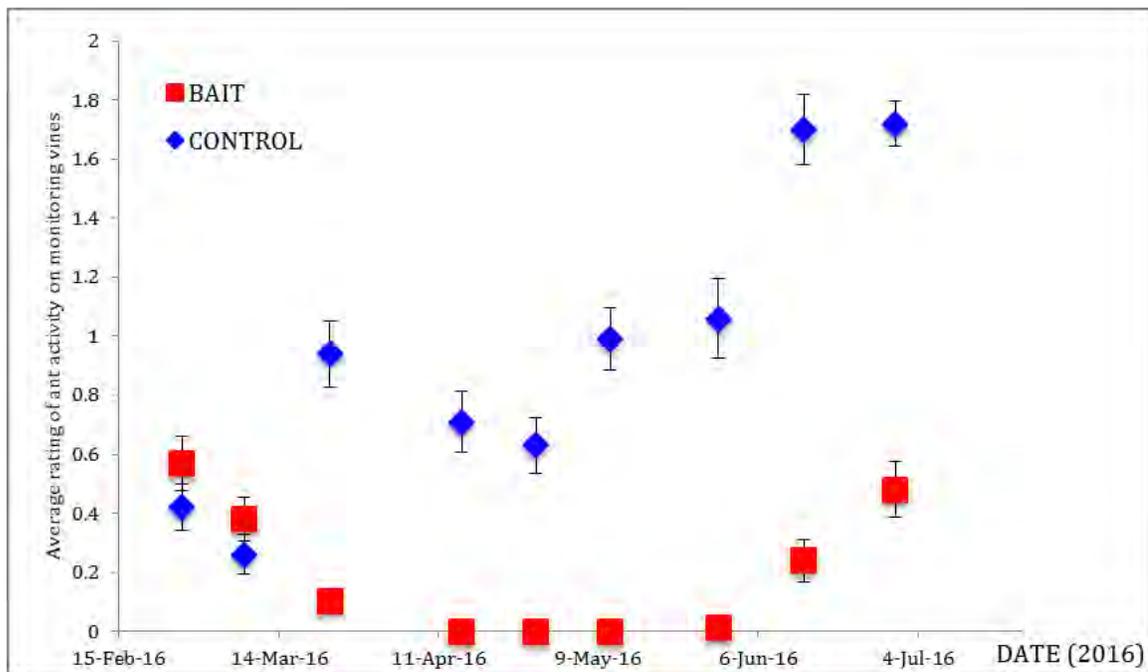


Figure 3. Ant activity (on a 0 to 3 scale) at 90 monitoring vines per treatment for blocks C1 and C2. Data are presented as the average rating for all vines, by treatment. Thiamethoxam (0.0006%) in polyacrylamide crystals was applied on March 16 and April 14, at a rate of 10 pounds per acre.

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**IMPROVING VINE MEALYBUG WINTER AND SPRING CONTROLS:
I. BIOASSAYS. II. USING HIGH PRESSURE LIQUID CHROMATOGRAPHY TO FOLLOW
INSECTICIDE MOVEMENT IN THE VINE**

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ABSTRACT

The vine mealybug (*Planococcus ficus*) has become one of the most important insect pests of California vineyards. Researchers, pest control advisors, and farmers 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. Our objectives are to improve pre- or post-harvest controls that target the winter-spring vine mealybug population and better determine the spring emergence of vine mealybug crawlers to better time foliar applications. In 2016, research focused on the field application bioassays and movement of Movento®, or more correctly its metabolites, in the vine, using high pressure liquid chromatograph (HPLC) methodology. We have confirmed that spirotetramat is rapidly converted in the leaves to a metabolite called enol-spirotetramat, and this metabolite can remain in the leaves for most of the season. The enol can change to other metabolites such as enol-glycoside and ketohydroxy as some of the primary metabolites found, but it is the enol metabolite that is most important for killing the mealybugs. There is a gradual decline in the amount of enol-spirotetramat, but we found spirotetramat in leaves 184 days after application, suggesting that this material might still yet be converted to enol-spirotetramat.

LAYPERSON SUMMARY

The vine mealybug (*Planococcus ficus*) has become one of the most important insect pests of California vineyards. Researchers, pest control advisors, and farmers 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. Our objectives are to improve pre- or post-harvest controls that target the winter-spring vine mealybug population and better determine the spring emergence of vine mealybug crawlers to better time foliar applications. In 2016, research focused on the field application bioassays and movement of Movento®, or more correctly its metabolites, in the vine, using high pressure liquid chromatograph (HPLC) methodology. Preparing samples and running the HPLC can be time consuming and we have processed less than 10% of the 6,000 samples collected. We have confirmed that spirotetramat is rapidly converted in the leaves to a metabolite called enol-spirotetramat, and this metabolite can remain in the leaves for most of the season. The enol-spirotetramat can change to other metabolites such as enol-glycoside and ketohydroxy, but it is the enol-spirotetramat that is most important for killing the mealybugs. There is a gradual decline in the amount of enol-spirotetramat, but we found spirotetramat in leaves 184 days after application, suggesting that this material might still yet be converted to enol-spirotetramat. As we process more of the samples we will be better able to determine the metabolic pathways of spirotetramat and what influence vineyard conditions and application methodology has on the effectiveness of Movento®.

INTRODUCTION

The vine mealybug (*Planococcus ficus*) has become one of the most important insect pests of California vineyards, threatening economic production and sustainable practices in this multi-billion-dollar 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., 2013). Researchers, pest control advisors, and farmers 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. 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 (Gutierrez et al., 2008) and can be the most difficult to control even with systemic insecticide applications (Daane, personal observation). Moreover, mealybugs can remain on even the remnant pieces of vine roots after vineyard removal, hosting pathogens and infesting new vines after replanting the vineyard (Bell et al., 2009).

Insecticides with systemic action are the best materials to control this protected population, but their proper use can vary among vineyards and regions. Moreover, vineyards with mealybug damage typically have large overwintering 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.

A delayed dormant (typically in February) application of chlorpyrifos (Lorsban®) was the standard post-harvest or pre-season control that targeted mealybugs on the trunk and cordon (Daane et al., 2006). The best in-season insecticide for vine mealybug that moves from the trunk and cordon to the leaves, canes, and fruit has been an application of Movento® (Bayer Crop Science), with the active ingredient spirotetramat, which may also help control root-feeding nematodes (Mike McKenry, personal communication). Still, the effectiveness of any systemic material will depend on application timing, soil moisture, vine condition and age, and commodity (for example, post-harvest application timing). Our objectives are to improve controls that target the winter-spring vine mealybug population and to better determine the spring emergence of vine mealybug crawlers to better time foliar applications. Specifically, we are conducting field bioassays to determine the effect of application timing, soil moisture, vine condition and age, and commodity (for example, post-harvest application timing, wine vs. raisin management practices) on systemic insecticide effectiveness. We plan to work with all vineyard-registered insecticide materials, but this past year's work has focused on field application bioassays and movement of Movento® in the vine. To follow the movement of Movento® we are collecting vine samples and using high pressure liquid chromatography (HPLC) to determine the amounts of different metabolites associated with Movento® in different parts of the vine. For example, two of the questions we plan to address is whether spirotetramat converts to the metabolite enol-spirotetramat (which is the primary toxicant) similarly under different conditions, such as vine nutrient status or cultivar, and where on the vine the metabolites move to and in what concentration are the metabolites found on different vine sections, such as the leaves versus the roots. We will also use our protocols to help confirm the presence of spirotetramat metabolites in the root system, in support of A. Westphal's proposal.

OBJECTIVES

The project 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. Bioassay
 - a. Investigate the population dynamics and controls for overwintering vine mealybugs.
 - b. Determine the temperature relationship of vine mealybug and grape mealybug to better predict spring emergence and spray timing.
2. Using HPLC to follow the movement of Movento® in the vine:
 - a. Improve the protocols to determine levels of spirotetramat and its first metabolite, the enol form, in vine tissue samples.
 - b. Investigate the dissipation and transformation mechanisms of the active ingredient of the pesticide Movento® after application.

RESULTS AND DISCUSSION

Objective 1. Bioassay: Insecticide controls for vine mealybug.

During 2015 and 2016, we used bioassays (visual counts of mealybugs) to look at control effectiveness across vineyards in different regions and with different management practices or vine structures. 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 wine grape region (San Joaquin County) with three vineyards near Lodi (one Cabernet Sauvignon, one Pinot Noir, and one Chardonnay); and the North Coast wine grape 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 Center that represent wine and table grape blocks undergoing studies for nitrogen, irrigation, and wine grape cultivars. At each site, we have counted mealybug densities on the vine, measured cluster damage, and taken vine fresh tissue samples before and after Movento® applications (sections from the leaf, cane, and trunk) (**Figure 1**).

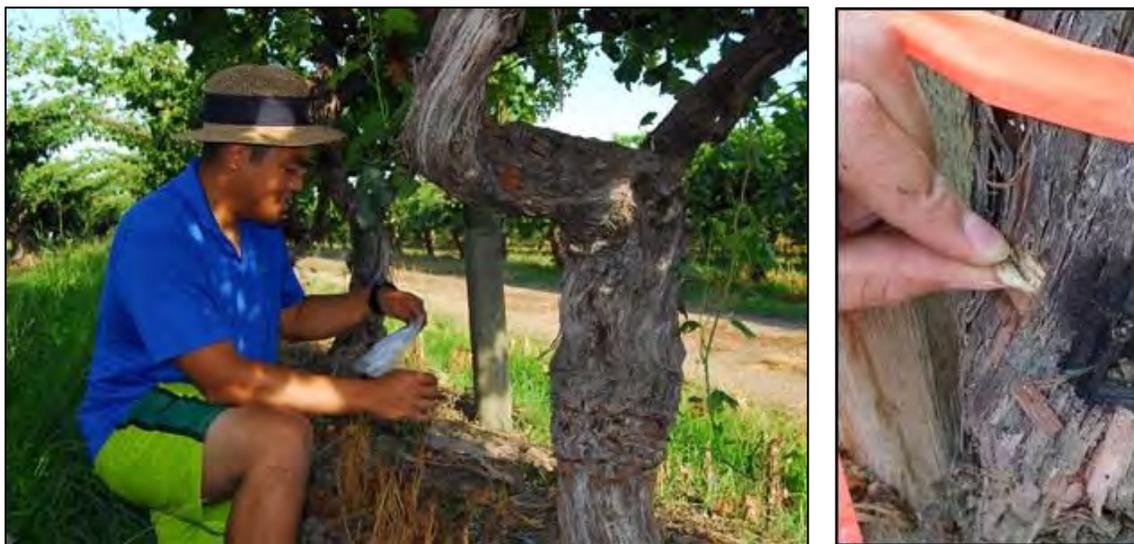


Figure 1. Sampling trunk live tissue, leaves and petioles, canes, cordons, trunk (above and below girdle, when present), and roots.

Pre-treatment mealybug counts were taken using a timed count. In brief, on each sampled vine an experienced sampler searched for mealybugs for a one-minute period. The areas of the vine searched changed 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 was then used to block treatments against density because vine mealybug populations can be clumped. In 2016, the visual count of mealybugs took place from April to October. This allowed us to monitor mealybug populations at different phenological stages of the crop. We monitored when the grape clusters were not ready to be harvested, when they were ready to be harvested, and after they were harvested.

We applied the insecticide Movento® at different 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 summer, fall (completed), and will continue this in the coming spring (**Figure 2**). A standardized application method was used across all vineyards so that surfactant and application rate would not be an influence. At each site there are 15 replicates (individual vines) per treatment per vineyard, with treatments placed in a complete randomized design.

We also have completed a measurement of economic damage on five clusters on each vine using a 0 to 3 scale: 0 means no mealybug damage, 1 means honeydew present but the bunch is salvageable, 2 means honeydew and mealybugs present but at least part of the bunch is salvageable, and 3 means a total loss. The economic damage of

clusters took place from June 2016 through harvest. We evaluated the clusters when the grape clusters were not ready to be harvested and when they were ready to be harvested.



Figure 2. Applying Movento®.

Taking into consideration all the sample areas, approximately 600 vines were sampled for mealybug counts and for cluster evaluation. Together, the treated vineyards include several factors that could be affecting the pesticide efficiency, such as the age of the vineyards, irrigation type, commodity (table, raisin, and wine grapes), the presence of a girdle, and geographical area.

Much of the data remains to be analyzed, especially the late season (just before harvest) and post-harvest sprays that will need additional sampling in spring and summer 2017 to determine treatment impact. Moreover, in our commercial fields the overall density of the mealybug was very low, making treatment comparisons difficult. One clear result was that vines sprayed with Movento® in May (the recommended standard treatment would be eight ounces in April or May) had less fruit damage compared to the untreated and the mid-July spray treatments (**Figure 3**; Chi Square $P < 0.001$). Even though mealybugs were found in low numbers throughout all the sampling areas, combining these same treatments across the different vineyards sampled in the Central Valley indicated that the spray treatment had a statistically significant effect on the numbers of individuals found in each developmental stage ($F_{2,2} = 5.3586, P = 0.004$).

We have yet to analyze the post-harvest treatments, but by the end of the season vines treated from mid- to late-May had fewer vine mealybugs compared to untreated vines; however, there was no significant difference between May and mid-July treatments (**Figure 4**). These results indicate that the metabolites of Movento® are moving through the vine and killing mealybugs even in the pre-harvest application treatment, but the earlier treatments are killing the mealybugs before they get into the fruit.

In our bioassay studies the low number of mealybugs found in all the monitoring sites and the low constant damage recorded suggest that visual counts and cluster damage evaluation alone were not sufficient tools to evaluate details of the vine mealybug population's response to pesticide applications. One problem is their clumped distribution in the host plant, which requires a great number of samples to get an accurate estimate of population response. There was also a repeated issue of grower overspray on the control plots, reducing our number of control replicates.

Data from the Napa Valley and Lodi Woodbridge vineyards has not yet been analyzed.

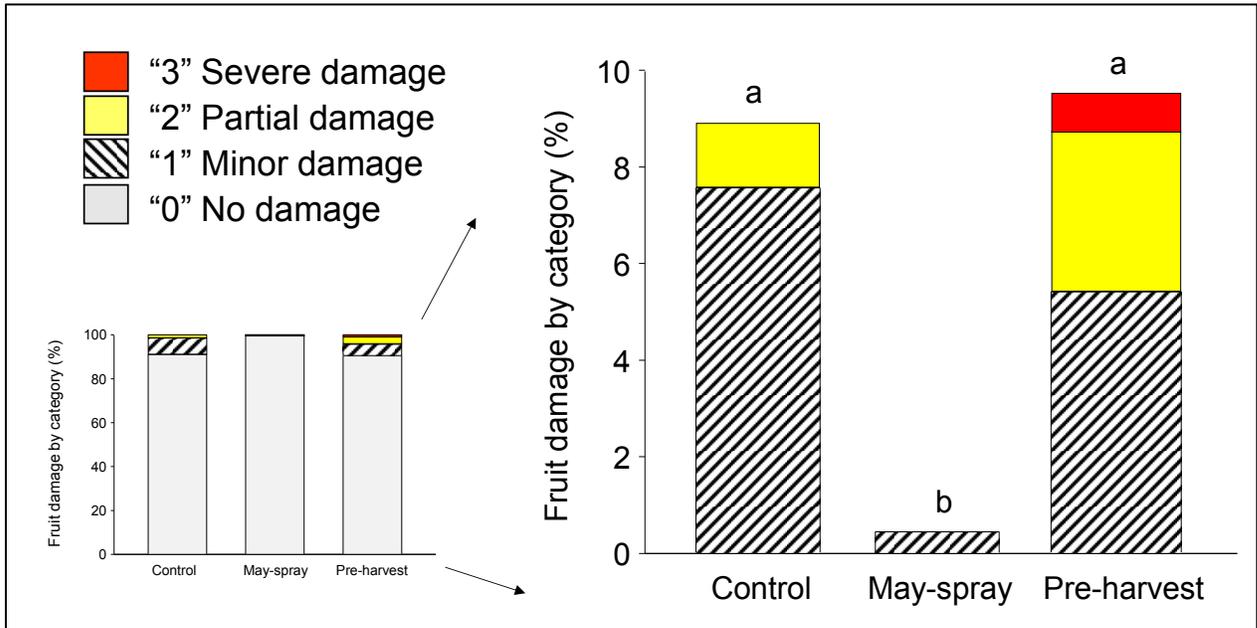


Figure 3. In the sampled San Joaquin Valley vineyards, results show that the May treatment (farmer standard treatment) had significantly less fruit damage than the control or pre-harvest (mid-July) treatment, as would be expected (the smaller graph includes “0 - no damage” fruit and is included to show that more than 90% of all fruit was clean across all treatments).

In the San Joaquin Valley vine treatments, what is interesting is that while there was more fruit damage in the pre-harvest treatment, there was some reduction in the number of adult vine mealybugs as compared to the control. **Figure 4** shows the average number of nymphs, adults, and ovisacs on vines treated in mid- late-May (farmer standard treatment), pre-harvest, and a no-spray control. There was significant difference between the mid-May and pre-harvest (mid-July) treatments in total numbers of mealybugs during the sample periods.

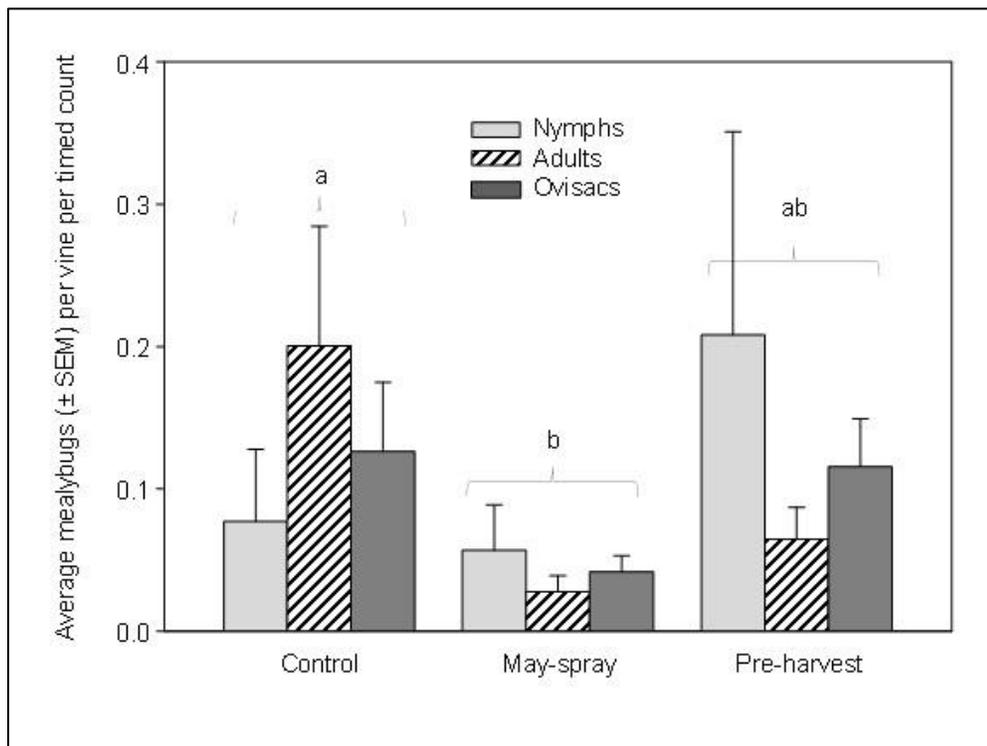


Figure 4. Average number of nymphs, adults, and ovisacs on vines treated in mid- late-May (farmer standard treatment), pre-harvest, and a no-spray control.

Objective 2. HPLC to follow the movement of systemic insecticides.

To study how the pesticide Movento® moves through the vines the pesticide uptake and movement of key metabolites in the plant was followed by means of high pressure liquid chromatograph (HPLC) methodology. To better understand our purpose a description of how Movento® works to kill mealybugs is needed. Spirotetramat is sprayed onto the leaves, where it has translaminar activity and gets absorbed. It is not the spirotetramat that primarily kills the mealybug, but the first breakdown product or metabolite called “enol” (Figure 5, from Bayer CropScience). The enol can change to other metabolites such as enol-glycoside and ketohydroxy as some of the primary metabolites found, but it is the enol metabolite that is most important for killing the mealybugs. The change from spirotetramat to enol appeared to be most effective in the leaf tissue, as described in Bayer-sponsored studies in apple, cotton, and other crops. Whereas some translaminar pesticides remain in the leaves, spirotetramat and its metabolites can be transported by the phloem (and to some extent the xylem) to other plant parts, and this is key in moving the product to where the mealybugs are.

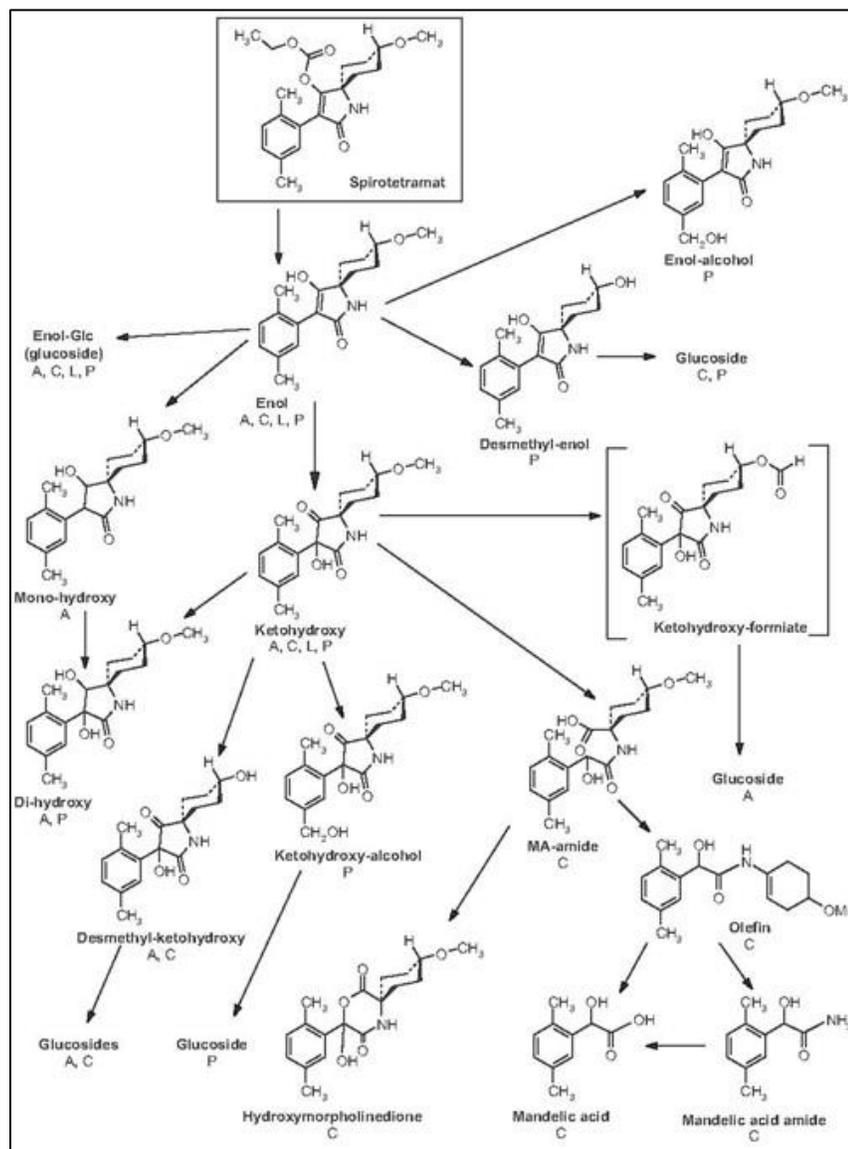


Figure 5. Breakdown products and metabolites of spirotetramat (from Bayer CropScience).

We used the HPLC to obtain the concentration of the active ingredient of Movento® (spirotetramat) and its three primary metabolites (spirotetramat-enol) and enol-glycoside and ketohydroxy (the latter two metabolites are not active against mealybugs as far as we know). To analyze the quantity of spirotetramat, enol, and other metabolites in leaves, the extraction method “QuEChERS” (Quick Easy Cheap Effective Rugged Safe) was followed. This

methodology allows the preparation and analyses of several samples at one time and provides extracts of several structurally different substances with good efficiencies.

We are currently adapting this QuEChERS extraction methodology to our samples to achieve the most trustful results. Adapting this method includes trying different solvents and mobile phases to clean and extract the desired compounds and testing various elution times. Afterwards the obtained results are compared to a standard curve for the desired compound (**Figures 6 and 7**). In this process, the most appropriate and reproducible cleaning and extraction process was determined for leaves, canes, and roots. We are still perfecting a process for smaller bark samples (<10 g) that can be completed without the addition of a “mass spectrophotometer” (MS).

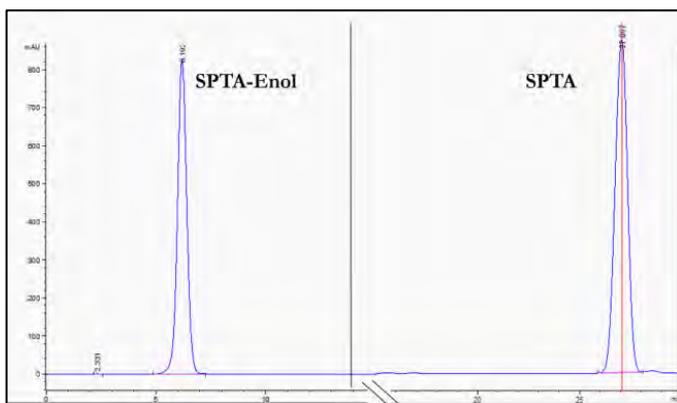


Figure 6. Example of known “standards” of spirotetramat-enol (SPTA-enol) and spirotetramat (SPTA) elution time. These compounds were eluted at 6.14 minutes and 27 minutes, respectively, and are compared with vine tissue samples.

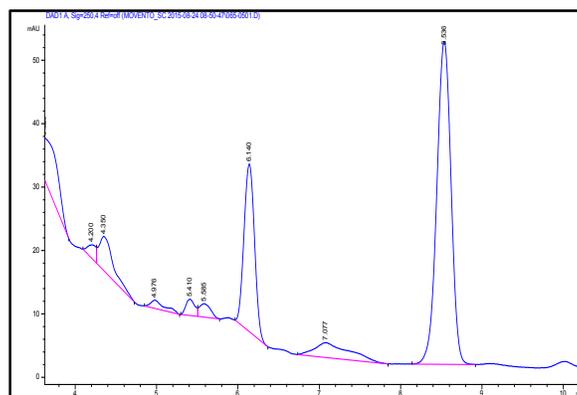


Figure 7. Example of a leaf sample processed by HPLC showing a peak that eluted at 6.14 minutes, matching the standard for SPTA-enol (see **Figure 5**) and indicating its presence in the sampled leaf.

Our analyzed samples are collected in association with our field bioassays. After counting mealybugs (see bioassay above), the following 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, samples from the bottom and middle part of the trunk were taken. If arm is not applicable, an upper part of the trunk was sampled. This fresh tissue sampling effort in 2016 resulted in approximately 6,000 samples being collected, which are being analyzed using the HPLC technology.

Results from leaf tissue analyses show that spirotetramat is quickly converted into enol (remember that enol is the metabolite responsible for killing the mealybugs), and a portion of the enol is also rapidly converted to enol-glucoside (we found this within five hours after spraying) (**Figure 8**). Note that the Y-axis is using a log scale so there are great differences in the amounts of metabolites. Most important was that some spirotetramat and enol was found in the leaf tissue up to 184 days after treatment. It is still unclear from our studies if the spirotetramat found long after the application will eventually convert to enol or if this conversion process slows, as it is surprising that the initial conversion to enol and enol-glycoside is so rapid but we still find spirotetramat unconverted five months after spray treatments. These tested vines will continue to be sampled until leaf drop, and other vine tissue (e.g., bark) will be sampled up to a year after the spray application. What surprised us in these leaf tissue analyses was that enol-glucoside was the most abundant and consistent (over time) of the four metabolites tested (**Figure 8**). It has been reported that under the right circumstances the enol-glycoside can revert to enol, although how common this occurs in vines is not known. At this point we assume that enol found after three to five months is from either relatively stable enol remaining in the leaves or spirotetramat that in the leaves is much later (in time) converted to enol. Note also that we found the ketohydroxy metabolite only on the last sample date, and at a very low amount (**Figure 8**).

When looking closer at the amount of spirotetramat and enol in leaf tissue over the sampling period it's clear that the amount of spirotetramat is reduced quickly, from about 100 parts per billion (ppb) five hours after spray to about 40 ppb after one to three days, and less than five ppb after one month (**Figure 9A**). There is not a

corresponding increase in enol, which is lower than spirotetramat initially but shows a more stable presence during the five-month sampling period (around 20 ppb; **Figure 9B**). Note that on two sample dates (1 and 110 days) we did not detect any enol; this analysis will be repeated with stored samples to determine if this unusual finding (especially at one day) was a data entry error. What is needed now is a field bioassay on the amount of enol in the plant that is toxic to mealybugs, and for how long the mealybug must feed to acquire this lethal dose.

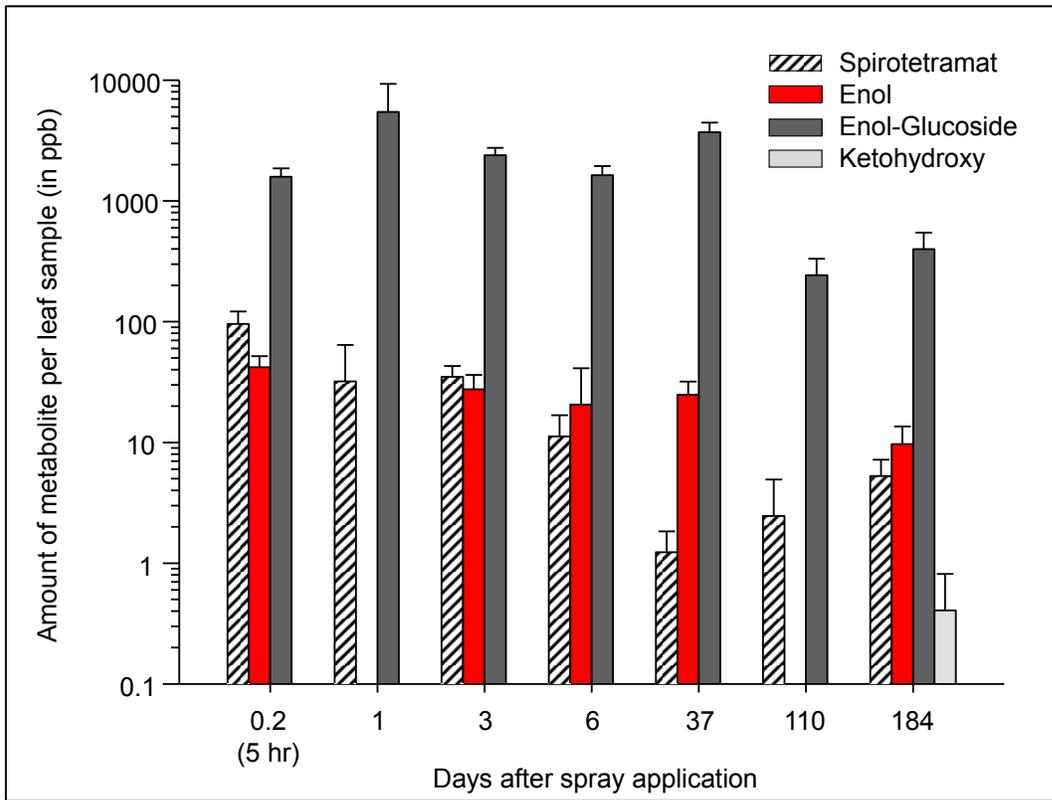


Figure 8. Mean concentration (parts per billion) of spirotetramat and three of its metabolite in leaf samples from five hours after spray to five months after spray.

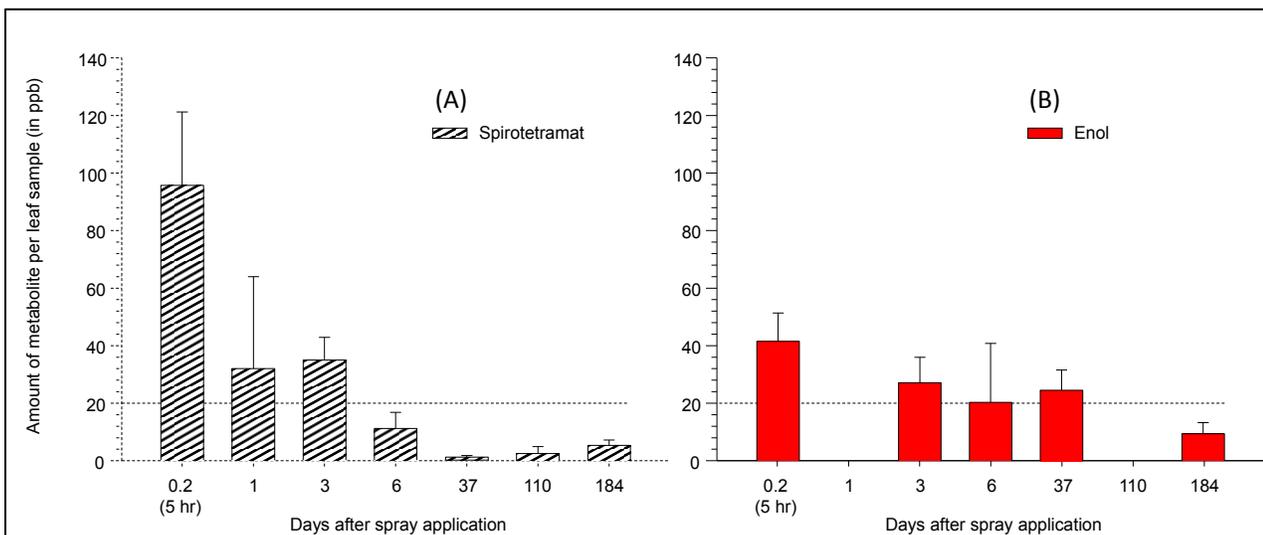


Figure 9. Spirotetramat (A) and Enol (B) content in samples leaves (in parts per billion) at different time after being treated with a label rate (8 oz per acre) of Movento® in May.

Our initial results from the trunk tissue analyses show that spirotetramat is found soon after spray application in the bark tissue. Here we looked at samples from six to seven days after spraying, and it was found 37 days later as well (**Figure 10**). In these samples the enol (the primary mealybug killing metabolite) was not found, whereas enol-glucoside and ketohydroxy were found after six days of spraying. These are preliminary results because only a relatively few bark samples ($n = 70$) have been processed, and these came from a single site. Still, it shows that the metabolites are moving with the phloem from the leaves to other vine sections. One question this does raise is whether the spirotetramat found in the trunk is easily converted to enol. We assume that the metabolites flow passively in the phloem and so it is possible that, depending on vine needs, the metabolites could be carried back to the leaves.

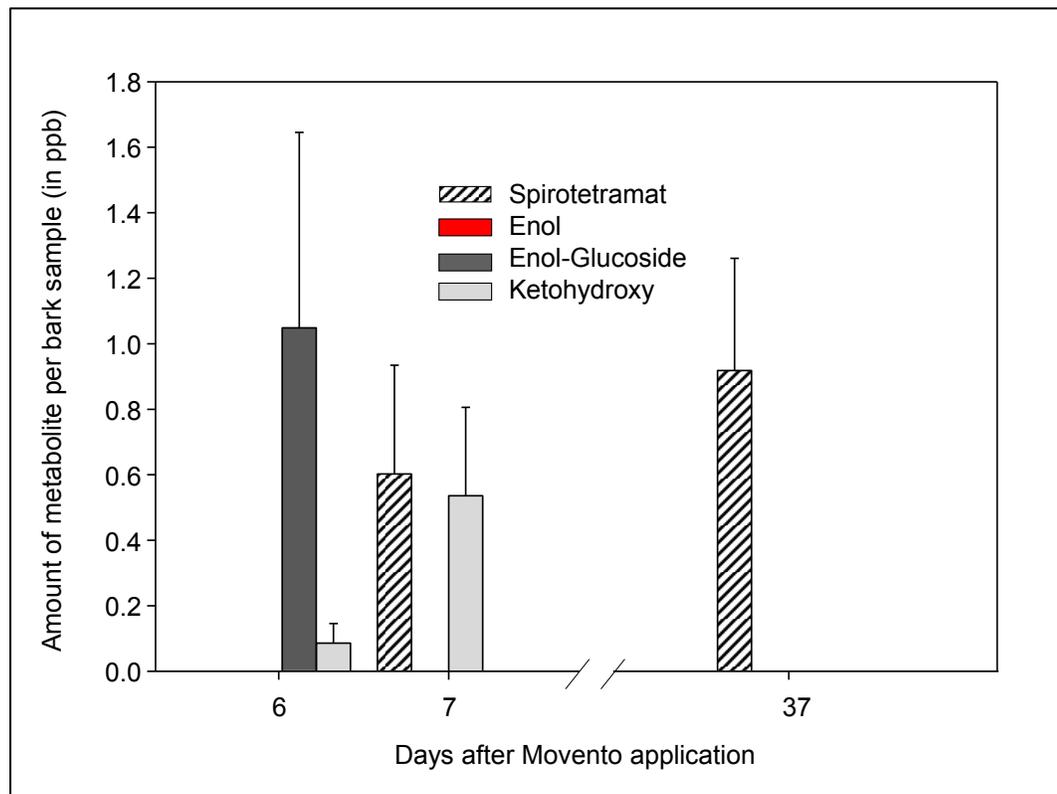


Figure 10. Mean concentration (ppb) of spirotetramat and three of its metabolite in leaf samples from six to seven and 37 days after Movento® was applied to the leaves at label rate (eight ounces per acre).

Temperature development of vine mealybug.

These data have not yet been analyzed.

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BROWN MARMORATED STINK BUG RISK AND IMPACTS IN WESTERN VINEYARDS

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ABSTRACT

Brown marmorated stink bug (*Halyomorpha halys*; BMSB) was found in increasing numbers in vineyards in Oregon from 2013 to 2016. In California, BMSB was found in areas closely bordering vineyards, but not in any vineyards to date. Temperatures above and below feeding thresholds (Low = 6°C, High = 26°C) result in cessation of feeding. Older life stages such as adults were found to result in a significant increase in feeding damage on winegrape berries. The feeding activity in relation to berry quality parameters is given for 2015. Increased feeding activity resulted in a significant increase in stylet sheaths per berry. There was a numerical reduction in berry weight and diameter with increasing feeding levels. The number of stylet sheaths in relation to degree days (DD) per day was used to create a feeding factor for BMSB. This feeding factor was significantly correlated with the number of stylet sheaths per berry. Feeding data collected during 2015 and 2016 in Oregon, combined with weather data collected from California will be used to create a BMSB risk index for each of the grape growing regions. These findings are preliminary pending additional analysis and data collection.

INTRODUCTION

Brown marmorated stink bug (*Halyomorpha halys*; BMSB) is becoming increasingly prevalent in Oregon and is rapidly becoming an economic concern for western vineyards (Oregon Department of Agriculture, 2011; Wiman et al., 2014a). This pest can feed on vegetative tissues, grapes, and can potentially cause contamination of the crop, leading 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 BMSB in important viticulture regions of Oregon (Walton et al., unpub.). BMSB was first found on the west coast in 2004 in Portland (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, BMSB is increasingly causing agricultural issues for growers (**Figure 1**). Since 2012, BMSB has increasingly been encountered by growers and can be found in wine grape vineyards of the Willamette Valley during the harvest period (Wiman et al., 2014a). Winemakers have recently reported finding dead BMSB in fermenting wines and infestation of winery buildings by BMSB.

Immature and adult BMSB 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 BMSB may cause direct crop loss due to berry necrosis. Berry feeding may also result in secondary pathogen infection and provide entry points for spoilage bacteria. Vectoring and facilitation of pathogen proliferation by BMSB is not unrealistic because true bugs (Heteroptera) such as BMSB share feeding behaviors with homopterans implicated as disease vectors in vineyards (Cilia et al., 2012; Daugherty, 1967; Mitchell, 2004; Wiman et al., 2014a). BMSB itself is a demonstrated vector of at least one phytoplasma disease (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 BMSB feeding intensity and associated pathogen infection is directly related to temperature (Wiman et al., 2014b), potentially making this pest more damaging in western production regions than on the east coast.

BMSB 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 wine grapes (Nielsen et al., 2008; Nielsen and Hamilton, 2009; Leskey et al., 2012a; 2012b; Pfeiffer et al.; 2012). However, unlike other pentatomids, BMSB 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, wine grapes are among the last crops to be harvested and this may increase the potential for late-season infestation and damage by BMSB.

Contamination of grape clusters by BMSB at harvest is a major concern. Adult BMSB have been observed to lodge themselves between the grapes during harvest. Other researchers are evaluating physical removal of BMSB from clusters, as well as removal by chemical cleanup sprays, blowers, and electronic sorters. However, some BMSB may remain in grape clusters and release defensive compounds during processing, causing taint in finished wine (E. Tomasino, pers. comm.). These taints are persistent, and may result in major market losses. Work conducted on Pinot noir has shown that trans-2-decenal, a defense compound produced by BMSB, is a contaminant present in wine that is processed with BMSB.

As in Oregon, many important wine grape growing regions of California are in close proximity to major urban centers where BMSB populations tend to increase and become sources for further spread. Little is known about BMSB 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 BMSB are occurring (Wiman, unpublished). In Oregon, BMSB has dispersed from Portland to northern Willamette Valley vineyards within a short period. It is important to survey the wine grape growing regions of Napa, Sonoma, and Lodi because these regions are geographically close to San Francisco and Sacramento, both areas with known BMSB infestation.

Feeding intensity of different life stages of BMSB in vineyards has not been fully determined. To date, most studies have focused on adults, even though nymphs are potentially more damaging. When BMSB egg masses are laid in vineyards the nymphs are more confined to feeding 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 BMSB in vineyards and feeding intensity may reflect environmental suitability. An observation from orchard crops is that the worst BMSB damage tends to occur on the borders (Joseph et al., 2014). Similarly, vineyard borders appear to be more susceptible to BMSB infiltration from surrounding vegetation. Grapevines located close to vineyard borders may provide a better environment for the bugs due to microclimate effects of shading by surrounding vegetation.

This study will help determine the potential for BMSB to cause direct damage to wine grape 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 BMSB 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 BMSB 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. Direct crop impact may be more pronounced under more optimal temperature regimes with different varieties, and with longer feeding periods by nymphs to more realistically simulate crop infestation by reproductive BMSB, as is found in vineyards in Oregon and presumably California.

OBJECTIVES

1. Survey key Oregon and California viticulture areas for BMSB presence.
2. Determine BMSB temperature-related field feeding intensity, impact, and regional risk index.
3. Provide Extension for identification, distribution, and importance of BMSB in western vineyards.

RESULTS AND DISCUSSION

Objective 1. Survey key Oregon and California viticulture areas for BMSB presence.

Methods. 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 BMSB pheromone traps were placed in the center of each row selected for beat sheet sampling. BMSB 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 BMSB into commercial vineyards. The vineyard regions sampled were California's north coast wine grape region (Mendocino, Napa, and Sonoma counties), Lodi-Woodbridge wine grape region, and San Joaquin Valley (Fresno County). All vineyard surveys were conducted in concert with other ongoing studies, with outreach to participating farmers on BMSB 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 wine grape 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 BMSB 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 BMSB were found during these field visits in California.

Sampling in Oregon included seven vineyards in the northern Willamette Valley. There were no clear differences between sampling sites, and data from all vineyards were pooled for the respective seasons. Work in Oregon is currently being completed for the 2016 season. This was the fourth year of sampling in these vineyards and data is presented as BMSB per pyramid trap over a two-week period (**Figure 1**; 2016 data not shown).

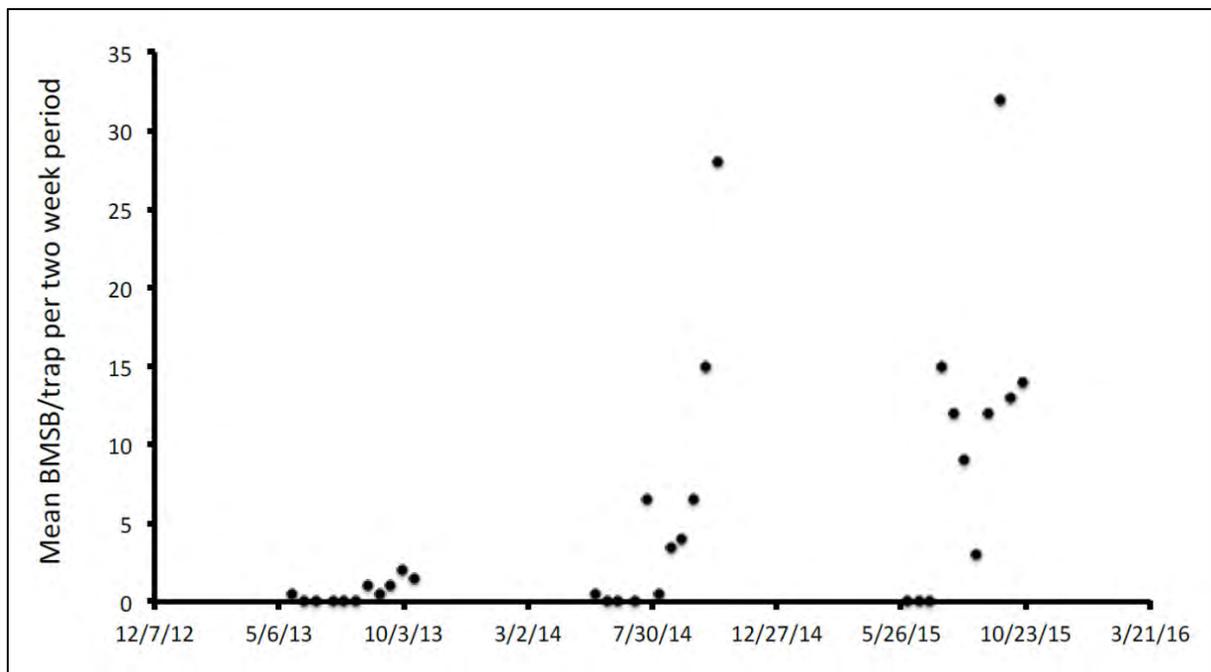


Figure 1. Number of BMSB per trap (seven traps) per two-week period in the northern Willamette Valley, Oregon, during 2013-2015.

Results. In all of the seven locations, BMSB was found in low numbers during the early part of summer in Oregon. The number of BMSB increased to ca. 30 BMSB per trap per two-week period during September through October of 2014 and 2015. The total cumulative number of BMSB trapped per trap during the whole period increased from 34 (2013) to 101 (2015) BMSB per trap collected during the respective seasons. Data collection

for 2016 is not yet complete for the Oregon trial sites. Preliminary information from 2016 data indicates similar population trends to that of 2015.

In California, at the UC Berkeley lab (Daane Laboratory) starting in October 2015 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 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, or squash) at each site every other week. From these samples no BMSB were found, but Say's stink bug (*Chlorochroa sayi*) and bagrada bug (*Bagrada hilaris*) were collected. There was one report of a possible BMSB find from a home garden in Napa County during 2016, but the identification of the specimen needs to be confirmed.

UC Davis (Zalom Laboratory) BMSB 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. BMSB 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 histrionica*, and *Nezara viridula*, but BMSB 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 UCCE Farm Advisor Jhalendra Rijal to discuss plans for collaboratively sampling community gardens and landscape plantings in the vicinity of previous finds in Stockton (San Joaquin County) and Modesto (Stanislaus County) in the coming year. We intend to use findings of BMSB 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 BMSB 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 BMSB temperature-related field feeding intensity, impact, and regional risk index.

Methods - Feeding intensity. In Oregon, we deployed portable electronic feeding monitors (Wiman et al., 2014b) during 2016 in order to determine in-vineyard feeding intensity. Portable feeding monitors consisting of an open circuit enclosed onto a section of the grapevine will be 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 BMSB. The insects were replaced once per week. The relative risk and intensity of BMSB 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.

Results. Data from this work showed clear feeding activity patterns on a daily basis (**Figures 2a** and **2b**) with a decrease in feeding at temperatures below and above 6°C and 26°C, respectively. These data support the estimated lower (3°C - 6°C) and upper (26°C - 29°C) threshold ranges of temperature-related feeding activity of BMSB (Wiman et al 2014).

Feeding impact. Feeding exclusion sleeves (48.0 cm x 39.5 cm, Premier Paint Roller, Richmond Hill, NY, item 60597) were placed over wine grape clusters in a commercial vineyard with known BMSB infestation in the northern Willamette Valley. The trial was maintained for a four and three-week period, respectively, from August 21 to September 21, 2015, and August 22 to September 21, 2016. There were four treatments: 1) no BMSB; 2) a partial egg mass with 10 hatching eggs; 3) three BMSB nymphs; 4) three adult BMSB. All treatments were enclosed in a single sleeve. 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). BMSB insects were exposed to clusters during the period when BMSB are typically found in vineyards in the Willamette Valley. Dead insects were replaced every week with BMSB 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 BMSB was determined by counting the number of stylet sheaths per berry at the end of the exposure period. 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 BMSB damage in different vineyard regions (Ruppel, 1983; Froissart et al., 2010; Wiman et al., 2014a; 2014b).

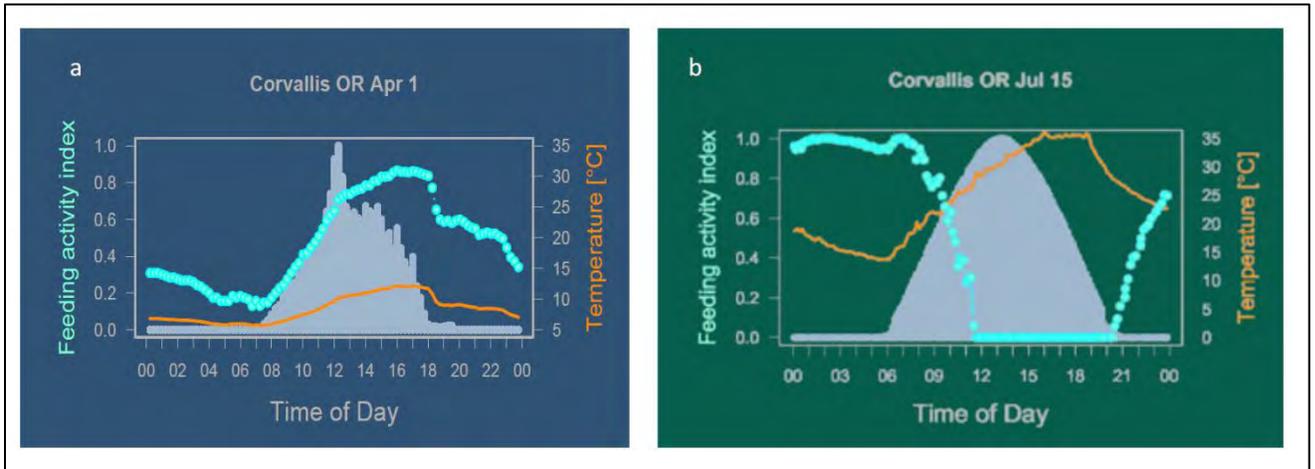


Figure 2. Adult BMSB feeding activity on Pinot noir winegrapes during cool (a) and warm (b) days in Corvallis, Oregon during 2016 using electric feeding monitors (adapted from Wiman et al., 2014b).

Results. During 2015 there were significantly higher temperatures recorded in locations that received higher temperature exposure levels compared to 2016 (**Figures 3a** and **3b**). Mean temperatures ranged from 12.3°C - 23.8°C during the experimental periods. Temperatures ranged from 23.5°C - 28.2°C on days when there was full sun exposure to virtually indistinguishable on cloudy days. These trends were, however, not found during 2016 where the mean sunny (18.0°C) and shady (18.4°C) regimes were statistically similar ($F_{2, 53} = 0.01$, $p = 0.99$).

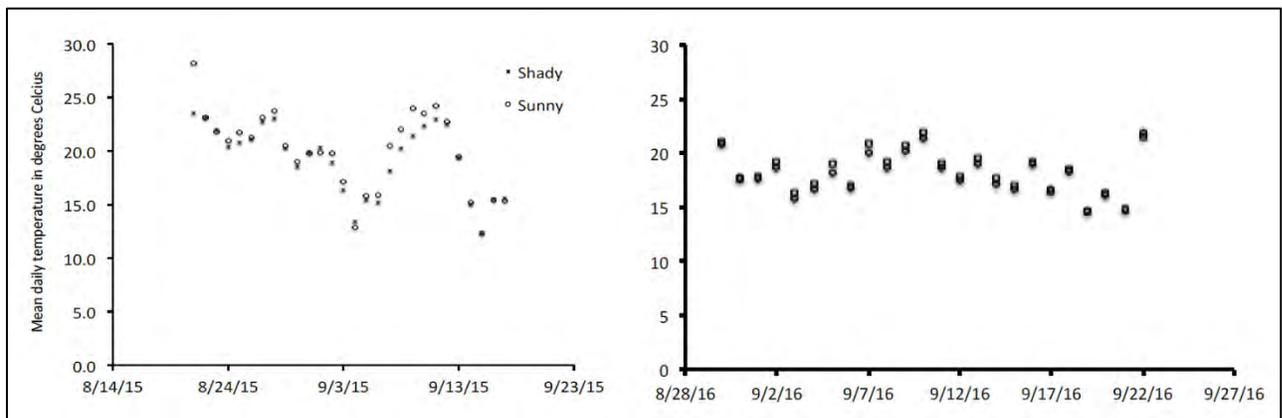


Figure 3a. Mean daily-recorded temperatures from each of shady and sun-exposed locations on Pinot noir preceding the harvest period on vines in Corvallis, Oregon during 2015 and 2016.

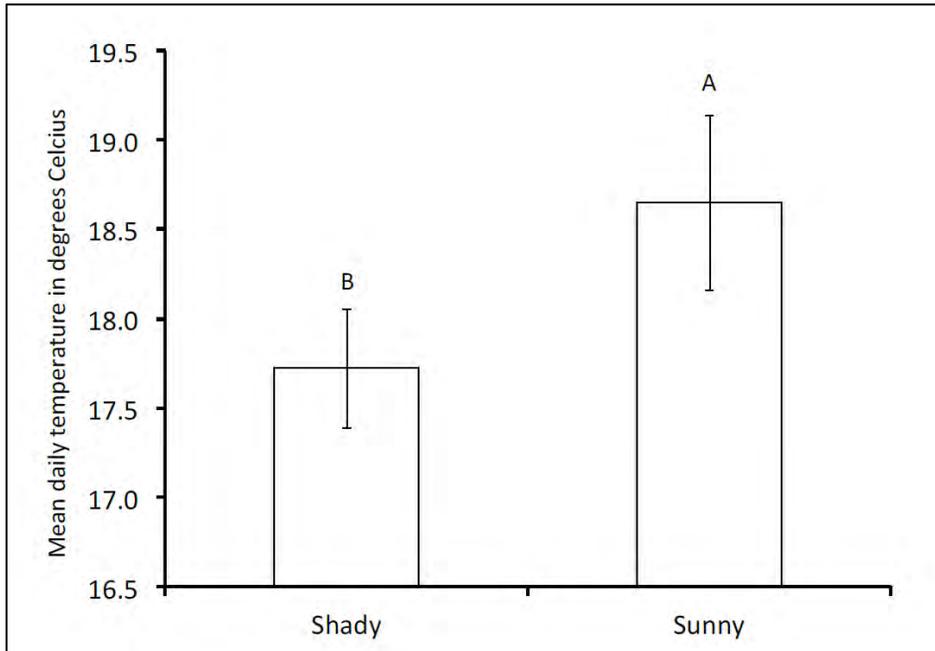


Figure 3b. Mean temperatures recorded in each of two sun-exposed locations on Pinot noir vines in Corvallis, Oregon during 2015 for a one-month period. Different letters indicate significantly different temperatures.

During 2015 there were significantly higher levels of stylet sheaths between sunny and shady locations in vines ($F_{1, 4074} = 45.079$, $p < 0.01$; **Figure 4**), and there were higher levels of stylet sheaths in treatments with adults compared to immature BMSB life stages. Feeding activity of BMSB still needs to be determined for 2016, but the trends found during 2015 appeared consistent with those found during 2016.

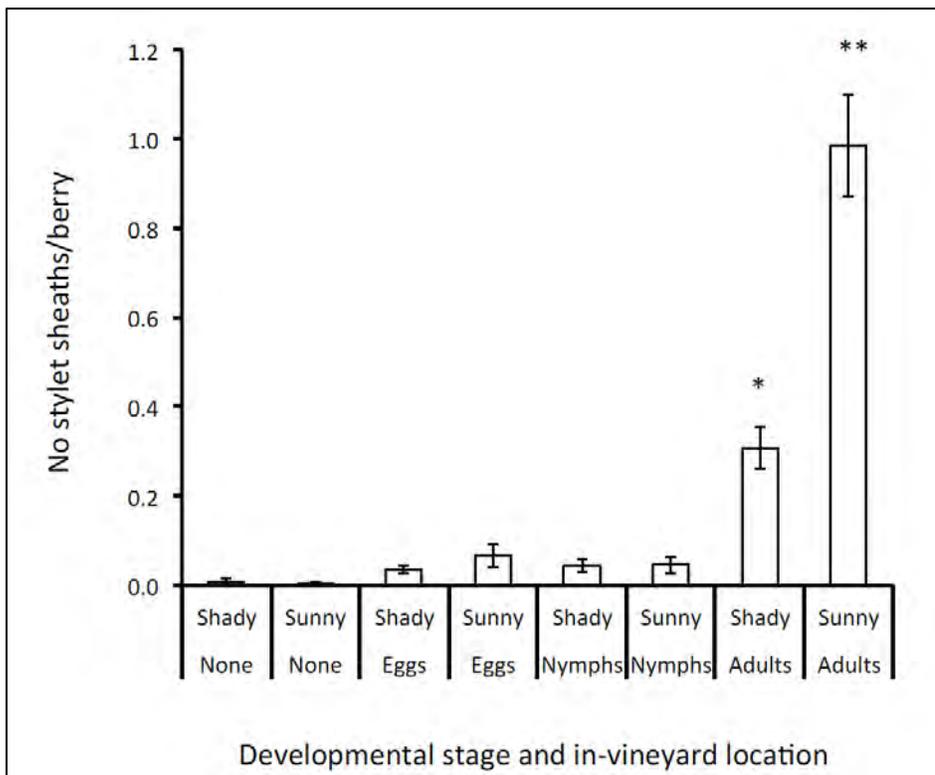


Figure 4. Number of stylet sheaths per berry on Pinot noir in Corvallis, Oregon during 2015. Bars with no, one, and two asterisks (*) are significantly different from other bars.

In order to determine if there were differences in BMSB feeding days (insect-days, Ruppel, 1983) between sunny and shaded locations during 2015 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 BMSB were placed on vines. For 2015, the winegrape quality parameters (**Tables 1 and 2**) showed statistically lower berry and cluster weights, lower berry diameter, less berries per cluster, and more stylet sheaths per berry (**Table 1**) between climate regimes. There was a numerical decrease in berry and cluster weights and lower berry diameter with increasing age of BMSB life stage, as well as warmer temperatures (**Table 2**).

Table 1. Mean berry characteristics of Pinot noir grapes and BMSB feeding activity (\pm SE) for temperature regimes (N = 40) and life stage treatments (N = 20) during 2015 in Corvallis, Oregon.

Group	Berry Weight (Grams)	Cluster Weight (Grams)	Berry Diameter (mm)	Berries/Cluster	Stylet Sheaths/Berry	°Brix
Shady	1.3 \pm 0.03a	90.0 \pm 4.9a	12.1 \pm 0.1a	72.4 \pm 3.9a	6.8 \pm 3.0b	21.6 \pm 0.04a
Sunny	1.1 \pm 0.03b	56.7 \pm 4.9b	11.6 \pm 0.1b	50.2 \pm 3.9b	12.8 \pm 3.0a	22.0 \pm 0.04a
Control	1.2 \pm 0.005a	75.9 \pm 5.8a	-	-	0.009 \pm 0.006b	21.5 \pm 0.3a
Eggs	1.1 \pm 0.002a	69.7 \pm 6.5a	-	-	0.047 \pm 0.012b	22.0 \pm 0.3a
Nymphs	1.2 \pm 0.003a	80.8 \pm 9.2a	-	-	0.060 \pm 0.018b	21.6 \pm 0.2a
Adults	1.2 \pm 0.013a	67.1 \pm 9.4a	-	-	0.781 \pm 0.177a	22.1 \pm 0.5a

Table 2. Mean grape berry characteristics of Pinot noir and BMSB feeding activity (\pm SE) for temperature regimes and life stage treatments (N = 10) during 2015 in Corvallis, Oregon.

Treatment	Cluster Weight (Grams)	Stylet Sheaths/Berry	°Brix
Shady Control	87.1 \pm 5.2a	0.013 \pm 0.013c	21.3 \pm 0.4a
Shady Egg	89.0 \pm 6.3a	0.028 \pm 0.012c	22.2 \pm 0.5a
Shady Nymph	94.0 \pm 14.4a	0.072 \pm 0.031c	21.9 \pm 0.2a
Shady Adult	89.9 \pm 14.6a	0.335 \pm 0.092b	21.1 \pm 0.6a
Sunny Control	64.8 \pm 9.4a	0.006 \pm 0.004c	21.7 \pm 0.5a
Sunny Egg	50.3 \pm 7.4a	0.066 \pm 0.200c	21.9 \pm 0.5a
Sunny Nymph	67.6 \pm 10.5a	0.047 \pm 0.018c	21.3 \pm 0.4a
Sunny Adult	44.3 \pm 6.8a	1.226 \pm 0.282a	23.0 \pm 0.5a

BMSB feeding was correlated based on BMSB life stage and temperature (Ruppel, 1983). For life stages, a factor of 1 was attributed to control treatments, 5.22 for eggs, 6.67 for nymphs, and 86.78 for adults. These factors were obtained by dividing the number of stylet sheaths found for each life stage by the number of stylet sheaths found in the control treatments (0.009) over the two seasons. We assume, based on the electronic feeding monitors, that no stylet sheaths are found in situations where temperatures are below 6°C and above 26°C respectively. These zero values of feeding were used in the fitting of a non-parametric curve in order to describe the lower and upper thresholds of BMSB feeding. The effect of temperature was determined by estimating the number of degree-days (DD) per day for each temperature regime. The DD/day were estimated using the lower and upper thresholds of 14°C and 34°C (Nielsen et al., 2008), respectively. Based on the relative number of DD/day in each regime, the corresponding factor was attributed to each of the regimes. The life stage factor was multiplied by DD/day to create a feeding factor (**Table 3**).

Table 3. BMSB feeding factor based on life stage, and number of Degree-Days/day (DD/day). BMSB feeding activity was acquired using an electronic feeding monitor as well as over two seasons on Pinot noir during 2015 and 2016 in Corvallis, Oregon.

BMSB life stage	Year	Temperature regime	Numerical BMSB life stage factor	DD/day	Feeding factor
None	2015	Shady	1	7.07	7.07
None	2015	Sunny	1	10.03	10.03
None	2016	Shady	1	7.115	7.115
None	2016	Sunny	1	8.59	8.59
Eggs	2015	Shady	5.22	7.07	36.9054
Eggs	2015	Sunny	5.22	10.03	52.3566
Eggs	2016	Shady	5.22	7.115	37.1403
Eggs	2016	Sunny	5.22	8.59	44.8398
Nymphs	2015	Shady	6.67	7.07	47.1569
Nymphs	2015	Sunny	6.67	10.03	66.9001
Nymphs	2016	Shady	6.67	7.115	47.45705
Nymphs	2016	Sunny	6.67	8.59	57.2953
Adults	Feeding monitor	Cold	86.78	0	0
Adults	2015	Shady	86.78	7.07	613.5346
Adults	2015	Sunny	86.78	10.03	870.4034
Adults	2016	Shady	86.78	7.115	617.4397
Adults	2016	Sunny	86.78	8.59	745.4402
Adults	Feeding Monitor	Hot 1	86.78	10.88	944.1664
Adults	Feeding Monitor	Hot 2	86.78	12.33	1069.9974

For the BMSB feeding correlation, the regression of stylet sheaths/berry on the feeding factor resulted in a significant fit using the function $y = (0.0000089) * x * (x - 143.717) * ((1028.8) - x) * \exp(1 / (-0.12867))$ ($R^2 = 0.71$; $F = 6.33$; $df = 1, 4$; $p < 0.003$, **Figure 5**).

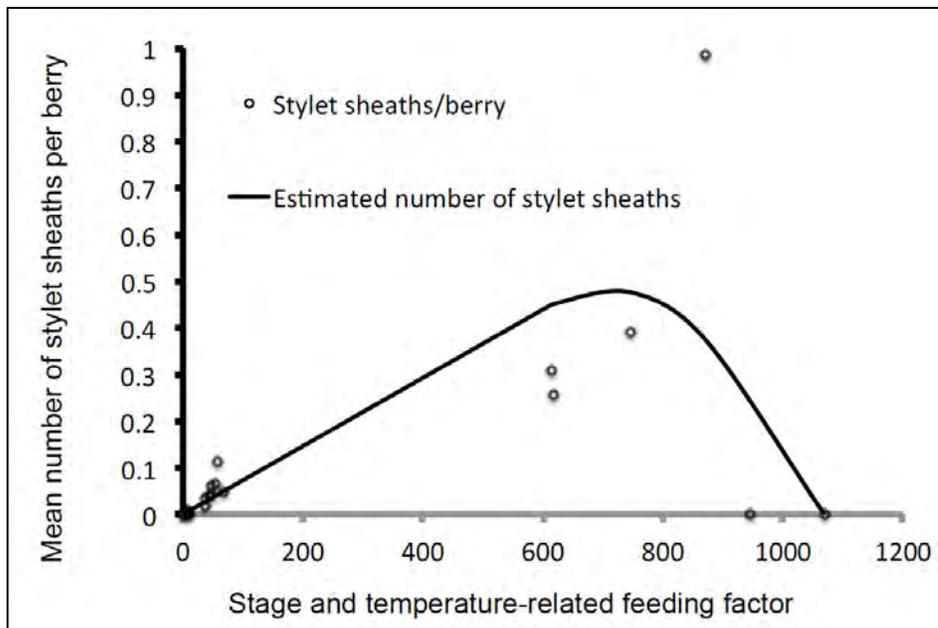


Figure 5. BMSB stylet sheaths per berry over feeding factor. The feeding factor was estimated based on life stage and number of Degree-Days/day (DD/day). BMSB feeding activity was acquired using an electronic feeding monitor during 2016 and also on Pinot noir wine grapes during 2015 and 2016 in Corvallis, Oregon.

Objective 3. Provide Extension for identification, distribution, and importance of BMSB in western vineyards.

Methods. Because BMSB may first be seen in small organic gardens and ornamental trees we also began outreach to 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 counties 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 Agricultural Commissioner, allowing us access to survey and sample small diversified farms. No BMSB have been found at these sites.

Results. In Oregon we presented results of earlier and 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 wine grape region, Lodi-Woodbridge wine grape region, and San Joaquin Valley (Fresno County) vineyards and small vegetable farms, no BMSB were found. While this is only the initial study, BMSB have been found in the Lodi-Woodbridge region in ornamental trees, but have yet to be found near the vineyards sampled. During 2016 there was a report of BMSB found in Napa in a home garden. In Oregon, BMSB were found in increasing numbers from 2013 to 2016 (2016 data not shown) in each of the seven vineyards sampled.

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SEARCHING FOR POTENTIAL VECTORS OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT

Grapevine red blotch-associated virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other grape leafroll diseases (GLDs). There has been some initial laboratory evidence that leafhoppers are potential vectors of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards. Our goal is to identify and 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. To date, we have tested leafhoppers (*Erythroneura elegantula*, *E. variabilis*, *E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), blue-green sharpshooter (*Graphocephala atropunctata*), and foliar-form grape phylloxera (*Daktulosphaira vitifoliae*). So far none of these insects have moved the pathogen from an infected plant or plant material to a clean plant in laboratory studies. More recently there has been evidence that a membracid may transmit GRBaV (Bahder et al., 2016) and transmission experiments evaluating the three-cornered alfalfa hopper (*Spissistilus festinus*) are still in progress. Our field studies have surveyed insects and potential non-crop reservoirs in vineyards with suspected movement of red blotch. None of the herbivores in this survey have tested positive for the virus responsible for red blotch, although many samples are still being tested in the laboratory. We have also conducted detailed mapping of red blotch in vineyards where movement of the virus is suspected in order to evaluate spatial trends related to virus spread. Similarly, we are also mapping GRBaV titers levels within the vine itself to help with the identification of novel vectors which may preferentially feed on regions of the vine where the virus is localized.

LAYPERSON SUMMARY

Grapevine red blotch-associated virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other grape leafroll diseases (GLDs). There has been some initial laboratory evidence that leafhoppers are potential vectors of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards and recent work at UC Davis identified an insect called a treehopper as a likely vector. Our goal is to identify and 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. To date, we have tested many leafhoppers (which are common in vineyards), grape whitefly, mealybugs (which are also commonly found in vineyards), blue-green sharpshooter, and foliar-form grape phylloxera. None of these insects have moved the pathogen from an infected plant or plant material to a clean plant in laboratory studies. We have begun transmission experiments evaluating a treehopper (three-cornered alfalfa hopper) to determine its efficiency. Our field studies have surveyed insects and potential non-crop reservoirs in vineyards with suspected movement of red blotch. None of the herbivores in this survey have tested positive for the virus responsible for red blotch, although many samples are still being tested in the laboratory. We have also conducted detailed mapping of red blotch in vineyards where movement of the virus is suspected in order to evaluate spatial trends related to virus spread. Similarly, we are also mapping GRBaV titer levels within the vine itself to help with the identification of novel vectors which may preferentially feed on regions of the vine where the virus is localized.

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 (UC Davis Viticulture Extension Specialist), Ed Weber (former 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 UC Davis Foundation Plant Services. Test results were most often negative for known 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 grapevine leafroll-associated viruses 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 family 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).

This project 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 transmit GRBaV (Poojari et al., 2013) and better evidence that a membracid may transmit the pathogen (Bahder et al., 2016). However, there has been mixed evidence of GRBaV field spread in association with leafhoppers. Concern for 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.

Our proposed work will screen all common vineyard arthropods as well as the “long shots” that are potential GRBaV 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 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

The overall objective is 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. Objectives for this research program are as follows:

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.

Table 1. Arthropods targeted for GRBaV tests

Common name	Scientific Name	Common Distribution
western grape leafhopper	<i>Erythroneura elegantula</i>	North Coast (north of Tehachapi Mtns.)
variegated leafhopper	<i>Erythroneura variabilis</i>	Central Valley (San Joaquin Co. to So. Cal.)
Virginia creeper leafhopper	<i>Erythroneura ziczac</i>	Northern CA
potato leafhopper	<i>Empoasca</i> sp.	Sporadic vineyard populations
vine mealybug	<i>Planococcus ficus</i>	California vineyards
grape mealybug	<i>Pseudococcus maritimus</i>	North Coast and San Joaquin Valley
obscure mealybug	<i>Pseudococcus viburni</i>	Central and North Coast
blue-green sharpshooter	<i>Graphocephala atropunctata</i>	Northern CA
European fruit lecanium scale	<i>Parthenolecanium corni</i>	North Coast
grape phylloxera	<i>Daktulosphaira vitifoliae</i>	North Coast, Sacramento Delta, Foothills
grape whitefly	<i>Trialeurodes vittatas</i>	California
mites	<i>Tetranychus</i> spp.	California

RESULTS AND DISCUSSION

Objective 1. Screen common vineyard insects and mites as potential vectors of GRBaV.

2013-2014: Initial transmission trials with potted vines.

In 2013 and 2014 we prioritized the screening of leafhoppers (western grape leafhopper and Virginia creeper leafhopper), grape whitefly, mealybugs (vine mealybug and grape mealybug), and blue-green sharpshooter because of the published work by Poojari et al. (2013), their prevalence in California vineyards, and/or their phloem feeding (this category of viruses [Geminiviridae] are 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 are 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 rupestris stem pitting (RSP) (UC Berkeley and Foundation Plant Services 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 Foundation Plant Services 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 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) and Pierce's disease (Almeida and Purcell, 2003 a, 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. A total of 20 test vines were inoculated for each of the above insect species in the 2014 trials.

Results from the 2013/2014 trials have not indicated that any of these insects (i.e., leafhoppers [western grape leafhopper and Virginia creeper leafhopper], grape whitefly, mealybugs [vine mealybug and grape mealybug], and blue-green sharpshooter 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. All insects that fed on infected plant material in these trials have tested negative as well. That said, we have recently begun to redesign our insect testing procedures in order to improve the sensitivity and accuracy of these laboratory tests. Insects from the 2013/2014 trials are being re-tested using new protocols that have been developed and verified.

2015 – Improved “bouquet” transmission trials.

In 2015 and 2016 protocols for these transmission experiments were modified due to concerns about (a) potentially low virus titer levels in the potted vines grown from cuttings of GRBaV-positive vines at vineyard field sites, and (b) the small number 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 that were not sprayed with insecticides. Each bouquet consists of ten mature grape leaves held in a 16-ounce plastic container that contains moist perlite. Ten leaves were collected from each of ten GRBaV-positive vines (nodes one to five) 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). Bouquet degradation was initially evaluated by testing petioles for GRBaV six to 48 hours after collection. Results indicated no degradation of the petioles. Finally, each trial now contains at least 100 insects/replicate (when possible) and 10 replicates per treatment.

Since July 2015 we have completed trials using the bouquets with 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 collected for GRBaV testing as well as a subsample of the candidate vectors (10-50 insects per replicate). After the 72 hour IAP, another subsample of the candidate vectors was 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 maintained 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 having elevated virus titer levels. Negative control source vines were one years 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 subsamples 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. So far, our 2015 and 2016 “bouquet” trials have shown no transmission of GRBaV by either the Virginia creeper leafhopper or the vine mealybug. Similarly, the trial with foliar-form grape phylloxera on two-year-old GRBaV-positive vines did not show any transmission.

Testing plant material for GRBaV.

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 one to five, and 0.1 g of tissue was macerated in 1.8 ml Grape 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 centrifuged for 10 minutes at 13,200 rpm at 20°C. One ml of the supernatant was pipetted into 1.5 ml Eppendorf tubes and stored at -20°C. After briefly vortexing, the DNA extracts were denatured prior to performing qPCR; 8 uL of extract was denatured in 99 uL of GES Denaturing Buffer plus 1 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 (Al Rwahnih et al., 2013). 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 (Al Rwahnih et al., 2013), 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 results. 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, accounting for the Ct values, melting temperatures, and component curves.

Testing insects for GRBaV.

All insects used in these studies were frozen (-80°C) and later tested 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; 25 mg 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, 1 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 were prepared, they were 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) Initial Denaturation at 98°C for 30 seconds, 2) Cycle of denaturing step at 98°C for 10 seconds, annealing step at 62°C for 30 seconds, and extension step at 72°C for 30 seconds, repeated 30 times, and 3) Final Extension at 72°C for 10 minutes. To visualize PCR product, a 2% agarose gel was used in 1x TAE buffer. A Qiagen GelPilot100bp Plus ladder was used. The gel was stained with ethidium bromide, and visualized on a GelDoc XR using the Quantity One program under UV light.

Conclusion – No transmission observed to date.

We have evaluated a total of seven vector candidates: grape leafhopper, Virginia creeper leafhopper, grape whitefly, vine mealybug, grape mealybug, blue-green sharpshooter, and foliar-form grape phylloxera. In 2015 and 2016 we modified experimental protocols that were designed to overcome perceived limitations in previous transmission experiments from 2013-2014. This led to the re-evaluation of two candidates, Virginia creeper leafhopper and vine mealybug, as well as evaluation of a new candidate, foliar-form grape phylloxera.

To date, none of the candidate vectors have tested positive for GRBaV and no transmission has been observed, although testing of insect and plant material from these experiments is ongoing. In summer and fall 2016 we plan to continue testing other candidate vectors listed in Table 1 as well as novel vectors identified from field collections in objective 2 (see below).

Objective 2. Screen uncommon organisms that feed on vines as potential vectors for GRBaV.

Vineyard insect survey.

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, groundcovers, and non-crop vegetation in the surrounding landscape using a combination of sweep-nets (on groundcovers, five samples per site, 30 sweeps per sample) and a D-Vac type suction sampling machine (on grapevines and non-crop vegetation), which consisted of a 25 cc gas blower/vacuum (Craftsman) fitted with a 5-gallon (18.9 liter) bucket on the vacuum tube to create a 1 ft² (0.093 m²) sampling cone. Each D-Vac sample consisted of five thrusts with the D-Vac running at full speed (five samples of grapevine per site, 5-10 samples of non-crop vegetation). All samples were held in a cooler and brought to the laboratory for immediate processing. Specimens were incapacitated using CO₂ gas, sorted and identified to species or genus, and then stored in 95% EtOH and stored at -80° 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., *Scaphytopius* spp., as well as the species *Deltocephalus fuscinevrosus*, *Dikrella californica*, and *Euscelidius schenki*. Other organisms include members of the families Acanaloniidae, Cixidae, Membracidae, Miridae, Lygaeidae, Psyllidae, and Tingidae.

Many novel insects have been collected from vineyard sites where movement of GRBaV is suspected, but to date none have tested positive for GRBaV, although many specimens are still in the process of being tested, and as mentioned above, we are still in the process of refining our laboratory techniques to improve sensitivity of detection for insect material.

Non-crop plant survey.

As a complement to the insect collection and testing, plant material was also collected from non-crop vegetation and tested for GRBaV in order to identify plant species that serve as reservoirs of GRBaV outside of the vineyard. Plant material was sampled from maple (*Acer* sp.), California buckeye (*Aesculus californica*), alder (*Alnus rhombifolia*), madrone (*Arbutus menziesii*), manzanita (*Arctostaphylos* sp.), coyotebrush (*Baccharis pilularis*), Oregon ash (*Fraxinus latifolia*), English ivy (*Hedera helix*), toyon (*Heteromeles arbutifolia*), California walnut (*Juglans californica*), wild cucumber (*Marah macrocarpa*), olive (*Olea europaea*), plum (*Prunus* sp.), coast oak (*Quercus agrifolia*), blue oak (*Q. douglasii*), valley oak (*Q. lobata*), wild rose (*Rosa californica*), blackberry (*Rubus* spp.), willow (*Salix* sp.), elderberry (*Sambucus* sp.), California bay (*Umbellularia californica*), periwinkle (*Vinca major*), wild grape (*Vitis californica*) and various vineyard groundcovers and weedy vegetation (*Artemisia douglasiana*, *Avena fatua*, *A. sativa*, *Brassica* spp., *Calendula officinalis*, *Conium maculatum*, *Convolvulus arvensis*, *Foeniculum vulgare*, *Malva parviflora*, *Raphanus sativa*, *Taraxacum officinale*, *Vicia fava*, and *Vigna* sp.). To date, most of this plant material has tested negative for GRBaV, with the exception of wild grape which has tested positive fairly consistently across multiple sites. It should be noted that “wild grape” at these sites may actually be a hybrid form *Vitis californica* x *V. vinifera* due to its proximity to commercial vineyards.

Vineyard insect and plant survey – Preliminary findings.

The insect and non-crop plant survey concluded in May 2016, marking one full year of monthly insect and plant sampling in five vineyards with suspected spread of GRBaV. As mentioned, testing of plant and insect material is ongoing, but here we present some preliminary summaries of the data based on findings to date. In our surveys, the only non-crop plant species to test positive for GRBaV has been wild grape (*V. californica* x *V. vinifera*), indicating a potential role of this plant in the spread of GRBaV into commercial vineyards. Here we present a summary of the insect community found on wild grapes in our survey (**Table 2**). Diptera (flies) and western grape leafhopper make up >50% of the insects found on wild grape and >90% of organisms are represented when we include the parasitic Aprocita (parasitoid wasps), spiders, Formicidae (ants), *Empoasca* spp., Coleoptera (beetles), *Chrysoperla* sp. (green lacewings), variegated leafhopper, *Osbornellus* sp., Psocoptera (book lice), Trichoptera (caddisflies), aphids and Miridae. From this group, only western grape leafhopper, *Empoasca* spp., variegated leafhopper, *Osbornellus* sp., aphids, and the Miridae are likely to feed directly on wild grape tissue and only western grape leafhopper and variegated leafhopper are known to successfully reproduce on it.

Evaluating insect community overlap between wild and wine grape could help identify novel insect vectors of GRBaV. Organisms that were found on both wild and wine grape include aphids, Berytidae, *Chrysoperla* sp., Coleoptera, *Deltocephalus fuscinevrosus*, Diptera, *Empoasca* spp., western grape leafhopper, variegated leafhopper, Formicidae, Galerucinae, parasitic Aprocrita, Lepidoptera, Lygaeidae, three-cornered alfalfa hopper (*Spissistilus festinus*), Miridae, *Orius* sp., Psocoptera, Psyllidae, *Scaphytopius* spp., spiders, Thysanoptera, Trichoptera, and a small number of unknown Cicadellids. Of these organisms that co-occur on both wild and wine grape, *Deltocephalus fuscinevrosus*, *Empoasca* spp., western grape leafhopper, variegated leafhopper, Lygaeidae, Miridae, Psyllidae, *Scaphytopius* spp., three-cornered alfalfa hopper, Thysanoptera, and the unknown Cicadellids will likely feed directly on grape plant tissue and only western grape leafhopper and variegated leafhopper are known to reproduce on these species. The most commonly encountered organism on cultivated wine grape was western grape leafhopper (35%), followed by variegated leafhopper (11%), Thysanoptera (5%), aphids (2%), and Lygaeidae (1%). All other organisms represented <1% of the community found on wine grapes. From this group of likely feeders that occur on both wild and wine grape, we have conducted GRBaV transmission experiments with western grape leafhopper and variegated leafhopper, which represent some of the commonly encountered organisms on both wild and wine grape. Results from these trials have not indicated any ability of these insects to transmit the virus.

Table 2. Arthropod community on wild grapes and cultivated wine grapes. Data shows mean annual abundance per sample \pm SEM and percentage of total arthropods found on the plant.

Order	Family	Genus/Species	Wild Grape		Wine Grape		
			Abundance	%	Abundance	%	
Araneae			0.39 \pm 0.12	6%	0.02 \pm 0.02	2%	
Coleoptera	Galerucinae		0.02 \pm 0.02	<1%	0.01 \pm 0.01	<1%	
	Cantharidae		-	-	<0.01	<1%	
	Other		0.18 \pm 0.09	3%	0.08 \pm 0.02	2%	
Dermaptera			0.04 \pm 0.03	1%	-	-	
Diptera	Syrphidae		-	-	<0.01	<1%	
	Other		2.80 \pm 0.68	41%	1.24 \pm 0.14	28%	
Hemiptera	Acanaloniidae		0.02 \pm 0.02	<1%	-	-	
	Alydidae		-	-	<0.01	<1%	
	Anthocoridae	<i>Orius</i> sp.	0.04 \pm 0.04	1%	0.03 \pm 0.01	<1%	
	Aphididae		0.08 \pm 0.05	1%	0.09 \pm 0.02	2%	
	Berytidae		0.04 \pm 0.03	1%	<0.01	<1%	
	Cicadellidae	<i>Acinopterus angulatus</i>		-	-	0.01 \pm 0.01	<1%
		<i>Deltocephalus fuscineruosus</i>		0.02 \pm 0.02	<1%	0.02 \pm 0.01	<1%
		<i>Dikraneura rufula</i>		-	-	<0.01	<1%
		<i>Dikrella</i> sp.		0.02 \pm 0.02	<1%	-	-
		<i>Empoasca</i> spp.		0.22 \pm 0.13	3%	<0.01	<1%
		<i>Erythroneura elegantula</i>		0.80 \pm 0.43	12%	1.51 \pm 0.44	35%
		<i>Erythroneura variabilis</i>		0.14 \pm 0.07	2%	0.47 \pm 0.19	11%
		<i>Graphocephala atropunctata</i>		-	-	<0.01	<1%
		<i>Macrosteles quadrilineatus</i>		-	-	<0.01	<1%
		<i>Osbornellus</i> sp.		0.12 \pm 0.10	2%	-	-
		<i>Scaphytopius</i> spp.		0.02 \pm 0.02	<1%	0.02 \pm 0.01	<1%
		<i>Sophonia</i> sp.		-	-	<0.01	<1%
		Unknown		0.04 \pm 0.03	1%	0.01 \pm 0.01	<1%
	Geocoridae	<i>Geocoris</i> sp.		-	-	<0.01	<1%
	Lygaeidae		0.06 \pm 0.05	1%	0.06 \pm 0.04	1%	
	Membracidae	<i>Spissistilus festinus</i>		0.02 \pm 0.02	<1%	0.02 \pm 0.01	<1%
	Miridae		0.08 \pm 0.05	1%	<0.01	<1%	
	Psyllidae		0.02 \pm 0.02	<1%	0.02 \pm 0.01	<1%	
Rhopalidae		0.02 \pm 0.02	<1%	-	-		
Tingidae		-	-	0.01 \pm 0.01	<1%		
Hymenoptera	Apoidea (non- <i>Apis</i>)		-	-	0.02 \pm 0.01	<1%	
	Aprocrita (parasitic)		0.57 \pm 0.17	9%	0.17 \pm 0.03	4%	
	Formicidae		0.37 \pm 0.12	6%	0.01 \pm 0.01	<1%	
	Vespidae		0.02 \pm 0.02	<1%	-	-	
Ixodida	Ixodidae		0.04 \pm 0.04	1%	-	-	
Lepidoptera			0.04 \pm 0.04	1%	<0.01	<1%	
Neuroptera	Chrysopidae	<i>Chrysoperla</i> sp.	0.14 \pm 0.12	2%	0.01 \pm 0.01	<1%	
Orthoptera			0.02 \pm 0.02	<1%	-	-	
Psocoptera			0.08 \pm 0.05	1%	0.07 \pm 0.02	2%	
Thysanoptera			0.04 \pm 0.03	1%	0.22 \pm 0.08	5%	
Trichoptera			0.08 \pm 0.05	1%	<0.01	<1%	

While it is notable that three-cornered alfalfa hopper, a known vector of GRBaV (Bahder et al., 2016), was found on both wild and wine grapes, on both plant species they represented <1% of total organisms. Regardless of the overall low populations encountered in vineyards, data on host plant associations of three-cornered alfalfa hopper (**Figure 1**) provides new information on population dynamics in vineyards. This species was primarily found in the late spring on groundcovers in and around the vineyard, which included various weedy grasses as well as overwintering grass/legume cover crops. As groundcovers died down, three-cornered alfalfa hopper was intermittently found in low abundance on wild grape, wine grape, toyon (*Heteromeles arbutifolia*) and coast oak (*Quercus agrifolia*) throughout the growing season. These are not necessarily reproductive hosts for this species

and further work is needed to better understand the life cycle of three-cornered alfalfa hopper on the non-crop habitats in and around vineyards.

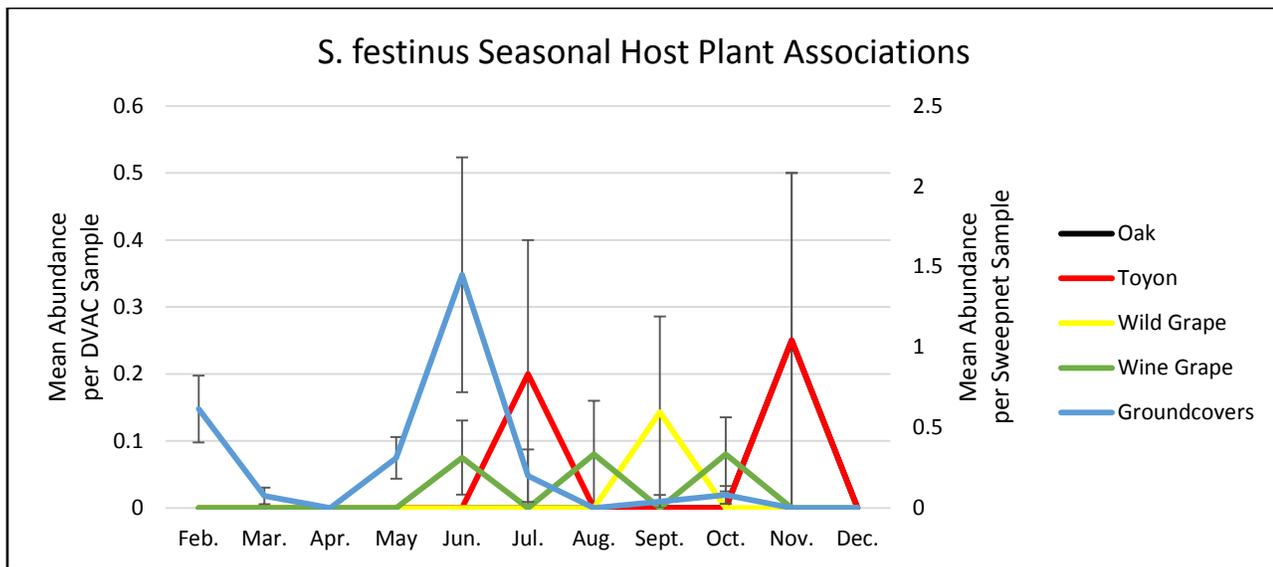


Figure 1. Seasonal host plant associations of *S. festinus* in North Coast vineyards. High densities of *S. festinus* were found on groundcovers in the late spring and then intermittently on wild grape, wine grape, coast oak and toyon. Plant species shown are not necessarily reproductive hosts. Right Y-axis denotes abundance on groundcovers, left Y-axis denotes abundance on all other plants.

Establishing colonies of novel vectors.

Due to the low abundance of novel candidate vectors (e.g. *Empoasca* spp., three-cornered alfalfa hopper, *D. fuscinervosus*), we have been working to establish colonies of these insects at the UC Berkeley greenhouse facilities in order to rear a large enough population suitable for GRBaV transmission experiments, which typically require >200 individuals per trial. Data is scant for many of these species and information on reproductive hosts is limited. As such, this spring we collected candidate species from vineyards and introduced them into cages containing various potential host plants. So far we have seen successful reproduction of *Aceratagallia* sp. and *Euscelidius schenki* on select host plants. We also collected large populations of three-cornered alfalfa hopper from alfalfa fields and are now seeing reproduction in our colonies.

Transmission experiment with three-cornered alfalfa hopper.

A GRBaV transmission experiment was conducted with field collected three-cornered alfalfa hoppers in July 2016. Individuals were collected from an organic alfalfa field and introduced into cages with GRBaV positive or negative vines. Each cage contained a single potted vine (11 cages each with a single GRBaV-positive vine and nine cages each with a single GRBaV-negative vine) and received 20 three-cornered alfalfa hopper adults. Adults were allowed to feed for 48 hours (AAP), after which the GRBaV-positive/negative vine was removed and a GRBaV-negative vine was introduced into each cage. The adults were allowed to feed on the negative vine for 48 hours (IAP) and were then removed from the vine. As with previous transmission experiments, the vines are now being held for a two-year period and will be tested for GRBaV every four months. While it has been demonstrated that three-cornered alfalfa hopper can vector GRBaV (Bahder et al., 2016), our goal is to first confirm these findings and then begin evaluating transmission efficiency of this species under laboratory and field conditions.

Objective 3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

Large block mapping (one site, 2009-present).

We have been studying grapevine leafroll disease (GLD) movement at one particular site in Napa Valley, beginning in 2009. The block is a 20 hectare newly planted (in 2008) block of Cabernet Sauvignon. Each year in September, incidence of GLD and more general “red leaf” symptoms were mapped at this site and location recorded with GPS. As early as 2009 many of the vines displayed “red leaf” symptoms but tested negative for

grapevine leafroll-associated virus (GLRaV). In our subsequent surveys these symptoms appeared to spread through the vineyard, although a majority of these “red leaf” symptom vines continued to test negative for GLRaV over this period. We began testing vines for both GLRaV and grapevine red blotch-associated virus (GRBaV) in 2014 and found that 136 vines tested positive for red blotch, nine tested positive for leafroll, and 11 tested positive for both red blotch and leafroll. Plant material from the 2015 survey is still in the process of being tested, but we recorded about 250 “red leaf” symptomatic vines, all of which had tested negative for GLRaV in 2014. With the development of new and more complete primers for both leafroll and red blotch, we are now in the process of re-testing plant material from the 2009-2013 survey to verify whether or not GRBaV is present in the “red leaf” symptom vines that previously tested negative for GLRaV.

Small block mapping (five sites, 2015-present).

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 collected from each vine for diagnostic testing. The idea is to return to these same blocks in September 2016 and 2017 to repeat this detailed mapping in order to evaluate if the virus appears to be spreading from vine to vine. In October 2015 we learned that one of these established vineyard sites was going to be removed due to intolerable levels of GRBaV incidence. In November 2015 we located an alternate site to replace the lost site and conducted the same detailed mapping protocol. Results from this new mapping effort will not be available until follow-up mapping in fall 2016.

Red blotch titers survey.

Concerns about the possibility of low GRBaV titer levels in potted vines used in the transmission trials (see objective 1) led us to initiate a broader survey to quantify GRBaV titer levels throughout grapevines over the course of the year. Starting in April 2015 plant material is collected each month from various parts (roots, trunk, canes, etc.) of at least 10 GRBaV positive vines at each of three vineyard sites in Napa Valley. The goal is to understand whether or not the virus localizes in certain regions of the grapevine during the year. If this is the case it could improve the focus of our search for novel vectors (i.e., vectors that preferentially feed on parts of the vine with high GRBaV titer levels).

CONCLUSIONS

Findings from this research help improve our understanding of GRBaV transmission and field epidemiology in order to develop better recommendations and control programs for commercial growers. Greenhouse trials to evaluate GRBaV transmission by both suspected and novel insects aim to clarify which, if any, insects can transmit this virus and, if so, how efficiently they do so. Similarly, screening insects from field sites with suspected spread of GRBaV allows us to identify additional novel vectors for subsequent evaluation in greenhouse trials. Testing plant material from non-crop species in the natural habitats surrounding vineyards provides new information on potential reservoirs of GRBaV outside of the vineyard. Closer evaluation of the insects associated with non-crop reservoirs of GRBaV will further reinforce efforts to identify novel vectors. Detailed mapping of GRBaV at multiple sites where spread of this virus has been suspected will allow us to confirm if this is actually the case as well as evaluate spatial trends of infected vines relative to pertinent landscape features, such as riparian habitats or adjacent vineyard blocks with high levels of GRBaV infection. Finally, quantifying GRBaV titer levels throughout the vine will aid in the search for novel vectors that may feed on specific areas of the vine where the virus is concentrated.

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BIOLOGY AND SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT

Grapevine red blotch-associated virus (GRBaV) is present in diseased grapevines affected by red blotch disease, a newly recognized threat to the grape and wine industry (Cieniewicz et al., 2016b; Sudarshana et al., 2015). By producing and using a full-length infectious clone of a representative isolate of each of the two phylogenetic clades previously identified (Krenz et al., 2014; Al Rwahnih et al., 2015), we showed systemic GRBaV infection in healthy grapevines following agroinoculation and the manifestation of typical disease symptoms, i.e., interveinal reddening on red-berried cultivars Cabernet Sauvignon, Cabernet franc, Syrah, and Pinot noir, and chlorotic and necrotic leaf areas on the white-berried cultivar Chardonnay. Infection was latent in rootstocks 110R and 3309C, except in SO4, which exhibited foliar chlorosis and cupping. This work demonstrated that GRBaV is the etiological agent of red blotch disease. Analysis of the spatiotemporal incidence of GRBaV in a selected vineyard of Cabernet franc in California over two consecutive years was consistent with the occurrence of virus spread. Clustering of diseased vines was confirmed by ordinary runs analysis. In contrast, no evidence of virus spread was obtained by monitoring a diseased Merlot vineyard in New York. GRBaV isolates spreading in California corresponded to phylogenetic clade II. A survey of riparian areas in proximity to the diseased Cabernet franc vineyard showed that free-living grapevines are infected with GRBaV (Perry et al., 2016). The GRBaV isolates from free-living grapevines, including hybrids of *Vitis californica* x *Vitis vinifera* cv. Sauvignon blanc, belonged to phylogenetic clade II, as did most of the GRBaV-infected vines in adjacent Cabernet franc and Merlot vineyards (Perry et al., 2016). The presence of GRBaV in free-living grapevines close to diseased commercial vineyards suggested the existence of a hemipteran vector. Insect sticky traps placed in the section of the Cabernet franc vineyard with extensive clustering of diseased vines from April to November showed a diversity of insect species that visited the vineyard, among which, the majority of specimens of four species consistently tested positive for GRBaV in PCR. These four species are vector candidates and their potential at transmitting GRBaV in controlled conditions in the greenhouse is investigated. Among the four vector candidates, the three-cornered alfalfa hopper (*Spissistilus festinus*) was shown to transmit GRBaV from infected to healthy vines in the greenhouse (Cieniewicz et al., 2016a). This finding revealed the potential of this treehopper as a vector of epidemiological significance in vineyards.

LAYPERSON SUMMARY

Red blotch is a newly recognized viral disease of grapevines that is widely distributed in U.S. vineyards. We showed that grapevine red blotch-associated virus (GRBaV) causes red blotch disease, regardless of its genetic makeup and variability. Limited information is available on the spread of this virus. Similarly, limited information is available on the association between virus variability and pathogenicity. Studying changes in virus prevalence over time in selected vineyards in California and New York revealed increased virus incidence in the California vineyard 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 virus reservoirs. Among insects visiting the California vineyard four species were found to carry the virus, suggesting a potential role as vectors. Subsequent work in the greenhouse showed that one of these vector candidates, the three-cornered alfalfa hopper (*Spissistilus festinus*), transmits GRBaV from infected to healthy vines, revealing that this treehopper is a vector of epidemiological importance in vineyards.

INTRODUCTION

Red blotch is a recently recognized disease of grapevines (Calvi 2011; Cieniewicz et al., 2016b; 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 wine grapes show red specks and blotches 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 wine grapes 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 (Sudarshana et al., 2015). 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).

GRBaV is isolated from grapevines affected by red blotch disease (Cieniewicz et al., 2016b; Sudarshana et al., 2015). This virus is a putative member of a new genus tentatively named *Grablovirus* (Zerbini, personal communication) in the family *Geminiviridae* (Cieniewicz et al., 2016b; Sudarshana et al., 2015; Varsani et al., 2014). GRBaV has a single-stranded DNA genome that codes for seven open reading frames (Al Rwahnih et al., 2013; Krenz et al., 2012; Perry et al., unpublished; Poojary et al., 2013; Seguin et al., 2014).

GRBaV was documented in major grape-growing U.S. States (Krenz et al., 2014). The virus was also reported in British Columbia and Ontario (Poojari et al., 2016) in Canada, and in a *Vitis* germplasm collection (Al Rwahnih et al., 2015a), indicating its widespread presence in North America. GRBaV was found in table grapes, wine grapes, French-American interspecific hybrids, and rootstocks (Al Rwahnih et al., 2015a; Sudarshana et al., 2015). 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 virus was also found in an archival sample collected in Sonoma County in the 1940s (Al Rwahnih et al., 2015b). 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 II and recombination is underlying some of the variation seen among GRBaV genomes within clade I.

Most vineyard managers and vintners report ripening issues with GRBaV-infected wine grapes. 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 wine grapes such as Cabernet franc and Cabernet Sauvignon (Calvi 2011; Cieniewicz et al., 2016b; 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.

Free-living grapevines proximal to vineyards were found infected with GRBaV (Bahder et al., 2016a; Perry et al., 2016). The GRBaV isolates in free-living grapevines were genetically related to clade II isolates in proximal Cabernet franc and Merlot vineyards (Perry et al., 2016). The presence of the virus in an alternate host that is at least 150 feet away from the natural host suggested the existence of a hemipteran vector. The Virginia creeper or ziczac leafhopper (*Erythroneura ziczac*) was claimed to transmit GRBaV from vine to vine in the greenhouse (Poojari et al., 2013); so was the three-cornered alfalfa hopper (*Spissistilus festinus*) (Bahder et al., 2016), but a vector of GRBaV of epidemiological significance in vineyards remains to be identified.

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 the 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 1**). 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 (Cieniewicz et al., 2016a). 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 II that was previously reported (Krenz et al., 2014).

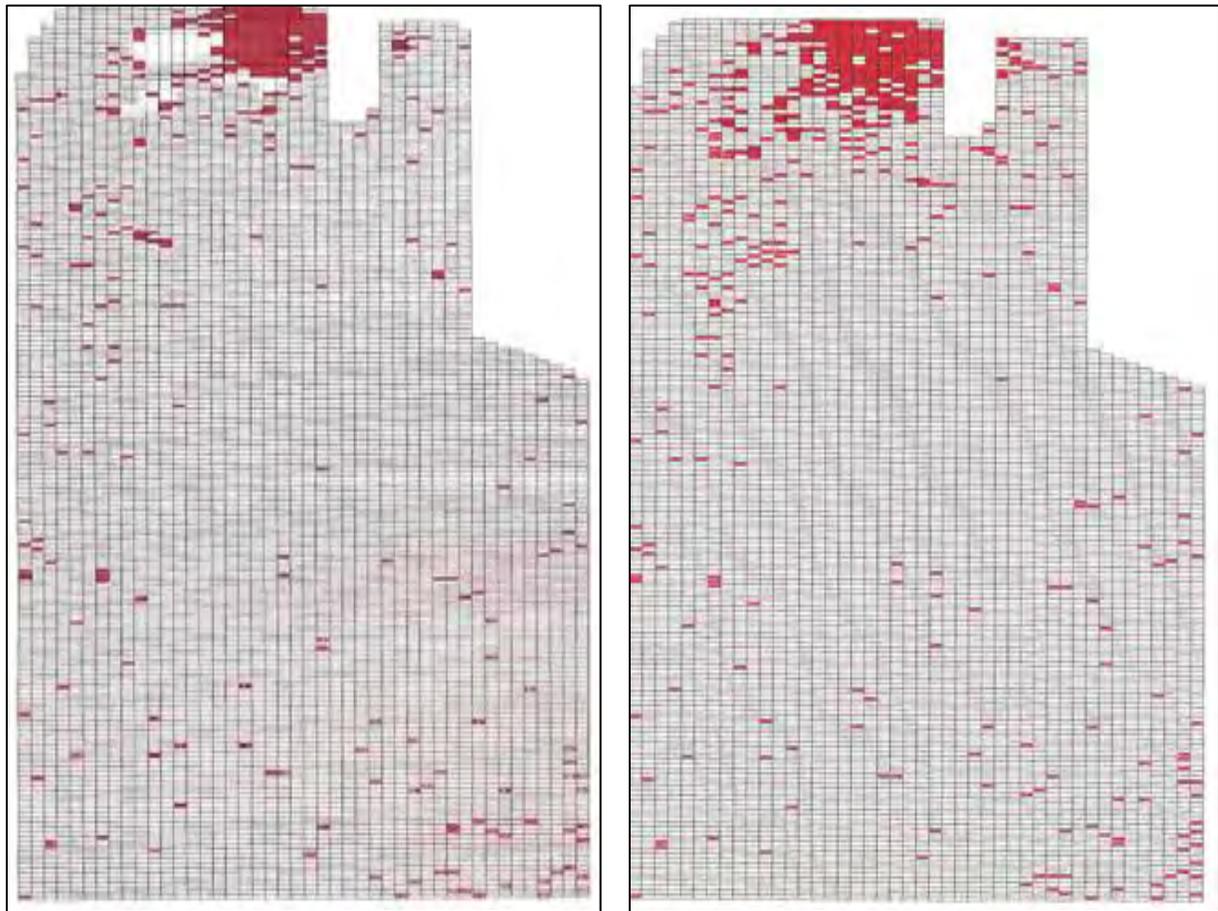


Figure 1. Spatial distribution of vines showing red blotch symptoms (in red) in a Cabernet franc vineyard in California in 2014 (left) and 2015 (right).

Spread of GRBaV was further studied in the vineyard area with extensive clustering of symptomatic vines (top middle area of the maps in **Figure 1**). This area consists of 10 consecutive rows of 25 vines each (**Figure 2**).

Symptomatic and asymptomatic vines were mapped in this area in 2013, 2014, and 2015. In addition, the presence or absence of GRBaV was confirmed in individual vines by PCR in spring and winter by using leaf and cane material, respectively (**Figure 2**). Data showed an increase of symptomatic vines from 47% (118 of 250 vines) in 2014 to 67% (168 of 250) in 2015. The presence of GRBaV was confirmed in all symptomatic vines. Similarly, the absence of GRBaV was confirmed in most of the asymptomatic vines with a few exceptions (7 of 250 vines). Based on our monitoring of vines in 2014 and 2015, it is anticipated that the seven asymptomatic vines that tested positive for GRBaV will become symptomatic in 2016. Altogether, these results further support the occurrence of short distance spread of GRBaV in the California vineyard (Cieniewicz et al., 2016a).

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 eventually 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.

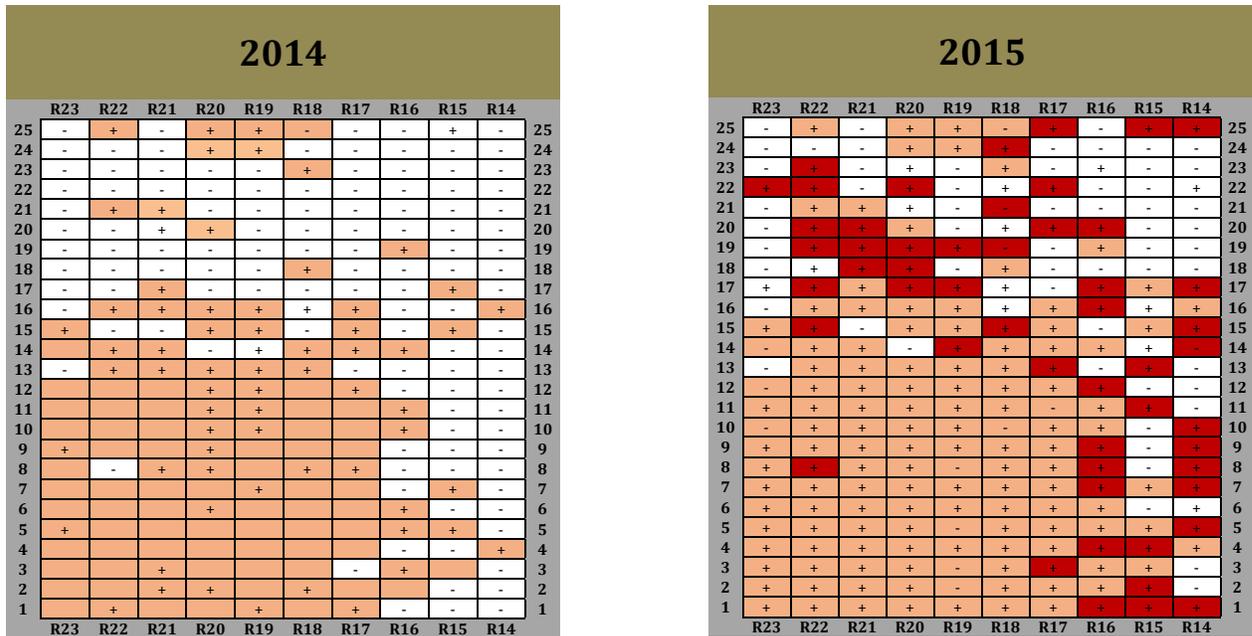


Figure 2. Distribution of GRBaV in a select area of a Cabernet franc vineyard in California. Each cell indicates a single vine. (+) indicates that a vine tested positive for GRBaV by PCR, (-) indicates a PCR-negative result. Salmon colored cells were symptomatic in 2014 and 2015, red cells were newly symptomatic in 2015, and white cells are asymptomatic.

Close to 100 sentinel vine (i.e., healthy vines for which the mother stocks from which scion budwood and rootstock canes were collected tested negative for GRBaV) were planted in the Cabernet franc vineyard in California in spring 2015. 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 infection status. The presence of GRBaV will be tested in sentinel vines in fall 2016. 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 1**) provided an incentive to investigate the existence of potential vectors.

Insect sticky traps were placed in the area of the selected vineyard in California where extensive clustering of diseased vines is occurring. 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 (Cieniewicz et al., 2016a). 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 subset 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 four species, among more than 50 species of Diptera, Apocrita, Coleoptera, Cicadellidae, Thysanoptera, Aphidae, Fulgoroidea, Phylloxera, Aleyrodidae, Membracidae, Blissidae/Lygaeidae, Psylloidea, Psocoptera, and Miridae that were caught on sticky traps, consistently carried genetic elements of GRBaV (**Table 1**).

These four species are members of the Membracidae (three-cornered alfalfa hopper), Cicadellidae (*Colladonus reductus* and *Osbornellus* sp.), and Cixiidae (unidentified species) (**Table 1**). These findings suggest that these four species can acquire GRBaV in the vineyard (Cieniewicz et al., 2016a). Populations of the four insect vector candidates were very low compared to populations of some typical grape pests, such as phylloxera, western grape

leafhopper, variegated leafhopper, and thrips. The vector candidate populations peaked in July (three-cornered alfalfa hopper and Cixiidae species) and September (*Colladonus reductus* and *Osbornellus* sp.) (**Figure 3**). The four vector candidates are phloem-feeders, as would be expected for a GRBaV transmitter. Of the four species that are able to acquire GRBaV in the vineyard, none is considered a pest of grapevines. Testing the capacity of these hemipteran insects at transmitting the virus to healthy grapevines in the greenhouse is critical to ascertaining their role as vectors.

Table 1. Detection of GRBaV by PCR in insects from 2015 sticky card survey in Napa Valley, California.

Species/Family	Common Name	Number Tested	GRBaV detected	Percent Positive
<i>Spissistilus festinus</i>	Three cornered alfalfa treehopper	25	12	48%
Cixiidae	Cixiid planthoppers	8	4	50%
<i>Colladonus reductus</i>	<i>Colladonus reductus</i>	23	14	61%
<i>Osbornellus</i> sp.	<i>Osbornellus</i> sp.	31	13	42%
Thysanoptera	Thrips	12	0	0%
Aleyrodidae	Whiteflies	52	0	0%
Psylloidea	Psyllids	25	0	0%
<i>Deltocephalus</i> sp.	<i>Deltocephalus</i> sp.	15	0	0%
<i>Erythroneura elegantula</i>	Western grape leafhopper	41	0	0%
<i>Erythroneura variabilis</i>	Variegated leafhopper	22	0	0%
<i>Euscelis</i> sp.	Brown leafhopper	33	0	0%
<i>Daktulosphaira vitifoliae</i>	Grape phylloxera (winged adults)	22	0	0%
<i>Sophonia orientalis</i>	Two-spotted leafhopper	5	0	0%
<i>Draeculacephala minerva</i>	Grass sharpshooter	4	0	0%
Aphididae	Aphids	46	1	2%
<i>Scaphytopius magdalensis</i>	Sharp-nosed leafhopper	45	3	7%
<i>Empoasca</i> sp.	Potato leafhopper	28	1	4%
<i>Graphocephala atropunctata</i>	Blue-green sharpshooter	23	1	4%

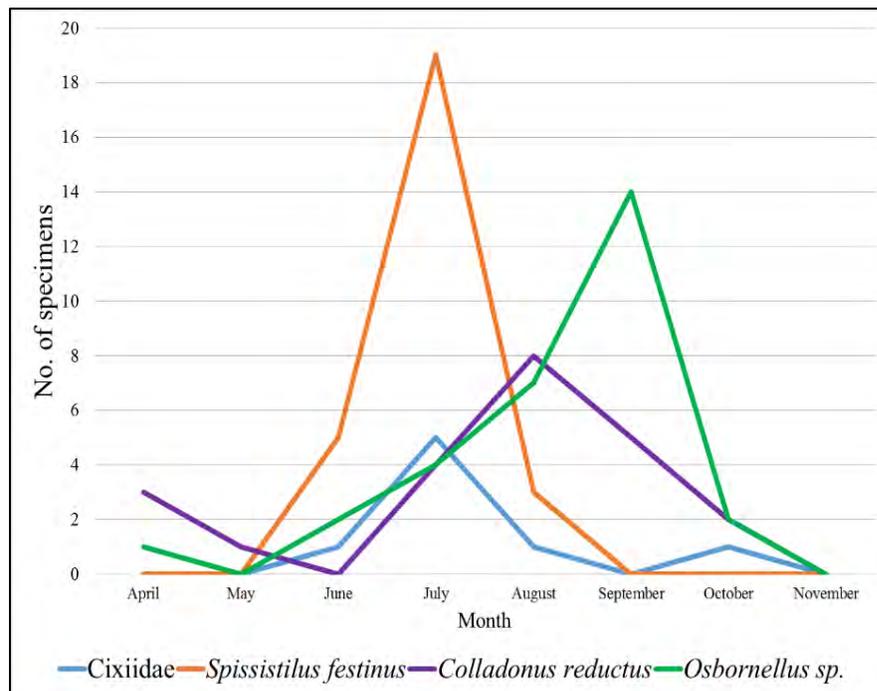


Figure 3. Specimen counts of GRBaV insect vector candidates from sticky cards during the 2015 growing season in a California vineyard.

The vectoring capacity of the four vector candidates in the greenhouse was initiated with the three-cornered alfalfa hopper (Cieniewicz et al., 2016). First, specimens of three-cornered alfalfa hopper from alfalfa fields in Yolo County and Fresno County in California were collected and established on alfalfa seedlings at Cornell. Then, groups of five to ten individuals were deposited on GRBaV-infected potted vines that were obtained by agroinoculation. After one to eight days of acquisition, groups of two to four individuals were transferred to healthy potted vines and allowed to feed for five to six days. Transmission assays were replicated three times with groups of 10-25 recipient plants. Subsets of three-cornered alfalfa hoppers were tested for the presence of GRBaV after the acquisition and transmission steps.

Data showed that all three-cornered alfalfa hopper specimens tested positive for GRBaV in multiplex PCR after the acquisition step (100%, 19 of 19) whereas those from alfalfa tested negative for GRBaV (0%, 0 of 17). Also, some specimens tested positive for GRBaV two to three weeks after the transmission step (80%, 12 of 15), indicating that the three-cornered alfalfa hopper can acquire the virus from infected vines in the greenhouse and keep it for extended time after acquiring it. This is consistent with a persistent transmission of GRBaV, as expected. In addition, three to six months post-transmission, recipient plants (6 of 42) became infected with GRBaV, supporting the capacity of the three-cornered alfalfa hopper at acquiring and transmitting GRBaV (Cieniewicz et al., 2016). These results, based on the use of a colony of three-cornered alfalfa hoppers established in the laboratory, confirmed the recent findings of Bahder et al. (2016b) who used vineyard specimens. The recipient vines are being monitored for disease symptom development. Together with our insect trap studies, the control transmission experiments revealed the three-cornered alfalfa hopper as a vector of epidemiological importance for GRBaV.

To address objective 2 and improve diagnostics for GRBaV, a robust real-time PCR methodology was developed using infected and healthy vines grown in the greenhouse and in vineyards. This assay is useful for characterizing the titer of the virus in infected plants and determining the optimal plant tissue and time of the year to collect samples for a reliable diagnosis. In parallel, strategies to produce an antiserum are refined through RNAseq approaches. This work is critical in providing insights into the expression strategies of the GRBaV genome during the infection process. It is anticipated that this knowledge will help us understand how viral genes are expressed in infected plants because efforts to develop an antiserum useful for diagnosis by using synthetic peptides directed against the coat protein or by overexpressing the coat protein coding region in bacteria cells failed so far (Perry and Fuchs, unpublished). In parallel, we contributed to the development of an AmplifyRP® Acceler8® assay for GRBaV. This is a rapid amplification and detection platform designed for testing of grapevine samples for GRBaV using a crude sample extract (Li et al., 2016). Amplified products are tested in a detection chamber where test outputs are read visually on a lateral flow strip. The entire testing process is specific and sensitive, and can be completed in as little as 30-60 minutes (Li et al., 2016).

To address objective 3 and determine if either of the two groups of GRBaV isolates are pathogenic in grapevines, we engineered infectious clones of a representative GRBaV isolate of each of the two phylogenetic clades. 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*. Isolate NY175 from *V. vinifera* cv. Merlot and isolate NY358 from *V. vinifera* cv. Cabernet franc belong to GRBaV phylogenetic clades I and II, 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, as well as vines of rootstock genotypes SO4, 110R, and 3309C that tested negative for GRBaV by PCR. 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 *Agrobacterium* 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 in grapevine tissue. 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 $25\pm 2^\circ\text{C}$ and $33\text{-}45\text{ mEm}^{-2}\text{sec}^{-1}$ (16-hr 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 a multiplex PCR (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.

Several Cabernet Sauvignon, Cabernet franc, Syrah, Pinot noir, Pinot gris, and Chardonnay vines 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. Agroinoculated SO4 became symptomatic (chlorosis and cupping) only after one dormancy period, whereas agroinoculated 3309C and 110R remained asymptomatic (Fuchs et al., unpublished). Some of the vines 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 GFLV-derived constructs (0 of 476) or untreated plants (0 of 56) exhibited red blotch-like symptoms, nor did they test positive for GRBaV in PCR. Sequencing the progeny in some infected vines indicated a 99.6% to 99.9% nucleotide sequence 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 (Fuchs et al., unpublished). 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 were communicated to farm advisors, extension educators, crop consultants, researchers, vineyard managers, and regulators at winter school meetings in California, New York, Oregon, New Jersey, and Virginia. The targeted venues were (i) the Virginia Vineyards Association on February 6, 2015 in Charlottesville, VA (250 participants); (ii) the Grape Expectations on February 28, 2015 in Cranberry, NJ (150 participants); (iii) the Eastern Winery Exposition on March 19, 2015 in Syracuse, NY (120 participants); (iv) the Rogue Valley Grape Growers Association on August 25, 2015 in Central Point, OR (50 participants); (v) the North American Grape Breeders Meeting on August 29, 2015 in Geneva, NY (60 participants); (vi) the Cornell Recent Advances in Viticulture and Enology conference on November 4, 2015 at the IRL Conference Center in Ithaca, NY (60 participants); (vii) the Napa Continuing Education Class Series 3 on November 10, 2015 in Yountville, CA (250 participants); (viii) a webinar on Grapevine Red Blotch Disease: What You Need to Know organized by Regional IPM Centers on February 26, 2016 (participants = 310); (ix) the Business, Enology and Viticulture New York conference on March 5, 2016 in Rochester, NY (160 participants); (x) a webinar on Viral Diseases Transmitted through Nursery Stock in the East: Grapevine Leafroll Disease, Tomato Ringspot, and Grapevine Red Blotch; Clean Plants for the Future of the Eastern Wine and Grape Industry, organized by Cornell University on March 17 in Geneva, NY (250 participants); and (xi) Long Island Grape Growers Association on March 4, 2016 in Riverhead, NY (15 participants).

CONCLUSIONS

Isolates of each of the two phylogenetic clades of GRBaV cause red blotch disease symptoms in *Vitis vinifera* following agroinoculation, confirming their etiological role, while infection is latent in rootstocks with the exception of SO4. Analysis of the spatiotemporal distribution of symptomatic, infected vines documents spread of GRBaV in a vineyard of Cabernet franc in California but not in New York. Some free-living grapevines proximal to the diseased vineyard in California are infected with GRBaV. The analysis of a subset of insect species caught on sticky traps for the presence of GRBaV enabled us to identify four vector candidates, among which the three-cornered alfalfa hopper was shown to acquire the virus from infected vines and transmit it to healthy vines. This finding suggests the three-cornered alfalfa hopper is a GRBaV vector of epidemiological importance.

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TIMING OF FIELD TRANSMISSION OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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Reporting Period: The results reported here are from work conducted July 1, 2016 to September 30, 2016.

ABSTRACT

The goal of this project is to determine when grapevine red blotch associated virus (GRBaV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBaV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBaV has been spreading are being used in this study. One vineyard has a riparian zone adjacent to it, with most virus spread occurring near the edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard has an alfalfa field adjacent to it and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field and within vineyard rows. The third vineyard has most disease spread adjacent to a recently disturbed wooded area. In each vineyard, every plant has a unique number and the location of each plant is being mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants are placed in each vineyard each month starting April 15 and going through September 15. After one month in the field the plants are returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants will be tested for GRBaV in late October and then the plants will be overwintered and retested in the spring of 2017 and 2018.

LAYPERSON SUMMARY

The goal of this project is to determine when grapevine red blotch-associated virus (GRBaV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBaV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBaV has been spreading are being used in this study. One vineyard has a riparian zone adjacent to it, with most virus spread occurring near the edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard has an alfalfa field adjacent to it, and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field and within vineyard rows. The third vineyard has most disease spread adjacent to a recently disturbed wooded area. In each vineyard, every plant has a unique number and the location of each plant is being mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants are placed in each vineyard each month starting April 15 and going through September 15. After one month in the field the plants are returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants will be tested for GRBaV in late October and then the plants will be overwintered and retested in the spring of 2017 and 2018.

INTRODUCTION

In 2012, a new virus was identified in Cabernet Franc grapevines in New York's Finger Lakes region and also in Cabernet Sauvignon grapevines in the Napa Valley. These plants exhibited leafroll-like symptoms but tested negative for leafroll viruses. At a meeting of the International Committee on the Study of Viruses and Virus-like Diseases of Grapevine in October 2012, the name grapevine red blotch-associated virus (GRBaV) was agreed upon for this new virus.

This research aims to determine when GRBaV is spreading in the field. So far, the three-cornered alfalfa hopper (*Spissistilus festinus*) has been shown to transmit GRBaV, but this vector is very minor in many vineyards where the virus is spreading. Movement of GRBaV in vineyards after planting has been documented and can be quite rapid, which clearly indicates the presence of an efficient vector, or a vector that is present in very high numbers.

An increase in the incidence of GRBaV over time in young, healthy vineyards that are adjacent to infected vineyards also suggests the existence of a vector. There has been much work done on trying to identify the vector(s) of GRBaV. Efforts looking at suspected vectors in California have resulted in the identification of the three-cornered alfalfa hopper as a vector early in 2016. Regardless, if this is the only vector or one of multiple vectors, the timing of transmission will be important information in developing a vector management plan.

If we know when the virus moves, efforts at vector control can be targeted to a specific timeframe rather than throughout the growing season. Also, knowing when the virus is moving in the vineyards will help focus on transient insects, which may be present in vineyards for only a short period of time, or insects that feed on grapevines but have other preferred hosts. In either case these vectors could escape detection and identification in standard insect surveys. If transmission is more efficient in riparian areas adjacent to vineyards it will provide clues as where one should look to identify potential vectors.

This project was started in March using in-house (USDA ARS) funds to ensure we could get the first year of field work done in 2016. Funding from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board became available July 1, 2016 and is being used for the remainder of the project. Three hundred grapevines (Merlot on 3309 rootstock) were obtained (donated) from Duarte nursery, repotted into three-gallon pots, and held in a screenhouse until being used in the field, or held in a canyand near Corvallis that is isolated from vineyards. Plants were tested for GRBaV prior to use in the field experiment. All plants tested negative for GRBaV in PCR assays using two sets of primers. Beginning April 15 plants were placed in each of three vineyards for a one-month period (45 plants each month total). Then in mid-May these plants were returned to Corvallis, treated with a systemic insecticide, and stored in a screenhouse. The second set of plants was taken to the vineyards in mid-May, and the process was repeated each month through September. The last set of plants will be collected from the vineyards in mid-October. There was a total of six sets of plants in each vineyard for a total of 270 trap plants, with an additional 30 plants that have not been taken to a vineyard and remained in the screenhouse or canyand during the summer. After the last set of plants is collected all 300 plants will be tested for GRBaV in mid-October. The plants will be retested in the spring of 2017.

OBJECTIVES

The objective of this project is to determine the timing of field transmission of GRBaV.

RESULTS AND DISCUSSION

Three hundred plants were provided by Duarte Nursery for this work. All plants were tested for GRBaV prior to the start of the experiment. Plants were potted in three-gallon pots and maintained in a canyand prior to taking them to the field. When plants were brought back to Corvallis from the fields they were treated with a systemic insecticide and maintained in a screenhouse.

The three vineyards were selected because of documented spread of GRBaV in these vineyards in previous years. Vineyard #1 was near Jacksonville in southern Oregon and has a small riparian area adjacent to the east edge of the vineyard. The trap plants were placed in a grassy area between the riparian zone and the vineyard. Vineyard #2 was near Medford in southern Oregon with the trap plants placed within the vineyard between every third plant in three rows near the west edge of the vineyard. There was an alfalfa field along the west edge of the vineyard. The third vineyard is in the Willamette Valley near Yamhill, Oregon. In this vineyard the spread is occurring throughout the vineyard, with high rates of spread along the east edge of the vineyard where there has been recent removal of adjacent woodlands. In this case the trap plants were place between plants in a single row of the vineyard near the edge of where symptoms were observed.

Each plant was numbered, 1-300 and the location of each plant and the month it was in the vineyard has been recorded. Thus, if GRBaV spread is happening from the alfalfa field, we will know which plants were nearest the source as well as which month the plants were in the field and exposed to potential GRBaV transmission.

All plants will be tested for GRBaV in late October of 2016 and held in a screenhouse over winter for retesting in the spring of 2017 and again in the fall of 2017. The experiment will be repeated in 2017, with new trap plants.

The experimental setup went according to plan and plant rotation went smoothly. We had feeding damage similar to that observed with three-cornered alfalfa hopper in one vine during the course of exposure in the vineyards. We placed sticky cards in the vineyard in the Willamette Valley and did not catch any three-cornered alfalfa hoppers. The last set of plants will be collected from the field the week of October 11. All 300 plants will be tested for GRBaV during the second half of October.

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EDUCATION AND OUTREACH FOR THE CALIFORNIA GRAPEVINE REGISTRATION AND CERTIFICATION PROGRAM, AND AN ASSESSMENT OF RECENTLY ESTABLISHED PRODUCTION VINES FROM INCREASE BLOCKS

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ABSTRACT

Virus diseases of plants are deleterious to California agriculture and can be introduced and propagated in a production system via planting material. The California Grapevine Registration and Certification Program was established in the 1950s in order to offer the voluntary option to growers of virus-screened planting material (nursery stock) (Alley and Golino, 2000). Because vectored viruses such as grapevine leafroll-associated virus 3 can also be spread within and between grape blocks by their vectors, in addition to being transmitted by propagation, there is uncertainty in some cases as to the source of viruses detected in production blocks. In addition, nursery increase blocks may become contaminated by vectors arriving from outside the nursery over time. These factors combine to increase the level of uncertainty in the industry as to the meaning and value of certification. The aim of this project is to provide a focused outreach effort and grower education on the topic of virus disease management, the use of clean plant material, and the function of the Registration and Certification Program. The core of the outreach will be evidence-based professional development materials built on the last five years of research and outreach by our team with both nurseries and grape production commodity groups in California. Our aim is to raise the overall level of knowledge among the grower population as to the value of clean plant material and help growers understand the functioning of the Registration and Certification Program, manage their expectations accordingly, and help to restore and build confidence in the program.

LAYPERSON SUMMARY

Upon initiation, when detrimental viruses in grapevines were thought to only be distributed via propagative material, the California Grapevine Registration and Certification Program intended to provide “virus free” material to growers. Since that time some regulated viruses have been shown to be transmitted by vectors, and additional viruses have been discovered. Although the California Grapevine Registration and Certification Program no longer uses the terminology “virus-free,” the industry still does, which can lead to mistrust in the supply chain between growers and plant nurseries. An example of this occurred recently due to the discovery of grapevine red blotch-associated virus and its presence in some certified material. Extension and outreach programs are needed to provide the appropriate explanation of the certification program, as well as demonstrate its value to the consumer. Additionally, further study is needed in terms of demonstrating the background infection as well as the reinfection rate in registered increase blocks where certified material is sourced from in order to provide protocols appropriate to disease spread.

INTRODUCTION

Certified grapevine nursery stock consumers (i.e., grape producers) are concerned that the quality of the product they are purchasing from the clean plant program does not meet the standard they believe it should. Much of this concern stems from the expectation that certification offers something greater, in terms of freedom from virus contamination, than it scientifically can. With the discovery that grapevine leafroll-associated virus 3 is spreading in California, in addition to the discovery of grapevine red blotch-associated virus (Al Rwahnih et al., 2013; Golino et al., 2008), grape producers question the quality of certified vines. There is good evidence that clean plant programs work and that they have large economic benefits that can be shared by all actors in the supply chain (Fuller et al., 2015), but, as with all supply chains, in order for clean plant programs to work well they require mutual trust among the actors in the chain. By defining the term “certified” according to the scientific sampling procedure and educating growers of the meaning of this term, we can bridge the current gap in perceptions that exists between the clean plant system and the purchasers of its products. However, because some viruses can be spread, unless a complete census of all certified vines is carried out every year, it is impossible for

any certification program to reduce virus incidence to zero. The meaning of the term “certified” must be defined in relation to the statistical performance of the actual sampling plan used. In order for grower trust in the system to build, that meaning must be clearly articulated and appropriate expectations established for disease incidence in planting material emerging from a program using the definition. Additionally, it is unclear at this time what level of background infection per year occurs in nursery increase blocks, and there is a lack of understanding of the potential reinfection of increase blocks between sampling rotations. The intentions of this project are to provide quantifiable outreach and extension involving the certification program while addressing the background infection in nursery increase blocks and the potential reinfection in increase blocks between sampling bouts.

OBJECTIVES

1. Develop a grower information packet and slide presentation to summarize the California Grapevine Registration and Certification Program.
2. Hold grower meetings in key grape-growing regions of California to explain the functioning, efficacy, and limitations of the certification program.
3. Quantify the impact of education and outreach by issuing pre-test and post-test surveys at grower meetings.
4. Assess the level of potential contamination or reinfection in newly-established vineyard blocks when material is sourced from increase blocks.
5. Assess the level of reinfection of leafroll 3 and red blotch viruses in increase blocks between certification sampling bouts.

RESULTS AND DISCUSSION

Since the project’s initiation in October of 2016 efforts have been made to collaborate with farm advisors and industry-related personnel across California. Because the project began only two months ago there are no results to discuss at this time.

CONCLUSIONS

Because this project began in October of this year, no conclusions can be made at this time.

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AUTHOR INDEX

Almeida, R.....	3, 7, 13, 267	Maloney, K.....	130
Azad, M.....	121	Martin, R.....	285
Backus, E.....	237	Mazzoni, V.....	212
Blaisdell, K.....	267	McElrone, A.....	152
Boissésou, C.....	190	McRoberts, N.....	288
Burbank, L.....	188	Mowery, P.....	15
Burr, T.....	15	Nieri, Rachele.....	212
Byrne, F.....	230	Perring, T.....	221
Cantu, D.....	139, 152, 178	Perry, K.....	277
Cervantes, F.....	237	Powell, A.....	109
Chen, J.....	189	Prabhaker, N.....	221
Cooper, M.....	241, 247, 257, 267	Redak, R.....	230
Cursino, L.....	15	Rock, C.....	121
Daane, K.....	247, 257, 267	Rolshausen, P.....	130
Dandekar, A.....	25, 35, 54, 59	Roper, C.....	130, 139, 152
Daugherty, M.....	195	Shugart, H.....	237
Dervishian, G.....	247	Sisterson, M.....	192
Falk, B.....	199	Soto, D.....	195
Fuchs, M.....	277	Stenger, D.....	188, 192
Gabriel, D.....	43	Stone-Smith, B.....	75
Gilchrist, D.....	49, 54, 59	Sukumaran, S.....	121
Golino, D.....	67, 267, 277	Sun, Q.....	152
Gordon, S.....	212	Traore, S.....	121
Hao, L.....	15	Tricoli, D.....	59, 158
Haviland, D.....	75	Van Zyl, S.....	247
Hochman Adler, V.....	247	Varela, L.....	241, 257
Jacques, M.....	190	Walker, A.....	167, 178
Kamita, S.....	205	Wallis, C.....	238
Kirkpatrick, B.....	49	Walton, V.....	257
Krugner, R.....	212, 238	White, B.....	230
Labavitch, J.....	109	Wilson, H.....	267
Lin, H.....	191	Wiman, N.....	257
Lincoln, J.....	59	Zalom, F.....	257
Lindow, S.....	59, 81, 94		

